Immunodominant CD8+ T cell Responses to HIV-1 Infection – 'The Good and the Bad'

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Thesis Submitted for the Degree of Doctor of Philosophy

Christ Church
Trinity Term 2011

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Acknowledgements

First and foremost I would like to thank my supervisors Prof. Sarah Rowland-Jones and Dr Tao Dong. Their knowledge, advice and support throughout the course of my D.Phil have been invaluable and have enabled me to progress a great deal as a scientist during my time working in their lab. I would also like to acknowledge the vast amount of help I have received from many members of the SRJ/TD lab both past and present.

In particular I would like to thank Srinika Ranasinghe for passing on a wealth of knowledge about cell culture techniques and assays at a time she herself was working hard to complete her D.Phil. I would also like to thank Yannis Hodges-Mameletzis for his help with molecular techniques as well as for his unfailing ability to put a smile on my face. Much thanks go to YanChun Peng who taught me, amongst many things, how to make MHC class I tetramers and looked after me so well on both of my research trips to China. Marie-Eve Blais has been a constant source of advice and encouragement throughout my D.Phil studies and has taught me much about what it means to be a good scientist. I would also like to acknowledge the contribution Julie Glanville has made to this thesis, her phlebotomy skills, technically advice, cell-sitting and scientific discussions have been invaluable.

There are also countless members of the support staff that work at the WIMM who have been of great help during the completion of these studies. I would like to thank the staff in the FACS facility in particular, Craig Waugh for his help with cell sorting. I would also like to thank Katalin de Gleria for her production of numerous peptides as well as Tim Rostron and John Franklin from the HLA typing and DNA sequencing lab.

Many collaborators from outside of the University of Oxford have also helped to make this work possible. I would like to thank Professors Huiping Yan, Keyi Xu and Hao

Wu from Beijing's YouAn hospital for recruiting SM cohort patients as well as allowing me to use their facilities to carry out experiments. Many extremely helpful members of Prof Huiping Yan's lab also deserve my thanks for the hours they gave to assist me in the experiments carried out in Beijing. I would also like to thanks Prof Doug Richman of UCSD for contributing samples from patient A794.

Many scientists I encountered before I started my D.Phil at the University of Oxford have also helped to bring me to the point I find myself now. I would like to thank the members of staff at the University of Edinburgh who were involved in teaching on the biological sciences (immunology) honours course the year I completed it. They gave me a solid base of immunology knowledge that has served me well over the last four years. Special thanks go to Prof Steve Anderton who took me on as a summer student when I didn't know which end of a pipette was up and gave me my first experience of scientific research.

Lastly, I would like to acknowledge my wonderful family and lovely friends for everything they have done over the past four years to always make me believe the end result was possible. Thank you to my dancing girls; Fallyn, Beckie, Lorna, Rodina and Ali for giving me such a fantastic place to escape from the stresses that come with doing a D.Phil. Thank you to Jen and Claire for visiting Oxford, having me to stay in London and always encouraging me along the way. Thank you to Jo for helping me out when I had FPLC emergencies and for being at the end of the corridor when I was having a bad day. Thank you to Kirsty for the tea breaks, for the CD107a assay, for all the discussions about science (amongst other topics) and for being on this journey with me. Finally thank you to my parents, Chris and Julie, who always told me that I could do anything if I just put my mind to it.

Abstract

Many lines of evidence indicate that CD8+ T cells are important in the control of HIV-1 infection and this has led to much vaccine research focused at eliciting virus specific CTL. However to date, the few large-scale trials of HIV-1 vaccines designed to elicit CD8+ T cells have produced disappointing results. This has highlighted our incomplete knowledge of the factors that determine if such cells are capable of viral control. The aim of this thesis is to further characterise qualitative aspects of HIV-1 specific CTL that are associated with both good and bad anti-viral activity.

HIV-1 specific CTL responses were investigated in three ways. Firstly, by longitudinally analysing an immunodominant HLA-B*08 restricted CD8+ T cell response in a single rapid progression patient. Secondly, HLA-B*40 restricted CTL responses to HIV-1 where characterised within a Chinese slow progressor cohort. Lastly, factors that affect the processing and presentation of certain overlapping HIV-1 specific CD8+ T cell epitopes were examined.

The results of these studies reveal that subtle variations in both host and viral proteins can have a substantial impact on virus specific CTL and in turn may impact on the outcome of disease. The generation of HIV-1 specific CD8+ T cells is a complex process affected by many variables including the viral sequence of epitope flanking regions as well as polymorphisms in the proteins involved in antigen processing and presentation. To add a further layer of complexity, it appears that HIV-1 virus specific CTL can modulate their functionality throughout the course of infection. Such factors should therefore be taken into account during HIV-1 vaccine design.

Declaration

I declare that the work presented in this DPhil thesis entitled “Immunodominant CD8+ T cell responses to HIV-1 - The good and the bad” is entirely my own work, except for where the contributions of my collaborators have been clearly acknowledged. No part of my thesis has been submitted for any degree or other qualification in this University or elsewhere.

Trinity Term 2011

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Abbreviations

AIDS	Acquired immunodeficiency Virus
ANN	Artificial neural network
APC	Antigen presenting cell
	Allophycocyanin
ART	Antiretroviral therapy
bp	Base pair
β2M	β2Microglobulin
CA	Capsid
CAF	CD8+ T cell antiviral factor
CDR	Complementarity determining region
CMV	Cytomegalovirus
^{51}Cr	^{51}Chromium
CRF	Circulating recombinant form
CTL	Cytotoxic T lymphocyte
cVL	Cell-associated viral load
CyPA	Cyclophilin A
D	Diversity
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EBV	Epstein Barr Virus
ER	Endoplasmic reticulum
ERAP	ER aminopeptidase
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FP	Fusion protein
FPLC	Fast liquid protein chromatography
GALT	Gut-associated lymphoid tissue
GWAS	Genome-wide association study
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HTLV	Human T-lymphocyte leukaemia virus
IFN	Interferon
IL	Interleukin
IN	Integrase
J	Joining
KIR	Killer immunoglobulin-like receptor
LAP	Leucine aminopeptidase
LD	Linkage disequilibrium
LTNP	Long term non-progressor
LTR	Long terminal repeat
MA	Matrix
MHC	Major histocompatibility complex
MHR	Major homology region
MIP	Macrophage inflammatory protein
NC	Nucleocapsid
NES	Nuclear export signal
NLS	Nuclear localisation signal
NK	Natural killer
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	peridinin chlorophyll protein
PFU	Plaque forming units
PHA	Phytohaemagglutinin
PIC	Pre-integration complex
PLC	Protein loading complex
pMHC	Peptide-MHC
PR	Protease
pVL	Plasma viral load
RAG	Recombinase activating gene
RNA	Ribonucleic acid
RSS	Recombination signal sequence
RRE	Rev response element
RT	Reverse transcriptase
rVV	Recombinant vaccinia virus
SD_{50}	Sensitising dose 50
SFU	Spot forming units
SIV	Simian immunodeficiency virus
SNP	Single nucleotide polymorphism
SU	Surface component
TAP	Transporter associated with
	antigen processing
$TCID_{50}$	50% Tissue culture infective dose
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl
	transferase
TGF	Transforming growth factor
TLR	Toll-like receptor
TM	Transmembrane component
TNF	Tumor necrosis factor
TPP	Tripeptidyl peptidase
V	Variable
VL	Viral load
WHO	World health organisation

Contents

Chapter 1 – Introduction

1.1 The HIV epidemic

The spread of human immunodeficiency virus (HIV), the causative agent behind acquired immunodeficiency syndrome (AIDS), is one of the most devastating and challenging problems currently facing the global health community. According to the world health organisation (WHO) there are at present an estimated 33.3 million people living with HIV worldwide, the vast majority of which live in resource-poor countries. In 2009 alone HIV caused 2.6 million new infections and 1.8 million deaths.

AIDS was first described in 1981 when 4 previously healthy gay men presented with several diseases indicative of immunosuppression, such as *Pneumocystis carinii* pneumonia, extensive mucosal candidiasis, multiple viral infections and Kaposi's sarcoma (Gottlieb, Schroff et al. 1981). Two years later the causative agent behind AIDS was identified as a lentivirus related to Human T-Lymphocyte leukaemia virus (HTLV), later named HIV-1 (Barre-Sinoussi, Chermann et al. 1983; Levy, Hoffman et al. 1984; Popovic, Sarngadharan et al. 1984).

1.2 The genetic diversity of HIV

AIDS results from infection with one of two closely related retroviruses denoted HIV-1 and HIV-2. These pathogens are the product of the zoonotic transmission of lentiviruses from two distinct non-human primate species. HIV-1 originates from the chimpanzee resident simian immunodeficiency virus $(SIV)_{cpz}$ whereas HIV-2 is derived from $SIV_{sm,}$ a virus that infects sooty mangabeys (Holmes 2001). Infection with HIV-2, mainly endemic to West Africa, is characterised by longer AIDS-free survival and slower CD4+ T cell decline as compared to infection with HIV-1 (de Silva, Cotten et al. 2008). HIV-1 has a much more global prevalence and is the causative agent responsible for the majority of worldwide AIDS causes.

HIV-1 can be divided into three groups; major (M), outlier (O) and non-major/non-outlier (N) that are the result of three separate zoonotic transmission events of related retroviruses from chimpanzees into humans (Korber, Muldoon et al. 2000; Keele, Van Heuverswyn et al. 2006).

Group M is responsible for the majority of global infections. Within group M there are nine subtypes or clades that are represented by the letters A-D, F-H, J and K (Robertson, Anderson et al. 2000). Genetic variation between viruses within the same clade is around 15-20% whereas variation between subtypes can be as much as 25-35% depending on the viral subtypes and HIV-1 proteins compared (Korber, Gaschen et al. 2001).

Recombinant viruses also exist and are termed circulating recombinant forms (CRFs). An example of such a virus is CRF02_AG, this was the second CRF to be

defined. It is common in areas of Africa and contains regions that resemble A and G subtypes (Robertson, Anderson et al. 2000).

HIV-1 subtypes are not evenly distributed throughout the world and certain subtypes are more or less prevalent in particular geographical locations as outlined in figure 1.1 Subtype B is the most common subtype in Western Europe and North American whilst subtype C is the most common in southern Africa and India. Consequently subtype C is the most prevalent subtype worldwide (Hemelaar, Gouws et al. 2006).

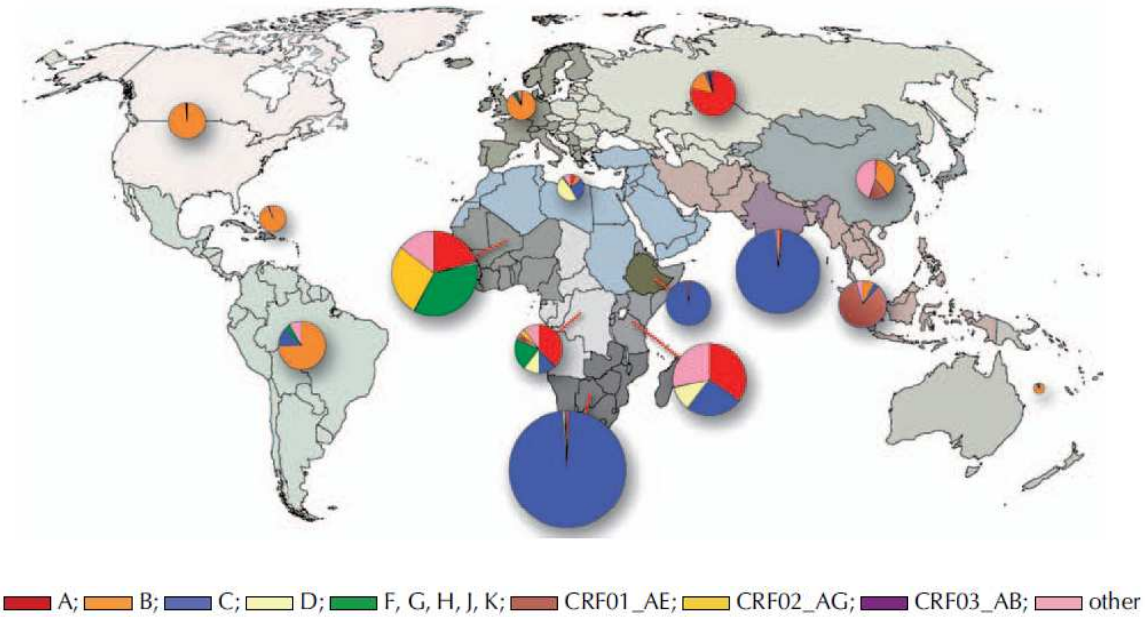


Figure 1.1: The regional distribution of HIV-1 subtypes (Hemelaar, Gouws et al. 2006)

1.3 The organisation of the HIV-1 genome and virion

The genome of HIV-1 consists of two copies of single stranded RNA, 9kb in length. Each strand encodes 9 open reading frames (ORF) which can be translated into 15 proteins (figure 1.2). Six of these are structural proteins comprising the majority of the mature virion and giving it its form, three are enzymes that are necessary for virus replication and a further 6 are accessory proteins that play various essential roles in ensuring the infectivity of the virus.

The *gag* ORF encodes four structural proteins; matrix (MA), capsid (CA), nucleocapsid (NC) and p6. MA lines the inner surface of the host derived plasma membrane that surrounds the virion. CA forms the core of the virus particle, encapsulating the viral genome as well as several viral proteins. NC coats the genomic RNA ensuring delivery to the assembling virion and protecting it from nucleases. Two further structural proteins are encoded by *env*, surface component (SU-gp120) and transmembrane component (TM-gp41). These are arranged at the surface of the virion and are involved in binding and entry of target cells.

The ORF *pol* encodes three enzymes; protease (PR), reverse transcriptase (RT) and integrase (IN). These enzymes have essential roles in the virus replication cycle and along with several accessory proteins can be found within the virus core (Frankel and Young 1998).

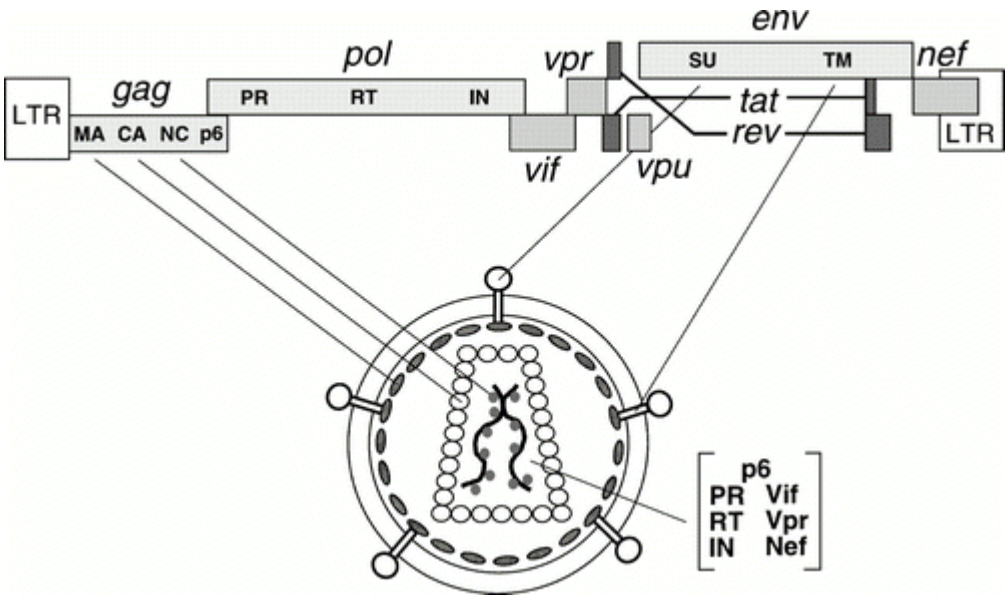


Figure 1.2: Organisation of the HIV-1 genome and virion (Frankel and Young 1998).

1.4 The HIV-1 life cycle

The life cycle of HIV-1 is a multi-step process, as outlined in figure 1.3. HIV-1 gains entry into host cells via the fusion of gp120 with the costimulatory molecule CD4. Gp120 must also bind a coreceptor, most commonly either CCR5 or CXCR4, two chemokine receptors often present on CD4+ cells. Engagement of gp120 with CD4 and a coreceptor leads to a conformational change exposing gp41 which can then facilitate fusion of the viral membrane with the cellular membrane.

Once inside the cell the HIV-1 core disassembles rapidly and reverse transcriptase produces double-stranded DNA from viral RNA. The reverse transcribed viral DNA then enters the nucleus of the host cell via a nuclear pore and becomes integrated into the host genome through the action of the viral enzyme integrase.

Viral RNA is then transcribed from the integrated provirus and transported into the cytoplasm. Here it is translated into viral proteins or transported directly to the cell membrane where viral proteins and RNA assemble to form virions that bud from the cell surface and mature into infectious particles (Lever and Jeang 2006).

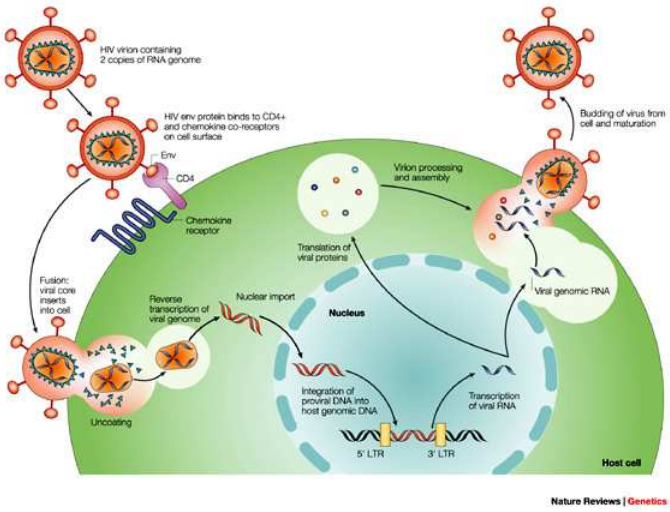


Figure 1.3: The HIV-1 life cycle (Rambaut, Posada et al. 2004)

1.5 HIV-1 proteins

1.5.1 Matrix

MA, also known as p17, is a *gag* encoded structural protein. It consists of five α-helices with the four N-terminal helices forming a large positively charged face that interacts with the phospholipids in the plasma membrane. This allows multimerised trimeric MA molecules to form a protective shell attached to the inner surface of the plasma membrane of the virus.

MA carries out several other important functions during the life cycle of HIV-1. As part of the uncleaved gag polyprotein ($Pr55^{Gag}$) MA directs unspliced viral RNA to the site of virus assembly. Once all the viral components are localised to the plasma membrane MA, still a component of $Pr55^{Gag}$, interacts with the viral RNA and the env proteins in order to coordinate the production of infectious particles (Fiorentini, Marini et al. 2006).

MA has a role to play earlier in the virus life cycle. It contains two nuclear localisation signals (NLS) and is found along with integrase, vpr, RT, NC and viral DNA within the pre-integration complex (PIC). MA plays a nonessential role in ensuring the efficient transport of the PIC into the nucleus of non-dividing cells (Reil, Bukovsky et al. 1998). MA also contains a nuclear export signal (NES) that is required to counteract the NLS thus ensuring the cytoplasmic availability of the components required for virion assembly (Dupont, Sharova et al. 1999).

MA has also been found to share helical topology with IFN-γ (Matthews, Barlow et al. 1994) and synergises with IL-2 to induce a T_H-1 cytokine profile and proliferation in PBMCs acting on NK cells as well as T cells (De Francesco, Caruso et al. 1998; Vitale, Caruso et al. 2003). Proinflammatory cytokines enhance HIV-1 replication within CD4+ target cells suggesting that the homology between MA and IFN-γ may be a viral strategy to boost propagation.

1.5.2 Capsid

In the mature virion, CA (also termed p24) forms a shell around the viral RNA genome and the core-associated proteins. It is made up of two domains, an N-terminal and a C-terminal domain. The C-terminal domain contains a major homology region (MHR) with 4 highly conserved residues Gln155, Gly156, Glu159 and Arg167 that form a stabilising hydrogen bond network (Freed 1998). Mutation of the MHR leads to defects in viral assembly, maturation and infectivity indicating that CA possesses several functions essential for effective viral replication (Mammano, Ohagen et al. 1994).

CA has been shown to be important in the incorporation of gag-pol polypeptide into the virions, this is vital for virus propagation as it ensures that RT, IN and PR are present within the virus particles (Smith, Srinivasakumar et al. 1993).

CA also binds to a host protein called cyclophilin A (CyPA), via a proline residue at position 90 in the N-terminal domain, incorporating the protein into HIV-1 virions (Thali, Bukovsky et al. 1994). The exact role the peptidyl-prolyl isomerase carries out during HIV-1 infection is unclear, however virus that lacks CyPA can infect cells but are

not reverse transcribed (Braaten, Franke et al. 1996). It is therefore hypothesised that it may play a role in viral uncoating.

1.5.3 Nucleocapsid

NC (p7) contains two cysteine rich domains that are structurally and functionally similar to zinc-finger motifs found in many DNA binding proteins (South, Blake et al. 1990). In the mature virion, NC is found within the core closely associated with the viral RNA.

During viral replication NC ensures the efficient incorporation of the viral genome into the virion. The process of encapsidating the viral genome favours un-spliced viral RNA as opposed to spliced viral RNA or cellular RNA. This specificity is lost if the zinc-finger domains of NC are mutated. NC also confers specificity for the encapsulation of the HIV-1 viral genome as opposed to genomes from other retroviruses (Zhang and Barklis 1995). In addition NC facilitates the stabilisation and dimerisation of the viral RNA prior to encapsidation.

NC also plays a role in virus assembly. When gag is truncated at its C-terminal, thus removing NC, assembly is abrogated (Dorfman, Luban et al. 1993). NC has been shown to mediate the tight packing (Bennett, Nelle et al. 1993) as well as the multimerisation (Franke, Yuan et al. 1994) of gag and loss of these functions could be the reason for the reduction in efficient assembly seen in its absence.

NC is involved in the reverse transcription of the viral genome. It ensures effective binding of the tRNA primer and the initiation of the enzymatic process (Freed 1998).

1.5.4 p6

The furthest most C-terminal protein encoded by *gag* is p6, the smallest known lentiviral protein, composed of 52 amino acids. It contains two late assembly (L-) domains that are involved in the efficient release of assembled virions from the cell surface. This is illustrated by the fact that truncation of p6 results in the tethering of virus particles to the plasma membrane of infected cells (Gottlinger, Dorfman et al. 1991; Pornillos, Garrus et al. 2002).

In addition to its involvement in virus release, p6 mediates the incorporation of the accessory protein vpr into virus particles during virion assembly (Freed 1998).

Lastly, p6 contains a conserved serine residue at position 40 that is involved in the cleavage of CA from the gag polyprotein. Mutation of this residue results in defective CA processing and altered morphology of the viral core, consequently leading to reduced infectivity (Votteler, Neumann et al. 2011).

1.5.5 gp120 and gp41

Glycosylated proteins encoded by the HIV-1 *env* gene are located within the host cell derived lipid bilayer that surrounds mature virions. Initially synthesised in the ER as

a polypeptide precursor denoted gp160, cleavage mediated by cellular protease in the golgi body generates the mature env proteins gp120 and gp41. These two molecules assemble into trimers facilitated by interactions between the ectodomain of gp41 and various discontinuous NH_2- and COOH-terminal gp120 structures. Following the addition of complex sugars in the golgi body the env glycoproteins are transported to the cell surface where they are incorporated into budding virions. At the surface of the virion gp120 forms the exterior domain of the env glycoprotein while gp41 comprises the transmembrane domain (Wyatt and Sodroski 1998).

The primary function of the env glycoproteins is to mediate binding of virions to target cells and instigate fusion between the viral and host cell membranes. This process begins with the binding of gp120 to CD4 which induces a conformational change in the viral protein exposing the chemokine receptor binding site (Sattentau and Moore 1991). The binding of gp120 to the coreceptors CCR5 and CXCR4 is dependent on one of 5 variable domains present in gp120 called the V3 domain (Speck, Wehrly et al. 1997). This coreceptor binding initiates a further conformational change that results in the exposure of gp41. The gp41 glycoprotein then facilitates host-viral membrane fusion by inserting its hydrophobic N-terminal fusion peptide (FP) into the lipid bilayer of the target cell.

Due to the exposed nature of the env glycoproteins they are important targets for anti-viral antibodies, however certain features of the molecule help them evade recognition. Although several regions of env are functionally constrained and therefore remain conserved, exposure of these domains to recognition by antibodies is restricted. The moieties involved in gp120-gp41 interactions are buried in the interior of the env

structure (Wyatt, Desjardin et al. 1997). The CD4 binding site is recessed within gp120 and flanked by heavily glycosylated regions (Wyatt, Sullivan et al. 1993). The chemokine receptor binding site is masked by the variable loops (Cao, Sullivan et al. 1997). In addition, as previously alluded to, gp120 is heavily glycosylated and as most carbohydrates are seen as self by the immune system this dramatically reduces the recognition of gp120 by antibodies (Kwong, Wyatt et al. 1998).

1.5.6 Reverse transciptase

RT is an asymmetric heterodimer composed of two subunits, p51 and p66. Whilst p66 is responsible for the enzymatic activity of RT, p51 has a role in maintaining the structure of the molecule.

RT has two enzymatic activities. It can function as a DNA polymerase and an RNase H. These two functions allow RT to convert the single-stranded RNA that comprises the viral genome into double-stranded DNA that can be integrated into the host genome. The DNA polymerase activity of RT functions to use the RNA genome as a template to produce an RNA/DNA duplex that is cleaved by the RNase H. The DNA polymerase then completes the process of creating the double stranded viral DNA that can then be integrated into the host genome (Sarafianos, Marchand et al. 2009).

HIV-1 RT does not possess 3'- 5' proofreading activity (Roberts, Bebenek et al. 1988) and therefore is prone to making errors during both RNA and DNA dependent DNA synthesis (Boyer, Bebenek et al. 1992). This contributes to the vast amount of

genetic diversity displayed by the virus on a global scale as well as aiding the virus in escaping immune recognition within an individual patient.

1.5.7 Integrase

Integration of the viral genome into the host cell DNA is catalysed by IN. IN is a 288-amino acid protein enzyme encoded in *pol* that has three domains; an N-terminal domain, a C-terminal domain and a central catalytic domain. Two reactions are required for the integration of viral DNA into the host genome. Firstly IN binds to the LTRs and catalyses an endonucleotide cleavage in which a dinucleotide is removed from each end of the viral DNA. The second reaction, also catalysed by IN, is a strand-transfer that results in the insertion of the viral DNA into the host genome. As well as mediating the integration of viral DNA into the host genome IN also has certain non-enzymatic activities. For example it is required for the stabilisation and nuclear transport of the PIC (Delelis, Carayon et al. 2008).

1.5.8 Protease

Once the viral genome has been integrated into the host genome viral proteins can be produced. Initially gag and gag-pol polyproteins are generated. These must be cleaved in order to produce the structural proteins (MA, CA, and NC) and the enzymes (RT, IN, PR) which then go on to produce mature virions. This cleavage is carried out by PR, a member of the aspartyl protease family. PR functions as a dimer with the active site

composed of six amino acids (triads AspThrGly found in each monomer) in positions 25 to 27 and 25' to 27' (Mager 2001).

1.5.9 Vpu

Vpu is a membrane-associated phosphoprotein that is localised to the Endoplasmic reticulum (ER) and expressed late in the virus replication cycle. It has several functions that aid in viral infectivity.

Firstly, vpu reduces the amount of CD4 at the cell surface by targeting it for degradation. It achieves this by associating with the cytoplasmic domain of CD4 when it is localised in the ER and initiating the addition of ubiquitin via E3 ubiquitin ligase activity. This has several positive consequences for viral replication, it prevents superinfection as there are fewer receptors at the cell surface for viral entry. It also enhances virion release as CD4 has an inhibitory effect on this process due to its capacity to bind to budding viruses through the env proteins. CD4 downregulation also allows for release of env, bound to such molecules in the ER, to form virions that can then bud from the cell surface (Nomaguchi, Fujita et al. 2008).

Secondly, vpu downregulates major histocompatibility complex (MHC) class I (Kerkau, Bacik et al. 1997) and class II molecules (Hussain, Wesley et al. 2008). These molecules are the means by which CD8+ and CD4+ T cells recognise infected cells, therefore loss of expression leads to the abrogation of immune response towards the virus and in turn results in an increase in viral replication.

Thirdly, vpu is involved in counteracting the activity of a host viral restriction factor called tetherin. Tetherin is a membrane bound protein molecule that causes the retention of fully formed virions at the cell surface. This retention only occurs in the absence of vpu, similarly vpu is only required for virion release from cells that possess tetherin (Neil, Zang et al. 2008).

1.5.10 Nef

Nef, like vpu is responsible for a reduction in the number of MHC class I molecules present on the surface of virally infected cells. Nef does not affect the synthesis of MHC class I molecules or their transport from the ER to the golgi body but rapidly internalises surface molecules facilitating their degradation in endosomal vesicles (Schwartz, Marechal et al. 1996). Nef also blocks transport of MHC class I molecules from the golgi to the cell surface. Interaction between nef and the cytoplasmic tail of MHC class I molecules is required for downregulation to occur (Williams, Roeth et al. 2002).

This downregulation of MHC class I molecules is an immune evasion strategy to reduce the recognition of virally infected cells by cytotoxic T lymphocytes (CTL). Indeed it has been shown that *in vitro* CTL are more efficient at killing target cells infected with a nef defective virus as opposed to a virus possessing a functional nef gene (Collins, Chen et al. 1998).

As a counter strategy to overcome viral downregulation of MHC molecules natural killer (NK) cells possess receptors that relay inhibitory signals when triggered by

MHC class I binding, therefore in the absence of such molecules virally infected cells are susceptible to killing. HIV-1 has in turn overcome this obstacle by selectively downregulating certain MHC class I molecules, HLA-A and HLA-B, but not significantly effecting the expression of others, HLA-C and HLA-E, thus protecting HIV-1 infected cells from NK cell mediated lysis (Cohen, Gandhi et al. 1999).

Similarly to Vpu, nef can also mediate CD4 down-regulation from the cell surface ensuring that viral budding can occur effectively. This process involves an interaction between nef and the cytoplasmic tale of CD4 (Harris and Neil 1994).

1.5.11 Vif

Vif (viral infectivity factor) is required for effective viral replication *in vitro* (Fisher, Ensoli et al. 1987), however the reliance of HIV-1 on vif for efficient propagation was shown to be dependent on the cell line under investigation (Gabuzda, Lawrence et al. 1992). This requirement for vif was found to be due to the presence or absence of the host viral restriction factor APOBEC3G (discussed later) whereby the viral protein is only necessary for replication when APOBEC3G is also present (Sheehy, Gaddis et al. 2002). Vif inhibits the antiviral activity of APOBEC3G by targeting it for degradation by the proteasome (Conticello, Harris et al. 2003).

1.5.12 Vpr

Vpr is a small 14kDa protein with several important functions in the life cycle of HIV-1. Vpr has cytostatic properties that cause infected cells to arrest in the G2 phase of the cell cycle (Jowett, Planelles et al. 1995). The biological significance of this block on cellular replication has proven difficult to determine, however it has been observed that HIV-1 long terminal repeats (LTR) which promote transcription of viral proteins are highly active during the G2 phase (Goh, Rogel et al. 1998). In addition peak HIV-1 translational activity has also been shown to occur in this phase of the cell cycle (Brasey, Lopez-Lastra et al. 2003). Vpr therefore acts to increase the production of HIV-1 proteins through both increased transcription and translation brought about by holding the infected cell in the G2 phase.

Vpr has been shown to facilitate the nuclear import of the viral pre-integration complex (Heinzinger, Bukinsky et al. 1994), this is essential for HIV-1 replication in non-dividing cells where the nuclear membrane remains intact at all times. Vpr exerts this activity through the presence of a NLS. It isn't however the only protein in the PIC to possess such a signalling component, as discussed earlier MA is also involved in the nuclear import of the viral genome. Therefore it is likely that a certain degree of redundancy exists with regards to this function of vpr.

Vpr can transactivate HIV-1 LTRs thus adding to the increase in viral gene transcription brought about by the G2 cell cycle arrest and increasing viral protein production. Vpr also alters the expression of several host genes and has been implicated

in modulating levels of several cytokines and chemokines as well as certain costimulatory molecules (Casey, Wen et al. 2010).

1.5.13 Rev

Rev is required for the expression of the HIV-1 proteins gag, pol and env from the integrated pro-viral DNA. Unspliced RNA encoding gag and gag-pol as well as singly spliced RNA encoding env possess a rev-response element (RRE). When rev binds to the RRE it targets the RNA for nuclear export thus preventing further splicing, enhancing cytoplasmic levels of these RNAs and increasing the expression of the encoded proteins.

Rev has also been shown to inhibit integration of viral DNA into the host genome thus reducing the likelihood of superinfection. The functional relevance of this is as yet unclear (Grewe and Uberla 2010).

1.5.14 Tat

Tat is involved in ensuring the efficient transcription of the integrated HIV-1 genome by inducing epigenetic modification of the LTR promoter, allowing RNA polymerase II to function effectively (Deng, de la Fuente et al. 2000). Tat has also been shown to modulate the expression of certain host genes. For example tat has been found to upregulate the expression levels of several cytokines, the HIV-1 co-receptor CCR5 and the IL-2 receptor. It has also been found to down-regulate certain genes, such as the MHC class I molecules (Romani, Engelbrecht et al. 2010).

1.6 HIV-1 pathogenesis and disease course

HIV-1 infection can be divided into three phases (figure 1.4). The acute or primary phase is characterised by a sharp increases in viral load to peak level quickly followed by a decrease to set point which is preceded by the initiation of the anti-viral immune response. A reduction in the number of CD4+ T cells found in peripheral blood also occurs in the acute phase of infection. This is followed by a rebound in CD4+ T cell numbers bringing them back up to near pre-infection levels. During the chronic asymptomatic phase there is a gradual increase in viral load accompanied by a gradual decrease in CD4+ T cell numbers. The symptomatic phase then occurs when the immune system can no long fight opportunist infections and AIDS ensues (Centlivre, Sala et al. 2007).

It is the reliance of HIV-1 on CD4 and either of the chemokine receptors CCR5 or CXCR4 for the initiation of viral entry into host cells that leads to CD4+ T cells being the predominant cell type infected by the virus. This tropism also allows for the infection of macrophages and dendritic cells (DCs) but it is the progressive depletion and dysfunction of CD4+ T cells occurring throughout the course of HIV-1 infection that gives rise to the inability of the immune system to prevent the opportunistic infections and cancers that comprise the symptoms of AIDS.

Work done utilising the simian model of HIV-1 infection SIV has shown that the reduction in CD4+ T cells seen in the peripheral blood during acute HIV-1/SIV infection is paralleled by a massive reduction in the number of CD4+ T cells that reside in mucosa-associated lymphoid tissue, in particular in the gut-associated lymphoid tissue (GALT)

(Smit-McBride, Mattapallil et al. 1998; Veazey, DeMaria et al. 1998; Vajdy, Veazey et al. 2001; Veazey, Marx et al. 2003).

This depletion of CD4+ T cells from the GALT was also observed in HIV-1 infected patients (Brenchley, Schacker et al. 2004; Mehandru, Poles et al. 2004) and is not simply caused by a down-regulation of CD4 as no increase in the CD3+CD4-CD8- population within the GALT is observed (Veazey, DeMaria et al. 1998). It is also not due to the redistribution of CD4+ T cells to elsewhere within the body as a reduction in such cells is also seen in organised lymph nodes and the peripheral blood (Schneider, Jahn et al. 1995; Clayton, Snow et al. 1997). This disproportionately large reduction in gut-associated CD4+ T cells may be due to the high percentage of memory CD4+ T cells that are found in the gut, such cells express CCR5 as well as being relatively activated (Veazey, Marx et al. 2003; Brenchley, Schacker et al. 2004), presenting HIV-1 with ideal target cells.

The exact mechanisms that lead to the massive depletion of mucosal CD4+ T cells and the gradual decline of peripheral CD4+ T cells during HIV-1 infection are far from fully understood. Infection of CD4+ T cells by HIV-1 can be directly cytopathic (Barre-Sinoussi, Chermann et al. 1983; Levy, Hoffman et al. 1984; Popovic, Sarngadharan et al. 1984) and several HIV-1 proteins have been implicated in mediating infected cell killing. Env proteins can bind to CD4 and coreceptor molecules in the endoplasmic reticulum, such fusogenic domains are then transported to the cell surface where they interfere with the integrity of the cell membrane leading to cell death (LaBonte, Patel et al. 2000). Vpr may also contribute to infected cell killing by activating

caspases and inducing apoptosis (Stewart, Poon et al. 1997). Infected CD4+ T cells can also be depleted via the killing activity of virus-specific CD8+ T cells.

However the number of cells depleted during HIV-1 infection outnumbers those that are infected suggesting that uninfected cells also undergo apoptosis and are depleted (Meyaard, Otto et al. 1992). One mechanism involved in bystander cell killing involves the upregulation of FasL induced by nef. If engagement then occurs between FasL on the infected cell and Fas expressed by an adjacent uninfected cell apoptosis will be triggered, thus leading to the death of the uninfected cell (Xu, Screaton et al. 1997; Geleziunas, Xu et al. 2001).

Depletion of uninfected CD4+ T cells may occur as a consequence of the massive amount of immune activation that occurs during HIV-1 infection (Appay and Sauce 2008). Several observations link this immune disregulation to HIV-1 and SIV pathogenesis. It has been noted that despite comparable viral loads, natural hosts of SIV sooty mangabeys do not progress to immunodeficiency whereas rhesus macaques develop AIDS when infected with SIV. One notable difference between SIV infections in these two non-human primate species is that sooty mangabeys present with a much lower level of immune activation (Silvestri, Sodora et al. 2003). This situation is also paralleled in humans infected with HIV-1 and those who contract the less pathogenic HIV-2, whereby the latter virus is associated with a lower level of immune activation in comparison to the former (Sousa, Carneiro et al. 2002).

These lines evidence suggest that the depletion of bystander CD4+ T cells by mechanisms involving excessive immune activation have an important role in driving HIV-1 and SIV pathogenesis and that viral replication and removal of infected cells alone

may not lead to immunodeficiency. The causes of the disproportionate immune activation observed during HIV-1 infection are not fully delineated but several mechanisms are thought to be involved. Antigenic stimulation of T cells by the virus throughout the course of infection contributes to the inflammatory environment. Certain HIV-1 gene products can directly act on lymphocytes and macrophages to induce the production of proinflammatory cytokines and chemokines (Merrill, Koyanagi et al. 1989; Simmons, Aluvihare et al. 2001). The rebound of other viral infections such as Epstein Barr virus (EBV) and cytomegalovirus (CMV) that occurs due to immunodeficiency caused by HIV-1 can also contribute to immune activation (Doisne, Urrutia et al. 2004). Microbial translocation caused by the disruption of the mucosal barrier due to the depletion of gut-associated immune cells has also been implicated contributing to the proinflammatory environment observed during HIV-1 infection (Brenchley, Price et al. 2006).

As well as the reduction in CD4+ T cells by the mechanisms described above the situation is compounded by the fact that during HIV-1 infection there is a deregulation of haematopoiesis resulting in a lower number of progenitor cells and a decrease in their ability to produce new cells (Moses, Nelson et al. 1998). There is also a decline in thymic output thought to be caused by direct infection of thymocytes (Schnittman, Denning et al. 1990) as well as the proinflammatory induction of direct thymosuppression and fibrosis in lymphatic tissue (Schacker, Nguyen et al. 2002).

In conclusion many mechanisms are at play during HIV-1 infection that cause the depletion of infected and uninfected CD4+ T cells as well as reducing the immune systems capacity to replace such cells eventually resulting in an inability to fight opportunistic infections and causing AIDS.

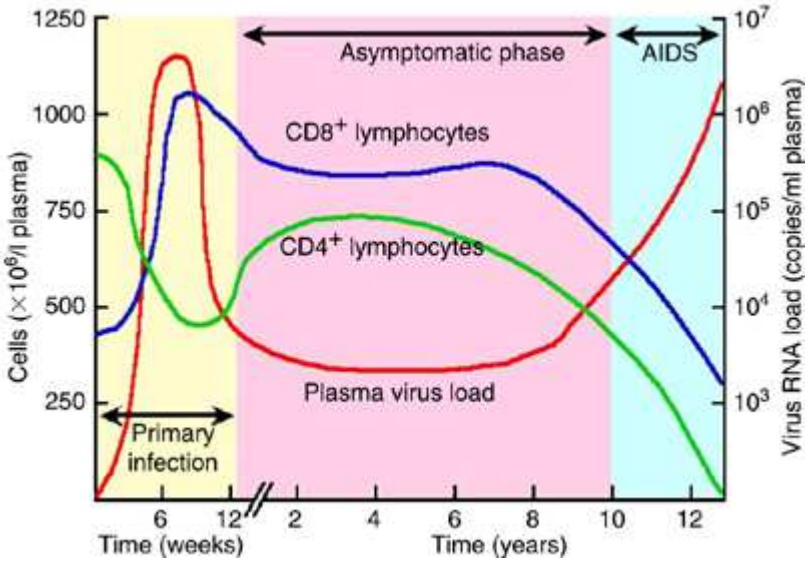


Figure 1.4: The disease course of HIV-1 (Munier and Kelleher 2007).

1.7 Heterogeneity in HIV-1 disease progression

The time between acquisition of HIV-1 and the onset of AIDS varies greatly from person to person. In the absence of ART, typical progressors acquire AIDS following around 6-8 years of infection (Pantaleo and Fauci 1996). Around 10% of those infected with HIV-1 progress to AIDS in under 3 years, these individuals are termed rapid progressors (Phair 1994). There are also rare individuals that are capable of controlling HIV-1 replication and remain AIDS free for many years. These patients have become the focus of intense research in the hope that such control can be mimicked by a vaccine.

HIV-1 controllers can be defined in several ways. About 1-5% of infected individuals fall under the definition of a long term non-progressor (LTNP), maintaining a CD4+ T cell count above 500 cells/mm^3 in the absence of anti-retroviral therapy (ART) for 10 years or more (Easterbrook 1999). Individuals that control HIV-1 can also be categorised on the basis of viral load. Elite control of HIV-1 occurs in around 1 out of every 300 infected individuals and is defined as the maintenance of viral load below 50 copies/ml in the absence of therapy for a year a more. Viremic controllers are those that maintain a viral load of between 50 and 2000 copies/ml for a year or more without therapy (Walker 2007). Patients identified by the HIV controller consortium that satisfy these criteria have a median duration of infection of 12 years suggesting that elite controllers and LTNPs are overlapping groups of patients.

1.8 The host anti-HIV-1 immune response

Despite the fact that infection with HIV-1 almost inevitably leads to AIDS, the immune system manages to control viral replication to some extent in practically all who become infected. As discussed above certain individuals are better than others at controlling HIV-1 and several arms of the immune system have been implicated in this control.

1.8.1 Innate immune responses to HIV-1

DCs are among the first cells to come into contact with HIV-1 following mucosal transmission. As well as playing a role in the adaptive immune response through their function as antigen presenting cells (APCs) DCs can have a more direct and innate anti-viral effect on HIV-1. They can recognize HIV-1 through ligation of ssRNA to toll-like receptors (TLRs) 7 and 8 producing a cascade of cytokines with anti-viral activities initiated by the production of interferon (IFN)-α (Chang and Altfeld 2010). IFN-α itself can act on the virus is several ways and has been shown to reduce HIV-1 replication *in vitro* (Baca-Regen, Heinzinger et al. 1994).

A further effect of IFN-α is that it strongly up-regulates intrinsic anti-viral restriction factors such as TRIM5α and APOBEC3G (Chakrabarti and Simon 2010). TRIM5α in the rhesus monkey completely blocks HIV-1 infection while low-level inhibition of HIV-1 replication is mediated by human TRIM5α (Stremlau, Owens et al. 2004). The mechanism of action of TRIM5α is unclear but it is thought to interfere with

the incoming capsid bringing about premature uncoating and disrupting reverse transcription (An and Winkler 2010).

APOBEC3G belongs to a class of protein cytidine deaminase enzymes. It functions by deaminating cytidines in single-stranded viral DNA resulting in lethal guanosine to adenosine mutations in the provirus (An and Winkler 2010). As discussed earlier HIV-1 has evolved a strategy to counteract this restriction factor, it possesses the accessory protein Vif which targets APOBEC3G for degradation via the ubiquitination pathway (Yu, Yu et al. 2003).

A further innate cell type that has been implicated in the control of HIV-1 is the NK cell. Such cells are capable of killing virally infected cells through the same mechanisms employed by CD8+ T cells (further discussed later), namely perforin/granzyme or death receptor dependent pathways. However unlike CTL, NK cells do not rely on rearranged receptors but instead use germline encoded molecules to recognise virally infected cells. As alluded to earlier these receptors can be both inhibitory and activating and it is the balance between the signals received by the NK cell that determines if it lyses a target cell or not.

NK cells are expanded during the acute stage of HIV-1 infection (Alter, Teigen et al. 2007). This expansion occurs prior to the development of the adaptive immune response giving NK cells the opportunity to act on the virus before it has replicated extensively and therefore when it is more susceptible to immune control. Evidence that NK cells may indeed play a role in control of HIV-1 replication comes from a study that demonstrates a certain genotype of NK cell receptor called a killer immunoglobulin-like receptor (KIR), in combination with its MHC class I ligand, is associated with slow HIV-

1 disease progression. Expression of KIR3DS1 in tandem with HLA alleles that belong to the HLA-Bw4-80I group are related to an increase in the time to onset of AIDS (Martin, Gao et al. 2002).

1.8.2 The Humoral immune responses to HIV-1

Antibodies mediate anti-viral activity in two ways, by binding to free virus as well as infected cells. Binding of antibodies to virus particles can inhibit them from infecting further target cells (neutralisation), it can also induce complement mediated lysis of the virion. Antibody recognition of virally infected cells has several mechanisms of disrupting viral replication such as; infected cell lysis, inhibition of viral replication and/or release and inhibition of cell-cell transmission (Burton 2002).

The first detectable B cell response to HIV-1 infection occurs around 8 days following the detection of plasma viral load, in the form antibody complexes. 5 days later the first free plasma anti-HIV-1 antibodies, which are specific for gp41, appear. After a further 14 days gp120-specific antibodies can be detected (Tomaras, Yates et al. 2008).

However these antibodies are non-neutralising, it takes 3 to 12 months following infection for antibodies that can neutralise autologous virus to appear (Gray, Moore et al. 2007). The range of epitopes bound by these first neutralising antibodies is narrow and often restricted to certain viral epitopes (Moore, Gray et al. 2008).

Whereas most patients produce neutralising antibodies of some description (Alter and Moody 2010), broadly neutralising antibody responses capable of neutralising a wide range of viral isolates are rarer (Gray, Madiga et al. 2009) and take longer to appear.

Such antibodies are not usually detected until 20 to 30 months post infection (McMichael, Borrow et al. 2010).

Efforts to correlate the presence of broadly neutralising antibodies with HIV-1 control have produced mixed results. It appears that the maintenance of such antibodies requires the presence of antigen, consequently they are rarely found in patients that control HIV-1 to a low or undetectable level (Lambotte, Ferrari et al. 2009; Doria-Rose, Klein et al. 2010; Sajadi, Guan et al. 2011). It is therefore difficult to conclude the exact role they might play in viral control.

The rarity of antibodies capable of neutralising a wide range of viral isolates during natural infection coupled with the highly variable nature of HIV-1 env proteins has compounded efforts to find useful targets for an antibody-inducing HIV-1 vaccine. To date no vaccine candidates have reliably induced broadly neutralising antibodies (Alter and Moody 2010).

1.8.3 The CD4+ T cell response to HIV-1

The generation of long-lived antiviral CD8+ T cell memory requires CD4+ T cell help (Sun and Bevan 2003; Sun, Williams et al. 2004). Whilst priming of CD8+ T cells can occur in the absence of CD4+ T cell help, the recall antigen response is impaired (Matloubian, Concepcion et al. 1994). This could have particular significance in the context of HIV-1 infection, as during the chronic phase of disease CD8+ T cells become exhausted and CD4+ T cells are limited in number.

The role of CD4+ T cells in the control of HIV-1 replication has been given limited attention in recent years. This could in part be due to the depleted and apparently dysfunctional nature of such cells during HIV-1 infection. There is however mounting evidence that they could be involved in viral control.

After an initial depletion of CD4+ T cells following HIV-1 infection, virus specific cells can be detected during the reduction in viral load to set point. Furthermore escape mutations within CD4+ T cell epitopes have been observed during early HIV-1 infection (Rychert, Saindon et al. 2007) suggesting that such cells are applying enough pressure on the virus to warrant a need to avoid recognition. In addition, HIV-1 controllers possess CD4+ T cells that proliferative efficiently as well as maintaining IL-2 (Rosenberg, Billingsley et al. 1997; Boaz, Waters et al. 2002; Younes, Yassine-Diab et al. 2003; Potter, Lacabaratz et al. 2007) and IL-21 production (Chevalier, Julg et al. 2011). This is not the case for viremic patients. However given that the virus is responsible for depleting CD4+ T cell numbers it is possible that the detection of an effective helper T cell response is just an effect and not a cause of a reduction in viral load.

That being said, IL-21 produced by CD4+ T cells from controllers can enhance perforin production by HIV-1 specific CD8+ T cells from chronic progressors (Chevalier, Julg et al. 2011), demonstrating that an effective CD4+ T cell response has the potential to have a positive effect on disease outcome.

The idea of producing a vaccine that induces a specific CD4+ T cell response is approached with caution by researchers. Simply due to their specificity, virus specific cells are more likely to be in prolonged contact with actively replicating HIV-1 as it is

their cognate antigen. This leads to HIV-1 specific CD4+ T cells being preferentially infected with virus *in vivo* (Douek, Brenchley et al. 2002). Consequently such a vaccine strategy could lead to the generation of a high number of cells that are particularly susceptible to targeting by HIV-1, potentially enhancing viral replication should infection occur.

Another subset of CD4+ T cells that may have a role to play during HIV-1 infection is Foxp3+CD25+ T regulatory cells. Due to the likely importance of immune activation in HIV-1 pathogenesis such cells have the potential to positively impact on disease outcome. However, conflicting data exists as to whether increased numbers of CD4+ T regulatory cells are associated with good or bad viral control. It is also possible that regulatory cells contribute to HIV-1 pathogenesis by dampening HIV-1 specific immune responses. Further work is required in order to fully delineate the role of CD4+ T regulatory cells during HIV-1 and to establish whether increasing or decreasing their numbers may be a useful therapy (Mason, De Rose et al. 2008).

1.8.4 The CD8+ T cell response to HIV-1

1.8.4.1 Mechanisms of CD8+ T cell anti-viral action

CD8+ T cells act to contain HIV-1 replication via various mechanisms. HIV-1 specific CD8+ T cells can suppress viral replication in CD4+ T cells without killing the infected cells (Walker, Erickson et al. 1991). Transwell experiments demonstrated that this anti-viral activity is mediated by one or more soluble factors (Walker and Levy 1989) that are now termed CD8+ T cell antiviral factor (CAF). CAF has been shown to function by suppressing HIV-1 LTR mediated transcription (Mackewicz, Blackbourn et al. 1995).

CD8+ T cells also secrete several chemokines and cytokines that have antiviral activity. The beta-chemokines MIP-1α, MIP-1β and RANTES are produced by CD8+ T cells in response to viral infection. These are ligands for the HIV-1 co-receptor CCR5 and therefore reduce viral entry into new target cells by binding to their receptor. Several cytokines with anti-viral properties are also secreted by CD8+ T cells during viral infection such as IFN-γ, TGF-β and TNF-α. How much these contribute to the control of HIV-1 replication is not fully understood (Tomaras and Greenberg 2001).

CD8+ T cells can also directly lyse HIV-1 infected targets preventing then from releasing viral progeny and reducing virus dissemination (Klenerman, Phillips et al. 1996). Cells that mediate this mechanism of action are termed cytotoxic T lymphocytes (CTL). Upon T cell receptor (TCR) recognition of target cells preformed granules containing lytic proteins, predominantly perforin and granzymes are release by CTL via exocytosis (Shiver and Henkart 1991; Shiver, Su et al. 1992). Granzymes are a group of

serine proteases of which granzymes A and B are the most abundant (Pasternack and Eisen 1985). Perforin is responsible for the entry of granzymes into the target cell by forming pores in the target cell membrane through which the proteases can pass (Pipkin and Lieberman 2007). Once inside a target cell such molecules induce apoptosis (Pasternack and Eisen 1985). A second cytotoxic pathway also exists, whereby the engagement of Fas on the target cell with FasL on the CTL leads to cell death through the induction of classical caspase dependent apoptosis (Henkart 1994).

1.8.4.2 How CD8+ T cells recognise virally infected cells

Virus-specific CTL mediation of these effector functions requires signalling through the TCR following recognition of virally infected cells. This process involves many intracellular and extracellular molecules expressed by both the target and effector cell.

1.8.4.2.1 CD8+ T cells recognise peptides bound to MHC class I molecules

CD8+ T cells display TCRs that bind to peptide antigen presented in MHC class I molecules (Doherty, Blanden et al. 1976; Townsend, Ohlen et al. 1989). MHC class I molecules are found on the surface of nearly all nucleated cells and when TCR recognition occurs in the presence of co-stimulation CD8+ T cells become activated and display the effector functions described above.

MHC class I molecules are encoded by three highly polymorphic genes located on the short arm of chromosome 6. This leads to each individual possessing up to six different MHC class I molecules, three inherited from each parent.

MHC class I molecules bind peptide antigens of between 8 and 12 amino acids in length that are anchored to the peptide binding groove at both the C- and N-termini as well as via other key residues in between (Bouvier and Wiley 1994). Certain MHC class I molecules require that specific amino acids are present at particular positions in order for binding to occur, such preferences are termed peptide binding motifs (Madden, Garboczi et al. 1993). Amino acids within a peptide that are not involved in contacting the MHC class I molecule can be altered without affecting binding thus allowing each molecule to bind to a very wide variety of peptides. Given that an individual has up to six different MHC class I alleles, presentation of truly diverse set of antigens can take place. The importance of which is emphasised during HIV-1 infection, as shown by the poor disease outcome associated with being homozygous at any HLA-loci (Carrington, Nelson et al. 1999).

1.8.4.2.2 T cell receptors are produced through the rearrangement of genomic DNA

In order to recognise the vast array of peptide-MHC (p-MHC) class I complexes the TCR must also be extremely diverse. This diversity is achieved in two ways, via both combinatorial and junctional mechanisms.

Combinatorial diversity occurs because the TCR α and β chains are encoded in the germline DNA by several non-continuous gene segments that are rearranged in T

cells before they are translated into protein (Davis and Bjorkman 1988), as illustrated in figure 1.5. The TCR α chain consists of 70-80 variable (V) segments and 61 joining (J) segments, whilst the β chain consist of 52 variable (V) segments, 2 diversity (D) segments and 13 joining (J) segments. Different combinations of these segments can be rearranged creating a vast amount of diversity. A further level of variation is added due to the pairing of different α and β chains.

To ensure that the DNA rearranges in the correct manner each segment is either followed or preceded by a recombination signal sequence (RSS). This consists of a heptamer 5'CACAGTG3' and a nonamer 5'ACAAAAACC3' separated by either a 23 or a 12 base pair (bp) spacer. A sequence followed by a 23 bp spacer can only recombine with one preceded by a 12 bp spacer and vice versa (Akira, Okazaki et al. 1987). The enzymes involved in recombination are recombinase activating gene (RAG)-1 and -2, these are expressed in developing lymphocytes only (Fugmann, Lee et al. 2000).

Junctional diversity occurs due to addition and subtraction of nucleotides at the junction between gene segments. P-nucleotides are palindromic sequences that occur because RAG enzymes generate DNA hairpins at the ends of the V, D and J segments (Lewis 1994; Lewis 1994). These are cleaved by an enzyme called Artemis at a random point leaving a single stranded tail of a few amino acids. The second strand is than added by DNA repair enzymes (Ma, Pannicke et al. 2002). N-nucleotides are non-template coded nucleotides added at the junctions of TCR gene segments as a result of imprecise joining (Siu, Kronenberg et al. 1984). This process is mediated by the enzyme terminal deoxynucleotidyl transferase (TdT).

The complementary determining regions (CDR) of the TCR α and β chains are hypervariable flexible domains that are responsible for interacting with the p-MHC complex. The CDR3 region comprises the most polymorphic part of the protein spanning the V(D)J junction and is predominantly involved in contacting the peptide whilst the CDR1 and 2 regions bind to the MHC molecule, although all three regions can be involved in contacting either the peptide or the MHC (Nikolich-Zugich, Slifka et al. 2004).

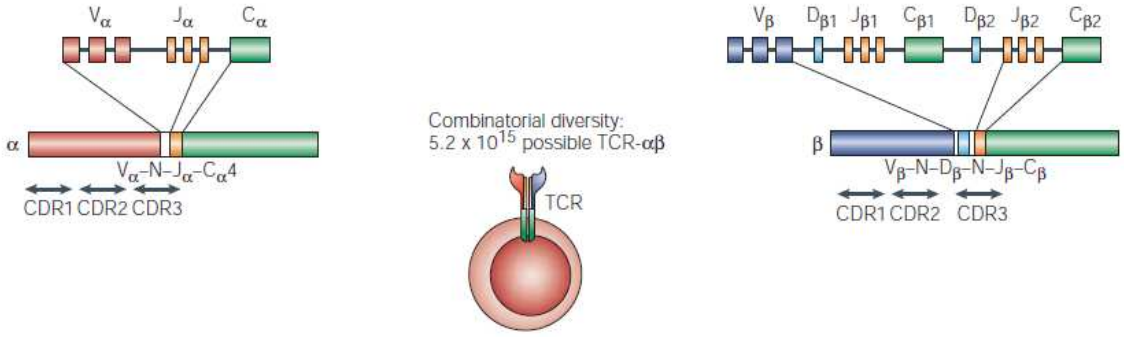


Figure 1.5 : T cell receptor rearrangement (Nikolich-Zugich, Slifka et al. 2004)

1.8.4.2.3 Antigen processing and presentation by MHC class I molecules

Peptides presented in MHC class I molecules are predominantly derived from intracellular expressed proteins. This allows the immune system to monitor the intracellular environment, detecting any abnormalities that could be indicative of viral or bacteria infection. Producing peptide fragments bound to MHC class I molecules at the cell surface from intracellular proteins requires many stages as illustrated by figure 1.6.

The first step in the production of antigenic peptides is the targeting of cytosolic protein for intracellular degradation. This occurs through the addition of multiple

ubiquitin moieties to the substrate protein via a cascade of catalytic events initiated by the E1-activating enzyme (Groothuis, Griekspoor et al. 2005).

The purpose of this ubiquitin tag is to allow for recognition of proteins destined for degradation by the proteasome. The proteasome is an abundant and ubiquitous protease that is required for the generation of most but not all antigenic peptides (Rock, Gramm et al. 1994). It is composed of a central barrel shaped 20s subunit and one or two optional caps known as 19s subunits. The 19s subunit binds to the polyubiquitinated protein facilitated by the E4 protein. It then removes the ubiquitin moieties and unfolds the protein allowing it to fit into the catalytic chamber of the proteasome within the 20s subunit. Catalytic threonine residues then orchestrate the breakage of peptide bonds thus cleaving the substrate.

There are two forms of proteasome, a constitutively expressed version and the immunoproteasome. The difference between these two molecules lies in the β-domains that make up the 20s subunit and contain the threonine residues involved in protein cleavage. IFN-γ induces the constitutive β-1, β-2 and β-5 domains to be replaced by the β-1i, β-2i and β-5i domains (Groothuis, Griekspoor et al. 2005). This leads to a change in the cleavage preference of the proteasome, in turn altering the repertoire of peptides being presented by the MHC class I molecules (Chapiro, Claverol et al. 2006).

The entry of a protein into the antigen presentation pathway can in theory occur at anytime during its lifespan, however it has been generally accepted that the majority of peptide antigens are derived from the degradation of proteins that have come to the end of their life. An alternative theory is that the degradation of newly synthesised proteins that are unable to carry out their biological function due to premature termination of

translation or defective folding also significantly contributes to the antigenic peptide repertoire. Degradation and presentation of these defective ribosomal products (DRiPs) could be important in the setting of a viral infection as it would allow for the recognition of infected cells directly following viral protein synthesis instead of when viral proteins are turned over (Shastri, Cardinaud et al. 2005).

Peptides produced by the proteasome range from 3 to 22 amino acids in length (Kisselev, Akopian et al. 1999), in order for some of these peptides to bind to MHC class I molecules they must be further trimmed by aminopeptidases in the cytosol or in the ER. Examples of such molecules are leucine aminopeptidase (LAP) a cytosolic enzyme upregulated by IFN-γ, tripeptidyl peptidase II (TPPII) a cytosolic peptidase that removes amino acids in blocks of three from the amino terminal of a peptide and ER aminopeptidase (ERAP1) that cleaves peptides that are greater than 7 amino acids in length (Yewdell, Reits et al. 2003).

In order for peptides generated by the proteasome to be loaded into MHC class I molecules they must move from the cytosol in to the ER. Crossing of the ER membrane is facilitated by the transporter for antigen processing (TAP) – an ER resident heterodimeric peptide transporter. TAP binds to peptides in the cytosol and by hydrolysing two molecules of ATP it creates a pore in the ER membrane and expels the peptide into the ER lumen.

Once inside the ER the peptide can be loaded into an MHC class I molecule. To ensure efficient peptide loading TAP is located along with the MHC class I molecule and several chaperone molecules in a structure known as the peptide loading complex (PLC). One such chaperone is called tapasin and has a critical role in MHC class I antigen

processing. Tapasin binds directly with the MHC class I molecule facilitating its association with TAP and mediating optimal peptide loading (Ortmann, Copeman et al. 1997). Other chaperones involved in loading peptide into MHC class I molecules are calreticulin and ERp60. Once a peptide has been successfully loaded into an MHC class I molecule it then enters the secretory pathway and is trafficked via the golgi body to the cell surface where it is available for recognition by the TCR.

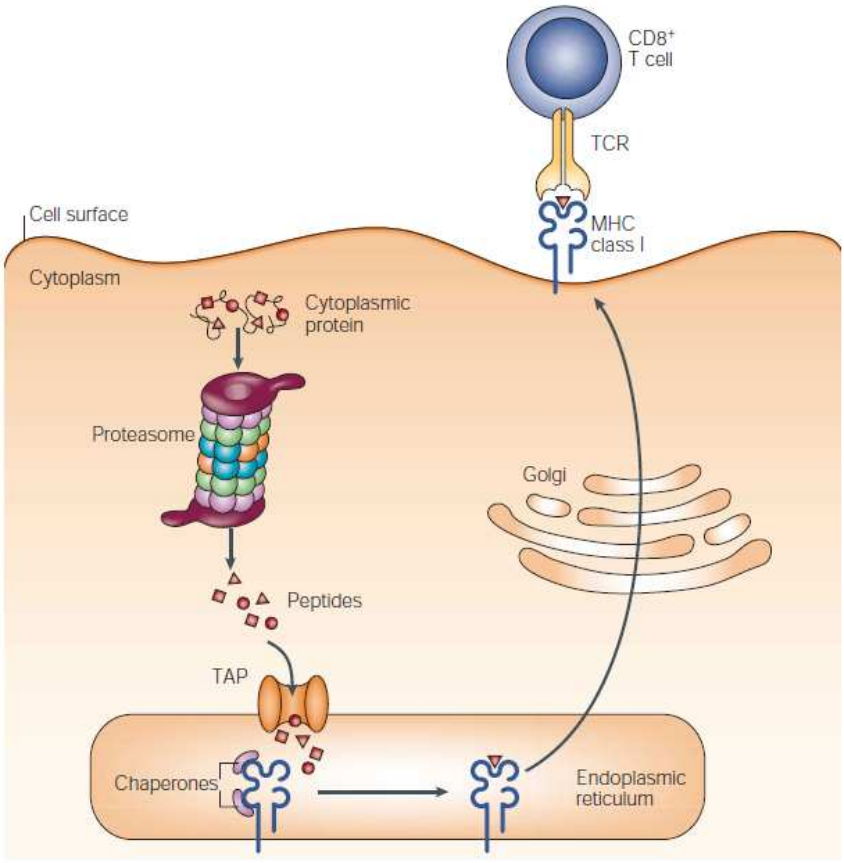


Figure 1.6: The antigen processing pathway (Yewdell, Reits et al. 2003).

1.8.4.3 The importance of CTL in HIV-1 infection

Given their crucial role in containing viral infection it is perhaps unsurprising that many lines of evidence demonstrate the importance of CTL in controlling HIV-1 replication. During acute infection there is a temporal association between the appearance of virus specific CTL and the fall in viremia from peak levels to set point, with the peak of the CTL response coinciding with the initiation of the decrease in viral load (Borrow, Lewicki et al. 1994; Koup, Safrit et al. 1994).

It has also been demonstrated that CTL play an active role in suppressing SIV during non-human primate models of infection. Rhesus macaques depleted of CD8+ T cells prior to challenge with SIV fail to control replication of the virus, as well as control animals, during the acute stage of the disease (Schmitz, Kuroda et al. 1999). Depletion of CD8+ T cells in chronically infected rhesus macaques also leads to an increase in viral load (Jin, Bauer et al. 1999).

Several MHC class I alleles, which function to present peptides to CD8+ T cells, have been associated with either fast or slow progression to AIDS. HLA-B*57 displays perhaps the most consistent and significant link to late onset of disease (Haynes, Pantaleo et al. 1996; Kaslow, Carrington et al. 1996; Klein, van der Burg et al. 1998) whilst HLA-B*35 has been related to fast development of AIDS (Carrington, Nelson et al. 1999). Further investigation revealed that only non-HLA-B*3501 subtypes, that present peptides with a -P-----X motif, and not HLA-B*3501 (-P------Y) are linked to faster onset of AIDS (Gao, Nelson et al. 2001). This suggests it is the peptide presenting function of the HLA allele that is responsible for its association with poor disease outcome.

Genome wide association studies (GWAS) that attempt to associate the possession of single nucleotide polymorphisms (SNPs) with outcome of HIV-1 infection have confirmed the link between certain HLA alleles and disease progression. One GWAS identified the polymorphism most significantly associated with low viral load set point to be a SNP located in the HCP5 gene on chromosome 6. Possession of one copy of this SNP was found to be associated with a reduction in viral load by a log. The protective version of this polymorphism was observed to be in high linkage disequilibrium (LD) with HLA-B*5701, as previously discussed an HLA allele association with good viral control (Fellay, Shianna et al. 2007). Another GWAS comparing elite controllers with progressors, found over 300 SNPs significantly associated with either good or bad HIV-1 control all of which were within the MHC locus on chromosome 6. This study also confirmed the SNP within the HCP5 gene as being associated with HIV-1 control (Pereyra, Jia et al. 2010).

Linking of particular HLA alleles to HIV-1 disease progression doesn't directly implicate CTL in the control of viral replication as such molecules are also involved in interactions with other cell types, such as NK cells. However, the observation that the link between HLA-B*35 and rapid disease progression is dependent on the subtype of the MHC and the motif of the peptides it presents does somewhat indicate that antigen specific MHC - CTL interactions are at play.

Furthermore the appearance of mutations within known CTL epitopes suggests that significant pressure is exerted on the virus to evade recognition by CTL. A longitudinal study of HIV-1 infected patients demonstrated an accumulation of amino acid variation in or near CTL epitopes within the proviral HIV-1 gag gene that could

provide a mechanism for immune escape (Phillips, Rowland-Jones et al. 1991). Another study observed mutations in a HLA-B*08 restricted nef epitope that emerged during acute HIV-1 infection, such sequence changes were found to either diminish or remove recognition by CTL (Price, Goulder et al. 1997). Further evidence that HIV-1 viral evolution is driven by pressure to escape the HLA-restricted immune response comes from a study that involved sequencing the RT gene from a large population of HLA diverse, HIV-1 infected individuals. It showed that polymorphisms within RT were frequently associated with the presence of particular HLA molecules (Moore, John et al. 2002).

1.8.4.4 Neither breadth nor magnitude of the HIV-1 specific CTL response predicts disease outcome

Given the wealth of evidence implicating CTL in the control of HIV-1 it is perhaps surprising that neither an increase in the breadth nor in the magnitude of the HIV-1 specific CD8+ T cell response can be consistently correlated with either higher CD4 counts, lower viral loads or better disease outcome.

An inverse correlation has been shown between the percentage of CD8+ T cells binding to a HLA-A*0201 gag specific tetramer and plasma viral load in a group of untreated patients at diverse disease stages (Ogg, Jin et al. 1998). However a positive correlation was observed between plasma viral load and HIV-, env- and nef- specific CD8+ T cell frequency, as measured using overlapping 15-mer peptides in an IFN-γ enzyme linked immunospot (ELISpot) assay (Betts, Ambrozak et al. 2001). Another

study utilising overlapping peptides in an IFN-γ ELISpot assay found that plasma viral load did not correlate with breadth or magnitude of total HIV-1-specific CD8+ T cell responses (Addo, Yu et al. 2003).

In order to unify these somewhat conflicting findings it has been suggested that certain CTL responses are 'driver' responses contributing to viral control whereas other CTL responses are merely 'passengers' unable to reduce viral replication and proliferating in response to the amount of antigen present (Klenerman, Wu et al. 2002; Zafiropoulos, Barnes et al. 2004).

With this concept in mind, a Peruvian cohort of therapy naïve HIV-1 clade B infected patients was investigated, is a similar fashion to two of the studies mentioned above, using IFN-γ ELISpot assays and overlapping peptides spanning the entire HIV-1 genome. The analysis was however approached in a different way. The relative contribution of individual HIV-1 protein specific responses to the total virus specific response was compared to the viral load and CD4 count of the patients. An inverse correlation was observed between viral load and the proportion of gag-specific, in particular p24-reactive, CTL responses among the total virus specific CTL response. A positive correlation was seen between the same parameter and CD4 count. This relationship was not observed when other HIV-1 gene products were studied (Zuniga, Lucchetti et al. 2006).

These findings illustrate that the method with which you detect virus specific responses in conjunction with how the data is analysed affects whether or not a driver, a passenger or a mixed response is being investigated. This could explain the discordant

correlations observed as a 'driver' response would inversely correlate with viral load whilst a 'passenger' response would positively correlate.

1.8.4.5 Quality plays a more important role than quantity in determining the efficacy of a HIV-1 specific CTL response

It is now widely acknowledged that it is the quality and not the quantity of a given response that determines if it contributes to the reduction of HIV-1 replication. Several highly interrelated factors are involved in influencing whether particular CTL comprise a 'driver' response capable of controlling viral replication.

1.8.4.5.1 HLA restriction

As stated earlier, several studies have shown that HLA class I molecules have an impact on disease outcome (Haynes, Pantaleo et al. 1996; Kaslow, Carrington et al. 1996; Klein, van der Burg et al. 1998; Carrington, Nelson et al. 1999; Gao, Nelson et al. 2001). This observation suggests that there are qualitative differences between MHC class I restricted HIV-1 specific CTL responses, adding weight to the theory that not all CTL are equally capable of controlling viral replication.

Given its significant correlation with slow disease progression it is perhaps not surprising that HLA-B*57 has been found to be enriched amongst elite controllers (Migueles, Sabbaghian et al. 2000; Lambotte, Boufassa et al. 2005; Emu, Sinclair et al.

2008; Pereyra, Addo et al. 2008; Sajadi, Constantine et al. 2009; Kosmrlj, Read et al. 2010) further emphasising its role in effective control of HIV-1.

1.8.4.5.2 Viral protein targeted

The positive effect exerted by certain HLA alleles on HIV-1 disease progression could in part be due to the location of the epitopes they present. As highlighted by certain studies mentioned above (Ogg, Jin et al. 1998; Zuniga, Lucchetti et al. 2006) as well as other investigations (Edwards, Bansal et al. 2002; Novitsky, Gilbert et al. 2003; Kiepiela, Ngumbela et al. 2007) targeting a response towards HIV-1 gag appears to be beneficial. One such study went on to show that an increase in the breadth of gag-specific responses correlates with a reduction in viremia (Kiepiela, Ngumbela et al. 2007). It has also been shown that HLA alleles associated with slow progression to AIDS preferentially present epitopes encoded by gag, in particular the p24 protein (Borghans, Molgaard et al. 2007).

Reasons for the beneficial nature of targeting gag are far from fully elucidated but it may be down to the fact that CD8+ gag-specific T cells can recognise virally infected CD4+ T cells as soon as 2 hours post-infection allowing for rapid lysis of infected cells. Gag is the earliest viral gene product to be detected during infection and this recognition occurs before viral DNA integration and protein expression (Sacha, Chung et al. 2007) as well as before nef mediated downregulation of HLA-A and B molecules.

The benefits of a eliciting a gag specific and in particular a p24 specific response could also be attributed to the fact that certain epitopes with this protein are highly conserved. This is due to p24 being the most functionally and structurally constrained

protein in HIV-1 therefore mutations with this protein result in a high fitness cost to the virus in turn reducing the chances of CTL escape (Schneidewind, Brockman et al. 2007; Miura, Brockman et al. 2009).

1.8.4.5.3 Efficient target cell killing

The most important mechanism by which CTL control HIV-1 replication and limit viral dissemination is through the eradication of infected cells. Differences in the ability of CTL to effectively load lytic granules and efficiently deliver their contents to the cell membrane of target cells have been observed.

In vitro stimulation of HIV-1 specific CD8+ T cells from LTNPs for 6 days leads to a significantly higher percentage of granzyme B and perforin positive cells than when equivalent cells from progressors are stimulated (Migueles, Laborico et al. 2002; Migueles, Osborne et al. 2008). In addition, a significantly higher quantity of granzyme B was delivered to target cells after 6 days *in vitro* incubation with CD8+ T cells from controllers as compared with cells from viremic progressors (Migueles, Osborne et al. 2008).

The antibody used to detect perforin in the previously described studies recognises the molecule in its granule-associated form. Use of an antibody capable of binding to several perforin conformations has allowed for the detection of newly synthesised perforin directly following antigen stimulation (Hersperger, Makedonas et al. 2008). This revealed that HIV-1 specific CD8+ T cells from elite controllers display an

enhanced ability to express perforin directly *ex vivo* as compared to cells from patients with progressive disease (Hersperger, Pereyra et al. 2010).

1.8.4.5.4 Functional avidity

The concentration of peptide epitope that induces the half-maximal effector response, as measured by cytokine release or specific target cell lysis, is used as a marker for CTL sensitivity to antigen and is termed functional avidity. This concentration of peptide that elicits 50% of the maximum response is sometimes referred to as the sensitising dose 50 (SD_{50}) and has been shown to have a bearing on the anti-viral efficacy of a given CTL response.

In vitro stimulation of specific CTL from previously immunised mice utilising low concentrations of peptide generates cells with high functional avidity, namely CTL that require lower amounts of peptide to produce an effector response. Conversely high dose peptide stimulation results in the generation of CTL with low functional avidity. Adoptive transfer experiments demonstrated that high avidity CTL were more effective at viral clearance in comparison to low avidity CTL, despite the fact that both could control viral replication *in vitro* (Alexander-Miller, Leggatt et al. 1996).

This ability to control viral replication better can be attributed to two mechanisms. Firstly, highly avid CTL can recognise lower antigen densities in comparison to low avidity CTL, thus allowing them to lyse infected target cells earlier in the course of infection. Secondly, highly avid CTL initiate lysis of target cells more rapidly than low avidity cells at any given antigen density (Derby, Alexander-Miller et al. 2001).

In the context of HIV-1 infection it has been demonstrated that the avidity of immunodominant virus specific CD8+ T cells positively correlates with cell associated viral load (cVL), with dominant CTL responses requiring low concentrations of peptide to induce half maximal IFN-γ release found in patients with low cVL (Almeida, Price et al. 2007). Also an inverse relationship has been demonstrated between the functional avidity of HIV-1 specific CTL and their ability to kill infected cells efficiently *in vitro* with a threshold in peptide concentration below which a steep increase in killing is observed (Bennett, Ng et al. 2007).

Suppression of HIV-1 *in vitro,* as measured by a reduction in target cells positive for p24 antibody, has also been shown to correlate with CTL functional avidity. Highly avid T cells induce the greatest decrease in virus following 3 days incubation with infected primary CD4+ T cells (Almeida, Sauce et al. 2009). Furthermore the functional avidity of HIV-1 specific CD8+ T cells is higher during acute infection than during chronic infection, leading to the hypothesis that this failure to maintain highly avid CD8+ T cells contributes to the ultimate inability of the immune system to control HIV-1 infection (Lichterfeld, Yu et al. 2007).

However immunodominant highly functionally avid HIV-1 specific CTL have been observed in patients with chronic progressive infection suggesting that the presence of such cells is not sufficient to control HIV-1 infection (Draenert, Verrill et al. 2004). Further studies have also cast doubt on the importance of functional avidity in controlling HIV-1 infection. The ability of HIV-1 specific CTL clones to kill virally infected cells as well as suppress viral replication *in vitro* has been shown to be more dependent on epitope specificity than functional avidity with gag- and nef-specific clones consistently

performing better than RT specific cells regardless of SD_{50} (Yang, Sarkis et al. 2003). Another study found no correlation between the functional avidity of gag- and env-specific CTL and their ability to inhibit viral replication *in vitro* (Chen, Piechocka-Trocha et al. 2009).

1.8.4.5.5 Polyfunctionality

The ability of an HIV-1 specific CD8+ T cell response simultaneously to produce several effector functions, namely the production of IFN-γ, TNF-α, MIP-1β, IL-2 and degranulation of lytic granules, has been associated with good viral control. Polyfunctional HIV-1 specific CD8+ T cells, that possess 4 or more of these functions, are significantly enriched in HIV-1 non-progressors as compared to progressors. Also the magnitude and proportion of HIV-1 specific CD8+ T cells that possess 5 functions inversely correlates with plasma viral load (pVL) (Betts, Nason et al. 2006). Several other studies have established a link between polyfunctional virus specific CD8+ T cell responses and control of viral replication (Critchfield, Young et al. 2008; Turk, Gherardi et al. 2008; Ferre, Hunt et al. 2009) but determining whether such cells are actively suppressing viral replication or are merely an effect of there being less viral antigen is difficult.

It has been shown that polyfunctionality of virus specific CD8+ T cells is lost over the course of HIV-1 infection if the viral antigen persists but maintained if there is a reduction in antigen load brought about either through the use of drug therapy or by the appearance of an escape mutation (Rehr, Cahenzli et al. 2008; Streeck, Brumme et al.

2008). This suggests that polyfunctionality may merely result from CD8+ T cells being exposed to a reduced level of viral antigen and is not directly responsible for controlling viral replication.

1.8.4.5.6 Differentiation state

On encounter with antigen, naïve CD8+ T cells undergo a process of differentiation in order to become effector cells. The steps in this process can be defined using the expression of two surface markers, the co-stimulatory molecules CD27 and CD28. Naïve and early differentiated T cells express CD27 and CD28 whilst both are down-regulated in fully differentiated T cells. This down-regulation occurs in a step-wise manner with CD28 first to be lost, leading to CD27+CD28- T cells representing an intermediate phenotype (Hamann, Roos et al. 1999).

During the chronic phase of HIV-1 infection most HIV-specific CD8+ T cells are of the CD28-CD27+ intermediate phenotype, these cells express lower levels of perforin than fully differentiated cells and are impaired in cytolytic activity (Appay, Nixon et al. 2000; Appay, Papagno et al. 2002). Some HIV-1 specific CD8+ T cells can become fully differentiated effector cells and it has been shown that high percentages of CD27- HIV-1 specific CD8+ T cells correlate with delayed disease progression, suggesting that CD27- effector cells may be important in controlling virus replication (van Baarle, Kostense et al. 2002).

However it has also been shown that the percentage of the total CD8+ T cell population that possesses the fully differentiated CD27-CD28- phenotype is increased in

patients with chronic progressive disease (Papagno, Spina et al. 2004). It remains a contentious issue as to whether highly differentiated CD8+ T cells represent effector-type cells capable of controlling viral replication or ageing lymphocytes that have lost their ability to proliferate.

1.8.4.5.7 Recognition of escape mutation

The ability of a CTL response to recognise common escape variants impacts on its ability to contain viral replication. HLA-B*08 restricted nef specific CTL from 4 long term non-progressor patients were found to possess TCRs with an extended CDR3 loop that were capable of recognising a common mutation that leads to escape from recognition by other CTL (Dong, Stewart-Jones et al. 2004). It has also been shown that a HLA-B*5301 restricted gag specific CTL response in a long term asymptomatic HIV-1 infected child was capable of recognising 8 out of 10 common escape variants both through the use of promiscuous TCRs and by utilising a polyclonal T cell population (Buseyne and Riviere 2001).

1.8.4.5.8 The complexity of CTL quality in HIV-1 infection

Elucidating which of the factors discussed above are most important in determining the quality of a CTL response to HIV-1 is complicated by their highly inter-related nature. For example the HLA allele plays a role in determining the epitopes that are presented and in turn the viral proteins targeted. It is therefore not always straight

forward to establish whether a given response is protective due to the specificity of the epitope targeted, down to some other intrinsic factor associated with the presenting HLA allele or indeed a combination of both.

Another example of the interwoven nature of the factors surrounding CTL quality comes from investigating polyfunctionality. The polyfunctionality of a given CD8+ T cell response is related to its functional avidity. A correlation exists between the SD_{50} of CTL clones and the percentage of cells displaying 3 or more functions when stimulated with peptide antigen (Almeida, Sauce et al. 2009). The epitope targeted can also impact on polyfunctionality by modulating the persistence of antigen, as certain functionally constrained regions are less liable to escape. As discussed earlier, a reduction in antigen stimulation brought about by immune escape leads to the maintenance of cells possessing more functions (Streeck, Brumme et al. 2008). This makes it complicated to determine how much each of these factors contributes to viral control as well as highlighting the problem of determining cause and effect.

The issue of cause and effect is a central theme when discussing the quality of HIV-1 specific CTL response. As alluded to several times already, it is very hard to confirm that the presence of factors associated with 'good' virus specific CTL are directly involved in viral control and not just a product of a reduction in viral replication that had come about through other immune mechanisms.

The situation is further complicated by the fact that viral factors as well as host factors impact on the CTL response. HLA-B*27+ children infected with HIV-1 containing an escape mutation in the protective immunodominant HLA-B*27 restricted epitope from their HLA-B*27+ mothers failed to control viral replication (Goulder,

Brander et al. 2001). This suggests that in this situation it is the protective nature of the specific HLA-restricted response and not simply the possession of the protective allele that is responsible for control.

This theory is reiterated by the observation that although HLA-B*57 is highly associated with HIV-1 viral control and enriched in cohorts of elite controllers/LTNPs some HLA-B*57+ HIV-1 patients progress to AIDS normally (Altfeld, Addo et al. 2003; Migueles, Laborico et al. 2003). The reasons why HLA-B*57 and HLA-B*27 are not protective in all HIV-1 patients are far from fully understood and are most likely determined by many factors.

Several other features of HIV-1 specific CTL responses that are associated with good clinical outcome have also been observed in patients that fail to control viral replication. As discussed previously highly functionally avid T cells are observed in HIV-1 progressors (Draenert, Verrill et al. 2004). In addition, Gag responses have been found in normal progressor and rapid progressor patients (Klein, van Baalen et al. 1995; Gea-Banacloche, Migueles et al. 2000). Similarly, possessing a response to a conserved epitope does not guarantee viral control (van der Burg, Klein et al. 1997).

Factors that robustly segregate ‘good’ CTL from ‘bad’ CTL are hard to come by and it is likely that a combination of several factors determines whether or not a CTL response is capable of successful viral control. Any steps towards further understanding these factors will greatly aid in the design of a successful HIV-1 vaccine.

1.9 HIV-1 and ART

Despite the concerted effort of the immune system to contain HIV-1 replication it can not prevent the continued loss of CD4+ T cells and the eventual arrival of AIDS. However not long after the discovery of HIV-1 as the cause of AIDS the first ART became available for use. This substantially reduced the death rate of infection and turned a lethal disease into a chronic manageable condition. Despite the continuing development of new and effect drugs to target HIV-1 several barriers to ART successfully halting the spread of the virus remain.

Firstly, life-long therapy is required as once a patient discontinues ART viral load rebounds and disease progression continues due to latent viral DNA incorporated into the host genome that can become reactivated. Secondly extended use of some drugs is accompanied by toxic side-effects affecting patient quality of life and reducing drug adherence. Thirdly the emergence of drug-resistant strains of HIV-1 leads to some drugs being ineffective at controlling certain viral strains (Broder 2009). Lastly the cost of ART prevents many HIV-1 infected persons in resource poor countries from having access to the drugs. This final point is perhaps the largest barrier to the success of ART as most HIV-1 infected people live in developing countries. According to the WHO, at the end of 2009 64% of HIV-1 infected people living in low- to middle-income countries who satisfied the criteria for the initiation of ART did not have access to the drugs.

1.10 The current HIV-1 vaccine situation

Given the above outlined issues associated with ART it is widely acknowledged that a safe and effective vaccine is the best hope for bringing the HIV-1 epidemic to an end. Several factors have compounded efforts to develop such a vaccine, the mucosal route of transmission, the rapid establishment of a persistent reservoir of latently infected cells, the preference for CD4+ T cells as targets for infection, the vast genetic diversity displayed by the virus and the rarity of the induction of neutralising antibodies.

Since 1987 more than 30 candidate HIV-1 vaccines, that displayed varying degrees of protection in nonhuman primate models of infection, have been tested in human trials. However, only 3 of these candidates made it as far as large scale international phase IIb or III studies.

These were a recombinant bivalent HIV-1 monomeric envelope gp120 vaccine named AIDSVAX B/B or B/E (2003), a replication-incompetent adenovirus 5 vector containing HIV-1 gag, pol and nef genes (STEP trial) (Buchbinder, Mehrotra et al. 2008) and finally canarypox vectored HIV-1 gag, env and protease plus AIDSVAX B/E (RV144 trial).

Only the RV144 trial, consisting of priming with the canarypox-HIV-1 vector and boosting with recombinant HIV-1 envelope gp120 subunit proteins, showed any level of efficacy. It was found to reduce infection rates by 31% in low risk individuals (Rerks-Ngarm, Pitisuttithum et al. 2009). To date, no vaccine candidate trialled in humans has been found to lower set-point viral load or improve the outcome of infection should it occur.

Trying to engineer protection against HIV-1 is a difficult task given that there is still an incomplete understanding of the events that lead to natural protection on the rare occasions it is achieved. It is widely believed that a better understanding of the host immune response to HIV-1 infection will allow for more focused vaccine research and ultimately an effective HIV-1 vaccine.

1.11 The aims of this study

With the aim of further characterising CD8+ T cell responses to HIV-1 and in order to establish aspects of CTL responses that may be associated with good and bad viral control the following studies were carried out:

1. Longitudinally analysis of the TCR usage, functionally properties and ability to suppress HIV-1 virus *In Vitro* of HLA-B*08 restricted nef specific CTL from a single rapid progressor patient was undertaken to determine qualitative changes that occur in virus specific CD8+ T cells throughout the course of infection.
2. Characterisation of CD8+ T cell responses restricted by two closely related HLA subtypes within a cohort of Chinese slow progressor patients was carried out to assess the affect of small changes in MHC class I molecules on the specificity and functionality of HIV-1 CTL as well as disease outcome.
3. The recognition of overlapping HIV-1 epitopes was investigated with respect to the sequence of the epitope flanking regions as well as the presence of competing HLA molecules in order to investigate factors that impact on efficient CD8+ T cell antigen processing and presentation.

Chapter 2 - Materials and Methods

2.1 Patient cohorts and ethics approval

Individuals studied in this project were enrolled in one of three patient cohorts. One patient (A794) was a member of a high risk cohort based at the San Diego AIDS treatment center. A second cohort of typical progressor patients based at a clinic in Oxford was also utilised. The remaining samples originated from a cohort of slow progressors in Henan province, China. Ethical approval was obtained from Beijing YouAn Hospital and the University of Oxford Tropical Ethics Committee (OXTREC).

2.2 Preparation of peripheral blood mononuclear cells (PBMCs)

Venous blood was taken and placed in to 50ml falcon tubes containing 40µl of heparin (CP pharmaceuticals ltd.). 25 ml of whole blood was diluted with 15ml of RPMI (Sigma, UK) and separated by Ficoll-Hypaque density centrifugation. The diluted blood was transferred into a leucosep tube (Greiner, Germany) containing 15ml of Ficoll-Hypaque (Sigma, UK) and centrifuged without brake for 20 minutes at 2000 rpm. Cells at the interface between the Ficoll-Hypaque and the plasma were carefully removed and washed twice in RPMI centrifuging at 1500 rpm for 5 minutes. Cells were then suspended in R10 medium (RPMI supplemented with 10% foetal calf serum (FCS; Sigma, UK), 2mM L-glutamine and 50U/ml penicillin/streptomycin).

2.3 B cell line, cytotoxic T cell line and clone generation

To produce transformed B cell lines 0.5 x 10^6 PBMCs were washed in 2ml RPMI, centrifuged at 1500 rpm for 5 minutes and resuspended in 100µl EBV culture supernatant before incubation at 37°C 5% CO_2 for 3 hours. 100µl of R15 (RPMI supplemented with 15% foetal calf serum (FCS; Sigma, UK), 2mM L-glutamine and 50 U/ml penicillin/streptomycin) containing 2µg/ml cyclosporin A (CsA; SANDIMMUN) was added and the cells were placed in one well of a 96 well round bottom plate. After 2 days 100µl of the media was replaced with 100µl of fresh R15 + 2µg/ml CsA. After 2 weeks the CsA was omitted.

To produce short term CTL lines 2-3 x 10^6 PBMCs were washed in 2ml RPMI, centrifuged at 1500 rpm for 5 minutes and resuspended in 100µl of peptide at 20µg/ml before incubation at 37°C 5% CO_2 for 1-2 hours. 1ml of H10 (RPMI supplemented with 10% human AB serum (National Blood service, UK), 2mM L-glutamine and 50 U/ml penicillin/streptomycin) was added and the cells were placed in 1 well of a 24 well plate. On day 3 post generation the CTL lines were given 1ml of H10 + 200U/ml IL-2.

CTL clones were either produced from CTL lines or directly from frozen PBMCs. CTL lines were stimulated with matching epitopes and stained with MHC class I tetramer for 15 minutes at 37°C 5% CO_2. Frozen PBMCs were defrosted, washed and counted before being stained with MHC class I tetramer in the same manner as the CTL lines. The cells were then washed once with FACS wash buffer (PBS, 0.5% bovine serum albumin (BSA; Sigma, UK), 0.5mM EDTA) and stained with anti-CD8- allophycocyanin (APC) at room temperature for 15 minutes. The cells were washed a final time before being

resuspended in 500µl of H10 for sorting. Sorted cells were expanded using irradiated allogeneic PBMCs containing 50µg/ml phycohaemagutinin (PHA). Cells were cloned using limited dilution.

2.4 ^{51}Cr release assay

Healthy B cells (up to 5 x 10^6) were pelleted and the last drop of supernatant was removed with a Pasteur pipette. The cells were then resuspended in 200µl (7.4 Mbq) of ^{51}Chromium (^{51}Cr; PerkinElmer) before being incubated at 37°C 5% CO_2 for 1 hour. The cells were then washed 3 times in RPMI before being counted and resuspended at a concentration of 10^5/ml in R10. 50µl of these target cells were added to each well of a 96 well round bottomed plate along with 100µl of CTL clone at desired concentration and 16.7µl of peptide at 10x final concentration required. The control wells were target cells plus 116.7µl RPMI for background release and target cells plus 100µl of 5% Triton-X and 16.7µl of RPMI for total release. The plate was then incubated for 4-6 hours at 37°C 5% CO_2. 40µl of supernatant was removed from each well and added to a Beta-plate (PerkinElmer) along with 150µl supermix solution (PerkinElmer). The radioactivity was measured using a gamma-counter (Microbeta Jet, PerkinElmer). Specific lysis was calculated as follows:

$$\text{Specific lysis} = \frac{\text{experimental counts-media control}}{\text{detergent control-media control}} \times 100$$

2.5 Degranulation assay

48µl/well of CTL clones at a concentration of 5 x 10^5/ml were added to a 96 well plate along with 2µl/well of anti-CD107a Phycoerythrin (PE) -conjugated antibody (BD Biosciences, San Jose, CA). The plate was incubated at 4°C for 30 minutes. 40µl/well of peptide pulsed or recombinant vaccinia virus (rVV) infected, MHC-matched B cell targets were then added to the plate followed by incubation for 1 hour at 37°C, 5% CO_2. 10µl of Brefaldin A at a concentration of 100µg/ml (final concentration 10µg/ml) was added to each well before a further 3 hours incubation at 37°C, 5% CO_2. 2µl/well of anti-CD8 APC-conjugated antibody (BD Biosciences, San Jose, CA) was added to each well followed by 20 minutes incubation at 37°C, 5% CO_2. The cells were then washed twice in FACS wash buffer and resuspended in 200µl of cell fix. Samples were read on a CyAn ADP flow cytometer and analysed using FlowJo software (Tree Star Inc).

2.6 IFN-γ ELISpot

96 well MultiScreen filter plates (MAIP S45, Millipore, MA) were coated with 15µg/ml of anti-IFN-γ capture antibody (1-DIK, Mabtech, Sweden) and incubated at 4°C overnight. The following day the MultiScreen plates were washed 6 times with 200µl/well of RPMI and blocked by the addition of 200µl/well of R10 followed by 2 hour incubation at 37°C 5% CO_2.

ELISpot assays were used to test IFN-γ release by both *ex vivo* PBMCs and CTL clones. When testing PBMCs, 100µl/well of cells at a concentration of 1 x 10^6/ml was

added to the plates along with optimal epitope peptides at a final concentration of 1μg/ml. R10 alone and PHA at a final concentration of 5μg/ml (Murex Biotech Ltd, UK) were used as negative and positive controls respectively. When testing CTL clones, 50μl/well of cells at a concentration of 8 x 10^3/ml (400 cells/well) was added to the plate along with 50μl/well of HLA-matched target B cells pulsed with the optimal peptide epitope or infected with rVV and at a concentration of 5 x 10^5/ml.

Plates were then incubated for 18 hours at 37°C 5% CO_2 before being washed 6 times with 200μl/well phosphate buffered saline (PBS). 50μl/well of biotinylated anti-IFN-γ mAb (Mabtech, Sweden) at a concentration 1μg/ml was added to plates before 2 hours incubation at room temperature. A further 6 washes with PBS were performed before 50μl/well of streptavidin-conjugated alkaline phosphatase antibody (Mabtech, Sweden) was added to the plates. A final incubation of 1 hour at room temperature was performed before the plates were washed 6 times with PBS and developed using an alkaline phosphate conjugate substrate kit (Bio-rad laboratories, Hercules, CA) to detect cytokine producing cells. Developed plates were read using an AID ELISpot reader system (Autoimmune Diagnostika, GmbH).

A positive ELISpot was defined at any result over 20 Spot forming units (SFU)/million PBMCs and greater that three times background.

Peptide	**Abbreviation**	**Location**
AEWDRVHPV	AEW	p24
GELDRWEKI	GEL	p17
IEELRQHL	IEE	RT
IEIKDTKEAL	IEI	p17
IETVPVKL	IET	RT
KEKGGLEGL	KEK	Nef
KELYPLTSL	KEL	p2p7p1p6
KETINEEAA	KET	p24
REPHNEWTL	REP	Vpr
SEGATPQDL	SEG	p24
TERQANFL	TER	p2p7p1p6

Table 2.1 HIV-1 peptides used in *ex vivo* ELISpot assay on PBMCs from HLA-B*40+ patients, along with their location within the viral genome. All are known HLA-B*40 restricted epitopes.

2.7 Infection of B cell lines with recombinant vaccinia viruses

Recombinant vaccinia viruses expressing HIV-1 Nef protein (rVV-Nef) from seven group M subtypes of HIV-1 were acquired from the NIH AIDS Research and Reference Reagent Program: HIV-$1_{92UG037.1}$(A), HIV-1_{MN} (B), HIV-$1_{96ZM651}$ (C), HIV-$1_{94UG114.1}$ (D), HIV-$1_{93BR020}$ (F), HIV-$1_{92NG83.2}$ (G) and HIV-$1_{90CF056}$ (H).

These rVV were used to infect EBV-transformed B cell lines that were then used in various ELISpot and degranulation assays. A defined amount (plaque forming units (PFU)/10^6 target cells) of rVV was used to resuspend pellets of target cells previously washed once in RPMI. The cells were then incubated for 1 hour at 37°C, 5% CO_2. 1ml of R10 was then added before the cells were incubated in the same conditions for a further 2 hours. Finally, the cells were washed three times and resuspended at the required concentration to be used in the assay.

2.8 Generation of peptide-MHC tetramers

MHC class I heavy chain and β2M were expressed by transforming HMS174 bacteria (Novagen) with expression plasmid PET23. The protein was then purified from inclusion bodies and urea solubilised. β2M (10mg), the required peptide (10mg) and MHC class I heavy chain (30mg) were added to refolding buffer (100mM Tris, 400mM L-arginine, 2mM EDTA, 5mM reduced glutathione, 0.5mM oxidised glutathione, 0.1mM PMSF in 1 litre of H_2O) and incubated with continuous stirring for 48 hours at 4°C. The protein was concentrated to a volume of 5-10ml using an ultrafiltration membrane

(Millipore, UK) at 4°C and 50psi pressure. Buffer exchange was performed using PD1 columns (GE healthcare, UK) before the protein was biotinylated using BirA enzyme (Avidity, Aurora, CO). Fast liquid protein chromatography (FPLC) using a G75 superdex column was used to purify the protein. Following further concentration the protein was frozen in 50µg aliquots. The protein was tetramerised by conjugation to ExtrAvidin®-R-phycoerythrin (PE) conjugate (Sigma, UK) before use.

HLA-B*4001	**HLA-B*4002**
IEELRQHLL	AEWDRVHPV
IEIKDTKEAL	IEIKDTKEAL
KEKGGLEGL	KEKGGLEGL
KELYPLTSL	KELYPLTSL
REPHNEWTL	KETINEEAA
	SEGATPQDL

Table 2.2 Peptides folded with the HLA-B*4001 and the HLA-B*4002 heavy chain.

2.9 Tetramer staining

MHC class I tetramer staining of whole blood, PBMCs and CTL clones was undertaken. 150µl of whole blood or 10^6 cells were stained with 1µl of PE-conjugated tetramer at 37°C, 5% CO_2 for 15 minutes. In the case of whole blood 2ml of red cell lysis buffer (BD pharmingen) was then added followed by mixing and incubation at room temperature for 15 minutes. All sample types were washed with FACS wash buffer before the addition of other surface staining antibodies; 2µl of CD27-APC, 4µl of CD8-peridinin chlorophyll protein (PerCP) and 10µl of CD28-fluorescein isothiocyanate (FITC; BD Biosciences, San Jose, CA) were added to the cells prior to incubated at room temperature in the dark for 15 minutes. A final wash step was performed before the cells were fixed in 200µl of cell fix (BD Biosciences). Samples were read on a CyAn ADP flow cytometer and analysed using FlowJo software (Tree Star Inc).

2.10 T cell receptor landscaping

Freshly-thawed PBMC were stained with MHC class I tetramer and anti-CD8 APC and sorted as described above. RNA was extracted from the sorted cells using an RNAqueous® Kit (Ambion, Austin, TX) and single stranded cDNA was generated using the cloned AMV first strand synthesis kit (Invitrogen, Carlsbad, CA). The cDNA was then sent to TcLand for 'landscaping', whereby quantitative PCR is used to determine the relative proportion of the epitope specific CD8+ T cell response that utilises each Vβ region of the TCR.

2.11 Vβ antibody staining

CTL clones were stained with 2µl each of a panel of FITC-conjugated antibodies specific for 22 different variable domains of the TCR β chain. After 20 minutes incubation in the dark at room temperature the cells were washed twice in FACS wash buffer and fixed in 200µl of cell fix (BD Biosciences). Samples were read on a CyAn ADP flow cytometer and analysed using FlowJo software (Tree Star Inc).

2.12 PCR and sequencing

RNA was extracted from CTL clones using an RNAqueous® Kit (Ambion, Austin, TX) and single stranded cDNA was generated using the cloned AMV first strand synthesis kit (Invitrogen, Carlsbad, CA). Using the cDNA as a template, polymerase chain reactions (PCR) were performed with pfu enzyme (Invitrogen, Carlsbad, CA). The primers used were 5' TGAATGCCCCAACAGCTCTC 3' (Vβ7 specific, forward) and 5' TGTCTCTACCCCAGGCCT 3' (Cβ specific, reverse) and the reaction conditions were as follows:

Step	Temperature	Time	No. of cycles
Hot Start	94°C	240 seconds	
Denaturing Annealing Extension	94°C 55°C 68°C	60 seconds 60 seconds 120 seconds	35
Denaturing	94°C	60 seconds	
Annealing	55°C	60 seconds	
Extension	68°C	420 seconds	

Table 2.3 PCR conditions

Amplified PCR samples were electorphoresed through agarose gels and purified with a PCR purification kit (Qiagen). Sequencing was then performed by John Franklin at the Human Immunology Unit (HIU) sequencing facility at the Weatherall Institute of Molecular Medicine (WIMM) using an automated ABI-377 DNA sequencer (Applied Biosciences Inc., USA).

2.13 Cytokine quantification

Supernatant from CTL clones cultured for 24 hours with MHC-matched target cells and specific peptide was analysed for cytokine concentration using an x-plex bead array system (Bio-Rad, Hercules, CA). The cytokines assayed for were IL-2, IL-4, IL-10, IL-13, TNF-α, MIP-1β, IFN-γ and RANTES. The assay was carried out as per the manufacturer's instructions. In brief, antibody-bound fluorescent beads were added to a 96-well filtration plate containing supernatant, standards or blanks in a final volume of

100µl. Plates were incubated on a plate shaker at 300 rpm for 30 minutes at room temperate. Biotinylated anti-cytokine detection antibodies were added following a wash step and incubated for 30 minutes at room temperature with continuous shaking at 300 rpm. The beads were again washed and streptavidin-PE was added for an incubation of 10 minutes at room temperate with continuous shaking at 300 rpm. A final wash step was performed before the samples were read on a luminex 100 analyzer (Bio-Rad, Hercules, CA).

2.14 Viral Suppression assay

C8166 cells were incubated with $TCID_{50}$ HIV-1 MN for 90 minutes at 37°C 5% CO_2. The cells were then washed twice in 10ml of RPMI, spinning at 1500rpm for 5 minutes. They were then resuspended in H10 at the desired concentration before 100µl was added to each well of a 96 well flat-bottomed plate along with 100µl of CTL clones in H10+IL-2 (200U/ml). The plate was then incubated at 37°C 5% CO_2 for 5 days before the supernatant was used to perform a p24 Capture ELISA (ImmunoDiagnostics, Inc., USA) as per the manufacturer's instructions.

Briefly, supernatant was diluted in dilution buffer and added to the pre-coated ELISA plate along with the p24 standards. The plate was incubated for 1 hour at room temperature. Three wash steps were performed before the detector reagent was added. The plate was again incubated at room temperature for 1 hour and washed three times. 100µl/well of the TMB substrate was then added and left for 10 minutes before the

reaction was stopped with 100µl/well of stop solution. The plate was read on an ELISA plate reader at 450nm.

Chapter 3: Longitudinal analysis of an immunodominant HLA-B*08 restricted nef specific CTL response from a single rapid progressor patient

3.1 Background

A794 was a male patient who experienced symptomatic viral illness following high risk sexual exposure to HIV-1 and was subsequently referred to the San Diego AIDS treatment center for assessment. Patient A794 was classified as a rapid progressor and was placed on ART upon diagnosis of acute HIV-1. Samples were taken at various time points throughout acute and early chronic infection, allowing for longitudinal analysis of the CTL response towards the virus.

Whilst the reason some HIV-1 infected patients rapidly progress to AIDS is not fully understood several factors have been correlated with poor viral control. As is the case for slow disease progression, possession of certain HLA alleles has been strongly associated with rapid progression. As previously mentioned HLA-B*35 is linked to fast onset of AIDS (Carrington, Nelson et al. 1999), as are HLA-A*68, -B*53 and -Cw*04 (Dean, Carrington et al. 2002). Being homozygous at 1 or more HLA loci is also correlated with reduced time to AIDS (Carrington, Nelson et al. 1999) suggesting that presenting a wide variety of antigenic peptides is of use during HIV-1 infection.

Ineffective innate immune mechanisms have also been implicated in poor viral control and rapid HIV-1 progression. Low numbers of NK cells in the blood of HIV-1

infected individuals have been associated with progression to AIDS within 6 years of seroconversion (Bruunsgaard, Pedersen et al. 1997). Furthermore polymorphisms in Toll-like receptor (TLR) 9, a pattern recognition receptor involved in sensing bacterial and viral infections by binding to CpG DNA motifs have also been associated with rapid HIV-1 disease progression (Bochud, Hersberger et al. 2007).

Polymorphisms in the molecules involved in HIV-1 cell entry have been implicated in rapid disease progression. HIV-1 infected patients homozygous for a structural variant of the co-receptor CX_3CR1 progress to AIDS more rapidly than patients with other haplotypes (Faure, Meyer et al. 2000). In addition, polymorphisms in the promoter region of the co-receptor CCR5 are associated with poor disease outcome in HIV-1 infection (Martin, Dean et al. 1998).

Associations between certain viral factors and rapid disease progression have also been observed. It has been shown that dual infection with two HIV-1 strains leads to a rapid decline to AIDS (Gottlieb, Nickle et al. 2004). Also infection with subtype D HIV-1 as compared to other subtypes present has been associated with poor disease outcome in both a Ugandan and a Tanzanian cohort of infected patients (Vasan, Renjifo et al. 2006; Baeten, Chohan et al. 2007).

Whilst most of the factors described above have been observed in single studies examining small numbers of patients, the association of particular HLA alleles with rapid disease progression comes from several studies investigating much larger cohorts of patients. As is the case with slow disease progression, HLA alleles are the factor most commonly and robustly associated with fast disease progression. This strongly implicates defective CTL responses as a contributing factor in rapid decline to AIDS.

In order to aid in focused and successful vaccine design it is not only important to understand why certain CTL responses are effective at controlling HIV-1 replication but also why certain CTL responses control viral replication poorly if at all. With the aim of taking a step towards this goal, the immunodominant CD8+ T cell response from patient A794 was examined at various time points during both acute and chronic infection.

Throughout the HIV-1 epidemic immune responses that occur during acute viral infection have been investigated much less frequently than those that take place during the chronic stage of disease. This is mainly due to the fact that diagnosis of HIV-1 infection predominantly occurs during chronic infection, making patient samples acquired during early infection hard to come by. However in recent years, through an increase in public health initiatives to screen high risk individuals and the establishment of global consortia, many more acute phase HIV-1 samples have become available for study. This phenomenon was driven by and has reiterated the fact that events that occur during early HIV-1 infection have a bearing on the outcome of infection.

After the initial peak in viremia that occurs during acute HIV-1 infection a viral load set point is established. The level of virus present at this point has been robustly associated with outcome of infection (Mellors, Rinaldo et al. 1996; Mellors, Margolick et al. 2007). The reduction in viral load that occurs during acute infection temporally coincides with the appearance of the first virus-specific CD8+ T cell responses. These CD8+ T cells are directed against a relatively small number of epitopes and during chronic infection an increase in the breadth and magnitude of the CTL response occurs. However this does not lead to an increase in viral control. This suggests that if the CTL response present during acute infection is indeed responsible for the reduction in viral

load that takes place at this time then early induced CD8+ T cells may possess certain properties not displayed by CTL during chronic infection (Streeck and Nixon 2010).

Examining an immunodominant HIV-1 specific CD8+ T cell response from a rapid progressor patient during both acute and chronic infection identified a particular TCR clonotype that was present during acute infection and predominated during chronic infection. CD8+ T cell clones that utilised this TCR and generated using both acute and chronic PBMC samples were found to differ in functional avidity by 1 log with the lower avidity clone originating from the chronic sample. This reduction in functional avidity was associated with a reduction in polyfunctionality and the secretion of the immunosuppressive cytokine IL-10.

These results indicate that the presence of an immunodominant highly avid HIV-1 specific CTL response during acute infection is not enough to ensure viral control. It also suggests that a CTL response that displays high avidity during acute infection may lose the ability to recognise low amounts of antigen as HIV-1 infection persists. Therefore factors other than functional avidity should be taken into account when designing vaccines to elicit anti-viral CTL responses.

3.2 Results

3.2.1 The HIV-1 specific CTL response elicited by patient A794 is dominated by HLA-B*08 restricted nef specific CD8+ T cells

MHC class I tetramers matched to A794's HLA background (outlined in table 3.1) refolded with immunodominant HIV-1 peptide epitopes were used to stain patient A794's PBMCs at various time points throughout the course of infection. Of all the tetramers tested, the HLA-B*08 tetramer specific for the nef epitope FLKEKGGL (FLK) bound to the highest percentage of CD8+ T cells at all time points (figure 3.1). These experiments were carried out by a former lab member, Pokrath Hansasuta.

A locus		B locus		C locus	
HLA-A*01	HLA-A*03	HLA-B*07	HLA-B*08	HLA-Cw*0701	HLA-Cw*0702

Table 3.1 HLA type of patient A794

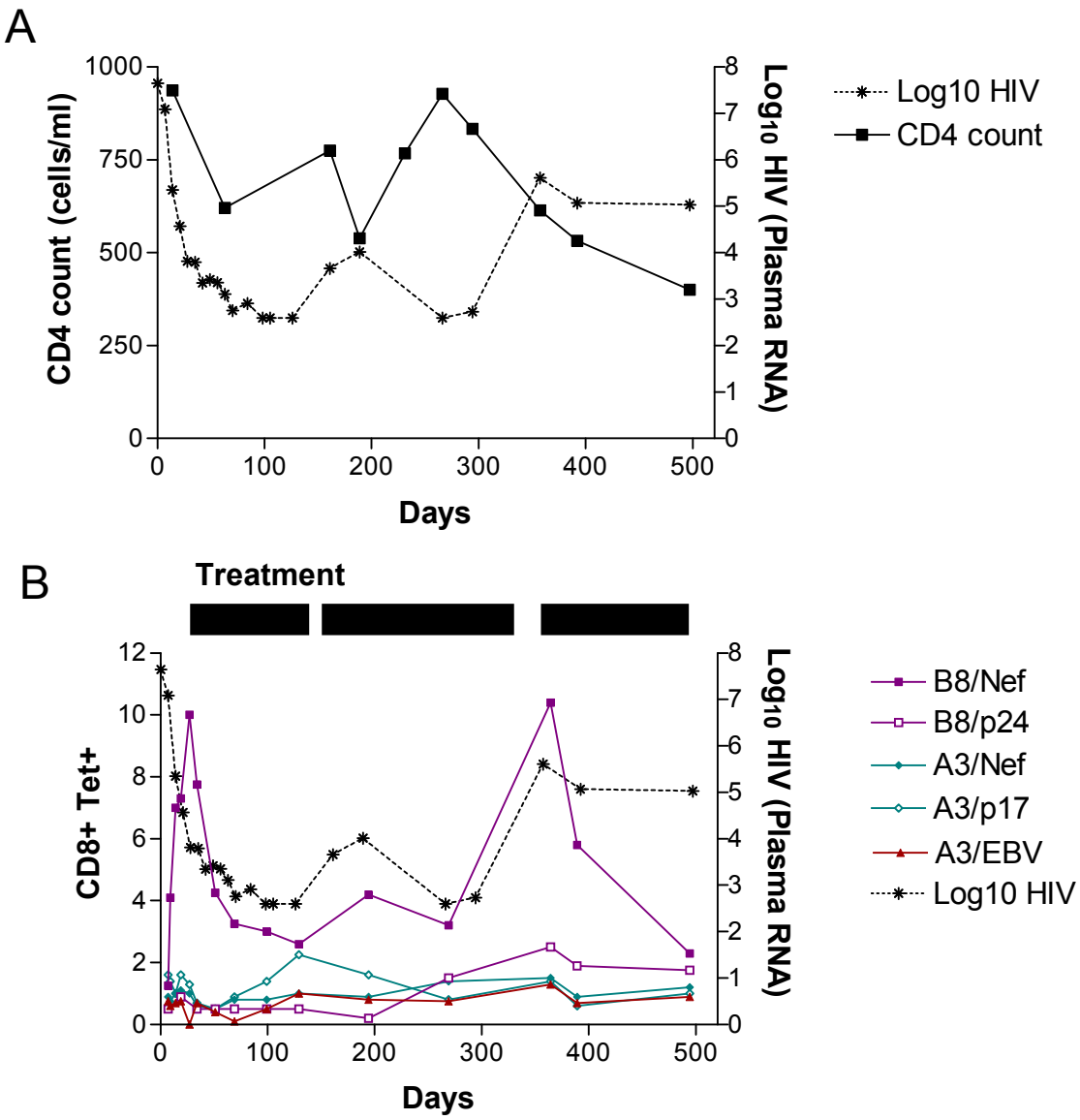


Figure 3.1 Patient A794 has an immunodominant HLA-B*08 restricted Nef specific CD8+ T cell response at all time points measured. (A) CD4 count and plasma viral load of patient A794 throughout the course of acute and early chronic infection, days indicates time post presentation with symptomatic acute HIV-1 infection. (B) The kinetics of the HIV-specific CTL response of patient A794 as shown by the percentage of CD8+ T cells binding to four HIV-1-specific MHC class I tetramers. The tetramers used in this study were as follows: HLA-B*08-FLKEKGGL (B8/Nef), HLA-B*08-DIYKRWII (B8/p24), HLA-A*03-QVPLRPMTYK (A3/Nef), HLA-A*03-KIRLRPGGK (A3/p17) and HLA-A*03-RLRAEAQVK (A3/EBV). Patient A794 was on ART as indicated by the black bar above figure B. Drug regime throughout the course of treatment was AZT/ZDV/Zidovudine retrovir, Indinavir/IDV/Crixivan and Lamivudine/3TC/Epivir.

3.2.2 The T cell receptor repertoire of the HLA-B*08 restricted FLK specific response narrows over time with T cells utilising Vβ7 predominating during chronic infection

Utilising quantitative PCR on DNA extracted from HLA-B*08 FLK tetramer sorted cells from patient A794, TCR landscaping was performed by TcLand Biotech. This technique was used to elucidate the TCR Vβ usage and CDR3 length of the FLK specific CD8+ T cells at various time points during infection as well as the frequency of each Vβ with the specific population.

As illustrated by figure 3.2 the TCR repertoire is heterogeneous during acute infection with six Vβ domains (2, 5.2, 6.1, 6.2, 7 and 13.1) used by substantial populations of FLK specific CD8+ T cells. This heterogeneity is lost over the course of infection and by day 359 most FLK specific CD8+ T cells display TCR utilising Vβ7, with a small population using Vβ13.1.

Heterogeneity is also reduced within specific Vβ+ populations as shown in figure 3.3. During acute infection there are two predominant populations of Vβ7+ cells with different CDR3 regions, one two amino acids longer than the other. During chronic infection T cells expressing Vβ7+ TCR with the longer CDR3 region are present at a much lower frequency.

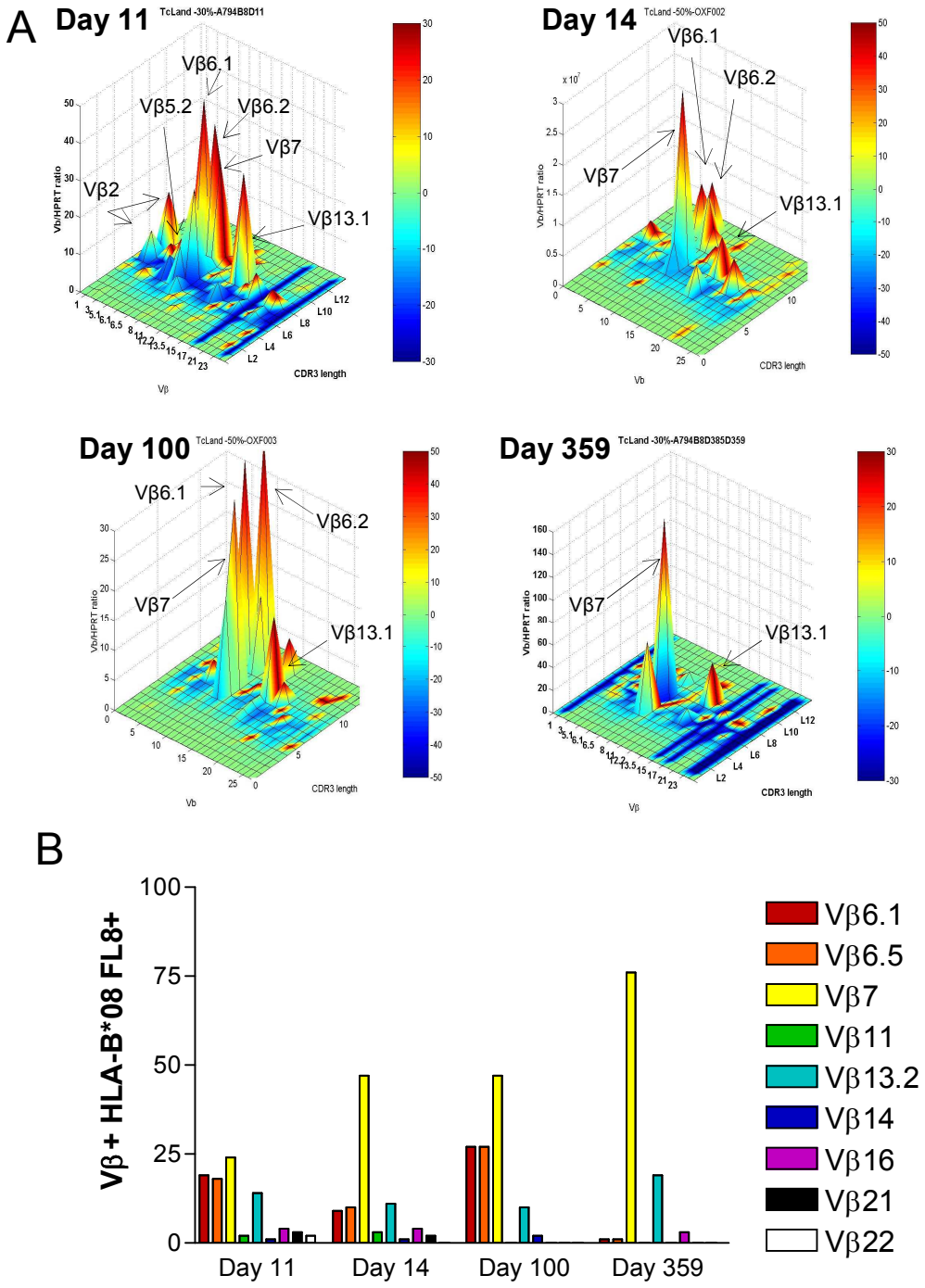


Figure 3.2 The TCR usage of the HLA-B*08 FLK CD8+ T cells changes throughout the course of disease. TCR landscape data was generated by performing quantitative PCR of the TCR β chain on HLA-B*08 FLK specific CD8+ T cells extracted from patient A794 at the time points shown. (A) Heat plots indicate the dominant Vβ used throughout the disease course. (B) A bar graph displaying the percentage of the total HLA-B*08 FLK specific T cells expressing of the most common TCR Vβ chains at stated time.

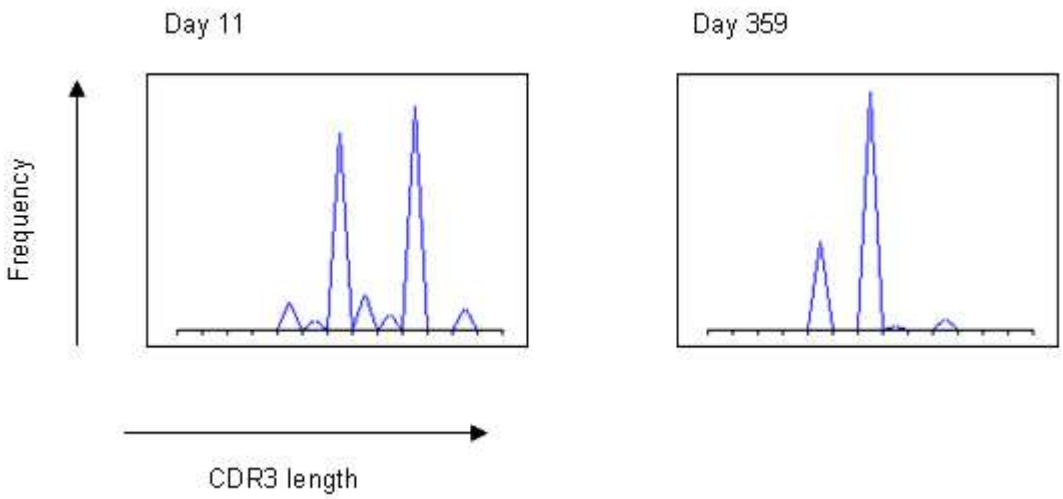


Figure 3.3 Changes in the CDR3 length of the Vβ7+ HLA-B*08 FLK specific cells between acute and chronic infection. TCR landscape data was generated by performing quantitative PCR of the TCR β chain on HLA-B*08 FLK sorted CD8+ T cells extracted from patient A794 at the time points shown.

3.2.3 HLA-B*08 restricted FLK specific CTL clones expressing the same TCR but isolated at different time points during infection differ in their functional avidity

HLA-B*08 FLK tetramers were used to sort CD8+ T cells from PBMCs isolated from patient A794 during both acute and chronic disease. These cells were then cultured in limiting dilution to produce CTL clones. The clones were stained with a panel of Vβ specific antibodies. This revealed that 5 out of 9 (56%) clones produced from PBMCs isolated during acute infection (day 11 post presentation with symptomatic viral illness) were positive for a Vβ7.2 antibody. 9 out of 15 (60%) clones produced from a chronic PBMC sample (day 359) were positive for the same antibody. This fits with the TCR landscaping data that showed TCR utilising the Vβ7 domain to be present throughout infection, predominating in the latter stages.

The sequence of the CDR3 region of the TCR β chain was determined for several clones using PCR and is outlined in table 3.2. Two Vβ7.2+ clones from acute infection were found to possess the same CDR3 region, whilst a third acute clone was found to utilise a different CDR3 region two amino acids longer. Both chronic clones for which the TCR sequence was determined also used the shorter β chain CDR3 region. This corroborates the landscaping data showing two predominating CDR3 lengths within the Vβ7+ FLK specific CD8+ T cells, one two amino acids longer than the other.

Clone	Vβ	CDR3	Jβ
11-7	7.2	SHVTSGGSRLTGELF	2.2
11-11	7.2	SQDGGQMNTEAF	1.1
11-16	7.2	SHVTSGGSRLTGELF	2.1
359-6	7.2	SQDGGQMNTEAF	1.1
359-9	7.2	SQDGGQMNTEAF	1.1

Table 3.2 Sequence analysis of the CDR3 regions of the T cell receptor-β chains of the FLK-specifc CTL clones derived from samples taken from patient A794 at two time-points post-presentation, day 11 and day 359.

An IFN-γ ELISpot assay was used to determine the functional avidity of the clones (figure 3.4). Clones were incubated with HLA-B*08+ target cells and decreasing amount of FLK peptide. The functional avidity was defined as the concentration of peptide that elicited half the maximal IFN-γ release. Despite using the same TCR, clone 11-11 has a functional avidity of 0.065μg/ml, around half a log lower than both 359-6 and 359-9 (table 3.3). Clone 11-16 has the lowest avidity, requiring the greatest amount of peptide (0.55μg/ml) to produce half maximal IFN-γ release.

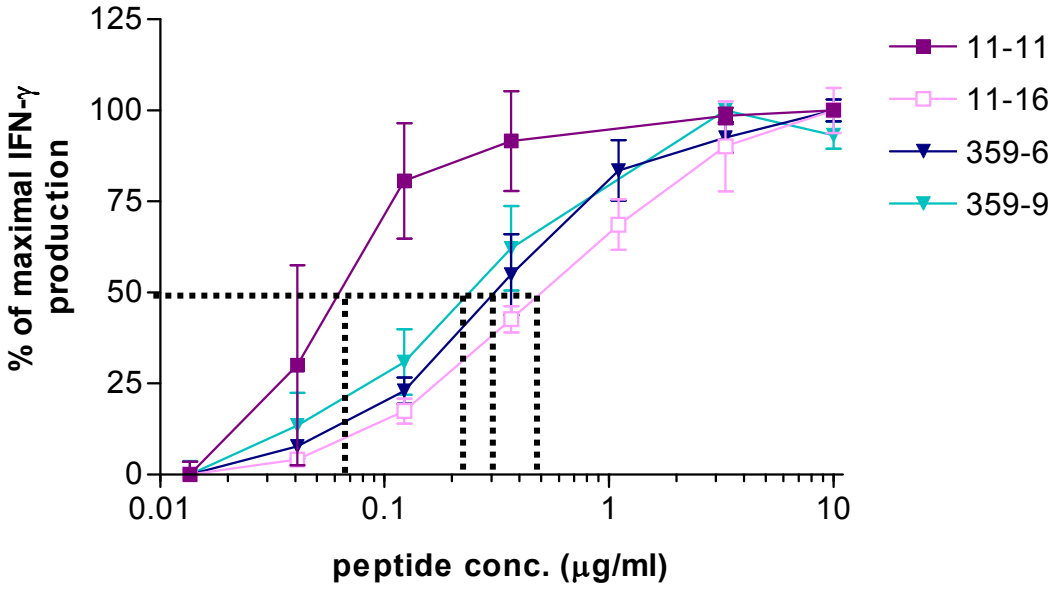


Figure 3.4 The HLA-B*08 restricted FLK specific clones display a range of functional avidities. Clones were incubated with HLA-B*08+ B cells and decreasing amounts of FLK peptide in an IFN-γ ELISpot assay. The functional avidity of the clones is defined as the concentration of peptide that induced half the maximal IFN-γ production.

Clone	Disease stage	CDR3	Functional Avidity (µg/ml)
11-11	acute	short	0.065
11-16	acute	long	0.55
359-6	chronic	short	0.3
359-9	chronic	short	0.25

Table 3.3 Properties of HLA-B*08 FLK clones

3.2.4 The difference in functional avidity between the clones does not stem from a difference in TCR avidity for peptide-MHC or a difference in CD8 expression

The avidity of the TCR expressed by the clones for p-MHC was assessed by measuring the percentage of CD8+ cells that bound to the HLA-B*08 FLK tetramer at decreasing concentrations. At all concentrations tested there was no difference between clones in the percentage of CD8+ cells that bound to the tetramer indicating no variation in the TCR avidity for p-MHC (figure 3.5).

It has been shown that the surface expression of the CD8 coreceptor molecule can modulate the functional avidity of a CTL response (Kroger and Alexander-Miller 2007), therefore clones 11-11, 11-16 and 359-6 were stained with anti-CD8 to determine expression levels. The difference in functional avidity between the FLK specific clones does not stem from a difference in CD8 surface expression as the anti-CD8 antibody stained all three clones comparably (figure 3.6).

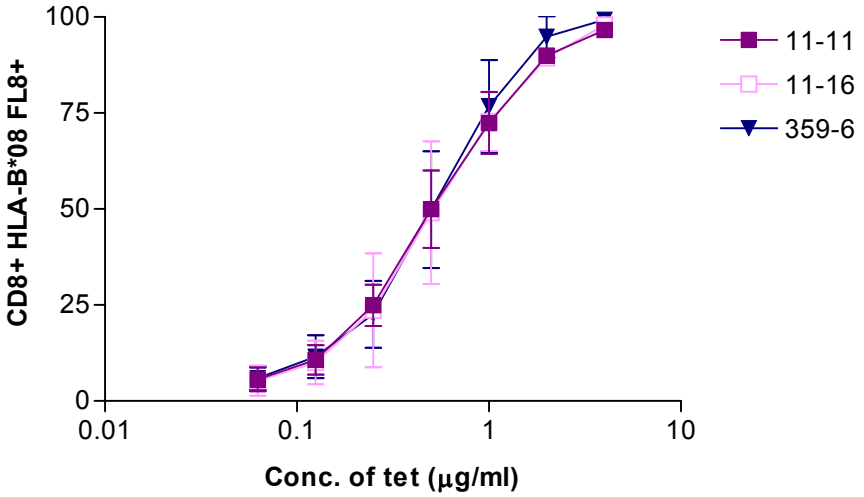


Figure 3.5 The TCR avidity of the clones for peptide-MHC does not differ. Clones were stained with HLA-B*08 FLK tetramers of decreasing concentrations and anti-CD8 antibody. Results are expressed as the percentage of CD8+ cells that stained positive for the tetramer.

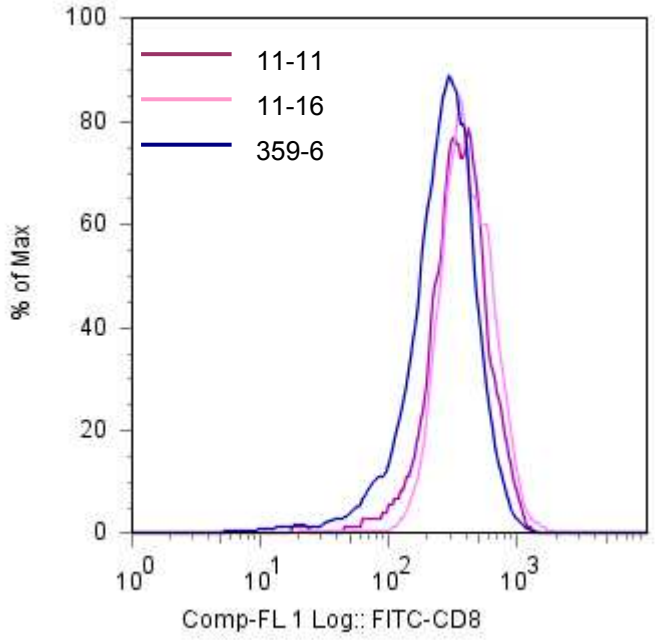


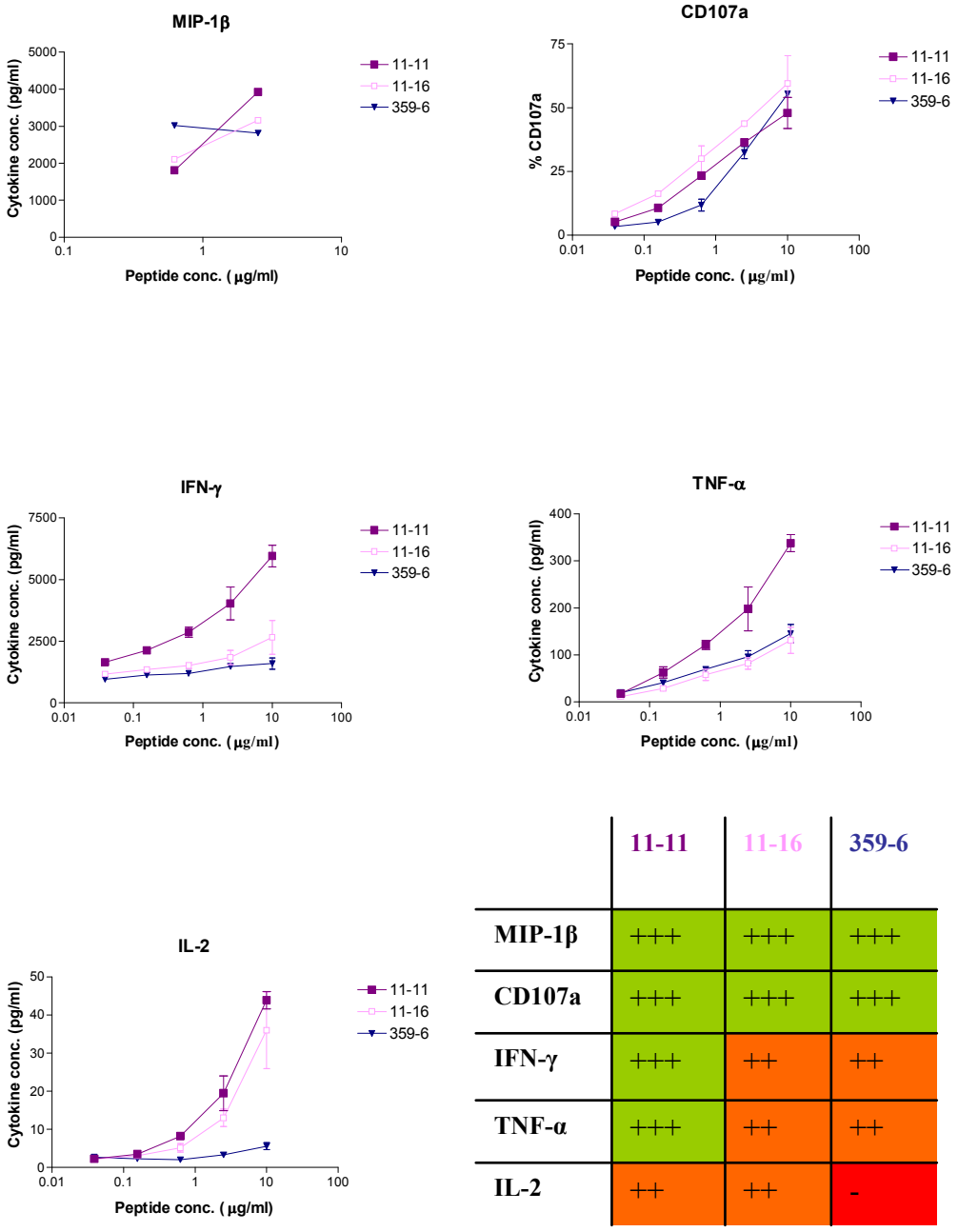
Figure 3.6 The FLK clones do not differ in their expression of CD8. The above histogram shows the expression of CD8 on clones 11-11, 11-16 and 359-6 stained with CD8-FITC on day 10 following re-stimulation.

3.2.5 Clones 11-11 and 11-16 are capable of secreting IL-2 upon antigen stimulation and are hence more polyfunctional than clone 359-6 that does not secrete this cytokine when stimulated

Polyfunctionality is defined as the ability of a cell to produce several effector functions, namely to secrete IFN-γ, MIP-1β, TNF-α, IL-2 and to degranulate (Betts, Nason et al. 2006). It has been shown to correlate with functional avidity, with highly avid clones being more polyfunctional than those that require higher amounts of peptide to elicit a half maximal effector response.

In this study the bulk polyfunctionality of the HLA-B*08 FLK clones was assessed using a bead array system to measure the amount of IFN- γ, MIP-1β, TNF-α and IL-2 in culture supernatant. Clones were incubated with HLA-B*08+ B cells and increasing concentrations of peptide for 24 hours before the supernatant was removed and assayed for cytokine concentration. CD107a expression, a maker of cellular degranulation, was measured per cell following stimulation of the clones for 4 hours with peptide-pulsed B cells and then staining with a CD107a specific antibody.

These tests revealed 11-11 to be more polyfunctional than 359-6, producing more IFN-γ and TNF-α at higher peptide concentrations and retaining the ability to produce IL-2. 359-6 does not produce any IL-2, even following stimulation with high concentrations of peptide. The least functionally avid of the tested clones, 11-16 was also observed to be more polyfunctional than 359-6, being capable of secreting IL-2 upon stimulation (figure 3.7).



	11-11	11-16	359-6
MIP-1β	+++	+++	+++
CD107a	+++	+++	+++
IFN-γ	+++	++	++
TNF-α	+++	++	++
IL-2	++	++	-

Figure 3.7 Polyfunctionality of the clones does not correlate with functional avidity. Whilst all clones still produce MIP-1β, CD107a, IFN-γ and TNF-α at high peptide concentrations 359-6 does not produce IL-2. Clones were incubated with a peptide pulsed HLA-B*08+ B cell line for 4 hours and then stained for CD107a or for 24 hours before the culture supernatant was assayed for cytokine concentration using the Bio-rad luminex system.

3.2.6 Upon antigenic stimulation clone 11-11 produces type 2 cytokines and clone 359-6 produces IL-10

Supernatant taken from co-culturing clone 11-11 with peptide pulsed B cells for 24 hours was observed to contain the type 2 cytokines IL-4 and IL-13 at higher concentrations, as measured by a bead array system, in comparison to the supernatant taken from co-culturing clones 11-16 and 359-6 with peptide pulsed B cells (figure 3.9). The concentration of both cytokines increased in a peptide dependent manner. Both 11-16 and 359-6 produced small amounts of IL-4 upon stimulation with high concentrations of peptide antigen and constitutively secrete relatively high levels of IL-13. Under the same co-culture conditions clone 359-6 secretes IL-10 in a peptide dependent manner which is not the case for the other clones tested (figure 3.8).

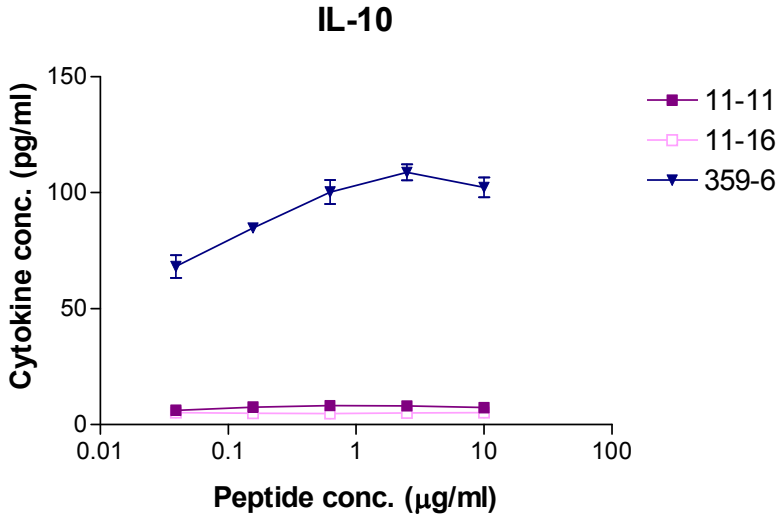


Figure 3.8 359-6 produces IL-10 when stimulated with antigen. Clones were incubated with a peptide pulsed HLA-B*08+ B cell line for 24 hours before the culture supernatant was assayed for cytokine concentration using the Bio-rad luminex system.

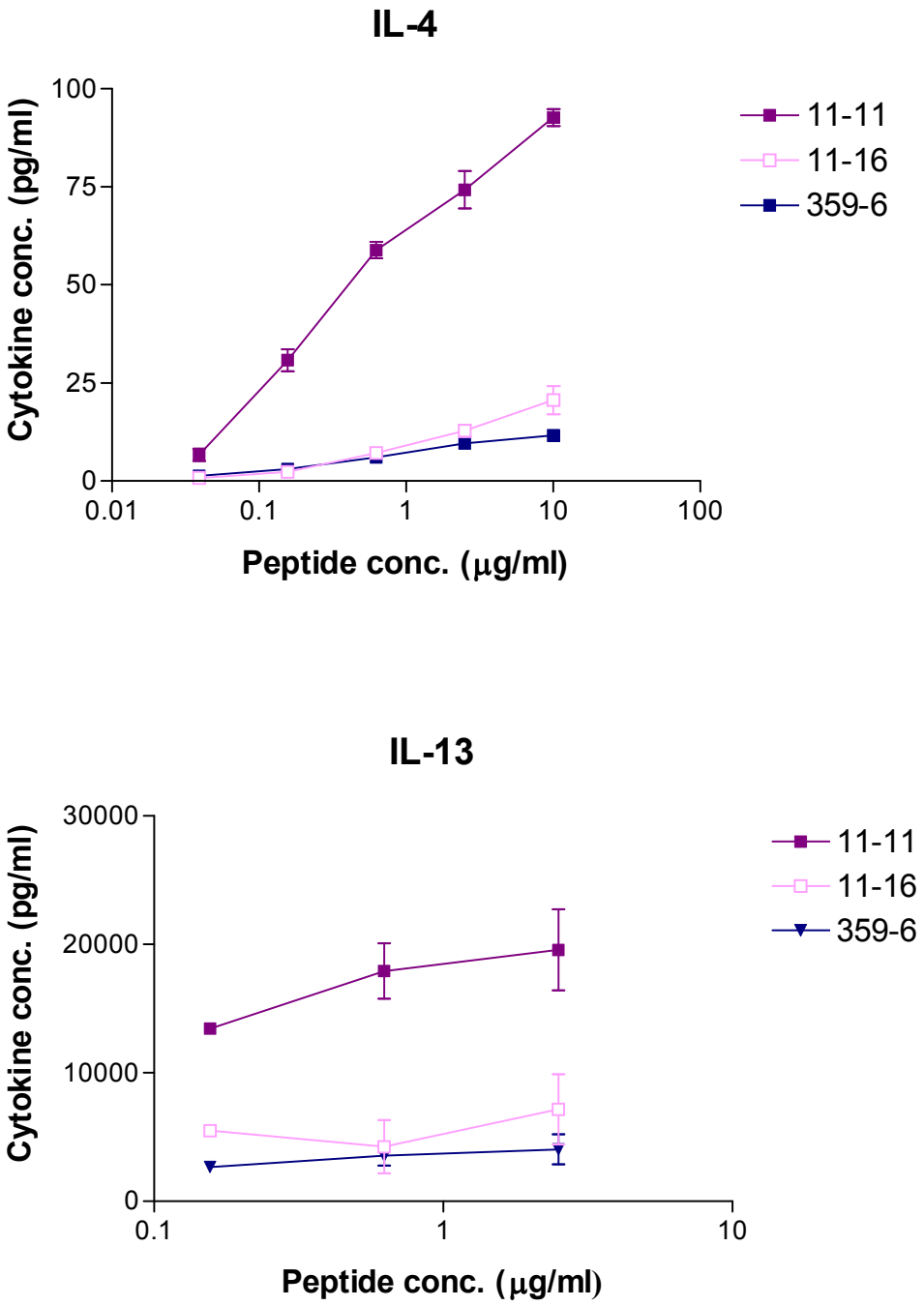


Figure 3.9 11-11 produces type 2 cytokines upon antigen stimulation. Clones were incubated with a peptide pulsed HLA-B*08+ B cell line for 24 hours before the culture supernatant was assayed for cytokine concentration using the Bio-rad luminex system.

3.2.7 *In vitro* viral suppression of lab strain HIV-1 MN does not differ between the HLA-B*08 restricted FLK specific CTL clones

The ability of the HLA-B*08 restricted FLK specific CTL clones to suppress viral replication *in vitro* was assessed by co-culturing the clones for 4 days with C8166, an HLA-B*08+ CD4+ T cell line, infected with lab strain HIV-1 MN. Supernatant was then removed and the HIV-1 p24 concentration was determining by ELISA. At the effector to target ratios of 1:10 and 1:20 clones 11-11, 11-16 and 359-6 all suppressed viral replication to near undetectable levels (figure 3.10). This suppression was HLA dependent as an HLA-B*07 restricted nef specific clone (J69 RP9) only marginally reduced the amount of HIV-1 p24 in the culture supernatant as compared to infected cells cultured with no clone at all.

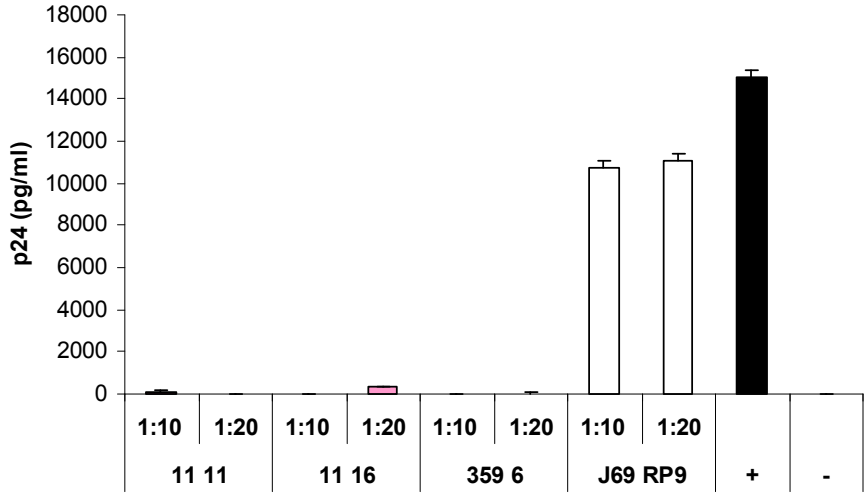


Figure 3.10 All clones suppress replication of HIV-1 *in vitro*. Clones were incubated with a C8166 CD4+ cell line infected with lab strain HIV-1 MN for 4 days at the effector:target ratios of 1:10 and 1:20 before 100µl of the supernatant was assayed by ELISA for amount of HIV-1 p24 present. J69 RP9 is an HLA mismatched clone specific for a HLA-B*07 restricted nef epitope. The positive control was infected cells incubated without clones and the negative control was uninfected cells.

3.2.8 During chronic infection the appearance of virus quasispecies containing FLK escape mutations occurs

Sequencing of plasma virus was carried out by Caroline Ignacio, a member of Prof. Doug Richman's group at The University of California San Diego. It revealed that the FLK epitope remained wild type until the last sample tested at day 497 (table 3.4). At this time point the sequencing trace revealed two peaks at certain nucleotide positions indicating the presence of viral quasispecies. Some of these possible nucleotide changes resulted in amino acid substitutions as outlined in table 4.4, two of which fall within the FLK epitope. These are an E to D substitution at position 4 and a K to E substitution at position 5.

The three possible mutant epitopes (FLKDKGGL, FLKEEGGL and FLKDEGGL) were tested for recognition by the HLA-B*08 restricted FLK specific clones in an IFN-γ ELISpot. Neither 11-11, 11-16 nor 359-6 could recognise any of the mutant peptides (figure 3.11).

Day	Sequence
7	VGFPVRPQVPLRPMTYKGAVDLSHFLKEKGGLEGLVHSQKRQEILDLWVYHTQGYFP
105	VGFPVRPQVPLRPMTYKGAVDLSHFLKEKGGLEGLVHSQKRQEILDLWVYHTQGYFP
126	VGFPVRPQVPLRPMTYKGAVDLSHFLKEKGGLEGLVHSQKRQEILDLWVYHTQGYFP
497	VGFPVRPQVPLRPMTYKGAVDLSHFLKEKGGLEGLVHSQKKQEILDLWIYHTQGYFP K N A DE IY R

Table 3.4 The sequence of the FLK epitope remains wildtype throughout the course of infection. At the last time point certain nucleotide positions display two peaks on the sequencing trace and hence are represented by ambiguity codons. This indicates the presence of viral quasispecies with any combination of the point mutations highlighted in purple.

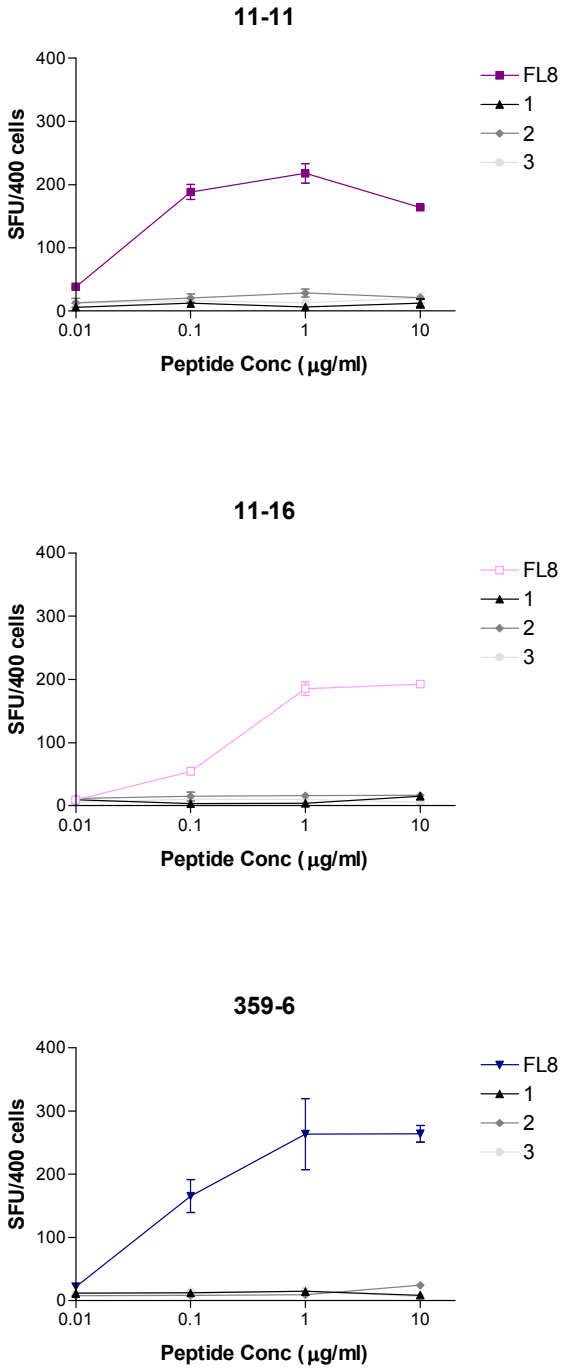


Figure 3.11 None of the clones recognise any of the possible escape mutations. IFN-γ ELISpot assays were carried out to measure the responses of clones 11-11, 11-16 and 359-6 to the three possible mutant epitopes (1=FLKDKGGL, 2=FLKEEGGL, 3=FLKDEGGL). All clones responded to the wild type epitope whilst no response to any of the mutant peptides could be measured

3.3 Discussion

Functional avidity, the concentration of peptide antigen required to induce a half maximal effector function, is a critical determinant of CD8+ T cell anti-viral activity. CTL capable of exerting effector functions in response to lower amounts of antigen can lyse infected target cells at earlier time points *in vitro* (Derby, Alexander-Miller et al. 2001) and are more efficient at controlling viral infections *in vivo* (Alexander-Miller, Leggatt et al. 1996), as compared to CD8+ T cells that required higher concentrations of peptide to produce a response. During HIV-1 infection it has been demonstrated that high avidity immunodominant virus specific CD8+ T cells are found within patients that possess lower cVL (Almeida, Price et al. 2007).

The data presented in this chapter demonstrates that two HLA-B*08 restricted nef specific CTL clones that share the same TCR but were generated from PBMC samples taken from a single patient at different time points almost one year apart during HIV-1 infection differ in functional avidity by almost one log. The clone generated from a sample obtained during chronic infection (359-6) requires a higher concentration of peptide epitope to induce half maximal IFN-γ release as compared to a CTL clone generated from an acute sample (11-11).

Clones 11-11 and 359-6 share the same TCR β chain, it is therefore possible that they originate from the same naïve precursor cell, although such an assumption cannot be proven definitively and sequencing of the α chain would be required to ensure that the TCR was fully shared between the clones. Clone 11-11 presumably represents the TCR clonotype soon after priming whilst clone 359-6 is representative of the response

following several rounds of expansion. There are two non-mutually exclusive mechanisms that would result in the difference in functional avidity displayed by these two clones. Either at the time of priming the precursor cell gave rise to progeny of differing functional avidity or the original expansion was of T cells with the same functional avidity that then went on to change in avidity over time due to antigenic exposure events and the cytokine environments encountered.

It has been shown *in vitro* that CD8+ T cells can modulate their functional avidity after priming. Induction of highly avid CTL can be achieved via stimulation with low concentrations of peptide, however subsequent stimulation with low or high doses of antigen can lead to a modulation in the functional avidity of a given CTL response (Kroger and Alexander-Miller 2007). Given that clone 359-6 was isolated from PBMCs extracted during chronic infection it can be assumed that it had undergone a greater amount of antigen stimulation as compared to clone 11-11 which may have led to a reduction in functional avidity.

Determinants of CTL functional avidity are far from fully understood and several factors have been implicated in influencing the responsiveness of a CD8+ T cell to its cognate antigen. These include the affinity of the TCR for p-MHC, surface expression of co-receptors such as CD8 (Kroger and Alexander-Miller 2007) and adhesion molecules, as well as the effectiveness of the signal transduction pathway (Slifka and Whitton 2001). The difference in functional avidity seen between clones 11-11 and 359-6 can not be explained by an increase in the affinity of the TCR for p-MHC as both clones possess the same TCR and bind to FLK specific tetramer with identical avidities. The expression of CD8 is also comparable between the two clones.

It is possible however that clone 11-11 is more efficient at transducing the TCR signal upon p-MHC binding, therefore leading to an effector response being achieved at lower concentrations of antigen. Slifka and Whitton (2001) demonstrated that expression of the signalling molecule Lck is associated with increased IFN-γ production and hypothesised that highly avid CTL are 'hard-wired' to respond to low dose antigen stimulation by optimising their TCR signal transduction machinery.

359-6 produces the immunosuppressive cytokine IL-10 upon antigenic stimulation. IL-10 production has been linked to expression of the inhibitory molecule PD-1 (Jin, Anderson et al. 2010) and could indicate that clone 359-6 is showing signs of exhaustion, a phenomenon well documented during chronic viral infections (Shin and Wherry 2007). CD8+ T cells that undergo excessive antigen stimulation lose their ability to exert effector functions and proliferate and thus become unable to control viral replication. A study that examined the gene expression profiles of acute phase virus specific CD8+ T cells and chronic phase T cells displaying signs of exhaustion found several differences, including major changes in the TCR signalling pathway (Wherry, Ha et al. 2007). Clone 359-6 may display reduced functional avidity due to suboptimal TCR signal transduction brought about by excess antigen stimulation that has led to exhaustion.

The reduced functional avidity displayed by 359-6 is also associated with a reduction in polyfunctionality as demonstrated by the inability of this clone to produce IL-2 upon antigen stimulation. The attributes associated with polyfunctionality, degranulation as measured by CD107a expression and the secretion of MIP-1β, IFN-γ, TNF-α and IL-2, occur in a step-wise fashion with each successive function requiring

higher amounts of antigen stimulation to be induced. The first function to be lost when the level of antigen recognition drops is IL-2 production, followed by TNF-α secretion, IFN-γ release, degranulation and MIP-1β secretion (Almeida, Sauce et al. 2009). Therefore at any given antigen concentration highly avid CTL will display more effector functions in comparison to CTL with low functional avidity.

The loss of effector functions associated with CD8+ T cell exhaustion also occur in a stepwise manner similar to that seen in the reduction in polyfunctionality between high and low avidity CTL. IL-2 production is lost first, followed by TNF-α release, then cytotoxicity is reduced and finally IFN-γ production ceases (Streeck and Nixon 2010). It is possible that the loss of such functions is associated with a need for increased antigenic stimulation to induce a response because exhausted CTL are undergoing progressive loss of functional avidity. This may be the case for clone 356-9.

It is of note however that clone 11-16 displays a reduced level of antigen sensitivity as compared to clone 359-6 yet still retains that ability to produce IL-2. This suggests that the level of functional avidity displayed by clone 359-6 is capable of resulting in IL-2 production and points to factors other than antigen sensitivity in determining polyfunctionality.

The reduction in functional avidity seen between CD8+ T cells within the immunodominant virus specific CTL response and possessing a dominant TCR clonotype may contribute to a loss of viral control. Whilst it was not possible to detect a difference in *in vitro* viral suppression as a result of this reduction in avidity it has been demonstrated that highly functionally avid CTL control viral replication more efficiently

in vivo even when no *in vitro* differences are observed (Alexander-Miller, Leggatt et al. 1996).

It is also possible that a reduction in functional avidity is a mechanism employed by CTL to avoid exhaustion by reducing their responsiveness to peptide in the face of continuous antigen stimulation. Selective depletion has been shown to occur amongst highly avid virus specific CTL during early HIV-1 infection (Lichterfeld, Yu et al. 2007). A successful anti-viral CTL response is most likely the result of a balancing act between functional avidity that is high enough to exert immune pressure on the virus and avoid deletion by neglect and not so high that it undergoes massive clonal expansion resulting in deletion due to exhaustion. Perhaps a heterogeneous CTL response with regards functional avidity is a strategy to ensure effective viral control with highly functional avid CTL present to effectively control replication and less functionally avid cells being present as a second layer of defence should such cells become exhausted.

The fact that FLK specific CTL utilising the TCR displayed by clone 11-16 appear to be out-competed by those possessing the short loop TCR displayed by clones 11-11 and 359-6 could be due to its lack of antigen responsiveness leading to death by neglect. Another possibility is that cells possessing the TCR clonotype present in clone 11-16 underwent a reduction in functional avidity over the course of infection in the same way that cells possessing the clonotype displayed by clones 11-11 and 359-6 appear to have done. Given that cells with the long loop TCR were starting from a much lower functional avidity a reduction would rapidly lead to unresponsiveness to the amount of antigen present and replication of the cells would no longer be maintained.

As with the difference in functional avidity seen between clones 11-11 and 359-6, the reduced functional avidity displayed by clone 11-16 is not associated with a reduction in TCR affinity or CD8 expression. It could however be the result of a decrease in the efficiency of the TCR signal transduction pathway. One difference that was observed between clones 11-11 and 11-16 was the ability of clone 11-11 to produce the type two cytokines IL-4 and IL-13 upon antigenic stimulation. The significance of this is not clear and whether or not this has a bearing on the persistence of the TCR clonotype possessed by 11-11 would be difficult to establish.

The production of IL-4 by HIV-1 specific CD8+ T cells has been previously reported and was found to be associated with reduced cytotoxicity. IL-4 was observed to be capable of modulating the cytokine profile of CD8+ T cells suggesting that its release could lead to general CD8+ T cell dysfunction (Maggi, Manetti et al. 1997). The production of IL-4 by clone 11-11 could be a reason why the CTL response generated by patient A794 was apparently ineffective at controlling viral replication *in vivo*.

As the magnitude of the FLK specific response as measured by tetramer staining closely mirrors patient A794's viral load it is possible that it represents a passenger response merely replicating as a result of viral antigen load and not controlling viral replication. However, during the chronic stage of disease nef sequencing revealed that viral quasispecies were present and that escape mutations with the FLK epitope were beginning to appear. This indicates that the FLK specific CTL response exerts enough pressure to provide virus variants expressing escape mutations with a selective advantage.

Whether these escape mutations appeared before or after the reduction in the functional avidity seen within the FLK specific response using the short β CDR3 region

is unclear. It is therefore not possible to determine if the reduction is functional avidity is the result of a reduction in antigen stimulation caused by the appearance of viral quasispecies possessing the FLK escape mutation. However as previously alluded to, stimulation of highly avid CTL with low concentrations of antigen results in the maintenance of high antigen sensitivity (Kroger and Alexander-Miller 2007). It has also been demonstrated that a reduction in antigen stimulation brought about by the appearance of escape mutations results in the maintenance of CTL polyfunctionality (Streeck, Brumme et al. 2008). This suggests that a reduction in antigen stimulation due to the appearance of viral quasispecies containing escape mutations may not lead to a reduction in functional avidity.

It is possible that a reduction in the avidity of the FLK specific CTL population using the short loop TCR led to a reduction in viral control, thus allowing the virus to escape. Whilst it could be argued that highly avid CTL are more likely to drive immune escape due to their superior viral control, it is also possible that highly avid CTL are so efficient at killing viral infected cells that they do not allow such cells to release viral progeny, therefore preventing the dissemination of virus that may contain mutations.

The data presented here suggests that induction of an immunodominant highly avid HIV-1 specific CTL response during acute infection is not enough to ensure viral control. It also suggests that a CTL response that is high avidity during acute infection may lose the ability to recognise low amounts of antigen as HIV-1 infection persists. This could have implications for the design of vaccines that aim to induce anti-viral CTL responses, suggesting that factors other than functional avidity should be taken into account when attempting to elicit a successful anti-viral CD8+ T cell response.

Chapter 4: Characterisation of immune responses within the HLA-B*40+ patients in a Chinese slow progressor cohort

4.1 Background

4.1.1 HIV-1 in China

In 2009 UNAIDS estimated that 740,000 people in China were living with HIV-1. In the same year it was estimated that 26,000 Chinese citizens died from AIDS related illnesses and that around 80,000 became newly infected with HIV-1. Figure 4.1 illustrates how the number of people in China living with HIV-1 has risen since 1990.

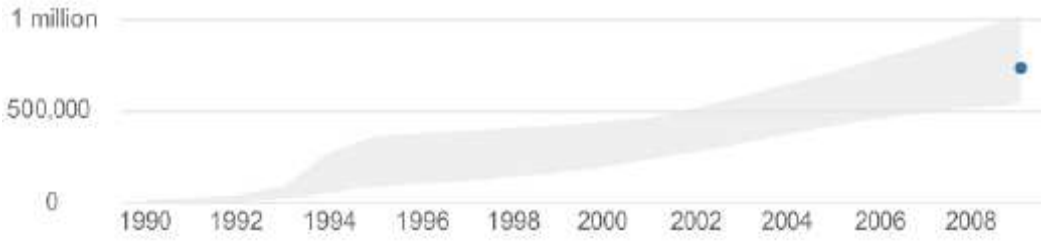


Figure 4.1: Increase in Chinese people living with HIV-1 since 1990 (UNAIDS/WHO, 2009)

The first reported cases of AIDS in China occurred in Beijing in 1985, mainly in foreigners and Chinese people who had travelled abroad (Zhang Kl and Ma 2002). In 1989 HIV-1 was identified among injecting drug users in Yunnan, a province of China that borders Myanmar, Vietnam and Laos. Around 1994 the virus began to spread beyond Yunnan province with a substantial number of cases reported in both drug users and

commercial plasma donors. By 1996 HIV-1 had reached every province in China (Lu and Li 2003). Rates of infection are now increasing in people outside high risk groups such as injecting drug users, sex workers and men who have sex with men, with the main route of transmission now sex between heterosexual partners. This is a worrying trend indicating that more needs to be done to prevent a major HIV/AIDS epidemic in China.

4.1.2 The Shuang Miao (SM) village cohort

As alluded to above a number of HIV-1 infected Chinese people contracted the virus by participating in plasma donation schemes. Between 1994 and 1995 an estimated 407 adults living in the village of Shuang Miao in Henan province became infected with HIV-1 by participating in such a scheme. The exact route of transmission is not known but it is thought that those who contracted the virus do so through the use of contaminated equipment or via pooled red blood cell replacement.

The Shuang Miao village is an isolated rural community comprising a relatively small number of family groups. There is limited migration in and out of the village and most marriages occur between members of the community. These factors have led to patients enrolled in the SM cohort having a comparatively narrow genetic background.

Of the 407 suspected initially infected individuals 258 survived without ART until the cohort was established in 2004, indicating that the patients enrolled in this study are enriched for slow progressors. Clinical data revealed that 20% of the cohort fit the definition of a LTNP, maintaining a CD4+ T cell count of above 500 cells/mm^3 in the

absence of therapy for 10 years (Easterbrook 1999). The percentage of LTNPs in most other cohorts of HIV-1+ patients is 1-5%.

Phylogenetic analysis of viral sequences derived from patients in the SM cohort revealed that infections were the result of exposure to a narrow source virus. Together these factors give the SM cohort several distinctive aspects (outlined in table 5.1) that provide a unique setting for studying HIV-1, removing many of the variables that can often complicate the analysis of data in other cohorts.

1.	The participants belong to an isolated rural community and are therefore relatively closely related
2.	Infection of all subjects occurred within a short time frame and probably by the same route
3.	Infection was the result of exposure to a narrow source virus
4.	The cohort contains an unusually high proportion of LTNPs

Table 4.1 Unique features of the SM cohort.

4.1.3 HLA-B*40 in the SM cohort

HLA-B*40 is the most common HLA-B allele in the SM cohort. HLA types were determined for 229 study participants, of which 60 are HLA-B*40+. This gives HLA-B*40 a population frequency of 26.2%. Whilst HLA-B*40 wasn't found to be associated with either poor or good viral control its high prevalence within the SM cohort deems patients expressing this allele worthy of further study.

Due to the reliance of any vaccine designed to elicit CD8+ T cells on an individual's own HLA molecules to present peptide and initiate a response it may be important to consider immunodominant responses to common MHC class I molecules

within a given population. This could allow for the immunogens contained within the vaccine to be tailored to ensure the maximum amount of responders within that population.

By performing IFN-γ ELISpot assays and tetramer staining of whole blood immunodominant HLA-B*40 restricted responses were identified. Furthermore, when the SM cohort members were divided based on HLA-B*40 subtype it was revealed that the immunodominance hierarchy of recognised peptide epitopes differ substantially between HLA-B*4001+ and HLA-B*4002+ patients. This resulted in immunodominant responses to different HIV-1 gene products which also differed in their differentiation state as measured by CD27 and CD28 expression. This may be responsible for a difference in viral control, as a trend for HLA-B*4002+ patients to possess higher CD4 counts and lower viral loads as compared to HLA-B*4001+ patients was observed.

These results show the importance of taking into account closely related HLA subtypes when determining immunodominant CD8+ T cell responses within a population. CTL responses restricted by related HLA subtypes can differ in their specificity and phenotype and therefore are likely to differ in their functionality. This may lead to a difference in disease outcome and should be considered when designing a CD8+ T cell based HIV-1 vaccine.

4.2 Results

4.2.1 The immunodominance hierarchy of HLA-B*40 restricted HIV-1 epitopes differs between HLA-B*4001+ and HLA-B*4002+ patients

PBMCs from 23 HLA-B*40+ HIV-1 infected patients were separated from whole blood and tested directly *ex vivo* in an IFN-γ ELISpot assay with 11 known HLA-B*40 restricted HIV-1 epitopes (outlined in table 2.1). For the analysis patients were separated based on HLA-B*40 subtype, eight patients were HLA-B*4001+ whilst 15 possessed the HLA-B*4002 subtype. The HLA type, clinical data and drug treatment regime for all patients is outline in table A.1 in appendix 5. Most patients were drug naïve when these studies were carried out in 2008. However, a small number had a brief period of therapy in 2003 when the government issued free ART but all such patients discontinued drug use shortly after starting therapy and no patients were on ART between 2003 and 2008.

This experiment revealed a difference in the immunodominance hierarchy of epitopes recognised by HLA-B*4001+ and HLA-B*4002+ patients (figure 4.2). The epitopes most frequently recognised by HLA-B*4001+ patients were KEK (nef) and TER (gag-p2p7p1p6). Whilst some HLA-B*4002+ patients recognised these two peptides, KET (gag-p24) and AEW (gag-p24) were the epitopes most frequently recognised by this group of patients. Neither of these two peptides were recognised by any HLA-B*4001+ patients.

These results are similar to those obtained from a previous study carried out by YongHong Zhang, a former member of the lab, who also observed KEK to be the

immunodominant epitope in HLA-B*4001+ patients and AEW and KET to be frequently recognised by HLA-B*4002+ patients (see appendix 1).

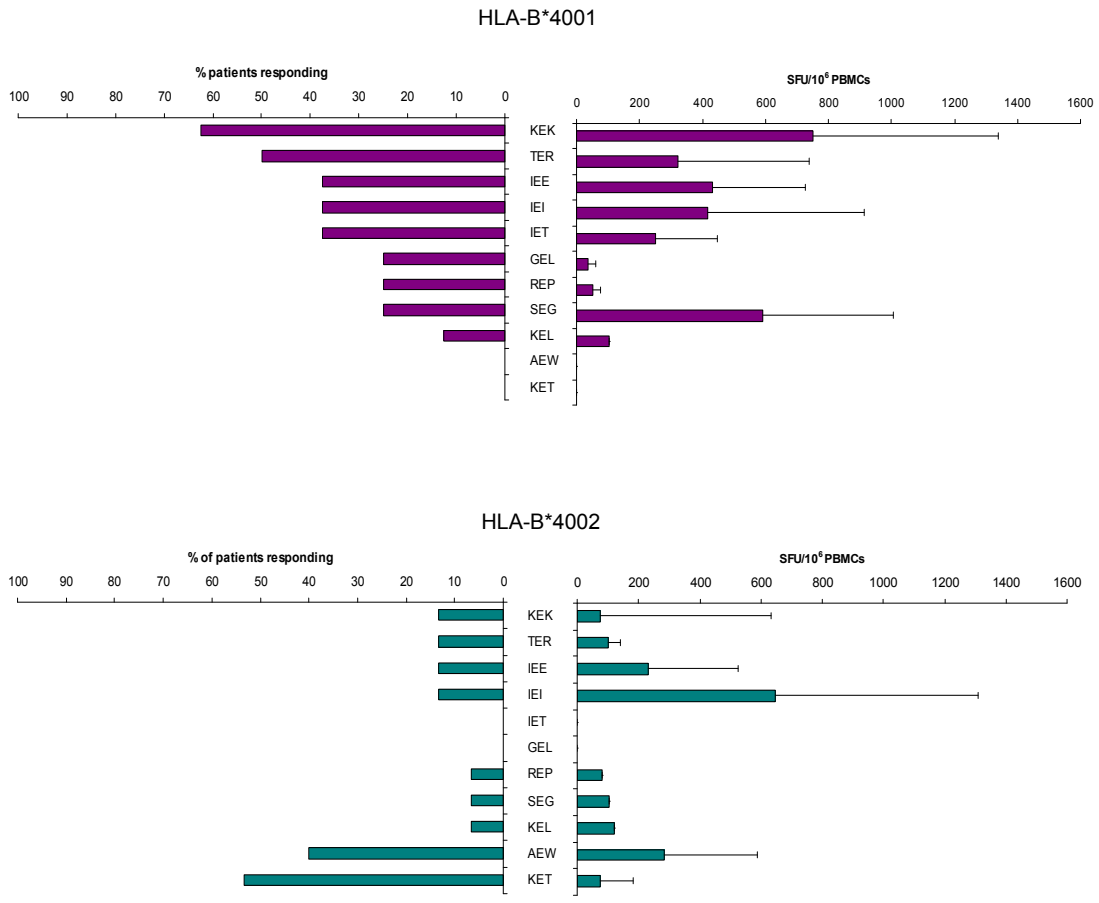


Figure 4.2 The immunodominance hierarchy of recognised known HLA-B*40 restricted epitopes differs between HLA-B*4001+ and HLA-B*4002+ patients. The percentage of 8 HLA-B*4001+ and 15 HLA-B*4002+ patients displaying a positive IFN-γ ELISpot result to the 11 known epitopes (left hand panel-see table 2.1) and the average magnitude of the response (SFU/ million PBMCs) (Right hand panel). Patients tested are outlined in table A.1 (appendix 5)

4.2.2 Tetramer staining of whole blood confirms that the immunodominant responses observed in the IFN-γ ELISpot assay are restricted by HLA-B*4001 and HLA-B*4002

Whole blood from HLA-B*4001+ and HLA-B*4002+ patients was stained with anti-CD8 PerCP and PE conjugated MHC class I tetramers. The HLA-B*4001 heavy chain was refolded with β2M and the 5 most immunodominant HLA-B*40 restricted epitopes in HLA-B*4001+ patients as determined by the previous experiments carried out by YongHong Zhang. The HLA-B*4002 heavy chain was refolded with β2M and 6 known HLA-B*40 restricted epitopes, chosen for their immunodominance within the HLA-B*4002+ members of the SM cohort (see table 2.2).

The tetramer staining results complement the IFN-γ ELISpot assay results (figure 4.3) with the most commonly recognised HLA-B*4001 tetramer being that which is specific for the KEK epitope. In addition, the AEW and KET specific tetramers were most frequently recognised by the HLA-B*4002+ patients. The average percentage of the tetramer positive cells was higher for the immunodominant responses as compared to responses to subdominant epitopes suggesting immunodominance within individual patients as well as at a population level.

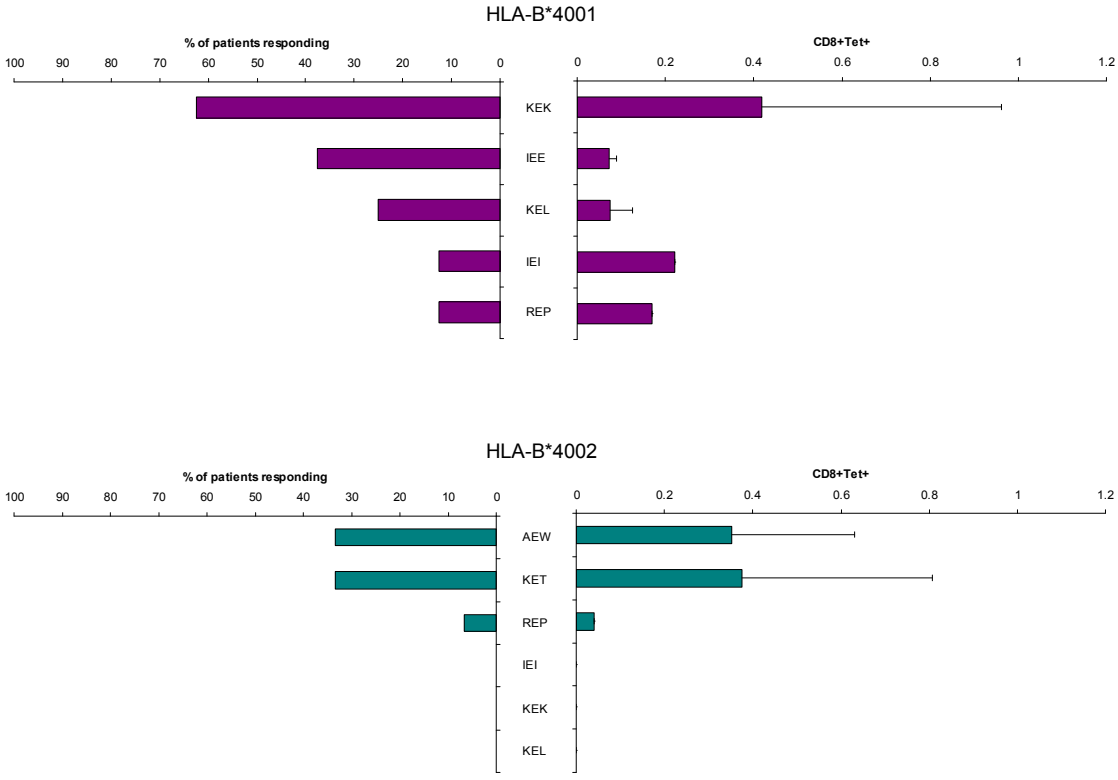


Figure 4.3 Staining of whole blood with HLA-B*4001 and HLA-B*4002 tetramers confirms the immunodominance patterns seen by in the IFN-γ ELISpot assay. The percentage of 8 HLA-B*4001+ and 15 HLA-B*4002+ patients responding to several MHC class I tetramers is shown on the right hand side and the average percentage of lymphocytes staining positive for CD8 and the stated tetramer is on the left. Patients tested are outlined in table A.1 (appendix 5)

4.2.3 Mutations found to be associated with the presence of HLA-B*40 and within known HLA-B*40 epitopes are enriched in patients with the subtype where an immunodominant response exists

Sequences of nef, gag and pol from several SM cohort members were determined and any amino acids that deviated from consensus were assessed for association with a particular HLA allele. Several mutations were found to be associated with HLA-B*40, some within known HLA-B*40 restricted epitopes (Dong, Zhang et al. 2011). Mutations within the immunodominant HLA-B*4001 restricted epitope KEK were found to be associated with HLA-B*40. The two epitopes immunodominantly recognised by HLA-B*4002+ patients, AEW and KET, were also found to contain mutations associated with HLA-B*40.

These mutations are outlined in figure 4.4. The frequency of these amino acid substitutions in HLA-B*4001+ and HLA-B*4002+ patients was determined (figure 4.4) and it was observed that the likelihood of possessing a HLA-B*40 associated mutation follows the same subtype pattern as immunodominance. There is a statistically significant increase in the number of HLA-B*4002+ patients with HLA-B*40 associated mutations within the KET and AEW epitopes as compared to HLA-B*4001+ patients ($p = 0.0143$ and $p = 0.0223$ respectively). There is also a trend for more HLA-B*4001+ patients to possess mutations within the KEK epitope as compared to HLA-B*4002+ patients but this did not reach significance, possibly due to the number of sequences analysed.

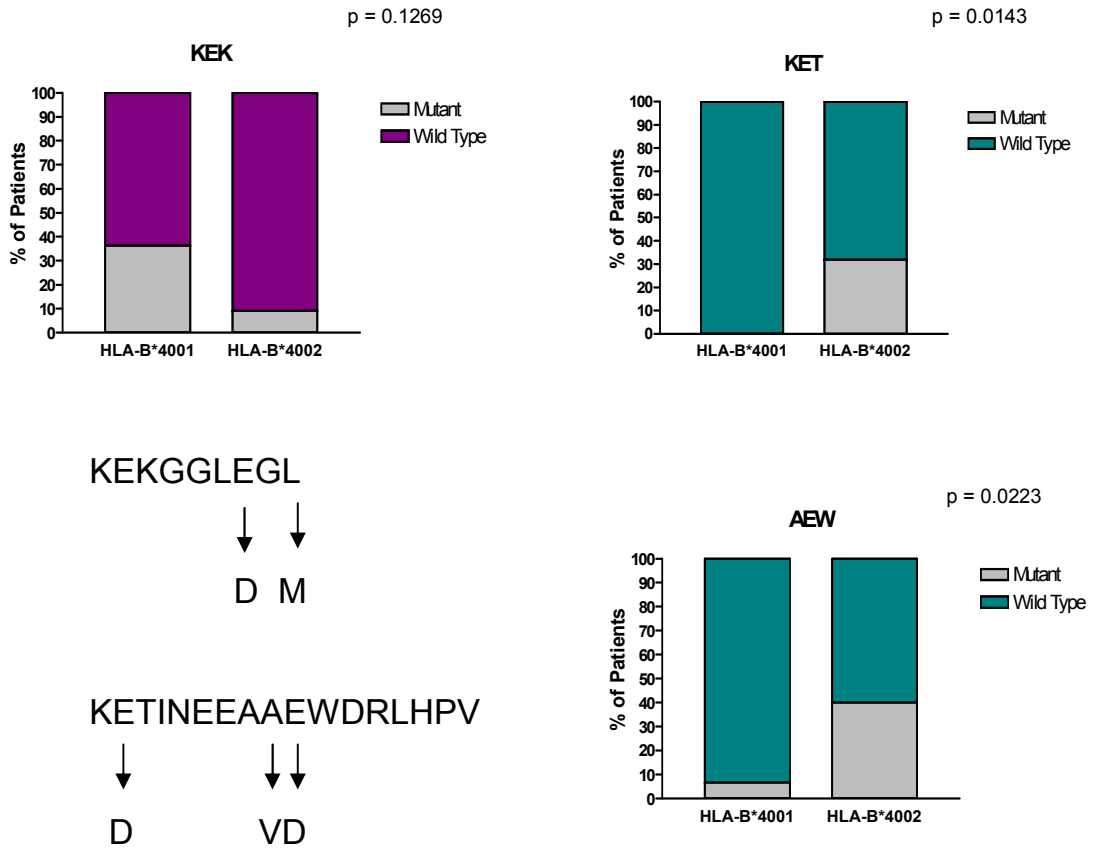


Figure 4.4 Mutations associated with the presence of HLA-B*40 and within HLA-B*40 restricted epitopes are significantly increased in patients with the subtype coupled to an immunodominant response. Percentage of HLA-B*4001+ and HLA-B*4002+ patients that possess mutations associated with HLA-B*40 (outlined above) within 3 known HLA-B*40 epitopes, KEK which is immunodominant in HLA-B*4001+ patients and AEW and KET which are immunodominant in HLA-B*4002+ patients. P values calculated using a chi^2 test.

4.2.4 Modelling of HLA-B*4001 and HLA-B*4002 using SWISS-MODEL shows that certain amino acid that differ between the subtypes are within the peptide binding groove and have the potential to impact on peptide binding and TCR recognition

The sequences of HLA-B*4001 and HLA-B*4002 were obtained from the EMBL-EBI IMGT/HLA database (figure 4.5). Utilising the solved structure of HLA-B*4103, an HLA allele with sequence homology to HLA-B*4001 and HLA-B*4002, SWISS-MODEL was then used to model the structure of both of these molecules (figure 4.5).

There are 8 amino acid substitutions between HLA-B*4001 and HLA-B*4002, 4 of which are hypothesised to have little impact on peptide binding. It is likely that the valine to methionine change at position 12 will have limited impact on the peptide binding groove as the side chains of both amino acids point outwards away from the peptide. The amino acid at positions 177, 178 and 180 do not comprise part of the peptide binding groove and so it is probable that changes in these residues will have little influence on epitope binding.

However, four amino acid substitutions between HLA-B*4001 and HLA-B*4002 have the potential to influence peptide binding. Differences in amino acids at position 11, 97, 143 and 147 exist between HLA-B*4001 and HLA-B*4002, all of which contribute to the binding groove. It is therefore possible that such amino acid substitutions could affect not only which peptides bind to the respective HLA molecules but also the conformation of any peptides that do bind.

Two of these amino acid substitutions, those at positions 143 and 147, are within the F pocket of the peptide binding groove and are involved in contacting the C terminal of the peptide epitope. This has the potential to alter the peptide binding motif of the HLA-B*40 subtypes with regards to the amino acid present at this position. The IFN-γ ELISpot data shows that the peptide epitopes immunodominantly recognised by HLA-B*4001+ and HLA-B*4002+ do indeed differ in the amino acid that is present at the C terminal as outlined in table 4.2.

HLA-B*4001		**HLA-B*4002**	
Peptide motif	**Immunodominance**	**Peptide motif**	**Immunodominance**
-E------L	+++	-E------A	+++
-E------I	+	-E------V	+++
		-E------L	+

Table 4.2 The amino acids at position 2 and at the C terminal of the peptides that are recognised by HLA-B*4001+ and HLA-B*4002+ patients as determine by the IFN-γ ELISpot assay outlined in figure 5.1

```
                  10         20         30         40         50         60         70         80         90        100
B*40:01:01    GSHSMRYFHT AMSRPGRGEP RFITVGYVDD TLFVRFDSDA TSPRKEPRAP WIEQEGPEYW DRETQISKTN TQTYRESLRN LRGYYNQSEA GSHTLQRMYG
B*40:02:01    ---------- SV-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------S---

                 110        120        130        140        150        160        170        180        190        200
B*40:01:01    CDVGPDGRLL RGHNQYAYDG KDYIALNEDL RSWTAADTAA QISQRKLEAA RVAEQLRAYL EGECVEWLRR YLENGKDKLE RADPPKTHVT HHPISDHEAT
B*40:02:01    ---------- ---------- ---------- ---------- --T---W--- ---------- ---------- ------ET-Q ---------- ----------

                 210        220        230        240        250        260        270        280        290        300
B*40:01:01    LRCWALGFYP AEITLTWQRD GEDQTQDTEL VETRPAGDRT FQKWAAVVVP SGEEQRYTCH VQHEGLPKPL TLRWEPSSQS TVPIVGIVAG LAVLAVVVIG
B*40:02:01    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                 310        320        330
B*40:01:01    AVVAAVMCRR KSSGGKGGSY SQAACSDSAQ GSDVSLTA
B*40:02:01    ---------- ---------- ---------- --------
```

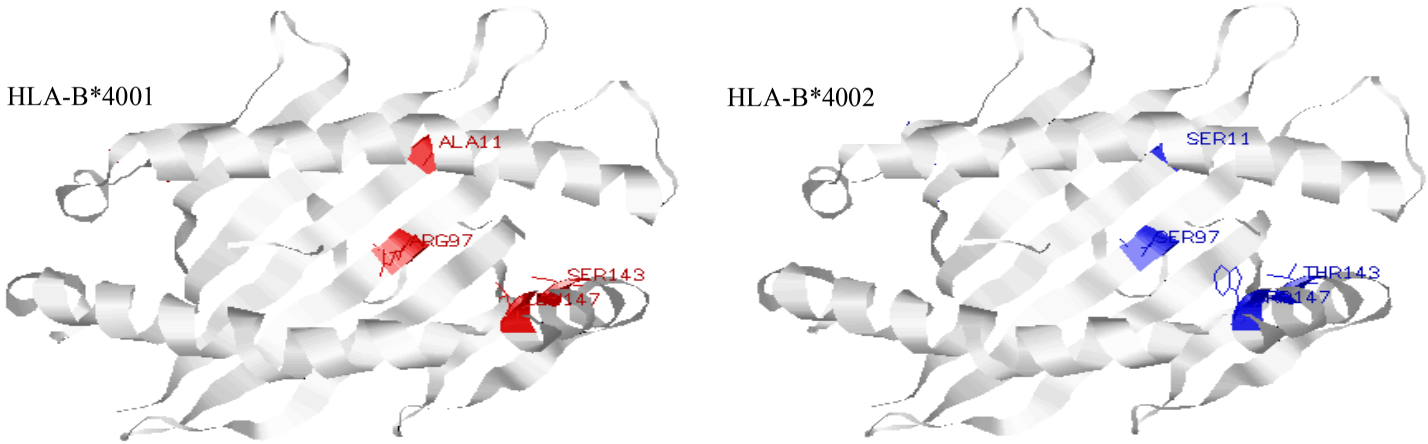


Figure 4.5 Modelling of HLA-B*4001 and HLA-B*4002 using SWISS-MODEL shows that certain amino acids that differ between the subtypes are within the peptide binding groove and have the potential to impact on peptide binding and TCR recognition. Sequences obtained from the EMBL-EBI IMGT/HLA database (outlined above) revealed 8 amino acid substitutions between HLA-B*4001 and HLA-B*4002. These sequences were then used to model the structure of the HLA molecules.

4.2.5 CD8+ T cell clones specific for KEK and restricted by HLA-B*4001 do not recognise their cognate antigen when it is presented by target cells expressing HLA-B*4002

Due to the differences in the peptide binding groove that exist between HLA-B*4001 and HLA-B*4002 it is possible that binding of the same peptide epitope to both molecules could result in peptide-MHC complexes that differ greatly in topology. To test this theory CD8+ T cell clones specific for the immunodominant nef epitope KEK and restricted by HLA-B*4001 were generated and their ability to lyse HLA-B*4001+ and HLA-B*4002+ B cells pulsed with the KEK peptide was tested in a chromium release assay.

The clones were able to lyse HLA-B*4001+ B cells pulsed with KEK but no specific lysis of the peptide pulsed HLA-B*4002+ B cells was observed (figure 4.6). This is despite the fact that several lines of evidence suggest that KEK does bind to HLA-B*4002. Some HLA-B*4002+ patients from the SM cohort produce positive IFN-γ ELISpot responses to the KEK peptide. KEK can be refolded with both the HLA-B*4001 and the HLA-B*4002 heavy chain to produce peptide-MHC tetramers (see appendix 2). There are also a published study indicating that KEK is recognised by HLA-B*4002+ patients (Sabbaj, Bansal et al. 2003).

Further evidence for a lack of cross reactivity between KEK specific CTL restricted by HLA-B*4001 and their cognate antigen presented by HLA-B*4002 comes from the inability of KEK specific HLA-B*4002 tetramers to bind to HLA-B*4001 restricted KEK specific CTL clones (see appendix 3).

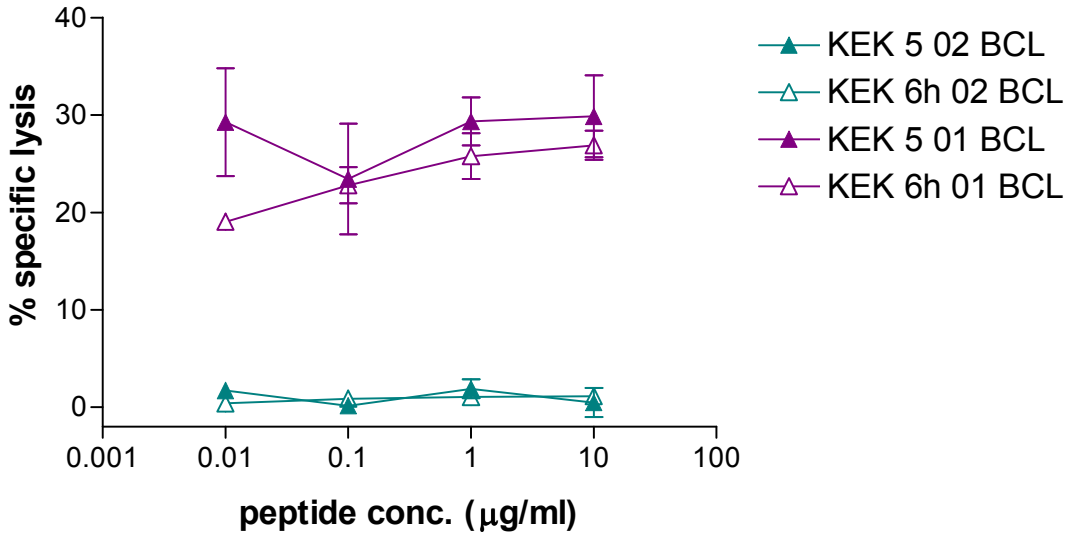


Figure 4.6 HLA-B*4001 restricted KEK specific CD8+ T cell clones do not recognise HLA-B*4002+ B cells pulsed with KEK peptide. Two KEK specific clones (KEK 5, KEK 6h) generated from a HLA-B*4001+ donor were tested in a chromium release assay with HLA-B*4001+ (purple symbols) and HLA-B*4002+ (green symbols) B cells pulsed with various concentrations of KEK peptide. Specific lysis was only observed when the HLA-B*4001 B cells were present.

4.2.6 CD8+ T cell clones specific for AEW and restricted by HLA-B*4002 do not recognise their cognate antigen when it is presented by target cells expressing HLA-B*4001

To determine whether HLA-B*4002 restricted CD8+ T cells could recognise the immunodominant epitope AEW when presented by HLA-B*4001, clones specific for this peptide were generated from a HLA-B*4002+ patient. These clones were then tested in a chromium release assay with both HLA-B*4001+ and HLA-B*4002+ B cells pulsed with the AEW peptide.

As was the case for the HLA-B*4001 restricted clones no cross-reactivity was observed. The AEW specific clones were able to lyse HLA-B*4002+ B cells pulsed with AEW but not peptide pulsed HLA-B*4001+ B cells (figure 4.7). There is less evidence to suggest that AEW is capable of binding HLA-B*4001. Only one HLA-B*4001+ patient from the SM cohort tested for recognition of AEW gave a positive IFN-γ ELISpot response which was relatively week (see appendix 4). The Los Alamos HIV sequence database lists AEW as restricted by HLA-B*4002 and HLA-B*40, however no studies have shown that this epitope is capable of binding to HLA-B*4001.

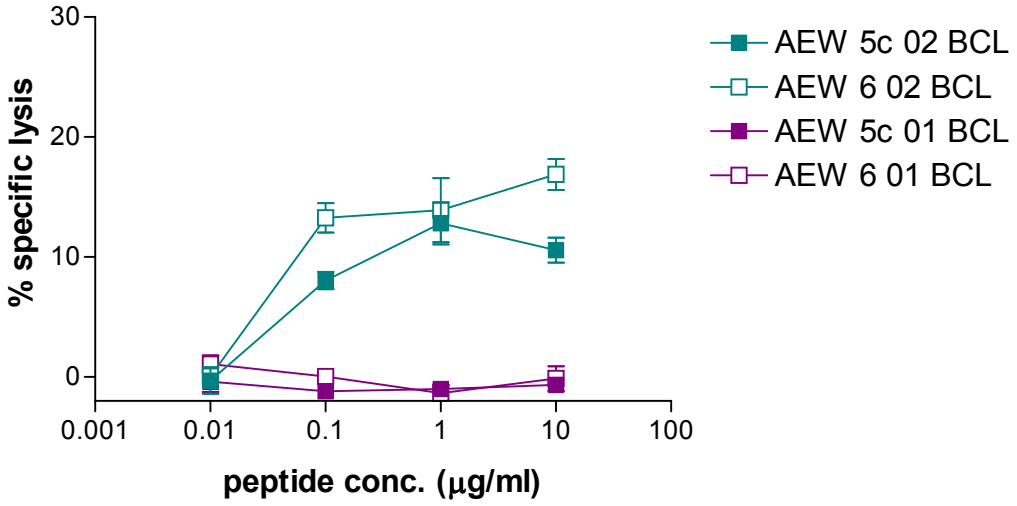


Figure 4.7 HLA-B*4002 restricted AEW specific CD8+ T cell clones do not recognise HLA-B*4001+ B cells pulsed with AEW peptide. Two AEW specific clones (AEW 5c, AEW 6) generated from a HLA-B*4002+ donor were tested in a chromium release assay with HLA-B*4001+ (purple symbols) and HLA-B*4002+ (green symbols) B cells pulsed with various concentrations of AEW peptide. Specific lysis was only observed when the HLA-B*4002 B cell was present.

4.2.7 The immunodominance pattern of epitopes recognised by HLA-B*4001 and HLA-B*4002 can not be explained solely by the predicted binding affinity of the peptide for the HLA molecule

The web-based server MetaMHCI was used to predict the binding affinity of the HLA-B*40 restricted peptide epitopes, used in the IFN-γ ELISpot assay, to both HLA-B*4001 and HLA-B*4002. An artificial neural network (ANN) was used to predict an IC50 for each 9mer and the one 10mer (IEI) to estimate their binding affinity to both HLA subtypes. No algorithm was available to predict the binding affinity of the three 8mers (TER, IEE and IEI) to the HLA molecules.

The peptide binding affinity of eight of the HLA-B*40 epitopes for both HLA-B*4001 and HLA-B*4002 are outlined in table 4.3 along with their relative immunodominance within each group of patients. This reveals that the pattern of immunodominance as determined by the IFN-γ ELISpot assay is not solely determined by HLA molecule binding affinity. However the immunodominant HLA-B*4001 restricted peptide KEK is predicted to have the highest binding affinity for this HLA-B*40 subtype.

HLA-B*4001			HLA-B*4002		
Epitope	**IC50 nM**	**immunodominance rank**	**Epitope**	**IC50 nM**	**immunodominance rank**
KEK	11.9	1	KEL	23.5	=7
KEL	15.1	9	AEW	65.4	2
REP	61.5	=6	KEK	97.5	=3
GEL	151.7	=6	IEI	162.4	=3
AEW	284.4	No responders	REP	301.6	=7
IEI	402.8	=3	KET	314.4	1
SEG	972.5	=6	GEL	350.2	No responders
KET	4791.1	No responders	SEG	887.3	=7

Table 4.3 The predicted binding affinity of 8 HLA-B*40 restricted epitopes for both HLA-B*4001 and HLA-B*4002 as determined by an ANN using the web-based server MetaMHCI

4.2.8 HLA-B*4001 restricted responses display a more differentiated phenotype as compared to HLA-B*4002 restricted responses

Whole blood was stained using PE conjugated p-MHC class I tetramers, anti-CD8 PerCP, anti-CD27 APC and anti-CD28 FITC. The percentage of each CD8+, tetramer specific population that displayed either an early, intermediate or late state of differentiation was determined by examining expression of CD27 and CD28. Representative FACS plots are shown in figure 4.8. CD27 and CD28 are two co-stimulatory molecules that are both present on naïve CD8+ T cells and are down-regulated in a step-wise fashion, with CD28 lost first, as the cells become fully differentiated.

These experiments revealed a statistically significant increase in the percentage of HLA-B*4002 restricted AEW specific CD8+ T cells that possess an intermediate phenotype as compared to HLA-B*4001 restricted KEK specific CD8+ T cells. A trend was also observed for an increase in the percentage of fully differentiated KEK specific CD8+ T cells as compared with AEW specific CD8+ T cells (figure 4.9 – top panel).

When HLA-B*4001 and HLA-B*4002 restricted responses were considered as a whole, a statistically significant increase in the number of fully differentiated HLA-B*4001 restricted CD8+ T cells was observed. In addition, a trend for a higher percentage of intermediately differentiated HLA-B*4002 restricted CD8+ T cells was also observed (figure 4.9 – lower panel).

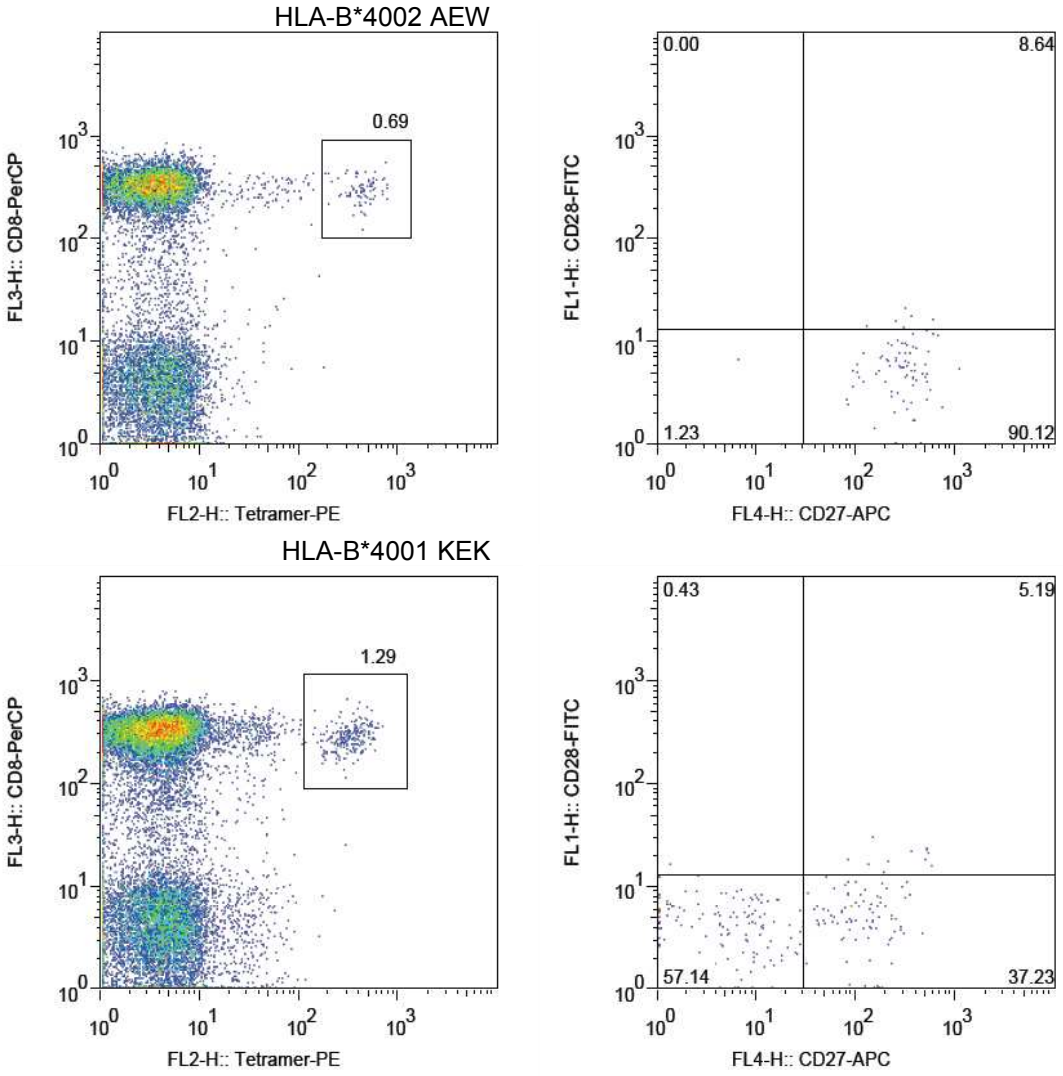


Figure 4.8 Representative FACS plots displaying the percentage of p-MHC class I tetramer positive cells that stain positive for CD27-FITC and CD28-APC. The left hand plots display the percentage of HLA-B*4002 AEW+ (top) and HLA-B*4001 KEK+ (bottom) cells contained with in the lymphocyte population of an HLA-B*4002+ and an HLA-B*4001+ patient, respectively. The right hand panel shows the percentage of these cells that are CD27+CD28+ (early differentiated), CD27+CD28- (intermediately differentiated) or CD27-CD28- (fully differentiated).

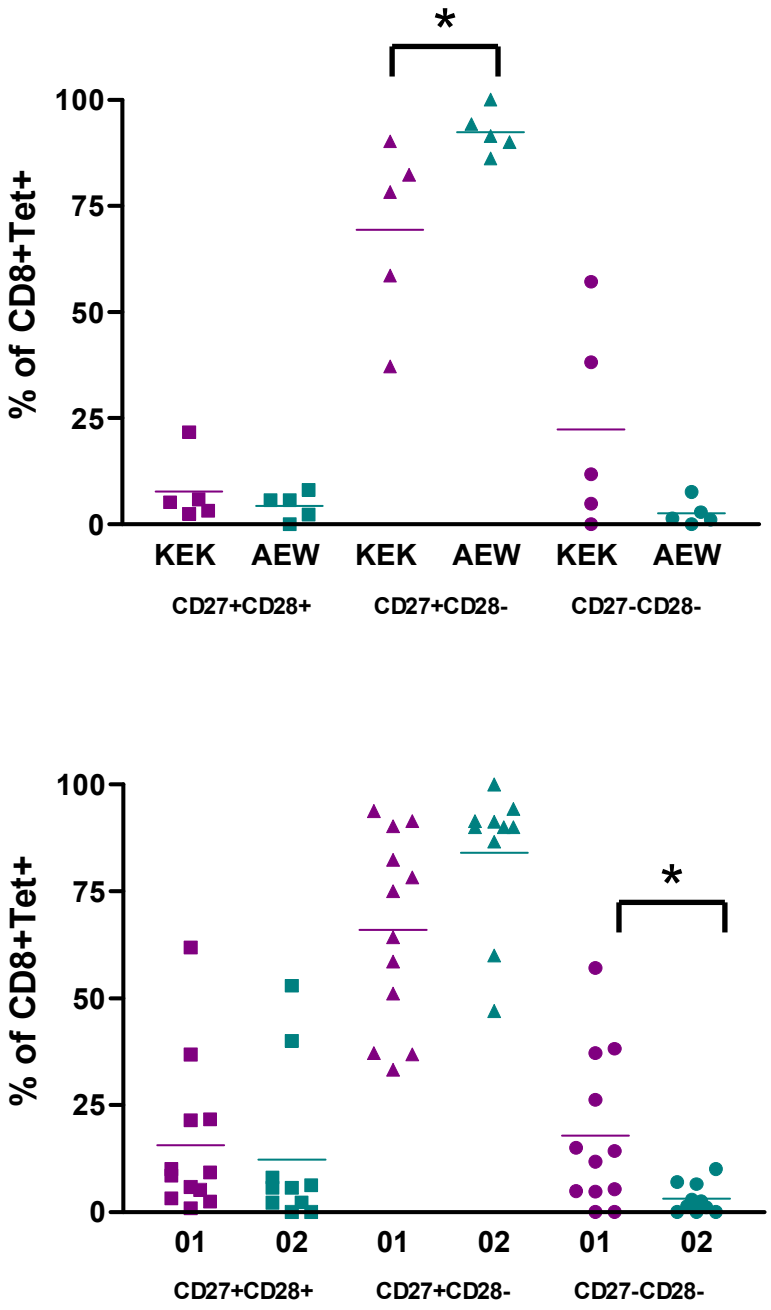


Figure 4.9 HLA-B*4001 restricted responses display a more differentiated phenotype in comparison to HLA-B*4002 restricted responses. Whole blood was stained with PE conjugated p-MHC class I tetramers, anti-CD8 PerCP, anti-CD27 APC and anti-CD28 FITC. The percentage of the CD8+Tet+ population that possessed each differentiation phenotype (CD27+CD28+, CD27+CD28- and CD27-CD28-) was determined and is displayed above. * = $p<0.05$ as determined by a Mann Whitney U test.

4.2.9 The difference in differentiation state observed between HLA-B*4001 and HLA-B*4002 restricted CD8+ T cells is not a result of the gene product targeted

The results of the differentiation phenotype experiment described above were further analysed to compare the expression of CD27 and CD28 between nef and gag specific responses. All of the nef specific responses were restricted by HLA-B*4001 and specific for the KEK epitope. The gag specific responses were restricted by both HLA-B*4001 (epitopes IEI and KEL) and HLA-B*4002 (epitopes AEW and KET).

Results revealed that whilst there is a trend for nef specific response to contain a higher percentage of fully differentiated cells, this difference is not significant. There was also no significant difference in the percentage of naïve or intermediately differentiated cells between the two groups (figure 4.10).

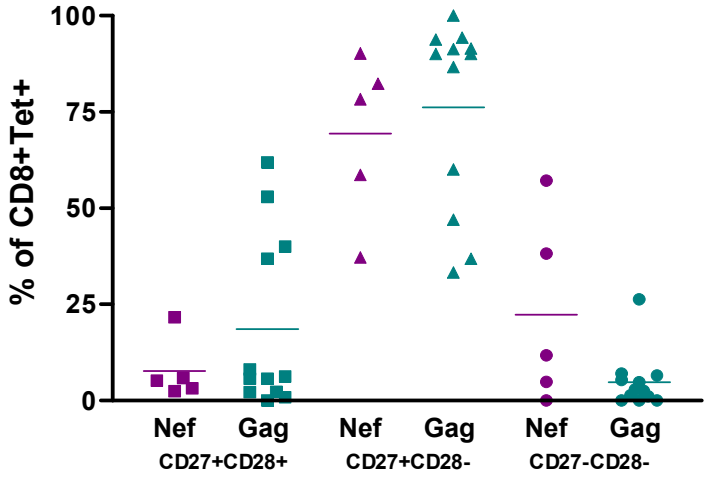


Figure 4.10 There are no significant differences in the differentiation state of nef specific HLA-B*40 restricted CD8+ T cell responses as compared with gag specific HLA-B*40 restricted CD8+ T cell responses. Whole blood was stained with PE conjugated p-MHC class I tetramers, anti-CD8 PerCP, anti-CD27 APC and anti-CD28 FITC.

4.2.10 A trend exists for HLA-B*4002+ members of the SM cohort to possess lower viral loads and higher CD4 counts as compared to HLA-B*4001+ patients

The CD4 count for 90 drug naïve members of the SM cohort was determined in 2005. This group of patients contained 10 HLA-B*4001+ cohort members and 19 that possess the HLA-B*4002 subtype. These patients were divided based on whether they had a CD4 count of below 200, between 200 and 350 or above 350 cells/mm^3 (figure 4.11-A). A higher frequency of HLA-B*4002 patients possessed a CD4 count of above 350 cells/mm3 and a higher frequency of HLA-B*4001+ patients displayed a CD4 count of below 200 cells/mm3. These differences did not reach significance possibly due to the small sample size of patients.

The Median of the CD4 counts was determined for these two groups of patients and compared to the median CD4 count of non-HLA-B*40+ patients in the cohort (figure 4.11-B). The median CD4 count of HLA-B*4002+ patients is higher than that for the non-HLA-B*40 patients whilst the HLA-B*4001+ patients displayed a reduced median CD4 count as compared to this group of patients. Again these results failed to reach significance.

The viral load was determined for several HLA-B*40+ patients in 2007 and 2008 and the patients were divided based on those that possess a viral load of below 2000 copies/mm^3 and those with a viral load above 10000 copies/mm^3 (figure 4.11-C). A higher frequency of HLA-B*4002+ patients displayed viral loads of below 2000 copies/mm3 than HLA-B*4001+ patients at both time points tested. These results also failed to reach significance.

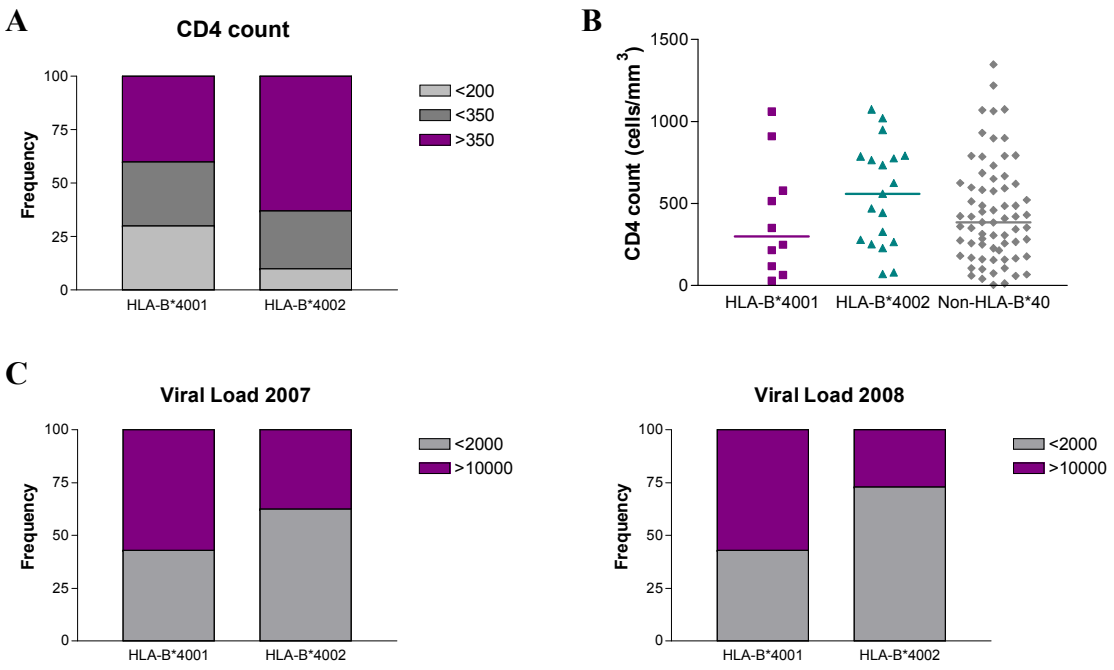


Figure 4.11 There is a trend for HLA-B*4002+ patients to possess higher CD4 counts and lower viral load as compared to HLA-B*4001+ patients. CD4 counts were determined for 90 drug naïve patients from the SM cohort in 2005. The frequency of HLA-B*4001+ and HLA-B*4002+ patients that displayed CD4 counts of below 200 cells/mm^3, between 200 and 350 cells/mm^3 and above 350 cells/mm^3 were determined and are shown in part A. The median of the CD4 counts was also compared between the two subtypes along with that for the non-HLA-B*40+ members of the cohort (B). The viral load for several HLA-B*4001+ and HLA-B*4002+ patients was determined in both 2007 and 2008 and patients were divided based on whether their viral load was below 2000 copies of RNA/mm^3 or above 10000 copies of RNA/mm^3 (C).

4.3 Discussion

The CD8+ T cell response against HIV-1 generated by a given individual often focuses on a few viral epitopes and within a cohort of infected people those who share the same HLA type often respond to the same antigens. This phenomenon, known as immunodominance, is well described for many viral and other infections but the underlying mechanisms that lead to the focussing of the immune response in this way are incompletely understood.

The data presented in this chapter shows that members of the HIV-1 infected SM cohort that posses two closely related HLA-subtypes, HLA-B*4001 and HLA-B*4002, produce immunodominant CD8+ T cell responses to different known HLA-B*40 epitopes. Viral sequence analysis revealed that HLA-B*40 associated mutations that occur within some of these epitopes are present at a significantly higher frequency in patients that posses the HLA-B*40 subtype associated with an immunodominant response to the given epitope. This suggests such immunodominance patterns have been present throughout infection and are not the result of loss of certain responses due to the acquisition of escape mutations.

Analysis to determine associations between HLA types and viral control within the SM cohort revealed that the average viral load and CD4 T cell count for HLA-B*40+ patients did not significantly differ from that of the cohort as a whole. This indicates that HLA-B*40 is associated with neither good nor bad viral control, a finding that reiterates published data stating that HLA-B*40 possesses a relative hazard score of 1 (Dean, Carrington et al. 2002).

However, when the patients are divided based on HLA subtype it was revealed that HLA-B*4002+ patients to have an increased CD4 count as compared to non-HLA-B*40 patients and HLA-B*4001+ patients. HLA-B*4001+ patients displayed a slightly reduced median CD4 count as compared to the non-HLA-B*40 members of the cohort. Whilst these differences did not reach significance, possibly due to the small sample size of patients analysed, it does suggest that HLA subtype should be taken into consideration when assessing the impact of MHC class I molecules on the outcome of HIV-1 disease as well as other infections.

There are in fact several published examples of HLA subtype affecting the outcome of HIV-1 infection. HLA-B*3501 presents peptides with a proline at position two and a tyrosine at the C terminal. HLA-B*3502 and HLA-B*3503 differ from HLA-B*3501 by only three and one amino acid, respectively. These amino acid substitutions occur within the F pocket of the peptide binding groove and lead to these two subtypes preferentially binding peptides with small hydrophobic amino acids at their C terminal instead of tyrosine. In addition to the differences in the HIV-1 epitopes that bind to these HLA-B*35 subtypes, differences in disease outcome have also been observed. HLA-B*3502 and HLA-B*3503 are associated with rapid progression to AIDS whilst no such association exists for HLA-B*3501 (Goulder and Watkins 2008).

A study of HIV-1 infected individuals in Durban, South Africa found HLA-B*5801 to be associated with low viral load and HLA-B*5802 to be associated with high viral load (Kiepiela, Leslie et al. 2004). Again these HLA-subtypes differ by only three amino acids, a difference that also translated into a divergence in peptide binding specificity. Interestingly HLA-B*5801 shows a preference for two gag epitopes that are

also presented by the protective allele HLA-B*57 whilst the immunodominant HLA-B*5802 restricted response is towards an env specific epitope (Goulder and Watkins 2008). These data suggest that it is the peptide presenting function of the HLA molecule and possibly the quality of the CTL response that is subsequently elicited that is responsible for any association with good or bad HIV-1 control. As well as indicating that it is important to consider the HLA subtype when establishing links between MHC class I molecules and HIV-1 disease outcome.

The two HLA-subtypes investigated in this study differ by eight amino acids, five of which are in the peptide binding groove. Four of these amino acids have side chains that point into the peptide binding groove and three are at positions which contact the bound peptide, namely 97, 143 and 147. The amino acid at position 97 of the HLA-molecule is involved in contacting the amino acids at position 6 and 7 of the peptide epitope. Those amino acids at positions 143 and 147 of the HLA molecules form links with the C terminal of the bound peptide. This could in part explain the altered immunodominace seen between the two HLA subtypes, as the peptides most commonly recognised by both groups of patients differ in the amino acid that is present at the C terminal. The immunodominant HLA-B*4001 restricted epitope has a leucine at the C terminal whilst the most frequently recognised epitopes by the HLA-B*4002+ patients have either a valine or an alanine at the C terminal.

However the peptide binding properties of MHC class I molecules are not the sole determinant of CD8+ T cell epitope immunodominance and several other factors could be involved in influencing which HIV-1 epitopes are dominantly recognised. This is reiterated by the fact that the predicted binding affinities of the peptides used in these

experiments for both HLA subtypes do not wholly explain the immunodominance pattern seen.

A factor known to impact on the immunodominance hierarchy of CD8+ T cell responses to HIV-1 is variation within the viral sequence. It has been described that perinatally infected HLA-B*27+ babies that contract HIV-1 containing an escape mutation within an immunodominant epitope fail to mount a response to this epitope. Instead their CD8+ T cells target an otherwise subdominant HLA-B*27 restricted epitope and subsequently they fail to control HIV-1 replication (Goulder, Brander et al. 2001). For the patients studied in this chapter, viral sequence variation is unlikely to be the cause of the difference in the CD8+ T cell epitope immunodominance hierarchy seen between the two HLA subtypes as both groups of patients became infected with the same narrow source virus.

The availability of different HIV-1 gene products for entry into the antigen processing pathway could also impact on whether certain epitopes are immunodominantly targeted or not. It has been demonstrated *in vitro* that gag-specific CTL can recognise virally infected cells two hours post infection (Sacha, Chung et al. 2007). This suggests that *de novo* synthesis of this protein is not required for the initiation of an immune response and could lead to gag-specific CD8+ T cell responses having an advantage over responses targeting other HIV-1 gene products in terms of immunodominance. However, immunodominant CTL responses do exists to other HIV-1 proteins.

The process of generating of peptide antigens for recognition by CD8+ T cells is a multistep pathway with each step affecting the peptides that will be presented at the cell

surface. Digestion of protein fragments by the immunoproteasome is required for the generation of most peptide epitopes and the sites at which cleavages occur influence the repertoire of peptides available for transport into the ER and MHC class I binding. Other proteases are also involved in protein cleavage both in the cytoplasm and the ER, these also have the potential to impact on the peptides that will be available at the cell surface for CD8+ T cell recognition.

TAP is responsible for transporting peptides from the cytosol into the ER. At this stage of the pathway the peptides must have the correct C terminus for MHC class I binding as only N-terminal cleavage can occur in the ER. TAP is located in the ER membrane in a peptide loading complex (PLC) associated with an MHC class I molecule as well as several other chaperone molecules in order to ensure efficient peptide loading.

One such chaperone is tapasin, a molecule that has been implicated in editing the MHC class I peptide repertoire towards the binding of high-affinity peptides. Tapasin binds to MHC class I molecules in a peptide sensitive manner and is dissociated by peptide binding allowing the p-MHC class I complex to exit the ER. This dissociation is more efficient when higher affinity peptides are bound suggesting that tapasin could be responsible to setting an affinity threshold for peptides that reach the cell surface and go on to elicit immune responses (Raghavan, Del Cid et al. 2008).

Studies have shown that HLA subtypes can differ in their dependency on tapasin for efficient peptide loading. For example, HLA-B*4402 depends heavily on tapasin for peptide loading, whereas HLA-B*4405 is comparatively tapasin independent (Williams, Peh et al. 2002; Zernich, Purcell et al. 2004). This could affect the repertoire of peptides that bind to each subtype in turn influencing immunodominance patterns. It is unknown

whether HLA-B*4001 and HLA-B*4002 differ in their dependence on tapasin but it is evident that small changes in MHC class I molecules can alter various steps in the antigen processing pathway.

Once a p-MHC class I molecule has made it to the cell surface, in order for a response to ensue the CD8+ TCR repertoire needs to contain receptors capable of recognising it. Differences in HLA subtypes could affect the process of positive and negative selection that occurs within the thymus during CD8+ T cell development thus altering the TCR repertoire and in turn impacting on epitope immunodominance.

Although the repertoire of TCRs among naïve CD8+ T cells is subject to a certain degree of degeneracy and has indeed evolved to react to as many foreign antigens as possible, small changes within the p-MHC complex can determine whether or not a specific TCR can recognise the molecule or not. This is highlighted by the observation stated in this chapter that HLA-B*4001 restricted KEK specific CTL clones only recognise their cognate peptide when it is presented by HLA-B*4001 and not HLA-B*4002.

Given the amino acid substitutions that are present between HLA-B*4001 and HLA-B*4002 it is indeed possible that the peptide epitope KEK binds to both molecules in different conformations. Crystal structures of HLA-B*2705 and HLA-B*2709 bound to the same self peptide (pVIPR) revealed that the epitope bound to HLA-B*2705 in a dual conformation whereas only one of the binding modes was observed in the case of HLA-B*2709 (Hulsmeyer, Fiorillo et al. 2004). The study authors hypothesised that since HLA-B*2705+ people often have pVIPR specific cells in their peripheral blood and

HLA-B*2709+ people rarely possess pVIPR specific CD8+ T cells, efficient thymic deletion must occur in the HLA-B*2709+ subjects.

Therefore whilst the binding affinity of certain peptides for the HLA-B*40 subtypes may preclude them from becoming immunodominant epitopes, other epitopes that do bind well to both molecules may be prevented from dominance due to changes in the CD8+ TCR repertoire. These results also suggest that the conformation of a p-MHC class I complex and not merely the sequence of the bound peptide are important for T cell recognition.

As well as producing immunodominant responses to different peptide epitopes, the differentiation phenotype of the immunodominant CD8+ T cells restricted by HLA-B*4001 and HLA-B*4002 also differ. There is a trend for HLA-B*4001 restricted KEK specific CD8+ T cells to contain a higher percentage of CD27-CD28- fully differentiated cells as compared to HLA-B*4002 restricted AEW specific cells. This difference becomes significant when all HLA-B*4001 restricted responses as measured by tetramer staining are compared to all HLA-B*4002 restricted responses. This difference does not stem solely from the fact that the immunodominant HLA-B*4001 restricted response is towards a nef epitope whilst the two most commonly recognised HLA-B*4002 restricted responses are directed against gag epitopes.

HLA-B*4001 restricted virus specific CD8+ T cells are atypical amongst HIV-1 specific CTL as generally such cells display an intermediate differentiation phenotype (Appay, Nixon et al. 2000). However, the significance of such differences in terms of CD8+ T cell functionality and HIV-1 disease outcome has proved difficult to establish.

Some researchers view intermediately differentiated CD8+ T cells as an appropriate HIV-1 specific response and see fully differentiated CTL as cells being pushed towards senescence. During acute infection when HIV-1 replication is controlled, potentially by CD8+ T cells, such cells display an intermediate differentiation phenotype (Appay, Papagno et al. 2002). Furthermore, an increase in the proportion of total CD8+ T cells that are CD27-CD28- is associated with progression to AIDS and a lack of CD27 expression on CD8+ T cells is linked to the expression of CD57 which is a marker of senescence (Papagno, Spina et al. 2004). In keeping with this, virus specific CD8+ T cells from patients infected with HIV-2, typically classified as LTNPs, predominantly display an early differentiation phenotype. In addition, CD28 expression correlates with CD4 count in these patients (Leligdowicz, Onyango et al. 2010).

However, others in the field believe that the lack of differentiation that occurs in the HIV-1 specific CD8+ T cell population represents a malfunctioning of the immune system and contributes to the inability of such cells to control HIV-1 infection. In has been demonstrated that an increase in the percentage of CD27- HIV-1 specific CD8+ T cells correlates with a reduction in viral load and an increase in AIDS free survival. The same study also revealed a link between a lack of fully differentiated EBV-specific CD8+ T cells and the development of EBV-associated non-Hodgkin lymphoma (van Baarle, Kostense et al. 2002).

The data presented in this chapter suggests that, given the trend for better viral control within the HLA-B*4002+ patients and the intermediate state of differentiation displayed by the immunodominant CD8+ T cells from these subjects, it may be beneficial to possess CTL that are not fully differentiated. However, other factors could also be

involved in the trend for better viral control displayed by the HLA-B*4002+ patients in the SM cohort. For example, gag specific CD8+ T cells have been associated with good HIV-1 disease outcome (Ogg, Jin et al. 1998; Edwards, Bansal et al. 2002; Novitsky, Gilbert et al. 2003; Zuniga, Lucchetti et al. 2006; Kiepiela, Ngumbela et al. 2007) and the two immunodominant CTL responses targeted by HLA-B*4002+ patients are towards epitopes located in gag whilst the most frequently recognised epitope by the HLA-B*4001+ patients is located in nef.

The reasons that lie behind the apparent block in maturation frequently displayed by HIV-1 specific CD8+ T cells are unknown. One factor that could be involved is the antigen dose that a given CTL response is exposed to, with cells that come into contact with higher levels of stimulation being pushed towards full maturation and senescence. One observation that came out of the work described in this chapter was that all the HLA-B*4001 restricted KEK specific clones tested displayed a higher functional avidity as compared to the HLA-B*4002 restricted AEW specific clones. This could lead to the KEK specific CTL receiving greater amounts of antigenic stimulation and therefore becoming more differentiated than the AEW specific CD8+ T cells. However, this observation is based on data using clones generated from only two patients, one HLA-B*4001+ and one HLA-B*4002+. It would have to be determined that this increase in functional avidity was a general feature of all KEK specific CTL before any further conclusions can be drawn.

The data presented in this chapter highlight the importance of taking into consideration closely related HLA subtypes when characterising immunodominant CD8+ T cell responses within a HIV-1 infected cohort of patients. This work has shown that

CTL responses restricted by related HLA subtypes can differ in their specificity and phenotype. Such differences could lead to distinct CD8+ T cell functionalities and may in turn lead to disparate disease outcomes. This is not surprising given that the existence of MHC class I polymorphisms provides evidence that significant differences in disease outcome occur as a result. Therefore, it may be necessary to consider the HLA subtypes within a given population when designing HIV-1 vaccine intended to elicit CD8+ T cells.

Chapter 5 – Factors that affect the processing and presentation of overlapping HIV-1 specific CD8+ T cell epitopes

5.1 Background

The error-prone nature of HIV-1 reverse transcription combined with its high replicative ability has resulted in the virus exhibiting a vast amount of genetic diversity. Group M HIV-1 comprises of several clades, sub-clades and circulating recombinant forms which can possess sequences that differ from each other by up to 30%. In addition viruses from within the same clade can also show substantial sequence differences, sometimes up to 15% (Korber, Gaschen et al. 2001). This presents those attempting to design a vaccine, which will be effective against a wide range of HIV-1 subtypes and within several infected populations, with a difficult task.

It therefore seems logical, that when devising a vaccine to elicit CD8+ T cells, concentrating on epitopes that remain conserved between several HIV-1 clades would be a good strategy. However, sequence conservation of the epitope itself doesn't always ensure that a response will be generated towards a given antigen. Other factors affect the processing and presentation of a peptide epitope. Previous work from our group has shown that the flanking sequence of a conserved HLA-B*08 restricted nef epitope affects the processing of the peptide and that certain amino acids upstream of the epitope are important in determining if the epitope is processed or not (Ranasinghe, Kramer et al. 2010).

Variable factors within the antigen presenting cell may also impact on the processing and presentation of a given peptide antigen. These include polymorphisms in the proteins involved in degrading peptides in the cytosol, transporting them into the ER and loading them into MHC class I molecules. Also the presence of certain HLA molecules that do not restrict a given epitope can affect the presentation of the peptide by competing with the restricting HLA molecule for binding to proteins within the PLC or peptide fragments in the ER.

The presence of HLA molecules that compete for binding of overlapping peptide epitopes has been shown to influence whether or not a given antigen proceeds fully through the antigen processing pathway and induces a CD8+ T cell response. It has been shown that an HLA-B*08+ donor failed to produce a CTL response to an immunodominant HLA-B*08 restricted flu epitope but did produce a response to an overlapping HLA-B*27 restricted epitope. It was concluded that the two HLA molecules were competing for binding to the same peptide fragment in the ER and that it was presence of HLA-B*27 that abrogated that HLA-B*08 restricted response (Tussey, Rowland-Jones et al. 1995).

The experiments outlined in this chapter were carried out to investigate further the affect of both flanking region sequence and antigen presenting cell HLA type on efficient epitope processing. Firstly, the processing of an HLA-B*40 restricted nef epitope that substantially overlaps with the HLA-B*08 restricted epitope described above was examined. Secondly, the processing of two overlapping HLA-A*03 and HLA-B*07 restricted nef epitopes in the presence of either one or both of these HLA molecules was also explored.

The results showed that the pattern of antigen processing can differ substantially between epitopes that overlap by several amino acids. A shift of just two amino acids can lead to both a reduction and an increase in the efficiency of processing of a peptide epitope as compared to the epitope beginning two amino acids previously. It was also demonstrated that the efficiency of processing of an HLA-B*07 restricted nef specific epitope differed within two B cell lines that possess divergent HLA types.

5.2 Results

5.2.1 Two HIV-1 nef epitopes restricted by HLA-B*40 and HLA-B*08 that overlap by 6 amino acids rely on different amino acids in their flanking regions for efficient processing

HLA-B*40 restricted clones specific for a nef epitope (KEK) were generated. This epitope overlaps with the HLA-B*08 restricted epitope described in Ranasinghe *et al* (2011) by 6 amino acids (figure 5.1). These clones were then used to determine whether or not the KEK epitope is processed and presented when a HLA-B*40+ B cell line was infected with several recombinant vaccinia viruses (rVV) containing HIV-1 nef proteins of varying sequences (table 5.1). The infected cells were tested for recognition by the KEK specific clones in an IFN-γ ELISpot assay. In addition, the presence of the KEK epitope on the surface of the B cell line was also determined by measuring the percentage of CD107a+ clones following incubation with the infected B cells (Figure 5.2). The results of these experiments are outline in table 5.1, depending on the nef sequence inserted into the rVV either one, both or neither of the epitopes were processed.

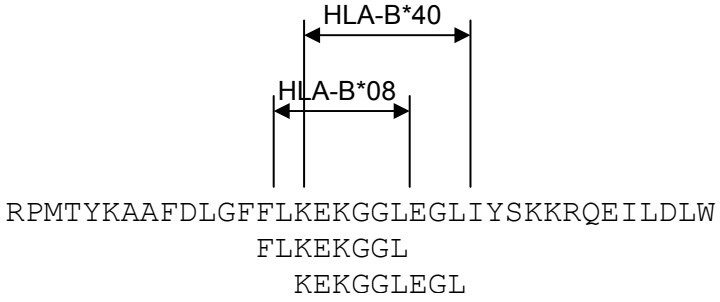


Figure 5.1 A region of HIV-1 nef contains two epitopes restricted by HLA-B*08 and HLA-B*40 that overlap by 6 amino acids

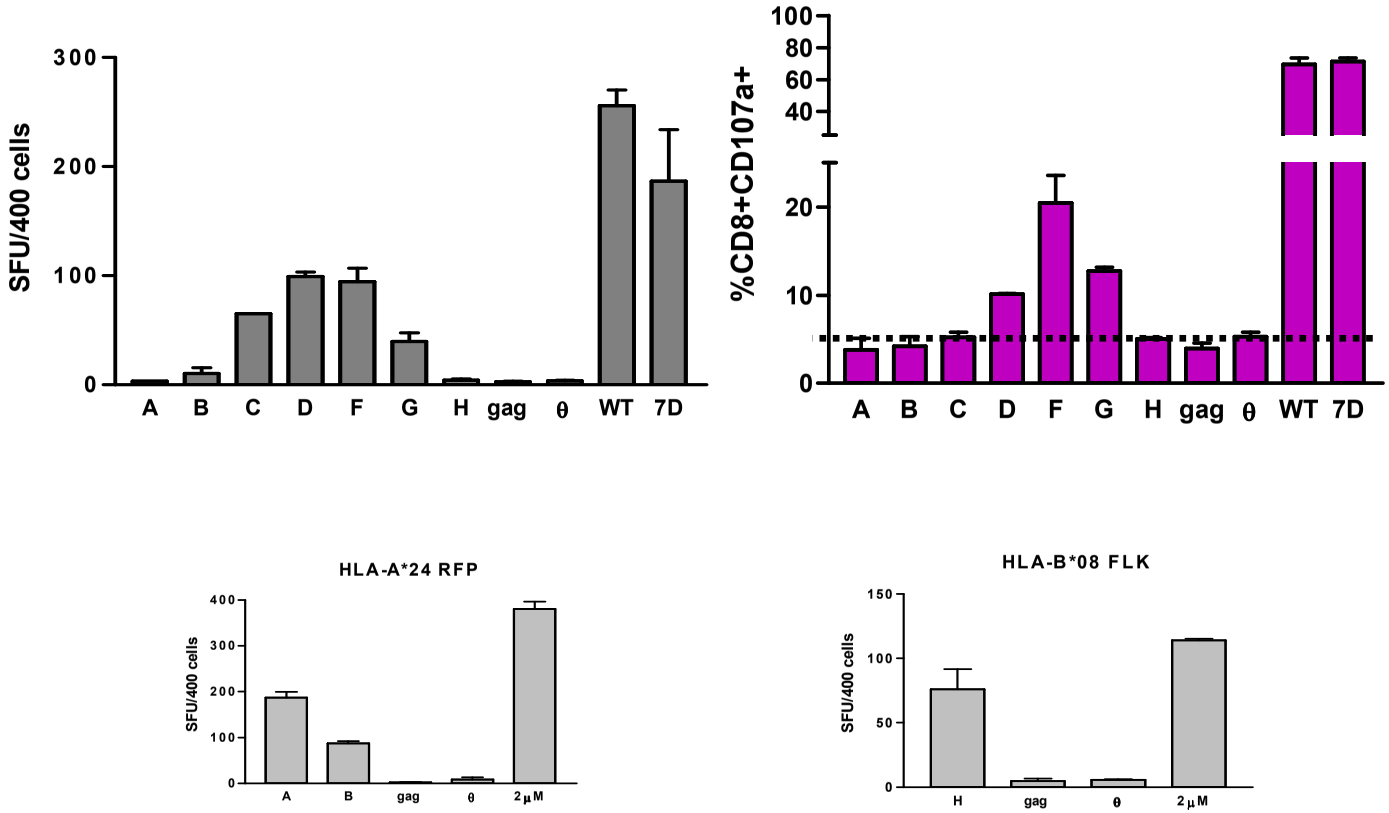


Figure 5.1 An HLA-B*40 restricted KEK specific clone recognises an HLA-B*40+ B cell line infected with rVV containing certain HIV-1 nef sequence but not others. rVV containing nef sequences derived from 7 different clades (A-D, F-H) were used to infect an HLA-B*40+ B cell line. An HLA-B*40 restricted KEK specific clone was used to determine the presence of the epitope at the cell surface in both an IFN-γ ELISpot assay and a CD107a assay. B cells infected with an rVV containing HIV-1 gag or pulsed with 2μM of peptide (WT = KEKGGLEGL, 7D = KEKGGLDGL) were used as negative and positive controls. The bottom panel shows recognition of infected B cells by an HLA-A*24 restricted clone and an HLA-B*8 restricted clone as a control to ensure infectivity of the rVV when the HLA-B*40 epitope was not processed and presented.

rVV-Nef HIV isolates	Clade	Viral sequence for FLK/KEK epitope and flanking region	FLK recognised*	KEK recognised
HIV-$1_{92UG037}$	A	VRPQVPLRPMTYKAAFDLGFFLKEKGGLDGLIYSKKRQEILDLWVYHT	-	-
HIV-1_{MN}	B	VKPQVPLRPMTYKAALDLSHFLKEKGGLDGLIYSQKRQDILDLWVYHT	++	-
HIV-$1_{96ZM651}$	C	VRPQVPLRPMTYKAAVDLSFFLKEKGGLEGLIYSKKRQEILDLWVYHT	+	++
HIV-$1_{94UG114}$	D	VRPQVPLRPMTYKEAVDLSHFLKEKGGLEGLVWSPKRQEILDLWVYHT	+++	+++
HIV-1_{CM235}	AE	VRPQVPLRPMTYKGAFDLSFFLKEKGGLEGLIYSKRRQEILDLWVYNT	-	ND
HIV-$1_{93BR020}$	F	VRPQVPLRPMTYKGAVDLSHFLKEKGGLEGLIYSKRRQEILDLWVYHT	++	+++
HIV-$1_{92NG083}$	G	VRPQVPLRPMTYKAAFDLSFFLKEKGGLDGLIYSKRRQDILDLWVYNT	-	++
HIV-$1_{90CF056}$	H	VRPQVPLRPMTYKGAFDLSHFLKEKGGLDGLIYSKQRQDILDLWVYNT	++	-

Table 5.1 The Nef sequences with the rVV, spanning the FLK and KEK epitopes and their flanking regions along with the HIV-1 isolate from which the sequences originates. The right hand two columns indicate whether or not infection with the rVV results in the presentation of the FLK and KEK epitopes. *As shown in Ranasinghe et al 2011.

5.2.2 The apparent absence of efficient KEK processing following infection with certain rVV containing nef sequences that possess a mutation at position 7 within the epitope does not stem from a lack of recognition of the mutant epitope or a reduction in functional avidity

Infection of HLA-B*40+ B cells with the rVV containing nef sequences derived from clades A, B and H viral isolates does not result in the processing and presentation of the KEK epitope. However, the nef sequences contained within these rVV include a glutamic acid to an aspartic acid substitution at position 7 within the KEK epitope. The clone used in the experiments outlined above was tested for its ability to recognise this 7D epitope variant by using the mutant peptide to pulse HLA-B*40+ B cells and assaying for IFN-γ release and CD107a expression (figure 5.1). In both instances recognition occurred, a point reiterated by the observation that infection with the rVV containing the clade G HIV-1 nef sequence which also contains the 7D variant KEK epitope results in the recognition of the epitope.

It is however possible that the KEK specific clone recognises this mutant epitope with a reduced functional avidity as compared to the wild type peptide and therefore a greater amount of peptide and in turn more efficient antigen processing is required in order to induce a response. The functional avidity of the HLA-B*40 restricted KEK specific clone for both the wild type and the 7D mutant peptide was determined using an IFN-γ ELISpot assay. The peptide concentration that elicited the half maximal response was the same for both peptides (figure 5.2) so whilst the 7D mutation may have an impact

on antigen processing it does not affect the recognition of the epitope with respect to the KEK specific clone used for these experiments.

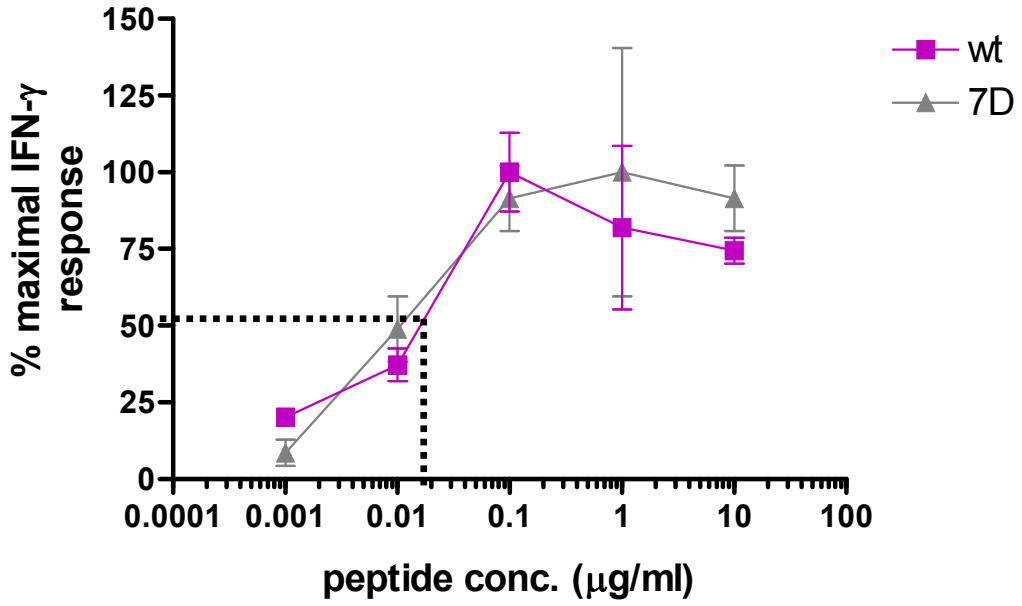


Figure 5.2 The HLA-B*40 restricted KEK specific CD8+ T cell clone recognises the wild type and the 7D mutant KEK peptide with the same functional avidity. A HLA-B*40+ B cell line was pulsed with various concentrations of both the wild type and the 7D mutant KEK peptide and incubated with the KEK specific clone in an IFN-γ ELISpot assay.

5.2.3 The processing of an HLA-A*03 restricted nef epitope is more efficient than that of an HLA-B*07 restricted epitope that it overlaps by 6 amino acids

The rVV described above were used to infect an HLA-A*03 and HLA-B*07+ B cell line which was then used in an IFN-γ ELISpot assay. CD8+ T cell clones, one restricted by HLA-A*03 and one restricted by HLA-B*07, were used to determine if two overlapping nef epitopes (figure 5.3) were processed and presented by the rVV infected B cell line. Infection of the B cell line with all of the tested rVV resulted in the processing and presentation of the HLA-A*03 restricted epitope to some extent whilst only one of the rVV was recognised by the HLA-B*07 restricted clone (Figure 5.4).

Whilst all the rVV contained the wild type sequence of the HLA-A*03 restricted epitope QVP, the HLA-B*07 restricted RPM epitope varied at positions 7 and 9. Some degree of variation in the recognition of the mutant epitopes by the HLA-B*07 restricted clone was seen (figure 5.5), however most peptides were recognised better than the peptide ending in AAL which represents the epitope contained with in the rVV possessing the clade B derived nef sequence. The results of these experiments are outlined in table 5.2.

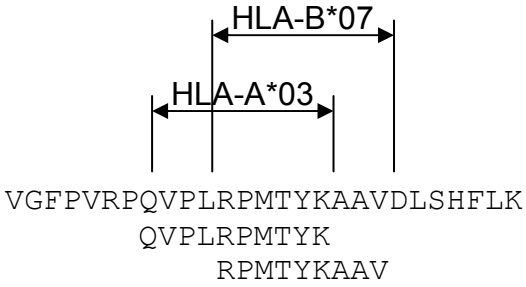


Figure 5.3 A region of HIV-1 nef contains two epitopes restricted by HLA-A*03 and HLA-B*07 that overlap by 6 amino acids

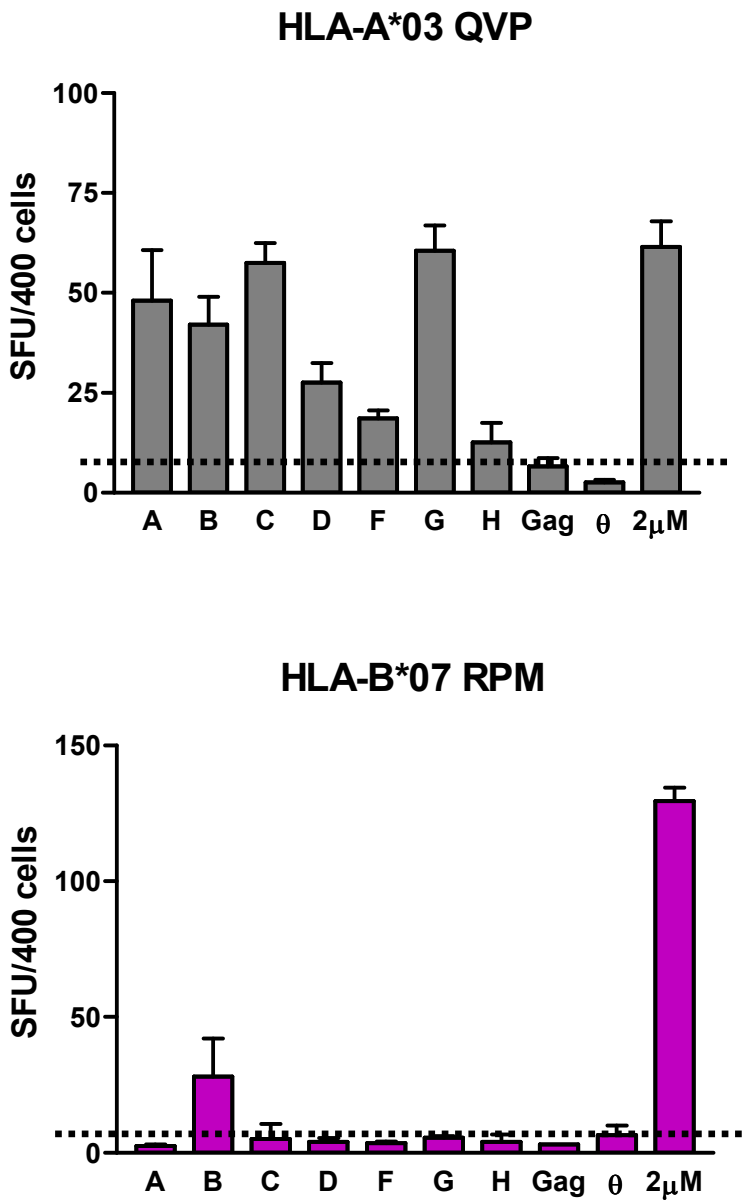


Figure 5.4 Infection of an HLA-A*03/HLA-B*07+ B cell line with 7 rVV containing varying HIV-1 nef sequences results in the processing and presentation of an HLA-A*03 restricted nef epitope whilst only the rVV containing the clade B derived nef sequence is recognised by an HLA-B*07 restricted nef specific clone. An HLA-A*03/HLA-B*07+ B cell line was infected with several rVV containing differing HIV-1 nef sequences. These cells were then tested for recognition by an HLA-A*03 restricted and an HLA-B*07 restricted nef specific clone in an IFN-γ ELISpot assay. B cells infected with rVV containing HIV-1 gag and 2μM peptide were used as + and - controls. Peptides used were QVPLRPMTYK and RPMTYKAAV.

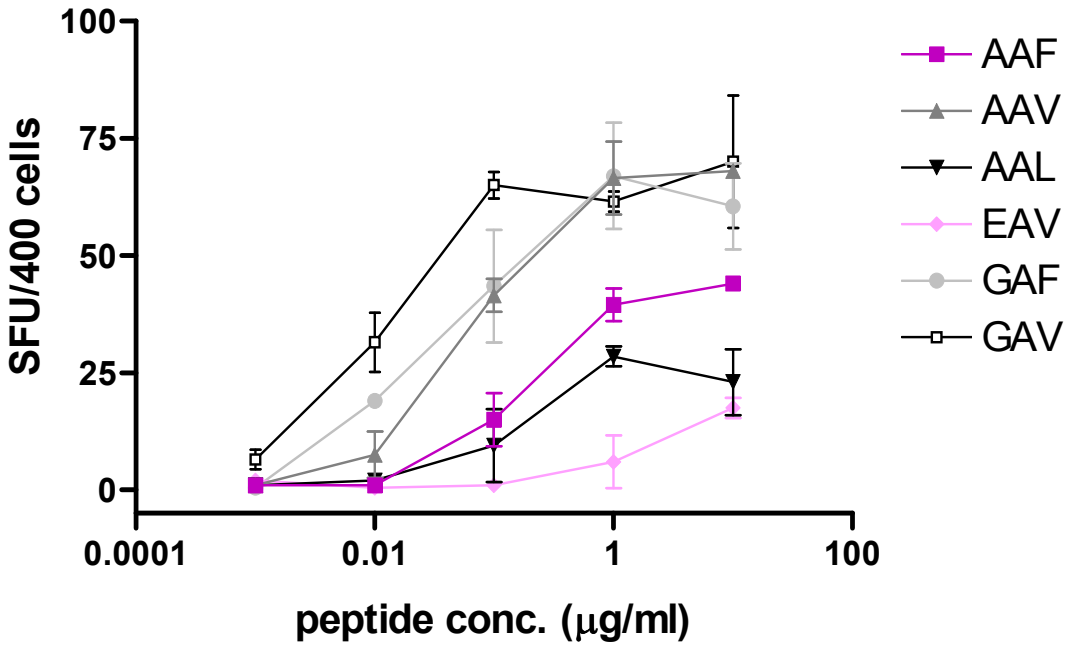


Figure 5.5 All the peptides that match the RPM epitope sequences found within the rVV are recognised by the HLA-B*07 restricted RPM specific clone. However, the peptides ending in AAF, AAV and EAV are less well recognised than the other peptides. Various concentrations of peptides beginning with the sequence RPMTYK and ending in the three amino acids outlined above were used to pulse an HLA-B*07+ B cell line that was then used in an IFN-γ ELISpot assay with an RPMTYKAAV specific CD8+ T cell clone.

rVV-Nef HIV isolates	Clade	Viral sequence for QVP/RPM epitope and flanking region	QVP rVV	QVP peptide	RPM rVV	RPM peptide
HIV-$1_{92UG037}$	A	-EEEEVGFPVRPQVPLRPMTYKAAFDLGFFLKEKGG	+++	+++	-	++
HIV-1_{MN}	B	-EEEEVGFPVKPQVPLRPMTYKAALDLSHFLKEKGG	+++	+++	+	++
HIV-$1_{96ZM651}$	C	-EEEEVGFPVRPQVPLRPMTYKAAVDLSFFLKEKGG	+++	+++	-	+++
HIV-$1_{94UG114}$	D	-EEEEVGFPVRPQVPLRPMTYKEAVDLSHFLKEKGG	++	+++	-	+
HIV-$1_{93BR020}$	F	-EEDEVGFPVRPQVPLRPMTYKGAVDLSHFLKEKGG	+	+++	-	+++
HIV-$1_{92NG083}$	G	EEDSDVGFPVRPQVPLRPMTYKAAFDLSFFLKEKGG	+++	+++	-	++
HIV-$1_{90CF056}$	H	EDGEEVGFPVRPQVPLRPMTYKGAFDLSHFLKEKGG	+	+++	-	+++

Table 5.2 The Nef sequence contained in the rVV, spanning the QVP and RPM epitopes and their flanking regions along with the HIV-1 isolate from which the sequences originates. The right hand four columns indicate whether or not infection with the rVV results in the presentation of the QVP and RPM epitopes as well as indicating if the peptide matching that of the epitope contained within the rVV is recognised.

5.2.4 The processing of the HLA-B*07 restricted RPM epitope differs in two different B cell lines with distinct HLA types whilst the processing of the HLA-A*03 restricted QVP is remain unchanged within two B cell lines

Two different B cell lines (J66 and J69) were infected with various amounts of the rVV containing the nef sequence derived from a clade B HIV-1 isolate. These cells were then used as target cells in an IFN-γ ELISpot where the efficiency of the RPM epitope processing was detected using a HLA-B*07 restricted RPM specific CD8+ T cell clone.

When 2 PFU/ target cell of the rVV was used to infect both cell lines the number of spot forming units/400 CTL clones was comparable. However, when 0.2 PFU/target cell of rVV was used to infect the B cell lines more spot forming units were observed when the J66 B cell line (HLA-A*03-/HLA-B*07+) was present. No different was seen in the response of the HLA-A*03 restricted QVP specific CTL clone to two B cell lines (J38 and J69) at any of the concentrations of rVV tested (figure 5.6).

The above described experiment was also repeated using the rVV containing both the clade C and the clade G derived nef sequences. Again the B cell line used had no bearing on the processing and presentation of the QVP epitope (figure 5.7). The HLA-A and B molecules present in the B cell lines used in these experiments are outlined in table 5.3.

J38	J66	J69
HLA-A*03 HLA-A*68 HLA-B*35 HLA-B*44	HLA-A*01 HLA-A*24 **HLA-B*07** HLA-B*57	HLA-A*01 **HLA-A*03** **HLA-B*07** HLA-B*44

Table 5.3 The HLA background of the B cell lines used in the experiments shown in figures 6.6 and 6.7

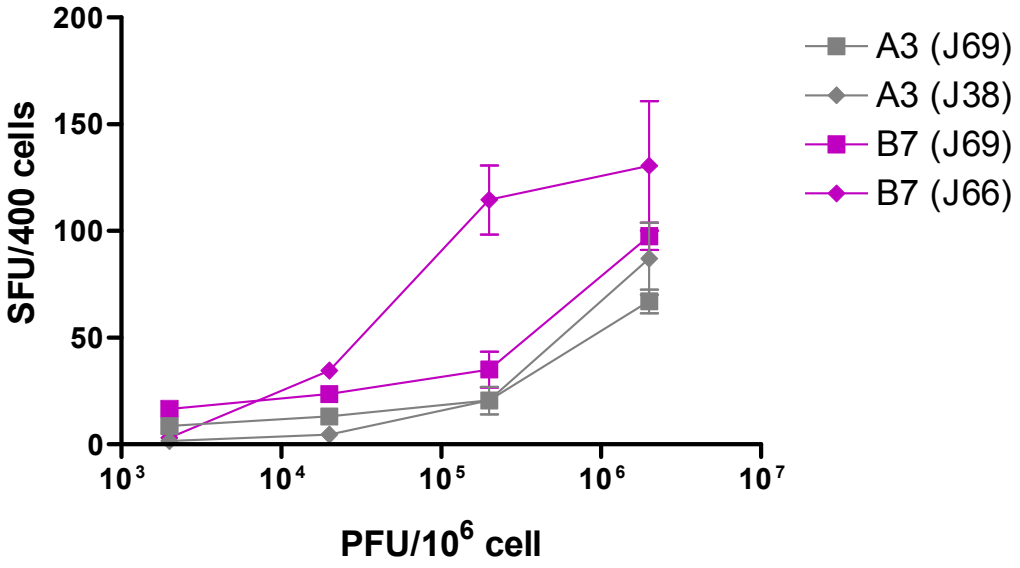


Figure 5.6 The B cell line used affects the processing of an HLA-B*07 restricted nef epitope. Three B cell lines (J38, J66 and J69) were infected with various amounts of the rVV containing the clade B derived nef and were subsequently used as target cells in an IFN-γ ELISpot assay. An HLA-A*03 restricted QVP specific clone and an HLA-B*07 restricted RPM specific clone were used to determine the efficacy of the processing and presentation of the QVP and RPM epitopes.

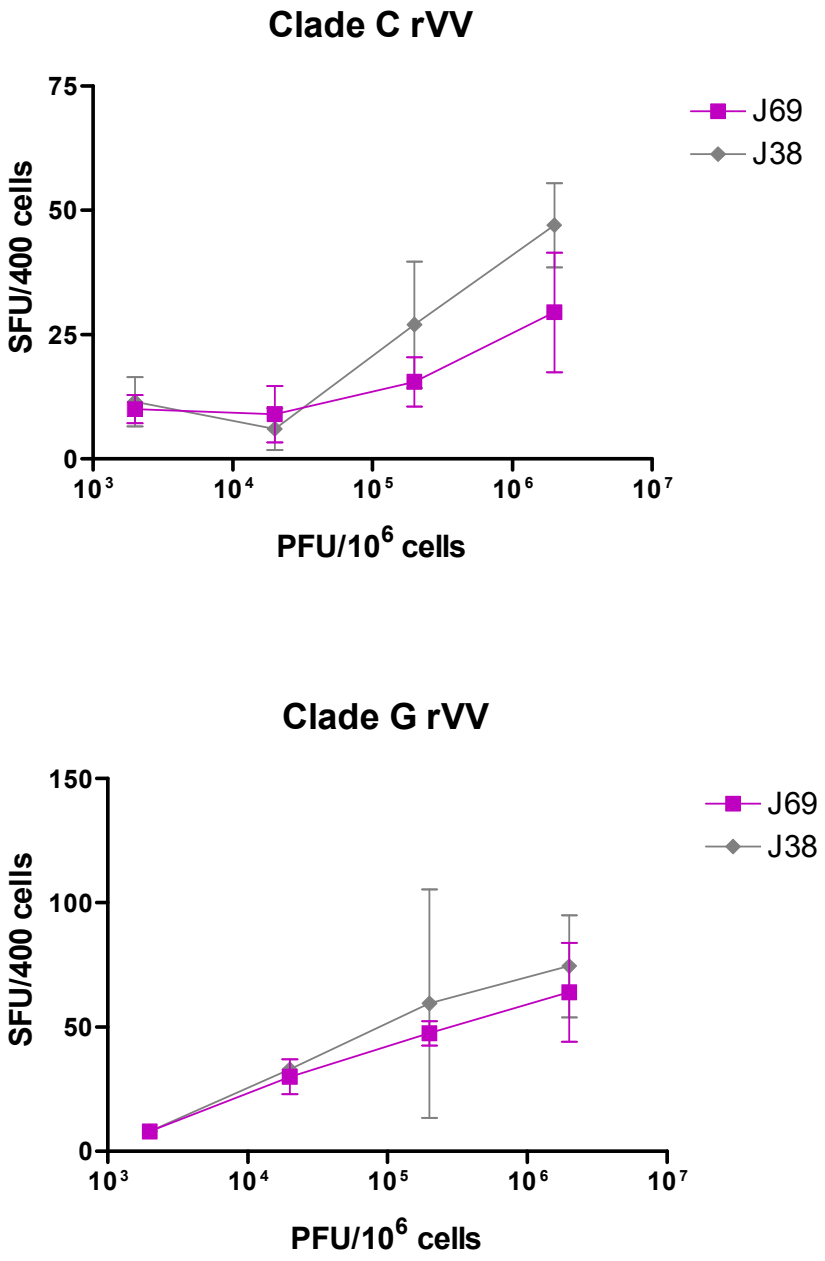


Figure 5.7 The B cell line used does not affect the processing of an HLA-A*03 restricted nef epitope. Two B cell lines (J38 and J69) were infected with various amounts of the rVV containing the clade C (top panel) or the clade G (bottom panel) derived nef sequences and were subsequently used as target cells in an IFN-γ ELISpot assay. An HLA-A*03 restricted QVP specific clone was used to determine the efficacy of the processing and presentation of the QVP epitope.

5.2.5 Both the HLA-A*03 restricted and the HLA-B*07 restricted nef specific clones recognise the different B cell lines pulsed with peptide with the same functional avidity

The ability of both the J66 and the J69 B cells to present the RPM epitope when pulsed with varying concentrations of peptide was assessed using a standard Cr^{51} release assay. No difference in the functional avidity of the HLA-B*07 restricted RPM specific clone was observed when both B cell lines were used in the assay. The same was true of the HLA-A*03 restricted QVP specific clone when incubated with both J38 and J69 B cells pulsed with peptide (figure 5.8).

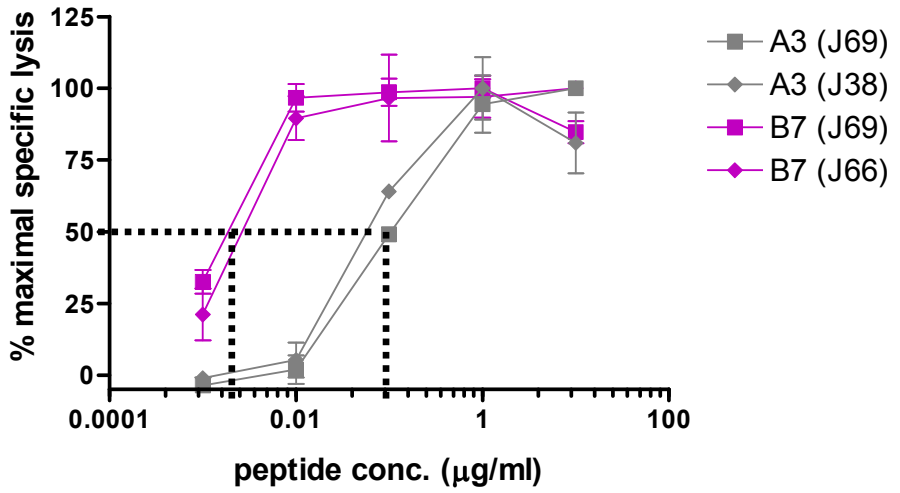


Figure 5.8 The HLA background of the peptide pulsed B cells does not affect their recognition by the CTL clones. Two B cell lines (J38 and J69) were pulsed with varying concentrations of QVPRPMTYK and incubated with an HLA-A*03 restricted clone in a standard Cr^{51} release assay. In addition, the J69 B cell lines along with the J66 B cell line were pulsed with varying concentrations of RPMTYKAAV peptides and incubated with an HLA-B*07 restricted clone in a standard Cr^{51} release assay.

5.3 Discussion

Many studies aimed at characterising HIV-1 specific CD8+ T cells make use of exogenously loaded peptide antigen. However, testing for recognition of a peptide sequence does not take into account the multiple steps that must occur in order to generate an epitope presented in an HLA molecule at the cell surface from a HIV-1 protein in the cytosol. Many variables are involved in determining if this process is efficient including the viral sequence of the epitope and its flanking regions, as well as features of the antigen presenting cell such as polymorphisms in the proteins involved in antigen processing.

The data presented in this chapter highlights that both the viral sequence and the antigen presenting cell can impact of the efficiency of HIV-1 epitope processing. Results outlined above describe how an HLA-B*40 restricted nef epitope relies on different amino acids for efficient processing as compared to an HLA-B*08 restricted epitope with which it overlaps by six amino acids. The processing of the HLA-B*08 restricted epitope FLK is heavily influenced by the amino acid directly preceding its N terminal. Efficient processing does not occur when a phenylalanine is present at this position (Ranasinghe, Kramer et al. 2010). As the HLA-B*40 restricted KEK epitope is situated two amino acids further along the nef sequence and has a conserved leucine directly preceding its N terminal it could be hypothesised that more efficient processing of the epitope occurs. However, as outlined in table 5.1, a conserved leucine instead of a phenylalanine at position -1 does not ensure processing of the KEK epitope.

It is perhaps unsurprising that the efficient processing and presentation of an epitope cannot be ensured by the presence of one specific amino acid. Only the generation of particular peptide fragments will result in the presentation of a given antigen. The correct C terminal of the antigen must be produced before TAP transport occurs as only N-terminal peptidases are present in the ER (Cascio, Hilton et al. 2001; Saric, Chang et al. 2002). Therefore subtle changes in peptide sequence that lead to even minor alterations in the fragments produced by the proteasome and other cytosolic proteases could result in the abrogation of processing of a given epitope.

Whilst the presence of a phenylalanine directly preceding the FLK epitope was highlighted as reducing the efficiency of the processing of the epitope, experiments using lab strain HIV-1 isolates revealed that even when a histidine is present at this position effective processing of the antigen isn't guaranteed (Ranasinghe, Kramer et al. 2010). Similarly, comparing the peptide sequences of the nef genes within the rVV whereby infection results in presentation of the KEK epitope with those that weren't recognised by the KEK specific clone reveal that no one particular amino acid can be used to segregate the two groups.

Due to the complexity involved in the process of antigen generation one specific amino acid alone cannot guarantee that an antigen is processed. However, as previously discussed small changes in amino acid sequence have the ability to have a large impact on epitope generation. It appears that the presence of certain individual amino acids may be able to explain the efficacy of processing both the HLA-A*03 and the HLA-B*07 restricted epitopes investigated in this chapter. Infection with only one rVV resulted in the processing of the RPM epitope, this virus is the only one to contain a nef sequence

with a lysine and not an arginine six amino acids preceding the epitope. Whilst this vaccinia is also the only one to contain a nef sequence with a leucine at the C terminal of the epitope, many studies have identified the sequence RPMTYKAAV as an HLA-B*07 restricted epitope suggesting that a valine at this position also allows for efficient antigen processing.

It also appears that one particular amino acid may be integral to the efficient processing of the HLA-A*03 restricted epitope. Nef sequences with an alanine at the amino acid directly following the C terminal of the epitope are always efficiently processed. However, infection with the rVV containing a nef sequence with a glutamic acid at this position results in a reduction in the efficacy of QVP processing. Furthermore, the two rVV that contain a nef sequence with a glycine at this position are the least well recognised by the QVP specific clone.

The viral sequence is not the only variable that impacts on the processing and presentation of HIV-1 epitopes. An experiment outlined in this chapter demonstrated that the efficiency of processing of the RPM epitope differed depending on the B cell line infected with the rVV. This observation is unlikely to stem from differences in the surface expression of the HLA-B*07 molecule and other proteins involved in TCR engagement as both B cell lines are equally efficient at presenting exogenously loaded peptide. One known difference between the two B cell lines tested was the HLA type of the donors that the cells were derived from. Both patients were HLA-A*01+ and HLA-B*07+ but whilst patient J69 possess HLA-A*03 and HLA-B*44, patient J66 is HLA-A*24+ and HLA-B*57+.

The processing of the RPM epitope is less efficient within the HLA-A*03+ J69 B cell line as compared to the HLA-A*03- J66 B cell line. It could therefore be hypothesised that this HLA molecule is competing with HLA-B*07 in the ER for binding of peptide fragments that contain the overlapping QVP and RPM epitopes thus reducing the amount of the RPM epitope binding to HLA-B*07 and being presented at the cell surface.

Peptides within the ER that have the potential to become the RPM antigen presented at the cell surface must contain the correct C terminal of this epitope but can be N-terminally extended. Whilst peptide fragments comprising an N-terminally extended RPM epitope will contain the QVP epitope it is improbable that these peptides will bind to HLA-A*03. Class I MHC molecules have a peptide binding site that is closed at both ends (Yaneva, Schneeweiss et al. 2009) meaning that HLA-A*03 is unlikely to bind to a C terminally extended QVP peptide. However, one study has solved the crystal structure of HLA-A*02 bound to an epitope extended by one amino acid at the C terminal. This revealed that the carboxyl-terminal residue was positioned outside the peptide binding site (Collins, Garboczi et al. 1994). It is therefore not impossible to conceive that HLA-A*03 may compete with HLA-B*07 for binding of RPM epitope containing peptides in the ER, although as the phenomenon described by Collins *et al* (1994) appears to be a rare event it remains unlikely.

It is also unlikely that HLA-B*44, also present in the J69 B cell line, will compete for binding of peptide fragments with the correct RPM C terminal. This is due to the fact that the peptide binding motif of HLA-B*44 requires a glutamic acid at position two and the amino acids 7, 8 and 9 positions upstream of the RPM C terminal are a proline, an

arginine and a leucine, respectively. It is however, conceivable that MHC class II molecules could be competing for binding of RPM containing peptide fragments, however no known MHC class II epitopes that contain the correct N terminal of this epitope have been characterised (http://www.hiv.lanl.gov/content/immunology).

Other properties of the MHC class I molecules besides those that govern peptide binding could be involved in the reduction in efficiency of RPM processing seen in the J69 B cell line. For example, different HLA molecules have been shown to differ with respect to tapasin-mediated interactions with TAP, thus modulating the incorporation of particular MHC class I molecules into the PLC (McCluskey, Rossjohn et al. 2004). Therefore if one of the HLA molecules present in the J69 B cell line and not the J66 B cell line displays a particularly strong association with tapasin and TAP this may prevent HLA-B*07 from incorporating into many PLCs and would thus reduce the efficiency of processing of HLA-B*07 restricted epitopes. This would be especially apparent under conditions of limiting peptide supply and given that the difference in antigen processing efficiency of the RPM epitope only becomes apparent when a low dose of rVV was used to infect the B cell lines, competition between HLA molecules for proteins involved in the PLC could be a possible reason for the differential processing displayed by the two B cell lines.

Aside from the polymorphisms present in HLA molecules, many other proteins involved in antigen processing and presentation are also polymorphic. There are six alleles of human TAP subunit 1 and four alleles of human TAP subunit 2. In addition, two alleles of tapasin that differ by one amino acid also exist (McCluskey, Rossjohn et al. 2004). Similarly, polymorphisms are present in proteins that comprise subunits of the

proteasome (Lim, Hunter et al. 1999) as well as the ER resident N-terminal peptidase ERAAP (Blanchard and Shastri 2008). However, whilst it is possible that the B cell lines J66 and J69 possess different alleles of these molecules no functional differences in the generation of CD8+ T cell epitopes have been attributed to different alleles of these proteins. On the other hand, some of these polymorphisms have been associated with disparate outcomes to certain infections or the presence of autoimmune disease (Dai, Ning et al. 2005; Burton, Clayton et al. 2007; Soundravally and Hoti 2008; Feng, Yin et al. 2009) suggesting that functional differences between the alleles may exist. Indeed, the fact that polymorphisms in these genes are maintained in the population implies some degree of functional relevance.

These observations emphasise the complexity of the antigen processing pathway and the many variables that can affect the processing and presentation of peptide epitopes. This complexity makes choosing antigens to target with a HIV-1 vaccine designed to elicit CD8+ T cells a very difficult task. Measuring recognition by vaccine induced CD8+ T cells of exogenously loaded peptide is not sufficient to elucidate that during natural infection that same epitope will be available for detection by the same cells. Many highly variable factors will be involved in determining this.

Inducing the CD8+ T cells in the first instance is also no easy task. Vaccines that contain CD8+ T cell epitopes removed from their original flanking sequences may not be processed efficiently enough to induce a CTL response. However as highlighted by work in this chapter, epitopes within their natural flanking regions are not always effectively processed either. Furthermore, polymorphisms within the vaccinated population in various proteins involved in antigen processing and presentation will also affect the

efficacy of epitope generation. Therefore further work needs to be carried out in order to identify HIV-1 antigens that are less susceptible to changes that affect antigen processing and are hence efficiently processed under most conditions. Such epitopes may make good vaccine targets.

Chapter 6: Overall discussion and future directions

The numerous studies that have indicated a role for CD8+ T cells in the control of HIV-1 have led to much vaccine research to be targeted at eliciting virus specific CTL. However, the results of the STEP trial involving a HIV-1 vaccine designed to generate HIV-1 specific CD8+ T cells called into question the effectiveness of this strategy as the trial was halted due to interim analysis revealing that efficacy endpoints would not be met (Gray, Buchbinder et al. 2010). Whilst other constructs have demonstrated more efficient generation of CTL in animal models (Liu, Ewald et al. 2008; Hansen, Vieville et al. 2009; Hansen, Ford et al. 2011) participants that received the STEP trial vaccine did generate virus specific CD8+ T cells. However, they were not protected from acquiring HIV-1 infection (Priddy, Brown et al. 2008).

One of the major obstacles in the bid to create an efficient HIV-1 vaccine that functions by eliciting CD8+ T cells is an incomplete understanding of the determinants of good or bad viral control by naturally generated virus-specific CTL. This has led to a consensus in the field that establishing characteristics of CTL that are associated with effective and poor HIV-1 control should be a priority and will in turn lead to more focused and hopefully more successful vaccine research.

The results presented in this thesis demonstrate that the generation of HIV-1 specific CD8+ T cells is a complex process that can be considerably affected by even subtle changes in viral sequence as well as self proteins that are involved in epitope processing and presentation. Even small changes in the amino acids that comprise the peptide binding groove of an HLA molecule, such that exist between closely related HLA

subtypes, can affect the specificity and in turn impact on the phenotype of CD8+ T cells that recognise epitopes restricted by these molecules. Polymorphisms in other proteins involved in peptide processing and presentation also exist, such changes also have the potential to impact on the efficient processing of viral epitopes. In addition, changes in amino acid sequence of the flanking regions of HIV-1 epitopes influence the efficient processing and presentation of the antigen. This means that even if CD8+ T cells are generated within an individual towards a given HIV-1 epitope by a vaccine, the same epitope may not be generated during natural infection if the flanking region of the antigen within the virus contains amino acids that can abrogate its processing and presentation. This would therefore render the vaccine elicited CD8+ T cells specific for that antigen incapable of controlling viral infection.

Even if all of these factors combine to result in the generation of a CD8 + T cell response towards a given HIV-1 epitope that is then present during natural infection, this does not guarantee that viral control will ensue. A substantial number of studies have demonstrated that not all HIV-1 specific CTL are equally capable of controlling HIV-1 replication. This is highlighted by work presented in this thesis demonstrating that a rapid progressor possessed a high quantity HLA-B*08 restricted CTL response throughout the course of acute and early chronic infection yet failed to control the virus. Furthermore, a trend for differences in the clinical parameters of HIV-1 infection were observed when comparing HLA-B*4001+ and HLA-B*4002+ patients that generated very different CD8+ T cell responses in terms of specificity and phenotype.

An extra level of complexity and heterogeneity in CTL responses to HIV-1 may also exist whereby the functionality of a given response can be modulated throughout the

course of infection. Data presented in chapter 3 demonstrates that two HLA-B*08 restricted nef specific CD8+ T cell clones with the same TCR substantially differ in functional avidity. Whilst these clones presumably stem from the same naïve precursor priming event, they were generated from patient samples obtained at different time points during infection. The clone generated from an acute phase sample is more functionally avid than the clone generated from a chronic phase sample. The chronic phase clone also produced IL-10 upon antigenic stimulation, possibly indicating an exhausted phenotype as a result of continued antigenic stimulation throughout the course of infection.

A small number of studies have been carried out to investigate the role of IL-10 production by HIV-1 specific CD8+ T cells (Elrefaei, Barugahare et al. 2006; Elrefaei, Burke et al. 2009; Torheim, Ndhlovu et al. 2009). However, further work investigating the existence of a correlation between the production of IL-10 by HIV-1 specific CD8+ T cells and the clinical parameters of infection could indicate if secretion of the cytokine has a detrimental effect on the control of viral replication or not. This is turn could led to the investigation of anti-IL-10 therapies for the control of chronic viral infections.

The effect this cytokine has on various cell populations during HIV-1 infection also requires further investigation. It may be of interest to carry out the IFN-γ ELISpot assay used to determine the functional avidity of the clones from patient A794 in the presence of an IL-10 blocking antibody. This will help to establish if the IL-10 secreted by clone 359-6 is having an autocrine effect, reducing the responsiveness of the clone itself to low levels of antigenic stimulation or if production of cytokine only exerts its inhibitory effects on other cell types. The production of IL-10 may not be causally linked to the reduction in functional avidity shown by clone 356-9, indeed clone 11-16 also

displays a reduced functional avidity as compared to clone 11-11 and does not produce IL-10 upon antigenic stimulation.

Examining the signal transduction pathway that is initiated in these clones following stimulation with peptide may hold the key to why they respond differently to low levels of antigen. It has been observed that high avidity CTL require lower amounts of p-MHC to reach the threshold of CD3ζ phosphorylation that is required for TCR signalling (Sharma and Alexander-Miller 2010). Assays to determine the efficiency of phosphorylation of the numerous molecules involved in TCR signal transduction could provide a mechanism for clone 11-16's ability to respond to low doses of peptide.

If CD8+ T cells that are generated from the same naïve precursor can differ in functionality, it is therefore unsurprising that CD8+ T cells restricted by different HLA molecules display substantial differences. Data presented in chapter 4 outlines how HIV-1 epitopes restricted by two closely related HLA subtypes differ in terms of specificity and phenotype, possibly resulting in divergent disease outcomes. To further investigate the causes of such differences investigating the TCR repertoire within both groups of patients would be of use. Positive and negative selection in the thymus shapes the naïve repertoire of T cells in the periphery and these processes are heavily influenced by the HLA molecules present (Starr, Jameson et al. 2003). The presence of one of the HLA-B*40 subtypes during T cell development may lead to the deletion of CD8+ T cell capable of recognising an HIV-1 epitope that is immunodominantly recognised by patients that possess the other subtype.

If however, T cells are present that are capable of recognising a given epitope and that epitope is also able to bind to the HLA-B*40 subtype in question, events that take

place during antigen processing and presentation may be affecting the ability of patients with this subtype to produce an immunodominant response to the antigen. Polymorphisms in any of the many proteins involved in antigen processing and presentation could be in linkage disequilibrium with either of the HLA-B*40 subtypes thus affecting the generation of epitopes that are efficiently processed by cells possessing the other subtype.

For example the presence of other HLA molecules that could compete with the HLA-B*40 molecule for binding to peptide fragments or molecules involved in peptide loading. This hypothesis could be investigated by infecting B cell lines possessing various combinations of HLA-B*4001 or HLA-B*4002 and other MHC class I molecules with the rVV used in chapter 5 and testing for the presence of specific HLA-B*40 restricted epitopes at the cell surface. However, if the HLA-B*40 subtypes are in linkage disequilibrium with other HLA molecules generating such B cell lines will be difficult.

Reasons why CD8+ T cells restricted by HLA-B*4001 and HLA-B*4002 may differ in their control of HIV-1 also requires further investigation. The ability of clones specific for immunodominant epitopes restricted by both HLA-B*40 subtypes could be tested for their ability to suppress HIV-1 replication *in vitro*. This would require a target cell line that is positive for both HLA subtypes, fortunately one such patient exists within the SM cohort. PBMCs from this patient could be used to generate CD4+ T cell lines that could then be infected with lab strain HIV-1 and used in viral suppression assays with HLA-B*4001 restricted and HLA-B*4002 restricted clones.

Furthermore, other functional properties of clones restricted by the different HLA-B*40 subtypes could be investigated. HLA-B*4001 restricted HIV-1 specific

CD8+ T cells are generally more differentiated than HIV-1 specific CD8+ T cells restricted by HLA-B*4002. This could result from such cells having received an increased amount of antigen stimulation and may mean that the HLA-B*4001 restricted cells are being pushed towards exhaustion. Both HLA-B*4001 and HLA-B*4002 restricted clones could be tested for the release of various cytokines, such as IL-10, upon antigenic stimulation.

The results shown in chapter 5, demonstrate that antigen processing and presentation is a complex procedure potentially affected by many variables. Several proteins are involved in turning cytosolic protein into peptide antigen presented in MHC class I molecules at the cell surface (Yewdell, Reits et al. 2003). Therefore, when differences in the efficiency of epitope processing and presentation are observed it is difficult to elucidate the exact cause. The efficiency of processing of the HLA-B*07 restricted RPM epitope was found to differs within two B cell lines that possess different HLA molecules.

This observation could arise for many reasons but may be the result of other MHC class I molecules competing with HLA-B*07 for binding of peptide fragments or proteins within the PLC. This hypothesis can be tested by investigating the efficiency of processing of other HLA-B*07 restricted epitopes in the B cell lines used in chapter 5. If HLA molecules that are present when the processing of RPM is less efficient are competing with HLA-B*07 for binding of proteins in the PLC then the processing of other epitopes restricted by this MHC class I molecule will also be affected. If they are competing for binding of peptide fragments containing the RPM epitope then potentially only the processing of this epitope will be affected. In order to determine which HLA

molecule present is possibly responsible for reducing the efficiency of presentation of the epitope, the processing of RPM could be determined within several B cell lines with various combinations of other HLA molecules.

Thus far, all HIV-1 vaccines used in large scale trials have contained whole virus proteins either in DNA or mature protein form (Barouch and Korber 2010). As was the case with the STEP trial, this approach does result in the generation of CD8+ T cells but as yet has failed to result in significant protection from acquisition of HIV-1. It may therefore be necessary to design a vaccine that takes a more targeted approach and is designed to elicit specific HIV-1 epitopes that are known to be able to control viral replication. However, this strategy necessitates the identification of such antigens.

The data presented in this thesis highlights the vast amount of factors that should be taken into account when choosing epitopes that may be good targets for an HIV-1 vaccine. Such antigens should be relatively resistant to a reduction in processing efficiency as a result of flanking sequence variation. In addition, their processing and presentation should be unaffected by the presence of competing HLA molecules and polymorphisms in other proteins involved in this pathway. In may also be of benefit to choose peptide antigens that are recognised by all common subtypes of the restricting HLA molecule. Also, the functionality and phenotype of the subsequently generated CD8+ T cells should be considered when deciding on potential HIV-1 vaccine targets.

This is by no means an exhaustive list of all the factors that contribute to determining whether or not a given viral antigen is a suitable target for an HIV-1 vaccine to elicit. Much research is required to further determine the characteristics of CD8+ T cells that are capable of viral control as well as those that can be generated by a

substantial proportion of individuals within a given population. With further understanding of such factors comes the knowledge that will hopefully lead to the development of a safe and widely effective HIV-1 vaccine.

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Appendices

Appendix 1 – IFN-γ ELISpot assay carried out by YongHong Zhang testing 8 HLA-B*4001+ and 16 HLA-B*4002+ patients from the SM cohort for recognition of 10 known HLA- B*40 restricted epitopes

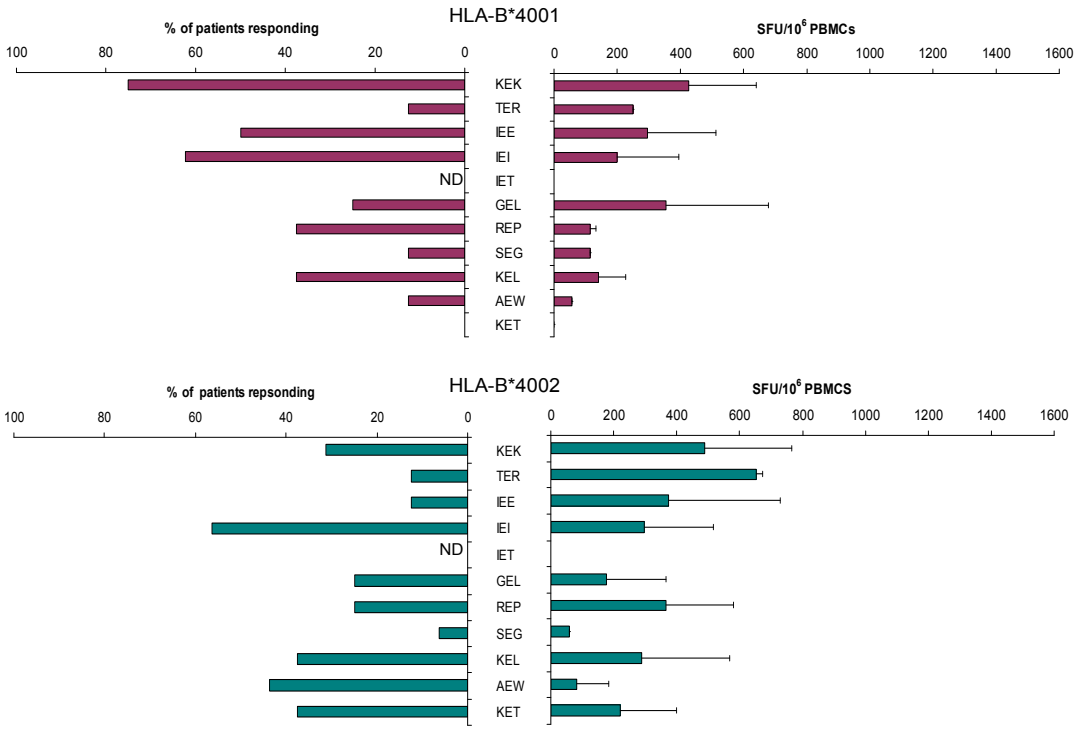

Figure A.1 The immunodominance hierarchy of recognised known HLA-B*40 restricted epitopes for HLA-B*4001+ and HLA-B*4002+ patients. Percentage of HLA-B*4001+ and HLA-B*4002+ patients displaying a positive IFN-γ ELISpot result to the 10 known epitopes (left hand panel-see table 2.1) and the average magnitude of the response (Spot forming units (SFU)/ million PBMCs) (Right hand panel).

Appendix 2 – The KEK peptide can be refolded with HLA-B*4002 heavy chain and β2M to produce a heterotrimer that can be tetramerised and stains ILT-2 cells

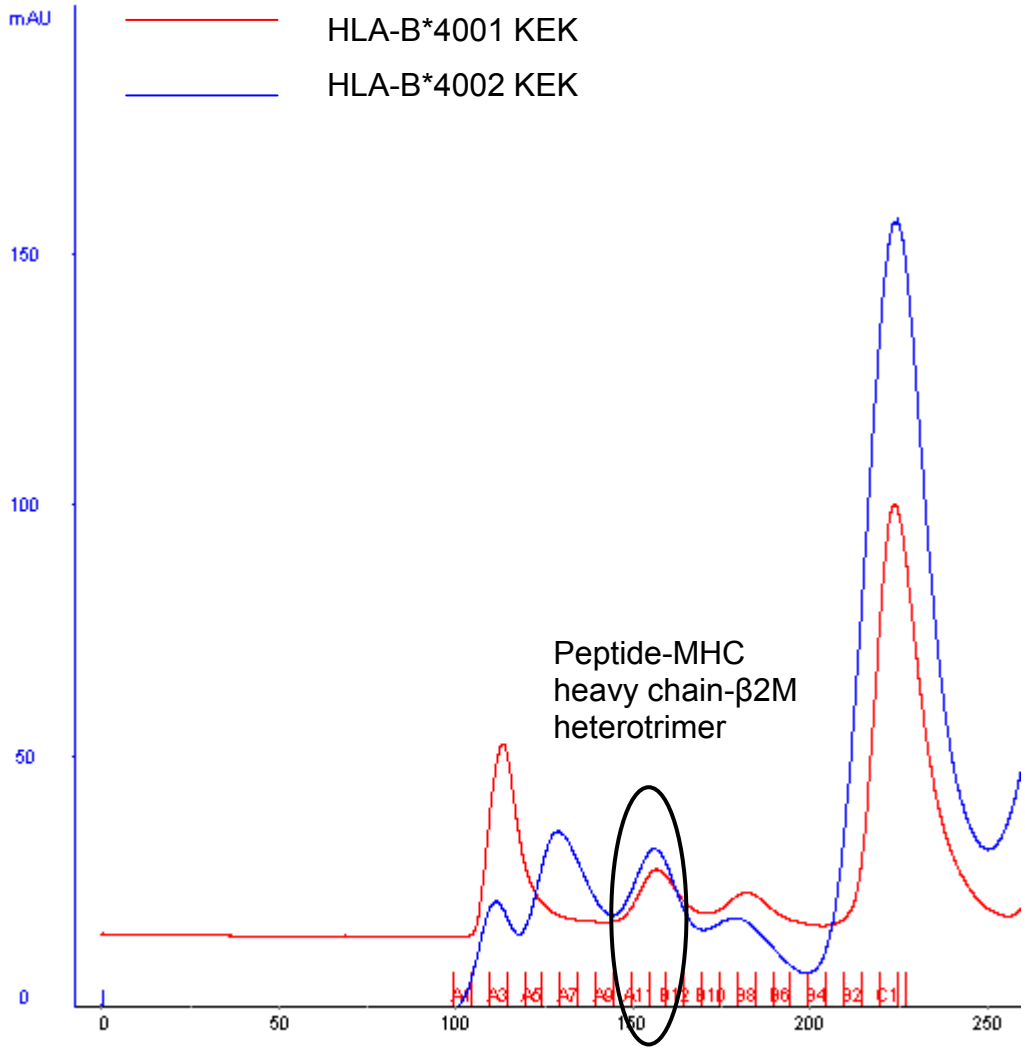

Figure A.2 FPLC purification of the KEK peptide refolded with both the HLA-B*4001 and HLA-B*4002 heavy chain and β2M results in the isolation of heterotrimers for both subtypes.

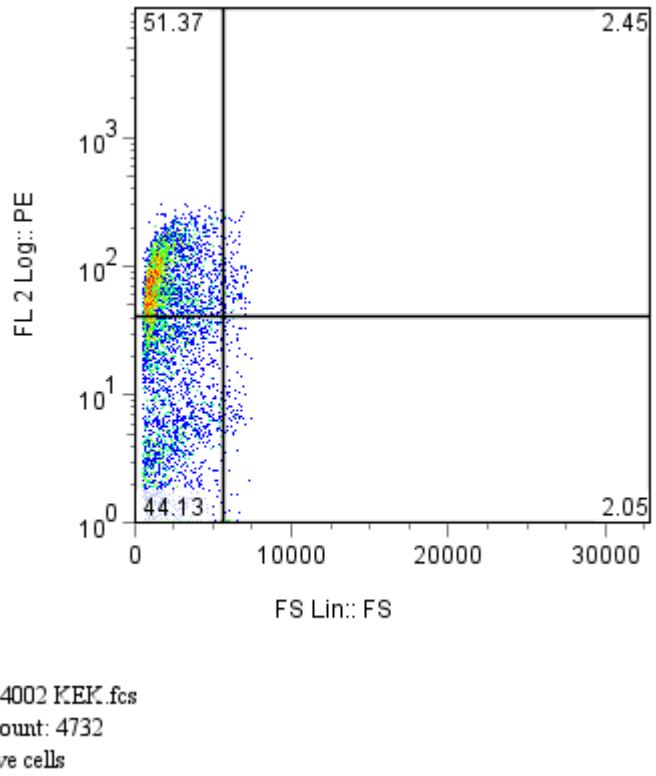

Figure A.3 Tetramerised heterotrimers composed of HLA-B*4002, β2M and KEK stain ILT-2 cells indicating that the components of the tetramers are present in the right conformation.

Appendix 3 – HLA-B*4001 restricted KEK specific clones do not bind to HLA-B*4002 KEK specific tetramers

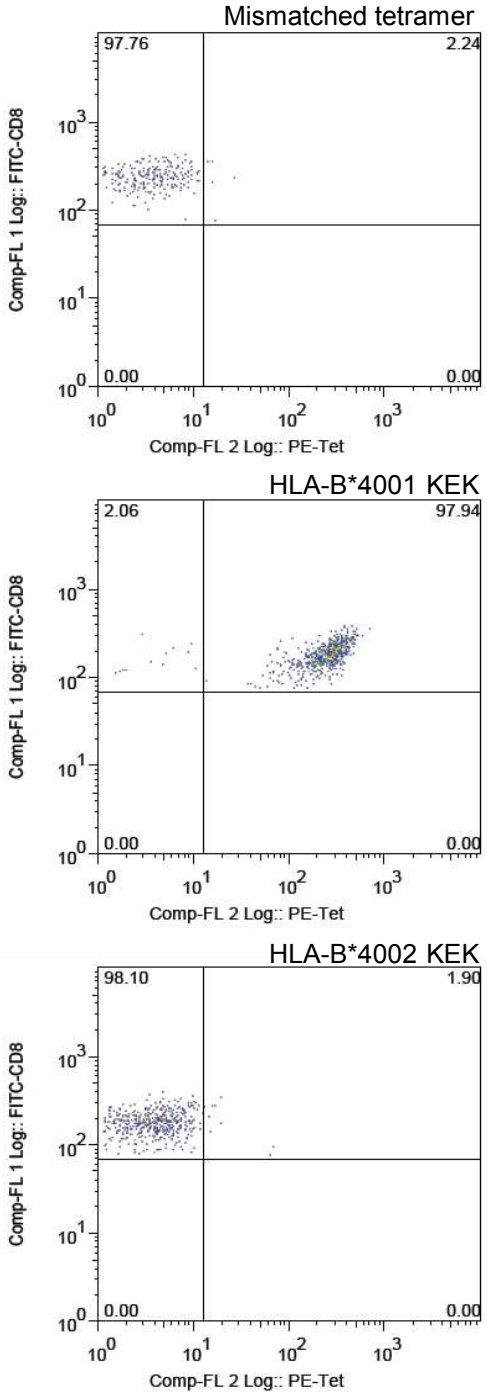

Figure A.4 An HLA-B*4001 restricted KEK specific clones binds to HLA-B*4001-KEK tetramers but not HLA-B*4002-KEK tetramers.

Appendix 4 – One HLA-B*4001+ patient from the SM cohort for which an IFN-γ ELISpot assay was carried out produced a positive response to the AEW peptide

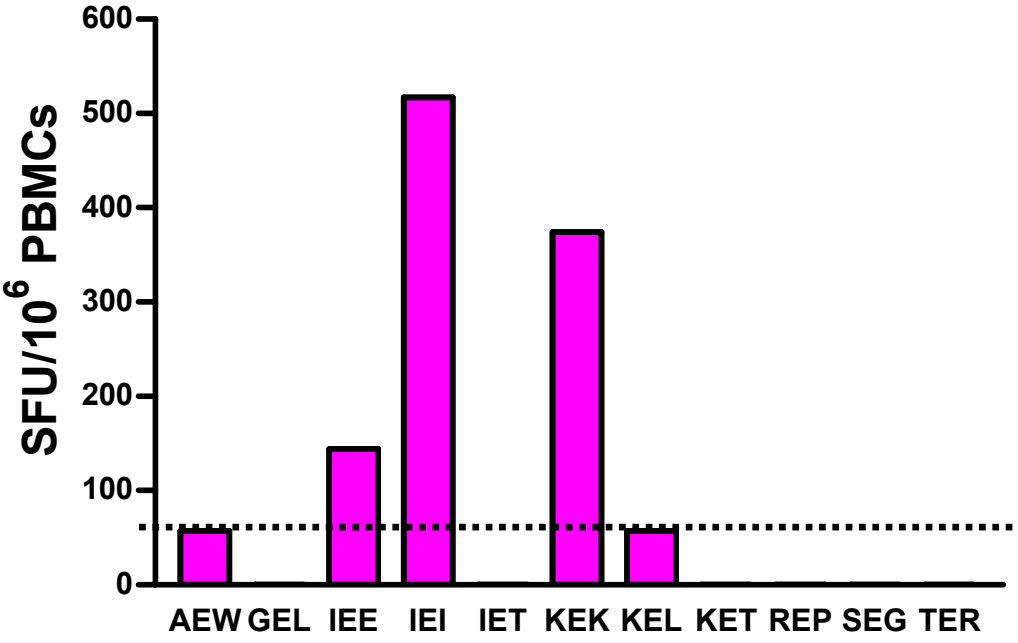

Figure A.5 Patient SM-191 is HLA-B*4001+ and produces an IFN-γ ELISpot response to the AEW peptide that is exactly three times background (dotted line) and above 20 SFU/10^6 PBMCs. Carried out by YongHong Zhang.

Appendix 5 – HLA type, clinical data and drug treatment information for SM cohort patients studied in chapter 4

SMID	HLA-B*40 subtype	HLA-A	HLA-B	HLA-C	CD4 count /mm^3 2008	VL 2008 copies/mm^3	HAART in 2003	HAART 2003-2008
006	02	A24, A24	B15, B40	Cw8, Cw8	331	9800	Yes	No
007	02	A1, A2	B40, B57	Cw6. Cw8	406	380	No	No
031	02	A11, A30	B13, B40	Cw6. Cw8	423	190	No	No
034	02	A24, A24	B40, B58	Cw3, Cw3	ND	900	No	No
035	01	A2, A33	B40, B58	Cw3, Cw7	491	1300	No	No
057	02	A32, A33	B40, B44	Cw4, Cw8	430	<LDL	Yes	No
175	02	A11, A33	B7, B40	Cw7, Cw8	134	38000	No	No
182	02	A2, A30	B13, B40	Cw6, Cw8	184	140	No	No
188	01	A2, A30	B13, B40	Cw6, Cw7	227	4400	No	No
203	02	A2, A30	B13, B40	Cw6, Cw8	291	<LDL	No	No
210	01	A2, A30	B40, B40	Cw3, Cw6	54	3200	Yes	No
211	01	A2, A2	B13, B40	Cw3, Cw3	200	10000	Yes	No
217	02	A2, A31	B40, B40	Cw3, Cw15	540	810	Yes	No
221	02	A32, A33	B38, B40	Cw7, Cw8	1268	440	No	No
222	02	A2, A11	B40, B54	Cw1, Cw8	480	<LDL	No	No
225	01	A2, A2	B35, B40	Cw3. Cw3	388	6800	No	No
226	02	A11, A24	B15, B40	Cw3, Cw3	422	<LDL	No	No
324	02	A11, A33	B13, B40	Cw7, Cw8	139	23000	No	No
335	02	A30, A31	B12, B40	Cw3, Cw6	414	10000	No	No
339	01	A2, A24	B40, B40	Cw7, Cw15	615	660	No	No
355	01	A2, A11	B7, B40	Cw7, Cw7	590	<LDL	Yes	No
356	01	A2, A33	B40, B58	Cw3, Cw7	196	ND	No	No
437	02	A11, A24	B38, B40	Cw7, Cw8	ND	<LDL	No	No

Table A.1 HLA type, clinical data and drug treatment information for SM cohort patients studied in chapter 4. PBMCs from these patients were used in the IFN-γ ELISpot outlines in figure 4.2 and whole blood was used for the tetramer staining shown in figure 4.3.