

NATURAL AND THERAPY-INDUCED IMMUNE CONTROL OF HIV-1

A thesis submitted for the Degree of
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By

Nicole Yager

St Cross College

Department of Clinical Medicine

University of Oxford

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ABSTRACT

The human immunodeficiency virus (HIV)-specific cytotoxic T-lymphocyte (CTL) response is important in the control of HIV-1 infection. Due to the virus having a high rate of mutation, immune pressure can select for variants that are no longer recognised by CTLs to dominate the viral quasispecies. This is similar to how antiretroviral resistance emerges. HIV-1 is therefore adapting to both human leukocyte antigen (HLA)-restricted immune responses and antiretroviral therapy. This thesis initially focused on the natural CTL response to an HLA-B*51-restricted epitope in integrase. This HLA class I allele is associated with slow progression to AIDS; however, as no CTL-driven escape mutation has been fully defined within this integrase epitope, we cannot determine what contributes to the association of HLA-B*51 and natural control of infection. By longitudinally studying a cohort of early HIV-infected individuals, we observed the emergence of polymorphisms that abrogate a CTL response to this epitope. CTL escape may also prove to be the downfall of current immunotherapy strategies attempting to combat HIV infection. T cell receptors (TCRs) have been genetically modified to enhance their binding affinity to an HLA-A*02-peptide complex and transduced into CD8⁺ cells to create an HIV adoptive therapy. We demonstrate through *in vitro* selection pressure assays that escape from these cells may be a difficult task for the virus given that the TCR is able to recognise the majority of variants of this epitope. Antigen processing mutations may represent the only option for escape. How this may translate clinically will only be determined through *in vivo* studies, which must be meticulously monitored. Finally, when this high affinity TCR was fused to an anti-CD3 single chain variable fragment to create proteins capable of redirecting non-HIV-specific CTLs to HIV-infected cells, we found that the result was specific lysis. These proteins may supersede the use of TCR-transduced cells when used in synergy with antiretroviral therapy.

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CONTRIBUTIONS TO THIS THESIS

Chapter 3

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ABBREVIATIONS

Ad5	Adenovirus type 5
AIDS	Acquired Immune Deficiency Syndrome
APC	Allophycocyanin
AZT	Azidothymidine
BCR	B cell receptor
bp	Base pair
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CDR	Complementarity determining region
CFSE	Carboxyfluorescein succinimidyl ester
CI	Confidence interval
CMV	Cytomegalovirus
CRF	Circulating recombinant form
CTL	Cytotoxic T-lymphocyte
Da	Dalton
EBV	Epstein-Barr virus
EC ₅₀	50% maximal effective concentration
ELISpot	Enzyme-linked immunosorbent spot
ER	Endoplasmic reticulum
ERAAP	ER aminopeptidase
E:T	Effector:Target
FATAL	Fluorometric assessment of T lymphocyte antigen specific lysis

FCS	Foetal calf serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
gp	Glycoprotein
GvHD	Graft-versus-host disease
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
ImmTAC	Immune Mobilising mTCR Against Cancer
kb	Kilobase
Kd	Dissociation constant
LANL	Los Alamos National Laboratory
LCMV	Lymphocytic choriomeningitis virus
LTNP	Long-term nonprogressors
LTR	Long terminal repeat
mAb	monoclonal antibody
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MOI	Multiplicity of infection
mTCR	Monoclonal T cell receptor
nAb	neutralising antibody
NK	Natural killer

NNRTI	Non-nucleoside analogue reverse transcriptase inhibitor
NRTI	Nucleoside analogue reverse transcriptase inhibitor
PBMC	Peripheral blood mononuclear cell
PHA	<i>Phaseolus vulgaris</i> agglutinin
PCR	Polymerase chain reaction
PE	Phycoerythrin
R5	CCR5-tropic
RANTES	Regulated upon activation, normal T expressed and secreted
RT	Reverse transcriptase
scFv	Single chain variable fragment
SEM	Standard error of the mean
SFU	Spot forming units
SG-PERT	SYBR Green product enhanced reverse transcriptase
SIV	Simian immunodeficiency virus
SPARTAC	Short Pulse AntiRetroviral Therapy At HIV seroconversion
SPR	Surface Plasmon resonance
TAA	Tumour-associated antigen
TAP	Transporter associated with antigen processing
TCID ₅₀	50% tissue culture infective dose
T _H	T helper cell
TNF	Tumour necrosis factor
UDPS	Ultradeep pyrosequencing
vRNA	Viral ribonucleic acid
X4	CXCR4-tropic

For my parents

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CHAPTER 1

Introduction

1.1 Human immunodeficiency virus

1.1.1 The pandemic

In the early 1980s, world media attention focused on a growing emergence of cases involving previously healthy individuals affected with a failing immune system, leading to a multitude of manifestations and eventually death. It was designated Acquired Immune Deficiency Syndrome (AIDS). Competitive research culminated in the discovery and isolation of the aetiological agent, human immunodeficiency virus (HIV) (Barre-Sinoussi et al., 1983, Popovic et al., 1984, Gallo et al., 1984). Thirty years on, substantial progress has been made in the HIV field in terms of understanding the virus and how it interacts with the immune system. However, neither a vaccine nor therapeutic eradication are at present imminently foreseeable.

HIV is transmitted through unprotected sexual intercourse, sharing of contaminated needles, transfusion of HIV-infected blood, and via mother-to-child during pregnancy, childbirth and breastfeeding. HIV prevention efforts consisting of education, condoms, access to male circumcision, needle-exchange programs, blood donor screening and treating pregnant/postpartum women, are still not enough to control the epidemic. According to UNAIDS, there were estimated to be 33.3 million people living with HIV globally at the end of 2009 (UNAIDS, 2010) (Figure 1.1).

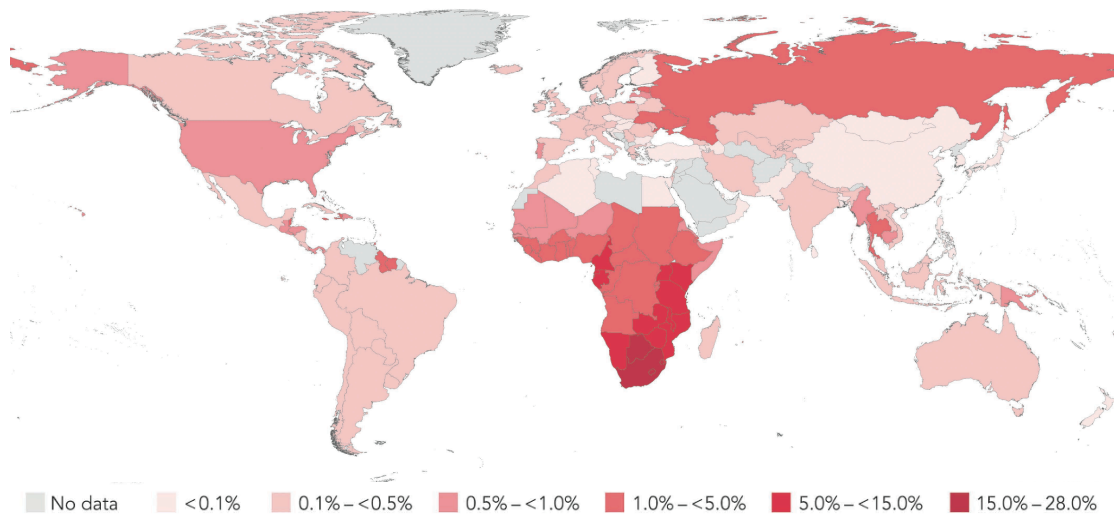


Figure 1.1 The worldwide prevalence of HIV in 2009 (UNAIDS, 2010).

During clinical latency of an HIV-infected individual, a period of roughly 10 to 15 years, CD4 count gradually declines and the infected individual is left with immune dysfunction which in turn increases susceptibility to opportunistic infections which healthy individuals are usually able to control. At this point, the individual is classified as having AIDS. In 2009, an estimated 1.8 million people worldwide died due to an AIDS-related illness (UNAIDS, 2010).

1.1.2 Viral structure and replication

HIV causes a persistent infection that cannot be cleared naturally by the human immune system or by current therapy. Instead, it remains latent in a reservoir of infected cells that may become activated at a later stage to produce new virions. HIV belongs to the retroviridae family; these viruses are able to reverse transcribe their single-stranded RNA genome into complementary DNA (cDNA) and then into a double-stranded DNA intermediate. The root of persistency stems from HIV being able to integrate this DNA

copy of its genome into the host cell's DNA, becoming a provirus, and exploiting the host cell's machinery to produce new virions.

The HIV virion is enveloped by the viral-encoded proteins gp120 (a surface glycoprotein) and gp41 (a transmembrane glycoprotein) attached to a lipid bilayer derived from a host cell. Associated with the membrane is the virion matrix composed of p17 molecules, which in turn surrounds a bullet-shaped capsid made from ~2,000 p24 molecules. This compartment contains two copies of a positive-sense single-stranded RNA genome of ~9.3 kb tightly bound to nucleocapsid proteins. Also packaged are the viral-encoded proteins reverse transcriptase (p66/p51), integrase (p31), protease (p11) and accessory proteins (Nef, Vif, Vpr) (Figure 1.2).

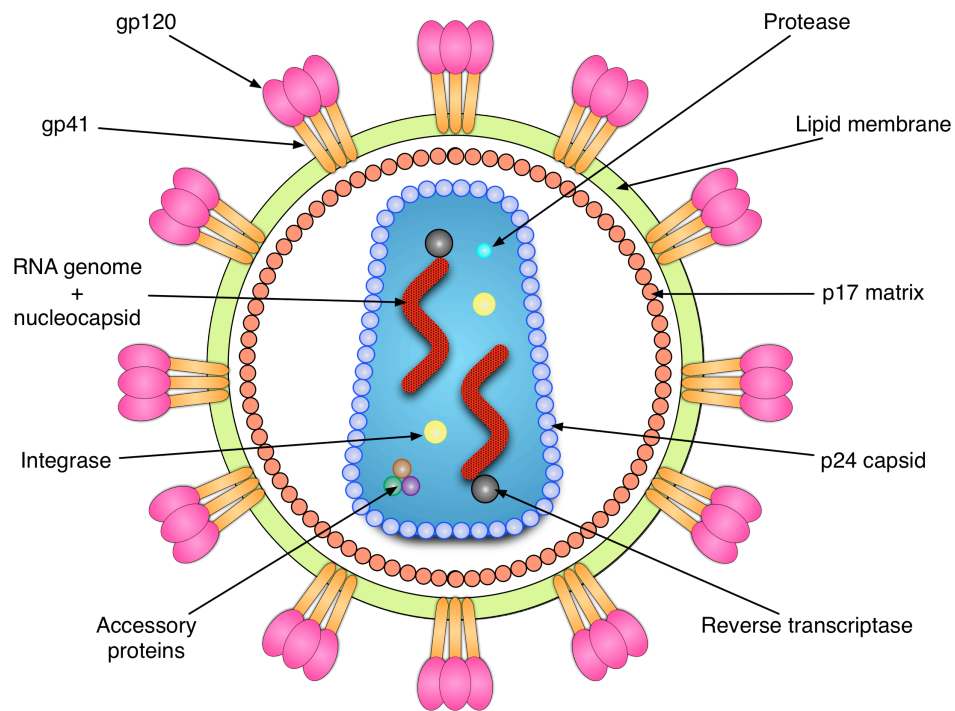
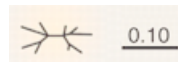


Figure 1.2 The HIV virion.

1.1.3 Diversity of HIV

HIV has extensive genetic diversity due to a high mutation rate and a high frequency of recombination (Coffin, 1986) (Figure 1.3). The genetic variability of HIV is also associated with the level of viral replication, which in a treatment-naïve individual can be 10^9 to 10^{12} new virions produced daily (Ho et al., 1995, Perelson et al., 1996).

(A)



(B)

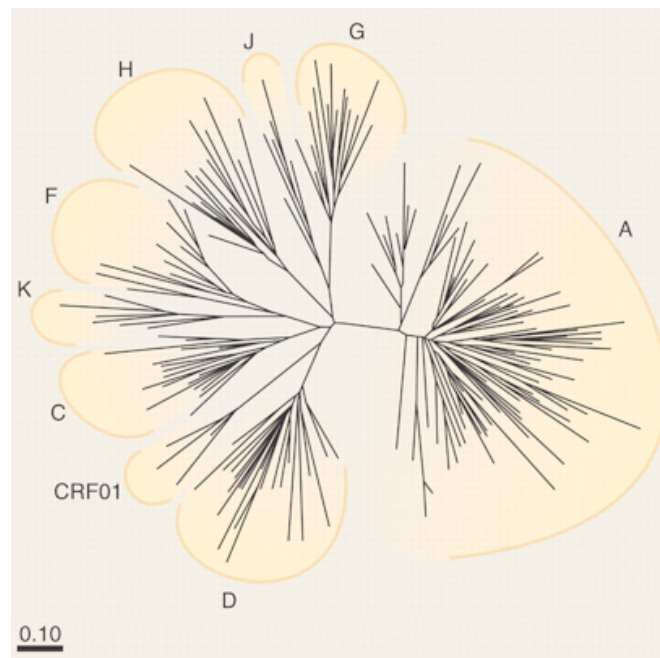


Figure 1.3 HIV diversity. (A) Phylogenetic analysis of 9 *env* (V2-C5) sequences from a single HIV subtype B-infected individual 73 months post-seroconversion; (B) phylogenetic analysis of *env* (V2-C5) sequences from 193 HIV-infected individuals from the Democratic Republic of Congo in 1997 (Korber et al., 2001). By permission of Oxford University Press.

The mutation rate of HIV is in the order of 10^{-4} to 10^{-5} mutations per nucleotide per replication cycle (Mansky and Temin, 1995). Mutations arise as a consequence of three enzymes: (i) host DNA-dependent DNA polymerase, which has a high fidelity with an error rate during replication of DNA chromosomes of $<10^{-9}$ mutations/bp/cycle (Loeb, 1991) and so has a negligible effect on the overall HIV mutation rate; (ii) viral reverse transcriptase; and (iii) host DNA-dependent RNA polymerase II. It is not known to what degree RNA pol II contributes to the overall HIV mutation rate; current assays indicate that reverse transcriptase is the major contributing factor (O'Neil et al., 2002). The error-prone nature reverse transcriptase possesses is brought on by a lack of 3'-5' proofreading activity, which is crucial for replication fidelity (Roberts et al., 1988). The mutations may be point mutations where a single nucleotide is altered and leads to a nonsynonymous (amino acid substitution) or synonymous (no change in amino acid) mutation, insertions or deletions.

Recombination can occur because two variant HIV genomes can be copackaged into the same virion creating a heterozygous particle. Once this virion has infected a new cell, recombination occurs as reverse transcriptase switches to homologous regions between the viral RNA templates (Clavel et al., 1989). This is a high frequency process, occurring with at least 2.8 crossovers during an *in vitro* single cycle assay (Zhuang et al., 2002). However, this does not take into account the probability of coinfection and so *in vivo* the recombination rate has been estimated to be 1.4×10^{-5} recombinations per site per cycle (Neher and Leitner, 2010) – a similar rate to the mutation rate.

With these high rates of mutation and recombination along with a high replication rate, the virus exists within an individual not with a single clonal genome, but as heterogeneous populations of related genomes known as a viral quasispecies (Domingo et al., 1985). This is a fluid state where viral variants may proliferate under the selective pressure of the environment. In addition, defective virions are also produced and have even been found to accumulate *in vivo* (Sanchez et al., 1997).

1.1.3.1 Classification of HIV

Phylogenetic analysis has identified two types of HIV: HIV-1 and HIV-2 (Clavel et al., 1986); both zoonotic. HIV-1 can be grouped into four groups: M, N, O and P, with >90% of infections being group M. Group M can be further divided into subtypes or clades: A, B, C, D, F, G, H, J, K and circulating recombinant forms (CRFs). Subtype B is the main subtype found in Western populations; subtypes A, C, D and CRF01_A/E are mainly found in sub-Saharan Africa and Asia.

HIV-2 can be grouped into eight groups: A – H. HIV-2 is less pathogenic than HIV-1, which may be why it is only endemic to West Africa.

1.2 Antiretroviral therapy

In 1987, zidovudine (azidothymidine, AZT) received US Food and Drug Administration (FDA) approval for use against HIV infection (Mitsuya et al., 1985). This nucleoside analogue reverse transcriptase inhibitor (NRTI) was the first of many antiretroviral drugs to come to market. At present, there are five classes of antiretroviral drugs

licensed named after the phase of the HIV life cycle they target: reverse transcriptase inhibitors (NRTI and non-nucleoside analogues, NNRTI); protease inhibitors; entry/fusion inhibitors; and integrase inhibitors (Figure 1.4). Antiretroviral therapy has increased the survival time of an HIV-infected individual as well as reducing the number of mother-to-child transmissions (Palella et al., 1998, Guay et al., 1999).

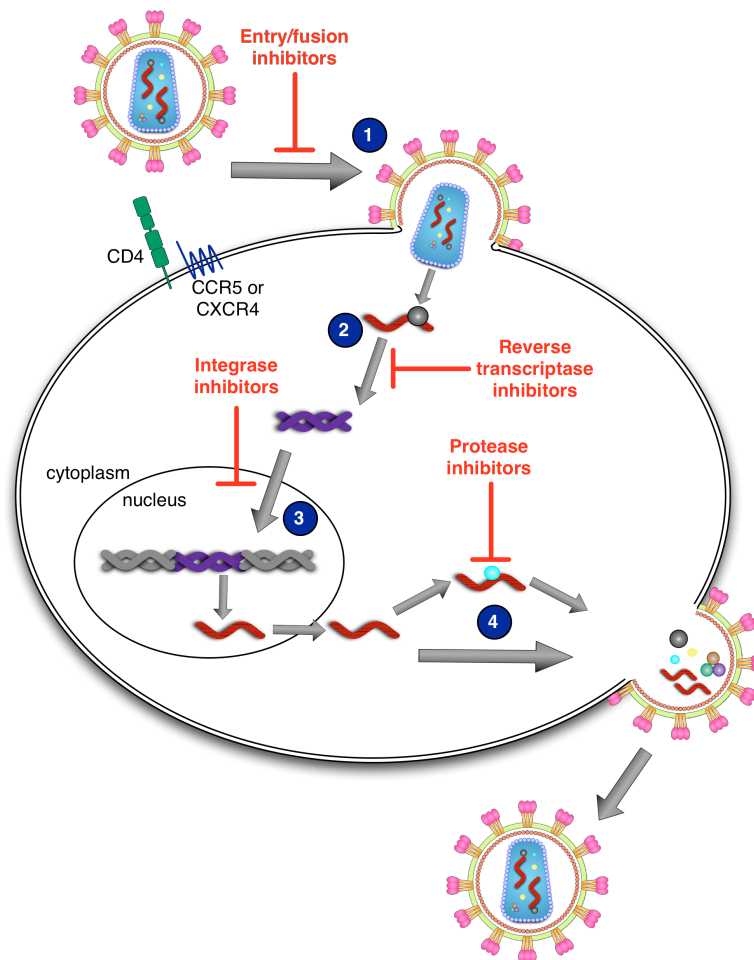


Figure 1.4 Schematic of the HIV life cycle and current antiretroviral targets. 1: The virion attaches to a host cell via the CD4 molecule and a coreceptor allowing membrane fusion. 2: The viral RNA is reverse transcribed into cDNA. 3: The viral genome is integrated into the chromosomal DNA, becoming proviral DNA. 4: The proviral DNA is transcribed and is either packaged as vRNA or translated and proteolytically processed into viral proteins. Once all the components are packaged, the virion buds and matures.

Although initially successful, the use of a single antiretroviral drug led to rapid drug resistance (Larder and Kemp, 1989). With FDA approval of the first protease inhibitor (saquinavir) and NNRTI (nevirapine), antiretroviral monotherapy was replaced by highly active antiretroviral therapy (HAART) in 1996. This entailed patients receiving a combination of three drugs, commonly two NRTIs with either a NNRTI or a protease inhibitor. The primary objective of HAART is to suppress viral replication so the viral load is below the limit of current diagnostic detection (<50 RNA copies/ml) whilst slowing the rate of CD4 count decline and extending the time to progression to AIDS.

HIV infection is now seen as a manageable chronic disease with the use of lifelong HAART therapy: the average life expectancy of a 20-year old HIV-infected individual is two-thirds of that of the general population (ART-CC, 2008). However, HAART has yet to eradicate HIV from an infected human. Even with an undetectable viral load for a considerable period of time, the withdrawal of HAART results in rapid plasma viral load rebounding within days (de Jong et al., 1997, Harrigan et al., 1999).

Initially, mathematical models predicted that the use of HAART would eliminate HIV from most viral compartments after 2.3 to 3.1 years (Perelson et al., 1997). This did not account for HIV persisting in latent reservoirs during HAART (Finzi et al., 1997, Wong et al., 1997, Chun et al., 1997) thus impeding eradication. In 1999, an estimate of 60.8 years of HAART would be required for eradication if the individual's viral reservoir contained 1×10^5 cells (Finzi et al., 1999) – a disheartening figure given the average HIV-infected individual's lifespan.

1.2.1 Antiretroviral resistance

It should be kept in mind that an individual does not need to be on therapy to generate a mutation that confers drug resistance: mutations are spontaneous events. Within the viral quasispecies, the dominance of a variant depends on its viral fitness, i.e. its capacity to replicate in a specific environment. In a treatment-naïve environment, a drug-resistant variant with an impaired replicative capacity would not dominate but may remain as a minor variant at a low frequency. In the presence of antiretroviral therapy, despite the drug-resistant variant having a lower replicative capacity previously, it is greater than the wildtype variant in this drug-pressurised environment; the drug-resistant variant is now the fitter virus and subsequently dominates the quasispecies (Coffin, 1995). To restore the replicative capacity of a drug-resistant variant, compensatory mutations may be necessary. Antiretroviral therapy causes a selection pressure on minor drug-resistant variants to emerge as the major variants and the eventual consequence is therapy failure.

Mutations in the HIV genome can alter the structure of the encoded proteins. Drug resistance occurs when these structural changes affect the binding or activity of an antiretroviral drug. A single mutation can sometimes generate high-level antiretroviral resistance. For example, the M184V polymorphism in reverse transcriptase occurs from a single nucleotide mutation, impairing the enzyme's ability to incorporate an analogue (i.e. the NRTIs lamivudine and emtricitabine) into the viral DNA, and results in high-level resistance (Gao et al., 1993). Alternatively, the accumulation of mutations may confer resistance. Etravirine is a NNRTI that received FDA approval in 2008 and has a high genetic barrier to developing drug resistance *in vitro* (Vingerhoets et al., 2005). All

currently known antiretroviral-resistance mutations are documented and meticulously monitored (Stanford, 2010).

Antiretroviral drug regimens are optimised so that a patient's viral load does not drastically increase; resistant mutations should not be present at treatment onset. As the rate of viral mutation is associated with the rate of viral replication, an effective regimen that suppresses viral replication is associated with a low probability of generating a resistance mutation (Coffin, 1995). This is why it is imperative that antiretroviral therapy is adhered to: the skipping of doses in an individual can lead to increased viral replication and therefore subsequent mutation. HAART has reduced the probability of drug resistance emerging because the likelihood of generating three spontaneous antiretroviral-resistant mutations in a treatment-naïve patient is extremely low. In comparison to monotherapy, HAART-induced suppression of plasma viraemia reduces the probability of developing drug-resistant mutations against all three drugs thereby prolonging their use.

In 1994, multiple mutations in *pol* resulted in the emergence of multidrug resistance to the NRTIs zidovudine and didanosine (ddI) when used as a combination therapy in patients (Shirasaka et al., 1993, Shafer et al., 1994). In a clinical study involving HIV-infected patients receiving either a NRTI combination of zidovudine+lamivudine, the protease inhibitor indinavir alone, or a combination of all three drugs, a statistically greater proportion of patients had a viral load of <500 copies/ml in the latter group after 6 months. In terms of mutations conferring drug resistance, 26/31 (84%) and 10/19 (53%) in the zidovudine+lamivudine group and indinavir alone group had resistance

mutations, respectively; as the majority of patients in the three-drug regimen had a viral load of <50 copies/ml, sequencing data were not determined (Gulick et al., 1997) (Figure 1.5).

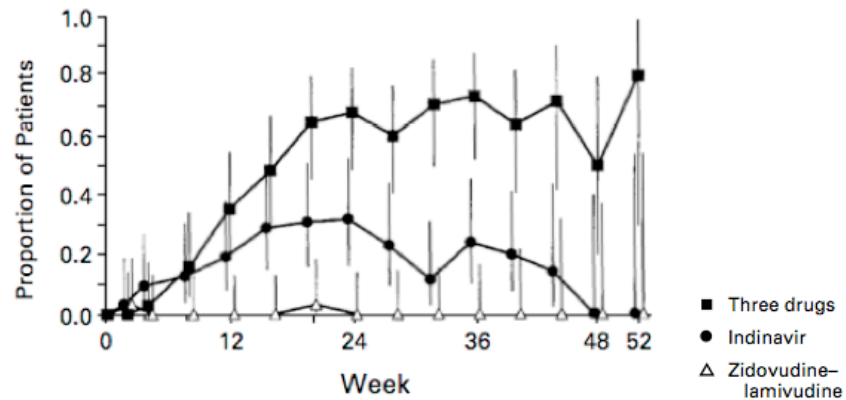


Figure 1.5 Effect of multidrug antiretroviral therapy on viral load. The proportion of patients with HIV RNA levels <50 copies/ml depends on the type of drug regimen (Gulick et al., 1997).

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Given that HAART is indefinite, multidrug resistance must be avoided at the population level. Once generated within a patient, it will persist as part of the viral quasispecies even when therapy has been changed, albeit possibly at a low frequency. As new antiretrovirals are used in these individuals, and resistance against these emerge, therapy options decrease. Transmission of multidrug-resistant strains is of major concern, especially as the median time to lose transmitted detectable drug-resistant variants ranges from 4.1 years to longer than an individual's lifetime (Little et al., 2008).

1.3 The immune system

The immune system is essential for human survival in a world dominated by microscopic infectious agents such as bacteria and viruses. It has evolved into a highly

complex network able to defend against a plethora of pathogens whilst having to distinguish between foreign (non-self) and host (self). In addition, the role of the immune system in cancer is a rapidly growing field, with tumour antigens being potential targets for cancer immunotherapy.

The immune response has two collaborative arms: an innate response and an adaptive response. The innate response is the first line of defence, occurring immediately following infection. It is nonspecific and has no immunological memory, involving cells such as monocytes/macrophages, granulocytes and natural killer (NK) cells. The adaptive immune response is subdivided into a humoral response and a cell-mediated response. The former involves the secretion of antibodies from terminally differentiated B lymphocytes that protect against extracellular pathogens; the latter requires T lymphocytes and protects against intracellular pathogens. The adaptive response is specific and immunological memory is generated once an antigen has been encountered. On a timescale, the adaptive response occurs after the innate response, with the cell-mediated response arriving before the humoral response.

1.3.1 Major histocompatibility complex

The major histocompatibility complex (MHC) is a genomic region that encodes for proteins that are expressed on the surface of cells and present antigen fragments. It is the most polymorphic section in the human genome. MHC class I proteins present intracellular antigens including viral peptides on the surface of all nucleated cells; MHC class II proteins present peptides digested from extracellular pathogens on professional antigen presenting cells such as dendritic cells, macrophages and B lymphocytes.

MHC class I proteins consist of a 45-kDa glycosylated heavy chain of three α domains non-covalently linked to β_2 -microglobulin. The α_1 and α_2 domains have the most structural variance and form a groove into which peptide is loaded. The amino acids that line the groove determine its shape and form pockets that can accommodate certain peptide residue side chains. Thus only a limited number of peptides, 8 to 11 amino acids in length, may be bound. The peptide is held in the binding groove by particular anchor residues, found at the C-terminal end and near the N-terminal end of the peptide; without these anchor residues the peptide cannot fit in the groove and will not bind to the MHC class I protein. The central residues of the peptide tend to protrude from the groove and are more accessible for T cell receptor (TCR) recognition (Figure 1.6).

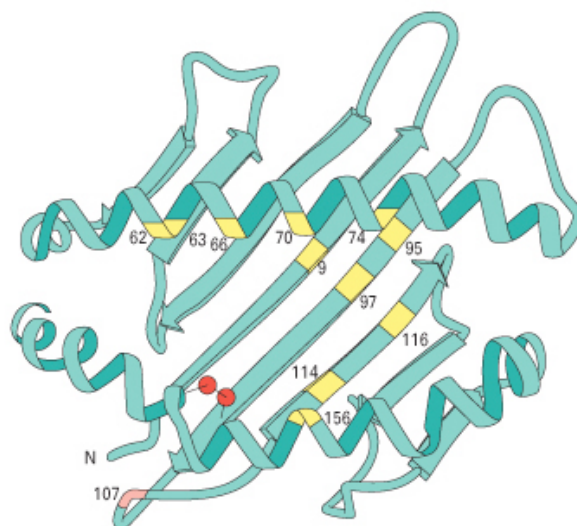


Figure 1.6 The HLA-A*02 peptide binding groove (Male et al., 2006). *Copyright Elsevier.*

In humans, the MHC encodes human leukocyte antigens (HLA). The three classical HLA class I molecules are HLA-A, -B and -C. A combination of these HLA alleles is inherited as a haplotype from each parent so that every individual can express a

maximum of six different classical HLA class I proteins on a cell's surface. As the peptides presented are HLA-restricted, individuals with HLA heterozygosity present a broader range of peptides than those with homozygosity. Different HLA haplotypes are found in different geographical locations due to populations being exposed to disparate pathogens over vast periods of time.

1.3.2 Antigen processing

In order to be presented by MHC class I molecules, self and non-self antigens must be produced in the appropriate size. This stage of synthesising endogenous peptide fragments is known as antigen processing. The cell's immunoproteasome has proteolytic activity and so is able to cleave both self and non-self proteins into peptides approximately 5 to 15 amino acids in length, with the final C-terminus of the peptide being generated. Peptides are transported into the endoplasmic reticulum (ER) by the cellular proteins transporter associated with antigen processing 1 and 2 (TAP1 and TAP2). Once in the ER, additional N-terminal trimming of the peptide occurs by the ER aminopeptidase (ERAAP) (Serwold et al., 2002). The MHC class I protein is then loaded with the peptide and this MHC-peptide complex is transported to the cell surface via the Golgi apparatus. It is now capable of presentation to a TCR.

1.3.3 Lymphocytes

There are three types of lymphocytes: NK cells, B cells and T cells. NK cells are a constituent of the innate immune response, inducing apoptosis of virus-infected cells and tumour cells that have downregulated MHC expression. This is extremely important in viral infection as viruses can manipulate a cell to downregulate MHC as a mechanism

of evading immune surveillance; there is an inverse correlation between MHC class I expression and NK cell killing. In HIV-1 infection, the viral protein Nef causes downregulation of HLA-A and -B whilst maintaining expression of HLA-C and -E to protect the cells from NK cell lysis (Cohen et al., 1999).

Specific immune responses are carried out by the B and T lymphocytes. Both originally derive from haematopoietic stem cells in the bone marrow, with T cells having migrated and matured in the thymus. Similarly, both B cells and T cells express an antigen-specific cell surface receptor: a B cell receptor (BCR; membrane-bound immunoglobulin) on B cells; a TCR on T cells. Self antigens are usually tolerated by the immune system, and if not, leads to autoimmune disease.

Once a B or T cell has encountered its antigen, the cell is activated and proliferates. This clonal expansion of a lymphocyte able to recognise the same antigen produces an effective immune response.

1.3.3.1 T cell receptor

Recognition of the MHC class I-peptide complex is via the TCR, found on the surface of all T cells with most expressing an $\alpha\beta$ TCR and a small subset expressing a $\gamma\delta$ TCR. The $\alpha\beta$ TCR is a heterodimer consisting of an α and β polypeptide chain both anchored in the cell membrane and linked by a disulphide bond. The diversity of these receptors is huge, culminating in a broad TCR repertoire: each TCR is specific for one or a few MHC-peptide complexes. This heterogeneity is due to somatic recombination of the

TCR genes V (variable), J (joining), and D (diversity; only with the β chain) during thymic development.

TCRs are positively selected in the thymus so that only those with an intermediate affinity to self MHC may further develop (those with high or low affinities undergo apoptosis). The cells are then negatively selected so that those that do not recognise self antigen continue in development. In contrast, B cell development involves immunoglobulin genes undergoing somatic recombination *and* somatic hypermutation. T cells do not undergo this process of random mutations being selected for binding ability (known as affinity maturation), possibly to prevent recognition of self antigens.

Within the variable domains of both the TCR α and β chains, are hypervariable regions known as complementarity determining regions (CDRs). It is these regions that form the antigen-binding site and so are the most diverse. CDR1 and CDR2 loops are approximately positioned over the anchored C- and N-terminus of the bound peptide, whilst the CDR3 loops of both chains are at the centre of the binding site and therefore are in contact with the protruding bound peptide; CDR3 is the most diverse CDR (Davis et al., 1998, Willcox et al., 1999).

The binding of TCRs to a MHC-peptide complex is characterized by a low affinity ($K_d = 10^{-4}$ to 10^{-5} M) (Matsui et al., 1991, Weber et al., 1992), which derives from a slow association rate (k_{on}) coupled with a fast dissociation rate (k_{off}) (Matsui et al., 1994). A

single MHC-peptide complex on a target cell may sometimes be enough to effectively activate a T cell cytolytic response (Sykulev et al., 1996).

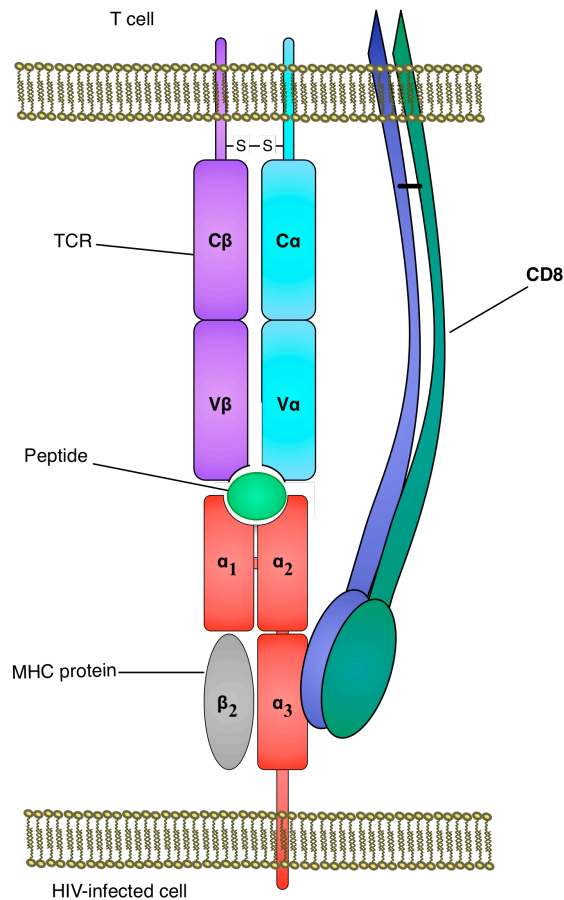


Figure 1.7 TCR recognition of a MHC-peptide complex. The CD8 coreceptor interacts with the α_3 domain of the MHC class I molecule.

1.3.3.2 T cell subsets

T cells can have different roles as determined by their cell lineage. We can identify these population subsets by the functionally important cell surface markers known as the cluster of differentiation (CD): CD3 is an element of the TCR complex; CD4 a marker of T helper (T_H1 , T_H2 and T_H17) cells and regulatory T (Treg) cells; and CD8 a

marker of cytotoxic T lymphocytes (CTLs). CD4 and CD8 proteins are important for engaging with the MHC-peptide-TCR complex with CD4 and CD8 binding MHC class II and I, respectively (Figure 1.7). The presence of CD8 has been shown to stabilise the MHC-peptide-TCR complex by a factor of 2 (Wooldridge et al., 2005) although the primary role of CD4 and CD8 seems to be in the recruitment of Lck to the TCR-CD3 complex, thereby initiating the signalling cascade (Artyomov et al., 2010).

As their name implies, CTLs have cytotoxic activity, killing host cells infected with intracellular pathogens through a variety of different pathways: using the granules that are a constituent of the cells or through direct signalling pathways such as Fas (Figure 1.8). In the first pathway, when a CTL had been activated, its granules polarise to the immunological synapse and are released into the extracellular space between the CTL and its target cell. Within these granules is perforin (a protein that forms pores in the target cell membrane) and granzymes (serine proteases that cleave proteins leading to apoptosis of the cell). In the second pathway, CTLs expressing the ligand FasL (CD95L) interact with the receptor Fas (CD95) found on the cell surface of target cells. Fas has death domains in its cytoplasmic tail and so this Fas-FasL interaction leads to trimerisation of Fas and recruitment and activation of proteins through the death domains that results in caspase-dependent apoptosis. Other pathways involve similar cascades occurring with other TNF receptors.

HIV has a cell tropism for cells of the immune system: T helper cells and macrophages (Gartner et al., 1986). This tropism is due to gp120 binding the CD4 antigen (Dalglish

et al., 1984), initiating a sequence of events enabling the virus to enter the cell and ultimately resulting in integration and production of virus progeny.

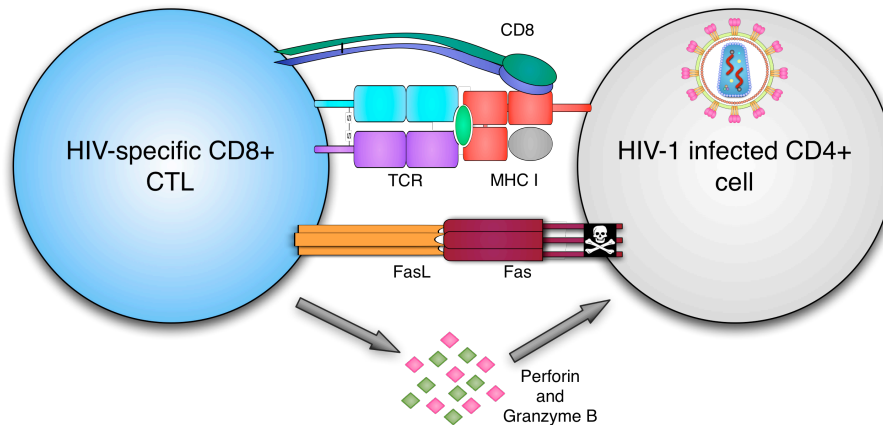


Figure 1.8 The cytotoxic activity of CTLs.

1.4 HIV-specific CTLs

HIV-specific CTLs were first detected in 1987 (Walker et al., 1987) and since that time, research on this cell population has been abundant. HIV-1-specific CTLs may be directed against any of the virus-encoded proteins, with often multiple epitopes, which are the HLA-restricted antigenic determinants, found in each protein.

During acute HIV infection, there is a large increase in viral load that peaks and then rapidly declines; it appears that viral replication is at this stage under the influence of immune control (Clark et al., 1991, Daar et al., 1991). In 1994, it was observed that individuals with primary HIV-1 infection and who mounted an early, strong HIV-1-specific CTL response coincided with better control of viraemia compared with those

individuals with weaker or delayed responses (Borrow et al., 1994, Koup et al., 1994). Neutralising antibodies (nAbs) are not detected until after this viraemia decline and so it is unlikely that it is this response that is responsible for the control of primary viraemia in acute HIV infection (Ariyoshi et al., 1992). The temporal association between CD8⁺ CTL responses and control of viraemia in primary HIV-1 infection may hold clues as to what responses need to be generated with a HIV vaccine.

The data observed in humans infected with HIV provides circumstantial evidence of the role of CTLs in controlling infection. We cannot ethically deplete the CD8⁺ cells in a human and monitor the outcome. The role of animal models fills this gap. The similarities between HIV infection in humans and simian immunodeficiency virus (SIV) infection in monkeys allows for more detailed assays to be carried out. The association between CD8⁺ CTL responses and control of viraemia in primary infection was also observed in SIV infection (Yasutomi et al., 1993). Total depletion of CD8⁺ T cells from the blood and near total depletion from the lymph nodes of SIV-infected rhesus macaques using a CD8-specific monoclonal antibody (mAb) resulted in a less profound decrease from peak viraemia during primary infection compared to macaques that were administered a control mAb. There was a significant difference in survival time between CD8-depleted and control macaques – survival time was shorter in the former (Schmitz et al., 1999).

CD8⁺ cells can also control viral replication through noncytolytic mechanisms (Walker et al., 1986). CCR5 is a natural receptor for RANTES (regulated upon activation, normal T expressed and secreted), MIP-1 α and MIP-1 β (macrophage inflammatory

proteins). These proteins are inhibitors of HIV-1 replication in macrophages and are secreted by CD8⁺ T cells (Cocchi et al., 1995); they may be released after HLA-restricted antigen-specific activation of the CTL (Yang et al., 1997).

1.4.1 CTL escape

We have known of viral CTL escape for 20 years. One of the first papers to demonstrate this *in vivo* used a mouse model of lymphocytic choriomeningitis virus (LCMV) where they found selection of virus mutants that were resistant to CTL recognition (Pircher et al., 1990).

CTL escape may occur in three different ways: escape from antigen processing; escape from MHC binding; and escape from TCR binding. During the antigen processing process, a change in one or multiple amino acids may prevent proteasomal cleavage, transport by TAP or ERAAP trimming (Eisenlohr et al., 1992). Peptide loading onto MHC molecules may be eliminated if amino acid changes alter the peptide anchor residues. Alternatively, polymorphisms within the epitope may abrogate TCR recognition or result in lower binding affinity, although the latter could still lead to some binding and induce T cell activation.

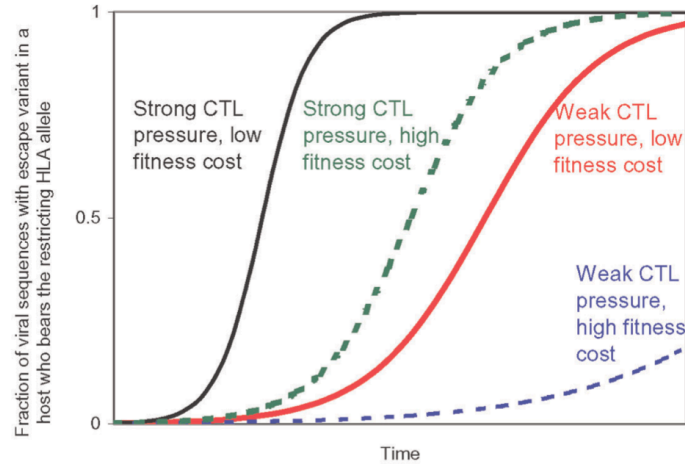
CTL escape happens in a similar way to antiretroviral resistance except the selective pressure comes from the CTLs. As previously discussed, the dominance of a variant within the viral quasispecies is dependent on its capacity to replicate in a specific environment. In a human host that has a HLA able to present a particular viral epitope, an escape mutation has a selective advantage because it avoids CTL recognition. This is

counterbalanced by a growth disadvantage in the form of a possible reduction in replication capacity. The extent of these growth advantages/disadvantages results in the circulating epitope variant in the viral quasispecies (Peyerl et al., 2004) (Figure 1.9A). Escape variants do not generally emerge as the dominant variant if there is no CTL pressure, if the impaired replication capacity is greater than the CTL pressure, or if there is insufficient replication for mutation (due to antiretroviral therapy or other immunodominant CTL responses). If the epitope is no longer presented because the virus has been transmitted to a host with a discordant HLA allele, there is no advantage of avoiding CTL recognition. The rate of reversion to wildtype sequence is therefore proportional to the fitness cost of the mutation (Figure 1.9B). As transmitted CTL escape mutations revert at different rates in the absence of immune pressure in new hosts, it indicates that regions of the viral genome are under different structural and functional constraints (Kent et al., 2005).

1.4.2 HIV-specific CTL escape

Following HIV infection, CTL responses arise early but are insufficient to prevent progression to AIDS: HIV escapes from epitope-specific CTL recognition. Variation causing loss of CTL recognition was first noted in Gag epitopes from longitudinally sampled HIV-infected individuals, indicating that the virus had evaded immune surveillance (Phillips et al., 1991).

(A)



(B)

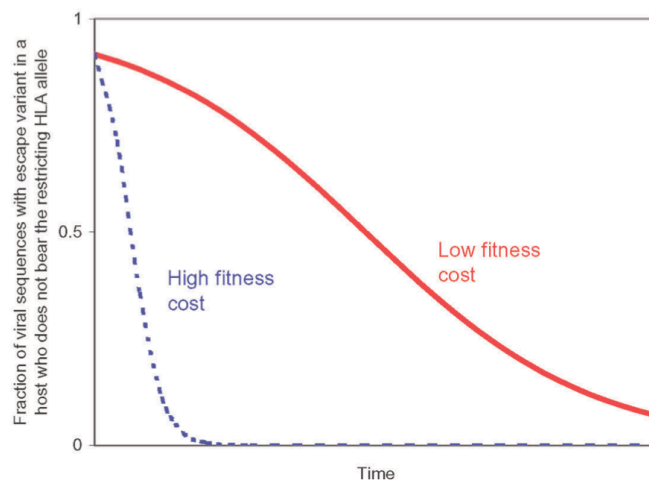


Figure 1.9 Models of CTL escape and reversion. (A) Model of CTL escape in terms of selection pressure and viral fitness in a host with the restricting HLA allele. (B) Model of reversion of a CTL escape variant in a host without the restricting HLA allele (Asquith et al., 2006).

In 1997, two papers published in *Nature Medicine* describe in detail escape from an immunodominant CTL response at different stages of disease. The first of these papers looked at primary infection with the immunodominant HLA-B*44-restricted epitope AENLWVTY (AY9, *env* gp160 31-39): at day 16 following the onset of symptoms, a

HIV-infected patient had a high frequency of CTLs against this epitope and no intra-epitope mutations; from day 30, mutations were observed including at the anchor position E32; and by day 136, all clones were fixed as either E32G or E32A and no longer recognisable by the patient's CTLs (Borrow et al., 1997). The second paper focused on late escape to the immunodominant HLA-B*27-restricted epitope KRWIIMGLNK (KK10, *gag* 263-272, p24 131-140). Two HLA-B*27 positive patients had CTL responses to this epitope for over 9 years; however, a fall in CD4 count and rise in viral load were observed concomitant with the R246K mutation found at the anchor position of this epitope (Goulder et al., 1997a). Both these papers demonstrated that a mutation at the anchor residue constitutes as CTL escape and may happen at any point of infection.

The first study of reversion in humans centred on the HLA-B*57/5801-restricted epitope TSTLQEQUIAW (TW10, *gag* 240-249, p24 108-117). The epitope TW10 is under positive selection from HLA-B*57/5801-restricted CTLs, and it was hypothesised that the T242N mutation reverts in the absence of HLA-B*57/5801 pressure. In a cross-sectional study of 311 chronically-infected individuals, the T242N mutation was seen in 0/187 HLA-B*57/5801 negative subjects. In the group's examination of reversion in the absence of immune pressure, two mother-to-child transmission pairs where the child was HLA-B*57/5801 negative were analysed. Longitudinal data from one mother-to-child transmission pair showed that the HLA-B*57 positive mother carried the T242N variant postpartum for 8 years; her HLA-B*57/5801 negative child retained the escape mutation 2 months postpartum, but by 5 months, 12/21 clones contained the escape variant and after 8 years only 3/17 contained the variant. The escape variant's

dominance had diminished, with reversion occurring as early as 5 months following transmission. In one longitudinally-studied, acutely-infected HLA-B*57/5801 negative subject, reversion was also seen, although at a much slower rate (Table 1.1) (Leslie et al., 2004).

Time point	Genetic material	T	S	T	L	Q	E	Q	I	G	W	Number of clones
Day 0	RNA	-	-	N	-	-	-	-	-	-	-	6/7
Pre-sero	RNA	-	-	N	-	-	-	-	M	-	-	1/7
Day 43	DNA	-	-	N	-	-	-	-	-	-	-	8/8
Day 818	RNA	-	-	N	-	-	-	-	-	-	-	2/7
	RNA	-	-	-	-	-	-	-	-	-	-	5/7
Day 983	RNA	-	-	N	-	-	-	-	-	-	-	3/14
	RNA	-	-	-	-	-	-	-	-	-	-	11/14
Day 1541	RNA	-	-	-	-	-	-	-	-	-	-	4/4

Table 1.1 An example of CTL reversion. Longitudinal samples of a HIV subtype B-infected individual (HLA-B*07 and -B*44) were taken since seroconversion. Partial reversion of the TW10 epitope occurs at some point between day 43 and 818 (Leslie et al., 2004). Reprinted by permission from Macmillan Publishers Ltd.

1.5 Adoptive T cell therapy

Adoptive T cell therapy is the transference of antigen-specific immune cells from a donor to a recipient in order to generate immunity in the latter. In the 1950s, Peter Medawar demonstrated the ability of an individual to overcome the rejection normally encountered when tissues are transplanted from one to another: Medawar and his team had discovered ‘acquired immunological tolerance’ (Billingham et al., 1953, Billingham et al., 1954). Since that time, research has incorporated the adoptive transfer of leucocytes, T cell lines or clones to fight against cancer and infectious diseases.

1.5.1 Adoptive transfer following stem cell transplant

Individuals undergoing an allogeneic hematopoietic stem cell transplant must go through a conditioning process by which chemotherapy and radiation are used to destroy the existing bone marrow, cancerous cells, and to prevent the individual's immune system from rejecting the transplant. Immunosuppressants are given post-transplant to prevent graft-versus-host disease (GvHD). The upshot of this process is that the recipient is susceptible to reactivation of latent viruses, such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV), which can be life-threatening for these patients.

1.5.1.1 Transfer of CMV-specific T cells

Even with antiviral drug therapy, reactivation of latent CMV infection in transplant patients can cause morbidity and mortality (Meyers et al., 1986). In order to restore CMV immunity, a pilot study of adoptively transferred human CMV-specific CD8⁺ CTL clones isolated and propagated from the donor following bone marrow transplantation limited CMV reactivation (Riddell et al., 1992). These positive results in a few patients led to a phase I study where 14 patients received prophylactic infusions ranging from 10⁷ to 10⁹ clones following transplantation. CMV viraemia and disease were not detected and the clones persisted for at least 12 weeks. However, CTL activity did decline in those recipients that were unable to also develop a CMV-specific CD4⁺ T_H response (Walter et al., 1995).

As a therapeutic therapy, adoptive transfer of 10⁶ CMV-specific CD4⁺ T cell lines in transplant recipients as a treatment for CMV infection failing antiviral therapy was

undertaken in 7 patients. CMV viraemia decreased significantly, with 5 patients clearing the virus (Einsele et al., 2002).

1.5.1.2 Transfer of EBV-specific T cells

EBV-driven lymphoproliferative disease can develop after transplant, caused by latent EBV-infected B cells from the donor being activated in the recipient. Activation causes uncontrolled B-cell proliferation which may produce lymphoma (Hanto et al., 1981).

The first attempt of adoptive transfer for the treatment of EBV lymphoproliferative disease was using polyclonal lymphocytes. It was thought that there would be EBV-specific CTLs within this polyclonal population and although there was clinical remission in the surviving patients, there was also GvHD (Papadopoulos et al., 1994). It has been postulated that there is a high GvHD risk when unselected lymphocytes are transferred due to alloreactive donor CTLs, less risk with enriched CTLs and the least amount of risk with CTL clones.

In a later attempt, EBV-specific CTL lines were gene-marked to determine the longevity of the transferred cells. The combination of both CD4⁺ and CD8⁺ cells being infused enabled CTL precursors to persist for 18 months and re-established an EBV immune response (Heslop et al., 1996). This methodology is able to produce an EBV response as a prophylactic and therapeutic to EBV-driven lymphoma (Rooney et al., 1998).

1.5.2 Adoptive transfer of HIV-1 specific T cells

With positive results achieved with the persistent viral infections CMV and EBV, it follows that research should focus on using adoptive transfer for other persistent infections, such as HIV.

In 1993, a phase I study involved the adoptive transfer of CD8⁺ cell lines in patients with AIDS or AIDS-related complex. Autologous CD8⁺ cells were enriched and *ex vivo* propagated. Individuals received 5 doses of 10⁸ to 10¹⁰ cells over 4 months as well as IL-2 with the final infusion. To track the CD8⁺ cells, a proportion were ¹¹¹In-labelled enabling total body imaging. The labelled-transferred cells initially accumulated in the lungs with <10% in circulation; after 24 h, the labelled cells were found mostly in the liver, spleen and bone marrow. CD4⁺ and CD8⁺ counts did fluctuate following infusion, however, clinically patients improved or remained stable (Whiteside et al., 1993, Ho et al., 1993, Torpey et al., 1993).

In 1996, persistence of autologous CD8⁺ Gag-specific CTL clones was studied in HIV-seropositive patients by modifying the cells to express an inducible suicide gene. The patients were to receive 4 doses of infusions, however, 5/6 patients cleared the cells following the third and fourth infusion, implying that an immune response was generated against these cells in these immunocompromised individuals (although this was against the modified part of the clones used to monitor persistence) (Riddell et al., 1996). Alternatively, the disappearance may be due to migration to specific organs, or due to lack of CD4⁺ T_H cells.

Knowing how antigen-specific CD4⁺ T_H cells may be important for transferred CTL persistence, but also that CD4⁺ cells are the target cells for HIV infection, expanding CD4⁺ cells posed a risk to expanding infected cells. It was found that there was no need to deplete T cell lines of CD4⁺ cells as following expansion, HIV could not be cultured (Lieberman et al., 1995). A pilot study involved 6 HIV-1 seropositive patients receiving an infusion of 1 billion *ex vivo*-expanded CTLs consisting of CD4⁺ and CD8⁺ cells (4.2% and 85%, respectively). None of the patients clinically progressed. In fact, within the first 2 weeks CD4 counts increased for all individuals and viraemia decreased for 5/6, although not greater than 3-fold (Lieberman et al., 1997). The problem with this was the lack of specificity of the CTLs infused; Env, Gag and Nef peptides were used in the expansion protocol, plus there would have been nonspecific CTLs in the infusion. No sequencing of circulating HIV was performed post-infusion, it was presumed that the breadth of the polyclonal cell line would reduce the chance of viral escape.

1.6 Eradication of HIV

Eradication of HIV can either be through curing all those already infected, or preventing spread of infection by use of a vaccine – with eventual eradication dependent on vaccine uptake.

1.6.1 A cure for HIV?

In order for HIV to infect a cell, not only is CD4 required, but also a coreceptor is needed for cell fusion. Macrophage-tropic HIV strains (R5) infect cells with the chemokine receptor CCR5 (Deng et al., 1996, Dragic et al., 1996), expressed on T_H1

cells and macrophages; T-cell tropic HIV strains (X4) infect cells with the chemokine receptor CXCR4 (Feng et al., 1996), expressed on naïve T cells. It has been documented that having a rare inherited homozygous defect in the gene that encodes CCR5, a 32 base pair deletion ($\Delta 32$), results in a dysfunctional protein that is no longer presented and the individual is highly resistant to R5 virus infection (Liu et al., 1996, Samson et al., 1996). In addition, individuals that are heterozygous for this mutation result in delayed progression to AIDS (Eugen-Olsen et al., 1997). In 2007, the first CCR5 antagonist (maraviroc), a HIV fusion inhibitor, was approved by the FDA.

Long-term control of HIV-1 has been achieved following successful transplantation of allogeneic hematopoietic stem cells from an HLA-matched donor with the $\Delta 32/\Delta 32$ CCR5 allele into an HIV-1 positive individual with acute myeloid leukemia. By 2009, 27 months after transplant, the patient's viral load had remained below 50 copies/ml despite discontinued use of antiretroviral therapy. This case is the first to show that eradicating HIV from primary target cells may sufficiently prevent viral rebound (Hutter et al., 2009a, Hutter et al., 2009b). This one case restores hope that scientific advances may one day eradicate HIV infection. The feasibility of using this particular method is tainted by difficulties in finding a donor that is not only homozygous for this mutation, but also HLA-matched; the $\Delta 32/\Delta 32$ CCR5 allele is prevalent in only ~1% Caucasians (Lucotte, 2002). In addition, life-threatening adverse events from transplantation pose further barriers.

1.6.2 HIV vaccine

A vaccine against HIV would ideally elicit an immune response capable of preventing infection; however, if not achievable, a vaccine that significantly lowered the viral load set point and therefore delayed disease would be desirable. Viral load is a strong predictor of progression to AIDS (Mellors et al., 1995, Mellors et al., 1996). As a viral load set point is achieved early in infection, it seems that those immune responses early in infection determine subsequent progression.

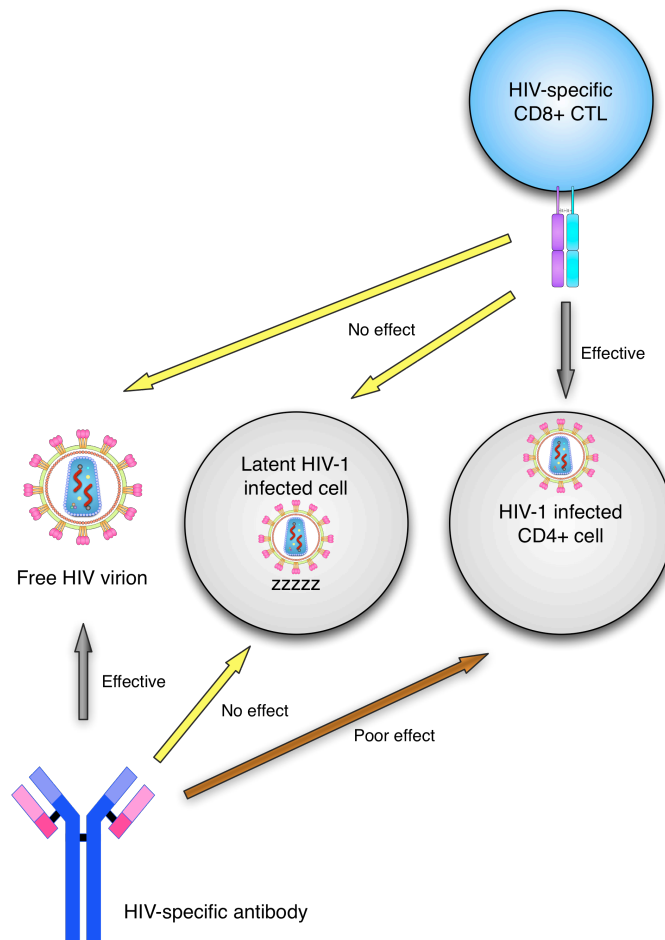


Figure 1.10 The immune response's effectiveness against HIV-1 infection.

HIV vaccine design is incorporating both the induction of humoral and cell-mediated responses (Figure 1.10). An HIV vaccine would ideally prevent virion infectivity by generating a nAb response that coats free virions and therefore blocks binding and entry into host cells. To date, nAb responses against circulating HIV strains have been ineffective. As previously discussed, the appearance of the HIV-specific CTL response and the decline in peak viraemia has led to the trialling of T cell vaccines that may lower the viral load set point. However, in order for this type of vaccine to be efficacious, we must determine what constitutes an effective HIV-specific CTL response and how the virus has evolved to counteract this in terms of CTL escape.

Much research has looked at defining correlates of immune protection and progression to AIDS. We currently assess the CTL response in terms of magnitude, although the breadth of the response may be just as important. Correlates of immune protection in nonhuman primates, such as SIV in the sooty mangabey where infection is non-pathogenic, are valuable although may not necessarily predict protection in humans. Studies on HIV progressors, HIV controllers and high-risk seronegative individuals may provide insights into what constitutes as an effective CTL response.

HIV controllers may be termed long-term nonprogressors (LTNPs; maintain CD4 count within the 'normal' range with no signs of disease progression over a long period of HIV infection) or elite controllers (maintain viral loads below the limit of detection, 50 copies/ml, without antiretroviral therapy) (Buchbinder et al., 1994). In a group of LTNPs, strong CTL and weak nAb responses were detected in those with the lowest viral loads and stable nonprogressive infection (Harrer et al., 1996, Rinaldo et al.,

1995). High-risk seronegative individuals provide evidence that exposure to HIV does not always equate to infection. A study of uninfected but HIV-exposed Gambian women found HIV-specific CTL that were able to kill virus-infected cells (Rowland-Jones et al., 1995).

The first HIV vaccine to enter clinical studies was VaxSyn in 1987, and was based on recombinant HIV gp160 protein with the aim of eliciting nAb. Since that time, numerous vaccines have entered clinical trials. Two of the most recent large-scale clinical studies were the Merck STEP trial and the RV144 Thai trial. The STEP trial involved the vaccination of subjects with 3 adenovirus type 5 (Ad5) vectors containing either *gag*, *pol* or *nef* genes with the aim of generating a cell-mediated immune response. The results from STEP showed that there was no protection from HIV infection or lowering of viral load set point – this was despite 77% of the HIV positive vaccinees generating HIV-specific CTL response before infection. Factors associated with the greatest risk of HIV infection were uncircumcised male subjects receiving the vaccine with pre-existing immunity to Ad5 (Buchbinder et al., 2008, McElrath et al., 2008).

The RV144 Thai trial tested the prime-boost combination of the ALVAC priming vaccine (a recombinant canarypox vector encoding subtype E gp120 and subtype B gp41 and gag/pro) and the AIDSVAX B/E vaccine (a recombinant subtype B and E gp120 subunit vaccine used as the boost). The trial investigators claimed that the regimen had a ‘modest’ protective effect: the vaccine lowered the rate of HIV infection by 31.2% compared to placebo ($p = 0.04$) in the modified intention-to-treat population

(Figure 1.11); by 26.2% ($p = 0.16$) in the per protocol population; and by 26.4% ($p = 0.08$) in the intention-to-treat population. The prime-boost vaccine had no significant effect on the viral load set point (Rerks-Ngarm et al., 2009). Current studies are investigating the correlates of protection from this trial so that armed with this data we may attempt future studies that may produce better than ‘modest’ results, with the ultimate aim of approving an efficacious HIV vaccine to be administered to those most at risk.

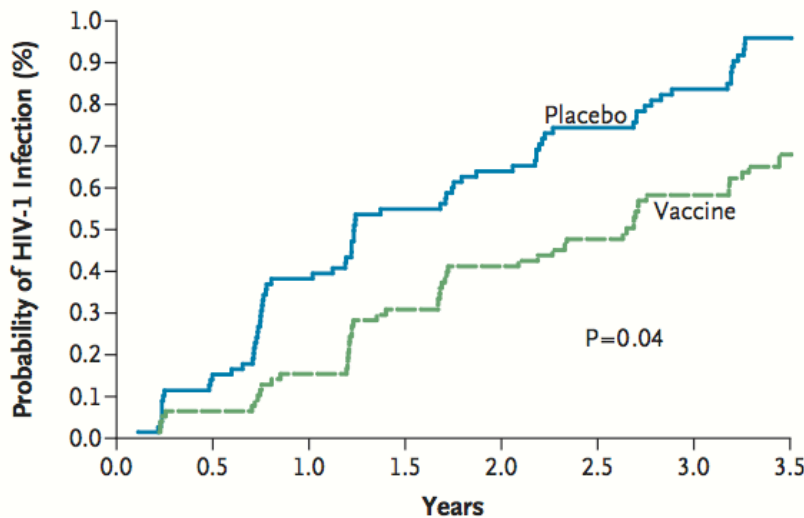


Figure 1.11 Hope of a future HIV vaccine. Kaplan-Meier cumulative rate of infection according to the modified intention-to-treat analysis from the RV144 Thai phase III study (Rerks-Ngarm et al., 2009). Reproduced with permission from Massachusetts Medical Society.

1.7 Rationale

This current work is a study of cell-mediated responses of past, present and future: past in terms of looking at natural CTL escape seen *in vivo* and using this for HIV vaccine design; present in terms of genetically modifying the immune response and predicting

potential therapy-induced escape *in vitro*; and future in terms of producing a specific, artificial immune response with any CD8⁺ cell capable of cytotoxic activity.

Chapter 3 uses longitudinal clinical trial data to examine an HIV epitope that is restricted by an HLA allele associated with slow progression to AIDS. By determining the CTL response to this epitope and its escape variants, we may further expand our knowledge in order to generate an efficacious T cell vaccine.

Chapter 4 focuses on adoptive transfer therapy that has had hugely positive results in studies of cancer, EBV and CMV. Based on *in vitro* studies, genetically modified HIV-1-specific CTLs have entered clinical studies. If as successful as antiretroviral therapy, can we pre-empt clinical results by predicting possible resistance in a similar manner to antiretroviral resistance?

Finally, chapter 5 uses a novel approach of combining specific CTL recognition with the ability to redirect cells that have the potential to lyse HIV-infected cells. This hypothesis-driven research is highly unpredictable but may be a form of adoptive transfer that circumvents the need for HIV-specific cell infusion.

HIV-1 is adapting to both HLA-restricted immune responses and antiretroviral therapy. It is important to understand the HIV response to both natural and therapy-induced CTL pressure if we are to ever eradicate this virus that has led to the deaths of millions.

CHAPTER 2

Materials and Methods

2.1 Patients

The study included 118 HIV-1 subtype B-infected subjects from Europe, with longitudinal samples obtained from St. Mary's Hospital (London, UK) and through the SPARTAC (Short Pulse AntiRetroviral Therapy At HIV seroConversion) trial. Baseline samples were taken around the time of seroconversion; they will be referred to as 'early' infection samples as they are within 6 months of transmission. Of the 118 patients, 12 expressed HLA-B*5101 (Table 2.1) and the remaining 106 patients were classed as HLA-B*5101-negative subjects.

Patient	HLA				Baseline		Year 1		HAART
	A1	A2	B1	B2	Viral load	CD4 count	Viral load	CD4 count	
K117	0201	2301	1537	5101	46,786	772	3,024	868	Y
K131	0101	1101	5101	5501	500,000	210	188	450	N
K151	0201	0201	4101	5101	40,338	486	350,074	349	Y
K152	2402	2601	4501	5101	103,838	537	-	-	Y
K39	0201	6801	2705	5101	40,200	659	11,894	611	Y
K59	0205	2402	4901	5101	11,327	657	-	-	Y
K61	3101	3201	4001	5101	1,690	580	10,192	720	Y
K9	0201	1101	0702	5101	130,004	540	24,742	710	N
M059375	0201	2402	4402	5101	196,332	250	220,107	310	Y
M084439	0301	2402	4402	5101	40,588	600	40,358	500	Y
M091699	0201	1101	5101	1501	2,077,438	420	139,810	270	Y
M094491	2402	3101	5101	5101	2,495,530	340	500,000	170	Y

Table 2.1 HIV subtype B-infected HLA-B*51 positive subjects. HLA, viral load (copies/ml), CD4 count (cells/ μ l) and whether on HAART following baseline are shown; - = no sample.

HIV-1 non-subtype B-infected subjects from St. Mary's Hospital and through the SPARTAC trial have also been sequenced but are not included in the subtype B analysis. If discussed, the subtype will be clearly stated.

2.2 PBMCs

Venous blood was collected from HLA-typed HIV-naïve or HIV-infected individuals. Peripheral blood was diluted with PBS and PBMCs isolated by centrifugation on a Lymphoprep (Nycomed) gradient. PBMCs were washed 3 times with PBS before being resuspended in R10 (RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM glutamine and 50 U/ml penicillin-streptomycin).

Cryopreserved PBMC samples were thawed in the presence of 27.3 Kunitz units RNase-free DNase I (Qiagen), spun down and resuspended in 5 ml R10. Cells were rested overnight before use in any assay.

2.2.1 CD8⁺ cell isolation

Highly purified CD8⁺ cells were isolated using CD8 microbeads according to the manufacturers instructions.

With the Dynal CD8 negative isolation kit (Invitrogen), PBMCs were resuspended in isolation buffer 1 (PBS containing 2% FCS, 2 mM EDTA) at 1×10^8 cells/ml. FCS and the kit's antibody mix were added at 20 μ l/ 1×10^7 cells, mixed and incubated at 4°C for 20 min. Cells were washed and resuspended in 800 μ l isolation buffer 1. Depletion

Dynabeads were added at 200 $\mu\text{l}/1 \times 10^7$ cells and incubated at 4°C for 15 min. 1 ml isolation buffer was added and the tube placed in a magnet for 2 min. The negatively-isolated CD8⁺ cells were poured into a fresh tube, spun and resuspended in R10.

With the EasySep Human CD8⁺ T cell enrichment kit (Stem cell technologies), PBMCs were resuspended in isolation buffer 2 (PBS containing 2% FCS) at 5×10^7 cells/ml. The kit's antibody cocktail was added at 50 $\mu\text{l}/5 \times 10^7$ cells, mixed and incubated at room temperature for 10 min. Magnetic nanoparticles were added at 50 $\mu\text{l}/5 \times 10^7$ cells, mixed and incubated at room temperature for 10 min. The cell suspension was made to a total volume of 2.5 ml with isolation buffer 2 and placed in a magnet for 5 min. The negatively-selected, enriched CD8⁺ cells were poured into a fresh tube, spun and resuspended in R10.

2.3 Cell lines

All cell lines were maintained in R10. The HIV-1 permissive cells 174xCEM.T0, 174xCEM.T1 and 174xCEM.T2 express CD4 and HLA-A*02 (Figure 2.1); T2 cells are TAP deficient.

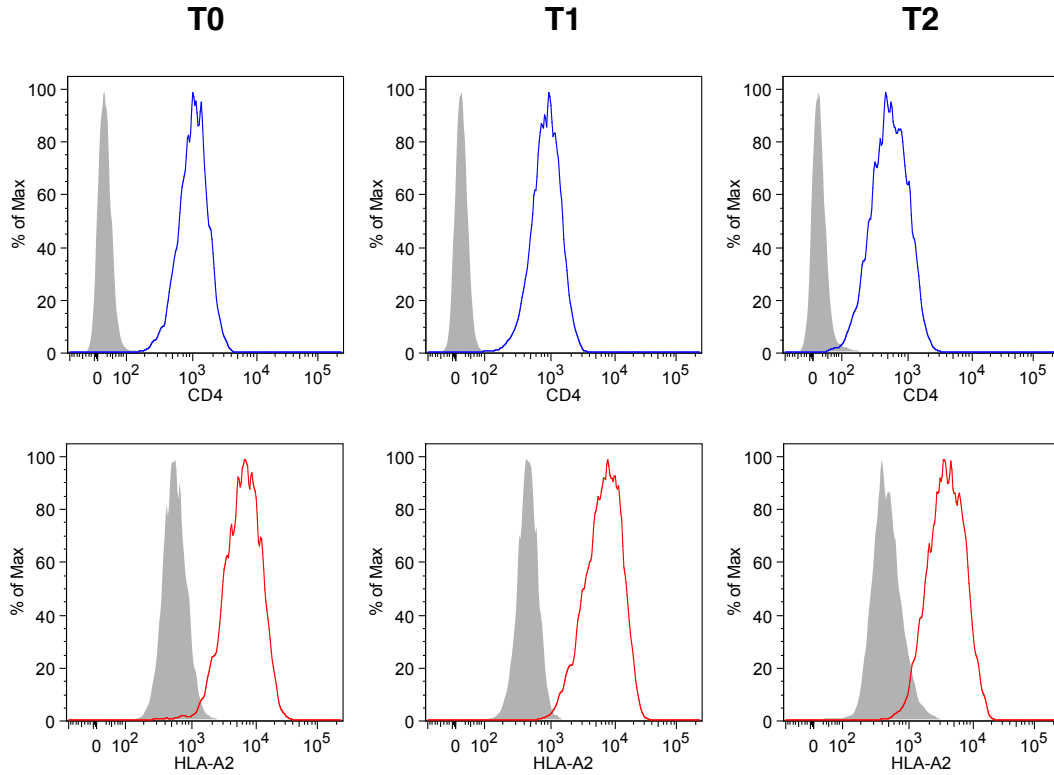


Figure 2.1 Phenotype of T0, T1 and T2 cells. Gray histogram bars show unstained control cells.

2.4 TCR-transduced $CD8^+$ cells

Adaptimmune generated all TCR-transduced cells according to the method outlined by Varela-Rohena et al., with details of phage display given in Appendix D (Varela-Rohena et al., 2008).

2.5 ImmTAC reagents

Immunocore generated all ImmTAC reagents. TCRs were produced as detailed in Appendix D and C-terminally fused with an anti-CD3 single chain variable fragment (scFv) to form an ImmTAC reagent. A list of all ImmTAC reagents with epitope targets

is given in Table 2.2; gp100 and NY-ESO-1 ImmTACs were used as controls. ImmTACs targeting SL9 differ by the CDR mutated and type of modification.

ImmTAC	Target	CDR mutations
a11b6	SLYNTVATL	$3\alpha + 2\beta$
a12b40	SLYNTVATL	$1\alpha + 2\beta + 3\beta$
a15b40	SLYNTVATL	$1\alpha + 2\alpha + 2\beta + 3\beta$
a15b26	SLYNTVATL	$1\alpha + 2\alpha + 2\beta$
NY-ESO-1	SLLMWITQV	n/a
gp100	YLEPGPVTV	n/a

Table 2.2 SL9-specific and control ImmTACs assayed.

2.6 Viral RNA extraction and cDNA synthesis

Viral RNA was extracted using the QIAamp Viral RNA Mini kit (Qiagen) as outlined in 2.6.1 or 2.6.2.

cDNA was synthesised from 10 μ l viral RNA using 400 ng random decamers (Abgene) and 500 μ M dNTPs, incubated at 65°C for 5 min then placed on ice for 2 min. To this mixture, the following made up a total reaction volume of 20 μ l: 1X First-Strand buffer; 5 mM DTT; 40 U RNaseOUT Recombinant Ribonuclease Inhibitor; and 200 U SuperScript III Reverse Transcriptase (Invitrogen). The reaction was run on a thermocycler using the following program: 25°C for 5 min; 50°C for 1 h; 70°C for 15 min.

2.6.1 From cell culture supernatants

560 µl Buffer AVL-carrier RNA was added to 140 µl cell culture supernatant, vortexed, and incubated at room temperature for 10 min. 560 µl 100% ethanol was added and vortexed. 630µl of this solution was added to a QIAfilter column and centrifuged at 8,000 rpm for 1 min; the flow-through was discarded. The remaining 630 µl solution was added to the column and centrifuged at 8,000 rpm for 1 min; the column placed into a clean collection tube. 500 µl Buffer AW1 was added and centrifuged at 8,000 rpm for 1 min; the column placed into a clean collection tube. 500 µl Buffer AW2 was added and centrifuged at 14,000 rpm for 3 min; the column placed into a clean collection tube. The column was centrifuged at 14,000 rpm for 1 min; the column placed into an Eppendorf. The viral RNA was eluted in 60 µl Buffer AVE and centrifuged at 8,000 rpm for 1 min.

2.6.2 From patient samples

1 ml of plasma sample was spun at 4°C for 1 h at 21,885 g. 860 µl of the fluid was discarded and the viral pellet resuspended. The protocol described in 2.6.1 was continued to isolate the viral RNA.

2.7 Polymerase Chain Reaction

A list of all the primers used is given in Table 2.3. Annealing temperatures in each polymerase chain reaction (PCR) were optimised dependent on the primer pairs used.

	Primer	Sequence	HXB2 Location
5' LTR	LTR_O_S	5'-GRAACCCACTGCTTAASSCTCAA-3'	506 → 528
	LTR_O_AS	5'-TGTTCTGGGCGCCACTGCTAGAGA-3'	626 ← 648
<i>Gag</i>	2F	5'-CCCGAACAGGGACTTGAAAGC-3'	641 → 661
	2R	5'-TCTGCAGCTTCCTCATTGATGGTC-3'	1398 ← 1421
	GagC5OP	5'-CTCTAGCAGTGGCGCCCGAA-3'	627 → 646
	GagC3OP	5'-TCCTTTCCACATTTCCAACAGCC-3'	2023 ← 2045
	GagC5IP	5'-ACTCGGCTTGCTGAAGTGC-3'	696 → 714
	GagC3IP	5'-CAATTTCTGGCTATGTGCCC-3'	1984 ← 2003
<i>Pol</i>	6F	5'-ACTCCATCCTGATAAATGGACAG-3'	3248 → 3270
	8R	5'-CACTAGGCAAAGGTGGCTTTATC-3'	5514 ← 5536
	7F	5'-AATTAGGAAAAGCAGGATATGTTACTG-3'	3901 → 3927
	7R	5'-AGTTTGTATGTCTGTTGCTATTATGTCTAC-3'	4830 ← 4859
BMV genome	SG-PERT_F	5'-GGTCTCTTTTAGAGATTTACAGTG-3'	n/a
	SG-PERT_R	5'-CGTGGTTGACACGCAGACCTCTTAC-3'	n/a

Table 2.3 List of primers.

2.7.1 Amplification of integrase from patient samples

A cDNA fragment encoding part of the HIV-1 *pol* gene was amplified using a nested PCR protocol. A reaction volume of 50 µl was comprised of 2 µl sample DNA, 0.4 µM of each primer, 200 µM dNTPs, 1.5 mM MgCl₂, 1 U of Platinum *Taq* DNA polymerase and 1X buffer (Invitrogen). Nest I PCR amplification, using primers 7F and 8R or primers 6F and 7R, was carried out using the following program: one cycle of 95°C for 1 min; 40 cycles of 94°C for 15 s, 53°C for 30 s, and 72°C for 3 min; one cycle of 72°C for 7 min. Nest II PCR amplification, using primers 7F and 7R, was carried out using the following program: one cycle of 95°C for 1 min; 40 cycles of 94°C for 15 s, 53°C for 30 s, and 72°C for 1.5 min; one cycle of 72°C for 7 min.

2.7.2 Amplification of Gag p17 from cell culture samples

A cDNA fragment encoding part of the HIV-1 *gag* gene was amplified using either *Taq* or *PfuUltra* DNA polymerase.

2.7.2.1 Taq DNA polymerase PCR

A reaction volume of 50 µl was comprised of 2 µl sample DNA, 0.4 µM primer 2F, 0.4 µM primer 2R, 200 µM dNTPs, 1.5 mM MgCl₂, 1 U of Platinum *Taq* polymerase and 1X buffer (Invitrogen). One round of PCR amplification was carried out using the following program: one cycle of 95°C for 1 min; 40 cycles of 94°C for 15 s, 64°C for 30 s, and 72°C for 1.5 min; one cycle of 72°C for 7 min.

2.7.2.2 PfuUltra DNA polymerase PCR

A reaction volume of 50 µl was comprised of 2 µl sample DNA, 0.4 µM primer 2F, 0.4 µM primer 2R, 200 µM dNTPs, 1 mM MgSO₄, 2.5 U of *PfuUltra* polymerase and 1X buffer (Agilent Technologies). One round of PCR amplification was carried out using the following program: one cycle of 95°C for 2 min; 30 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 1 min; one cycle of 72°C for 10 min.

2.7.3 PCR purification

PCR products were resolved by electrophoresis at 80 V for 30 min on an ethidium bromide 1% agarose gel. The band was cut out of the gel over UV light and purified using the QIAquick gel extraction kit (Qiagen).

2.8 TOPO TA cloning

PCR products were cloned using the TOPO TA Cloning kit for sequencing (Invitrogen). *Taq* polymerase-amplified purified PCR products were cloned directly; however, in order to clone a *PfuUltra* polymerase-amplified purified PCR product, an additional step of attaching an A-tail was performed. The A-tailing reaction volume of 10 µl was comprised of 6.5 µl purified *PfuUltra* polymerase-amplified PCR fragment, 0.2 mM dATP (Promega), 5 U *Taq* polymerase and 1X *Taq* polymerase-specific buffer including MgCl₂ (Roche). This was incubated at 72°C for 15 min, with cloning started immediately afterwards.

4 µl *Taq* polymerase-amplified PCR product or A-tailed *PfuUltra* polymerase-amplified PCR product was added to 10 ng pCR4-TOPO and salt solution (200 mM NaCl, 10 mM MgCl₂). These were mixed and incubated at room temperature for 15 min. This ligation mixture was added to One Shot TOP10 chemically competent *E. coli* on ice and left for 10 min. The cells were heat-shocked at 42°C for 30 s and immediately put back on ice. 250 µl SOC medium was added and cells shaken for 1 h at 37°C, 300 rpm. 150 µl of this transformation was spread on LB agar plates (100 µg/ml ampicillin) and incubated at 37°C overnight.

Either the Montage 96-well plasmid preparation kit (Millipore) or the QIAprep Spin Miniprep kit (Qiagen) was used to isolate plasmid DNA.

2.8.1 Montage 96-well plasmid preparation kit

1 ml LB broth (50 µg/ml kanamycin) was added to each well of a 96-well deep well culture block; a single colony was picked off the LB agar plate and added to each well. The plate was incubated overnight at 37°C, 220 rpm. Plates were centrifuged for 10 min at 2,500 rpm at room temperature; supernatant discarded. 100 µl Solution 1 (cell resuspension buffer plus RNase A) was added and vortexed. 100 µl Solution 2 (cell lysis buffer) was added, vortexed, and left for 5 min. 100 µl Solution 3 (neutralisation buffer) was added and vortexed. The spin plate consisting of a clearing plate, plasmid plate, and collection plate was assembled and 200 µl of neutralised product transferred to the clearing plate. The plate was centrifuged at 2,500 rpm for 40 min; waste was discarded from the collection plate and clearing plate removed. 200 µl Solution 4 (wash buffer) was added to the plasmid plate and centrifuged at 2,500 rpm for 10 min; waste was discarded from the clearing plate. The plasmid was eluted in 50 µl ddH₂O by incubating at room temperature for 30 min. A fresh 96-well plate was placed under the plasmid plate and centrifuged at 2,500 for 10 min.

2.8.2 QIAprep Spin Miniprep kit

3 ml LB broth (100 µg/ml ampicillin) was added to a 14 ml round-bottom tube and a single colony picked off the LB agar plate and added. The tube was incubated overnight at 37°C, 220 rpm. Bacterial cells were harvested by centrifugation for 10 min at 4,000 rpm at 4°C; supernatant discarded. 250 µl Buffer P1 (cell resuspension buffer plus RNase A) was added, vortexed, then transferred to a microcentrifuge tube. 250 µl Buffer P2 (cell lysis buffer) was added, inverted 6 times, and left for 5 min. 350 µl Buffer P3 (neutralisation buffer) was added, inverted 6 times, then centrifuged for 10

min at 13,000 rpm. The supernatant was applied to a QIAprep spin column, and spun for 1 min at 13,000 rpm; flow-through discarded. 500 µl Buffer PB was added and spun for 1 min at 13,000 rpm; flow-through discarded. 750 µl Buffer PE was added and spun for 1 min at 13,000 rpm; flow-through discarded. Spin columns were spun for a further 1 min at 13,000 rpm before the plasmid was eluted in 50 µl ddH₂O (preheated to 70°C).

2.9 Sequencing

Direct PCR population sequencing was performed on all samples. The sequencing primer used was dependent on the PCR product; primers are listed in Table 2.3. Sequencing reaction volumes of 10 µl were used which contained 3 µl of purified PCR product, 3.3 µM primer, 1.5 µl 5X BigDye dilution buffer and 0.7 µl ABI Big Dye (Applied Biosystems). Samples were run on a thermocycler using the following program: one cycle of 96°C for 30 s; 30 cycles of 96°C for 20 s, 50°C for 10 s, and 60°C for 4 min.

2.9.1 Clean-up

Sequencing products were cleaned-up using an ethanol/sodium acetate precipitation protocol. To each well, 2 µl sodium acetate (3 M stock), 50 µl 100% ethanol and 10 µl ddH₂O were added, incubated at room temperature for 15 min then centrifuged for 80 min at 4,000 rpm at 4°C. Plates were inverted, spun briefly, and 200 µl 70% ethanol added to each well then centrifuged for 10 min at 4,000 rpm at 4°C. Again, plates were inverted, spun briefly, and 200 µl 70% ethanol added to each well then centrifuged for 10 min at 4,000 rpm at 4°C. Plates were inverted, spun briefly and incubated at 95°C for

1 min to remove excess ethanol. Plates were analysed by the University of Oxford Zoology sequencing facility; sequences were edited using Sequencher Version 4.8 (Gene Codes) and aligned manually using Se-Al software.

2.10 Ultradeep pyrosequencing

Cell culture viral RNA was prepared as described in 2.6, and the following RT-PCR reaction set up in a 50 µl volume using the AffinityScript One-Step RT-PCR Kit (Agilent Technologies): 1X Master mix (containing Herculase II fusion DNA polymerase), 0.4 µM GagC5OP primer, 0.4 µM GagC3OP primer, 1 µl AffinityScript RT/RNase block and 10 µl viral RNA. Samples were run on a thermocycler using the following program: one cycle of 50°C for 10 min; one cycle of 92°C for 1 min; 30 cycles of 92°C for 20 s, 55°C for 20 s, and 72°C for 1 min; one cycle of 72°C for 5 min. A second round of PCR in a reaction volume of 50 µl was performed in duplicate comprising of 250 µM dNTPs, 0.2 µM GagC5IP primer, 0.2 µM GagC3IP primer, 1 µl *PfuUltraII* DNA polymerase, 1X *PfuII* buffer and 5 µl first round PCR product. Samples were run on a thermocycler using the following program: one cycle of 95°C for 1 min; 30 cycles of 95°C for 20 s, 54°C for 20 s, and 72°C for 30 s; one cycle of 72°C for 3 min.

PCR products were resolved by electrophoresis at 80 V for 30 min on a crystal violet 1% agarose gel. The band was cut out of the gel over white light and duplicate products pooled and purified using the QIAquick gel extraction kit (Qiagen). Purified PCR

products were quantified in terms of concentration and purity using the NanoDrop 1000 spectrophotometer (Thermo Scientific).

2.10.1 Rapid Library preparation

To shear the DNA into a 500 bp fragment library, purified PCR products (500 ng in 120 µl TE buffer) were fragmented using the S220 sonicator (Covaris). DNA fragments were purified using the MinElute PCR purification kit (Qiagen) and eluted in 16 µl TE buffer. To repair the DNA ends, the Rapid Library Preparation kit part 2 (454 sequencing, Roche) was used involving the addition of 1X RL buffer, 1 mM ATP, 0.4 mM dNTP mix, 1 U T4 polymerase, 1 µl T4 polynucleotide kinase and 5U *Taq* polymerase in a total volume of 25 µl. Samples were run on a thermocycler using the following program: one cycle of 25°C for 20 min; one cycle of 72°C for 20 min.

A different adaptor (1 µl) was added to each sample using the Rapid Library MID Adaptors kit (454 sequencing, Roche). 1 µl RL ligase was added to each tube, vortexed and incubated at 25°C for 10 min. To remove adaptor dimers and fragments <400 bp, calibrated Agencourt AMPure XP beads (Beckman Coulter) were used and DNA eluted in 50 µl TE buffer; libraries were quantitated using the TBS 380 Fluorometer (Turner Biosystems) and quality assessed using the Bioanalyzer (Agilent).

2.10.2 Pyrosequencing

PCR amplicons were pooled in equimolar concentrations and clonally amplified on capture beads in water-in-oil emulsion micro-reactors. Pyrosequencing was carried out

using the 454 Life Sciences platform (GS FLX Titanium, Roche) at the Wellcome Trust Centre for Human Genetics, University of Oxford.

2.11 Viral load quantitation

Extracted viral RNA from cell culture supernatants was quantitated by real-time RT-PCR by amplifying a region of the HIV long terminal repeat (LTR). The following RT-PCR reaction was set up in a 20 µl volume using the SuperScript III One-Step RT-PCR Kit (Invitrogen) on a LightCycler 480 Multiwell Plate (Roche): 1X reaction mix, 1 µM LTR_O_S primer, 1 µM LTR_O_AS primer, 1 µl LTR Taqman probe (AGTRGTGTGTGCCCCGTCTGTTG; 5'FAM, 3'BHQ1), 0.4 µl SuperScript III RT/Platinum *Taq* High Fidelity enzyme mix and 2 µl viral RNA. Plates were run on the LightCycler 480 (Roche) using the following program: one cycle of 50°C for 30 min; one cycle of 94°C for 2 min; 45 cycles of 94°C for 25 s, 55°C for 20 s, and 68°C for 30 s; one cycle of 94°C for 20 s; one cycle of 68°C for 5 min. HIV LTR standards were concurrently run and a calibration curve created to extrapolate the number of RNA templates per test. Viral RNA (copies/µl) in cell culture supernatants was calculated by multiplying the RNA templates in 1 µl by volume eluted in / volume extracted from.

2.12 Interferon-γ Enzyme-linked ImmunoSpot (IFNγ ELISpot) assay

The number of spots per well were quantified using the AID EliSpot automated plate reader (AID GmbH) and were multiplied to spot forming units (SFU)/10⁶ PBMCs depending on the number of PBMCs plated. Background (0 to 2 spots/well at 50,000

PBMC/well) was subtracted from all positive wells. A positive result required the following criteria: at least 3-fold greater than the background; a positive response to *Phaseolus vulgaris* agglutinin (PHA; Sigma); and tested wells had >50 SFU/10⁶ PBMCs.

Peptide	Sequence
LI9-WT	LPPVVAKEI
LI9-1	LPPIVAKEI
LI9-2	LPPIIAKEI
LI9-3	LPPVIAKEI
LI9-4	IPPVVAKEI
LI9-5	IPSVVAKEI
LI9-6	LPSIVAKEI
LI9 POOL	LI9-1 to LI9-6
TI8	TAFTIPSI
NI9	NANPDCKTI
SL9	SLYNTVATL
gp100	YLEPGPVTV

Table 2.4 Peptides used in IFN γ ELISpots

2.12.1 Patient ELISpots

96-well MultiScreen-IP plates (Millipore) were precoated with 0.5 μ g /100 μ l anti-IFN γ mAb (1-D1K; Mabtech) overnight at 4°C. Plates were washed five times with sterile PBS and blocked for 2 h with 200 μ l R10 at room temperature. Thawed patient PBMCs, prepared as in 2.2, were added to each well at a concentration of 45,000 to 70,000 cells per well with either the peptide pool (2 μ g/ml of each peptide), 1 μ g/ml LI9 optimal peptide or serial log dilutions of optimal peptide (Table 2.4); test wells were performed in duplicate when PBMCs were available. Negative control wells containing PBMCs

and R10 were run in duplicate; positive control wells containing PBMCs and 6.25 µg/ml PHA were run in duplicate. Plates were incubated for 16 h at 37°C, 5% CO₂.

Plates were washed seven times with PBS/0.05% Tween-20, then 0.05µg/100 µl of filtered anti-IFN-γ biotin (7-B6-1; Mabtech) added to each well and incubated for 2 h at room temperature. Plates were washed seven times with PBS/0.05% Tween-20, then 100 µl streptavidin-alkaline phosphatase added to each well (Mabtech) and incubated for 40 min at room temperature in the dark. Plates were washed three times with PBS/0.05% Tween-20 and twice with sterile PBS. The AP Conjugate substrate kit (Bio-Rad) was used to develop plates for 3 to 7 min, then plates were washed with dH₂O.

2.12.2 ELISpots with ImmTAC addition

The human IFNγ ELISPOT set (BD Biosciences) was used. 96-well MultiScreen-IP plates (Millipore) were precoated with 100 µl capture antibody (diluted according to Certificate of Analysis) overnight at 4°C. Plates were washed once with sterile R10 and blocked for 2 h with 200 µl R10 at room temperature. T2 cells were peptide pulsed with 1 nM SL9 or gp100 (Table 2.4) and incubated at 37°C, 5% CO₂ for 1 h. Cells were washed three times with R10 and plated at 50,000 cells per well. Serial log dilutions of a11b6, a12b40, a15b40 or a15b26 ImmTACs were added to the appropriate wells, followed by 8,000 CD8⁺ cells/well; test wells were performed in triplicate. Plates were incubated for 16 h at 37°C, 5% CO₂. Negative control wells containing peptide-pulsed T2 cells + CD8⁺ cells, or ImmTACs + CD8⁺ cells were run in duplicate; positive control wells containing peptide-pulsed T2 cells + ImmTACs + CD8⁺ cells + 5 µg/ml PHA were run in duplicate. Plates were incubated for 16 h at 37°C, 5% CO₂.

Plates were washed three times with PBS/0.05% Tween-20, then 100 μ l of detection antibody (diluted according to Certificate of Analysis) added to each well and incubated for 2 h at room temperature. Plates were washed three times with PBS/0.05% Tween-20, then 100 μ l streptavidin-horseradish peroxidase added to each well and incubated for 1 h at room temperature in the dark. Plates were washed three times with PBS/0.05% Tween-20 and twice with sterile PBS. The AEC substrate set (BD Biosciences) was used to develop plates for 3 to 5 min, then plates were washed with dH₂O.

2.13 Virus stocks

HIV-1_{LAI} stocks were generated by transfecting MT4 cells with pLAI.2 by electroporation (250 V, 950 μ F). Serial passaging in MT4 cells produced high titer HIV-1_{LAI} stocks; low passage virus stocks were frozen in aliquots at -80°C until use.

2.14 Viral titer determination

Viral titer (50% tissue culture infective doses (TCID₅₀)/ml) was determined by MAGI assay (Kimpton and Emerman, 1992). HeLa-CD4-LTR- β -gal cells (MAGI cells) were plated at 4×10^4 cells per well in a 24-well plate in DMEM culture media (10% FCS, 0.1 mg/ml G418 and 0.05 mg/ml hygromycin B), and incubated overnight at 37°C, 5% CO₂. The culture media was removed and 150 μ l of diluted virus, prepared in DMEM culture media, was added; 4 μ g/ml hexadimethrine bromide (Polybrene), a cationic polymer known to increase efficiency of infection, was added to each well. All dilutions

were tested in duplicate. Virus adsorbed for 2 h at 37°C, 5% CO₂, then 1 ml DMEM culture media added per well; plates were incubated for 48 h at 37°C, 5% CO₂. The culture medium was removed and 1 ml of fixing solution (1% formaldehyde, 0.2% glutaraldehyde) added for 5 min at room temperature. The fixing solution was removed, cells washed three times with PBS, and 250 µl staining solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM Mg₂Cl, 0.4 mg/ml X-gal) added and incubated at 37°C, 5% CO₂ for 50 min. Cells were washed twice with PBS before counting positive (blue) syncytia. Viral titer was calculated by the following:

$$\text{TCID}_{50}/\text{ml} = \text{number of positive syncytia} \times \text{virus dilution factor} \times 10$$

2.15 FATAL assay

The FATAL (Fluorometric assessment of T lymphocyte antigen specific lysis) assay was adapted from Sheehy et al. (Sheehy et al., 2001). HLA-A*02⁺ target cells were peptide pulsed with specified peptide at the stated concentration for 1 h at 37°C, 5% CO₂. The cells were washed three times with R10 and then stained with carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes) diluted to a final concentration of 0.67 µM for 10 min at room temperature. Cells were washed twice with R10 and plated at a predetermined cell concentration per well. Control, unpulsed HLA-A*02⁺ cells were stained with CellTracker Orange (Invitrogen) at a final concentration of 5 µM for 20 min at 37°C, washed once with R10, and plated at the same cell concentration per well as the CFSE-stained cells.

With the assays performed in chapter 4, TCR-transduced cells were stained with CellTracker Violet (Invitrogen) at a final concentration of 5 μ M for 20 min at 37°C, washed once with R10, and plated at an effector-to-target cell ratio of 1:1 resulting in each well containing: 2.5 x 10⁴ CFSE-stained peptide-pulsed T2 cells (Table 2.5); 2.5 x 10⁴ CellTracker Orange-stained unpulsed T2 cells; and 2.5 x 10⁴ CellTracker Violet-stained TCR-transduced cells. Plates were incubated overnight at 37°C, 5% CO₂. The next day, cells were stained with LIVE/DEAD fixable near-IR dead cell stain (Invitrogen) and then resuspended in PBS before being analysed on the LSRII (BD Biosciences).

		1	2	3	4	5	6	7	8	9
SLYNTVATL	SL9	S	L	Y	N	T	V	A	T	L
SL F NTVAT V	3F9V	-	-	F	-	-	-	-	-	V
SLYN A VATL	5A	-	-	-	-	A	-	-	-	-
SL F NTV A V L	3F8V	-	-	F	-	-	-	-	V	-
SLYNT I AL	6I	-	-	-	-	-	I	-	-	-
SL F NTVATL	3F	-	-	F	-	-	-	-	-	-
SL F NT I ATL	3F6I	-	-	F	-	-	I	-	-	-
SLYNTVAT I	9I	-	-	-	-	-	-	-	-	I
SL F NT I A V L	3F6I8V	-	-	F	-	-	I	-	V	-
SLYNTV A V L	8V	-	-	-	-	-	-	-	V	-
SLYN I VATL	5I	-	-	-	-	I	-	-	-	-
SLYNT I A V L	6I8V	-	-	-	-	-	I	-	V	-
SL F N A I A V L	3F5A6I8V	-	-	F	-	A	I	-	V	-
SLYNTVAIL	8I	-	-	-	-	-	-	-	I	-
SLYNTV V TL	7V	-	-	-	-	-	-	V	-	-
SL F N A V A V L	3F5A8V	-	-	F	-	A	-	-	V	-
SLYNTVAT F	9F	-	-	-	-	-	-	-	-	F
SLYNTVAT P	9P	-	-	-	-	-	-	-	-	P
ILKEPVHGV	IV9	I	L	K	E	P	V	H	G	V

Table 2.5 SL9 variant peptides and control peptide assessed for TCR recognition.

With the assays performed in chapter 5, PBMCs or isolated CD8⁺ cells from HIV-naïve individuals were stained with CellTracker Violet at a final concentration of 5 µM for 20 min at 37°C, washed once with R10, and plated at an effector-to-target cell ratio of 5:1. The a11b6 ImmTAC was added to each well at a final concentration of either 0.1 or 1 nM, and plates incubated overnight at 37°C, 5% CO₂. The next day, cells were stained with LIVE/DEAD fixable violet dead cell stain (Invitrogen) and then resuspended in PBS before being analysed on the LSRII.

Cytotoxicity was determined using the following equation, where spontaneous release is when no TCR-transduced cells or ImmTAC is added:

$$\text{Cytotoxicity (\%)} = 100 - \frac{\% \text{ CFSE of test peptide concentration}}{\% \text{ CFSE of spontaneous release}} \times 100$$

2.16 In vitro HIV infection

For *in vitro* HIV infection of T0, T1 or T2 cells, low passage HIV_{LAI} was added at a multiplicity of infection (MOI) of 0.001 or 0.01 in the presence of 4 µg/ml Polybrene and incubated for 4 h at 37°C, 5% CO₂. Cells were washed twice with R10 to remove excess virus. For *in vitro* HIV infection of PBMCs, cells were activated with 5 µg/ml PHA for 72 h prior to addition of low passage HIV-1_{LAI} at a MOI of 0.001 in the presence of 4 µg/ml Polybrene. PBMCs were incubated for 4 h at 37°C, 5% CO₂ and then washed twice with R10.

2.17 In vitro selection pressure assays

2.17.1 Continuous culture

T0 cells were infected with HIV_{LAI} at a MOI of 0.01 and plated in a 24-well plate at 2.5×10^5 cells/well. TCR-transduced cells were added to the HIV-infected T0 cells at an effector-to-target cell ratio of 1:10, 1:5 or 1:1; each well had a total volume of 2 ml with 10% IL-2 Cellkines (ZeptoMetrix) and plates were incubated at 37°C, 5% CO₂. All samples were performed in duplicate. At days 5, 9, 11, 15, 18 and 24 postinfection, culture media were replaced with 2.5×10^5 uninfected T0 cells/well in fresh R10 plus 10% IL-2 Cellkines. Cells were stained for flow cytometry and analysed on the LSRII at various time-points and viral supernatant stored at -80°C.

2.17.2 Passaging

T0 cells were infected with HIV_{LAI} at a MOI of 0.001 and incubated at 37°C, 5% CO₂. At day 2 postinfection, HIV-infected T0 cells were plated in a 24-well plate at 5×10^5 cells/well. TCR-transduced cells were added at an effector-to-target cell ratio of 1:10; each well had a total volume of 2 ml with 10% IL-2 Cellkines and plates were incubated at 37°C, 5% CO₂. Samples were performed in duplicate. At day 4 postinfection, 5×10^5 uninfected T0 cells were added to each well. Cells were stained for flow cytometry and analysed on the LSRII on days 4, 6 and 8 postinfection and viral supernatant stored at -80°C. Viral supernatant from the day 6 postinfection samples was thawed and 500 µl added to individual Falcon tubes containing 1×10^6 T0 cells in the presence of 4 µg/ml Polybrene and incubated for 4 h at 37°C, 5% CO₂. Cells were washed twice with R10 and plated in a 24-well plate. TCR-transduced cells were added to corresponding HIV-infected T0 cells at an effector-to-target cell ratio of 1:10; each well had a total volume

of 2 ml with 10% IL-2 Cellkines and plates incubated at 37°C, 5% CO₂. Cultures were fed twice a week, and at days 7 and 9 postinfection, culture media were replaced with 1 x 10⁶ uninfected T0 cells/well in fresh R10 plus 10% IL-2 Cellkines. The cells were stained for flow cytometry and analysed on the LSRII at days 5, 8 and 14 postinfection and viral supernatant stored at -80°C.

2.18 Redirected lysis of HIV-infected T1 cells

T1 and T2 cells were separately infected with HIV_{LAI} at a MOI of 0.001 and incubated at 37°C, 5% CO₂. At day 4 postinfection, uninfected T1 cells were stained with CFSE diluted to a final concentration of 0.67 µM for 10 min at room temperature. Cells were washed twice with R10 and plated at 25,000 cells/well. HIV-infected T2 cells were stained with CellTracker Orange at a final concentration of 5 µM for 20 min at 37°C, washed once with R10, and plated at 25,000 cells/well. Unstained HIV-infected T1 cells were also plated at 25,000 cells/well. Isolated CD8⁺ cells were stained with CellTracker Violet at a final concentration of 5 µM for 20 min at 37°C, washed once with R10, and plated at 100,000 cells/well. Each well contained 10 µM AZT and a specified concentration of ImmTAC reagent. Plates were incubated overnight at 37°C, 5% CO₂. The next day, cells were stained with LIVE/DEAD fixable near-IR dead cell stain and then resuspended in PBS before being analysed on the LSRII.

2.19 Redirected lysis of HIV-infected PBMCs

PHA-activated PBMCs from two different donors (HLA-A*02⁺ and HLA-A*02⁻) were separately infected with HIV_{LAI} at a MOI of 0.001 and incubated at 37°C, 5% CO₂. At day 4 postinfection, unstained, uninfected HLA-A*02⁻ PBMCs were plated at 20,000 cells/well. HIV-infected HLA-A*02⁻ PBMCs were stained with CellTracker Orange at a final concentration of 5 µM for 20 min at 37°C, washed once with R10, and plated at 20,000 cells/well. HIV-infected HLA-A*02⁺ PBMCs cells were stained with CellTracker Violet at a final concentration of 5 µM for 20 min at 37°C, washed once with R10, and plated at 20,000 cells/well. Each well contained 10 µM AZT and a specified concentration of ImmTAC reagent. Plates were incubated overnight at 37°C, 5% CO₂. The next day, cells were stained with LIVE/DEAD fixable near-IR dead cell stain and fluorochrome-conjugated antibodies, resuspended in PBS then analysed on the LSRII.

2.20 Flow cytometry

Following all staining steps, cells were washed twice in PBS; uninfected cells were resuspended in PBS and HIV-infected cells were resuspended in 2% formaldehyde before flow cytometry analysis. Flow cytometry was either performed on the FACSCalibur (Becton Dickinson) or the LSRII. Data were analysed using FlowJo (Tree Star) and gated using unstained cells, isotype controls or other appropriate methods.

The following fluorochrome-conjugated mouse monoclonal anti-human antibodies were used for cell surface staining and diluted to an optimised concentration: CD14-Pacific

Blue (Invitrogen); CD19-Pacific Blue (Invitrogen); CD4-Qdot 605 (Invitrogen); CD4-Phycoerythrin (PE or RD1; R&D Systems); CD4-Alexa Fluor 700 (BD Biosciences); CD8-PerCP (BD Biosciences); CD8-Alexa Fluor 700 (BD Biosciences); CD8-allophycocyanin (APC; R&D Systems); CD3-ECD (R-PE-Texas Red-X; Beckman Coulter); CD69-PE-Cy7 (eBioscience); and human V beta 5a TCR-fluorescein isothiocyanate (FITC; Pierce Biotech).

For intracellular staining, cells were first fixed and permeabilised using Leucoperm (AbD Serotec). Cells were intracellularly stained for p24 using either KC57-FITC or KC57-RD1 (Beckman Coulter). Uninfected cells were stained to determine the correct gating. It was found that p24 staining correlates well with p24 antigen in viral supernatants (Figure 2.2) as measured by the Innostest HIV antigen mAb (Innogenetics).

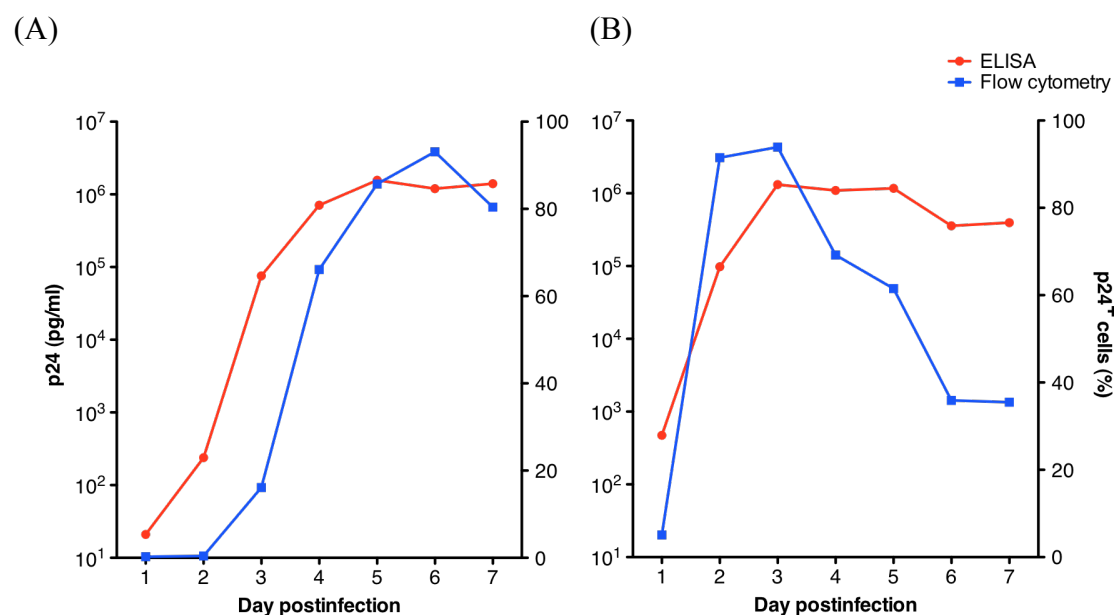


Figure 2.2 Comparison of flow cytometry staining HIV-infected cells with p24 ELISA of cell culture supernatant. ELISA measurements are on the left y-axis; flow cytometry measurements are on the right y-axis. (A) HIV-infected T0 cells; (B) HIV-infected MT4 cells.

2.21 SYBR Green product enhanced reverse transcriptase (SG-PERT) assay

Enzymatic activity of reverse transcriptase was detected in cell culture supernatants using a modified version of the SG-PERT assay (Pizzato et al., 2009). 5 μ l of undiluted viral supernatant or HIV RT standards (Ambion) was incubated for 15 min at room temperature with 0.125% Triton X-100, 25 mM potassium chloride, 50 mM TrisHCl (pH 7.4), 20% glycerol and 0.4 U RNasin ribonuclease inhibitor (Promega). Half of the lysed virus or RT standard was mixed with 0.2 μ M SG-PERT_F primer, 0.2 μ M SG-PERT_R primer, 12 ng Brome mosaic virus (BMV) RNA and 1X LightCycler 480 SYBR Green I Master (Roche). Plates were run on the LightCycler 480 using the following program: one cycle of 37°C for 30 min; one cycle of 95°C for 5 min; 45 cycles of 95°C for 5 s, 55°C for 5 s, 72°C for 15 s, and 83°C for 7 s; one cycle of 94°C for 20 s; one cycle of 68°C for 5 min. A calibration curve was created using the HIV RT standards to extrapolate the RT activity.

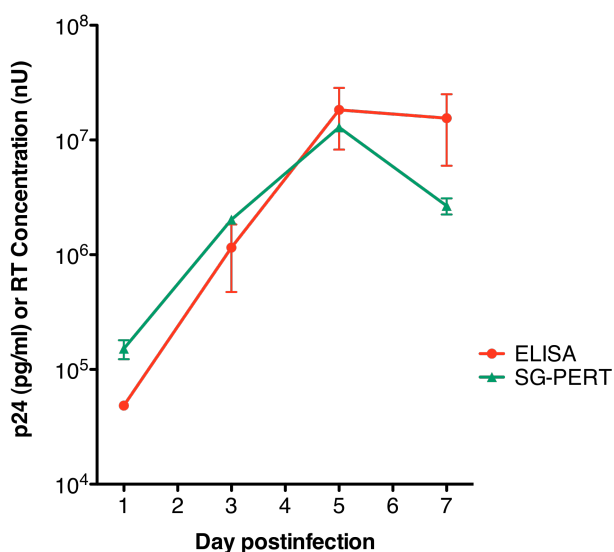


Figure 2.3 Comparison of p24 ELISA with SG-PERT of HIV-infected T0 cell culture supernatants. Each was performed in duplicate; error bars are SEM.

It was found that RT activity correlates well with p24 antigen in viral supernatants (Figure 2.3) as measured by the Innostest HIV antigen mAb. There is a fall in RT activity following peak infection indicating that residual p24 still present following peak infection may not correlate with new virion production.

2.22 Statistical and phylogenetic analysis

To test for significant difference between variables, Fisher's exact test or the unpaired t test was performed. A value of $P < 0.05$ was considered statistically significant; * = 0.01 to 0.05; ** = 0.001 to 0.01; *** = < 0.001 . EC_{50} values were calculated using GraphPad Prism software with the log(agonist) vs. response equation. Linear regression was also calculated using GraphPad Prism; R^2 and P values are shown.

HIV subtypes were confirmed using the Recombinant Identification Program (RIP) available on the Los Alamos HIV sequence database (<http://www.hiv.lanl.gov/>). Phylogenetic analysis of sequences was performed using PhyML (Phylogenetic inferences using Maximum Likelihood) and the general time reversible (GTR) model of evolution (Guindon and Gascuel, 2003). Trees were visualised using Figtree v1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>).

CHAPTER 3

Longitudinal analysis of the HLA-B*51-restricted epitope LI9 in HIV-1 integrase

Introduction

HIV-1 integrase is an essential enzyme in the HIV life cycle as it inserts a DNA copy of the viral genome into the host cell's DNA, thereby allowing HIV to utilise the host's DNA replication machinery. The enzyme must first cleave two nucleotides from the 3' end of the viral DNA, and then transfer these strands into the target DNA forming new covalent bonds (Engelman et al., 1991). In 2007, the first integrase inhibitor, raltegravir, was approved by the FDA (Merck & Co, 2007). This class of antiretroviral drug abrogates the incorporation of the HIV DNA by blocking the joining of the viral DNA ends with the cellular DNA.

Encoded by the C-terminal end of the *pol* gene, HIV-1 integrase is 288 amino acids in length. The protein consists of 3 domains: an N-terminal domain (residues 1-49) that contains a His-His-Cys-Cys (HHCC) motif enabling zinc binding, which in turn enhances tetramerisation (Zheng et al., 1996); a catalytic core domain (residues 50-212) containing the acidic Asp-Asp-Glu (DDE) motif (Engelman and Craigie, 1992); and a C-terminal domain (residues 213-288) that binds both viral and host DNA (Vink et al., 1993). Despite raltegravir's recent market use, integrase inhibitor resistance mutations

have already been selected within the catalytic core domain (Stanford, 2010). The need for an HIV vaccine remains.

When selecting HIV antigens to incorporate in a CTL vaccine, research has turned to both specific epitopes and the protein source of those epitopes. It has been found that independent of HLA type, Gag-specific CTL responses to HIV-1 subtype C infection are associated with lower viraemia and Env-specific CTL responses are associated with higher viraemia; the Pol-specific response was not associated with either a lower or higher viraemia (Kiepiela et al., 2007). This does not mean that Pol-specific HIV responses do not contribute to HIV control; within a patient, the individual CTL response to a Pol epitope may be of similar or greater magnitude than that found against a Gag epitope. In two HLA-identical siblings, one made an HLA-A*02 response against the Gag epitope SLYNTVATL, whilst the other made an HLA-A*02 response against the Pol epitope ILKEPVHGV (Goulder et al., 1997b). In a cohort of 27 chronic HIV-infected individuals, only Gag- and Pol-specific CTL responses were correlated markers of progression to AIDS; IFN γ ELISpot responses against Gag peptides inversely correlated with viral load and directly correlated with absolute CD4⁺ cell count; Pol responses directly correlated with absolute CD4⁺ cell count (Edwards et al., 2002). The correlation with the Pol response was heavily dependent on the response to integrase peptides (Figure 3.1). In general, Gag, Pol and Nef peptides are often the main antigens included in CTL vaccine design; however, the case may be that emphasis should be placed on the epitope itself compared to its protein source (Chen et al., 2011).

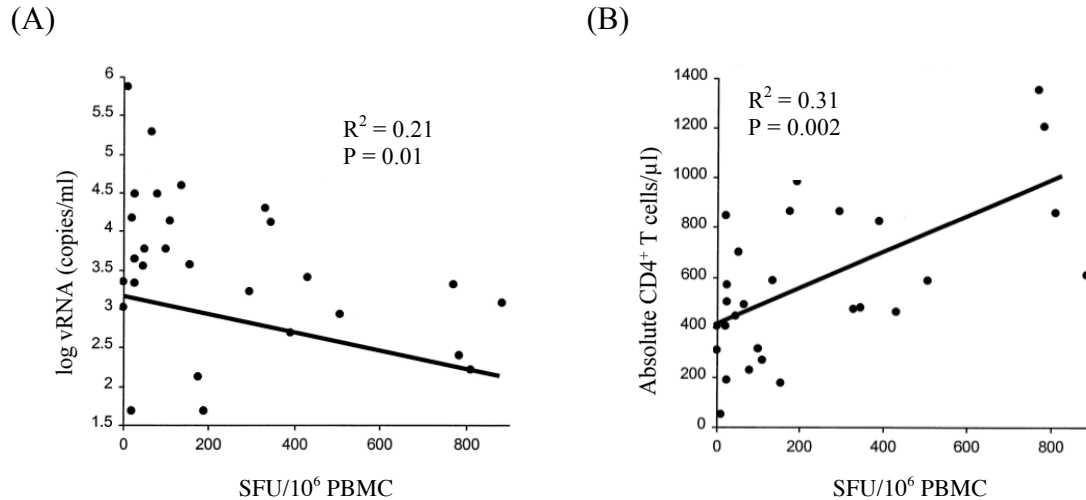


Figure 3.1 HIV-1 integrase-specific immune responses correlate with (A) vRNA and (B) absolute CD4⁺ cell count (Edwards et al., 2002).

In 2009, HIV_{NL4-3} recombinant viruses that encoded Gag-protease sequences from elite controllers had a reduced *in vitro* replication capacity compared to progressors (Miura et al., 2009). The same effect was published 2 years later in recombinant viruses encoding RT-integrase sequences; this was most pronounced in HLA-B*51 and -B*57 elite controllers (Brumme et al., 2011). This reduced replication capacity suggests that CTL escape mutations in both Gag and RT-integrase impact on viral fitness, which may contribute to the HIV-infected elite controller phenotype.

Specific HLA alleles are associated with different rates of progression to AIDS. HLA-B*27, -B*57 and -B*51 have the lowest relative hazard values and so are associated with slower progression to AIDS; HLA-B*35, -B*08 and -Cw*04 have the highest relative hazard values and are associated with rapid progression to AIDS (Kaslow et al., 1996, O'Brien et al., 2001). A plethora of research has focused on the protective alleles

HLA-B*27 and HLA-B*57; however, HLA-B*51 has been identified as a protective allele and yet in comparison, little research on this allele has been undertaken. This is despite HLA-B*51 being more prevalent than HLA-B*27 and -B*57 in Europe and Asia (Meyer et al., 2007).

HLA-B*51 is part of the HLA-B7 supertype (along with HLA-B*0702, -B*3501, -B*4201, -B*5301, and -B*5401) and so has a binding preference for 9 mers with generally a proline at position 2 (P2) and a leucine, isoleucine or valine at the C-terminal position; an alanine at P2 may also be tolerated. Secondary anchors positions 1 (P1) and 3 (P3) may also play a part in terms of peptide binding affinity (Sidney et al., 1996). The B7 supertype is associated with a significantly higher viral load (Trachtenberg et al., 2003) and yet HLA-B*51 is associated with slower progression to AIDS. This contradictory association adds further weight as to why HLA*51 should be studied.

To date, 20 HLA-B*51-restricted CTL epitopes have been identified and of those, three are of most interest: NANPDCKTI (NI9, *gag* 325-333 p24, 193-201); TAFTIPSI (TI8, *pol* 283-290, RT 128-135); and LPPVVAKEI (LI9, *pol* 743-751, integrase 28-36) (Yusim et al., 2009). LI9, situated in the N-terminal domain of integrase, was found to be the strongest binder to HLA-B*5101 (Tomiyama et al., 1999).

A known interclade difference is present and can be clearly seen within the LI9 epitope at residue 31: the consensus is Val (V) and Ile (I) in HIV-1 subtype B and C, respectively. The dominant circulating LI9 variant in HIV-1 subtype B is LPPVVAKEI

followed by a large proportion of subtype C consensus variant (LPPV**I**AKEI), then varying proportions of minor variants (**I**PPVVAKEI > LPPV**I**AKEI > other variants) (Figure 3.2). Sequence polymorphisms have been found at all positions in subtype B LI9 variants except P2, position 6 (P6) and 9 (P9).

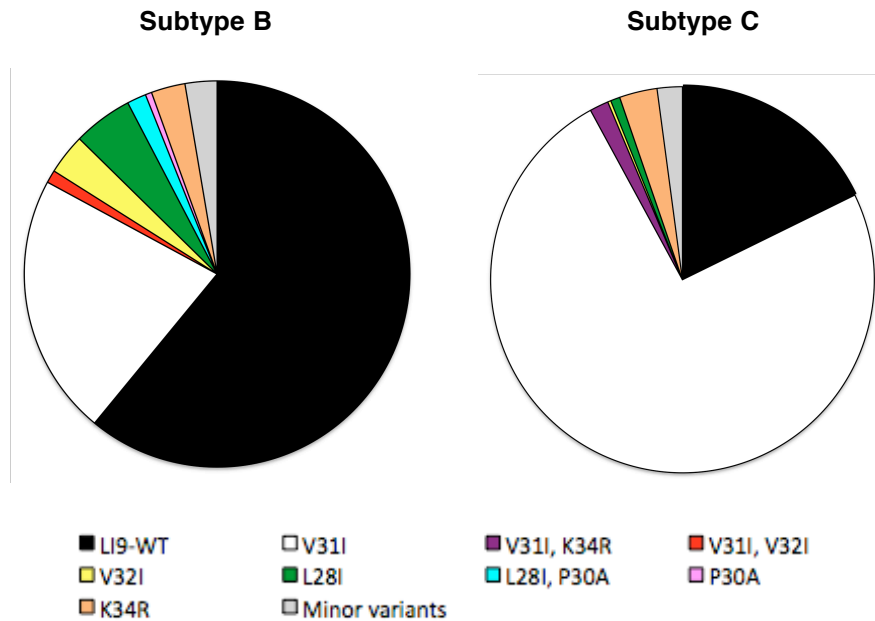


Figure 3.2 Variant integrase LI9 epitopes according to HIV-1 subtype. The frequency of each variant including minor variants is shown in Appendix A.

In 2005, Leslie et al. published data indicating that I31V in LI9 is an HLA-B*51 negatively associated subtype B polymorphism, or a ‘negatope’. This simply means that there is an association between HLA-B*51 expression and I31V which is now the consensus residue in subtype B strains. In a cross-sectional study of chronically infected HIV-1 subtype B patients, 150/246 (60.1%) HLA-B*51 negative and 35/40 (87.5%) HLA-B*51 positive subjects had consensus 31V dominating ($P = 0.0011$). It was

inferred from these results that HLA-B*51 selection pressure was driving the escape variant 31V to form the consensus in subtype B infection (Leslie et al., 2005). The interclade difference seen in Figure 3.2 supports this hypothesis as HLA-B*51 is a rare allele type in South Africa where 31I is the consensus.

In an HIV-1 subtype-B-infected horizontal transmission pair, where the donor expressed HLA-B*51, the sequenced viral RNA from the first time-point detected 31I in 22/22 clones; 18 months later this had stably mutated into 31V (11/11 clones). The 31V variant was transmitted and remained 8 months post-transmission. This example provided further evidence that there is a negative association in HIV subtype B infection between HLA-B*51 expression and I31V due to the positive selection of an escape mutation that does not revert once transmitted to disparate-HLA type recipients (Leslie et al., 2005).

Although the transmission pair data published by Leslie et al. is correct, the full LI9 epitope clonal sequencing data for this transmitter pair displays more viral evolution than just at residue 31. Table 3.1 details the frequency of clones with LI9 variants at the different time-points for patient P32 (HLA-A*0301, -A*0101, -B*5101, -B*0801) and S32 (HLA-A*0101 homozygous, HLA-B*0801 homozygous). The first time-point of chronically-infected patient P32 had circulating virus bearing not only the polymorphism V31I, but also V32I (22/22 clones); 18 months later, clonal sequencing showed that indeed the V31I polymorphism had reverted to consensus, but also that the V32I polymorphism remained. A further 3 months later and three variants were

detected: one with the V32I polymorphism, one with an additional L28I polymorphism, and one with an additional P30A polymorphism.

	P32 (HLA-B*51 ⁺)			S32 (HLA-B*51 ⁻)		
	21-Sep-1999	08-Mar-2001	01-Jun-2001	21-Aug-2001	08-Oct-2001	04-Apr-2002
LPPVVAKEI	0/22	0/11	0/18	0/12	0/16	23/23
LPPIIAKEI	22/22	0/11	0/18	0/12	0/16	0/23
IPPVIAKEI	0/22	0/11	9/18	0/12	0/16	0/23
LPPVIAKEI	0/22	11/11	7/18	12/12	16/16	0/23
LP A VIAKEI	0/22	0/11	2/18	0/12	0/16	0/23
Viral load (copies/ml)	54,433	50,517	25,221	910,600	816,964	1,289,725
CD4 count (cells/ μ l)	unknown	610	590	350	290	260

Table 3.1 Clonal sequencing of the LI9 epitope from the P32/S32 transmitter pair. Phylogenetic analysis of these clones against subtype reference sequences is given in Appendix B.

Patient P32 infected the HLA-B*5101-negative patient S32 with the first sample of this acute HIV-infected individual showing that the circulating viral variant was that of the V32I polymorphism. This variant persisted for at least 48 days. The final sample taken was 6 months later, at this time reversion to consensus had occurred.

These other polymorphisms found within LI9 need to be addressed as may have a greater impact on CTL escape than just residue 31 in HLA-B*51 positive individuals. As CTL escape occurred within two years and reversion in less than one year post-transmission, it seemed viable to expand on the data by examining a large cohort of longitudinally-sampled acute/early HIV-1 subtype B-infected patients. Most CTL studies of HIV infection are of chronically-infected individuals and are cross-sectional.

This offers a snapshot of an individual's immune response and may compare widely different time-points of infection. If similar results to the P32/S32 transmitter pair were observed in this longitudinal cohort, it would provide large-scale evidence as to how the virus escapes this CTL response and the rapidity of reversion would provide insight into its viral fitness cost. From the P32/S32 data, we hypothesised that P1, P3 and/or position 5 (P5) were required for CTL recognition, and that there would be reversion of escape mutations at these positions in the HLA-B*51 negative patients within one year due to an associated fitness cost. Wildtype LI9 would therefore persist in circulating virus sequences and may be useful in HIV vaccine design.

A CTL-driven HLA-B*51-restricted LI9 escape variant has not been fully categorised. Without a clear escape variant defined, we cannot interpret polymorphisms in terms of immune selection pressure, nor can reversion from escape be put into real context. The data presented in this chapter utilises samples from individuals with early HIV-1 infection, with longitudinal sampling providing the unique opportunity of monitoring the adapting CTL response over time starting from a period close to the transmission event.

Results

3.1 Diversity of LI9 epitope variants in early HIV-1 infection

HIV-infected individuals are most infectious during the first few weeks of infection when viral load peaks, and in the final stages of infection when the individual has progressed to AIDS. It is at this earlier time-point when a person may not know they have been infected that there is the highest probability of exposing others to the virus, hence why studying early infection is so important. Longitudinal samples were obtained from an Open study at St. Mary's Hospital and through the SPARTAC trial. Participants in the SPARTAC trial are classified at baseline as being acute or early HIV-1 infected as enrolment was within 6 months of infection; patients were generally within stage IV – VI of primary HIV infection (Fiebig et al., 2003). Randomly assigned into three arms, participants either: did not receive HAART; received HAART for 12 weeks; or received HAART for 48 weeks after baseline samples had been taken.

Direct PCR population sequencing was performed on samples from 118 acute/early HIV-1 subtype B-infected patients at baseline. The amplified region of HIV-1 *pol* encoding integrase contained the HLA-B*51-restricted epitope LI9. The authenticity of further time-points was confirmed by phylogenetic analysis (Figure 3.3).

negative individuals follows a similar pattern to those listed on the Los Alamos database (Figure 3.5) with less diversity; this was expected given the sampling size. Samples that were also of the B7 supertype and with a phenotype frequency > 0.1 , i.e. HLA-B*0702 and -B*3501, were also studied for comparison (Appendix C). All variants at baseline detected in this cohort had been previously listed on Los Alamos, except the **IPSVVAKEI** variant in a HLA-B*51 negative patient. This variant will be analysed later in this chapter.

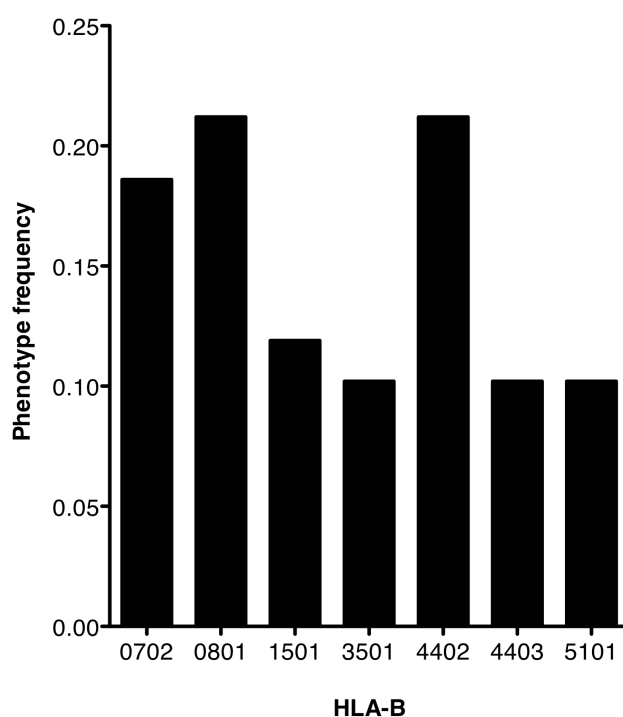


Figure 3.4 Phenotype frequency of HLA-B alleles in the 118 patients sampled at baseline. Only those with a frequency > 0.1 are shown.

Variant	L	P	P	V	V	A	K	E	I	B*51 ⁺		B*51 ⁻		Total	
										n	%	n	%	n	%
LI9	.	.	.	.	.	.	.	.	.	6	50.0	72	67.9	78	66.1
V31I	.	.	.	I	.	.	.	.	.	2	16.7	25	23.6	27	22.9
L28I	I	.	.	.	.	.	.	.	.	2	16.7	5	4.7	7	5.9
V31I, V32I	.	.	.	I	I	.	.	.	.	2	16.7	0	0.0	2	1.7
K34R	.	.	.	.	.	.	R	.	.	0	0.0	2	1.9	2	1.7
P30A	.	.	A	.	.	.	.	.	.	0	0.0	1	0.9	1	0.8
L28I, P30S	I	.	S	.	.	.	.	.	.	0	0.0	1	0.9	1	0.8
Total n (variants)										12	(4)	106	(6)	118	(7)

Table 3.2 Variant integrase LI9 epitopes at baseline and with respect to HLA-B*51 expression of the HIV subtype B-infected participant. All population sequencing data were compared to the HIV subtype B consensus sequence.

In terms of HLA-associated sequence polymorphisms in this cohort, the LPP**I**VAKEI variant was only found in patients expressing HLA-B*51. There is an association between this double mutant and HLA-B*51 expression ($P = 0.0096$). At baseline, there was no evidence of an association between L28I, P30X or V31I and HLA-B*51 expression ($P = 0.188, 1.0$ and 0.486 , respectively, Fisher's exact test).

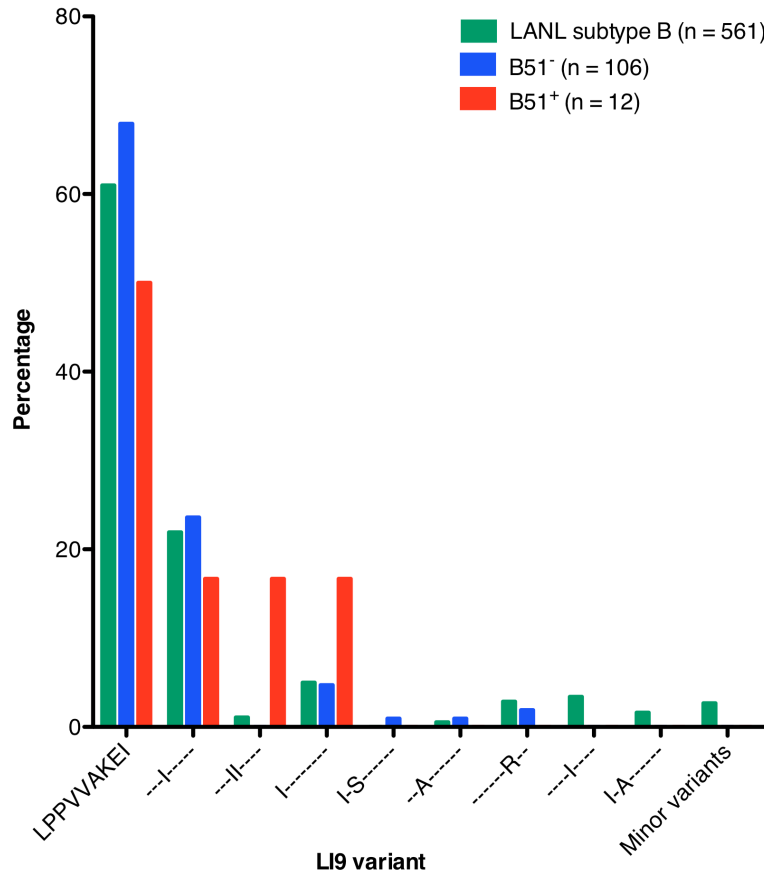


Figure 3.5 Variant integrase LI9 epitopes for SPARTAC/Open study HIV subtype B-infected patients at baseline according to HLA-B*51 expression compared to untyped samples on Los Alamos.

3.2 HLA-B*51-associated selection pressure on LI9

Of the 118 patients, a further time-point one year later was examined for 10 and 83 of the HLA-B*51 positive and negative subjects, respectively, enabling intra-host HIV-1 evolution to be monitored (Table 3.3). In the HLA-B*51-positive group, there are some interesting things to consider. We have already noted the association of LPPVVAKEI in the HLA-B*51 positive patients, but we can also see the emergence of LPPVVAKEI and LPSIVAKEI, with the disappearance of LPPVVAKEI at year 1. An analysis of the

upstream and downstream residues of LI9 in integrase was undertaken. No clear compensatory mutations could be determined.

(A)

Variant	L	P	P	V	V	A	K	E	I	Baseline		Year 1	
										n	%	n	%
LI9	.	.	.	.	.	.	.	.	.	5	50.0	4	40.0
V31I	.	.	.	I	.	.	.	.	.	2	20.0	0	0.0
V32I	.	.	.	.	I	.	.	.	.	0	0.0	2	20.0
L28I	I	.	.	.	.	.	.	.	.	1	10.0	1	10.0
V31I, V32I	.	.	.	I	I	.	.	.	.	2	20.0	2	20.0
P30S, V31I	.	.	S	I	.	.	.	.	.	0	0.0	1	10.0
Total n (variants)										10	(4)	10	(5)

(B)

Variant	L	P	P	V	V	A	K	E	I	Baseline		Year 1	
										n	%	n	%
LI9	.	.	.	.	.	.	.	.	.	56	67.5	56	67.5
V31I	.	.	.	I	.	.	.	.	.	19	22.9	19	22.9
L28I	I	.	.	.	.	.	.	.	.	4	4.8	4	4.8
K34R	.	.	.	.	.	.	R	.	.	2	2.4	2	2.4
P30A	.	.	A	.	.	.	.	.	.	1	1.2	1	1.2
L28I, P30S	I	.	S	.	.	.	.	.	.	1	1.2	1	1.2
Total n (variants)										83	(6)	83	(6)

Table 3.3 Longitudinal data of HIV-1 subtype B LI9 variants with respect to HLA-B*51 expression. Population sequencing of baseline and year 1 samples are given for both (A) HLA-B*51 positive and (B) HLA-B*51 negative subjects.

Of the 10 HLA-B*51 positive individuals, four had a change in the dominating LI9 variant from baseline to year 1 as determined by direct PCR population sequencing. These mutated epitopes were: LPPVVAKEI → LPPVIAKEI; LPPIVAKEI →

LPPV**I**AKEI; LPP**I**VAKEI → LPP**I**IAKEI; and LPP**I**IAKEI → LPS**I**VAKEI. No change of the dominating variant was detected by direct PCR population sequencing for the 83 HLA-B*51 negative individuals (Figure 3.6). There is a statistically significant association between HLA-B*5101 expression and an evolving LI9 sequence from baseline to year 1 ($P < 0.0001$).

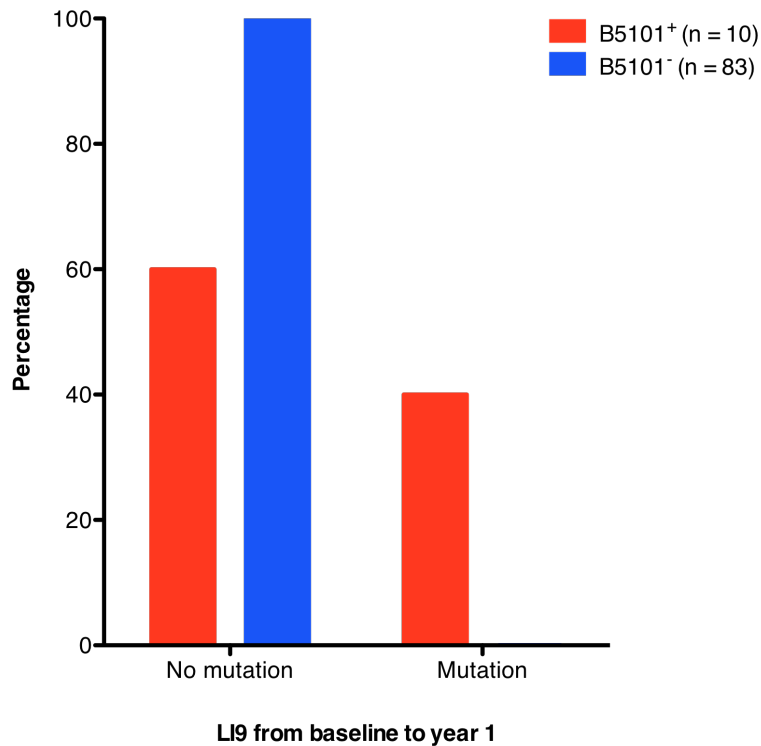


Figure 3.6 Change in the dominating LI9 variant from baseline to year 1 with respect to HLA-B*5101 expression. A mutating LI9 sequence was associated with HLA-B*5101 expression ($P < 0.0001$, Fisher's exact test).

The proportion of patients with wildtype LI9 was analysed according to HLA-B*51 expression. If escape from wildtype (the consensus sequence in HIV-1 subtype B infection) were occurring, we would expect the proportion of patients with

LPPVVAKEI to decrease over time. LI9 was not statistically more polymorphic in HLA-B*51 positive subjects than in negative subjects at baseline (50% versus 32.5%; $P = 0.304$) or at year 1 (60% versus 32.5%; $P = 0.158$). We repeated the comparison Leslie et al. documented in terms of I31V being a negative association; if 31V is a HLA-B*51-selected escape mutation, we would expect the HLA-B*51 positive patients with 31I to select for the I31V polymorphism over time. The mean proportion of patients with 31V between the HLA groups was not significantly different at either baseline or year 1 ($P = 0.257$ and 0.700 , respectively, Figure 3.7).

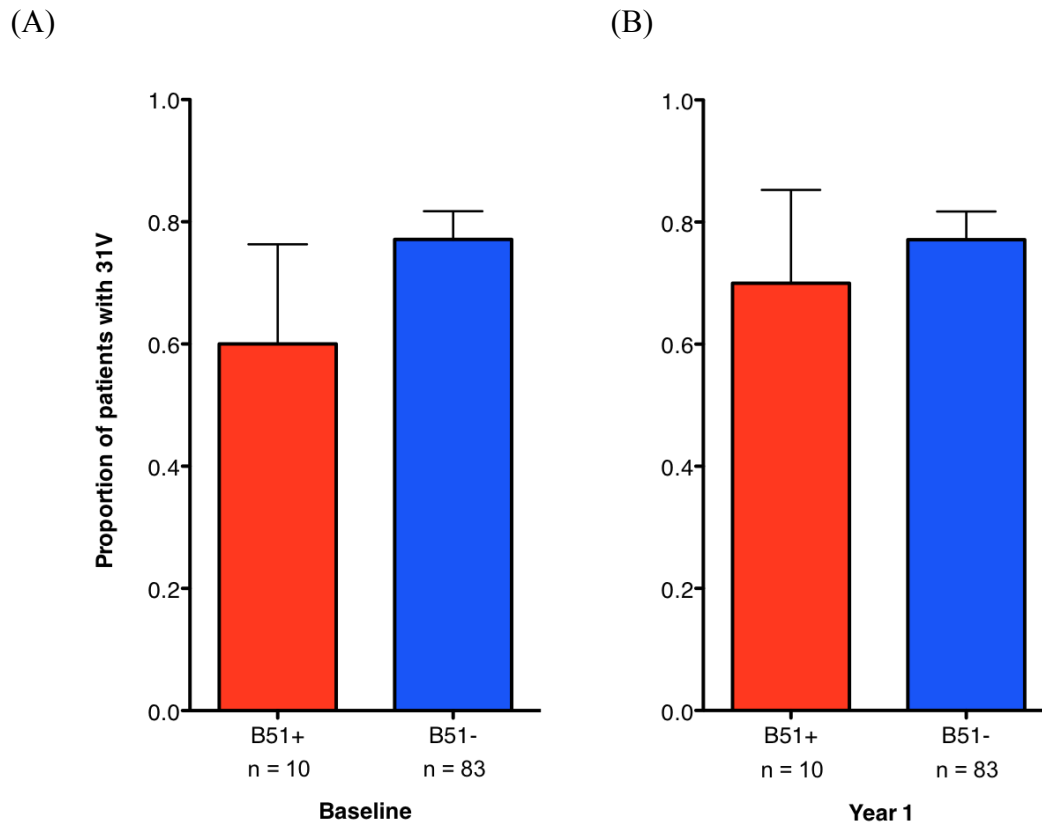


Figure 3.7 Proportion of subtype B 31V variants found in the study in a time-dependent manner, with respect to HLA-B*51 expression at (A) baseline and (B) year 1. The standard error of the mean (SEM) is shown; there is no statistical significant difference between HLA-B*5101 expression and 31V.

However, we have determined a strong association between HLA-B*51 expression and a mutating LI9 epitope over one year (Figure 3.6). With reference to the clonal sequences found in patient P32 (Table 3.1), we postulated that residue 31 of integrase may not be an important factor in terms of early CTL escape.

The mean proportion of patients with LPPVVAKEI or LPPV**I**AKEI between the HLA-B*51 positive and negative subjects significantly differs between baseline and year 1 ($P = 0.0936$ and < 0.0006 , respectively) (Figure 3.8).

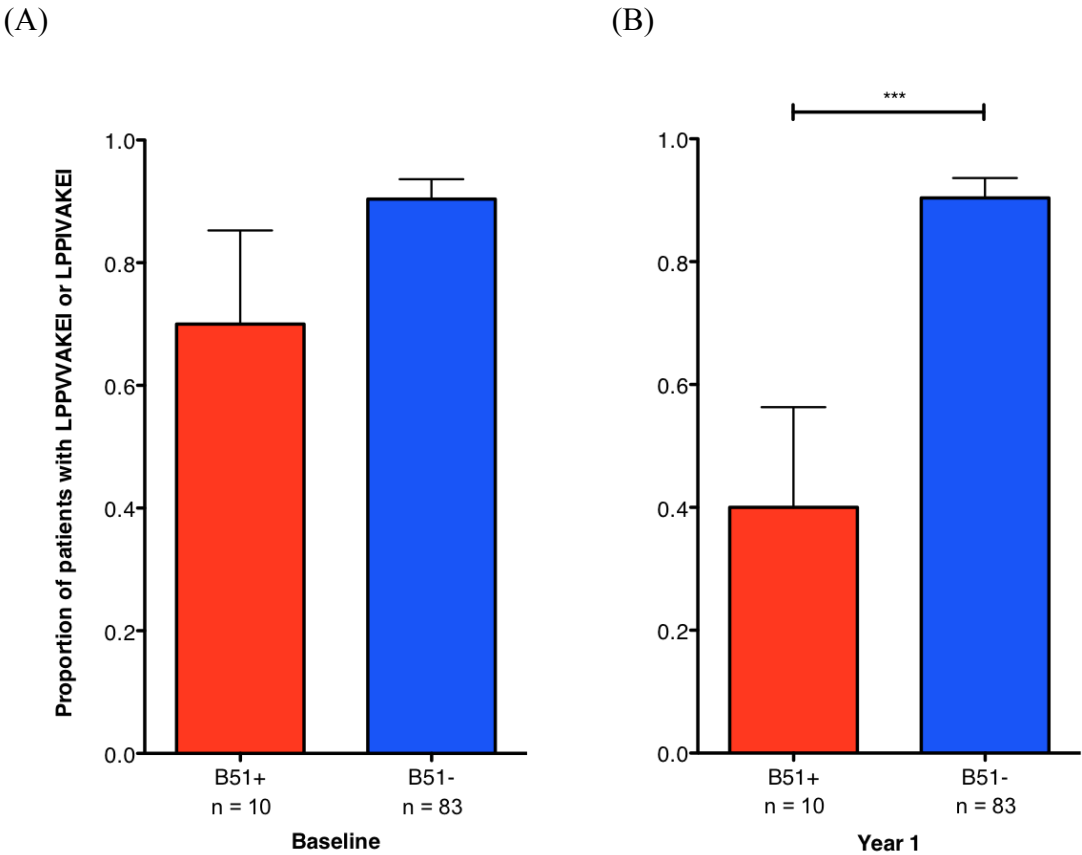


Figure 3.8 Proportion of subtype B LPPVVAKEI or LPPV**I**AKEI variants found in the study in a time-dependent manner, with respect to HLA-B*51 expression at (A) baseline; and (B) year 1. The SEM is shown; there is a statistical significant association between expressing HLA-B*5101 and not having LPPVVAKEI or LPPV**I**AKEI by year 1 ($P < 0.0006$, Fisher's exact test).

We also direct PCR population sequenced further time-points for the HLA-B*51 positive subjects that are interesting to note. One patient with LPPVVAKEI at baseline and no year 1 sample, had LPPV**I**AKEI dominating at week 96; another had LPPVVAKEI dominating at baseline, month 6 and year 1 and 2, then at year 3 LPPV**I**AKEI emerged. The individual with **I**PPVVAKEI at baseline and year 1 had a mixture of **I**PSVVAKEI and **I**PAVVAKEI at year 2. Similarly to the earlier time-points shown in Table 3.3, these three HLA-B*51 positive subjects have had mutations emerge in LI9 at P3 and P5.

*3.2.1 Cellular response to HLA-B*51-restricted epitopes*

Of the HLA-B*51 positive subjects with PBMCs available, only one patient made no positive response at baseline to LI9; this patient also made no response to TI8 or NI9; no further time-points were available. Of the five HLA-B*51 positive subjects with IFN γ ELISpot data at both baseline and week 24, all five made a response to LI9; 4/5 subjects made a response to TI8 at baseline and 3/5 at week 24; only 1 participant had a NI9 response (Figure 3.9). LI9 was the most frequently targeted HLA-B*51 epitope. Depending on the individual and the time-point of infection, either TI8 or LI9 were the immunodominant response; the latter was typically when no response to TI8 was generated (Figure 3.10).

3.2.2 Cellular response to LI9 and variants

Sequencing changes in the HLA-B*5101 positive patients infer that the LI9 epitope is under immune pressure. To assess the LI9-specific CTL response of the cohort, IFN γ ELISpot assays were carried out using subject PBMCs and LI9 variant peptides.

The purpose of using different peptide concentrations in an ELISpot is to represent the amount of epitope counts there may be on a cell. Most papers report ELISpot data after cells have been in the presence of 1 μ M peptide – this a high concentration of peptide and may not represent a natural response.

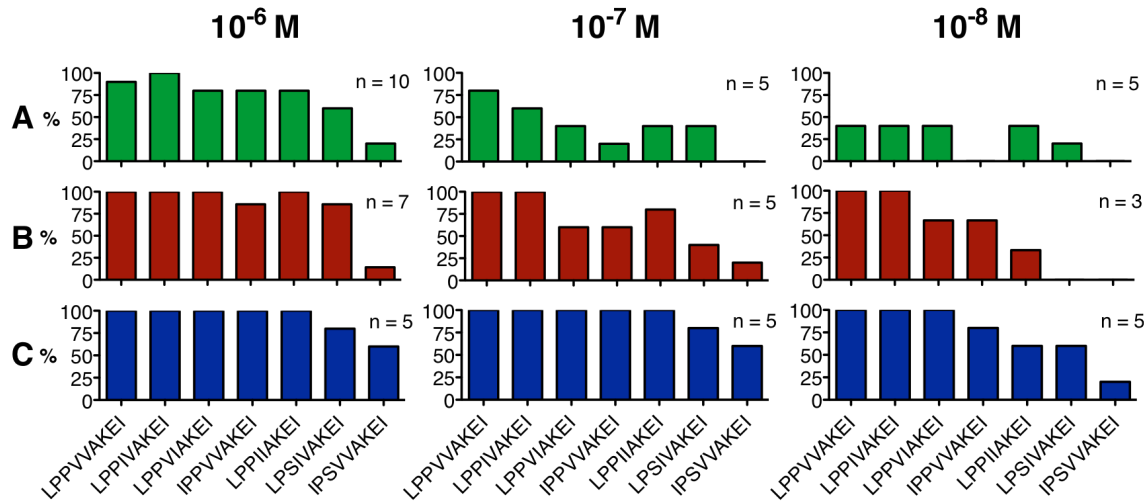


Figure 3.11 Longitudinal analysis of HLA-B*51-restricted LI9-specific CTL responses. The percentage of HLA-B*51 positive subjects with a positive IFN γ ELISpot response (>50 SFU/10⁶ PBMCs) in the presence of serial log dilutions of LI9 and variants is shown at (A) baseline to 3 months, (B) year 1 and (C) year 2.

The percentage of subjects expressing HLA-B*51 with a positive response to LI9 and its variants increased over time and decreased as the peptide was titrated to lower concentrations. At 10⁻⁸ M peptide-pulsing, only 2/5 participant's PBMCs had a positive response at baseline to LI9 and some of the variant peptides assayed although these were all <500 SFU/10⁶ PBMCs; no positive response to **IPPVVAKEI** and **IPSVVAKEI** was observed (Figure 3.11). A positive response to **IPSVVAKEI** was rarely seen, and if observed, only at the higher peptide concentrations and always a weak response.

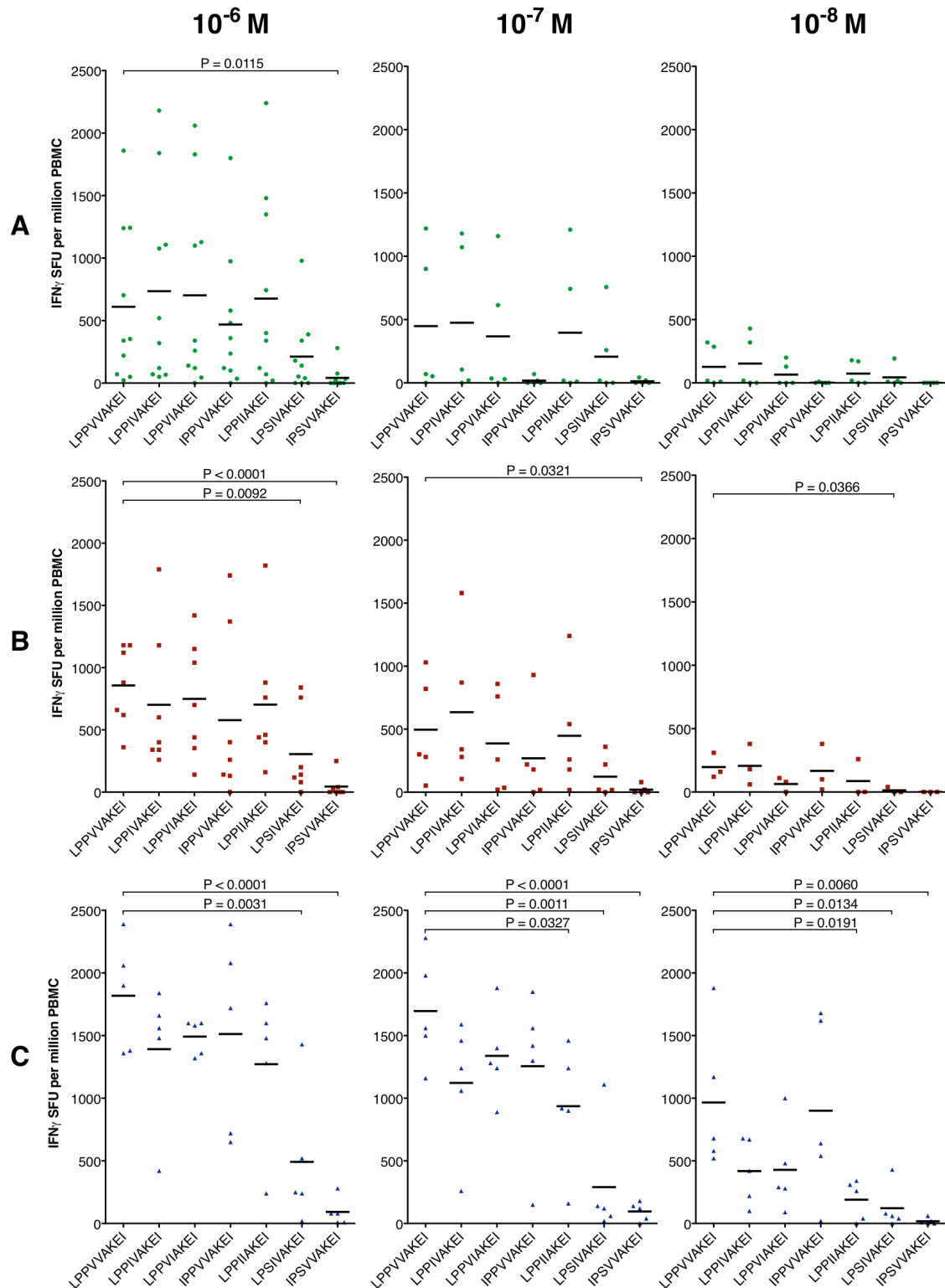


Figure 3.12 Longitudinal analysis of HLA-B*51-restricted LI9-specific CTL responses. IFN γ ELISpot data of HIV subtype B-infected patient PBMCs using serial log dilutions of peptide (10^{-6} , 10^{-7} and 10^{-8} M) is shown at (A) baseline to 3 months, (B) year 1 and (C) year 2.

The magnitude of the response for the patients in Figure 3.11 was evaluated in Figure 3.12. The magnitude changes dependent on the time-point taken and the peptide concentration used; year 2 > year 1 > baseline, and $10^{-6} > 10^{-7} > 10^{-8}$ M as would be expected. At all time-points studied, responses to 10^{-6} M peptide were generally <500 SFU/ 10^6 PBMCs for LP**SI**VAKEI and **IP**S**S**VAKEI. A statistical significant difference was observed between LPPVVAKEI and LP**SI**VAKEI at year 1 (10^{-6} and 10^{-8} M) and year 2 (10^{-6} , 10^{-7} and 10^{-8} M); between LPPVVAKEI and **IP**S**S**VAKEI at baseline (10^{-6} M), year 1 (10^{-6} and 10^{-7} M) and year 2 (10^{-6} , 10^{-7} and 10^{-8} M). In addition, a statistical significant difference in magnitude was observed between LPPVVAKEI and LPP**II**AKEI at year 2 (10^{-7} and 10^{-8} M).

Figure 3.11 and Figure 3.12 suggest that the presence of **IP**S**S**VAKEI, LP**SI**VAKEI, LPP**II**AKEI or **IP**PPVVAKEI variant may prevent an effective CTL response either in terms of it being absent, or very weak.

Functional avidity may be assessed by determining the EC_{50} value, which is the peptide concentration that induces 50% of the peak IFN γ response. Although it appears from Figure 3.13 that LPPVVAKEI has the highest functional avidity, there is no statistically significant difference between LI9 and LPP**I**VAKEI, LPPV**I**AKEI or **IP**PPVVAKEI. The variants LPP**II**AKEI and LP**SI**VAKEI have significantly larger EC_{50} values than wildtype LI9 ($P = 0.0016$ and 0.0218 , respectively); **IP**S**S**VAKEI was omitted from this analysis due to only one EC_{50} value (1.847×10^{-6} M) being available. In terms of clinical outcome, there was a correlation between viral load and the EC_{50} value for

wildtype LI9 ($R^2 = 0.448$, $P = 0.0485$). Those patients with a higher functional avidity to LPPVVAKEI correlated with a lower viral load.

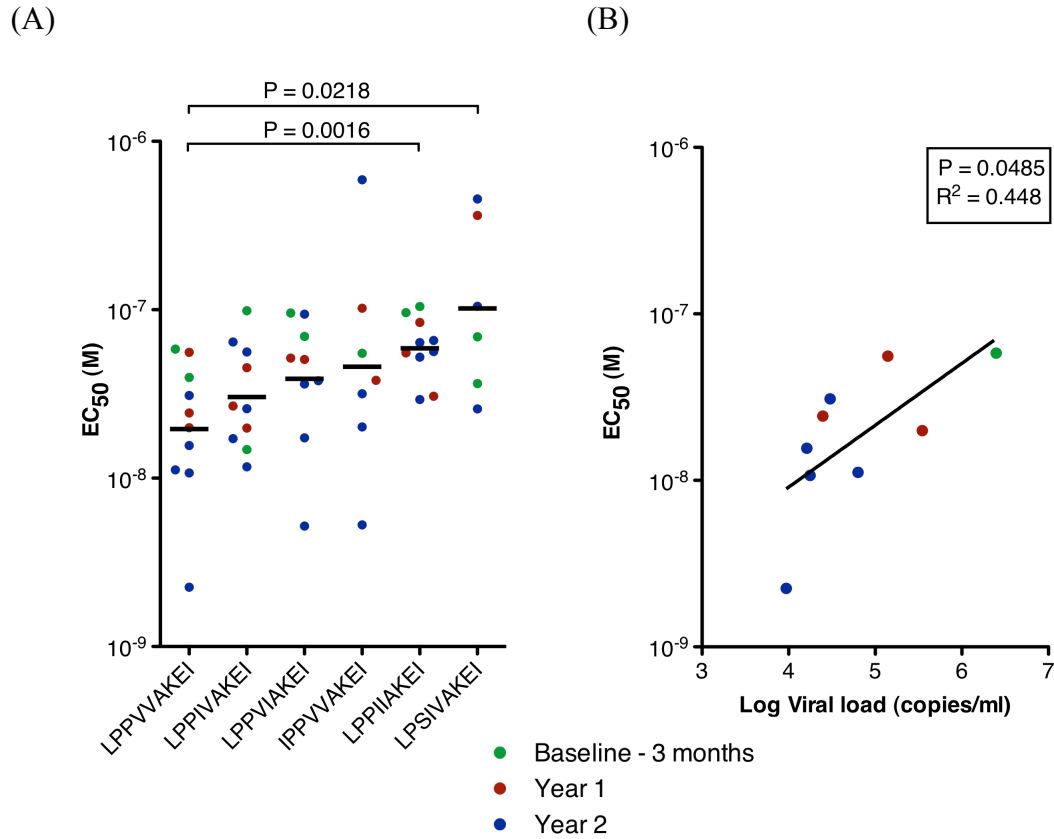


Figure 3.13 (A) Functional avidity of HLA-B*51-restricted LI9- and variant-specific CTL. The geometric mean is shown as a horizontal bar for each variant. (B) Functional avidity of HLA-B*51-restricted LPPVVAKEI versus viral load. PBMCs from subjects with positive responses were analysed. EC_{50} values were only included if the $R^2 > 0.8$ and the curve had converged.

3.3 Case studies

The sequencing results shown in Table 3.2 and Table 3.3 are of the dominant variants in the viral quasispecies. During the analysis of these population sequences, ambiguous bases were often detected, especially within the LI9 epitope of the HLA-B*51 positive

subjects. It is highly probable minor variants are present in samples that would only be detected using other sequencing methods. Clonal sequencing on some of these patients was undertaken.

*3.3.1 Emergence of LPPVIAKEI and LPSVIAKEI in a HLA-B*51 positive subject*

Subject K151 is male and was ~36 years of age when infected with HIV-1 subtype B. His viral load (RNA copies/ml) and CD4 count (cells/ μ l) were at baseline: 40,338 and 486; at year 1: 350,074 and 349; and at year 2: 227,438 and 310, respectively. HLA typing revealed him as HLA-A*0201 homozygous, HLA-B*5101 and HLA-B*4101.

Direct PCR population sequencing of integrase for K151 showed: at baseline, LPPVVAK~~E~~I; at month 6, LPPVVAK~~E~~I; at year 1, LPPV~~I~~AK~~E~~I; and at year 2, LPPV~~I~~AK~~E~~I with less intense base peaks altering residues 30, 32 and 35. With the emergence of the V32I polymorphism at year 1 in addition to the mixed bases detected at year 2, clarification of the HIV-1 variants in K151 was of particular interest. Clonal sequencing data were obtained at three time-points for K151 (Table 3.4). Similar results to the population sequencing were observed except at year 2, we were able to note that the P30S polymorphism was expanding within the viral quasispecies (4/29 clones); some other variants may also be present or be *Taq* DNA polymerase-induced error as they were detected in only 1 clone. The LPS~~V~~I~~A~~K~~E~~I variant is not listed on the Los Alamos HIV database (Appendix A).

		28	29	30	31	32	33	34	35	36	Number of Clones
		L	P	P	V	V	A	K	E	I	
K151	Baseline	.	.	.	.	.	.	.	.	.	31/31
	Year 1	.	.	.	.	I	.	.	.	.	25/26
		P	.	.	.	I	.	.	.	.	1/26
	Year 2	.	.	.	.	I	.	.	.	.	22/29
		.	.	S	.	I	.	.	.	.	4/29
		.	.	.	A	I	.	.	.	.	1/29
		.	.	.	.	M	.	.	.	.	1/29
		.	.	.	.	.	.	Q	.	.	1/29

Table 3.4 Longitudinal LI9 clonal sequencing data for subject K151.

An IFN γ ELISpot was performed on PBMCs from subject K151 at year 1 using multiple LI9 peptide variants titrated at serial log dilutions. Unfortunately, the LPSVIAKEI clonal variant was only detected late in this analysis and the variant peptide was not included in the ELISpot assay. Figure 3.14 shows that out of the seven variants examined, a strong response to LPPVVAKEI, LPPIVAKEI and IPPVVAKEI were observed, with IFN γ release even in the presence of 10^{-8} M peptide. The LPPIIAKEI peptide produced an intermediate response in these PBMCs, although no response was observed in the presence of 10^{-8} M peptide; whilst LPPVIAKEI and LPSIVAKEI induced a weak response only in the presence of 10^{-6} M peptide. No positive response was observed with the peptide IPSVVAKEI at any concentration assayed. This data supports the clonal sequencing results: if the transmitted variant to patient K151 was LPPVVAKEI, the strong CTL response to this epitope has driven escape to the simplest escape variant which is the V32I variant. This has further mutated into LPSVIAKEI (4/29 clones), which we predict would produce a similar IFN γ response as IPSVVAKEI or LPSIVAKEI.

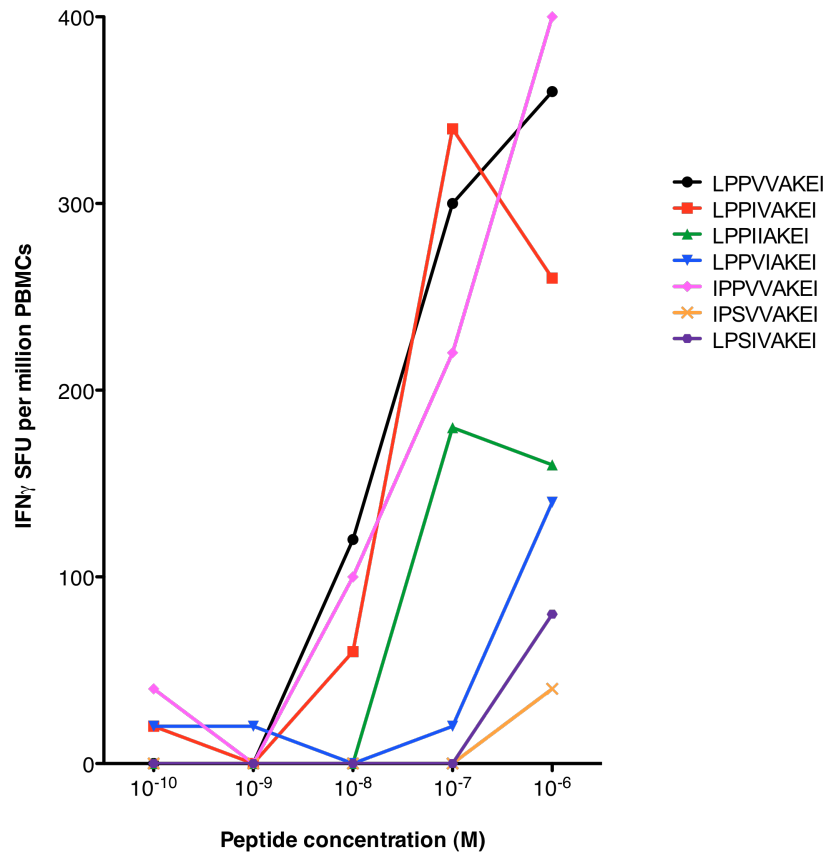


Figure 3.14 IFN γ ELISpot for subject K151 at year 1 using serial log dilutions of LI9 variant peptides. The magnitude of a response is following subtraction of background; a positive response is regarded as >50 SFU/10⁶ PBMCs.

3.3.2 Emergence of LPPIIAKEI as the dominating variant in a HLA-B*51 positive subject

Subject K39 is male (HLA-A*0201, -A*6801, -B*2705, -B*5101) and was ~38 years of age when he contracted HIV-1. His viral load (HIV RNA copies/ml) and CD4 count (cells/ μ l) were: at baseline, 40,200 and 659; at year 1, 11,894 and 611; at year 2, 16,217 and 524, respectively. This is a somewhat unusual participant as although the integrase sequence sits within the subtype B branching of the phylogenetic tree (Figure 3.3) and the Recombinant Identification Program (RIP) designated it as a pure subtype B virus, it

has recently been determined to be a recombinant virus with the Env sequence returning a subtype A designation. It has been kept in for subtype B analysis of integrase.

Direct PCR population sequencing of integrase for subject K39 showed: at baseline, LPP**I**VAKEI; at week 12, LPP**I**VAKEI; at month 6, LPPVVAKEI; at year 1, LPP**I**VAKEI; and at year 2, LPP**I**VAKEI. Mixed base peaks were observed at residues 31 and 32 at baseline and year 1. The dominating variant at year 2 has a dubious mixed base peak at residue 31. Clonal sequencing revealed that the baseline sample has an equal amount of LPP**I**VAKEI and LPP**I**VAKEI (Table 3.5).

		28	29	30	31	32	33	34	35	36	Number of clones
		L	P	P	V	V	A	K	E	I	
K39	Baseline	.	.	.		.	.	.	.	.	14/28
		.	.	.			.	.	.	.	14/28
	Year 1	.	.	.			.	.	.	.	24/30
		.	.	.	.	.	.	.	.	.	5/30
		.	.	.	A	.	.	.	.	.	1/30

Table 3.5 Longitudinal LI9 clonal sequencing data for subject K39.

IFN γ ELISpot was performed on PBMCs from subject K39 both at week 12 and year 1 using multiple LI9 peptide variants titrated at serial log dilutions in duplicate. Figure 3.15 demonstrates that the CTL response becomes stronger over time. At both week 12 and year 1, a strong response to LPPVVAKEI, LPP**I**VAKEI, LPP**I**VAKEI, LPPV**I**AKEI and LPS**I**VAKEI were observed, with EC₅₀ values between 10⁻⁸ and 10⁻⁷ M. An IFN γ -producing response to **I**PPVVAKEI was only seen at the higher peptide concentrations, and no positive response to **I**PSVVAKEI was observed.

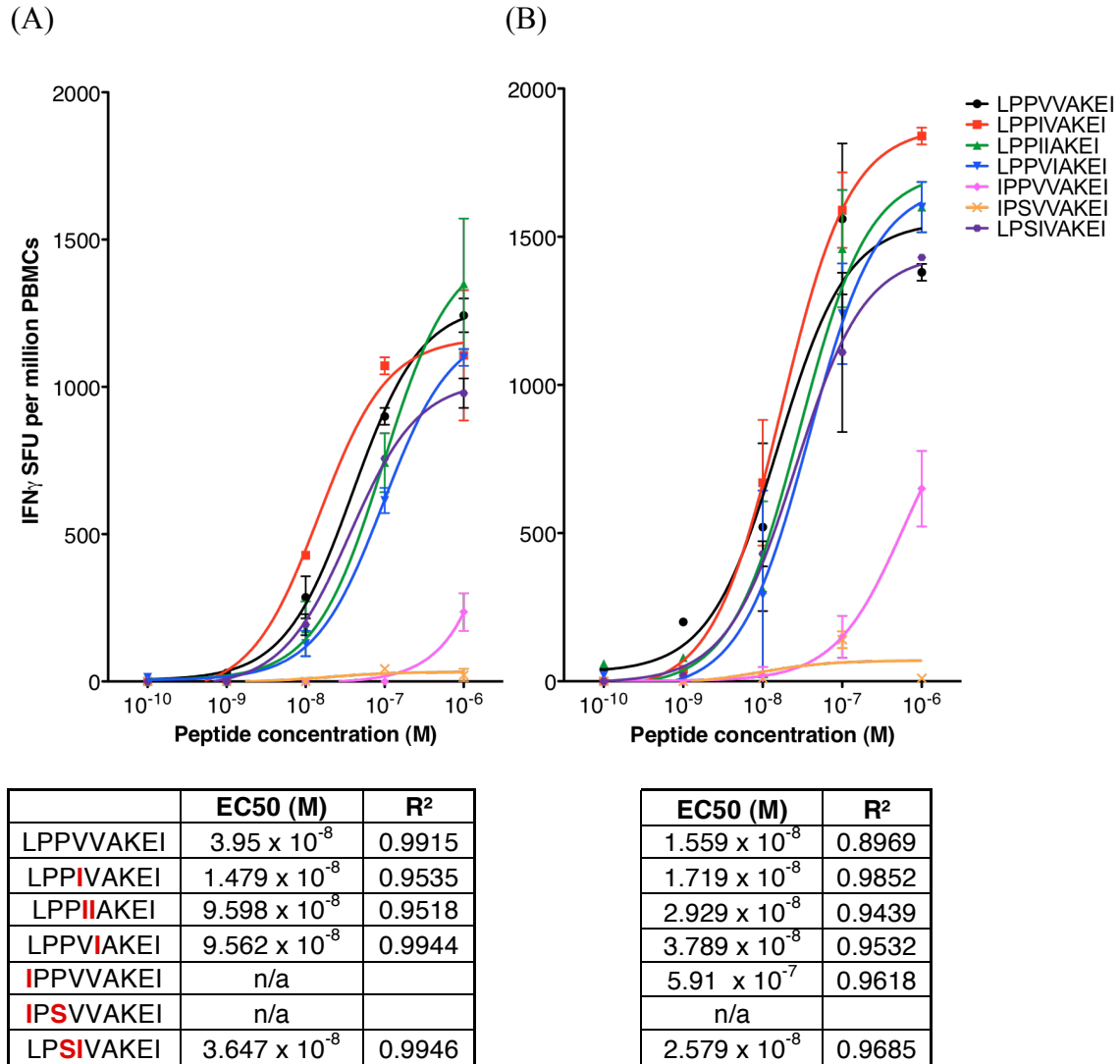


Figure 3.15 IFN γ ELISpot for subject K39 at (A) week 12 and (B) year 1 using serial log dilutions of LI9 variant peptides. The magnitude of a response is following subtraction of background. Each well was performed in duplicate except for (B) at concentrations 10^{-9} and 10^{-10} due to a lack of PBMCs. Error bars are SEM. EC₅₀ values and R² values are given for the curves that have converged.

In order for CTL escape to occur in subject K39, the only true escape variant would be **IPSVVAKEI**, and to a lesser degree **IPPVVAKEI**. We can at this time only postulate as to why **IPSVVAKEI** and **IPPVVAKEI** were not found to emerge within subject K39.

Perhaps some unidentified compensatory mutation(s) are required for the L28I mutation to occur. No L28I polymorphism in the dominating variant was seen over time in any of the HLA-B*51 positive patients analysed; those with this polymorphism had it present at baseline. Even in subject P32 in Table 3.1, this L28I polymorphism only emerged after at least 18 months and from the LPPV**I**AKEI variant.

*3.3.3 Emergence of P30X as the dominating LI9 variant in a HLA-B*51 positive subject*

Subject M084439 is male and 26 years of age when infected with HIV-1 subtype B. His viral load (RNA copies/ml) and CD4 count (cells/ μ l) were at baseline: 40,558 and 600; at year 1: 40,358 and 500; and at year 2: 16,763 and 720. HLA typing revealed him to be HLA-A*0301, -A*2402, -B*4402 and -B*5101.

Direct PCR population sequencing showed: at baseline, LPP**I**AKEI with mixed peaks at P3, P4 and P5; at year 1, LP**S**I**I**VAKEI with mixed peaks at P3; at year 2, LP**S**I**I**VAKEI; and at year 3.5, LP**H**VV**I**AKEI. P30H had not been previously detected (Appendix A) and so we were keen to determine how this mutation emerged. Clonal sequencing was carried out on baseline and year 1 samples (Table 3.6). Whilst the dominating variant at baseline was LPP**I**AKEI, LP**S**I**I**VAKEI was found to a somewhat lesser extent and LP**H**I**I**VAKEI already present. By year 1, LP**S**I**I**VAKEI and LP**H**I**I**VAKEI were the only variants observed, and we postulate that LP**H**VV**I**AKEI emerged from LP**H**I**I**VAKEI and outgrew LP**S**I**I**VAKEI at a time-point between year 2 and 3.5.

		28 L	29 P	30 P	31 V	32 V	33 A	34 K	35 E	36 I	Number of clones
M084439	Baseline	.	.	.	I	I	.	.	.	.	13/30
		.	.	S	I	.	.	.	.	.	10/30
		.	.	H	I	.	.	.	.	.	4/30
		.	.	.	.	.	.	.	.	.	2/30
		.	.	.	.	.	R	.	.	.	1/30
	Year 1	.	.	S	I	.	.	.	.	.	24/30
		.	.	H	I	.	.	.	.	.	6/30

Table 3.6 Longitudinal LI9 clonal sequencing data for subject M084439.

Unfortunately the only PBMCs still available for this subject was at 1 month post-baseline; the IFN γ -ELISpot response to all the peptides were very weak at 10⁻⁶ M and only those against LPP**I**VAKEI, LPP**I**IAKEI, LPPVVAKEI were just on the border of a positive response; a strong positive response to PHA was observed.

3.3.4 Reversion of the IPSVVAKEI variant in a HLA-B*51 negative subject

Subject M103765 is male and was 30 years of age when infected with HIV-1 subtype B. HLA typing revealed as HLA-A*0201, -A*0301, -B*0702, and -B*4402. His viral load (RNA copies/ml) was: at baseline: 317,155; at year 1: 43,740; and at year 2: 233,250.

Direct PCR population sequencing of integrase for M103765 showed: at baseline, **I**PSVVAKEI; at year 1, **I**PSVVAKEI; and at year 2, **I**PPVVAKEI. With the S30P polymorphism at year 2, we wanted to assess the rapidity of this reversion. Clonal sequencing data were obtained at three time-points (Table 3.7). Similar results to the population sequencing were observed with 30P in 0/20 clones at baseline, and 5/32 clones at year 1; by year 2, no 30S variant remained.

		28	29	30	31	32	33	34	35	36	Number of clones
		L	P	P	V	V	A	K	E	I	
M103765	Baseline	I	.	S	.	.	.	.	.	.	19/20
		I	.	S	.	.	.	E	.	.	1/20
	Year 1	I	.	S	.	.	.	.	.	.	27/32
		I	.	.	.	.	.	.	.	.	4/32
		I	.	.	A	.	.	.	.	.	1/32
	Year 2	I	.	.	.	.	.	.	.	.	30/32
		I	S	.	.	.	.	.	.	.	1/32
		I	.	.	.	.	.	.	.	V	1/32

Table 3.7 Longitudinal LI9 clonal sequencing data for subject M103765.

There may be two explanations for this S30P mutation: (i) it is reversion of a B*5101-driven mutation in a disparate HLA host; (ii) it is a mutation in a B7 supertype host that may or may not recognise the LI9 epitope. Of the other 21 HLA-B*0702 individuals in this early HIV-infected cohort, no change in dominant variant was observed from baseline to year 1, and of the 14 individuals with a sequenced year 2 sample, again no change in the dominating variant was observed. An IFN γ ELISpot assay was carried out on 3-month and year 1 PBMCs with the following peptides: LPPVVAKEI, LPPIVAKEI, LPPIIAKEI, LPPVIAKEI, IPPVVAKEI, IPSVVAKEI and LPPIVAKEI. No positive response at 10⁻⁶ M was observed against any of the peptides at both time-points; a strong positive response to PHA was observed.

The only other participant (HLA-A*0201 homozygous, HLA-B*1501, and -B*4402) of the SPARTAC study with 30S at baseline was infected with a circulating recombinant form. The LPSVVAKEI variant dominated at baseline and month 6; however, at year 1, the S30P mutation had occurred according to direct PCR population sequencing.

Discussion

An effective HIV CTL vaccine that significantly lowers the viral load set point and delays progression to AIDS has not been achieved despite decades of research and clinical trials. Its success is dependent on the antigens included to generate a HIV-specific response. By studying HIV epitopes restricted by HLA alleles associated with slow progression to AIDS, we may gain a better understanding of how CTL escape and reversion influence viral fitness and progression. With the majority of CTL research centred on Gag epitopes, there has been a neglect of individual epitopes in Pol and other HIV genes that may be equally as important. We focused on an integrase epitope restricted by an HLA allele associated with slow progression to AIDS – the HLA-B*51-restricted LI9 epitope.

Most research on LI9 CTL escape has concerned integrase residue 31, position 4 (P4) of the epitope, with I31V branded a negatively associated subtype B polymorphism (Leslie et al., 2005). The paper concluded that LI9/31V is a HLA-B*51 selection pressure-driven escape variant; however, this was deduced from sequencing data alone and the statement implies that LPPVVAKEI has escaped from LPPVVAKEI to become the subtype B consensus. This emphasis on residue 31 has overshadowed other HLA-B*51-associated LI9 polymorphisms that may have a significant impact on the immunodominant LI9 cellular response. We aimed to fully define LI9 variants that evade CTL recognition. We hypothesised that mutations at P1, P3 and/or P5 would weaken or prevent CTL recognition, and so we predicted escape would be observed at

these positions in HLA-B*51 positive patients and reversion in HLA-B*51 negative patients in early HIV infection.

By sequencing 118 acute/early HIV subtype B-infected subjects at baseline, LPP**I**VAKEI was found to be significantly associated with HLA-B*51 expression. The two HLA-B*51 positive subjects with LPP**I**VAKEI at baseline were sampled two years apart and do not sit close to each other on the phylogenetic tree. This association at baseline indicates that either this variant was transmitted or escape had occurred prior to baseline sampling. In this cohort, 'baseline' samples actually represent any new infection up to 6 months. This is past peak viraemia, when the CTL response initiates, and so we may have missed the transmission sequences of these HLA-B*51 positive patients.

A corresponding 93/118 samples at year 1 were sequenced. Whilst the LPP**I**VAKEI variant did in fact disappear from dominance in the 10 HLA-B*51 positive individuals, there was not an increase in LPPVVAKEI. Instead, the emergence of LPPV**I**AKEI and LPS**I**VAKEI was observed, with a significant association between HLA-B*5101 expression and LI9 mutation involving a change at P3 and P5, but not at P1, over one year. In addition, polymorphisms at P3 and P5 were observed in three other HLA-B*51 positive patients at time-points past year 1. LP**H**I**V**AKEI and LP**S**V**I**AKEI were also variants detected through clonal sequencing; these and **I**PSVVAKEI had not been previously noted on Los Alamos.

In this smaller cohort of early HIV-infected individuals, we did not find that I31V was negatively associated with HLA-B*51 expression. Supporting this finding, Bhattacharya et al. found that the interpretation of the Leslie et al. data were confounded by viral lineage founder effects. The paper outlines multiple pieces of evidence against the hypothesis that 31V is an HLA-associated escape mutation that has been selected over time due to the frequency of HLA-B*51 in a given population. Firstly, this so-called CTL escape mutation was in the founder virus of subtype B, i.e., 31V has dominated the subtype since the earliest ancestor. Secondly, the frequency of both 31V and 31I has remained constant over the years. Comparing samples grouped from 1983 to 1989, with samples grouped from 1990 to 2004, there has been no statistically significant change in the frequency of 31V/31I ($P = 0.30$). And lastly, 31I dominates in Thailand, 18/21 (85.7%) of CRF01 patients, despite a high frequency of the HLA-B*51 allele in the population; Thailand has a HLA-B*51 frequency of 10%, which is comparable to the UK (Bhattacharya et al., 2007).

In 2009, a paper was published by Kawashima et al. that showed HIV-1 is adapting to the HLA-B*51-restricted TI8 response in populations of high HLA-B*51 prevalence, and that LI9-I31V (%) correlated with HLA-B*51 prevalence ($P = 0.001$) (Kawashima et al., 2009). Although data from the London acute study (HLA-B*51 prevalence ~12%) was available for TI8, it was unavailable for LI9. From the 118 patients sequenced in this study, 89 patients (75.4%) have the I31V variant dominating; this fits well with the model of HIV-1 LI9 residue 31 adapting to HLA-B*51. However, as with the Leslie et al. 2005 paper, the Kawashima LI9 data is based on cross-sectional sequencing data and is irrespective of other LI9 polymorphisms. So although the I31V

variant may be present in populations with a greater prevalence of HLA-B*51, the positive IFN γ response to both the LPPVVAKEI and LPP**I**VAKEI peptides in this study indicates that it is not the I31V polymorphism alone that is driving LI9 escape. In addition, Figure 3.7 shows that in this acute/early cohort, there was no significant difference in the proportion of patients with 31V between HLA-B*51 positive and negative subjects; in fact a higher proportion on HLA-B*51 negative subjects had the 31V variant at both baseline and year 1. This is the opposite of what was found when looking at TI8 in the acute cohort. If both these HLA-B*51-restricted immunodominant epitopes do not revert often (Duda et al., 2009), then it is surprising that the I31V variant is found in 77.1% of the HLA-B*51 negative patients in the cohort whilst TI8-I135X is found in 42%.

Reversion of the P30S polymorphism was observed in the only HLA-B*51 negative HIV subtype B-infected individual with 30S in this study prior to year 1 sampling, and in a HLA-B*51 negative HIV non-subtype B-infected subject at year 1 post-study. This indicates that there is an associated fitness cost with the P30S polymorphism that results in reversion when HLA-B*51 pressure is absent. The lack of the LI9 variants LPPV**I**AKEI, LPS**S**VIAKEI, LPP**I**IAKEI, LPS**I**VAKEI and LP**H**I**I**VAKEI in HLA-B*51 negative individuals, and the reversion of **I**PSVVAKEI to **I**PPVVAKEI, indicates that HLA-B*51 pressure selects for a multitude of LI9 variants that may ultimately lead to LI9 CTL escape although result in a virus with a reduced replication capacity. Of note, the 30A and 34R variants observed in HLA-B*51 negative subjects (1 and 2 patients, respectively) remained stable for up to 2 years; no further time-points were available.

These HLA-B*51-associated polymorphisms at P3 and P5 led us to undertake IFN γ ELISpots to confirm which variants were capable of inducing a cellular response. A positive response to IPSVVAKEI was a rare occurrence and when found, it was always a weak response; mean responses to LPSIVAKEI at all time-points and peptide concentrations were <500 SFU/10⁶ PBMCs. In addition, the magnitude of the CTL response to LPPIIAKEI at year 2 (peptide concentrations 10⁻⁷ and 10⁻⁸ M) was significantly lower than that for LPPVVAKEI.

The functional avidity of CD8⁺ T cells matures during the early stages of viral infection (Slifka and Whitton, 2001). We found no significant difference between functional avidity at baseline, year 1 and year 2. In fact, of the two patients with EC₅₀ values determined at two different time-points, there was no clear trend of the later time-point having a higher functional avidity. This indicates that either the functional avidity of LI9-specific CTLs was fully mature by the time of sampling, or they may need a longer period to develop during HIV infection. In general, the magnitude of response to LPPIVAKEI, LPPVIAKEI and IPPVVAKEI were good; when functional avidity was assessed, these variant LI9 peptides had EC₅₀ values that were not significantly different from LPPVVAKEI. Only responses to LPPIIAKEI and LPSIVAKEI had greater EC₅₀ values than wildtype LI9, implying that they have a significant lower functional avidity and may be less well recognised by LI9-specific CTLs. The overall EC₅₀ values are lower than those reported against HLA-B*27 epitopes (Almeida et al., 2007, Payne et al., 2010); there is a stronger functional avidity to LI9 and its variants. As viral load is a predictor of progression to AIDS, the fact that we found that functional avidity to

wildtype LI9 inversely correlates with viral load, suggests that strong CTL recognition of LI9 is associated with a lower viral load.

According to previously published papers and Los Alamos, LI9 is an overlapping epitope with MASDFNLPPV (MV10; *pol* 737-746, integrase 22-31) and RAMASDFNL (RA9; *pol* 735-743, integrase 20-28). These peptides were found to be intermediate binders of the A2 supertype. However, IFN γ and cytotoxic responses were only produced by memory resting cells following *in vitro* expansion with 10 μ M peptide stimulation and IL-2; no memory effector CD8⁺ response was detected for both these peptides in an IFN γ ELISpot assay (Propato et al., 2001). The predicted HLA-A*0201-restricted epitope RA9 was used to generate a weak CTL response in HIV-1-naïve individuals (Reche et al., 2006); there has been no data on this individual peptide in terms of cytotoxic activity, nor responses to this peptide in HIV-infected donors. In this early HIV-1 subtype B-infected cohort of 93 longitudinally-sampled patients, there are 55 HLA-A*0201 positive patients, with 8 HLA-A*0201 homozygous; no change in LI9 was observed in HLA-A*0201 positive/HLA-B*51 negative individuals except the reversion found in patient M103765.

In addition, the LI9 epitope has been found to be recognised by the B7-supertype alleles HLA-B*4201, HLA-B*0702 (Leslie et al., 2006) and HLA-B*0705 (Kiepiela et al., 2007). In this current study, only one subject was HLA-B*4201 positive and had the subtype B consensus LI9 sequence at baseline, year 1 and year 2 with no mixed bases

according to direct PCR population sequencing. There were no HLA-B*0705 individuals in this cohort and HLA-B*0702 has been evaluated in chapter 3.3.4.

We hypothesised that polymorphisms at P1, P3 and/or P5 may abrogate CTL recognition by no longer binding with the TCR, and after gathering data from a longitudinally-sampled, early HIV-infected cohort, we conclude that the main factor in HLA-B*51-associated LI9 escape is P3. Due to the fitness cost of mutating P3, compensatory mutations within the epitope at P1, P4 or P5, or unidentified mutations outside the epitope, may be required. Unfortunately, we did not assess the response of the PBMCs to LP**S**V**I**AKEI (as seen in patient K151) or LP**A**V**I**AKEI (as seen in patient P32). Escape of CTL recognition at P3 of an epitope is also observed in the HLA-B*57/5801-restricted epitope TW10, resulting in the T242N mutation (Table 1.1). Similarly to the reversion we have seen with the P30S mutation in LI9, reversion of the T242N mutation in an HIV-infected HLA-B*57/5801 negative subject was observed at an early time-point of infection (Leslie et al., 2004).

In summary, we propose that LI9 would be a good candidate to be included in an HIV CTL vaccine as there is generally a positive response to the four most frequent subtype B LI9 variants of a strong magnitude, plus it is difficult to gain full LI9 escape in early HIV infection. With the reversion of P30S in HLA-B*51 negative subjects, LI9 and variants that induce strong CTL responses would persist in circulating viral sequences and so may prove beneficial in HIV vaccine design.

CHAPTER 4

Difficulty of HIV-1 escape from CD8⁺ T cells expressing enhanced TCR *in vitro*

Introduction

From the studies of the 1990s, adoptive transfer of *ex vivo*-expanded autologous HIV-1-specific CTLs into HIV-1-infected individuals had minimal effects on clinical outcome. Many factors may have contributed to these results. HIV-specific CD8⁺ cells are unable to proliferate well *ex vivo* without IL-2 or CD4⁺ T_H cells (Lichterfeld et al., 2004), and so may be required to be infused as part of the adoptive therapy.

Failure may be due to rapid apoptosis of the infused cells. This was observed when two CD8⁺ CTL clones, against the HLA-A*0201-restricted epitopes SLYNTVATL (SL9, *gag* p17 77-85) and VIYQYMDDL (VL9, *pol* 334-342, RT 179-187), were expanded from an AIDS patient and infused back. There was no significant change in CD4/CD8 count or viral load. Staining of the CD8⁺, HLA-A*02-SL9 tetramer⁺ PBMCs showed that these cells were rapidly apoptosing, with 92.3% staining Annexin-V positive after 48 h with the CTLs infused compared to 29.3% before infusion (Tan et al., 1999).

Failure of HIV adoptive therapy may also be due to CTL-induced viral escape from the infused cells. When autologous CD8⁺ CTL clones against the HLA-A*0301-restricted epitope QVPLRPMTYK (QK10, *nef* 73-82) were infused into an AIDS patient, the

primary objective of prolonging survival by delaying clinical progression was not achieved. Viral sequencing was carried out both before and after the CTL infusions. Prior to adoptive transfer, the patient carried HIV variants with the sequences QVPLRPMTYK and QIPLRPMTYK; the V74I polymorphism was not predicted to abrogate CTL recognition. Following adoptive transfer, viral clones from plasma and cultured CD4⁺ cells showed a diverse amount of *nef* deletions within the QK10 epitope (Koenig et al., 1995). It appears that adoptively transferred CTL pressure selected escape mutant variants.

So although the concept of adoptively transferring *ex vivo*-expanded autologous HIV-1-specific CTLs was valid, an advanced approach was necessary to produce a significant effect on clinical outcome. Cancer studies that manipulated antigen-specific TCRs and transfected these into lymphocytes provided the starting point for further HIV studies. TCRs that recognise tumour-associated antigens (TAAs), such as the HLA-A*02 restricted TAAs MART-1, gp100 and NY-ESO-1, may be isolated, cloned into retroviral vectors, and transduced into primary T cells obtained from patients. An example of this type of therapy can be seen with MART-1 TCR-transduced cells. The TCR α and β chain genes from a MART-1-specific CTL were isolated from a metastatic melanoma patient that had displayed tumour regression, and cloned into a retroviral vector ready for transduction into primary T cells. Out of 15 HLA-A*0201 positive patients with progressive metastatic melanoma that were infused with MART-1 TCR-transduced autologous cells, 2 patients had sustained tumour regression and were clinically disease-free for over 20 months; both had persisting TCR-transduced cells detected at one year postinfusion (Hughes et al., 2005, Morgan et al., 2006). As not all

15 patients had tumour regression, optimisation of the transduction procedure to obtain higher levels of TCR gene expression and/or use of a higher affinity TCR may improve the success rate.

The first attempt of transducing HIV-specific TCRs into cells was in 2000 where different levels of TCR transduction efficiency were achieved; this TCR was directed against the HLA-A*03-restricted epitope RLRPGGKKK (RK9, *gag* p17 20-28). Specific lysis of HIV-infected HLA-A*03⁺CD4⁺ Jurkat cells was dependent on TCR expression. At an E:T ratio of 1:1, high (96%), intermediate (94%) and low (65%) expressed TCR-transduced CD8⁺ cells led to ~90%, 70% and 50% specific lysis, respectively; at an E:T ratio of 1:10, ~40%, 25% and 15% specific lysis were observed, respectively (Cooper et al., 2000). The difficulty of transducing cells to the highest TCR expression status was not detailed in the paper, although we might suppose it to be difficult given that no follow-up ensued.

In 2004, the sequential transduction of TCR α and β chains specific for the HLA-B*3501-restricted epitope IPLTEEAEL (IL9, *pol* 448-456, RT 293-301) into CD8⁺ cells resulted in lysis of a HIV_{LAI}-infected CD4⁺ EBV-transformed B cell line. Although an *in vitro* positive outcome, >15% specific lysis was only seen at an E:T ratio of 8:1. The specificity of the TCR-transduced cells in comparison to the parental CTL clone was assessed by coculturing with cells peptide-pulsed with natural variants of the epitope: **V**PLTEEAEL, **A**PLTEEAEL and ILPT**K**EAEL; the TCR-transduced cells recognised to a similar degree only the same variant peptides as the parental clone (Ueno et al., 2004).

These early studies were inefficient as separate retroviral vectors containing each TCR chain (one for α and one for β) needed to be transduced. To resolve this issue, the self-cleaving peptide 2A was introduced between the TCR chain genes enabling insertion into a retroviral vector as a single transcript. Once translated, peptide 2A causes cleavage which produces equal amounts of TCR α and β chains (Szymczak et al., 2004). This simpler methodology was used to transduce HIV-specific TCRs in 2008. Joseph et al. describe the efficient transduction of TCR genes into CD8⁺ cells leading to the production of CTLs with HIV-1 inhibitory activity. A TCR specific for HLA-A*0201-SL9 was isolated from a CTL clone and the α and β chains cloned into a lentiviral vector as a single transcript linked by peptide 2A; the resultant K_d value of the TCR-transduced cells was 6×10^{-10} M. When T2 cells were peptide pulsed with 1 μ M SL9 or ILKEPVHGV (HLA-A*02-restricted IV9, *pol* 464-472, RT 309-317) and incubated with the TCR-transduced CD8⁺ cells at an E:T ratio of 50:1, ~50% lysis was observed against the SL9-pulsed cells; ~10% lysis was seen when incubated with the IV9-pulsed T2 cells (Joseph et al., 2008). This large E:T ratio that resulted in only ~40% specific lysis above control-pulsed cells at a high peptide concentration would not translate clinically well.

Later that year, Varela-Rohena et al. published an innovative paper that received media headlines such as “‘Assassin’ cells home in on HIV” (BBC, 2008). This group had enhanced an HIV-specific TCR, transduced it into CD8⁺ cells and produced significant *in vitro* specific lysis. Using phage display (Appendix D) on the SL9-specific CTL line from patient 868 (HLA-A*02, -A*24, -B*27, -B*35), a natural, wildtype TCR (wt;

bearing the TCR chain V β 5) was isolated that bound to the SL9 antigen with a K_d value of 1.43×10^{-7} M and a binding half-life of <1 min. This TCR in itself was of a higher affinity than most TCRs binding HLA-peptide complexes which is usually $\sim 10^{-5}$ M (Matsui et al., 1991, Weber et al., 1992). Site-directed mutagenesis of the CDR in both the TCR α and β chains resulted in the production of the high affinity TCR a11b6 with a K_d value of $<4 \times 10^{-10}$ M and a binding half-life >2.5 h. Wildtype and a11b6 TCR-transduced primary CD8⁺ cells were >85% SL9-tetramer positive and 40% V β 5 positive (Varela-Rohena et al., 2008).

To assess the cytotoxicity of these TCR-transduced cells, PHA-activated HLA-A*02⁺ purified CD4⁺ T cells were infected with HIV_{NL4-3} (X4 strain; SLYNTIAVL) and cocultured with non-, wt or a11b6 TCR-transduced CD8⁺ cells for 8 days at an E:T ratio of 1:20: 47%, 39% and 7.3% of the viable CD8⁺ cells were p24⁺, respectively. In a similar assay but using HIV_{Bal} (R5 strain; SLYNTVATL), 11.1%, 9.2% and 2.2% of the cells were p24⁺, respectively. Although the wt TCR-transduced cells had some cytotoxic effect ($\sim 17\%$ and 17% against HIV_{NL4-3}- and HIV_{Bal}-infected cells, respectively), the high-affinity a11b6 TCR-transduced cells were far superior at killing their target ($\sim 85\%$ and 80% against HIV_{NL4-3}- and HIV_{Bal}-infected cells, respectively) at this low E:T ratio (Varela-Rohena et al., 2008).

As already observed by Ueno et al., a wt TCR-transduced cell can only recognise the same variants as its parental CTL clone (Ueno et al., 2004). Targeting SL9 is complicated by the fact that the epitope has many natural polymorphisms (Table 4.1);

an ideal SL9-specific TCR would recognise all of these variants. The TCR chosen by Joseph et al. from CTL clone 18030D23, was found to not recognise SLYN**L**VAVL variants and barely suppressed viruses containing SL**F**NTVATL or SLYNT**I**AVL (Yang et al., 2003). Originating from patient 868 (proviral sequence: SLYNT**I**AVL, 11/12 clones; SLYNT**I**ATL, 1/12 clones) the 868 CTL line is able to recognise many natural variants of the SL9 epitope. Chromium release assays involving these CTLs and EBV-transformed B cells pulsed with natural variants of SL9 at an E:T ratio of 3:1 showed that a strong CTL response (specific lysis at peptide concentrations <1 nM) was induced by: SL9, 6I8V, 6I and 3F6I peptide-pulsed cells. Only at the higher peptide concentrations (>10 nM) did the following trigger specific lysis: 3F8V, 3F, 3F6I8V, 3S, 3L, 3C and SLYAAAAAL; 3F5A, 3H, 3H5A8V and SLYGGGGGL peptide-pulsed cells elicited no CTL response (Sewell et al., 1997, Whelan et al., 1999, Goulder et al., 1997b).

Variant	S	L	Y	N	T	V	A	T	L	LANL Subtype B	
										n	%
SL9	.	.	.	.	.	.	.	.	.	269	27.8
3F	.	.	F	.	.	.	.	.	.	133	13.7
8V	.	.	.	.	.	.	.	V	.	123	12.7
6I8V	.	.	.	.	.	I	.	V	.	71	7.3
3F8V	.	.	F	.	.	.	.	V	.	60	6.2
6I	.	.	.	.	.	I	.	.	.	47	4.9
3F6I8V	.	.	F	.	.	I	.	V	.	37	3.8
3F6I	.	.	F	.	.	I	.	.	.	26	2.7
3F5A8V	.	.	F	.	A	.	.	V	.	18	1.9
V2	.	V	.	.	.	.	.	.	.	16	1.7
5A8V	.	.	.	.	A	.	.	V	.	13	1.3
Minor variants (<1% each)										155	16.0
Total n (variants)										968	(101)

Table 4.1 Gag p17 SL9 variants and their frequencies are provided from those listed on the LANL HIV Molecular Immunology Database (Yusim et al., 2009).

The wt and a11b6 TCR-transduced cells were tested for their ability to recognise natural SL9 variants. K562 cells (HLA-A*02⁺) were peptide-pulsed with 50 pg/ml SL9 or the SL9 variants 3F, 3F8V or 3F6I8V. The MIP-1 β and IFN γ response for all the SL9 variants in the presence of either wt or a11b6 TCR-transduced cells were comparable to SL9-pulsed targets. PHA-activated HLA-A*02⁺ purified CD4⁺ T cells were infected with the viral isolates 92BR-028 (SLYNTVATL), 92BR-017 (SLYNTVA**V**L) or 92BR-018 (SL**F**NTVA**V**L) and cocultured with either wt or a11b6 TCR-transduced cells at an E:T ratio of 1:10. 5-20% reduction of p24⁺ cells was observed for all three isolates with the wt TCR-transduced cells; the percentage of p24⁺ cells was reduced to <40% for all three isolates cocultured with the a11b6 TCR-transduced cells. Furthermore, at an E:T ratio of 1:50, the high affinity TCR-transduced cells caused the percentage of p24⁺ cells to be reduced to ~40%, 70% and 50% for isolates 92BR-028, -017 and -018, respectively (Varela-Rohena et al., 2008). Surface Plasmon resonance (SPR) binding analysis showed that the wt and a11b6 TCR are able to recognise the 8 most frequent SL9 variants with K_d values all below 10⁻⁵ M (Table 4.2). Varela-Rohena et al. concluded their paper with the following remark:

“HIV-1 will have to devise new ways to escape from CD8 T cells transduced with supraphysiologic SL9-specific TCR”.

This chapter probes further into the results generated using high affinity TCR-transduced CD8⁺ cells. Our primary objective was to determine whether HIV-1 could escape from these cells capable of recognising all the common variants of SL9. As TCR binding was so strong, we hypothesised that the virus would escape MHC binding by

mutating at an anchor residue, provided this did not result in a defective virus, or escape antigen processing by mutating at a residue in proximity to the epitope. Alternatively, if unable to escape in these ways, the question was whether it is beneficial to the virus to mutate to present epitope variants that are less well recognised than SL9, i.e. would there be a shift in the dominating viral variant in relation to TCR affinity? Utilising the *in vitro* selection pressure assays that have been previously detailed in antiretroviral resistance and CTL escape studies, we aimed to resolve these unknowns. We also wanted to confirm the ability of the TCRs to recognise variants other than those listed in Table 4.2.

SL9 Epitope	wildtype TCR	a11b6 TCR	a868b6 TCR
SLYNTI I ATL (6I)	68 nM	151 pM	2.3 nM
SLYNTVATL (SL9)	150 nM	365 pM	4.9 nM
SLYNTI A V L (6I8V)	167 nM	454 pM	6.3 nM
SLYNTV A V L (8V)	385 nM	1.7 nM	19 nM
SL F NTIATL (3F6I)	1.2 μ M	5.4 nM	69 nM
SL F NTI A V L (3F6I8V)	2.7 μ M	27 nM	239 nM
SL F NTVATL (3F)	2.9 μ M	21 nM	252 nM
SL F NTV A V L (3F8V)	2.7 μ M	69 nM	718 nM

Table 4.2 Kd values of the wildtype and high affinity TCR to SL9 variants as determined by BIAcore SPR binding analysis. The a11b6 TCR has both α and β chains enhanced; the a868b6 TCR has a wildtype α chain and an enhanced β chain (Varela-Rohena et al., 2008). *Adapted by permission from Macmillan Publishers Ltd.*

This wt and a high affinity (a868b6, also known as a6) TCR are currently in a phase I clinical trial by the University of Pennsylvania and the biotechnology company Adaptimmune Ltd. HIV-1-infected HLA-A*0201 positive patients will be given escalating doses of autologous T cells modified with either the wt or a6 TCR; safety and tolerability are the primary objectives being assessed, with antiviral effect and persistence as secondary outcome measures (ClinicalTrials.gov, 2010). By determining how HIV may escape these TCR-transduced cells *in vitro*, we may translate these findings into the clinic, anticipate *in vivo* resistance outcome, and therefore act sooner in its prevention.

Results

We aimed to confirm and expand on the studies conducted by Varela-Rohena et al. using the TCRs from the phase I clinical trial: the wt TCR and the a868b6 TCR. This high affinity TCR has the wildtype α chain (a868) and an enhanced β chain (b6); the TCR will be known throughout this chapter as a6. SPR binding analysis for the a6 TCR is shown in Table 4.2. Adaptimmune transduced and cultured all TCR-transduced cells used in the following assays.

4.1 Effect of genetically modified TCR-transduced cells on HIV-infected PBMCs

The effect of a6 TCR-transduced cells *in vitro* on HIV_{LAI}-infected PHA-activated HLA-A*02⁺ PBMCs was first evaluated. This was dissimilar to Varela-Rohena et al. where purified HLA-A*02⁺CD4⁺ T cells were infected with the X4 strain HIV_{NL4-3}. These discrepancies were purposely made to confirm that the presence of other cell populations would not affect the TCR-transduced cells, and to ensure that the HIV strain had the SL9 subtype B consensus sequence (HIV_{LAI}: SLYNTVATL; HIV_{NL4-3}: SLYNTI**AVL**). As an additional control, influenza-specific TCR (JM22)-transduced cells that recognise the HLA-A*02-restricted epitope GILGFVFTL were included.

The TCR-transduced cells had variable levels of CD8 purity (Figure 4.1): the wt and a6 TCR-transduced cells had a high CD8⁺ cell content (86.7% and 86.3%, respectively); the JM22- and non-transduced cells had a lower CD8⁺ cell purity (54.5% and 68.0%, respectively). These cells all express HLA-A*02.

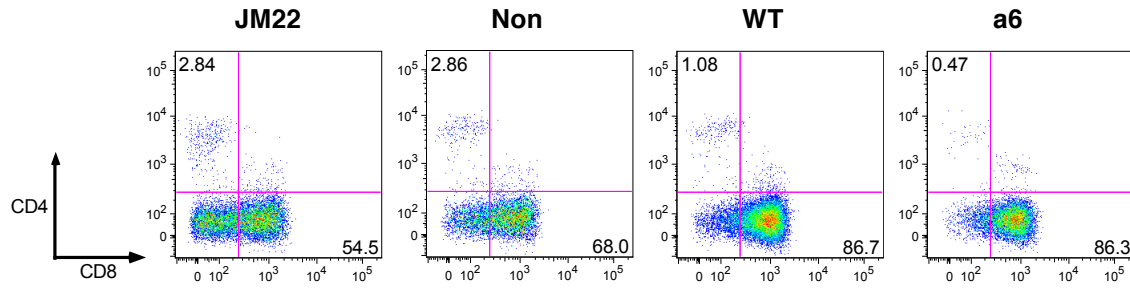


Figure 4.1 CD8 purity of the viable TCR-transduced cells.

The first assay used autologous PBMCs to the TCR-transduced cells. At day 2 postinfection, the TCR-transduced cells were added at an E:T ratio of 1:10, and p24 intracellular staining performed daily until 6 days postinfection. An example of the flow cytometry plots is given in Figure 4.2A. As seen by Varela-Rohena et al., p24 expression was highest in the PBMCs cocultured with non-transduced cells, followed by those with the wt TCR-transduced cells, and the least amount of p24 expression was when cocultured with the high affinity TCR-transduced cells.

The cell cultures were analysed daily by flow cytometry to follow the progress of HIV infection. HIV-infected PBMCs *in vitro* are difficult to gain 100% infection of the CD4⁺ cells compared to cell lines, and so there tends to be a peak day of infection. Figure 4.2B shows that with the control, which was not cocultured with any other cells, the percentage of p24⁺ cells reaches its peak around day 5 postinfection. This was not the case with the PBMCs cocultured with the wt or a6 TCR-transduced cells; the slope of the curves indicates their peak infection may be on day 6 or 7. Therefore, the addition of these SL9-specific cells delayed HIV progression *in vitro*.

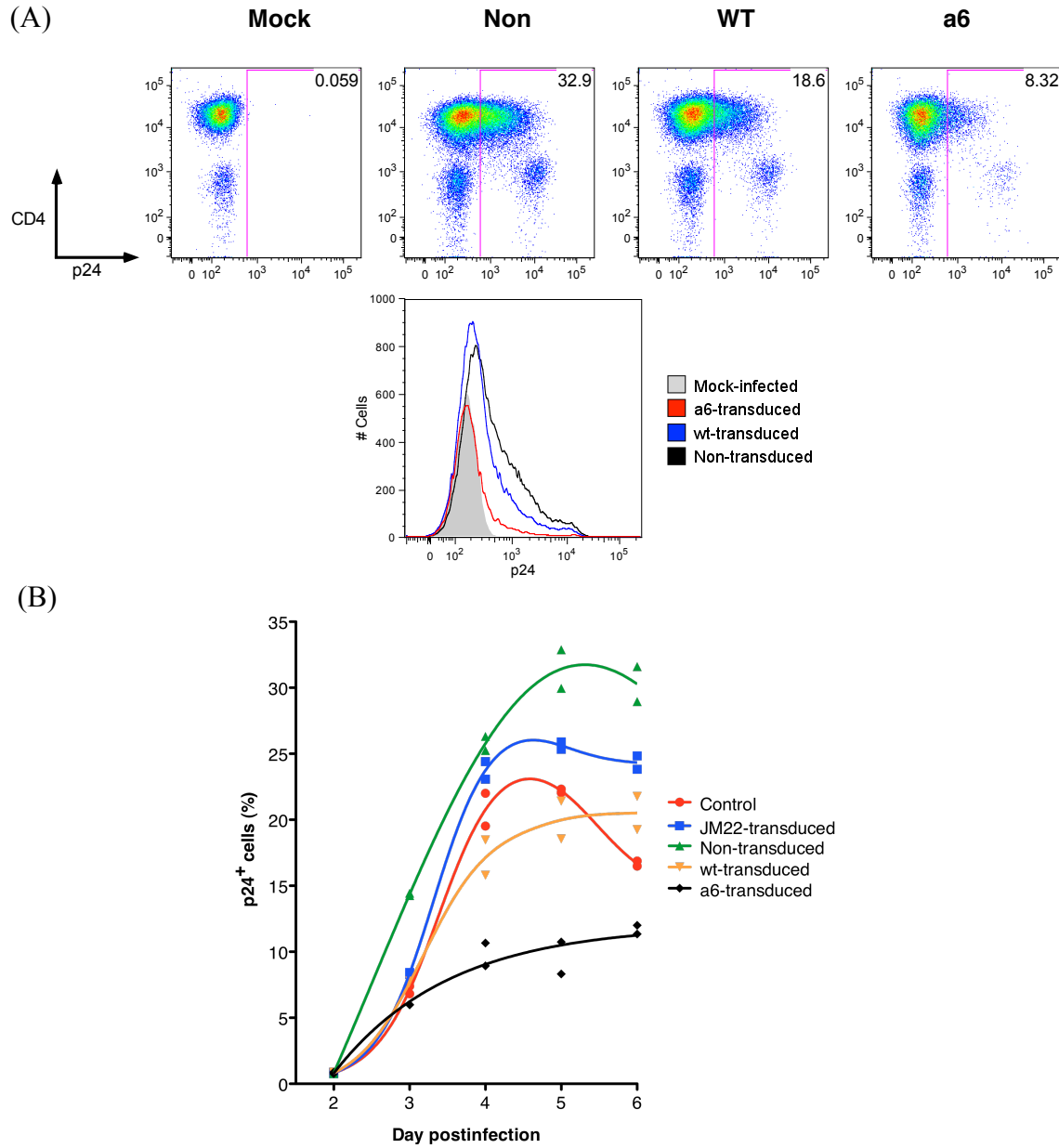


Figure 4.2. Effect of TCR-transduced cells on HIV-1-infected autologous HLA-A*02⁺ PBMCs. (A) Day 5 postinfection stains gated on viable CD14⁻CD19⁻CD3⁺CD8⁻ cells. The ICS p24 expression is compared to the filled histogram showing mock-infection. **(B)** The percentage of p24⁺ cells each day postinfection following the addition of TCR-transduced CD8⁺ cells is shown. The control is HIV-infected HLA-A*02⁺ PBMCs without the addition of any other cells.

Furthermore, although the wt TCR-transduced cells had a minor effect on the magnitude of p24⁺ cells in these PBMC cultures, the high affinity a6 TCR-transduced cells significantly reduced HIV infection. At day 5 postinfection, the mean percentage of p24⁺ cells was 22.1% ± 0.2%, 25.6% ± 0.4%, 31.4 ± 2.1%, 20.0% ± 2.0% and 9.5% ± 1.7% for the control, JM22-, non-, wt and a6 TCR-transduced cells, respectively. This equates to a 36.4% and 69.7% significant reduction in the percentage of p24⁺ cells when comparing the wt and a6 TCR-transduced cells to the addition of non-transduced cells (P = 0.0305 and 0.0074), respectively; there was a 57.0% decrease when a6 TCR-transduced cells were compared to the control infected PBMCs (P = 0.0091). At no time-point was the percentage of p24⁺ cells when cocultured with wt TCR-transduced cells significantly lower than the control infected PBMCs; the a6 TCR-transduced cells lowered the percentage of p24⁺ cells by 52.9% and 30.0% compared to the control infected PBMCs at day 4 and 6 postinfection (P = 0.0184 and 0.0058), respectively.

A surprising result was that coculturing with non- and JM22-transduced cells resulted in a greater proportion of PBMCs becoming infected at peak infection compared to control (P = 0.0243 and 0.0081, respectively). The lack of a high CD8 purity (Figure 4.1) may have provided additional targets for HIV infection, providing a possible explanation for this increase in p24 expression.

The assay was repeated using allogeneic HLA-A*02⁺ PBMCs, i.e. the TCR-transduced cells were not from the same donor as the PBMCs that were infected. HIV_{LAI} infection was slower in these PBMCs with peak infection not determined by the end of the assay (Figure 4.3). However, the data clearly demonstrates that in a similar fashion to that

seen in the infected autologous PBMCs, the a6 TCR-transduced cells have a superior effect on decreasing HIV infection *in vitro*. At day 5 postinfection, the mean percentage of p24⁺ cells was 9.7% ± 0.1%, 9.6% ± 0.7%, 10.8% ± 0.2%, 6.3% ± 0.2% and 3.6% ± 0.7% for the control, JM22-, non-, wt and a6 TCR-transduced cells, respectively. This equates to a 41.9% and 67.2% significant reduction in the percentage of p24⁺ cells when comparing the wt and a6 TCR-transduced cells to the addition of non-transduced cells (P = 0.002 and 0.0046); there is a 35.4% and 63.5% decrease in p24⁺ cells when comparing the wt and a6 TCR-transduced cocultures to the control infected PBMCs (P = 0.0024 and 0.006), respectively.

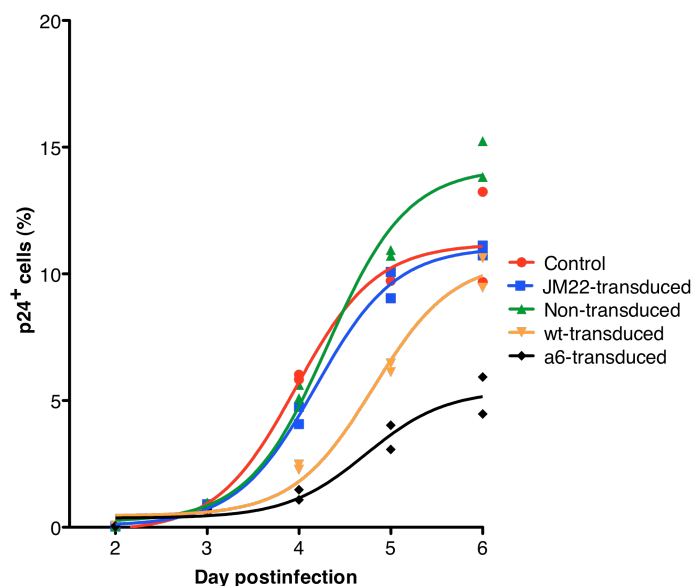


Figure 4.3 Effect of TCR-transduced cells on HIV-1-infected allogeneic HLA-A*02⁺ PBMCs. The percentage of p24⁺ cells each day postinfection following the addition of TCR-transduced CD8⁺ cells gated on viable CD14⁺CD19⁺CD3⁺CD8⁺ cells is shown. The control is HIV-infected HLA-A*02⁺ PBMCs without the addition of any other cells.

As with the autologous cocultures, the addition of the non-transduced cells to the allogeneic HIV-infected PBMCs resulted in a higher percentage of p24⁺ cells at day 5 postinfection compared to control (P = 0.0109); however, there was no significant difference between the control and infected PBMCs cocultured with JM22-transduced cells.

Varela-Rohena et al. found that after 8 days in culture the wt and high affinity a11b6 TCR-transduced cells resulted in a ~17% and 85% decrease in the percentage of p24⁺ cells in HIV_{NL4.3}-infected CD4⁺ cells, respectively, compared to coculture with non-transduced cells. In our current assays, the wt TCR-transduced cells were more effective at controlling *in vitro* HIV infection, and the high affinity a6 TCR-transduced cells was less effective. Differences may be due to the different HIV lab strain used, PBMCs instead of purified CD4⁺ cells as targets, the different time-points analysed, and the fact that our high affinity TCR was enhanced in only one of its TCR chains. Figure 4.2 and Figure 4.3 demonstrate that the β chain-enhanced a6 TCR-transduced cells significantly reduced the percentage of gag⁺ cells in both autologous and allogeneic PBMCs *in vitro*. We surmise that the a6 TCR is a good candidate to have been chosen for the phase I clinical trial.

4.2 *In vitro* selection pressure assays

To predict whether SL9 escape variants will emerge *in vivo* when under pressure from wt or a6 TCR-transduced cells, a set of *in vitro* assays were undertaken. Two different methodologies were employed to select for SL9 escape variants. The first was similar to

many antiretroviral drug selection pressure assays that utilise a long-term culture assay with continuous antiretroviral presence. Detected antiretroviral resistance mutations that have emerged *in vitro* often correlate with those that emerge *in vivo* (Gao et al., 1992). The second was similar to a selection pressure assay where HIV-infected cells were passaged in the presence of CTLs. Detected CTL resistance mutations correlate with the ability of a CTL clone to recognise epitope variants (Yang et al., 2003).

4.2.1 Continuous culture selection pressure assay

The cell line used in this assay was the CD4⁺ 174xCEM.T0 cell line that expresses HLA-A*02. T0 cells were infected with HIV_{LAI} and the TCR-transduced cells added at an E:T ratio of 1:10, 1:5 and 1:1 to provide variable degrees of selection pressure. The TCR-transduced cells were stained to determine CD8 purity and TCR transduction efficiency. The wt and a6 TCR-transduced cells were of a high CD8 purity (>96%) and had high TCR Vβ5 expression (>60%); the JM22- and non-transduced cells were >90% CD8⁺ and had a low percentage of endogenous TCR Vβ5 (<4%) (Figure 4.4). Uninfected T0 cells were added on days 5, 9, 11, 15, 18, and 24 to provide fresh targets for HIV infection. The cells were analysed by flow cytometry at various time-points and viral RNA extracted for sequencing.

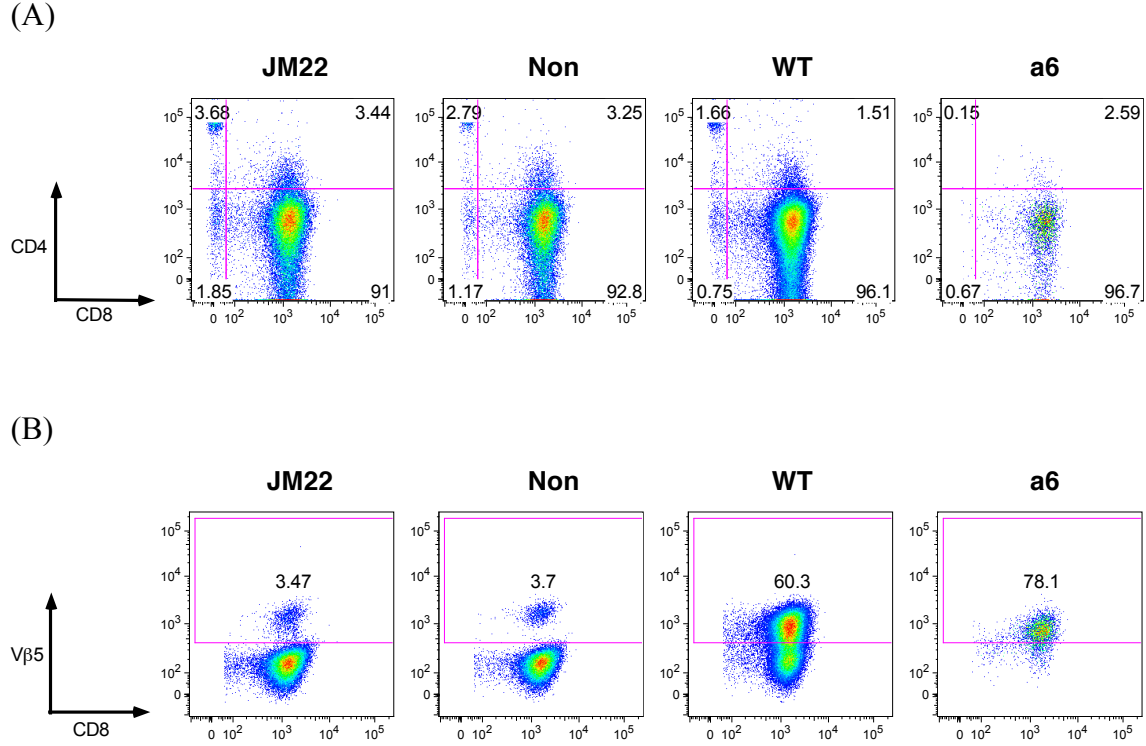


Figure 4.4 TCR-transduced cells prior to coculturing with infected T0 cells. (A) CD8 purity and (B) TCR transduction efficiency as analysed by expression of TCR V β 5. Lymphocytes were gated on viable CD14⁻CD19⁺CD3⁺ cells.

The E:T ratios chosen were aimed to exert enough pressure on the virus to select for escape variants. The a6 TCR-transduced cells were able to control HIV infection in this cell line to a greater degree than expected at all three E:T ratios; the wt TCR-transduced were highly effective at the higher E:T ratios (Figure 4.5A). Figure 4.5B shows that the majority of the cells were p24⁺ on days 8 and 14 postinfection in the absence of TCR-transduced cells and in the presence of JM22- and non-transduced cells. At day 8 postinfection, the percentage of p24⁺ T0 cells was ~55% and 5% when cocultured with wt and a6 TCR-transduced cells, respectively; at day 14 postinfection, ~20% and 28% were p24⁺, respectively.

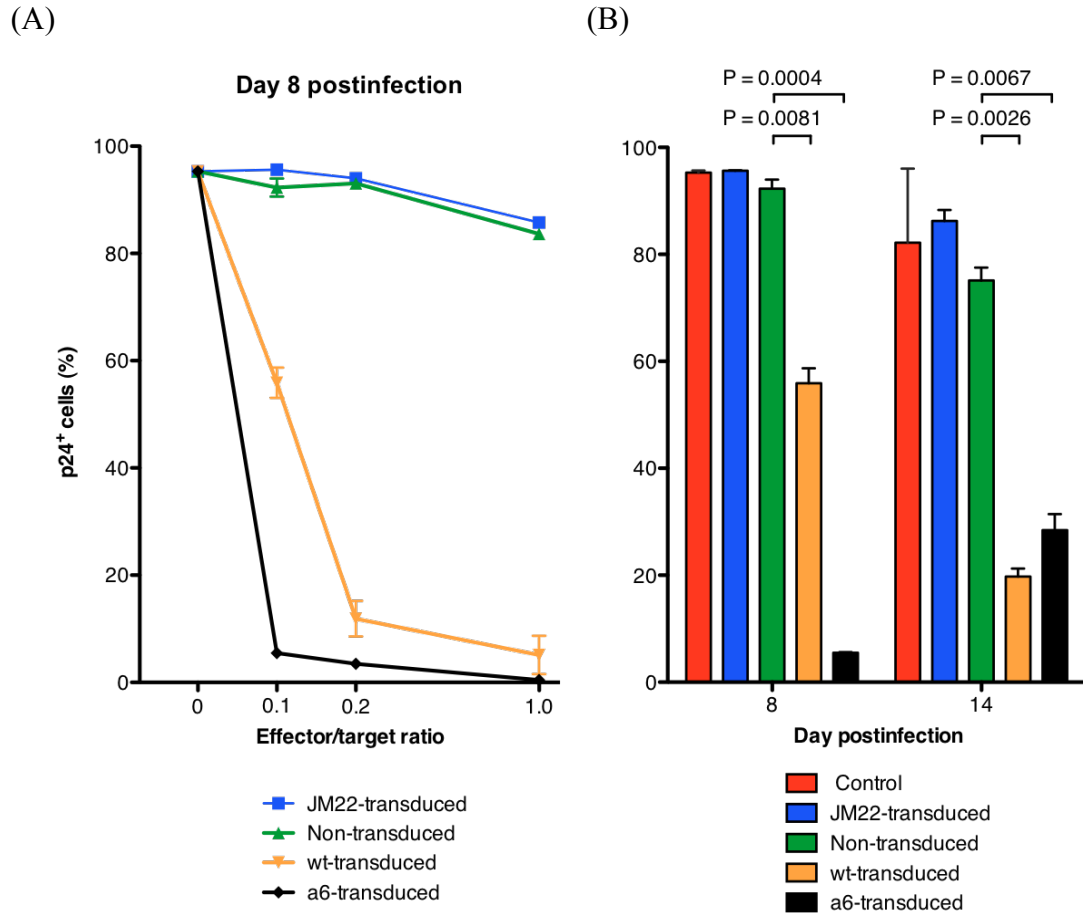


Figure 4.5 Effect of TCR-transduced cells on HIV-1-infected HLA-A*02⁺ T0 cells. (A) The percentage of p24⁺ cells at day 8 postinfection when cocultured with TCR-transduced cells at different E:T cell ratios. **(B)** The percentage of p24⁺ cells at day 8 and 14 postinfection following coculture with TCR-transduced cells at an E:T ratio of 1:10. Cells were gated on viable CD8⁺ events. Each well was performed in duplicate; error bars are SEM.

The wt and a6 TCR-transduced cells were capable of controlling HIV_{LAI} infection in a T0 cell culture at both day 8 and day 14 postinfection compared to the non-transduced cells. At an E:T ratio of 1:10, the wt TCR-transduced cells reduced the percentage of gag⁺ cells by 39.5% and 73.7% (P = 0.0081 and 0.0026) at day 8 and 14 postinfection,

respectively; the a6 TCR-transduced cells by 94.1% and 62.1% ($P = 0.0004$ and 0.0067), respectively (Figure 4.5).

4.2.1.1 Analysis of long-term persistence in vitro

The persistence of TCR-transduced cells in adoptive transfer is of high importance as it may significantly influence clinical outcome. We analysed the infected T0 cell cocultures at day 31 to determine whether the TCR-transduced cells had persisted when grown in R10 media supplemented with IL-2. The $CD8^+$ cells were still viable and expression of TCR V β 5 had either remained stable or slightly increased for the wt and a6 TCR-transduced cells (Figure 4.6) compared to day 0 (Figure 4.4).

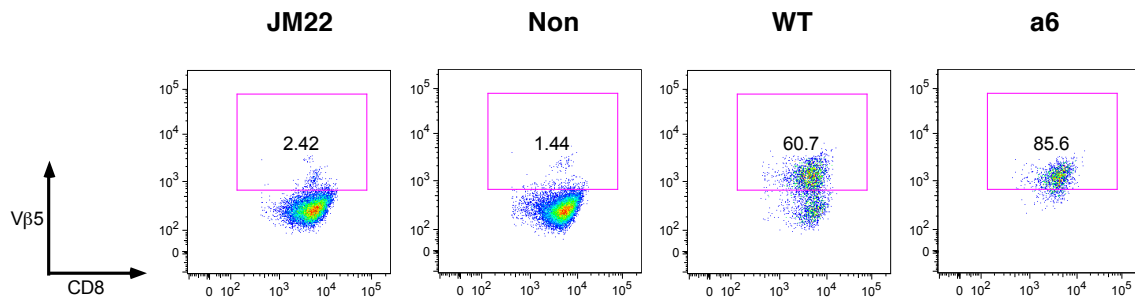


Figure 4.6 Longevity of the TCR-transduced cells cocultured at an E:T ratio of 1:10 at day 31 postinfection. Lymphocytes were gated on viable $CD8^+$ cells.

4.2.1.2 Analysis of viral sequences

Direct PCR population sequencing of day 8, 14 and 31 postinfection samples gave identical Gag p17 sequences to HIV_{LAI} for all samples at all E:T ratios. No mixed peaks were observed on the chromatograms indicating that there were probably no escape variants within the cultures that were prevalent above 10 to 20% (Palmer et al., 2005).

Clonal sequencing on selected samples was carried out to detect minor variants. *Taq* DNA polymerase-amplified PCR products were cloned into a TOPO TA vector and sequenced; the clonal sequences are shown in Appendix E. No polymorphism was detected in more than 2 clones. Due to the error rate of *Taq* DNA polymerase (8×10^{-6} mutations/bp/amplification cycles), a 40-cycle amplification of a 780-bp amplicon may result in $\sim 8/32$ clones with a PCR-induced mutation. As the variants we observed were mainly only in 1 clone, we cannot conclude that they are real variants at low prevalence or due to PCR error. Only one of the samples cloned had the same polymorphisms in 2 clones: HIV-infected T0 cells cocultured with wt TCR-transduced cells at an E:T ratio of 1:10 for 31 days. Two polymorphisms in the same two clones were observed: the first, a C $\rightarrow$ T resulting in H89Y; and the second, an A $\rightarrow$ G resulting in E107G.

To verify which polymorphisms were due to PCR error, sample cDNA was amplified with *PfuUltra* DNA polymerase, the PCR product was A-tailed and this cloned into a TOPO TA vector, then sequenced. As this DNA polymerase has a lower error rate (0.4×10^{-6} mutations/bp/amplification cycles), less than 1/32 clones would contain a PCR-induced mutation under similar conditions with the same primers. None of the variants detected using *Taq* DNA polymerase were observed using *PfuUltra* DNA polymerase (Table 4.3). No significant minor variants emerged in this continuous culture assay when cocultured with either wt or a6 TCR-transduced cells in the samples that were cloned.

(A)

	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	
	S	E	E	L	R	S	L	Y	N	T	V	A	T	L	Y	C	V	H	Q	
wt-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	28/28
a6-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	26/26

(B)

	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	
	S	E	E	L	R	S	L	Y	N	T	V	A	T	L	Y	C	V	H	Q	
wt-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	30/30
a6-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	29/30
	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1/30

Table 4.3 Clonal sequencing of (A) day 14 and (B) day 31 postinfection samples cocultured at an E:T ratio of 1:10. *PfuUltra* DNA polymerase (error rate = 0.4×10^{-6} mutations/bp/PCR cycles) was used to amplify the cDNA. The number of clones sequenced is given for each sample.

4.2.2 Selection pressure in a passaging assay

Similar to the continuous culture assay, T0 cells were infected with HIV_{LAI} and at day 2 postinfection, the TCR-transduced cells were added at an E:T ratio of 1:10. Uninfected T0 cells were added on day 4 postinfection to provide fresh targets for HIV infection. The cells were analysed by flow cytometry at various time-points, cell culture supernatant stored and viral RNA extracted for sequencing.

Figure 4.7 shows that less than half of the viable CD8⁺ cells were p24⁺ on day 6 postinfection for all cultures; ~27% and 15% of the T0 cells cocultured with wt and a6 TCR-transduced cells were p24⁺. Only the addition of a6 TCR-transduced cells resulted in a significant reduction in p24⁺ cells compared to coculturing with non-transduced cells (54.8% decrease, $P = 0.0294$). At day 8 postinfection, >95% of the cultures were

p24⁺ for all samples regardless of whether the SL9-specific TCR-transduced cells were present.

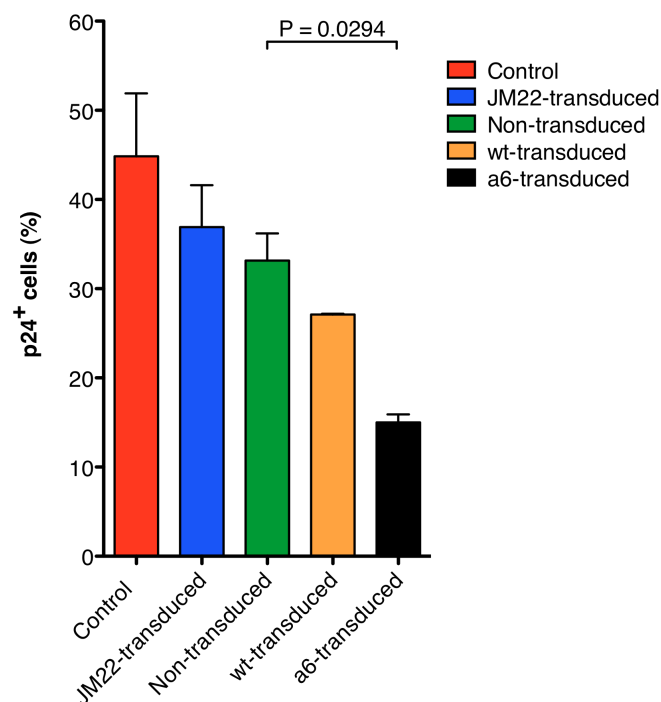


Figure 4.7 Effect of TCR-transduced cells on HIV-1-infected HLA-A*02⁺ T0 cells. The percentage of p24⁺ cells at day 6 postinfection following coculture with TCR-transduced cells gated on viable CD8⁺ cells is shown. Each well was performed in duplicate; error bars are SEM.

Direct population sequencing of day 6 and 8 postinfection samples gave identical Gag p17 sequence to HIV_{LAI} for all samples; no mixed peaks were observed on the chromatograms. Clonal sequencing showed little diversity that could positively be classed as non-PCR error (Table 4.4).

	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	
	S	E	E	L	R	S	L	Y	N	T	V	A	T	L	Y	C	V	H	Q	
wt-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	30/32
	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1/32
	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1/32
wt-transduced B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	30/32
	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1/32
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	1/32
a6-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	32/32
a6-transduced B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	29/30
	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	1/30

Table 4.4 Clonal sequencing of passage 1 day 6 postinfection samples. *Taq* DNA polymerase (error rate = 8×10^{-6} mutations/bp/PCR cycles) was used to amplify the cDNA. The number of clones sequenced is given for each sample.

The viral supernatant from passage 1 day 6 postinfection was used to infect fresh T0 cells. For example, the viral supernatant from infected T0 cells cocultured with a6 TCR-transduced cells was used to infect fresh T0 cells that were also cocultured with a second batch of a6 TCR-transduced cells at the same E:T ratio. Uninfected T0 cells were added on day 7 and 9 postinfection to provide fresh targets for HIV infection. Cells were analysed by flow cytometry at days 5, 8 and 14 postinfection, and viral RNA extracted for sequencing.

Figure 4.8 shows that those samples that were cocultured with the a6 TCR-transduced cells had 86.1% and 95.9% ($P = 0.0053$ and 0.0001 , respectively) less p24⁺ cells than those cocultured with non-transduced cells at day 5 and 8 postinfection, respectively. By day 14 postinfection, the percentage of p24⁺ cells had increased to levels of those

cultures not under selection pressure. The wt TCR-transduced cells caused a 14.3% reduction ($P = 0.0156$) in $p24^+$ cells at day 8 compared to non-transduced cells.

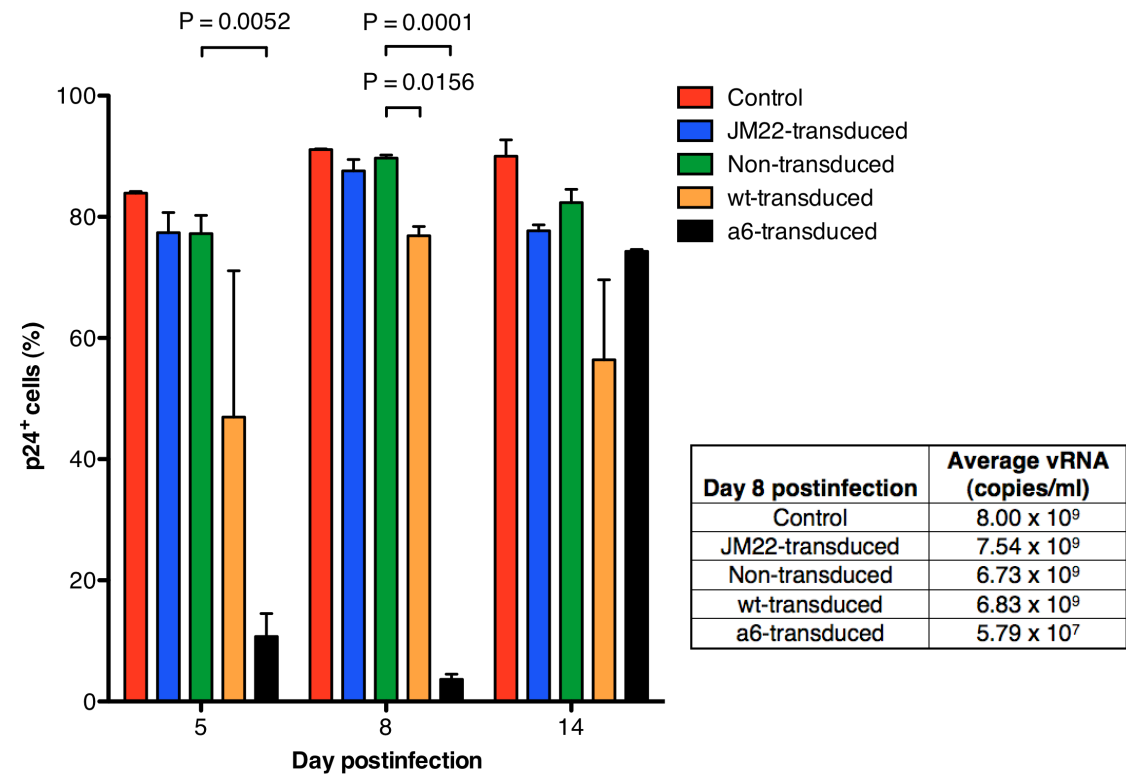


Figure 4.8 Effect of TCR-transduced cells on HIV-1-infected HLA-A*02⁺ T0 cells after passaging. The percentage of $p24^+$ cells at day 5, 8 and 14 postinfection following the addition of TCR-transduced cells gated on viable CD8⁺ cells is shown. Each well was performed in duplicate; error bars are SEM. The average viral load in the cell culture supernatants is given.

Direct PCR population sequencing of passage 2 day 8 and 14 postinfection samples gave identical Gag p17 sequences to HIV_{LAI} for all samples; no mixed peaks were observed on the chromatograms. As with the continuous culture assay, no clonal sequence was detected in more than 2 clones other than that identical to HIV_{LAI}. Only one cloned sample, infected T0 cells cocultured with wt TCR-transduced cells, had a C→G resulting in the L75V polymorphism observed in 2 clones (Appendix E). We

cannot conclude that samples with one variant clone from *Taq* DNA polymerase-amplified PCR products are low prevalent variants or due to PCR error. No variant sequences observed using *Taq* DNA polymerase were found when using *PfuUltra* DNA polymerase when cloning (Table 4.5). No significant minor variants emerged during this passaging assay when cocultured with wt or a6 TCR-transduced cells.

	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	
	S	E	E	L	R	S	L	Y	N	T	V	A	T	L	Y	C	V	H	Q	
Non-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	28/29
	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1/29
wt-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	31/31
wt-transduced B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	32/32
a6-transduced B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	32/32

Table 4.5 Clonal sequencing of passage 2 day 8 postinfection samples. *PfuUltra* DNA polymerase (error rate = 0.4×10^{-6} mutations/bp/PCR cycles) was used to amplify the cDNA. The number of clones sequenced is given for each sample.

4.3 Ultradeep pyrosequencing

Direct PCR population sequencing and molecular clonal Sanger sequencing have limited sensitivity in detecting minor HIV variants. Ultradeep pyrosequencing (UDPS; 454 Life Sciences) enables massively parallel amplification and pyrosequencing (pyrophosphate-based sequencing) of single DNA molecules in picolitre-volume wells (Margulies et al., 2005). To detect low prevalent HIV variants and verify non-PCR error variants observed through cloning, UDPS was performed on selected samples. Two samples from each *in vitro* selection pressure assay method were chosen. From the continuous culture assay at day 31 postinfection, using an E:T ratio of 1:10: a sample

cocultured with wt TCR-transduced cells was chosen to confirm or reject the H89Y and E107G polymorphisms; and a sample cocultured with a6 TCR-transduced cells. From the passaging culture assay at passage 2 day 8 postinfection: a sample cocultured with non-transduced cells was sequenced as a control where no selection pressure was on the virus and so no escape variants above the HIV mutation rate should emerge; and a sample cocultured with wt TCR-transduced cells to verify the L75V polymorphism and other minor variants.

Nested PCR was used to generate ~1,300-bp products with *PfuUltra* II DNA polymerase. These were fragmented into a 500-bp fragment library using sonication (Covaris), ligated to adaptors, amplified on capture beads in water-in oil emulsion microreactors and UDPS. Reads were aligned using the Smith-Waterman algorithm against the HIV_{LAI} *gag* reference sequence.

	Sample	Viral load (copies/ml)	Total number of reads	Number of reads after exclusion
1	wt TCR-transduced A	2.45×10^8	17,396	15,797
2	a6 TCR-transduced A	7.83×10^7	44,297	40,627
3	non-transduced A	6.64×10^9	18,224	16,653
4	wt TCR-transduced B	7.14×10^9	33,115	29,986

Table 4.6 Number of sequencing reads generated by UDPS. Samples 1 and 2 are day 31 postinfection samples from 4.2.1; samples 3 and 4 are passage 2 day 8 postinfection samples from 4.2.2. Calculated viral loads may be lower than actual cell culture supernatant viral loads as the vRNA was freeze-thawed since UDPS.

The 454 pyrosequencing GS FLX Titanium platform generated a mean of 28,258 (range: 17,396 – 44,297) reads per sample prior to noise reduction. Following exclusion of reads that contain an ambiguous base call and reads shorter than 150 bases and longer than 500 bases, the depth of reads was still vast, ranging from 15,797 to 40,627 reads (Table 4.6).

We used the conservative mismatch error rate 0.004 determined in homopolymeric regions (Wang et al., 2007) to distinguish UDPS errors from real variants at low prevalence. UDPS of the four *in vitro* generated virus samples produced some interesting points to note. Firstly, the non-transduced A culture from the passage 2 day 8 postinfection assay did not produce any significant variants. This control acts as a reference point of the rate the virus mutates under no selection pressure in the *in vitro* assay. Note, the mutation rate of HIV is 10^{-4} to 10^{-5} mutations per nucleotide per replication cycle (Mansky and Temin, 1995).

From the continuous culture assay, the presence of the wt TCR-transduced cells for a month produced no significant variants, indicating that the H89Y and E107G polymorphisms were PCR error. Coculturing with a6 TCR-transduced cells for a month produced a synonymous mutation (T→A, nucleotide 204) in Gag p17 that is in approximately 7.8% of the sequence reads ($P < 0.001$, Table 4.7). As the viral load was $>10^7$ copies/ml, there appears to be copious viral replication; this synonymous mutation indicates the capacity for mutation to occur under this selection pressure. From the passaging assay, by day 8 postinfection at passage 2, coculturing with wt TCR-transduced cells produced a nonsynonymous mutation in 2.9% of the reads ($P < 0.001$,

Table 4.7). This was the same nucleotide substitution observed when cloning a *Taq* DNA polymerase-amplified fragment, i.e. C→G resulting in a L75V polymorphism.

	Variant	Number of reads with variant	Read depth at position	% of reads with variant	P value
D8 wt TCR-transduced B passage 2	L75V	224	7,699	2.9%	3.04E-104
D31 a6 TCR-transduced A continuous	L68L	786	10,141	7.8%	0.0

Table 4.7 Significant UDPS misbase reads spanning Gag p17 nt 199-285. A mismatch error rate for homopolymeric regions of 0.0044 was used to calculate P values (Wang et al., 2007).

4.3.1.1 L75V polymorphism

Within Gag p17, residue 75 is 2 amino acids upstream of the SL9 epitope and so a polymorphism at this position is a potential antigen processing escape mutation. Proteasomes cleave self and non-self proteins into peptide fragments, and are either found in a constitutive form, or induced by IFN γ to form an immunoproteasome (Akiyama et al., 1994); both forms may have different cleavage specificities. *In vitro* constitutive proteasomal and immunoproteasomal digestion assays using p17 peptides spanning bases 68 to 92, containing 75L, found that the N-terminus of SL9 epitope precursors were either 76R/K or the uncleaved 68L (Tenzer et al., 2009). Therefore, as proteasomal cleavage of SL9 epitope precursors occurs C-terminally to 75L, we hypothesised that selection pressure from the wt TCR-transduced cells led to the emergence of this L75V proteasomal cleavage or ERAAP trimming escape mutation.

Supporting this theory, L75V is an uncommon polymorphism, found in 0.6% of HIV-1 subtype B sequences (Kuiken et al., 2010). Three proteasome prediction software methods were used to analyse cleavage sites using a peptide sequence spanning bases 68 to 89 and either containing 75L or 75V. The 75L was predicted to be a proteasomal cleavage site in all three programs; however, the results for 75V were contradictory. Two of the programs predicted 75V as a cleavage site, whilst the third had no cleavage at this position using an algorithm based on human erythrocyte proteasome cleaving an experimental dataset of enolase and ovalbumin peptides (Appendix F).

4.4 TCR-transduced cell recognition of SLYNTVATL variants

With no significant intraepitope diversity emerging in both *in vitro* selection pressure assays, we aimed to determine whether the wt and a6 TCR are capable of recognising not only those variants analysed by SPR (Table 4.2), but also other natural SL9 variants and artificial variants. The TCR-transduced cells were assessed for specific lysis of peptide-pulsed cells using a FATAL assay. The cell line used in this assay was the CD4⁺ 174xCEM.T2 cell line that expresses HLA-A*02 but does not present endogenously synthesised peptides due to the deletion of MHC class II-encoded genes for TAP1 and TAP2 (Cerundolo et al., 1990); this enables them to be loaded efficiently with exogenous peptide. T2 cells were pulsed with SL9 variant peptides or the control peptide IV9 (Table 2.5). The concentration of peptide chosen for loading was 1×10^{-8} M so that the difference in specific lysis of the variants compared to SL9 could be observed: enough peptide needed to be loaded for lysis to occur, but not excessive

amounts leading to maximal lysis. The peptide-pulsed T2 cells were cocultured with TCR-transduced cells at an E:T ratio of 1:1.

The wt TCR-transduced cells were initially compared to the JM22-transduced cells. As the HLA-A*02-restricted epitope that JM22 TCR recognises, GILGFVFTL, shares only 33.3% identity to SL9 (position 6, 8 and 9), it should not lyse SL9 or its variants. The TCR-transduced cells had a CD8 purity and V β 5 expression of 80.1% and 5.59% for the JM22-transduced cells, respectively, and 78% and 61.8% for the wt TCR-transduced cells, respectively.

When specific lysis against all the SL9 variants was grouped together, the mean value for the JM22-transduced cells was 4.85% (95%CI: 0.19% to 9.5%) whilst that for the wt TCR-transduced cells was 22.4% (95%CI: 14.22% to 30.59%) (Figure 4.9). Overall, the wt TCR-transduced cells had a significantly greater magnitude of specific lysis of the SL9-loaded cells and variants ($P = 0.0004$). The following peptides caused significantly higher specific lysis than SL9: 5A (2.5-fold, $P = 0.0102$), 3F6I (2.5-fold $P = 0.0473$), 3F6I8V (2.3-fold, $P = 0.0004$), 3F5A6I8V (1.1-fold, $P = 0.0271$), and 8I (1.2-fold, $P = 0.0320$); the following peptides caused significantly less specific lysis than SL9: 7V ($P = 0.0238$), 3F5A8V ($P = 0.0007$), 9F ($P = 0.0048$), and IV9 ($P = 0.0089$).

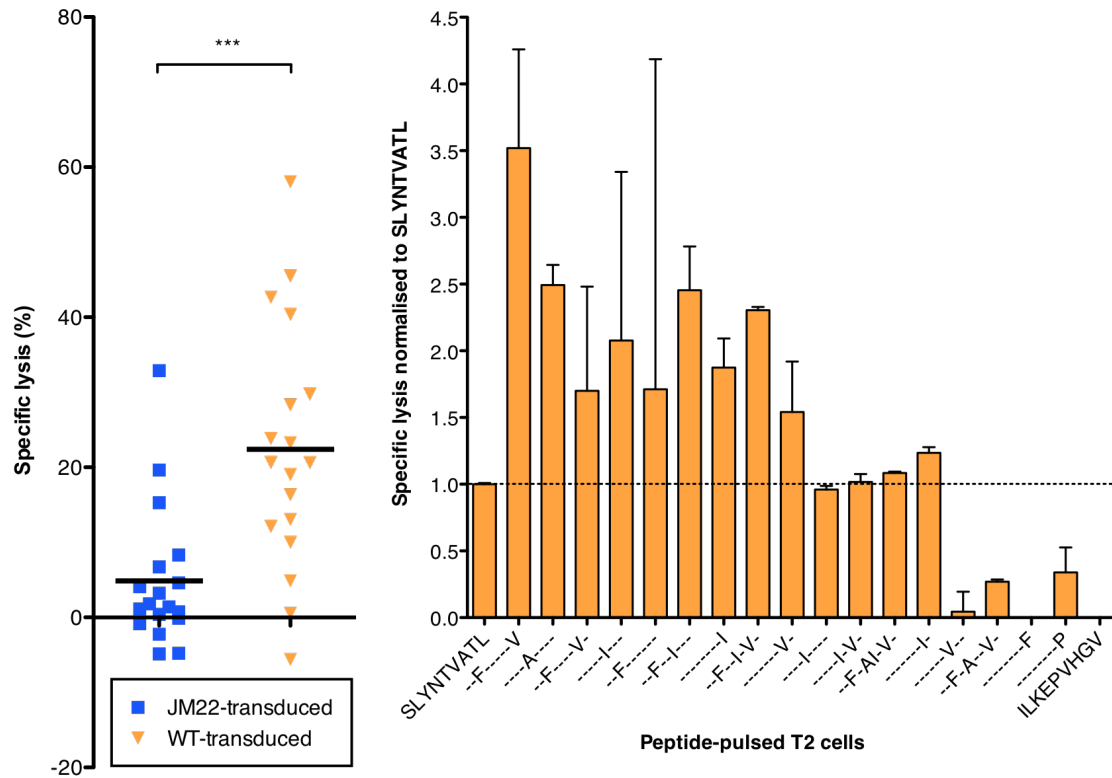


Figure 4.9 FATAL assay to detect cytotoxicity of T2 cells pulsed with 10^{-8} M SL9 or variant peptide in the presence of either JM22- or wt TCR-transduced cells at an E:T ratio of 1:1. All assays were done in duplicate; error bars are SEM. Specific lysis was calculated via the following: $100 - (\% \text{ CFSE of test SL9 variant} / \% \text{ CFSE of spontaneous release}) \times 100$. Values were normalised to SLYNTVATL = 1.0 by subtracting the average JM22-transduced cytotoxicity and dividing by that induced by wt TCR-transduced cells + SLYNTVATL peptide-pulsed T2 cells.

Next, the wt TCR-transduced cells were compared to the $\alpha 6$ TCR-transduced cells in their ability to specifically lyse SL9- and variant-pulsed T2 cells. Non-transduced cells were used as an additional control; however, these surprisingly resulted in a mean specific lysis of -7.0% (95% CI: -8.6% to -5.3%) (Figure 4.10). This negative mean value indicates that the CFSE-stained peptide-pulsed T2 cells actually increased in percentage compared to background control.

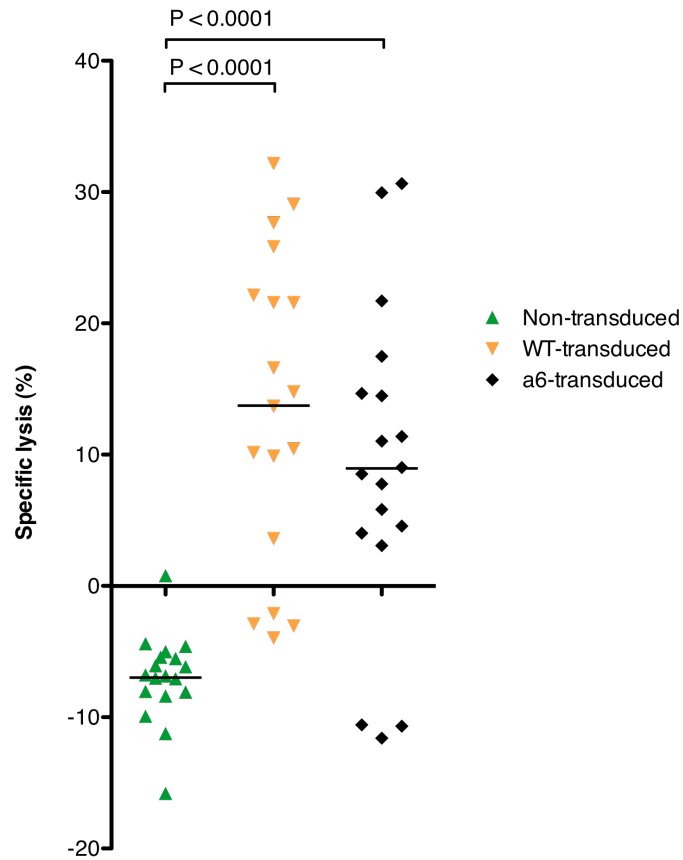


Figure 4.10 FATAL assay to detect cytotoxicity of T2 cells pulsed with 10^{-8} M SL9 or variant peptide in the presence of either non-, wt or a6 TCR-transduced cells at an E:T ratio of 1:1. All assays were done in duplicate. Specific lysis was calculated via the following: $100 - (\% \text{ CFSE of test SL9 variant} / \% \text{ CFSE of spontaneous release}) \times 100$.

When the specific lysis against all the SL9 variants was grouped together, the mean specific lysis value for the wt TCR-transduced cells was 13.7% (95% CI: 7.8% to 19.6%), whilst that for the a6 TCR-transduced cells was 9.0% (95% CI: 2.9% to 15.0%) (Figure 4.10). These mean values are significantly greater than that for the non-transduced cells ($P < 0.0001$); there was no statistically significant difference between the mean specific lysis value for the wt and a6 TCR-transduced cells.

In this assay, no peptide variant caused significantly higher specific lysis than SL9 by wt TCR-transduced cells; the following led to significantly less specific lysis than SL9: 8V ($P = 0.0439$), 5I ($P = 0.0182$), 6I8V ($P = 0.0025$), 3F5A6I8V ($P = 0.0021$), 8I ($P = 0.0015$), 7V ($P = 0.0030$), 3F5A8V ($P = 0.0038$), 9F ($P = 0.0005$), 9P ($P = 0.0006$) and IV9 ($P = 0.0008$). Only the 3F8V variant led to significantly higher specific lysis than SL9 by $\alpha 6$ TCR-transduced cells (1.7-fold, $P = 0.0365$); the following led to significantly less specific lysis than SL9: 9I ($P = 0.0238$), 3F5A6I8V ($P = 0.0482$), 8I ($P = 0.0055$), 9F ($P = 0.0093$), 9P ($P = 0.0368$) and IV9 ($P = 0.0117$) (Figure 4.11).

Differences in wt TCR-transduced cell cytotoxicity magnitude in Figure 4.9 and Figure 4.11 may be due to the batch of cells used which may have a change in CD8 purity, transduction efficiency or health of the cells. Consistently, no lysis was observed when the TCR-transduced cells were cocultured with T2 cells pulsed with the control peptide IV9, signifying the specificity of these cells for their target epitope. The variants 9F and 9P are not predicted to bind HLA-A*0201 using the NetMHC3.2 prediction program (Lundegaard et al., 2008), and the absence of lysis when T2 cells were pulsed with these peptides, confirms that either the peptides were not bound, or were not recognised by the TCRs. The $\alpha 6$ TCR recognised the same SL9 variant peptides as the wt TCR (except for the artificial 8I variant) plus to some degree the 7V and 3F5A8V variants that were not recognised by the wt TCR.

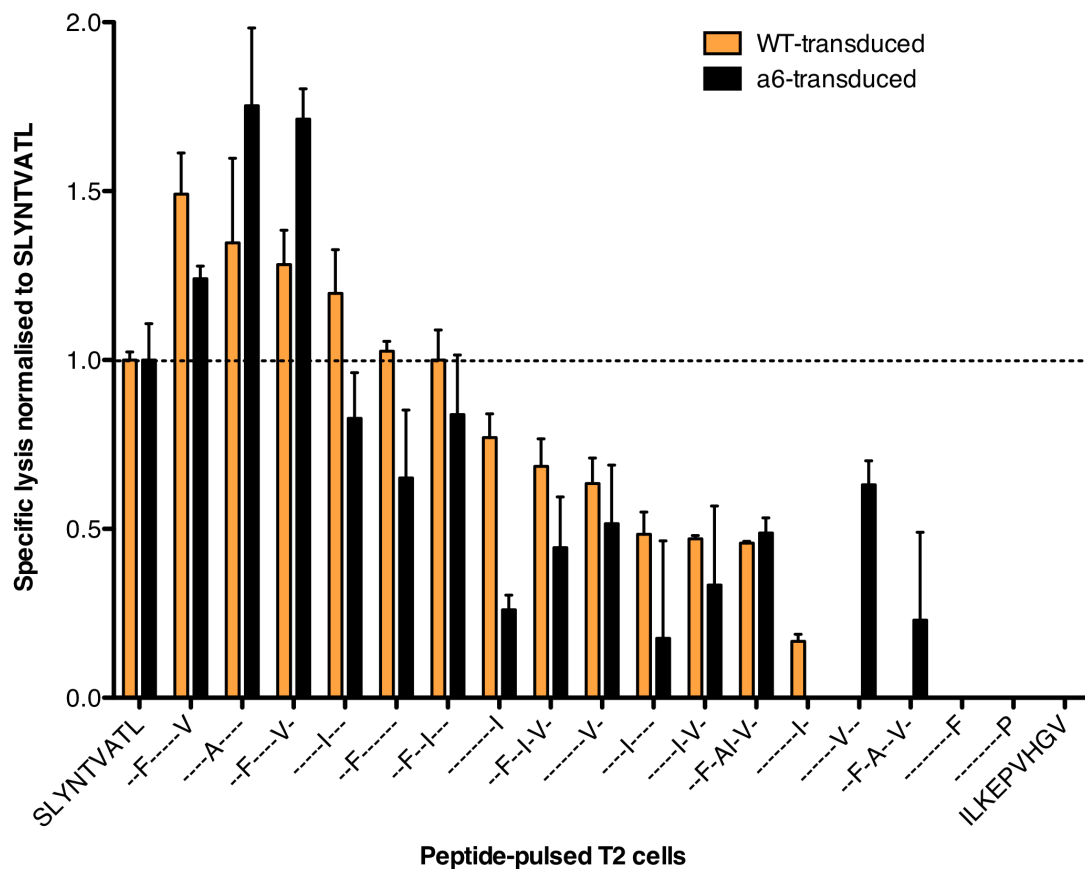


Figure 4.11 FATAL assay to detect cytotoxicity of T2 cells pulsed with 10^{-8} M SL9 or variant peptide in the presence of either wt or a6 TCR-transduced cells at an E:T ratio of 1:1. All assays were done in duplicate; error bars are SEM. Cytotoxicity values were normalised to SLYNTVATL = 1.0.

There was no correlation between TCR binding affinity and specific lysis for both the wt and a6 TCR (Figure 4.12). Although we may have expected increased specific lysis to be associated with a lower K_d value, this was not seen when target cells were pulsed with 10^{-8} M SL9 or variant peptide. Schmid et al. have found that above an optimal affinity threshold, T cell function was at its maximum and could not be improved; for a NY-ESO-1-specific TCR, this K_d value was determined to be $\sim 5 \mu\text{M}$, (Schmid et al.,

2010). All K_d values for the SL9-specific wt and a6 TCR assessed were $<5 \mu\text{M}$ (Table 4.2).

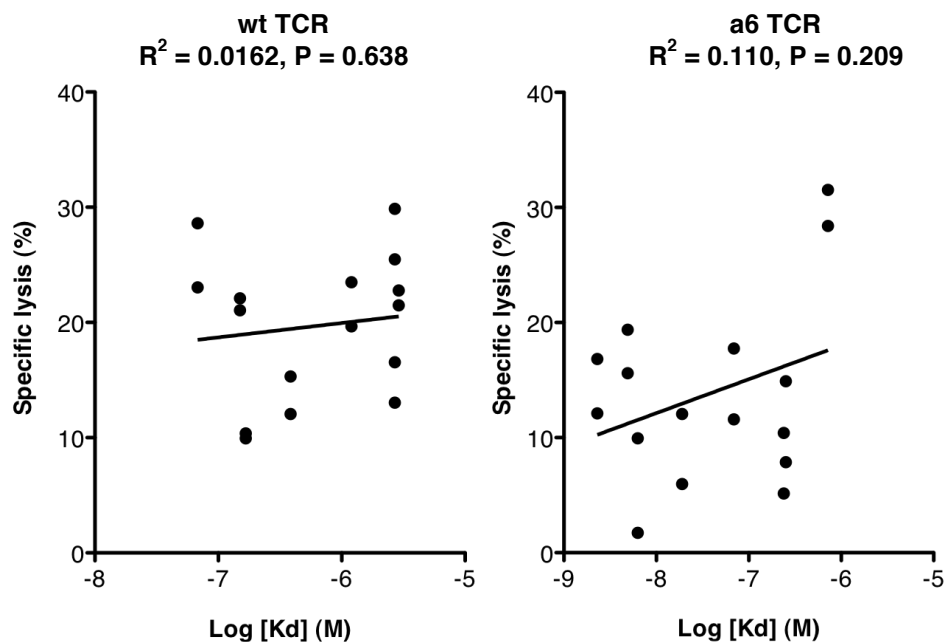


Figure 4.12 No correlation of TCR binding affinity against specific lysis. The K_d values against the eight most frequent SL9 variants determined using SPR binding analysis (Table 4.2) were plotted against their corresponding specific lysis percentages as determined by the FATAL assay shown in Figure 4.10. Each replicate is shown.

Discussion

Adoptive transfer of cells transduced with either a natural or enhanced SL9-specific TCR is currently being evaluated in a phase I clinical trial with HIV-infected patients. HIV has consistently been able to avoid immune surveillance and resist antiretroviral therapy due to its high mutation and recombination rate. We hypothesised that to escape SL9-specific TCR-transduced cells with enhanced binding, SL9 variants would emerge that either no longer bound to HLA-A*02 or were not processed correctly. Based on antiretroviral resistance and CTL escape *in vitro* selection pressure assays, we sought to determine potential escape variants by subjecting the virus to SL9-specific TCR-transduced cell pressure and sequencing the resultant virion genomes. The strength of the selection pressure balanced with the fitness cost to the virus would contribute to which, if any, variant would emerge to avoid TCR recognition.

Using the models of Asquith et al., we hypothesised that the presence of wt TCR-transduced cells exerting a strong CTL pressure would produce escape variants despite a possible fitness cost to the virus and therefore act as a positive control in the assay. With the high affinity a6 TCR-transduced cells exerting an even stronger CTL pressure but with the capability of recognising more SL9 variants, escape would be generated at an even higher fitness cost, and so it would be difficult for a dominant variant to fully emerge (Figure 4.13).

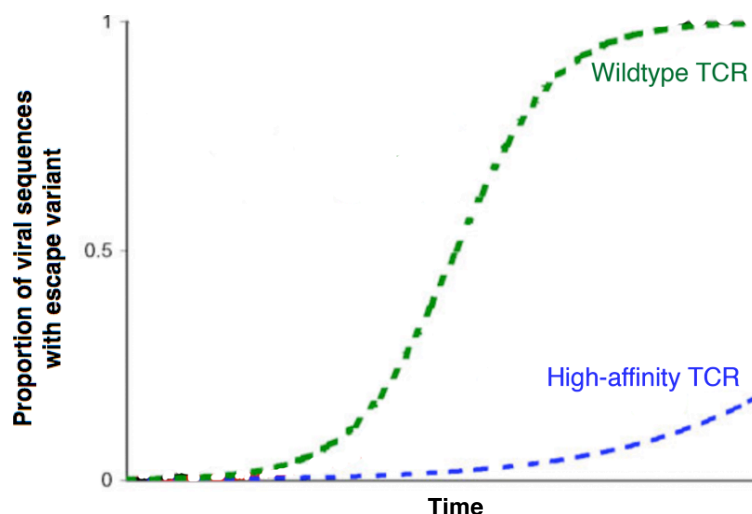


Figure 4.13 Predicted model of SL9-specific TCR-transduced cell escape in an HLA-A*02⁺ host based on a previously published model (Asquith et al., 2006).

First, the a6 TCR-transduced cells were confirmed to be more cytotoxic than the wt TCR-transduced cell when cocultured with HIV-infected PBMCs *in vitro*. This was found in both autologous and allogeneic HLA-A*02⁺ PBMCs. If the clinical trial using autologous cells is successful, TCR-transduced allogeneic cell transfer may circumvent the need to use an HIV-infected patient's own cells. An allogeneic CTL cell line transduced with the HLA-A*02-restricted MART-1 TCR was found to be safe when used in a phase I clinical trial of 15 HLA-A*02 positive patients with metastatic melanoma (Duval et al., 2006). In addition, the use of partially HLA-matched third-party EBV-specific CTLs has been documented in transplant patients without *de novo* GvHD occurring (Barker et al., 2010, Melenhorst et al., 2010).

In our *in vitro* selection pressure assays, the a6 TCR-transduced cells were again more cytotoxic than the wt TCR-transduced cells at E:T ratios of 1:10, 1:5 and 1:1. The

longevity of TCR-transduced cells in an HIV-infected culture had not been assessed previously. We found that the cells persisted *in vitro* for at least one month in the presence of IL-2. When HIV-infected haplotyped-matched human-PBL-SCID mice were administered an expanded CD8⁺ CTL clone against the HLA-B*27-restricted epitope IRLRPGGKK (IK9, *gag* p17 19-27), despite an initial decline in mean viral RNA, viraemia had rebounded by day 24. The IK9-specific CTL clones had rapidly disappeared in the HIV-infected mice, possibly due to not proliferating and interacting with their targets; IK9-specific CTLs were long-lived in uninfected mice, as were control CTLs in both HIV-infected and uninfected mice (McKinney et al., 1999). CD4⁺ T_H cells or IL-2 may need to be transferred/administered simultaneously as part of the adoptive therapy strategy in order to establish a persistent CTL response (Lichterfeld et al., 2004). EBV adoptive therapy can persist *in vivo* for as long as 18 months with the infusion of both CD4⁺ and CD8⁺ cells (Heslop et al., 1996).

Direct PCR population sequencing at all assayed E:T ratios and at various time-points of the continuous culture and passaging culture found that no major HIV escape variant had emerged under wt or a6 TCR selection pressure. In addition, molecular clonal sequencing could not definitively identify minor HIV variants. Only through UDPS was the L75V polymorphism confirmed in 2.9% of reads, emerging under wt TCR-transduced cell pressure. UDPS has previously been used to detect minor antiretroviral drug resistance mutations that are not detected by direct PCR population sequencing (Le et al., 2009, Simen et al., 2009). At only two residues upstream of the SL9 epitope, L75V may represent an N-terminal processing mutation.

Antigen processing escape mutations in the flanking region of an epitope have been documented for other HLA-restricted CTL epitopes (Allen et al., 2004, Draenert et al., 2004, Milicic et al., 2005). Without *in vitro* proteasomal cleavage assays, we can only state that the prediction programs gave contradictory results with respect to the L75V polymorphism. Although no lab-based research has been carried out on the L75V polymorphism, L75X has been assessed. When virus constructs were made encoding patient-derived Gag sequences with nonconsensus SL9 flanking residues, including one with L75F, the epitope was processed efficiently. Interestingly, in a laboratory worker accidentally infected with HIV-1_{IIIB}, the only dominating change in p17 to occur 6 years postinfection was the L75Y polymorphism; however, this was also not found to block antigen processing (Brander et al., 1999).

Antiretroviral resistance is an example of the importance of determining possible escape mutations as soon as possible. In HAART-naïve individuals, the prevalence of resistance in transmitted or primary HIV-1 infection is >8% in developed countries (Weinstock et al., 2004, Vercauteren et al., 2009); an estimated 76% of HIV-infected patients in the US are resistant to one or more antiretroviral drug (Richman et al., 2004). Selection of antiretroviral-resistant variants *in vitro* has generated similar results to what occurs *in vivo* for many years. In 1992, selection of variants resistant to AZT and ddI *in vitro* were detected when increasing concentrations of drug were added to HIV-infected cell cultures for 41 days (Gao et al., 1992); as no intermediate time-points were sequenced, it is not known at what stage the resistance mutations emerged or to what frequency. Of note, this paper was in hindsight to *in vivo* results.

Selection pressure assays were not always successful in detecting drug-resistance mutations. Rooke et al. were not able to detect AZT-resistant mutations *in vitro* despite exposing HIV-infected cells to various concentrations of AZT for at least 500 days (Rooke et al., 1990). Their paper ended with the incorrect remark:

“Our inability to isolate AZT-resistant mutants of HIV-1 *in vitro* may further be an indication that such mutants are unlikely to always emerge under conditions of long-term AZT therapy”

Rapid *in vitro* selection of a CTL escape mutation has been observed with a Rev-specific CTL cocultured with HIV-infected PBMCs at an E:T ratio of 1:10. On day 11 postinfection, an HLA-B*14-restricted intraepitope mutation dominated the viral quasispecies, which was not recognised by the CTL (Van Baalen et al., 1998). Our passaging selection pressure assay was based on the method of Yang et al. to detect *in vitro* CTL escape: they analysed three different CTL clones that recognise the HLA-A*02-SL9 complex. Proviral sequencing demonstrated that intraepitope mutation occurred after two passages in 2 out of 3 CTL clone cultures. One of these CTL clones was used to inhibit viral replication of cells infected with HIV_{NL4-3} containing point mutations in the SL9 epitope. The CTL clone was unable to suppress replication of the variant that emerged during the *in vitro* selection pressure assay (Yang et al., 2003).

What should be kept in mind is that mutation is a spontaneous event. What emerges in one culture *in vitro* or one HIV-infected individual may not necessarily be replicated in another. These *in vitro* selection pressure assays cannot encompass exactly what might

happen in an HIV-infected individual, and so we can only conclude that the L75V polymorphism is one of a many possible evolution events that may occur *in vivo*. Despite the lack of CTL escape in our assays compared with that found by Van Baalen et al. and Yang et al., we would not like to make the same mistake as Rooke et al. and conclude that escape mutants are unlikely to emerge under conditions of long-term TCR-transduced cell adoptive therapy. We may only state that full CTL escape was not found *in vitro* under these particular conditions in the timeframe analysed.

TCR-transduced cell resistance mutations did not dominate because either: the selection pressure wasn't great enough; fitness costs incurred by any mutations were too high; the cultures were not analysed for a long enough duration; or there was insufficient viral replication. The difficulty of the current *in vitro* assays is getting the TCR-transduced cell pressure correct: too little and escape may not need to occur; too much and there may not be enough viral replication for escape to happen. Choosing the effector-to-target cell ratio can be compared to selecting the antiretroviral drug concentration. The situation is complicated by the availability of the cells, the general health of the cells, and the cell longevity. It may be that the E:T ratio was not high enough for the wt TCR-transduced cells to select for escape variants, and the E:T ratio was too high for the a6 TCR-transduced cells and so there was not enough viral replication for the escape to occur. Viral RNA quantitation was carried out on cell culture supernatants from both *in vitro* assays with the minimum viral load found to be 10^7 copies/ml, indicating abundant viral replication even in the presence of the a6 TCR-transduced cells. Alternatively, the cultures may have needed to be assessed for a longer period.

A limitation of this current study was the lack of a positive control; we had assumed that the wt TCR-transduced cells would act in this manner. However, the weakest affinity for a SL9 variant using the wildtype 868 TCR is 2.9 μ M against SLFNTVATL (Table 4.2). This is still a strong Kd value given that most TCR binding affinities have Kd values ranging between 10^{-4} and 10^{-5} M (Matsui et al., 1991, Weber et al., 1992), and so we would predict TCR binding to the most common SL9 variants to still occur.

With no intraepitope escape emerging in the selection pressure assays, confirmation of SL9 variant recognition was undertaken. T2 cells were pulsed with 10 nM SL9 or variant peptide; at this concentration, ~55 SL9 peptides are estimated to be presented by a cell (Varela-Rohena et al., 2008). Similarly to the chromium release assays using the parental 868 CTL line (Sewell et al., 1997, Whelan et al., 1999, Goulder et al., 1997b), the wt and a6 TCR-transduced cells lysed the same tested SL9 variant peptides. There was no specific lysis by the wt and a6 TCR-transduced cells of the T2 cells pulsed with SLYNTVATF or SLYNTVATP; these anchor residue mutations may confer CTL escape by no longer binding MHC. However, SLYNTVATP has been shown to be a nonviable variant (Ali et al., 2004) with the L85R mutation shown to block virion production (Freed et al., 1994). This indicates that the 9P variant, and possibly the 9F variant, will not emerge under TCR-transduced cell pressure as the variants are not replication competent due to a severe fitness cost. The SLYNTVVTL and SLFNAVAVL variants were also not recognised by the wt TCR; the 3F5A8V variant is the ninth most common SL9 variant (Table 4.1) and so patients with this circulating in their viral quasispecies may not respond to this TCR-transduced cell adoptive therapy.

An important point to note was that the addition of the non-transduced cells actually resulted in consistently negative specific lysis values. We cannot account for these negative values, but they may be due to the non-transduced cells causing early spontaneous release of the fluorescent dyes used despite flow cytometry analysis always kept to within 24 h of cell staining. It seems unlikely that non-transduced cells would specifically lyse the CellTracker Orange-stained unpulsed T2 cells, or aid specifically the proliferation of CFSE-stained peptide-pulsed T2 cells. We also observed that the non-transduced cells led to increased HIV infection in PBMCs that we are unable to fully explain, although we hypothesised it may be due to a lack of CD8 purity.

We had aimed to translate the data obtained in our *in vitro* assays into what may transpire under clinical conditions *in vivo*. Our primary objective, to determine whether HIV-1 could escape from TCR-transduced cells capable of recognising all the common variants of SL9, has resulted in no dominating escape *in vitro*. The L75V polymorphism, if indeed an escape mutation, may prove to be one mechanism of wt TCR-transduced cell resistance that could prevent a positive clinical outcome. We also confirmed the ability of the TCRs to recognise most SL9 variants. If the TCR-transduced cells are infused into patients that have low abundant 3F5A8V variants, these may expand in the viral quasispecies and lead to escape. Ultimately, *in vivo* results will now confirm which, if any, escape variants may arise due to TCR-transduced cell selection pressure.

CHAPTER 5

TCR-anti-CD3 fusion protein redirected CTL-mediated lysis of HIV-1-infected cells *in vitro*

Introduction

Adoptive transfer of enhanced TCR-transduced cells may encounter many difficulties *in vivo* other than escape that leads to failure. It is unknown where the infused cells will migrate to, how many infusions are required, how long they will persist, and whether CD4⁺ T_H cells, IL-2 or some other cytokine needs to be infused as well. Furthermore, the efficiency of the TCR transduction and *ex vivo* expansion of the cells may affect clinical outcome case-by-case. This patient-specific process is lengthy, unpredictable and costly. It would be favourable to circumvent these complications whilst retaining the same biological outcome.

With similar problems found in cancer adoptive therapy studies involving cells transduced with TCRs specific for TAAs, alternative strategies have been utilised with resultant anti-cancer candidates already in clinical studies. The biopharmaceutical company Micromet Inc focuses on T cell based therapies for the treatment of cancer using their BiTE (Bispecific T Cell Engager) antibody platform technology (Micromet, 2011). The BiTE antibody is specific for both CD3 and a TAA, enabling transient redirection of a T cell to a tumour cell by forming a TAA-BiTE-CD3 complex. Once the T cell is in close proximity, T cell activation may occur and lead to apoptosis of the

tumour cell (Figure 5.1). Micromet's lead BiTE candidate blinatumomab (MT103; MEDI-538) is in phase I/II clinical studies for acute lymphoblastic leukaemia and non-Hodgkin's lymphoma. Blinatumomab consists of two immunoglobulin variable domains that recognise CD19, found on the surface of most B cells, and two variable domains that recognise CD3. A dose of 60 $\mu\text{g}/\text{m}^2$ blinatumomab per day in NHL patients led to either complete or partial tumour regression in 7/7 patients (Bargou et al., 2008).

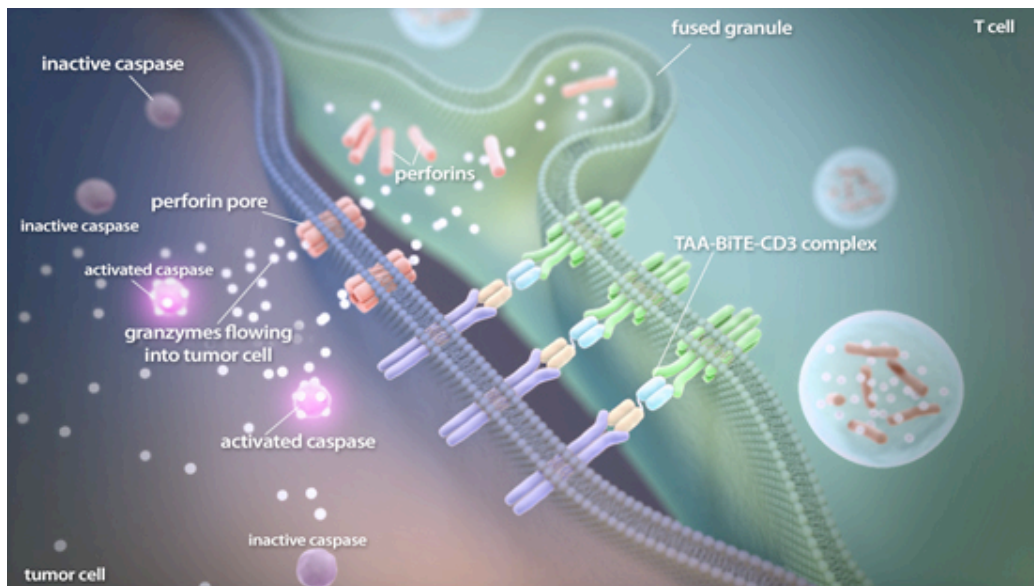


Figure 5.1 The BiTE antibody bridges the space between a T cell and tumour cell, subsequently leading to apoptosis of the tumour cell (Micromet, 2011).

Cancer studies have shown that the concept of redirected lysis is plausible and suggest no reasons against it being applied as a treatment for other diseases. The biotechnology company Immunocore Ltd has designed high affinity monoclonal T cell receptors (mTCRs) that can target intracellular proteins unable to be accessed by mAbs. These

have been fused to an anti-CD3 single chain variable fragment (scFv) domain (Figure 5.2) to create their ImmTAC (Immune Mobilising mTCR Against Cancer) technology platform capable of redirecting T cell lysis. A major advantage of this technology is that in comparison to bispecific antibodies, there is no limitation in terms of antigen. While antibodies can only target cell surface antigens, Immunocore's ImmTAC reagents are able to target intracellular proteins therefore increasing the possible number of antigens. ImmTAC technology is being used against cancer, with the lead product candidate IMCgp100 in a phase I clinical trial against advanced metastatic melanoma. When a HLA-A*02⁺ melanoma tumour cell presents the gp100 peptide YLEPGPVTV, the TCR component of IMCgp100 binds gp100 with a high affinity and for a sufficient amount of time for the anti-CD3 scFv component of IMCgp100 to bind a CD3 molecule on a non-melanoma specific CD8⁺ T cell. This enables redirection and activation of that cell to kill the tumour cell (Immunocore, 2011).

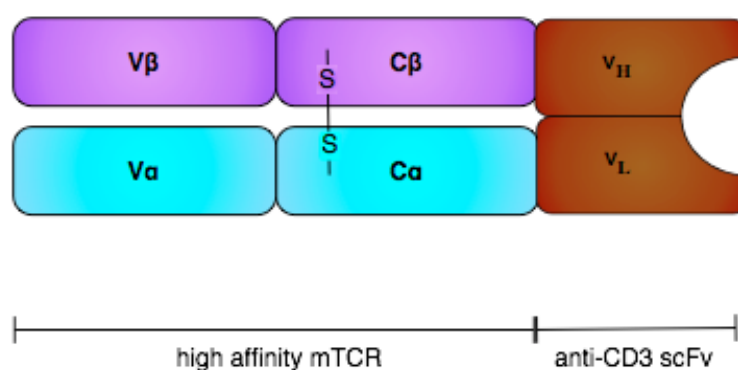


Figure 5.2 Schematic of an ImmTAC.

Although originally targeting tumours, the ImmTAC technology is also in early-stage development for autoimmune diseases and viral diseases such as HIV and hepatitis C

virus infection. Utilising similar high affinity SL9-specific TCRs to that used to transduce cells detailed in chapter 4, Immunocore has fused these to an anti-CD3 scFv of a lower affinity (~25 nM). The HIV gag ImmTAC binds strongly through the high affinity TCR with a long binding half-life to an HIV-infected cell presenting the HLA-A*0201-SL9 complex. Any CD8⁺ cell within the vicinity is recruited via the anti-CD3 scFv with the ImmTAC bridging the immunological synapse. This SL9-ImmTAC-CD3 complex is predicted to cause CD8⁺ cell activation and redirected lysis of the HIV-infected cell (Figure 5.3).

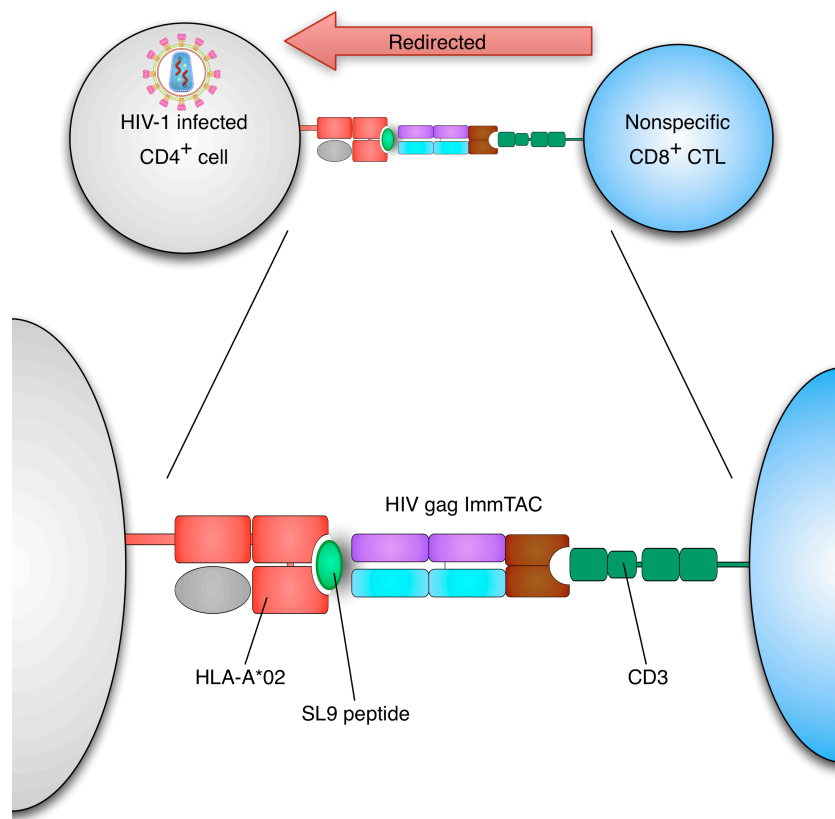


Figure 5.3 Schematic diagram of how the HIV gag ImmTAC causes redirected lysis of an HIV-infected cell.

The primary objective of this chapter was to determine whether HIV gag ImmTAC reagents do redirect T cells against HIV-infected cells *in vitro*. Building on the assays used in chapter 4, we would assess the ImmTACs in both HIV-infected HLA-A*02-expressing cell lines and PBMCs. We hypothesised that similarly to when wt and a6 TCR-transduced cells are cocultured with HIV-infected cells, the HIV gag ImmTACs would significantly reduce the proportion of gag⁺ cells *in vitro*, thereby controlling HIV infection.

Results

5.1 Redirected lysis of SL9-pulsed T0 cells with a11b6 ImmTAC

As the high affinity a11b6 TCR-transduced cells were significantly effective at controlling HIV-1 infection *in vitro* (Table 4.2) (Varela-Rohena et al., 2008), this TCR, with both an enhanced α and β chain, was the first to be fused to an anti-CD3 scFv to form a HIV gag ImmTAC. All ImmTAC reagents were provided by Immunocore and are known throughout this chapter by the TCR.

In order to test the a11b6 ImmTAC, we titrated serial log dilutions of SL9 peptide to ensure the ImmTAC was capable of redirecting CD8⁺ cells to targets presenting concentrations of peptide that may correlate with the number of epitopes presented by a HIV-infected cell. A FATAL assay was set up where the peptide-pulsed target 174xCEM.T0 cells that express HLA-A*02 were stained with CFSE and cocultured with unpulsed, CellTracker Orange-stained T0 cells and CellTracker Violet-stained CD8⁺ cells at an E:T ratio of 5:1. The a11b6 ImmTAC was added at a final concentration of 1 nM and incubated overnight. An example of the flow cytometry plots is given in Figure 5.4.

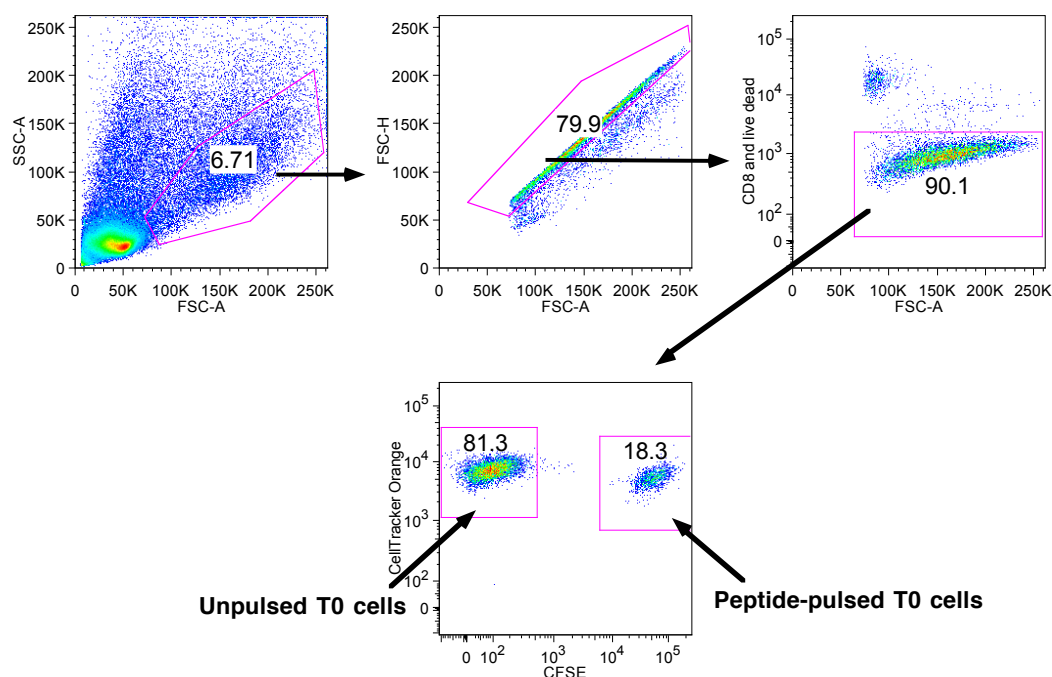
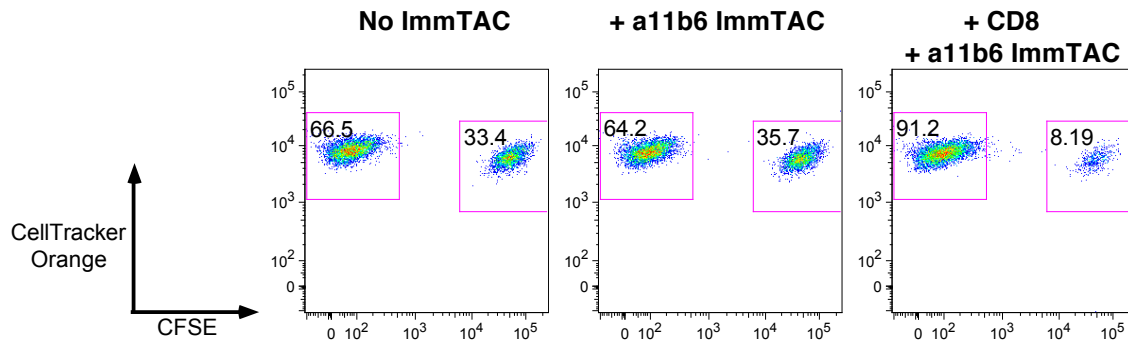


Figure 5.4 Gating strategy of the FATAL assay. Target HLA-A*02⁺ T0 cells were gated on singlet, viable, CD8⁺ cells; CFSE⁺ cells were peptide-pulsed whilst CellTracker Orange⁺ cells were unpulsed.

When 1 nM a11b6 ImmTAC alone was added to the coculture of peptide-pulsed and unpulsed T0 cells, there was no change in the proportions of stained T0 cells. When cocultured with effector CD8⁺ cells in the presence of 1 nM a11b6 ImmTAC, specific lysis was observed at SL9 peptide concentrations as low as 10^{-10} M. At a peptide concentration of 10^{-9} M, specific lysis reached almost 50%; the curve plateaus from 10^{-8} M peptide-pulsing at a maximal $\sim 75\%$ lysis (Figure 5.5). The EC₅₀ value using 1 nM a11b6 ImmTAC reagent is 5.17×10^{-10} M SL9 peptide ($R^2 = 0.9906$).

(A)



(B)

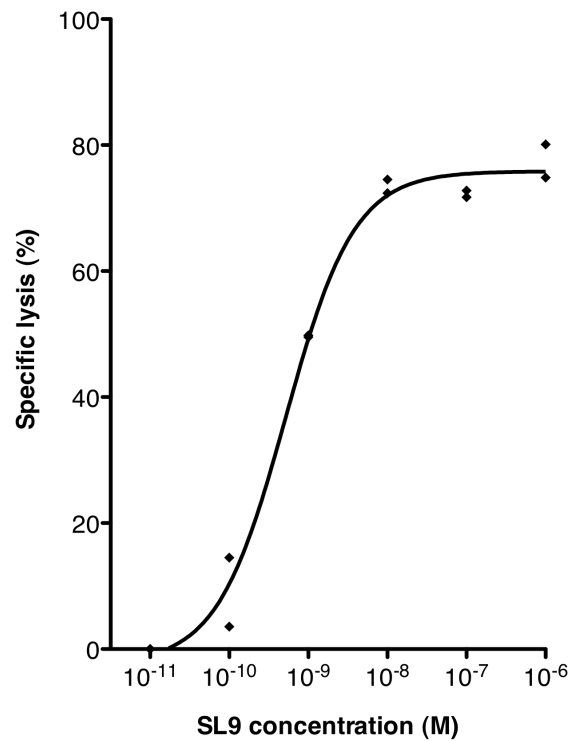


Figure 5.5 FATAL assay to detect cytotoxicity of T0 cells pulsed with SL9 peptide in the presence of 1 nM a11b6 ImmTAC and CD8⁺ cells at an E:T ratio of 5:1. (A) Example of cells pulsed with 10⁻⁸ M SL9 peptide; (B) Cells pulsed with serial log dilutions of SL9 peptide. All assays were done in duplicate. Specific lysis was calculated via the following: $100 - (\% \text{ CFSE of test SL9 concentration} / \% \text{ CFSE of spontaneous release}) \times 100$.

A similar FATAL assay was carried out except instead of purified CD8⁺ cells added, PBMCs from a healthy donor were used as effector cells. As the percentage of CD8⁺ cells within a PBMC population can vary, ranging from ~10 to 30%, we can estimate that the E:T ratio of CD8⁺ effector cells:target T0 cells is 1:1 to 2:1. When 0.1 nM a11b6 ImmTAC was added to the peptide-pulsed T0 cell/unpulsed T0 cell/PBMC coculture, maximal specific lysis was not achieved with the SL9 peptide concentrations assayed. Only at 10⁻⁶ M SL9 peptide was almost 40% specific lysis observed. When 1 nM a11b6 ImmTAC was added, the specific lysis curve was calculated to plateau at ~69% (Figure 5.6) with an EC₅₀ value of 1.259 x 10⁻⁷ M SL9 peptide (R² = 0.9938).

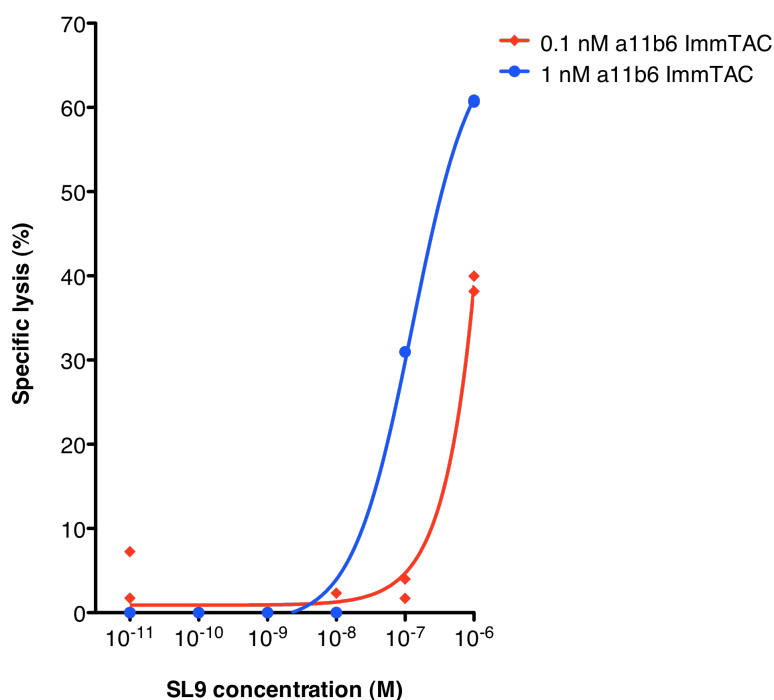


Figure 5.6 FATAL assay to detect cytotoxicity of T0 cells pulsed with serial log dilutions of SL9 peptide in the presence of 0.1 or 1 nM a11b6 ImmTAC and PBMCs. All assays were done in duplicate.

This 250-fold difference in EC_{50} value when comparing the assays shown in Figure 5.5 and Figure 5.6 was probably due to the change in $CD8^+$ E:T cell ratio, and the presence of other cell types in the PBMC population may have reduced the ease of redirected lysis. Overall, the a11b6 ImmTAC was capable of redirecting $CD8^+$ effector cells to peptide-pulsed HLA-A*02⁺ target cells leading to their apoptosis.

5.2 Effect of a11b6 ImmTAC on HIV-infected PBMCs

The a11b6 ImmTAC was tested in HIV-infected PBMCs in the same assay as in chapter 4.1. HLA-A*02⁺ PBMCs from two different donors were PHA-activated and 3 days later infected with HIV_{LAI}. At day 2 postinfection, a11b6 ImmTAC was added at a final concentration of 0.1, 0.3 or 1 nM, and p24 intracellular staining performed daily until 6 days postinfection. As an additional control, the cancer-specific NY-ESO-1 ImmTAC was used; this ImmTAC has an anti-CD3 scFv fused to an HLA-A*02-restricted NY-ESO-1-specific TCR that recognises the peptide SLLMWITQV. This assay did not require the addition of $CD8^+$ cells as those already present in the PBMCs should act as internal effector cells; of the $CD3^+$ cells, the proportion of $CD8^+$ cells ranged from 10 to 20%. An example of the flow cytometry plots is given in Figure 5.7A.

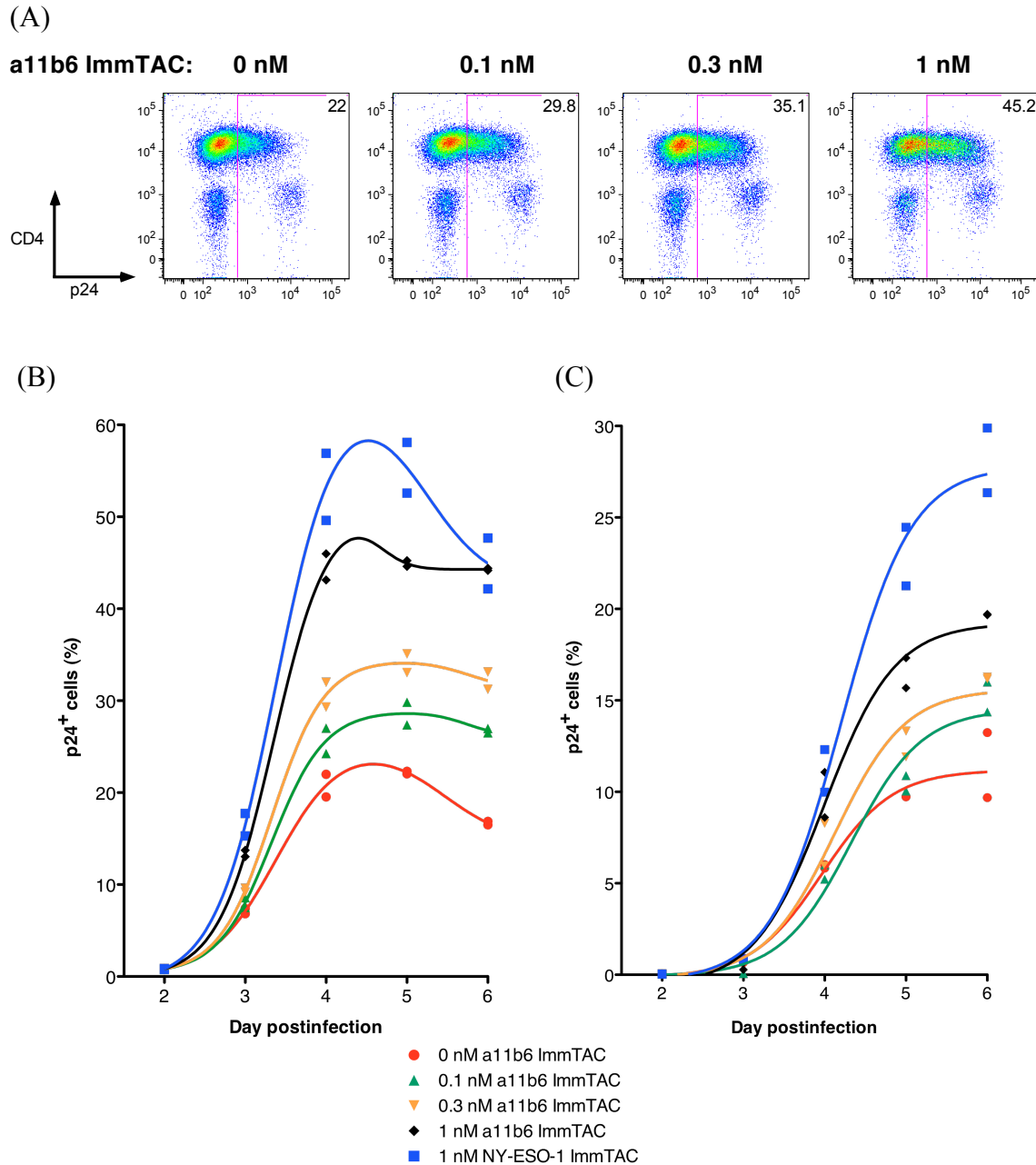


Figure 5.7 (A) Effect of a11b6 ImmTAC on HIV-1-infected HLA-A*02⁺ PBMCs day 5 postinfection. Plots of CD4 versus intracellular p24 are gated on viable CD14⁻CD19⁻CD3⁺CD8⁻ cells. **(B)** The percentage of p24⁺ cells at each day postinfection following the addition of ImmTACs in HIV-infected PBMCs from donor 1. **(C)** The percentage of p24⁺ cells at each day postinfection following the addition of ImmTACs in HIV-infected PBMCs from donor 2.

Figure 5.7B shows that with the control, which did not receive any ImmTAC, the percentage of p24⁺ cells reaches its peak around day 5 postinfection in PBMCs from donor 1. There was no change in the day that peak infection was observed when PBMCs were cocultured with the a11b6 or NY-ESO-1 ImmTACs. However, whereas the high affinity a6 TCR-transduced cells in chapter 4.1 significantly decreased the percentage of gag⁺ cells (Figure 4.2), the addition of the high affinity ImmTACs had the opposite effect. At day 5 postinfection, the mean percentage of p24⁺ cells was 22.2% ± 0.2%, 28.6% ± 1.7%, 34.1% ± 1.4%, 44.9% ± 0.4% and 55.3% ± 3.9% for the control, 0.1 nM a11b6 ImmTAC, 0.3 nM a11b6 ImmTAC, 1 nM a11b6 ImmTAC and 1 nM NY-ESO-1 ImmTAC, respectively. This equates to a 1.2-, 1.5- and 2.0-fold significant increase in the percentage of p24⁺ cells when comparing no ImmTAC addition to 0.1, 0.3 and 1.0 nM a11b6 ImmTAC addition (P = 0.0353, 0.0073 and 0.0002), respectively. This a11b6 ImmTAC dose-dependent increase in p24⁺ cells indicates that more cells became infected at higher concentrations of a11b6 ImmTAC. The addition of 1 nM NY-ESO-1 ImmTAC caused the largest increase in p24⁺ cells in PBMCs from donor 1 at day 5 postinfection, 2.4-fold more than the control (P = 0.0069).

This unexpected result was also observed when the ImmTACs were added to HIV-infected PBMCs from donor 2. Figure 5.7C shows that although HIV_{LAI} growth was slower in these PBMCs, the presence of ImmTACs increased the percentage of p24⁺ cells. At day 5 postinfection, the mean percentage of p24⁺ cells was 9.7% ± 0.01%, 10.47% ± 0.6%, 12.62% ± 1.0%, 16.5% ± 1.2% and 22.9% ± 2.3% for the control, 0.1 nM a11b6 ImmTAC, 0.3 nM a11b6 ImmTAC, 1 nM a11b6 ImmTAC and 1 nM NY-ESO-1 ImmTAC, respectively. This equates to a 1.7- and 2.3-fold significant increase

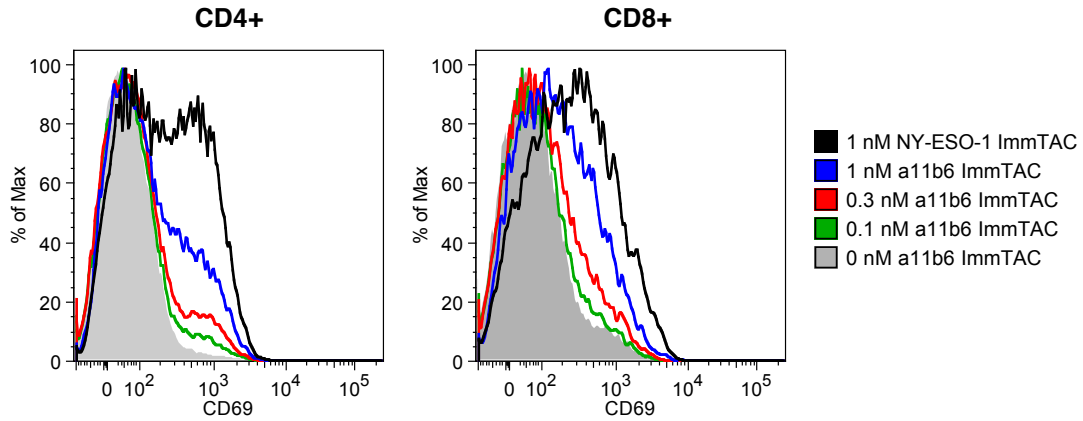
in the percentage of p24⁺ cells when comparing no ImmTAC addition to 1 nM a11b6 ImmTAC and 1 nM NY-ESO-1 ImmTAC (P = 0.0144 and 0.0145), respectively. There was no significant difference between the percentage of p24⁺ cells when comparing no ImmTAC to 0.1 or 0.3 nM a11b6 ImmTAC addition at any day postinfection analysed.

Varela-Rohena et al. found that after 8 days *in vitro* the high affinity a11b6 TCR-transduced cells resulted in an 85% decrease in the percentage of p24⁺ cells in HIV_{NL4-3}-infected CD4⁺ cell cultures compared to coculture with non-transduced cells (Varela-Rohena et al., 2008). When the same TCR was fused to an anti-CD3 scFv to form an ImmTAC with the aim of redirecting lysis, there was an increase in HIV infection *in vitro*.

As HIV preferentially infects activated CD4⁺ T cells (Gowda et al., 1989), we assessed the expression of CD69 on the T cell subsets in our HIV-infected PBMC cultures. CD69 is an early activation marker of lymphocytes, expressed within a few hours of stimulation. It is still expressed for over 18 h after the stimulus has been removed, before gradually decreasing (Hara et al., 1986, Testi et al., 1988, Cebrian et al., 1988).

Figure 5.8A show an example of CD69 expression at day 4 postinfection on both CD4⁺ and CD8⁺ cells. The ImmTACs were added on day 2 postinfection and it can be seen that peak CD69 expression was on day 3 or 4 and gradually decreases over time (Figure 5.8B). This was independent of HIV infection as a similar pattern of ImmTAC dose-dependent increase in CD69 expression was observed in uninfected PBMC cultures.

(A)



(B)

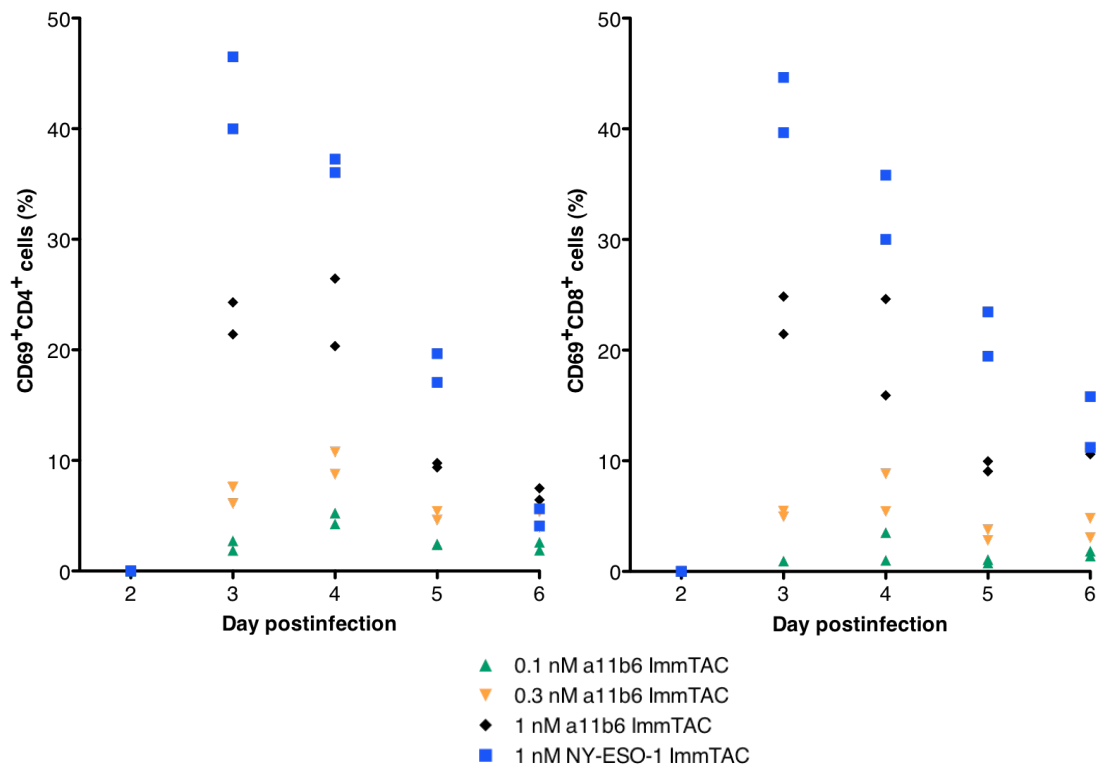


Figure 5.8 Activation status of HIV-infected PBMCs from donor 1. (A) Day 4 postinfection stains gated on viable CD14⁻CD19⁻CD3⁺ cells. Expression of CD69 is shown for either CD4⁺ or CD8⁺ cells. Gray histogram bars show HIV-infected PBMCs without ImmTAC addition. (B) The percentage of CD69⁺ cells at each day postinfection following the addition of ImmTACs. Each graph is gated on either CD4⁺ or CD8⁺ cells after subtraction of the expression of CD69 without ImmTAC addition.

The activation status of the cells in this assay can also be compared to when the non-, JM22-, wt and a6 TCR-transduced cells were added to the same HIV-infected PBMCs from donor 1 in chapter 4.1. Of the five time-points analysed by flow cytometry, CD69⁺CD4⁺ expression was <1.2%, whilst CD69⁺CD8⁺ was <5.2%.

As increasing concentrations of the ImmTACs caused dose-dependent expression of an activation marker on CD4⁺ cells, we therefore concluded that the ImmTACs enhanced the susceptibility of CD4⁺ cells to HIV infection.

5.3 New ImmTACs

Despite the a11b6 TCR having a K_d value of 3.65 x 10⁻¹⁰ M (Table 4.2) and a binding half-life >2.5 h to HLA-A*02-SL9, these properties could be improved by more site-directed mutagenesis of the CDR in both the TCR α and β chains. Through this methodology, Immunocore produced three new ImmTACs: a12b40, a15b40 and a15b26; the K_d and binding half-life values of these SL9-specific TCRs are 2 x 10⁻¹¹ M and 31 h, 1.8 x 10⁻¹¹ M and 59 h, and 2.65 x 10⁻¹⁰ M and 34 h, respectively. Fusion of the TCR with the anti-CD3 scFv did not greatly affect these binding properties (Appendix G).

5.3.1 Effect on effector cells cocultured with SL9-pulsed T2 cells

The new ImmTACs were titrated in an IFN_γ ELISpot assay to determine their potency when redirecting lysis. The gp100 peptide (YLEPGPVTV) was used as a control to detect nonspecific release of IFN_γ. Serial log dilutions of a11b6, a12b40, a15b40 and

a15b26 ImmTACs were plated with 1 nM SL9 or gp100 peptide-pulsed T2 cells. Effector CD8⁺ cells were added at an E:T ratio of 1:6.25 and incubated overnight. The plates were developed to detect IFN γ release.

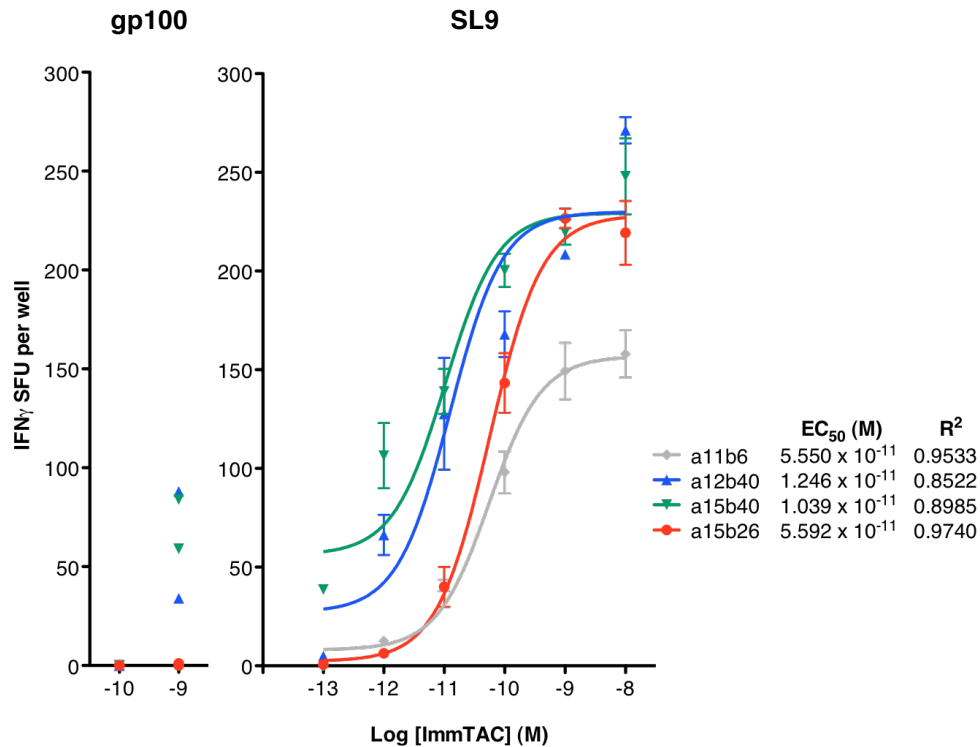


Figure 5.9 IFN γ responses of CD8⁺ cells redirected to target cells using serial log dilutions of HIV-specific ImmTACs. Each well contained 50,000 target cells pulsed with either 1 nM SL9 or gp100, and 8,000 effector cells. Each well was performed in triplicate; error bars are SEM.

Figure 5.9 shows that all the ImmTACs were able to activate effector cells to produce IFN γ in the presence of 1 nM SL9-pulsed target cells, with a maximal response achieved with 1 nM ImmTAC. The a11b6 and a15b26 ImmTACs had similar EC₅₀ values of 5.6 x 10⁻¹¹ M, although at the higher ImmTAC concentrations the maximal production of IFN γ was higher for the a15b26 ImmTAC. The a12b40 and a15b40

ImmTACs were ~5-fold more potent at activating IFN γ release from effector cells (EC₅₀ values of $\sim 1 \times 10^{-11}$ M). However, when target cells were pulsed with control peptide, sharing only 22% identity with SL9, nonspecific IFN γ release was observed with 1 nM a12b40 and a15b40 ImmTACs. This was equivalent to the IFN γ detected when 0.001 nM a12b40 and a15b40 ImmTACs were plated with 1 nM SL9-pulsed T2 cells. This nonspecific IFN γ release was not observed when 0.1 nM a12b40 and a15b40 ImmTACs were plated with the gp100-pulsed T2 cells.

5.3.2 *Effect on HIV-infected PBMCs*

Potency was decided to be a more important factor than off-target activity, especially given that no nonspecific IFN γ release was observed at lower ImmTAC concentrations. The two most potent ImmTACs, a12b40 and a15b40, were tested in HIV-infected PBMCs. PHA-activated HLA-A*02⁺ PBMCs were infected with HIV_{LAI} and at day 2 postinfection, the ImmTACs were added at a final concentration of 0.01, 0.1 and 1 nM. Flow cytometry was performed 24 and 48 h later to assess intracellular p24. The HLA-A*02-restricted gp100 ImmTAC was used as a control, as were HIV-infected PBMCs that were not incubated with any ImmTAC. As before, this assay did not require the addition of CD8⁺ cells as those already present in the PBMCs should act as internal effector cells; the PBMCs were from donor 2 as used in chapter 4.1 and 5.2.

As previously found with the a11b6 ImmTAC (Figure 5.7), there was no decrease in the percentage of p24⁺ cells when a12b40 and a15b40 ImmTACs were added to HIV-infected PBMCs (Figure 5.10). At day 3 and 4 postinfection, the mean percentage of p24⁺ cells was $7.6\% \pm 0.1\%$ and $10.0\% \pm 3.6\%$, respectively, when no ImmTACs were

added. There was a significant increase in the mean percentage of p24⁺ cells at day 3 in the presence of 0.1 and 1 nM a12b40 (P = 0.0277 and 0.0086) and 1 nM a15b40 (P = 0.0048); the mean percentage of p24⁺ cells at day 4 postinfection was 27.2% ± 0.1% and 33.8% (not in duplicate) in the presence of 1 nM a12b40 and a15b40 ImmTAC, respectively. Only with 1 nM gp100 ImmTAC was a significant 1.2-fold increase seen at day 3 postinfection (P = 0.0225).

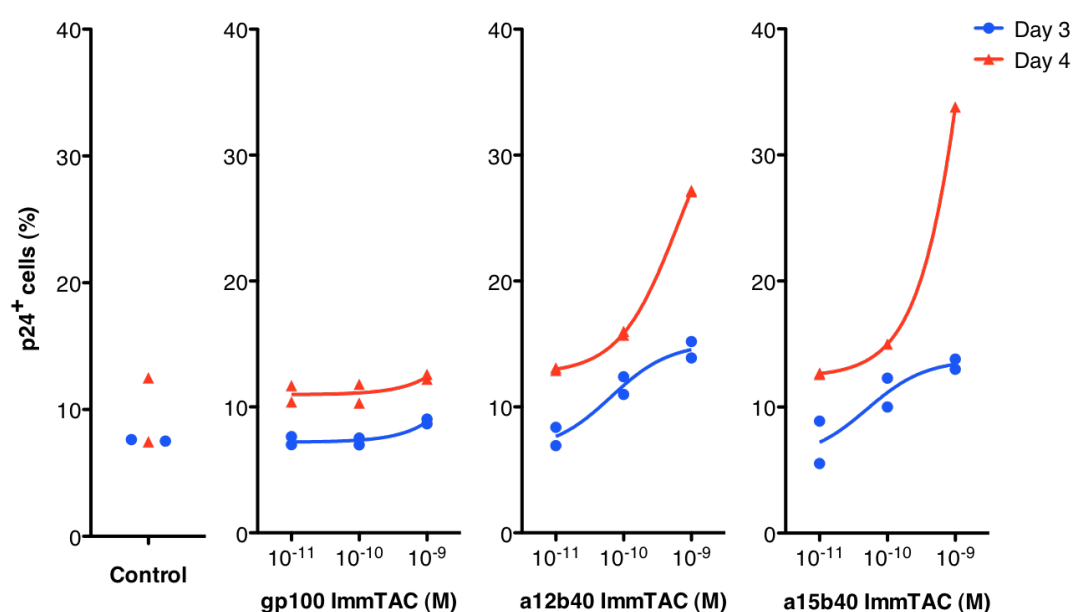


Figure 5.10 Effect of ImmTACs on HIV-1-infected HLA-A*02⁺ PBMCs at day 3 and 4 postinfection. The percentage of intracellular p24⁺ cells gated on viable CD14⁺CD19⁺CD8⁺ cells is shown. The control is HIV-1-infected HLA-A*02⁺ PBMCs without ImmTAC addition.

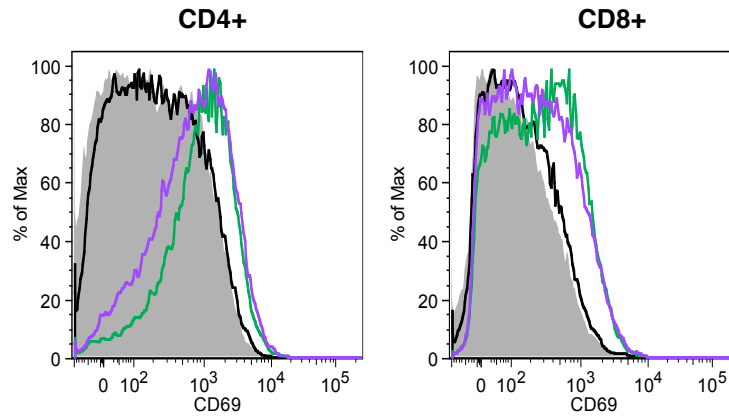
Furthermore, this increase in the percentage of gag⁺ cells was confirmed to correspond to virion release in the PBMC culture supernatant. A real-time reverse transcriptase assay was performed on thawed cell-free viral supernatant from day 4 postinfection gp100 and a12b40 ImmTAC samples (Appendix H). There was no significant

difference between the reverse transcriptase activity from the no ImmTAC supernatant and any concentration of the gp100 ImmTAC or 0.01 nM a12b40 ImmTAC; at 0.1 and 1 nM concentrations, the a12b40 ImmTAC led to a 5.3- and 7.9-fold increase in RT activity compared to no ImmTAC addition ($P = 0.0008$ and 0.0452), respectively.

Next, the expression of CD69 on the T cell subsets in our HIV-infected PBMC cultures was assessed. In the presence of 0.1 and 1 nM a12b40 and a15b40 ImmTAC, there was increased expression of CD69 on CD4⁺ and CD8⁺ cells. There was no significant difference between expression of CD69 when the gp100 ImmTAC was added compared to control (Figure 5.11).

This a12b40 and a15b40 ImmTAC dose-dependent increase of gag⁺ cells accompanied by a dose-dependent increase in CD69 expression on CD4⁺ cells indicates that the activated cells became more infected at increased concentrations of ImmTAC. Unlike the NY-ESO-1 ImmTAC, the gp100 ImmTAC did not cause a large increase in p24⁺ cells nor an increase in CD69. This implies that it is not the anti-CD3 scFv domain alone causing increased cell activation and HIV infection *in vitro*.

(A)



(B)

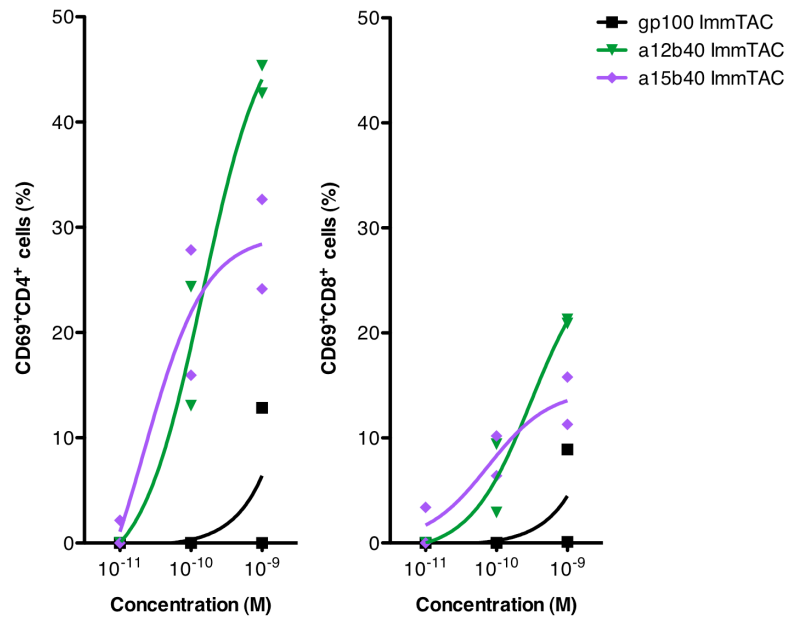


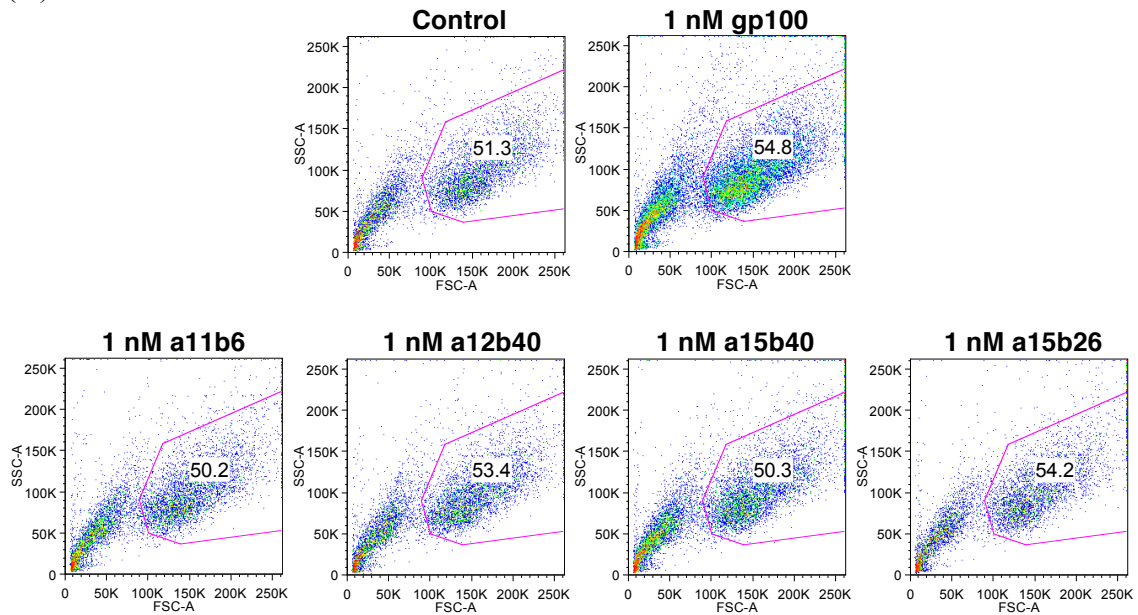
Figure 5.11 Activation status of the HIV-infected PBMCs at day 3 postinfection. (A) Gated on viable CD14⁺CD19⁻ cells when in the presence of 1 nM ImmTAC. Expression of CD69 is shown for either CD4⁺ or CD8⁺ cells. Gray histogram bars show HIV-infected PBMCs without ImmTAC addition. (B) The percentage of CD69⁺ cells following the addition of ImmTACs. Each graph is gated on either CD4⁺ or CD8⁺ cells after subtraction of the expression of CD69 without ImmTAC addition.

5.3.3 *Effect on HIV-infected T1 cells*

To investigate further this increase in T cell activation and HIV infection observed with a11b6, a12b40 and a15b40 ImmTACs in PBMCs, these ImmTACs plus a15b26 and gp100 ImmTACs were tested in the CD4⁺ 174xCEM.T1 cell line that expresses HLA-A*02. T1 cells were infected with HIV_{LAI} and on day 2 postinfection, 0.1 or 1 nM ImmTAC and effector cells (>95% CD8⁺) were added at an E:T ratio of 5:1. Flow cytometry was performed 24 h later to assess intracellular p24.

The effect of the ImmTACs on uninfected T1 cells alone was first assessed. There was no change in the proportion of live T1 cells in the presence of any ImmTAC. This shows that the ImmTACs alone caused no toxicity to the T1 cells (Figure 5.12A). However, when CD8⁺ effector cells were cocultured with the uninfected T1 cells, the a12b40 and a15b40 ImmTACs led to a decrease in the percentage of live T1 cells even at a concentration of 0.1 nM. This was not a consequence of the effector cells proliferating, but due to nonspecific loss of uninfected T1 cells. The same effect was not seen with a11b6 or a15b26 ImmTACs at a final concentration of 0.1 nM (Figure 5.12B); and at final concentration of 1 nM, the percentage of gated T1 cells was 23.8% and 18.9%, respectively.

(A)



(B)

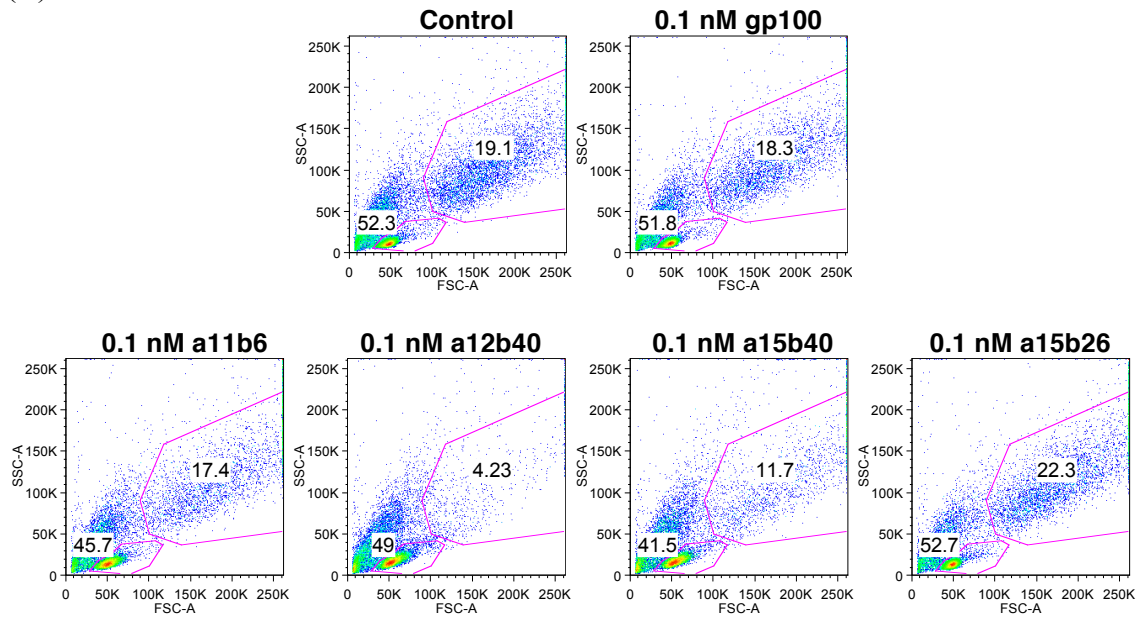


Figure 5.12 Effect of ImmTACs on uninfected T1 cells. (A) With no effector cells added. (B) Cocultured with CD8⁺ cells at an E:T ratio of 5:1.

Next, the expression of CD69 on the CD8⁺ effector cells in our uninfected T1 cell cultures was analysed. In the presence of 0.1 nM a12b40 and a15b40 ImmTACs, CD69

expression increased compared to no ImmTAC addition, gp100 and a15b26 ImmTAC addition (Figure 5.13); this increased even further with 1 nM a12b40 and a15b40 ImmTACs. The CD4⁺ T1 cells do not have upregulated expression of CD69; T1 cells do not express on their cell surface CD3 so may not behave like CD4⁺ cells isolated from PBMCs. As all the ImmTACs have the same anti-CD3 scFv, this difference in activation and nonspecific lysis must be due to the mTCR.

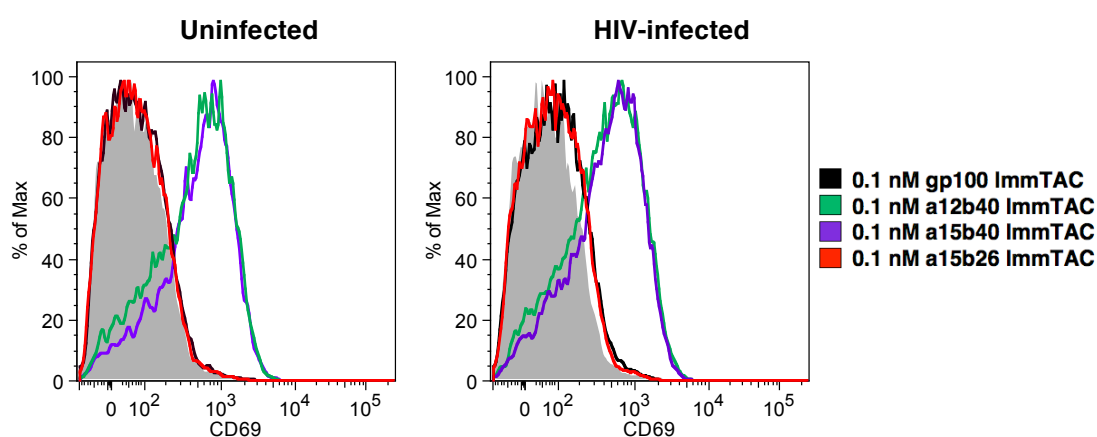


Figure 5.13 Effect of ImmTAC reagents on CD69 expression on CD8⁺ cells in the presence of uninfected or infected T1 cells. Cells were gated on viable, CD8⁺ cells in the lymphocyte population. Gray histogram bars show without ImmTAC addition.

A similar pattern was observed in the HIV-infected T1 cells, where without effector cells there was no ImmTAC-mediated impact on the cells. The a12b40 and a15b40 ImmTACs were removed from subsequent analysis because the number of live T1 cells had dropped to such low levels that the number of events was <200 at both 0.1 and 1 nM concentrations. There was no significant difference in the percentage of gag⁺ cells

when a11b6 or a15b26 ImmTAC were added to the HIV-infected T1/CD8⁺ cell cocultures compared to no ImmTAC addition (Figure 5.14).

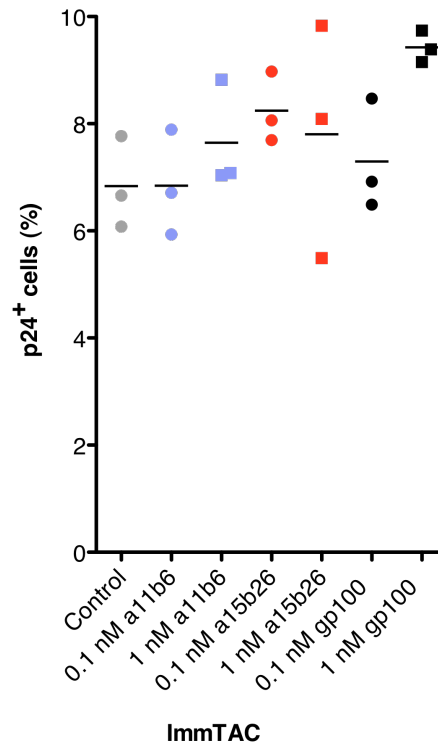


Figure 5.14 Effect of ImmTACs on HIV-infected T1 cells at day 3 postinfection in the presence of CD8⁺ cells at an E:T ratio of 5:1. The percentage of intracellular p24⁺ cells gated on viable CD8⁺ cells is shown. The ImmTAC reagents a12b40 and a15b40 were removed from this analysis as there were not enough collected events for these samples. The control is without ImmTAC addition.

5.4 Redirected lysis of HIV-infected cells

As the ImmTACs appeared incapable of controlling the spread of HIV infection in the same manner as the SL9-specific TCR-transduced cells in chapter 4, we evaluated their ability to specifically target HIV-infected HLA-A*02⁺ cells that were presenting SL9.

5.4.1 *Specific redirected lysis of HIV-infected T1 cells*

This assay involved three HLA-A*02⁺CD4⁺ cell populations mixed together with the aim of specifically redirecting effector cells to only lyse the CD4⁺ cells presenting SL9. T1 cells and T2 cells were separately infected with HIV_{LAI} and at day 4 postinfection, unstained HIV-infected T1 cells were mixed with CellTracker Orange-stained HIV-infected T2 cells and CFSE-stained uninfected T1 cells. AZT to a final concentration of 10 μ M was added plus CellTracker Violet-stained effector cells (>80% CD8⁺) at an E:T ratio of 4:1. The ImmTACs a12b40, a15b40, a15b26 and gp100 were added at a final concentration of 1 nM and the cocultures incubated overnight, then analysed by flow cytometry.

An example of a flow cytometry plot in terms of which stained population relates to which cell population is shown in Figure 5.15. When no ImmTAC was added to the cocultures, a mean of 34.7% of the cells were HIV-infected target T1 cells (after effector cell exclusion). The gp100 ImmTAC was used as a non-HIV-specific ImmTAC control, resulting in a mean value of 33.6% HIV-infected target T1 cells. When 1 nM a15b26 ImmTAC was added to the coculture, the mean percentage of HIV-infected target T1 cells significantly decreased to 23.5%, equivalent to 32.2% specific lysis ($P = 0.0007$) (Figure 5.16).

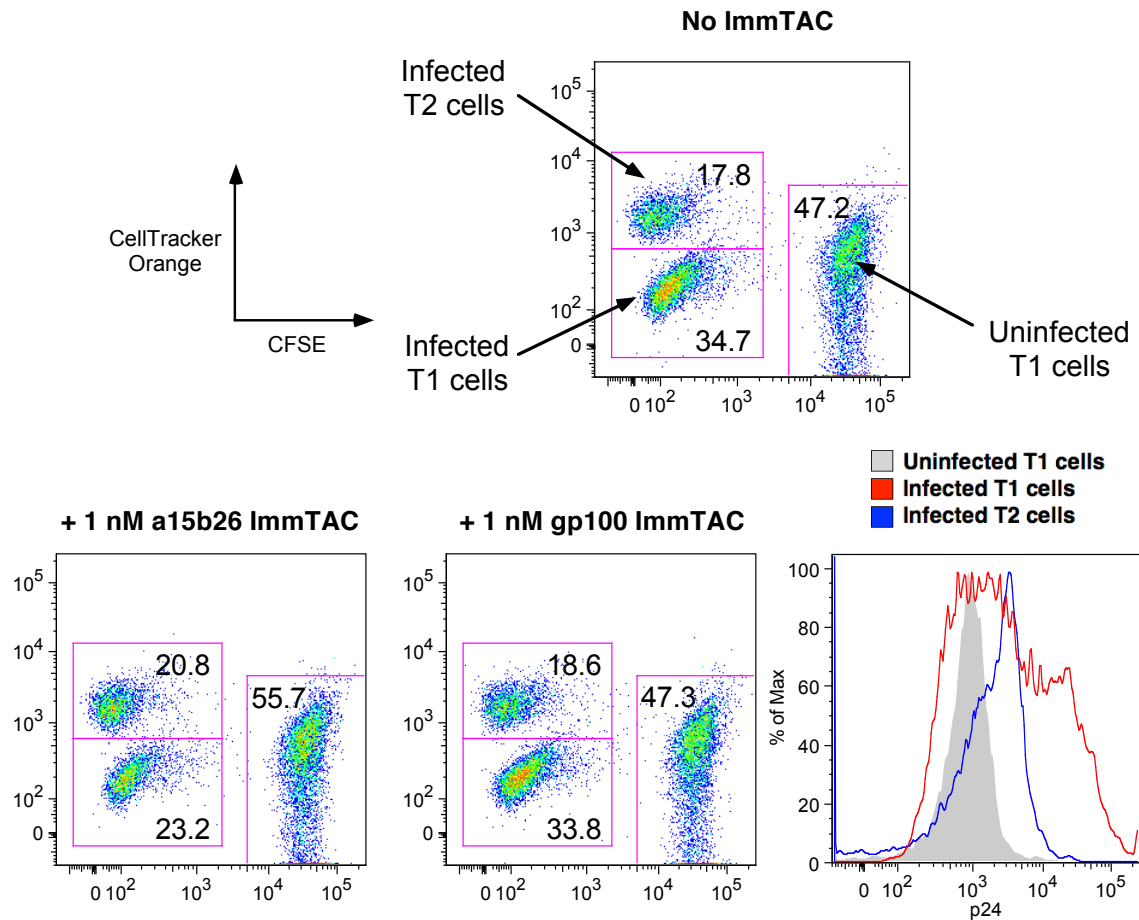


Figure 5.15 Flow cytometry plots of unstained HIV-infected T1 cells mixed with CellTracker Orange-stained HIV-infected T2 cells and CFSE-stained uninfected T1 cells. Gated on viable, CellTracker Violet⁺ cells. A histogram plot confirming the HIV infection status of the T1 and T2 cells is also shown.

As AZT was in the cocultures, uninfected cells remain so and therefore will not present SL9 and should not be targeted. The HIV-infected T2 cells are TAP-deficient so do not present SL9 and should also not be targeted. Figure 5.15 and Figure 5.16 show that 1 nM a15b26 ImmTAC was able to specifically redirect the effector cells to HIV-infected target T1 cells that present SL9 and lyse them.

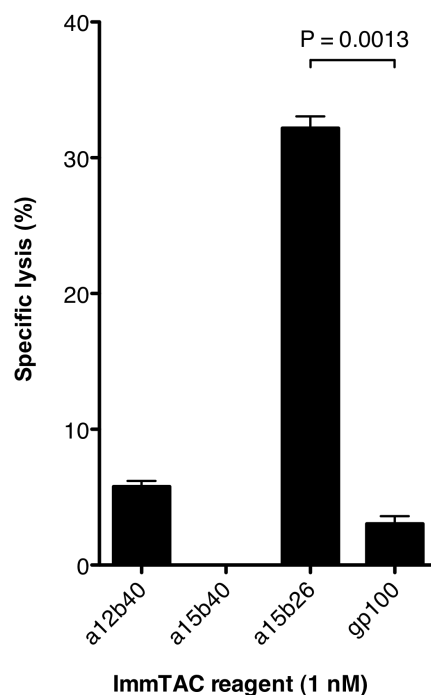
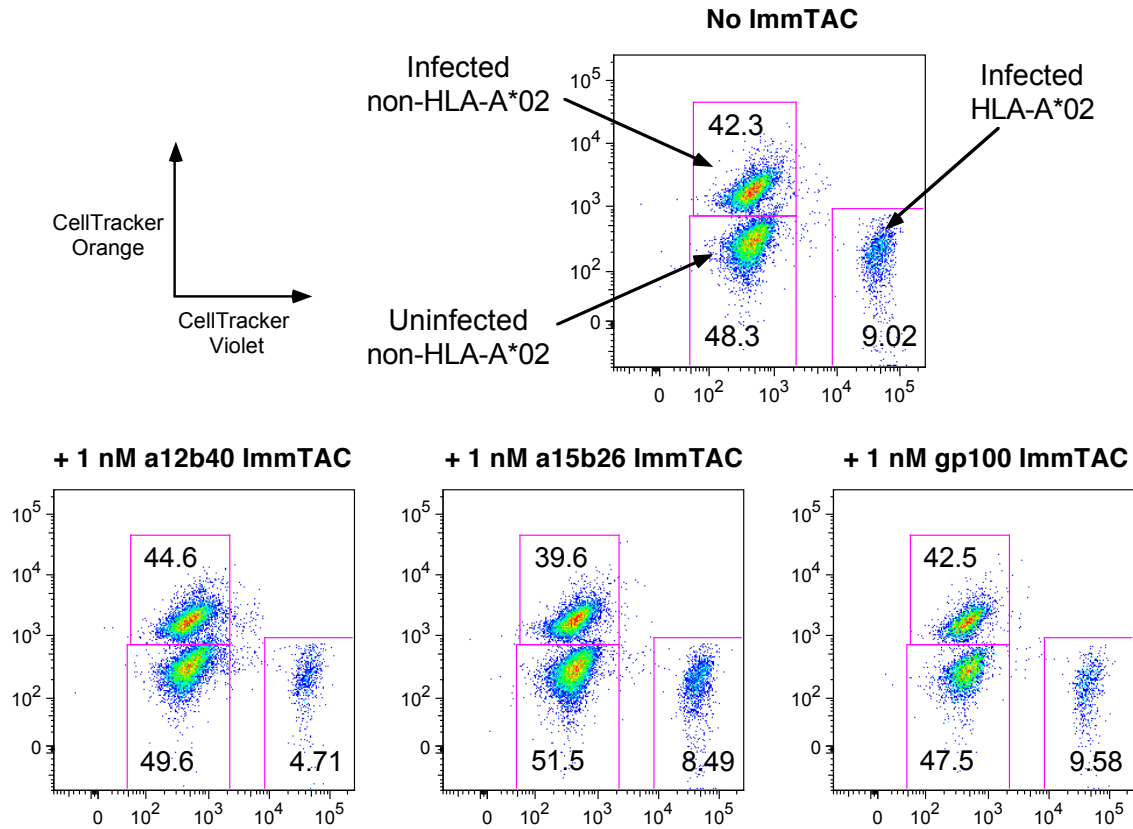


Figure 5.16 Specific lysis of HIV-infected T1 cells compared to lysis of the internal controls: uninfected T1 cells and infected T2 cells. Each well was performed in duplicate; error bars are SEM.

5.4.2 *Specific redirected lysis of HIV-infected PBMCs*

A similar assay to the previous was undertaken except PBMCs used instead of a cell line with added effector cells. PBMCs from HLA-A*02⁺ and HLA-A*02⁻ donors were separately infected with HIV_{LAI}. At day 4 postinfection, unstained uninfected HLA-A*02⁻ PBMCs were mixed with CellTracker Orange-stained HIV-infected HLA-A*02⁻ PBMCs and CellTracker Violet-stained HIV-infected HLA-A*02⁺ PBMCs. AZT to a final concentration of 10 μ M was added plus a12b40, a15b40, a15b26 or gp100 ImmTAC at a final concentration of 1 nM. The cocultures were incubated overnight and then analysed by flow cytometry.

(A)



(B)

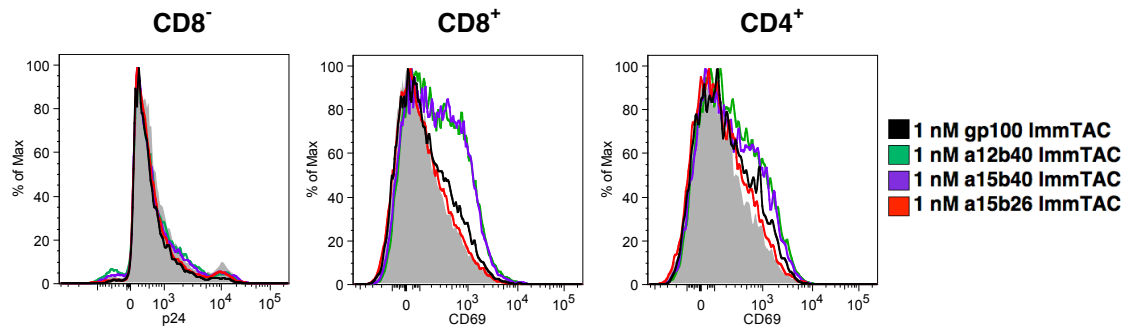


Figure 5.17 Flow cytometry plots of unstained, uninfected HLA-A*02⁻ PBMCs mixed with CellTracker Orange-stained HIV-infected HLA-A*02⁻ PBMCs and CellTracker Violet-stained HIV-infected HLA-A*02⁺ PBMCs. (A) Gated on viable, singlet, CD3⁺CD8⁻ cells. Double positive CellTracker Orange and CellTracker Violet cells have been gated out. (B) Gated on viable, singlet, CD3⁺ cells. Gray histogram bars show without ImmTAC addition.

An example of a flow cytometry plot in terms of which stained population relates to which cell population is shown in Figure 5.17A. When no ImmTAC was added to the cocultures, a mean of 8.7% of the cells were HIV-infected HLA-A*02⁺ target cells. The gp100 ImmTAC was used as a non-HIV-specific ImmTAC control, and this led to a mean of 10.2% HIV-infected HLA-A*02⁺ target cells.

Figure 5.17B shows that the cocultures had similar levels of intracellular p24 unlike that seen in chapter 5.3.2. This was because the ImmTACs were added in combination with AZT, so despite the CD4⁺ and CD8⁺ cells being activated by 1 nM a12b40 and a15b40 ImmTACs, there was no increase in p24 expression, i.e. no increase in HIV infection.

Only when 1 nM a12b40 or a15b40 ImmTACs were added to the cocultures did the mean percentage of HIV-infected HLA-A*02⁺ target cells significantly decrease to 5.1% and 5.2%, respectively; this is equivalent to 41.4% and 40.1% specific lysis ($P = 0.0187$ and 0.0071), respectively. Therefore, in an HIV-infected PBMC culture, a12b40 and a15b40 ImmTACs were able to specifically redirect internal CD8⁺ cells to HIV-infected CD4⁺ cells causing cell lysis. 1 nM a15b26 ImmTAC did not significantly redirect the effector cells to HIV-infected HLA-A*02⁺ target cells in this system (Figure 5.18).

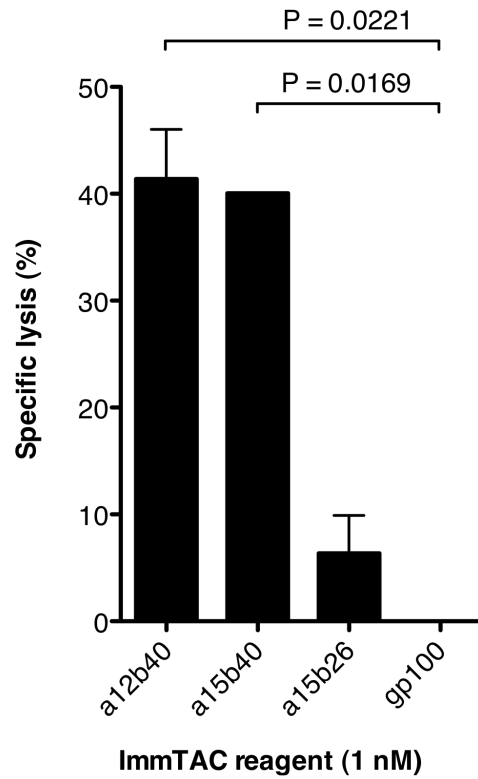


Figure 5.18 Specific lysis of HIV-infected CD3⁺CD8⁻ cells from a HLA-A*02⁺ donor compared to lysis of the internal controls: uninfected HLA-A*02⁻ cells and HIV-infected HLA-A*02⁻ cells. Each well was performed in duplicate; error bars are SEM.

Discussion

A high affinity SL9-specific mTCR was fused to an anti-CD3 scFv to create a HIV gag ImmTAC with the aim of specifically lysing HIV-infected cells and controlling infection. These reagents were designed to supersede the use of adoptive transfer with enhanced SL9-specific TCR-transduced cells that result in a significant reduction in gag⁺ cells *in vitro*. The ImmTACs, with a high affinity mTCR domain, would strongly bind to the HLA-A*02-SL9 complex on an HIV-infected cell with a long half-life, and the anti-CD3 scFv domain would redirect passing CD3⁺CD8⁺ cells to the HIV-infected cell, causing cell activation and lysis. Based on the assays in chapter 4, we sought to determine whether the HIV gag ImmTACs are able to redirect T cells against HIV-infected cells. We hypothesised that these ImmTACs would significantly reduce the proportion of gag⁺ cells *in vitro* to the same degree as their TCR-transduced cell counterparts.

The first HIV gag ImmTAC candidate was a11b6 ImmTAC; this TCR successfully reduced the proportion of gag⁺ cells *in vitro* when transduced into cells (Varela-Rohena et al., 2008). When HLA-A*02⁺ cells were pulsed with SL9 peptide and cocultured with CD8⁺ effector cells and 1 nM a11b6 ImmTAC, we confirmed that the ImmTAC was able to redirect CD8⁺ T cells to HLA-A*02-SL9 expressing cells resulting in their lysis; half the maximal lysis was observed at a SL9 concentration of 5.17×10^{-10} M. A similar effect was observed when PBMCs were used instead of purified CD8⁺ effector cells albeit at higher SL9 peptide concentrations. This difference was an indicator that a

pronounced effect in HIV-infected PBMCs may not be as easily achieved with the ImmTACs due to variations in effector-to-target cell ratio.

When increasing concentrations of a11b6 ImmTAC were tested in HIV-infected HLA-A*02⁺ PBMCs in the same assay as in chapter 4.1, a dose-dependent increase in gag⁺ cells was observed; 1 nM a11b6 ImmTAC caused the proportion of gag⁺ cells to approximately double. So contradictory to our hypothesis, the a11b6 ImmTAC did not reduce the proportion of gag⁺ cells *in vitro*. Aggregation of the ImmTAC may have been a factor in this negative result, enabling them to act like anti-CD3 antibodies, which cause cellular activation and therefore increased HIV infection *in vitro*.

Three new ImmTACs were produced, a12b40, a15b40 and a15b26, with even stronger binding affinities and longer binding half-lives than a11b6 ImmTAC. All three were able to induce CD8⁺ effector cells to release IFN γ in the presence of 1 nM SL9 peptide-pulsed HLA-A*02⁺ cells, signifying that redirection was occurring; a12b40 and a15b40 ImmTACs were considered to be the most potent. However, nonspecific IFN γ release in the presence of control peptide-pulsed cells with 1 nM a12b40 and a15b40 ImmTACs indicated an off-target activity. When increasing concentrations of a12b40 and a15b40 ImmTACs were tested in HIV-infected HLA-A*02⁺ PBMCs, a dose-dependent increase in gag⁺ cells was observed, similar to that found with the a11b6 ImmTAC. When increasing concentrations of a11b6 and a15b26 ImmTACs were tested in HIV-infected T1 cells cocultured with CD8⁺ cells, there was no change in the percentage of gag⁺ cells. These results implied that the HIV gag ImmTACs could not control the

percentage of gag⁺ cells in HIV-infected cell cultures in the same way as the TCR-transduced cells did in chapter 4.

It was also found that increasing concentrations of the a11b6, a12b40 and a15b40 ImmTACs in PBMC cultures caused an upsurge in the early activation marker CD69, not only on CD8⁺ cells, but also on CD4⁺ cells; this pattern was observed in both infected and uninfected PBMCs. The gp100 ImmTAC did not cause an increase in CD69 expression. As HIV preferentially infects activated CD4⁺ T cells, we postulated that the ImmTACs were increasing the capacity for HIV infection hence the dose-dependent increase in gag⁺ cells. This increase in CD4⁺CD69⁺ cells was not observed in our cell line cultures, possibly to due to an inability to express CD69; the addition of Staphylococcal Enterotoxin B to HIV-infected T0 cells also does not upregulate CD69 expression.

Although unable to control HIV infection *in vitro*, the question beckoned as to whether the ImmTACs were able to lead to specific lysis of HIV-infected cells. The a15b26 ImmTAC in the presence of 10 μ M AZT and CD8⁺ effector cells, specifically lysed HIV-infected target T1 cells over uninfected T1 cells and HIV-infected, TAP-deficient control cells. However, in HIV-infected PBMC cultures with AZT present, this ImmTAC did not have a significant effect on the HIV-infected target cells, and it was in fact a12b40 and a15b40 ImmTACs that were able to redirect CD8⁺ cells to lyse target cells. These contradictory results may be due to the use of cell lines versus PBMCs as the E:T ratio in a PBMC culture is less than 5:1, plus the presence of other cell subsets within the culture may have lessened the effect of the ImmTACs on the CD8⁺ cells.

The problem of nonspecificity is highly unlikely due to the anti-CD3 scFv. When gp100, a11b6 and a15b26 ImmTACs were added to uninfected T1 cell/effector cell cocultures, no change in live T1 cell proportions were observed, whilst the addition of a12b40 and a15b40 ImmTACs resulted in a decrease in the number of live HLA-A*02⁺ cells. As all the ImmTACs have the same anti-CD3 scFv, the difference in nonspecific lysis must be due to the mTCR.

The a12b40 and a15b40 ImmTACs have the strongest TCR binding affinities, with K_d values of 24 and 27 pM, respectively. When NY-ESO-1-specific TCRs with binding affinities ranging from 4 μM to 26 pM were transduced into CD8⁺ cells, despite showing high antigen specificity to HLA-A*02 tetramers, there was a loss of specificity when incubated with peptide-pulsed T2 cells at the higher TCR affinities. It appeared that specificity for HLA-A*02 overrode specificity for the antigen in a TCR affinity-dependent manner (Zhao et al., 2007). The antigen specificity threshold of this NY-ESO-1-specific TCR in CD8⁺ cells was predicted to be within a binding affinity of 450 and 280 nM (Robbins et al., 2008). So whilst T cells need the presence of self MHC molecules in the periphery for long-term survival, they should not be binding to a level that triggers cell activation (Kirberg et al., 1997). It appears that the TCR affinities of a12b40 and a15b40 ImmTACs are so high they bind and trigger T cell activation nonspecifically, leading to cell lysis.

When the a11b6 TCR, with a K_d value of 365 pM, was transduced into CD8⁺ cells and fused to the anti-CD3 scFv, it retained antigen specificity. However, while the a11b6 TCR-transduced CD8⁺ cells are able to control *in vitro* HIV-infection in PBMCs, when

in ImmTAC form, they intensified HIV infection. As HIV infection actually increased compared to control, this different *in vitro* outcome is not down to redirected lysis being slower than direct lysis by TCR-transduced cells. The a15b26 TCR, with a K_d value of 265 pM, showed similar results to a11b6 ImmTAC in HIV-infected T1 cells. As this cell line does not surface stain positive for CD3, we hypothesised that the increase in gag⁺ cells in HIV-infected PBMCs is due to a combination of activating CD3⁺CD4⁺ cells and redirecting them to the infected target cell, therefore spreading infection (Figure 5.19).

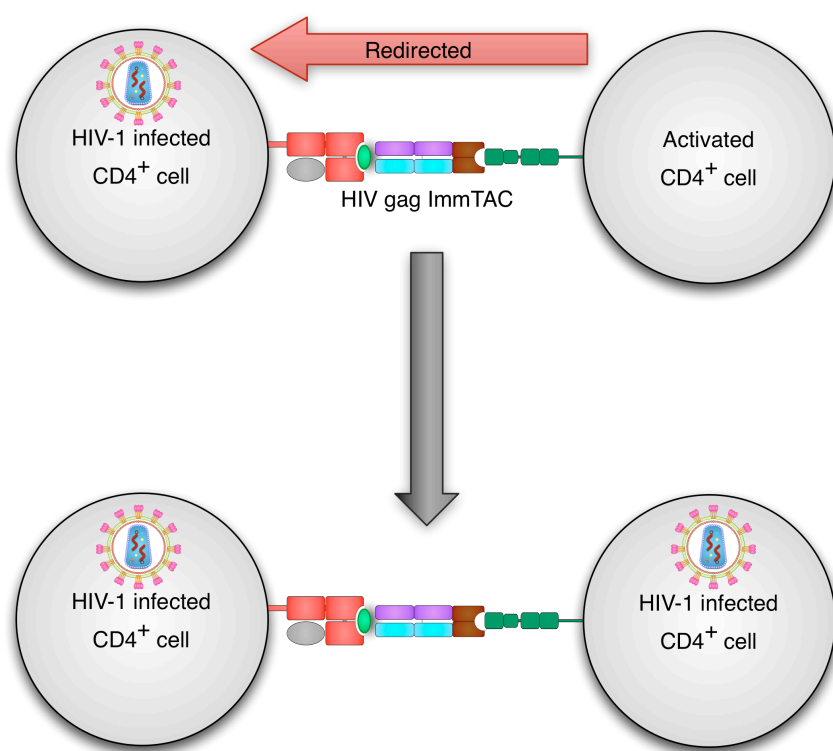


Figure 5.19 Schematic diagram of an alternative role of the HIV-specific ImmTACs. The anti-CD3 scFv domain of the ImmTAC is able to bind to CD3 molecules on CD4⁺ cells, therefore bridging the immunological synapse between an HIV-infected CD4⁺ cell and an activated uninfected CD4⁺ cell.

In the HLA-A*02⁺ cell line not staining positive for CD3 and without activation, the lack of control of HIV infection is possibly due to the rate of infection being greater than the rate of redirected lysis, whereas with the TCR-transduced cells, the rate of infection appears to be slower than TCR-transduced cell-mediated cytotoxicity. Within the HIV-infected PBMC cultures, the CD4/CD8 ratio was high and so the ImmTACs possibly came into contact with more CD3⁺CD4⁺ cells than CD3⁺CD8⁺ cells, increasing the spread of infection. So, although we have shown that the HIV-specific ImmTACs do redirect T cells to HIV-infected cells, this includes CD4⁺ T cells as well as CD8⁺ T cells, therefore providing more opportunity for HIV spread. To prevent this situation, the HIV-specific ImmTAC must be used in synergy with HAART *in vivo*. This would readdress the balance in favour of HIV-infected cell lysis, with no spread of infection (Figure 5.20). If also combined with an HIV latent cell activator, such as a histone deacetylase inhibitor, elimination of HIV from an infected individual may become a realistic outcome in the future.

The premise of the ImmTACs was to manipulate the host immune system as HIV is able to manipulate the host cell machinery. ImmTACs were to behave like satellite navigation devices for effector cells, programmed to direct them to the predetermined destination of an HIV-infected cell. However, it appears that other cell subsets have had their journey reprogrammed with harmful consequences. This concept bears similarities to how HIV uses dendritic cells *in vivo* to increase the spread of infection. We suggest that the HIV-infected cells hitchhike with the HIV-specific ImmTAC to target cells, which can be compared with HIV virions hijacking to dendritic cells through DC-SIGN.

In the latter model, the virions are transported to lymph nodes that are enriched with CD4⁺ cells (Cameron et al., 1992, Geijtenbeek et al., 2000).

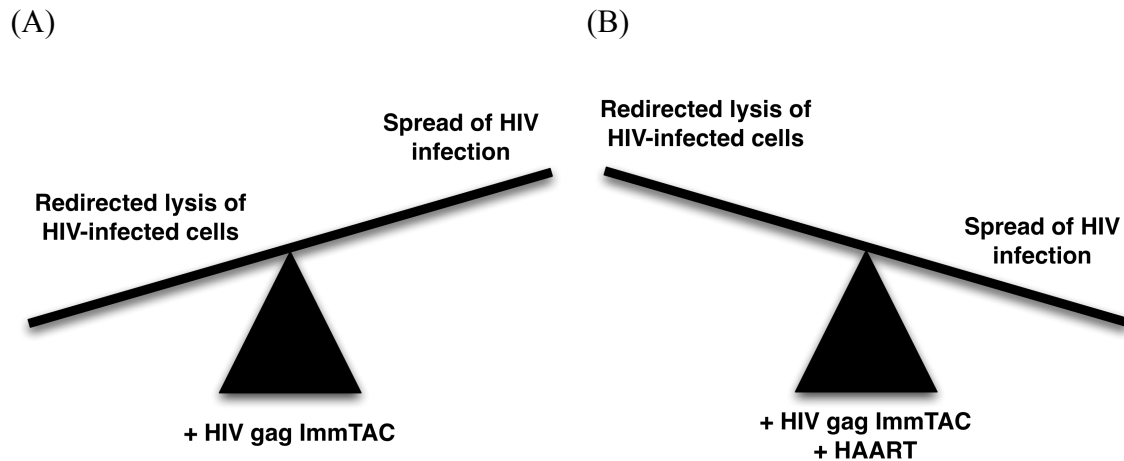


Figure 5.20 Balance of the HIV gag ImmTAC bridging the immunological synapse between a CD8⁺ CTL enabling cell killing or CD4⁺ cell with the potential for spread of infection. (A) With just the addition of the ImmTAC, the balance is in favour of spread of HIV infection. (B) In combination with HAART, we postulate that the balance is in favour of control of infection and elimination of HIV-infected cells.

In summary, we have found that HIV-specific ImmTACs are able to redirect T cells against SL9-presenting cells; however, in HIV-infected cultures without antiretroviral drug assistance, there is increased spread of infection. The ImmTACs alone are inferior to the TCR-transduced cells at controlling HIV infection *in vitro*. A key point to bear in mind is that this is a novel HIV therapeutic and optimisation of a successful HIV-specific ImmTAC may take many years and will need to be carefully evaluated for safety. The use of a humanised mouse model that can be infected with HIV and express HLA-A*02 would be valuable in the further assessment of the ImmTACs.

CHAPTER 6

Conclusive remarks and future perspectives

There is currently no HIV vaccine or therapeutic mechanism of eradicating HIV from an infected individual. Recent vaccines have resulted in only a modest protective effect (Rerks-Ngarm et al., 2009), whilst the one case of eradicating HIV to below the limit of detection involved transplantation of $\Delta 32/\Delta 32$ CCR5 stem cells (Hutter et al., 2009a, Hutter et al., 2009b) and so unviable on a mass level. This thesis focuses on how the CTL response may be utilised in a vaccine or as part of an immunotherapy. The importance of the CTL response originates from its temporal association with control of viraemia in primary HIV-1 infection (Borrow et al., 1994, Koup et al., 1994), CD8⁺ cell depletion studies in macaques (Schmitz et al., 1999), associations of HLA alleles with varying rates of disease progression (Kaslow et al., 1996, O'Brien et al., 2001), and from studies in LTNPs (Harrer et al., 1996, Rinaldo et al., 1995) and uninfected but highly HIV-exposed individuals (Rowland-Jones et al., 1995). Over 1000 papers have been published on the CTL response to HIV infection; step-by-step we are learning how to harness this response to its maximum potential.

Initially, we focused on studying natural CTL escape in HIV-infected patients. Most studies have looked at CTL responses to Gag epitopes, HLA-B*57- or -B*27-restricted epitopes, or have been cross-sectional in chronically-infected individuals. Not wanting to simply repeat previous studies, the P32/S32 transmitter pair data (Table 3.1) were used as the basis of our hypothesis-driven research investigating escape from the HLA-B*51-restricted epitope LI9 in integrase, in longitudinally-sampled early HIV-infected

patients. This HLA allele is associated with slow progression to AIDS; however, is far less studied than HLA-B*57 or -B*27. Research on the LI9 epitope has mainly been sequencing-oriented with I31V predicted to be a negatively-associated polymorphism (Leslie et al., 2005, Kawashima et al., 2009). Although we did not find this negative association in our early HIV-infected cohort, we did observe the emergence of the V32I and P30S polymorphisms over a one year period. This latter polymorphism was the only way of consistently avoiding or weakening CTL recognition in HLA-B*51 positive subjects as determined by IFN γ ELISpot assays. Reversion at this position within a year in disparate HLA hosts indicates that the mutation may be associated with a viral fitness cost. Therefore, the data implies that even if P30S was selected within an HLA-B*51 positive individual due to CTL pressure, these variants might have a low replicative capacity and so progression to AIDS may still be a slow process.

Confirming the significance of studying this integrase epitope, functional avidity to wildtype LI9 was found to inversely correlate with viral load. This implies that stronger CTL recognition of wildtype LI9 in HLA-B*51 positive patients correlates with a lower viral load. Future HIV vaccine design incorporating LI9 may contribute to a better clinical outcome in HLA-B*51 positive subjects. As wildtype LI9 or the V31I variant are the most frequent across HIV-1 subtypes, their presence in a vaccine might provide cross-subtype protection.

Population genetics of HLA alleles in different geographical locations must also be taken into account when designing a CTL vaccine. In Europe, 11%, 6.8% and 6.8% of the population express HLA-B*51, -B*27 and -B*57, respectively; in South-East Asia,

9.2%, 5% and 0.6%, respectively; in sub-Saharan Africa, 4.8%, 2.2% and 8.6%, respectively (Meyer et al., 2007). Although it is important to study these HLA class I alleles to determine factors causing this association with slow disease progression, the prevalence of HLA alleles in a given population will also affect the success of a vaccine. It may be that specific vaccines for specific geographical locations, or mixtures of vaccines may be required.

We next studied an adoptive therapy presently in a phase I clinical trial based on the infusion of CD8⁺ cells that have been transduced with a high affinity TCR enabling recognition and specific lysis of HIV-infected cells, into HIV-infected patients (Varela-Rohena et al., 2008, ClinicalTrials.gov, 2010). This TCR was specific for the HLA-A*02-restricted epitope SL9; HLA-A*02 has a prevalence of 57.8% in Europe, 49% in South-East Asia and 38.8% in sub-Saharan Africa (Meyer et al., 2007). Despite the high mutation rate of HIV-1 and high viral loads generated within our *in vitro* assays, no selection of significant intraepitope mutations occurred following incubation of HIV-infected cells with either wildtype or enhanced SL9-specific TCR-transduced cells. Only the nonsynonymous mutation L75V, that may represent an N-terminal processing mutation, provided a potential mechanism for the virus to escape wildtype TCR recognition; this was not observed with high affinity TCR-transduced cell pressure. In addition, both the wildtype and enhanced TCR were able to recognise a wide range of natural and artificial SL9 variants.

These assays indicate that escape from SL9-specific TCR-transduced cells may not be a rapid event within recipients and would be dependent on minor variants already within

the viral quasispecies. For example, the presence of the 3F5A8V variant within the viral quasispecies may lead to its selection, as the TCR-transduced cells are unable to recognise this SL9 variant. However, if only the wildtype SL9 variant was present, the probability of generating all three mutations would reduce the chances of escape occurring in this manner. The hurdles facing those with multi-antiretroviral drug resistance should act as a warning of what may occur with immunotherapy. Although we were unable to detect significant immunotherapy resistance *in vitro*, this does not equate to what may occur *in vivo*. It will therefore be necessary to monitor for resistance mutations within individuals during any clinical trials.

On the basis of previous HIV CTL adoptive therapy attempts, persistence and the frequency of infusions will have the most direct effect on the TCR-transduced cell therapy's success. Furthermore, as with transplantation of $\Delta 32/\Delta 32$ CCR5 stem cells, the TCR-transduced cell adoptive therapy is faced with the problem that it would be unfeasible to do for every HIV-infected individual. To bypass the need for transduction, expansion and infusion of cells, the high affinity SL9-specific TCR was fused to an anti-CD3 scFv, creating an HIV gag ImmTAC. Although this ImmTAC was able to redirect CTLs to HIV-infected cells and cause specific lysis, it was unable to control HIV infection and actually increased the spread of infection in the absence of antiretroviral drugs. This type of therapy could therefore only be used in synergy with antiretroviral therapy, perhaps when multidrug resistance has limited patient choices.

It should be kept in mind that even if these SL9-specific TCR-transduced cells or HIV gag ImmTACs are effective *in vivo*, all potential recipients must be HLA typed as these

current methods are only applicable to HLA-A*02 positive individuals. Further HLA types and epitopes that this type of immunotherapy could be applied to would need to be decided; the key would be to target as many individuals in a population using the minimum number of HLA alleles. In addition, if resistance to this type of therapy were to eventually emerge, multi-TCR-transduced CD8⁺ cells or multi-HIV gag ImmTACs would be an option, acting in a similar fashion to HAART. This means that TCRs that recognise other HLA-A*02-restricted epitopes, such as IV9 or VL9, would need to be isolated and enhanced. Any future HIV-specific TCR development should take care in not losing specificity when strengthening the TCR binding affinity. As both antigen specificity and TCR binding affinity probably have optimal thresholds, the aim would be to achieve maximal CD8⁺ T cell function whilst retaining specificity.

Future studies need to focus on latent HIV reservoirs if we are to ever eradicate the virus. These reservoirs are unaffected by HAART, and the TCR-transduced cells and ImmTACs may have little effect. Current research has focused on reactivating latently infected cells using histone deacetylase inhibitors, such as valproic acid (Lehrman et al., 2005), which induce HIV expression from an integrated virus. If used in combination with HAART or HIV-specific TCR-transduced cells/ImmTACs, this may provide a potential mechanism of eradicating the virus from an individual.

To summarise, this thesis has analysed two different methods of how the CTL response may be used to control HIV infection. Building on previous studies and attempting completely novel research in both a natural and therapy-induced manner may finally win the fight against HIV.

APPENDICES

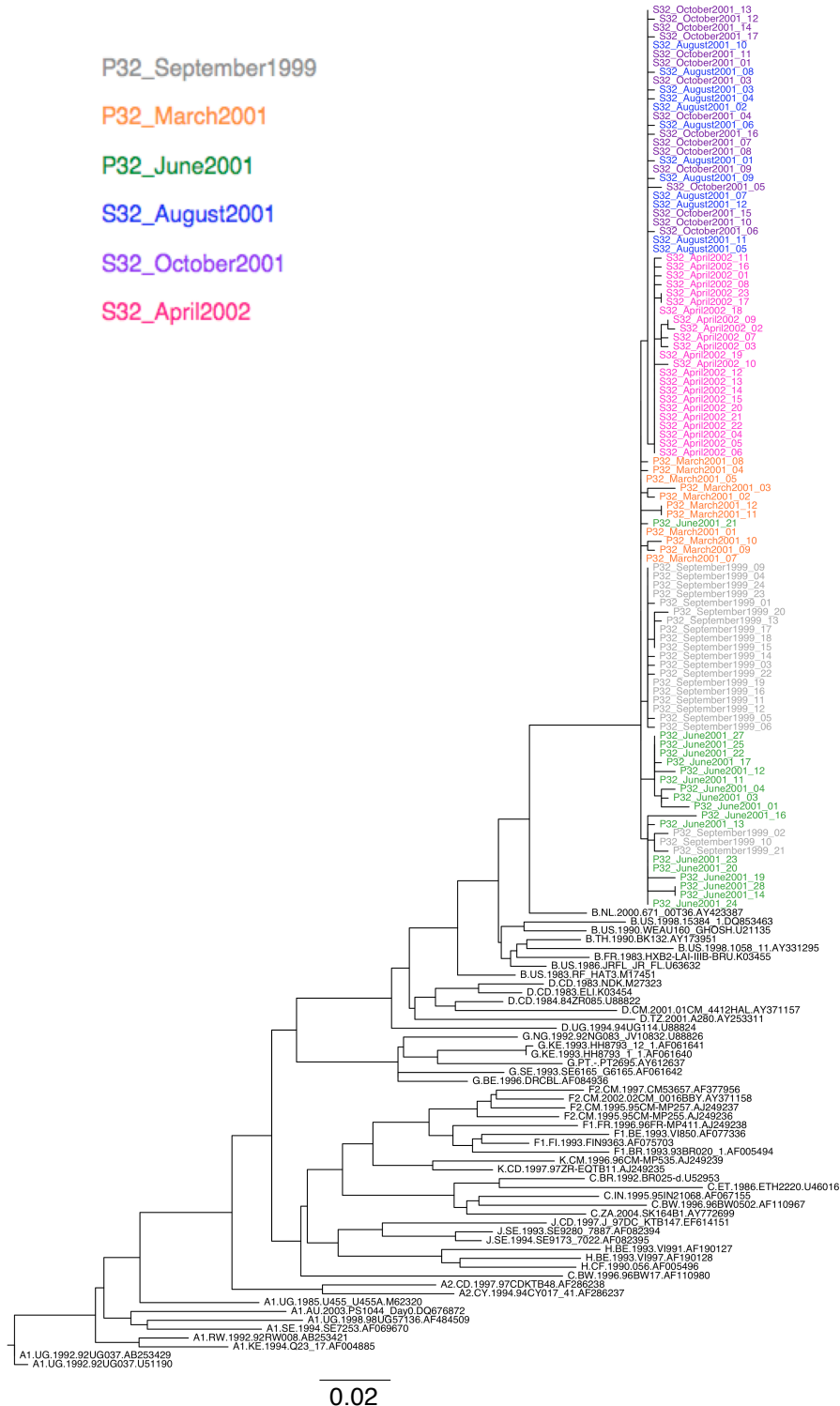
Appendix A

Integrase LI9 variants and their frequencies are provided from those listed on the LANL HIV Molecular Immunology Database (Yusim et al., 2009).

Variant	L	P	P	V	V	A	K	E	I	LANL Subtype B		LANL Subtype C	
										n	%	n	%
LI9	.	.	.	.	.	.	.	.	.	342	61.0	67	17.8
V31I	.	.	.	I	.	.	.	.	.	123	21.9	280	74.3
V32I	.	.	.	.	I	.	.	.	.	19	3.4	1	0.3
L28I	I	.	.	.	.	.	.	.	.	28	5.0	3	0.8
V31I, V32I	.	.	.	I	I	.	.	.	.	6	1.1	0	0.0
K34R	.	.	.	.	.	.	R	.	.	16	2.9	12	3.2
P30A	.	.	A	.	.	.	.	.	.	3	0.5	0	0.0
L28I, P30A	I	.	A	.	.	.	.	.	.	9	1.6	0	0.0
V31I, K34R	.	.	.	I	.	.	R	.	.	0	0.0	6	1.6
V31I, E35D	.	.	.	I	.	.	.	D	.	2	0.4	0	0.0
E35K	.	.	.	.	.	.	.	K	.	1	0.2	0	0.0
P30S, V31I	.	.	S	I	.	.	.	.	.	2	0.4	1	0.3
L28R	R	.	.	.	.	.	.	.	.	1	0.2	0	0.0
L28I, A33P	I	.	.	.	.	P	.	.	.	0	0.0	1	0.3
L28M	M	.	.	.	.	.	.	.	.	1	0.2	0	0.0
V32L	.	.	.	.	L	.	.	.	.	1	0.2	0	0.0
L28I, V31I	I	.	.	I	.	.	.	.	.	1	0.2	1	0.3
V31I, K34R, E35G	.	.	.	I	.	.	R	G	.	0	0.0	1	0.3
V31I, I36M	.	.	.	I	.	.	.	.	M	0	0.0	1	0.3
I36V	.	.	.	.	.	.	.	.	V	0	0.0	1	0.3
V31M	.	.	.	M	.	.	.	.	.	0	0.0	1	0.3
L28I, P30T	I	.	T	.	.	.	.	.	.	1	0.2	0	0.0
V32I, K34R	.	.	.	.	I	.	R	.	.	1	0.2	0	0.0
L28M, V31I	M	.	.	I	.	.	.	.	.	1	0.2	0	0.0
P30L	.	.	L	.	.	.	.	.	.	0	0.0	1	0.3
L28I, V31I, V32I	I	.	.	I	I	.	.	.	.	1	0.2	0	0.0
E35Q	.	.	.	.	.	.	.	Q	.	2	0.4	0	0.0
Total n (variants)										561	(20)	377	(14)

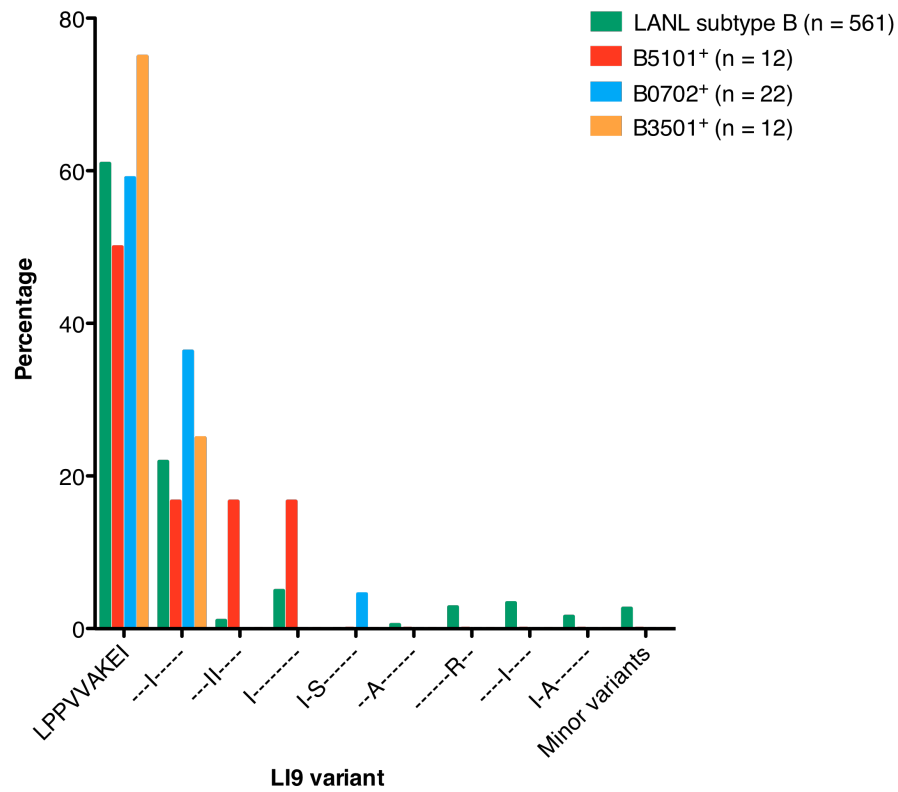
Appendix B

Phylogenetic analysis of the transmitter pair P32 and S32 aligned with subtype reference sequences from the Los Alamos HIV sequence database.



Appendix C

Variant integrase LI9 epitopes for SPARTAC/Open study HIV subtype B-infected patients at baseline according to HLA-B expression compared to untyped samples on Los Alamos.



Appendix D

As a CTL clone could not be isolated and grown from patient 868 (HLA-A*02, -A*24, -B*27, B*35), phage display was used on the parental 868 HLA-A*0201-restricted SL9-specific CTL line. The methodology was originally detailed in 2005 (Li et al., 2005).

Phage display to isolate a natural SL9 TCR, further TCR development and transductions were carried out by Adaptimmune. In brief, RNA was prepared from the 868 CTL line and cDNA synthesised. Broad TCR primer sets were used to amplify the V α and V β chains present. The amplified V α and V β products were pooled, respectively, and a second round of PCR was set up using flanking primers, and the pools of V α and V β chains were randomly assembled into pairs. These were cloned into a phagemid vector and transformed into *E. coli* to generate a library of 10⁸ clones. When released, these phage particles displayed the V α and V β chains as part of their coat. The SL9 peptide was used so that only those phage displaying the HLA-A*02-SL9-specific V α and V β chains would bind. CDR2 and CDR3 mutations were introduced into a chosen phagemid to produce a high-affinity SL9 TCR (Varela-Rohena et al., 2008).

Appendix E

Clonal sequencing of day 31 postinfection samples from the continuous culture selection pressure assay. *Taq* DNA polymerase was used to amplify the cDNA.

	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	
	S	E	E	L	R	S	L	Y	N	T	V	A	T	L	Y	C	V	H	Q	
JM22-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	24/26
	.	.	.	.	K	.	.	.	H	.	.	.	.	.	.	.	.	.	.	1/26
	.	.	K	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1/26
Non-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	28/28
wt-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	28/32
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1/32
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	1/32
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	.	2/32
wt-transduced B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	31/32
	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1/32
a6-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1/30
	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	1/30
a6-transduced B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	28/28

Clonal sequencing of day 8 postinfection, passage 2 samples from the passaging culture selection pressure assay. *Taq* DNA polymerase was used to amplify the cDNA.

	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	
	S	E	E	L	R	S	L	Y	N	T	V	A	T	L	Y	C	V	H	Q	
Control A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	25/25
JM22-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	26/30
	P	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1/30
Non-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	31/31
wt-transduced A	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	26/28
	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	1/28
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1/28
wt-transduced B	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	26/31
	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	1/31
	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	2/31
	.	.	.	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.	1/31
a6-transduced A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	30/30
a6-transduced B	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	22/24
	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	1/24
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1/24

Appendix F

Proteasomal cleavage prediction programs:

1. NetChop: predicts cleavage sites based on *in vitro* digestion data and sequence signal from the boundaries of naturally processed MHC class I ligands (Kesmir et al., 2002).

Predicts both 75L (score = 0.957) and 75V (score = 0.901) to be cleavage sites.

2. MAPPP (MHC-I Antigenic Peptide Processing Prediction): predicts 20S proteasomal cleavage sites based on cleavage determining amino acid motifs (Hakenberg et al., 2003).

Predicted both 75L and 75V to be cleavage sites.

3. PProC (Prediction Algorithm for Proteasomal Cleavages): predicts cleavage site based on *in vitro* digestion data (Nussbaum et al., 2001).

Type I (based on cleavages in enolase) predicted that both 75L and 75V with a strong cleavage strength.

Type II (based on cleavages in enolase and ovalbumin peptides) predicted 75L with a strong cleavage strength. No cleavage was predicted with 75V.

Type III (based on cleavages in enolase and other ovalbumin peptides) predicted 75L with a strong cleavage strength; a medium cleavage strength was predicted with 75V.

Appendix G

High-affinity SL9 TCRs were C-terminally fused with an anti-CD3 scFv to form HIV gag ImmTACs; Immunocore generated all ImmTAC reagents and carried out binding analyses.

Binding kinetics of high affinity TCRs to SLYNTVATL as determined by BIAcore SPR binding analysis:

	a12b40	a15b40	a15b26
K_{on} (1/Ms)	3.19×10^5	1.84×10^5	2.13×10^4
K_{off} (1/s)	6.22×10^6	3.24×10^6	5.64×10^6
K_d	20 pM	18 pM	265 pM
t_{1/2}	31 h	59 h	34 h

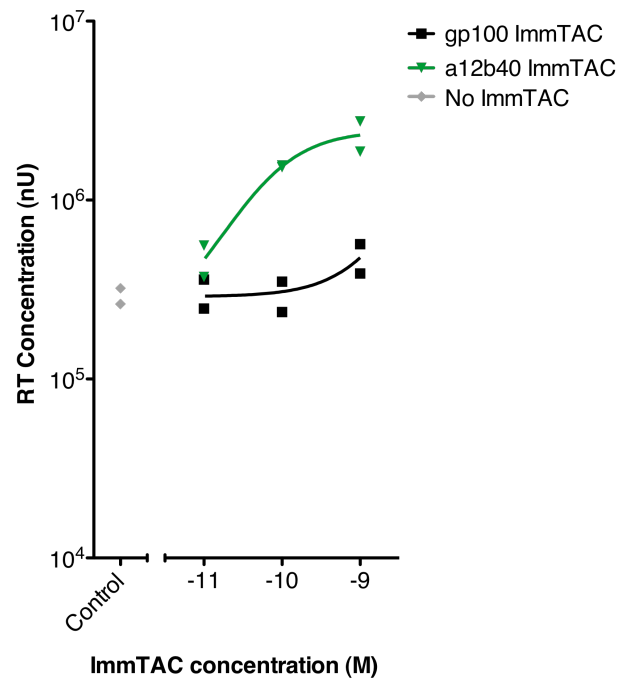
The effect of fusing a high affinity TCR with an anti-CD3 scFv to create an ImmTAC does not greatly alter the K_d or t_{1/2} values:

	a12b40 ImmTAC	a15b40 ImmTAC	a15b26 ImmTAC
K_{on} (1/Ms)	3.04×10^5	1.16×10^5	2.30×10^4
K_{off} (1/s)	7.23×10^6	3.15×10^6	4.29×10^6
K_d	24 pM	27 pM	213 pM
t_{1/2}	27 h	61 h	45 h

Appendix H

Effect of ImmTACs on HIV-1-infected HLA-A*02⁺ PBMCs at day 4 postinfection.

Reverse transcriptase activity is shown as determined by SG-PERT.



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