A STUDY OF THE PHENOTYPE AND FUNCTION OF HLA-C RESTRICTED CD8 T CELLS IN HIV-1 INFECTION

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Thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

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A recent study showed that a polymorphism ~35kb upstream of the HLA-C gene (-35 SNP) correlates with host control of HIV-1 in Caucasians, with the minor allele (*C*) associating with significantly lower set point viral loads than the major allele (*T*). A link between viral load and HLA-C is suggested by linkage of the two SNP alleles with different HLA-C alleles and by the fact that HLA-C, in contrast to HLA-A and -B, is not down-regulated by HIV-1 Nef protein. In addition, the -35*C* variant has been shown to associate with higher HLA-C messenger RNA expression in EBV-transformed B cell lines. We initially propose that increased surface expression of HLA-C in subjects with the protective SNP leads to increased breadth and magnitude of HLA-C restricted T cell responses, explaining the decrease in viral load in these subjects.

This study initially investigates whether the -35 SNP correlates with the surface level of HLA-C using the monoclonal antibodies DT9, which recognises both HLA-C and HLA-E, and 3D12, which is specific for HLA-E. The lymphocytes from -35 *CC* subjects expressed a significantly higher level of surface HLA-C when compared to those from -35 *TT* subjects, but this difference in HLA-C expression can be attributed primarily to the very low expression of a single allelic product, HLA-Cw*07.

Increased surface HLA-C should translate to functional differences between *CC* and *TT* subjects. This study confirmed that HLA-C restricted CD8 T cell responses against HIV-1 do exist, even for HLA-Cw*07, but represent a minority of total T cell responses. They were detected in all -35 SNP genotypes but there were no functional differences, making it unlikely that the protective effect of this SNP on viral load set point could be accounted for solely by HLA-C restricted T cell responses.

Finally, a viral suppression assay was used to investigate the capacity of CD8 T cells to suppress HIV-1 replication in Caucasian and African subjects. We provide evidence that the -35 SNP effect on viral load is indeed T cell mediated. However, we suggest that the protective effect of the -35 SNP on viral load set point manifests as a result of linkage disequilibrium of this polymorphism with both favourable and unfavourable HLA-B and HLA-C alleles.

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen presenting cell
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
β2M	Beta-2 microglobulin
BCL	B cell line
BSA	Bovine serum albumin
CAF	CD8 antiviral factor
CCR5	Chemokine (C-C motif) receptor type 5
CD	Cluster of differentiation
CHAVI	Center for HIV/AIDS Vaccine Immunology
CMV	Cytomegalovirus
CRF	Circulating recombinant form
CSA	Cyclosporin A
CXCR4	Chemokine (C-X-C motif) receptor type 4
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELF	Epitope Location Finder
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
ER	Endoplasmic reticulum
FCS	Foetal calf serum
GALT	Gut-associated lymphoid tissue

GC	Germinal centre
HAART	Highly Active Antiretroviral Therapy
HIV-1	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
ICAM	Intercellular adhesion molecule
ICS	Intracellular cytokine staining
IEF	Isoelectric focussing
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
INT	Integrase
KIR	Killer cell immunoglobulin-like receptor
LANL	Los Alamos National Laboratory
LCMV	Lymphocytic Choriomeningitis Virus
LTNP	Long term nonprogressor
LTR	Long terminal repeat
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
mRNA	Messenger ribonucleic acid
nAb	Neutralising antibody
NC	Nucleocapsid
NK	Natural killer cell
NKT	Natural killer T cell
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
pI	Isoelectric point
PR	Protease
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
	Also known as CCL5 [Chemokine (C-C motif) ligand 5]
RNA	Ribonucleic acid
RT	Reverse transcriptase
SDF	Stromal-cell derived factor
SFU	Spot-forming unit
SIV	Simian immunodeficiency virus
SNP	Single nucleotide polymorphism
STCL	Short-term cell line
TAP	Transporter associated with antigen processing
TCL	T cell line
TCID	Tissue culture infectious dose
TCR	T cell receptor
T_H	T helper cell
TNF	Tumour necrosis factor
TRIM5	Tripartite motif-containing protein 5
UTR	Untranslated region

Three- and one-letter abbreviations of 20 naturally encoded amino acids.

Amino acid	Three-letter abbreviation	One-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Chapter 1: INTRODUCTION

1.1 The Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS) Epidemic

The human immunodeficiency virus (HIV) was first identified as the aetiological agent for acquired immunodeficiency syndrome (AIDS) in 1983 (Barre-Sinoussi, Chermann et al. 1983; Levy, Hoffman et al. 1984; Popovic, Sarngadharan et al. 1984) and continues to persist as a lethal pathogen. AIDS is arguably the most serious infectious disease to have affected humankind (Rambaut, Posada et al. 2004). In 2009, the United Nations estimated that 33.2 million people worldwide were living with HIV infection and that 2.6 million had been newly infected, with sub-Saharan Africa bearing the largest burden of disease (UNAIDS 2010). The number of annual AIDS-related deaths worldwide is steadily decreasing from a peak of 2.1 million in 2004 to an estimated 1.8 million in 2009. Although combination antiretroviral therapy (often referred to as highly active antiretroviral therapy – HAART) has transformed the management and prognosis of HIV infection in industrialised countries (Girardi, Lauria et al. 2005), it's roll-out in developing countries has been problematic with only 36% of the 15 million people in need in low- and middle-income countries receiving HAART at the end of 2009 (UNAIDS 2010). These drugs suppress viral replication but cannot cure infection and additionally problems of drug toxicity, patient adherence and drug resistance raise questions about the long-term efficacy of drug treatment. Thus the demand for an effective vaccine has never been more urgent as it represents the best long-term solution to ending the HIV epidemic.

1.2 Structure and organisation of the HIV genome

1.2.1 The Virus

HIV is a retrovirus within the subgroup of lentiviruses. Viruses within this subgroup, including the simian immunodeficiency viruses (SIVs) from which HIV evolved (Hahn, Shaw et al. 2000), all establish long term chronic infections. There are two main types of HIV; HIV-1, which is though to have originated as a result of zoonotic transmission from chimpanzees (*Pan troglodytes troglodytes*) in Central Africa (Gao, Bailes et al. 1999; Santiago, Rodenburg et al. 2002); and HIV-2, thought to have been acquired from sooty mangabey monkeys (*Cercocebus atys*) in West Africa (Gao, Yue et al. 1992). Of these two strains HIV-1 is predominantly responsible for the HIV pandemic and as such is the focus of this thesis. HIV-1 is characterized by extensive genetic diversity and is phylogenetically divided into 3 groups, M (main), N (new) and O (outlier), which are thought to represent separate introduction into humans from chimpanzees (Gao, Bailes et al. 1999; Sharp and Hahn 2010) (Figure 1.1A). The M group is further split into 9 subtypes or clades (A-D, F-H, J and K) and at least 15 circulating recombinant forms (Rambaut, Posada et al. 2004). The M group of HIV-1 has a near global distribution, with its different clades predominating in different geographical regions. Strains belonging to the same clade can differ by up to 20% in their envelope proteins and between-clade differences can approach 35%, with this diversity continually growing (Gaschen, Taylor et al. 2002). While clade C in Southern Africa and India comprise the highest number of HIV-1 infections globally (Figure 1.1B), the majority of HIV-1 research has disproportionately focused on clade B infection in North America and Western Europe.

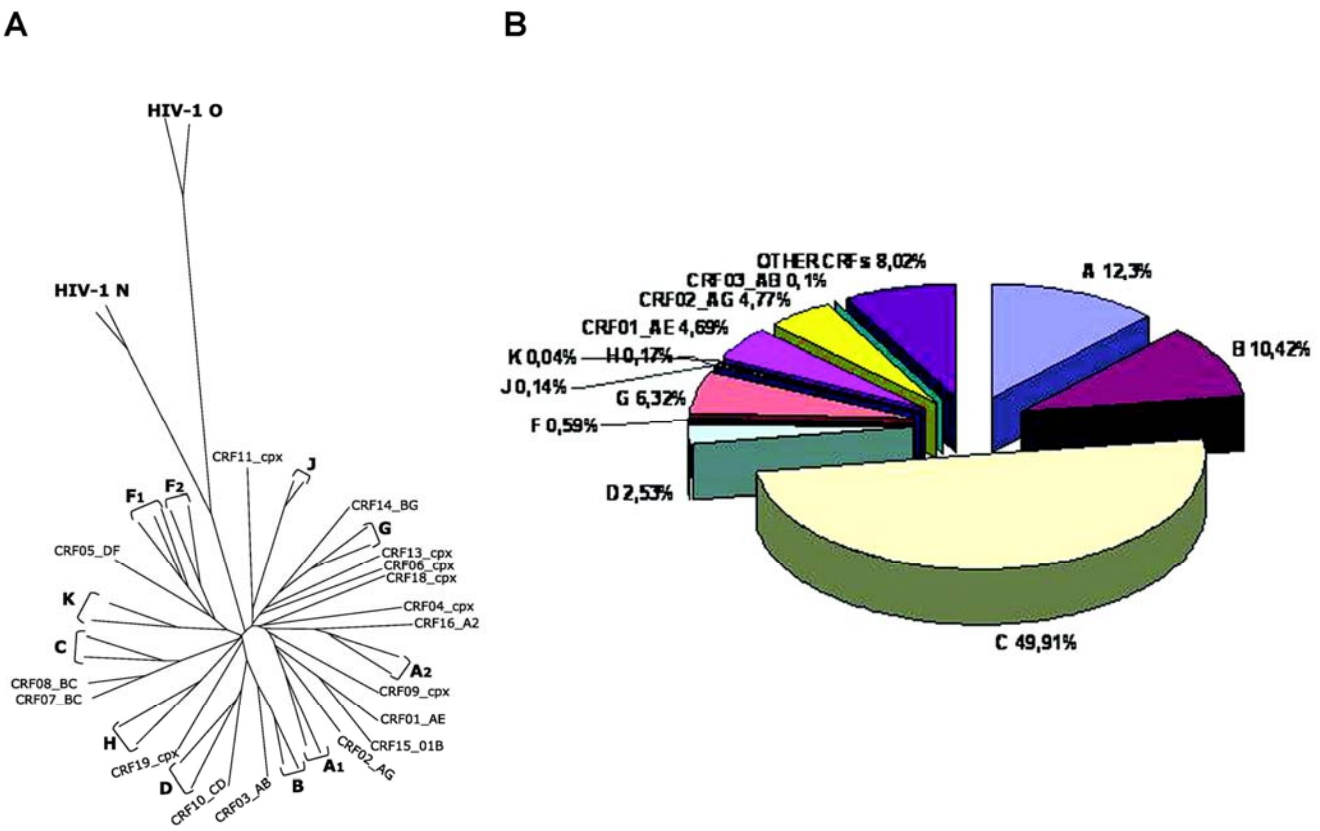


Figure 1.1. The genetic diversity and global distribution of HIV-1.
(A) The phylogenetic tree showing HIV-1 groups M, N, and O and subtypes (clades) and circulating recombinant forms (CRFs) within the M group. (B) Global prevalence of HIV-1 clades and CRFs within the M group. The global prevalence of each form, expressed as a percentage of the total number of HIV-1 isolates identified worldwide, is shown. Reproduced from Buonaguro *et al.* (Buonaguro, Tornesello et al. 2007).

1.2.2 Structure

HIV-1 consists of two copies of a single-stranded positive-sense ribonucleic acid (RNA) genome complexed with nucleocapsid proteins (p7/p9). This ribonucleoprotein core is encased, together with the three viral enzymes (protease, reverse transcriptase, integrase) and other accessory and regulatory proteins, inside a characteristic bullet shaped protein core formed from the p24 capsid protein, which is in turn surrounded by the matrix layer, derived from the p17 protein. The matrix protein is located underneath the virion envelope. The virion envelope consists of a lipid bilayer, derived from the host cell membrane, and a virally encoded tetrameric envelope protein complex, of which each subunit consists of two non-covalently-linked membrane proteins, gp120 (the outer envelope protein) and gp41 (the transmembrane protein) (Figure 1.2A) (Barre-Sinoussi 1996). Approximately 14 envelope spikes, composed of trimeric gp120 and gp41 subunits, protrude from the virion surface (Zhu, Liu et al. 2006). Contrary to common depictions of HIV-1, such as that shown in Figure 1.2.A, these envelope spikes are not evenly distributed across the surface of the virus, but rather tend to cluster together (Zhu, Liu et al. 2006).

1.2.3 Genome

The HIV-1 genome is approximately 9.7 kilobases long and consists of 9 genes encoding 15 proteins (Figure 1.2B). At either end of the genome are identical sequences, referred to as long terminal repeats (LTRs), which are involved in transcription and proviral deoxyribonucleic acid (DNA) integration. As with all retroviruses HIV-1 possesses 3 principal genes: *gag, pol* and *env*. The *gag* gene encodes the structural proteins p17, p24 and p7, which form the viral matrix, capsid and nucleocapsid respectively, and p6, a linker protein involved in viral budding

(Greene and Peterlin 2002). The *pol* gene encodes the viral enzymes protease (PR), reverse transcriptase (RT) and RNase H, and integrase (INT); RT is the hallmark of a retrovirus and this enzyme is responsible for transcribing its genomic RNA into a double-stranded DNA copy (provirus) which serves as a template for generation of new viral RNA genomes after integration into the host genome. The *env* gene encodes the surface glycoproteins gp120 and gp41, which are involved in target cell binding and entry. In addition, HIV-1 encodes a further 6 accessory genes, viral infectivity factor (*vif*), viral protein R (*vpr*), viral protein U (*vpu*), transcriptional activator (*tat*), regulator of viral gene expression (*rev*), and negative effector (*nef*) that regulate its replication cycle and interact with and modulate the host cell (Greene and Peterlin 2002). Although *in vitro* analysis did not indicate a convincing role for the accessory genes in viral replication, their essential nature was first highlighted by the finding that SIV variants lacking a functional *nef* gene replicate poorly and do not cause disease in adult monkeys (Kestler, Ringler et al. 1991). The roles of the various HIV-1 proteins are summarized in Table 1.1.

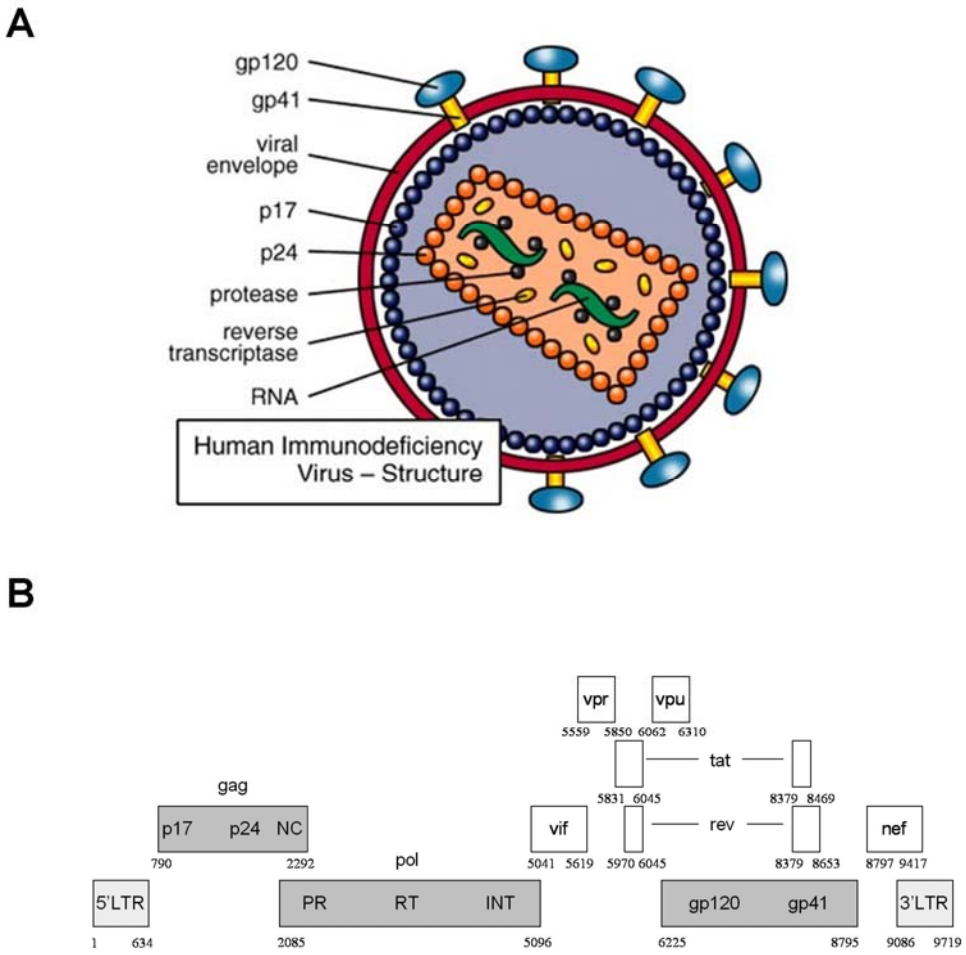


Figure 1.2. The structure and genome of HIV-1.
(A) A schematic representation of the structure of HIV-1. (B) Organisation of the HIV-1 genome. HIV-1 is comprised of 9 genes; three main genes (*gag*, *pol* and *env*, shown by grey boxes), and six accessory genes (*vif*, *tat*, *rev*, *vpu*, *vpr* and *nef*, shown by white boxes). At either end of the genome are long terminal repeat sequences (LTR). The gag gene encodes proteins that form the viral matrix (p17) and capsid (p24) and nucleocapsid (NC); *pol* encodes the protease (PR), reverse transcriptase (RT) and integrase (INT) enzymes; *env* encodes the gp120 and gp41 surface glycoproteins.

Table 1.1. HIV-1 protein functions.

GENE	PROTEIN(S)	FUNCTION(S)
Gag	p17, Matrix (MA)	Forms viral matrix layer; targeting gag polyprotein to lipid rafts; nuclear import of provirus
	p24, Capsid (CA)	Forms protective viral capsid, binds cyclophilin A to protect virus from host cell derived restriction factors such as TRIM5-α
	p7, Nucleocapsid (NC)	Binds to viral RNA
	p6	Interacts with vpr; involved in viral budding
Pol	p10, Protease (PR)	gag/pol cleavage and maturation
	p66, Reverse transcriptase (RT) and RNAse H	Reverse transcription
	p32, Integrase (INT)	Integration of the provirus into the host genome
Env	gp120	Binds to CD4 and chemokine receptors CCR5 and CXCR4
	gp41	Mediates cell fusion
Tat	p14, Transcriptional activator	Viral transcription factor that upregulates transcription from the viral promoter within the long terminal repeats
Rev	p19, Regulator of viral gene expression	Inhibits viral RNA splicing and promotes nuclear export of incompletely spliced viral RNAs which act as template for the translation of *gag*/*pol*/*env* proteins
Vif	p23, viral infectivity factor	Accelerates virion maturation and infectivity; inhibits host derived restriction factor APOBEC3G
Vpr	p15, Viral protein R	Nuclear import of provirus; increases virus production; promotes cell-cycle arrest
Vpu	Viral protein U	Promotes CD4 degradation and viral budding; vpu antagonises the antiviral activity of tetherin which causes retention of fully formed virions on infected cell surfaces
Nef	p24, Negative effector	Downregulates CD4 and HLA class I molecules; blocks apoptosis; increases T cell activation; increases infectivity

1.2.4 HIV-1 life cycle

The life cycle of HIV-1 is summarized in Figure 1.3. In brief, HIV-1 entry into cells is facilitated by the envelope glycoprotein gp160, comprised of surface and transmembrane subunits, gp120 and gp41 respectively. Initially gp120 binds to its primary cell surface receptor CD4 and then, following certain conformational changes, to its coreceptor CCR5 or CXCR4 (Choe, Farzan et al. 1996; Dragic, Litwin et al. 1996; Feng, Broder et al. 1996). Binding of these two receptors exposes gp41, which then mediates cell fusion and internalization of the viral core into the host cell cytoplasm, which is central to the infection process.

Co-receptor usage is determined by the third variable region (V3 loop) of gp120 (Hartley, Klasse et al. 2005) and dictates the cell type infected. R5 or macrophage-tropic viruses, which predominate during the acute and asymptomatic stages of infection, bind the CCR5 co-receptor, expressed at high levels on activated/memory CD4 T-cells as well as macrophages and dendritic cells (Stevenson 2003; Lusso 2006). X4 or T-cell-tropic viruses on the other hand, more commonly found in the late stages of disease as patients clinically progress to AIDS, infect via the CXCR4 co-receptor, which is found on a broader range of cells including naïve/resting T-cells, macrophages and thymic precursors (Lusso 2006). A naturally occurring 32-base pair deletion in the gene encoding CCR5 (CCR5-Δ32 variant) results in these individuals being highly resistant to infection by primary HIV-1 isolates (Dean, Carrington et al. 1996; Liu, Paxton et al. 1996; Samson, Libert et al. 1996; Martinson, Chapman et al. 1997). The mutant CCR5 receptor never reaches the cell surface in individuals homozygous for this mutation (1-2% of the Caucasian population), resulting in a near complete resistance to HIV-1 infection (Liu, Paxton et al. 1996; Martinson, Chapman

et al. 1997). Although heterozygosity for this mutation does not prevent infection, it does slow progression in both untreated and treated individuals (Dean, Carrington et al. 1996). When published associations related to HIV-1 disease control/progression outside of the major histocompatibility complex (MHC) were specifically tested in genome-wide association studies, only variants in the CCR5-CCR2 locus, including CCR5-Δ32 variant, replicated with statistical significance (Fellay, Ge et al. 2009; Pereyra, Jia et al. 2010). These findings emphasise the pivotal role played by CCR5 in the person-to-person spread of HIV-1.

Once inside the cell, the RNA genome is converted into DNA by the viral RT enzyme. The double stranded DNA proviral genome generated by reverse transcription is then actively transported into the nucleus and integrates randomly in the host genome. This process is thought to be facilitated by the Vpr, Int and p17 proteins (Heinzinger, Bukinsky et al. 1994; Gomez and Hope 2005). Once integrated, the HIV provirus is either transcribed using host cell machinery to produce new viral proteins, or persists as a latent infection through mechanisms that remain unclear (Chun, Carruth et al. 1997; Marcello 2006).

Transcription of the proviral genome is carried out by host cell RNA polymerases, and this transcript is spliced in various ways to form the viral proteins described above (Kim, Byrn et al. 1989; Jones and Peterlin 1994). The Tat, Rev and Nef proteins are produced first from completely spliced messenger RNA (mRNA) templates, followed by Gag, Pol, Env and the accessory proteins Vpr, Vpu and Vif, which are encoded by unspliced mRNAs (Klotman, Kim et al. 1991). The order with which the various HIV-1 genes are transcribed is known to play an important role in

determining the cellular immune responses raised against the virus (van Baalen, Guillon et al. 2002; Probst, Tschannen et al. 2003). However, Gag-specific CD8 T cells have been shown to recognise infected CD4 T cells as early as two hours postinfection, before proviral DNA integration, viral protein synthesis and Nef-mediated MHC class I downregulation (Sacha, Chung et al. 2007). These viral proteins are transported to the cell membrane where they are assembled to form a viral particle, which then surrounds itself with a lipid bilayer membrane as it buds out from the host cell. Following budding, final processing of the Gag and Pol proteins by encapsulated viral proteases produce mature, infectious viral particles (Kaplan, Krogstad et al. 1994).

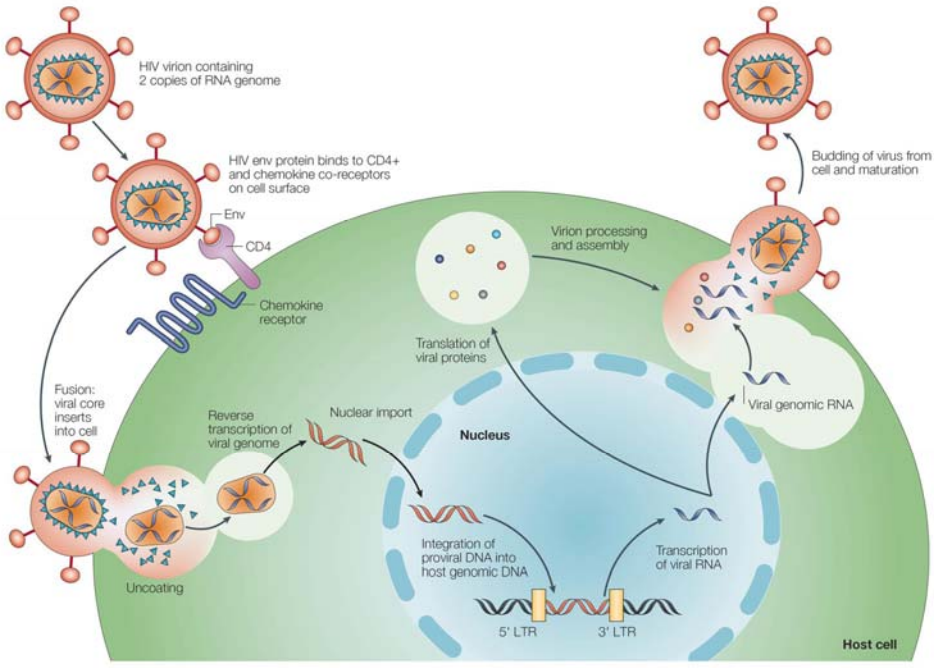


Figure 1.3. Key aspects of the HIV-1 life cycle.
Reproduced from Rambaut *et al.* (Rambaut, Posada et al. 2004).

1.2.5 Evolution

HIV-1 is one of the fastest evolving of all organisms, with an evolutionary rate of approximately 0.0024 substitutions per base pair per year (for gp160 envelope) (Korber, Muldoon et al. 2000), roughly a million times faster than that of its human host (Kumar and Subramanian 2002). This phenomenal mutation rate is the consequence of three factors, namely mutation, recombination and replication (Malim and Emerman 2001). First, the HIV-1 RT lacks a proof-reading function and thus is extremely error prone, making between 0.2-0.3 errors per genome per replication cycle (Preston, Poiesz et al. 1988; Mansky and Temin 1995). Mutations are also introduced into the genome through posttranslational editing of viral DNA by the human antiviral apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like (APOBEC) cytidine deaminases APOBEC3G and APOBEC3F, that induce lethal guanine (G) to adenine (A) hypermutation (Bishop, Holmes et al. 2004). The antiviral effect of APOBEC3 can also be deaminase-independent through interference with virus transcription or integration. The HIV-1 vif protein has evolved to block the action of APOBEC3 proteins by targeting them for proteasomal degradation, through a ubiquitination pathway that involves host proteins, thus maintaining the infectivity of viral progeny (Yu, Yu et al. 2003; Kremer and Schnierle 2005). However, HIV-1 clones that have undergone G to A hypermutation can be isolated from HIV-1 infected individuals (Kieffer, Kwon et al. 2005), suggesting that the suppression of APOBEC3G by vif is not complete, and thus that APOBEC3G induced mutation still represents an important source of HIV-1 variation.

Second, there are two copies of the HIV-1 RNA genome, allowing RT to jump from one strand to another when it encounters lesions (Coffin 1979). HIV-1 is thought to

be particularly prone to such recombination, with 3 recombination events estimated to occur per genome per replication cycle (Zhuang, Jetzt et al. 2002). As cells are often infected by more than one virus (Jung, Maier et al. 2002) the two RNA strands contained within a virion can come from different parent viruses. Thus recombination can lead to the formation of new chimerical HIV-1 genomes and represents a further source of genetic variation. Importantly, and unlike the incremental accumulation of sequence changes that occurs through copying errors, recombination has the potential to introduce large numbers of genetic changes simultaneously. Most variation generated by mutation and recombination involves nucleotide substitution, but insertions and deletions are also observed (Larder, Bloor et al. 1999), explaining why HIV-1 genomes can vary in length.

Finally, HIV-1 possesses an extraordinary reproductive capacity, producing anywhere from between 10^{10} to 10^{12} new virions per day (Wei, Ghosh et al. 1995; Perelson, Neumann et al. 1996). Together these factors predict that every single point mutation within the HIV-1 genome will be produced every day (Coffin 1995). Thus HIV-1 represents a near perfect substrate for the process of natural selection, a factor that has undoubtedly contributed to its phenomenal success as a pathogen.

1.3 Natural history of HIV-1 infection

HIV-1 is transmitted via the exchange of bodily fluid containing infectious viral particles. There are three main routes of transmission. The first of these is sexual exposure through the genital tract or rectal mucosa, by far the most common route of transmission globally (Shattock and Moore 2003). Secondly, by parenteral transmission usually through the sharing of needles among intravenous drug users, but

also less commonly through contaminated blood products. Finally, by mother-to-child transmission which can arise *in utero*, intra-partum and post-partum through breastfeeding. For the purposes of this discussion, sexual transmission is focused on. Although it is not possible for practical and ethical reasons to study the very first events following HIV-1 transmission in humans *in vivo*, some understanding has been gained from *in vitro* work infecting mucosal explants (Hu, Frank et al. 2004; Veazey, Klasse et al. 2005; Margolis and Shattock 2006). In addition, much understanding has been gained from the nonhuman primate model of transmission of HIV-1 by infecting rhesus macaques with SIV *in vivo* (Zhang, Schuler et al. 1999; Hu, Gardner et al. 2000; Miller, Li et al. 2005).

The hallmark of HIV-1 infection is the destruction of CD4 T cells and subsequent loss of immune competence (Stevenson 2003). However, this does not occur immediately and normal HIV-1 infection can be divided into three distinct phases; the acute phase that immediately follows infection, the asymptomatic or chronic phase, and finally the onset of full-blown AIDS. These 3 phases and their characteristic features are shown in Figure 1.4. The acute phase is defined as the period between infection and seroconversion, when anti-HIV-1 antibodies can first be detected in the blood. This can take anywhere from 3-12 weeks and is often associated with flu-like symptoms, fever and characteristic rash; the clinical symptoms are referred to as seroconversion illness. The acute phase is also characterized by a huge burst of viral replication, with viral load detected in the blood often exceeding 10^7 copies per ml of plasma (Kahn and Walker 1998; Hecht, Busch et al. 2002; Kassutto and Rosenberg 2004).

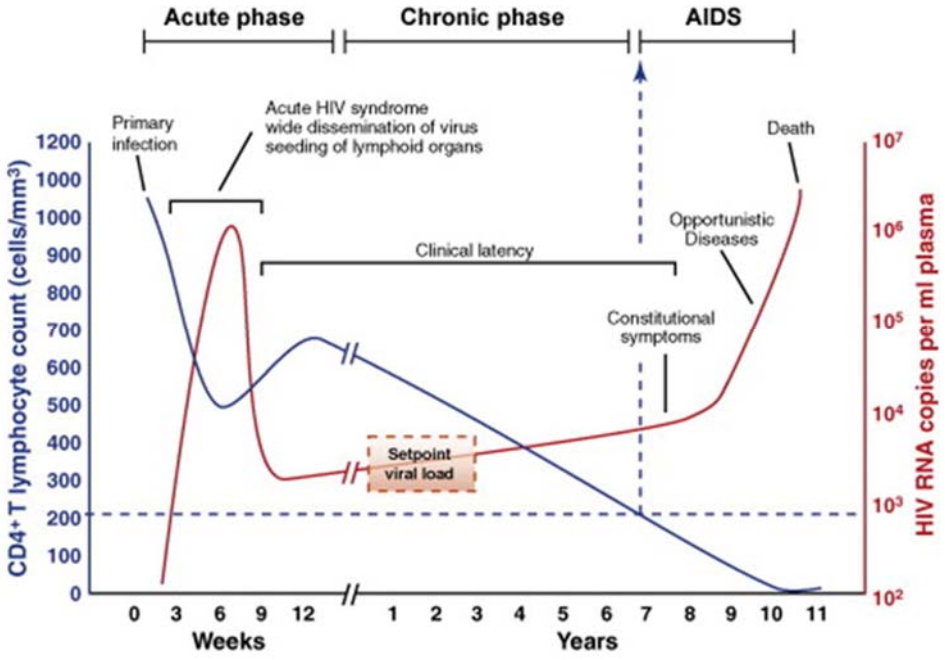


Figure 1.4. Characteristic natural course of HIV-1 infection.
In the acute phase, flu-like symptoms last 6-12 weeks, there is a peak in virus load and drop in CD4 T cells. Following the acute phase virus replication reaches a steady level known as the viral load set point. The chronic asymptomatic phase lasts on average 7-10 years. AIDS onset is associated with increasing virus replication and declining CD4 cell counts <200/mm^3. Reproduced from An and Winkler (An and Winkler 2010).

Transmission of HIV-1 infection requires the dissemination of virus from sites of infection at mucosal surfaces to T cell zones in secondary lymphoid organs, where extensive viral replication occurs in CD4 T helper cells (Fauci 1996). These cells express both CD4 and the chemokine receptor CCR5, which together form the receptor complex required for entry by R5 viral isolates that are prevalent early after infection (Dragic, Litwin et al. 1996; Lu, Berson et al. 1997; Littman 1998). The X4 viruses are rarely transmitted and generally appear only late in infection. Following sexual transmission of HIV-1, a single virus or virus-infected cell establishes productive clinical infection in the majority of cases (Derdeyn, Decker et al. 2004; Keele, Giorgi et al. 2008; Salazar-Gonzalez, Salazar et al. 2009). This was

demonstrated by sequencing viruses in heterosexual transmission pairs and in acute HIV-1 infection and provided evidence that a single virus (or virus-infected cell) initiated productive infection in close to 80% of the individuals tested, and two to five viruses in the other 20%. Virus can cross the mucosal epithelial barrier within hours (Hu, Gardner et al. 2000) to establish a small founder population of infected CD4 cells (Zhang, Schuler et al. 1999; Miller, Li et al. 2005). The virus infects mucosal CD4+CCR5+ T cells and remains localised in genital/rectal mucosa and draining lymph nodes for approximately ten days (referred to as the eclipse phase before viral RNA is detectable in plasma). This founder population undergoes local expansion during the first week of infection to generate sufficient virus and infected cells to disseminate and establish a self-propagating systemic infection throughout the secondary lymphoid organs (Miller, Li et al. 2005).

At the end of the eclipse phase, virus and/or virus-infected cells reach the draining lymph node, where they meet activated CD4+CCR5+ T cells which serve as targets for further infection. This process is augmented by dendritic cells (DCs) residing in the skin and mucosal surfaces, which are professional antigen presenting cells (APCs) that serve to capture antigens efficiently and possess the unique capacity to subsequently migrate to the T cell areas of secondary lymphoid organs. HIV-1 is thought to subvert the trafficking capacity of DCs to gain access to the CD4 T cell compartment in the lymphoid tissues (Rowland-Jones 1999). HIV-1 binds specifically to these cells through the interaction of gp120 with DC-specific ICAM3-grabbing non-integrin (DC-SIGN), which functions as a unique HIV-1 *trans* receptor, facilitating virus transfer to activated T cells (Geijtenbeek, Kwon et al. 2000). The virus then replicates rapidly and spreads throughout the body to other lymphoid

tissues, particularly gut-associated lymphoid tissue (GALT), where CD4+CCR5+ cells are present in high numbers (Brenchley, Schacker et al. 2004; Brenchley, Price et al. 2006; Brenchley, Paiardini et al. 2008). It is estimated that up to 80% of CD4 T cells in the gut-associated lymphoid tissue are depleted in the first three weeks of HIV-1 infection (Veazey, DeMaria et al. 1998; Schacker, Little et al. 2001; Brenchley, Schacker et al. 2004; Mattapallil, Douek et al. 2005). While the virus is replicating in the lymphoid tissues, plasma viraemia increases exponentially to reach a peak of over a million virus copies per ml of plasma at 21-28 days following HIV-1 infection in humans. The CD4 T cell numbers are low at the time of peak viraemia but later return to near normal levels in the blood but not in the GALT, with up to half of the body's memory CD4 T cells estimated to be destroyed during acute viraemia (Veazey, DeMaria et al. 1998; Brenchley, Schacker et al. 2004; Li, Duan et al. 2005; Mattapallil, Douek et al. 2005). It remains uncertain how this peak viraemia of acute infection is controlled. On the one hand, mathematical modelling of infection suggests that the peak viraemia is limited by the availability of new CD4 T cells to infect (Phillips 1996; Guadalupe, Reay et al. 2003; Petravic, Loh et al. 2008) and on the other work, in SIV-infected macaques showed that peak viraemia was dependent on the presence of CD8 T cells (Reimann, Tenner-Racz et al. 1994; Matano, Shibata et al. 1998; Jin, Bauer et al. 1999; Letvin, Schmitz et al. 1999; Schmitz, Kuroda et al. 1999).

Virus level then falls over 12-20 weeks to a stable level known as the viral load set point (Ho, Neumann et al. 1995; Schacker, Hughes et al. 1998; Rodriguez, Sethi et al. 2006). Concomitant with the appearance of HIV-1-specific CD8 T cells (Borrow, Lewicki et al. 1994; Koup, Safrit et al. 1994), this peak viraemia is resolved and

levels out at the viral load set point at a median of approximately 30,000 HIV-1 RNA copies/ml plasma (Lyles, Munoz et al. 2000). However, this viral load set point varies widely between individuals and is a known (though partial) predictor of time to progression to AIDS (Ho 1996; Mellors, Rinaldo et al. 1996; Mellors, Munoz et al. 1997; Blattner, Oursler et al. 2004; Mellors, Margolick et al. 2007). Viral load is then maintained at or around the viral load set point for the duration of the chronic phase. Despite often low plasma viral loads, the chronic phase of infection is characterized by rapid viral replication of up to 10^{12} virions per day (Wei, Ghosh et al. 1995), resulting in a high turnover of CD4 T cells (Ho, Neumann et al. 1995), and chronic immune activation (Sousa, Carneiro et al. 2002; Papagno, Spina et al. 2004). Probably as a consequence of this rapid turnover and chronic immune activation, a gradual decline in circulating CD4 T cells is observed during the chronic phase (Grossman, Meier-Schellersheim et al. 2002; Stevenson 2003), at a rate of approximately 50-70 cells/mm^3/year. The onset of AIDS is associated with a drop in CD4 cell count below 200 cells per mm^3 of blood, after which point an individual becomes progressively more immunocompromised and, in the absence of antiretroviral therapy, will usually succumb to opportunistic infections within a year. The average time from infection to death, in the absence of HAART, varies depending on environment but is a median of 10 years (Bacchetti and Moss 1989). However, a great deal of heterogeneity exists, with on the one hand about 10% of individuals developing symptoms of AIDS within 5 years (rapid progressors) (Phair, Jacobson et al. 1992), and on the other about 5% of individuals maintaining normal CD4 counts and suppressing viral loads to undetectable levels for more than 10 years (long term nonprogressors; LTNP) (Hogan and Hammer 2001; Deeks and Walker 2007). The mechanisms underlying the differences in rates of disease progression

remain unclear, but, as discussed in the following section, there is growing evidence that the immune response to HIV-1 plays a crucial role.

1.4 Antigen Presentation

1.4.1 The Major Histocompatibility Complex (MHC)

The MHC was shown to have a physiological role in the immune response to infection (Zinkernagel and Doherty 1974). In those experiments mice were infected with lymphocytic choriomeningitis virus (LCMV) and their splenocytes were tested for their ability to lyse chromium-labelled infected targets. Only spleen preparations from mice sharing a H-2 (equivalent to MHC region in humans) allele with the LCMV-infected target cells were lysed (Zinkernagel and Doherty 1974). Soon after this, the recognition of influenza virus-infected cells by human CD8 T cells was shown to be MHC class I-restricted (McMichael, Ting et al. 1977). Subsequently, it was demonstrated that these CD8 T cells recognised and interacted with antigenic peptide in the peptide-binding groove of presenting MHC class I molecules (Townsend, Gotch et al. 1985; Townsend, Rothbard et al. 1986; Bjorkman, Saper et al. 1987), only after intracellular processing of antigen (Cerundolo, Alexander et al. 1990).

The MHC region is a large cluster of genes, located on the short arm of chromosome 6, and contains the human leucocyte antigen (HLA) class I and II genes that are the most polymorphic loci in humans (Carrington and O'Brien 2003; Mungall, Palmer et al. 2003). It plays an essential role in the innate and adaptive arms of the immune system by regulating presentation of peptide fragments to CD8 T cells and by serving

as ligands for killer immunoglobulin-like receptors (KIRs) expressed on natural killer (NK) cells respectively, and is characterised by high gene density, high polymorphism and high linkage disequilibrium. Linkage disequilibrium is defined as the non-random association of alleles from two independent loci, owing to their close physical proximity and a lack of recombination between them. There are three major HLA class I loci in humans with HLA-B the most polymorphic (1601 molecules), followed by HLA-A (1119 molecules) and finally HLA-C (750 molecules), as of January 2011 (http://www.ebi.ac.uk/imgt/hla/stats.html) (Robinson, Mistry et al. 2011). The strong linkage disequilibrium reflects the presence of common conserved haplotypes within the entire MHC region including the HLA class I locus (Degli-Esposti, Leaver et al. 1992; Dawkins, Leelayuwat et al. 1999; de Bakker, McVean et al. 2006). These MHC haplotypes are composed of genomic blocks, often spanning over 100 kilobases in size and multiple genetic loci, that exhibit limited recombination and high levels of polymorphism, thus allowing an almost perfect transfer of sequence between generations (Dawkins, Leelayuwat et al. 1999). MHC haplotypes observed in the population therefore reflect the highly conserved nature of these 'frozen' genomic blocks, as well as the relatively frequent occurrence of recombination events between blocks that generate recombinant haplotypes (Degli-Esposti, Leaver et al. 1992). In this context, there is strong linkage disequilibrium between the HLA-B and HLA-C loci reflecting their carriage within a single genomic block so that recombination within HLA-B/C haplotypes is relatively rare (Degli-Esposti, Leaver et al. 1992). The inheritance of these two HLA class I loci critically shapes the capacity for generating HLA-restricted responses to viral antigens, as indicated by studies implicating these HLA alleles in modulating the host immune response to HIV-1 infection (Moore, John et al. 2002; Trachtenberg, Bhattacharya et al. 2009).

1.4.2 Structure of MHC class I molecules

Human class I MHC molecules are composed of a ~44kDa transmembrane heavy chain glycoprotein, a 12kDa soluble protein called β2-microglobulin (β2M), and a short peptide of 8-10 residues derived from endogenous proteins as well as virus- and tumour-specific proteins. The class I heavy chain is encoded by three polymorphic genes within the MHC. A large solvent-exposed groove in the class I heavy chain consisting of two long α-helices (α1 and α2 domains) forms a binding site for antigenic peptides (Bjorkman, Saper et al. 1987). The α3 domain is packed against the underside of the α1 and α2 domains and anchors the class I heavy chain to the cell membrane. The protein β2M is noncovalently associated with the class I heavy chain and is proximal to the cell membrane. The antigenic peptide binds in the groove in an extended conformation and interacts with conserved and polymorphic MHC residues along the binding site through its main- and side-chain atoms. These interactions are important to maintain class I MHC molecules stably assembled (Madden, Garboczi et al. 1993; Bouvier and Wiley 1994). Some of the peptide side-chains, particularly those from the middle positions of the sequence are orientated upward toward the solvent. It is this region of the peptide that is presented by class I MHC molecules at the surface of infected cells for recognition by T cell receptors (TCRs) (Bjorkman, Saper et al. 1987).

1.4.3 MHC class I antigen presentation pathway

The recognition of antigens in the peptide-binding groove of surface expressed MHC class I molecules by specific TCRs is central to CD8 T cell activation. For MHC

class I molecules the goal is to report on intracellular events (such as viral infection, the presence of intracellular bacteria or cellular transformation) to CD8 T cells and cells displaying peptides recognised as foreign are destroyed (Shresta, Pham et al. 1998). Individual cells can express up to six different MHC class I alleles and each MHC molecule of a given allele is estimated to potentially bind billions of distinct peptides (Shastri, Cardinaud et al. 2005). Generating this complex repertoire of antigenic peptides and loading them on to these MHC molecules for presentation on the cell surface is accomplished by the antigen processing and presentation pathway (Bijlmakers and Ploegh 1993; Yewdell, Reits et al. 2003; Lilley and Ploegh 2005; Shastri, Cardinaud et al. 2005; Jensen 2007; Vyas, Van der Veen et al. 2008). These events can be summarised into the following six steps: 1, acquisition of antigenic proteins (for example, by virus infection); 2, tagging of the antigenic protein for destruction by ubiquitination; 3, proteolysis by the proteasome; 4, delivery of peptides by the transporter associated with antigen processing (TAP) to the endoplasmic reticulum (ER); 5, binding of peptides to MHC class I molecules; and 6, display of peptide-MHC class I complexes on the cell surface (Figure 1.5). Each successive step of MHC class I complex biosynthesis is either a known or potential target for viral immunoevasins.

Most cells exclusively present peptides derived from endogenous proteins on MHC class I molecules. However, exogenous peptides can also be presented on MHC class I molecules via cross-presentation (Bevan 1976; Bevan 1976). Cross-presentation requires the requisite peptide precursors to gain access to the cytosol for processing by the proteasome, followed by their active transport into the ER where newly assembled MHC class I molecules are found. Dendritic cells appear to be uniquely

equipped for cross-presentation as they can take up and degrade infected non-immune cells or cell-derived fragments and subsequently deliver the peptide fragments to the MHC class I pathway for display on the cell surface to CD8 T cells (Albert, Sauter et al. 1998; Guermonprez and Amigorena 2005).

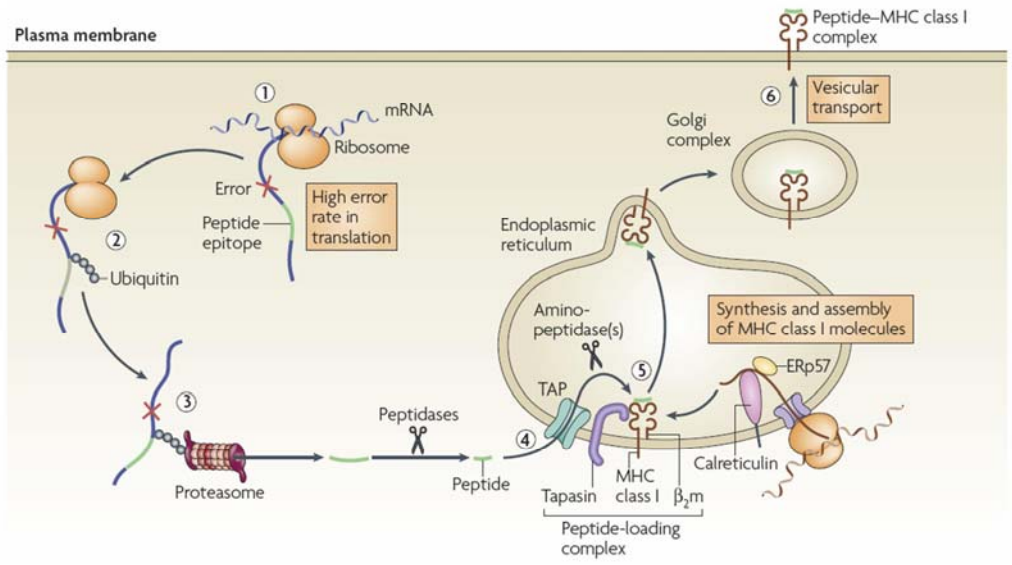


Figure 1.5. MHC class I antigen presentation pathway.
Antigen processing and presentation by MHC class I molecules can be divided into six discrete steps. Step 1: acquisition of antigens from proteins with errors (for example, due to premature termination or misincorporation). Step 2: misfolded proteins are tagged with ubiquitin for degradation. Step 3: the proteasome degrades these ubiquitylated proteins into peptides. Step 4: the peptides are delivered to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) complex. Step 5: peptide is loaded onto nascent MHC class I molecules; this process is facilitated by members of the peptide-loading complex, such as tapasin and two housekeeping ER proteins, known as calreticulin and ERp57. Step 6: peptide-loaded MHC class I molecules are transported via the Golgi complex to the cell surface. Reproduced from Vyas *et al.* (Vyas, Van der Veen et al. 2008).

1.4.4 Structure of MHC class II molecules and their antigen presentation pathway

MHC class II molecules are composed of two transmembrane glycoprotein chains of 34kDa and 29kDa, with each chain containing two domains respectively (α1 and α2, β1 and β2). These molecules possess a similar three-dimensional structure to that of class I molecules and the allelic differences are also attributed to major polymorphism within and around the peptide binding groove. A key difference between the two classes of MHC molecule is that the peptide-binding grooves are more open in class II molecules, such that the ends of peptides are extended along the groove. The peptide-binding groove can therefore accommodate peptides that are variable in length, frequently 13-17 amino acids long, though peptides of unlimited length can bind. In addition, the binding pockets are more permissive in their accommodation of different amino acid side chains. Therefore, in comparison with class I molecules, it can be difficult to define anchor residues and predict peptide binding motifs.

For MHC class II molecules, the goal is to sample the extracellular milieu and present antigens to CD4 T cells, which in turn regulate antiviral immune responses and aid in the generation of CD8 T cells. The MHC class II pathway is constitutively active only in 'professional' APCs, including DCs, B cells, macrophages and thymic epithelial cells. Proteins that enter the endocytic pathway are degraded by endosomal proteases. The resulting peptides are loaded onto class II MHC αβ dimers and are displayed at the cell surface to CD4 T cells (Cresswell 1996; Bryant, Lennon-Dumenil et al. 2002; Watts 2004). MHC class II molecules rely on a specialised transmembrane chaperone protein, the invariant chain (Ii), for stable assembly in the ER. The Ii prevents premature peptide loading in the ER by occupying the peptide

groove. The Ii then routes the class II MHC complex to acidic compartments, where Ii is removed and peptide loading occurs. The mature class II MHC molecules are then transported to the cell surface and interact with the TCR complex on CD4 T cells.

1.5 The host immune response to HIV-1

1.5.1 The cellular immune response

1.5.1.1 CD8 T cells

HIV-1-specific CD8 T cells are detected early in acute infection, prior to seroconversion, and at their peak can constitute over 10% of all circulating CD8 T cells (Borrow, Lewicki et al. 1994; Koup, Safrit et al. 1994). CD8 T cells recognize infected cells through the interaction of their TCRs with pathogen-derived peptides bound to HLA class I molecules on the cell surface. On recognition of their cognate antigen, HIV-1-specific CD8 T cells secrete a range of cytokines, including IFN-γ and TNF-α, and chemokines, including RANTES, macrophage inflammatory protein-1 (MIP1)-α and MIP1-β. These molecules have both direct and indirect antiviral effects that will be discussed in a later section. The most important role of CD8 T cells in HIV-1 control however, is probably the direct killing of HIV-1-infected cells (Lichterfeld, Yu et al. 2004). HIV-1-specific CD8 T cells kill HIV-1-infected CD4 T cells both *in vitro* (Yang, Kalams et al. 1996; Yang, Kalams et al. 1997), and *ex vivo* (Walker, Chakrabarti et al. 1987; Lichterfeld, Yu et al. 2004; Saez-Cirion, Lacabaratz et al. 2007). In addition, they effectively inhibit viral replication in other cell types permissive to HIV-1 infection, including monocytes and dendritic cells (Severino,

Sipsas et al. 2000). Upon TCR engagement with peptide-MHC class I complexes, CD8 T cells display multiple effector functions including cytotoxicity and cytokine secretion (Harty, Tvinnereim et al. 2000; Gulzar and Copeland 2004). Cytotoxicity is mediated in two ways; by the release of perforin and GranzymeB, the former punching holes in the plasma membrane, allowing the latter to enter and induce cell death by activation of the caspase cascade (Shankar, Xu et al. 1999); and through the upregulation of Fas-ligand, which binds to Fas, itself upregulated on infected cells, and induces cell death (Katsikis, Wunderlich et al. 1995; Westendorp, Frank et al. 1995; Hadida, Vieillard et al. 1999). This apoptosis is not limited to cells that are infected by, or specific for, HIV-1 but occurs predominantly in bystander cells (Finkel, Tudor-Williams et al. 1995). Recently, a novel mechanism of perforin-mediated CD8 T cell cytotoxicity that bypasses the traditional model of cytotoxic granule exocytosis from activated T cells was described (Makedonas, Banerjee et al. 2009), with rapid upregulation of perforin *de novo* after antigen stimulation without the requirement for proliferation. This newly synthesised perforin can be immediately transported to the immunological synapse, both in association with and independent of cytotoxic granules, to promote cytotoxicity (Makedonas, Banerjee et al. 2009).

The hallmark of chronic viral infections like HIV-1 is the inability of the immune system to completely clear infection, resulting in viral persistence. Under these conditions of chronic antigen persistence, virus-specific CD8 T cells gradually lose their ability to proliferate and there is an increasing degree of functional impairment marked by dysfunctional cytotoxic activity and a reduction in cytokine secretion profile (Appay, Nixon et al. 2000), eventually leading to exhaustion and/or anergy of these cells (Zajac, Blattman et al. 1998) and ultimately resulting in an inability to

clear the persisting infection. A number of studies have described the accumulation of functional impairments of HIV-1-specific CD8 T cells from early to chronic stages of infection. These CD8 T cells develop abnormally (Champagne, Ogg et al. 2001), their cytotoxic function can be deficient in chronically infected (Appay, Nixon et al. 2000; Zhang, Shankar et al. 2003) as well as in acutely infected individuals (Koup, Safrit et al. 1994; Safrit, Andrews et al. 1994) and there are significant differences in their ability to proliferate and secrete cytokines with HIV-1-specific CD8 T cells from LTNPs exhibiting a greater proliferative capacity and higher levels of cytotoxicity and cytokine secretion against autologous infected CD4 T cells than those from individuals with progressing disease (Migueles, Laborico et al. 2002; Lichterfeld, Kaufmann et al. 2004; Horton, Frank et al. 2006; Migueles, Osborne et al. 2008; Rehr, Cahenzli et al. 2008).

Although not conclusive, there is a great body of evidence to suggest that CD8 T cells play the pivotal role in controlling HIV-1 replication. First, the temporal association between the appearance of HIV-1-specific CD8 T cell responses and the decrease in viral load during acute infection suggests the importance of CD8 T cells in the establishment of viral load set point (Borrow, Lewicki et al. 1994; Koup, Safrit et al. 1994). This observation was confirmed by studies in the rhesus macaque/SIV model, in which depletion of circulating CD8 T cells, using anti-CD8 monoclonal antibodies, resulted in a sudden burst of viral replication, which persisted until antibodies wore off and the CD8 T cells returned (Matano, Shibata et al. 1998; Jin, Bauer et al. 1999; Schmitz, Kuroda et al. 1999). Subsequent studies in macaques showed that vaccines that stimulate CD8 T cell responses partially protect against challenge with SIV (Gallimore, Cranage et al. 1995; Kent, Zhao et al. 1998; Liu, O'Brien et al. 2009).

Complete protection is rare, but reduction of virus load compared with controls after SIV challenge is seen consistently (Allen, O'Connor et al. 2000; Barouch, Craiu et al. 2000; Liu, O'Brien et al. 2009; Hansen, Ford et al. 2011).

A second line of evidence to support the role of CD8 T cells in immune control of HIV-1 is the association between certain HLA class I molecules and disease outcome. Several independent studies have identified highly significant associations between the expression of certain HLA alleles and either slow or rapid progression to AIDS (Carrington, Nelson et al. 1999; O'Brien, Gao et al. 2001; Carrington and O'Brien 2003; Fellay, Shianna et al. 2007; Pereyra, Jia et al. 2010). The precise mechanism behind these associations remains elusive, and indeed because HLA molecules are also recognized by NK cells, some groups have suggested that they could be related to NK rather than CD8 T cell activity (Flores-Villanueva, Yunis et al. 2001; Martin, Gao et al. 2002; Lopez-Vazquez, Mina-Blanco et al. 2005). However, on close examination several of the HLA associations appear to be independent of NK activity (Martin, Gao et al. 2002; Kiepiela, Leslie et al. 2004), and thus are likely to be a consequence of the CD8 T cell response. This is supported by the fact that HLA association with disease progression appears to work on a more general level, one that is certainly independent of NK activity. HLA homozygosity has been shown to increase the rate of disease progression (Carrington, Nelson et al. 1999; Tang, Costello et al. 1999), and in an additive manner, such that individuals homozygous for more than one HLA allele progress faster than those homozygous for just a single allele (Carrington, Nelson et al. 1999). There is also some evidence to suggest that the frequency of an HLA allele within a population is predictive of time to AIDS,

with common alleles generally associated with faster progression than those that are rare (Trachtenberg, Korber et al. 2003; Scherer, Frater et al. 2004).

A third line of evidence suggesting the importance of CD8 T cells in control of HIV-1 infection is the demonstration that the selection of particular viral escape mutations within CD8 T cell-restricted epitopes can precipitate loss of immune control of both HIV-1 and SIV infection (Phillips, Rowland-Jones et al. 1991; Goulder, Phillips et al. 1997; Barouch, Kunstman et al. 2002; Feeney, Tang et al. 2004). CD8 T cell escape mutation describes the positive selection of mutations in HIV-1 that allow it to evade the T cell immune response. Following the peak in the CD8 T cell response, the virus sequence starts to change dramatically and rapid selection of mutations at discrete sites in the virus genome occur as viraemia declines to the viral set point (Bernardin, Kong et al. 2005; Salazar-Gonzalez, Salazar et al. 2009; Boutwell, Rolland et al. 2010). The first report of such escape in HIV-1 came in 1991 when Philips *et al.* noted the occurrence of variation within HLA-B*08-restricted Gag epitopes that affected their recognition by autologous CD8 T cells (Phillips, Rowland-Jones et al. 1991), although no direct link between the variants and CD8 T cell selection pressure was established. This came later with experiments linking the adoptive transfer of *in vitro* expanded CD8 T cells specific for an epitope in Nef with the occurrence of mutation within that epitope (Koenig, Conley et al. 1995). Subsequently, examples of escape mutation occurring in natural infection were reported during acute infection (Borrow, Lewicki et al. 1997; Price, Goulder et al. 1997; Jones, Wei et al. 2004; Liu, McNevin et al. 2006; Goonetilleke, Liu et al. 2009; Streeck, Jolin et al. 2009; Turnbull, Wong et al. 2009). Goonetilleke *et al.* performed a comprehensive analysis of the primary T cell response in the earliest stages of HIV-1 infection in four

individuals and demonstrated that the earliest escapes from the founder virus occurred within ten days of infection (Goonetilleke, Liu et al. 2009). These CD8 T cell-selected mutations were then followed by sequential selection of escape mutations at different epitopes with the pattern continuing throughout the course of infection. These sequence changes were not restricted to epitopes recognised by CD8 T cells but also involved amino acids upstream of the epitope itself that play a role in antigen processing (Draenert, Le Gall et al. 2004; Jones, Wei et al. 2004; Tenzer, Wee et al. 2009). The earliest T cell responses were often specific for Env and Nef while responses to other viral proteins, including the conserved Gag and Pol proteins, typically arose later and therefore likely play more of a significant role in maintaining the viral load set point than controlling early viraemia (Goonetilleke, Liu et al. 2009; Turnbull, Wong et al. 2009; Wang, Li et al. 2009). This HLA class I mediated selection pressure on viral sequence continues into the chronic stages of HIV-1 infection (Koup 1994; Price, Goulder et al. 1997; Kelleher, Long et al. 2001; Cao, McNevin et al. 2003; Leslie, Pfafferott et al. 2004; Allen, Altfeld et al. 2005; Martinez-Picado, Prado et al. 2006; Bhattacharya, Daniels et al. 2007; Crawford, Prado et al. 2007; Frater, Brown et al. 2007; Brumme, Brumme et al. 2008; Keele, Giorgi et al. 2008; Matthews, Prendergast et al. 2008) with mathematical modelling estimating that only 2% of virus-infected cell death is attributable to these late CD8 T cells recognising a single epitope (Asquith, Edwards et al. 2006). This may be the result of replicative fitness costs of the escape mutation on the virus such that mutant viruses grow much slower than the founder virus. Experimental evidence supporting a relationship between the selection of CD8 T cell escape mutations and reduced viral fitness include the reversion of escape mutations to a more ancestral sequence following transmission to an HLA-mismatched recipient who cannot target the

epitope (Allen, Altfeld et al. 2004; Friedrich, Dodds et al. 2004; Leslie, Pfafferott et al. 2004; Barouch, Powers et al. 2005; Li, Gladden et al. 2007; Crawford, Lumm et al. 2009), as well as reduced plasma viral load at set point following the transmission of certain escape variants from donors who expressed protective HLA alleles (Chopera, Woodman et al. 2008; Goepfert, Lumm et al. 2008). More recently, population based methods have demonstrated that escape mutation is common, widespread and selected by CD8 T cell responses restricted by a wide array of different HLA molecules (Moore, John et al. 2002; Kawashima, Pfafferott et al. 2009; John, Heckerman et al. 2010).

The compelling evidence highlighted above suggests a central role for the CD8 T cell response in controlling HIV-1 infection. However, strong IFN-γ producing CD8 T cell responses are detectable in patients with progressive HIV-1 infection (Draenert, Verrill et al. 2004), implying that not all anti-HIV-1 CD8 T cell responses are capable of controlling viral replication *in vivo*. There are several possible reasons behind this apparent paradox. By preferentially infecting and killing CD4 T cells, HIV-1 probably interferes with the CD4 T cell help needed to prime an effective memory CD8 T cell response (Janssen, Lemmens et al. 2003). Therefore, the CD8 T cells observed in progressive HIV-1 disease may be dysfunctional due to a lack of CD4 T cell help (Lichterfeld, Kaufmann et al. 2004). In addition, it is gradually becoming apparent that not all HIV-1-specific CD8 T cells possess the same antiviral efficacy, either measured by *in vitro* replication assay (Loffredo, Rakasz et al. 2005; Chung, Lee et al. 2007; Vojnov, Reed et al. 2010), or by ability to exert sufficient selection pressure to select for escape mutation *in vivo* (Leslie, Price et al. 2006). Therefore,

only a proportion of the total HIV-1-specific CD8 T cell response observed may actually contribute to the control of HIV-1 replication *in vivo*.

1.5.1.2 CD4 T cells

CD4 or T-helper cells are also antigen specific, being activated when their TCR recognises its cognate antigen in the context of the correct HLA class II molecule. This T cell help is known to be important for several reasons. Firstly, CD4 T cells play a role in regulating CD8 T cells by priming CD8 T cells, maintaining CD8 T cell memory and maturing CD8 T cell function. Evidence that CD4 T cells are important in priming CD8 T cells comes from murine models that pinpoint CD40L, expressed on activated CD4 T cells, as crucial in triggering DCs to produce the cytokine IL-12, which in turn is central in initiating the CD8 T cell response (Ridge, Di Rosa et al. 1998). The proliferation and maintenance of memory CD8 T cell responses, capable of full secondary expansion, is dependent on the production of IL-2 by CD4 T cells (Sun and Bevan 2003; Sun, Williams et al. 2004; Williams, Tyznik et al. 2006). Indeed, there is evidence that the CD8 T cell response in HIV-1 non-controllers may be suffering from a lack of CD4 T cell help, and that this can be restored through the adoptive transfer of autologous CD4 T cells or simply by the addition of exogenous IL-2 (Lichterfeld, Kaufmann et al. 2004). However, the long survival of particular CD8 T cell clones for many years in patients chronically infected with HIV-1 (Moss, Rowland-Jones et al. 1995), when CD4 T cell help is known to be damaged, suggests that naturally activated CD8 T cells may be able to survive better in the presence of diminished help. The role for CD4 T cells in regulating CD8 T cell function has been shown in animal models where absence of CD4 T cells resulted in a CD8 T cell response that was numerous in terms of HLA tetramer-stained cells, but lacking in

function (Zajac, Blattman et al. 1998). In chronic HIV-1 infection, less than 15% of HIV-1-specific cells contained perforin, which was reflected in poor *ex vivo* killing of appropriate target cells, compared with CMV-specific cells from the same donors, 50% of which expressed perforin and killed well (Appay, Nixon et al. 2000).

A second role for CD4 T cells involves the activation of the humoral arm of the anti-HIV-1 immune response through the production of cytokines such as IL-4. Miller and Mitchell described in murine models the bone marrow origin of antibody-producing cells (B cells) and the thymic origin of the help (T cells) needed to activate these B cells to produce antibodies (Miller and Mitchell 1968; Mitchell and Miller 1968). Since the ground-breaking work by Mosmann and Coffman showing that long-term CD4 T cell lines could be subdivided into two groups, those that make IFN-γ as their signature cytokine (T_H1) and those that produce IL-4 (T_H2) (Mosmann, Cherwinski et al. 1986; Stevens, Bossie et al. 1988; Mosmann and Coffman 1989), it has been realised that CD4 T cells are not an exclusive set of cells, but represent a series of distinct cell populations with different functions. One such population, called follicular helper CD4 T cells (T_{FH}), with a unique phenotype expressing high levels of CXCR5, are the specialised providers of B cell help (Breitfeld, Ohl et al. 2000; Schaerli, Willimann et al. 2000; Kim, Rott et al. 2001). In secondary lymphoid organs, lymphocytes are segregated on the basis of chemokine sensitivity into discrete B cell zones (follicles) and T cell zones. After being activated by antigen, B cells increase their expression of CCR7 and migrate to the interface between the zones, where they encounter cognate T cells. This encounter results in a burst of proliferation in the outer follicle (Coffey, Alabyev et al. 2009). These B cells have three potential fates; they can become plasmablasts and migrate to extrafollicular sites

producing a transient wave of relatively low-affinity, antigen-specific antibodies; they can return to the centre of the B cell follicle and proliferate further to form a germinal centre (GC) (Allen, Okada et al. 2007); they can assume a memory phenotype (early memory B cells) that enter the circulation (Blink, Light et al. 2005). Proliferating B cells in a GC (centroblasts) initiate somatic hypermutation and thereby diversify their antigen receptors to enable further clonal selection on the basis of affinity. These GCs also contain T_{FH} cells that upregulate CXCR5 expression to enable its follicular localisation, and antigen presenting cells such as DCs, macrophages and specialised cells known as follicular dendritic cells (FDCs). GC B cells with potentially altered affinity for antigens then stop proliferating and become centrocytes that are selected according to their ability to bind antigen complexes on FDCs and to elicit help from cognate T_{FH} cells. Thus GC T_{FH} cells initiate GC formation, provide cell survival and proliferation signals (via multiple pathways, including CD40L, IL-4, IL-21 and programmed death 1) to GC B cells, stimulate the process of somatic hypermutation in the GC B cells, and then subsequently select GC B cells of appropriately high affinity to stop being GC B cells and instead differentiate into either plasma cells or memory B cells and exit the GC (Crotty 2011).

Finally, HIV-1-specific CD4 T cells are known to have some direct antiviral effects, as on activation they produce many of the same cytokines and chemokines as CD8 T cells, and in certain instances can directly kill HIV-1 infected cells (Appay, Zaunders et al. 2002; Norris, Moffett et al. 2004).

Activated CD4 T cells are the primary target for HIV-1 infection. The ensuing failure of T cell help may be central in the pathogenesis of HIV-1 infection and could be the

critical feature that ultimately undermines immune control. This is compounded by the fact that HIV-1 has been shown to preferentially infect activated HIV-1-specific CD4 T cells (Douek, Brenchley et al. 2002; Li, Duan et al. 2005; Mattapallil, Douek et al. 2005).

1.5.1.3 Natural Killer (NK) and NKT cells

Similar to most viral infections, NK and NKT cells become activated during acute HIV-1 infection (Alter, Teigen et al. 2007; Borrow and Bhardwaj 2008). Prior to the peak in viraemia, blood NK cells proliferate and show enhanced activity when tested *ex vivo* (Alter, Teigen et al. 2007). These activated cells can control HIV-1 replication through cytolysis of virally infected cells (in a similar way to CD8 T cells) and the production of antiviral cytokines and chemokines including IFN-γ, TNF-α, RANTES, and MIP1-α and -β. In addition, they can interact with DCs and thereby influence T cell responses (Fauci, Mavilio et al. 2005; Lanier 2005). NK cells express a diverse array of activating and inhibitory receptors, including KIRs that can recognise specific groups of HLA class I molecules. Alleles of the HLA-A and HLA-B loci form the Bw4/Bw6 cluster defined by two different amino acid sequences at residues 77-83 in the carboxyl-terminal end of the α1 helix of the HLA class I peptide binding groove; there are four Bw4 motif sequences, which differ only at positions 77 (asparagine, aspartic acid or serine) and 80 (isoleucine or threonine). This region of the HLA molecule interacts with NK cells via their KIRs. Under normal conditions, the inhibitory signals are dominant and therefore prevent the destruction of normal cells by NK cells. Viral infected cells and certain tumour cells are known to downregulate HLA class I molecules in order to evade the CD8 T cell response

(Peterlin and Trono 2003). However, inhibitory KIRs such as KIR2DS1 bind to HLA class I molecules, and the removal of this dominant inhibitory signal results in NK cell activation and killing of the cell. HIV-1 appears to have somewhat circumvented this immune mechanism by only downregulating certain HLA class I molecules (HLA-A and -B), and leaving others on the surface (HLA-C and -E) (Cohen 2003; Bonaparte and Barker 2004; Ward, Bonaparte et al. 2004).

An analysis of HLA-B alleles in HIV-1 infected individuals has shown that the presence of homozygosity for HLA-Bw4 (the ligand for KIR3DL1 and putatively KIR3DS1) is associated with a profound suppression of viraemia, delay in progression to AIDS and a slower decline in CD4 T cell count (Flores-Villanueva, Yunis et al. 2001). Further to this, large genetic studies have shown that the activating KIR3DS1 allele in combination with HLA-Bw4 alleles with an isoleucine at position 80 (HLA-B Bw4-80Ile) (Martin, Gao et al. 2002; Qi, Martin et al. 2006) and that the inhibitory KIR3DL1 alleles in combination with HLA-Bw4 (Martin, Qi et al. 2007) both associate with control of viraemia at set point as well as with protection against progression to AIDS. These seemingly inconsistent observations can be explained by a model in which activation of NK cells confers protection from HIV-1 disease progression either through the loss of a strong inhibitory signal to KIR3DL1 resulting from HLA-B downregulation on HIV-1 infected cells or by triggering the activating KIR3DS1 receptors. Intriguingly, HLA-B*27 and HLA-B*57, the most protective HLA class I alleles in terms of disease progression, belong to the group of HLA-B Bw4 alleles. However, the protective KIR-HLA-B Bw4 effect has been shown to be independent of the presence of HLA alleles (HLA-B*27, -B*51, -B*57, -

B*8 and -B*35) that could potentially confound the result (Flores-Villanueva, Yunis et al. 2001; Martin, Qi et al. 2007).

Hence enhanced NK cell activity can contribute to the control of viral replication. There is growing evidence that HIV-1 can evade this NK cell-mediated immune pressure by selecting for escape variants that modulate the recognition of infected cells by KIR. Previous *in vitro* studies have demonstrated that sequence variations in HLA class I-presented epitopes can modulate the binding of KIR (Malnati, Peruzzi et al. 1995; Peruzzi, Parker et al. 1996; Hansasuta, Dong et al. 2004; Stewart-Jones, di Gleria et al. 2005; Thananchai, Gillespie et al. 2007), providing a potential mechanism for virus-sequence-dependent recognition of infected cells by NK cells. However, it remains unknown whether NK cells can directly mediate antiviral immune pressure *in vivo* in humans. Brackenridge *et al.* followed early-emerging mutations that mapped to the immunodominant TW10 (TSTLQEQIGW [Gag residues 240-249]) epitope in two HLA-B*5703-positive individuals and two mutations emerged, a glycine-to-glutamate change at position 9 (G9E) and a threonine-to-asparagine change at position 3 (T3N) (Brackenridge, Evans et al. 2011). The T3N mutation escaped T cell recognition while the G9E mutation failed to successfully evade TW10-specific T cells. They then tested whether the G9E mutation influenced the binding of HLA-B*5703 to soluble KIR3DL1 protein and found that this variant severely reduced interaction between KIR3DL1 and HLA-B*5703. They suggest that during HIV-1 infection, some early-emerging variants like G9E could affect KIR-HLA interaction and possibly represent NK cell escape mutations (Brackenridge, Evans et al. 2011). More recently, Alter *et al.* described several amino acid polymorphisms within the HIV-1 clade B sequence that were significantly associated

with the expression of KIR2DL2 on the population level (Alter, Heckerman et al. 2011). They went on to demonstrate in *in vitro* functional studies that HIV-1 selects for sequence polymorphisms in KIR2DL2-positive individuals that lead to an enhanced binding of this inhibitory KIR to infected cells, resulting in the inhibition of NK cell function and thereby enabling HIV-1 to escape the potential protective role of this KIR (Alter, Heckerman et al. 2011).

These data suggest that NK cells may play an important role in the immune control of HIV-1. However, the timing of NK cell antiviral effects remains uncertain. NK cells do not contribute to the selection of virus escape mutants before peak viraemia, although it is possible that they account for some of the unexplained mutations that appear together with those that are selected by early T cell responses as viraemia decreases to reach the set point (Goonetilleke, Liu et al. 2009). Alternatively, the antiviral effects of NK and/or NKT cells might have a greater influence at later time points.

1.5.2 The humoral immune response

Functional antibodies are necessary for the protection afforded by most successful vaccines. Almost all HIV-1 infections elicit abundant HIV-1-specific antibodies, but their role, if any, in the immune control of HIV in unclear. The initial antibody response to the viral envelope is non-neutralising and does not select for viral escape (Tomaras, Yates et al. 2008). Unlike CD8 T cell responses, they are unlikely to be involved in the resolution of primary viraemia, as the first neutralizing antibodies (nAb) do not appear until 3-6 months after infection, when viral load has generally already reached set point (Ariyoshi, Harwood et al. 1992; Wei, Decker et al. 2003).

In addition, unlike with CD8 T cells, the depletion of B cells with monoclonal antibody from macaques had no effect on SIV viraemia, despite significantly slowing the emergence of nAb (Schmitz, Kuroda et al. 2003).

The reason for the apparent failure of antibodies to control HIV-1 infection is primarily due to the ability of the virus to mutate and conceal itself from these responses (Burton, Desrosiers et al. 2004; Haynes and Montefiori 2006). Neutralizing antibodies target the surface glycoproteins gp120 and gp41 to block target cell binding and entry. HIV-1 escapes these antibody responses in three ways; point mutation within the epitopes that prevent antibody binding (McKeating, Gow et al. 1989); concealing epitopes within deep recesses or shielding them by flexible tertiary folds of the gp120 molecule (Mascola and Montefiori 2003); and finally hiding epitopes behind constantly shifting glycan shields (Wei, Decker et al. 2003). Antibody escape in this way is an ongoing process during HIV-1 infection, with the host constantly generating new antibodies and the virus constantly changing to evade them. The nature of this struggle between host and pathogen is demonstrated by the fact that antibodies isolated from a given host are often effective against virus isolated from earlier time points, but rarely neutralize contemporary viral strains (Richman, Wrin et al. 2003; Wei, Decker et al. 2003; Skrabal, Saragosti et al. 2005).

Nonetheless, some lines of evidence support a role for nAb in HIV-1. There are several studies showing the potential protective effects of anti-HIV-1 nAb *in vitro* (Haigwood and Stamatatos 2003), and passive transfer experiments have demonstrated that nAb can prevent SIV infection in rhesus macaques (Shibata, Igarashi et al. 1999; Baba, Liska et al. 2000; Mascola, Stiegler et al. 2000; Veazey,

Shattock et al. 2003; Ferrantelli, Rasmussen et al. 2004; Hessell, Poignard et al. 2009). In humans, Trkola *et al.* showed a delay of virus rebound after cessation of HAART, through passive transfer of broadly neutralising human monoclonal antibodies (2G12, 2F5 and 4E10) (Trkola, Kuster et al. 2005). In addition, a number of studies have suggested that LTNPs possess strong, cross-reactive nAb (Cao, Qin et al. 1995; Montefiori, Pantaleo et al. 1996; Pilgrim, Pantaleo et al. 1997; Zhang, Fracasso et al. 1997; Carotenuto, Looij et al. 1998). These patients maintain high CD4 T cell counts in the absence of HAART but have low yet detectable viral loads (Cao, Qin et al. 1995; Martinez, Costagliola et al. 2005). More recently, analysis of the ability of longitudinal serum samples, from 40 HIV-1 clade C seropositive individuals, to neutralise a large panel of HIV-1 envelope variants, established that after 3 years of infection, fewer than 20% of individuals developed antibodies with cross-neutralising activity (Gray, Madiga et al. 2011). These heterologous nAb targeted different regions on the HIV-1 envelope and first appeared in some individuals as early as one year postinfection but peaked at 4 years, with no increases thereafter (Gray, Madiga et al. 2011). Interestingly, the emergence of these nAb correlated with low CD4 T cell count and high viral load at set point (defined as 6 months postinfection), suggesting that antigen-driven selection is crucial in the development of these antibodies (Gray, Madiga et al. 2011). This correlation between neutralisation breadth and high viral load set point had previously been shown (Piantadosi, Panteleeff et al. 2009; Sather, Armann et al. 2009). Therefore, whilst the role of HIV-1 nAb in natural infection remains unclear, they should not be dismissed, and indeed their induction remains a central goal in HIV-1 vaccine design.

1.5.3 Other host defence mechanisms

1.5.3.1 Soluble factors

Activation of the immune system by HIV-1 infection leads to the production of a range of soluble factors that can inhibit viral replication (Walker, Moody et al. 1986; Jassoy, Harrer et al. 1993; Price, Johnson et al. 1995). The most abundant of these is probably IFN-γ, which is secreted by both activated T cells (particularly CD8 T cells) and NK cells (Kedzierska, Crowe et al. 2003). IFN-γ has been shown by several groups to have direct antiviral activity *in vitro* (Emilie, Maillot et al. 1992; Denis and Ghadirian 1994; Fan, Turpin et al. 1994; Dhawan, Heredia et al. 1995), although the importance of this activity *in vivo* is unclear (McMichael and Rowland-Jones 2001). In addition, IFN-γ has several non-specific effects that heighten the general "awareness" of the immune system; including upregulation of HLA class I and II expression, formation and upregulation of the immunoproteasome, enhancement of NK cell activity, and T and B cell activation (Kedzierska, Crowe et al. 2003). Other important soluble factors include the natural chemokine ligands for the HIV-1 coreceptor CCR5, RANTES and MIP-1α and MIP-1β, all of which have been shown to potently inhibit viral growth of R5 HIV-1 strains *in vitro* (Cocchi, DeVico et al. 1995; Price, Sewell et al. 1998; Wagner, Yang et al. 1998). Furthermore, separate genetic studies have shown that naturally occurring mutations and variations in gene copy number that reduce production of RANTES and MIP-1α are associated with increased spread of HIV-1 *in vivo* and accelerated progression to AIDS (An, Nelson et al. 2002; Gonzalez, Kulkarni et al. 2005). It was subsequently shown that variation in the MIP-1α gene copy number does not have any major effects on the control of HIV-1 (Urban, Weintrob et al. 2009). These chemokines are secreted by activated CD8 T

cells, and antigen specific CD4 T cells, as well as by NK cells and γδ T cells (DeVico and Gallo 2004). In a similar fashion, the natural ligand for CXCR4, stromal cell-derived factor 1 (SDF-1), is known to inhibit the growth of X4 strains of HIV-1 (Bleul, Farzan et al. 1996). Finally, CD8 T cells produce further, as yet undefined, soluble factors that appear to suppress HIV-1 growth, collectively termed CD8 antiviral factor (CAF) (Walker, Moody et al. 1986; Mackewicz and Levy 1992; Levy, Mackewicz et al. 1996; DeVico and Gallo 2004). HIV-1-specific CD8 T cells secrete these antiviral factors at sites of virus replication, efficiently inhibiting virus replication *in vitro* (Yang, Kalams et al. 1997).

1.5.3.2 Other cellular defences

Humans possess a number of additional innate defence mechanisms to protect against retroviral infection. Three that have caused much interest in the HIV-1 field in recent years are the antiviral proteins APOBEC3G, tripartite motif-containing protein 5-α (TRIM5-α) and tetherin. These intracellular intrinsic restriction factors are constitutively expressed but also inducible by interferon.

APOBEC3G induces G to A hypermutation by interfering with the reverse transcription step in the retrovirus life cycle (Bishop, Holmes et al. 2004). However, HIV-1 is able to effectively block the activity of APOBEC3 through the binding of the Vif protein (Kremer and Schnierle 2005).

TRIM5-α on the other hand potently blocks HIV-1 infectivity by binding to the capsid protein and interfering with the uncoating step early in the viral life cycle

(Stremlau, Owens et al. 2004; van Manen, Rits et al. 2008). Again however, HIV-1 has found a way to circumvent this restriction factor, by binding a second host derived protein, Cyclophilin A, which blocks the TRIM5-α binding site (Towers, Hatziioannou et al. 2003).

Tetherin is a transmembrane protein that restricts the release of fully formed progeny virions from infected cells, presumably by a direct retention mechanism that is independent of any viral protein target (Neil, Zang et al. 2008; Tokarev, Skasko et al. 2009). These retained virions are endocytosed and subsequently degraded. A major function of the HIV-1 Vpu protein is to facilitate virus spread by antagonising tetherin-induced particle retention (Neil, Zang et al. 2008; Tokarev, Skasko et al. 2009).

The fact that HIV-1 has side-stepped these three host defence mechanisms serves as another sobering reminder of the immense adaptability this virus possesses.

1.6 The role of the MHC class I region in HIV-1 control

The HLA class I locus, with its extensive polymorphism, has the strongest effects on HIV-1 disease progression, greater than any other genetic locus identified to date (Carrington and O'Brien 2003; Fellay, Shianna et al. 2007). In fact the HLA class I and CCR5 loci were the only regions to display significant associations with plasma viral load at the genome-wide level emphasising the potentially central role of class I-restricted CD8 T cells in HIV-1 control (Fellay, Ge et al. 2009; Pereyra, Jia et al. 2010). Due to this extensive polymorphism of HLA molecules, different individuals are able to respond to specific immunodominant HIV-1 epitopes depending on their

HLA background. Since disease progression is influenced by an individual's HLA background, some CD8 T cell responses apparently control the virus better than others. This polymorphism is focussed primarily on those residues that line the peptide-binding groove of HLA class I molecules and therefore define the peptide-binding motif for a given HLA molecule (Sette and Sidney 1999; Marsh, Parham et al. 2000). Differences in the amino acid sequences of the HLA class I peptide binding groove have been shown to affect the binding of peptides, particularly in the B and F pockets of the HLA molecule, which interact with the second amino acid residue and the carboxy-terminal amino acid residues (at position 9) of bound peptides, respectively (Bouvier and Wiley 1994; Barber, Gillece-Castro et al. 1995). In the setting of HIV-1 infection, single amino acid sequence differences in closely related HLA molecules can have crucial consequences in terms of disease outcome. For example, HLA-B*35 subtypes can be divided into two groups based on peptide-binding specificity, with HLA-B*35-Px alleles associated with accelerated progression to AIDS while HLA-B*35-PY alleles correspond to slower progression in Caucasians (Gao, Nelson et al. 2001). The HLA-B*35-PY group (HLA-B*3501 and -B*3508) bind epitopes with proline in position 2 and tyrosine in position 9 while the HLA-B*35Px group (HLA-B*3502, -B*3503, -B*3504 and -B*5301) similarly bind to proline in position 2 but can bind several different amino acids (not including tyrosine) at position 9 (Gao, Nelson et al. 2001). HLA-B*3501 differs from -B*3502 and -B*3503 by only three and a single amino acid respectively (Gao, Nelson et al. 2001). Another example involves HLA-B*5801 and -B*5802. These two alleles differ by only three amino acids resulting in a substantial difference in peptide binding specificity (Marsh, Parham et al. 2000). In a study of clade C infection in South Africa, there was a strong association between HLA-B*5801 and low viral

load, whereas HLA-B*5802 was associated with high viral load (Kiepiela, Leslie et al. 2004). Finally, when the same epitope was presented by different HLA alleles within the HLA-B7 supertype (HLA-B*0702, -B*4201 and -B*8101 which are identical in amino acid sequence in the residues forming their B and F primary binding pockets), there were substantial differences in the ability of CD8 T cells to select for escape mutations, distinct TCR repertoires were recruited and there were significant differences in the functional avidity of responding CD8 T cells, all suggesting that the unique peptide-MHC complexes generated were qualitatively different (Leslie, Price et al. 2006).

Further evidence implicating the nature of the HLA-peptide interaction as the major factor modulating control of HIV-1 infection came from a genome-wide association study that revealed amino acids of the MHC complex associate with the rate of progression to AIDS (Pereyra, Jia et al. 2010). They performed genome-wide association analysis in a multi-ethnic cohort of HIV-1 controllers and progressors and analysed the effects of individual amino acids within the classical HLA proteins. The study confirmed and extended previous HLA association data in HIV-1 infected Caucasians (Carrington and O'Brien 2003) by finding that HLA-B*5701, -B*2705 and -B*14 were protective, whereas types -B*35 and -Cw*07 were associated with progression to AIDS. Examination of all the polymorphic amino acids in HLA class I revealed that amino acid positions 67, 70, and particularly 97, in the peptide binding groove of HLA-B had stronger statistical associations with HIV-1 protection than any single HLA molecule, including HLA-B*5701, suggesting that conformational differences in peptide presentation at these sites contribute to the protective or susceptible nature of the various HLA-B alleles (Pereyra, Jia et al. 2010). Valine,

asparagine and tryptophan occurred at position 97 in the HLA-B*5701, -B*2705 and -B*14 protective haplotypes, respectively, whereas arginine occurred in risk alleles like HLA-B*35, -B*53 and -Cw*07 (Pereyra, Jia et al. 2010). This implies that discrete amino acids at a single position within the peptide binding groove influence disease outcome.

1.6.1 Evidence for the importance of the HLA-B locus in HIV-1 control

There is a wealth of theoretical evidence suggesting that the polymorphism in the HLA complex has been driven by infectious diseases, with the HLA-B locus under higher selection pressure than HLA-A and HLA-C (Jeffery and Bangham 2000; Prugnolle, Manica et al. 2005; Meyer, Single et al. 2006). In the context of HIV-1, biological evidence for this comes from several studies.

The alleles HLA-B*5701 and HLA-B*5703 have the clearest protective effects against HIV-1 in Caucasians and Africans, respectively (Pelak, Goldstein et al. 2010). The majority of detectable HIV-1 specific CD8 T cell responses are restricted by HLA-B alleles (Kiepiela, Leslie et al. 2004; Bihl, Frahm et al. 2006; Kiepiela, Ngumbela et al. 2007). In a cohort of 375 HIV-1 infected individuals in South Africa, Kiepela *et al.* showed that HLA-B alleles contribute significantly more towards the total HIV-1-specific CD8 T cell response than either HLA-A or HLA-C, and that, through variation in viral load set point, these are the alleles principally associated with diverse outcomes following HIV-1 infection (Kiepiela, Leslie et al. 2004).

The strongest selection pressure on HIV-1 is exerted by HLA-B restricted CD8 T cell responses (Kiepiela, Leslie et al. 2004; Matthews, Prendergast et al. 2008; Rousseau, Daniels et al. 2008). Matthews *et al.* addressed the role of HLA-mediated selection of viral escape mutants that revert post-transmission by analysing viral Gag, Pol and Nef sequences from 710 infected individuals (Matthews, Prendergast et al. 2008). They found that the number of reverting polymorphisms following transmission was correlated with a reducing viral load, particular for HLA-B-associated polymorphisms in Gag, supporting the hypothesis that the CD8 T cell responses driving immune control are those that select escape mutations that inflict a fitness cost upon the virus and that therefore revert post-transmission (Matthews, Prendergast et al. 2008). These studies confirmed that host HLA alleles provide differential impacts on the virus and found that HLA-B alleles, followed by HLA-C, were the most commonly associated with amino acid changes.

The mechanism for the central role of HLA-B in the control of HIV-1 replication remains unclear. One possibility is that a more diverse selection of peptide-binding motifs is offered by HLA-B alleles. Amino acids binding the B pocket of HLA-A alleles are broadly hydrophobic residues (does not however bind proline). In contrast, the B pocket of HLA-B alleles can variously accommodate hydrophobic residues (including proline), positively and negatively charged residues, and histidine and glutamine (Marsh, Parham et al. 2000; Kiepiela, Leslie et al. 2004).

Another possibility may be that the protein specificity of the HIV-1-specific CD8 T cell response is related to successful immune control. A prospective study of 38 asymptomatic HIV-1-infected individuals showed that a Gag-specific, but not an Env-

specific, CD8 T cell response was protective against disease progression (Riviere, McChesney et al. 1995). Several other cross-sectional studies have demonstrated that preferential targeting of the structurally conserved Gag protein is associated with lower viral loads (Edwards, Bansal et al. 2002; Patke, Langan et al. 2002; Novitsky, Gilbert et al. 2003; Masemola, Mashishi et al. 2004; Zuniga, Lucchetti et al. 2006; Borghans, Molgaard et al. 2007; Kiepiela, Ngumbela et al. 2007; Streeck, Lichterfeld et al. 2007; Rolland, Heckerman et al. 2008; Ndongala, Peretz et al. 2009). Kiepela *et al.* demonstrated in a cohort of 578 clade C infected subjects that an increasing number of Gag-specific T cell responses correlated with decreasing viral loads, in contrast to Env-specific T cell responses, which were associated with higher viraemia (Kiepiela, Ngumbela et al. 2007). The protective effect of these broad Gag-specific CD8 T cell responses remained irrespective of HLA type (Borghans, Molgaard et al. 2007; Kiepiela, Ngumbela et al. 2007). Preferential targeting of the highly variable envelope protein was also observed in HLA-B*5802-positive individuals and correlated with higher viraemia (Ngumbela, Day et al. 2008). Protective HLA-B alleles restrict CD8 T cell responses that impose a strong selection pressure on a few specific Gag p24 epitopes, resulting in escape mutations for which fitness costs have been demonstrated (Frater, Brown et al. 2007; Streeck, Lichterfeld et al. 2007). Thus it would appear that Gag-specific but not non-Gag-specific CD8 T cell responses are associated with control in HIV-1 infection. However, it is clear that the targeting of conserved regions alone is insufficient to account for differences in immune control, as targeting of the conserved Pol protein and conserved regions within the central part of Nef protein were not associated with effective immune control as measured by viral load (Kiepiela, Ngumbela et al. 2007).

Another reason why Gag may be an important target for CD8 T cells is that Gag-specific CD8 T cells may be able to recognise and kill virus-infected cells sooner after infection than their non-Gag-specific counterparts. Recent studies show that Gag and Pol-specific CD8 T cells recognise SIV-infected targets within two hours of infection, whereas Tat-, Env- and Nef-specific CD8 T cells only recognise virus-infected target cells following *de novo* synthesis of viral proteins (Sacha, Chung et al. 2007; Sacha, Chung et al. 2007). The implication is that Gag-specific CD8 T cells therefore have a window of approximately 10 hours between expression of Gag on the cell surface and the impact of newly Nef-mediated downregulation of MHC class I molecules (Cohen, Gandhi et al. 1999; Sacha, Chung et al. 2007).

In contrast to the discussions above are the following observations. Some MHC class I alleles associated with slower disease progression, such as HLA-B*51 in humans and Mamu-B*08 and Mamu-B*17 in macaques, do not immunodominantly target Gag, suggesting that targeting of some other regions of the virus may also be capable of eliciting viral control (Altfeld, Kalife et al. 2006; Loffredo, Friedrich et al. 2007; Loffredo, Bean et al. 2008). Additionally, high frequency HIV-1-specific CD8 T cell responses are usually detectable in infected individuals who have developed AIDS (Hay, Ruhl et al. 1999; Draenert, Verrill et al. 2004). Kiepela *et al.* have demonstrated that the majority of HLA class I alleles seem to have little impact on the disease outcome of HIV-1 infection and that most selected viral escape mutants do not significantly affect viraemia (Kiepiela, Leslie et al. 2004; Kiepiela, Ngumbela et al. 2007).

A third explanation for the central role of HLA-B in the control of HIV-1 replication relates to the fact that certain combinations of HLA-B alleles and KIR allotypes on NK cells are associated with slower progression to HIV-1 disease (Altfeld and Goulder 2007; Borrow and Bhardwaj 2008; Carrington, Martin et al. 2008). This has been discussed earlier in Section 1.5.1.3 of this thesis. A final possible mechanism for the protective effect of the HLA-B locus is that HLA-B-restricted CD8 T cell responses are more polyfunctional than HLA-A or HLA-C-restricted T cell responses, even when the same peptide is presented (Harari, Cellerai et al. 2007), although this mechanism fails to explain the association of alleles like HLA-B*35 and HLA-B*5802 with high viral load set points. Polyfunctionality refers to the ability of a virus-specific CD8 T cell to effect more than one function in response to antigen. Classically, five CD8 T cell effector functions are assessed after stimulation with cognate peptides by multiparametric flow cytometry and include degranulation (CD107a mobilisation) and cytokine (IFN-γ, TNF-α and IL-2) and chemokine (MIP-1β) production. Betts *et al.* compared the ability of HIV-1 specific T cells to mount these five distinct functions simultaneously in response to antigen, between HIV-1 LTNPs and chronic progressors (Betts, Nason et al. 2006). They found that polyfunctionality in the virus-specific T cells was significantly more frequent in LTNPs than those with progressive infection. A further suggestion that polyfunctionality in CD8 T cells correlates with their antiviral capacity was made by Almeida *et al*. They found that CD8 T cells specific to the immunodominant HLA-B27 KK10 epitope in Gag (KRWIILGLNK, residues 263-272) were more frequently polyfunctional than were CD8 T cells specific to subdominant epitopes (Almeida, Price et al. 2007). However, an important caveat centres on the issue of cause-and-effect. It is unclear whether HIV-1-specific CD8 T cell polyfunctionality actually

drives a low viral load or if a polyfunctional profile is merely a byproduct of a low antigenic presence.

1.6.2 The HLA-C locus and HIV-1 control

A genome-wide association study of European Caucasians identified polymorphisms that explain nearly 15% of the variation of HIV-1 viral set-point (Fellay, Shianna et al. 2007). The most significant of these was located in the *HLA* complex *P5* (*HCP5*) gene which was in high linkage disequilibrium with *HLA-B*5701* (r^2=1). The second most significant polymorphism (rs9264942) accounted for 6.5% of total variation and was located 35kb upstream of the *HLA-C* gene (-35 SNP) (Figure 1.6), with the minor allele (*C*) associating with lower viral load set point than the major allele (*T*). Despite minor linkage disequilibrium (r^2=0.05) between *HLA-B*5701* and the -35 SNP, there was an independent effect of each of these variants on viral load set point. In addition, the -35*C* variant was also shown to associate with higher HLA-C mRNA expression in EBV-transformed B cell lines (Stranger, Forrest et al. 2005; Fellay, Shianna et al. 2007), suggesting an inverse correlation between HLA-C expression and viral load. The -35 SNP association with viral load set point was subsequently replicated in a larger cohort by the same group (Fellay, Ge et al. 2009) and with other independent Caucasian cohorts (Catano, Kulkarni et al. 2008; Dalmasso, Carpentier et al. 2008; Limou, Le Clerc et al. 2009; Trachtenberg, Bhattacharya et al. 2009; van Manen, Kootstra et al. 2009; Pereyra, Jia et al. 2010). The -35 SNP is also present in African-American populations but does not correlate with viral load set point (Shrestha, Aissani et al. 2009; Pelak, Goldstein et al. 2010).

The potential role of HLA-C in low viral load set point had previously been raised following a number of key findings. The HIV-1 Nef protein, which contributes significantly to HIV-1 pathogenesis, selectively down-regulates surface HLA-A and HLA-B but not HLA-C (and HLA-E) on infected cells (Collins, Chen et al. 1998; Cohen, Gandhi et al. 1999; Collins and Baltimore 1999; Williams, Roeth et al. 2002; Lewis, Balamurugan et al. 2008). In addition HLA-C molecules play a role in innate immunity by serving as ligands for certain inhibitory KIRs. All HLA-C alleles can be broadly divided into two groups based on a polymorphism at position 80 of the heavy chain. Those alleles with asparagine at position 80 (HLA-C1 group; Cw*01/03/07/08) bind the inhibitory KIR2DL2 and KIR2DL3 while those with lysine at position 80 (HLA-C2 group; Cw*02/04/05/06) bind the inhibitory KIR2D1 (Lanier 2005; Parham 2005), implicating this locus as the most important in terms of KIR regulation of NK cell activity. In fact certain HLA-C1 and KIR2DL3 combinations have been shown to clear virus in acute hepatitis C virus infection (Khakoo, Thio et al. 2004), although no such effect has been demonstrated in HIV-1 infection (Flores-Villanueva, Yunis et al. 2001). The infected cells, though relatively resistant to HLA-A and HLA-B-restricted CD8 T cell lysis, continue to express HLA-C and therefore retain HLA-C-restricted CD8 T cell activity and are able to inhibit NK cells.

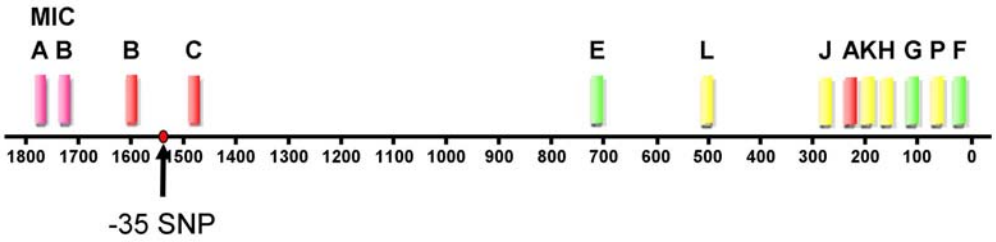


Figure 1.6. Map of the HLA class I region showing the position of the -35 SNP. This map only shows the class I genes and related genes on chromosome 6. HLA-H, -J, -K, -L and -P are complete class I genes that are not expressed (pseudogenes). The principal class I genes encoding the heavy chains of the six class I isoforms HLA-A, -B, -C, -E, -F and -G are shown. The MHC class I-related chain (MIC) gene family consists of five genes of which MICA and MICB are expressed.

1.6.2.1 Distinctive features of the HLA-C molecule

The analysis of responses restricted by HLA-C is challenging as HLA-C alleles are often in strong linkage disequilibrium with HLA-B alleles, making it difficult to distinguish HLA-C from HLA-B-restricted responses. This raises the possibility that HLA-C epitopes might mistakenly have been identified as restricted by HLA-A or HLA-B. HLA-C shares sequence homology with HLA-A and HLA-B molecules but it also differs from these HLA-A antigens on several levels. HLA-C alleles are closely related to each other and their α1 domain is unusually conserved and therefore characteristic of HLA-C. The KYRV (lysine, tyrosine, arginine, valine) motif of residues 66, 67, 69 and 76 in the α1 helix is conserved in all HLA-C molecules and absent in all HLA-A and HLA-B molecules except HLA-B*46 (Zemmour and Parham 1992). In addition, a conserved glycine at amino acid 45 (α1 helix), the presence of four HLA-C-unique residues in the α2 domain and the reduced diversity at the B pocket of the antigen recognition site are particularly striking of HLA-C and correlate with binding of a restricted set of self-peptides in comparison with HLA-A and HLA-B antigens, emphasising the distinctive character of this antigen (Neisig, Melief et al. 1998; Turner, Ellexson et al. 1998).

The main feature distinguishing HLA-C is its low cell surface expression, which is approximately 10% of the levels of HLA-A and HLA-B molecules (Snary, Barnstable et al. 1977). Explanations that have been offered to account for this low surface expression include low levels of mRNA (McCutcheon, Gumperz et al. 1995), poor assembly with β2M and restricted peptide binding leading to the accumulation of misfolded intermediates in the ER (Neisig, Melief et al. 1998). McCutcheon *et al.* showed that reduced protein expression of HLA-C in B cells correlated with low

mRNA levels resulting from faster degradation of the HLA-C mRNA than HLA-A or HLA-B mRNA. In contrast other independent groups have detected similar levels of HLA-A/B/C mRNA in EBV-transformed B cell lines (Neefjes and Ploegh 1988; Tibensky, Decary et al. 1988). An explanation for these seemingly conflicting results comes from a study that showed that the gene expression profile of HLA-A, HLA-B and HLA-C varies according to the tissues examined, with HLA-C mRNA levels lowest in larynx mucosa compared to colon mucosa and peripheral blood lymphocytes, while HLA-C mRNA levels was comparable with that of HLA-A in peripheral blood lymphocytes (Garcia-Ruano, Mendez et al. 2010).

The association of class I heavy chain with β2M is a prerequisite for surface expression. HLA-C associates inefficiently with β2M, leading to an accumulation of free heavy chains in the ER (Neisig, Melief et al. 1998). Consequently, HLA-C heavy chains remain associated with calnexin, leading to their degradation in the ER (Stam, Spits et al. 1986; Neefjes and Ploegh 1988; Sibilio, Martayan et al. 2008). However, some HLA-C heavy chains do bind β2M and become properly assembled. An explanation for their poor expression at the cell surface is their prolonged association with TAP in the ER, resulting in slower exocytosis (Neisig, Melief et al. 1998). The retention of TAP depends on the KYRV motif in the α1 helix which restricts the range of acceptable self-ligands for HLA-C heavy chains by reducing the plasticity of the antigen recognition groove, hence prolonging the interaction with TAP while waiting for the appropriate peptide (Sibilio, Martayan et al. 2008).

1.6.2.2 Evidence for HLA-C restricted T cell responses in HIV-1 control

Similar to HLA-B, studies have shown evidence for HLA-C-restricted CD8 T cell responses driving the selection of escape mutations (Matthews, Prendergast et al. 2008; Rousseau, Daniels et al. 2008). In fact Matthews *et al.* highlighted a potential role for HLA-C restricted targeting of HIV-1 Pol in early infection and showed a strong correlation between the number of reverting mutations selected in HIV-1 Pol by HLA-C alleles and decreasing viral load (Matthews, Prendergast et al. 2008). Subsequent to this finding, a few studies have demonstrated functional evidence for HLA-C-selected viral mutations with a fitness cost.

Thananchai *et al.* studied the interactions of CD8 T cells and the KIR2DL1 receptor on NK cells with the immunodominant HLA-Cw*0401 restricted epitope SF9 (SFNCGGEFF [Env residues 375-383]) and its escape variant SL9 (a mutation at position 383 replacing phenylalanine with leucine) (Thananchai, Makadzange et al. 2009). They showed that HLA-Cw*0401 restricted CD8 T cell clones recognised the wild type SF9 epitope with high affinity, as shown by their ability to kill target cells pulsed with low peptide concentrations, whereas lysis of the target cells pulsed with the variant SL9 epitope was only observed at very high peptide concentrations. Using BIAcore and NK cell lines they demonstrated that the T cell escape variant SL9 forms a better ligand for KIR2DL1 when bound to HLA-Cw*0401 suggesting resistance to NK cell lysis. They therefore postulated that this escape mutant is selected because it enables infected cells to escape simultaneously from both the adaptive and innate arms of the immune system (Thananchai, Makadzange et al. 2009).

Honeyborne *et al.* studied the dominant HLA-Cw*03-restricted CD8 T cell response towards the YL9 epitope (YVDRFFKTL [Gag residues 296-304]) in individuals with chronic HIV-1 infection (Honeyborne, Codoner et al. 2010). They defined three mutations within the sequence driven by HLA-Cw*03 selection. The sequence changes detected involved threonine at position 303 mutating to alanine, isoleucine and valine (T303A, T303I and T303V). *In vitro* studies revealed that introduction of the changes T303A and T303I significantly reduced both CD8 T cell recognition and viral replicative capacity. However, subsequent selection of the valine-303 variant, which is the most common variant in the population, restored both viral fitness and CD8 T cell recognition. These data suggest that for most Cw*03 individuals, there is a balance between the selection of the T303I/A variants which are poorly recognised by CD8 T cells but significantly reduce viral fitness (as evidenced by reduction in viral replicative capacity) and the selection of T303V where viral escape is modest but fitness cost is minimal too.

Honda *et al.* identified two novel HLA-Cw*1202-restricted Pol specific CD8 T cell epitopes, KY9 (KQNPDIVIY [Pol residues 328-336]) and IY10 (ILKEPVHGVY [Pol residues 464-473]) (Honda, Zheng et al. 2011). CD8 T cell responses against these epitopes were detected in 25-40% of chronically HIV-1 infected individuals who expressed HLA-Cw*1202 and suppressed HIV-1 replication *in vitro*. However only IY10 selected an escape mutant, valine to alanine at position 472, with ensuing fitness cost (Honda, Zheng et al. 2011).

Cao *et al.* assessed T cell responses within an infected individual who remained antiretroviral therapy-free for four years and reported an unusual escape mutation

(Cao, McNevin et al. 2008). In addition to several other responses, they demonstrated the presence on an immunodominant HLA-Cw*0102-restricted CD8 T cell response against the NL8 epitope (NSPTRREL [Pol residues 24-31]) which arose in chronic infection. As the CD8 T cell response to this epitope peaked, viral escape ensued via a three amino acid SPT (serine, proline, threonine) repeat within the epitope. Interestingly, viral genotypes with this insertion are known to confer resistance to the nucleoside reverse transcriptase inhibitor group of antiretroviral drugs. They go on to speculate that the presence of this insertion in diverse HIV-1 subtypes may play some functional role in influencing viral replication capacity (Cao, McNevin et al. 2008).

The findings discussed above suggest the possibility that some HLA-C restricted CD8 T cells control HIV-1 *in vivo*. However, despite all of these data, the contribution of these responses to HIV-1 control is still unknown. Therefore, further analyses of HLA-C restricted CD8 T cell responses are needed to clarify the role of this HLA locus in HIV-1 infection.

1.7 Thesis Aims

The observation that the effect of the -35 SNP may be due to differences in *HLA-C* gene expression (Stranger, Forrest et al. 2005; Fellay, Shianna et al. 2007), together with the observation that recognition of infected cells by HLA-C restricted CD8 T cells remains unaffected by HIV-1 *Nef* expression (Adnan, Balamurugan et al. 2006), suggest a possible mechanism for the effect of this polymorphism on viral load set point. The higher surface HLA-C expression could benefit the adaptive and innate arms of the immune system by enhancing antigen presentation to CD8 T cells and by

resulting in stronger NK cell responses (through stronger engagement of inhibitory KIRs during their maturation process), respectively.

This thesis focuses solely on the potential role of HLA-C in the adaptive immune system. The hypothesis being examined is that the increased levels of surface HLA-C in individuals that harbour the -35*C* variant result in an increased breadth and/or magnitude of HLA-C restricted CD8 T cell responses following HIV-1 infection, leading to better control of viral replication and negating the immune-evasionary effects of *Nef*-mediated downregulation of HLA-A and HLA-B molecules.

With this hypothesis in mind, the aims of this thesis are to:-

- Surface stain lymphocytes from genotyped Caucasian volunteers for levels of HLA-C to investigate whether individuals homozygous for the -35 SNP protective allele (*C*) have higher surface levels of HLA-C than those homozygous for the non-protective allele (*T*). This will confirm the existing expression data and is essential given that post-translational regulation of all HLA class I molecules may complicate the relationship between levels of *HLA-C* messenger RNA and protein (McCutcheon, Gumperz et al. 1995; Johnson 2000).
- Determine whether differences in surface expression translate to measurable differences in CD8 T cell function in HIV-1 infected individuals. The breadth and magnitude of HLA-C restricted CD8 T cell responses will be examined via the IFN-γ ELISpot assay, predicting that -35 *CC* individuals, with their higher levels of HLA-C, will have a greater breadth and magnitude of responses.

- Demonstrate that the -35 SNP effect on viral load in Caucasians is indeed mediated through T cells. The viral suppression assay, which measures the ability of CD8 T cells to suppress viral replication in autologous CD4 T cells, was selected to evaluate the antiviral capacity of CD8 T cells. The ability of CD8 T cells from -35 *CC* and *TT* individuals to suppress cells infected with HIV-1 (as opposed to cells presenting exogenously added peptide) will be compared.
- Study the -35 SNP in a different ethnic group, focussing on Africans.

Chapter 2: MATERIALS AND METHODS

2.1 Subjects and samples

2.1.1 HIV-1 seronegative cohort

One hundred and forty-seven healthy Caucasian laboratory workers from the Weatherall Institute of Molecular Medicine (WIMM, University of Oxford) and the Jenner Institute (University of Oxford and the Institute for Animal Health) were screened. Three millilitres of blood was drawn in ethylenediaminetetraacetic acid (EDTA)-coated tubes, deoxyribonucleic acid (DNA) extracted using the Puregene DNA isolation kit (Gentra) and the -35 SNP genotype determined by polymerase-chain reaction (PCR). Subjects homozygous for *CC* and *TT* had peripheral blood mononuclear cells (PBMC) isolated by standard density gradient centrifugation from heparinised blood samples. PBMC were viably cryopreserved in fetal calf serum supplemented with 10% dimethyl sulfoxide (DMSO) and stored in vapour phase liquid nitrogen. Written informed consent was obtained from all volunteers under appropriate university regulations.

2.1.2 HIV-1 seropositive cohorts

There was collaboration with Steve Deeks and Jeff Martin at University of California, San Francisco who provided cryopreserved PBMC from 39 subjects enrolled in their Study of the Consequences of the Protease Inhibitor Era (SCOPE) cohort of HIV-1 infected subjects. They had previously genotyped these subjects at the -35 SNP and selected only *CC* and *TT* subjects that were Caucasian. These subjects were male and

female, had a CD4 count above 350, were HAART-naïve or off therapy for at least six months.

The McMichael group is part of the Center for HIV/AIDS Vaccine Immunology (CHAVI) network. Therefore, there was access to PBMC from seropositive Caucasian and African subjects from the CHAVI 001 cohort. The PBMC from 15 Caucasian subjects who were HAART-naïve were studied. In addition blood pellets from 289 African subjects were made available for DNA extraction and -35 SNP genotyping. PBMC from 28 of these African subjects, who were either *CC* or *TT*, were used in subsequent studies.

Viral load set point was calculated by Statistical Center for HIV/AIDS Research and Prevention (SCHARP) using an algorithm defined by Fellay *et al.* (Fellay, Shianna et al. 2007), that used an average of viral loads within a set point window. The criteria for defining the set point window for a given subject included removing outlying viral loads based on three major categories: values from the initial up- or downswing of viraemia, values from the accelerating phase of the disease, and apparent outlying values during the set point interval. In addition, subjects that had less than two viral load measurements, subjects that had started HAART within six months of enrolment and subjects that had been enrolled for less than six months were excluded from the viral load set point estimation.

2.1.3 Transfectants

Peter Parham (Stanford University, California) donated the HLA class I deficient cell line 721.221 and derivative 721.221 cells stably transfected with copy DNA

expressing different HLA-C alleles (HLA-Cw*0102, *0202, *0304, *0401, *0601, *0701, *0801, *0802). Veronique Braud (Centre National de la Recherche Scientifique/Université de Nice-Sophia Antipolis, Valbonne, France) donated 721.221-AEH cell line which expresses HLA-E*0101 (Lee, Goodlett et al. 1998).

2.2 Molecular biology techniques

2.2.1 DNA extraction

Chromosomal DNA was extracted from whole blood using the Puregene DNA extraction kit (Gentra), as per manufacturer instructions. This was used for both HLA class I typing and -35 SNP genotyping.

2.2.2 -35 SNP genotyping

Genomic DNA was genotyped for the -35 SNP by PCR using a common forward primer (5'-GGGTGGTGCCAAGTATGAG-3') and alternative allele-specific reverse primers (5'-AGAAAGTCCCACAGTGCCTA-3' and 5'-AGAAAGTCCCACAGTGCCTG-3'). Genotyping reactions also contained control primers that amplify a constant region in the *DRB1* locus (Olerup and Zetterquist 1992). Thermocycling conditions were: 96°C for 1min; 5 cycles of 96°C for 25 seconds, 70°C for 45 seconds, 72°C for 45 seconds; 21 cycles of 96°C for 25 seconds, 65°C for 50 seconds, 72°C for 45 seconds; and 4 cycles of 96°C for 25 seconds, 55°C for 1 minute, 72°C for 2 minutes. Products were resolved on 2% agarose gels and visualised under ultraviolet with ethidium bromide. This PCR assay was validated by direct sequencing of a PCR product spanning the -35 SNP for the first 100 samples

genotyped. The primers and PCR protocol were designed and optimised by Simon Brackenridge and Tim Rostron (WIMM, University of Oxford).

2.2.3 HLA typing

This was performed by Tim Rostron (WIMM, University of Oxford) using the sequence-specific primer method adapted from Bunce (Bunce 2003), which uses allele-specific primer combination in PCR amplification to provide absolute HLA resolution to two digits and high-probability resolution to four digits.

2.3 Preparation and maintenance of primary cells and cell lines

2.3.1 Tissue culture

Unless otherwise stated, all cells were rested in an incubator at 37^{o}C with 5% CO_2. The following media were used for growing cells.

R10 RPMI 1640 (Sigma-Aldrich), 10% Fetal Calf Serum (FCS; Sigma-Aldrich), 2mM L-glutamine (1%; Sigma-Aldrich), 100mM sodium pyruvate (1%; Sigma-Aldrich) and 10mM Hepes buffer (1%; Sigma-Aldrich) with antibiotics (1%; 50U/ml penicillin-streptomycin)

R15 RPMI 1640 (Sigma-Aldrich), 10% FCS (Sigma-Aldrich), 2mM L-glutamine (1%; Sigma-Aldrich), 100mM sodium pyruvate (1%; Sigma-Aldrich) and 10mM Hepes buffer (1%; Sigma-Aldrich) with antibiotics (1%; 50U/ml penicillin-streptomycin)

RAB-10 RPMI 1640 (Sigma-Aldrich), 10% heat inactivated Human AB Serum (Sigma-Aldrich), 2mM L-glutamine (1%; Sigma-Aldrich), 100mM sodium pyruvate (1%; Sigma-Aldrich) and 10mM Hepes buffer (1%; Sigma-Aldrich) with antibiotics (1%; 50U/ml penicillin-streptomycin)

2.3.2 Generation of Short Term Cell Lines (STCL)

Two to three million PBMCs were stimulated with the peptide(s) of interest, at a final concentration of 2µg/ml, in RAB-10 medium supplemented with recombinant human interleukin 7 (rIL7) at a final concentration of 25ng/ml. After 3 days, rIL2 at a final concentration of $1.8x10^3$ U/ml was added. On day 7, fresh RAB-10 medium was added and rIL2 at a final concentration of $1.8x10^3$ U/ml. On day 10, the cells were washed four times with PBS and then rested for at least 30 hours in RAB-10 medium before use in cultured ELISpot assays.

2.3.3 Generation and maintenance of EBV-immortalised B lymphoblastoid cell lines (BCL)

Selected PBMC were plated out in 96-well plates containing 100µl of EBV supernatant at 0.5 million cells per well and incubated for 3 to 4 hours. Then 100µl of R15 medium containing 2µg/ml cyclosporin A (CSA) was added. After 48 hours, 100µl supernatant was replaced with fresh R15 medium containing 2µg/ml CSA. After 10-14 days, the rapidly dividing BCL were expanded by adding fresh R10 medium every 2-3 days.

2.3.4 Generation and maintenance of T cell lines (TCL)

This relied on using autologous BCL as antigen presenting cells. Two to three million PBMC from the selected subject were stimulated with 20µl of 100µM peptide for 1-2 hours in an incubator. Then 1ml of RAB-10 was added and the cells rested for 3 days in a 24-well plate. A further 1ml of RAB-10 containing 200U/ml rIL2 was then added and the cells rested. After 14 days the TCL was restimulated with autologous

BCL. The BCL was pulsed with 100μM of the relevant peptide for 1-2 hours at 37°C, washed twice, and irradiated. These irradiated cells were then mixed with PBMC at a ratio of 1:1 in RAB-10 and incubated for 3 days. Then RAB-10 containing 200U/ml rIL2 was added. The TCL were restimulated using peptide-pulsed, irradiated BCL every 14 days.

2.3.5 Generation of CD8-positive T cell clones

T cell clones were generated from TCL using a MACS IFN-γ cell enrichment and detection kit (Miltenyi Biotec) in combination with cell sorting. TCL were restimulated with peptide for 3-6 hours, labelled with IFN-γ cytokine catch reagent and detection reagent (as per manufacturer's protocol) and anti-CD8 antibody before proceeding to cell sorting. Lymphocytes that were both IFN-γ producing and CD8 positive were collected. These sorted T cells were then plated out in 96-well plates at 1-3 cells per well and cultured with 100, 000 irradiated allogeneic feeder PBMC (in RAB-10 medium and stimulated with phytohaemagglutinin (PHA) at final concentration of 50μg/ml) in a final volume per well of 100μl. On day 4, cells were fed with 100μl of RAB-10 medium supplemented with 200U/ml rIL2. Wells were screened for specific recognition of peptide-pulsed autologous BCL by ELISpot after 14 days in culture. Wells showing specific recognition of relevant peptide (no IFN-γ secretion in corresponding negative control well containing autologous BCL only) were transferred to 24-well plates for restimulation with 2 million irradiated allogeneic feeder PBMC (in RAB-10 medium and stimulated with PHA at final concentration of 50μg/ml) in a final volume per well of 1ml. On day 3, 1ml of RAB-10 medium supplemented with 200U/ml rIL2 was added. Clones were expanded with irradiated feeders being added every 14 days and rIL2 containing RAB-10 three days

later. All experiments with CD8 T cell clones were conducted with day 10-14 clones for maximal cytolytic capacity and cytokine release.

2.4 Flow cytometry

2.4.1 Monoclonal antibodies

A panel of directly conjugated and unconjugated monoclonal antibodies was purchased (Table 2.1). The monoclonal antibody DT9 was affinity purified on protein A-sepharose beads (Sigma-Aldrich) from a hybridoma supernatant by standard procedures and conjugated using a FluoroTag FITC conjugation kit (Sigma).

Table 2.1. Monoclonal antibodies used in flow cytometry.

ANTIBODY	CLONE	CONJUGATE	SOURCE
Anti-CD3	SK7	PerCP	Becton Dickinson
Anti-CD4	RPA-T4	PE	Becton Dickinson
Anti-CD8	SK1	APC	Becton Dickinson
Anti-CD8	SK1	FITC	Becton Dickinson
Anti-CD8	SK1	PE-Cy7	Becton Dickinson
Anti-CD19	SJ25C1	PE-Cy7	Becton Dickinson
Anti-IFN-γ	B27	FITC	Becton Dickinson
LIVE/DEAD		AQUA	Invitrogen
Goat anti-mouse IgG heavy chain	polyclonal	FITC	Sigma-Aldrich
Goat anti-mouse IgG		R-PE	Invitrogen
Rat anti-mouse kappa	187.1	FITC	Southern Biotech
Mouse IgG2b kappa isotype control	MPC-11	-	BioLegend
Mouse IgG2b isotype control	MOPC-141	FITC	Sigma-Aldrich
Anti-MHC class I	W6/32	FITC	Sigma-Aldrich
Anti-MHC class I	W6/32	-	BioLegend
Anti-HLA-E	MEM-E/06	-	Santa Cruz
Anti-HLA-E	MEM-E/08	-	Santa Cruz
Anti-HLA-E	3D12	-	BioLegend
Anti-p24	KC57	FITC	Beckman Coulter

APC (allophycocyanin); FITC (fluorescein isothiocyanate); R-PE (red-phycoerythrin); PerCP (peridinin chlorophyll protein); PE-Cy7 (phycoerythrin-cyanin 7); - unconjugated

2.4.2 Surface staining of PBMC

Cell surface staining was performed on cryopreserved PBMC that were thawed and rested for at least two hours. For all cell surface staining, 1 million cells were washed once with phosphate buffered saline (PBS; Sigma-Aldrich) and then stained with Live/Dead stain (Invitrogen) and incubated at room temperature for 20 minutes as per manufacturer's instructions. All subsequent wash steps used 0.5% bovine serum albumin in PBS (0.5% BSA/PBS). For direct antibody staining, cells were then incubated on ice for 15 minutes with appropriate antibodies. For indirect staining, cells were incubated with primary antibodies (15 minutes on ice), free secondary antibody binding sites were blocked with mouse serum (Southern Biotech), and then cells were incubated with secondary antibody and conjugated antibodies against surface markers. Cells were washed twice between each step with 0.5% BSA/PBS. After being stained, cells were washed again, fixed with Cell Fix Buffer (Becton Dickinson), and analysed using a CyAn flow cytometer and FlowJo software (Treestar, Inc.). Background (unstained cells) autofluorescence was subtracted from stained cells.

2.4.3 Characterisation of monoclonal antibodies

LABScreen Single Antigen Class I Combi beads (One Lambda), each coated with a single HLA allotype (Pei, Lee et al. 2003), were incubated with DT9, 3D12, MEM-E/06, MEM-E/08 and W6/32 antibodies for 30 minutes at room temperature, washed 3 times with PBS buffer, incubated at room temperature for 30 minutes with goat anti-mouse IgG-R-PE (Invitrogen), washed 3 times as before, and analysed on a LABScan 100 flow analyser.

The specificity of the monoclonal antibody panel to HLA-E was tested by binding to HLA-E on transfected 721.221 AEH cell (Lee, Goodlett et al. 1998).

2.4.4 Intracellular cytokine staining (ICS)

This technique was used to confirm that generated TCL were epitope-specific and responded by secreting IFN-γ. Cells that had not received antigenic stimulation for at least fourteen days were restimulated with antigenic peptides presented by autologous BCL for an hour before the addition of Brefeldin A (GolgiPlug from Becton Dickinson). A polyclonal stimulation with the superantigen *Staphylococcus aureus* enterotoxin B (SEB) was also used as positive control. The cells were then rested in the incubator for a minimum of 5 hours. The lymphocytes were then washed with PBS before adding Live/Dead stain for 20 minutes followed by surface stains (anti-CD4 and anti-CD8) for 15 minutes at room temperature. After washing, the lymphocytes were then fixed and permeabilised (Caltag Fix Perm kit) and anti-IFN-γ monoclonal antibody added for a further 15 minutes. There was a final wash step before cells were analysed using a CyAn flow cytometer and FlowJo software (Treestar, Inc.).

2.5 Isoelectric focusing (IEF)

This technique was used to separate different HLA molecules by their electric charge differences.

2.5.1 Metabolic labelling and Immunoprecipitation

Ten million cells were transferred to methionine-free RPMI medium (Sigma-Aldrich) with 10% FCS and rested in a humidified CO_2 incubator for an hour. The cells (still in 1ml media) were then labelled with 100µCi of ^{35}S-methionine (EasyTag Express from PerkinElmer) and returned to the incubator for either three hours (cell lines) or overnight (PBMC).

Labelling was then stopped by the addition of 20ml ice cold PBS. The cells in PBS were centrifuged at 4^{o}C, supernatant decanted and the pellet lysed in 500µl lysis buffer [0.5% NP40, 40mM Tris Acid, 40mM Tris Base, 5mM EDTA, 150mM NaCl, 0.05% azide] supplemented with protease inhibitor (Roche). After 20 minutes on ice, the cell lysates were centrifuged at 13000 rpm for 10 minutes at 4^{o}C to remove organelles and other debris. Lysates were precleared with 100µl washed 10% Pansorbin (Calbiochem) on a roller for one hour at 4^{o}C then centrifuged and the supernatant collected and divided into two equal aliquots for immunoprecipitation. Supernatant was incubated with DT9 (10µg) or W6/32 (10µg) for one hour at 4^{o}C under constant rotation, then 50µl Protein A-Sepharose beads (Sigma-Aldrich) added and the incubation continued for a further one hour. The Protein A-Sepharose beads were then washed three times in lysis buffer by spinning at 8000 rpm for 3 minutes at 4^{o}C. The beads were then incubated with 80µl neuraminidase (10 units/ml in 0.05M EDTA; Lorne laboratories Ltd.) at 37^{o}C for an hour with occasional agitation to resuspend the beads, and then washed once with lysis buffer. The immunoprecipitate was eluted from the beads by adding 70µl sample buffer (9.5M urea, 2% NP40, 2% ampholines pH 3.5-9.5, 5% 2-mercaptoethanol) and incubating at 37^{o}C for 15

minutes. The beads were sedimented by spinning briefly in a microcentrifuge and 60µl of the supernatant was removed for analysis by IEF.

2.5.2 One dimensional isoelectric focusing (1D-IEF)

The composition of the gel was 4.5% polyacrilamide gel (19:1 acrylamide:bisacrylamide; Biorad), 9M urea, 2% NP40, 4% ampholyte pH 5-8 (GE Healthcare) and 1% ampholyte pH 3-10 (Biorad), polymerised with 90µl 10% ammonium persulphate and 30 µl TEMED per 30ml gel solution. Gel plate dimensions were 18x16 cm with 0.75mm spacers and were run using a Hoefer SE600 tank. The upper buffer was 50mM sodium hydroxide and the lower buffer 20mM phosphoric acid. The gel was run for 14-16 hours at a constant voltage of 880V with current limited to 12mA and power to 8W (allowing maximum voltage to be achieved over several hours). The gels were then fixed in 10% acetic acid for 40 minutes, then incubated in Amplify (sodium salicylate; GE Healthcare)) for another 20 minutes. Finally they were vacuum-dried and exposed to film (or Phosphor-Imager plate) for 3-4 days (W6/32 immunoprecipitate) or 2-3 weeks (DT9 immunoprecipitate).

2.6 T cell epitope mapping

The viral peptides recognised by the primary HIV-1-specific T cell response in each subject were identified by *ex vivo* interferon-gamma enzyme-linked immunospot assay (IFN-γ ELISpot).

2.6.1 Peptides

There were 48 optimal HLA-C-restricted HIV-1 peptides (Appendix 1) described in the Los Alamos National Laboratory (LANL) database (Frahm, Linde et al. 2007). There were 398 18-mer peptides overlapping by 10 amino acids, corresponding to the entire HIV-1 2001 clade B consensus sequence. All peptides were synthesised in-house (Medical Research Council Human Immunology Unit, WIMM, University of Oxford). The peptides were determined to be greater than 95% pure by MALDI-TOF analysis.

The 48 optimal HLA-C restricted peptides were screened in a 2-dimensional peptide matrix comprising 12 pools of 8 peptides, allowing for the identification of the respective peptide by responses in the two corresponding pools. All 398 overlapping peptides spanning clade B HIV-1 proteome were included in four different peptide pools with Gag, Pol and Env as individual pools, and Nef, Rev, Tat, Vif, Vpr and Vpu peptides combined into a single accessory and regulatory protein (Nef/Acc) pool. The final concentration of each peptide within the HLA-C matrix pools was 2µg/ml. For the Gag, Pol, Env and Nef/Acc pools the final peptide concentrations were 2µg/ml, 1 µg/ml, 1.5µg/ml and 1.5µg/ml, respectively, ensuring a final DMSO concentration of 0.5%. The optimal peptide pool configuration in each matrix and the peptides to be re-tested in a second round of assays was designed using the 'Deconvolute-This' software (Roederer and Koup 2003). LANL Epitope Location Finder (ELF) software was used to identify HLA-matched known and predicted CD8 T cell epitopes (http://www.hiv.lanl.gov/content/sequence/ELF/epitope_analyzer.html). Epitopes were predicted when the amino acid sequence contained anchor residues that match HLA motifs but occurred in epitopes not found on the LANL database.

2.6.2 Enzyme-linked Immunospot Assay (ELISpot)

Thawed PBMC were plated in 96 well polyvinylidene plates (Millipore) that had each been pre-coated with 10ml (100µl/well) of 10µg/ml anti-IFN-γ monoclonal antibody, 1-DIK (Mabtech) in sterile PBS. The peptides were added in a volume of 50µl and PBMC were added at 75,000 – 150, 000 cells per well in a volume of 50µl R10 medium. Wells containing only PBMC served as negative controls while wells containing PHA (at a final concentration of 10µg/ml) and PBMC served as positive controls. The plates were incubated overnight and developed the following day. The captured IFN-γ produced in wells was detected with 1µg/ml (diluted in sterile 0.5% BSA/PBS) biotinylated mouse anti-human IFN-γ secondary antibody, 7-B6-1 (Mabtech), followed by a peroxidase-conjugated avidin-biotin complex (Vector labs). The resulting spots in each well were counted using an automated ELISpot plate reader (AID ELISpot reader systems; Autoimmun Diagnostika) and the number of specific T cells was calculated by subtracting the negative control values. Responses were regarded as positive if they had at least three times the mean number of spots in the control wells. For the mapping of HLA-C restricted responses, a plate was successful if it had a strong PHA response (demonstrating functional activity of the cryopreserved cells; > 300 spot forming units/million cells) and a positive response (> 50 spot forming units/million cells) in at least one consensus B pool. For a subject to be chosen for deconvolution of the HLA-C matrix, a low threshold of positivity (at least 30 spot forming units/million cells in at least two matrix pools) was applied to peptide pools to maximise sensitivity. In second round (deconvolution) ELISpots, each of the peptides was tested in triplicate, as well as peptide pools containing optimal HLA-A and HLA-B restricted epitopes (Frahm, Linde et al. 2007). A more

stringent definition of positive response was applied with at least 50 spot forming units/million cells or at least three times background counts (negative controls) required.

Cultured ELISpots were performed as described above but with a few modifications. PBMC were stimulated with pooled deconvoluted peptides (from first round matrix screening) for 10 days to create STCL. These cells were added to ELISpot plates at 40,000 per well.

T cell clones were also used in ELISpot assays. There were 2 modifications to the technique described above. Five hundred clones were added per well. Ten thousand autologous or HLA-matched BCL peptide-pulsed for one hour served as antigen presenting cells. Autologous or HLA-matched BCL without peptide were used as negative controls.

2.7 Chromium release killing assay

A standard cytolytic killing assay was used to assess the capacity of generated HLA-C restricted CD8 T cell clones to kill exogenous peptide-loaded antigen presenting cells. In brief, autologous BCL or 721.221 stably transfected with the appropriate HLA-C allele, were labelled with 200µl (7.4MBq) of chromium 51 (^{51}Cr) (Amersham Biosciences) for one hour at 37^{o}C. These target cells were then washed three times with RPMI and peptide-sensitised target cells were prepared by pulsing with 100µg/ml of relevant peptide for another hour at 37^{o}C. Targets were finally washed twice in RPMI and resuspended at 100, 000 cells/ml in RAB-10. Cytolytic activity was determined in standard chromium release assays in 96-well U-bottom plates

containing 5000 target cells incubated with serial dilutions of T cell clones resulting in varying effector to target ratios, in quadruplicate. The plates were incubated at 37^{o}C for four hours. Supernatants were then harvested for quantification of ^{51}Cr release with a microscintillation counter (Wallac MicroBeta). Controls included target cells without clones (spontaneous) and target cells with 5% Triton (Sigma-Aldrich) (maximal). Specific lysis was calculated as follows: [(experimental release – spontaneous release) / (maximal release – spontaneous release)] X 100%. Spontaneous release was less that 25% in all assays.

2.8 HIV-1 Viral Suppression Assay (VSA)

2.8.1 HIV-1 stocks

The clade B laboratory-adapted HIV-1_{BaL} (ARP118) and the clade C primary viral isolate HIV-$1_{ES\ X1936(C)}$(ARP1073) were obtained from the Centre for AIDS Reagents, NIBSC HPA UK, supported by the EC FP6/7 Europrise Network of Excellence, and NGIN consortia and the Bill and Melinda Gates GHRC-CAVD Project and were donated by Dr S Gartner (Courtesy of NIH AIDS Research and Reference Reagent Programme) and Dr Lucia Perez Alvarez (National Centre for Microbiology, Madrid, Spain), respectively.

High titre stocks of these two HIV-1 viruses were generated. Briefly, donor PBMC from a seronegative subject was resuspended in R10 medium with PHA (at a final concentration of 5μg/ml) at a cell density of 2 million cells/ml, and incubated for 3 days. The PHA-stimulated PBMC were washed three times with R10 and superinfected with HIV-1 by spinoculation (centrifuged at 2000rpm for 2 hours at

25°C) (O'Doherty, Swiggard et al. 2000). Approximately 80% of HIV-1 supernatant was discarded and cells were then resuspended in R10 with IL2 at 20IU/ml, at a density of 1 million cells/ml. Aliquots of infected cells were taken at 1, 3, 5 and 7 days post-infection and intracellular p24 antigen expression in CD4-positive cells determined. The highest proportion of infected cells was seen at day 5. Supernatant from this time point was frozen in 1ml aliquots at -80°C and used as viral stock in viral suppression assays.

2.8.2 Quantifying viral stocks and monitoring virus production

The accurate determination of HIV-1 infectious titre is critically important for studies on replication kinetics or competitive fitness of different HIV-1 isolates. Endpoint dilution of HIV-1 to determine tissue culture dose for 50% infectivity ($TCID_{50}$) is considered the standard method for measuring the level of replication-competent, infectious HIV-1 in culture supernatants (Reed and Muench 1938; Ball, Abraha et al. 2003). $TCID_{50}$ was determined by making 10-fold serial dilutions of supernatants of each stock of virus and performing triplicate infections of PHA and IL2-treated PBMC. After 6 days of culture, cells were stained for p24 antigen positivity and the endpoint calculated by the Reed-Muench accumulative method (Reed and Muench 1938). The $TCID_{50}$ titre was calculated by adding the proportional distance (PD) value between the two percentages above and below 50% to the exponent of the dilution which gave the next percentage of infected cultures above 50%. The PD was calculated as follows: [(% infected cultures above 50%) – 50 / (% infected cultures above 50%) – (% infected cultures below 50%)]. The $TCID_{50}$/ml for HIV-1_{BaL} and HIV-$1_{ES\ X1936}$ were $1.1x10^5$ and $1x10^{2.8}$ IU/ml, respectively.

2.8.3 Isolation of primary CD4 T cells

The CD4 T cell fraction from thawed PBMC was isolated by negative selection by magnetic bead separation using CD8 microbeads (Miltenyi Biotech). In brief, PBMC were incubated with anti-CD8 microbeads for 15 minutes at 4^{o}C and then washed with cold buffer (PBS containing 0.5% BSA and 2mM EDTA). Labelled cells were resuspended in wash buffer and the cell suspension run through a column in the magnetic field of a MACS separator (Miltenyi Biotech). The column was washed three times with small volumes of buffer and the collected effluent contained the greater than 95% pure unlabelled CD4 T cell population. The column was then removed from the separator and placed in a suitable collection tube. The magnetically labelled CD8 T cell fraction was collected by adding 1ml of buffer on to the column and firmly pushing the plunger into the column.

2.8.4 CD8 T cell antiviral suppression assay

The VSA used here was based on the quantification of p24 antigen (p24 Ag)-positive CD4 T cells following HIV-1 superinfection and culture with or without *ex vivo* unstimulated autologous CD8 T cells for up to 7 days. Cryopreserved PBMC from HIV-1 seropositive subjects were thawed and depleted of CD8 T cells by magnetic bead separation. The remaining CD4 T cells were stimulated with PHA (5μg/ml) in R10 for 3 days, washed three times with R10, and infected with either HIV-1_{BaL} at a multiplicity of infection (MOI; ratio of virus to target cells) of 0.01 or HIV-$1_{ES\ X1936}$ at an MOI of 0.001, by spinoculation. The superinfected CD4 T cells (50, 000 cells/well) were cultured in triplicate in R10 with IL2 at 20IU/ml in 96-well round bottomed plates, alone or together with autologous unstimulated *ex vivo* CD8 T cells

at a CD8:CD4 T cell ratio of 2:1. The CD8 T cells were obtained by positive microbead selection from a freshly thawed vial of PBMC on day 3. Cultures were harvested after 6 days of culture.

Cells were then stained with Aqua Live/Dead stain (Invitrogen), then fixed with 20μg/ml lysolecithin in 1% paraformaldehyde for 2 minutes at room temperature and permeabilised with cold 50% methanol (-10 to -20^{o}C) for 15 minutes at 4^{o}C, followed by 0.1% Nonidet P-40 for 5 minutes at 4^{o}C. Cells were then stained with p24 antibody (KC-57 FITC; Beckman Coulter) and cell surface antibodies (CD3-PerCP, CD4-PE and CD8-PE-Cy7; Becton Dickinson) and acquired on a CyAn flow cytometer. Data were analysed using FlowJo software (Treestar Inc.). CD8 T cell antiviral suppressive activity was expressed as percentage inhibition and determined as follows: [(fraction of p24+ cells in CD4 T cells cultured alone) – (fraction of p24+ in CD4 T cells cocultured with CD8 T cells)] / (fraction of p24+ cells in CD4 T cells cultured alone) X 100%.

2.9 Statistical methods

2.9.1 Measure of peptide variability

Peptide alignments containing one protein sequence per individual were downloaded from the LANL HIV database and aligned relative to HXB2 standards (http://www.hiv.lanl.gov/content/sequence/QUICK_ALIGN/QuickAlign.html).

Entropy is a measure of the amino acid variability at a given position that takes into account both the number of possible amino acids allowed and their frequency. A Shannon entropy score was calculated for each amino acid position in the peptide

alignment, using a programme written by Simon Brackenridge – Simon's Protein Entropy Widget. A peptide's entropy score was calculated as a mean of entropy scores of amino acid positions included in the peptide. Thus peptides with a high entropy score were variable while those with a low entropy score were more conserved.

2.9.2 HLA linkage disequilibrium

HLA haplotypes were inferred by linkage disequilibrium, using the LANL tool (http://www.hiv.lanl.gov/content/immunology/hla/hla_linkage.html), with significance measured by Fisher's exact test. The strength of association was measured by calculating D' [D' = D/D_{max}, where D = P(AB) – P(A)P(B) and D_{max} = min[P(A)P(b), P(a)P(B)], if D>0].

2.9.3 Statistical analysis

Statistical tests were performed with GraphPad Prism software, version 5.0. All p values <0.05 were considered significant, with two-tailed analysis used to compare the level of significance throughout.

Chapter 3: DOES THE -35 SNP CORRELATE WITH INCREASED SURFACE EXPRESSION OF HLA-C?

Published in the Journal of Virology 2011; 85(7):3367-74. Corrah TW et al. Reappraisal of the relationship between the HIV-1 protective single-nucleotide polymorphism 35 kilobases upstream of the HLA-C gene and surface HLA-C expression.

3.1 Introduction

A polymorphism located 35kb upstream of the *HLA-C* locus (-35 SNP) was identified as a major genetic determinant of HIV-1 viral load set point in Caucasians (Fellay, Shianna et al. 2007; Fellay, Ge et al. 2009). Transcriptional data suggested that the effect of this polymorphism may be due to differences in *HLA-C* gene expression with the -35*C* protective variant shown to associate with high *HLA-C* mRNA levels (Stranger, Forrest et al. 2005; Fellay, Shianna et al. 2007). However, extensive variation in the *HLA-C* mRNA sequence resulting in potential mismatches between labelled mRNA from cells and the probe on the detection array could reduce hybridisation (personal communication from David Goldstein, Duke University) and post-translational regulation of all HLA class I molecules (McCutcheon, Gumperz et al. 1995; Johnson 2000) may complicate this relationship. To better determine whether *HLA-C* was the causative gene of the -35 SNP, studies were designed to investigate whether the actual surface expression of HLA-C was consistent with the transcriptional data.

The culmination of similar studies were recently published by Thomas *et al.* concluding that the -35*C* allele is a proxy for high HLA-C surface expression (Thomas, Apps et al. 2009). Data presented in this chapter are not inconsistent with

their observations but the interpretation of the relationship between the two is different and is discussed further later in this chapter. They also extended the observations made by Fellay *et al.* (Fellay, Shianna et al. 2007; Fellay, Ge et al. 2009) by examining the effect of -35 SNP genotypes on HIV-1 viral load measurements and progression to AIDS in two independent cohorts. In a comparison of the two extreme viral load groups (<2000 versus >10000) in a cohort of 935 individuals, -35*CC* was very protective relative to -35*TT* with 62.3% of individuals with the protective genotype restricting the virus to mean viral loads of <2000, but only 15.1% of individuals with the -35*TT* genotype controlled the virus to this extent (odds ratio=0.23, $p=1x10^{-8}$) (Thomas, Apps et al. 2009). In a second cohort of 763 individuals, they determined whether this SNP had any effect on progression to AIDS by measuring progression to three outcomes of CD4 count <200, AIDS-defining illness and death. The -35*CC* genotype had a marginally significant protective effect on progression to a CD4 count <200 (p=0.01), but a stronger protective effect on progression to AIDS (p=0.001) and death (p=0.001), relative to -35*TT* (Thomas, Apps et al. 2009). Although the -35*C* variant had a protective effect on both, the effect on AIDS progression was not nearly as significant as its effect on viral load in the early stages of infection (Thomas, Apps et al. 2009).

The main aim of this chapter is to determine whether the two -35 SNP alleles correlate with surface expression of HLA-C. The study of individual surface HLA molecules is complicated by the scarcity of allele-specific antibodies. The monoclonal antibody DT9, originally raised against MHC class I proteins from cotton-top tamarins, is the only available HLA-C-specific antibody that does not extensively cross-react with HLA-A or HLA-B alleles (Braud, Allan et al. 1998).

However, immunoprecipitation and one-dimensional isoelectric focusing (1D-IEF) studies confirmed that DT9 also recognises the non-classical HLA-E protein (Braud, Allan et al. 1998). Therefore, in these studies, surface staining experiments were performed using this antibody and the contribution of HLA-E to DT9 binding was quantified. Surface HLA-C levels were measured by DT9 staining of EBV-immortalised B cell lines, staining of PBMC from HIV-1 seronegative Caucasian volunteers in the first instance and later confirmed in a group of HIV-1 infected individuals. To resolve HLA-C from HLA-E, metabolic labelling and subsequent immunoprecipitation with DT9 was combined with one-dimensional isoelectric focussing (1D-IEF). The traditional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique which allows separation of proteins according to their molecular weight could not resolve HLA-C from HLA-E. However, these two proteins of similar molecular weight, possess unique isoelectric points allowing their separation by 1D-IEF.

3.2 Results

3.2.1 The -35 SNP alleles are in linkage disequilibrium with *HLA-C* alleles

One hundred and forty-seven healthy Caucasian volunteers were HLA typed and genotyped for the -35 SNP with 58 (39.5%) homozygous for the non-protective allele (*TT*) and 14 (9.5%) homozygous for the protective allele (*CC*) (Figure 3.1), in concordance with the frequencies seen by the HapMap project when studying Utah Residents with Northern and Western European Ancestry (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=9264942) (Consortium

2003). It was immediately clear even in this small cohort that certain *HLA-C* alleles were in linkage disequilibrium with either allele of the -35 SNP (Table 3.1). In fact, it is now established that *HLA-C* alleles can be perfectly divided into two groups according to linkage disequilibrium with the -35*C* (*Cw*0102*, *-0202, -0302, -0501*, *-0602*, *-0801, -0802*, *-1202*, *-1203*, *-1402*) or -35*T* (*Cw*0303, -0304, -0401, -0701, -0702, -0704, -1502, -1504, -1505, -1506, -1601, -1602, -1604, -1701*) allele (Fellay, Ge et al. 2009; Thomas, Apps et al. 2009). The PBMC from a subset of these healthy volunteers were then stained with DT9.

Table 3.1. Linkage disequilibrium of -35 SNP and HLA-C alleles in Caucasians.

-35 SNP allele	HLA-C allele	p value [a]	Frequency [b]	D' [c]
T	*07	**1.27E-07**	0.3149	0.8919
C	*12	**3.95E-06**	0.0623	1
C	*05	**2.94E-05**	0.0761	1
C	*06	1.82E-04	0.1038	0.8202
T	*03	6.45E-03	0.1626	0.8198
C	*02	3.99E-02	0.0484	1
C	*08	3.99E-02	0.0311	1
T	*01	1.05E-01	0.0277	0.9
T	*04	1.09E-01	0.09	1
T	*14	2.05E-01	0.0069	1
T	*16	3.32E-01	0.0346	1
T	*15	3.34E-01	0.0208	1
T	*17		0.0104	1

[a] p values were calculated using Fisher's exact test; allowing for the number of comparisons made, p values of less than 8.68E-05 were deemed significant.
[b] Allelic frequency
[c] Linkage disequilibrium

This data was generated from 147 HIV-1 seronegative Caucasian donors using the HLA linkage disequilibrium analysis tool provided by the LANL database (http://www.hiv.lanl.gov/content/immunology/hla/hla_linkage.html). Two-digit HLA typing was used throughout the analysis given the small size of the cohort. The linkage disequilibrium between *HLA-C* alleles and either allele of the -35 SNP only reached statistical significance in three cases (bold type).

3.2.2 Determining the specificity and applicability of HLA-C and HLA-E antibodies

DT9 antibody binds to both HLA-C and HLA-E (Braud, Allan et al. 1998). Therefore, it was essential to examine cell surface expression of HLA-E by PBMC to better understand DT9 staining. Three commercially available monoclonal antibodies that are nominally specific for HLA-E (MEM-E/06, MEM-E/08 and 3D12) (Lee, Goodlett et al. 1998; Menier, Saez et al. 2003; Palmisano, Contardi et al. 2005) were tested using 220.221 cells transfected with HLA-E. These cells do not express class I molecules (Shimizu and DeMars 1989). The levels of staining measured by flow cytometry with all three antibodies were similar to that with DT9, confirming that they all recognise HLA-E, at least as expressed by transfected cells (Figure 3.2A). These antibodies were then used to stain PBMC from healthy volunteers by flow cytometry and revealed marked differences in the levels of staining. Consistent with previous reports (Lo Monaco, Sibilio et al. 2008; Thomas, Apps et al. 2009), there was no staining of PBMC with MEM-E/08 (Figure 3.2B). In contrast the level of staining detected with MEM-E/06 varied considerably from donor to donor, suggesting that this antibody may cross-react with other HLA proteins. Finally, there were fairly constant levels of staining of PBMC with 3D12.

The possibility that MEM-E/06 cross-reacts with other class I proteins prompted testing all of the antibodies used for binding specificity. To do this, the LABScreen system was employed (Pei, Lee et al. 2003). This is a Luminex assay that utilises a panel of colour-coded beads which are each individually coated with a purified single HLA allele, covering 97 common HLA class I alleles spanning HLAs A, B and C, for antibody detection. Monoclonal antibody was first incubated with LABScreen beads.

Then monoclonal antibody-bound beads were labelled with R-Phycoerythrin (PE)-conjugated goat anti-human IgG before being analysed using the LABScan 100 flow analyser and interpretation software. The analyser detects the fluorescent emission of PE from each bead and the software defines HLA specificity by comparing the reaction pattern of test monoclonal antibody to the lot-specific worksheet defining the antigen array. In addition to testing the three HLA-E antibodies, DT9 was also tested. Since there can be considerable bead-to-bead differences in the amount of HLA protein present on the individual antigen beads (personal communication from Susan Fuggle and Suzanne Page at the Oxford Transplant Centre), binding of the pan-class I antibody W6/32 (Barnstable, Bodmer et al. 1978) was used to normalise the results. While DT9 was highly specific for HLA-C and cross-reacted only with the rare HLA-A*8001 and HLA-B*7301 alleles [phenotype frequencies of 0-5.4% and 0-0.5% respectively among Caucasoid populations, (http://allelefrequencies.net) (Gonzalez-Galarza, Christmas et al. 2011)] (Figure 3.3A), confirming previous findings (Thomas, Apps et al. 2009), MEM-E/06 did indeed show extensive cross-reactions with many HLA-A, HLA-B and HLA-C proteins (Figure 3.3B), as previously reported (Menier, Saez et al. 2003; Lo Monaco, Sibilio et al. 2008), and there was good correlation with the levels of PBMC staining described above. To confirm that antibody cross-reaction patterns seen with the beads could be transferred to cell surface staining, transfected cell lines were employed. Cross-reaction of MEM-E/06 with HLA-A*0301 but not with HLA-A*0201 was confirmed by immunoprecipitation and 1D-IEF, using 220.221 cells transfected with these HLAs (Figure 3.4). Much less cross-reaction was seen for both MEM-E/08 and 3D12 (Figure 3.3C and D), and for 3D12, the level of binding seen with the beads did not translate into cross-reaction with these HLAs at the cell surface.

Although we cannot rule out cross-reaction with other non-classical HLA proteins, it appears that 3D12 is indeed specific for HLA-E and that HLA-E is present on the surfaces of cells at levels detectable by flow cytometry. It is not clear why MEM-E/08 stains HLA-E-transfected cells and not PBMC, but one possible explanation is that this antibody is actually specific for a misfolded HLA heavy chain which may be present at increased levels on the surfaces of transfected cells. A precedent for this has been set with the monoclonal antibodies HC10 and W/632 which both bind MHC class I heavy chains. HC10 binds to denatured free MHC class I heavy chains only (Stam, Spits et al. 1986; Carreno and Hansen 1994). The binding of beta-2 microglobulin causes a conformational change in the isolated heavy chains which allows W6/32 to bind (Parham, Barnstable et al. 1979; Neefjes and Ploegh 1988).

3.2.3 DT9 staining of EBV-immortalised B cell lines

The original transcriptional data showing a relationship between the -35 SNP and *HLA-C* expression was performed in EBV-immortalised B cell lines (Stranger, Forrest et al. 2005; Fellay, Shianna et al. 2007). In addition, BCLs express, on average, nine times as much cell surface class I molecules (HLA-A,B,C) as peripheral lymphocytes (McCune, Humphreys et al. 1975; Parham, Barnstable et al. 1979). Therefore, cell surface expression of HLA-C was first studied in BCLs. To examine whether the transcriptional data translated to surface expression of HLA-C, BCLs were stained with DT9. There were 20 BCLs available from a previous study carried out in the laboratory. These were predominantly from -35 *CT* and *TT* Caucasian individuals. Therefore five -35 *CC* individuals were chosen at random from the cohort of healthy volunteers and BCLs were generated (using protocol discussed in Materials and

Methods section of this thesis). Twenty-five BCLs were stained with DT9 and confirmed that there was a significantly higher level of DT9 binding from *CC* compared to *TT* individuals (p=0.0221; Mann-Whitney test) (Figure 3.5). The difference in DT9 staining was not statistically significant when comparing either the *CC* against *CT* or *CT* against *TT* groups (p=0.1466 and p=0.0572 respectively; Mann-Whitney test).

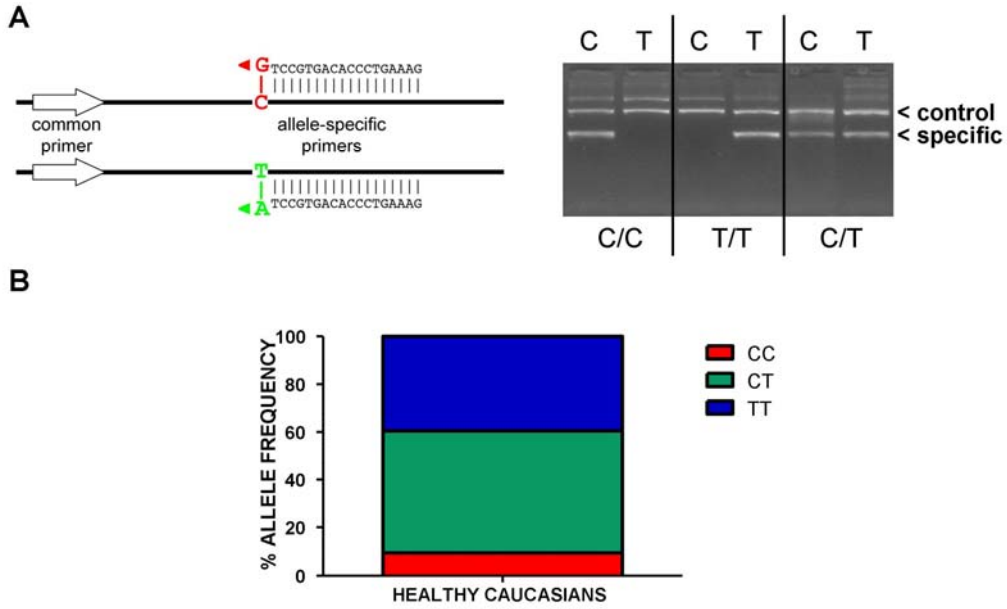


Figure 3.1. Distribution of the -35 SNP genotype in the healthy Caucasian cohort studied.
(A) Genomic DNA was amplified using -35 SNP allele-specific primers and a common control primer upstream of the polymorphism to resolve the *C* and *T* alleles into a single specific band 334kb in size and the common control region into a 796kb sized band. (B) The stacked graph shows the frequency of the -35 SNP genotype in this cohort of 147 individuals.

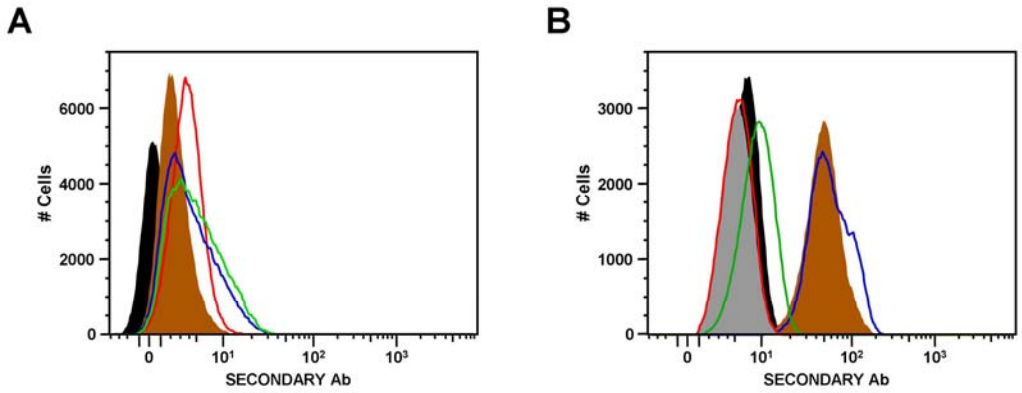


Figure 3.2. Staining of 220.221AEH and PBMC with DT9 and HLA-E specific antibodies.

(A) Staining of the 220.221AEH cell line: unstained cells (filled black), MEM-E/06 (filled brown), MEM-E/08 (red), 3D12 (green), and DT9 (blue). (B) Representative staining of $CD3^+$ lymphocytes (gating on appropriate forward and side scatter, live cells and CD3 expression) from a healthy volunteer (HLA-A*02, B*35, B*44, Cw*04, Cw*05): unstained cells (filled black), MEM-E/06 (filled brown), MEM-E/08 (red), 3D12 (green), DT9 (blue), and an IgG2b isotype control (filled grey).

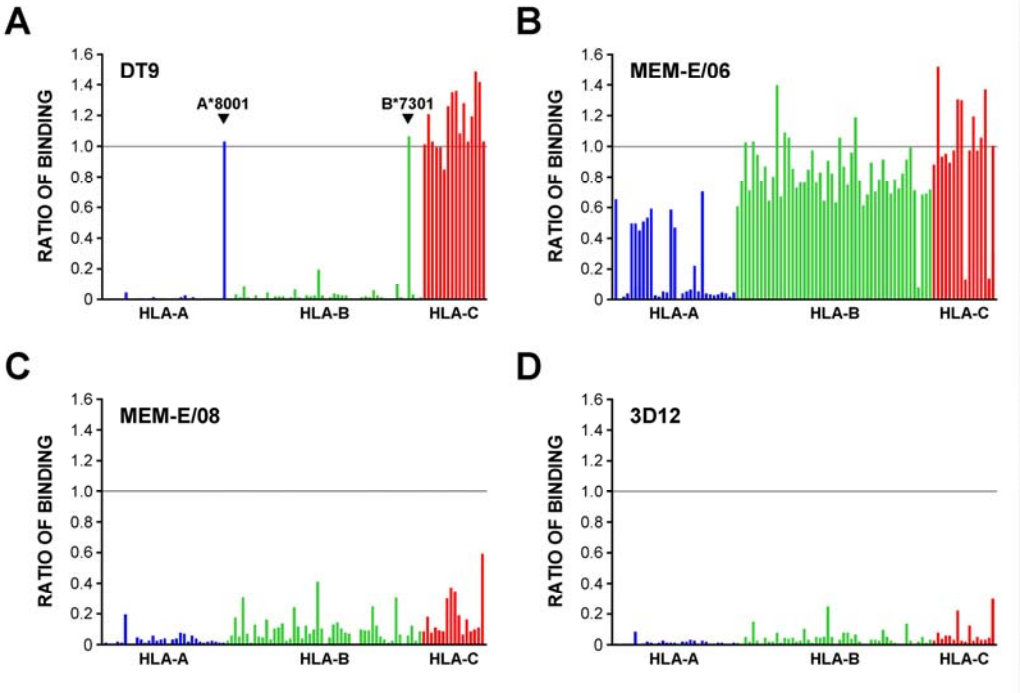


Figure 3.3. Characterisation of monoclonal antibodies DT9, 3D12, MEM-E/06 and MEM-E/08 reactivity against 97 common HLA class I allotypes.
Binding of (A) DT9, (B) MEM-E/06, (C) MEM-E/08, and (D) 3D12 to beads coated with recombinant HLA-A (blue), -B (green) and -C (red) protein, normalised relative to the binding of W6/32 (the horizontal line corresponds to the level of binding seen with W6/32). Values shown represent the average of three runs; the maximum standard errors of the mean were: 0.06 (DT9), 0.05 (MEM-E/06), 0.012 (MEM-E/08) and 0.01 (3D12). The order of the HLA allotypes tested along the x axis was: A*0101, 0201, 0203, 0206, 0301, 1101, 1102, 2301, 2402, 2403, 2501, 2601, 2901, 2902, 3001, 3002, 3101, 3201, 3301, 3303, 3401, 3402 3601, 4301, 6601, 6602, 6801, 6802, 6901, 7401; B*8001, 0702, 0801, 1301, 1302, 1401 1402, 1501, 1502, 1503, 1510, 1511, 1512, 1513, 1516, 1801, 2705, 2708, 3501, 3701, 3801, 3901, 4001, 4002, 4006, 4101, 4201, 4402, 4403, 4501, 4601, 4701, 4801, 4901, 5001, 5101, 5102, 5201, 5301, 5401, 5501, 5601, 5701, 5703, 5801, 5901, 6701, 7301, 7801, 8101, 8201; Cw*0102, 0202, 0302, 0303, 0304, 0401, 0501, 0602, 0702, 0801, 1203, 1402, 1502, 1601, 1701, 1802.

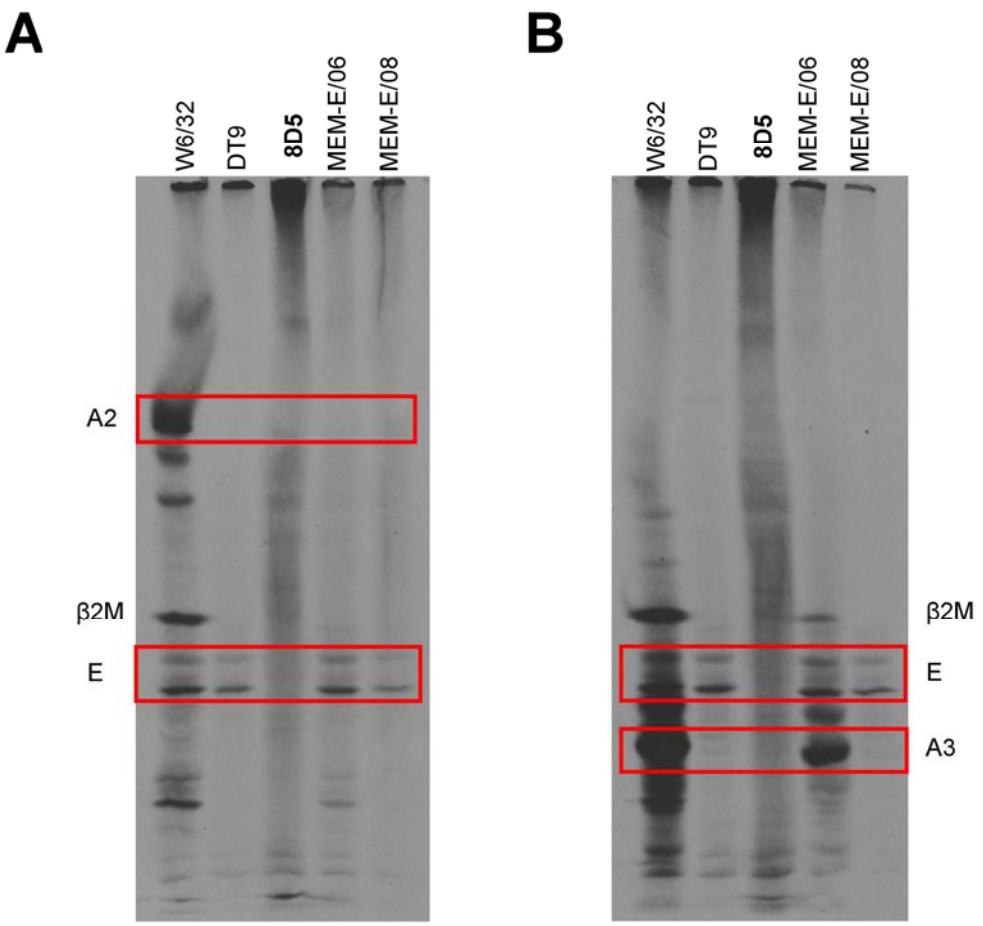


Figure 3.4. IEF analysis of the reactivity of W6/32, DT9, MEM-E/06 and MEM-E/08.

(A) HLA-A*02- and (B) HLA-A*03-transfected 220.221 cell lines were metabolically labelled with ^{35}S, the cells were then lysed, the lysate was immunoprecipitated with the indicated antibodies and then run on an IEF gel. MEM-E/06 pulls down HLA-A*03 but not HLA-A*02. Both transfected cell lines display two major HLA-E bands corresponding to the two alleles HLA-E*0101 and HLA-E*0103. 8D5 is a nominally HLA-C specific monoclonal antibody provided by a collaborator (Marco Colonna, Washington University School of Medicine). β2M is beta-2 microglobulin.

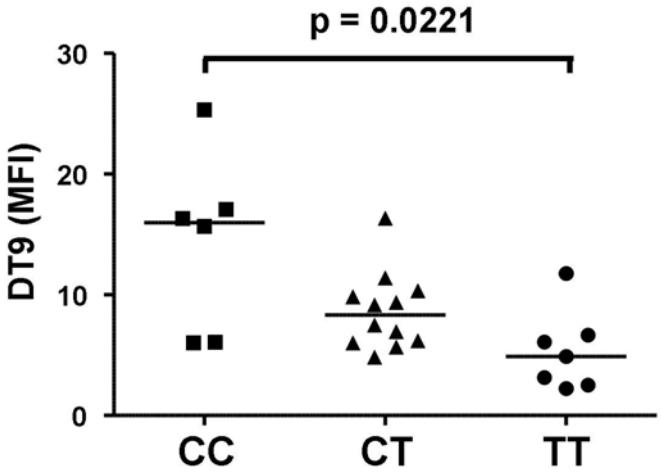


Figure 3.5. Direct DT9 staining of live CD19$^+$ EBV-immortalised B cell lines from individuals with different -35 SNP genotypes.
BCLs from HIV-1 seronegative individuals were stained with DT9. Cells were gated on appropriate forward and side scatter, live cells, CD3 expression and the B cell marker CD19. DT9 median fluorescence intensities (MFI; y axis) were corrected for background autofluorescence of unstained cells. -35 SNP genotypes are indicated on the x axis. Horizontal lines represent the median MFI value for each group. P values were calculated using the Mann-Whitney test.

3.2.4 DT9 staining of PBMC from healthy and HIV-1-infected subjects

DT9 was then used to stain cryopreserved PBMCs from twenty-eight HIV-1 seronegative subjects selected on the basis of homozygosity of their -35 SNP genotype (both *TT* and *CC*) and the absence of the HLA alleles (*A*8001* and *B*7301*) that cross-react with DT9. There was a significantly higher level of DT9 staining of lymphocytes (CD3 positive cells) from *CC* subjects compared with *TT* subjects (p=0.0459, Mann-Whitney test; Figure 3.6A). When the *TT* subjects were subdivided based on their HLA-C alleles, DT9 staining of *TT* subjects who were homozygous for *HLA-Cw*07* was significantly lower than both *CC* subjects who were all *HLA-Cw*07* negative (p=0.004) and *TT* subjects who were either heterozygous for, or who did not have, *HLA-Cw*07* (p=0.0007; Figure 3.6B). There was no significant difference in

the DT9 staining of PBMC from *TT* subjects who were not homozygous for *HLA-Cw*07* and *CC* subjects. These findings imply that low HLA-Cw*07 levels could account for the differences in HLA-C expression, previously described as high levels associated with the -35 *CC* genotype (Thomas, Apps et al. 2009).

Phytohaemagglutinin-stimulated PBMC were also stained with DT9 to replicate the increased activation of lymphocytes seen in acute HIV-1 infection (Fauci 1993; Appay, Papagno et al. 2002; Papagno, Spina et al. 2004). Stimulation resulted in upregulation of DT9 staining (Figure 3.6C and D). DT9 staining of *HLA-Cw*07* homozygous *TT* subjects remained significantly lower than that of both *CC* subjects ($p=0.0018$) and *TT* subjects who were either heterozygous for *HLA-Cw*07* or lacked this allele ($p=0.001$), consistent with non-stimulated *ex vivo* PBMC.

Lymphocytes from twenty-five HIV-1 infected subjects were also stained with DT9. As before, DT9 staining of *CC* subjects was just significantly higher than staining of *TT* subjects ($p=0.0413$; Figure 3.6E), and again this difference in staining was even more striking with *TT* subjects that were also homozygous for *HLA-Cw*07* (Figure 3.6F, $p=0.0043$). Furthermore, there was no significant difference in median fluorescence intensity values comparing HIV-1 uninfected and infected subjects, suggesting that, in chronically infected subjects at least, infection does not increase surface expression of HLA-C protein.

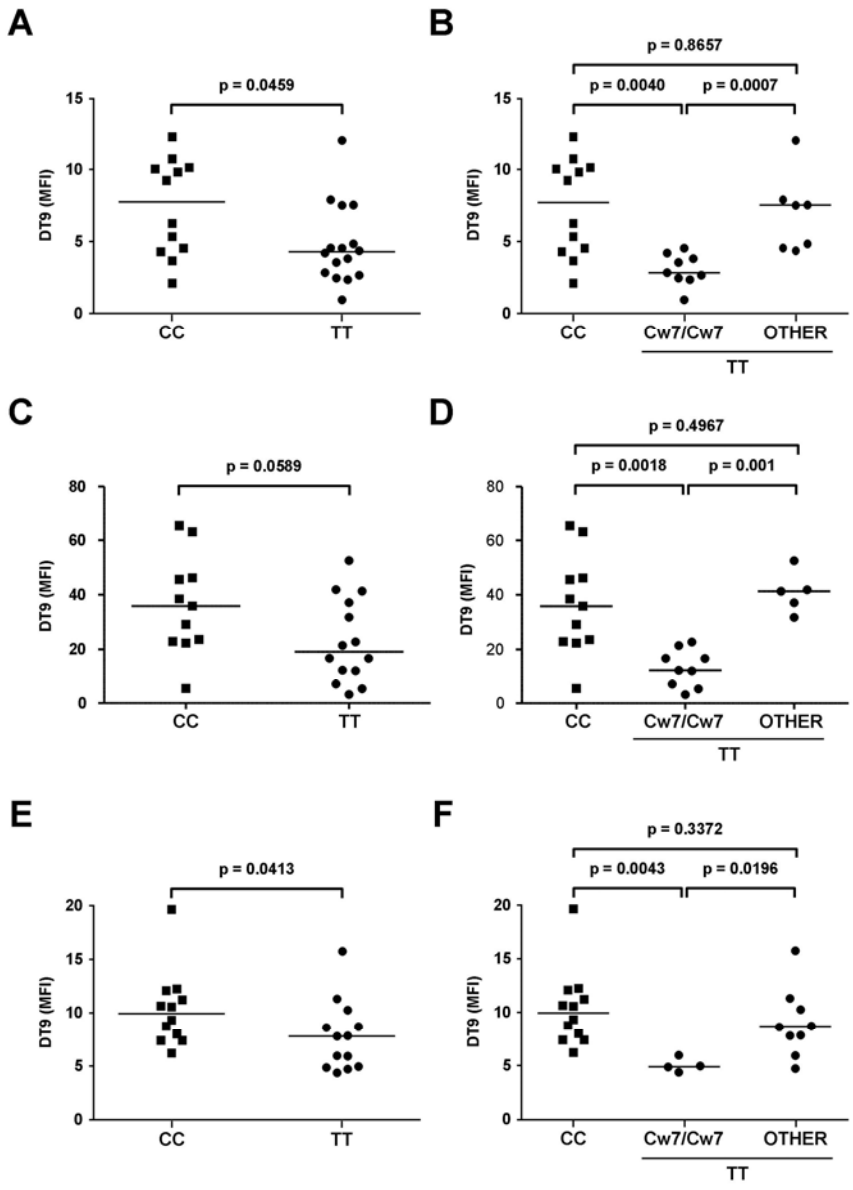


Figure 3.6. Direct DT9 staining of live CD3$^+$ T lymphocytes from -35 SNP homozygotes.

PBMC from HIV-1 uninfected subjects were stained with DT9 *ex vivo* (A and B) and after 2 days of PHA stimulation (C and D) to simulate the immune activation typical of acute HIV-1 infection. PBMC from chronically HIV-1 infected individuals were also stained with DT9 *ex vivo* (E and F). DT9 median fluorescence intensities (MFI; y axis) were corrected for background autofluorescence of unstained cells. -35 SNP genotypes are indicated on the x axis. In panels B, D and F, "other" refers to -35 *TT* individuals who were not homozygous for HLA-Cw*07. Horizontal lines represent the median MFI value for each group. P values were calculated using the Mann-Whitney test.

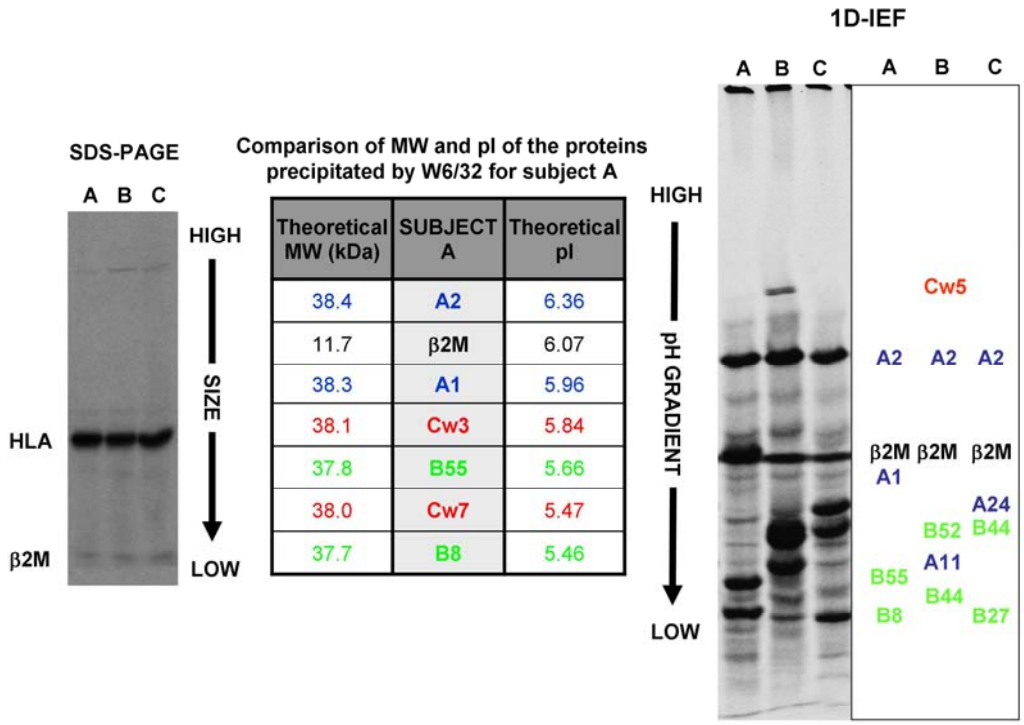


Figure 3.7. Immunoprecipitation of metabolically labelled MHC class I proteins. PBMC from three subjects (A, B and C) were metabolically labelled with ^{35}S, the cells were then lysed and the lysate was immunoprecipitated with W6/32. It was not possible to resolve immunoprecipitated class I proteins by size (SDS-PAGE), instead the proteins were resolved based on isoelectric point (pI) by IEF. The pI of most HLA-C alleles is similar to HLA-A and HLA-B alleles making it difficult to correctly identify HLA-C bands unless their pI is very high like HLA-Cw*05, shown in this example. The HLA type (and pI) for subjects B and C are as follows: subject B – A*02 (6.36), A*11 (5.77), B*44 (5.7), B*52 (5.85), Cw*05 (7.11), Cw*12 (5.91); subject C – A*02 (6.36), A*24 (5.91), B*27 (5.46), B*44 (5.70), Cw*02 (5.86), Cw*03 (5.84). The theoretical pI was calculated using the 'Compute pI/Mw' (molecular weight) tool provided by the Expert Protein Analysis System server at http://expasy.org/tools/pi_tool.html.

3.2.5 Quantifying HLA-C on the cell surface

Having established that there were differences in DT9 binding to the cell surface at the -35 SNP genotype, it was important to resolve the contribution HLA-C made to DT9 binding.

3.2.5.1 Resolving HLA proteins by one-dimensional isoelectric focusing

Metabolic labelling and subsequent immunoprecipitation with DT9 showed the relative amounts of HLA-C and HLA-E synthesised in cells. When these techniques were combined with traditional SDS-PAGE, there was no resolution of the different MHC class I proteins because there was little difference in their molecular weights (Figure 3.7). Therefore, IEF following immunoprecipitation, with DT9 and W6/32, of metabolically labelled PBMC (Figure 3.7) that resolves proteins by their unique isoelectric points was performed.

3.2.5.1.1 IEF of W6/32 and DT9 Immunoprecipitate from HLA-C-transfected 220 .221 cells

For ease of identification of HLA-C bands in healthy subjects, IEF was first performed on 220.221 cells, transfected with various *HLA-Cw* copy DNA (Figure 3.8). As expected, the positions of the bands were identical whether immunoprecipitated with DT9 or W6/32 antibodies, though the intensities were very different. This difference in intensity, having started with identical protein, suggests that the avidity of DT9 for protein was less than that of W6/32 resulting in less protein being pulled down with DT9. Several of the Cw proteins (including Cw*03, Cw*06

and Cw*07) resolved as two bands. This is most likely the result of post-translational modification, rather than alternative mRNA processing (Mizuno, Kang et al. 1989).

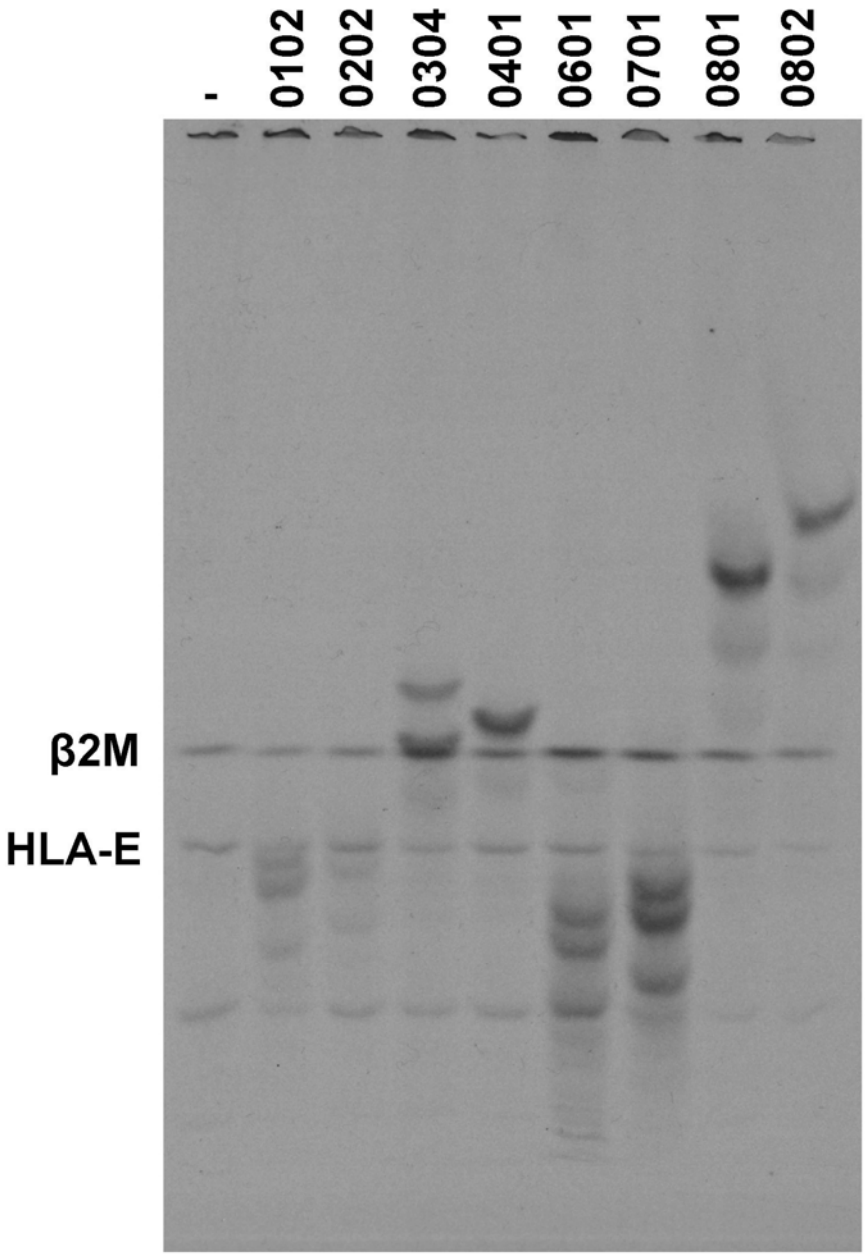


Figure 3.8. IEF of 220.221 cells transfected with various HLA-Cw copy DNA. Untransfected and transfected 220.221 cells were metabolically labelled with ^{35}S, the cells were then lysed, the lysate was immunoprecipitated with W6/32 and then run on an IEF gel. – refers to untransfected 220.221 cells; the numbers at the top of the lanes refer to the HLA-Cw allele transfected; β2M is beta-2 microglobulin.

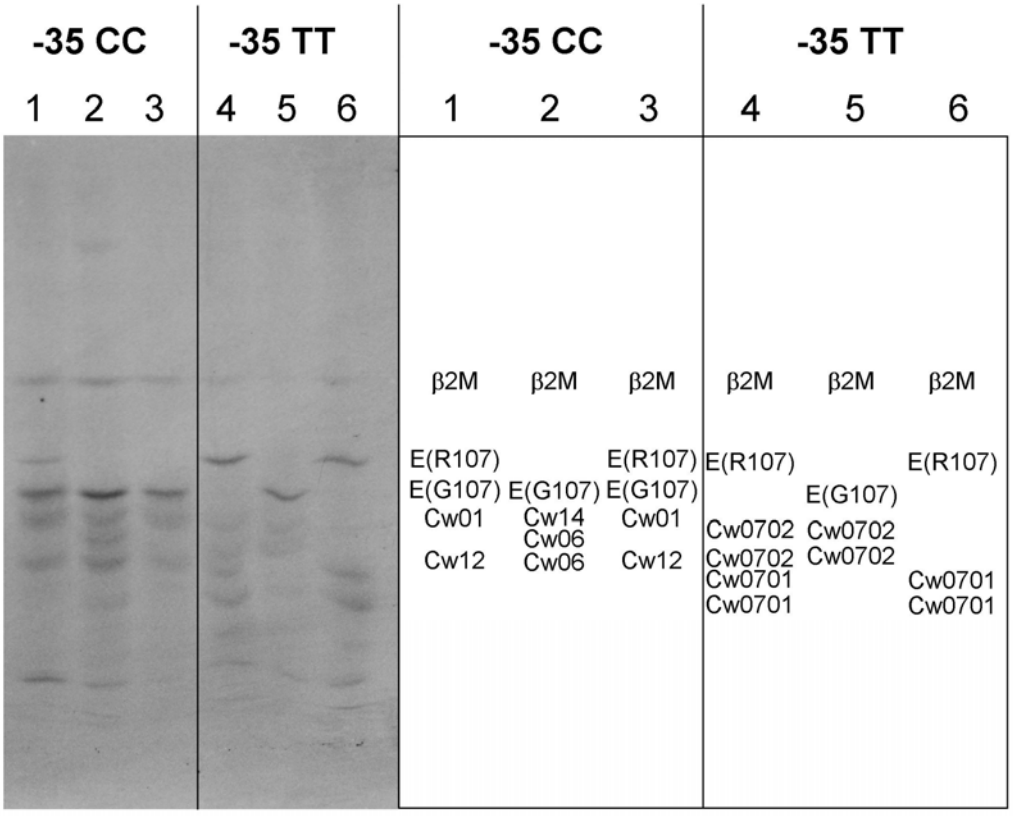


Figure 3.9. IEF of HLA-C and HLA-E proteins immunoprecipitated with DT9. PBMC from 6 subjects (3 -35 *CC* and 3 -35 *TT*) were metabolically labelled with ^{35}S, the cells were then lysed, the lysate was immunoprecipitated with DT9 and then run on an IEF gel. Some of the PBMC display the two major HLA-E alleles – HLA-E*0101 and HLA-E*0103. There is a single amino acid difference between these two alleles located at position 107 on the α2 domain of the HLA-E heavy chain, where an arginine in HLA-E*0101 [E(R107)] is substituted for a glycine in HLA-E*0103 [E(G107)]. Lanes 1-6 each represent PBMC from a different patient; β2M is beta-2 microglobulin.

3.2.5.1.2 IEF of HLA-C and HLA-E proteins immunoprecipitated with DT9

Finally, IEF was performed on PBMC from healthy subjects who were either -35 *CC* or *TT*. The IEF protocol was initially established with W6/32 immunoprecipitates because more protein was pulled down and therefore the gels were much clearer. When W6/32 immunoprecipitates were resolved, the bands corresponding to HLA-A and HLA-B alleles were easily identified (Figure 3.7). The lower expression level of HLA-C made the identification of the HLA-C bands more difficult (much fainter than HLA-A/B bands). In addition, only HLA-C alleles with unusually high isoelectric points (such as Cw*05; Figure 3.7) were easily identified because their bands were clearly distinct from HLA-A/B alleles which had much lower isoelectric points.

IEF of the protein immunoprecipitated with DT9 (Figure 3.9) negated the problem of HLA-A/B bands but lower expression of HLA-C meant that the gels had to be exposed to film for several weeks (as opposed to several days for W6/32 immunoprecipitate). As noted with the 220.221 transfectants, some of the Cw alleles resolved as multiple bands. The gels demonstrated that HLA-E was more highly expressed than HLA-C (HLA-E bands had greater intensity than HLA-C bands when compared by eye). Keeping in mind the small sample size (only 6 subjects), the ratio of HLA-C to HLA-E bands in *CC* and *TT* subjects appeared constant suggesting that there was no difference in HLA-C expression levels between the two groups.

An important caveat is that this technique measured only the total protein synthesised as opposed to protein on the cell surface. Having established a reliable IEF protocol, attempts were made at surface labelling of cells with biotin and fluorescent dyes before running DT9 immunoprecipitates of lysed cells on IEF gels to study surface proteins only. In theory, western blotting of the IEF gel should then allow detection

of the labelled protein with the relevant probe. Unfortunately, the IEF gels did not blot efficiently, presumably as a result of the high non-ionic detergent content of IEF gels. Additionally, these labelling methods target lysine residues, resulting in a loss of positive charge and ensuing change in isoelectric point. Therefore, a difference in surface HLA-C expression between -35 *CC* and *TT* subjects was not conclusively demonstrated using this technique.

3.2.5.2 Resolving the contribution of HLA-C to DT9 binding by saturation binding

Williams *et al*. had previously combined indirect antibody binding and metabolic labelling to calculate the number of binding sites for W6/32 (Williams 1977). This technique was adapted for flow cytometry. The use of saturating amounts of W6/32, DT9 and a truly HLA-E specific antibody, all detected by a common secondary antibody that binds with the same avidity to these primary antibodies, allowed the calculation of the contribution of HLA-C to DT9 binding.

The LABScreen beads staining data (Figure 3.3) revealed that 3D12 antibody was the most specific for HLA-E with minimal cross-reactivity (if any) to classical class I alleles. To confirm that the extremely low levels of binding seen in that assay did not translate to binding in cells, individuals who expressed three of the highest bead-binding alleles (HLAs B*40, Cw*05 and Cw*08) were selected and their PBMCs were used in IEF assays using 3D12 to immunoprecipitate proteins. The allele with the highest binding to beads was actually Cw*18 but this allele is extremely rare in Caucasian populations [phenotype frequency of 0-2.5% from

http://allelefrequencies.net, (Gonzalez-Galarza, Christmas et al. 2011)] and was not present in our cohort of healthy volunteers. 3D12 failed to bind to these three HLA alleles in cells (Figure 3.10), confirming that the bead binding seen was background staining.

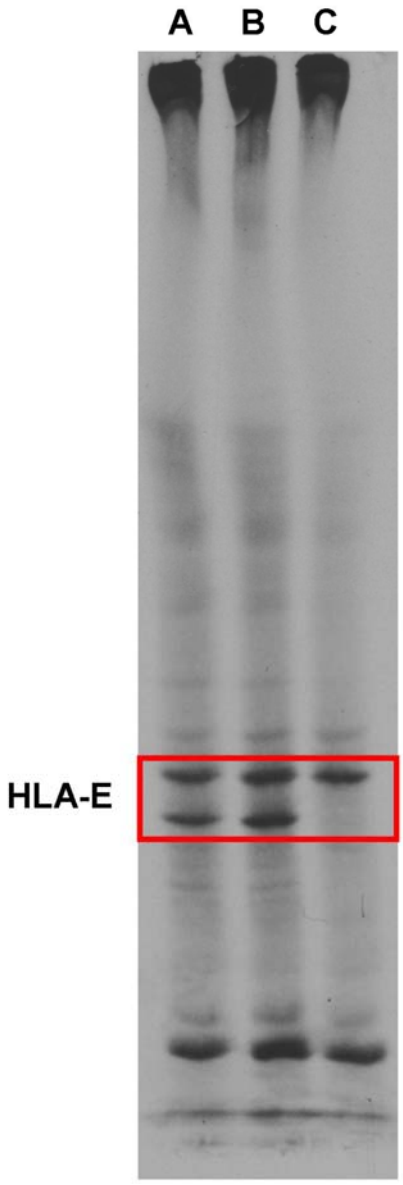


Figure 3.10. IEF of the reactivity of 3D12 monoclonal antibody.
PBMC from 3 subjects were metabolically labelled with ^{35}S, the cells were then lysed, the lysate was immunoprecipitated with 3D12 and then run on an IEF gel. Only HLA-E protein was pulled down. HLA typing for the subjects are as follows: subject A – A*01/32, B*14/40, Cw*03/08; subject B – A*02/11, B*44/52, Cw*05/12; subject C – A*02/68, B*15/40, Cw*03/03.

Thirty healthy volunteers were selected and their lymphocytes were stained with W6/32, DT9 and 3D12 in indirect antibody binding assays (Figures 3.11 and 3.12). As with the direct DT9 staining, the low levels of staining observed in these indirect assays are consistent with cell surface levels of HLA-C protein being ten-fold less than those of HLA-A and HLA-B proteins (Snary, Barnstable et al. 1977). Despite the consistently low staining with 3D12, there was a significant difference in median fluorescence intensity between unstained cells and cells stained with 3D12 ($p<0.0001$, Mann-Whitney) confirming surface expression of HLA-E (Figure 3.12A), and, when subjects were grouped by -35 SNP genotype, there was no significant difference in 3D12 binding (Figure 3.12B). Consistent with the observations made during direct antibody staining, differences in DT9 staining in this indirect staining assay are driven by the low levels of binding in *TT* individuals that are homozygous for *HLA-Cw*07* (Figure 3.12C).

There was no statistically significant correlation between 3D12 and DT9 binding (Spearman test; $r=0.3495$, $p=0.0583$; Figure 3.12D). These data imply that the differences seen in DT9 staining are the result of differences in HLA-C binding alone. Furthermore, the constant contribution of surface HLA-E to the DT9 binding means that the difference in HLA-C surface expression between the *CC* and *TT* subjects is greater than previously recognised. To better quantify this difference, the ratio of HLA-C (3D12 staining subtracted from DT9 staining) to total Class I protein on the cell surface (measured by W6/32 staining) was calculated. The proportion of actual HLA-C binding to W6/32 binding ranged between 1.0-4.6%, 1.0-3.4% and 0.4-2.6% for -35 *CC*, *CT* and *TT* subjects respectively. There was a significant difference in the ratio of HLA-C to W6/32 binding between -35 *CC* and *TT* subjects (Figure 3.12E;

p=0.0061, Mann-Whitney), but, again, this difference was exclusively driven by the low expression of HLA-Cw7 (p=0.0032, comparing -35 *CC* with -35 *TT* subjects homozygous for *HLA-Cw*07*). As before, there was no significant difference in the ratio of HLA-C to W6/32 between -35 *CC* and *TT* subjects not homozygous for *HLA-Cw*07* (p=0.0896). From these data, the conclusion is that -35 *CC* subjects express 1.76 times more HLA-C molecules than -35 *TT* subjects when considered as a whole, but five times more HLA-C than -35 *TT* subjects who are homozygous for *HLA-Cw*07*. Therefore, the protective -35 SNP genotype does not associate with increased expression of HLA-C. Rather, the non-protective allele associates with the greatly reduced expression of a single *HLA-C* allele, Cw*07.

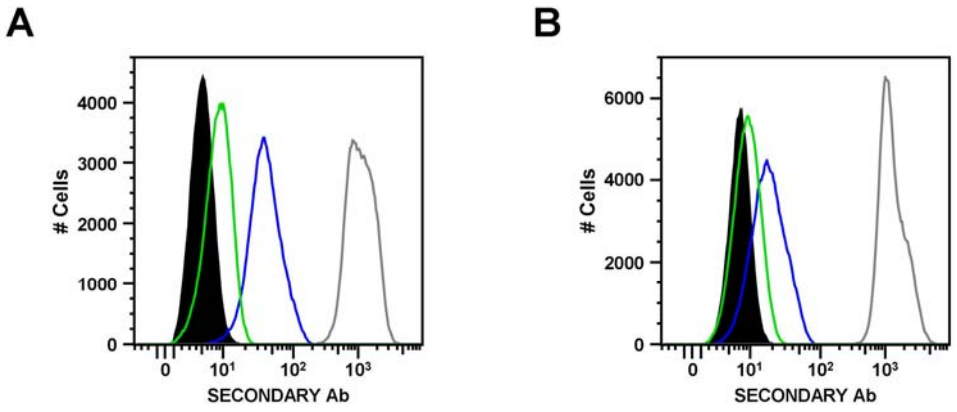


Figure 3.11. Indirect staining of lymphocytes with 3D12, DT9 and W6/32 antibodies.
Representative staining of $CD3^+$ lymphocytes (gated on appropriate forward and side scatter, live cells, and CD3 expression) from two HIV-1 uninfected subjects: (A) a -35 *TT* subject (homozygous for *HLA-Cw*07*), (B) a -35 *CC* subject (*HLA-Cw*05/06*). Unstained cells are shown filled black, 3D12 in green, DT9 in blue, and W6/32 in grey.

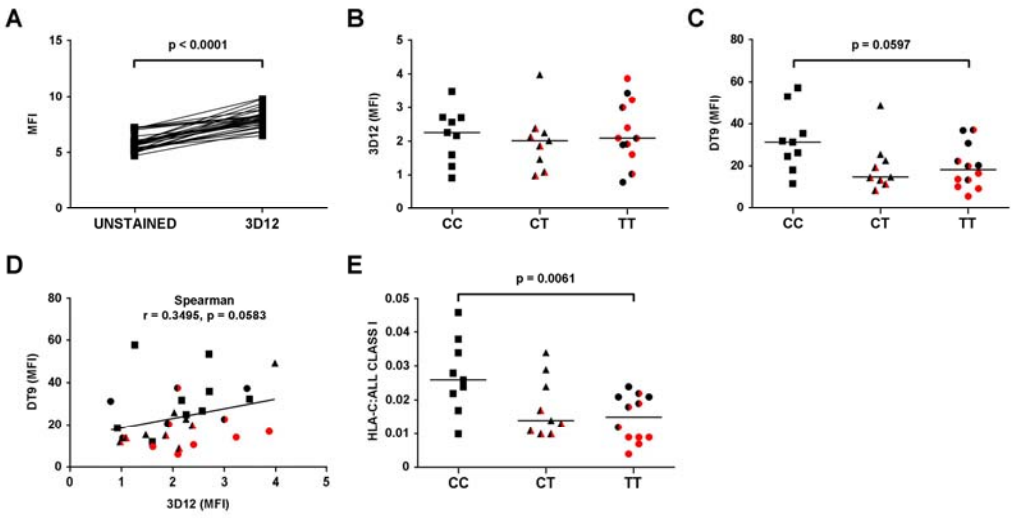


Figure 3.12. Indirect staining of lymphocytes from 30 healthy volunteers with 3D12 and DT9.
(A) Paired comparisons of the median fluorescence intensity (MFI) of unstained cells and cells stained with 3D12. (B and C) Indirect staining (with 3D12 and DT9, respectively) of live $CD3^+$ lymphocytes, corrected for background auto-fluorescence of unstained cells. (D) Correlation of background corrected DT9 and 3D12 staining. (E) Ratio of HLA-C levels (3D12 staining subtracted from DT9 staining) to total class I (W6/32 staining). For panels B–E, red and half red symbols denote individuals homozygous and heterozygous for *HLA-Cw*07*, respectively. Statistical significance calculated using the Mann-Whitney test.

3.3 Discussion

The focus of this chapter was to investigate whether the -35 SNP, shown to be responsible for maintaining effective control of HIV-1 in Caucasians by lowering viral load set point (Fellay, Shianna et al. 2007), associated with increased surface expression of HLA-C. Transcriptional studies had previously suggested that the -35*C* variant associates with higher *HLA-C* mRNA expression in both EBV-transformed B cell lines and lymphocytes (Stranger, Forrest et al. 2005; Fellay, Shianna et al. 2007; Thomas, Apps et al. 2009), initially suggesting an inverse correlation between *HLA-C* expression and viral load. The homology between HLA alleles makes it difficult to generate allele-specific antibodies. Consequently, the best available antibody for

HLA-C is DT9, which binds both HLA-C and HLA-E (Braud, Allan et al. 1998). This antibody was used to stain the surface of BCLs and lymphocytes from both seronegative and HIV-1 seropositive individuals. The contribution of HLA-C to the total DT9 binding was then determined using HLA-E-specific monoclonal antibodies.

In healthy Caucasian subjects, DT9 staining of BCLs and *ex vivo* and PHA stimulated lymphocytes showed consistently higher DT9 levels in *CC* compared with *TT* subjects. Only one other group have examined HLA-C expression in relation to the -35 SNP and they showed that the -35 *CC* genotype associates with significantly higher HLA-C cell surface expression than does the -35 *TT* genotype (Thomas, Apps et al. 2009). They discounted cross-reaction of DT9 with HLA-E as it was shown that the HLA-E specific antibody MEM-E/08 stained a HLA-E-transfected cell line but did not stain lymphocytes (Thomas, Apps et al. 2009). It has also previously been reported that MEM-E/08 cannot be used to detect HLA-E on PBMC by flow cytometry (Lo Monaco, Sibilio et al. 2008).

Consistent with these reports, the data presented here showed no staining of PBMC with this antibody. However, the HLA-E specific antibody 3D12, which was confirmed not to cross-react significantly with HLAs A, B or C (Figures 3.3 and 3.10), bound to lymphocytes. Thus HLA-E is expressed at low levels on the surfaces of lymphocytes, consistent with earlier reports showing that human cells express HLA-E (Pacasova, Martinozzi et al. 1999; King, Allan et al. 2000; Ishitani, Sageshima et al. 2003; Marin, Ruiz-Cabello et al. 2003; Menier, Saez et al. 2003; Coupel, Moreau et al. 2007).

Isoelectric focussing of DT9 immunoprecipitates from lysed lymphocytes was performed in an attempt to resolve the relative contributions of HLA-C and HLA-E to DT9 binding. Resolution of HLA-C alleles was possible but low HLA-C expression meant that quantification of individual alleles was extremely difficult. IEF showed no difference in relative HLA-C to HLA-E expression between *CC* and *TT* subjects when cells were lysed, suggesting that the differences in DT9 surface staining were most likely due to changes in HLA-C surface expression. Unfortunately, this technique could not be adapted to directly comment on surface levels of HLA-C and HLA-E.

Surface staining with 3D12 confirmed that HLA-E is expressed on the cell surface of lymphocytes. There was no significant difference in 3D12 staining when individuals were grouped according to their -35 SNP genotype. Therefore, these results confirm that the differences in DT9 staining associated with these genotypes are due to differences in HLA-C surface expression. In addition, the difference between HLA-C expression levels in donors with -35 *CC* and *TT* alleles is greater than previously recognised.

The cell surface expression of HLA-E requires the availability of a set of conserved nonameric peptides derived from the leader sequence of various HLA class I molecules (including HLAs A, B and C) (Braud, Jones et al. 1997; Lee, Goodlett et al. 1998), suggesting that HLA-E is expressed ubiquitously. In addition, there is evidence that HLA-E plays an important role in immunological recognition events involving NK cells by serving as a ligand for the inhibitory CD94/NKG2A and CD94/NKG2C receptors present on these cells, as well as a subset of T cells (Braud, Allan et al. 1998; Lee, Llano et al. 1998). The interaction of HLA-E with

CD94/NKG2A results in inhibition of NK cell-dependent lysis (Braud, Allan et al. 1998; Lee, Llano et al. 1998).

In fact, it has been proposed that HIV-1-mediated upregulation of HLA-E expression serves as an additional immune evasion strategy, specifically targeting the antiviral activities of NK cells (Nattermann, Nischalke et al. 2005). A similar mechanism had previously been shown in CMV infection (Tomasec, Braud et al. 2000). Natterman *et al.* showed enhanced expression of HLA-E on lymphocytes from HIV-1 infected individuals compared to healthy individuals. Using *in vitro* assays, they also showed that the HIV-1 peptide AISPRTLNA (Gag position 146-154) binds to HLA-E, resulting in enhanced surface expression of HLA-E and reduced susceptibility to NK cell lysis.

The DT9 staining data presented here also show an important difference from those reported previously (Thomas, Apps et al. 2009). Like Thomas *et al*. (Thomas, Apps et al. 2009), consistently higher DT9 staining of cells from -35 *CC* subjects compared with -35 *TT* subjects, both for healthy volunteers ($p=0.0459$; Mann-Whitney test) and individuals infected with HIV-1 ($p=0.0413$; Mann-Whitney test) was observed; there was also considerable overlap in the range of DT9 staining levels between *CC* and *TT* subjects, reflecting a wide spread of HLA-C expression across the different alleles. However, in contrast, cells from *TT* subjects homozygous for *HLA-Cw*07* had by far the lowest DT9 staining (Figures 3.6 and 3.12). Indeed, the difference in staining levels between *CC* subjects and *TT* subjects homozygous for *HLA-Cw*07* was highly significant ($p=0.004$ for healthy volunteers, $p=0.0043$ for HIV-1 infected individuals; Mann-Whitney test). The difference in staining between *TT* subjects homozygous for

*HLA-Cw*07* and all other *TT* subjects was highly significant for healthy volunteers (p=0.0007; Mann-Whitney test), but less significant for the HIV-1 infected individuals (p=0.0196; Mann-Whitney test). Importantly, there was no statistically significant difference in staining between *CC* subjects and *TT* subjects when *HLA-Cw*07* homozygotes were excluded, both for the seronegative and the HIV-1 infected subjects. Although not commented on by the authors, a similar pattern of particularly low DT9 antibody binding to HLA-Cw*07 was seen in the staining data of HIV-1 seronegative subjects presented by Thomas *et al* (Thomas, Apps et al. 2009).

Even when the contribution of the constant background of surface HLA-E expression to the level of DT9 staining was taken into account, this picture did not change. Although the significance of the difference in expression levels increased between *CC* and *TT* subjects (p=0.0061; Mann-Whitney test) (Figure 3.12E), this difference was still driven entirely by the very low staining of cells from the *HLA-Cw*07* homozygotes (*CC* vs *TT Cw*07/Cw*07* p=0.0032; *CC* vs *TT* non-*Cw*07* homozygous p=0.0896). Overall, the levels of surface HLA-C expression were estimated to be approximately 1.76 fold greater for PBMC from *CC* subjects compared with *TT* subjects but this difference was almost all accounted for by the approximately 5 fold decreased expression of HLA-C in -35 *TT* donors who were also homozygous for *HLA-Cw*07*, compared with -35 *CC* subjects. Therefore, the -35 SNP is actually most strongly associated with intrinsically low expression of HLA-Cw*07, rather than high expression of several *-35C* associated HLA-C allotypes. However, the possibility remains that other HLA-C alleles may also have different intrinsic levels of expression that could contribute to the overall levels of surface staining.

The low surface expression of HLA-Cw*07 immediately raised three questions. Firstly, is this allele truly expressed at such low levels or could the low expression demonstrated be an experimental design error? Afterall, surface staining experiments were performed using DT9 antibody and it could simply be that this antibody has very low avidity for HLA-Cw*07. The LABScreen bead staining data refutes this (Figure 3.3A). DT9 bound to the HLA-Cw*07-coated bead with greater avidity than W6/32 (ratio of DT9 to W6/32 binding greater than 1). In fact of the 16 HLA-C allele-coated beads, only 4 other HLA-C alleles had a higher ratio of DT9 to W6/32 binding. This confirms that HLA-Cw*07 is indeed expressed at significantly lower levels on the surface of lymphocytes.

The second question raised, having confirmed low HLA-Cw*07 expression, was why is this allele expressed at such low levels and how does it differ from other HLA-C alleles? The presence of MHC class I molecules at the cell surface is a complex process involving the formation of a complex composed of heavy chain, light chain and peptide in the groove (Bijlmakers and Ploegh 1993). These MHC class I molecules are spontaneously endocytosed via coated pits and transferred from endosomes to the golgi bodies for recycling back to the cell surface (Tse and Pernis 1984; Machy, Truneh et al. 1987; Dasgupta, Watkins et al. 1988). The steady-state expression of MHC class I molecules on the cell surface is therefore a result of the equilibrium between the rate of newly presented molecules and their internalisation rate. The cytoplasmic tail of MHC class I molecules contributes to their expression (Vega and Strominger 1989; Davis, Reyburn et al. 1997; Gruda, Achdout et al. 2007; Schaefer, Williams et al. 2008) and accounts for about two-thirds of the protein

turnover at the cell surface (Davis, Reyburn et al. 1997). This 30-33 amino acid long cytoplasmic tail is highly conserved and in encoded in exons 5-7 in humans (Malissen, Malissen et al. 1982; Guild and Strominger 1984).

It was hypothesised that the lower cell surface expression of HLA-Cw*07 was due to differences in its cytoplasmic tail compared to other HLA-C molecules. Therefore, the cytoplasmic tail sequence of all HLA-C molecules was aligned and compared for significant amino acid differences (Figure 3.13). The most striking difference was the number of cysteine residues, with HLA-Cw*07 molecules possessing a unique fourth cysteine residue at position 326 in the cytoplasmic tail. Interestingly, the number of cysteine residues in the cytoplasmic tail of different MHC class I proteins also varies with HLA-A proteins containing one at position 340, HLA-B proteins containing either one or two at positions 309 and 326, while all HLA-C proteins contain three or four cysteines at positions 309, 321, 326 and 340 (Gruda, Achdout et al. 2007). Mutagenesis studies revealed that cysteine residues are important for proper MHC class I assembly and egress from the endoplasmic reticulum and for correct conformation at the cell surface (Gruda, Achdout et al. 2007). The postulated mechanism involves *S*-palmitoylation, a posttranslational modification in which the fatty acid palmitate is covalently conjugated to cysteine residues through a thioester linkage, which increases the hydrophobicity of proteins and contributes to their membrane anchoring (Kaufman, Krangel et al. 1984; Bijlmakers and Marsh 2003; Gruda, Achdout et al. 2007). It is feasible that the extra cysteine residue present in the cytoplasmic tail of HLA-Cw*07 is responsible for the low cell surface expression of this molecule.

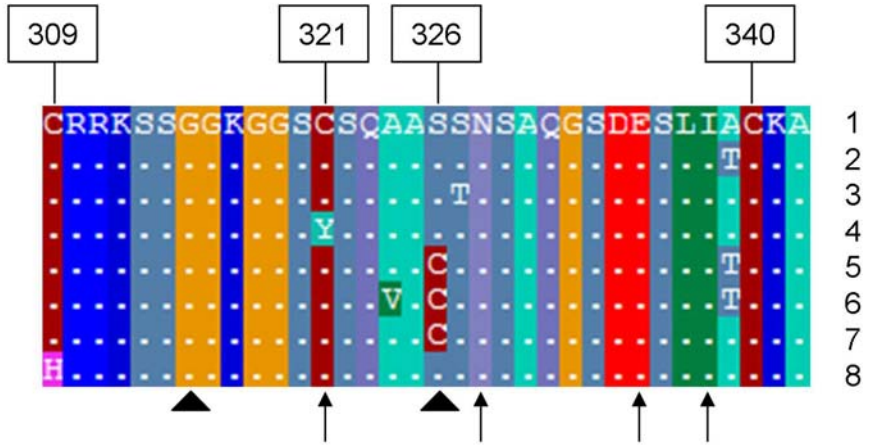


Figure 3.13. Protein sequences of the cytoplasmic tail domain region of all human HLA-C alleles.
Information on 158 HLA-C protein sequences was obtained from the GenBank database. The cytoplasmic tails were aligned and eight distinct groups (1-8) were seen. [1] Cw*01(except 24)/02/03/04(except 30)/05/06/08(except 22)/12/14/15/16/18; [2] Cw*0124; [3] Cw*0430; [4] Cw*0822; [5] Cw*0701/02/03/04/26/29/30/42/46/49/52/57/59; [6] Cw*0706/18/19; [7] Cw*0711; [8] Cw*1701/03/05. The location of cysteine residues is indicated in boxes. The HLA-Cw*07 proteins were all unique in containing an extra cysteine residue at position 326. A dash indicates homology with the consensus sequence while a letter represents a change at that position. The two large arrow heads denote exon boundaries while the four arrows indicate amino acid differences unique to HLA-C (compared to HLA-A and HLA-B cytoplasmic tails).

A compelling alternative explanation for the differential levels of HLA-C allotype expression involves microRNAs, a class of non-protein-coding RNAs that bind to specific sites in the 3' untranslated region (UTR) resulting in posttranscriptional regulation (Kulkarni, Savan et al. 2011). MicroRNA-148a was shown to regulate the expression of HLA-C in an allele-specific manner by differential binding to its binding-site, a single base pair insertion/deletion at position 263 in the *HLA-C* 3'UTR (Kulkarni, Savan et al. 2011). Binding of microRNA-148a to alleles marked by 263

insertion (for example *Cw*0702*, *Cw*0303*, *Cw*0401*, *Cw*0701*) resulted in relatively lower surface expression of these alleles while binding to those alleles marked by 263 deletion (for example *Cw*0602*) resulted in high expression of HLA-C alleles (Kulkarni, Savan et al. 2011). The continuous gradient of HLA-C expression demonstrated above suggests that microRNA-148a regulation is not the sole mechanism involved. It has been suggested that additional *cis*-acting factors may fine tune HLA-C expression in an allotype-specific manner and *trans*-acting factors unlinked to the *HLA-C* locus may also affect expression levels in a manner that is independent of HLA-C allotype (Kulkarni, Savan et al. 2011).

From the data presented above, the lower staining of the -35 *TT* group was wholly due to significantly lower DT9 staining in individuals homozygous for *HLA-Cw*07*. In this cohort of 147 individuals, this allele was very common and the homozygous haplotype was present in 10% of individuals. This raised the third question of whether the -35 SNP association with viral load set point is driven by the presence of this allele. Fortunately, the group responsible for the initial observation are also part of the larger CHAVI network. They were approached with a specific question – is the *Cw*07/Cw*07* haplotype responsible for the association of the -35 *TT* genotype with higher viral load set point?

Jacques Fellay reanalysed the data in 1340 individuals from two Caucasian cohorts (Fellay, Ge et al. 2009) using a regression model including age, gender and principal components axes (to correct for population stratification). *HLA-Cw*07* homozygosity strongly associated with viral load set point ($p=5x10^{-5}$). However, once the -35 SNP was added to the model, *Cw*07* homozygosity was no longer significant (p=0.07; vs.

p=1.6x10^{-13} for the SNP alone). The exclusion of individuals homozygous for *HLA-Cw*07* did not abolish the association of the -35 SNP with control of HIV-1 infection. In addition, there was a significant difference in viral load set point comparing homozygosity for *Cw*07* (n=104) against homozygosity for all other HLA-C alleles (n=91) (p=2.5x10^{-5}), and this difference was even more significant comparing homozygosity for *Cw*07* against other HLA-C homozygous alleles that are in linkage disequilibrium with the -35*C* SNP allele (n=45; p=1.2x10^{-6}). However, there was no significant difference in viral load set point comparing homozygous *Cw*07* individuals to individuals homozygous for other HLA-C alleles that are in linkage disequilibrium with -35*T* (mostly *Cw*03* and *Cw*04* with n=38; p=0.06). This suggests that lower expression of HLA-Cw*07, a particularly common allele in people of European descent [phenotype frequency of 26.0-66.2% from http://allelefrequencies.net, (Gonzalez-Galarza, Christmas et al. 2011)], contributes only a part of the protective effect marked by the -35 SNP. Therefore, the -35 SNP appears to be a marker for several causal variants, most likely HLA-C alleles, with *HLA-Cw*07* undoubtedly a part of the picture but probably not alone.

The role of HLA molecules in the adaptive immune response is to present viral peptides to TCRs that ultimately activate CD8 T cells leading to lysis of infected cells. The diversity of self peptides presented in the thymus shapes the characteristics of the mature T cell repertoire. A thymocyte (immature CD8 T cell) emerges from the thymus as a mature CD8 T cell if it's TCR binds to at least one self-peptide-MHC class I molecule with an affinity that exceeds the positive selection threshold and does not interact with the same complex more strongly than the negative selection threshold (Marusic-Galesic, Stephany et al. 1988; Marusic-Galesic and Pavelic 1990).

It is possible that low expression results in positive selection of fewer HLA-Cw*07-restricted T cells in the thymus during maturation, resulting in lower numbers of these cells in the periphery. Recently, *in silico* thymic selection experiments predicted that the relative ability of protective and non-protective HLA-B alleles to control HIV-1 replication correlates with their peptide-binding characteristics during thymic development (Kosmrlj, Read et al. 2010). Protective alleles like HLA-B*57 bind fewer self-peptides in the thymus, and result in B*57-restricted CD8 T cells that are more cross-reactive to point mutants of targeted HIV-1 peptides and therefore control viral replication better (Kosmrlj, Read et al. 2010). Carrying on this analogy, it is possible that HLA-Cw*07-restricted CD8 T cells are less flexible in the sequence of HIV-1 peptides that they bind, resulting in mutated peptides easily escaping their action. Low level expression of HLA-Cw*07 could therefore result in less effective presentation of peptides to CD8 T cells and in less control of HIV-1 replication.

Consistent with these possibilities, *HLA-Cw*07* was significantly associated with the highest mean viral load set point of all the HLA-C alleles (Fellay, Ge et al. 2009). This was recently confirmed in a larger cohort in the International HIV Controllers Study which implicated seven HLA class I alleles with control of HIV-1 and showed that *HLA-Cw*07* is a risk allele associated with high viraemia and more rapid disease progression (Pereyra, Jia et al. 2010).

In conclusion, it is extremely unlikely that the association of the -35 SNP with control of HIV-1 infection is a direct result of the SNP itself, and the role of increased expression of HLA-C allotypes associated with the *-35C* SNP allele (Fellay, Shianna et al. 2007; Thomas, Apps et al. 2009; Pereyra, Jia et al. 2010) appears uncertain. The

data presented here imply that it is the particularly low expression of the -*35T*-associated HLA-Cw*07 that contributes substantially to the low HLA-C expression associated with -*35T* SNP allele, and possibly to the relatively high risk of disease progression.

Chapter 4: DOES SURFACE HLA-C EXPRESSION RELATE TO DIFFERENCES IN T CELL FUNCTION?

4.1 Introduction

In the previous chapter, DT9 antibody was used to demonstrate a difference in surface HLA-C expression at the -35 SNP genotype, although the difference between -35 *CC* and *TT* individuals was driven largely by the low expression of *HLA-Cw*07* in *TT* individuals. This chapter, describes experiments performed in parallel that investigate whether the homozygous *CC* and *TT* alleles of the -35 SNP are associated with measurable differences in immune function.

It was hypothesised that higher levels of surface expression may have several knock-on effects. The higher levels of HLA-C may mean that T cells in *CC* individuals are presented with greater numbers of HIV-1 epitopes and/or lower avidity circulating T cells may be expanded. This could in turn produce greater number of responses (breadth) and strength (magnitude) of responses by CD8 restricted T cells resulting in lower viral load. Cellular immune responses play a pivotal role in the control of HIV-1 infection. Comprehensive screening techniques with IFN-γ-based ELISpot or intracellular cytokine staining assays and overlapping peptides have allowed the assessment of T cell responses against the entire expressed HIV-1 genome (Betts, Ambrozak et al. 2001; Addo, Yu et al. 2003). Addo *et al.* used an IFN-γ ELISpot assay and overlapping peptides spanning the HIV-1 proteome to analyse the T cell response to all potential epitopes in 57 HIV-1 infected individuals at different stages of infection. They demonstrated that all HIV-1 proteins are targeted by HIV-1-specific CD8 T cells and importantly, showed that the responses detected by this

method were, with few exceptions, the result of CD8 T cell activity. In addition, they also showed that the breadth and magnitude of virus-specific T cell responses varied significantly among individuals at different stages of infection, with the broadest and strongest responses detectable for individuals with untreated chronic infection (Addo, Yu et al. 2003). These broad and strong IFN-γ-positive CD8 T cell responses were still detectable in the presence of high viral loads typical of progressive disease course (Draenert, Verrill et al. 2004). However, there was no correlation between the viral load and the total breadth and/or magnitude of CD8 T cell responses against the entire HIV-1 proteome (Betts, Ambrozak et al. 2001; Addo, Yu et al. 2003; Frahm, Korber et al. 2004; Streeck, Brumme et al. 2008). One limitation of the Addo *et al.* study was that the ELISpot assay commented on cellular cytokine function but gave no information on the cytolytic activity of CD8 T cells.

A greater breadth of responses in *CC* individuals could mean that they are more likely to target critical sites in the HIV-1 proteome. One of the major factors limiting the effectiveness of CD8 T cell responses is the propensity for HIV-1 to evade these responses through sequence evolution or viral escape (Borrow, Lewicki et al. 1997; Price, Goulder et al. 1997; Allen, Altfeld et al. 2005). Whereas CD8 T cell escape mutations provide a benefit to the virus to enable the evasion of host immune pressure, some of these mutations may come at a substantial cost to viral replication. The enforced escape mutations in these critical sites in *CC* subjects could result in a fitness cost to the virus and result in lower viral load. Several lines of evidence support a relationship between the selection of CD8 T cell escape mutations and reduced HIV-1 fitness. These include the reversion of escape mutations to a more ancestral sequence following transmission to an HLA-mismatched recipient who

cannot target the epitope (Allen, Altfeld et al. 2004; Friedrich, Dodds et al. 2004; Leslie, Pfafferott et al. 2004; Barouch, Powers et al. 2005; Li, Gladden et al. 2007; Crawford, Lumm et al. 2009), as well as reduced plasma viral load at set point following the transmission of certain escape variants from donors who expressed protective HLA alleles (Chopera, Woodman et al. 2008; Goepfert, Lumm et al. 2008). The more rapidly reverting mutations have been observed to preferentially occur at conserved residues, indicating that structurally conserved regions of HIV-1 may be particularly refractory to sequence change (Li, Gladden et al. 2007). More recently, *in vitro* studies have used a recombinant viral assay encoding single or multiple escape mutations in Gag and Pol to show reduced replicative capacity of the virus (Miura, Brumme et al. 2009; Brockman, Brumme et al. 2010; Miura, Brumme et al. 2010; Wright, Brumme et al. 2010). However, it is clear that the targeting of conserved regions alone is insufficient to account for differences in immune control, as targeting of the conserved Pol protein and conserved regions within the central part of Nef protein were not associated with effective immune control as measured by viral load (Kiepiela, Ngumbela et al. 2007).

Thus, it seems reasonable to assume that eliciting T cell responses that are efficient, preserved and not evaded by HIV-1 would be beneficial. These T cells would target conserved regions of the proteome and escape mutations to these constrained epitopes would result in a fitness cost to the escaping virus with reduced replicative capacity. The Shannon entropy score can be used to measure amino acid variability at a given position, and takes into account both the number of possible alternative amino acids at that position and their frequency, with highly conserved epitopes having a low entropy score. The entropy of the amino acids within the T cell epitopes in HIV-1

proteins can be calculated using pre-aligned sequences from HIV-1 infected individuals listed in the LANL database. This approach has been used to show that an inverse correlation exists between protein sequence variability and the presence of HIV-1 specific CD8 T cell epitopes (Yusim, Kesmir et al. 2002; Fontaine Costa, Rao et al. 2010). Yusim *et al*. observed that regions in the HIV-1 proteome with a low density of CD8 T cell epitopes were more variable than regions with a high epitope density. They further hypothesised that HIV-1 had escaped CD8 T cell epitopes predominantly in the variable protein regions and that large-scale adaptation of the ancestral HIV-1 viral sequence to the human population had resulted in the apparent clustering of CD8 T cell epitopes that is observed in current-day HIV-1 sequences (Yusim, Kesmir et al. 2002). This hypothesis of epitope clustering was opposed when it was demonstrated that the vast majority of HIV-1, hepatitis C virus and influenza proteins had a predicted CD8 T cell epitope distribution that was indistinguishable from a random distribution and that the epitope distribution in these three viral proteomes was similar to that of proteins of comparable size in the human proteome (Schmid, Kesmir et al. 2009). The entropy of HIV-1 epitopes targeted by HLA-A, -B and -C alleles across the entire clade B proteome was measured and found that those targeted by HLA-B alleles were significantly more conserved than residues targeted by HLA-A ($p<0.01$; Mann-Whitney test) (Fontaine Costa, Rao et al. 2010).

To address functional differences in IFN-γ secretion, two pilot studies were performed in treatment-naïve HIV-1 infected Caucasian individuals. As a result of the data from Addo *et al.* (Addo, Yu et al. 2003), chronically infected subjects were chosen for these studies. They involved mapping responses by *ex vivo* IFN-γ ELISpot assays in a peptide matrix system against a panel of optimally defined HLA-C restricted

epitopes and against a panel of overlapping peptides spanning the entire clade B proteome.

The ability of CD8 T cells to recognise and kill HIV-1 infected cells is dependent on the specific recognition of viral peptides presented by HLA class I molecules (Townsend, Rothbard et al. 1986; Nixon, Townsend et al. 1988). This cytotoxic activity can be measured by the chromium-release assay. This *ex vivo* assay depends on co-culturing CD8 T cells with radioactively labelled target cells. When T cells kill their targets, radioactivity is released into the supernatant and can be measured using a gamma counter. The level of radioactivity measured is proportional to cytolytic activity. To study the cytolytic activity of CD8 T cells, a panel of HLA-C restricted CD8 T cell clones were generated for use in peptide presentation studies. The hypothesis to be tested was that higher levels of surface HLA-C in B cell lines from *CC* individuals would result in increased killing as measured by the chromium release assay while lower levels of HLA-C in *TT* individuals presenting the same epitope to the same CD8 T cell clone would result in less killing due to differences in epitope presentation.

The observations by Thomas *et al.* (Thomas, Apps et al. 2009) and confirmed in the previous chapter of this thesis that the -35 SNP is in strong linkage-disequilibrium with certain HLA-C alleles does not negate these studies as different HLA-C alleles bind distinct peptide binding motifs (Rammensee, Bachmann et al. 1999) and consequently present different epitopes to T cells. It remains a possibility that certain HLA-C alleles may present peptides that are more or less prone to escape, explaining the association of the -35 SNP with viral load set point. This was explored by

comparing entropy scores of defined optimal HLA-C restricted HIV-1 epitopes with the hypothesis that epitopes presented by HLA-C alleles associated with -35*C* would be more conserved and lead to immunodominant CD8 T cell responses that control viral replication.

4.2 Results

4.2.1 Functional differences in IFN-γ secretion at the -35 SNP

The matrix approach is an epitope screening method that employs peptide pools in a matrix array in ELISpot assays (Roederer and Koup 2003). The identity of individual recognised peptides can be deduced from the pattern of recognised peptide pools. These individual peptides are then confirmed in second round (deconvolution) ELISpot assays, leading to a positive result with the minimal use of cells.

This approach was used to study HLA-C restricted responses in two HIV-1 infected Caucasian cohorts. In all cases, the ELISpot assays were performed while blinded to the -35 SNP genotype of the individuals being studied.

4.2.1.1 Mapping of T cell responses against optimal HLA-C restricted epitopes

The LANL database was scanned for previously described optimal HLA-C epitopes (Frahm, Linde et al. 2007) and these epitopes were synthesized for screening in a peptide matrix system. These 48 epitopes (Appendix I) were assigned to 12 peptide pools (8 peptides per pool) with each epitope appearing in two separate peptide pools. As an additional control, peptides spanning the entire HIV-1 proteome split into 4

peptide pools (Gag; Env; Pol; Nef and Rev/Tat/Vpu/Vpr/Vif in one Nef/accessory 'acc' pool) were screened (Table 4.1) in *ex vivo* ELISpot assays. Cultured ELISpot assays were also used to confirm that responses detected *ex vivo* after deconvolution were true positives.

Twenty-five HIV-1 infected individuals from the SCOPE cohort, who were either -35 *CC* or *TT*, were screened for HLA-C-restricted T cell responses (Table 4.2). Nineteen of the 25 individuals were *CC* at the -35 SNP. Six of these 25 individuals had at least two positive responses (as defined in Materials and Methods section) in the 12 matrix pools (containing described optimal HLA-C epitopes) and went on to be deconvoluted (Figure 4.1). Putative HLA-C restricted peptide responses were confirmed in four out of these six individuals (Figure 4.2).

There were sufficient cells in only three of the six individuals that were deconvoluted to set up short term cell lines (STCLs). These STCLs were used in a cultured ELISpot assay and confirmed the deconvolution *ex vivo* ELISpot results, i.e. SCOPE 1242 and SCOPE 1534 responded to the same peptide they had *ex vivo* while SCOPE 1213 did not make a HLA-C restricted peptide response (Figure 4.3). Only one of the four optimal HLA-C restricted peptides matched the individual's HLA-C alleles (Table 4.3). None of these putative HLA-C restricted responses were confirmed by HLA restriction.

There was a low frequency of putative HLA-C restricted T cells in chronically infected HIV-1 patients with only 4 out of 25 (16%) SCOPE individuals recognising putative HLA-C restricted peptides (Table 4.3). Three subjects were homozygous for the protective allele (-35 *CC*) and clearly there were insufficient numbers to determine

differences in the response rate between *CC* and *TT* alleles. The HLA-C restricted T cell responses contributed only a small proportion of overall T cell response against clade B consensus peptides covering the entire HIV-1 proteome (Figure 4.1).

Table 4.1. Composition of clade B peptide pools.

PEPTIDE POOL	No. OF PEPTIDES PER POOL
Gag	68
Pol	125
Env	111
Nef/Accessory	94

Nef/Accessory pool contained Nef, Rev, Tat, Vif, Vpr and Vpu peptides. All the peptides in each pool were 18mers overlapping by 10 amino acids.

Table 4.2. Summary of SCOPE subjects studied with IFN-γ ELISpot assays.

SCOPE SUBJECT	-35 SNP	HLA-B*27 OR HLA-B*57	[a]SUCCESSFUL R1	[b]SELECTED FOR R2	SUFFICIENT CELLS FOR CULTURED ELISPOT	HLA-C RESTRICTED RESPONSES
1037	CC	YES	YES	NO		
1074	CC	NO	YES	NO		
1086	CC	YES	YES	NO		
1111	CC	YES	YES	NO		
1120	CC	NO	YES	YES	NO	YES
1175	CC	YES	YES	NO		
1189	CC	NO	YES	NO		
1204	CC	YES	YES	NO		
1213	CC	NO	YES	YES	YES	NO
1218	CC	YES	YES	NO		
1242	CC	NO	YES	YES	YES	YES
1255	CC	NT	YES	NO		
1278	CC	YES	YES	NO		
1291	CC	YES	YES	NO		
1296	CC	YES	YES	NO		
1510	CC	NO	YES	NO		
1517	CC	YES	YES	NO		
1529	CC	YES	YES	NO		
1534	CC	NO	YES	YES	YES	YES
1072	TT	NO	YES	YES	NO	NO
1127	TT	NO	YES	NO		
1151	TT	NO	YES	YES	NO	YES
1198	TT	NO	YES	NO		
1209	TT	NO	YES	NO		
1258	TT	NT	YES	NO		

[a] PHA response > 300 spot forming units/million cells (SFU/M) and a positive response (>50 SFU/M) in at least one clade B consensus pool; R1 = first round ELISpot

[b] Positive response (>30 SFU/M) in at least 2 HLA-C peptide pools; R2 = second round (deconvolution) ELISpot

NT = not HLA typed

Table 4.3. SCOPE subjects that recognised putative HLA-C epitopes.

SUBJECT	-35 SNP	HLA-A	HLA-A	HLA-B	HLA-B	HLA-C	HLA-C	PEPTIDE ID	LANL HLA-C RESTRICTION
1120	CC			3910	4405	0202	1203	P802	0802
1151	TT			4101	4402	0501	1701	P3944	0802
1242	CC			1402	3901	***0802***	1203	P916	***0802***
1534	CC	2403	3301	1402	5001	0602	0802	P922	03

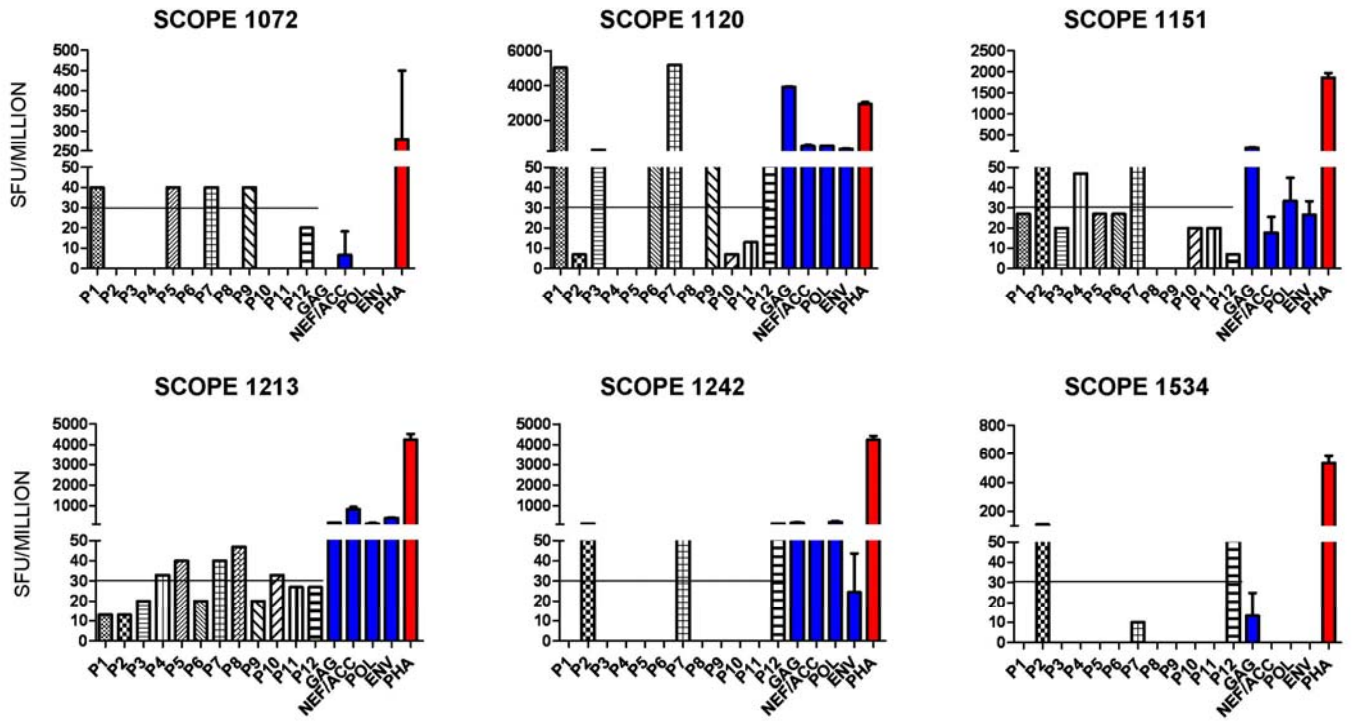


Figure 4.1. First round *ex vivo* ELISpot assays of the 6 SCOPE subjects selected for deconvolution.
PBMC were stimulated with pools of previously defined optimal HLA-C restricted CD8 T cell peptides arranged in a matrix format (P1-P12), clade B consensus peptides spanning the entire proteome grouped by protein (blue bars) and phytohaemagglutinin (PHA) which served as a positive control (red bars). Cells were then assayed for IFN-γ production.
Data are expressed as background-subtracted spot forming units per million cells (SFU/million). Error bars indicate the standard error of the mean. The black line at 30 SFU/million shows the threshold above which responses were considered positive.

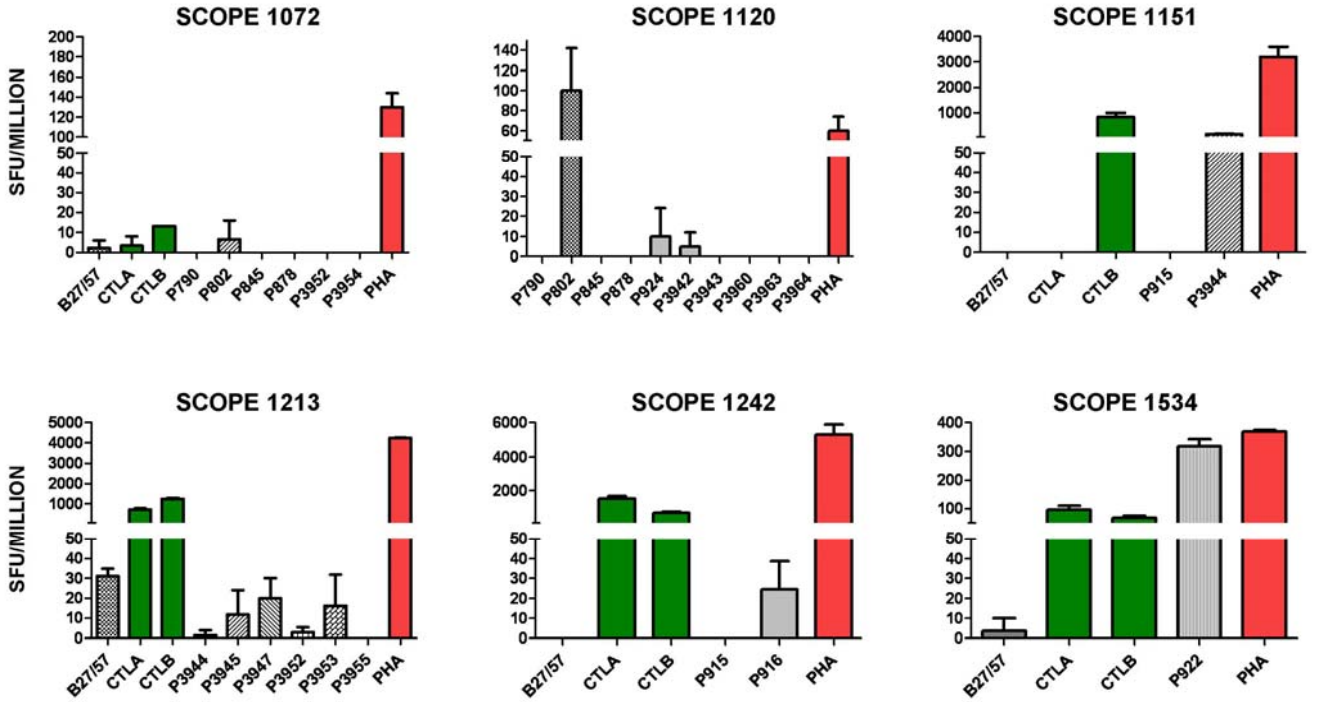


Figure 4.2. Deconvoluted *ex vivo* ELISpot assays against individual HLA-C restricted peptides.
PBMC were stimulated with pools of previously defined optimal HLA-B*27/B*57 peptides (B27/57), optimal HLA-A and HLA-B restricted peptides (CTLA and CTLB; green bars), the putative HLA-C restricted peptide and phytohaemagglutinin (PHA) which served as a positive control (red bars). Cells were then assayed for IFN-γ production. Four subjects (SCOPEs 1120, 1151, 1242, 1534) had responses to individual HLA-C restricted peptides. There were insufficient cells for SCOPE 1120 to stimulate with CTLA and CTLB pools.
Data are expressed as background-subtracted spot forming units per million cells (SFU/million). Error bars represent the standard error of the mean.

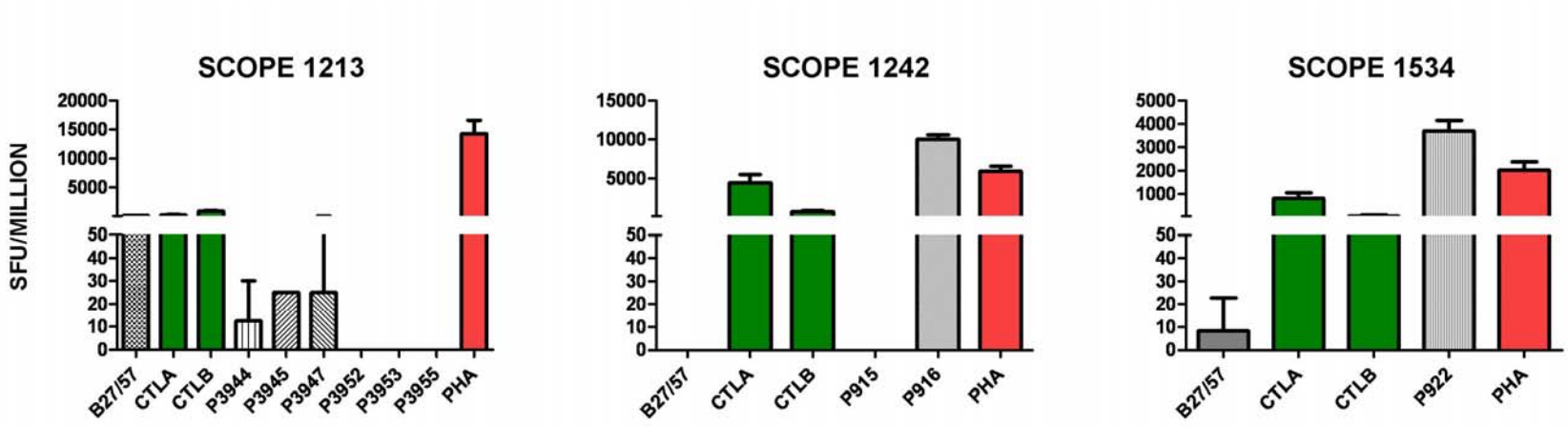


Figure 4.3. Cultured ELISpot assays against individual HLA-C restricted peptides.
PBMC were cultured with the relevant peptide for 10 days in the presence of recombinant interleukin 2 and 7. The cells were then washed and rested for 30 hours before being used in ELISpot assays for IFN-γ production. The rested cells were re-stimulated with previously defined optimal HLA-B*27/B*57 peptides (B27/57), optimal HLA-A and HLA-B restricted peptides (CTLA and CTLB; green bars), the relevant HLA-C restricted peptide and phytohaemagglutinin (PHA) which served as a positive control (red bars). These cultured ELISpot assays confirmed the response of SCOPE 1242 and SCOPE 1534 to the HLA-C restricted peptide initially detected by *ex vivo* deconvolution ELISpot assays.
Data are expressed as background-subtracted spot forming units per million cells (SFU/million). Error bars represent the standard error of the mean.

4.2.1.2 Mapping of T cell responses against the entire HIV-1 proteome

The approach of screening for responses against defined epitopes described above had one major limitation – HLA-C restricted T cell epitopes that had not been previously defined in the literature were missed. Therefore 15 HIV-1 infected individuals (9 *CT* and 6 *TT*) from the CHAVI 001 cohort were mapped for T cell responses directed against the entire expressed HIV-1 genome (Table 4.4), by using overlapping peptide pools covering the entire clade B proteome. The *CT* genotype appears to be intermediate to *CC* and *TT* with a single -35*C* copy conferring protection (Fellay, Shianna et al. 2007; Fellay, Ge et al. 2009; Thomas, Apps et al. 2009). In fact, each copy of the -35*C* allele associates with a 0.3log lower viral load set point (Fellay, Ge et al. 2009). In the small cohort presented here, *CT* individuals had a significantly lower viral load set point than *TT* individuals (p=0.0176; Mann-Whitney test) (Figure 4.4), suggesting that there may be T cell differences between the two genotypes.

There were 398 18mer peptides (overlapping by 10 amino acids) assigned to 120 pools (with 10 peptides per pool) with each peptide appearing in three separate pools. All T cell responses to 18mer peptides identified by the matrix approach were subsequently reconfirmed individually in a second assay. Within a responding peptide, the optimal epitope and HLA restriction were inferred from the LANL database using the Epitope Location Finder tool (http://www.hiv.lanl.gov/content/sequence/ELF/epitope_analyzer.html). Short term cell lines were generated and the 18mer peptides, together with the described optimal epitopes within them, were confirmed in cultured ELISpot assays.

Table 4.4. CHAVI 001 subjects screened for T cell responses against the entire HIV-1 clade B proteome.

SUBJECT	-35 SNP	HLA-A	HLA-A	HLA-B	HLA-B	HLA-C	HLA-C	VIRAL LOAD SET POINT (copies/ml)	No. of 18MER RESPONSES [a](DESCRIBED, PREDICTED)	No. of 18MER RESPONSES WITH NO DESCRIBED OPTIMAL
700010094	CT	0301	2902	1801	4901	0501	0701	22, 909	3 (0,2)	1
700010111	CT	0301	0301	0702	4002	0202	0702	14, 125	8 (0,6)	2
700010174	CT	1101	2601	3801	5601	0102	1203	*566, 667*	7 (1,3)	4
700010271	CT	0201	3001	1302	4001	0302	0605	*49, 447*	3 (1,2)	0
700010516	CT	0101	3202	5601	5701	0102	0602	6, 310	4 (0,4)	1
700010802	CT	0201	3101	1302	1501	0302	0602	1, 738	10 (0,10)	2
709020528	CT	0101	0101	0801	5701	0605	0701	*14, 231*	5 (1,4)	0
709020620	CT	0201	1101	0702	5201	0702	1202	*10, 838*	7 (1,6)	1
709020645	CT	0301	2402	4402	5101	0501	1502	*4, 368*	7 (0,2)	3
700010742	TT	0301	2301	4403	5301	0401	1601	123, 027	5 (0,4)	3
700010783	TT	1101	3201	1801	3501	0401	0701	*63, 386*	12 (1,9)	2
700010791	TT	0201	0301	0702	0702	0702	0702	*35, 964*	9 (1,8)	1
701010114	TT	0101	0101	0702	0801	0701	0702	*146, 775*	13 (1,12)	3
701010186	TT	0201	0301	1501	4403	0302	1601	*56, 540*	4 (0,0)	0
701010359	TT	0301	1101	0702	3501	0401	0702	*186, 771*	21 (2,17)	5
T0TAL	15								118 (9,89)	28

[a] Previously described and predicted (based on peptide-binding motifs) HLA-C restricted optimal epitopes from LANL database.
The viral load set point values in italics were calculated by averaging 3-6 consecutive viral load readings while those in normal font were calculated by the more accurate Fellay *et al.* (Fellay, Shianna et al. 2007) method defined in Materials and Methods.

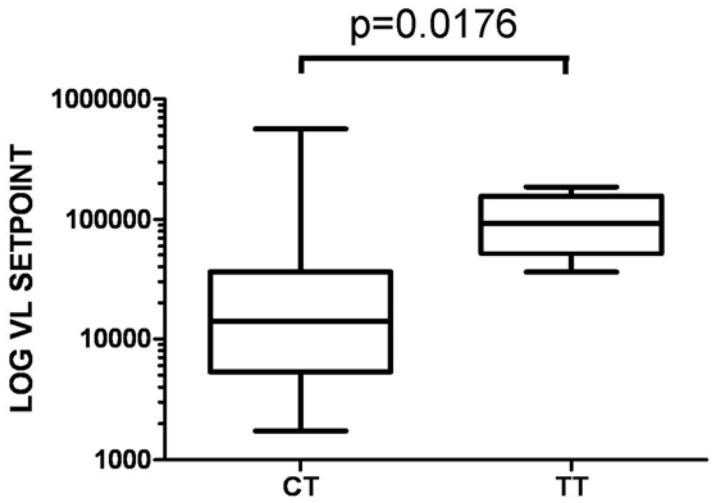


Figure 4.4. -35 SNP *CT* genotype is associated with lower viral load set point than *TT* genotype.
The box and whisker plots represent the minimum and maximum values (whiskers) and the 25th percentile, median and 75th percentile (box). There were 9 *CT* and 6 *TT* HIV-1 seropositive individuals studied. Statistical analysis performed using the Mann-Whitney test.

Across all the individuals studied, 69 18mer peptides, spanning all HIV-1 proteins except Rev, were targeted by T cells, with Nef (13 of 25 [52%]) and Gag (17 of 68 [25%]) containing the most widely targeted peptides (Figure 4.5), as previously described (Addo, Yu et al. 2003). Representative second line and cultured ELISpot assays for one of the studied individuals are shown in Figure 4.6. There was no statistically significant difference in the number of peptides recognised by -35 *CT* and *TT* individuals (median 7, range 3-10; and median 10.5, range 4-21, respectively; p=0.1094, Mann-Whitney test) (Figure 4.7A). Similarly there was no statistically significant difference in the magnitude of responses against the entire proteome at the -35 SNP genotype (median 2317 sfu/million, range 733-4135 sfu/million for *CT* group; and median 3225 sfu/million, range 656-10605 sfu/million for *TT* group; p=0.3277, Mann-Whitney test) (Figure 4.7B). Interestingly, the median breadth and magnitude of responses was greater in *TT* individuals and it may simply be that failure to detect statistically significant differences between the two groups was a result of too small a cohort to assess what may be subtle allelic differences. However, the magnitude of the total T cell responses correlated significantly with the breadth of responses (Spearman r=0.7871, p=0.0005) (Figure 4.7C).

Within these 69 targeted 18mer peptides there were 75 previously described optimal epitopes. However, there were no described optimal epitopes within some of these 18mers (Table 4.4), suggesting that the number of true optimal epitopes within this study cohort is underestimated. Some 18mers and optimal epitopes were recognised by only one individual while others were targeted by multiple individuals independent of their HLA types (Figure 4.5). Of these 75 optimal epitopes, only 5 (6.6%) were restricted by HLA-C alleles. Table 4.4 also lists the number of predicted (not already

described on LANL database) HLA-C optimal epitopes within the 18mer based on each individual's HLA type. It is important to note that peptide binding motifs for HLA-A and HLA-B alleles also expressed by the subjects were present within these 18mers. Unfortunately, the cost of manufacturing all these 'potential' optimal epitopes and the vast number of cells needed precluded the confirmation of HLA restrictions. Even if all HLA restrictions had been confirmed, the proportion of HLA-C restricted responses is unlikely to have increased given that new HLA-A and HLA-B restricted responses would also be detected.

While data was available for only 40 Caucasian subjects across the two cohorts studied, it suggested that HLA-C restricted responses, either *CC*, *CT* or *TT*, were not immunodominant whether measured by breadth or magnitude of response. This is consistent with literature that HLA-B (followed by HLA-A) restricted T cell responses are dominant in HIV-1 infection (Kiepiela, Leslie et al. 2004; Bihl, Frahm et al. 2006; Kiepiela, Ngumbela et al. 2007). Discussions with the biostatistician, Dr Peter Gilbert (SCHARP, Washington DC) based on these data and the known frequency of the two genotypes in Caucasian populations, suggested that at least 400 HIV-1 seropositive individuals would need to be screened to have sufficient power to detect a difference (probably small) in HLA-C restricted responses between *CC* and *TT* individuals. These studies were not pursued further because it was not feasible to find this number of untreated HIV-1 seropositive individuals to study. In addition, it did not seem likely that such a significant population-wide effect by the -35 SNP could be accounted for by T cell responses limited to at best 16% (4/25 individuals had a putative HLA-C restricted response) of the population especially since, even in

the small numbers studied here, these HLA-C restricted T cell responses were detected in all -35 SNP (*CC*, *CT*, *TT*) genotypes.

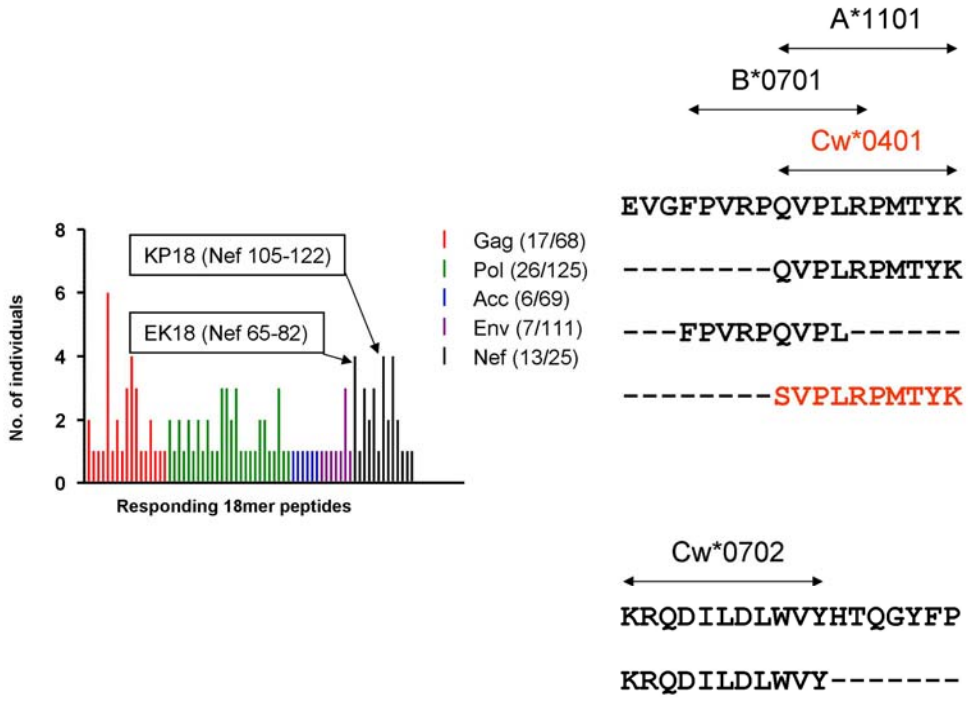
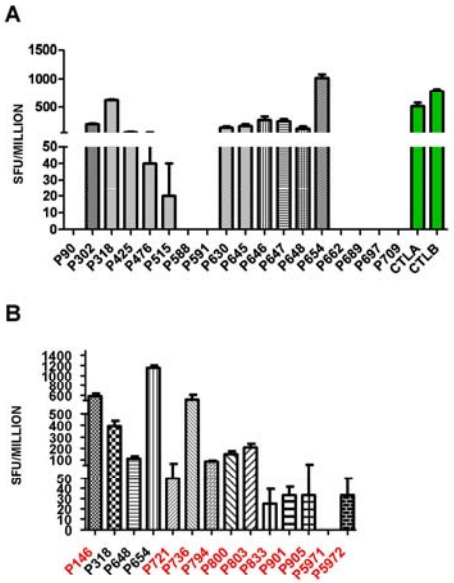


Figure 4.5. Positive 18mer peptide responses across the entire expressed HIV-1 genome.
PBMC from 15 individuals were stimulated with pools of overlapping 18mer peptides covering the HIV-1 clade B proteome arranged in a matrix format and responding peptides in these pools were then confirmed individually by assaying for IFN-γ production. The 69 responding peptides are represented on the *x* axis and the corresponding number of individuals with a response to the individual peptide are represented on the *y* axis. The sequences of two peptides are highlighted to demonstrate that one or more optimal epitopes may exist within an 18mer and that certain epitopes can be presented by more than one HLA molecule.



PEPTIDE	SEQUENCE	HLA RESTRICTION
302	EVGFPVRPQVPLRPMTYK	
5971	---FPVRPQVPL------	B*0702
5972	------RPQVPLRPM---	B*0702
736	--------QVPLRPMTYK	A*0301
318	HMARELHPEYYKDC	
	NO DESCRIBED OPTIMAL	
425	TACQGVGGPGHKARVLAE	
803	-------GPGHKARVLA-	B*07
630	RAILHIPRRIRQGLERAL	
794	-----IPRRIRQGL----	B*0702
645	KRQDILDLWVYHTQGYFP	
146	KRQDILDLWVY-------	Cw*0702
646	FPDWONYTPGPGIRYPLT	
800	-------TPGPGVRYPL-	B*0702
647	PGPGIRYPLTFGWCFKLV	
833	------YPLTFGWCY---	B*0702
901	------YPLTFGWCF---	B*0702
721	-------PLTFGWCYKL-	A*0201
905	--------LTFGWCFKL-	A*0201
648	LTFGWCFKLVPVEPEKVE	
	LTFGWCFKL---------	A*0201
654	FDSRLAFHHMARELHPEY	
	-----AFHHVAREL----	A*0201

Figure 4.6. Representative ELISpot assays for one individual (700010791) confirming 18mer peptide and optimal epitope responses.
(A) PBMC were stimulated with the putative 18mer peptides, pools of previously defined optimal HLA-A and HLA-B restricted peptides (CTLA and CTLB; green bars), and phytohaemagglutinin (PHA) which served as a positive control (magnitude 4825 sfu/million). Cells were then assayed for IFN-γ production.
(B) PBMC were cultured with the responding 18mer peptides for 10 days in the presence of recombinant interleukin 2 and 7. The cells were then washed and rested for 30 hours before being used in ELISpot assays for IFN-γ production. The rested cells were re-stimulated with 18mer peptides and optimal epitopes within these (where available) and PHA which served as a positive control (magnitude 6200 sfu/million).
Peptides in black represent 18mer peptides while those in red are optimal epitopes. Data are expressed as background-subtracted spot forming units per million cells (SFU/million). Error bars represent the standard error of the mean.

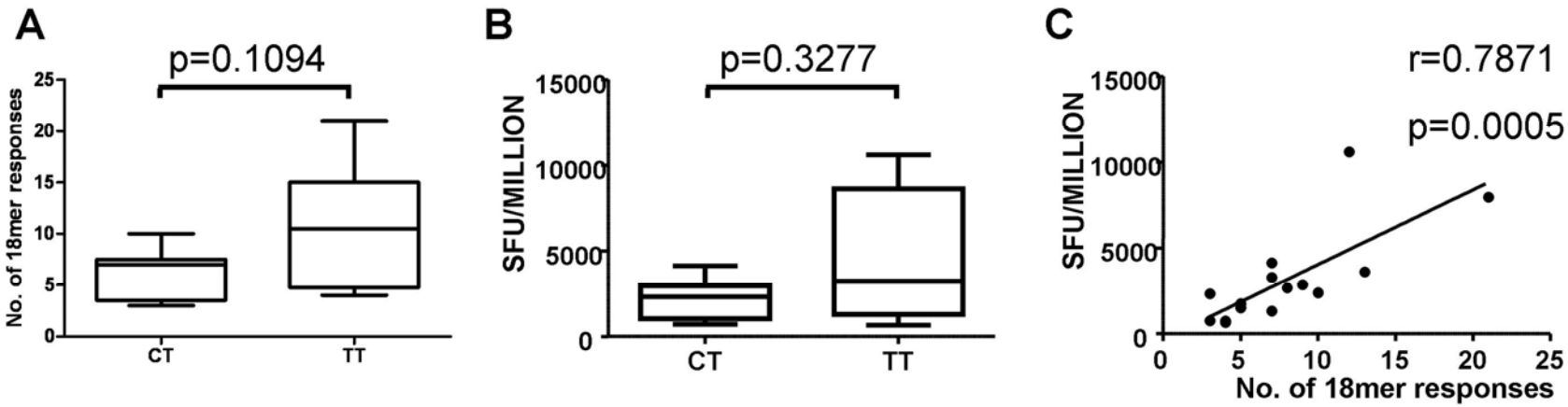


Figure 4.7. Comparison of breadth and magnitude of 18mer peptide responses between -35 *CT* and *TT* individuals.
Panel A shows that there is no difference in breadth of responses against HIV-1 peptides at the -35 SNP genotype. Panel B similarly shows no difference in the magnitude (given in spot forming units per million, sfu/million) of T cell responses to the entire expressed HIV-1 genome at the -35 SNP genotype. However, there is strong positive correlation between the breadth and total magnitude of HIV-1 specific T cell responses (Panel C). The box and whisker plots in panels A and B represent the minimum and maximum values (whiskers) and the 25th percentile, median and 75th percentile (box). Statistical analysis performed with the Mann-Whitney test for panels A and B, and Spearman rank test for panel C.

4.2.2 Functional differences in antigen presentation at the -35 SNP

HLA-C restricted CD8 T cell clones were generated to determine whether the levels of HLA-C expression affect HIV-1 antigen presentation. In brief, PBMCs from HIV-1 infected individuals with previously mapped *ex vivo* ELISpot T cell responses to HLA-C restricted epitopes were used to generate BCLs and STCLs against these selected epitopes. The ELISpot results were confirmed in IFN-γ intracellular cytokine staining assays using the STCLs. The STCLs against these selected epitopes were then cell sorted on CD8 and IFN-γ positive cells and these double positive cells were then expanded to generate HLA-C restricted CD8 T cell clones by limiting dilution. Finally, the clones were tested in *ex vivo* IFN-γ ELISpot assays against the individual epitopes used to generate the STCL and used in chromium release assays against autologous BCLs to confirm peptide specificity and cytolytic activity respectively of the generated clones.

In total, 9 HLA-Cw*0102, 5 HLA-Cw*0801 and 4 HLA-Cw*0802 restricted CD8 T cell clones were established from three HIV-1 infected individuals. Unfortunately, it was not possible to establish HLA-Cw*07-restricted clones which tended to die out rapidly. Figure 4.8 demonstrates the various stages leading to the generation of a Cw*0102-restricted CD8 T cell clone. In an attempt to quantify antiviral activity of CD8 T cells, the functional avidity of exogenously added peptides is used as a surrogate for endogenously produced viral epitopes (Alexander-Miller, Leggatt et al. 1996; Yang, Sarkis et al. 2003). This functional avidity is expressed as the sensitising dose of exogenously added peptide resulting in half-maximal CD8 T cell triggering against uninfected target cells and is measured by assessing cytolysis of target cells or release of cytokine over various concentrations of added peptide. The HLA-C

restricted clones generated here showed 100-fold differences in functional avidity depending on the antigenic specificity of the clones and despite these different antigenic specificities kill exogenously peptide-loaded cells with similarly high efficiency (Figure 4.9). In addition, these HLA-C restricted clones had comparable avidity and cytolytic activity as measured by chromium release assay to other HLA-A and HLA-B restricted clones in the literature (Yang, Kalams et al. 1996; Yang, Sarkis et al. 2003; Bennett, Ng et al. 2007), suggesting that despite their lower surface expression, HLA-C restricted CD8 T cells are capable of playing a functional role in controlling HIV-1 replication *in vivo*.

Unfortunately, the strong linkage disequilibrium of the two -35 SNP alleles with groups of HLA-C alleles (see previous chapter) made it impossible to perform these presentation experiments as initially designed (outlined in the introduction to this chapter). However, the possibility remains that certain common alleles such as HLA-Cw*07 (-35*T* associated) on the one hand and HLA-Cw*02 (-35*C* associated) on the other, which respectively are associated with different rates of HIV-1 disease progression (Carrington and O'Brien 2003), may present epitopes that are more or less prone to escape and this was therefore measured.

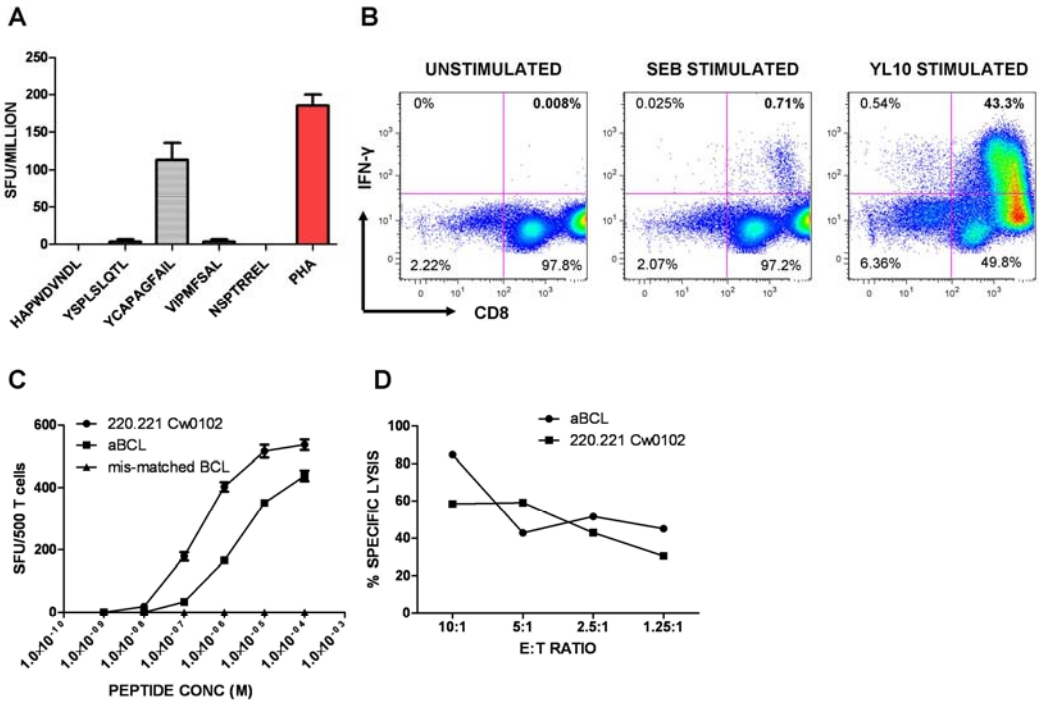


Figure 4.8. The generation of Cw*0102-restricted CD8 T cell clones against the epitope YCAPAGFAIL (YF10, Env 217-226).

(A) PBMC from an HIV-1 infected individual were stimulated with previously described Cw*0102 restricted epitopes in an IFN-γ ELISpot assay and the YL10 epitope responded with 110 sfu/million. (B) Short term T cell lines were generated by culturing PBMC with YL10 peptide for 10 days in the presence of recombinant IL2 and IL7. These T cell lines were then restimulated with YL10 and staphylococcal endotoxin B (SEB) for an hour. Brefeldin A was then added and the cells rested for at least 5 hours before staining for cell surface markers. The cells were then permeabilised and finally stained with anti-IFN-γ monoclonal antibody. SEB served as a positive control and an unstimulated control was also set up. The T cell line secreted IFN-γ on antigen stimulation and double positive cells (CD8 positive and IFN-γ secreting) were subsequently used to generate T cell clones by limiting dilution. These clones were tested in an IFN-γ ELISpot assay (C) and a 4 hour chromium release assay with peptide-pulsed autologous B cell line (aBCL) or HLA-matched transfected cell line targets (D). To confirm HLA restriction of the clones, a BCL which was matched for all HLA alleles except Cw*0102 was also used to present peptide to clones (C).

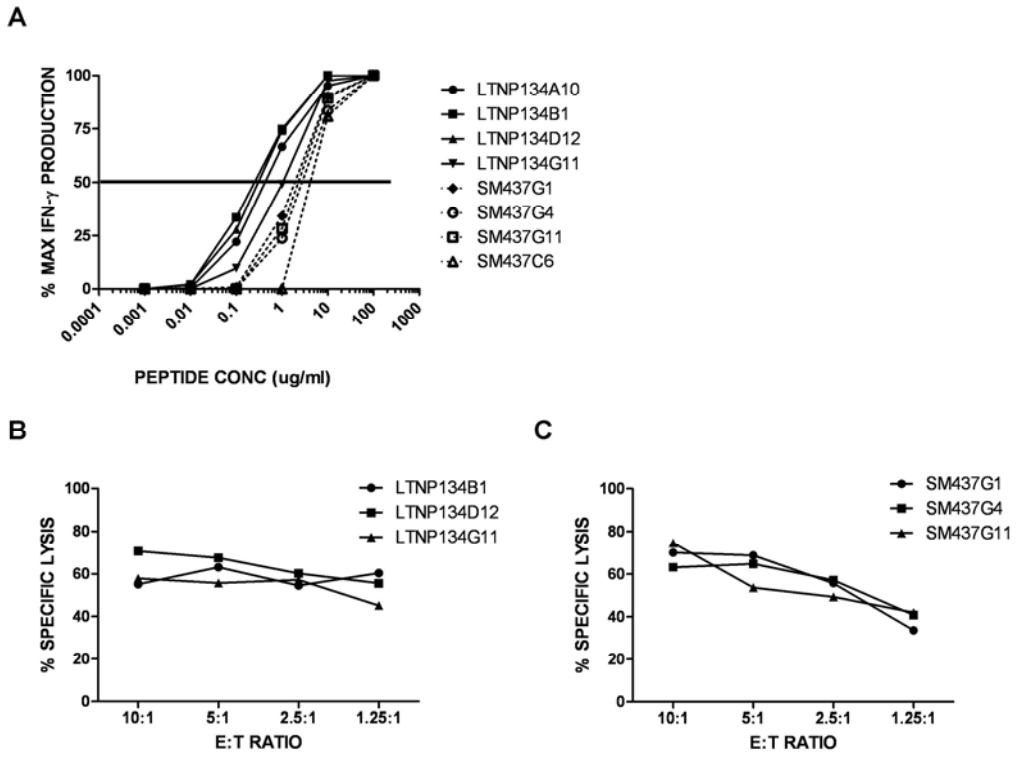


Figure 4.9. Functional avidity and killing capacity of CD8 T cell clones.
Several clones were generated against the Cw*0801 restricted epitope NCSFNISTSI (Env 156-165; subject SM437) and the Cw*0802 restricted epitope VTDSQYALGI (Pol 561-660; subject LTNP134). (A) An example demonstrates that the indicated clones differ markedly in functional avidity by peptide titration in the IFN-γ ELISpot assay. Despite these differences in avidity, Cw*0802 restricted clones (B) and Cw*0801 restricted clones (C) displayed similar cytolytic activity as measured in a standard 4 hour chromium release assay with peptide-pulsed autologous BCL targets.

4.2.3 Evaluation of amino acid conservation in epitopes presented by -35 SNP linked HLA-C alleles

A list of defined optimal HLA-C restricted epitopes was obtained from the LANL database (Frahm, Linde et al. 2007). After omitting epitope sequences that were near identical (one extra amino acid at either terminal), presented by both -35*C* and -35*T* associated HLA-C alleles and HLA-Cw*18 restricted epitopes (rare in Caucasians), there remained only 9 epitopes restricted by -35*C* associated HLA-C alleles and 3 epitopes restricted by -35*T* associated HLA-C alleles (Table 4.5). In order to assess the degree of conservation in all HLA-C restricted epitopes, the full collection of pre-aligned sequences from clade B HIV-1 infected individuals available in the LANL database were used to calculate the Shannon entropy score per amino acid residue within each epitope. These entropy scores for each amino acid position were then averaged to give an epitope entropy score and these epitope entropy scores were subsequently compared between the two groups. Highly conserved epitopes will have a low entropy score while variable epitopes, reflecting multiple amino acid substitutions within an epitope at the population level, will have a high entropy score. Entropy analysis showed no difference in conservation of epitopes presented by HLA-C alleles in linkage disequilibrium with the alleles of the -35 SNP ($p=0.8636$, Mann-Whitney test; Figure 4.10).

Table 4.5. List of epitopes analysed for Shannon entropy scores.

SEQUENCE	HIV PROTEIN/aa POSITION[a]	HLA-C ALLELE	-35 SNP ALLELE	No. OF SEQUENCES ANALYSED
VIPMFSAL	Gag 168-175	0102	C	3870
NSPTRREL	Pol 24-31	0102	C	4136
YVDRFFKTL	Gag 296-304	0303	T	3539
SFNCGGEFF	Env 375-383	0401	T	5852
AEQASQEVKNWM	Gag 306-317	05	C	3772
SAEPVPLQL	Rev 67-75	0501	C	2284
YFPDWQNYT	Nef 120-128	06	C	3153
KRQEILDLWVY	Nef 105-115	07	T	3087
TPQDLNTML	Gag 180-188	0802	C	3881
IVTDSQYAL	Pol 650-658	0802	C	2190
HLVWASREL	Gag 33-41	0804	C	4936
CCFHCQVC	Tat 30-37	12	C	1556

[a] The position refers to the position in HXB2 reference sequence.

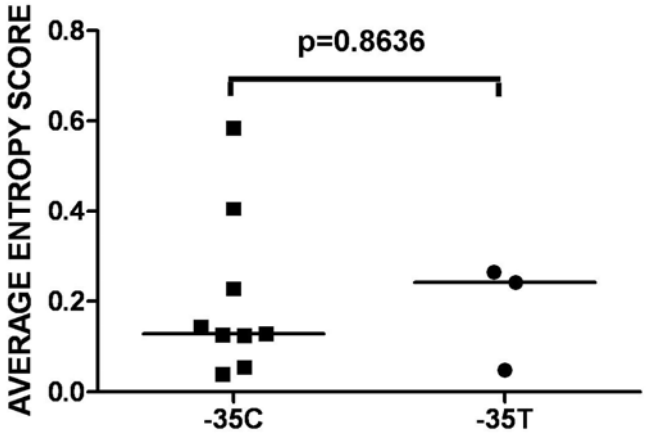


Figure 4.10. Average entropy scores for LANL listed HLA-C restricted epitopes. There is no significant difference in epitope entropy scores between -35*C* linked and -35*T* linked HLA-C alleles. The horizontal bar represents the median entropy score in each group. Statistical analysis was performed using the Mann-Whitney test.

4.3 Discussion

Having established a difference in surface HLA-C expression at the -35 SNP genotype, this chapter focussed on determining if this resulted in measurable differences in immune function. Immune function was measured by cytokine secretion (IFN-γ ELISpot assay) and cytolytic activity (chromium release assay). The conservation of epitopes recognised by HLA-C restricted T cells was also examined with the hypothesis that epitopes restricted by HLA-C alleles associated with the -35*C* allele would be the most conserved.

The IFN-γ ELISpot assay was used to detect responses against a panel of optimal HLA-C restricted epitopes. This strategy biases detected responses to those that have been previously reported, so in addition, all T cell responses against HIV-1 clade B proteome were mapped and attempts were made to determine those responses that were HLA-C restricted. The use of clade B consensus peptides is likely to favour the detection of responses in well-conserved areas of the genome, since differences between the autologous virus sequence and the consensus peptides are smallest within these regions, potentially underestimating detected responses. It has been demonstrated that the magnitude and breadth of HIV-1 specific T cell responses directed against p24 Gag, Vpr and Tat are greater with peptides spanning autologous sequences compared to consensus sequences, with 29% of peptide-specific responses detected by autologous virus sequences missed by the sole use of consensus peptides (Altfeld, Addo et al. 2003).

When mapping T cell responses against the panel of optimal epitopes, at best 16% of subjects had a detectable response to a HLA-C restricted peptide. This low response

could be due to the fact that mapping was against LANL-described optimal peptides. This database is not exhaustive and therefore the total breadth and magnitude of responses may be underestimated, as potential responses to as yet undefined epitopes will be missed. The subject SCOPE 1242 has a Cw*0802 allele and recognised the Nef epitope AL9 (P916) as predicted by the LANL database. SCOPE 1120 recognised the p24 epitope TL9 (P802). According to the LANL database, this epitope is restricted by B*07/B*39/B*42/B*53/B*81/Cw*0802. The subject carries the B*3910/Cw*0202/Cw*1203 alleles. It is likely that this response was restricted by B*39. This suggests that the percentage of HLA-C restricted T cell responses observed could be even lower. SCOPE 1151 and SCOPE 1534 recognised the p24 epitope RV9 (P3944) and the Nef epitope AL9 (P922) respectively. These subjects did not carry any of the class I alleles that the LANL database listed as restrictions for these peptides, raising the possibility of new HLA-C restricted responses (or even more likely new HLA-A or HLA-B responses).

The SCOPE cohort subjects studied were almost all LTNPs and were heavily enriched for the protective HLA-B*27 and HLA-B*57 alleles. Fellay *et al.* showed that *HLA-B*5701* is in linkage disequilibrium with the -35*C* allele (r^2=0.05, D'=0.84) (Fellay, Shianna et al. 2007), therefore this cohort was highly skewed toward the *CC* genotype. The concept of immunodomination refers to the ability of CD8 T cells specific for immunodominant epitopes to suppress the CD8 T cell response to subdominant epitopes (Yewdell and Bennink 1999). Altfeld *et al.* demonstrated in a cohort of 104 individuals with primary HIV-1 infection an active immunodomination of HLA-B*27 and HLA-B*57 restricted CD8 T cell responses over other HLA allotypes (Altfeld, Kalife et al. 2006). They had previously shown that the immunodominant HLA-B*57 restricted CD8 T cell response persisted over time

(Altfeld, Addo et al. 2003). This could be another explanation for the low frequency of HLA-C restricted responses detected in the present study.

When mapping responses against peptides spanning the entire consensus clade B proteome, optimal epitopes within responding 18mer peptides were inferred from the LANL database based on the HLA alleles expressed by the individual studied. This method resulted in some optimal epitopes being missed as not only were predicted epitopes based on HLA-C peptide binding motifs not tested, but there were also incidences of no described epitopes within several responding 18mers. Using this method, there were 75 immunodominant responses with HLA-C restricted responses accounting for approximately 7% of total responses. The limitation of these studies may be that measurements were in chronic infection and HLA-C restricted responses may be more immunodominant in early infection which may be critical to determining set point. However, data from Michael Liu (McMichael CHAVI Group, University of Oxford) looking at T cell responses in acute HIV-1 infection suggests that HLA-C restricted CD8 T cell responses are similarly rare at this early stage. They have mapped 20 subjects using overlapping 18mer peptides spanning autologous virus sequence within 6 months of HIV-1 infection. Within each responding 18mer, the optimal epitope, as predicted using the Epitope Location Finder tool, was synthesised and experimentally defined in IFN-γ ELISpot assays. To date, they have confirmed a total of 61 optimal epitopes (including some previously described epitopes) of which only 4 are HLA-C restricted. This approach is the closest there is to a systematic search for HLA-C restricted T cell epitopes.

The frequency of HLA-C restricted responses in Caucasians is similar to that observed in Africans. Previous studies have also used overlapping peptides spanning the entire HIV-1 proteome to map immunodominant epitope responses in infected African subjects (Kiepiela, Ngumbela et al. 2007; Matthews, Prendergast et al. 2008). In a cohort of 578 treatment naïve clade C infected subjects, 160 dominant CD8 T cell responses and HLA restrictions were defined with 13% of these responses being restricted by HLA-C alleles (Kiepiela, Ngumbela et al. 2007). When this cohort was extended to include another 681 subjects, there were 205 immunodominant responses, with the frequency of HLA-C restricted responses remaining unchanged (Matthews, Prendergast et al. 2008).

The data presented does not demonstrate a relationship between the breadth and magnitude of HLA-C restricted T cell responses (as measured by cytokine secretion) and -35 SNP genotype, at least in chronically infected HIV-1 individuals. It is well established that the immunological pressure exerted by CD8 T cells can select for HIV-1 viral variants that escape recognition with a fitness cost (Phillips, Rowland-Jones et al. 1991; Goulder and Watkins 2004; Peyerl, Barouch et al. 2004). There is also evidence that the CD8 T cell responses that are present in chronic HIV-1 infection may differ substantially from those that constitute the initial anti-viral response in acute infection (Goulder, Altfeld et al. 2001; Yu, Addo et al. 2002; Streeck, Jolin et al. 2009). HLA-C restricted CD8 T cell responses have been described in primary HIV-1 infection (Altfeld, Kalife et al. 2006; Goonetilleke, Liu et al. 2009). Goonetilleke *et al.* mapped the earliest T cell responses against autologous virus in three acutely infected HIV-1 subjects and detected the newly defined HLA-Cw*0401-restricted Env epitope QFRNKTIVF which escaped between 0 and 14 days

(Goonetilleke, Liu et al. 2009). It is therefore clear that the magnitude and breadth of HLA-C restricted CD8 T cell responses will vary depending on the stage of HIV-1 infection studied. However, the breadth and magnitude of these HLA-C restricted responses will remain low compared to HLA-A and HLA-B restricted responses.

The ability of CD8 T cells to clear virus-infected cells is dependent on the presentation of viral peptides processed intracellularly and displayed by MHC class I molecules. Although the ability of CD8 T cells to secrete multiple cytokines has been associated with long term non-progressive infection, this ability has not been directly linked to viral control (Migueles, Laborico et al. 2002; Betts, Nason et al. 2006). The ELISpot and chromium release assays rely on exogenously added peptides, a practice that does not account for differences that may be associated with antigen processing and presentation (Valentine, Piaskowski et al. 2008; Tenzer, Wee et al. 2009) and the kinetics of T cell lysis and viral replication (Yang, Kalams et al. 1996) in HIV-1 infected cells. In fact the effector function (cytokine secretion or cytotoxicity) a CD8 T cell elicits has been demonstrated to depend on the cognate peptide concentration (Betts, Price et al. 2004). Betts *et al.* examined the HLA-A*02 restricted response to the CMV NLVPMVATV epitope and the HLA-B*57 restricted response to the KAFSPEVIPMF (HIV-1 Gag 163-174) epitope using tetramers and showed that a large proportion of the tetramer positive cells responded to exogenous peptide by secreting cytokines or degranulating (their measure of cytotoxic activity). When peptide concentrations were varied they observed that at the highest peptide concentration, the majority of responding cells exhibited both functions. However, as the peptide concentration decreased, fewer cells produced cytokines, whereas many of those same cells continued to display cytolytic activity. Additionally, at the lowest

peptide concentrations at which a cytolytic response was detected, no cytokine secretion was detectable (Betts, Price et al. 2004). This mechanism could explain why the HLA-C restricted clones studied here displayed both cytokine secretion and cytolytic effector functions.

The evidence suggests that the control of viral replication *in vivo* is not simply a quantitative one (as measured by the frequency of virus-specific IFN-γ producing cells), suggesting that qualitative factors may also be involved. The ability of CD8 T cell lines or clones to inhibit/suppress HIV-1 replication in infected autologous or HLA-matched target cells is therefore a better measure of antiviral function. Recent studies of viral suppression by CD8 T cell clones derived from SIV-infected primates have shown marked differences in suppressive activity among multiple clones specific for the same epitopes, underscoring the importance of T cell receptor diversity, and that *in vitro* suppression of virus replication does not correlate with the ability to produce cytokines (including IFN-γ) (Chung, Lee et al. 2007; Loffredo, Burwitz et al. 2007). Similar studies in humans have shown that the detection of HIV-1 specific CD8 T cell cross-clade epitope responses by IFN-γ ELISpot assay does not correlate with suppression of HIV-1 viruses containing the same variant sequences (Bennett, Ng et al. 2008). Yang *et al.* adapted the chromium release assay to evaluate the interaction of HIV-1 specific CD8 T cell clones with acutely HIV-1 infected target cells by comparing targets that were directly infected with HIV-1 to targets that were loaded with exogenous peptide (Yang, Sarkis et al. 2003). They showed that HIV-1 specific CD8 T cell clones of different specificities and ranges of functional avidity lysed exogenously peptide-loaded cells with similarly high efficiency and that functional avidity has no effect on the efficiency of killing of HIV-1 infected cells.

Similar results were also obtained when these clones were tested for their ability to suppress viral replication, with inhibition more dependent on epitope specificity than functional avidity (Yang, Sarkis et al. 2003). Chen *et al.* compared the ability of five CD8 T cell clones of differing specificities derived from a single individual to inhibit virus in infected autologous CD4 T cells (Chen, Piechocka-Trocha et al. 2009). They showed that despite comparable potency by IFN-γ ELISpot and comparable killing in chromium release assays, clones differed markedly in their ability to inhibit HIV-1 replication, with the Gag-specific clones displaying the most potent suppressive activity (Chen, Piechocka-Trocha et al. 2009). Together, these studies indicate that at a clonal level, using primary viruses to infect target cells, there are marked differences in the ability of CD8 T cells to inhibit virus replication, despite comparable activity by ELISpot and chromium release assays.

The findings in the previous chapter that HLA-Cw*07 is expressed at very low levels and has a high population frequency in Caucasians led to a focus on whether this allele had any specific effects on T cell function. In the 15 CHAVI 001 individuals studied, 8 possessed at least a single HLA-Cw*07 allele and 4 demonstrated HLA-Cw*07-restricted IFN-γ ELISpot responses against the previously defined optimal epitope KRQDILDLWVY (KY11, Nef 105-115) (Table 4.4 and Figure 4.5). This epitope is the only HLA-Cw*07 restricted epitope defined in the LANL list of best characterised HIV-1 epitopes (Frahm, Linde et al. 2007). This probably reflects the fact that most work on T cell responses in HIV-1 has centred on HLA-B alleles. Indeed the data presented here suggests that there are potentially many more predicted HLA-Cw*07-restricted epitopes based on peptide binding motifs (Table 4.4). A limited number of immunodominant HLA-Cw*07-restricted HIV-1-specific T cell

responses have been previously defined (Nehete, Lewis et al. 1998; Mkhwanazi, Thobakgale et al. 2010).

In summary, this chapter explored whether there were CD8 T cell functional differences between -35 *CC* and *TT* genotypes based on their varying levels of HLA-C surface expression. It has been demonstrated that HLA-C restricted T cell clones are highly functional as measured by their ability to secrete IFN-γ and lyse cells in chromium release assays. These HLA-C restricted responses are in a minority but exist even for the low expression HLA-Cw*07 allele, as across the two Caucasian cohorts studied here HLA-C restricted T cell responses accounted for at best 16% of the total T cell response and there was no difference in T cell response between *CC*, *CT* and *TT* genotypes. There was no difference in the conservation of epitopes presented by -35*C* and -35*T* linked HLA-C alleles, although the lack of available data on HLA-C restricted epitopes makes it impossible to draw any meaningful conclusions. Given the low frequency of these responses, it seemed unlikely that HLA-C restricted CD8 T cells are solely responsible for the differences in viral load set point associated with the -35 SNP. In addition, there is no evidence at all that the -35 SNP protective effect on viral load set point is actually T cell mediated. Therefore in the next chapter, a different approach was taken, focussing specifically on whether T cells play a role in the protective effect attributed to the -35 SNP.

Chapter 5: IS THE -35 SNP EFFECT ON VIRAL LOAD SET POINT ACTUALLY T CELL MEDIATED?

5.1 Introduction

The work in human studies showing that the development of CD8 T cell responses correlates with the fall in viraemia at the end of acute infection (Borrow, Lewicki et al. 1994; Koup, Safrit et al. 1994), that the waning of these responses correlates with rising viraemia in late infection (Rinaldo, Huang et al. 1995), together with work in primate models showing that transient depletion of CD8 T cells in SIV-infected macaques results in increased viral replication (Matano, Shibata et al. 1998; Jin, Bauer et al. 1999; Schmitz, Kuroda et al. 1999) provide convincing qualitative evidence for an important role of CD8 T cells in controlling HIV-1 infection. The seminal observation that HIV-1 Nef protein has the ability to selectively downregulate HLA-A and HLA-B molecules to minimise CD8 T cell surveillance, while maintaining surface HLA-C expression (Collins, Chen et al. 1998) suggested that HLA-C restricted CD8 T cell responses could play a key role in controlling HIV-1 replication. This potential role was highlighted by the description of a single nucleotide polymorphism 35kb upstream of the transcriptional start site of HLA-C (the -35 SNP) that associated with HIV-1 control (Fellay, Shianna et al. 2007). Individuals with the protective genotype show increased expression of HLA-C surface protein (chapter 3 of this thesis) (Thomas, Apps et al. 2009), which correlates with better clinical status (CD4 counts and viral load) and delayed progression to AIDS (Thomas, Apps et al. 2009). It was therefore speculated that higher HLA-C expression results in a stronger HLA-C restricted T cell response which might play a

role in the control of HIV-1 replication in individuals with the protective variant (chapter 4 of this thesis). However these HLA-C restricted CD8 T cell responses were shown to be present at very low frequency (at best 16% of total T cell responses) and magnitude (chapter 4). In addition, HLA-C restricted T cells in chronic HIV-1 infection have equivalent cytotoxic activity *in vitro* relative to their HLA-A and HLA-B restricted counterparts (Goulder, Bunce et al. 1997; Adnan, Balamurugan et al. 2006). Like HLA-A and HLA-B restricted CD8 T cells, they share an intermediate memory T cell phenotype expressing high levels of CD27 and low levels of CD45RA and CD28 (Appay, Nixon et al. 2000; Makadzange, Gillespie et al. 2010) and do not differ in polyfunctionality (Mkhwanazi, Thobakgale et al. 2010).

Overall, HLA-B alleles more frequently induce detectable responses that are generally of greater magnitude than those restricted by either HLA-A or HLA-C alleles (Kiepiela, Leslie et al. 2004; Bihl, Frahm et al. 2006). In HIV-1 infection specifically, HLA-B restricted CD8 T cell responses constitute the bulk of the immune response (Kiepiela, Leslie et al. 2004). In addition, genetic studies looking at HLA allele associations with HIV-1 outcomes have mostly defined HLA-B alleles as having the strongest associations with HIV-1 disease progression (O'Brien, Gao et al. 2001; Carrington and O'Brien 2003; Pereyra, Jia et al. 2010).

Taken together, these data suggest that HLA-C restricted CD8 T cells are unlikely to be solely responsible for the -35 SNP effect on viral load set point. Therefore, the main aim of this chapter was to examine if there is any correlation between total CD8 T cell function and the -35 SNP.

The precise attributes of CD8 T cells that suppress viral production *in vivo* are not defined. HIV-1 specific CD8 T cells may recognise supraphysiologic exogenously added peptide on host cells but fail to recognise the same endogenously derived peptide in HIV-1 infected cells (Bennett, Ng et al. 2007; Bennett, Ng et al. 2008; Valentine, Piaskowski et al. 2008). Consequently, as discussed in the previous chapter, some of the currently used immunoassays may detect a CD8 T cell specific response that in reality may not correlate with *in vivo* suppression of HIV-1. Directly infecting autologous CD4 T cells with primary HIV-1 strains is the ideal method for identifying CD8 T cell correlates of protection and this technique (virus suppression assay) has recently gained support as a more accurate surrogate for immune control *in vivo* (Spentzou, Bergin et al. 2010; Makedonas and Betts 2011). This assay involves infecting CD4 target cells with an exogenous HIV-1 strain and viral replication is allowed to proceed for several days. Autologous CD8 T cells are added to the CD4 target cells and any ensuing reduction in p24 content over the time in culture represents the neutralising activity of the CD8 T cells. Thus, this assay gauges all the potential inhibitory mechanisms, direct target cell lysis (Yang, Kalams et al. 1997; Migueles, Osborne et al. 2008) as well as non-cytolytic mechanisms of virus inhibition including cytokine and chemokine secretion (Walker, Moody et al. 1986; Walker, Erickson et al. 1991; Cocchi, DeVico et al. 1995; Tomaras, Lacey et al. 2000), of the CD8 T cell mediated antiviral response elicited by HIV-1 infection.

This assay has been used to define differences in the viral suppressive properties of HIV-1 specific CD8 T cell responses focusing on single peptide-specific CD8 T cell clones or lines (Yang, Kalams et al. 1997; Yang, Sarkis et al. 2003; Chung, Lee et al. 2007; Loffredo, Burwitz et al. 2007; Chen, Piechocka-Trocha et al. 2009), on multiple

tetramer-sorted CD8 T cells from fresh PBMC (Vojnov, Reed et al. 2010), on HIV-1 antigen specificity (Julg, Williams et al. 2010) and on specific subpopulations of study subjects such as HIV-1 controllers (Saez-Cirion, Lacabaratz et al. 2007; Saez-Cirion, Sinet et al. 2009). The mechanism responsible for this antiviral activity remains undetermined. Several studies have shown that MHC class I blockade (by W6/32 antibody) negated CD8 T cell mediated suppression of HIV-1 (Spentzou, Bergin et al. 2010) and physical separation of the effector CD8 T cells from the target CD4 T cells by transwell chambers largely reversed the observed suppression (Yang, Kalams et al. 1997; Saez-Cirion, Lacabaratz et al. 2007; Spentzou, Bergin et al. 2010). Saez-Cirion *et al.* went on to demonstrate that this observed control of HIV-1 replication was achieved through the physical elimination of infected CD4 T cell targets by CD8 T cells. A marked suppression of HIV-1 infection was observed when unstimulated CD8 T cells were cocultured with autologous CD4 targets. The coculture was then split and CD8 T cells were depleted from one set of cocultures. The two cultures were subsequently rechallenged with HIV-1 and the CD8 depleted culture was readily superinfected whereas suppression was still observed in the non-depleted coculture (Saez-Cirion, Lacabaratz et al. 2007). They also assessed the capacity of CD8 T cells to control HIV-1 replication in autologous and heterologous CD4 T cells and showed that the same CD8 T cells that were able to control HIV-1 *in vitro* when cocultured with autologous CD4 T cells were ineffective when cocultured with heterologous CD4 T cells (Saez-Cirion, Lacabaratz et al. 2007). These data were consistent with the killing of target cells (though this remains to be formally demonstrated), as opposed to interfering with HIV-1 replication via non-cytolytic soluble mediators, and demonstrated that CD8 T cell antiviral suppression was HLA class I restricted and cell contact dependent.

A criticism of previously developed viral suppression assays used to test the antiviral efficacies of epitope-specific CD8 T cell lines or clones is that these cell types required culturing cells in IL2 and frequent restimulation using BCLs pulsed with supraphysiological concentrations of peptide, resulting in less physiologically relevant cell populations. These problems were avoided by using MHC class I tetramers to isolate CD8 T cells specific for 12 different MHC class I SIV epitopes restricted by Mamu-A*01, -A*02 and -B*08 and the virus suppressive ability of each of these cell populations was determined (Vojnov, Reed et al. 2010). They established an antiviral hierarchy among these 12 epitope specific CD8 T cells and, perhaps more importantly, demonstrated that almost all epitope-specific CD8 T cells have some capacity to suppress viral replication. The inhibition of viral production from infected CD4 cells by epitope-specific CD8 T cells was unrelated to the viral load of each animal and suppression did not correlate with disease status, the protein from which the epitopes were derived, epitope avidity or cytokine secretion (Vojnov, Reed et al. 2010).

In keeping with conditions that are more analogous to the *in vivo* situation, other studies have used polyclonal CD8 T cells which represent a heterogeneous and realistic population of circulating cells in various stages of activation, proliferation and differentiation (Saez-Cirion, Lacabaratz et al. 2007; Saez-Cirion, Sinet et al. 2009; Freel, Lamoreaux et al. 2010; Julg, Williams et al. 2010; Spentzou, Bergin et al. 2010)(Yang et al 2011, submitted manuscript). These T cells are therefore likely to be targeting multiple epitopes concurrently. Saez-Cirion *et al.* have focused on the suppressive activity of CD8 T cells from HIV-1 infected controllers (or LTNPs) and

have shown that these cells are able to control HIV-1 infection, compared to CD8 T cells from viraemic individuals and from antiretroviral therapy treated individuals with undetectable viral load (Saez-Cirion, Lacabaratz et al. 2007; Saez-Cirion, Sinet et al. 2009). In addition, this suppressive capacity was stable over time, as measured in multiple bleeds from 5 individuals over a 1-3 year period, and was partially effective even on other primate lentiviruses (Saez-Cirion, Sinet et al. 2009).

Studies using viral suppression assays have often used mitogen or cytokine stimulated CD8 T cells and have mostly relied on a secondary assay to quantify HIV-1 gene products in the culture supernatant (p24 ELISA). Most recently, this assay has been used to examine CD8 T cell suppressive capacity in chronically infected adults with diverse CD4 T cell counts and progression rates (Yang *et al.* 2011, submitted manuscript). In the viral suppression assay developed by Yang *et al.*, HIV-1 infected CD4 T cells were quantified directly by detection of intracellular p24 antigen using a flow cytometry-based approach. In addition, they used *ex vivo* unstimulated CD8 T cells to avoid bias in the HIV-1 specific T cell repertoire due to *in vitro* expansion and to minimise induction of non-specific suppressive activity as a result of polyclonal T cell activation. There was a strong correlation between the frequencies of infected CD4 T cells obtained by intracellular p24 staining and the concentration of p24 in their corresponding culture supernatants [Spearman's rank correlation test; $r=0.856$, $p<0.000001$ (Saez-Cirion, Shin et al. 2010); $r=0.82$, $p<0.0001$ (Yang *et al.* 2011, submitted manuscript)]. They then evaluated the antiviral suppressive capacity of *ex vivo* CD8 T cells from 30 antiretroviral therapy naïve HIV-1 seropositive individuals and showed that antiviral suppressive activity was present in all individuals and was inversely related to set point viraemia ($r=-0.72$, $p=0.0008$ for effector to target ratio of

2:1; Spearman's rank correlation test). Further, they observed a highly significant interaction between viral suppression and the CD4 T cell count gradient ($p<0.0001$; linear mixed model), with greater CD8 T cell suppressive activity being associated with slower CD4 T cell decline.

Thus the viral suppression assay was chosen as a measure of total CD8 T cell response to capture HLA-A, HLA-B and HLA-C restricted responses. Further justification for using this assay comes from studies showing that CD8 T cell suppression of HIV-1 replication correlates directly with clinical state with the strongest response seen in asymptomatic seropositive individuals and a reduction in the response seen concomitant with disease progression (Mackewicz, Ortega et al. 1991; Mackewicz and Levy 1992; Landay, Mackewicz et al. 1993; Blackbourn, Mackewicz et al. 1996). Preliminary SNP typing of a small number of individuals studied by Yang *et al.* (Yang *et al.* 2011, submitted manuscript) suggested a trend with CD8 T cells from -35 *CC* individuals suppressing heterologous HIV-1 infection (the clade B laboratory adapted strain HIV-1_{BaL}) more effectively than CD8 T cells from -35 *TT* individuals. Therefore, HIV-1 infected Caucasian individuals who were antiretroviral therapy naïve (the population in which the -35 SNP observation was made) were recruited, genotyped at the -35 SNP and viral suppression assays were performed to determine the ability of their CD8 T cells to suppress HIV-1 viral replication in autologous CD4 T cells infected with a heterologous strain of HIV-1 using the flow cytometric assay developed by Yang *et al.* (submitted manuscript).

5.2 Results

5.2.1 Assay optimisation and standardisation

PBMC from HIV-1 uninfected individuals were depleted of CD8 T cells by magnetic bead separation. This yielded an approximately 96% depletion of CD8 T cells (Figure 5.1). These CD4 T cells were activated with PHA and then infected with HIV-1_{BaL} before being used in a 10 day culture time course, in the absence of CD8 T cells, to determine the kinetics of HIV-1_{BaL} infection. The optimal infection of CD4 T cells peaked at day 5 (Figure 5.2).

5.2.2 CD8 T cell mediated viral suppression assay specificity

To determine the viral suppression assay specificity and criteria for assay positivity, HIV-1 inhibition mediated by CD8 T cells was assessed in 10 HIV-1 uninfected individuals. Uninfected activated CD4 T cells cultured alone were stained for intracellular p24 antigen to determine a cut-off for infection with HIV-1_{BaL}. The mean and median frequencies of p24 positive CD4 T cells were 0.481% and 0.385% respectively, with minimum and maximum values of 0.19% and 0.96%. Using three standard deviations from the mean as a cut-off, values above 1.5% were considered to indicate true infection of CD4 cells with HIV-1. CD8 T cell activity was expressed as percentage suppression of intracellular p24 antigen (defined in Materials and Methods). At day 7 of coculture, CD8 T cells from uninfected individuals had a median antiviral suppressive activity of 0% (range 0-12.26%). The cut-off for assay positivity was taken as the mean plus 3 standard deviations and suppression detected above this value of 15% was defined as HIV-1 specific. The data were assumed to be normally distributed.

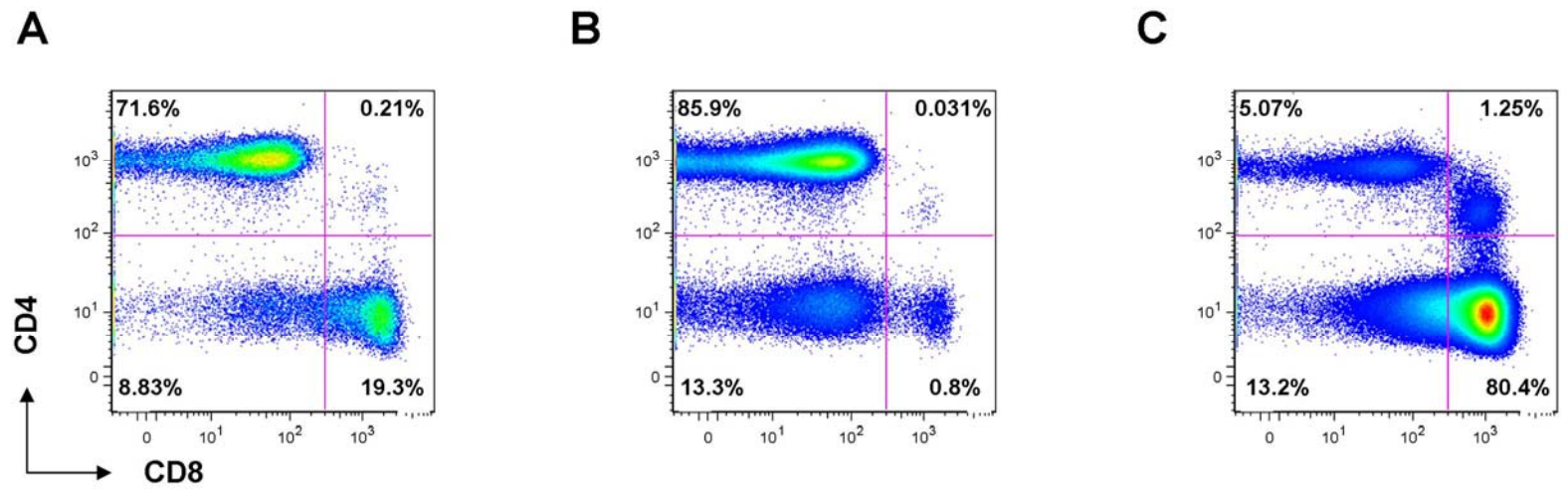


Figure 5.1. Purification of CD4 and CD8 cells from freshly isolated PBMC.
Representative CD4 and CD8 stains from a healthy volunteer of unseparated fraction (A), negatively selected CD4 fraction (B) and positively selected CD8 fraction (C). The cell separation was performed by incubating PBMC with CD8 antibody-coated magnetic beads for 15 minutes. The cell suspension was then run through a magnetic column. Unlabeled cells that passed through the column were collected and consisted of the CD4 fraction. The labelled CD8 positive cells that stuck to the magnetic column were then collected separately. All three fractions were stained with anti-CD3/4/8 monoclonal antibodies conjugated to separate fluorochromes before analysing in a flow cytometer. Lymphocytes were then selected by first gating on CD3 positive cells then plotting CD4 against CD8-postive T cells. This technique of negatively selecting CD4 cells yielded a purified cell fraction with >95% depletion of CD8 cells. These purified CD4 T cells were stimulated with phytohaemagglutinin for 3 days before being superinfected with HIV-1_{BaL} and used in viral suppression assays.

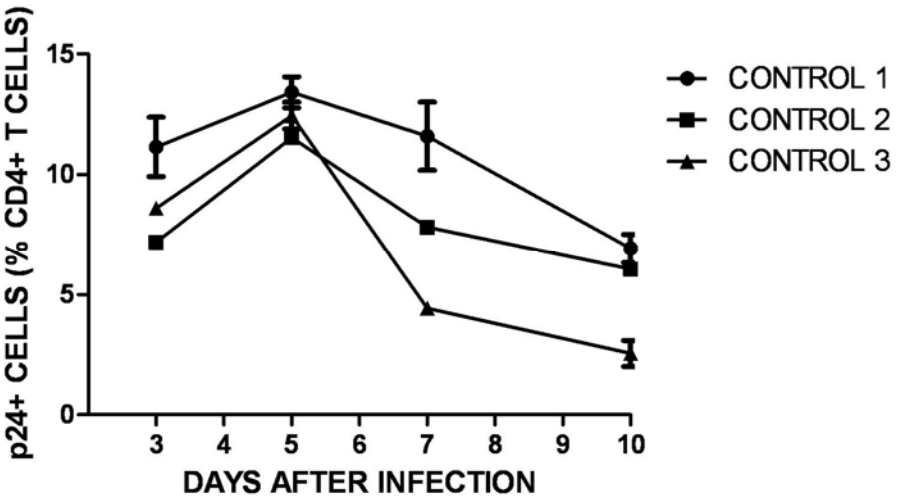


Figure 5.2. Time course of HIV-1_{BaL} superinfection.
The purified CD4 T cell fraction from 3 HIV-1 uninfected individuals (Controls 1-3) were stimulated with 5μg/ml PHA for 3 days and then superinfected with HIV-1_{BaL} at a multiplicity of infection of 0.01 and cultured in duplicate without autologous CD8 T cells for 10 days. At days 3, 5, 7 and 10 following superinfection, intracellular p24 antigen was quantified as a measure of HIV-1 infection. Maximal superinfection of CD4 T cells peaked at day 5. Each data point represents the mean of duplicate wells, with standard deviation indicated by error bars.

5.2.3 CD8 T cell mediated suppression of HIV-1_{BaL} replication

A cross-sectional analysis, at a single time-point, of CD8 T cell antiviral suppressive capacity in 46 chronically infected, antiretroviral therapy naïve Caucasian individuals was performed (Table 5.1). Antiviral suppression was assessed on day 6 of the coculture using a CD8:CD4 cell ratio of 2:1. This time-point was selected as the optimal infection of CD4 T cells likely occurred between days 5 and 7; additionally, day 6 was also used by Yang *et al.* (submitted manuscript) (Figure 5.2). HIV-1_{BaL} suppression was detectable in all but one individual (median, interquartile range: 63.22%, 41.92-79.27%) and was significantly greater than values obtained from 10 healthy controls ($p<0.0001$; Mann-Whitney test) (Figure 5.3). This CD8 T cell mediated suppression of viral replication was due to the absence of infected CD4 T cells in the coculture (Figure 5.4). The lack of CD8 T cell antiviral activity in a single individual was not due to an inability to superinfect autologous CD4 T cells (frequency of infected CD4 T cells was 5.6%). There was no correlation between HIV-1_{BaL} suppression and the frequency of infected CD4 cells (Spearman $r=0.05$, $p=0.7321$) (Figure 5.5), indicating that higher antiviral CD8 T cell activity was not simply a consequence of lower HIV-1 superinfection of autologous CD4 T cells *in vitro*. In 14 chronic progressor individuals, a second effector to target ratio of one CD8 T cell to 10 infected CD4 T cells was tested and showed significantly higher CD8 antiviral activity at the higher ratio ($p=0.0047$; Mann-Whitney test) (Figure 5.6). This result is consistent with a previous study that showed that the antiviral activity of CD8 T cells from viraemic individuals was rapidly lost when CD8 T cells were diluted (Saez-Cirion, Lacabaratz et al. 2007). Endogenous virus was detected by intracellular p24 antigen staining in 10 of 46 chronically infected individuals after PHA activation and culture of their CD4 T cells (Figure 5.4). This reactivation of

autologous HIV-1 was higher in another study with 15 of 35 reactivating (Spentzou, Bergin et al. 2010). This difference may be explained by different culture conditions. In Spentzou *et al.* they activated CD4 T cells for 7 days before culturing for a further 13 days prior to assaying for p24 antigen. Another study that employed identical CD4 activation and culture conditions to those used here had similar reactivation rates with 7 of 30 chronically infected individuals reactivating autologous HIV-1 (Yang *et al.* 2011, submitted manuscript). In addition, Yang *et al.* went on to compare CD8 T cell suppression of autologous virus and HIV-1_{BaL} superinfected CD4 T cells in 5 of these individuals and observed very similar levels of inhibition of both viruses, irrespective of the level of viraemia and of CD8 T cell antiviral activity, suggesting that suppression of heterologous HIV-1 replication by CD8 T cells is comparable to autologous virus inhibition (Yang *et al.* 2011, submitted manuscript). On the basis of this finding, these 10 individuals whose CD8 T cell suppressive ability as measured in this study included both autologous virus and HIV-1_{BaL} virus were included in subsequent analysis.

Table 5.1. Characteristics of Caucasian HIV-1 infected individuals studied in viral suppression assays.

SUBJECT[a]	GENDER	-35 SNP	VL GROUP[b]	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2	REACTIVATION[c]	P24% CD4[d]	% SUPPRESSION
IC130		CT								NO	10.07	98.5
IC145	M	CT		0201	0301	5701	1501	0303	0602	NO	15.4	98
IC137		CC		0101	3003	1401	1401	0802	0802	NO	6.27	94.3
IC150	M	CC		0301	3001	1302	1401	0602	0802	NO	8.93	94.2
709020620	M	CT	13675	0201	1101	0702	5201	0702	1202	YES	7.53	91
709020645	M	CT	5154	0301	2402	4402	5101	0501	1502	NO	1	90.5
IC142	M	CT		0101	0301	0702	2702	01	0702	NO	2.97	86.25
709020528	M	CT	15723	0101	0101	0801	5701	0605	0701	YES	4.56	86.2
S1296	M	CC				4402	5701	0501	0602	NO	2.43	84.19
S1534	M	CC	VC	2403	3301	1402	5001	0602	0802	NO	1.69	82.25
S1242	M	CC	VC			1402	3901	0802	1203	NO	1.58	80.57
S1184	M	CC	PC	301	2501	1402	2705	0102	0802	NO	1.59	78.83
IC149	M	TT		0201	0301	35	35	0401	0401	NO	8.13	74.55
IC144	M	CC		0201	2501	2702	5101	0202	1502	YES	3.4	74.35
700010111	M	CT	14125 [SP]	0301	0301	0702	4002	0202	0702	NO	2.78	72.11
IC125	M	TT		02	02	0702	4001	0304	0702	NO	1.3	67.7
S1514	M	CC	VC			2705	4402	0102	0501	NO	2.55	67.46
701010359	M	TT	245854	0301	1101	0702	3501	0401	0702	NO	4.5	65.95
700010802	M	CT	1738 [SP]	0201	3101	1302	1501	0302	0602	NO	9.4	65.63
700010791	M	TT	40084	0201	0301	0702	0702	0702	0702	NO	4.67	64.43
S1541	M	CT	VC			0702	1402	0702	0802	NO	1.68	64.23
IC119	M	TT								YES	8.03	64.1
S1185	M	CC	VC			5201	5701	0602	1202	NO	2.55	64.08
700010271	M	CT	105412	0201	3001	1302	4001	0302	0605	NO	7.04	62.36
700010094	M	CT	22909 [SP]	0301	2902	1801	4901	0501	0701	NO	2.09	58.1

SUBJECT[a]	GENDER	-35 SNP	VL GROUP[b]	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2	REACTIVATION[c]	P24% CD4[d]	% SUPPRESSION
S1086	M	CC	VC	0201	3201	4002	5701	0202	0602	NO	1.65	56.92
700010516	M	CT	6310 ^{SP}	0101	3202	5601	5701	0102	0602	NO	5	56.59
IC109	M	CT		0201	11			01	0501	NO	2.33	55.5
S1204	M	CC	EC			2705	5801	0202	0302	NO	2.38	49.73
S1529	M	CC	EC	0201	0301	1402	5701	0602	0802	NO	2.55	48.37
700010742	M	TT	123027	0301	2301	4403	5301	0401	1601	NO	3.7	46.78
701010186	M	TT	69764	0201	0301	1501	4403	0302	1601	NO	3.78	44.13
IC138	F	CT								NO	1.74	44
700010174	M	CT	>750000	1101	2601	3801	5601	0102	1203	YES	3.9	43.57
S1218	M	CC	PC			2705	4002	0102	0202	NO	2.44	43.23
709020500	M	TT		0101	0201	0201	0801	0303	0701	YES	6.53	38
IC104	F	TT		0101	2402	0801	55	0303	0701	YES	29.37	36.8
701010114	M	TT	169738	0101	0101	0702	0801	0701	0702	NO	2.1	35.36
700010135	M	TT	261350	0201	3303	5301	5301	0401	0401	YES	9.1	34.57
S1168	M	TT	VC	0301	1101			0304	0401	NO	4.46	31.81
S1517	M	CC	EC			1801	5701	0602	1203	NO	2.08	31.58
701010315	M	TT	26851	0301	3202	0702	3906	0702	1502	NO	4.68	30.49
S1041	M	TT	VC	0201	3101	1501	3501	0304	0401	YES	4.01	23.77
S1198	M	TT	EC			0702	3501	0401	0702	NO	3.34	19.26
700010662	M	TT	7672	0201	2402	3502	5601	0102	0401	NO	2.39	17.17
S1175	M	CC	PC			1302	2705	0102	0602	YES	5.6	0

All individuals assay were infected with clade B HIV-1 except IC125 who had clade C infection.

[a] All viral suppression assays for IC cohort individuals performed by Hongbing Yang.

[b] The SCOPE cohort individuals are LTNPs and their viral load data have been divided into 3 groups; elite controllers (EC) <75 copies/ml, viraemic controllers (VC) with viral load 75-2000 copies/ml and partial controllers (PC) with viral load 2000-10000 copies/ml. For other individuals studied, the viral load set point (number ^{SP}) is listed where calculated, or the viral load at the time point used for setting up viral suppression assay is given.

[c] Reactivation of an individual's autologous HIV-1 infection was determined by calculating the frequency of p24 positive cells in uninfected CD4 T cells. Values above 1.5% were deemed as reactivation of that individual's autologous virus (1% cut-off for assays performed by Hongbing Yang).

[d] Frequency of p24 positive cells in autologous CD4 T cells infected with HIV-1_{BaL} and cultured alone.

The p24 % of infected CD4 cells and % suppression were calculated as defined in Materials and Methods.

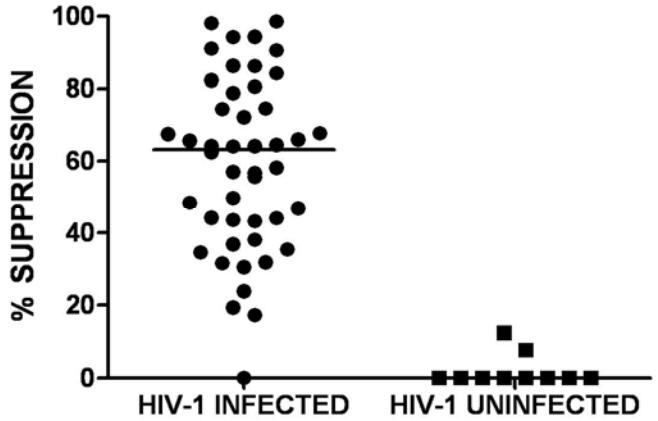


Figure 5.3. HIV-1 seropositive individuals produce consistently higher CD8 T cell suppression of heterologous HIV-1 replication in HIV-1_{BaL} superinfected autologous CD4 T cells than seronegative individuals.
In all 56 individuals, the purified CD4 T cell fraction was stimulated with 5μg/ml PHA for 3 days and then superinfected with HIV-1_{BaL} at a multiplicity of infection of 0.01 and cultured in triplicate with autologous CD8 T cells at an effector to target ratio of 2:1. Viral inhibition was determined after 6 days in culture by quantifying p24 antigen-positive cells and calculating percentage suppression as described in Materials & Methods. Horizontal lines represent median values.

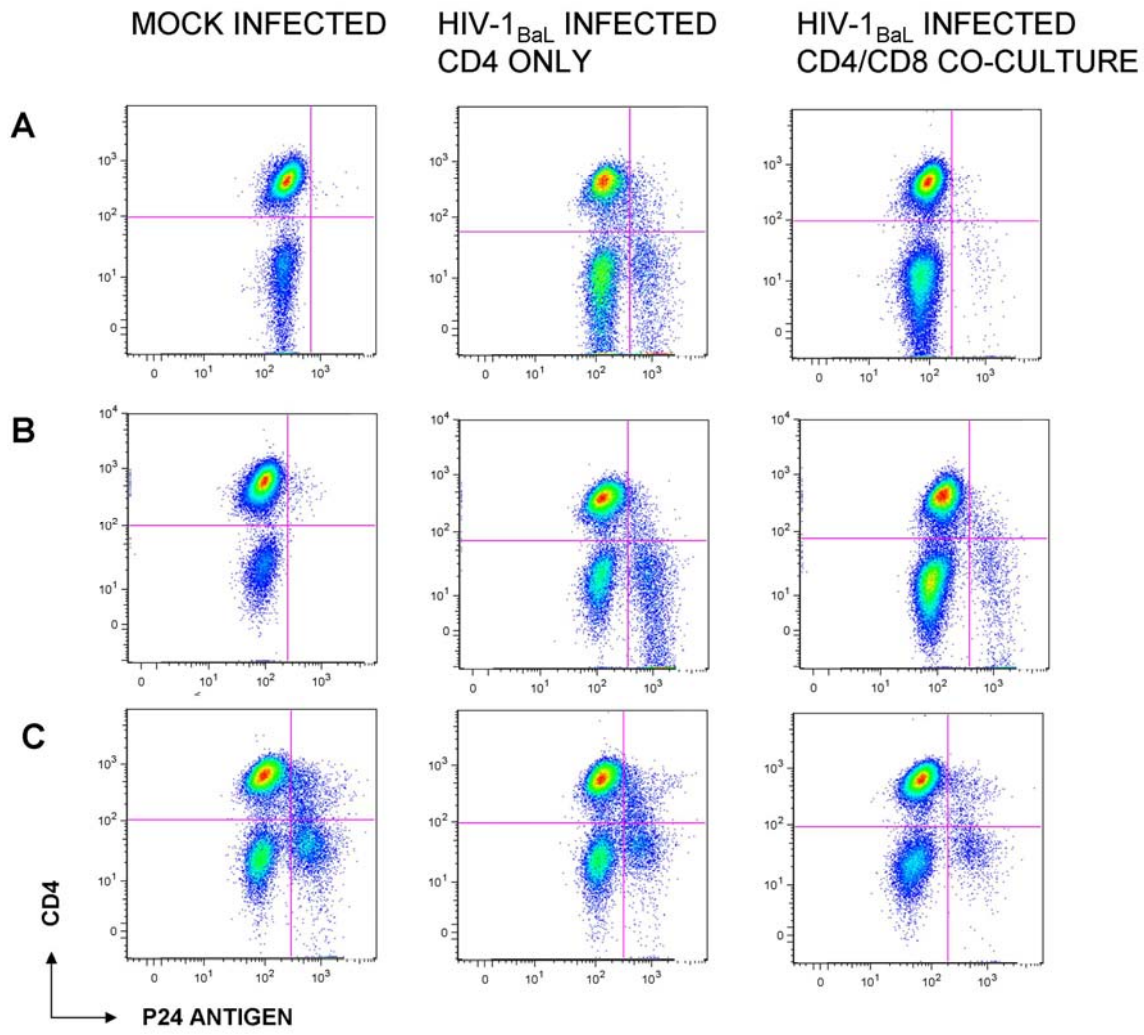


Figure 5.4. Representative flow cytometry plots showing detection of HIV-1_{BaL} infected (p24 antigen-positive) cells within the CD4 T cell population after gating on live CD3-positive, CD8-negative lymphocytes in 3 individuals with chronic HIV-1 infection.

The purified CD4 T cell fraction was stimulated with 5μg/ml PHA for 3 days and then superinfected with HIV-1_{BaL} at a multiplicity of infection of 0.01 and cultured in triplicate with autologous CD8 T cells at an effector to target ratio of 2:1. Viral inhibition was determined after 6 days in culture by quantifying p24 antigen-positive cells. Mock-infected (left), HIV-1_{BaL} superinfected CD4 T cells cultured alone (middle) or with autologous *ex vivo* unstimulated CD8 T cells (right) are shown.

Plot A demonstrates strong suppression of heterologous HIV-1 replication by autologous CD8 T cells and plot B demonstrates weak suppression of viral replication by CD8 T cells. Plot C demonstrates reactivation of the individual's autologous HIV-1 virus following PHA stimulation of CD4 cells.

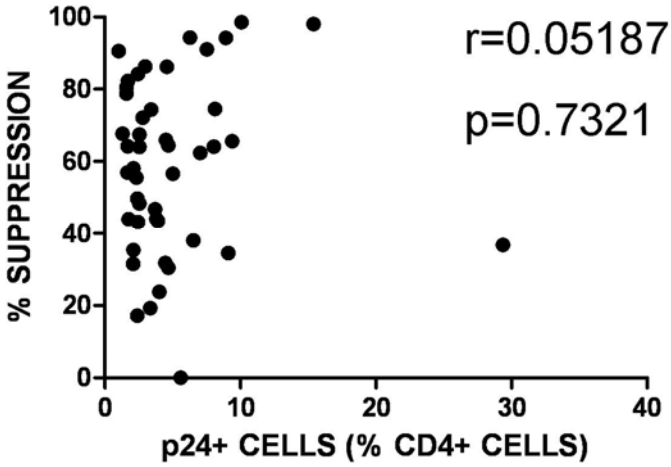


Figure 5.5. CD4 T cell superinfection levels with heterologous virus do not correlate with CD8 T cell –mediated suppression of HIV-1 replication.
Each data point represents the mean of triplicate wells. Statistical analysis was performed with the Spearman rank correlation test.

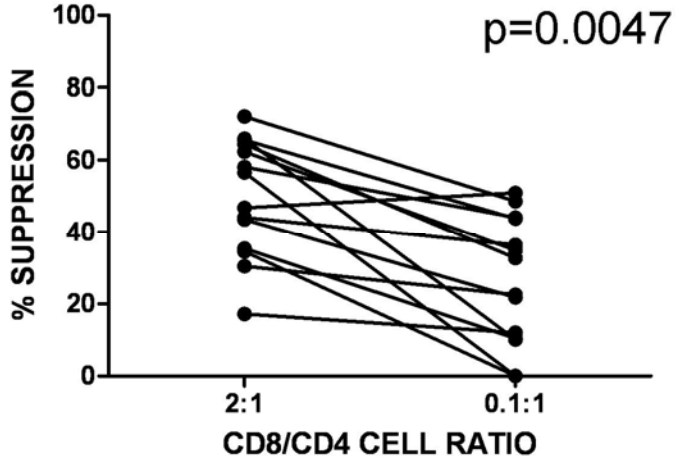


Figure 5.6. CD8 T cell antiviral suppressive activity titrates at lower effector to target ratios in HIV-1 infected individuals.
The purified CD4 T cells from 14 HIV-1 infected individuals were stimulated with 5μg/ml PHA for 3 days and superinfected with HIV-1_{BaL}. These superinfected CD4 T cells were co-cultured in triplicate wells with autologous CD8 T cells at CD8/CD4 ratios of 2:1 and 0.1:1 for 6 days. Viral inhibition was determined by quantifying p24 antigen-positive cells and calculating percentage suppression as described in Materials & Methods. There was a statistically significant reduction in suppressive activity at the lower effector to target ratio (Mann-Whitney test).

5.2.3.1 The protective -35 SNP effect in Caucasians is T cell-mediated

When CD8 T cell antiviral activity in these 46 individuals was grouped according to -35 SNP genotype (Figure 5.7), *CC* and *CT* individuals had significantly higher suppressive activity than *TT* individuals (p=0.0151 and p=0.0022, respectively; Mann-Whitney test). There was no difference in suppressive activity between *CC* and *CT* individuals (p=0.4307; Mann-Whitney test), confirming that possessing a single -35*C* allele was enough to confer strong CD8 T cell antiviral activity. Similar to the findings on surface HLA-C expression, there was a continuum of CD8 T cell antiviral activity that cannot be attributed completely to homozygosity of -35 SNP genotypes. However, this remains the first evidence suggesting that the HIV-1-protective -35 SNP effect is mediated through T cells.

5.2.3.2 The protective -35 SNP effect in Caucasians may actually be driven by HLA-B alleles

The International HIV Controllers Study implicated B*5701, B*2705, B*14/Cw*0802, B*52, A*25 as protective alleles and B*35 and Cw*07 as risk alleles in Caucasians (Pereyra, Jia et al. 2010). Previous genetic and functional studies have also implicated B*51 as a protective allele (Kaslow, Carrington et al. 1996; O'Brien, Gao et al. 2001; Tomiyama, Fujiwara et al. 2005). Therefore, the distribution of these 'good' and 'bad' HLA-B alleles within the 46 individuals with CD8 T cell antiviral activity was examined (Figures 5.7 and 5.8)

It was immediately clear that all these 'good' HLA-B alleles were present only in -35 *CC* and *CT* genotypes while 'bad' HLA-B alleles were over-represented in *TT*

individuals. In addition, when suppression was plotted against protective and risk alleles (Figure 5.8), as defined by the International HIV Controllers Study, individuals who possessed at least a single protective allele had significantly greater CD8 T cell antiviral suppressive activity compared to individuals lacking these alleles or individuals who possessed at least one risk allele (and no protective allele), p=0.0025 and p=0.0134 respectively (Mann-Whitney test). Therefore the association between pairs of HLA alleles was further investigated by combining the healthy laboratory volunteers and the SCOPE cohort of HIV-1 infected individuals (all previously genotyped at the -35 SNP). In total 414 Caucasian individuals were examined for linkage disequilibrium patterns between HLA-B and HLA-C alleles (Table 5.2), using the HLA frequency analysis tool on the LANL database (http://www.hiv.lanl.gov/content/immunology/hla/hla_linkage.html). This suggested that in Caucasians 'good' HLA-B alleles are in linkage disequilibrium with 'good' HLA-C alleles (e.g. HLA-B*5701 and HLA-Cw*0602) which are in turn in linkage disequilibrium with the -35*C* genotype. In similar fashion, 'bad' HLA-B alleles are in linkage disequilibrium with 'bad' HLA-C alleles (e.g. HLA-B*3501 and HLA-Cw*0401) which are also in linkage disequilibrium with the -35*T* genotype.

The -35 SNP genotype and HLA-B alleles were tested for association with HIV-1 suppression in separate linear regression models. When tested individually, both variables strongly associated with suppression (p=0.002 and p=0.007 for -35 SNP genotype and HLA-B allele, respectively). In a model already including the SNP genotype, adding in the HLA-B allele did not make any difference in the model (p=0.206). Similarly, in a model already including HLA-B alleles, adding in SNP genotype was also not significant (p=0.084). Therefore, in these linear regression

models, neither variable reached statistical significance ($p<0.05$) and it remains unclear, with the numbers available, which variable was 'driving' the signal of association.

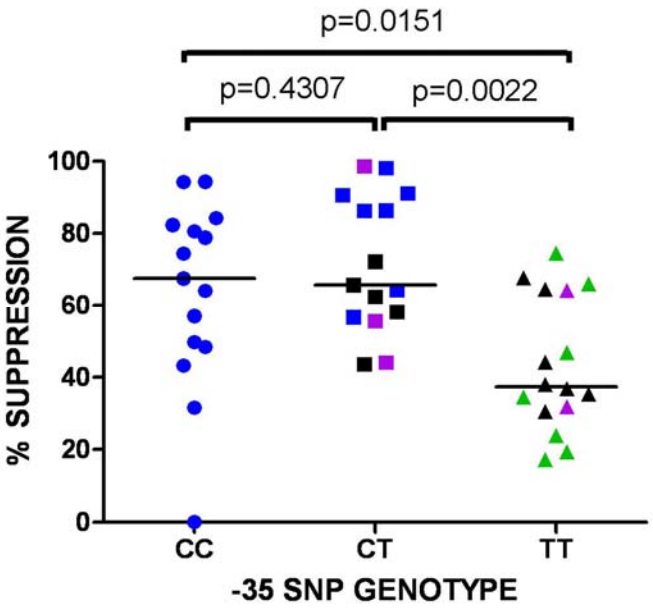


Figure 5.7. There is correlation between CD8 T cell antiviral suppressive capacity and the -35 SNP genotype.
The antiviral suppressive capacity of *ex vivo* CD8 T cells is strongly correlated with -35 SNP genotype. Blue data points are individuals who possess at least a single protective HLA-B allele (HLA-B*14/27/51/52/57), green individuals possess at least a single risk HLA-B allele (HLA-B*35 family), black data points are individuals who do not possess a protective or risk allele and purple data points correspond to the five individuals for whom there was no HLA typing data available. Statistical analysis was performed with the Mann-Whitney test.

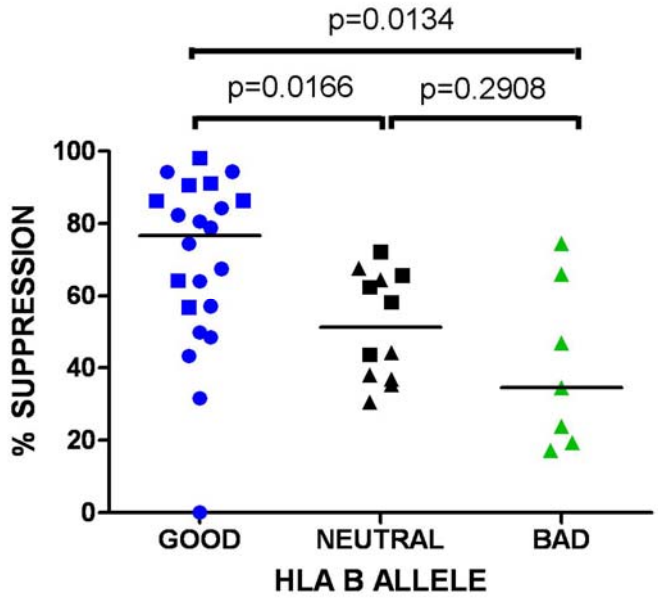


Figure 5.8. HLA-B alleles may play a significant role in the HIV-1 protective effect attributed to the -35 SNP.
The CD8 T cell antiviral suppressive activity for 41 individuals with full HLA typing was plotted against the presence of at least a single protective (good) HLA-B allele, the presence of at least a single risk (bad) HLA-B allele and the absence of both protective and risk alleles (neutral). Circles, squares and triangles reflect -35 *CC*, *CT* and *TT* individuals respectively. Protective and risk HLA-B alleles are as defined in the legend to Figure 5.7. Statistical analysis was performed with the Mann-Whitney test.

Table 5.2. Pairs of HLA alleles that are in linkage disequilibrium in the 414 Caucasian individuals studied.

HLA1	HLA2	P VALUE	-35 SNP	BOTH HLA	ONLY HLA1	ONLY HLA2	NEITHER	D PRIME
B1402	C0802	6.20E-28	*C*	19	0	6	389	1
A30	B1302	3.11E-11		6	0	4	404	1
B1302	C0602	4.90E-09	*C*	10	0	55	349	1
B1401	C0802	2.63E-08	*C*	6	0	19	389	1
B3801	C1203	2.63E-08	*C*	6	0	19	389	1
B4101	C1701	2.90E-08	*T*	4	0	3	407	1
B44	C16	1.64E-07	*T*	6	27	0	381	1
B5501	C0303	3.40E-07	*T*	5	0	18	391	1
B5801	C0302	3.41E-07	*C*	3	1	0	410	1
B5701	C0602	1.77E-28	*C*	32	1	33	348	0.9641
B3501	C0401	1.45E-24	*T*	24	1	24	365	0.9548
B0801	C0701	3.04E-38	*T*	41	3	19	351	0.9203
B15	C03	1.91E-17	*C/T*	19	2	31	362	0.8917
B4402	C0501	3.75E-41	*C*	41	7	8	358	0.8836
B55	C03	2.19E-07	*C/T*	8	1	42	363	0.8736
B5101	C1402	1.59E-11	*C*	7	5	1	401	0.8713
B38	C12	3.91E-08	*C*	6	1	14	393	0.8499
B4001	C0304	4.15E-13	*T*	12	2	20	380	0.8452
B0702	C0702	2.82E-40	*T*	39	7	7	361	0.8288
A25	B1801	5.06E-07		5	1	13	395	0.8258
A25	C1203	3.09E-06	*C*	5	1	20	388	0.8226
B4403	C1601	1.40E-17	*T*	13	5	4	392	0.754
B40	C03	1.22E-15	*C/T*	20	6	30	358	0.7375
B4002	C0202	3.90E-07	*C*	6	2	17	389	0.7353
A29	B4403	3.04E-14		11	4	7	392	0.7212
B51	C15	2.64E-07	*T*	5	8	2	399	0.705
B18	C12	2.17E-08	*C*	7	3	13	391	0.6848

HLA1	HLA2	P VALUE	-35 SNP	BOTH HLA	ONLY HLA1	ONLY HLA2	NEITHER	D PRIME
A29	C1601	1.48E-12	*T*	10	5	7	392	0.6524
B2705	C0202	7.41E-13	*C*	13	10	10	381	0.5396
B2705	C0102	2.21E-09	*C*	10	13	9	382	0.4985
B1501	C0303	4.02E-09	*T*	12	20	11	371	0.4817
B1801	C1203	7.93E-08	*C*	9	9	16	380	0.4679
B1501	C0304	3.62E-08	*T*	13	19	19	363	0.3565

The 414 individuals consisted of seronegative volunteers and HIV-1 seropositive individuals from the SCOPE cohort. This table was generated using the LANL HLA frequency analysis tool – inputted HLA types are used to return any linkage disequilibrium found by a 2-sided Fisher's exact test. HLA alleles in bold type are protective (HLA A*25, B*14/27/51/52/57/58) and risk (HLA B*35) alleles.

5.3 Discussion

It is well established that CD8 T cells are important in the initial decline of peak viraemia during acute HIV-1 infection and the subsequent determination of viral load set point during chronic infection. This vital role has been confirmed by experimental depletion of CD8 T cells in SIV-infected macaques (Matano, Shibata et al. 1998; Jin, Bauer et al. 1999; Schmitz, Kuroda et al. 1999) and in humans inferred from the temporal association between this decrease in viral load and the appearance of HIV-1 specific CD8 T cells (Borrow, Lewicki et al. 1994; Koup, Safrit et al. 1994), from the selection of particular viral mutants that escape CD8 T cell recognition (Borrow, Lewicki et al. 1997; McMichael and Phillips 1997; Price, Goulder et al. 1997; McMichael and Rowland-Jones 2001; Leslie, Pfafferott et al. 2004; Goonetilleke, Liu et al. 2009) and from the observation that specific HLA class I molecules are consistently associated with particular HIV-1 disease outcomes (Carrington and O'Brien 2003; Kiepiela, Leslie et al. 2004; Pereyra, Jia et al. 2010). These findings together with the close proximity of the HIV-1-protective -35 SNP to the *HLA-C* locus were highly suggestive that CD8 T cells played a mechanistic role in the SNP effect. The data presented here are the first direct evidence that the association between the -35 SNP and viral load set point operates through HIV-1 specific CD8 T cells.

The capacity of CD8 T cells, from 46 antiretroviral therapy naïve HIV-1 infected Caucasians, to suppress heterologous HIV-1 infection of autologous CD4 T cells was assessed with a viral suppression assay. Individuals were selected from a cohort of HIV-1 chronic progressors and long term non-progressors (enriched for the -35*CC* genotype). This showed that circulating CD8 T cells from different groups of HIV-1

infected individuals were able to efficiently control heterologous HIV-1 infection *ex vivo*. The antiviral CD8 T cell suppressive capacity ranged from 0-98.5%. Six of the 46 infected individuals studied showed potent suppressive activity greater than 90% inhibition. Interestingly, all six were chronic progressors. This conflicts with recent studies which suggest that potent antiviral activity is unique to HIV-1 long-term non-progressor individuals who typically possess protective HLA class I alleles (Saez-Cirion, Lacabaratz et al. 2007; Saez-Cirion, Sinet et al. 2009; Spentzou, Bergin et al. 2010).

When these 46 individuals were grouped by -35 SNP genotype, distinct patterns in CD8 T cell antiviral activity were observed. The -35 *CC* group as a whole possessed the highest CD8 T cell antiviral activity, despite the presence of an outlying individual with no CD8 T cell activity against HIV-1_{BaL}, while the *TT* group showed the lowest suppressive capacity (p=0.0151; Mann-Whitney test). The impact of a single -35*C* allele or two alleles on CD8 T cell antiviral activity was studied. The presence of at least a single protective allele showed significant difference with these individuals displaying greater CD8 T cell antiviral activity compared to *TT* individuals (p=0.0013; Mann-Whitney test). The presence of two -35*C* alleles did not significantly increase median suppression compared to a single allele, though suppression remained significantly higher than for *TT* individuals. This is evidence that the protective -35 SNP effect in HIV-1 disease is mediated through CD8 T cells. A potential criticism of this analysis is that the *CC* group was predominantly composed of long-term nonprogressors (13 of 15 individuals), which may bias the result given that this group of individuals have been shown to possess high CD8 T cell antiviral activity (Saez-Cirion, Lacabaratz et al. 2007; Saez-Cirion, Sinet et al.

2009; Spentzou, Bergin et al. 2010). Unfortunately, the strict criteria of being Caucasian and antiretroviral therapy naïve made it particularly difficult to enrol large numbers of HIV-1 infected chronic progressors who possessed the -35 *CC* genotype into the study. Interestingly, the two chronic progressors that were studied showed inhibition values of 94.3% and 94.2% which were higher than those recorded for all *CC* LTNPs. Another criticism is the cross-sectional nature of this study which makes it is impossible to separate cause and effect. Is the higher CD8 T cell antiviral activity seen in -35 *CC* individuals the cause or simply a consequence of lower viral load set point? A detailed longitudinal study of HIV-1 infected individuals will be necessary to answer this question, with the ensuing difficulty in recruiting sufficient numbers.

It was immediately clear that protective HLA-B alleles were over-represented in -35 *CC* and *CT* genotypes while risk HLA-B alleles were only present in the *TT* group. Analysis of HLA genotypes in 414 Caucasians revealed linkage disequilibrium between pairs of HLA-B and HLA-C alleles. An unexpected finding was that protective HLA-B alleles were always in linkage disequilibrium with HLA-C alleles that are in linkage disequilibrium with the -35*C* allele. The only exception to this rule was the linkage disequilibrium between protective HLA-B*5101 and -35*T*-associated HLA-Cw*1502. Interestingly, HLA-B*51 was not implicated as a protective allele in the largest genetic association study of HLA alleles that associate with HIV-1 control in Caucasians performed to date (Pereyra, Jia et al. 2010). Similarly, risk HLA-B alleles were in linkage disequilibrium with HLA-C alleles that are in linkage disequilibrium with the -35*T* allele. Identical linkage disequilibrium patterns between HLA-B and HLA-C alleles had previously been documented in a larger cohort of

1204 individuals (Fellay, Ge et al. 2009). This finding raised the possibility that the -35 SNP is in fact a marker for protective and risk HLA-B alleles.

The individuals who displayed potent suppressive activity along with those individuals who displayed high suppressive activity had one thing in common. They all possessed at least a single protective HLA-B allele (B*14/27/51/52/57). In addition when individuals were grouped by -35 SNP genotype, protective HLA-B alleles (and higher suppressive activity) were present in -35 *CC* and *CT* groups, whereas risk alleles (B*35; and lower suppressive activity) were only seen in *TT* group. These findings shed new light on the recent findings of Dinges *et al.* (Dinges, Richardt et al. 2010). They used IFN-γ ELISpot assays to screen for HIV-1 specific CD8 T cell responses to previously reported epitopes based on HLA genotype in individuals who possessed HLA-B*57/58, HLA-B*27 and HLA-B*35 alleles (the alleles with the strongest genetic associations with HIV-1 disease progression) and examined the relationships between these responses and the decline in CD4 T cell counts and HIV-1 viral load (Dinges, Richardt et al. 2010). They showed that CD8 T cell responses within B*57/58- and B*27-restricted alleles to conserved epitopes resulted in delayed declines in CD4 T cell counts and lower viral loads, while responses within B*35-restricted alleles to variable epitopes resulted in more rapid disease progression and higher viral loads. Additionally, the presence of certain B*57/58-, B*27- and B*35-restricted HIV-1 specific CD8 T cell responses after primary infection better defined disease progression than the HLA genotype alone, suggesting that it is the HIV-1 specific CD8 T cells and not the presence of a particular HLA allele that determine disease progression (Dinges, Richardt et al. 2010).

The data presented here surprisingly showed that the highest suppressive activity was detected in CD8 T cells from chronic progressors. A potential explanation for this may be the time-point tested in viral suppression assays. As a whole, the PBMCs tested from chronic progressors were collected within a few years of seroconversion. On the other hand, the PBMCs from LTNPs were sampled many years after seroconversion. It remains a distinct possibility that the antiviral activity of CD8 T cells diminishes with time. The only study to have looked at CD8 T cell antiviral activity in a longitudinal analysis focussed on cells from five long-term nonprogressors only and showed no loss in this HIV-1 suppressive capacity over the relatively short duration of between one and three years (Saez-Cirion, Sinet et al. 2009).

The antiviral CD8 T cell suppressive activity of cells from long-term nonprogressors ranged from 0-84%. Similarly, Saez-Cirion *et al.* observed weak HIV-1 suppressive capacity in 5 of 19 long-term nonprogressors studied (Saez-Cirion, Sinet et al. 2009). Although some underestimation of the CD8 T cell response may result from using a laboratory-adapted HIV-1 strain for HIV-1 suppression analyses, they showed that CD8 T cells from two of these weak suppressors had limited suppressive capacity even when autologous viruses were used, further supporting a truly weak CD8 T cell response in those individuals (Saez-Cirion, Sinet et al. 2009). These two studies are therefore in agreement with recent reports that there is significant heterogeneity in HIV-1 specific CD8 T cell response breadth, magnitude and polyfunctionality among long-term nonprogressors, and some have low to undetectable HIV-1 specific T cell responses (Emu, Sinclair et al. 2008; Pereyra, Addo et al. 2008).

There are several potential explanations for the weak suppressive activity in long-term nonprogressors observed in our cohort. It may be that control of viraemia is due to robust HIV-1 specific CD8 T cell responses in lymphoid tissues in these individuals. Support for this comes from Ferre and colleagues who have shown that HIV-1 long-term nonprogressors have polyfunctional HIV-1 specific T cell responses in rectal mucosa that were frequently stronger than in blood (Ferre, Hunt et al. 2009; Ferre, Lemongello et al. 2010). Another explanation may be that at the time point tested, CD8 T cell escape mutations had already occurred. Therefore, assuming HIV-1_{BaL} contained consensus/wild-type sequences, no suppressive antiviral activity would be detected in the assay. Despite the very low levels of ongoing viral replication in HIV-1 elite controllers, plasma viruses from these individuals display a substantial number of HLA-associated polymorphisms regardless of HLA class I allele types, indicating that viral escape from HIV-1 specific CD8 T cells is common even in elite controllers (Miura, Brumme et al. 2009). Finally, it may simply be that additional non-CD8 T cell factors account for other instances of decreased viral replication. To date, no clear pattern has emerged with regard to the immune response, host genetics or viral diversity that explains the long-term nonprogressor phenotype. One potential candidate remains the humoral immune response (Guan, Sajadi et al. 2009; Mahalanabis, Jayaraman et al. 2009; Scheid, Mouquet et al. 2009). Other recent reports have suggested that elevated levels of $CD4^+CD25^+$ regulatory T cells play a role by dampening HIV-1 replication by suppressing T cell activation during the acute phase of HIV-1 infection (Chase, Yang et al. 2008) and that elevated gamma-delta T cell levels play a role through an as yet undefined mechanism (Riedel, Sajadi et al. 2009).

The CD8 T cell suppression data presented here provide a possible functional explanation for the results of genome-wide association studies showing that the -35 SNP associates with HIV-1 viral load set point (Fellay, Shianna et al. 2007; Fellay, Ge et al. 2009). However, they give no indication of the specific HIV-1 proteins or epitopes targeted. These can be extrapolated from multiple cross-sectional studies which have shown that CD8 T cell responses, as measured by IFN-γ ELISpot assays, in individuals with low viral load and slow disease progression are preferentially directed against HIV-1 Gag (Riviere, McChesney et al. 1995; Edwards, Bansal et al. 2002; Addo, Yu et al. 2003; Martinez-Picado, Prado et al. 2006; Geldmacher, Currier et al. 2007; Kiepiela, Ngumbela et al. 2007; Streeck, Lichterfeld et al. 2007; Emu, Sinclair et al. 2008). Further, investigation of the specificity of the HIV-1 specific CD8 T cell response in individuals expressing protective, neutral or risk HLA class I alleles demonstrated that a highly conserved region within p24 Gag (amino acid residues 240 to 272, containing the known immunodominant responses HLA-B*57-TW10 and HLA-B*27-KK10) was highly targeted by HIV-1 specific CD8 T cells in those individuals expressing protective alleles but not in those expressing non-protective alleles during primary HIV-1 infection (Streeck, Lichterfeld et al. 2007; Emu, Sinclair et al. 2008). In addition, several potential epitopes restricted by other common HLA class I alleles such as HLA-A*02, -A*03, -B*07 and -B*08 are also contained within this area of p24 Gag (http://www.hiv.lanl.gov/content/immunology/old_maps/ctl/p24.html) and can be targeted in chronic infection (Streeck, Jolin et al. 2009). In contrast, non-Gag-specific CD8 T cell responses did not contribute to immune control and Env-specific CD8 T cell responses have been associated with elevated viraemia (Kiepiela, Ngumbela et al. 2007).

Further evidence that Gag-specific CD8 T cell responses are associated with better control of HIV-1 comes from viral suppression assays (Chen, Piechocka-Trocha et al. 2009; Julg, Williams et al. 2010). Chen *et al.* demonstrated that *in vitro* expanded Gag-specific CD8 T cell lines and clones from HIV-1 infected individuals resulted in far greater suppression of heterologous HIV-1 replication in autologous CD4 T cells than *in vitro* expanded Env-specific CD8 T cell lines and clones (Chen, Piechocka-Trocha et al. 2009). Julg *et al.* took this a bit further by using bulk CD8 T cells in their viral suppression assays. First they screened for Gag-specific responses using IFN-γ ELISpot assays in a large cohort of HIV-1 infected individuals and divided responders into high (six or more Gag-specific responses) and low (zero or one Gag-specific response) groups. After matching individuals in both groups for total magnitude of HIV-1 specific CD8 T cell responses and total peripheral CD4 counts, they compared the abilities of bulk CD8 T cells from both groups to inhibit HIV-1 replication *in vitro*. They showed that CD8 T cells from high Gag responders displayed a significantly stronger ability to suppress HIV-1 replication in autologous CD4 T cells than did individuals with narrow or no Gag-specific responses (Julg, Williams et al. 2010). In addition, protective HLA class I alleles did not differ significantly in their distributions among the two groups, suggesting that Gag specificity, rather than the particular HLA allele restricted responses, dictated superior antiviral control (Julg, Williams et al. 2010).

This raises the question of why CD8 T cell responses directed against this region of p24 Gag are more beneficial than responses directed against other HIV-1 regions. Firstly, Gag is a conserved protein and in particular the region between amino acid

residues 240 to 272 is highly conserved with more than 98.3% conservation per amino acid position according to the HIV-1 clade B sequences published in the LANL database (Streeck, Jolin et al. 2009). Secondly, previous studies have indicated that sequence variations within this region may incur a significant fitness cost to the virus (Leslie, Pfafferott et al. 2004; Martinez-Picado, Prado et al. 2006). Thirdly, there is early presentation of CD8 T cell epitopes in Gag, derived from the incoming capsid particle prior to *de novo* protein synthesis of other viral proteins (Sacha, Chung et al. 2007).

When the findings from these various studies are pieced together with the -35 SNP association with viral load set point, they are consistent with the hypothesis that a broad Gag-specific CD8 T cell response results in lower viral load set point potentially due to more potent inhibition of viral replication. The data presented here examine three CD8 T cell related mechanisms for the -35 SNP protective effect on viral load set point. Firstly, the protective effect is solely due to the HLA-C alleles in linkage disequilibrium with -35*C*. However, surface HLA-C expression studies and T cell mapping studies discussed in the previous chapters do not favour this mechanism. Secondly, the protective effect is solely due to HLA-B restricted CD8 T cells. There is a wealth of evidence in favour of the protective effect of the HLA-B locus in HIV-1 disease progression (Kiepiela, Leslie et al. 2004; Bihl, Frahm et al. 2006). The intricacy of the linkage disequilibrium pattern in the MHC region makes it difficult to define causal variants with certainty. It remains a strong possibility that the -35 SNP is a marker for protective and risk HLA-B alleles. The final mechanism involves a combination of class I restricted CD8 T cells. It may simply be that the SNP is a marker for combinations of protective HLA-A, -B and -C alleles. It is very well

established that pairs of HLA-B and HLA-C alleles are in linkage disequilibrium and that particular haplotypes can be either protective (B*14-Cw*0802 and B*57-Cw*06 in Caucasians; and B*3910-Cw*1203 and B*8101-Cw*0401 in Africans) or harmful (B*35-Cw*0401 in Caucasians) in the context of HIV-1 disease progression (Carrington, Nelson et al. 1999; Hendel, Caillat-Zucman et al. 1999; Fellay, Ge et al. 2009; Leslie, Matthews et al. 2010; Pereyra, Jia et al. 2010). In addition, HLA-Cw*07, recently defined as a risk allele (Pereyra, Jia et al. 2010), is in strong linkage disequilibrium with HLA-B*07 and HLA-B*08, and the latter is known to have an unfavourable impact on HIV-1 disease progression (Kaslow, Duquesnoy et al. 1990; McNeil, Yap et al. 1996; O'Brien, Gao et al. 2001; Carrington and O'Brien 2003; Turnbull, Lopes et al. 2006). The next chapter focuses on determining the contribution of HLA-B and HLA-C alleles to the protective -35 SNP effect by examining viral suppression in African individuals.

Chapter 6: THE -35 SNP IN AFRICAN POPULATIONS – IS THE SNP A TAG FOR PROTECTIVE HLA-B ALLELES?

6.1 Introduction

The previous chapter provided evidence that the -35 SNP protective effect on HIV-1 viral load set point in Caucasians is mediated through HIV-1-specific CD8 T cells which play a central role in controlling primary viraemia and the long-term suppression of viral replication. The HIV-1 specific CD8 T cells from -35 *CC* individuals displayed significantly greater antiviral suppressive activity compared to -35 *TT* individuals. In addition, due to distinctive linkage disequilibrium patterns, protective and risk HLA-B alleles clustered in *CC* and *TT* genotypes respectively.

It was becoming increasingly evident that the association of the -35 SNP with control of HIV-1 viral load set point in Caucasians was not a direct effect of the SNP itself. Personal communications from Jacques Fellay, David Goldstein and Mary Carrington that the -35 SNP effect did not associate with viral load at set point in African-American cohorts, further suggested the -35 SNP was a marker for an as yet undefined variant/s. The proximity of the -35 SNP to the HLA-C locus, the fact that the -35 SNP is in partial linkage disequilibrium with all *HLA-C* alleles and associates with protein expression levels of HLA-C (discussed in chapter 3 of this thesis), (Fellay, Shianna et al. 2007; Fellay, Ge et al. 2009; Thomas, Apps et al. 2009), made it appealing that this SNP was a marker of the effect of *HLA-C* expression on HIV-1 control. However, the functional studies detailed in chapter 4 of this thesis, suggest that *HLA-C* expression on its own is unlikely to be the causative marker. The

observation in the previous chapter that protective and risk HLA-B alleles clustered in -35 SNP genotypes together with the literature showing that the strongest HLA-associations with either slow or rapid HIV-1 disease progression are with HLA-B alleles (Carrington and O'Brien 2003; Kiepiela, Leslie et al. 2004) and that HLA-B restricted CD8 T cells exert the strongest selection pressure on the virus (Kiepiela, Leslie et al. 2004; Matthews, Prendergast et al. 2008; Rousseau, Daniels et al. 2008), led to the hypothesis that the -35 SNP is actually a marker for protective and risk HLA-B alleles. This hypothesis will be tested in a different ethnic population where a different HIV-1 viral subtype predominates.

Southern Africa remains the region most heavily affected by the HIV-1 epidemic with clade C virus being the predominant subtype (UNAIDS 2010). A study of C clade infected individuals from the region showed that most HIV-1 specific CD8 T cell responses were HLA-B restricted (Kiepiela, Leslie et al. 2004). Although certain HLA class I associations with control of HIV-1 infection cross the boundaries of ethnicity and viral subtype (e.g. HLA-B*57 protective in clade B infected Caucasians (Migueles, Sabbaghian et al. 2000; Altfeld, Addo et al. 2003) and clades A and C infected Africans (Costello, Tang et al. 1999; Lazaryan, Lobashevsky et al. 2006)), others remain confined by these boundaries (e.g. HLA-B*35 risk allele in Causcasians and African-Americans (Carrington, Nelson et al. 1999; Gao, Nelson et al. 2001) but has no appreciable effect on Africans (Tang, Tang et al. 2002)). Therefore, the literature was reviewed for protective and risk HLA alleles in African populations and these are presented in Table 6.1. The two largest genetic association studies were conducted in South Africa (n=1211) (Leslie, Matthews et al. 2010) and Zambia (n=784) (Tang, Malhotra et al. 2010). In the South African cohort five HLA alleles

were associated with a significantly higher or lower viral load following the application of the Bonferroni correction for multiple comparisons (B*5703 and B*5801 with low viral loads and B*5802, Cw*0602 and B*18 with high viral loads) (Kiepiela, Leslie et al. 2004; Leslie, Matthews et al. 2010). In the Zambian cohort stringent logistic regression analyses provided confirmatory findings for seven of ten HLA variants associated with HIV-1 viral load detected in generalised linear models (A*36 and DRB1*0102 were unfavourable alleles associating with high viral load, whereas A*74, B*57, B*81, Cw*18 and A*30-Cw*03 haplotype were favourable associating with low viral load) (Tang, Malhotra et al. 2010).

It was also clear from these African genetic association studies that the linkage disequilibrium pattern between HLA-B and HLA-C alleles were very different to those in Caucasians (Figure 6.1). The finding in chapter 5 of this thesis that -35*C* associated HLA-C alleles were in linkage disequilibrium with protective HLA-B alleles and that -35*T* associated HLA-C alleles were in linkage disequilibrium with risk HLA-B alleles did not hold up in the already published literature on HLA associations with HIV-1 viral load in African populations (Figure 6.1), assuming the -35 SNP association with HLA-C alleles remains the same in this ethnic group. Interestingly, in the South African cohort, reanalysis of HLA associations with high/low viral load and CD4 counts indicated that the apparent associations through HLA-A and HLA-C alleles were explained by linkage disequilibrium with HLA-B alleles (Kiepiela, Leslie et al. 2004). These initial observations added weight to the hypothesis that the -35 SNP is a marker for protective and risk HLA-B alleles.

Table 6.1. Protective and risk alleles/haplotypes against HIV-1 in African populations.

ALLELE	GOOD/BAD[a]	GENETIC STUDY REFERENCES	FUNCTIONAL STUDY REFERENCES
B57	GOOD		(Goulder, Phillips et al. 1997; Migueles, Sabbaghian et al. 2000; Altfeld, Addo et al. 2003; Kiepiela, Leslie et al. 2004; Frater, Brown et al. 2007; Goepfert, Lumm et al. 2008; Crawford, Lumm et al. 2009)
B5801	GOOD		(Leslie, Pfafferott et al. 2004; Frater, Brown et al. 2007; Navis, Schellens et al. 2008)
B5802	BAD		(Ngumbela, Day et al. 2008)
B1302	GOOD		(Harrer, Bergmann et al. 2005; Honeyborne, Prendergast et al. 2007; Prado, Honeyborne et al. 2009)
B8101	GOOD		(Frater, Brown et al. 2007)
A74	GOOD		(Matthews, Adland et al. 2011)
A29	GOOD	(Tang, Tang et al. 2002; Tang, Shao et al. 2008; Koehler, Walsh et al. 2010; Tang, Malhotra et al. 2010)	
A36	BAD	(Tang, Tang et al. 2002; Tang, Shao et al. 2008; Koehler, Walsh et al. 2010; Tang, Malhotra et al. 2010)	
B1801	BAD	(Kiepiela, Leslie et al. 2004)	
B4201	GOOD	(Kiepiela, Leslie et al. 2004)	
B4501	BAD	(Tang, Tang et al. 2002; Kiepiela, Leslie et al. 2004; Tang, Shao et al. 2008; Leslie, Matthews et al. 2010; Tang, Malhotra et al. 2010)	
Cw0602	BAD	(Kiepiela, Leslie et al. 2004)	
Cw16	BAD	(Tang, Malhotra et al. 2010)	
Cw18	GOOD	(Tang, Malhotra et al. 2010)	

ALLELE	GOOD/BAD[a]	GENETIC STUDY REFERENCES	FUNCTIONAL STUDY REFERENCES
A23-Cw07	BAD	(Tang, Malhotra et al. 2010)	
A7401-B5703 A30-Cw03 B14-Cw08 B3910-Cw1203 B8101-Cw0401	GOOD	(Tang, Tang et al. 2002; Kiepiela, Leslie et al. 2004; Tang, Shao et al. 2008; Leslie, Matthews et al. 2010; Tang, Malhotra et al. 2010)	

[a] Good – these are HLA alleles that have been shown to associate with lower HIV-1 viral load set point and /or slower progression to AIDS in genetic studies; CD8 T cells expressing these alleles have also typically been shown to exert a selection pressure (through functional data) on the virus leading to alterations in viral sequence and ultimately escape from a viral epitope response with ensuing viral fitness cost.
Bad – these are HLA alleles that have been shown to associate with higher HIV-1 viral load set point and/or rapid progression to AIDS in genetic studies; functional studies have to show that epitopes recognised by this allele do not exert selection pressure on the virus. The only example found in the literature was in a cohort of > 1000 clade C infected subjects where virus from HLA-B*5801 and HLA-B*5802 individuals was sequenced and showed strong selection pressure mediated through B*5801 but not B*5802 (Ngumbela, Day et al. 2008).

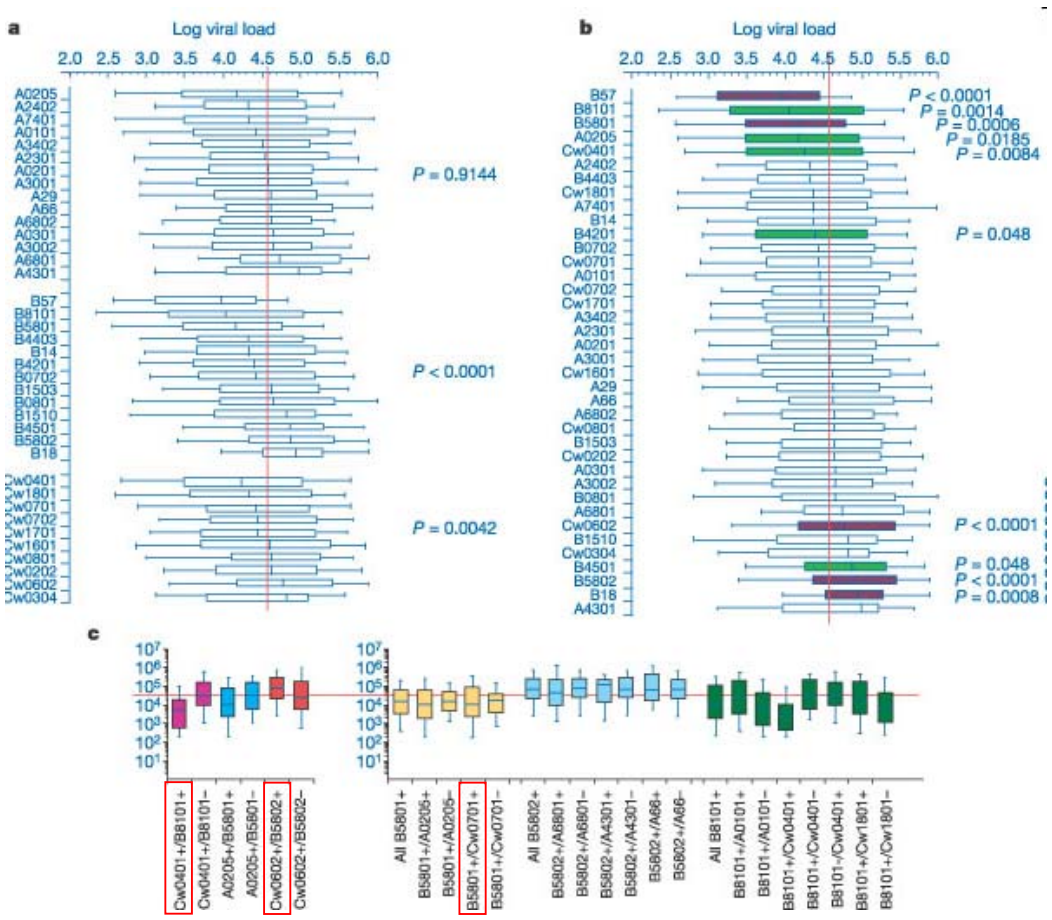


Figure 6.1. HLA class I molecule expression and viral load in chronically infected Zulu/Xhosa (n=706).

a, Contribution of class I loci to viral load variation. Viral loads shown as box plots (95th, 75th, 50th, 25th and 5th centiles); vertical red line indicates cohort median viral load. **b**, Association of individual HLA alleles with differential viral loads. Purple shading: $P < 0.001$. Green shading: $0.05 > P > 0.001$. **c**, Effect of linkage disequilibrium on the associations observed in **b**. Left panel: viral load associations of HLA-Cw*0401, A*0205 and Cw*0602 are lost. Right panel: viral load association of HLA-B*8101 is lost; however, HLA-B*5801, B*5802 (shown), and B*57, B*4201 and B*4501 (not shown) associations are maintained irrespective of linkage disequilibrium effects. Note that in Africans -35*C* associated HLA-C alleles and -35*T* associated HLA-C alleles are not in linkage disequilibrium with protective and risk HLA-B alleles respectively as observed in Caucasians (red boxes). **[Figure adapted from (Kiepiela, Leslie et al. 2004)]**.

This chapter explores the -35 SNP in an African population and sets out to answer the following questions:

- Does the -35 SNP exist in Africans?
- Is the -35 SNP in linkage disequilibrium with HLA-C alleles?
- Does the -35 SNP associate with viral load set point in Africans in an identical pattern to that in Caucasians?
- Does CD8 T cell antiviral suppression capacity (as measured by the viral suppression assay) correlate with HLA-B alleles?

On the basis of the different linkage disequilibrium patterns between HLA-B and HLA-C alleles in this ethnic group, the prediction is that no association exists between the -35 SNP and viral load set point. In addition, based on the hypothesis that the -35 SNP is a marker for HLA-B alleles, individuals with protective HLA-B alleles and those with risk HLA-B alleles are predicted to demonstrate the highest and lowest antiviral suppressive activity respectively.

6.2 Results

6.2.1 Study Population

The CHAVI network has enrolled a total of 443 African individuals (HIV-1 uninfected and chronically HIV-1 infected antiretroviral therapy-naïve adults) across three Southern African countries (Malawi, Tanzania and South Africa) for various HIV-1 related studies. They have also collected stringent viral load set point data, using the definition described by Fellay *et al* (Fellay, Shianna et al. 2007) (defined in

Materials and Methods), for 323 individuals. Whole blood pellets from 289 HIV-1 infected individuals (129 Malawians, 141 South Africans and 19 Tanzanians) were available for DNA extraction. HLA typing and -35 SNP genotyping was performed on all these samples.

6.2.2 Different distribution of HLA alleles in African and Caucasian populations

The DNA from the 289 individuals represents 3 different countries and many more tribes from Southern Africa. On the basis of similar distribution of class I HLA alleles in the 3 countries (Figure 6.2), all samples were grouped as a homogenous African population for all further analysis. In contrast, there was a very different pattern of HLA allele distributions between the 289 Africans and the cohort of 414 Caucasians (Figure 6.3). Among the *HLA-A* alleles, *A*01* and *A*02* were expressed by 80% of Caucasians compared to 30% of Africans. *HLA-A*30* was the most frequent allele in Africans (approximately 40%, compared to 4% of Caucasians) and *A*74* was exclusive to Africans. Other alleles exclusive to Africans were *B*42*, *B*81* and *Cw*18*.

6.2.3 The -35 SNP in Africans

Similar to Caucasian populations, the -35 SNP occurs in Africans. There were 37 *CC* (12.8%), 127 *CT* (43.9%) and 125 *TT* (43.3%) individuals. These frequencies were different from the 5.3% and 60.2% for *CC* and *TT* respectively quoted by the HapMap project (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=9264942), most

likely because the African population studied in that cohort was the Yoruba tribe from Nigeria (West Africa).

6.2.3.1 The -35 SNP displays a different linkage disequilibrium pattern with *HLA-C* alleles in Africans

The 162 individuals who were homozygous for -35*C* and -35*T* alleles were examined for linkage disequilibrium with *HLA-C* alleles using the HLA frequency analysis tool (http://www.hiv.lanl.gov/content/immunology/hla/hla_linkage.html) on the LANL database. In this small cohort, linkage disequilibrium between the -35 SNP alleles and four *HLA-C* alleles reached statistical significance (Table 6.2). *HLA-Cw*06*, *HLA-Cw*08* and *HLA-Cw*12* were in linkage disequilibrium with -35*C* and *HLA-Cw*04* was in linkage disequilibrium with -35*T*. Other *HLA-C* alleles were expressed by both -35 *CC* and *TT* individuals (Table 6.3). In particular, the frequency of *HLA-Cw*07* in both *CC* and *TT* individuals was similar, whereas *HLA-Cw*16/17/18* was mostly expressed by *TT* individuals. This differs from linkage disequilibrium patterns in Caucasians, where *HLA-C* alleles can be divided into 2 separate groups based on linkage with -35*C* or -35*T* and *HLA-Cw*07* allele is always associated with -35*T* (Fellay, Ge et al. 2009; Thomas, Apps et al. 2009).

6.2.3.2 *HLA-B* and *HLA-C* allele linkage disequilibrium in Africans is different to the pattern seen in Caucasians

Perhaps unsurprisingly, given the different frequencies of HLA alleles in the two populations, the pattern of *HLA-B* and *HLA-C* alleles that are in linkage disequilibrium differs between African and Caucasian populations (Table 6.4). Four haplotypes - *B*07-Cw*07*, *B*13-Cw*06*, *B*14-Cw*08* and *B*35-Cw*04* - were seen

in both populations although the strength of linkage disequilibrium varied. Protective *HLA-B* alleles in Africans were in linkage with both -35*C* and -35*T* associated *HLA-C* alleles. The protective alleles *B*42*, *B*57* and *B*81* were in linkage disequilibrium with *Cw*17*, *Cw*18* and *Cw*18* respectively, all of which are predominantly seen in association with -35*T* allele (Table 6.3). The protective allele *B*13* was in linkage disequilibrium with the -35*C* associated *Cw*06* (Table 6.2). Similarly, risk *HLA-B* alleles in Africans were in linkage with both -35*C* and -35*T* associated *HLA-C* alleles. HLA-*B*5802* was in linkage with *Cw*06* (-35*C* associated), *B*45* and *B*18* were in linkage with *Cw*16* (predominantly -35*T* associated) and *Cw*07* (both -35*C* and -35*T* associated) respectively. This is in stark contrast to the findings in Caucasians, in whom protective and risk *HLA-B* alleles were linked to -35*C* and -35*T* associated *HLA-C* alleles respectively. All the linkages demonstrated in this small cohort have been replicated in larger studies focussing on South African and Zambian populations (Kiepiela, Leslie et al. 2004; Leslie, Matthews et al. 2010; Tang, Malhotra et al. 2010).

6.2.3.3 The -35 SNP does not correlate with viral load set point in Africans

Having established an association between the -35 SNP and HLA alleles, the next step was to determine whether the association between the -35 SNP and viral load set point was upheld in African populations. There were 172 Africans who were genotyped at the -35 SNP and also had viral load set point data available. There was no association between viral load set point and -35 SNP genotype (Figure 6.4). This suggests that the protective effect of the -35 SNP is limited to Caucasians.

Table 6.2. Linkage disequilibrium between HLA-C alleles and -35 SNP alleles from 162 Africans.

HLA-C ALLELE	-35 ALLELE	FREQUENCY	D'	p value
06	C	0.3649	1	1.23E-18
04	T	0.2280	1	3.08E-07
08	C	0.1081	1	3.91E-06
12	C	0.0810	1	1.02E-04

p values are for a 2-sided Fisher's exact test; D' = linkage disequilibrium

Table 6.3. Frequencies of HLA-C alleles and -35 SNP alleles that did not reach statistical significance in 162 Africans.

HLA-C ALLELE	No. in *CC* (n=74)	FREQUENCY IN *CC*	No. in *TT* (n=250)	FREQUENCY IN *TT*
02	7	0.0946	32	0.1280
03	8	0.1081	21	0.0840
05	1	0.0135	0	0
07	11	0.1486	48	0.1920
14	0	0	1	0.0040
15	0	0	3	0.0120
16	1	0.0135	23	0.0920
17	2	0.0270	36	0.1440
18	3	0.0405	29	0.1160

Table 6.4. Pairs of HLA alleles that are in linkage disequilibrium in the 289 African individuals studied.

HLA1	HLA2	P VALUE	-35 SNP	BOTH HLA	ONLY HLA1	ONLY HLA2	NEITHER	D PRIME
B42	C17	4.37E-50	C/T	54	2	5	228	0.9551
B5802	C6	8.02E-36	C	44	0	22	223	1
B14	C8	1.16E-24	C	29	3	17	240	0.8885
B45	C16	5.48E-20	C/T	21	5	10	253	0.7846
B39	C12	2.09E-18	C	12	2	1	274	0.9191
B53	C04	1.52E-14	T	28	3	53	205	0.8655
B57	C18	2.01E-14	C/T	18	3	24	244	0.8329
B15	C2	6.01E-14	C/T	46	52	15	176	0.6279
B81	C18	1.51E-11	C/T	18	8	24	239	0.6370
B35	C04	2.54E-11	T	18	0	63	208	1
A30	B42	4.13E-10		44	76	12	157	0.6336
A30	C17	2.08E-9	C/T	45	75	14	155	0.5942
A34	B44	2.99E-9		16	9	27	237	0.5771
B13	C06	8.76E-9	C	12	0	54	223	1
B07	C07	2.91E-8	C/T	23	6	68	192	0.6980
A74	B35	1.93E-7		12	30	6	241	0.6100
B18	C07	2.60E-7	C/T	21	6	70	192	0.6756
A01	B81	5.96E-7		10	10	16	253	0.4506
A29	B44	5.64E-6		15	18	28	228	0.3592
A01	C18	1.11E-5	C/T	11	9	31	238	0.4735
B44	C04	3.23E-5	T	24	19	57	189	0.3861

This table was generated using the LANL HLA frequency analysis tool – inputted HLA types are used to return any linkage disequilibrium found by a 2-sided Fisher's exact test. HLA alleles in bold type are protective and risk alleles are in italic.

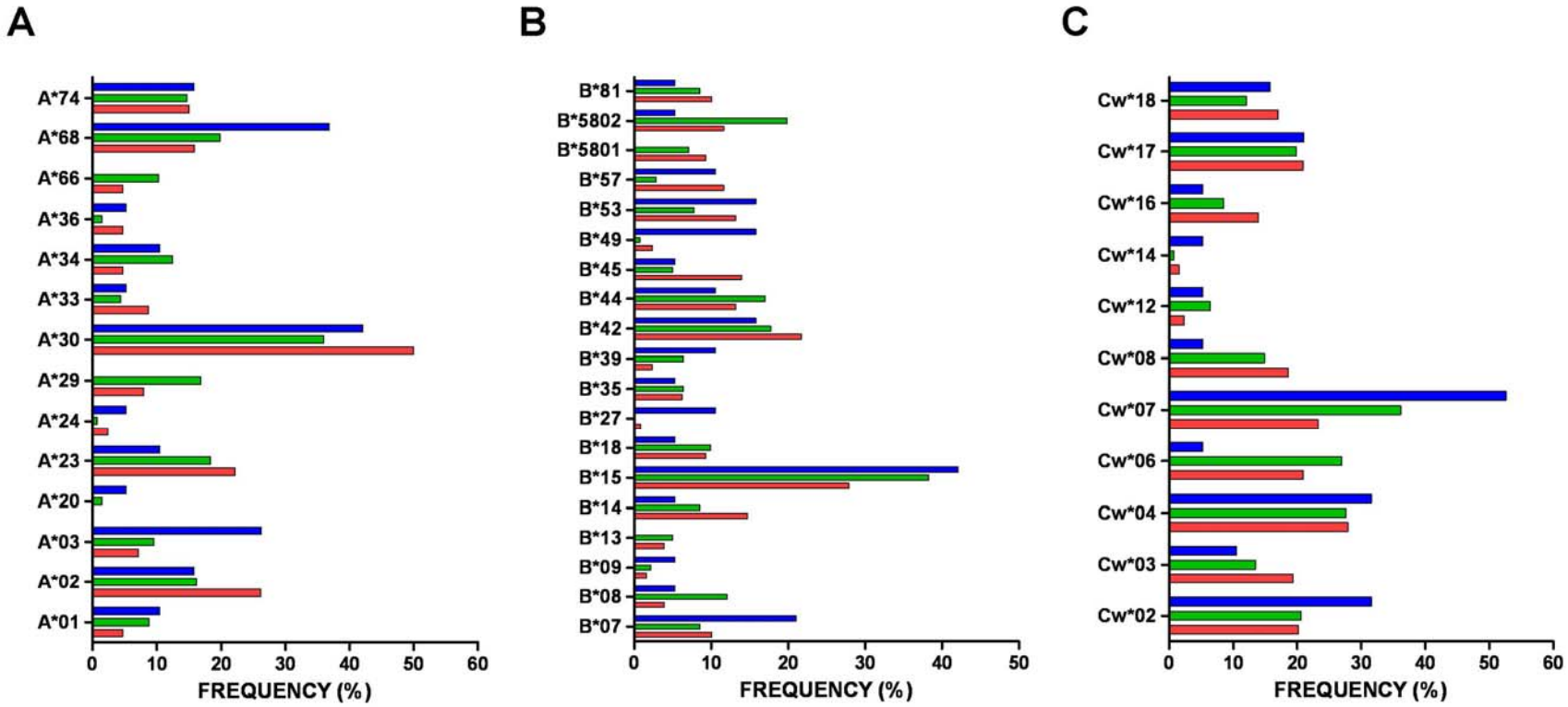


Figure 6.2. Population frequencies of HLA class I alleles among 289 Africans from Malawi (129), South Africa (141) and Tanzania (19). (A) HLA-A allele frequencies; (B) HLA-B allele frequencies; (C) HLA-C allele frequencies. The x-axis shows the percentage population frequency and the y-axis shows the HLA alleles. Red, green and blue bars represent Malawi, South Africa and Tanzania respectively.

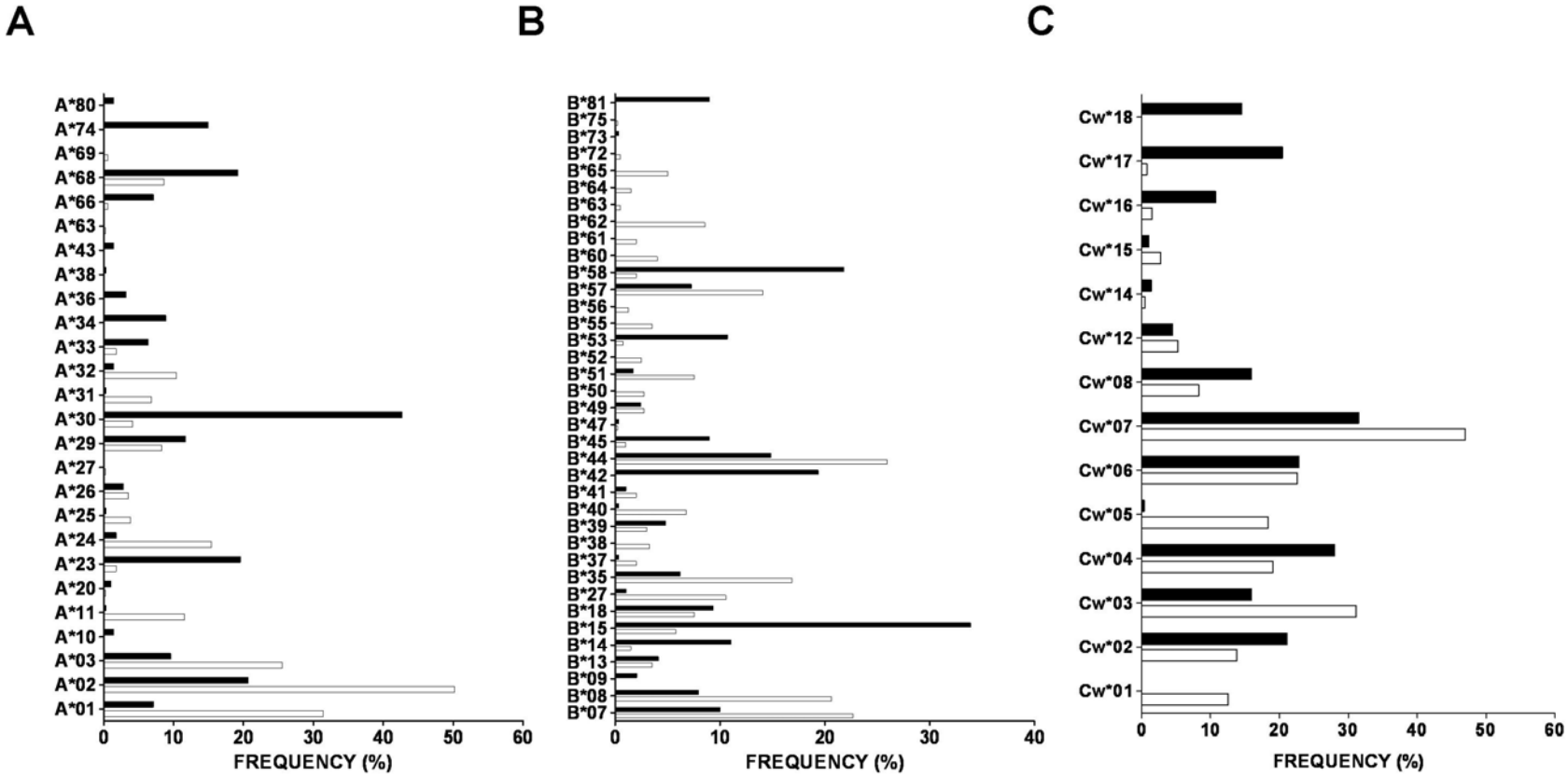


Figure 6.3. Comparison of population frequencies of HLA class I alleles among 289 Africans and 414 Caucasians.
(A) HLA-A allele frequencies; (B) HLA-B allele frequencies; (C) HLA-C allele frequencies. The x-axis shows the percentage population frequency and the y-axis shows the HLA alleles. Black and white bars represent the African and Caucasian populations respectively.

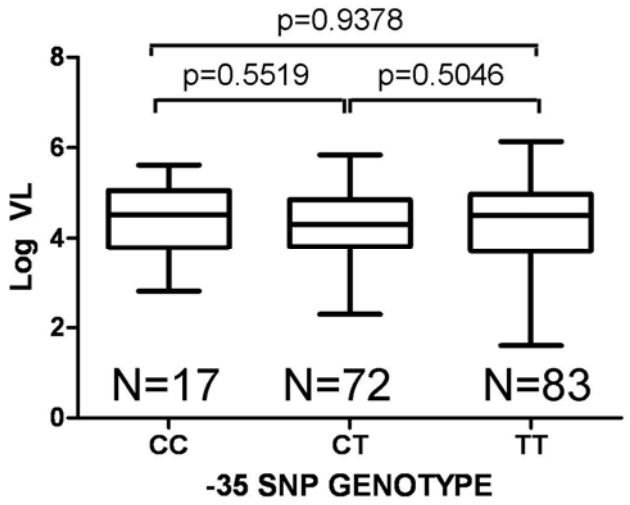


Figure 6.4. HIV-1 viral load at the set point does not correlate with the -35 SNP genotype in Africans.
The viral load set point data are presented as box and whisker plots (maximum value, 75th centile, 50th centile, 25th centile and minimum value) for the respective genotypes. The p values were calculated using the Mann-Whitney test.

6.2.4 CD8 T cell mediated suppression of HIV-1 replication in Africans

The lack of association between the -35 SNP and viral load in Africans provides further evidence that the -35 SNP is not causal of the differences in viral load observed in Caucasians. To test the hypothesis that the -35 SNP is a marker for HLA-B alleles, the antiviral suppressive activity of CD8 T cells from chronically HIV-1 infected Africans was determined using the previously described viral suppression assay. All individuals were antiretroviral therapy naïve. Since all the African individuals were infected with clade C virus, HIV-$1_{ES\ X1936}$ isolate was used as the heterologous HIV-1 strain in all assays. This is a CCR5-tropic clade C primary isolate originally obtained from plasma sampling of a Spanish woman during acute infection with HIV-1 (Fernandez-Garcia, Cuevas et al. 2009).

As before, the kinetics of HIV-$1_{ES\ X1936}$ infection was determined by infecting activated CD4 T cells and culturing these infected cells alone in a 10 day time-course (Figure 6.5). The optimal infection of CD4 T cells peaked at day 5 and day 7 respectively for the two control subjects. Therefore, for chronically HIV-1 infected individuals, cultures were harvested after six days and intracellular p24 staining performed. This maintained consistency with the time-point for optimal infection of CD4 T cells seen with HIV-1_{BaL} in Caucasians. A CD8 to CD4 cell ratio of 2:1 was used for all assays in these chronically infected individuals.

PBMC from five HIV-1 seronegative individuals were used to determine the viral suppression assay specificity and criteria for assay positivity. The mean and median frequencies of p24 positive CD4 T cells cultured alone were 0.414% and 0.37%

respectively, with minimum and maximum values of 0.2% and 0.88%. These data were used to define the background level of p24 staining, with values above 1.24% (three standard deviations from the mean) considered to indicate true infection of CD4 T cells with HIV-1. Next, coculture of CD8 and HIV-$1_{ES\ X1936}$-infected CD4 T cells was used to define non-specific CD8 T cell antiviral suppressive activity. CD8 T cells from these seronegative controls had a median antiviral suppressive activity (defined in Material and Methods) of 0% (range 0-7.74%). Again three standard deviations from the mean was used to define assay positivity and suppression detected above 12% was defined as HIV-1-specific. This analysis assumes that the variables measured are normally distributed.

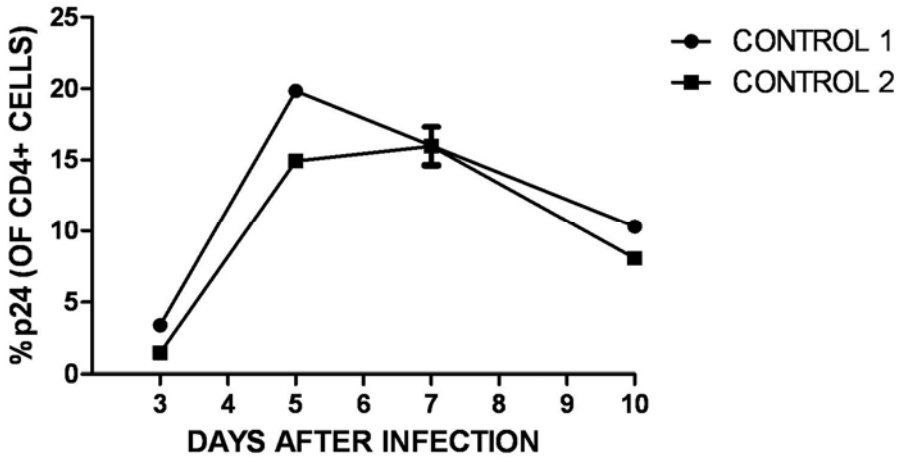


Figure 6.5. Time course of HIV-$1_{ES\ X1936}$ superinfection.
The purified CD4 T cell fraction from 2 HIV-1 uninfected individuals (Controls 1 and 2) were stimulated with 5μg/ml PHA for 3 days and then superinfected with HIV-$1_{ES\ X1936}$ at a multiplicity of infection of 0.001 and cultured in duplicate without autologous CD8 T cells for 10 days. At days 3, 5, 7 and 10 following superinfection, intracellular p24 antigen was quantified as a measure of HIV-1 infection. Maximal superinfection of CD4 T cells peaked at day 5 for one individual and day 7 for the other. Each data point represents the mean of duplicate wells, with standard deviation indicated by error bars.

6.2.4.1 The ability of CD8 T cells from chronically infected HIV-1 individuals to suppress heterologous virus replication is clade-specific

The viral suppression assay directly assesses the breadth of autologous CD8 T cell mediated antiviral activity against a heterologous HIV-1 strain. It relies on pre-existing effector and memory CD8 T cells recognising specific epitopes. There is sequence diversity among HIV-1 clades and therefore the heterologous virus used in suppression assays could potentially impact on CD8 T cell antiviral activity. The clade-specific HIV-1 consensus sequences reflect viral amino acid sequences that are most frequently found in the circulating viral species of a distinct geographic region. The extreme diversity of HIV-1 was addressed by Fischer *et al.* using polyvalent vaccine antigens, comprised of sets of mosaic proteins assembled from fragments of natural sequences via a computational optimisation method (Fischer, Perkins et al. 2007). These mosaic peptide sets maximised the coverage of potential T cell epitopes for a viral population. To evaluate inter- and intra-clade cross reactivity, they calculated the fraction of all perfect nonamer amino acid matches between natural sequences and the vaccine antigens in the entire Gag protein and central parts of Nef protein in clades B and C sequences. Focussing on four mosaic antigen sets optimised on C clade viruses, they found that 80-85% of these nonamers perfectly matched clade C sequences but the coverage of these same mosaic sets fell to 50-60% when compared to clade B sequences (Fischer, Perkins et al. 2007).

Thus the consensus C clade sequence was used as a surrogate for the infecting virus in all individuals studied. The amino acid sequences of the two heterologous viruses - the laboratory-adapted HIV-1_{BaL} (clade B) and the primary isolate HIV-$1_{ES\ X1936}$

(clade C) - were compared (Table 6.5). The two sequences were identical at only 58% of amino acid sites. In addition, the amino acid sequence of Gag, Pol, Nef and Env proteins were compared between clade C consensus, HIV-$1_{ES\ X1936}$ and HIV-1_{BaL} (Table 6.5). As expected the HIV-$1_{ES\ X1936}$/consensus clade C sequence comparison had more amino acids in common than the cross-clade HIV-1_{BaL}/consensus clade C comparison, with sequence homologies of 83.3-96.3% and 77.3-92.1% respectively.

To determine if these amino acid sequence differences would impact the antiviral suppressive activity of CD8 T cells, in a subset of 12 chronically infected individuals (Table 6.6), autologous CD4 T cells were infected with both heterologous viruses (Figure 6.6). Although there was no difference in the ability of the two viruses to superinfect autologous CD4 T cells ($p=0.5693$, Wilcoxon signed rank test) (Figure 6.6A), there was a statistically significant difference in the ability of autologous CD8 T cells to suppress viral replication in infected CD4 T cells ($p=0.0005$, Wilcoxon signed rank test) (Figure 6.6B). The autologous CD8 T cells were better at suppressing C clade HIV-$1_{ES\ X1936}$ replication than B clade HIV-1_{BaL}. This justified the decision to use the clade C virus to superinfect CD4 T cells in all individuals studied by viral suppression assay.

Table 6.5. Amino acid sequence comparison of HIV-1 isolates.

SEQUENCE 1	SEQUENCE 2	LENGTH	GAPS	IDENTITY	% IDENTITY
BaL ALL	X1936 ALL	4921	976/4921	2854/4921	58
BaL GAG	X1936 GAG	506	14/506	417/506	82.4
BaL POL	X1936 POL	1010	11/1010	905/1010	89.6
BaL NEF	X1936 NEF	210	4/210	154/210	73.3
BaL ENV	X1936 ENV	868	20/868	641/868	73.8
BaL GAG	C CON GAG	503	13/503	427/503	84.9
BaL POL	C CON POL	1007	7/1007	927/1007	92.1
BaL NEF	C CON NEF	208	1/208	167/208	80.3
BaL ENV	C CON ENV	859	32/859	664/859	77.3
X1936 GAG	C CON GAG	498	5/498	473/498	95
X1936 POL	C CON POL	1003	4/1003	966/1003	96.3
X1936 NEF	C CON NEF	210	3/210	175/210	83.3
X1936 ENV	C CON ENV	868	34/868	747/868	86.1

Sequences were obtained from the European Bioinformatic Institute and LANL databases and aligned using the EMBOSS water bioinformatics tool found at http://emboss.bioinformatics.nl/cgi-bin/emboss/water.
Identity = the number of identical matches between the two sequences over the reported aligned region (including any gaps in the length).
ALL = entire amino acid sequence of named HIV-1 isolate.

Table 6.6. Subset of 12 HIV-1 infected antiretroviral therapy naïve African individuals who were superinfected with HIV-1_{BaL} (clade B) and HIV-$1_{ES\ X1936}$ (clade C).

SUBJECT	GENDER	-35 SNP	% P24 BaL[a]	% SUPPRESSION BaL[b]	%P24 X1936[a]	% SUPPRESSION X1936[b]
704010171	F	TT	4.94	91.89	20.07	97.81
704010612	M	CC	6.9	41.17	7.71	82.71
702010514	F	CC	4.69	25.35	2.83	73.33
706010139	F	CC	1.7	46.75	2.13	71.72
704010285	F	CC	9.9	0	5.4	69.4
704010503	F	TT	5.56	60.97	2.9	68.68
702010477	M	TT	3.8	22.28	14.66	65.2
707010370	F	CC	4.85	43.11	4.28	60.3
707010071	F	TT	3.93	0	2.15	54.79
703010345	M	TT	11.07	0	10.97	49.25
704010445	M	TT	2.91	43.7	2.68	43.72
707010060	M	CC	5.02	0	2.74	6.45

[a] Frequency of p24 positive cells in autologous CD4 T cells infected with either HIV-1_{BaL} or HIV-$1_{ES\ X1936}$ and cultured alone (as defined in Materials and Methods).

[b] Assessment of CD8 T cell antiviral activity (% suppression as defined in Materials and Methods) after addition of autologous CD8 T cells to HIV-1 superinfected autologous CD4 T cells; an effector to target ratio of 2:1 was used throughout.

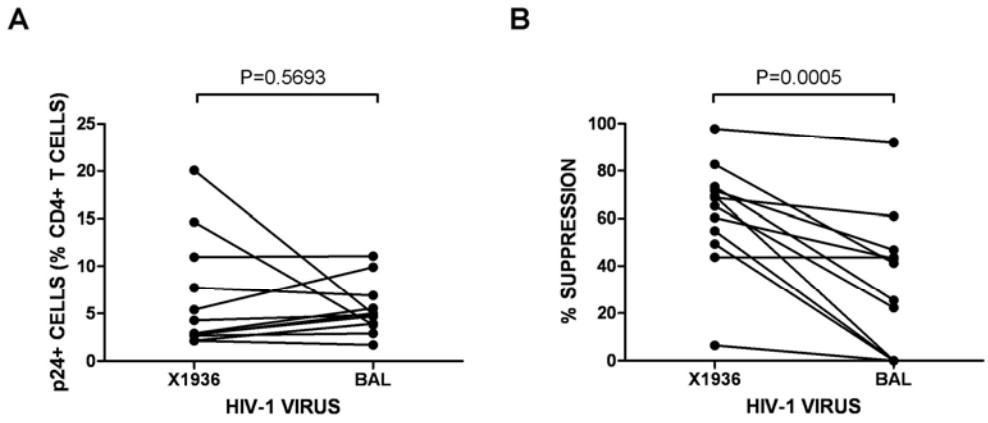


Figure 6.6. The antiviral suppressive activity of CD8 T cells is HIV-1 clade-specific.

The purified CD4 T cells from 12 HIV-1 infected individuals were stimulated with 5μg/ml PHA for 3 days and superinfected with HIV-1_{BaL} and HIV-$1_{ES\ X1936}$. These superinfected CD4 T cells were co-cultured in triplicate wells with autologous CD8 T cells at a CD8/CD4 ratio of 2:1 for 6 days. Viral inhibition was determined by quantifying p24 antigen-positive cells and calculating percentage suppression as described in Materials & Methods. (A) CD4 T cells were superinfected by both viruses. (B) CD8 T cell antiviral activity directed against HIV-$1_{ES\ X1936}$ (clade C) was significantly greater than that against HIV-1_{BaL} (clade B). Statistical analysis was performed using the Wilcoxon signed rank test.

6.2.4.2 There is no correlation between the -35 SNP and CD8 T cell antiviral activity in Africans

A cross-sectional analysis of CD8 T cell antiviral suppressive activity in 28 randomly selected chronically infected individuals across a wide range of plasma viral loads was performed (Table 6.7). The individuals studied were evenly distributed between *CC* and *TT* genotypes. Endogenous virus was detected by intracellular p24 antigen staining in 18 of 28 (60%) individuals after activation and culture of their CD8-depleted T cells. This differs with the findings in Caucasians where the reactivation rate of endogenous virus was a third this value (10 of 46 individuals assayed). The reasons for this difference are unclear but may simply be due to differences in biological phenotype of clades B and C virus. There was no correlation between HIV-$1_{ES\ X1936}$ suppression and the frequency of superinfected autologous CD4 T cells, indicating that potent antiviral CD8 T cell activity was not an artefact of lower superinfection of CD4 T cells *in vitro* ($r=0.1161$, $p=0.5565$; Spearman rank correlation test) (Figure 6.7A). When CD8 T cell antiviral activity was grouped according to -35 SNP genotype (Figure 6.7B), there was no difference in suppressive activity between *CC* and *TT* individuals ($p=0.4763$, Mann Whitney test).

Table 6.7. Characteristics of 28 HIV-1 infected antiretroviral therapy naïve African individuals studied in viral suppression assays.

SUBJECT	GENDER	-35 SNP	VIRAL LOAD[a]	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2	REACTIVATION[b]	P24% CD4[c]	% SUPPRESSION[d]
704010171	F	TT	1094	01	34	15	81	02	18	NO	20.07	97.81
705010377	M	CC	20796	03	80	1303	1801	02	06	NO	15.94	88.89
705010110	M	CC	49795	66	66	3910	5802	06	1203	NO	2.48	85.95
704010612	M	CC	1883	29	68	1302	5802	06	06	YES	7.71	82.71
704010330	M	TT	49189	34	68	42	44	04	17	NO	3.68	73.82
702010514	F	CC	11751	01	30	42	81	17	18	NO	2.83	73.33
704010414	M	CC	274819	34	68	3910	5802	06	1203	YES	14.2	72.83
706010139	F	CC	120149	66	68	14	5801	07	08	NO	2.13	71.72
705010868	M	CC	-	30	7401	15	4501	02	16	NO	3.05	70.79
704010285	F	CC	3585	01	02	5802	5802	06	12	YES	5.4	69.4
704010503	F	TT	788	01	23	81	07	07	18	NO	2.9	68.68
702010477	M	TT	200	30	33	42	81	17	18	NO	14.66	65.2
707010370	F	CC	4374	02	30	2703	3910	02	1203	YES	4.28	60.3
704010316	F	TT	9848	23	43	08	15	04	07	YES	13.25	56.29
704010558	F	TT	28683	30	7401	1801	35	04	07	YES	5.26	55.7
707010071	F	TT	12429	36	7403	15	4501	07	16	YES	2.15	54.79
702010710	F	TT	188036	02	68	15	53	04	04	YES	6.86	49.83
703010345	M	TT	72421	30	68	07	53	04	07	NO	10.97	49.25
704010445	M	TT	3747	30	30	4202	53	04	17	YES	2.68	43.72
704010532	F	TT	10142	23	32	07	07	01	07	YES	5.36	29.94
705010608	M	CC	27735	68	68	5802	5802	06	06	YES	1.43	26.28
705010154	F	TT	111938	24	24	07	07	07	07	YES	6.21	24.05
705010782	M	CC	-	03	30	3910	5802	06	1203	YES	4.66	15.21
702010088	M	TT	304672	30	7401	15	45	02	16	YES	7.38	11.56
704010408	M	TT	19166	30	30	53	5703	04	18	YES	8.77	10.06
707010060	M	CC	420512	30	68	15	5703	03	18	YES	2.74	6.45

SUBJECT	GENDER	-35 SNP	VIRAL LOAD[a]	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2	REACTIVATION[b]	P24% CD4[c]	% SUPPRESSION[d]
704010028	F	CC	8681	68	68	14	5801	06	08	YES	4.9	0
705010497	F	CC	44590	02	66	5801	5802	06	07	YES	6.02	0

All individuals were infected with clade C virus. The primary isolate HIV-$1_{ES\ X1936}$ (clade C) was used to superinfect CD4 T cells.

[a] Viral load at set point.

[b] Reactivation of an individual's autologous HIV-1 infection was determined by calculating the frequency of p24 positive cells in uninfected autologous CD4 T cells as defined in Materials and Methods (1.5% was the cut-off for reactivation of autologous virus).

[c] Frequency of p24 positive cells in autologous CD4 T cells infected with HIV-$1_{ES\ X1936}$ and cultured alone (as defined in Materials and Methods).

[d] Assessment of CD8 T cell antiviral activity (% suppression as defined in Materials and Methods) after addition of autologous CD8 T cells to HIV-1 superinfected autologous CD4 T cells; an effector to target ratio of 2:1 was used throughout.

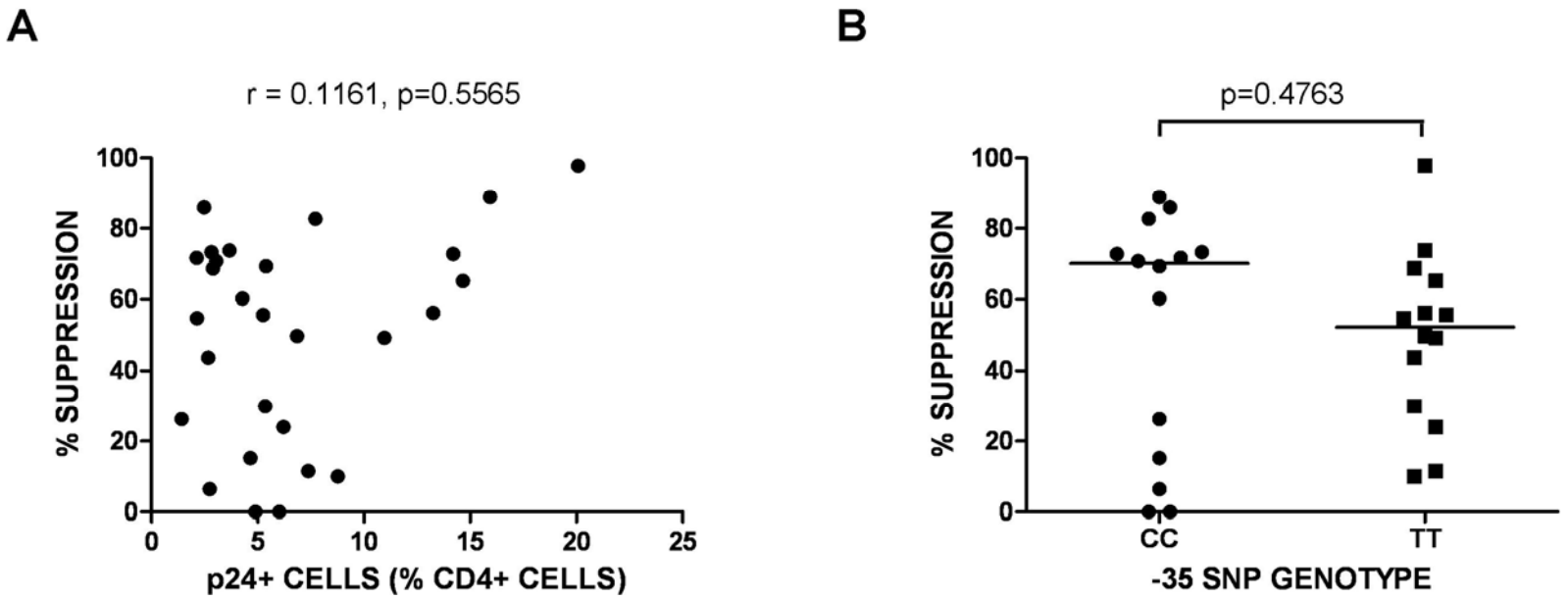


Figure 6.7. CD8 T cell suppression of HIV-$1_{ES\ X1936}$ does not correlate with the frequency of superinfected CD4 T cells or -35 SNP genotype.

(A) CD4 T cell superinfection levels with HIV-$1_{ES\ X1936}$ do not correlate with CD8 T cell-mediated suppression of HIV-1 replication. Each data point represents the mean of triplicate wells. Statistical analysis was performed with the Spearman rank correlation test. (B) The antiviral suppressive capacity of *ex vivo* CD8 T cells does not correlate with the -35 SNP in Africans. Statistical analysis was performed with the Mann-Whitney test.

6.2.4.3 HLA-B alleles drive CD8 T cell antiviral activity in Africans

The next step was to determine if CD8 T cell antiviral activity correlated with HIV-1 viral load set point (Figure 6.8). In the small number of individuals studied, the antiviral CD8 T cell suppressive capacity was inversely related to set point viraemia but this trend failed to reach statistical significance (r=-0.2589, p=0.1834; Spearman rank correlation test). Interestingly, the highest suppressive CD8 T cell activity was seen in individuals who possessed protective HLA-B alleles (B*1302, B*4201, B*5703, B*5801, B*8101 and the B*3910-Cw*1203 haplotype) while individuals with risk HLA-B alleles (B*1801, B*4501, B*5802) tended to display lower CD8 T cell suppressive activity (Figure 6.9). The presence of at least a single protective HLA-B allele countered the effect of risk alleles in individuals who expressed both and conferred strong CD8 T cell antiviral activity. Interestingly, individuals with protective HLA-B alleles were divided into two groups - those whose CD8 T cells displayed strong suppressive activity (65.2-97.81%) and those whose CD8 T cells displayed weak to no suppression (0-15.21%). The four lowest CD8 T cell suppressive activity were measured in individuals who expressed HLA-B*57/5801 (these two alleles are closely related and present the same epitopes). In Caucasians the CD8 T cell suppressive activity of individuals with protective HLA-B alleles was more widespread with one outlying individual whose T cells failed to suppress HIV-1 replication.

When CD8 T cell antiviral activity in these 28 individuals was grouped according to possession of at least a single protective HLA-B allele and absence of a protective HLA-B allele (Figure 6.10), there was no statistically significant difference between the two groups (p=0.1567, Mann Whitney test).

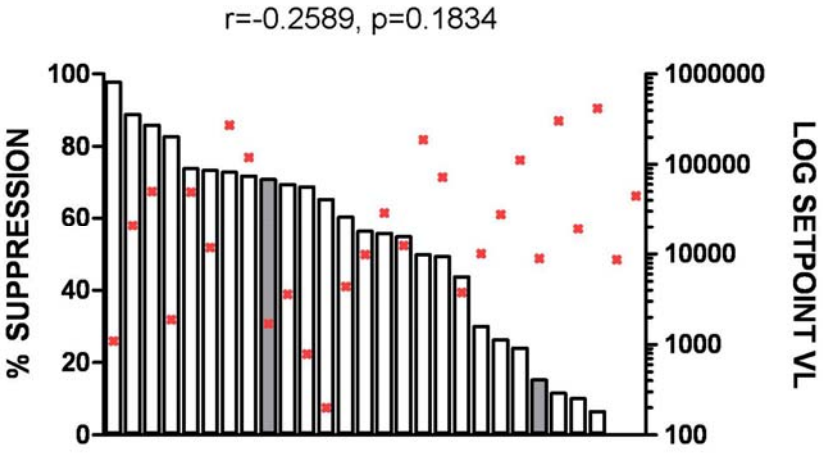


Figure 6.8. Correlation between CD8 T cell antiviral suppression and viral load set point in chronic HIV-1 infection.
In the 28 African individuals studied, there was a trend toward an inverse relationship between suppression of HIV-1 replication and control of viraemia. The columns represent suppressive capacity and the red crosses represent viral load set point for each individual. For two individuals (grey columns), there were insufficient data to calculate viral load set point – therefore viral load at the time of the assay was plotted. Statistical analysis was performed with the Spearman rank correlation test.

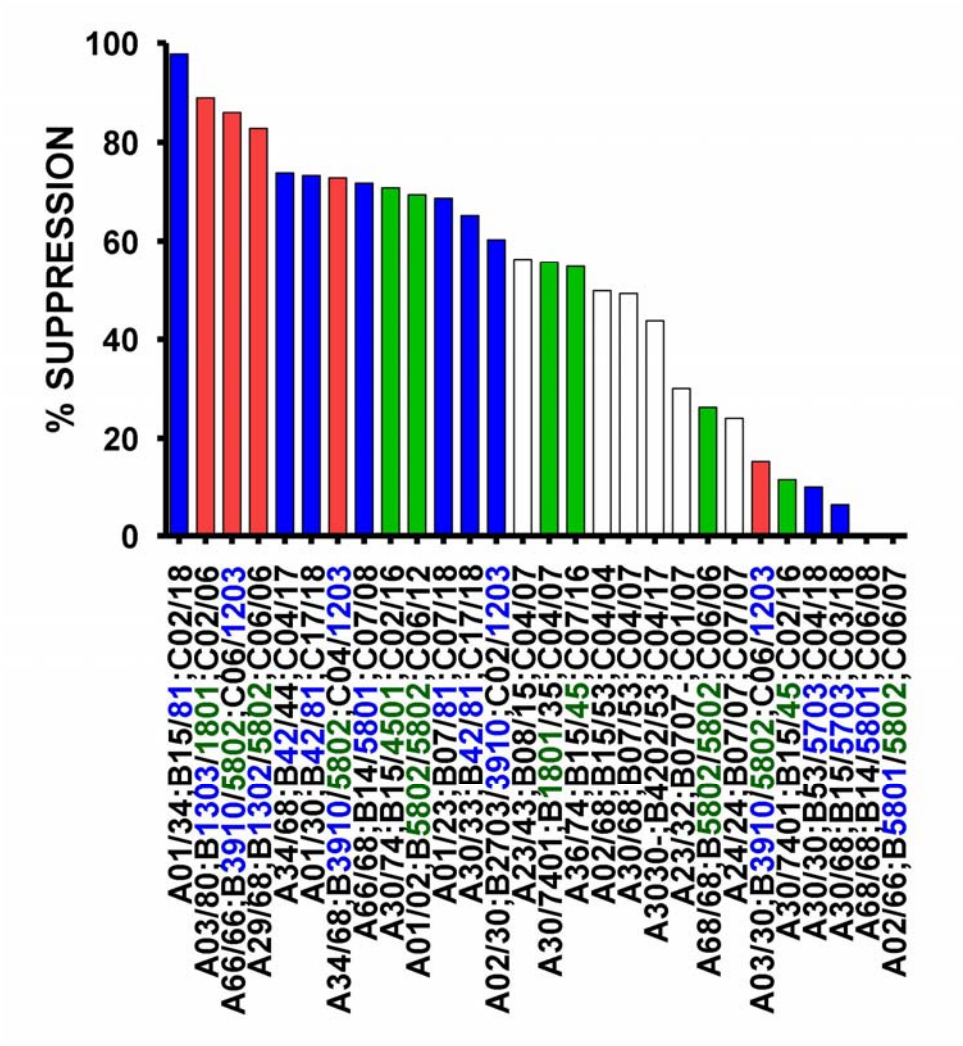


Figure 6.9. The HLA-B allele expressed impacts on CD8 T cell antiviral suppressive capacity.

Protective and risk HLA-B alleles for HIV-1 progression were plotted against CD8 T cell suppressive activity. The HLA typing for the 28 African individuals studied are listed on the x-axis. Blue columns represent individuals who possess at least a single protective HLA-B allele (B*1302, B*4201, B*5703, B*5801, B*8101 and B*3910-Cw*1203 haplotype). Green columns represent individuals who possess at least a single risk HLA-B allele (B*1801, B*4501, B*5802). Red columns represent individuals who possess both protective and risk HLA-B alleles. Clear columns represent individuals who possess neutral HLA-B alleles (not shown to be protective or risk alleles in genetic association studies).

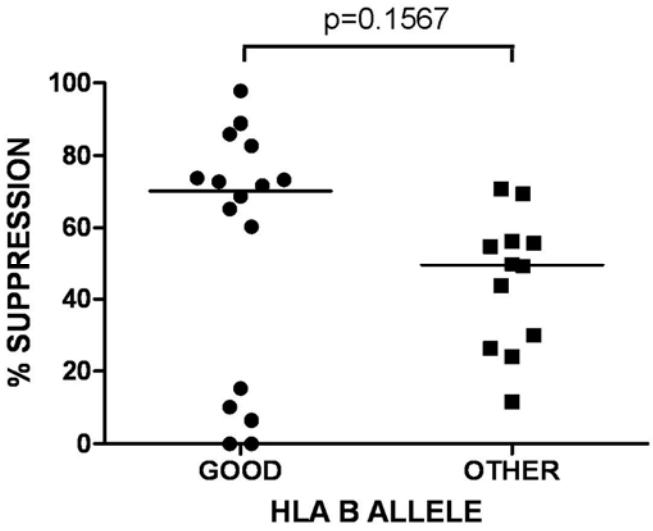


Figure 6.10. Protective HLA-B alleles are not solely responsible for CD8 T cell antiviral activity in Africans.
The 28 African individuals were divided into two groups based on expression of at least a single protective HLA-B allele (B*1302, B*4201, B*5703, B*5801, B*8101 and B*3910-Cw*1203 haplotype). There was no difference in CD8 T cell suppressive activity between the two groups. 'Good' refers to those individuals possessing protective HLA-B alleles and 'Other' refers to individuals who did not possess protective HLA-B alleles.

6.3 Discussion

An association between the -35 SNP and HIV-1 viral load set point is well established with the -35*C* allele correlating with low viral load set point and the -35*T* allele with high viral load set point in Caucasian populations (Fellay, Shianna et al. 2007; Fellay, Ge et al. 2009; Thomas, Apps et al. 2009). HLA-C restricted CD8 T cells were postulated to be the mechanism of viral control (Fellay, Shianna et al. 2007; Thomas, Apps et al. 2009). A small scale T cell study showed no differences in the frequency or magnitude of ELISpot responses to a panel of HLA-C restricted epitopes between -35*CC* and *TT* individuals (chapter 4 of this thesis). However, the only evidence that HIV-1 specific CD8 T cells play a mechanistic role in the association between the -35 SNP and viral load set point was demonstrated using viral suppression assays (chapter 5 of this thesis). These two findings led to the hypothesis that the -35 SNP is actually a marker for protective and risk HLA-B alleles.

This hypothesis was tested in a different ethnic group. Samples from HIV-1 clade C chronically infected individuals from Malawi, South Africa and Tanzania were available for study. To increase the sample size, these individuals were grouped as a homogenous African population on the basis of similar distribution of HLA alleles. This is potentially one criticism of the study design as the HLA alleles were not identical across the three groups and certain over- or under-represented HLA alleles could potentially skew the results.

The -35 SNP association with viral load set point was assessed in 172 Africans. There was no statistically significant difference in set point between *CC*, *CT* and *TT* individuals. This finding has recently been validated by the first genome-wide search

for genetic variants associated with differences in susceptibility to HIV-1 infection in an African population (Petrovski, Fellay et al. 2011). They compared genotypes of HIV-1 infected and non-infected individuals attending sexually-transmitted infection clinics in Malawi and found no SNP that had a genome-wide statistically significant association with viral load at set point (Petrovski, Fellay et al. 2011). When the impact of the -35 SNP was assessed in 121 African-American adolescents with chronic HIV-1 infection, the *CC* genotype was associated with reduced viral load set point (Shrestha, Aissani et al. 2009). However, a genome wide association study performed in 515 HIV-1 infected African-Americans found no single SNP with a statistically significant association with viral load at set point (Pelak, Goldstein et al. 2010). These findings provide further evidence that the -35 SNP is not causal and is instead tagging a causative variant or variants.

Another difference between African and Caucasian populations was the linkage disequilibrium patterns within the major histocompatibility complex. Given the small numbers studied, linkage disequilibrium between 2-digit HLA-C types and the two alleles of the -35 SNP were studied (Tables 6.2 and 6.3). Similar patterns to that observed in Caucasians was seen. The most striking exception involved *HLA-Cw*07* which was in linkage with both -35*C* and -35*T* alleles – the numbers were too small to detect which 4-digit HLA types were responsible for individual linkage patterns. The pattern of HLA-B and HLA-C haplotypes in linkage disequilibrium was also different in the African population (Table 6.4). In contrast to the observation in Caucasians, protective HLA-B alleles were not always associated with -35*C* linked HLA-C alleles and similarly risk HLA-B alleles were not always associated with -35*T* linked HLA-C alleles. Assuming that HLA-B restricted CD8 T cells do indeed play a significant role

in controlling HIV-1 replication, this difference in HLA-B/C linkage disequilibrium (from that observed in Caucasians) provides an explanation for the lack of correlation between the -35 SNP and viral load set point in Africans.

The HIV-1 clade C subtype is predominant in African populations. The ability of CD8 T cells to inhibit HIV-1 of different clades was assessed in twelve antiretroviral therapy naïve individuals using HIV-1_{BaL} (clade B) and HIV-$1_{ES\ X1936}$ (clade C) in viral suppression assays. These individuals controlled clade C superinfection significantly better than clade B infection ($p=0.0005$, Wilcoxon signed rank test). A possible explanation for this finding is that HIV-1 specific CD8 T cells target conserved HIV-1 epitopes (Goulder, Altfeld et al. 2001; Yusim, Kesmir et al. 2002; Bansal, Sabbaj et al. 2003; Smith, Amara et al. 2005; Yu, Lichterfeld et al. 2005) and amino acid homology is likely to be higher in viruses within the same clade than viruses across clades. These data contrast with a previous study which reported that CD8 T cells from HIV-1 seropositive individuals efficiently inhibited laboratory and primary isolates of HIV-1 clades A/D, B and C with CXCR4 or CCR5 coreceptor tropisms (Spentzou, Bergin et al. 2010). The likely explanation for the difference observed lies in the methodology. Spentzou *et al.* used autologous seven-day antibody-expanded CD8 T cells in their viral suppression assay, whereas this study used *ex vivo* unstimulated CD8 T cells to minimise induction of non-specific suppressive activity as a result of polyclonal T cell activation. On the basis of this finding the remaining sixteen individuals had their CD4 T cells superinfected with the clade C virus HIV-$1_{ES\ X1936}$. There was an inverse correlation between HIV-1 viral load set point and CD8 T cell antiviral activity, although this correlation did not reach

statistical significance in the small number of individuals studied (r=-0.2589, p=0.1834; Spearman rank correlation test).

As predicted, there was no association between CD8 T cell antiviral activity and the -35 SNP. The CD8 T cell HIV-1 suppressive activity was then plotted against the presence or absence of protective HLA-B alleles to test the hypothesis that the -35 SNP simply tags 'good' and 'bad' HLA-B alleles. There was no association between possessing a protective HLA-B allele and CD8 T cell antiviral activity (p=0.1567, Mann Whitney test). This suggested that the different linkage disequilibrium patterns in the major histocompatibility complex region in Africans had resulted in the causal site no longer being tagged by the -35 SNP variants. However closer inspection of the five outlying individuals with at least a single protective HLA-B allele and poor CD8 T cell suppressive activity revealed that four of them possessed HLA-B*5703/5801. It is not clear why there are such outliers but one possibility might be as follows. HLA-B*57 is consistently associated with slower disease progression in HIV-1 infection, particularly the B*5701 and B*5703 alleles which relate to better clinical outcome in Caucasian and African populations respectively (Klein, Keet et al. 1994; Costello, Tang et al. 1999; Migueles, Sabbaghian et al. 2000; Tang, Tang et al. 2002). A number of immunodominant HLA-B*57 epitopes which map to the Gag protein have been described (Goulder, Bunce et al. 1996), with KF11 (KAFSPEVIPMF, Gag 162-172) prevailing during chronic HIV-1 infection (Gillespie, Kaul et al. 2002; Ferrari, Currier et al. 2004; Gillespie, Stewart-Jones et al. 2006). In the context of HLA-B*5701, this epitope is associated with a limited tendency for viral escape mutations (Migueles, Laborico et al. 2003). However, KF11 variants have been described in naturally circulating viral strains and in particular have been shown to

associate with greater sequence variation in geographic regions (Africa) where the HLA-B*5703 subtype dominates (Gillespie, Kaul et al. 2002; Ferrari, Currier et al. 2004; Currier, Harris et al. 2005; Yu, Lichterfeld et al. 2007). Two variants were significantly more frequently encountered in HLA-B*57-expressing clade C infected individuals compared to the clade C-infected HLA-B*57-negative background population. These variants involved single amino acid alterations at position 2 [A2(G/S/N)] and a second variant which combined amino acid changes at positions 2 and 4 [A2G-S4(N/K)]. The [A2G-S4(N/K)] variant represented the most common KF11 variant found in HLA-B*5703 expressing individuals. Yu *et al.* sequenced the viral region encoding the KF11 epitope in a cohort of 426 HIV-1 clade C infected individuals and found the wild-type sequence in 76.9% of all individuals but in only 44.6% of HLA-B*5703-positive individuals (Yu, Lichterfeld et al. 2007). Cross-sectional studies of CD8 T cell function in HLA-B*57 expressing individuals have shown that HLA-B*5701 individuals with proviral DNA with wild type KF11 sequence cross-recognise all the common KF11 variants (Gillespie, Kaul et al. 2002; Ferrari, Currier et al. 2004; Currier, Harris et al. 2005). Three different patterns of response are apparent among HLA-B*5703 individuals depending on the epitope sequence of the proviral DNA. Individuals carrying wild-type and [A2(G/S/N)] variants have similar patterns of cross-reactivity with responses detected against all variants while individuals with provirus with the [A2G-S4(N/K)] variant showed responses specific for the index epitope but little or no response to any of the other variants (Gillespie, Kaul et al. 2002; Ferrari, Currier et al. 2004; Currier, Harris et al. 2005). In addition, the data also indicate that the observed paucity of KF11 variants in HLA-B*5701-expressing individuals is associated with a highly conserved KF11-specific T cell receptor repertoire that is able to cross-recognise the major naturally

occurring KF11 epitope variants. This is in contrast to individuals expressing HLA-B*5703, which differs from HLA-B*5701 only in two amino acid residues, who recruit an entirely different KF11-specific T cell receptor repertoire that frequently fails to cross-recognise specific KF11 variants that arise commonly *in vivo* in clade C infected HLA-B*5703-expressing individuals (Gillespie, Stewart-Jones et al. 2006; Yu, Lichterfeld et al. 2007). The heterologous virus HIV-$1_{ES\ X1936}$ used in the viral suppression assays contains the KF11 wild-type sequence. It is reasonable to suggest therefore that the four individuals with the lowest measured CD8 T cell suppressive activity (all expressed B*57/5801) possessed CD8 T cells that did not recognise the wild-type KF11 sequence present in HIV-$1_{ES\ X1936}$. Work is currently ongoing in the laboratory to confirm the KF11 sequence in all HLA-B*57/5801 individuals assayed; if this proves to be the explanation, it would be reasonable to exclude these four individuals from the analysis, in which case the difference between the two groups in Figure 6.10 would become statistically significant.

The observation that CD8 T cells from individuals with protective HLA-B alleles possessed strong antiviral activity in two ethnic groups adds further weight to previous studies that have identified a central role for HLA-B alleles in influencing immune control of HIV-1 infection (Kiepiela, Leslie et al. 2004; Bihl, Frahm et al. 2006; Harari, Cellerai et al. 2007; Kiepiela, Ngumbela et al. 2007). The small number of individuals studied in the viral suppression assays described above and the known linkage disequilibrium between HLA alleles makes it impossible to conclude for certain that the -35 SNP tags only HLA-B alleles. However, a cohort of over 1200 chronically HIV-1 clade C infected and treatment-naïve individuals has recently been investigated for correlation between individual HLA type and HIV-1 control as

measured by viral load and absolute CD4 count (Leslie, Matthews et al. 2010) and provides some interesting insights. The dominant effect of HLA-B on viral load and CD4 count was maintained even after the main protective (B*57 and B*5801) and risk (B*5802 and B*18) alleles were removed from the analysis. The HLA-C alleles Cw*0401 and Cw*1203 remained significant contributors to variation in viral load and CD4 count after HLA-B*57/58/18 alleles were accounted for. They also identified three protective HLA haplotypes - B*3910-Cw*1203, B8101-Cw*0401 and A*7401-B*5703 - in which protection was either dependent on or enhanced by co-expression of the HLA-B and non-B alleles. The B*3910-Cw*1203 haplotype was detected in the small cohort of 28 African individuals detailed in this thesis. Leslie *et al.* conclude that although single HLA alleles do have a strong effect on disease control, an individual's disease status is likely the result of the additive effect of some or all of the HLA alleles that individual possesses rather than a dominant effect of a single protective or risk HLA allele (Leslie, Matthews et al. 2010).

In conclusion, this chapter focussed on the effect of the -35 SNP on viral load set point in an African population. There was no correlation between viral load set point and -35 SNP genotype confirming that this SNP is not causal. In the small number of individuals studied there was however a trend toward an inverse correlation between viral load set point and CD8 T cell antiviral activity. The evidence for the -35 SNP tagging protective and risk HLA-B alleles is inconclusive and more likely tags a combination of protective and risk HLA-B and HLA-C haplotypes.

Chapter 7: DISCUSSION

7.1 Overall Discussion

Previous studies in Caucasians have observed that a single nucleotide polymorphism 35kb upstream of the *HLA-C* gene associates with control of viral load set point (Fellay, Shianna et al. 2007; Fellay, Ge et al. 2009), as well as slower progression to a CD4 count below 200 and increased time to death (Thomas, Apps et al. 2009). Transcriptional data showing that this SNP associates with differences in *HLA-C* expression levels and the linkage of -35 SNP alleles with *HLA-C* alleles (Fellay, Ge et al. 2009; Thomas, Apps et al. 2009) have all been taken as evidence that this SNP is a marker for the *HLA-C* locus, suggesting a possible link between viral load and HLA-C. As a result, there has been much speculation on a role for HLA-C in controlling HIV-1 infection. HIV-1 selectively down-regulates surface HLA-A and -B, but not HLA-C and -E, via the action of the Nef protein (Collins, Chen et al. 1998; Cohen, Gandhi et al. 1999; Collins and Baltimore 1999; Williams, Roeth et al. 2002). The infected cells may therefore be relatively resistant to HLA-A and HLA-B restricted CD8 T cell lysis but continue to express HLA-C. While it has been shown that recognition of infected cells by HLA-C restricted CD8 T cells is unaffected by HIV-1 Nef expression (Adnan, Balamurugan et al. 2006), these responses are relatively weak (Kiepiela, Leslie et al. 2004; Bihl, Frahm et al. 2006) and are consistently associated with high viraemia in HIV-1 infected subjects, even when targeting the Gag protein (Kiepiela, Ngumbela et al. 2007). This suggests that they contribute little to viral control *in vivo*. HLA-C molecules also act as ligands for the inhibitory KIR2DL receptors (Lanier 2005; Parham 2005), so infected cells expressing high levels of HLA-C may be less vulnerable to NK cell recognition (Collins, Chen et al. 1998;

Collins and Baltimore 1999; Adnan, Balamurugan et al. 2006). However, it is not known whether the level of HLA-C expressed by the target cell is critical, and, in addition, NK cells lacking inhibitory receptors for HLA-C and HLA-E molecules would still be able to kill HIV-1 infected T cells (Bonaparte and Barker 2004). Thus, studies were designed to address whether the -35*C*/*T* alleles are associated with differences in surface HLA-C levels in Caucasians, and to explore mechanisms by which HLA-C expression may affect the efficiency of HIV-1 control by CD8 T cells. Parallel studies examining whether HLA-C expression impacted on NK cell control of HIV-1 were performed by collaborators from the Persephone Borrow (University of Oxford) group.

The prevailing model for the effects of the protective -35*C* allele is that it is associated with higher cell surface levels of HLA-C protein than the nonprotective -35*T* allele. In chapter 3, it is shown that the median level of staining with the antibody DT9 (recognises both HLA-C and HLA-E) of lymphocytes from both seronegative and HIV-1 seropositive -35 *TT* Caucasian individuals is significantly lower than that of *CC* individuals ($p=0.0459$ and $p=0.0413$ respectively; Mann-Whitney test). However, this difference results entirely from low staining in individuals homozygous for HLA-Cw*07. To determine the true difference in surface HLA-C expression on lymphocytes from -35 *CC* and *TT* individuals, indirect saturation staining of lymphocytes with 3D12 (specific for HLA-E) and DT9 antibodies was performed. HLA-C levels (DT9 minus 3D12) were then normalised to the total level of MHC class I (W6/32 staining) and a significant difference between *CC* and *TT* individuals in the ratio of HLA-C to total MHC class I was detected ($p=0.0061$; Mann-Whitney test), with *CC* individuals expressing 1.76 times more

HLA-C than *TT* individuals. This difference was again exclusively driven by the low expression of HLA-Cw*07 (p=0.0032, comparing *CC* individuals with *TT* individuals homozygous for HLA-Cw*07; Mann-Whitney test), with *CC* individuals expressing 5 times more HLA-C than *TT* individuals homozygous for HLA-Cw*07. Importantly, there was no significant difference in the ratio of HLA-C to total MHC class I in *CC* and *TT* individuals not homozygous for HLA-Cw*07 (p=0.0896; Mann-Whitney test). Therefore, the protective -35 SNP genotype does not associate with increased expression of HLA-C. Rather, the non-protective allele associates with the greatly reduced expression of a single HLA-C allele, HLA-Cw*07. This finding raised the question of whether this allele alone accounted for the higher virus load in *TT* individuals. Fellay *et al.* confirmed that of all the HLA-C alleles, HLA-Cw*07 is significantly associated with the highest mean viral load set point (Fellay, Ge et al. 2009), yet exclusion of individuals homozygous for this allele does not abolish the association of the -35 SNP with control of HIV-1 infection. There remains a distinct possibility that other HLA-C alleles with different intrinsic levels of expression could contribute to the overall levels of surface staining. Evidence for this comes from a study showing that variation within the 3' UTR of *HLA-C* regulates binding of the microRNA (miR)-148a to its target site, resulting in relatively low surface expression of alleles that bind this microRNA and high expression of HLA-C alleles that escape posttranscriptional regulation (Kulkarni, Savan et al. 2011).

In chapter 4, the association between -35 SNP genotype and set point viral load was explored further. It was hypothesised that enhanced HLA-C expression could also drive an increase in the breadth and/or magnitude of HLA-C restricted CD8 T cell responses. Therefore two pilot studies were performed to investigate this in

treatment-naïve HIV-1 infected Caucasians, the population in which the original SNP observation was made. The first study involved screening for IFN-γ positive CD8 T cell responses by ELISpot assay against 48 previously described optimal HLA-C epitopes in a peptide matrix system. To ensure no potential responses were missed, the second study mapped responses by IFN-γ ELISpot assay using overlapping peptides spanning the entire clade B proteome. In the 40 individuals screened in these two studies, there were no differences in the frequency or magnitude of HLA-C restricted CD8 T cell responses between -35 *CC* and *TT* individuals. At best, only 16% of T cell responses were HLA-C restricted. This is consistent with the literature on HLA-C restricted T cell responses in HIV-1 infected individuals. Kiepela ***et al.*** studied treatment-naïve clade C infected South African individuals and found that 13% of immunodominant CD8 T cell responses were HLA-C restricted (Kiepiela, Ngumbela et al. 2007). Discussions with a biostatistician suggested that 400 seropositive individuals would need to be screened to have sufficient power to detect a difference in HLA-C restricted T cell responses between *CC* and *TT* individuals, which was not feasible.

It was hypothesised that epitopes presented by HLA-C alleles in linkage disequilibrium with -35*C* would be more conserved and lead to strong CD8 T cell responses resulting in lower viral load set point. These conserved epitopes possibly carry a significant viral replicative fitness cost. In contrast, by being less conserved and by implication carrying less viral fitness cost, those epitopes presented by HLA-C alleles in linkage disequilibrium with -35*T* will be able to escape the actions of CD8 T cells resulting in higher viral load set points. The Shannon entropy score was used as a measure of amino acid conservation when comparing thousands of clade B viral

sequences from HIV-1 infected individuals and showed no statistically significant difference in conservation in the two groups of epitopes. An important caveat is that the amino acid sequences chosen for comparison were the described optimal epitopes from the LANL database and therefore the numbers compared were small (9 epitopes restricted by -35*C*-linked HLA-C alleles and 3 epitopes restricted by -35*T*-linked HLA-C alleles). The assumption made at the outset in the epitopes studied was that HLA class I restricted CD8 T cells were driving entropy, with low entropy scores reflecting the net effect of escape mutations and subsequent reversion at a population level. Another explanation for low entropy scores could simply be that these epitopes are not under enough selection pressure by CD8 T cells to mutate. It has not been possible to explore which of the two scenarios described is more likely as this would require analysis of large-scale transmission data. The only study to explore which of these two scenarios is more likely measured the degree of conservation of HIV-1 residues targeted by HLA-B versus HLA-A alleles, and found that those targeted by HLA-B alleles were more conserved (Fontaine Costa, Rao et al. 2010). To determine whether the higher degree of conservation of HLA-B-restricted epitopes was the local net effect of escape mutations and subsequent reversion they analysed data published by Wang *et al*. of near full-length viral genomes from 98 chronically infected individuals containing 76 HLA class I-associated mutations (within and flanking regions of described and predicted epitopes) (Wang, Li et al. 2009). Their analyses revealed that the number of escapes and reversions associated with HLA-B was significantly enriched when compared with HLA-A ($p=0.002$; Chi-squared test) (Fontaine Costa, Rao et al. 2010). The reason why HLA-B proteins target conserved regions is largely unknown but may be one of the factors contributing to the immunodominance of HLA-B restricted CD8 T cell responses and their stronger

impact on HIV-1 disease progression (Kiepiela, Leslie et al. 2004; Bihl, Frahm et al. 2006; Kiepiela, Ngumbela et al. 2007).

A panel of HLA-C restricted CD8 T cell clones was generated and they were shown to possess cytolytic activity as measured by the chromium release assay. Although the cytolytic activity of these clones was not directly compared to those restricted by HLA-A and/or HLA-B alleles, several studies have confirmed that HLA-C restricted clones have comparable avidity and cytolytic activity to their HLA-A and HLA-B counterparts (Yang, Kalams et al. 1996; Yang, Sarkis et al. 2003; Bennett, Ng et al. 2007). The distinct linkage disequilibrium patterns between groups of HLA-C alleles and -35*C*/*T* precluded a direct comparison of epitope presentation to T cell clones from *CC* and *TT* individuals. The low frequency of HLA-C restricted CD8 T cell responses, the lack of functional differences between these T cells from individuals with *CC* and *TT* genotypes, coupled with the fact that these T cells are functionally identical to their HLA-A and HLA-B-restricted counterparts make it unlikely that the -35 SNP exerts its protective effect on viral load set point through HLA-C restricted CD8 T cells as initially proposed. Similarly, no difference in the NK receptor repertoire, levels of NK receptor expression, NK licensing or total NK responsiveness to activating stimuli in *CC* and *TT* HIV-1-seronegative Caucasian individuals was found (personal communication from Persephone Borrow).

To understand the mechanisms underlying better control of HIV-1 by certain HLA alleles, Mkhwanazi *et al.* investigated the functional characteristics of CD8 T cell responses restricted by either HLA-B*57/5801 (protective) or HLA-Cw*07 (risk allele) alleles within 9 individuals who possessed both B*57/5801 and Cw*07-

restricted T cell responses concurrently. The immunodominant HLA-B*57/5801-restricted responses were to four epitopes while in contrast only one HLA-Cw*07-restricted response was targeted, KY11. The overall magnitude of the IFN-γ producing CD8 T cell responses restricted by HLA-Cw*07 was significantly higher than responses restricted by HLA-B*57/5801 (p=0.0012, Mann-Whitney test). There was no significant difference in polyfunctional CD8 T cell responses (evaluated by simultaneous measurement of five functions – IFN-γ, TNF-α, IL-2, MIP-1β and CD107a – using multicolour flow cytometry) mediated by the two alleles within individuals with both responses. In addition, they evaluated whether the low magnitude of HLA-B*57/5801-restricted responses compared to greater magnitude HLA-Cw*07-restricted responses was due to sequence variation in the epitopes presented to CD8 T cells. HLA-B*57/5801-restricted epitopes had high sequence variation compared to HLA-Cw*07-restricted epitopes (p=0.05, Fisher's exact test) which may partly account for the low magnitude of HLA-B*57/5801- compared to HLA-Cw*07-restricted responses. Another explanation for the lower magnitude of HLA-B*57/5801-restricted responses may be the small sample size and relatively advanced stage of disease in the individuals studied (Mkhwanazi, Thobakgale et al. 2010). Their data on the conservation of amino acids within the KY11 epitope is consistent with the data presented in chapter 4 of this thesis (Figure 4.10). The average entropy score for this -35*T* linked epitope was lower than that of some -35*C* linked epitopes. The limited literature on T cell responses restricted by the non-protective HLA-Cw*07 allele do not support the notion that these T cells are limited functionally (Mkhwanazi, Thobakgale et al. 2010).

Only one other study has focussed on determining whether there are functional differences in T cell responses between *CC* and *TT* individuals (Specht, Telenti et al. 2010). Although the average set point viral load in individuals with the *CC* 'high HLA-C expression' genotype are significantly lower than those in individuals in the *TT* genotype, the distributions overlap. This suggests that some individuals with the protective genotype are not able to mount effective HLA-C mediated immune responses. Specht *et al.* hypothesised that high set point viral loads in individuals with the *CC* genotype are associated with an increased capability of Nef to downregulate HLA-C. To investigate a possible association between Nef function and set point viral loads in individuals with different -35 SNP genotypes, they selected 49 HIV-1 seropositive individuals representing both *CC* and *TT* genotypes who controlled their virus to various degrees and amplified their respective *nef* genes. Their results revealed no evidence for such a scenario. However, detailed functional analyses identified a set of Nef functions known to influence the interaction of antigen presenting cells (APC) with CD4 helper T cells that were enhanced in *CC* (but not *TT*) individuals who failed to control their virus. Specifically, *nef* alleles from individuals with the protective *CC* genotype and high set point viral load were significantly more effective in downregulating CD4, CD28 and CXCR4 and in upregulating the invariant chain than those from *CC* individuals with low set point viral loads. Downregulation of CD4 may negatively affect APC-T cell interaction because CD4 is a co-receptor for the TCR-CD3 complex, while lack of the CD28 costimulatory signal will prevent lasting T cell activation and may render the HIV-1 infected T cells anergic (Weiss and Littman 1994). CXCR4 downregulation inhibits T cell migration in response to the chemokine stromal cell-derived factor 1 (Geyer, Fackler et al. 2001), and high surface levels of invariant chain impair MHC class II

antigen presentation by APCs (Stumptner-Cuvelette, Morchoisne et al. 2001). These data suggest that efficient inhibition of costimulatory signals and T cell activation by Nef is advantageous for effective viral persistence in individuals with the protective *CC* but not the susceptible *TT* genotype (Specht, Telenti et al. 2010). However, this effective Nef-mediated counteraction of improved HLA-C mediated immunity by complex indirect mechanisms is obviously the exception because most individuals with the *CC* genotype efficiently control HIV-1 (Thomas, Apps et al. 2009). They also found that individuals with the *TT* genotype and low set point viral loads expressed levels of HLA-C mRNA that were as high as those detected in individuals with the protective *CC* genotype (Specht, Telenti et al. 2010). These results suggest that high levels of HLA-C expression may facilitate control of HIV-1 irrespectively of the -35 SNP genotype (Specht, Telenti et al. 2010).

At this point, the data presented in this thesis had demonstrated a difference in HLA-C expression at the -35 SNP genotype but failed to show a functional difference in HLA-C restricted CD8 T cells. There was still no functional explanation for the SNP effect on viral load set point. However, there is overwhelming evidence that HIV-1-specific CD8 T cells play a central role in resolution of primary viraemia and the long-term suppression of viral replication (Goulder and Watkins 2008; McMichael, Borrow et al. 2010). An alternative explanation for the correlation of the -35 SNP with viral load set point is that the alleles of the -35 SNP are markers for 'good' and 'bad' HLA-A/HLA-B/HLA-C haplotypes. Therefore a broader approach was taken to correlate -35 SNP genotype with total CD8 T cell function. The viral suppression assay is likely to be a more important and physiologically relevant composite measure of CD8 T cell antiviral activity (Fauce, Yang et al. 2007). In addition the CD8 T cell

antiviral suppressive capacity has previously been shown to correlate inversely with HIV-1 viral load set point and disease progression (Mackewicz, Ortega et al. 1991; Landay, Mackewicz et al. 1993; Blackbourn, Mackewicz et al. 1996) (Yang *et al.* 2011, submitted manuscript). In chapter 5, the CD8 T cell antiviral suppressive activity of 46 chronically HIV-1 infected antiretroviral therapy-naïve Caucasian individuals was measured using the viral suppression assay to determine whether there is a measurable difference in the ability of T cells from -35 *CC* and *TT* individuals to control virus replication in HIV-1 infected CD4 T cells. This is the first evidence to show that the -35 SNP protective effect on viral load set point is mediated through CD8 T cells. When CD8 T cell antiviral activity was grouped according to -35 SNP genotype, *CC* and *CT* individuals had significantly higher suppressive activity than *TT* individuals ($p=0.0151$ and $p=0.0022$ respectively; Mann-Whitney test). It was also apparent that traditionally protective HLA-B alleles (B*14/27/51/52/57/58) were present only in -35 *CC* and *CT* genotypes while risk HLA-B alleles (B*35) were over-represented in the *TT* genotype. These protective HLA-B alleles were shown to be in linkage disequilibrium with -35*C* linked HLA-C alleles while risk HLA-B alleles were in linkage disequilibrium with -35*T* linked HLA-C alleles. It was therefore hypothesised that in Caucasians, the protective -35 SNP effect on viral load is driven by HLA-B restricted CD8 T cell responses. This is perhaps not surprising given that HLA-B alleles have been shown to be more frequently inducing detectable responses that are generally of greater magnitude than either HLA-A or HLA-C alleles (Kiepiela, Leslie et al. 2004; Bihl, Frahm et al. 2006).

From these data, there appeared to be an association between the -35 SNP, HLA-C expression, presence of protective/risk HLA-B alleles, CD8 T cell suppressive activity

and control of HIV-1. Kulkarni *et al.* concluded that miR-148a regulates the expression of HLA-C in an allele-specific manner that is dependent on variation in the miR-148a-binding site of the *HLA-C* 3' UTR. This binding site (deletion/insertion at position 263 in 3' UTR) is also in strong linkage disequilibrium with the -35 SNP and was shown to have an effect on HIV-1 control that is independent of individual HLA-A, B or C alleles in a cohort of 2,527 seropositive Caucasians ($p=2x10^{-14}$, odds ratio 0.33 with 95% confidence interval of 0.25-0.43; in a logistic regression analysis model) (Kulkarni, Savan et al. 2011). When the viral suppression assay data was plotted against the miR-148a binding site variant, those individuals with 263 deletion/deletion (and high HLA-C expression) displayed higher CD8 T cell suppressive activity than individuals with 263 insertion/insertion (and low HLA-C expression) ($p=0.0175$, Mann-Whitney test) (Figure 7.1). This difference remained statistically significant even after correcting for multiple comparisons using the same data set. Therefore it is clear that the -35 SNP is not the causal variant for differential HLA-C expression and HIV-1 control, but rather is marking another polymorphism(s) that directly affects levels of HLA-C and control of HIV-1.

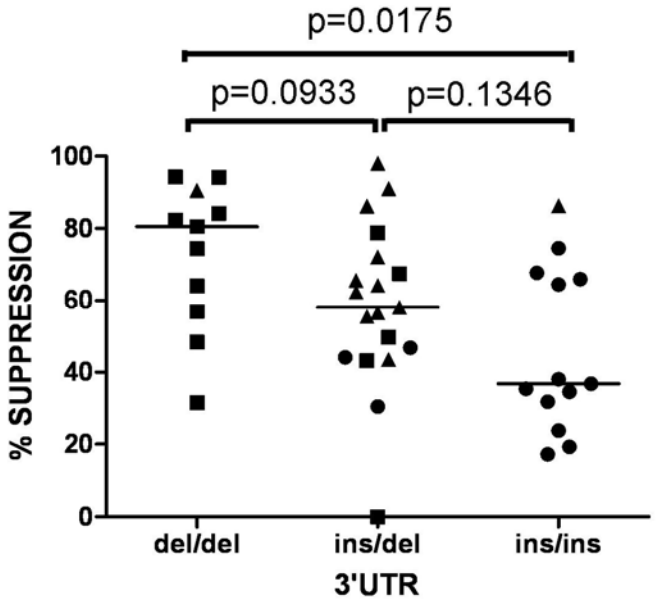


Figure 7.1. There is correlation between CD8 T cell antiviral suppressive capacity and HLA-C 3' UTR 263 deletion/insertion genotype.
The 263 deletion/insertion variant was extrapolated from the data from Kulkarni *et al.* (Kulkarni, Savan et al. 2011). HLA-C alleles 0102, 0302, 0303, 0304, 0401, 0701, 0702, 0704, 1402 and 1701 are in strong linkage disequilibrium with 3' UTR 263 insertion variant (D' 1, $p<0.0001$); HLA-C alleles 0202, 0501, 0602, 0801, 0802, 0804, 1202, 1203, 1502, 1505, 1506, 1601, 1602 and 1604 are in strong linkage disequilibrium with 3' UTR 263 deletion variant (D' 1, $p<0.0001$). Each data point is representative of a single individual; square=*CC*, triangle=*CT* and circle=*TT*. Statistical analysis was performed with the Mann-Whitney test.

To test the hypothesis that HLA-B restricted CD8 T cells are responsible for the -35 SNP protective effect on viral load, a different ethnic group was studied. In chapter 6, HLA-B/HLA-C linkage disequilibrium patterns, the association between the -35 SNP genotype and viral load set point and CD8 T cell antiviral suppressive capacity were studied in a cohort of clade C infected seropositive African individuals. In this ethnic group, protective (B*1302, B4201, B*5703, B*5801, B*8101 and B*3910-Cw*1203 haplotype) and risk (B*1801, B*4501, B*5802) HLA-B alleles are different to those defined in Caucasians (Leslie, Matthews et al. 2010; Tang, Malhotra et al. 2010).

Unlike in Caucasians, HLA-C alleles could not be divided into two separate groups based on linkage with -35*C* or -35*T*. In addition there was a distinct pattern of HLA-B/HLA-C linkage disequilibrium to that noted in Caucasians, with protective HLA-B alleles no longer always linked to -35*C*. Similarly risk HLA-B alleles in Africans were not always in linkage disequilibrium with -35*T*. Accordingly, in contrast to what is seen in Caucasians, there was no difference in the viral load set points of 172 African *CC*, *CT* or *TT* HIV-1 infected individuals. This is consistent with results reported for an African-American and another African cohort (Pelak, Goldstein et al. 2010; Petrovski, Fellay et al. 2011). There was no measurable difference in the ability of T cells from *CC* and *TT* infected African individuals to suppress HIV-1 replication ($p=0.4763$; Mann-Whitney test). In these 28 African individuals, CD8 T cells from those with protective HLA-B alleles tended to have higher antiviral suppressive activity than T cells from those with risk HLA-B alleles, though this difference did not reach statistical significance in these small numbers. It will require much larger numbers to confirm the hypothesis that the -35 SNP protective effect is driven by HLA-B restricted T cells. Therefore chapter 6 concluded by proposing that although particular HLA alleles, particularly those of the *HLA-B* locus, can have a strong individual impact, overall HIV-1 control is likely to be influenced by the combination of all HLA alleles present.

Indeed, it is becoming increasingly clear that genome-wide association studies in human diseases can be hampered by the complexity of linkage disequilibrium patterns in the MHC region. These studies capture the majority of common variants in the genome that explain only a small proportion of heritability, while low frequency polymorphisms, each conferring an intermediate increase in risk, can explain a

significant proportion of the genetic susceptibility to common diseases (Frazer, Murray et al. 2009; Goldstein 2009). Recently Dickson *et al*. argued that rare variants can create synthetic association signals in genome wide association studies, by occurring more often in association with one of the alleles of a SNP, which would therefore synthetically confer an increased risk for disease (Dickson, Wang et al. 2010). It is therefore suggested that the *-35 SNP* effect is an example of just such a synthetic association, arising as a compound effect of linkage disequilibrium (Figure 7.2).

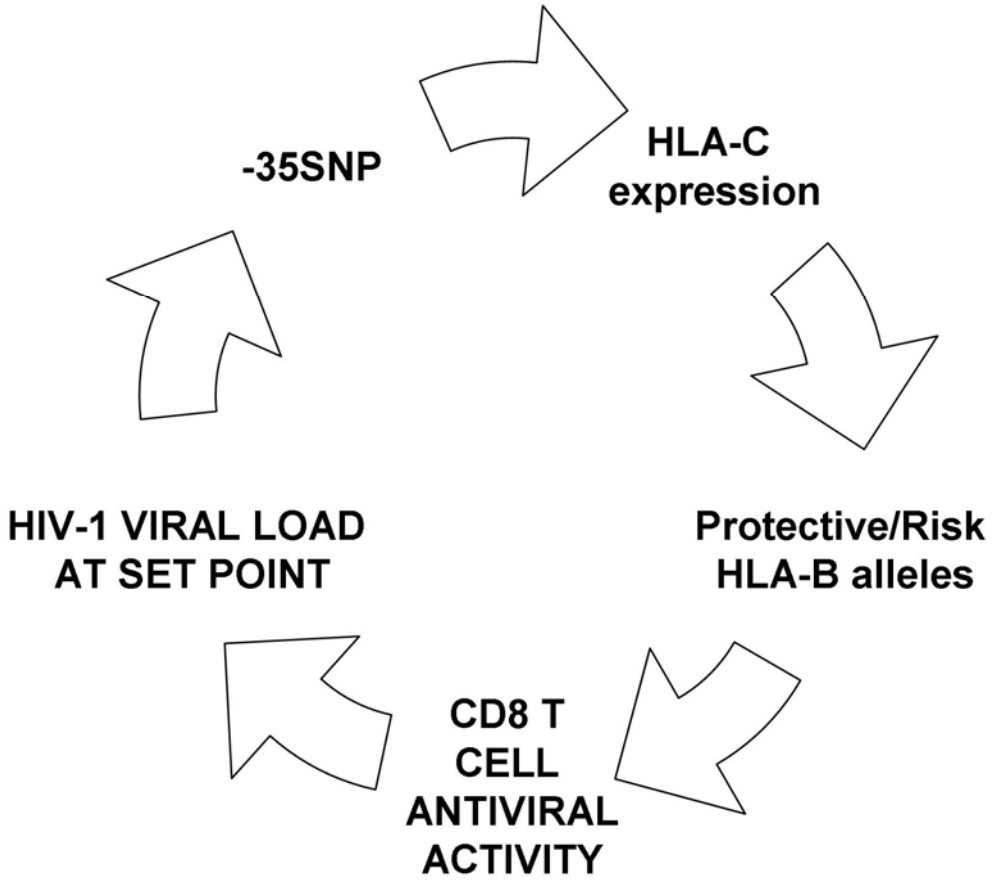


Figure 7.2. The -35 SNP is not causal, but is tagging another polymorphism(s), that directly affects HIV-1 control.

7.2 Future directions

Several additional points arise from the data presented here. An original aim had been to determine if there is a difference in antigen presentation resulting from the low or high HLA-C expression associated with the -35 SNP. It will be interesting to study T cell recognition of directly HIV-1 infected antigen presenting cells by comparing HLA-C-restricted CD8 T cell clones to HLA-A and HLA-B-restricted clones. HLA-C alleles are expressed on the cell surface at approximately 10% of the levels of HLA-A and HLA-B (Snary, Barnstable et al. 1977). This will assess the effect of low HLA expression on T cell recognition of virus-infected cells and also the effect of Nef (by using viruses with intact or deleted *nef* genes) in setting the balance between HLA-A, -B and -C-restricted responses.

The results from these initial viral suppression assay studies will be carried further by increasing the numbers of individuals studied. The data from Caucasians discussed here was heavily skewed toward LTNPs. It will be interesting to determine if the association between CD8 T cell antiviral activity and -35 SNP genotype is maintained in a larger number of chronic progressors. Similarly, the numbers of Africans assayed will be increased to determine if the trend seen in 28 individuals, that CD8 T cells from individuals with protective HLA-B alleles have greater antiviral suppressive activity than those from individuals with risk HLA-B alleles, achieves significance. Finally as alluded to in chapter 6, CD8 T cell antiviral suppressive activity in individuals expressing HLA-B*57/5801 could be divided into two distinct groups, one with high and the other low suppression. The Gag epitope KF11 is immunodominant in chronic infection and is highly variant in Africans expressing HLA-B*5703 (Gillespie, Kaul et al. 2002; Ferrari, Currier et al. 2004; Yu, Lichterfeld

et al. 2007). The heterologous virus used in the viral suppression assays contained wild-type KF11 variant. It was postulated that in the group with high CD8 T cell suppressive activity, the individuals were infected with a clade C virus that contained wild-type KF11 sequence and the memory CD8 T cells recognised this variant and mounted a strong immune response. However in the group with little or no suppressive activity, the predominant autologous virus strain contained a KF11 escape variant and the memory CD8 T cells failed to recognise wild-type KF11 in the heterologous virus used in the assay. Therefore, the *Gag* gene in all HLA-B*57/5801 possessing Caucasian and African individuals will be sequenced to determine if CD8 T cell antiviral suppressive activity correlates with mutations in the KF11 epitope. The studies mentioned above are currently ongoing in the McMichael CHAVI group.

REFERENCES

Addo, M. M., X. G. Yu, A. Rathod, D. Cohen, R. L. Eldridge, D. Strick, M. N. Johnston, C. Corcoran, A. G. Wurcel, C. A. Fitzpatrick, M. E. Feeney, W. R. Rodriguez, N. Basgoz, R. Draenert, D. R. Stone, C. Brander, P. J. Goulder, E. S. Rosenberg, M. Altfeld and B. D. Walker (2003). "Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load." J Virol **77**(3): 2081-92.

Adnan, S., A. Balamurugan, A. Trocha, M. S. Bennett, H. L. Ng, A. Ali, C. Brander and O. O. Yang (2006). "Nef interference with HIV-1-specific CTL antiviral activity is epitope specific." Blood **108**(10): 3414-9.

Albert, M. L., B. Sauter and N. Bhardwaj (1998). "Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs." Nature **392**(6671): 86-9.

Alexander-Miller, M. A., G. R. Leggatt and J. A. Berzofsky (1996). "Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy." Proc Natl Acad Sci U S A **93**(9): 4102-7.

Allen, C. D., T. Okada and J. G. Cyster (2007). "Germinal-center organization and cellular dynamics." Immunity **27**(2): 190-202.

Allen, T. M., M. Altfeld, S. C. Geer, E. T. Kalife, C. Moore, M. O'Sullivan K, I. Desouza, M. E. Feeney, R. L. Eldridge, E. L. Maier, D. E. Kaufmann, M. P. Lahaie, L. Reyor, G. Tanzi, M. N. Johnston, C. Brander, R. Draenert, J. K. Rockstroh, H. Jessen, E. S. Rosenberg, S. A. Mallal and B. D. Walker (2005). "Selective escape from CD8+ T-cell responses represents a major driving force of human immunodeficiency virus type 1 (HIV-1) sequence diversity and reveals constraints on HIV-1 evolution." J Virol **79**(21): 13239-49.

Allen, T. M., M. Altfeld, X. G. Yu, K. M. O'Sullivan, M. Lichterfeld, S. Le Gall, M. John, B. R. Mothe, P. K. Lee, E. T. Kalife, D. E. Cohen, K. A. Freedberg, D. A. Strick, M. N. Johnston, A. Sette, E. S. Rosenberg, S. A. Mallal, P. J. Goulder, C. Brander and B. D. Walker (2004). "Selection, transmission, and reversion of an antigen-processing cytotoxic T-lymphocyte escape mutation in human immunodeficiency virus type 1 infection." J Virol **78**(13): 7069-78.

Allen, T. M., D. H. O'Connor, P. Jing, J. L. Dzuris, B. R. Mothe, T. U. Vogel, E. Dunphy, M. E. Liebl, C. Emerson, N. Wilson, K. J. Kunstman, X. Wang, D. B. Allison, A. L. Hughes, R. C. Desrosiers, J. D. Altman, S. M. Wolinsky, A. Sette and D. I. Watkins (2000). "Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia." Nature **407**(6802): 386-90.

Almeida, J. R., D. A. Price, L. Papagno, Z. A. Arkoub, D. Sauce, E. Bornstein, T. E. Asher, A. Samri, A. Schnuriger, I. Theodorou, D. Costagliola, C. Rouzioux, H. Agut, A. G. Marcelin, D. Douek, B. Autran and V. Appay (2007). "Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover." J Exp Med **204**(10): 2473-85.

Alter, G., D. Heckerman, A. Schneidewind, L. Fadda, C. M. Kadie, J. M. Carlson, C. Oniangue-Ndza, M. Martin, B. Li, S. I. Khakoo, M. Carrington, T. M. Allen

and M. Altfeld (2011). "HIV-1 adaptation to NK-cell-mediated immune pressure." Nature **476**(7358): 96-100.

Alter, G., N. Teigen, R. Ahern, H. Streeck, A. Meier, E. S. Rosenberg and M. Altfeld (2007). "Evolution of innate and adaptive effector cell functions during acute HIV-1 infection." J Infect Dis **195**(10): 1452-60.

Altfeld, M., M. M. Addo, E. S. Rosenberg, F. M. Hecht, P. K. Lee, M. Vogel, X. G. Yu, R. Draenert, M. N. Johnston, D. Strick, T. M. Allen, M. E. Feeney, J. O. Kahn, R. P. Sekaly, J. A. Levy, J. K. Rockstroh, P. J. Goulder and B. D. Walker (2003). "Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection." Aids **17**(18): 2581-91.

Altfeld, M., M. M. Addo, R. Shankarappa, P. K. Lee, T. M. Allen, X. G. Yu, A. Rathod, J. Harlow, K. O'Sullivan, M. N. Johnston, P. J. Goulder, J. I. Mullins, E. S. Rosenberg, C. Brander, B. Korber and B. D. Walker (2003). "Enhanced detection of human immunodeficiency virus type 1-specific T-cell responses to highly variable regions by using peptides based on autologous virus sequences." J Virol **77**(13): 7330-40.

Altfeld, M. and P. Goulder (2007). "'Unleashed' natural killers hinder HIV." Nat Genet **39**(6): 708-10.

Altfeld, M., E. T. Kalife, Y. Qi, H. Streeck, M. Lichterfeld, M. N. Johnston, N. Burgett, M. E. Swartz, A. Yang, G. Alter, X. G. Yu, A. Meier, J. K. Rockstroh, T. M. Allen, H. Jessen, E. S. Rosenberg, M. Carrington and B. D. Walker (2006). "HLA Alleles Associated with Delayed Progression to AIDS Contribute Strongly to the Initial CD8(+) T Cell Response against HIV-1." PLoS Med **3**(10): e403.

An, P., G. W. Nelson, L. Wang, S. Donfield, J. J. Goedert, J. Phair, D. Vlahov, S. Buchbinder, W. L. Farrar, W. Modi, S. J. O'Brien and C. A. Winkler (2002). "Modulating influence on HIV/AIDS by interacting RANTES gene variants." Proc Natl Acad Sci U S A **99**(15): 10002-7.

An, P. and C. A. Winkler (2010). "Host genes associated with HIV/AIDS: advances in gene discovery." Trends Genet **26**(3): 119-31.

Appay, V., D. F. Nixon, S. M. Donahoe, G. M. Gillespie, T. Dong, A. King, G. S. Ogg, H. M. Spiegel, C. Conlon, C. A. Spina, D. V. Havlir, D. D. Richman, A. Waters, P. Easterbrook, A. J. McMichael and S. L. Rowland-Jones (2000). "HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function." J Exp Med **192**(1): 63-75.

Appay, V., L. Papagno, C. A. Spina, P. Hansasuta, A. King, L. Jones, G. S. Ogg, S. Little, A. J. McMichael, D. D. Richman and S. L. Rowland-Jones (2002). "Dynamics of T cell responses in HIV infection." J Immunol **168**(7): 3660-6.

Appay, V., J. J. Zaunders, L. Papagno, J. Sutton, A. Jaramillo, A. Waters, P. Easterbrook, P. Grey, D. Smith, A. J. McMichael, D. A. Cooper, S. L. Rowland-Jones and A. D. Kelleher (2002). "Characterization of CD4(+) CTLs ex vivo." J Immunol **168**(11): 5954-8.

Ariyoshi, K., E. Harwood, R. Chiengsong-Popov and J. Weber (1992). "Is clearance of HIV-1 viraemia at seroconversion mediated by neutralising antibodies?" Lancet **340**(8830): 1257-8.

Asquith, B., C. T. Edwards, M. Lipsitch and A. R. McLean (2006). "Inefficient cytotoxic T lymphocyte-mediated killing of HIV-1-infected cells in vivo." PLoS Biol **4**(4): e90.

Baba, T. W., V. Liska, R. Hofmann-Lehmann, J. Vlasak, W. Xu, S. Ayehunie, L. A. Cavacini, M. R. Posner, H. Katinger, G. Stiegler, B. J. Bernacky, T. A. Rizvi,

R. Schmidt, L. R. Hill, M. E. Keeling, Y. Lu, J. E. Wright, T. C. Chou and R. M. Ruprecht (2000). "Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection." Nat Med **6**(2): 200-6.

Bacchetti, P. and A. R. Moss (1989). "Incubation period of AIDS in San Francisco." Nature **338**(6212): 251-3.

Ball, S. C., A. Abraha, K. R. Collins, A. J. Marozsan, H. Baird, M. E. Quinones-Mateu, A. Penn-Nicholson, M. Murray, N. Richard, M. Lobritz, P. A. Zimmerman, T. Kawamura, A. Blauvelt and E. J. Arts (2003). "Comparing the ex vivo fitness of CCR5-tropic human immunodeficiency virus type 1 isolates of subtypes B and C." J Virol **77**(2): 1021-38.

Bansal, A., S. Sabbaj, B. H. Edwards, D. Ritter, C. Perkins, J. Tang, J. J. Szinger, H. Weiss, P. A. Goepfert, B. Korber, C. M. Wilson, R. A. Kaslow and M. J. Mulligan (2003). "T cell responses in HIV type 1-infected adolescent minorities share similar epitope specificities with whites despite significant differences in HLA class I alleles." AIDS Res Hum Retroviruses **19**(11): 1017-26.

Barber, L. D., B. Gillece-Castro, L. Percival, X. Li, C. Clayberger and P. Parham (1995). "Overlap in the repertoires of peptides bound in vivo by a group of related class I HLA-B allotypes." Curr Biol **5**(2): 179-90.

Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. Williams and A. Ziegler (1978). "Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis." Cell **14**(1): 9-20.

Barouch, D. H., A. Craiu, M. J. Kuroda, J. E. Schmitz, X. X. Zheng, S. Santra, J. D. Frost, G. R. Krivulka, M. A. Lifton, C. L. Crabbs, G. Heidecker, H. C. Perry, M. E. Davies, H. Xie, C. E. Nickerson, T. D. Steenbeke, C. I. Lord, D. C. Montefiori, T. B. Strom, J. W. Shiver, M. G. Lewis and N. L. Letvin (2000). "Augmentation of immune responses to HIV-1 and simian immunodeficiency virus DNA vaccines by IL-2/Ig plasmid administration in rhesus monkeys." Proc Natl Acad Sci U S A **97**(8): 4192-7.

Barouch, D. H., J. Kunstman, M. J. Kuroda, J. E. Schmitz, S. Santra, F. W. Peyerl, G. R. Krivulka, K. Beaudry, M. A. Lifton, D. A. Gorgone, D. C. Montefiori, M. G. Lewis, S. M. Wolinsky and N. L. Letvin (2002). "Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes." Nature **415**(6869): 335-9.

Barouch, D. H., J. Powers, D. M. Truitt, M. G. Kishko, J. C. Arthur, F. W. Peyerl, M. J. Kuroda, D. A. Gorgone, M. A. Lifton, C. I. Lord, V. M. Hirsch, D. C. Montefiori, A. Carville, K. G. Mansfield, K. J. Kunstman, S. M. Wolinsky and N. L. Letvin (2005). "Dynamic immune responses maintain cytotoxic T lymphocyte epitope mutations in transmitted simian immunodeficiency virus variants." Nat Immunol **6**(3): 247-52.

Barre-Sinoussi, F. (1996). "HIV as the cause of AIDS." Lancet **348**(9019): 31-5.

Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum and L. Montagnier (1983). "Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS)." Science **220**(4599): 868-71.

Bennett, M. S., H. L. Ng, A. Ali and O. O. Yang (2008). "Cross-clade detection of HIV-1-specific cytotoxic T lymphocytes does not reflect cross-clade antiviral activity." J Infect Dis **197**(3): 390-7.

Bennett, M. S., H. L. Ng, M. Dagarag, A. Ali and O. O. Yang (2007). "Epitope-dependent avidity thresholds for cytotoxic T-lymphocyte clearance of virus-infected cells." J Virol **81**(10): 4973-80.

Bernardin, F., D. Kong, L. Peddada, L. A. Baxter-Lowe and E. Delwart (2005). "Human immunodeficiency virus mutations during the first month of infection are preferentially found in known cytotoxic T-lymphocyte epitopes." J Virol **79**(17): 11523-8.

Betts, M. R., D. R. Ambrozak, D. C. Douek, S. Bonhoeffer, J. M. Brenchley, J. P. Casazza, R. A. Koup and L. J. Picker (2001). "Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection." J Virol **75**(24): 11983-91.

Betts, M. R., M. C. Nason, S. M. West, S. C. De Rosa, S. A. Migueles, J. Abraham, M. M. Lederman, J. M. Benito, P. A. Goepfert, M. Connors, M. Roederer and R. A. Koup (2006). "HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells." Blood **107**(12): 4781-9.

Betts, M. R., D. A. Price, J. M. Brenchley, K. Lore, F. J. Guenaga, A. Smed-Sorensen, D. R. Ambrozak, S. A. Migueles, M. Connors, M. Roederer, D. C. Douek and R. A. Koup (2004). "The functional profile of primary human antiviral CD8+ T cell effector activity is dictated by cognate peptide concentration." J Immunol **172**(10): 6407-17.

Bevan, M. J. (1976). "Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay." J Exp Med **143**(5): 1283-8.

Bevan, M. J. (1976). "Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming." J Immunol **117**(6): 2233-8.

Bhattacharya, T., M. Daniels, D. Heckerman, B. Foley, N. Frahm, C. Kadie, J. Carlson, K. Yusim, B. McMahon, B. Gaschen, S. Mallal, J. I. Mullins, D. C. Nickle, J. Herbeck, C. Rousseau, G. H. Learn, T. Miura, C. Brander, B. Walker and B. Korber (2007). "Founder effects in the assessment of HIV polymorphisms and HLA allele associations." Science **315**(5818): 1583-6.

Bihl, F., N. Frahm, L. Di Giammarino, J. Sidney, M. John, K. Yusim, T. Woodberry, K. Sango, H. S. Hewitt, L. Henry, C. H. Linde, J. V. Chisholm, 3rd, T. M. Zaman, E. Pae, S. Mallal, B. D. Walker, A. Sette, B. T. Korber, D. Heckerman and C. Brander (2006). "Impact of HLA-B alleles, epitope binding affinity, functional avidity, and viral coinfection on the immunodominance of virus-specific CTL responses." J Immunol **176**(7): 4094-101.

Bijlmakers, M. J. and M. Marsh (2003). "The on-off story of protein palmitoylation." Trends Cell Biol **13**(1): 32-42.

Bijlmakers, M. J. and H. L. Ploegh (1993). "Putting together an MHC class I molecule." Curr Opin Immunol **5**(1): 21-6.

Bishop, K. N., R. K. Holmes, A. M. Sheehy and M. H. Malim (2004). "APOBEC-mediated editing of viral RNA." Science **305**(5684): 645.

Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger and D. C. Wiley (1987). "The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens." Nature **329**(6139): 512-8.

Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger and D. C. Wiley (1987). "Structure of the human class I histocompatibility antigen, HLA-A2." Nature **329**(6139): 506-12.

Blackbourn, D. J., C. E. Mackewicz, E. Barker, T. K. Hunt, B. Herndier, A. T. Haase and J. A. Levy (1996). "Suppression of HIV replication by lymphoid tissue CD8+ cells correlates with the clinical state of HIV-infected individuals." Proc Natl Acad Sci U S A **93**(23): 13125-30.

Blattner, W. A., K. A. Oursler, F. Cleghorn, M. Charurat, A. Sill, C. Bartholomew, N. Jack, T. O'Brien, J. Edwards, G. Tomaras, K. Weinhold and M. Greenberg (2004). "Rapid clearance of virus after acute HIV-1 infection: correlates of risk of AIDS." J Infect Dis **189**(10): 1793-801.

Bleul, C. C., M. Farzan, H. Choe, C. Parolin, I. Clark-Lewis, J. Sodroski and T. A. Springer (1996). "The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry." Nature **382**(6594): 829-33.

Blink, E. J., A. Light, A. Kallies, S. L. Nutt, P. D. Hodgkin and D. M. Tarlinton (2005). "Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization." J Exp Med **201**(4): 545-54.

Bonaparte, M. I. and E. Barker (2004). "Killing of human immunodeficiency virus-infected primary T-cell blasts by autologous natural killer cells is dependent on the ability of the virus to alter the expression of major histocompatibility complex class I molecules." Blood **104**(7): 2087-94.

Borghans, J. A., A. Molgaard, R. J. de Boer and C. Kesmir (2007). "HLA alleles associated with slow progression to AIDS truly prefer to present HIV-1 p24." PLoS One **2**(9): e920.

Borrow, P. and N. Bhardwaj (2008). "Innate immune responses in primary HIV-1 infection." Curr Opin HIV AIDS **3**(1): 36-44.

Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw and M. B. Oldstone (1994). "Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection." J Virol **68**(9): 6103-10.

Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone and G. M. Shaw (1997). "Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus." Nat Med **3**(2): 205-11.

Boutwell, C. L., M. M. Rolland, J. T. Herbeck, J. I. Mullins and T. M. Allen (2010). "Viral evolution and escape during acute HIV-1 infection." J Infect Dis **202 Suppl 2**: S309-14.

Bouvier, M. and D. C. Wiley (1994). "Importance of peptide amino and carboxyl termini to the stability of MHC class I molecules." Science **265**(5170): 398-402.

Brackenridge, S., E. J. Evans, M. Toebes, N. Goonetilleke, M. K. Liu, K. di Gleria, T. N. Schumacher, S. J. Davis, A. J. McMichael and G. M. Gillespie (2011). "An early HIV mutation within an HLA-B*57-restricted T cell epitope abrogates binding to the killer inhibitory receptor 3DL1." J Virol **85**(11): 5415-22.

Braud, V., E. Y. Jones and A. McMichael (1997). "The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9." Eur J Immunol **27**(5): 1164-9.

Braud, V. M., D. S. Allan, C. A. O'Callaghan, K. Soderstrom, A. D'Andrea, G. S. Ogg, S. Lazetic, N. T. Young, J. I. Bell, J. H. Phillips, L. L. Lanier and A. J. McMichael (1998). "HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C." Nature **391**(6669): 795-9.

Braud, V. M., D. S. Allan, D. Wilson and A. J. McMichael (1998). "TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide." Curr Biol **8**(1): 1-10.

Breitfeld, D., L. Ohl, E. Kremmer, J. Ellwart, F. Sallusto, M. Lipp and R. Forster (2000). "Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production." J Exp Med **192**(11): 1545-52.

Brenchley, J. M., M. Paiardini, K. S. Knox, A. I. Asher, B. Cervasi, T. E. Asher, P. Scheinberg, D. A. Price, C. A. Hage, L. M. Kholi, A. Khoruts, I. Frank, J. Else, T. Schacker, G. Silvestri and D. C. Douek (2008). "Differential Th17 CD4 T-cell depletion in pathogenic and nonpathogenic lentiviral infections." Blood **112**(7): 2826-35.

Brenchley, J. M., D. A. Price and D. C. Douek (2006). "HIV disease: fallout from a mucosal catastrophe?" Nat Immunol **7**(3): 235-9.

Brenchley, J. M., T. W. Schacker, L. E. Ruff, D. A. Price, J. H. Taylor, G. J. Beilman, P. L. Nguyen, A. Khoruts, M. Larson, A. T. Haase and D. C. Douek (2004). "CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract." J Exp Med **200**(6): 749-59.

Brockman, M. A., Z. L. Brumme, C. J. Brumme, T. Miura, J. Sela, P. C. Rosato, C. M. Kadie, J. M. Carlson, T. J. Markle, H. Streeck, A. D. Kelleher, M. Markowitz, H. Jessen, E. Rosenberg, M. Altfeld, P. R. Harrigan, D. Heckerman, B. D. Walker and T. M. Allen (2010). "Early selection in Gag by protective HLA alleles contributes to reduced HIV-1 replication capacity that may be largely compensated for in chronic infection." J Virol **84**(22): 11937-49.

Brumme, Z. L., C. J. Brumme, J. Carlson, H. Streeck, M. John, Q. Eichbaum, B. L. Block, B. Baker, C. Kadie, M. Markowitz, H. Jessen, A. D. Kelleher, E. Rosenberg, J. Kaldor, Y. Yuki, M. Carrington, T. M. Allen, S. Mallal, M. Altfeld, D. Heckerman and B. D. Walker (2008). "Marked epitope- and allele-specific differences in rates of mutation in human immunodeficiency type 1 (HIV-1) Gag, Pol, and Nef cytotoxic T-lymphocyte epitopes in acute/early HIV-1 infection." J Virol **82**(18): 9216-27.

Bryant, P. W., A. M. Lennon-Dumenil, E. Fiebiger, C. Lagaudriere-Gesbert and H. L. Ploegh (2002). "Proteolysis and antigen presentation by MHC class II molecules." Adv Immunol **80**: 71-114.

Bunce, M. (2003). "PCR-sequence-specific primer typing of HLA class I and class II alleles." Methods Mol Biol **210**: 143-71.

Buonaguro, L., M. L. Tornesello and F. M. Buonaguro (2007). "Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: pathogenetic and therapeutic implications." J Virol **81**(19): 10209-19.

Burton, D. R., R. C. Desrosiers, R. W. Doms, W. C. Koff, P. D. Kwong, J. P. Moore, G. J. Nabel, J. Sodroski, I. A. Wilson and R. T. Wyatt (2004). "HIV vaccine design and the neutralizing antibody problem." Nat Immunol **5**(3): 233-6.

Cao, J., J. McNevin, U. Malhotra and M. J. McElrath (2003). "Evolution of CD8+ T cell immunity and viral escape following acute HIV-1 infection." J Immunol **171**(7): 3837-46.

Cao, J., J. McNevin, M. McSweyn, Y. Liu, J. I. Mullins and M. J. McElrath (2008). "Novel cytotoxic T-lymphocyte escape mutation by a three-amino-acid insertion in the human immunodeficiency virus type 1 p6Pol and p6Gag late domain associated with drug resistance." J Virol **82**(1): 495-502.

Cao, Y., L. Qin, L. Zhang, J. Safrit and D. D. Ho (1995). "Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection." N Engl J Med **332**(4): 201-8.

Carotenuto, P., D. Looij, L. Keldermans, F. de Wolf and J. Goudsmit (1998). "Neutralizing antibodies are positively associated with CD4+ T-cell counts and T-cell function in long-term AIDS-free infection." Aids **12**(13): 1591-600.

Carreno, B. M. and T. H. Hansen (1994). "Exogenous peptide ligand influences the expression and half-life of free HLA class I heavy chains ubiquitously detected at the cell surface." Eur J Immunol **24**(6): 1285-92.

Carrington, M., M. P. Martin and J. van Bergen (2008). "KIR-HLA intercourse in HIV disease." Trends Microbiol **16**(12): 620-7.

Carrington, M., G. W. Nelson, M. P. Martin, T. Kissner, D. Vlahov, J. J. Goedert, R. Kaslow, S. Buchbinder, K. Hoots and S. J. O'Brien (1999). "HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage." Science **283**(5408): 1748-52.

Carrington, M. and S. J. O'Brien (2003). "The influence of HLA genotype on AIDS." Annu Rev Med **54**: 535-51.

Catano, G., H. Kulkarni, W. He, V. C. Marconi, B. K. Agan, M. Landrum, S. Anderson, J. Delmar, V. Telles, L. Song, J. Castiblanco, R. A. Clark, M. J. Dolan and S. K. Ahuja (2008). "HIV-1 disease-influencing effects associated with ZNRD1, HCP5 and HLA-C alleles are attributable mainly to either HLA-A10 or HLA-B*57 alleles." PLoS ONE **3**(11): e3636.

Cerundolo, V., J. Alexander, K. Anderson, C. Lamb, P. Cresswell, A. McMichael, F. Gotch and A. Townsend (1990). "Presentation of viral antigen controlled by a gene in the major histocompatibility complex." Nature **345**(6274): 449-52.

Champagne, P., G. S. Ogg, A. S. King, C. Knabenhans, K. Ellefsen, M. Nobile, V. Appay, G. P. Rizzardi, S. Fleury, M. Lipp, R. Forster, S. Rowland-Jones, R. P. Sekaly, A. J. McMichael and G. Pantaleo (2001). "Skewed maturation of memory HIV-specific CD8 T lymphocytes." Nature **410**(6824): 106-11.

Chase, A. J., H. C. Yang, H. Zhang, J. N. Blankson and R. F. Siliciano (2008). "Preservation of FoxP3+ regulatory T cells in the peripheral blood of human immunodeficiency virus type 1-infected elite suppressors correlates with low CD4+ T-cell activation." J Virol **82**(17): 8307-15.

Chen, H., A. Piechocka-Trocha, T. Miura, M. A. Brockman, B. D. Julg, B. M. Baker, A. C. Rothchild, B. L. Block, A. Schneidewind, T. Koibuchi, F. Pereyra, T. M. Allen and B. D. Walker (2009). "Differential neutralization of human immunodeficiency virus (HIV) replication in autologous CD4 T cells by HIV-specific cytotoxic T lymphocytes." J Virol **83**(7): 3138-49.

Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P. D. Ponath, L. Wu, C. R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard and J. Sodroski (1996). "The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates." Cell **85**(7): 1135-48.

Chopera, D. R., Z. Woodman, K. Mlisana, M. Mlotshwa, D. P. Martin, C. Seoighe, F. Treurnicht, D. A. de Rosa, W. Hide, S. A. Karim, C. M. Gray and C. Williamson (2008). "Transmission of HIV-1 CTL escape variants provides HLA-mismatched recipients with a survival advantage." PLoS Pathog **4**(3): e1000033.

Chun, T. W., L. Carruth, D. Finzi, X. Shen, J. A. DiGiuseppe, H. Taylor, M. Hermankova, K. Chadwick, J. Margolick, T. C. Quinn, Y. H. Kuo, R. Brookmeyer, M. A. Zeiger, P. Barditch-Crovo and R. F. Siliciano (1997). "Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection." Nature **387**(6629): 183-8.

Chung, C., W. Lee, J. T. Loffredo, B. Burwitz, T. C. Friedrich, J. P. Giraldo Vela, G. Napoe, E. G. Rakasz, N. A. Wilson, D. B. Allison and D. I. Watkins (2007). "Not all cytokine-producing CD8+ T cells suppress simian immunodeficiency virus replication." J Virol **81**(3): 1517-23.

Cocchi, F., A. L. DeVico, A. Garzino-Demo, S. K. Arya, R. C. Gallo and P. Lusso (1995). "Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells." Science **270**(5243): 1811-5.

Coffey, F., B. Alabyev and T. Manser (2009). "Initial clonal expansion of germinal center B cells takes place at the perimeter of follicles." Immunity **30**(4): 599-609.

Coffin, J. M. (1979). "Structure, replication, and recombination of retrovirus genomes: some unifying hypotheses." J Gen Virol **42**(1): 1-26.

Coffin, J. M. (1995). "HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy." Science **267**(5197): 483-9.

Cohen, G. B., R. T. Gandhi, D. M. Davis, O. Mandelboim, B. K. Chen, J. L. Strominger and D. Baltimore (1999). "The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells." Immunity **10**(6): 661-71.

Cohen, J. (2003). "HIV. Escape artist par excellence." Science **299**(5612): 1505-8.

Collins, K. L. and D. Baltimore (1999). "HIV's evasion of the cellular immune response." Immunol Rev **168**: 65-74.

Collins, K. L., B. K. Chen, S. A. Kalams, B. D. Walker and D. Baltimore (1998). "HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes." Nature **391**(6665): 397-401.

Consortium, T. I. H. (2003). "The International HapMap Project." Nature **426**(6968): 789-96.

Costello, C., J. Tang, C. Rivers, E. Karita, J. Meizen-Derr, S. Allen and R. A. Kaslow (1999). "HLA-B*5703 independently associated with slower HIV-1 disease progression in Rwandan women." Aids **13**(14): 1990-1.

Coupel, S., A. Moreau, M. Hamidou, V. Horejsi, J. P. Soulillou and B. Charreau (2007). "Expression and release of soluble HLA-E is an immunoregulatory feature of endothelial cell activation." Blood **109**(7): 2806-14.

Crawford, H., W. Lumm, A. Leslie, M. Schaefer, D. Boeras, J. G. Prado, J. Tang, P. Farmer, T. Ndung'u, S. Lakhi, J. Gilmour, P. Goepfert, B. D. Walker, R. Kaslow, J. Mulenga, S. Allen, P. J. Goulder and E. Hunter (2009). "Evolution of HLA-B*5703 HIV-1 escape mutations in HLA-B*5703-positive individuals and their transmission recipients." J Exp Med **206**(4): 909-21.

Crawford, H., J. G. Prado, A. Leslie, S. Hue, I. Honeyborne, S. Reddy, M. van der Stok, Z. Mncube, C. Brander, C. Rousseau, J. I. Mullins, R. Kaslow, P.

Goepfert, S. Allen, E. Hunter, J. Mulenga, P. Kiepiela, B. D. Walker and P. J. Goulder (2007). "Compensatory mutation partially restores fitness and delays reversion of escape mutation within the immunodominant HLA-B*5703-restricted Gag epitope in chronic human immunodeficiency virus type 1 infection." J Virol **81**(15): 8346-51.

Cresswell, P. (1996). "Invariant chain structure and MHC class II function." Cell **84**(4): 505-7.

Crotty, S. (2011). "Follicular helper CD4 T cells (TFH)." Annu Rev Immunol **29**: 621-63.

Currier, J. R., M. E. Harris, J. H. Cox, F. E. McCutchan, D. L. Birx, S. Maayan and G. Ferrari (2005). "Immunodominance and cross-reactivity of B5703-restricted CD8 T lymphocytes from HIV type 1 subtype C-infected Ethiopians." AIDS Res Hum Retroviruses **21**(3): 239-45.

Dalmasso, C., W. Carpentier, L. Meyer, C. Rouzioux, C. Goujard, M. L. Chaix, O. Lambotte, V. Avettand-Fenoel, S. Le Clerc, L. D. de Senneville, C. Deveau, F. Boufassa, P. Debre, J. F. Delfraissy, P. Broet and I. Theodorou (2008). "Distinct genetic loci control plasma HIV-RNA and cellular HIV-DNA levels in HIV-1 infection: the ANRS Genome Wide Association 01 study." PLoS One **3**(12): e3907.

Dasgupta, J. D., S. Watkins, H. Slayter and E. J. Yunis (1988). "Receptor-like nature of class I HLA: endocytosis via coated pits." J Immunol **141**(8): 2577-80.

Davis, D. M., H. T. Reyburn, L. Pazmany, I. Chiu, O. Mandelboim and J. L. Strominger (1997). "Impaired spontaneous endocytosis of HLA-G." Eur J Immunol **27**(10): 2714-9.

Dawkins, R., C. Leelayuwat, S. Gaudieri, G. Tay, J. Hui, S. Cattley, P. Martinez and J. Kulski (1999). "Genomics of the major histocompatibility complex: haplotypes, duplication, retroviruses and disease." Immunol Rev **167**: 275-304.

de Bakker, P. I., G. McVean, P. C. Sabeti, M. M. Miretti, T. Green, J. Marchini, X. Ke, A. J. Monsuur, P. Whittaker, M. Delgado, J. Morrison, A. Richardson, E. C. Walsh, X. Gao, L. Galver, J. Hart, D. A. Hafler, M. Pericak-Vance, J. A. Todd, M. J. Daly, J. Trowsdale, C. Wijmenga, T. J. Vyse, S. Beck, S. S. Murray, M. Carrington, S. Gregory, P. Deloukas and J. D. Rioux (2006). "A high-resolution HLA and SNP haplotype map for disease association studies in the extended human MHC." Nat Genet **38**(10): 1166-72.

Dean, M., M. Carrington, C. Winkler, G. A. Huttley, M. W. Smith, R. Allikmets, J. J. Goedert, S. P. Buchbinder, E. Vittinghoff, E. Gomperts, S. Donfield, D. Vlahov, R. Kaslow, A. Saah, C. Rinaldo, R. Detels and S. J. O'Brien (1996). "Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study." Science **273**(5283): 1856-62.

Deeks, S. G. and B. D. Walker (2007). "Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy." Immunity **27**(3): 406-16.

Degli-Esposti, M. A., A. L. Leaver, F. T. Christiansen, C. S. Witt, L. J. Abraham and R. L. Dawkins (1992). "Ancestral haplotypes: conserved population MHC haplotypes." Hum Immunol **34**(4): 242-52.

Denis, M. and E. Ghadirian (1994). "Interleukin 13 and interleukin 4 protect bronchoalveolar macrophages from productive infection with human immunodeficiency virus type 1." AIDS Res Hum Retroviruses **10**(7): 795-802.

Derdeyn, C. A., J. M. Decker, F. Bibollet-Ruche, J. L. Mokili, M. Muldoon, S. A. Denham, M. L. Heil, F. Kasolo, R. Musonda, B. H. Hahn, G. M. Shaw, B. T. Korber, S. Allen and E. Hunter (2004). "Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission." Science **303**(5666): 2019-22.

DeVico, A. L. and R. C. Gallo (2004). "Control of HIV-1 infection by soluble factors of the immune response." Nat Rev Microbiol **2**(5): 401-13.

Dhawan, S., A. Heredia, L. M. Wahl, J. S. Epstein, M. S. Meltzer and I. K. Hewlett (1995). "Interferon-gamma-induced downregulation of CD4 inhibits the entry of human immunodeficiency virus type-1 in primary monocytes." Pathobiology **63**(2): 93-9.

Dickson, S. P., K. Wang, I. Krantz, H. Hakonarson and D. B. Goldstein (2010). "Rare variants create synthetic genome-wide associations." PLoS Biol **8**(1): e1000294.

Dinges, W. L., J. Richardt, D. Friedrich, E. Jalbert, Y. Liu, C. E. Stevens, J. Maenza, A. C. Collier, D. E. Geraghty, J. Smith, Z. Moodie, J. I. Mullins, M. J. McElrath and H. Horton (2010). "Virus-specific CD8+ T-cell responses better define HIV disease progression than HLA genotype." J Virol **84**(9): 4461-8.

Douek, D. C., J. M. Brenchley, M. R. Betts, D. R. Ambrozak, B. J. Hill, Y. Okamoto, J. P. Casazza, J. Kuruppu, K. Kunstman, S. Wolinsky, Z. Grossman, M. Dybul, A. Oxenius, D. A. Price, M. Connors and R. A. Koup (2002). "HIV preferentially infects HIV-specific CD4+ T cells." Nature **417**(6884): 95-8.

Draenert, R., S. Le Gall, K. J. Pfafferott, A. J. Leslie, P. Chetty, C. Brander, E. C. Holmes, S. C. Chang, M. E. Feeney, M. M. Addo, L. Ruiz, D. Ramduth, P. Jeena, M. Altfeld, S. Thomas, Y. Tang, C. L. Verrill, C. Dixon, J. G. Prado, P. Kiepiela, J. Martinez-Picado, B. D. Walker and P. J. Goulder (2004). "Immune selection for altered antigen processing leads to cytotoxic T lymphocyte escape in chronic HIV-1 infection." J Exp Med **199**(7): 905-15.

Draenert, R., C. L. Verrill, Y. Tang, T. M. Allen, A. G. Wurcel, M. Boczanowski, A. Lechner, A. Y. Kim, T. Suscovich, N. V. Brown, M. M. Addo and B. D. Walker (2004). "Persistent recognition of autologous virus by high-avidity CD8 T cells in chronic, progressive human immunodeficiency virus type 1 infection." J Virol **78**(2): 630-41.

Dragic, T., V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cayanan, P. J. Maddon, R. A. Koup, J. P. Moore and W. A. Paxton (1996). "HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5." Nature **381**(6584): 667-73.

Edwards, B. H., A. Bansal, S. Sabbaj, J. Bakari, M. J. Mulligan and P. A. Goepfert (2002). "Magnitude of functional CD8+ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma." J Virol **76**(5): 2298-305.

Emilie, D., M. C. Maillot, J. F. Nicolas, R. Fior and P. Galanaud (1992). "Antagonistic effect of interferon-gamma on tat-induced transactivation of HIV long terminal repeat." J Biol Chem **267**(29): 20565-70.

Emu, B., E. Sinclair, H. Hatano, A. Ferre, B. Shacklett, J. N. Martin, J. M. McCune and S. G. Deeks (2008). "HLA class I-restricted T-cell responses may contribute to the control of human immunodeficiency virus infection, but such

responses are not always necessary for long-term virus control." J Virol **82**(11): 5398-407.
Fan, S. X., J. A. Turpin, J. R. Aronovitz and M. S. Meltzer (1994). "Interferon-gamma protects primary monocytes against infection with human immunodeficiency virus type 1." J Leukoc Biol **56**(3): 362-8.
Fauce, S. R., O. O. Yang and R. B. Effros (2007). "Autologous CD4/CD8 co-culture assay: a physiologically-relevant composite measure of CD8+ T lymphocyte function in HIV-infected persons." J Immunol Methods **327**(1-2): 75-81.
Fauci, A. S. (1993). "Multifactorial nature of human immunodeficiency virus disease: implications for therapy." Science **262**(5136): 1011-8.
Fauci, A. S. (1996). "Host factors and the pathogenesis of HIV-induced disease." Nature **384**(6609): 529-34.
Fauci, A. S., D. Mavilio and S. Kottilil (2005). "NK cells in HIV infection: paradigm for protection or targets for ambush." Nat Rev Immunol **5**(11): 835-43.
Feeney, M. E., Y. Tang, K. A. Roosevelt, A. J. Leslie, K. McIntosh, N. Karthas, B. D. Walker and P. J. Goulder (2004). "Immune escape precedes breakthrough human immunodeficiency virus type 1 viremia and broadening of the cytotoxic T-lymphocyte response in an HLA-B27-positive long-term-nonprogressing child." J Virol **78**(16): 8927-30.
Fellay, J., D. Ge, K. V. Shianna, S. Colombo, B. Ledergerber, E. T. Cirulli, T. J. Urban, K. Zhang, C. E. Gumbs, J. P. Smith, A. Castagna, A. Cozzi-Lepri, A. De Luca, P. Easterbrook, H. F. Gunthard, S. Mallal, C. Mussini, J. Dalmau, J. Martinez-Picado, J. M. Miro, N. Obel, S. M. Wolinsky, J. J. Martinson, R. Detels, J. B. Margolick, L. P. Jacobson, P. Descombes, S. E. Antonarakis, J. S. Beckmann, S. J. O'Brien, N. L. Letvin, A. J. McMichael, B. F. Haynes, M. Carrington, S. Feng, A. Telenti and D. B. Goldstein (2009). "Common genetic variation and the control of HIV-1 in humans." PLoS Genet **5**(12): e1000791.
Fellay, J., K. V. Shianna, D. Ge, S. Colombo, B. Ledergerber, M. Weale, K. Zhang, C. Gumbs, A. Castagna, A. Cossarizza, A. Cozzi-Lepri, A. De Luca, P. Easterbrook, P. Francioli, S. Mallal, J. Martinez-Picado, J. M. Miro, N. Obel, J. P. Smith, J. Wyniger, P. Descombes, S. E. Antonarakis, N. L. Letvin, A. J. McMichael, B. F. Haynes, A. Telenti and D. B. Goldstein (2007). "A whole-genome association study of major determinants for host control of HIV-1." Science **317**(5840): 944-7.
Feng, Y., C. C. Broder, P. E. Kennedy and E. A. Berger (1996). "HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor." Science **272**(5263): 872-7.
Fernandez-Garcia, A., M. T. Cuevas, M. Munoz-Nieto, A. Ocampo, M. Pinilla, V. Garcia, E. Serrano-Bengoechea, M. J. Lezaun, E. Delgado, M. Thomson, M. Gonzalez-Galeano, G. Contreras, R. Najera and L. Perez-Alvarez (2009). "Development of a panel of well-characterized human immunodeficiency virus type 1 isolates from newly diagnosed patients including acute and recent infections." AIDS Res Hum Retroviruses **25**(1): 93-102.
Ferrantelli, F., R. A. Rasmussen, K. A. Buckley, P. L. Li, T. Wang, D. C. Montefiori, H. Katinger, G. Stiegler, D. C. Anderson, H. M. McClure and R. M. Ruprecht (2004). "Complete protection of neonatal rhesus macaques against oral exposure to pathogenic simian-human immunodeficiency virus by human anti-HIV monoclonal antibodies." J Infect Dis **189**(12): 2167-73.
Ferrari, G., J. R. Currier, M. E. Harris, S. Finkelstein, A. de Oliveira, D. Barkhan, J. H. Cox, M. Zeira, K. J. Weinhold, N. Reinsmoen, F. McCutchan, D. L. Birx,

S. Osmanov and S. Maayan (2004). "HLA-A and -B allele expression and ability to develop anti-Gag cross-clade responses in subtype C HIV-1-infected Ethiopians." Hum Immunol **65**(6): 648-59.

Ferre, A. L., P. W. Hunt, J. W. Critchfield, D. H. Young, M. M. Morris, J. C. Garcia, R. B. Pollard, H. F. Yee, Jr., J. N. Martin, S. G. Deeks and B. L. Shacklett (2009). "Mucosal immune responses to HIV-1 in elite controllers: a potential correlate of immune control." Blood **113**(17): 3978-89.

Ferre, A. L., D. Lemongello, P. W. Hunt, M. M. Morris, J. C. Garcia, R. B. Pollard, H. F. Yee, Jr., J. N. Martin, S. G. Deeks and B. L. Shacklett (2010). "Immunodominant HIV-specific CD8+ T-cell responses are common to blood and gastrointestinal mucosa, and Gag-specific responses dominate in rectal mucosa of HIV controllers." J Virol **84**(19): 10354-65.

Finkel, T. H., G. Tudor-Williams, N. K. Banda, M. F. Cotton, T. Curiel, C. Monks, T. W. Baba, R. M. Ruprecht and A. Kupfer (1995). "Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes." Nat Med **1**(2): 129-34.

Fischer, W., S. Perkins, J. Theiler, T. Bhattacharya, K. Yusim, R. Funkhouser, C. Kuiken, B. Haynes, N. L. Letvin, B. D. Walker, B. H. Hahn and B. T. Korber (2007). "Polyvalent vaccines for optimal coverage of potential T-cell epitopes in global HIV-1 variants." Nat Med **13**(1): 100-6.

Flores-Villanueva, P. O., E. J. Yunis, J. C. Delgado, E. Vittinghoff, S. Buchbinder, J. Y. Leung, A. M. Uglialoro, O. P. Clavijo, E. S. Rosenberg, S. A. Kalams, J. D. Braun, S. L. Boswell, B. D. Walker and A. E. Goldfeld (2001). "Control of HIV-1 viremia and protection from AIDS are associated with HLA-Bw4 homozygosity." Proc Natl Acad Sci U S A **98**(9): 5140-5.

Fontaine Costa, A. I., X. Rao, E. Lechenadec, D. van Baarle and C. Kesmir (2010). "HLA-B molecules target more conserved regions of the HIV-1 proteome." Aids **24**(2): 211-5.

Frahm, N., B. T. Korber, C. M. Adams, J. J. Szinger, R. Draenert, M. M. Addo, M. E. Feeney, K. Yusim, K. Sango, N. V. Brown, D. SenGupta, A. Piechocka-Trocha, T. Simonis, F. M. Marincola, A. G. Wurcel, D. R. Stone, C. J. Russell, P. Adolf, D. Cohen, T. Roach, A. StJohn, A. Khatri, K. Davis, J. Mullins, P. J. Goulder, B. D. Walker and C. Brander (2004). "Consistent cytotoxic-T-lymphocyte targeting of immunodominant regions in human immunodeficiency virus across multiple ethnicities." J Virol **78**(5): 2187-200.

Frahm, N., C. Linde and C. Brander (2007). Identification of HIV-Derived, HLA Class I Restricted CTL Epitopes: Insights into TCR Repertoire, CTL Escape and Viral Fitness. HIV Molecular Immunology 2006/2007. C. B. Bette TM Korber, Barton F Haynes, Richard Coup, John P Moore, Bruce D Walker and David Watkins., Los Alamos National Laboratory, Theoretical Biology and Biophysics, Los Alamos, New Mexico.**:** 3-28.

Frater, A. J., H. Brown, A. Oxenius, H. F. Gunthard, B. Hirschel, N. Robinson, A. J. Leslie, R. Payne, H. Crawford, A. Prendergast, C. Brander, P. Kiepiela, B. D. Walker, P. J. Goulder, A. McLean and R. E. Phillips (2007). "Effective T-cell responses select human immunodeficiency virus mutants and slow disease progression." J Virol **81**(12): 6742-51.

Frazer, K. A., S. S. Murray, N. J. Schork and E. J. Topol (2009). "Human genetic variation and its contribution to complex traits." Nat Rev Genet **10**(4): 241-51.

Freel, S. A., L. Lamoreaux, P. K. Chattopadhyay, K. Saunders, D. Zarkowsky, R. G. Overman, C. Ochsenbauer, T. G. Edmonds, J. C. Kappes, C. K. Cunningham,

T. N. Denny, K. J. Weinhold, G. Ferrari, B. F. Haynes, R. A. Koup, B. S. Graham, M. Roederer and G. D. Tomaras (2010). "Phenotypic and functional profile of HIV-inhibitory CD8 T cells elicited by natural infection and heterologous prime/boost vaccination." J Virol **84**(10): 4998-5006.

Friedrich, T. C., E. J. Dodds, L. J. Yant, L. Vojnov, R. Rudersdorf, C. Cullen, D. T. Evans, R. C. Desrosiers, B. R. Mothe, J. Sidney, A. Sette, K. Kunstman, S. Wolinsky, M. Piatak, J. Lifson, A. L. Hughes, N. Wilson, D. H. O'Connor and D. I. Watkins (2004). "Reversion of CTL escape-variant immunodeficiency viruses in vivo." Nat Med **10**(3): 275-81.

Gallimore, A., M. Cranage, N. Cook, N. Almond, J. Bootman, E. Rud, P. Silvera, M. Dennis, T. Corcoran, J. Stott and et al. (1995). "Early suppression of SIV replication by CD8+ nef-specific cytotoxic T cells in vaccinated macaques." Nat Med **1**(11): 1167-73.

Gao, F., E. Bailes, D. L. Robertson, Y. Chen, C. M. Rodenburg, S. F. Michael, L. B. Cummins, L. O. Arthur, M. Peeters, G. M. Shaw, P. M. Sharp and B. H. Hahn (1999). "Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes." Nature **397**(6718): 436-41.

Gao, F., L. Yue, A. T. White, P. G. Pappas, J. Barchue, A. P. Hanson, B. M. Greene, P. M. Sharp, G. M. Shaw and B. H. Hahn (1992). "Human infection by genetically diverse SIVSM-related HIV-2 in west Africa." Nature **358**(6386): 495-9.

Gao, X., G. W. Nelson, P. Karacki, M. P. Martin, J. Phair, R. Kaslow, J. J. Goedert, S. Buchbinder, K. Hoots, D. Vlahov, S. J. O'Brien and M. Carrington (2001). "Effect of a single amino acid change in MHC class I molecules on the rate of progression to AIDS." N Engl J Med **344**(22): 1668-75.

Garcia-Ruano, A. B., R. Mendez, J. M. Romero, T. Cabrera, F. Ruiz-Cabello and F. Garrido (2010). "Analysis of HLA-ABC locus-specific transcription in normal tissues." Immunogenetics **62**(11-12): 711-9.

Gaschen, B., J. Taylor, K. Yusim, B. Foley, F. Gao, D. Lang, V. Novitsky, B. Haynes, B. H. Hahn, T. Bhattacharya and B. Korber (2002). "Diversity considerations in HIV-1 vaccine selection." Science **296**(5577): 2354-60.

Geijtenbeek, T. B., D. S. Kwon, R. Torensma, S. J. van Vliet, G. C. van Duijnhoven, J. Middel, I. L. Cornelissen, H. S. Nottet, V. N. KewalRamani, D. R. Littman, C. G. Figdor and Y. van Kooyk (2000). "DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells." Cell **100**(5): 587-97.

Geldmacher, C., J. R. Currier, E. Herrmann, A. Haule, E. Kuta, F. McCutchan, L. Njovu, S. Geis, O. Hoffmann, L. Maboko, C. Williamson, D. Birx, A. Meyerhans, J. Cox and M. Hoelscher (2007). "CD8 T-cell recognition of multiple epitopes within specific Gag regions is associated with maintenance of a low steady-state viremia in human immunodeficiency virus type 1-seropositive patients." J Virol **81**(5): 2440-8.

Geyer, M., O. T. Fackler and B. M. Peterlin (2001). "Structure--function relationships in HIV-1 Nef." EMBO Rep **2**(7): 580-5.

Gillespie, G. M., R. Kaul, T. Dong, H. B. Yang, T. Rostron, J. J. Bwayo, P. Kiama, T. Peto, F. A. Plummer, A. J. McMichael and S. L. Rowland-Jones (2002). "Cross-reactive cytotoxic T lymphocytes against a HIV-1 p24 epitope in slow progressors with B*57." Aids **16**(7): 961-72.

Gillespie, G. M., G. Stewart-Jones, J. Rengasamy, T. Beattie, J. J. Bwayo, F. A. Plummer, R. Kaul, A. J. McMichael, P. Easterbrook, T. Dong, E. Y. Jones and

S. L. Rowland-Jones (2006). "Strong TCR conservation and altered T cell cross-reactivity characterize a B*57-restricted immune response in HIV-1 infection." J Immunol **177**(6): 3893-902.

Girardi, E., F. N. Lauria and G. Ippolito (2005). "HIV/AIDS in 2004: the epidemiologist's point of view." Cell Death Differ **12 Suppl 1**: 837-44.

Goepfert, P. A., W. Lumm, P. Farmer, P. Matthews, A. Prendergast, J. M. Carlson, C. A. Derdeyn, J. Tang, R. A. Kaslow, A. Bansal, K. Yusim, D. Heckerman, J. Mulenga, S. Allen, P. J. Goulder and E. Hunter (2008). "Transmission of HIV-1 Gag immune escape mutations is associated with reduced viral load in linked recipients." J Exp Med **205**(5): 1009-17.

Goldstein, D. B. (2009). "Common genetic variation and human traits." N Engl J Med **360**(17): 1696-8.

Gomez, C. and T. J. Hope (2005). "The ins and outs of HIV replication." Cell Microbiol **7**(5): 621-6.

Gonzalez-Galarza, F. F., S. Christmas, D. Middleton and A. R. Jones (2011). "Allele frequency net: a database and online repository for immune gene frequencies in worldwide populations." Nucleic Acids Res **39**(Database issue): D913-9.

Gonzalez, E., H. Kulkarni, H. Bolivar, A. Mangano, R. Sanchez, G. Catano, R. J. Nibbs, B. I. Freedman, M. P. Quinones, M. J. Bamshad, K. K. Murthy, B. H. Rovin, W. Bradley, R. A. Clark, S. A. Anderson, J. O'Connell R, B. K. Agan, S. S. Ahuja, R. Bologna, L. Sen, M. J. Dolan and S. K. Ahuja (2005). "The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility." Science **307**(5714): 1434-40.

Goonetilleke, N., M. K. Liu, J. F. Salazar-Gonzalez, G. Ferrari, E. Giorgi, V. V. Ganusov, B. F. Keele, G. H. Learn, E. L. Turnbull, M. G. Salazar, K. J. Weinhold, S. Moore, N. Letvin, B. F. Haynes, M. S. Cohen, P. Hraber, T. Bhattacharya, P. Borrow, A. S. Perelson, B. H. Hahn, G. M. Shaw, B. T. Korber and A. J. McMichael (2009). "The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection." J Exp Med **206**(6): 1253-72.

Goulder, P. J., M. A. Altfeld, E. S. Rosenberg, T. Nguyen, Y. Tang, R. L. Eldridge, M. M. Addo, S. He, J. S. Mukherjee, M. N. Phillips, M. Bunce, S. A. Kalams, R. P. Sekaly, B. D. Walker and C. Brander (2001). "Substantial differences in specificity of HIV-specific cytotoxic T cells in acute and chronic HIV infection." J Exp Med **193**(2): 181-94.

Goulder, P. J., M. Bunce, P. Krausa, K. McIntyre, S. Crowley, B. Morgan, A. Edwards, P. Giangrande, R. E. Phillips and A. J. McMichael (1996). "Novel, cross-restricted, conserved, and immunodominant cytotoxic T lymphocyte epitopes in slow progressors in HIV type 1 infection." AIDS Res Hum Retroviruses **12**(18): 1691-8.

Goulder, P. J., M. Bunce, G. Luzzi, R. E. Phillips and A. J. McMichael (1997). "Potential underestimation of HLA-C-restricted cytotoxic T-lymphocyte responses." Aids **11**(15): 1884-6.

Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael and S. Rowland-Jones (1997). "Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS." Nat Med **3**(2): 212-7.

Goulder, P. J. and D. I. Watkins (2004). "HIV and SIV CTL escape: implications for vaccine design." Nat Rev Immunol **4**(8): 630-40.

Goulder, P. J. and D. I. Watkins (2008). "Impact of MHC class I diversity on immune control of immunodeficiency virus replication." Nat Rev Immunol **8**(8): 619-30.

Gray, E. S., M. C. Madiga, T. Hermanus, P. L. Moore, C. K. Wibmer, N. L. Tumba, L. Werner, K. Mlisana, S. Sibeko, C. Williamson, S. S. Abdool Karim and L. Morris (2011). "The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4+ T cell decline and high viral load during acute infection." J Virol **85**(10): 4828-40.

Greene, W. C. and B. M. Peterlin (2002). "Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy." Nat Med **8**(7): 673-80.

Grossman, Z., M. Meier-Schellersheim, A. E. Sousa, R. M. Victorino and W. E. Paul (2002). "CD4+ T-cell depletion in HIV infection: are we closer to understanding the cause?" Nat Med **8**(4): 319-23.

Gruda, R., H. Achdout, N. Stern-Ginossar, R. Gazit, G. Betser-Cohen, I. Manaster, G. Katz, T. Gonen-Gross, B. Tirosh and O. Mandelboim (2007). "Intracellular cysteine residues in the tail of MHC class I proteins are crucial for extracellular recognition by leukocyte Ig-like receptor 1." J Immunol **179**(6): 3655-61.

Guadalupe, M., E. Reay, S. Sankaran, T. Prindiville, J. Flamm, A. McNeil and S. Dandekar (2003). "Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy." J Virol **77**(21): 11708-17.

Guan, Y., M. M. Sajadi, R. Kamin-Lewis, T. R. Fouts, A. Dimitrov, Z. Zhang, R. R. Redfield, A. L. DeVico, R. C. Gallo and G. K. Lewis (2009). "Discordant memory B cell and circulating anti-Env antibody responses in HIV-1 infection." Proc Natl Acad Sci U S A **106**(10): 3952-7.

Guermonprez, P. and S. Amigorena (2005). "Pathways for antigen cross presentation." Springer Semin Immunopathol **26**(3): 257-71.

Guild, B. C. and J. L. Strominger (1984). "Human and murine class I MHC antigens share conserved serine 335, the site of HLA phosphorylation in vivo." J Biol Chem **259**(14): 9235-40.

Gulzar, N. and K. F. Copeland (2004). "CD8+ T-cells: function and response to HIV infection." Curr HIV Res **2**(1): 23-37.

Hadida, F., V. Vieillard, L. Mollet, I. Clark-Lewis, M. Baggiolini and P. Debre (1999). "Cutting edge: RANTES regulates Fas ligand expression and killing by HIV-specific CD8 cytotoxic T cells." J Immunol **163**(3): 1105-9.

Hahn, B. H., G. M. Shaw, K. M. De Cock and P. M. Sharp (2000). "AIDS as a zoonosis: scientific and public health implications." Science **287**(5453): 607-14.

Haigwood, N. L. and L. Stamatatos (2003). "Role of neutralizing antibodies in HIV infection." Aids **17 Suppl 4**: S67-71.

Hansasuta, P., T. Dong, H. Thananchai, M. Weekes, C. Willberg, H. Aldemir, S. Rowland-Jones and V. M. Braud (2004). "Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific." Eur J Immunol **34**(6): 1673-9.

Hansen, S. G., J. C. Ford, M. S. Lewis, A. B. Ventura, C. M. Hughes, L. Coyne-Johnson, N. Whizin, K. Oswald, R. Shoemaker, T. Swanson, A. W. Legasse, M. J. Chiuchiolo, C. L. Parks, M. K. Axthelm, J. A. Nelson, M. A. Jarvis, M. Piatak, Jr., J. D. Lifson and L. J. Picker (2011). "Profound early control of

highly pathogenic SIV by an effector memory T-cell vaccine." Nature **473**(7348): 523-7.

Harari, A., C. Cellerai, F. B. Enders, J. Kostler, L. Codarri, G. Tapia, O. Boyman, E. Castro, S. Gaudieri, I. James, M. John, R. Wagner, S. Mallal and G. Pantaleo (2007). "Skewed association of polyfunctional antigen-specific CD8 T cell populations with HLA-B genotype." Proc Natl Acad Sci U S A **104**(41): 16233-8.

Harrer, E. G., S. Bergmann, K. Eismann, M. Rittmaier, A. Goldwich, S. M. Muller, B. M. Spriewald and T. Harrer (2005). "A conserved HLA B13-restricted cytotoxic T lymphocyte epitope in Nef is a dominant epitope in HLA B13-positive HIV-1-infected patients." Aids **19**(7): 734-5.

Hartley, O., P. J. Klasse, Q. J. Sattentau and J. P. Moore (2005). "V3: HIV's switch-hitter." AIDS Res Hum Retroviruses **21**(2): 171-89.

Harty, J. T., A. R. Tvinnereim and D. W. White (2000). "CD8+ T cell effector mechanisms in resistance to infection." Annu Rev Immunol **18**: 275-308.

Hay, C. M., D. J. Ruhl, N. O. Basgoz, C. C. Wilson, J. M. Billingsley, M. P. DePasquale, R. T. D'Aquila, S. M. Wolinsky, J. M. Crawford, D. C. Montefiori and B. D. Walker (1999). "Lack of viral escape and defective in vivo activation of human immunodeficiency virus type 1-specific cytotoxic T lymphocytes in rapidly progressive infection." J Virol **73**(7): 5509-19.

Haynes, B. F. and D. C. Montefiori (2006). "Aiming to induce broadly reactive neutralizing antibody responses with HIV-1 vaccine candidates." Expert Rev Vaccines **5**(4): 579-95.

Hecht, F. M., M. P. Busch, B. Rawal, M. Webb, E. Rosenberg, M. Swanson, M. Chesney, J. Anderson, J. Levy and J. O. Kahn (2002). "Use of laboratory tests and clinical symptoms for identification of primary HIV infection." Aids **16**(8): 1119-29.

Heinzinger, N. K., M. I. Bukinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M. A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson and M. Emerman (1994). "The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells." Proc Natl Acad Sci U S A **91**(15): 7311-5.

Hendel, H., S. Caillat-Zucman, H. Lebuanec, M. Carrington, S. O'Brien, J. M. Andrieu, F. Schachter, D. Zagury, J. Rappaport, C. Winkler, G. W. Nelson and J. F. Zagury (1999). "New class I and II HLA alleles strongly associated with opposite patterns of progression to AIDS." J Immunol **162**(11): 6942-6.

Hessell, A. J., P. Poignard, M. Hunter, L. Hangartner, D. M. Tehrani, W. K. Bleeker, P. W. Parren, P. A. Marx and D. R. Burton (2009). "Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques." Nat Med **15**(8): 951-4.

Ho, D. D. (1996). "Viral counts count in HIV infection." Science **272**(5265): 1124-5.

Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard and M. Markowitz (1995). "Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection." Nature **373**(6510): 123-6.

Hogan, C. M. and S. M. Hammer (2001). "Host determinants in HIV infection and disease. Part 1: cellular and humoral immune responses." Ann Intern Med **134**(9 Pt 1): 761-76.

Honda, K., N. Zheng, H. Murakoshi, M. Hashimoto, K. Sakai, M. A. Borghan, T. Chikata, M. Koyanagi, Y. Tamura, H. Gatanaga, S. Oka and M. Takiguchi (2011). "Selection of escape mutant by HLA-C-restricted HIV-1 Pol-specific

cytotoxic T lymphocytes carrying strong ability to suppress HIV-1 replication." Eur J Immunol **41**(1): 97-106.

Honeyborne, I., F. M. Codoner, A. Leslie, G. Tudor-Williams, G. Luzzi, T. Ndung'u, B. D. Walker, P. J. Goulder and J. G. Prado (2010). "HLA-Cw*03 -restricted CD8+ T-cell responses targeting the HIV-1 Gag Major Homology Region drive virus immune escape and fitness constraints compensated by intra-codon variation." J Virol.

Honeyborne, I., A. Prendergast, F. Pereyra, A. Leslie, H. Crawford, R. Payne, S. Reddy, K. Bishop, E. Moodley, K. Nair, M. van der Stok, N. McCarthy, C. M. Rousseau, M. Addo, J. I. Mullins, C. Brander, P. Kiepiela, B. D. Walker and P. J. Goulder (2007). "Control of human immunodeficiency virus type 1 is associated with HLA-B*13 and targeting of multiple gag-specific CD8+ T-cell epitopes." J Virol **81**(7): 3667-72.

Horton, H., I. Frank, R. Baydo, E. Jalbert, J. Penn, S. Wilson, J. P. McNevin, M. D. McSweyn, D. Lee, Y. Huang, S. C. De Rosa and M. J. McElrath (2006). "Preservation of T cell proliferation restricted by protective HLA alleles is critical for immune control of HIV-1 infection." J Immunol **177**(10): 7406-15.

Hu, J., M. B. Gardner and C. J. Miller (2000). "Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells." J Virol **74**(13): 6087-95.

Hu, Q., I. Frank, V. Williams, J. J. Santos, P. Watts, G. E. Griffin, J. P. Moore, M. Pope and R. J. Shattock (2004). "Blockade of attachment and fusion receptors inhibits HIV-1 infection of human cervical tissue." J Exp Med **199**(8): 1065-75.

Ishitani, A., N. Sageshima, N. Lee, N. Dorofeeva, K. Hatake, H. Marquardt and D. E. Geraghty (2003). "Protein expression and peptide binding suggest unique and interacting functional roles for HLA-E, F, and G in maternal-placental immune recognition." J Immunol **171**(3): 1376-84.

Janssen, E. M., E. E. Lemmens, T. Wolfe, U. Christen, M. G. von Herrath and S. P. Schoenberger (2003). "CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes." Nature **421**(6925): 852-6.

Jassoy, C., T. Harrer, T. Rosenthal, B. A. Navia, J. Worth, R. P. Johnson and B. D. Walker (1993). "Human immunodeficiency virus type 1-specific cytotoxic T lymphocytes release gamma interferon, tumor necrosis factor alpha (TNF-alpha), and TNF-beta when they encounter their target antigens." J Virol **67**(5): 2844-52.

Jeffery, K. J. and C. R. Bangham (2000). "Do infectious diseases drive MHC diversity?" Microbes Infect **2**(11): 1335-41.

Jensen, P. E. (2007). "Recent advances in antigen processing and presentation." Nat Immunol **8**(10): 1041-8.

Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson and D. D. Ho (1999). "Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques." J Exp Med **189**(6): 991-8.

John, M., D. Heckerman, I. James, L. P. Park, J. M. Carlson, A. Chopra, S. Gaudieri, D. Nolan, D. W. Haas, S. A. Riddler, R. Haubrich and S. Mallal (2010). "Adaptive interactions between HLA and HIV-1: highly divergent selection imposed by HLA class I molecules with common supertype motifs." J Immunol **184**(8): 4368-77.

Johnson, D. R. (2000). "Differential expression of human major histocompatibility class I loci: HLA-A, -B, and -C." Hum Immunol **61**(4): 389-96.

Jones, K. A. and B. M. Peterlin (1994). "Control of RNA initiation and elongation at the HIV-1 promoter." Annu Rev Biochem **63**: 717-43.

Jones, N. A., X. Wei, D. R. Flower, M. Wong, F. Michor, M. S. Saag, B. H. Hahn, M. A. Nowak, G. M. Shaw and P. Borrow (2004). "Determinants of human immunodeficiency virus type 1 escape from the primary CD8+ cytotoxic T lymphocyte response." J Exp Med **200**(10): 1243-56.

Julg, B., K. L. Williams, S. Reddy, K. Bishop, Y. Qi, M. Carrington, P. J. Goulder, T. Ndung'u and B. D. Walker (2010). "Enhanced anti-HIV functional activity associated with Gag-specific CD8 T-cell responses." J Virol **84**(11): 5540-9.

Jung, A., R. Maier, J. P. Vartanian, G. Bocharov, V. Jung, U. Fischer, E. Meese, S. Wain-Hobson and A. Meyerhans (2002). "Multiply infected spleen cells in HIV patients." Nature **418**(6894): 144.

Kahn, J. O. and B. D. Walker (1998). "Acute human immunodeficiency virus type 1 infection." N Engl J Med **339**(1): 33-9.

Kaplan, A. H., P. Krogstad, D. J. Kempf, D. W. Norbeck and R. Swanstrom (1994). "Human immunodeficiency virus type 1 virions composed of unprocessed Gag and Gag-Pol precursors are capable of reverse transcribing viral genomic RNA." Antimicrob Agents Chemother **38**(12): 2929-33.

Kaslow, R. A., M. Carrington, R. Apple, L. Park, A. Munoz, A. J. Saah, J. J. Goedert, C. Winkler, S. J. O'Brien, C. Rinaldo, R. Detels, W. Blattner, J. Phair, H. Erlich and D. L. Mann (1996). "Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection." Nat Med **2**(4): 405-11.

Kaslow, R. A., R. Duquesnoy, M. VanRaden, L. Kingsley, M. Marrari, H. Friedman, S. Su, A. J. Saah, R. Detels, J. Phair and et al. (1990). "A1, Cw7, B8, DR3 HLA antigen combination associated with rapid decline of T-helper lymphocytes in HIV-1 infection. A report from the Multicenter AIDS Cohort Study." Lancet **335**(8695): 927-30.

Kassutto, S. and E. S. Rosenberg (2004). "Primary HIV type 1 infection." Clin Infect Dis **38**(10): 1447-53.

Katsikis, P. D., E. S. Wunderlich, C. A. Smith and L. A. Herzenberg (1995). "Fas antigen stimulation induces marked apoptosis of T lymphocytes in human immunodeficiency virus-infected individuals." J Exp Med **181**(6): 2029-36.

Kaufman, J. F., M. S. Krangel and J. L. Strominger (1984). "Cysteines in the transmembrane region of major histocompatibility complex antigens are fatty acylated via thioester bonds." J Biol Chem **259**(11): 7230-8.

Kawashima, Y., K. Pfafferott, J. Frater, P. Matthews, R. Payne, M. Addo, H. Gatanaga, M. Fujiwara, A. Hachiya, H. Koizumi, N. Kuse, S. Oka, A. Duda, A. Prendergast, H. Crawford, A. Leslie, Z. Brumme, C. Brumme, T. Allen, C. Brander, R. Kaslow, J. Tang, E. Hunter, S. Allen, J. Mulenga, S. Branch, T. Roach, M. John, S. Mallal, A. Ogwu, R. Shapiro, J. G. Prado, S. Fidler, J. Weber, O. G. Pybus, P. Klenerman, T. Ndung'u, R. Phillips, D. Heckerman, P. R. Harrigan, B. D. Walker, M. Takiguchi and P. Goulder (2009). "Adaptation of HIV-1 to human leukocyte antigen class I." Nature **458**(7238): 641-5.

Kedzierska, K., S. M. Crowe, S. Turville and A. L. Cunningham (2003). "The influence of cytokines, chemokines and their receptors on HIV-1 replication in monocytes and macrophages." Rev Med Virol **13**(1): 39-56.

Keele, B. F., E. E. Giorgi, J. F. Salazar-Gonzalez, J. M. Decker, K. T. Pham, M. G. Salazar, C. Sun, T. Grayson, S. Wang, H. Li, X. Wei, C. Jiang, J. L. Kirchherr, F. Gao, J. A. Anderson, L. H. Ping, R. Swanstrom, G. D. Tomaras, W. A. Blattner, P. A. Goepfert, J. M. Kilby, M. S. Saag, E. L. Delwart, M. P. Busch, M. S. Cohen, D. C. Montefiori, B. F. Haynes, B. Gaschen, G. S. Athreya, H. Y. Lee, N. Wood, C. Seoighe, A. S. Perelson, T. Bhattacharya, B. T. Korber, B. H. Hahn and G. M. Shaw (2008). "Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection." Proc Natl Acad Sci U S A **105**(21): 7552-7.

Kelleher, A. D., C. Long, E. C. Holmes, R. L. Allen, J. Wilson, C. Conlon, C. Workman, S. Shaunak, K. Olson, P. Goulder, C. Brander, G. Ogg, J. S. Sullivan, W. Dyer, I. Jones, A. J. McMichael, S. Rowland-Jones and R. E. Phillips (2001). "Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses." J Exp Med **193**(3): 375-86.

Kent, S. J., A. Zhao, S. J. Best, J. D. Chandler, D. B. Boyle and I. A. Ramshaw (1998). "Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus." J Virol **72**(12): 10180-8.

Kestler, H. W., 3rd, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel and R. C. Desrosiers (1991). "Importance of the nef gene for maintenance of high virus loads and for development of AIDS." Cell **65**(4): 651-62.

Khakoo, S. I., C. L. Thio, M. P. Martin, C. R. Brooks, X. Gao, J. Astemborski, J. Cheng, J. J. Goedert, D. Vlahov, M. Hilgartner, S. Cox, A. M. Little, G. J. Alexander, M. E. Cramp, S. J. O'Brien, W. M. Rosenberg, D. L. Thomas and M. Carrington (2004). "HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection." Science **305**(5685): 872-4.

Kieffer, T. L., P. Kwon, R. E. Nettles, Y. Han, S. C. Ray and R. F. Siliciano (2005). "G-->A hypermutation in protease and reverse transcriptase regions of human immunodeficiency virus type 1 residing in resting CD4+ T cells in vivo." J Virol **79**(3): 1975-80.

Kiepiela, P., A. J. Leslie, I. Honeyborne, D. Ramduth, C. Thobakgale, S. Chetty, P. Rathnavalu, C. Moore, K. J. Pfafferott, L. Hilton, P. Zimbwa, S. Moore, T. Allen, C. Brander, M. M. Addo, M. Altfeld, I. James, S. Mallal, M. Bunce, L. D. Barber, J. Szinger, C. Day, P. Klenerman, J. Mullins, B. Korber, H. M. Coovadia, B. D. Walker and P. J. Goulder (2004). "Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA." Nature **432**(7018): 769-75.

Kiepiela, P., K. Ngumbela, C. Thobakgale, D. Ramduth, I. Honeyborne, E. Moodley, S. Reddy, C. de Pierres, Z. Mncube, N. Mkhwanazi, K. Bishop, M. van der Stok, K. Nair, N. Khan, H. Crawford, R. Payne, A. Leslie, J. Prado, A. Prendergast, J. Frater, N. McCarthy, C. Brander, G. H. Learn, D. Nickle, C. Rousseau, H. Coovadia, J. I. Mullins, D. Heckerman, B. D. Walker and P. Goulder (2007). "CD8+ T-cell responses to different HIV proteins have discordant associations with viral load." Nat Med **13**(1): 46-53.

Kim, C. H., L. S. Rott, I. Clark-Lewis, D. J. Campbell, L. Wu and E. C. Butcher (2001). "Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells." J Exp Med **193**(12): 1373-81.

Kim, S. Y., R. Byrn, J. Groopman and D. Baltimore (1989). "Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression." J Virol **63**(9): 3708-13.

King, A., D. S. Allan, M. Bowen, S. J. Powis, S. Joseph, S. Verma, S. E. Hiby, A. J. McMichael, Y. W. Loke and V. M. Braud (2000). "HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells." Eur J Immunol **30**(6): 1623-31.

Klein, M. R., I. P. Keet, J. D'Amaro, R. J. Bende, A. Hekman, B. Mesman, M. Koot, L. P. de Waal, R. A. Coutinho and F. Miedema (1994). "Associations between HLA frequencies and pathogenic features of human immunodeficiency virus type 1 infection in seroconverters from the Amsterdam cohort of homosexual men." J Infect Dis **169**(6): 1244-9.

Klotman, M. E., S. Kim, A. Buchbinder, A. DeRossi, D. Baltimore and F. Wong-Staal (1991). "Kinetics of expression of multiply spliced RNA in early human immunodeficiency virus type 1 infection of lymphocytes and monocytes." Proc Natl Acad Sci U S A **88**(11): 5011-5.

Koehler, R. N., A. M. Walsh, E. Saathoff, S. Tovanabutra, M. A. Arroyo, J. R. Currier, L. Maboko, M. Hoelsher, M. L. Robb, N. L. Michael, F. E. McCutchan, J. H. Kim and G. H. Kijak (2010). "Class I HLA-A*7401 is associated with protection from HIV-1 acquisition and disease progression in Mbeya, Tanzania." J Infect Dis **202**(10): 1562-6.

Koenig, S., A. J. Conley, Y. A. Brewah, G. M. Jones, S. Leath, L. J. Boots, V. Davey, G. Pantaleo, J. F. Demarest, C. Carter and et al. (1995). "Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression." Nat Med **1**(4): 330-6.

Korber, B., M. Muldoon, J. Theiler, F. Gao, R. Gupta, A. Lapedes, B. H. Hahn, S. Wolinsky and T. Bhattacharya (2000). "Timing the ancestor of the HIV-1 pandemic strains." Science **288**(5472): 1789-96.

Kosmrlj, A., E. L. Read, Y. Qi, T. M. Allen, M. Altfeld, S. G. Deeks, F. Pereyra, M. Carrington, B. D. Walker and A. K. Chakraborty (2010). "Effects of thymic selection of the T-cell repertoire on HLA class I-associated control of HIV infection." Nature **465**(7296): 350-4.

Koup, R. A. (1994). "Virus escape from CTL recognition." J Exp Med **180**(3): 779-82.

Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing and D. D. Ho (1994). "Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome." J Virol **68**(7): 4650-5.

Kremer, M. and B. S. Schnierle (2005). "HIV-1 Vif: HIV's weapon against the cellular defense factor APOBEC3G." Curr HIV Res **3**(4): 339-44.

Kulkarni, S., R. Savan, Y. Qi, X. Gao, Y. Yuki, S. E. Bass, M. P. Martin, P. Hunt, S. G. Deeks, A. Telenti, F. Pereyra, D. Goldstein, S. Wolinsky, B. Walker, H. A. Young and M. Carrington (2011). "Differential microRNA regulation of HLA-C expression and its association with HIV control." Nature.

Kumar, S. and S. Subramanian (2002). "Mutation rates in mammalian genomes." Proc Natl Acad Sci U S A **99**(2): 803-8.

Landay, A. L., C. E. Mackewicz and J. A. Levy (1993). "An activated CD8+ T cell phenotype correlates with anti-HIV activity and asymptomatic clinical status." Clin Immunol Immunopathol **69**(1): 106-16.

Lanier, L. L. (2005). "NK cell recognition." Annu Rev Immunol **23**: 225-74.

Larder, B. A., S. Bloor, S. D. Kemp, K. Hertogs, R. L. Desmet, V. Miller, M. Sturmer, S. Staszewski, J. Ren, D. K. Stammers, D. I. Stuart and R. Pauwels (1999). "A family of insertion mutations between codons 67 and 70 of human immunodeficiency virus type 1 reverse transcriptase confer multinucleoside analog resistance." Antimicrob Agents Chemother **43**(8): 1961-7.

Lazaryan, A., E. Lobashevsky, J. Mulenga, E. Karita, S. Allen, J. Tang and R. A. Kaslow (2006). "Human leukocyte antigen B58 supertype and human immunodeficiency virus type 1 infection in native Africans." J Virol **80**(12): 6056-60.

Lee, N., D. R. Goodlett, A. Ishitani, H. Marquardt and D. E. Geraghty (1998). "HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences." J Immunol **160**(10): 4951-60.

Lee, N., M. Llano, M. Carretero, A. Ishitani, F. Navarro, M. Lopez-Botet and D. E. Geraghty (1998). "HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A." Proc Natl Acad Sci U S A **95**(9): 5199-204.

Leslie, A., P. C. Matthews, J. Listgarten, J. M. Carlson, C. Kadie, T. Ndung'u, C. Brander, H. Coovadia, B. D. Walker, D. Heckerman and P. J. Goulder (2010). "Additive contribution of HLA class I alleles in the immune control of HIV-1 infection." J Virol **84**(19): 9879-9888.

Leslie, A., D. Price, P. Mkhize, K. Bishop, A. Rathod, C. Day, H. Crawford, I. Honeyborne, T. Asher, G. Luzzi, A. Edwards, C. M. Rousseau, J. Mullins, G. Tudor-Williams, V. Novelli, C. Brander, D. C. Douek, P. Kiepiela, B. Walker and P. Goulder (2006). "Differential selection pressure exerted on HIV by CTL targeting identical CTL epitopes but restricted by distinct HLA alleles from the same HLA-supertype." Journal of immunology **Accepted**.

Leslie, A., D. A. Price, P. Mkhize, K. Bishop, A. Rathod, C. Day, H. Crawford, I. Honeyborne, T. E. Asher, G. Luzzi, A. Edwards, C. M. Rousseau, J. I. Mullins, G. Tudor-Williams, V. Novelli, C. Brander, D. C. Douek, P. Kiepiela, B. D. Walker and P. J. Goulder (2006). "Differential selection pressure exerted on HIV by CTL targeting identical epitopes but restricted by distinct HLA alleles from the same HLA supertype." J Immunol **177**(7): 4699-708.

Leslie, A. J., K. J. Pfafferott, P. Chetty, R. Draenert, M. M. Addo, M. Feeney, Y. Tang, E. C. Holmes, T. Allen, J. G. Prado, M. Altfeld, C. Brander, C. Dixon, D. Ramduth, P. Jeena, S. A. Thomas, A. St John, T. A. Roach, B. Kupfer, G. Luzzi, A. Edwards, G. Taylor, H. Lyall, G. Tudor-Williams, V. Novelli, J. Martinez-Picado, P. Kiepiela, B. D. Walker and P. J. Goulder (2004). "HIV evolution: CTL escape mutation and reversion after transmission." Nat Med **10**(3): 282-9.

Letvin, N. L., J. E. Schmitz, H. L. Jordan, A. Seth, V. M. Hirsch, K. A. Reimann and M. J. Kuroda (1999). "Cytotoxic T lymphocytes specific for the simian immunodeficiency virus." Immunol Rev **170**: 127-34.

Levy, J. A., A. D. Hoffman, S. M. Kramer, J. A. Landis, J. M. Shimabukuro and L. S. Oshiro (1984). "Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS." Science **225**(4664): 840-2.

Levy, J. A., C. E. Mackewicz and E. Barker (1996). "Controlling HIV pathogenesis: the role of the noncytotoxic anti-HIV response of CD8+ T cells." Immunol Today **17**(5): 217-24.

Lewis, M. J., A. Balamurugan, A. Ohno, S. Kilpatrick, H. L. Ng and O. O. Yang (2008). "Functional adaptation of Nef to the immune milieu of HIV-1 infection in vivo." J Immunol **180**(6): 4075-81.

Li, B., A. D. Gladden, M. Altfeld, J. M. Kaldor, D. A. Cooper, A. D. Kelleher and T. M. Allen (2007). "Rapid reversion of sequence polymorphisms dominates early human immunodeficiency virus type 1 evolution." J Virol **81**(1): 193-201.

Li, Q., L. Duan, J. D. Estes, Z. M. Ma, T. Rourke, Y. Wang, C. Reilly, J. Carlis, C. J. Miller and A. T. Haase (2005). "Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells." Nature **434**(7037): 1148-52.

Lichterfeld, M., D. E. Kaufmann, X. G. Yu, S. K. Mui, M. M. Addo, M. N. Johnston, D. Cohen, G. K. Robbins, E. Pae, G. Alter, A. Wurcel, D. Stone, E. S. Rosenberg, B. D. Walker and M. Altfeld (2004). "Loss of HIV-1-specific CD8+ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4+ T cells." J Exp Med **200**(6): 701-12.

Lichterfeld, M., X. G. Yu, M. T. Waring, S. K. Mui, M. N. Johnston, D. Cohen, M. M. Addo, J. Zaunders, G. Alter, E. Pae, D. Strick, T. M. Allen, E. S. Rosenberg, B. D. Walker and M. Altfeld (2004). "HIV-1-specific cytotoxicity is preferentially mediated by a subset of CD8(+) T cells producing both interferon-gamma and tumor necrosis factor-alpha." Blood **104**(2): 487-94.

Lilley, B. N. and H. L. Ploegh (2005). "Viral modulation of antigen presentation: manipulation of cellular targets in the ER and beyond." Immunol Rev **207**: 126-44.

Limou, S., S. Le Clerc, C. Coulonges, W. Carpentier, C. Dina, O. Delaneau, T. Labib, L. Taing, R. Sladek, C. Deveau, R. Ratsimandresey, M. Montes, J. L. Spadoni, J. D. Lelievre, Y. Levy, A. Therwath, F. Schachter, F. Matsuda, I. Gut, P. Froguel, J. F. Delfraissy, S. Hercberg and J. F. Zagury (2009). "Genomewide association study of an AIDS-nonprogression cohort emphasizes the role played by HLA genes (ANRS Genomewide Association Study 02)." J Infect Dis **199**(3): 419-26.

Littman, D. R. (1998). "Chemokine receptors: keys to AIDS pathogenesis?" Cell **93**(5): 677-80.

Liu, J., K. L. O'Brien, D. M. Lynch, N. L. Simmons, A. La Porte, A. M. Riggs, P. Abbink, R. T. Coffey, L. E. Grandpre, M. S. Seaman, G. Landucci, D. N. Forthal, D. C. Montefiori, A. Carville, K. G. Mansfield, M. J. Havenga, M. G. Pau, J. Goudsmit and D. H. Barouch (2009). "Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys." Nature **457**(7225): 87-91.

Liu, R., W. A. Paxton, S. Choe, D. Ceradini, S. R. Martin, R. Horuk, M. E. MacDonald, H. Stuhlmann, R. A. Koup and N. R. Landau (1996). "Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection." Cell **86**(3): 367-77.

Liu, Y., J. McNevin, J. Cao, H. Zhao, I. Genowati, K. Wong, S. McLaughlin, M. D. McSweyn, K. Diem, C. E. Stevens, J. Maenza, H. He, D. C. Nickle, D. Shriner, S. E. Holte, A. C. Collier, L. Corey, M. J. McElrath and J. I. Mullins (2006). "Selection on the human immunodeficiency virus type 1 proteome following primary infection." J Virol **80**(19): 9519-29.

Lo Monaco, E., L. Sibilio, E. Melucci, E. Tremante, M. Suchanek, V. Horejsi, A. Martayan and P. Giacomini (2008). "HLA-E: strong association with beta2-

microglobulin and surface expression in the absence of HLA class I signal sequence-derived peptides." J Immunol **181**(8): 5442-50.

Loffredo, J. T., A. T. Bean, D. R. Beal, E. J. Leon, G. E. May, S. M. Piaskowski, J. R. Furlott, J. Reed, S. K. Musani, E. G. Rakasz, T. C. Friedrich, N. A. Wilson, D. B. Allison and D. I. Watkins (2008). "Patterns of CD8+ immunodominance may influence the ability of Mamu-B*08-positive macaques to naturally control simian immunodeficiency virus SIVmac239 replication." J Virol **82**(4): 1723-38.

Loffredo, J. T., B. J. Burwitz, E. G. Rakasz, S. P. Spencer, J. J. Stephany, J. P. Vela, S. R. Martin, J. Reed, S. M. Piaskowski, J. Furlott, K. L. Weisgrau, D. S. Rodrigues, T. Soma, G. Napoe, T. C. Friedrich, N. A. Wilson, E. G. Kallas and D. I. Watkins (2007). "The antiviral efficacy of simian immunodeficiency virus-specific CD8+ T cells is unrelated to epitope specificity and is abrogated by viral escape." J Virol **81**(6): 2624-34.

Loffredo, J. T., T. C. Friedrich, E. J. Leon, J. J. Stephany, D. S. Rodrigues, S. P. Spencer, A. T. Bean, D. R. Beal, B. J. Burwitz, R. A. Rudersdorf, L. T. Wallace, S. M. Piaskowski, G. E. May, J. Sidney, E. Gostick, N. A. Wilson, D. A. Price, E. G. Kallas, H. Piontkivska, A. L. Hughes, A. Sette and D. I. Watkins (2007). "CD8+ T cells from SIV elite controller macaques recognize Mamu-B*08-bound epitopes and select for widespread viral variation." PLoS One **2**(11): e1152.

Loffredo, J. T., E. G. Rakasz, J. P. Giraldo, S. P. Spencer, K. K. Grafton, S. R. Martin, G. Napoe, L. J. Yant, N. A. Wilson and D. I. Watkins (2005). "Tat(28-35)SL8-specific CD8+ T lymphocytes are more effective than Gag(181-189)CM9-specific CD8+ T lymphocytes at suppressing simian immunodeficiency virus replication in a functional in vitro assay." J Virol **79**(23): 14986-91.

Lopez-Vazquez, A., A. Mina-Blanco, J. Martinez-Borra, P. D. Njobvu, B. Suarez-Alvarez, M. A. Blanco-Gelaz, S. Gonzalez, L. Rodrigo and C. Lopez-Larrea (2005). "Interaction between KIR3DL1 and HLA-B*57 supertype alleles influences the progression of HIV-1 infection in a Zambian population." Hum Immunol **66**(3): 285-9.

Lu, Z., J. F. Berson, Y. Chen, J. D. Turner, T. Zhang, M. Sharron, M. H. Jenks, Z. Wang, J. Kim, J. Rucker, J. A. Hoxie, S. C. Peiper and R. W. Doms (1997). "Evolution of HIV-1 coreceptor usage through interactions with distinct CCR5 and CXCR4 domains." Proc Natl Acad Sci U S A **94**(12): 6426-31.

Lusso, P. (2006). "HIV and the chemokine system: 10 years later." Embo J **25**(3): 447-56.

Lyles, R. H., A. Munoz, T. E. Yamashita, H. Bazmi, R. Detels, C. R. Rinaldo, J. B. Margolick, J. P. Phair and J. W. Mellors (2000). "Natural history of human immunodeficiency virus type 1 viremia after seroconversion and proximal to AIDS in a large cohort of homosexual men. Multicenter AIDS Cohort Study." J Infect Dis **181**(3): 872-80.

Machy, P., A. Truneh, D. Gennaro and S. Hoffstein (1987). "Major histocompatibility complex class I molecules internalized via coated pits in T lymphocytes." Nature **328**(6132): 724-6.

Mackewicz, C. and J. A. Levy (1992). "CD8+ cell anti-HIV activity: nonlytic suppression of virus replication." AIDS Res Hum Retroviruses **8**(6): 1039-50.

Mackewicz, C. E., H. W. Ortega and J. A. Levy (1991). "CD8+ cell anti-HIV activity correlates with the clinical state of the infected individual." J Clin Invest **87**(4): 1462-6.

Madden, D. R., D. N. Garboczi and D. C. Wiley (1993). "The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2." Cell **75**(4): 693-708.

Mahalanabis, M., P. Jayaraman, T. Miura, F. Pereyra, E. M. Chester, B. Richardson, B. Walker and N. L. Haigwood (2009). "Continuous viral escape and selection by autologous neutralizing antibodies in drug-naive human immunodeficiency virus controllers." J Virol **83**(2): 662-72.

Makadzange, A. T., G. Gillespie, T. Dong, P. Kiama, J. Bwayo, J. Kimani, F. Plummer, P. Easterbrook and S. L. Rowland-Jones (2010). "Characterization of an HLA-C restricted CTL response in chronic HIV infection." Eur J Immunol.

Makedonas, G., P. P. Banerjee, R. Pandey, A. R. Hersperger, K. B. Sanborn, G. A. Hardy, J. S. Orange and M. R. Betts (2009). "Rapid up-regulation and granule-independent transport of perforin to the immunological synapse define a novel mechanism of antigen-specific CD8+ T cell cytotoxic activity." J Immunol **182**(9): 5560-9.

Makedonas, G. and M. R. Betts (2011). "Living in a house of cards: re-evaluating CD8(+) T-cell immune correlates against HIV." Immunol Rev **239**(1): 109-24.

Malim, M. H. and M. Emerman (2001). "HIV-1 sequence variation: drift, shift, and attenuation." Cell **104**(4): 469-72.

Malissen, M., B. Malissen and B. R. Jordan (1982). "Exon/intron organization and complete nucleotide sequence of an HLA gene." Proc Natl Acad Sci U S A **79**(3): 893-7.

Malnati, M. S., M. Peruzzi, K. C. Parker, W. E. Biddison, E. Ciccone, A. Moretta and E. O. Long (1995). "Peptide specificity in the recognition of MHC class I by natural killer cell clones." Science **267**(5200): 1016-8.

Mansky, L. M. and H. M. Temin (1995). "Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase." J Virol **69**(8): 5087-94.

Marcello, A. (2006). "Latency: the hidden HIV-1 challenge." Retrovirology **3**(1): 7.

Margolis, L. and R. Shattock (2006). "Selective transmission of CCR5-utilizing HIV-1: the 'gatekeeper' problem resolved?" Nat Rev Microbiol **4**(4): 312-7.

Marin, R., F. Ruiz-Cabello, S. Pedrinaci, R. Mendez, P. Jimenez, D. E. Geraghty and F. Garrido (2003). "Analysis of HLA-E expression in human tumors." Immunogenetics **54**(11): 767-75.

Marsh, S. G. E., P. Parham and L. D. Barber (2000). The HLA FactsBook, Academic Press.

Martin, M. P., X. Gao, J. H. Lee, G. W. Nelson, R. Detels, J. J. Goedert, S. Buchbinder, K. Hoots, D. Vlahov, J. Trowsdale, M. Wilson, S. J. O'Brien and M. Carrington (2002). "Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS." Nat Genet **31**(4): 429-34.

Martin, M. P., Y. Qi, X. Gao, E. Yamada, J. N. Martin, F. Pereyra, S. Colombo, E. E. Brown, W. L. Shupert, J. Phair, J. J. Goedert, S. Buchbinder, G. D. Kirk, A. Telenti, M. Connors, S. J. O'Brien, B. D. Walker, P. Parham, S. G. Deeks, D. W. McVicar and M. Carrington (2007). "Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1." Nat Genet **39**(6): 733-40.

Martinez-Picado, J., J. G. Prado, E. E. Fry, K. Pfafferott, A. Leslie, S. Chetty, C. Thobakgale, I. Honeyborne, H. Crawford, P. Matthews, T. Pillay, C. Rousseau, J. I. Mullins, C. Brander, B. D. Walker, D. I. Stuart, P. Kiepiela and P. Goulder (2006). "Fitness cost of escape mutations in p24 Gag in association

with control of human immunodeficiency virus type 1." J Virol **80**(7): 3617-23.

Martinez, V., D. Costagliola, O. Bonduelle, N. N'go, A. Schnuriger, I. Theodorou, J. P. Clauvel, D. Sicard, H. Agut, P. Debre, C. Rouzioux and B. Autran (2005). "Combination of HIV-1-specific CD4 Th1 cell responses and IgG2 antibodies is the best predictor for persistence of long-term nonprogression." J Infect Dis **191**(12): 2053-63.

Martinson, J. J., N. H. Chapman, D. C. Rees, Y. T. Liu and J. B. Clegg (1997). "Global distribution of the CCR5 gene 32-basepair deletion." Nat Genet **16**(1): 100-3.

Marusic-Galesic, S. and K. Pavelic (1990). "Dynamics of positive and negative selection in the thymus: review and hypothesis." Immunol Lett **24**(3): 149-54.

Marusic-Galesic, S., D. A. Stephany, D. L. Longo and A. M. Kruisbeek (1988). "Development of CD4-CD8+ cytotoxic T cells requires interactions with class I MHC determinants." Nature **333**(6169): 180-3.

Mascola, J. R. and D. C. Montefiori (2003). "HIV-1: nature's master of disguise." Nat Med **9**(4): 393-4.

Mascola, J. R., G. Stiegler, T. C. VanCott, H. Katinger, C. B. Carpenter, C. E. Hanson, H. Beary, D. Hayes, S. S. Frankel, D. L. Birx and M. G. Lewis (2000). "Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies." Nat Med **6**(2): 207-10.

Masemola, A., T. Mashishi, G. Khoury, P. Mohube, P. Mokgotho, E. Vardas, M. Colvin, L. Zijenah, D. Katzenstein, R. Musonda, S. Allen, N. Kumwenda, T. Taha, G. Gray, J. McIntyre, S. A. Karim, H. W. Sheppard and C. M. Gray (2004). "Hierarchical targeting of subtype C human immunodeficiency virus type 1 proteins by CD8+ T cells: correlation with viral load." J Virol **78**(7): 3233-43.

Matano, T., R. Shibata, C. Siemon, M. Connors, H. C. Lane and M. A. Martin (1998). "Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques." J Virol **72**(1): 164-9.

Mattapallil, J. J., D. C. Douek, B. Hill, Y. Nishimura, M. Martin and M. Roederer (2005). "Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection." Nature **434**(7037): 1093-7.

Matthews, P. C., E. Adland, J. Listgarten, A. Leslie, N. Mkhwanazi, J. M. Carlson, M. Harndahl, A. Stryhn, R. P. Payne, A. Ogwu, K. H. Huang, J. Frater, P. Paioni, H. Kloverpris, P. Jooste, D. Goedhals, C. van Vuuren, D. Steyn, L. Riddell, F. Chen, G. Luzzi, T. Balachandran, T. Ndung'u, S. Buus, M. Carrington, R. Shapiro, D. Heckerman and P. J. Goulder (2011). "HLA-A*7401-Mediated Control of HIV Viremia Is Independent of Its Linkage Disequilibrium with HLA-B*5703." J Immunol **186**(10): 5675-86.

Matthews, P. C., A. Prendergast, A. Leslie, H. Crawford, R. Payne, C. Rousseau, M. Rolland, I. Honeyborne, J. Carlson, C. Kadie, C. Brander, K. Bishop, N. Mlotshwa, J. I. Mullins, H. Coovadia, T. Ndung'u, B. D. Walker, D. Heckerman and P. J. Goulder (2008). "Central role of reverting mutations in HLA associations with human immunodeficiency virus set point." J Virol **82**(17): 8548-59.

McCune, J. M., R. E. Humphreys, R. R. Yocum and J. L. Strominger (1975). "Enhanced representation of HL-A antigens on human lymphocytes after

mitogenesis induced by phytohemagglutinin or Epstein-Barr virus." Proc Natl Acad Sci U S A **72**(8): 3206-9.

McCutcheon, J. A., J. Gumperz, K. D. Smith, C. T. Lutz and P. Parham (1995). "Low HLA-C expression at cell surfaces correlates with increased turnover of heavy chain mRNA." J Exp Med **181**(6): 2085-95.

McKeating, J. A., J. Gow, J. Goudsmit, L. H. Pearl, C. Mulder and R. A. Weiss (1989). "Characterization of HIV-1 neutralization escape mutants." Aids **3**(12): 777-84.

McMichael, A. J., P. Borrow, G. D. Tomaras, N. Goonetilleke and B. F. Haynes (2010). "The immune response during acute HIV-1 infection: clues for vaccine development." Nat Rev Immunol **10**(1): 11-23.

McMichael, A. J. and R. E. Phillips (1997). "Escape of human immunodeficiency virus from immune control." Annu Rev Immunol **15**: 271-96.

McMichael, A. J. and S. L. Rowland-Jones (2001). "Cellular immune responses to HIV." Nature **410**(6831): 980-7.

McMichael, A. J., A. Ting, H. J. Zweerink and B. A. Askonas (1977). "HLA restriction of cell-mediated lysis of influenza virus-infected human cells." Nature **270**(5637): 524-6.

McNeil, A. J., P. L. Yap, S. M. Gore, R. P. Brettle, M. McColl, R. Wyld, S. Davidson, R. Weightman, A. M. Richardson and J. R. Robertson (1996). "Association of HLA types A1-B8-DR3 and B27 with rapid and slow progression of HIV disease." Qjm **89**(3): 177-85.

Mellors, J. W., J. B. Margolick, J. P. Phair, C. R. Rinaldo, R. Detels, L. P. Jacobson and A. Munoz (2007). "Prognostic value of HIV-1 RNA, CD4 cell count, and CD4 Cell count slope for progression to AIDS and death in untreated HIV-1 infection." Jama **297**(21): 2349-50.

Mellors, J. W., A. Munoz, J. V. Giorgi, J. B. Margolick, C. J. Tassoni, P. Gupta, L. A. Kingsley, J. A. Todd, A. J. Saah, R. Detels, J. P. Phair and C. R. Rinaldo, Jr. (1997). "Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection." Ann Intern Med **126**(12): 946-54.

Mellors, J. W., C. R. Rinaldo, Jr., P. Gupta, R. M. White, J. A. Todd and L. A. Kingsley (1996). "Prognosis in HIV-1 infection predicted by the quantity of virus in plasma." Science **272**(5265): 1167-70.

Menier, C., B. Saez, V. Horejsi, S. Martinozzi, I. Krawice-Radanne, S. Bruel, C. Le Danff, M. Reboul, I. Hilgert, M. Rabreau, M. L. Larrad, M. Pla, E. D. Carosella and N. Rouas-Freiss (2003). "Characterization of monoclonal antibodies recognizing HLA-G or HLA-E: new tools to analyze the expression of nonclassical HLA class I molecules." Hum Immunol **64**(3): 315-26.

Meyer, D., R. M. Single, S. J. Mack, H. A. Erlich and G. Thomson (2006). "Signatures of demographic history and natural selection in the human major histocompatibility complex Loci." Genetics **173**(4): 2121-42.

Migueles, S. A., A. C. Laborico, H. Imamichi, W. L. Shupert, C. Royce, M. McLaughlin, L. Ehler, J. Metcalf, S. Liu, C. W. Hallahan and M. Connors (2003). "The differential ability of HLA B*5701+ long-term nonprogressors and progressors to restrict human immunodeficiency virus replication is not caused by loss of recognition of autologous viral gag sequences." J Virol **77**(12): 6889-98.

Migueles, S. A., A. C. Laborico, W. L. Shupert, M. S. Sabbaghian, R. Rabin, C. W. Hallahan, D. Van Baarle, S. Kostense, F. Miedema, M. McLaughlin, L. Ehler, J. Metcalf, S. Liu and M. Connors (2002). "HIV-specific CD8+ T cell

proliferation is coupled to perforin expression and is maintained in nonprogressors." Nat Immunol **3**(11): 1061-8.

Migueles, S. A., C. M. Osborne, C. Royce, A. A. Compton, R. P. Joshi, K. A. Weeks, J. E. Rood, A. M. Berkley, J. B. Sacha, N. A. Cogliano-Shutta, M. Lloyd, G. Roby, R. Kwan, M. McLaughlin, S. Stallings, C. Rehm, M. A. O'Shea, J. Mican, B. Z. Packard, A. Komoriya, S. Palmer, A. P. Wiegand, F. Maldarelli, J. M. Coffin, J. W. Mellors, C. W. Hallahan, D. A. Follman and M. Connors (2008). "Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control." Immunity **29**(6): 1009-21.

Migueles, S. A., M. S. Sabbaghian, W. L. Shupert, M. P. Bettinotti, F. M. Marincola, L. Martino, C. W. Hallahan, S. M. Selig, D. Schwartz, J. Sullivan and M. Connors (2000). "HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors." Proc Natl Acad Sci U S A **97**(6): 2709-14.

Miller, C. J., Q. Li, K. Abel, E. Y. Kim, Z. M. Ma, S. Wietgrefe, L. La Franco-Scheuch, L. Compton, L. Duan, M. D. Shore, M. Zupancic, M. Busch, J. Carlis, S. Wolinsky and A. T. Haase (2005). "Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus." J Virol **79**(14): 9217-27.

Miller, J. F. and G. F. Mitchell (1968). "Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes." J Exp Med **128**(4): 801-20.

Mitchell, G. F. and J. F. Miller (1968). "Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes." J Exp Med **128**(4): 821-37.

Miura, T., C. J. Brumme, M. A. Brockman, Z. L. Brumme, F. Pereyra, B. L. Block, A. Trocha, M. John, S. Mallal, P. R. Harrigan and B. D. Walker (2009). "HLA-associated viral mutations are common in human immunodeficiency virus type 1 elite controllers." J Virol **83**(7): 3407-12.

Miura, T., Z. L. Brumme, M. A. Brockman, P. Rosato, J. Sela, C. J. Brumme, F. Pereyra, D. E. Kaufmann, A. Trocha, B. L. Block, E. S. Daar, E. Connick, H. Jessen, A. D. Kelleher, E. Rosenberg, M. Markowitz, K. Schafer, F. Vaida, A. Iwamoto, S. Little and B. D. Walker (2010). "Impaired replication capacity of acute/early viruses in persons who become HIV controllers." J Virol **84**(15): 7581-91.

Mizuno, S., S. H. Kang, H. W. Lee, J. A. Trapani, B. Dupont and S. Y. Yang (1989). "Isolation and expression of a cDNA clone encoding HLA-Cw6: unique characteristics of HLA-C encoded gene products." Immunogenetics **29**(5): 323-30.

Mkhwanazi, N., C. F. Thobakgale, M. van der Stok, S. Reddy, Z. Mncube, F. Chonco, B. D. Walker, M. Altfeld, P. J. Goulder and T. Ndung'u (2010). "Immunodominant HIV-1-specific HLA-B- and HLA-C-restricted CD8+ T cells do not differ in polyfunctionality." Virology **405**(2): 483-91.

Montefiori, D. C., G. Pantaleo, L. M. Fink, J. T. Zhou, J. Y. Zhou, M. Bilska, G. D. Miralles and A. S. Fauci (1996). "Neutralizing and infection-enhancing antibody responses to human immunodeficiency virus type 1 in long-term nonprogressors." J Infect Dis **173**(1): 60-7.

Moore, C. B., M. John, I. R. James, F. T. Christiansen, C. S. Witt and S. A. Mallal (2002). "Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level." Science **296**(5572): 1439-43.

Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin and R. L. Coffman (1986). "Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins." J Immunol **136**(7): 2348-57.

Mosmann, T. R. and R. L. Coffman (1989). "TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties." Annu Rev Immunol **7**: 145-73.

Moss, P. A., S. L. Rowland-Jones, P. M. Frodsham, S. McAdam, P. Giangrande, A. J. McMichael and J. I. Bell (1995). "Persistent high frequency of human immunodeficiency virus-specific cytotoxic T cells in peripheral blood of infected donors." Proc Natl Acad Sci U S A **92**(13): 5773-7.

Mungall, A. J., S. A. Palmer, S. K. Sims, C. A. Edwards, J. L. Ashurst, L. Wilming, M. C. Jones, R. Horton, S. E. Hunt, C. E. Scott, J. G. Gilbert, M. E. Clamp, G. Bethel, S. Milne, R. Ainscough, J. P. Almeida, K. D. Ambrose, T. D. Andrews, R. I. Ashwell, A. K. Babbage, C. L. Bagguley, J. Bailey, R. Banerjee, D. J. Barker, K. F. Barlow, K. Bates, D. M. Beare, H. Beasley, O. Beasley, C. P. Bird, S. Blakey, S. Bray-Allen, J. Brook, A. J. Brown, J. Y. Brown, D. C. Burford, W. Burrill, J. Burton, C. Carder, N. P. Carter, J. C. Chapman, S. Y. Clark, G. Clark, C. M. Clee, S. Clegg, V. Cobley, R. E. Collier, J. E. Collins, L. K. Colman, N. R. Corby, G. J. Coville, K. M. Culley, P. Dhami, J. Davies, M. Dunn, M. E. Earthrowl, A. E. Ellington, K. A. Evans, L. Faulkner, M. D. Francis, A. Frankish, J. Frankland, L. French, P. Garner, J. Garnett, M. J. Ghori, L. M. Gilby, C. J. Gillson, R. J. Glithero, D. V. Grafham, M. Grant, S. Gribble, C. Griffiths, M. Griffiths, R. Hall, K. S. Halls, S. Hammond, J. L. Harley, E. A. Hart, P. D. Heath, R. Heathcott, S. J. Holmes, P. J. Howden, K. L. Howe, G. R. Howell, E. Huckle, S. J. Humphray, M. D. Humphries, A. R. Hunt, C. M. Johnson, A. A. Joy, M. Kay, S. J. Keenan, A. M. Kimberley, A. King, G. K. Laird, C. Langford, S. Lawlor, D. A. Leongamornlert, M. Leversha, C. R. Lloyd, D. M. Lloyd, J. E. Loveland, J. Lovell, S. Martin, M. Mashreghi-Mohammadi, G. L. Maslen, L. Matthews, O. T. McCann, S. J. McLaren, K. McLay, A. McMurray, M. J. Moore, J. C. Mullikin, D. Niblett, T. Nickerson, K. L. Novik, K. Oliver, E. K. Overton-Larty, A. Parker, R. Patel, A. V. Pearce, A. I. Peck, B. Phillimore, S. Phillips, R. W. Plumb, K. M. Porter, Y. Ramsey, S. A. Ranby, C. M. Rice, M. T. Ross, S. M. Searle, H. K. Sehra, E. Sheridan, C. D. Skuce, S. Smith, M. Smith, L. Spraggon, S. L. Squares, C. A. Steward, N. Sycamore, G. Tamlyn-Hall, J. Tester, A. J. Theaker, D. W. Thomas, A. Thorpe, A. Tracey, A. Tromans, B. Tubby, M. Wall, J. M. Wallis, A. P. West, S. S. White, S. L. Whitehead, H. Whittaker, A. Wild, D. J. Willey, T. E. Wilmer, J. M. Wood, P. W. Wray, J. C. Wyatt, L. Young, R. M. Younger, D. R. Bentley, A. Coulson, R. Durbin, T. Hubbard, J. E. Sulston, I. Dunham, J. Rogers and S. Beck (2003). "The DNA sequence and analysis of human chromosome 6." Nature **425**(6960): 805-11.

Nattermann, J., H. D. Nischalke, V. Hofmeister, B. Kupfer, G. Ahlenstiel, G. Feldmann, J. Rockstroh, E. H. Weiss, T. Sauerbruch and U. Spengler (2005). "HIV-1 infection leads to increased HLA-E expression resulting in impaired function of natural killer cells." Antivir Ther **10**(1): 95-107.

Navis, M., I. M. Schellens, P. van Swieten, J. A. Borghans, F. Miedema, N. A. Kootstra, D. van Baarle and H. Schuitemaker (2008). "A nonprogressive clinical course in HIV-infected individuals expressing human leukocyte antigen B57/5801 is associated with preserved CD8+ T lymphocyte responsiveness to the HW9 epitope in Nef." J Infect Dis **197**(6): 871-9.

Ndongala, M. L., Y. Peretz, S. Boulet, M. Doroudchi, B. Yassine-Diab, M. R. Boulassel, D. Rouleau, C. Tremblay, R. LeBlanc, J. P. Routy, R. P. Sekaly and N. F. Bernard (2009). "HIV Gag p24 specific responses secreting IFN-gamma and/or IL-2 in treatment-naive individuals in acute infection early disease (AIED) are associated with low viral load." Clin Immunol **131**(2): 277-87.

Neefjes, J. J. and H. L. Ploegh (1988). "Allele and locus-specific differences in cell surface expression and the association of HLA class I heavy chain with beta 2-microglobulin: differential effects of inhibition of glycosylation on class I subunit association." Eur J Immunol **18**(5): 801-10.

Nehete, P. N., D. E. Lewis, D. N. Tang, M. S. Pollack and K. J. Sastry (1998). "Presence of HLA-C-restricted cytotoxic T-lymphocyte responses in long-term nonprogressors infected with human immunodeficiency virus." Viral Immunol **11**(3): 119-29.

Neil, S. J., T. Zang and P. D. Bieniasz (2008). "Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu." Nature **451**(7177): 425-30.

Neisig, A., C. J. Melief and J. Neefjes (1998). "Reduced cell surface expression of HLA-C molecules correlates with restricted peptide binding and stable TAP interaction." J Immunol **160**(1): 171-9.

Ngumbela, K. C., C. L. Day, Z. Mncube, K. Nair, D. Ramduth, C. Thobakgale, E. Moodley, S. Reddy, C. de Pierres, N. Mkhwanazi, K. Bishop, M. van der Stok, N. Ismail, I. Honeyborne, H. Crawford, D. G. Kavanagh, C. Rousseau, D. Nickle, J. Mullins, D. Heckerman, B. Korber, H. Coovadia, P. Kiepiela, P. J. Goulder and B. D. Walker (2008). "Targeting of a CD8 T cell env epitope presented by HLA-B*5802 is associated with markers of HIV disease progression and lack of selection pressure." AIDS Res Hum Retroviruses **24**(1): 72-82.

Nixon, D. F., A. R. Townsend, J. G. Elvin, C. R. Rizza, J. Gallwey and A. J. McMichael (1988). "HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides." Nature **336**(6198): 484-7.

Norris, P. J., H. F. Moffett, O. O. Yang, D. E. Kaufmann, M. J. Clark, M. M. Addo and E. S. Rosenberg (2004). "Beyond help: direct effector functions of human immunodeficiency virus type 1-specific CD4(+) T cells." J Virol **78**(16): 8844-51.

Novitsky, V., P. Gilbert, T. Peter, M. F. McLane, S. Gaolekwe, N. Rybak, I. Thior, T. Ndung'u, R. Marlink, T. H. Lee and M. Essex (2003). "Association between virus-specific T-cell responses and plasma viral load in human immunodeficiency virus type 1 subtype C infection." J Virol **77**(2): 882-90.

O'Brien, S. J., X. Gao and M. Carrington (2001). "HLA and AIDS: a cautionary tale." Trends Mol Med **7**(9): 379-81.

O'Doherty, U., W. J. Swiggard and M. H. Malim (2000). "Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding." J Virol **74**(21): 10074-80.

Olerup, O. and H. Zetterquist (1992). "HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological

DR typing in clinical practice including donor-recipient matching in cadaveric transplantation." Tissue Antigens **39**(5): 225-35.

Pacasova, R., S. Martinozzi, H. J. Boulouis, Y. Szpak, M. Ulbrecht, F. Sigaux, E. H. Weiss and M. Pla (1999). "Cell surface detection of HLA-E gene products with a specific monoclonal antibody." J Reprod Immunol **43**(2): 195-201.

Palmisano, G. L., E. Contardi, A. Morabito, V. Gargaglione, G. B. Ferrara and M. P. Pistillo (2005). "HLA-E surface expression is independent of the availability of HLA class I signal sequence-derived peptides in human tumor cell lines." Hum Immunol **66**(1): 1-12.

Papagno, L., C. A. Spina, A. Marchant, M. Salio, N. Rufer, S. Little, T. Dong, G. Chesney, A. Waters, P. Easterbrook, P. R. Dunbar, D. Shepherd, V. Cerundolo, V. Emery, P. Griffiths, C. Conlon, A. J. McMichael, D. D. Richman, S. L. Rowland-Jones and V. Appay (2004). "Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection." PLoS Biol **2**(2): E20.

Parham, P. (2005). "MHC class I molecules and KIRs in human history, health and survival." Nat Rev Immunol **5**(3): 201-14.

Parham, P., C. J. Barnstable and W. F. Bodmer (1979). "Use of a monoclonal antibody (W6/32) in structural studies of HLA-A,B,C, antigens." J Immunol **123**(1): 342-9.

Patke, D. S., S. J. Langan, L. M. Carruth, S. M. Keating, B. P. Sabundayo, J. B. Margolick, T. C. Quinn and R. C. Bollinger (2002). "Association of Gag-specific T lymphocyte responses during the early phase of human immunodeficiency virus type 1 infection and lower virus load set point." J Infect Dis **186**(8): 1177-80.

Pei, R., J. H. Lee, N. J. Shih, M. Chen and P. I. Terasaki (2003). "Single human leukocyte antigen flow cytometry beads for accurate identification of human leukocyte antigen antibody specificities." Transplantation **75**(1): 43-9.

Pelak, K., D. B. Goldstein, N. M. Walley, J. Fellay, D. Ge, K. V. Shianna, C. Gumbs, X. Gao, J. M. Maia, K. D. Cronin, S. K. Hussain, M. Carrington, N. L. Michael and A. C. Weintrob (2010). "Host determinants of HIV-1 control in African Americans." J Infect Dis **201**(8): 1141-9.

Perelson, A. S., A. U. Neumann, M. Markowitz, J. M. Leonard and D. D. Ho (1996). "HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time." Science **271**(5255): 1582-6.

Pereyra, F., M. M. Addo, D. E. Kaufmann, Y. Liu, T. Miura, A. Rathod, B. Baker, A. Trocha, R. Rosenberg, E. Mackey, P. Ueda, Z. Lu, D. Cohen, T. Wrin, C. J. Petropoulos, E. S. Rosenberg and B. D. Walker (2008). "Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy." J Infect Dis **197**(4): 563-71.

Pereyra, F., X. Jia, P. J. McLaren, A. Telenti, P. I. de Bakker, B. D. Walker, S. Ripke, C. J. Brumme, S. L. Pulit, M. Carrington, C. M. Kadie, J. M. Carlson, D. Heckerman, R. R. Graham, R. M. Plenge, S. G. Deeks, L. Gianniny, G. Crawford, J. Sullivan, E. Gonzalez, L. Davies, A. Camargo, J. M. Moore, N. Beattie, S. Gupta, A. Crenshaw, N. P. Burtt, C. Guiducci, N. Gupta, X. Gao, Y. Qi, Y. Yuki, A. Piechocka-Trocha, E. Cutrell, R. Rosenberg, K. L. Moss, P. Lemay, J. O'Leary, T. Schaefer, P. Verma, I. Toth, B. Block, B. Baker, A. Rothchild, J. Lian, J. Proudfoot, D. M. Alvino, S. Vine, M. M. Addo, T. M. Allen, M. Altfeld, M. R. Henn, S. Le Gall, H. Streeck, D. W. Haas, D. R. Kuritzkes, G. K. Robbins, R. W. Shafer, R. M. Gulick, C. M. Shikuma, R.

Haubrich, S. Riddler, P. E. Sax, E. S. Daar, H. J. Ribaudo, B. Agan, S. Agarwal, R. L. Ahern, B. L. Allen, S. Altidor, E. L. Altschuler, S. Ambardar, K. Anastos, B. Anderson, V. Anderson, U. Andrady, D. Antoniskis, D. Bangsberg, D. Barbaro, W. Barrie, J. Bartczak, S. Barton, P. Basden, N. Basgoz, S. Bazner, N. C. Bellos, A. M. Benson, J. Berger, N. F. Bernard, A. M. Bernard, C. Birch, S. J. Bodner, R. K. Bolan, E. T. Boudreaux, M. Bradley, J. F. Braun, J. E. Brndjar, S. J. Brown, K. Brown, S. T. Brown, J. Burack, L. M. Bush, V. Cafaro, O. Campbell, J. Campbell, R. H. Carlson, J. K. Carmichael, K. K. Casey, C. Cavacuiti, G. Celestin, S. T. Chambers, N. Chez, L. M. Chirch, P. J. Cimoch, D. Cohen, L. E. Cohn, B. Conway, D. A. Cooper, B. Cornelson, D. T. Cox, M. V. Cristofano, G. Cuchural, Jr., J. L. Czartoski, J. M. Dahman, J. S. Daly, B. T. Davis, K. Davis, S. M. Davod, E. DeJesus, C. A. Dietz, E. Dunham, M. E. Dunn, T. B. Ellerin, J. J. Eron, J. J. Fangman, C. E. Farel, H. Ferlazzo, S. Fidler, A. Fleenor-Ford, R. Frankel, K. A. Freedberg, N. K. French, J. D. Fuchs, J. D. Fuller, J. Gaberman, J. E. Gallant, R. T. Gandhi, E. Garcia, D. Garmon, J. C. Gathe, Jr., C. R. Gaultier, W. Gebre, F. D. Gilman, I. Gilson, P. A. Goepfert, M. S. Gottlieb, C. Goulston, R. K. Groger, T. D. Gurley, S. Haber, R. Hardwicke, W. D. Hardy, P. R. Harrigan, T. N. Hawkins, S. Heath, F. M. Hecht, W. K. Henry, M. Hladek, R. P. Hoffman, J. M. Horton, R. K. Hsu, G. D. Huhn, P. Hunt, M. J. Hupert, M. L. Illeman, H. Jaeger, R. M. Jellinger, M. John, J. A. Johnson, K. L. Johnson, H. Johnson, K. Johnson, J. Joly, W. C. Jordan, C. A. Kauffman, H. Khanlou, R. K. Killian, A. Y. Kim, D. D. Kim, C. A. Kinder, J. T. Kirchner, L. Kogelman, E. M. Kojic, P. T. Korthuis, W. Kurisu, D. S. Kwon, M. LaMar, H. Lampiris, M. Lanzafame, M. M. Lederman, D. M. Lee, J. M. Lee, M. J. Lee, E. T. Lee, J. Lemoine, J. A. Levy, J. M. Llibre, M. A. Liguori, S. J. Little, A. Y. Liu, A. J. Lopez, M. R. Loutfy, D. Loy, D. Y. Mohammed, A. Man, M. K. Mansour, V. C. Marconi, M. Markowitz, R. Marques, J. N. Martin, H. L. Martin, Jr., K. H. Mayer, M. J. McElrath, T. A. McGhee, B. H. McGovern, K. McGowan, D. McIntyre, G. X. McLeod, P. Menezes, G. Mesa, C. E. Metroka, D. Meyer-Olson, A. O. Miller, K. Montgomery, K. C. Mounzer, E. H. Nagami, I. Nagin, R. G. Nahass, M. O. Nelson, C. Nielsen, D. L. Norene, D. H. O'Connor, B. O. Ojikutu, J. Okulicz, O. O. Oladehin, E. C. Oldfield, 3rd, S. A. Olender, M. Ostrowski, W. F. Owen, Jr., E. Pae, J. Parsonnet, A. M. Pavlatos, A. M. Perlmutter, M. N. Pierce, J. M. Pincus, L. Pisani, L. J. Price, L. Proia, R. C. Prokesch, H. C. Pujet, M. Ramgopal, A. Rathod, M. Rausch, J. Ravishankar, F. S. Rhame, C. S. Richards, D. D. Richman, B. Rodes, M. Rodriguez, R. C. Rose, 3rd, E. S. Rosenberg, D. Rosenthal, P. E. Ross, D. S. Rubin, E. Rumbaugh, L. Saenz, M. R. Salvaggio, W. C. Sanchez, V. M. Sanjana, S. Santiago, W. Schmidt, H. Schuitemaker, P. M. Sestak, P. Shalit, W. Shay, V. N. Shirvani, V. I. Silebi, J. M. Sizemore, Jr., P. R. Skolnik, M. Sokol-Anderson, J. M. Sosman, P. Stabile, J. T. Stapleton, S. Starrett, F. Stein, H. J. Stellbrink, F. L. Sterman, V. E. Stone, D. R. Stone, G. Tambussi, R. A. Taplitz, E. M. Tedaldi, A. Telenti, W. Theisen, R. Torres, L. Tosiello, C. Tremblay, M. A. Tribble, P. D. Trinh, A. Tsao, P. Ueda, A. Vaccaro, E. Valadas, T. J. Vanig, I. Vecino, V. M. Vega, W. Veikley, B. H. Wade, C. Walworth, C. Wanidworanun, D. J. Ward, D. A. Warner, R. D. Weber, D. Webster, S. Weis, D. A. Wheeler, D. J. White, E. Wilkins, A. Winston, C. G. Wlodaver, A. van't Wout, D. P. Wright, O. O. Yang, D. L. Yurdin, B. W. Zabukovic, K. C. Zachary, B. Zeeman and M. Zhao (2010). "The major

genetic determinants of HIV-1 control affect HLA class I peptide presentation." Science **330**(6010): 1551-7.

Peruzzi, M., K. C. Parker, E. O. Long and M. S. Malnati (1996). "Peptide sequence requirements for the recognition of HLA-B*2705 by specific natural killer cells." J Immunol **157**(8): 3350-6.

Peterlin, B. M. and D. Trono (2003). "Hide, shield and strike back: how HIV-infected cells avoid immune eradication." Nat Rev Immunol **3**(2): 97-107.

Petravic, J., L. Loh, S. J. Kent and M. P. Davenport (2008). "CD4+ target cell availability determines the dynamics of immune escape and reversion in vivo." J Virol **82**(8): 4091-101.

Petrovski, S., J. Fellay, K. V. Shianna, N. Carpenetti, J. Kumwenda, G. Kamanga, D. D. Kamwendo, N. L. Letvin, A. J. McMichael, B. F. Haynes, M. S. Cohen and D. B. Goldstein (2011). "Common human genetic variants and HIV-1 susceptibility: a genome-wide survey in a homogeneous African population." Aids **25**(4): 513-8.

Peyerl, F. W., D. H. Barouch and N. L. Letvin (2004). "Structural constraints on viral escape from HIV- and SIV-specific cytotoxic T-lymphocytes." Viral Immunol **17**(2): 144-51.

Phair, J., L. Jacobson, R. Detels, C. Rinaldo, A. Saah, L. Schrager and A. Munoz (1992). "Acquired immune deficiency syndrome occurring within 5 years of infection with human immunodeficiency virus type-1: the Multicenter AIDS Cohort Study." J Acquir Immune Defic Syndr **5**(5): 490-6.

Phillips, A. N. (1996). "Reduction of HIV concentration during acute infection: independence from a specific immune response." Science **271**(5248): 497-9.

Phillips, R. E., S. Rowland-Jones, D. F. Nixon, F. M. Gotch, J. P. Edwards, A. O. Ogunlesi, J. G. Elvin, J. A. Rothbard, C. R. Bangham, C. R. Rizza and et al. (1991). "Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition." Nature **354**(6353): 453-9.

Piantadosi, A., D. Panteleeff, C. A. Blish, J. M. Baeten, W. Jaoko, R. S. McClelland and J. Overbaugh (2009). "Breadth of neutralizing antibody response to human immunodeficiency virus type 1 is affected by factors early in infection but does not influence disease progression." J Virol **83**(19): 10269-74.

Pilgrim, A. K., G. Pantaleo, O. J. Cohen, L. M. Fink, J. Y. Zhou, J. T. Zhou, D. P. Bolognesi, A. S. Fauci and D. C. Montefiori (1997). "Neutralizing antibody responses to human immunodeficiency virus type 1 in primary infection and long-term-nonprogressive infection." J Infect Dis **176**(4): 924-32.

Popovic, M., M. G. Sarngadharan, E. Read and R. C. Gallo (1984). "Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS." Science **224**(4648): 497-500.

Prado, J. G., I. Honeyborne, I. Brierley, M. C. Puertas, J. Martinez-Picado and P. J. Goulder (2009). "Functional consequences of human immunodeficiency virus escape from an HLA-B*13-restricted CD8+ T-cell epitope in p1 Gag protein." J Virol **83**(2): 1018-25.

Preston, B. D., B. J. Poiesz and L. A. Loeb (1988). "Fidelity of HIV-1 reverse transcriptase." Science **242**(4882): 1168-71.

Price, D. A., P. J. Goulder, P. Klenerman, A. K. Sewell, P. J. Easterbrook, M. Troop, C. R. Bangham and R. E. Phillips (1997). "Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection." Proc Natl Acad Sci U S A **94**(5): 1890-5.

Price, D. A., A. K. Sewell, T. Dong, R. Tan, P. J. Goulder, S. L. Rowland-Jones and R. E. Phillips (1998). "Antigen-specific release of beta-chemokines by anti-HIV-1 cytotoxic T lymphocytes." Curr Biol **8**(6): 355-8.

Price, P., R. P. Johnson, D. T. Scadden, C. Jassoy, T. Rosenthal, S. Kalams and B. D. Walker (1995). "Cytotoxic CD8+ T lymphocytes reactive with human immunodeficiency virus-1 produce granulocyte/macrophage colony-stimulating factor and variable amounts of interleukins 2, 3, and 4 following stimulation with the cognate epitope." Clin Immunol Immunopathol **74**(1): 100-6.

Probst, H. C., K. Tschannen, A. Gallimore, M. Martinic, M. Basler, T. Dumrese, E. Jones and M. F. van den Broek (2003). "Immunodominance of an antiviral cytotoxic T cell response is shaped by the kinetics of viral protein expression." J Immunol **171**(10): 5415-22.

Prugnolle, F., A. Manica, M. Charpentier, J. F. Guegan, V. Guernier and F. Balloux (2005). "Pathogen-driven selection and worldwide HLA class I diversity." Curr Biol **15**(11): 1022-7.

Qi, Y., M. P. Martin, X. Gao, L. Jacobson, J. J. Goedert, S. Buchbinder, G. D. Kirk, S. J. O'Brien, J. Trowsdale and M. Carrington (2006). "KIR/HLA pleiotropism: protection against both HIV and opportunistic infections." PLoS Pathog **2**(8): e79.

Rambaut, A., D. Posada, K. A. Crandall and E. C. Holmes (2004). "The causes and consequences of HIV evolution." Nat Rev Genet **5**(1): 52-61.

Rammensee, H., J. Bachmann, N. P. Emmerich, O. A. Bachor and S. Stevanovic (1999). "SYFPEITHI: database for MHC ligands and peptide motifs." Immunogenetics **50**(3-4): 213-9.

Reed, L. J. and H. Muench (1938). "A simple method of estimating fifty per cent endpoints." Am J Hyg **27**: 493-497.

Rehr, M., J. Cahenzli, A. Haas, D. A. Price, E. Gostick, M. Huber, U. Karrer and A. Oxenius (2008). "Emergence of polyfunctional CD8+ T cells after prolonged suppression of human immunodeficiency virus replication by antiretroviral therapy." J Virol **82**(7): 3391-404.

Reimann, K. A., K. Tenner-Racz, P. Racz, D. C. Montefiori, Y. Yasutomi, W. Lin, B. J. Ransil and N. L. Letvin (1994). "Immunopathogenic events in acute infection of rhesus monkeys with simian immunodeficiency virus of macaques." J Virol **68**(4): 2362-70.

Richman, D. D., T. Wrin, S. J. Little and C. J. Petropoulos (2003). "Rapid evolution of the neutralizing antibody response to HIV type 1 infection." Proc Natl Acad Sci U S A **100**(7): 4144-9.

Ridge, J. P., F. Di Rosa and P. Matzinger (1998). "A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell." Nature **393**(6684): 474-8.

Riedel, D. J., M. M. Sajadi, C. L. Armstrong, J. S. Cummings, C. Cairo, R. R. Redfield and C. D. Pauza (2009). "Natural viral suppressors of HIV-1 have a unique capacity to maintain gammadelta T cells." Aids **23**(15): 1955-64.

Rinaldo, C., X. L. Huang, Z. F. Fan, M. Ding, L. Beltz, A. Logar, D. Panicali, G. Mazzara, J. Liebmann, M. Cottrill and et al. (1995). "High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors." J Virol **69**(9): 5838-42.

Riviere, Y., M. B. McChesney, F. Porrot, F. Tanneau-Salvadori, P. Sansonetti, O. Lopez, G. Pialoux, V. Feuillie, M. Mollereau, S. Chamaret and et al. (1995). "Gag-specific cytotoxic responses to HIV type 1 are associated with a decreased risk of progression to AIDS-related complex or AIDS." AIDS Res Hum Retroviruses **11**(8): 903-7.

Robinson, J., K. Mistry, H. McWilliam, R. Lopez, P. Parham and S. G. Marsh (2011). "The IMGT/HLA database." Nucleic Acids Res **39**(Database issue): D1171-6.

Rodriguez, B., A. K. Sethi, V. K. Cheruvu, W. Mackay, R. J. Bosch, M. Kitahata, S. L. Boswell, W. C. Mathews, D. R. Bangsberg, J. Martin, C. C. Whalen, S. Sieg, S. Yadavalli, S. G. Deeks and M. M. Lederman (2006). "Predictive value of plasma HIV RNA level on rate of CD4 T-cell decline in untreated HIV infection." Jama **296**(12): 1498-506.

Roederer, M. and R. A. Koup (2003). "Optimized determination of T cell epitope responses." J Immunol Methods **274**(1-2): 221-8.

Rolland, M., D. Heckerman, W. Deng, C. M. Rousseau, H. Coovadia, K. Bishop, P. J. Goulder, B. D. Walker, C. Brander and J. I. Mullins (2008). "Broad and Gag-biased HIV-1 epitope repertoires are associated with lower viral loads." PLoS One **3**(1): e1424.

Rousseau, C. M., M. G. Daniels, J. M. Carlson, C. Kadie, H. Crawford, A. Prendergast, P. Matthews, R. Payne, M. Rolland, D. N. Raugi, B. S. Maust, G. H. Learn, D. C. Nickle, H. Coovadia, T. Ndung'u, N. Frahm, C. Brander, B. D. Walker, P. J. Goulder, T. Bhattacharya, D. E. Heckerman, B. T. Korber and J. I. Mullins (2008). "HLA class I-driven evolution of human immunodeficiency virus type 1 subtype c proteome: immune escape and viral load." J Virol **82**(13): 6434-46.

Rowland-Jones, S. L. (1999). "HIV: The deadly passenger in dendritic cells." Curr Biol **9**(7): R248-50.

Sacha, J. B., C. Chung, E. G. Rakasz, S. P. Spencer, A. K. Jonas, A. T. Bean, W. Lee, B. J. Burwitz, J. J. Stephany, J. T. Loffredo, D. B. Allison, S. Adnan, A. Hoji, N. A. Wilson, T. C. Friedrich, J. D. Lifson, O. O. Yang and D. I. Watkins (2007). "Gag-specific CD8+ T lymphocytes recognize infected cells before AIDS-virus integration and viral protein expression." J Immunol **178**(5): 2746-54.

Sacha, J. B., C. Chung, J. Reed, A. K. Jonas, A. T. Bean, S. P. Spencer, W. Lee, L. Vojnov, R. Rudersdorf, T. C. Friedrich, N. A. Wilson, J. D. Lifson and D. I. Watkins (2007). "Pol-specific CD8+ T cells recognize simian immunodeficiency virus-infected cells prior to Nef-mediated major histocompatibility complex class I downregulation." J Virol **81**(21): 11703-12.

Saez-Cirion, A., C. Lacabaratz, O. Lambotte, P. Versmisse, A. Urrutia, F. Boufassa, F. Barre-Sinoussi, J. F. Delfraissy, M. Sinet, G. Pancino and A. Venet (2007). "HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype." Proc Natl Acad Sci U S A **104**(16): 6776-81.

Saez-Cirion, A., S. Y. Shin, P. Versmisse, F. Barre-Sinoussi and G. Pancino (2010). "Ex vivo T cell-based HIV suppression assay to evaluate HIV-specific CD8+ T-cell responses." Nat Protoc **5**(6): 1033-41.

Saez-Cirion, A., M. Sinet, S. Y. Shin, A. Urrutia, P. Versmisse, C. Lacabaratz, F. Boufassa, V. Avettand-Fenoel, C. Rouzioux, J. F. Delfraissy, F. Barre-Sinoussi, O. Lambotte, A. Venet and G. Pancino (2009). "Heterogeneity in

HIV suppression by CD8 T cells from HIV controllers: association with Gag-specific CD8 T cell responses." J Immunol **182**(12): 7828-37.

Safrit, J. T., C. A. Andrews, T. Zhu, D. D. Ho and R. A. Koup (1994). "Characterization of human immunodeficiency virus type 1-specific cytotoxic T lymphocyte clones isolated during acute seroconversion: recognition of autologous virus sequences within a conserved immunodominant epitope." J Exp Med **179**(2): 463-72.

Salazar-Gonzalez, J. F., M. G. Salazar, B. F. Keele, G. H. Learn, E. E. Giorgi, H. Li, J. M. Decker, S. Wang, J. Baalwa, M. H. Kraus, N. F. Parrish, K. S. Shaw, M. B. Guffey, K. J. Bar, K. L. Davis, C. Ochsenbauer-Jambor, J. C. Kappes, M. S. Saag, M. S. Cohen, J. Mulenga, C. A. Derdeyn, S. Allen, E. Hunter, M. Markowitz, P. Hraber, A. S. Perelson, T. Bhattacharya, B. F. Haynes, B. T. Korber, B. H. Hahn and G. M. Shaw (2009). "Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection." J Exp Med **206**(6): 1273-89.

Samson, M., F. Libert, B. J. Doranz, J. Rucker, C. Liesnard, C. M. Farber, S. Saragosti, C. Lapoumeroulie, J. Cognaux, C. Forceille, G. Muyldermans, C. Verhofstede, G. Burtonboy, M. Georges, T. Imai, S. Rana, Y. Yi, R. J. Smyth, R. G. Collman, R. W. Doms, G. Vassart and M. Parmentier (1996). "Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene." Nature **382**(6593): 722-5.

Santiago, M. L., C. M. Rodenburg, S. Kamenya, F. Bibollet-Ruche, F. Gao, E. Bailes, S. Meleth, S. J. Soong, J. M. Kilby, Z. Moldoveanu, B. Fahey, M. N. Muller, A. Ayouba, E. Nerrienet, H. M. McClure, J. L. Heeney, A. E. Pusey, D. A. Collins, C. Boesch, R. W. Wrangham, J. Goodall, P. M. Sharp, G. M. Shaw and B. H. Hahn (2002). "SIVcpz in wild chimpanzees." Science **295**(5554): 465.

Sather, D. N., J. Armann, L. K. Ching, A. Mavrantoni, G. Sellhorn, Z. Caldwell, X. Yu, B. Wood, S. Self, S. Kalams and L. Stamatatos (2009). "Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection." J Virol **83**(2): 757-69.

Schacker, T., S. Little, E. Connick, K. Gebhard, Z. Q. Zhang, J. Krieger, J. Pryor, D. Havlir, J. K. Wong, R. T. Schooley, D. Richman, L. Corey and A. T. Haase (2001). "Productive infection of T cells in lymphoid tissues during primary and early human immunodeficiency virus infection." J Infect Dis **183**(4): 555-62.

Schacker, T. W., J. P. Hughes, T. Shea, R. W. Coombs and L. Corey (1998). "Biological and virologic characteristics of primary HIV infection." Ann Intern Med **128**(8): 613-20.

Schaefer, M. R., M. Williams, D. A. Kulpa, P. K. Blakely, A. Q. Yaffee and K. L. Collins (2008). "A novel trafficking signal within the HLA-C cytoplasmic tail allows regulated expression upon differentiation of macrophages." J Immunol **180**(12): 7804-17.

Schaerli, P., K. Willimann, A. B. Lang, M. Lipp, P. Loetscher and B. Moser (2000). "CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function." J Exp Med **192**(11): 1553-62.

Scheid, J. F., H. Mouquet, N. Feldhahn, M. S. Seaman, K. Velinzon, J. Pietzsch, R. G. Ott, R. M. Anthony, H. Zebroski, A. Hurley, A. Phogat, B. Chakrabarti, Y. Li, M. Connors, F. Pereyra, B. D. Walker, H. Wardemann, D. Ho, R. T. Wyatt, J.

R. Mascola, J. V. Ravetch and M. C. Nussenzweig (2009). "Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals." Nature **458**(7238): 636-40.

Scherer, A., J. Frater, A. Oxenius, J. Agudelo, D. A. Price, H. F. Gunthard, M. Barnardo, L. Perrin, B. Hirschel, R. E. Phillips and A. R. McLean (2004). "From the Cover: Quantifiable cytotoxic T lymphocyte responses and HLA-related risk of progression to AIDS." Proc Natl Acad Sci U S A **101**(33): 12266-70.

Schmid, B. V., C. Kesmir and R. J. de Boer (2009). "The distribution of CTL epitopes in HIV-1 appears to be random, and similar to that of other proteomes." BMC Evol Biol **9**: 184.

Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin and K. A. Reimann (1999). "Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes." Science **283**(5403): 857-60.

Schmitz, J. E., M. J. Kuroda, S. Santra, M. A. Simon, M. A. Lifton, W. Lin, R. Khunkhun, M. Piatak, J. D. Lifson, G. Grosschupff, R. S. Gelman, P. Racz, K. Tenner-Racz, K. A. Mansfield, N. L. Letvin, D. C. Montefiori and K. A. Reimann (2003). "Effect of humoral immune responses on controlling viremia during primary infection of rhesus monkeys with simian immunodeficiency virus." J Virol **77**(3): 2165-73.

Sette, A. and J. Sidney (1999). "Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism." Immunogenetics **50**(3-4): 201-12.

Severino, M. E., N. V. Sipsas, P. T. Nguyen, S. A. Kalams, B. D. Walker, R. P. Johnson and O. O. Yang (2000). "Inhibition of human immunodeficiency virus type 1 replication in primary CD4(+) T lymphocytes, monocytes, and dendritic cells by cytotoxic T lymphocytes." J Virol **74**(14): 6695-9.

Shankar, P., Z. Xu and J. Lieberman (1999). "Viral-specific cytotoxic T lymphocytes lyse human immunodeficiency virus-infected primary T lymphocytes by the granule exocytosis pathway." Blood **94**(9): 3084-93.

Sharp, P. M. and B. H. Hahn (2010). "The evolution of HIV-1 and the origin of AIDS." Philos Trans R Soc Lond B Biol Sci **365**(1552): 2487-94.

Shastri, N., S. Cardinaud, S. R. Schwab, T. Serwold and J. Kunisawa (2005). "All the peptides that fit: the beginning, the middle, and the end of the MHC class I antigen-processing pathway." Immunol Rev **207**: 31-41.

Shattock, R. J. and J. P. Moore (2003). "Inhibiting sexual transmission of HIV-1 infection." Nat Rev Microbiol **1**(1): 25-34.

Shibata, R., T. Igarashi, N. Haigwood, A. Buckler-White, R. Ogert, W. Ross, R. Willey, M. W. Cho and M. A. Martin (1999). "Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys." Nat Med **5**(2): 204-10.

Shimizu, Y. and R. DeMars (1989). "Production of human cells expressing individual transferred HLA-A,-B,-C genes using an HLA-A,-B,-C null human cell line." J Immunol **142**(9): 3320-8.

Shresta, S., C. T. Pham, D. A. Thomas, T. A. Graubert and T. J. Ley (1998). "How do cytotoxic lymphocytes kill their targets?" Curr Opin Immunol **10**(5): 581-7.

Shrestha, S., B. Aissani, W. Song, C. M. Wilson, R. A. Kaslow and J. Tang (2009). "Host genetics and HIV-1 viral load set-point in African-Americans." Aids **23**(6): 673-7.

Sibilio, L., A. Martayan, A. Setini, E. Lo Monaco, E. Tremante, R. H. Butler and P. Giacomini (2008). "A single bottleneck in HLA-C assembly." J Biol Chem **283**(3): 1267-74.

Skrabal, K., S. Saragosti, J. L. Labernardiere, F. Barin, F. Clavel and F. Mammano (2005). "Human immunodeficiency virus type 1 variants isolated from single plasma samples display a wide spectrum of neutralization sensitivity." J Virol **79**(18): 11848-57.

Smith, J. M., R. R. Amara, L. S. Wyatt, D. L. Ellenberger, B. Li, J. G. Herndon, M. Patel, S. Sharma, L. Chennareddi, S. Butera, J. McNicholl, H. M. McClure, B. Moss and H. L. Robinson (2005). "Studies in macaques on cross-clade T cell responses elicited by a DNA/MVA AIDS vaccine, better conservation of CD8 than CD4 T cell responses." AIDS Res Hum Retroviruses **21**(2): 140-4.

Snary, D., C. J. Barnstable, W. F. Bodmer and M. J. Crumpton (1977). "Molecular structure of human histocompatibility antigens: the HLA-C series." Eur J Immunol **7**(8): 580-5.

Sousa, A. E., J. Carneiro, M. Meier-Schellersheim, Z. Grossman and R. M. Victorino (2002). "CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load." J Immunol **169**(6): 3400-6.

Specht, A., A. Telenti, R. Martinez, J. Fellay, E. Bailes, D. T. Evans, M. Carrington, B. H. Hahn, D. B. Goldstein and F. Kirchhoff (2010). "Counteraction of HLA-C-mediated immune control of HIV-1 by Nef." J Virol **84**(14): 7300-11.

Spentzou, A., P. Bergin, D. Gill, H. Cheeseman, A. Ashraf, H. Kaltsidis, M. Cashin-Cox, I. Anjarwalla, A. Steel, C. Higgs, A. Pozniak, A. Piechocka-Trocha, J. Wong, O. Anzala, E. Karita, L. Dally, F. Gotch, B. Walker, J. Gilmour and P. Hayes (2010). "Viral inhibition assay: a CD8 T cell neutralization assay for use in clinical trials of HIV-1 vaccine candidates." J Infect Dis **201**(5): 720-9.

Stam, N. J., H. Spits and H. L. Ploegh (1986). "Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products." J Immunol **137**(7): 2299-306.

Stevens, T. L., A. Bossie, V. M. Sanders, R. Fernandez-Botran, R. L. Coffman, T. R. Mosmann and E. S. Vitetta (1988). "Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells." Nature **334**(6179): 255-8.

Stevenson, M. (2003). "HIV-1 pathogenesis." Nat Med **9**(7): 853-60.

Stewart-Jones, G. B., K. di Gleria, S. Kollnberger, A. J. McMichael, E. Y. Jones and P. Bowness (2005). "Crystal structures and KIR3DL1 recognition of three immunodominant viral peptides complexed to HLA-B*2705." Eur J Immunol **35**(2): 341-51.

Stranger, B. E., M. S. Forrest, A. G. Clark, M. J. Minichiello, S. Deutsch, R. Lyle, S. Hunt, B. Kahl, S. E. Antonarakis, S. Tavare, P. Deloukas and E. T. Dermitzakis (2005). "Genome-wide associations of gene expression variation in humans." PLoS Genet **1**(6): e78.

Streeck, H., Z. L. Brumme, M. Anastario, K. W. Cohen, J. S. Jolin, A. Meier, C. J. Brumme, E. S. Rosenberg, G. Alter, T. M. Allen, B. D. Walker and M. Altfeld (2008). "Antigen load and viral sequence diversification determine the functional profile of HIV-1-specific CD8+ T cells." PLoS Med **5**(5): e100.

Streeck, H., J. S. Jolin, Y. Qi, B. Yassine-Diab, R. C. Johnson, D. S. Kwon, M. M. Addo, C. Brumme, J. P. Routy, S. Little, H. K. Jessen, A. D. Kelleher, F. M. Hecht, R. P. Sekaly, E. S. Rosenberg, B. D. Walker, M. Carrington and M. Altfeld (2009). "Human immunodeficiency virus type 1-specific CD8+ T-cell responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells." J Virol **83**(15): 7641-8.

Streeck, H., M. Lichterfeld, G. Alter, A. Meier, N. Teigen, B. Yassine-Diab, H. K. Sidhu, S. Little, A. Kelleher, J. P. Routy, E. S. Rosenberg, R. P. Sekaly, B. D. Walker and M. Altfeld (2007). "Recognition of a defined region within p24 gag by CD8+ T cells during primary human immunodeficiency virus type 1 infection in individuals expressing protective HLA class I alleles." J Virol **81**(14): 7725-31.

Stremlau, M., C. M. Owens, M. J. Perron, M. Kiessling, P. Autissier and J. Sodroski (2004). "The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys." Nature **427**(6977): 848-53.

Stumptner-Cuvelette, P., S. Morchoisne, M. Dugast, S. Le Gall, G. Raposo, O. Schwartz and P. Benaroch (2001). "HIV-1 Nef impairs MHC class II antigen presentation and surface expression." Proc Natl Acad Sci U S A **98**(21): 12144-9.

Sun, J. C. and M. J. Bevan (2003). "Defective CD8 T cell memory following acute infection without CD4 T cell help." Science **300**(5617): 339-42.

Sun, J. C., M. A. Williams and M. J. Bevan (2004). "CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection." Nat Immunol **5**(9): 927-33.

Tang, J., C. Costello, I. P. Keet, C. Rivers, S. Leblanc, E. Karita, S. Allen and R. A. Kaslow (1999). "HLA class I homozygosity accelerates disease progression in human immunodeficiency virus type 1 infection." AIDS Res Hum Retroviruses **15**(4): 317-24.

Tang, J., R. Malhotra, W. Song, I. Brill, L. Hu, P. K. Farmer, J. Mulenga, S. Allen, E. Hunter and R. A. Kaslow (2010). "Human leukocyte antigens and HIV type 1 viral load in early and chronic infection: predominance of evolving relationships." PLoS One **5**(3): e9629.

Tang, J., W. Shao, Y. J. Yoo, I. Brill, J. Mulenga, S. Allen, E. Hunter and R. A. Kaslow (2008). "Human leukocyte antigen class I genotypes in relation to heterosexual HIV type 1 transmission within discordant couples." J Immunol **181**(4): 2626-35.

Tang, J., S. Tang, E. Lobashevsky, A. D. Myracle, U. Fideli, G. Aldrovandi, S. Allen, R. Musonda and R. A. Kaslow (2002). "Favorable and unfavorable HLA class I alleles and haplotypes in Zambians predominantly infected with clade C human immunodeficiency virus type 1." J Virol **76**(16): 8276-84.

Tenzer, S., E. Wee, A. Burgevin, G. Stewart-Jones, L. Friis, K. Lamberth, C. H. Chang, M. Harndahl, M. Weimershaus, J. Gerstoft, N. Akkad, P. Klenerman, L. Fugger, E. Y. Jones, A. J. McMichael, S. Buus, H. Schild, P. van Endert and A. K. Iversen (2009). "Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance." Nat Immunol **10**(6): 636-46.

Thananchai, H., G. Gillespie, M. P. Martin, A. Bashirova, N. Yawata, M. Yawata, P. Easterbrook, D. W. McVicar, K. Maenaka, P. Parham, M. Carrington, T. Dong and S. Rowland-Jones (2007). "Cutting Edge: Allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B." J Immunol **178**(1): 33-7.

Thananchai, H., T. Makadzange, K. Maenaka, K. Kuroki, Y. Peng, C. Conlon, S. Rowland-Jones and T. Dong (2009). "Reciprocal recognition of an HLA-Cw4-restricted HIV-1 gp120 epitope by CD8+ T cells and NK cells." Aids **23**(2): 189-93.

Thomas, R., R. Apps, Y. Qi, X. Gao, V. Male, C. O'HUigin, G. O'Connor, D. Ge, J. Fellay, J. N. Martin, J. Margolick, J. J. Goedert, S. Buchbinder, G. D. Kirk, M. P. Martin, A. Telenti, S. G. Deeks, B. D. Walker, D. Goldstein, D. W. McVicar, A. Moffett and M. Carrington (2009). "HLA-C cell surface expression and control of HIV/AIDS correlate with a variant upstream of HLA-C." Nat Genet **41**(12): 1290-4.

Tibensky, D., F. Decary and T. L. Delovitch (1988). "HLA-C genes are transcribed in HLA-C blank individuals." Immunogenetics **27**(3): 220-4.

Tokarev, A., M. Skasko, K. Fitzpatrick and J. Guatelli (2009). "Antiviral activity of the interferon-induced cellular protein BST-2/tetherin." AIDS Res Hum Retroviruses **25**(12): 1197-210.

Tomaras, G. D., S. F. Lacey, C. B. McDanal, G. Ferrari, K. J. Weinhold and M. L. Greenberg (2000). "CD8+ T cell-mediated suppressive activity inhibits HIV-1 after virus entry with kinetics indicating effects on virus gene expression." Proc Natl Acad Sci U S A **97**(7): 3503-8.

Tomaras, G. D., N. L. Yates, P. Liu, L. Qin, G. G. Fouda, L. L. Chavez, A. C. Decamp, R. J. Parks, V. C. Ashley, J. T. Lucas, M. Cohen, J. Eron, C. B. Hicks, H. X. Liao, S. G. Self, G. Landucci, D. N. Forthal, K. J. Weinhold, B. F. Keele, B. H. Hahn, M. L. Greenberg, L. Morris, S. S. Karim, W. A. Blattner, D. C. Montefiori, G. M. Shaw, A. S. Perelson and B. F. Haynes (2008). "Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia." J Virol **82**(24): 12449-63.

Tomasec, P., V. M. Braud, C. Rickards, M. B. Powell, B. P. McSharry, S. Gadola, V. Cerundolo, L. K. Borysiewicz, A. J. McMichael and G. W. Wilkinson (2000). "Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40." Science **287**(5455): 1031.

Tomiyama, H., M. Fujiwara, S. Oka and M. Takiguchi (2005). "Cutting Edge: Epitope-dependent effect of Nef-mediated HLA class I down-regulation on ability of HIV-1-specific CTLs to suppress HIV-1 replication." J Immunol **174**(1): 36-40.

Towers, G. J., T. Hatziioannou, S. Cowan, S. P. Goff, J. Luban and P. D. Bieniasz (2003). "Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors." Nat Med **9**(9): 1138-43.

Townsend, A. R., F. M. Gotch and J. Davey (1985). "Cytotoxic T cells recognize fragments of the influenza nucleoprotein." Cell **42**(2): 457-67.

Townsend, A. R., J. Rothbard, F. M. Gotch, G. Bahadur, D. Wraith and A. J. McMichael (1986). "The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides." Cell **44**(6): 959-68.

Trachtenberg, E., T. Bhattacharya, M. Ladner, J. Phair, H. Erlich and S. Wolinsky (2009). "The HLA-B/-C haplotype block contains major determinants for host control of HIV." Genes Immun **10**(8): 673-7.

Trachtenberg, E., B. Korber, C. Sollars, T. B. Kepler, P. T. Hraber, E. Hayes, R. Funkhouser, M. Fugate, J. Theiler, Y. S. Hsu, K. Kunstman, S. Wu, J. Phair,

H. Erlich and S. Wolinsky (2003). "Advantage of rare HLA supertype in HIV disease progression." Nat Med **9**(7): 928-35.

Trkola, A., H. Kuster, P. Rusert, B. Joos, M. Fischer, C. Leemann, A. Manrique, M. Huber, M. Rehr, A. Oxenius, R. Weber, G. Stiegler, B. Vcelar, H. Katinger, L. Aceto and H. F. Gunthard (2005). "Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies." Nat Med **11**(6): 615-22.

Tse, D. B. and B. Pernis (1984). "Spontaneous internalization of Class I major histocompatibility complex molecules in T lymphoid cells." J Exp Med **159**(1): 193-207.

Turnbull, E. L., A. R. Lopes, N. A. Jones, D. Cornforth, P. Newton, D. Aldam, P. Pellegrino, J. Turner, I. Williams, C. M. Wilson, P. A. Goepfert, M. K. Maini and P. Borrow (2006). "HIV-1 epitope-specific CD8+ T cell responses strongly associated with delayed disease progression cross-recognize epitope variants efficiently." J Immunol **176**(10): 6130-46.

Turnbull, E. L., M. Wong, S. Wang, X. Wei, N. A. Jones, K. E. Conrod, D. Aldam, J. Turner, P. Pellegrino, B. F. Keele, I. Williams, G. M. Shaw and P. Borrow (2009). "Kinetics of expansion of epitope-specific T cell responses during primary HIV-1 infection." J Immunol **182**(11): 7131-45.

Turner, S., M. E. Ellexson, H. D. Hickman, D. A. Sidebottom, M. Fernandez-Vina, D. L. Confer and W. H. Hildebrand (1998). "Sequence-based typing provides a new look at HLA-C diversity." J Immunol **161**(3): 1406-13.

UNAIDS (2010). Report on the global AIDS epidemic 2010. (http://www.unaids.org/globalreport/documents/20101123_GlobalReport_full_en.pdf).

Urban, T. J., A. C. Weintrob, J. Fellay, S. Colombo, K. V. Shianna, C. Gumbs, M. Rotger, K. Pelak, K. K. Dang, R. Detels, J. J. Martinson, S. J. O'Brien, N. L. Letvin, A. J. McMichael, B. F. Haynes, M. Carrington, A. Telenti, N. L. Michael and D. B. Goldstein (2009). "CCL3L1 and HIV/AIDS susceptibility." Nat Med **15**(10): 1110-2.

Valentine, L. E., S. M. Piaskowski, E. G. Rakasz, N. L. Henry, N. A. Wilson and D. I. Watkins (2008). "Recognition of escape variants in ELISPOT does not always predict CD8+ T-cell recognition of simian immunodeficiency virus-infected cells expressing the same variant sequences." J Virol **82**(1): 575-81.

van Baalen, C. A., C. Guillon, M. van Baalen, E. J. Verschuren, P. H. Boers, A. D. Osterhaus and R. A. Gruters (2002). "Impact of antigen expression kinetics on the effectiveness of HIV-specific cytotoxic T lymphocytes." Eur J Immunol **32**(9): 2644-52.

van Manen, D., N. A. Kootstra, B. Boeser-Nunnink, M. A. Handulle, A. B. van't Wout and H. Schuitemaker (2009). "Association of HLA-C and HCP5 gene regions with the clinical course of HIV-1 infection." Aids **23**(1): 19-28.

van Manen, D., M. A. Rits, C. Beugeling, K. van Dort, H. Schuitemaker and N. A. Kootstra (2008). "The effect of Trim5 polymorphisms on the clinical course of HIV-1 infection." PLoS Pathog **4**(2): e18.

Veazey, R. S., M. DeMaria, L. V. Chalifoux, D. E. Shvetz, D. R. Pauley, H. L. Knight, M. Rosenzweig, R. P. Johnson, R. C. Desrosiers and A. A. Lackner (1998). "Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection." Science **280**(5362): 427-31.

Veazey, R. S., P. J. Klasse, S. M. Schader, Q. Hu, T. J. Ketas, M. Lu, P. A. Marx, J. Dufour, R. J. Colonno, R. J. Shattock, M. S. Springer and J. P. Moore (2005).

"Protection of macaques from vaginal SHIV challenge by vaginally delivered inhibitors of virus-cell fusion." Nature **438**(7064): 99-102.

Veazey, R. S., R. J. Shattock, M. Pope, J. C. Kirijan, J. Jones, Q. Hu, T. Ketas, P. A. Marx, P. J. Klasse, D. R. Burton and J. P. Moore (2003). "Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120." Nat Med **9**(3): 343-6.

Vega, M. A. and J. L. Strominger (1989). "Constitutive endocytosis of HLA class I antigens requires a specific portion of the intracytoplasmic tail that shares structural features with other endocytosed molecules." Proc Natl Acad Sci U S A **86**(8): 2688-92.

Vojnov, L., J. S. Reed, K. L. Weisgrau, E. G. Rakasz, J. T. Loffredo, S. M. Piaskowski, J. B. Sacha, H. L. Kolar, N. A. Wilson, R. P. Johnson and D. I. Watkins (2010). "Effective simian immunodeficiency virus-specific CD8+ T cells lack an easily detectable, shared characteristic." J Virol **84**(2): 753-64.

Vyas, J. M., A. G. Van der Veen and H. L. Ploegh (2008). "The known unknowns of antigen processing and presentation." Nat Rev Immunol **8**(8): 607-18.

Wagner, L., O. O. Yang, E. A. Garcia-Zepeda, Y. Ge, S. A. Kalams, B. D. Walker, M. S. Pasternack and A. D. Luster (1998). "Beta-chemokines are released from HIV-1-specific cytolytic T-cell granules complexed to proteoglycans." Nature **391**(6670): 908-11.

Walker, B. D., S. Chakrabarti, B. Moss, T. J. Paradis, T. Flynn, A. G. Durno, R. S. Blumberg, J. C. Kaplan, M. S. Hirsch and R. T. Schooley (1987). "HIV-specific cytotoxic T lymphocytes in seropositive individuals." Nature **328**(6128): 345-8.

Walker, C. M., A. L. Erickson, F. C. Hsueh and J. A. Levy (1991). "Inhibition of human immunodeficiency virus replication in acutely infected CD4+ cells by CD8+ cells involves a noncytotoxic mechanism." J Virol **65**(11): 5921-7.

Walker, C. M., D. J. Moody, D. P. Stites and J. A. Levy (1986). "CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication." Science **234**(4783): 1563-6.

Wang, Y. E., B. Li, J. M. Carlson, H. Streeck, A. D. Gladden, R. Goodman, A. Schneidewind, K. A. Power, I. Toth, N. Frahm, G. Alter, C. Brander, M. Carrington, B. D. Walker, M. Altfeld, D. Heckerman and T. M. Allen (2009). "Protective HLA class I alleles that restrict acute-phase CD8+ T-cell responses are associated with viral escape mutations located in highly conserved regions of human immunodeficiency virus type 1." J Virol **83**(4): 1845-55.

Ward, J. P., M. I. Bonaparte and E. Barker (2004). "HLA-C and HLA-E reduce antibody-dependent natural killer cell-mediated cytotoxicity of HIV-infected primary T cell blasts." Aids **18**(13): 1769-79.

Watts, C. (2004). "The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules." Nat Immunol **5**(7): 685-92.

Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong and G. M. Shaw (2003). "Antibody neutralization and escape by HIV-1." Nature **422**(6929): 307-12.

Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn and et al. (1995). "Viral dynamics in human immunodeficiency virus type 1 infection." Nature **373**(6510): 117-22.

Weiss, A. and D. R. Littman (1994). "Signal transduction by lymphocyte antigen receptors." Cell **76**(2): 263-74.

Westendorp, M. O., R. Frank, C. Ochsenbauer, K. Stricker, J. Dhein, H. Walczak, K. M. Debatin and P. H. Krammer (1995). "Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120." Nature **375**(6531): 497-500.

Williams, A. F. (1977). "Differentiation antigens of the lymphocyte cell surface." Contemp Top Mol Immunol **6**: 83-116.

Williams, M., J. F. Roeth, M. R. Kasper, R. I. Fleis, C. G. Przybycin and K. L. Collins (2002). "Direct binding of human immunodeficiency virus type 1 Nef to the major histocompatibility complex class I (MHC-I) cytoplasmic tail disrupts MHC-I trafficking." J Virol **76**(23): 12173-84.

Williams, M. A., A. J. Tyznik and M. J. Bevan (2006). "Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells." Nature **441**(7095): 890-3.

Wright, J. K., Z. L. Brumme, J. M. Carlson, D. Heckerman, C. M. Kadie, C. J. Brumme, B. Wang, E. Losina, T. Miura, F. Chonco, M. van der Stok, Z. Mncube, K. Bishop, P. J. Goulder, B. D. Walker, M. A. Brockman and T. Ndung'u (2010). "Gag-protease-mediated replication capacity in HIV-1 subtype C chronic infection: associations with HLA type and clinical parameters." J Virol **84**(20): 10820-31.

Yang, O. O., S. A. Kalams, M. Rosenzweig, A. Trocha, N. Jones, M. Koziel, B. D. Walker and R. P. Johnson (1996). "Efficient lysis of human immunodeficiency virus type 1-infected cells by cytotoxic T lymphocytes." J Virol **70**(9): 5799-806.

Yang, O. O., S. A. Kalams, A. Trocha, H. Cao, A. Luster, R. P. Johnson and B. D. Walker (1997). "Suppression of human immunodeficiency virus type 1 replication by CD8+ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms." J Virol **71**(4): 3120-8.

Yang, O. O., P. T. Sarkis, A. Ali, J. D. Harlow, C. Brander, S. A. Kalams and B. D. Walker (2003). "Determinant of HIV-1 mutational escape from cytotoxic T lymphocytes." J Exp Med **197**(10): 1365-75.

Yang, O. O., P. T. Sarkis, A. Trocha, S. A. Kalams, R. P. Johnson and B. D. Walker (2003). "Impacts of avidity and specificity on the antiviral efficiency of HIV-1-specific CTL." J Immunol **171**(7): 3718-24.

Yewdell, J. W. and J. R. Bennink (1999). "Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses." Annu Rev Immunol **17**: 51-88.

Yewdell, J. W., E. Reits and J. Neefjes (2003). "Making sense of mass destruction: quantitating MHC class I antigen presentation." Nat Rev Immunol **3**(12): 952-61.

Yu, X., Y. Yu, B. Liu, K. Luo, W. Kong, P. Mao and X. F. Yu (2003). "Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex." Science **302**(5647): 1056-60.

Yu, X. G., M. M. Addo, E. S. Rosenberg, W. R. Rodriguez, P. K. Lee, C. A. Fitzpatrick, M. N. Johnston, D. Strick, P. J. Goulder, B. D. Walker and M. Altfeld (2002). "Consistent patterns in the development and immunodominance of human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T-cell responses following acute HIV-1 infection." J Virol **76**(17): 8690-701.

Yu, X. G., M. Lichterfeld, S. Chetty, K. L. Williams, S. K. Mui, T. Miura, N. Frahm, M. E. Feeney, Y. Tang, F. Pereyra, M. X. Labute, K. Pfafferott, A. Leslie, H. Crawford, R. Allgaier, W. Hildebrand, R. Kaslow, C. Brander, T. M. Allen, E. S. Rosenberg, P. Kiepiela, M. Vajpayee, P. A. Goepfert, M. Altfeld, P. J. Goulder and B. D. Walker (2007). "Mutually exclusive T-cell receptor induction and differential susceptibility to human immunodeficiency virus type 1 mutational escape associated with a two-amino-acid difference between HLA class I subtypes." J Virol **81**(4): 1619-31.

Yu, X. G., M. Lichterfeld, B. Perkins, E. Kalife, S. Mui, J. Chen, M. Cheng, W. Kang, G. Alter, C. Brander, B. D. Walker and M. Altfeld (2005). "High degree of inter-clade cross-reactivity of HIV-1-specific T cell responses at the single peptide level." Aids **19**(14): 1449-56.

Yusim, K., C. Kesmir, B. Gaschen, M. M. Addo, M. Altfeld, S. Brunak, A. Chigaev, V. Detours and B. T. Korber (2002). "Clustering patterns of cytotoxic T-lymphocyte epitopes in human immunodeficiency virus type 1 (HIV-1) proteins reveal imprints of immune evasion on HIV-1 global variation." J Virol **76**(17): 8757-68.

Zajac, A. J., J. N. Blattman, K. Murali-Krishna, D. J. Sourdive, M. Suresh, J. D. Altman and R. Ahmed (1998). "Viral immune evasion due to persistence of activated T cells without effector function." J Exp Med **188**(12): 2205-13.

Zemmour, J. and P. Parham (1992). "Distinctive polymorphism at the HLA-C locus: implications for the expression of HLA-C." J Exp Med **176**(4): 937-50.

Zhang, D., P. Shankar, Z. Xu, B. Harnisch, G. Chen, C. Lange, S. J. Lee, H. Valdez, M. M. Lederman and J. Lieberman (2003). "Most antiviral CD8 T cells during chronic viral infection do not express high levels of perforin and are not directly cytotoxic." Blood **101**(1): 226-35.

Zhang, Y. J., C. Fracasso, J. R. Fiore, A. Bjorndal, G. Angarano, A. Gringeri and E. M. Fenyo (1997). "Augmented serum neutralizing activity against primary human immunodeficiency virus type 1 (HIV-1) isolates in two groups of HIV-1-infected long-term nonprogressors." J Infect Dis **176**(5): 1180-7.

Zhang, Z., T. Schuler, M. Zupancic, S. Wietgrefe, K. A. Staskus, K. A. Reimann, T. A. Reinhart, M. Rogan, W. Cavert, C. J. Miller, R. S. Veazey, D. Notermans, S. Little, S. A. Danner, D. D. Richman, D. Havlir, J. Wong, H. L. Jordan, T. W. Schacker, P. Racz, K. Tenner-Racz, N. L. Letvin, S. Wolinsky and A. T. Haase (1999). "Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells." Science **286**(5443): 1353-7.

Zhu, P., J. Liu, J. Bess, Jr., E. Chertova, J. D. Lifson, H. Grise, G. A. Ofek, K. A. Taylor and K. H. Roux (2006). "Distribution and three-dimensional structure of AIDS virus envelope spikes." Nature **441**(7095): 847-52.

Zhuang, J., A. E. Jetzt, G. Sun, H. Yu, G. Klarmann, Y. Ron, B. D. Preston and J. P. Dougherty (2002). "Human immunodeficiency virus type 1 recombination: rate, fidelity, and putative hot spots." J Virol **76**(22): 11273-82.

Zinkernagel, R. M. and P. C. Doherty (1974). "Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis." Nature **251**(5475): 547-8.

Zuniga, R., A. Lucchetti, P. Galvan, S. Sanchez, C. Sanchez, A. Hernandez, H. Sanchez, N. Frahm, C. H. Linde, H. S. Hewitt, W. Hildebrand, M. Altfeld, T. M. Allen, B. D. Walker, B. T. Korber, T. Leitner, J. Sanchez and C. Brander (2006). "Relative dominance of Gag p24-specific cytotoxic T lymphocytes is

associated with human immunodeficiency virus control." J Virol **80**(6): 3122-5.

APPENDIX

Appendix I - LANL list of optimal HLA-C restricted peptides

Peptide ID	PEPTIDE SEQUENCE	HIV PROTEIN	HLA RESTRICTION
P790	YFPDWQNYT	Nef	Cw*06
P802	TPQDLNTML	P24	Cw*0802
P825	YVDRFFKTL	P24	Cw*0303
P826	VTDSQYALGI	RT	Cw*08
P845	WASRELERF	P17	Cw*0602
P878	QASQEVKNW	P24	Cw*04, B*53
P883	ISPRTLNAW	P24	Cw*0602
P914	VIPMFSAL	P24	Cw*0102, Cw*01
P915	RAIEAQQHL	Gp160	Cw*0304
P916	AAVDLSHFL	Nef	Cw*0802
P917	HLVWASREL	P17	Cw*0602
P918	VRDQAEHL	Integrase	Cw*1801
P919	CCFHCQVC	Tat	Cw*1203
P920	LYNTVATL	P17	Cw*14
P921	FRDYVDRFF	P24	Cw*1801
P922	AALDLSHFL	Nef	Cw*03
P924	KRQEILDLWVY	Nef	Cw*0701, Cw*0702
P1687	IPIH**YCAPAGFAIL**KCNN	Env	Cw*0102
P3740	SKLMEMGH**HAPWDVNDL**	Vpu	Cw*0102
P3939	RAIEAQQM	Gp160	Cw*0801
P3940	GAFDLSFFL	Nef	Cw*0802
P3941	AKTIIVHL	Gp160	Cw*0602
P3942	KKQEILDLWVY	Nef	Cw*07
P3943	EKAFSPEV	P24	Cw*0602
P3944	RAEQASQEV	P24	Cw*0802
P3945	NSPTRREL	Gag_Pol_TF	Cw*0102
P3946	RAIEAQQHM	Gp160	Cw*0801
P3947	DLNTMLNTV	P24	Cw*08, B*1402
P3948	VRMYSPVSI	P24	Cw*1801
P3949	YLRDQQLLGIWGC	Gp160	Cw*07
P3950	SAEPVPLQL	Rev	Cw*05, Cw*08
P3951	TLRAEQATQD	P24	Cw*0304
P3952	RPGGKKKYKL	P17	Cw*04
P3953	KRYMIKHLV	P17	Cw*0602
P3954	NCSFNISTSI	Gp160	Cw*08
P3955	CKNVSTVQC	Gp160	Cw*0802
P3956	VYYGVPVWKEA	Gp160	Cw*07
P3957	QAISPRTL	P24	Cw*03
P3958	RQDILDLWVY	Nef	Cw*07
P3959	CTNVSTVQC	Gp160	Cw*08
P3960	QYDQIPIEI	Protease	Cw*0401

Peptide ID	PEPTIDE SEQUENCE	HIV PROTEIN	HLA RESTRICTION
P3961	YRLGVGALI	Gp160	Cw*1801
P3962	DILDLWIY	Nef	Cw*0701
P3963	IVTDSQYAL	RT	Cw*0802
P3964	FNCGGEFF	Gp160	Cw*04
P3965	KYRLKHLVW	P17	Cw*04
P4921	RQG**YSPLSLQTL**FPNPRG	Env	Cw*0102
P5095	RQG**YSPLSLQTL**PPNPRE	Env	Cw*0102

For 4 peptides, 18mers containing the optimal peptide (**in bold**) were used in ELISpot assays. This list of best defined CD8 T cell epitopes was downloaded from the LANL database (http://www.hiv.lanl.gov) and was published in 2007 (Frahm, Linde et al. 2007).