HETEROCLITIC PEPTIDES ENHANCE IL-2 PRODUCTION AND RECOGNITION BREADTH OF HIV-SPECIFIC $CD8^+$ T CELLS

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by

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A thesis submitted to the School of Graduate Studies
in partial fulfillment of the requirements for the degree of
Master of Science in Medicine

Immunology and Infectious Diseases /Biomedical Sciences/ Faculty of Medicine
Memorial University of Newfoundland

September 2012

St. John's Newfoundland

Abstract

Heteroclitic peptides are peptide sequence variants with enhanced immunogenicity relative to reference or wild type peptides. In therapeutic cancer vaccines, heteroclitic peptides dramatically augment cell-mediated immunity against tumour antigens by activating cytotoxic T lymphocytes (CTL) against tumour-associated self-peptides. Heteroclitic peptides could likewise contribute to human immunodeficiency virus (HIV) immunotherapy by broadening CTL reactivity and/or by selectively stimulating interleukin (IL)-2-production (correlated with disease control and non progression). To investigate this possibility, we screened peripheral blood mononuclear cells (PBMC) from HIV-infected subjects for interferon-γ (IFN-γ) and IL-2 production by enzyme-linked immunosorbent spot (ELISPOT) assay with peptide pools spanning the major HIV antigens. Pools stimulating IL-2 production were deconvoluted with peptide matrices to identify the individual 15mers responsible. Nef 83→91 (A2-7), Nef 135→143 (A2-8), Gag 18→ 27 (A3-2) and Gag 77→85 (A2-Gag) were confirmed as optimally defined 9mer peptides stimulating IL-2 production by PBMC from two or more HIV-infected individuals in our cohort. Twenty-four potentially heteroclitic variants were generated with conservative and semi-conservative amino acid substitutions at positions 3, 5 and 7 of these 9mers. An additional 24 variants were generated in a similar fashion with Gag147→155 (B57-1), Gag433 (A2-9), and non-HIV peptides, A2-Flu (GILGFVFTL) and irrelevant peptide, A2-IP (LLDVPTAAV). Eighty-six HIV-infected and six HIV-uninfected subjects were then tested for $CD8^+$ T cell reactivity against one or more of the variant sets by ELISPOT assay, at one or more time points. Heteroclitic

peptides enhanced IL-2 production by $CD8^+$ T cells in 50% of cases in which an IL-2 response was detected and enhanced IFN-γ production in 25% of cases in which an IFN-γ response was detected. Variants that increased IFN-γ and/or IL-2 production in comparison to index peptides were further tested for heteroclitic properties in cell culture. Proliferation, differentiation and breadth of reactivity were assessed by carboxyfluorescein succinimidyl ester (CFSE) dilution, surface and intracellular flow cytometry and cytotoxicity assays. Heteroclitic peptides stimulated broader reactivity of peptide-stimulated $CD8^+$ T cells, reduced the exhausted phenotype of responding $CD8^+$ T cells, and, in some cases, enhanced proliferation of responding T cells. Heteroclitic properties of particular peptides varied between individuals and, in most cases, within individuals at different time points. Although we do not know the mechanisms by which these peptide act, it appears that these peptides stimulate the same subsets of $CD8^+$ T cells as reference peptides but somehow exert differential signaling effects. Further insight into these mechanisms would provide information on heteroclitic peptide design for HIV $CD8^+$ T cell epitopes and contribute to a basic understanding of the relationship between T cell receptor: peptide interactions and T cell function.

Acknowledgements

Special thanks to my supervisor, Dr. Michael Grant, for giving me the opportunity to work in his lab and for all his guidance, support and patience throughout this project. I thank the members of my committee, Dr. Rodney Russell and Dr. Sheila Drover, for this critiques and encouragement. Thank you to all the contributing members on this this project, Natasha Hollett, Julia Pohling, and Katrin Zipperlen. Thank you to my lab mates, Katrin Zipperlen, Stephen Penney, Maureen Gallant, Natalie Campbell, Staci Stapleton, and Kayla Harris, for making the lab and the other room enjoyable working environments. Thanks to my classmates, Lisa LeShane and Alex Dancyger, for always celebrating the good times and listening during the hard times (Go Tripod Unit!). Very special thanks to Adam Woodland for listening to all of my science mumble jumble and for all his love and support. Lastly, I thank and dedicate this thesis to my family, Mom, Dad, Eric, Erin and Rebecca, for their continued love and support in everything I do.

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List of Abbreviations

aa	amino acid
ACD	acid-citrate-dextrose
AIDS	Acquired Immunodeficiency Syndrome
AIED	acute infection early disease
AIRE	autoimmune regulator
ALP	alkaline phosphatase
APC	antigen presenting cell
ART	antiretroviral therapy
ATP	adenosine triphosphate
BLCL	EBV-transformed B lymphoblastoid cells
bp	base pair
C	constant
CDR	complementarity-determining region
CFSE	carboxyfluorescein succinimidyl ester
cssDNA	complementary single stranded DNA
CTEC	cortical thymic epithelial cells
CTL	cytotoxic T lymphocyte
DC	dendritic cells
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive

DRiPs	defective ribosomal products
ds	double stranded
EBV	Epstein-Barr Virus
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
env	envelope
ER	endoplasmic reticulum
ERAAP	ER aminopeptidase associated with antigen processing
EU	HIV-exposed but uninfected individuals
Fab	antigen binding fragment
FADD	Fas-Associated protein with Death Domain
FCS	fetal calf serum
gag	group-specific antigen
gp	glycoprotein
HAART	highly active ART
HCL	hydrochloric acid
HEV	high endothelial venule
HHV-8	human herpes virus 8
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif

lck	lymphocyte-specific protein tyrosine kinase
LMP	the low molecular protein
LTNP	long-term non-progressor
LTR	long terminal repeat
MECL	multicatalytic endopeptidase complex subunit
MHC	Major Histocompatibility Complex
MIP	macrophage inflammatory protein
mRNA	messenger RNA
mϕ	macrophage
nef	negative-regulation factor
NK	natural killer
NNRTI	non-nucleoside RT inhibitors
NRTI	nucleotide RT inhibitor
PBC	peptide-binding complex
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pMHC	peptide:MHC complex
pol	polymerase
rev	regulator of viral expression
RNA	ribonucleic acid
RT	reverse transcriptase
SIV	simian immunodeficiency virus
SIVmac	SIV rhesus macaque

ss	single stranded
SSP	Sequence Specific Primer
TAP	transporter associated with antigen processing
tat	transactivator
TCM	central memory T cells
TCR	T cell receptor
TEM	effector memory T cells
TGF	transforming growth factor
TNF	tumor necrosis factor
V	variable
vif	viral infectivity
vpr	viral protein R
vpu	viral protein U
WHO	World Health Organization
Zap-70	zeta-chain-associated protein kinase 70

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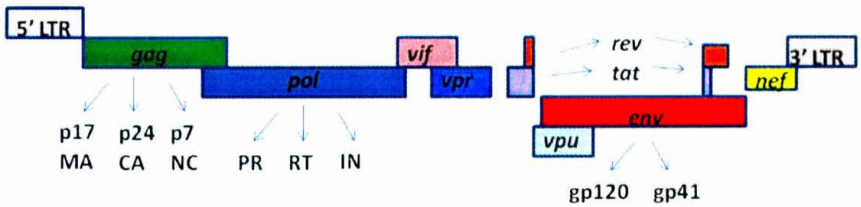
Chapter 1 Introduction

1.1 HIV-1 summary

The World Health Organization (WHO) estimates that 33 million people worldwide are infected with HIV [1]. Infection with HIV leads to a gradual loss of immune competence allowing opportunistic infections that are not normally pathogenic to become life threatening and allowing certain cancers to occur. Most untreated individuals infected with HIV progress over time to acquired immunodeficiency syndrome (AIDS) [2]. Transmission of HIV occurs through transfer of infected bodily fluids, namely blood, semen, vaginal fluid and breast milk, within which HIV can be present as free virus or contained within an infected immune cell. This transfer can occur through unsafe sex, contaminated needles, contaminated breast milk, perinatal transmission or through infected blood products, although the latter is not as common now due to improvements in HIV screening. Two types of HIV, HIV-1 and HIV-2, have been identified. HIV-1 is more prominent worldwide and leads to a more virulent infection while HIV-2 is confined to West Africa and is not associated with immunosuppression [3]. This thesis will focus on HIV-1.

1.1.1 Structure

HIV is encoded for by 9 genes (Figure 1.1.1) [4]. Envelope (*env*), group-specific antigen (*gag*) and polymerase (*pol*) genes are common to all retroviruses and are required to make the structural and enzymatic proteins, respectively, for the formation of new virus particles. Approximately fourteen "spikes" [5], trimers of the *env*-encoded glycoproteins (gp) gp120 and gp41, are embedded within the viral envelope (Figure 1.1.2). gp120 is important for docking of virus particles onto host cells [6], while gp41 is required for viral fusion and internalization [7, 8]. Beneath the outer envelope lies the matrix, which maintains the integrity of the virus particle and separates the viral envelope from the capsid. The viral capsid is made up of ~2,000 copies of *gag*-encoded p24 and contains two single strands of HIV ribonucleic acid (RNA) that are tightly bound to the nucleocapsid protein, p7, and the *pol*-encoded enzymes, reverse transcriptase (RT) and integrase. Each strand of RNA has a complete copy of the virus' genes – *env*, *gag*, and *pol*, and the remaining 6 regulatory genes, transactivator (*tat)*, regulator of viral expression (*rev)*, negative-regulation factor (*nef)*, viral infectivity (*vif)*, viral protein R (*vpr)* and viral protein U (*vpu*) (Figure 1.1.1). The regulatory genes encode proteins that control infectivity, replication and pathogenesis of HIV. The long terminal repeat (LTR) at either end of each strand of RNA is involved in control of HIV replication. The *pol*-encoded enzymes, reverse transcriptase, protease and integrase are responsible for copying the viral RNA genome into deoxyribo-nucleic acid (DNA), processing immature gag and gag/pol proteins and integrating the DNA copy of the HIV genome into the host DNA, respectively.



Gene		Gene product/function
gag	Group-specific antigen	Encodes matrix (MA), capsid (CA), nucleocapsid (NC), and p2 proteins
pol	Polymerase	Encodes reverse transcriptase (RT), protease (PR) and integrase (IN) enzymes
env	Envelope	Cleaved into two proteins: gp120 binds CD4 and CCR5/CXCR4 allowing viral attachment; gp41 mediates virus fusion and internalization
tat	Transactivator	Positive regulator of viral RNA transcription and promotes CD4 lymphocyte activation
rev	Regulator of viral expression	Allows export of unspliced and partially spliced viral RNA
vif	Viral infectivity	Overcomes inhibitions by the host factors APOBEC3G and promotes more stable reverse transcriptase complexes
vpr	Viral protein R	Facilitates infection of macrophages and promotes cell-cycle arrest
vpu	Viral protein U	Promotes intracellular degradation of CD4 and enhances virion release
nef	Negative-regulation factor	Promotes CD4 lymphocyte activation, blocks cell suicide, enhances infectivity and is associated with disease progression

Figure 1.1.1 HIV Gene Structure. HIV's RNA genome encodes 9 genes, which are flanked by LTRs. Each strand of RNA has a complete copy of the virus' genes – the 3 structural genes *env*, *gag*, and *pol*, and the remaining 6 regulatory genes, *tat*, *rev*, *nef*, *vif*, *vpr* and *vpu*. The regulatory genes encode proteins that control infectivity, replication and pathogenesis of HIV. The LTRs at either end of each strand of RNA are involved in control of HIV replication. Adapted from [9, 10].

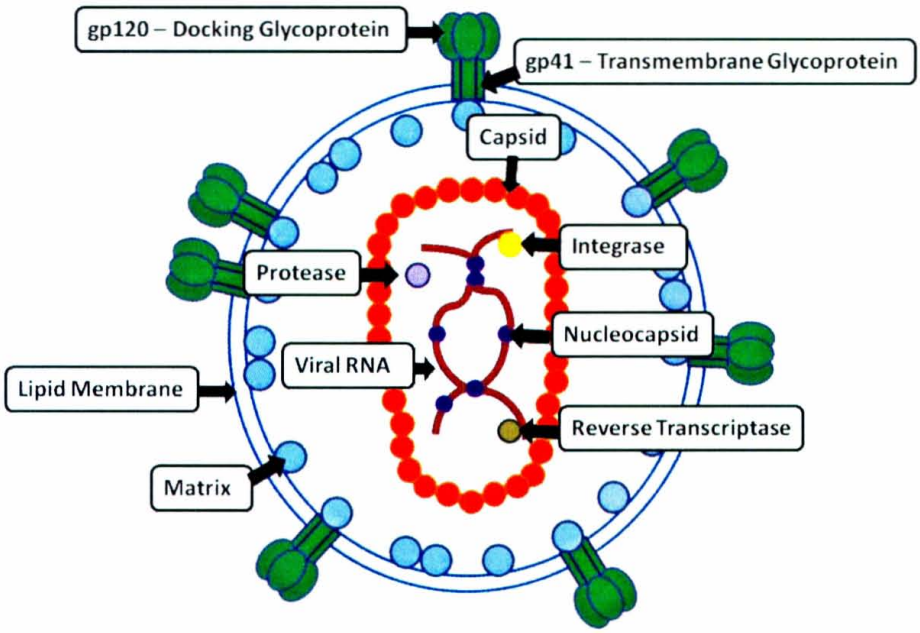


Figure 1.1.2 The structure of HIV. The viral envelope is composed of a phospholipid bilayer, taken from the membrane of a human cell when a newly formed virus particle buds. Embedded throughout the viral envelope are trimers of *env*-encoded gp120 and gp41, which mediate viral attachment and fusion to host cells. The matrix lays beneath the viral envelope. It maintains the integrity of the virus particle and separates the viral envelope from the capsid. The viral capsid is made up of ~2,000 copies of *gag*-encoded p24 and contains two single strands of HIV RNA that are tightly bound to the nucleocapsid proteins and the *pol*-encoded enzymes, RT and integrase. Each strand of RNA has a complete copy of the virus' genes. Adapted from [11].

1.1.2 Tropism

HIV tropism is dependent on the host cell receptors required for viral attachment. The CD4 receptor, expressed on $CD4^+$ T cells, macrophages (mϕ), and dendritic cells (DC), acts as the primary receptor for gp120-mediated binding of HIV to target cells [12, 13]. In 1996, it was discovered that HIV relies on the use of co-receptors, CCR5 and CXCR4, to help mediate virus fusion and penetration into the target cell [14-16]. The importance of CCR5 as the predominant co-receptor in HIV-1 infection [14, 15] was highlighted upon the observance that polymorphisms in CCR5, particularly a homozygous 32 base pair (bp) deletion in the coding region (CCR5-Δ32), reduced the risk of HIV infection regardless of the mode of transmission [17-19]. However, a few HIV^+ CCR5-Δ32 individuals with a slower course of disease existed, indicating viral use of an additional co-receptor, CXCR4. These CXCR4-using variant viruses usually have altered tropism and pathogenic properties. They are common in late-stage subtype B HIV-1 infection, in which there is often a co-receptor switch from CCR5 to CXCR4 [20, 21]. HIV can thus be *CCR5-tropic (R5), CXCR4-tropic (X4)* or *dual-mix tropic (R5-X4)*.

1.1.3 Life cycle

The HIV life cycle (Figure 1.1.3) begins with virus entry into the host cell. Viral gp120 binds to integrin α4β7, the gut mucosal homing receptor on peripheral T cells, activating adhesion molecules to establish the virological synapse [22]. gp120 binding of

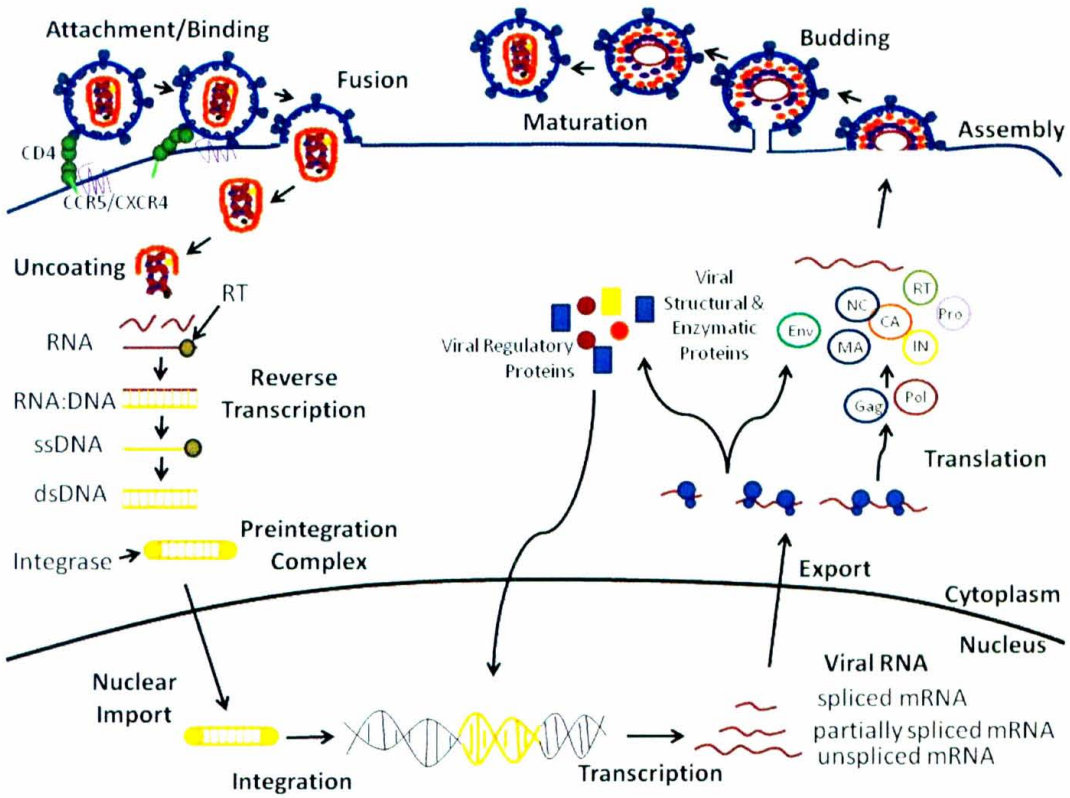


Figure 1.1.3 The Life Cycle of HIV. The life cycle of HIV begins with fusion of the virus particle to the host cell surface, mediated by the interactions of gp120, gp41, CD4 and CCR5 and/or CXCR4. After fusion, the viral capsid and its contents are released into the host cell cytoplasm. Viral DNA is formed by reverse transcription of the RNA genome. Once formed, the viral DNA is transported into the nucleus and integrates into the host DNA. Upon activation of the cell, the DNA is transcribed to make new viral RNA, which will be used as genomic RNA and to make new viral proteins. These move to the cell surface and budding begins. During maturation of the viral particle, individual HIV proteins are released cleaved and reassemble to form the matrix, capsid, nucleocapsid, RT and integrase, completing maturation of the virus particle. Adapted from [23-25].

CD4 induces a conformational change in gp120 that allows interaction with CCR5 or CXCR4 and allows gp41-mediated fusion to the host membrane [26]. Upon fusion, the viral capsid is released into the cytoplasm [27] and the single-stranded (ss) RNA genome is released. The viral RT reverse-transcribes the ssRNA into a complementary ss deoxyribonucleic acid (cssDNA) copy. The RT has RNase activity [28] that degrades the viral RNA during this process and has DNA-dependent DNA polymerase activity [29] that synthesizes a *sense* strand from the *antisense* cssDNA to form a double-stranded (ds) DNA copy of the HIV genome. The dsDNA migrates to the nucleus and is integrated into the host genome becoming an integrated provirus [30].

Activation of $CD4^+$ T cells induces translocation of transcription factors NFκB and NFAT to the nucleus [31, 32], which bind to promoters in the viral LTR of the integrated provirus and initiate transcription of the viral genome [33]. The first viral transcripts, which encode several of the regulatory proteins, are multiply spliced, then exported and translated in the cytoplasm. Singly spliced and unspliced transcripts are translated into the RT, integrase, viral protease and the structural proteins env (polyprotein gp160), gag, and nef. The structural proteins form the new virus particle around additional unspliced transcripts that constitute the HIV genome. After translation of the viral proteins, the virus is assembled. gp160 is cleaved into gp41 and gp120 by proteases in the Golgi complex and transported to the plasma membrane [34]. The gag and pol polyproteins associate with the HIV RNA at the plasma membrane and budding begins. During maturation of the viral particle, these polyproteins are cleaved and

reassemble to form the matrix, capsid, nucleocapsid, RT and integrase, completing maturation of the virus particle [35].

1.1.4 Diagnosis of HIV infection

Unless there is a high index of suspicion, HIV infection is not normally diagnosed immediately. Those infected may experience a flu-like illness (fever, headache, tiredness, and enlarged lymph nodes) in the acute phase of infection, which usually disappears within 1-4 weeks and is easily mistaken for another viral infection [36]. HIV-infected individuals are highly infectious during this stage due to the abundance of virus in the peripheral blood and genital fluids. At seroconversion, HIV-specific antibodies in the blood can be detected by the enzyme-linked immunosorbent assay (ELISA) and Western blot [36].

The ELISA is generally used to screen donated blood products and for general surveillance of infection [36]. If the serum contains antibodies for specific HIV antigens they will bind to the HIV antigen-coated ELISA plate. Because of the variability of this procedure, the lack of a comparable global standard and the concern of false positive responses, a single ELISA test cannot be used to confirm HIV infection. The Western blot test, like the ELISA, is an antibody detection test [37]. If HIV-specific antibodies are present in the serum they will attach to some of the HIV proteins on the membrane and an image of bands will be displayed on the photographic film. A positive result is one in

which at least one viral band corresponding to each gag, pol and env proteins is present. If fewer than 3 bands are detected, the results are inconclusive and another test is needed.

If the person is recently infected (1-3 months) a sufficient amount of antibodies for these tests may not yet be present. In this case, HIV RNA or DNA can be detected using a polymerase chain reaction (PCR)-based method to confirm the presence of HIV genetic material in the blood [36]. This method, in quantitative form, is normally used to track clinical progression of HIV infection and to monitor the effect of antiretroviral therapy but can be used to confirm a new infection in high risk individuals. A high viral load is strongly correlated to disease progression and increased risk of transmission [38]. HIV infection is confirmed when a sequence of tests, including a repeat of the initial ELISA test combined with the Western blot test and PCR test for viral load, confirm HIV infection [36].

1.1.5 Pathogenesis

HIV infects cells that express CD4 and CCR5 or CXCR4, particularly $CD4^+$ T cells. $CD4^+$ T cells are key players of our immune system. They interact with the innate immune system and are required for activation of B cells and CTL. In the acute phase of HIV infection, there is an abundance of virus in the peripheral blood and genital fluids, a significant decrease in the number of circulating $CD4^+$ T cells and a marked increase in antibody production and in the number of $CD8^+$ T cells that recognize and kill HIV-infected cells. Following seroconversion, when HIV-specific antibodies are detectable in

the blood, the acute phase of viremia has passed and there is a rebound in the number of circulating $CD4^+$ T cells. During this asymptomatic period, HIV replication continues and the number of $CD4^+$ T cells gradually declines. When the number of $CD4^+$ T cells falls below 500 cells/μl of blood the symptomatic phase of infection begins, marked by the appearance of opportunistic infections and other symptoms. When HIV-infected individuals become ill with one or more of the clinical or indicator illnesses, they have AIDS [39].

The mechanism by which HIV causes $CD4^+$ T cell depletion has caused much debate over the last fifteen years. Ho *et al.* [40] showed that in the presence of the protease inhibitor ritonavir, there was a rapid reduction in the amount of free virus in the plasma and a significant increase in the number of circulating $CD4^+$ cells. As drug-resistant mutations evolved, the viral load increased and the number of circulating $CD4^+$ cells decreased [40, 41], suggesting that HIV causes $CD4^+$ T cell depletion by direct cytopathic effect. Later, it was observed that African non-human primates infected with their species-specific strain of simian immunodeficiency viruses (SIVs) did not show progressive $CD4^+$ T cell declines and manifestations of immunodeficiency despite chronic viral replication [42]. Schindler *et al.* [43] reported that $CD4^+$ T cell depletion was not observed in these hosts because nef proteins from these SIV strains down-regulate expression of the T cell receptor (TCR)-CD3 complex leading to decreased cell activation and apoptosis whereas nef proteins from HIV-1 and the chimpanzee strain of SIV had no effect on TCR-CD3 surface expression. This suggested that increased immune activation and apoptosis leads to the demise of $CD4^+$ T cells.

Other mechanisms of HIV pathogenesis have also been suggested. $CD8^+$ T cell-mediated killing of infected $CD4^+$ T cells has long been considered one of these mechanisms [44, 45] but cytotoxic killing of infected $CD4^+$ T cells may only be effective during a certain "window period" of infection [46, 47]. Cell cycle dysregulation, caused by chronic T cell activation, accelerated cell turnover and an imbalance in cytokine production, also leads to the depletion of uninfected $CD4^+$ and $CD8^+$ T cells by causing perturbations in protein metabolism [48]. Circulating $CD4^+$ T cells may also be sequestered in secondary lymphoid organs [49]. The relative contributions of each of these mechanisms on HIV pathogenesis and $CD4^+$ T cell depletion remains unclear.

1.1.6 Diagnosis of AIDS

In Canada, if an individual has received a positive result from HIV testing and has one or more of the clinical illnesses, or indicator diseases, such as pulmonary tuberculosis, recurrent pneumonia and invasive cervical cancer, that person has AIDS [39]. Symptoms of opportunistic infections common with AIDS include coma, extreme fatigue, fever, nausea, vomiting, severe persistent diarrhea, vision loss, weight loss and mental symptoms such as confusion and forgetfulness [50]. Kaposi's sarcoma, caused by the human herpesvirus-8 (HHV-8), is the most common AIDS-related cancer. It causes reddish-purple lesions on the skin that were a hallmark feature of AIDS patients in the 1980's. Other opportunistic infections that can occur in AIDS patients include, but are not limited to candidiasis, toxoplasmosis, cytomegalovirus, herpes simplex, *Pneumocystis*

jiroveci, tuberculosis and recurrent pneumonia [39, 50]. In untreated HIV infection, progression to AIDS takes about ten years on average. However, since the introduction of antiretroviral therapy (ART), particularly highly active ART (HAART), progression to AIDS can be delayed for several decades or completely prevented.

1.1.7 Antiretroviral therapy

Before ART was available, people with AIDS were unlikely to live for more than a few years. Since the introduction of ART in 1987 [51] and HAART in 1996 [52] mortality and morbidity in HIV-infected patients has dramatically decreased [53]. ART therapies have come a long way since they were first introduced. Antiretroviral drugs are more numerous, diverse with respect to antiretroviral class, more potent and less toxic than before. Current HAART is a combination therapy consisting of a cocktail of at least 3 different antiretroviral drugs that belong to at least 2 different classes of antiretroviral agents. Currently, seven classes of antiretroviral agents exist. These include entry inhibitors, CCR5 antagonists, nucleoside and nucleotide RT inhibitors (NRTIs), non-nucleoside RT inhibitors (NNRTIs), protease inhibitors, integrase inhibitors [54] and maturation inhibitors [55]. Entry inhibitors interfere with the binding, fusion and entry of the virus into the host cell. CCR5 antagonists bind to CCR5 blocking gp120 binding. NRTIs are modified DNA nucleotides that lack a 3' hydroxyl group. These are incorporated into the viral DNA during cDNA synthesis and result in DNA chain termination as no additional nucleotides can be attached to them. NNRTIs bind directly to

the RT and interfere with its ability to synthesize the HIV cDNA strand from the viral RNA. Protease inhibitors prevent cleavage of polyproteins needed to produce infectious viral particles while integrase inhibitors block HIV DNA integration into its host DNA. Maturation inhibitors are still in development [55]. They could potentially inhibit proper assembly of the virion and prevent maturation of the particle.

Despite the effectiveness of HAART, many issues such as the generation of drug-resistance mutations, non-adherence, unwanted short and long-term side effects, cost, and high pill burden exist. Non-adherence, due to poor access, drug abuse, pill burden, and unwanted side effects, allows viral replication to rebound leading to the generation of immune escape and drug resistance mutations [56-58]. However, in many regions where individuals have access to state-of-the-art HIV care, the incidence and risk of antiretroviral resistance and incomplete viral suppression is declining [59]. Up until the recent HIV Prevention Trials Network (HPTN) 052 randomized trial [60], it was thought that even while HIV-infected individuals had HAART-suppressed viral loads, they were could still pass the virus on to others. However, results of the HPTN 052 trial showed that early initiation of ART is efficacious in preventing HIV transmission [60, 61].

1.1.8 Viral persistence

Despite effective therapy, HIV persists and continues to infect new hosts, particularly in areas where ART is unavailable or treatments are not initiated early in infeciton. HIV persistence is mainly due to the number of mutations that can be generated

in minimal time periods and to its ability to maintain latent infection as a provirus. The viral RT lacks proofreading capacity allowing a high error rate and multiple mutations to occur [62] thus leading to the rapid development of viral quasispecies including drug-resistant and immune escape variant strains. In some cases, a single mutation can confer resistance to some drugs [63], while a sequential accumulation of multiple mutations is required to confer resistance for others [64]. The mutations generated in the presence of one drug may lead to cross-resistance in which the virus becomes resistant to drugs to which it has never been exposed [65, 66]. This usually confers resistance within one class of inhibitor but is of great concern as it significantly limits the combinations of ART that can be used. However, in regions where state-of-the-art ART is available, these problems are not as common.

1.2 $CD8^+$ T cells

Hematopoietic thymocyte precursor cells that migrate from the bone marrow to the thymus mature there and become T cells. Both $CD4^+$ and $CD8^+$ T cells mature in the thymus and are distinguished from each other by the expression of their respective co-receptors (CD4 or CD8) and by their different effector functions. $CD4^+$ T cells are divided into several subgroups (T_H1, T_H2, T_H17, T_{regs}, etc.) based on their different cytokine production and effector functions. Activated $CD8^+$ T cells (CTL) kill virus-infected and tumor cells. The following section will address many aspects of $CD8^+$ T cell

development and function including how these cells recognize antigen, how they develop in the thymus, and most importantly their role in HIV infection.

1.2.1 The T cell receptor (TCR)

Each T cell has ~30,000 clonotypic TCRs on its surface [67]. Two different TCR heterodimers, the α:β TCR and γ:δ TCR exist. The α:β TCR, the focus of this section, is expressed on most T cells and is homologous to the antigen binding fragment (Fab) of immunoglobulin (Ig) [68]. The γ:δ TCRs represent a minor subset of TCRs, which have different antigen-recognition properties from the α:β TCRs. Each TCR consists of two receptor chains, which are disulphide-linked variable trans-membrane glycoproteins anchored in the membrane (Figure 1.2.1). The membrane proximal region of each chain is termed the constant region (C), while the membrane distal region of each chain is the variable region (V). The amino-terminal variable region is the site of antigen binding.

Crystal structures of the α:β TCR [69-73] have revealed that all but the Cα domain of the TCR are similar in structure to the domains of Igs. The Ig-like domains of the TCR are constructed from two β sheets linked by disulfide bridges to form a β barrel. Four loops of β strands at the outer edge of the β barrel form the hypervariable regions of the V region of each chain. These loops form a complementary surface to a particular antigen and as such are called complementarity-determining regions (CDRs). The Cα domain differs from the others in that half of it is connected to the Cβ domain forming a β sheet while the other half is composed of loosely packed strands joined to an α-helix by

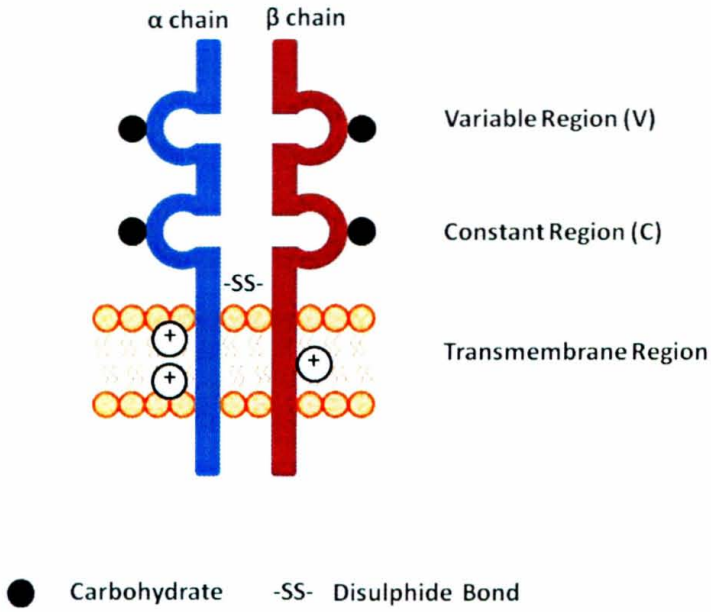


Figure 1.2.1 The structure of the α:β TCR. The TCR is composed of two transmembrane gp chains, α and β. The membrane proximal region of the extracellular portion of each chain is termed the constant region (C), while the membrane distal region of the extracellular portion of each chain is the variable region (V). The amino-terminal variable region is the site of antigen binding. Both chains are anchored in the membrane via a short stalk segment. This segment contains a cysteine residue that forms a disulfide bond, linking the two chains. The hydrophobic transmembrane segment of each chain contains positively charged residues. Adapted from [74].

a disulfide bond. Gene rearrangements, junction diversity and random pairing of α and β chains result in high variability of TCRs, particularly in the highly variable CDR3 region that interacts directly with the antigenic peptide. CDR1 and CDR2 are less variable as they are required for interaction with self-MHC molecules and thus, must have conserved regions to maintain recognition [68].

1.2.2 Peptide processing and loading onto MHC class I

$CD8^+$ T cells recognize peptide antigens through the TCR only in the context of self major histocompatibility complex (MHC) class I molecules (human leukocyte antigens (HLA) in humans). MHC class I is expressed on nearly all nucleated cells as well as platelets. During the inflammatory response, cytokines such as IFN-γ and tumor necrosis factor (TNF)-α increase the expression of MHC class I molecules on the cell surface. The majority of the peptides presented by MHC Class I are derived from newly synthesized proteins in the cytosol although a smaller fraction is derived from mature proteins [75, 76]. These proteins are degraded through the proteolytic activity of the proteasome, a cylindrical structure formed by the adenosine triphosphate (ATP)-dependent association of the proteolytic core particle and the regulatory particles that make up the base and the lid [77].

The proteasome exists in two forms: 1) The constitutive proteasome, which is present in all somatic cells and 2) the immunoproteasome, which is present only in antigen-presenting cells (APCs) and inflamed tissues. When APC are stimulated with

IFN-γ, the low molecular protein (LMP) 2, LMP7 and multicatalytic endopeptidase complex subunit (MECL)-1 of the immunoproteasome are synthesized. These replace constitutive subunits in the newly synthesized proteasome [78]. The ATP-dependent activity of the immunoproteasome is regulated by PA28, which binds to the terminal rings of the proteasome to form a protease-activator complex that opens the proteolytic core particle, strongly regulating the degradation and release of endogenously-synthesized peptides [79]. The oligopeptides released range from 4-20 amino acid residues in length and terminate mostly with basic and hydrophobic residues. Such C-termini are favoured for uptake by the transporter associated with antigen processing (TAP) and are required for binding to MHC class I molecules [78].

Once released from the proteasome, the majority of the peptides are further degraded for use in protein synthesis or energy production. The remainder bind to chaperones, which protect them from degradation until translocation into the lumen of the endoplasmic reticulum (ER). Transport into the ER is achieved in an ATP-dependent manner by the heterodimeric TAP complex, an integral ER membrane protein whose expression is significantly enhanced in the presence of IFN-γ [80]. The TAP complex preferentially transports peptides 8-16 residues in length, with peptides of the appropriate length for binding to MHC class I (8-11 residues) most efficiently transported. Once inside the ER, peptide precursors that are not at the appropriate MHC class I-binding length are trimmed at the N-terminus by the ER aminopeptidase associated with antigen processing (ERAAP) to generate peptides that can be presented by MHC class I [81].

MHC class I molecules are synthesized and loaded with peptide in the ER. This

process is mediated by several molecular chaperones such as calnexin and calreticulin, which prevent degradation of properly folded proteins and allow stable assembly of the MHC class I: peptide complex (pMHC). ER-resident proteins, including ERp57, the TAP-associated glycoprotein (tapasin), and the TAP heterodimer interact with the newly formed complex. Tapasin, which has an ER retention signal in its cytoplasmic domain [82], mediates the critical interaction of the MHC class I heterodimer with TAP, allowing peptide binding [83]. After transport into the ER via TAP, peptides are loaded in the $\alpha_1\alpha_2$ domain of MHC class I molecules. Low affinity peptides do not cause the release of the MHC from the peptide-binding complex (PBC), while high affinity peptides may cause conformational changes that complete the folding of the MHC class I molecule resulting in its release from the PBC and subsequent display at the cell surface [84].

Each individual carries a set of 6 classical MHC Class I alleles; one A, one B and one C allele from each parent. These alleles are highly polymorphic, particularly in the amino acids forming the peptide-binding groove. Such polymorphisms are likely the result of evolutionary pressure to maximize peptide binding diversity in the presence of multiple emerging pathogens. Each MHC class I molecule has a preferred peptide-binding motif, however, with the lack of structural data these are not all known. All, however, are functionally complex; they interact at different sites with peptides, β_2-m, the α:β TCR, the CD8 coreceptor and natural killer cell (NK) inhibitory molecules [85].

1.2.3 Peptide recognition and activation of $CD8^+$ T cells

MHC class I molecules present peptides in the peptide binding groove, formed by two flanking α-helices and a floor of antiparallel β-strands [86]. Peptide anchor residues interact with the MHC class I binding cleft in specificity pockets through a series of hydrogen bonds and ionic interactions leaving the upward pointing side chains available for interaction with the TCR [87, 88]. Normally, MHC class I presents autologous peptides derived by degradation of defective ribosomal products (DRiPs) and mature proteins that have reached the end of their natural life. Since T cell precursors that recognize self-peptides are eliminated in the thymus, the presentation of these autologous peptides by MHC class I is typically ignored by T cells. In contrast, T cells bearing receptors that recognize foreign peptides bound to self-MHC class I mature and are highly specific for foreign antigens in the context of self-MHC [89].

The TCR alone allows for peptide/MHC (pMHC) recognition but is not sufficient for intracellular signalling. Other molecules, CD3γ, CD3δ, and CD3ε (CD3 complex) and the TCRζ chain, which contain conserved immunoreceptor tyrosine-based activation motifs (ITAMs), are required for the expression of the TCR on the surface of the cell and for the signalling cascade that follows antigen recognition [90-92]. The interaction of the TCR complex (TCR and CD3 complex) with pMHC is stabilized by the CD8 co-receptor on $CD8^+$ T cells [93]. pMHC recognition by the TCR enhances lymphocyte-specific protein tyrosine kinase (Lck) recruitment to the TCR complex [94]. Lck phosphorylates the ITAMs of the CD3 chains and the TCRζ chain, which allows the ζ-chain-associated

protein kinase 70 (Zap-70) to bind. ZAP-70 is then phosphorylated by Lck initiating an intracellular signalling cascade involving many other proteins that ultimately results in cytokine production, proliferation, differentiation and/or activation-induced cell death [95, 96]. This process is also dependent on costimulatory receptors such as CD28 [97] and other auxiliary adhesion molecules such as integrins [98], which allow formation of the immunological synapse. CD28 is expressed on T cells and is ligated by B7 on APCs. This ligation provides an essential co-stimulatory signal that promotes TCR-induced tyrosine phosphorylation of the TCRζ chain and ZAP-70, which increases T cell proliferation by augmenting IL-2 production and prevents the induction of anergy and cell death.

Once activated through this mechanism, $CD8^+$ T cells become effector CTL. CTL mediate cytotoxic killing of virus-infected and tumor cells through two dominant contact-dependent mechanisms: granule exocytosis and the receptor-mediated Fas-Associated protein with Death Domain (FADD) pathway (reviewed in [99]). Activation of $CD8^+$ T cells results in the upregulation of cytokine receptors and induces the expression of granule components such as perforin and granzymes (serine proteases). Perforin is a Ca^{2+}-dependent pore-forming protein that multimerizes in membranes and is responsible for the entry of granzymes into target cells, however, the mechanism by which this occurs is unclear [100]. Granzymes A and B are the most abundant granzymes and are each capable of proteolytically activating cell death pathways, which ultimately cause fragmentation of the target cell's DNA and subsequent cell death [101, 102]. The perforin pathway is the predominant pathway used for CTL-mediated killing of target cells likely

due to its efficiency. Cytotoxic granules are released within minutes of target cell recognition and can be reoriented after each encounter allowing CTL-mediated killing of multiple target cells [103]. The FADD-mediated pathway which includes members of the TNF receptor pathway, TNFR1, TRAILR and most commonly Fas [104-106], is also involved in CTL-mediated killing of target cells. However, unlike in the granule exocytotis pathway, very little ligand (e.g. FasL) is stored and so there is a greater lag time (1-2 h) between recognition and killing of each target cell [99]. Activation of this pathway relies on the release of certain ligands (e.g. FasL) which bind to their receptors, resulting in the recruitment of FADD and caspase 8. This leads to the activation of a cascade of proteases, particularly caspases, which then cleave various substrates responsible for the changes associated with apoptosis such as nuclear membrane breakdown and DNA cleavage [107].

1.2.4 T cell selection

T cells are derived from hematopoietic stem cells in the bone marrow that migrate through the blood to mature in the thymus, a lymphoepithelial organ in the upper anterior thorax [108]. Maturation requires the stepwise completion of several processes including rearrangement of TCR genes, formation of a complete TCR, and positive and negative selection that ensures self-MHC restriction and self-tolerance (Figure 1.2.3) [109]. Tolerance is the state of immunological nonresponsiveness in the presence of a particular antigen. It is achieved through the deletion of autoreactive T cells during maturation

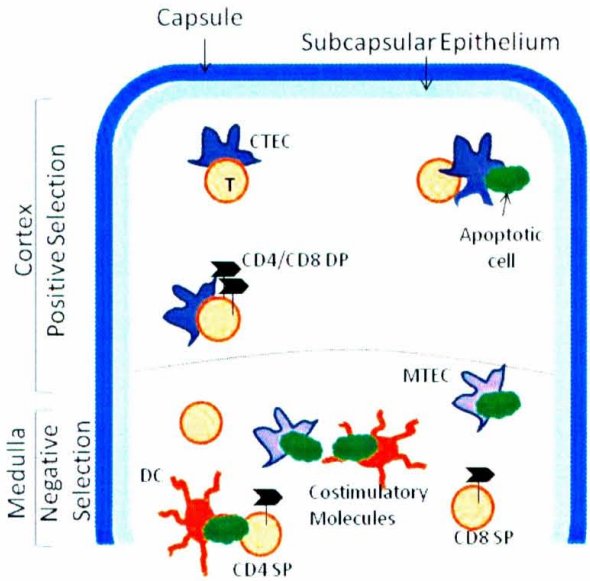


Figure 1.2.2 Positive and negative selection in the thymus. CD4/CD8 double positive (DP) thymocytes undergo TCR gene rearrangements in the cortex. DP cells expressing appropriate TCRs receive a "rescue signal" through the self-pMHC:TCR interaction with cortical thymic epithelial cells (CTEC), which induces DP maturation to the $CD4^{+}8^{-}$ or $CD4^{-}8^{+}$ single positive (SP) stage, while cells expressing TCRs that lack specificity for self-MHC undergo apoptosis. Positively selected cells enter the medulla. In the medulla, high-avidity TCR/self-peptide:MHC ligations with medullary thymic epithelial cells (MTEC) or DC leads to negative selection of self-reactive thymocytes. Adapted from [109].

(central tolerance) and the suppression of autoreactive T cells that have escaped elimination in the periphery (peripheral tolerance).

On entering the thymus, immature lymphocytes committed to the thymocyte lineage interact with the thymic stroma in the cortex and begin differentiation along the T cell lineage pathway. At the end of this phase (~1 week), the thymocytes express distinctive markers of the T cell lineage such as CD2 but do not express markers of mature T cells (CD3, CD4 or CD8). These cells are called double-negative (DN) thymocytes because they lack CD4 and CD8. The DN phase is further subdivided into four stages (DN1, DN2, DN3 and DN4) based on the presence and absence of certain surface molecules and the state of TCR chain gene rearrangement [110]. If gene rearrangement of the β chain locus is unsuccessful in the DN3 stage, the cells die by apoptosis. Successful gene rearrangement results in the expression of a complete pre-TCR, a combination of the β chain and a surrogate pre-TCR α chain, at the cell surface [110]. The cells then enter the DN4 stage in which proliferation occurs. The assembly of the pre-TCR with the CD3 molecules arrests further β chain rearrangement and leads to the expression of both CD4 and CD8 on the cell surface. When these double positive (DP) thymocytes cease to proliferate, the α chain locus rearranges and a complete α:β TCR is produced.

The fate of each DP cell depends on the ability of its new TCR to interact with a self-pMHC complex, a process known as positive selection. This process occurs in the cortex of the thymus and is mediated by cortical thymic epithelial cells (CTEC). Receptor editing, in which sequential rounds of rearrangement at the TCR α locus occurs, at this

stage increases the likelihood of successful self-MHC restriction [111]. DP cells expressing appropriate TCRs receive a "rescue signal" through the self-pMHC:TCR interaction, which induces DP maturation to the $CD4^+8^-$ or $CD4^-8^+$ single positive (SP) stage [112], while cells expressing TCRs that lack specificity for self-MHC (~95%) undergo apoptosis [113]. Chemokines that direct the T cell for further maturation within the thymic medulla are also produced during this time [114]. Negative selection, in which cells expressing high avidity self-reactive TCR are deleted, occurs throughout thymic development [115], but is mostly concentrated in the medulla [111]. In the medulla, this process is dependent on the high avidity TCR:self-peptide:self-MHC interaction and costimulatory signals from APC, particularly the interaction of CD28 (on thymocytes) and B7 (on DCs) [116, 117]. Other costimulatory signals through CD40 provided by medullary thymic epithelial cells and DCs [118], and through CD5 and CD43 on T cells [119] also have a role in clonal deletion. Negative selection occurs to a lesser extent in the thymic cortex, where there is a lack of costimulatory APCs. Here, clonal deletion of semimature T cells with strong TCR binding in the absence of costimulation is Fas-dependent [120].

In order for successful clonal deletion of potentially self-reactive T cells to occur, there must be a complete representation of self-antigen within the thymus. The expression of many of these tissue-specific antigens in the thymic medulla is controlled by genes such as the autoimmune regulator (AIRE) gene [121]. AIRE deficiency in mice and in humans results in autoimmunity, highlighting its importance in clonal deletion and central tolerance [122]. If a cell is tolerant to tissue-specific antigens and survives both

positive and negative selection, it leaves the thymus and circulates in the periphery as an immunocompetent single positive (SP) mature T cell. This means that selection of the T cell repertoire is based on the recognition of one or a few self-MHC molecules modified by the binding of a vast array of self-peptides. Thus, the recognition of self-peptides below a certain threshold of avidity is ultimately responsible for positive selection of T cells. The diverse repertoire of T cells that is selected recognizes foreign peptides bound to the same self-MHC molecule as the selecting self-peptide in the periphery. This means that foreign peptides that are recognized in the periphery are actually all heteroclitic variants of self-peptides. Heteroclitic variants are slight alterations of naturally-occurring peptides that enhance TCR recognition [123]. We will apply this concept to generate potentially heteroclitic variants of HIV peptides for possible use in therapeutic vaccines (discussed further below).

1.2.5 $CD8^+$ T cell response in HIV infection

Several findings suggest an important role for $CD8^+$ T cells in controlling HIV infection. Studies by both Koup *et al.* [124] and Borrow *et al.* [125] indicated CTL control of HIV replication during acute infection by monitoring the level of HIV-specific CTL activity in relation to plasma viremia in HIV-infected study participants. In both studies, participants who showed an increase in CTL activity during acute infection showed a concomitant decline in viremia, whereas those who had low or undetectable CTL activity had prolonged symptoms and persistently higher levels of viremia. $CD8^+$ T

cell-mediated control of HIV-infection was also suggested from *in vivo* $CD8^+$ T cell depletion studies in the SIV rhesus macaque (SIVmac) model of AIDS. These studies showed a rapid and marked increase in viremia in acute [126, 127] and chronic [127] infection when $CD8^+$ T cells were eliminated. However, upon the reappearance of SIV-specific $CD8^+$ T cells, the increase in viremia was suppressed [127]. The rapid generation of sequence mutations within immunodominant CTL epitopes [128-132] provides additional evidence of CTL pressure on HIV replication as does the association of particular HLA class I alleles (B27 and B57) with long term nonprogression (reviewed in [133, 134]).

Long-term non-progressors (LTNPs) exhibit natural immune control to HIV which allows stable normal $CD4^+$ T cell counts and continuous low to undetectable viral loads. Because of the lack of an animal model of protection, studies of LTNPs and HIV-exposed but uninfected individuals (EU) have been used to define correlates of protection. While vigorous $CD8^+$ T cell responses have been implicated in the maintenance of low viral load in LTNPs [135, 136] and seronegativity in EU [137-139], no single $CD8^+$ T cell function has proven to correlate with protection or control. LTNPs do, however, possess a greater proportion of polyfunctional HIV-specific $CD8^+$ T cells relative to HIV-infected progressors [140]. HIV-specific $CD8^+$ T cells from LTNPs can release several cytokines simultaneously (IFN-γ, IL-2, TNF-α, mϕ inflammatory protein (MIP)-1β, and perforin) [140, 141], have greater proliferative capacity [142, 143] and have the ability to dramatically upregulate granzyme B and perforin production after long-term culture [44, 144].

Other specific properties of $CD8^+$ T cells have been proposed as correlates of protective immunity in LTNPs and HIV controllers. Chen *et al.* [145] found that HLA-B27-restricted $CD8^+$ T cells in controllers were better able to inhibit viral replication through targeting of the immunodominant Gag epitope. This was also associated with distinct TCR clonotypes, characterized by superior control of HIV-1 replication in vitro, greater cross-reactivity to epitope variants and enhanced loading and delivery of perforin. Similarly, Turnbull *et al.* [146] have shown that epitope-specific responses with enhanced cross-recognition of sequence variants were most strongly correlated to delayed progression to disease. They also reported that the rate of disease progression is determined by the quality of responses to certain critical epitopes and that this is influenced by properties of the dominant TCRs used for epitope recognition. Importantly, IL-2 production by polyfunctional HIV-specific $CD8^+$ T cells has also been correlated with enhanced viral suppression [141]. Almeida *et al.* [147] reported that polyfunctionality and potent HIV-suppressive activity of HIV-specific $CD8^+$ T cells are a result of increased antigen sensitivity, while Harari *et al.* [148] argue that $CD8^+$ T cells with these attributes are equipped with low-avidity TCRs and thus have low antigen sensitivity. Such debates must be resolved in order to properly define $CD8^+$ T cell correlates of protection. It is important to note as well that $CD8^+$ T cell responses may not be the only protective measure in HIV protection. Emu *et al.* [149] compared HLA polymorphisms and HIV-specific $CD8^+$ T cell responses among LTNPs, non-controllers (high level viremia), and "ART suppressed" individuals (undetectable HIV RNA while on ART). In this study, while the majority of LTNPs did have higher HIV-specific $CD8^+$

T cell responses than the other groups, many LTNPs lacked protective HLA class I alleles and evidence of T cell-mediated control. It is, however, important to note that only gag, env and pol peptide pools were used to detect $CD8^+$ T cell responses. Thus it remains possible that these individuals have detectable $CD8^+$ T cell responses to other HIV proteins.

Defining how $CD8^+$ T cells inhibit HIV is one of the most critical questions that need to be addressed in order to develop efficacious protective and therapeutic vaccines. In addition, we must also determine which $CD8^+$ T cells are responsible for viral control. Several subsets of $CD8^+$ T cells including naive, effector and memory, exist. Upon activation, naive T cells become effector T cells (CTL) or differentiate into a diverse array of memory T cells. The process by which a naive cell enters into an effector lineage or memory lineage is not entirely clear. Chang *et al.* [150] show that a dividing T cell initially responding to a microbe asymmetrically divides into two daughter cells each with a different cell fate specification: a terminal effector lineage and a memory lineage. Sarkar *et al.* [151] suggest that cells that receive prolonged antigenic stimulation during the later stages of infection are more likely to become terminally differentiated effector cells, while those who do not endure prolonged antigen stimulation and have the ability to make IL-2 become memory T cells. Perhaps both of these mechanisms are involved or maybe the memory phenotype depends entirely on the antigenic history of each T cell.

CTLs produce effector molecules, such as perforin and granzyme B, and are responsible for the targeted destruction of virus-infected or tumor cells. They are terminally differentiated and short-lived, although 5-10% of these cells survive long-term

and can be reactivated for protection upon reinfection with the same pathogen [152, 153]. Memory T cells differ from both naive and effector T cells and can be further subdivided into effector memory T cells (T_{EM}) or central memory T cells (T_{CM}). These memory T cell subsets are defined by location, function and phenotype. T_{CM} are long-lived cells that, upon each exposure to their specific antigen, generate an accelerated immune response. They express CD62L and CCR7, which allows them to cross high endothelial venules (HEVs) and enter the lymph nodes from the bloodstream [154]. T_{CM} produce IL-2 which allows them to sustain their own survival but are poor producers of effector cytokines, such as IFN-γ, until they are reactivated [155]. T_{EM} are shorter-lived, reside in non-lymphoid tissues and express low levels of cytotoxic effector molecules such as granzyme B and perforin [155, 156]. A study by Freel *et al.* [157] that investigated the ability of cells from various differentiation stages to inhibit HIV replication showed that early memory stages through terminal effectors were able to effectively inhibit viral replication to similar degrees. However, given the strong correlation between the sustained presence of HIV-specific $CD8^+$ T_{CM} and long-term non progression [158, 159], it appears that $CD8^+$ T_{CM} are vital to HIV control and as such should be a target of both therapeutic and prophylactic vaccines.

1.3 Therapeutic vaccines

Two general classes of vaccines exist: preventative vaccines and therapeutic vaccines. Preventative vaccines are administered to uninfected individuals to provide

protection against future infection. These vaccines often consist of live attenuated or inactivated microorganisms, or parts of those microorganisms, that stimulate the body's immune system and prime immune cells to respond quickly against subsequent infection. Preventative vaccines are effective because of immunological memory, which allows the adaptive immune system to easily recognize and destroy pathogens it has already seen. Preventative vaccines are available for a number viral infections [160] and are responsible for the global eradication of smallpox [161] and near eradication of poliomyelitis [162].

Therapeutic vaccines, unlike preventative vaccines, modulate the ongoing immune response in unresolved or chronic conditions. They typically induce cell-mediated immunity rather than antibody- and complement-mediated immunity (although some vaccine initiatives aim for neutralizing responses) by enhancing existing or generating new immune responses towards chronic pathogens or tumor antigens. Therapeutic vaccination can be achieved through both passive transfer and active immunization to selectively reduce immunopathology. Their potential use in HIV infection and mechanisms by which an effective therapeutic HIV vaccine may be designed are discussed in the following subsections.

1.3.1 The need for a therapeutic HIV vaccine

For HAART to be effective, strict adherence is required. Initially, HAART regimens significantly impacted daily life as special dietary and strict scheduling

requirements were demanded in order to prevent the development of drug-resistance mutations [56, 65, 66]. Nowadays, many drugs can be combined into a single pill, which reduces pill burden and scheduling requirements. However, not all combinations of drugs are available in this single dose, therefore, if a person develops several drug-resistance mutations they may have to resort to harsher HAART regimens. HAART is also very toxic and even short-term use is associated with the HIV-associated lipodystrophy syndrome [163], and cardiovascular and bone disease [164]. Therapeutic vaccination would reduce the need for toxic and expensive ART and would offer several advantages over current ART such as fewer short- and long-term side effects, longer lasting effects following a single administration, and a reduction in the number of ART-resistance mutations. Previous and current clinical trials suggest that the side effects associated with therapeutic vaccination would be similar to those of approved vaccines, such as inflammation at the site of injection and mild flu-like symptoms [165]. The ultimate goal of a therapeutic vaccine would be to generate an anti-HIV immune response that would completely clear the infection, however, most researchers think this is not possible in HIV infection due to the virus' ability to remain latent for decades [165]. An effective therapeutic HIV vaccine could, however, prevent or delay the need for ART by sustaining immunological fitness and the level and stability of $CD4^+$ T cells, thus allowing viral control without the added toxicity of ART.

While much research has been directed at therapeutic vaccination, there is no therapeutic vaccine approved for human use [166]. Several challenges face the development of an effective therapeutic HIV vaccine. The virus' ability to proliferate

quickly and remain quiescent for many years despite CTL pressure means a therapeutic vaccine could help control the infection but will never clear it. Also, due to the variation in HIV strains and limited resistance to closely related strains, a universal vaccine is unlikely. The disappointing MERCK Step trial [167], although a preventative vaccine trial, left us with two important questions that need to be answered for effective vaccine design – 1) Which CTL functions correlate with protection? 2) What magnitude of response in assays correlates to an effective CTL response in vivo? Answers to these questions will allow us to surpass current challenges and design better future vaccines.

1.3.2 Peptide-based vaccines

Several approaches to therapeutic vaccination for HIV infection have been and/or are currently being explored. These include the use of whole inactivated HIV [168], recombinant DNA and viral vectors [169-171], dendritic cells [172-174], protein subunits and peptides [175, 176]. Peptide-based vaccines, specifically epitope-based peptide vaccines, are of particular interest because they allow the ability to select the minimal immunogenic region of specific protein antigens that can be targeted by a specific cell type (primarily CTL) to induce effective and precise immune responses. Peptides are produced easily and safely, therefore, can be produced on a large scale, and quite economically. If injected, peptides cannot revert to a virulent form or integrate into host DNA, concerns associated with live and attenuated vaccines and DNA vaccines, respectively. Peptide-based vaccines can also be designed to contain multiple epitopes of

one or more pathogens, which, given the HLA-restriction of the epitopes, is a necessity if a broadly applicable vaccine is desired. Broad vaccination strategies should also include the incorporation of peptides that elicit immune responses across a variety of HIV clades, which may be achieved by basing peptide design on conserved regions in virus strains. One such strategy is the use of mosaic vaccine strategies which use a computational approach to design polyvalent T cell-based vaccines [177]. This strategy maximizes coverage of cross-clade HIV sequences.

Although there are many advantages to peptide-based vaccines, there are also many challenges. Such vaccines must take the presence of B and T cell epitopes and HLA-restriction into consideration. In cancer immunotherapy, approaches such as the Tübingen approach [178], which combines genomics, peptidomics, bioinformatics and T-cell immunology, allow the identification, selection and validation of large numbers of MHC/HLA class I-associated peptides from tumor-associated antigens for use in vaccines. Peptide stability, delivery and low immunogenicity also pose difficulties in generating effective peptide-based vaccines but these may be overcome through peptide modification. Peptide stability can be improved through the incorporation of β-amino-acids into the epitope, which increases MHC-binding affinity and allows protease-resistance [179, 180], and through modifications of the amino and carboxy termini, which also allow protease-resistance and improve the enzymatic stability of the peptide [181]. Peptide delivery strategies have been significantly improved through the use of recombinant cytokine adjuvants [182, 183], oil-emulsion-type adjuvants [184] and recent developments in liposome, microparticle and nanoparticle delivery systems (reviewed in

[185]). Improvements in delivery result in enhanced immunogenicity. Immunogenicity can also be improved through slight epitope modifications, in which one or several amino acids are substituted with others. Heteroclitic peptides, which are the focus of this research project, represent an approach to epitope modification.

1.3.3 Heteroclitic peptides - their use in therapeutic cancer vaccines and potential use in therapeutic HIV vaccines

The term heteroclitic is defined as "deviating from ordinary forms or rules" [186]. Heteroclitic peptides are slight alterations of naturally-occurring or index peptides that enhance TCR recognition [123]. They do not just deviate from the index peptide in terms of sequence but deviate from the index peptide in terms of their functionality. Heteroclitic peptides stimulate more potent immune responses against their respective index peptide than the index peptide itself [187]. To date, this phenomenon has been employed most effectively in therapeutic cancer vaccines to overcome suboptimal MHC/TCR affinities and T cell tolerance to tumour-associated antigens [188-191]. Tumour-associated antigens are generally self antigens that become overexpressed in transformed cells [189, 190]. These antigens seldom elicit an immune response as most T cells with high or even moderate avidity for self peptides are eliminated during T cell selection in the thymus. In animal models and in vitro human systems, heteroclitic peptides have been shown to break T cell tolerance and dramatically augment cell-mediated immunity against tumour-associated antigens [188-190]. Slansky *et al.* [192] attribute this enhanced antigen-

specific antitumor immunity to increased stability of the pMHC:TCR complex, which results in increased avidity and residence time of this complex. However, the ability of heteroclitic peptides to hyperstimulate T cells and generate high avidity interactions is not sufficient on its own to eliminate tumors. The T cells that become primed by high avidity heteroclitic peptides must be able to undergo further activation and expansion driven by the lower avidity, poorly immunogenic index peptides, allowing the selective destruction of transformed cells that overexpress the index self-peptide [190]. Results from current clinical trials [193, 194] highlight the promise of this approach. Based on these results we propose that the same principle may be applied to HIV infection to selectively augment HIV-specific $CD8^+$ T cell responses.

In a study by Sette *et al.* [188], potentially heteroclitic variants were generated from several HLA-A2 and A3-restricted tumor-associated peptides (9mers and 10mers) and from two HLA-A2-restricted viral peptides, HBV Pol.455 and HIV Pol.476 (both 9mers). Peptide analogues that demonstrated heteroclitic activity, regardless of index peptide origin, each had amino acid substitutions that were of conservative or semiconservative nature at positions 3, 5, or 7 but never at positions 1, 4, 6 or 8. The heteroclitic peptides identified, including two for the HIV Pol.476 index peptide, produced up to a 10^7-fold increase in T cell sensitivity to peptide stimulation and were associated with higher magnitude responses [188]. X-ray crystallography-inferred 3-dimensional structure of HLA-A2 and A3 pMHC:TCR complexes show that side chains of amino acids at positions 3, 5 and 7 in the HLA-A2 and A3 binding peptides interact directly with the CDR3 regions of the TCR α and β chains [195, 196] thus suggesting that

heteroclicity is a result of enhanced TCR recognition. The study by Sette *et al.* [188] also included a table of similarity scores for each amino acid in relation to the others. These relationships were quantified by averaging the rank coefficient score for tolerability of point mutations within a protein (Dayhoff PAM250), hydrophobicity (an average of Kyte/Doolittle and Fauchere/Pliska scales), and amino acid side chain volume (measured by H_20 displacement) for each amino acid pair. We propose that heteroclitic variants of HIV index peptides can be generated in the same manner as in the study by Sette *et al.* [188] and incorporated into therapeutic HIV vaccine design.

1.3.4 Central memory T cells as a target of therapeutic HIV vaccines

The sustained presence of HIV-specific $CD8^+$ T_{CM} cells in LTNP is strongly correlated with control of HIV replication and slower disease progression [158, 159]. The T_{CM} lineage is characterized by the $CD3^+CD8^+CD45RA^-CD28^+CCR7^+CD127^+$ phenotype [197]. T_{CM} produce IL-2, undergo self-renewal, have a broad TCR repertoire and serve as progenitors of new effector and memory T cells [198]. Most importantly, T_{CM} appear to be extremely rare in progressive HIV infection [144, 197]. Renewal of T_{CM} appears to be dependent on low-level antigen persistence and continued proliferation. However, in cases of chronic infection, when high viral loads persist, renewal is less frequent leading to a decline in T_{CM} populations [151, 197]. The requirements for commitment to the long-lived T_{CM} lineage is not fully understood but is likely designated early during T cells responses involving APC-priming [198]. Further insight into this

process of differentiation would allow us to design vaccines that could selectively target T_{CM}. Heteroclitic peptides enhance cell-mediated immune responses by exposing the immune system to peptides that are rare or non-existent in clinical HIV isolates leading to the activation of $CD8^+$ T cells that are cross-reactive with the naturally-occurring HIV peptides. We propose that heteroclitic peptides, which selectively target IL-2-producing T cells, may offer the appropriate level of antigen stimulation to be selectively recognized by or lead to the generation of $CD8^+$ T_{CM}. If this holds true, then heteroclitic peptides should be incorporated in therapeutic HIV vaccines as their ability to enhance recognition by, or generation of T_{CM} could result in improved control of HIV replication and slower disease progression.

1.4 Preliminary work

Preliminary work for this project was completed by Julia Pohling, Natasha Hollett and Katrin Zipperlen. The first step in this project was to identify HIV peptides that stimulated IL-2 production by $CD8^+$ T cells in HIV-infected individuals. PBMC from HIV-infected subjects were screened for IFN-γ and IL-2 production by ELISPOT assay with overlapping 15mer peptide sets spanning the major HIV clade B antigens (National Institutes of Health AIDS Reference Reagent Program). Individual peptides were pooled in sets of 49 (Nef), 123 (Gag), 125 (Pol1), 123 (Pol2), 128 (Env1), and 83 (Env2). Initially, more than 40 individuals were screened against the pools by ELISPOT assay to determine which pools elicited IL-2 responses. Twelve individuals from this initial

screening had >200 IL-2 spot forming units (SFU)/10^6 PBMC against one or more of the HIV peptide pools, with HIV Gag and Nef pools eliciting IL-2 responses most frequently. Those individuals contributed PBMC for follow-up studies to identify individual HIV peptides that stimulated this response. Pools stimulating IL-2 production (Gag and Nef) were then deconvoluted with peptide matrices by ELISPOT assay to identify individual 15mers responsible. The Gag matrix (Figure 1.4.1 A) consisted of 20 pools of 11 peptides, 2 pools of 12 peptides and 1 pool of 2 peptides (123 peptides total) in an 11 x 12 grid and the Nef matrix (Figure 1.4.1 B) consisted of 14 pools of 7 peptides (49 peptides total) in a 7 x 7 grid. Each peptide was present in two and only two pools. By comparing the pattern of IL-2 production against the peptide pools, the specific peptide or peptides responsible for IL-2 production were identified. Several of the 15mer peptides identified contained optimally defined $CD8^+$ T cell epitopes consistent with the HLA class I type of the participant tested. A2-7 (Nef 83→91), A2-8 (Nef 135→143), and A2-Gag (Gag 77→85) (all HLA-A2 restricted) and A3-2 (Gag18→27) (HLA-A3 restricted) were confirmed by ELISPOT assay as optimally defined 9mer peptides stimulating IL-2 production by PBMC from two or more HIV-infected individuals in our cohort. PBMC from an additional 60 subjects who expressed the appropriate HLA class I molecules were then screened by ELISPOT assay against these index peptides for IFN-γ and IL-2 production.

A)

Gag

peptide pools

peptide pools	1	2	3	4	5	6	7	8	9	10	11
12	7872	7873	7874	7875	7876	7877	7878	7879	7880	7881	7882
13	7883	7884	7885	7886	7887	7888	7889	7890	7891	7892	7893
14	7894	7895	7896	7897	7898	7899	7900	7901	7902	7903	7904
15	7905	7906	7907	7908	7909	7910	7911	7912	7913	7914	7915
16	7916	7917	7918	7919	7920	7921	7922	7923	7924	7925	7926
17	7927	7928	7929	7930	7931	7932	7933	7934	7935	7936	7937
18	7938	7939	7940	7941	7942	7943	7944	7945	7946	7947	7948
19	7949	7950	7951	7952	7953	7954	7955	7956	7957	7958	7959
20	7960	7961	7962	7963	7964	7965	7966	7967	7968	7969	7970
21	7971	7972	7973	7974	7975	7976	7977	7978	7979	7980	7981
22	7982	7983	7984	7985	7986	7987	7988	7989	7990	7991	7992
23	7993	7994									

B)

Nef

peptide pools

peptide pools	1	2	3	4	5	6	7
8	5139	5140	5141	5142	5143	5144	5145
9	5146	5147	5148	5149	5150	5151	5152
10	5153	5154	5155	5156	5157	5158	5159
11	5160	5161	5162	5163	5164	5165	5166
12	5167	5168	5169	5170	5171	5172	5173
13	5174	5175	5176	5177	5178	5179	5180
14	5181	5182	5183	5184	5185	5186	5187

Figure 1.4.1 The Gag and Nef matrices. A) The Gag matrix consists of 23 pools (pool number is highlighted in green). Pools 1, 2 and 12-22 each contain 11 peptides whose sequence is identified by an identification number (ex 7872). Pools 3-11 each contain 10 peptides and pool 23 contains 2 peptides (123 peptides total). B) The Nef matrix consists of 14 pools (pool number is highlighted in orange) each containing 7 peptides whose sequence is identified by an identification number (ex 5139). The pools were tested by ELISPOT assay. Each peptide was present in two and only two pools therefore by comparing the pattern of IL-2 production against the peptide pools the specific peptide or peptides responsible for IL-2 production were identified.

From A2-7, A2-8 and A3-2, eighteen potentially heteroclitic variants were designed by making conservative and semiconservative substitutions at positions 3, 5 and 7 as in the study by Sette [188]. The choice of amino acid to be substituted was based on the relationship to the index amino acid and rarity or absence among HIV-1 clade B sequences submitted to the Los Alamos HIV sequence database (www.hiv.lanl.gov/content/sequence/HIV/mainpage.html). Choosing variant peptide sequences that are rare or absent in natural HIV-1 infection is important. In chronic HIV infection, the infected individual's immune system, has been exposed to a variety of HIV epitopes but has not been able to clear or, in many cases, control the infection. The T cells that react typically recognize the immunodominant HIV epitopes, therefore T cells recognizing non-immunodominant epitopes do not expand. However, T cells recognizing immunodominant epitopes eventually become functionally exhausted therefore, in order to stimulate new T cell subsets or reactivate exhausted T cells, we must expose them to new epitopes. More than 10 individuals were screened against the potentially heteroclitic variants by ELISPOT assay and at least 6 responded to one or more of the variants with $\geq$ 50 IL-2 SFU/10^6 PBMC. This preliminary data formed the basis of the current study in which additional variant sets were generated and PBMC from additional subjects were screened against the original and the new variant sets. The specific aims of the current study are described below.

1.5 Specific aims

1.5.1 Identify peptides that selectively augment IL-2 production by HIV-specific $CD8^+$ T cells relative to index peptides

The first aim of this study was to identify potentially heteroclitic peptides that selectively augment IL-2 production by HIV-specific $CD8^+$ T cells relative to index peptides. IL-2 production was measured by ELISPOT assay, a simple and rapid test that allows the enumeration of cytokine-producing cells. At the beginning of this study, we speculated that the ability of HIV-specific $CD8^+$ T cells to produce IL-2 correlated with delayed disease progression and viral control. This correlate of protection was inferred based on the association of LTNP with the sustained presence of HIV-specific $CD8^+$ T_{CM} cells, which have the ability to produce their own IL-2 and sustain their own proliferation [158, 159]. In a recently published study by Akinsiku *et al.* [141] the capacity of HIV-specific $CD8^+$ T cells from progressors and controllers (neither on ART) to produce effector molecules (CD107a, IL-2, IFN-γ, TNF-α, perforin) and inhibit HIV replication in autologous $CD4^+$ T cells was measured. Results from this study showed a strong correlation of suppressive capacity of $CD8^+$ T cells with polyfunctional, IL-2 production. Although no single functional output of $CD8^+$ T cells can be used as a correlate of protection, this study validates our use of IL-2 production by HIV-specific $CD8^+$ T cells as a marker of enhanced immunogenicity and functionality of heteroclitic peptides relative to index peptides.

Because IL-2 responses are rare in progressive HIV infection, we also compared the ability of reference and variant peptides to stimulate IFN-γ production by $CD8^+$ T cells. In a setting of high viremia, IFN-γ secretion is the most resistant to functional exhaustion than all other T cell functions. In standard ELISPOT assays measuring HIV-specific immune responses, IFN-γ is the most commonly assessed cytokine [199, 200]. In addition to this, previous research has shown that heteroclitic peptides can increase production of IFN-γ and/or interleukin-10 (IL-10) relative to reference peptides [188].

1.5.2 Investigate whether IL-2-inducing heteroclitic HIV peptides augment proliferation and differentiation of $CD8^+$ T cells

The second aim of this study was to compare the capacity of IL-2-inducing heteroclitic peptides and index peptides to drive proliferation and differentiation of $CD8^+$ T cells. The ability of T_{CM} to produce IL-2 allows them to support their own proliferation [158] therefore we will test the ability of potentially heteroclitic HIV peptides to induce proliferation of these cells in the absence of any exogenous cytokines. PBMC were labelled with CFSE, stimulated with either potentially heteroclitic or index peptides in the absence of exogenous cytokines, and cultured for 7 days before staining for flow cytometry. Four color flow cytometry was also used to determine whether the IL-2 inducing peptides also drive differentiation of HIV-specific $CD8^+$ T cells in the absence or presence of exogenous cytokines.

1.5.3 Measure TCR/peptide avidity of IL-2 inducing heteroclitic HIV peptides relative to index peptides

T cell avidity is defined as the ability of T cells to respond to the lowest concentration of peptide in the context of the appropriate self-MHC molecule. High avidity $CD8^+$ T cells require lower amounts of peptide for triggering of effector functions while low avidity $CD8^+$ T cell require higher amounts of peptide. Several studies identify T cell avidity as an important parameter for cell-mediated control [201-203]. Almeida *et al.* [201] show that the presence of high avidity HIV-specific $CD8^+$ T cells, which are polyfunctional in nature, is strongly correlated with enhanced control of HIV replication. Using depletion studies, Lichterfeld *et al.* [203] showed that loss of high avidity HIV-specific $CD8^+$ T cells is strongly associated with disease progression in HIV-infected individuals. Based on the importance of TCR/peptide-HLA class I avidity for HIV control, we also tested the ability of IL-2 inducing heteroclitic peptides to enhance TCR/peptide-HLA class I avidity relative to index peptides.

1.5.4 Test the ability of IL-2 inducing heteroclitic HIV peptides to generate broadly reactive CTL

Heteroclitic peptides enhance cell-mediated immune responses by priming $CD8^+$ T cells with naturally rare or non-existent peptide leading to the activation of $CD8^+$ T cells cross-reactive with the naturally-occurring peptides. Because the HIV RT lacks

proofreading capacity, many viral quasispecies, including those with drug-resistance and immune escape mutations, can exist within a single individual [62]. In order for a heteroclitic HIV peptide to be effective in a therapeutic vaccine it must activate CTL that are broadly reactive against multiple variations of the HIV peptide which may occur in the host. Therefore, we also tested the ability of IL-2 inducing heteroclitic HIV peptides to generate CTL that are not only cross-reactive with the index peptide but broadly reactive with the entire variant set.

Chapter 2 Materials and Methods

2.1 Study participants

HIV-infected study participants (numbered 1 to 257) were recruited through the St. John's General Hospital HIV Clinic, St. John's, Newfoundland, Canada. Non-infected study participants (numbered 1000 and above) were recruited through the Faculty of Medicine, Memorial University of Newfoundland, St. John's, Canada. All participants provided informed consent for whole blood collection and immunological studies. PBMC from over 40 HIV-infected participants were initially screened against the major HIV antigens for IL-2 production to ultimately identify 9 mer peptides responsible for this response. Those HIV-infected participants who originally responded and an additional 52 individuals who expressed the appropriate HLA molecule (HLA-A2, A3, B18, B35, or B57) were selected for heteroclitic peptide testing against HIV and non-HIV peptide sets. Most HIV-infected study participants were on antiretroviral therapy and had achieved viral suppression to below limits of detection with clinical tests at the time of participation. Non-infected study participants expressing the appropriate HLA (HLA-A2) were selected for heteroclitic peptide testing against non-HIV peptide sets and used as negative controls against interesting heteroclitic HIV peptides eliciting IL-2 responses in HIV-infected study participants. Ethical approval for this study was granted by the Memorial University of Newfoundland Faculty of Medicine Human Investigation Committee.

2.2 PBMC isolation

Whole blood was collected by forearm venipuncture into acid-citrate-dextrose (ACD)-containing vacutainers to prevent coagulation during visits scheduled for clinical tests. After centrifugation at 400 *g* for 10 minutes, most of the plasma was removed, aliquotted and stored at -80°C. The remaining blood was diluted 1:4 in phosphate buffered saline (PBS) and PBMC were isolated by Ficoll-Hypaque (GE Healthcare Bio-Sciences) density gradient centrifugation. In short, a solution of Ficoll and sodium metrizoate of specific density was layered below the PBS-diluted blood and centrifuged at 400 *g* for 30 minutes. This caused sedimentation of the erythrocytes and granulocytes to the bottom of the tube allowing collection of PBMC from the plasma-Ficoll interface. PBMC were used fresh or cryopreserved in lymphocyte medium (RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 µg/ml streptomycin, 100 IU/ml penicillin, 2 mM L-glutamine, 10 mM HEPES buffer and 2 x 10^{-5} M 2-mercaptoethanol; all Invitrogen) with FCS increased to 20% and supplemented with 10% dimethylsulfoxide (DMSO) (Sigma).

2.3 Epstein-Barr virus (EBV) transformation of B cells

EBV transformation of B cells is an effective procedure for immortalization of human B lymphocytes [204]. The B95-8 cell line (EBV transformed marmoset leukocytes) is a marmoset cell line that releases high titres of transforming EBV and can

thus be used as a source of EBV to establish continuous B cell lines from human donors. B95-8 cells, established in culture, were centrifuged at 1100 rpm for 5 minutes. The supernatant was poured off into a sterile 15 ml tube and filtered through a 0.45 μM aerodisc syringe filter (PALL Corporation) to ensure complete removal of cells. 2.5 ml of filtered supernatant was added to 5 x 10^6 freshly isolated PBMC and incubated for 24 hours at 37ºC in 5% CO_2. After 24 hours, the cells were centrifuged and resuspended in 3 ml of lymphocyte medium with FCS increased to 25% and supplemented with 1 μg/ml cyclosporin A (Novartis). The cells were incubated at 37ºC in 5% CO_2 for two weeks before adding more lymphocyte medium. When a sufficient number of transformed cells was grown (6-8 weeks), as indicated by the appearance of large cellular masses that could be seen by the naked eye, a stock of 10 x 10^6 cells/vial were cryopreserved in liquid nitrogen. B cell lines were established for all participants in this study.

2.4 HLA typing

All samples received prior to September 2010 were HLA typed for Class I A and B antigens with the Lambda Monoclonal Typing Tray Second HLA Class I, Lot 6A (One Lambda). This kit is a serological typing method in which monoclonal antibodies to most HLA specificities and complement, predotted onto the microtest tray, are mixed with an equal volume (1 μl) of cells at 2-4 x 10^6 cells/ml. The reactions are read following a 1 hour incubation period. After September 2010, the Micro Sequence Specific Primer (SSP) HLA Class I DNA Typing Tray (One Lambda) was used for HLA typing. This

DNA-based tissue typing technique uses completely matched oligonucleotide primers to amplify a target sequence corresponding to a particular HLA allele. The primers and internal controls are pre-aliquoted into a 96-well format and require only the addition of 25-200 ng/µl DNA (100 ng/µl is optimal) and recombinant Taq polymerase (5 U/µl). PCR was performed using the PTC-100^{TM} Programmable Thermal Controller (MJ research, Inc.) as per kit instructions. After PCR, the DNA fragments were separated by gel electrophoresis, stained with ethidium bromide and exposed to UV light for visualization. The presence or absence of specific bands indicated the presence or absence of specific alleles.

2.5 ELISPOT assays

PBMC samples from more than 70 HIV-infected participants were stimulated with pools of overlapping 15mer peptide sets spanning the major HIV clade B antigens (National Institutes of Health AIDS reference reagent program). Individual peptides were dissolved in DMSO, then pooled in sets of 49 (Nef), 123 (Gag), 125 (Pol1), 123 (Pol2), 128 (Env1), and 83 (Env2) in unsupplemented RPMI 1640 at a final concentration of 1.0 µg/ml of each peptide. PBMC were used fresh or thawed and cultured overnight at 37°C/5% CO_2 in lymphocyte medium before counting viable cells using the trypan blue exclusion method. MultiScreen 96-well plates (Millipore) were pre-wetted with 50 µl of 35% ethanol, washed 4 times with PBS and coated overnight with 100 µl/well of 7.5 µg/ml IFN-γ mAb 1-D1K or 15 µg/ml IL-2 mAb IL2-I/249 (both Mabtech) at 4 °C. The

plate was washed 4 times in PBS then 2 x 10^5 or 4 x 10^5 cells at a concentration of 2 x 10^6 cells/ml in lymphocyte medium were added per well for IFN-γ and IL-2 detection, respectively, in single (IL-2) or dual (IFN-γ) replicates. Peptides (10 µl/pool for IFN-γ and 20 µl/pool for IL-2) were added at a final concentration of 0.1 µg/ml. Medium alone was used as a negative control and 5 µg/mL phytohemagglutinin was used as the positive control. After overnight incubation, the plates were washed 6 times with PBS then 100 µl/well of 1 ug/ml 7-B6-1-biotin or IL-2-II-biotin (both Mabtech) were added for IFN-γ and IL-2 detection, respectively, for 2 hours. Plates were washed 5 times with PBS and 100 µl/well of a 1/1000 dilution of streptavidin-alkaline phosphatase (ALP) (Mabtech) was added for 1 hour. Plates were subsequently washed 5 times with PBS and 100 µl/well of chromogenic ALP substrate (BioRad) was added. Streptavidin has a high affinity for biotin therefore any biotinylated antibodies used to detect either IFN-γ or IL-2 are bound by enzyme-conjugated streptavidin. ALP then converts the chromogenic substrate, a mix of nitroblue tetrazolium in aqueous dimethylformamide (DMF) containing magnesium chloride and 5-bromo-4-chloro-3-indolyl phosphate in DMF, into an iododye which is bound in place on the membrane forming a purple spot. Each spot is equivalent to one reactive cell. Spots were counted with an Immunoscan reader (Celluar Technology Limited). Quality control was performed manually and results were reported as IFN-γ SFU/10^6 PBMC or IL-2 SFU/10^6 PBMC. A positive response was considered to be at least 50 IL-2 SFU/10^6 PBMC or 100 IFN-γ SFU/10^6 PBMC and at least twice the background.

Pools stimulating IL-2 production (Gag and Nef) were deconvoluted with peptide matrices by ELISPOT assay (as above) to identify the individual 15mers responsible. The Gag matrix consisted of 20 pools of 11 peptides, 2 pools of 12 peptides and 1 pool of 2 peptides (123 peptides total) in an 11 x 12 grid and the Nef matrix consisted of 14 pools of 7 peptides (49 peptides total) in a 7 x 7 grid. Each peptide was present in two and only two pools. By comparing the pattern of IL-2 production against the peptide pools the specific peptide responsible for IL-2 production was identified. Several of the 15mer peptides identified contained optimally defined $CD8^+$ T cell epitopes consistent with the HLA class I type of the participant tested. A2-7 (Nef 83→91), A2-8 (Nef 135→143), and A2-Gag (Gag 77→85) (all HLA-A2 restricted) and A3-2 (Gag18→27) (HLA-A3 restricted) were confirmed as optimally defined 9mer peptides stimulating IL-2 production by PBMC from two or more HIV-infected individuals in our cohort. From these peptides, twenty-four potentially heteroclitic variants were generated (described below) and tested by ELISPOT assay as above except that 10 µl (IFN-γ) or 20 µl (IL-2) of 40 µg/ml peptide was added to each well for a final peptide concentration of 4 µg/ml.

Additive ELISPOT assays were used to determine if overlapping or different subsets of T cells respond to both reference and heteroclitic peptides. Additive ELISPOT assays were performed as above for IFN-γ detection in terms of plate coating and detection. 200,000 PBMC were added per well and stimulated with 4 µg/ml of reference peptide, 4 µg/ml of variant peptide or a combination of reference and one variant peptide at 4 µg/ml each peptide.

2.6 Heteroclitic peptides

Potentially heteroclitic peptides were generated by making conservative and semi-conservative substitutions at amino acid positions 3, 5 and 7. Substitutions were made based on a table of similarity scores for each amino acid in relation to the others. These relationships were quantified by averaging the rank coefficient score for tolerability of point mutations within a protein (Dayhoff PAM250), hydrophobicity (an average of Kyte/Doolittle and Fauchere/Pliska scales), and amino acid side chain volume (measured by H_20 displacement) for each amino acid pair [188]. In addition to aa relationships, substitutions were made based on rarity or absence among HIV-1 clade B sequences in the Los Alamos HIV sequence database (http://www.hiv.lanl.gov/content/sequence/QUICK_ALIGN/QuickAlign.html). Positions 3, 5 and 7 were selected for substitutions based on X-ray crystallography-inferred 3D structure of HLA-A2 or A3/peptide/TCR complex that revealed side chains of amino acid at these positions are most exposed to the CDR3 regions of the TCR α and β chains [195, 196]. Because the anchor residues for HLA-A2 and –A3-restricted peptides are at positions 2 and 9, substitutions at positions 3, 5 and 7 should not alter HLA-binding. Several of the peptides used were presented by HLA-A2, A3 and/or additional HLA molecules. Additional HIV variant peptides were made using Gag147→155 (B57-1; HLA-B57 restricted) and Gag433→440 (A2-9; HLA-A2 restricted) as reference peptides. HLA-B57 also has anchor residues at positions 2 and 9; therefore, B57-1 variants were synthesized as above. Because A2-9 is an 8mer, substitutions were made at positions 3, 5

and 8. Substitutions at position 8 may have disrupted HLA-A2-binding. HLA-A2–restricted non-HIV (A2-Flu and A2-IP) peptide variant sets were generated as above for HLA-A2-restricted peptides. Potential heteroclitic peptide sequences are shown in Table 2.6.1.

Binding predictions were performed using the Center for Biological Sequence Analysis (Technical University of Denmark) NetMHC 3.0 server, which predicts binding of peptides to a number of different HLA alleles using artificial neural networks (ANNs) and weight matrices (http://www.cbs.dtu.dk/services/NetMHC-3.0/). Unfortunately, this was performed after peptide synthesis. Many variant peptides enhanced binding or had similar binding to the appropriate HLA relative to the reference peptide. Few peptides weakened binding or did not bind. The results are shown in Table 2.6.2.

2.7 CTL assays

CTLs were generated by stimulating 5 x 10^6 PBMC, centrifuged and resuspended in approximately 100 µl of lymphocyte medium, with 100 µl of peptide at 200 µg/ml for a final peptide concentration of 100 µg/ml. Following 1 hour of incubation at 37ºC/5% CO_2, cells were resuspended at 2.5 x 10^6 PBMC/ml in lymphocyte medium supplemented with 25 ng/ml of recombinant human IL-7 (National Cancer Institute (NCI) Repository). On day 3, 20 U/ml exogenous IL-2 (NCI Repository) was added. CTL activity was assessed between days 8 and 11 by ^{51}Cr release assay, using peptide-pulsed autologous or HLA-matched EBV-transformed B lymphoblastoid cells (BLCL) as targets. 1 x 10^6

Table 2.6.1 A comparison of reference and corresponding variant peptide sequences

Reference Peptide (name, position: aa sequence)	Variant Peptides (name: aa sequence)	HLA Restriction
A2-7 (Nef83): AAVDLSHFL	A2-7-1: AA**L**DLSHFL* A2-7-2: AA**I**DLSHFL* A2-7-3: AAVD**I**SHFL A2-7-4: AAVD**V**SHFL A2-7-5: AAVDLS**Q**FL A2-7-6: AAVDLS**R**FL	A2 Bw60 Bw62
A2-8 (Nef135): YPLTFGWCF	A2-8-1: YP**I**TFGWCF A2-8-2: YP**V**TFGWCF A2-8-3: YPLT**Y**GWCF A2-8-4: YPLT**W**GWCF A2-8-5: YPLTFG**F**CF A2-8-6: YPLTFG**R**CF*	A2 B18 B35
A3-2 (Gag18): KIRLRPGGK	A3-2-1: KI**K**LRPGGK A3-2-2: KI**Q**LRPGGK A3-2-3: KIRL**K**PGGK* A3-2-4: KIRL**Q**PGGK A3-2-5: KIRLRP**A**GK A3-2-6: KIRLRP**V**GK	A3 B7 B27
A2-Gag (Gag77): SLYNTVATL	A2-Gag-1: SL**W**NTVATL A2-Gag-2: SL**L**NTVATL A2-Gag-3: SLYN**S**VATL* A2-Gag-4: SLYN**W**VATL A2-Gag-5: SLYNTV**G**TL A2-Gag-6: SLYNTV**M**TL*	A2
B57-1 (Gag147): LSPRTLNAW	B57-1: LS**I**RTLNAW B57-2: LS**N**RTLNAW B57-3: LSPR**P**LNAW B57-4: LSPR**N**LNAW B57-5: LSPRTL**D**AW B57-6: LSPRTL**T**AW	B57
A2-9 (Gag433): FLGKIWPS	A2-9-1: FL**S**KIWPS A2-9-2: FL**N**KIWPS A2-9-3: FLGK**T**WPS A2-9-4: FLGK**K**IWPS A2-9-5: FLGKIWP**V** A2-9-6: FLGKIWP**VS***	A2
A2-Flu: GILGFVFTL	A2-Flu-1: GI**V**GFVFTL* A2-Flu-2: GI**T**GFVFTL A2-Flu-3: GILG**Y**VFTL A2-Flu-4: GILG**H**VFTL A2-Flu-5: GILGFV**Y**TL* A2-Flu-6: GILGFVH**T**L	A2
A2-IP: LLDVPTAAV	A2-IP-1: LL**N**VPTAAV A2-IP-2: LL**I**VPTAAV A2-IP-3: LLDV**I**TAAV A2-IP-4: LLDV**N**TAAV A2-IP-5: LLDVPT**S**AV A2-IP-6: LLDVPT**I**AV	A2

Differences from the reference peptide are highlighted in red and underlined
* marks peptides that elicited IL-2 production by $CD8^+$ T cells

Table 2.6.2: HLA-Binding Predictions for Reference and Variant Peptides

Peptide Name: Sequence	Predicted Binding Affinity (nM)for corresponding HLA			Bind Level For given HLA		
	HLA-A2 (B60, B62 not available)			HLA-A2		
A2-7: AAVDLSHFL	544					
A2-7-1: AALDLSHFL*	140			WB		
A2-7-2: AATDLSHFL*	999					
A2-7-3: AAVDISHFL	259			WB		
A2-7-4: AAVDVSHFL	363			WB		
A2-7-5: AAVDLSQFL	1394					
A2-7-6: AAVDLSRFL	6309					
	HLA-A2	HLA-B18	HLA-B35	HLA-A2	HLA-B18	HLA-B35
A2-8: YPLTFGWCF	39166	19	8		SB	SB
A2-8-1: YPITFGWCF	41001	41	7		SB	SB
A2-8-2: YPVTFGWCF	43500	27	6		SB	SB
A2-8-3: YPLTYGWCF	37008	18	7		SB	SB
A2-8-4: YPLTWGWCF	35963	48	6		SB	SB
A2-8-5: YPLTFGFCF	38696	35	9		SB	SB
A2-8-6: YPLTFGRCF*	47774	199	14		WB	SB
	HLA-A3	HLA-B7	HLA-B27	HLA-A3	HLA-B7	HLA-B27
A3-2: KIRLRPGGK	64	28484	28124	WB		
A3-2-1: KIKLRPGGK	103	36189	28784	WB		
A3-2-2: KIQLRPGGK	138	36261	26403	WB		
A3-2-3: KIRLKPGGK*	110	33274	27026	WB		
A3-2-4: KIRLQPGGK	131	36320	29372	WB		
A3-2-5: KIRLRPAGK	48	28306	21710	SB		
A3-2-6: KIRLRPVGK	53	28228	23095	WB		
	HLA-A2			HLA-A2		
A2-Gag: SLYNTVATL	162			WB		
A2-Gag-1: SLWNTVATL	132			WB		
A2-Gag-2: SLLNTVATL	123			WB		
A2-Gag-3: SLYNSVATL*	52			WB		
A2-Gag-4: SLYNWVATL	15			SB		
A2-Gag-5: SLYNTVGTL	589					
A2-Gag-6: SLYNTVMTL*	73			WB		
	HLA-A2			HLA-A2		
B57-1: LSPRTLNAW	83			WB		
B57-1: LSTRTLNAW	80			WB		
B57-2: LSNRTLNAW	152			WB		
B57-3: LSPRPLNAW	1584					
B57-4: LSPRNLNAW	193			WB		
B57-5: LSPRTLDAW	204			WB		
B57-6: LSPRTLTAW	137			WB		
	HLA-A2			HLA-A2		
A2-9: FLGKIWPS	341			WB		
A2-9-1: FLSKIWPS	126			WB		
A2-9-2: FLNKIWPS	188			WB		
A2-9-3: FLGKTWPS	949					
A2-9-4: FLGKKIWPS	204			WB		
A2-9-5: FLGKIWPV	3			SB		
A2-9-6: FLGKIWPVS*	2984					
	HLA-A2			HLA-A2		
A2-Flu: GILGVFTL	17			SB		
A2-Flu-1: GIVGFVFTL*	43			SB		
A2-Flu-2: GITGFVFTL	69			WB		
A2-Flu-3: GILGYVFTL	11			SB		
A2-Flu-4: GILGHVFTL	36			SB		
A2-Flu-5: GILGFVYTL*	30			SB		
A2-Flu-6: GILGFVHTL	53			WB		
	HLA-A2			HLA-A2		

A2-IP: LLDVPTAAV	48	SB
A2-IP-1: LL<u>N</u>VPTAAV	99	WB
A2-IP-2: LL<u>T</u>VPTAAV	146	WB
A2-IP-3: LLDV<u>T</u>TAAV	29	SB
A2-IP-4: LLDV<u>N</u>TAAV	27	SB
A2-IP-5: LLDVPT<u>S</u>AV	89	WB
A2-IP-6: LLDVPT<u>T</u>AV	48	SB

Differences from the reference peptide are highlighted in red and underlined
* marks peptides that elicited IL-2 production by $CD8^+$ T cells
Strong binder is denoted by SB. SB threshold is 50 nM
Weak binder is denoted by WB. WB threshold is 500 nM
Non binders are indicated by a blank. Non binder threshold is >500 nM

BLCL were incubated with 100 µCi of sodium chromate ($Na_2{}^{51}CrO_4$), which was absorbed by passive transfer. Following a 90 minute incubation, cells were washed once in PBS, twice in PBS supplemented with 1% FCS and once in lymphocyte media. Viable cells were counted using the trypan blue exclusion method then resuspended at 1 x 10^5 cells/ml. 50 µl BLCL were added to a 96-well U-bottom microtest plate (Becton Dickinson) then pulsed with 50 µl of 40 µg/ml peptide or unsupplemented RPMI 1640 as a negative control, in duplicate, for one hour. 50 µl BLCL added to 250 µl 1 N hydrogen chloride (HCL) or 250 µl lymphocyte medium, in duplicate, was used to measure maximum ^{51}Cr release and spontaneous ^{51}Cr release, respectively. CTL were washed with lymphocyte media to remove excess IL-2 and counted using the trypan blue exclusion method. CTL were resuspended at 5 x 10^5 cells/ml and added at 20:1, 10:1, 5:1 and 2.5:1 effector:target ratios, in duplicate. Lymphocyte media was added for a final volume of 300 µl/well prior to a 5 hour incubation at 37ºC/5% CO_2. Peptide-specific CTL recognized BLCL-presented peptides and exerted effector functions, killing the BLCL, thus causing release of ^{51}Cr into the supernatant. 125 µl of supernatant was added to 50 µl of bleach in counting tubes (Fisherbrand) then placed in a 1480 WIZARD™ Automatic Gamma Counter (PerkinElmer) which read the amount of radioactivity present in each tube. The percent specific lysis was calculated from the amount of ^{51}Cr present in the supernatant of the test wells relative to the amount of ^{51}Cr present in the supernatant of the maximum and minimum release wells by the following formula:

$$\% \text{ specific lysis} = \frac{\text{test } {}^{51}Cr \text{ release} - \text{min } {}^{51}Cr \text{ release}}{\text{max } {}^{51}Cr \text{ release} - \text{min } {}^{51}Cr \text{ release}}$$

Cross-reactivity assays were performed by ^{51}Cr release assay in which autologous BLCL were pulsed with each peptide in the entire peptide set. Effector cells were added at an appropriate effector:target ratio determined in the previous assay. Results were plotted as percent specific lysis against each peptide. Avidity of the TCR/peptide interaction was also determined by ^{51}Cr release assay in which autologous BLCL were pulsed with serial dilutions (10 μM to 1 x 10^{-5} μM final) of the test peptide. Effector cells were added at an appropriate effector:target ratio based on the previous assay. The results were plotted as percent specific lysis against peptide concentration and the avidity was estimated as the concentration of peptide at which the percent specific lysis fell to 50% of the maximum.

2.8 Proliferation assays and flow cytometry

Proliferation assays were performed using the CellTrace™ CFSE Cell Proliferation Staining kit (Invitrogen). A 5 mM stock solution of CFSE was prepared by dissolving 50 μg CFSE in 18 μl DMSO. PBMC were resuspended at 1 x 10^6 cells/ml in pre-warmed PBS supplemented with 5 mM EDTA (Sigma) and 0.25 μM CFSE (Invitrogen) and incubated, in the dark, at 37ºC for 14 minutes. Following a 5 minute incubation on ice the cells were quenched with 5 volumes of ice-cold lymphocyte media and centrifuged to remove any unabsorbed CFSE. 2 x 10^6 to 5 x 10^6 PBMC (depending on availability) were centrifuged and resuspended in approximately 100 μl of lymphocyte medium, with 100 μl of peptide at 200 μg/ml for a final peptide concentration of 100

μg/ml. Following 1 hour of incubation at 37°C/5% CO_2, cells were resuspended at 2.5 x 10^6 PBMC/ml in lymphocyte medium. On day 7, cells were harvested and stained with a four color panel for surface and intracellular markers using anti-CD8-PerCP (HIT8a; BioLegend) and an appropriate color combination of anti-CCR7-APC (3D12; eBiosciences), anti-IFN-γ-APC (4S.B3; eBiosciences), anti-PD-1-APC (EH12.2H7; BioLegend), anti-perforin-APC/PE (dG9; BioLegend/B-D48; Santa Cruz), anti-IL-2-PE (MQ1-17H12; eBiosciences) and anti-CD45RA-PE (JS-83; eBiosciences). No FITC-conjugated antibodies were used because CFSE occupies the FITC channel. Additional flow cytometry experiments in which cells were not CFSE labelled involved appropriate color combinations of the previously described antibodies along with anti-IL-2-FITC (MQ1-17H12; Caltag), anti-IFN-γ-FITC (4S.B3, eBiosciences) and anti-CD3-FITC (UCHT1; eBiosciences). Staining for intracellular cytokines (IFN-γ and IL-2) was done after a 4 hour incubation of 1 x 10^6 peptide stimulated-PBMC with 100,000 reference or heteroclitic peptide-pulsed autologous BLCL in the presence of 10 μg/ml (final concentration) Brefeldin A. Appropriate isotype controls were used. Staining was performed as per IntraStain Kit (Dako) procedure. In short, 1 x 10^6 cells were washed in flow buffer (PBS supplemented with 5 mM EDTA, 0.2% sodium azide (both Sigma) and 0.5% FCS (Invitrogen) with the pH adjusted to 7-7.2), stained with antibodies to surface markers, washed, incubated with fixative containing 5-10% formaldehyde, washed, permeabilized and stained with antibodies to intracellular markers. After a final wash, cells were resuspended in 200 μl of 1% paraformadehyde and analyzed within one week

of staining with a FACSCalibur™ (BD Biosciences). Data analysis was performed using WINMDI 2.8 software.

2.9 Statistical Analysis

Statistical analysis was carried out with GraphPad Prism 4.03 Software. Normality of data distribution was assessed by D'Agostino and Pearson omnibus normality test. When data was normally distributed, means were presented for comparison. If data did not fit a normal distribution, medians were presented for comparison. One-Tailed Student's Paired *t* test was used to compare PD-1 expression on reference and heteroclitic peptide-stimulated $CFSE^{low}$ $CD8^+$ T cells. Probability values < 0.05 were considered significant.

Chapter 3 Results

3.1 A comparison of IFN-γ and/or IL-2 induction by reference and potentially heteroclitic peptides

The first aim of this study was to identify peptides that selectively augmented IL-2 production by HIV-specific $CD8^+$ T cells relative to reference peptides. Because the production of IL-2 is rare in progressive HIV infection, we also tested the ability of potentially heteroclitic HIV peptides to enhance IFN-γ production by HIV-specific $CD8^+$ T cells relative to reference peptides. Cytokine production was measured by ELISPOT assay in which PBMC from 87 HIV-infected and 5 HIV-uninfected individuals were screened in single (IL-2) or duplicate test (IFN-γ) against one or more of the 8 peptide sets used in this study (Table 2.6.1) at one or more time points. Each of the peptides is 9aa in length, with the exception of A2-9 and variants A2-9-1 through A2-9-5, which are 8mers. The number of people tested against each peptide set and the number of people who had a positive response (at least twice background and $\geq$ 100 IFN-γ SFU/10^6 PBMC or $\geq$ 50 IL-2 SFU/10^6 PBMC) to one or more peptides are indicated in Table 3.1.1. In most cases (A2-7, A2-8, A2-9, A3-2, B57-1, A2-Flu, and A2-IP), less than 50% of the subjects tested responded by IFN-γ production and fewer than 15% of those tested responded by IL-2 production. For one peptide set, A2-Gag, 60% of those tested responded by IFN- γ production, while 21% responded by IL-2 production.

These results are fairly descriptive so in order to better understand them, there are a few things to keep in mind. Firstly, a positive response is at least twice background and $\geq$ 100 SFU/10^6 PBMC for IFN-γ or $\geq$ 50 SFU/10^6 PBMC for IL-2. In comparing IFN-γ responses, two peptides were considered similar if there was $<$ 100 IFN-γ SFU/10^6 PBMC difference. A peptide was considered better than another if there was $\geq$ 100 IFN-γ SFU/10^6 PBMC with a difference of 100-300 IFN-γ SFU/10^6 PBMC representing a slightly better peptide, a difference of 301-700 IFN-γ SFU/10^6 PBMC representing a moderately better peptide and a difference of $>$ 700 IFN-γ SFU/10^6 PBMC representing a much better peptide. These ranges were chosen arbitrarily based on the variation in responses observed. For IL-2 responses, two peptides were considered similar if there was $\leq$ 25 IL-2 SFU/10^6 PBMC difference. A peptide was considered better than another if there was $>$ 25 more SFU//10^6 PBMC. The results are described in comparing the number of cases (reference and heteroclitic peptide pair comparisons) for each category. The total number of cases is based on the number people and their respective peptide-pair comparisons that elicited positive responses.

Table 3.1.1 A summary of the number of people with positive IFN-γ and IL-2 responses to one or more peptides in each peptide set.

Peptide Set	A2-7	A2-8	A2-9	A2-Gag	A3-2	B57-1	A2-Flu	A2-IP
Number of People Tested (150 total)	21	26	15	37	19	7	10+ 5-	7+ 3-
Number of People with Positive IFN-γ Responses (60 total)	7	11	5	21	5	3	4+ 3-	1+ 0-
Number of People with Positive IL-2 Responses (15 total)	3	2	2	5	2	0	0+ 1-	0+ 0-

- + refers to HIV-infected individuals
- - refers to HIV-uninfected individuals
- Peptide set refers to both reference and variant peptides
- A positive response is one that is at least twice the background and at least ≥100 IFN-γ SFU/10^6 PBMC or ≥50 IL-2 SFU/10^6 PBMC
- Some individuals were tested against one or more peptide sets, therefore, there is overlap in the total number of people tested and with positive responses

In ~50% of those individuals who responded to one or more peptides by IFN-γ production, the reference peptide stimulated ≥ 100 IFN-γ SFU/10^6 PBMC more than all other variants within their respective peptide set (Representative examples shown in Figure 3.1.1). In the majority of cases (19/29) the reference was only slightly better than the variants (100-300 IFN-γ SFU/10^6 PBMC difference; Figure 3.1.1 A). In 5 cases, the reference was moderately better than the variants (301-700 IFN-γ SFU/10^6 PBMC difference; Figure 3.1.1 B) and in an additional 5 cases, the reference was much better than the variants (> 700 IFN-γ SFU/10^6 PBMC difference; Figure 3.1.1 C). Thirty-seven percent of those individuals who responded by IFN-γ production (22/60) had similar IFN-γ responses (< 100 IFN-γ SFU/10^6 PBMC difference) to the reference peptide and at least 1, but up to 5, variant peptides within a set (Representative examples shown in Figure 3.1.2). For A2-7, a common trend was observed in which the reference peptide and variants A2-7-1 to A2-7-4 stimulated similar IFN-γ responses in 3 of the 7 who responded to this peptide set (#34, #125 and #138; Figure 3.1.3 A). A common trend was also observed for peptide set A2-8 (Representative examples shown in Figure 3.1.3 B). Three of the eleven individuals who responded to A2-8, responded only to the reference peptide. Of the 8 remaining individuals who responded to at least one of the variant peptides, A2-8-6 rarely stimulated a response. In cases where a response was generated, it was less than 20% of the next highest response to any of the peptides within that set.

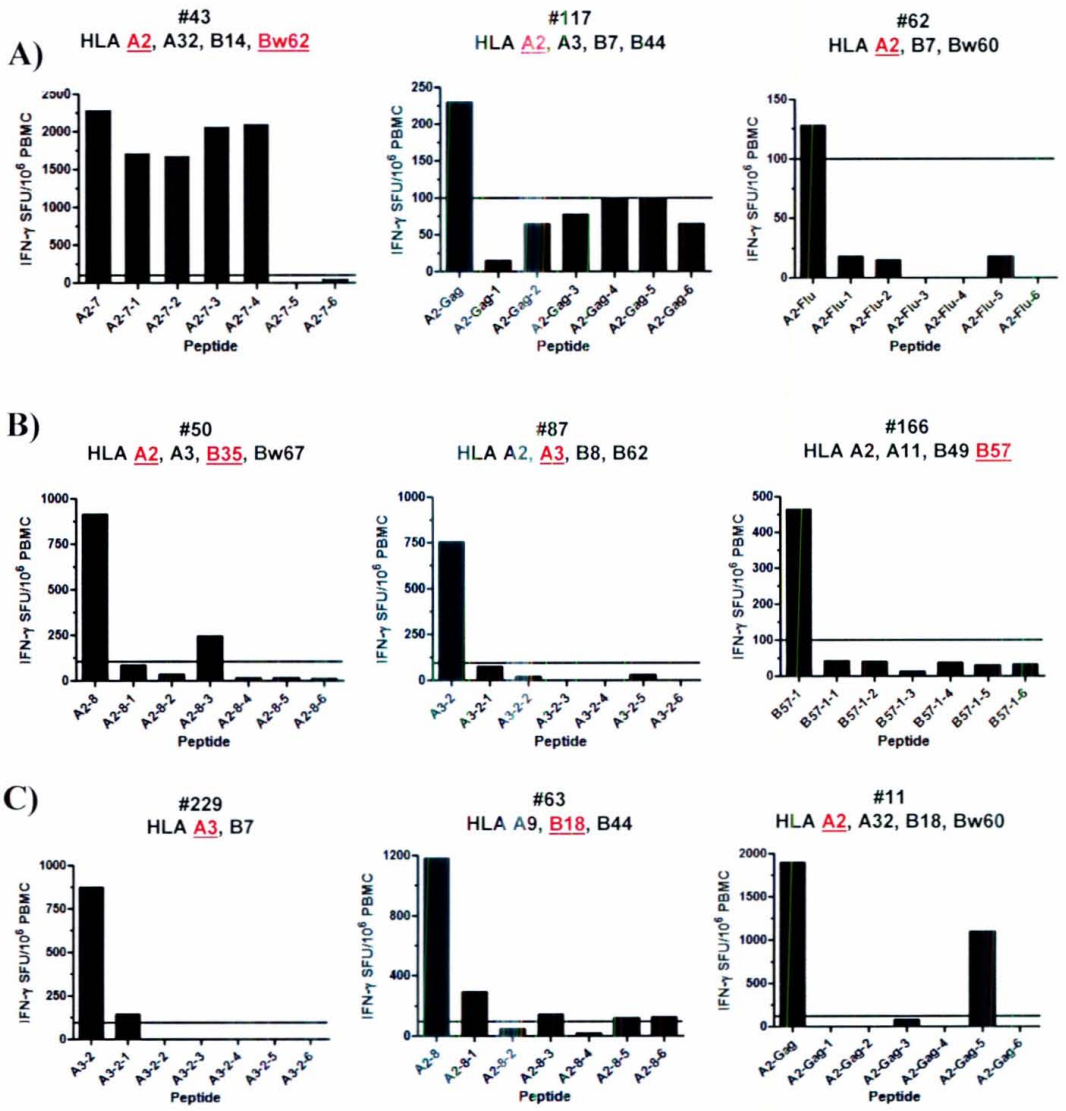

A)
#43
HLA A2, A32, B14, Bw62
#117
HLA A2, A3, B7, B44
#62
HLA A2, B7, Bw60
IFN-γ SFU/10^6 PBMC
Peptide
A2-7
A2-7-1
A2-7-2
A2-7-3
A2-7-4
A2-7-5
A2-7-6
A2-Gag
A2-Gag-1
A2-Gag-2
A2-Gag-3
A2-Gag-4
A2-Gag-5
A2-Gag-6
A2-Flu
A2-Flu-1
A2-Flu-2
A2-Flu-3
A2-Flu-4
A2-Flu-5
A2-Flu-6
B)
#50
HLA A2, A3, B35, Bw67
#87
HLA A2, A3, B8, B62
#166
HLA A2, A11, B49 B57
A2-8
A2-8-1
A2-8-2
A2-8-3
A2-8-4
A2-8-5
A2-8-6
A3-2
A3-2-1
A3-2-2
A3-2-3
A3-2-4
A3-2-5
A3-2-6
B57-1
B57-1-1
B57-1-2
B57-1-3
B57-1-4
B57-1-5
B57-1-6
C)
#229
HLA A3, B7
#63
HLA A9, B18, B44
#11
HLA A2, A32, B18, Bw60

Figure 3.1.1 Comparison of reference and variant stimulation of IFN-γ production I. PBMC from HIV-infected and/or HIV-uninfected individuals were tested in duplicate against one or more peptide sets for IFN-γ production by ELISPOT assay. Duplicates were within 20% of each other. Duplicates were averaged and the background was subtracted. A positive response is at least 100 IFN-γ SFU/10^6 PBMC and at least twice background. Representative examples in which reference peptides stimulated A) 100-300 B) 301-700 or C) > 700 IFN-γ SFU/10^6 PBMC more than all variants are shown. The HLA class I allele that presents each peptide set is highlighted in red and underlined.

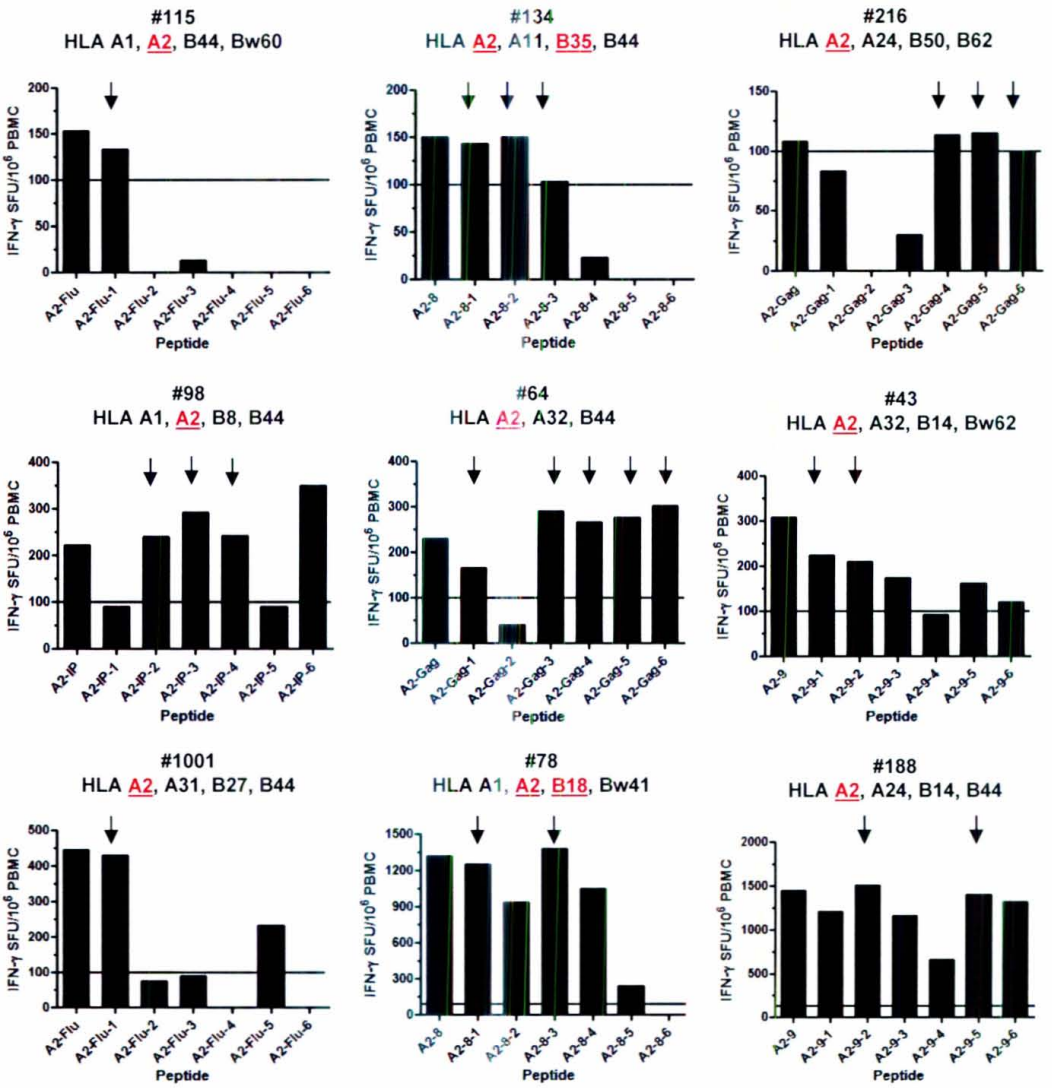

#115
HLA A1, A2, B44, Bw60
IFN-γ SFU/10^6 PBMC
A2-Flu
A2-Flu-1
A2-Flu-2
A2-Flu-3
A2-Flu-4
A2-Flu-5
A2-Flu-6
Peptide
#134
HLA A2, A11, B35, B44
IFN-γ SFU/10^6 PBMC
A2-8
A2-8-1
A2-8-2
A2-8-3
A2-8-4
A2-8-5
A2-8-6
Peptide
#216
HLA A2, A24, B50, B62
IFN-γ SFU/10^6 PBMC
A2-Gag
A2-Gag-1
A2-Gag-2
A2-Gag-3
A2-Gag-4
A2-Gag-5
A2-Gag-6
Peptide
#98
HLA A1, A2, B8, B44
IFN-γ SFU/10^6 PBMC
A2-iP
A2-iP-1
A2-iP-2
A2-iP-3
A2-iP-4
A2-iP-5
A2-iP-6
Peptide
#64
HLA A2, A32, B44
IFN-γ SFU/10^6 PBMC
A2-Gag
A2-Gag-1
A2-Gag-2
A2-Gag-3
A2-Gag-4
A2-Gag-5
A2-Gag-6
Peptide
#43
HLA A2, A32, B14, Bw62
IFN-γ SFU/10^6 PBMC
A2-9
A2-9-1
A2-9-2
A2-9-3
A2-9-4
A2-9-5
A2-9-6
Peptide
#1001
HLA A2, A31, B27, B44
IFN-γ SFU/10^6 PBMC
A2-Flu
A2-Flu-1
A2-Flu-2
A2-Flu-3
A2-Flu-4
A2-Flu-5
A2-Flu-6
Peptide
#78
HLA A1, A2, B18, Bw41
IFN-γ SFU/10^6 PBMC
A2-8
A2-8-1
A2-8-2
A2-8-3
A2-8-4
A2-8-5
A2-8-6
Peptide
#188
HLA A2, A24, B14, B44
IFN-γ SFU/10^6 PBMC
A2-9
A2-9-1
A2-9-2
A2-9-3
A2-9-4
A2-9-5
A2-9-6
Peptide

Figure 3.1.2 Comparison of reference and variant stimulation of IFN-γ production II. PBMC from HIV-infected and/or HIV-uninfected individuals were tested in duplicate against one or more peptide sets for IFN-γ production by ELISPOT assay. Duplicates were within 20% of each other. Duplicates were averaged and the background was subtracted. A positive response is at least 100 IFN-γ SFU/10^6 PBMC and at least twice background. Representative examples in which reference and one or more variant peptides stimulated responses within 100 IFN-γ SFU/10^6 PBMC of each other are shown. Variant peptides that fit this criterion are indicated by arrows. The HLA class I allele that presents each peptide set is highlighted in red and underlined.

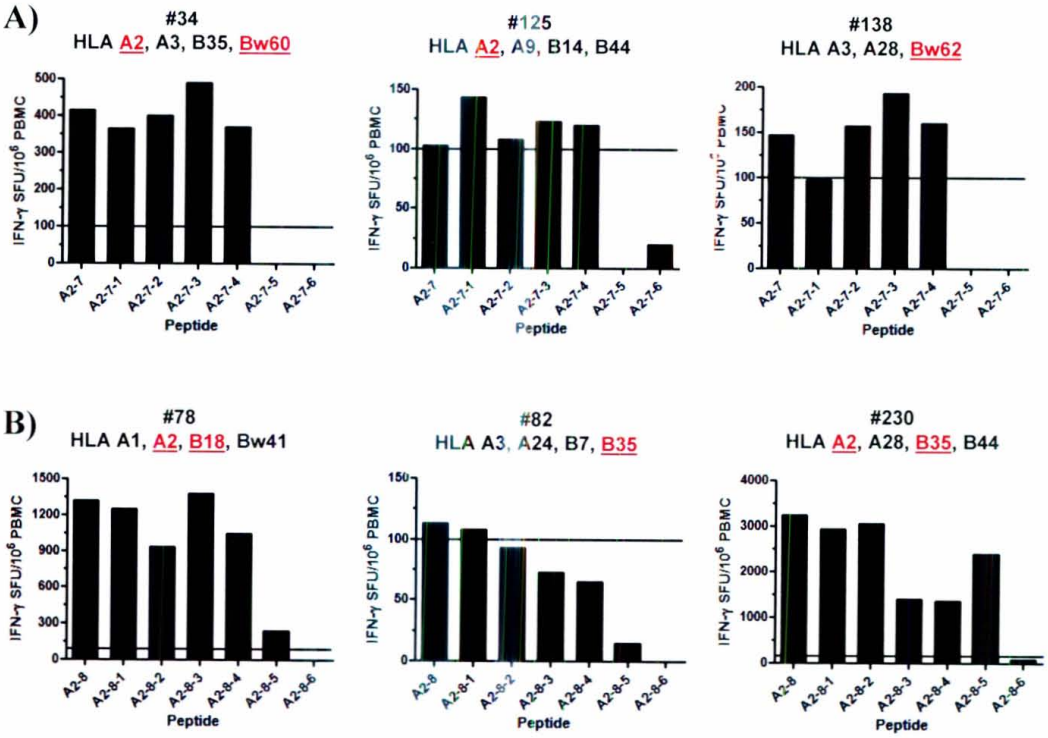

A)
#34
HLA A2, A3, B35, Bw60
#125
HLA A2, A9, B14, B44
#138
HLA A3, A28, Bw62
IFN-γ SFU/10^6 PBMC
Peptide
A2-7
A2-7-1
A2-7-2
A2-7-3
A2-7-4
A2-7-5
A2-7-6
B)
#78
HLA A1, A2, B18, Bw41
#82
HLA A3, A24, B7, B35
#230
HLA A2, A28, B35, B44
A2-8
A2-8-1
A2-8-2
A2-8-3
A2-8-4
A2-8-5
A2-8-6

Figure 3.1.3 Comparison of reference and variant stimulation of IFN-γ production III. PBMC from HIV-infected individuals were tested in duplicate against A2-7 and/or A2-8 peptide sets for IFN-γ production by ELISPOT assay. Duplicates were within 20% of each other. Duplicates were averaged and the background was subtracted. A positive response is at least 100 IFN-γ SFU/10^6 PBMC and at least twice background. A) Representative examples of the trend observed for peptide set A2-7 in which similar responses to the reference peptide (< 100 IFN-γ SFU/10^6 PBMC difference) were observed for variants A2-7-1, -2, -3 and -4 while variants A2-7-5 and -6 stimulated weak or no responses. B) Representative examples of the trend observed for peptide set A2-8 in which variant A2-8-6 rarely stimulated a response. In cases where a response was generated it was < 20% of the next highest response. The HLA class I allele that presents each peptide set is highlighted in red and underlined.

In 9/60 responding individuals, variant peptides stimulated more IFN-γ production than their respective reference peptides by ≥ 100 IFN-γ SFU/10^6 PBMC (Figure 3.1.4). In 4 of the 9 individuals who responded in this way, 2 of the variant peptides within a peptide set fit this criterion. Therefore, a total of 13 reference and variant peptide pairs in which variant peptides stimulated more IFN-γ exist. In 8/13 pairs, the variant was slightly better than the reference (100-300 IFN-γ SFU/10^6 PBMC difference; Figure 3.1.4 A) with 4 additional variants being moderately better than their corresponding reference peptides (301-700 IFN-γ SFU/10^6 PBMC difference; Figure 3.1.4 B) and 1 variant being much better than its reference peptide (> 700 IFN-γ SFU/10^6 PBMC difference; Figure 3.1.4 C). These results show that variant peptides induced similar or higher IFN-γ responses to those induced by reference peptides in ~50% of responding individuals.

As shown by the MERCK STEP trial [167], IFN-γ responses alone are not a strong correlate of protection in HIV infection. IL-2 responses, however, are associated with the presence of HIV-specific $CD8^+$ T_{CM} cells [158, 159] and are strongly correlated with enhanced suppressive capacity of $CD8^+$ T cells [141]. Therefore, we tested the ability of reference and variant peptides to induce IL-2 production by $CD8^+$ T cells. IL-2 responses were measured by ELISPOT assay in single tests at the same time points as IFN-γ responses were measured. We used selective IL-2 production by $CD8^+$ T cells as a marker of enhanced immunogenicity and functionality of heteroclitic peptides relative to index peptides. The number of IL-2 producing cells can also be used to estimate the number of HIV-specific $CD8^+$ T_{CM}, since these cells produce their own IL-2 to sustain

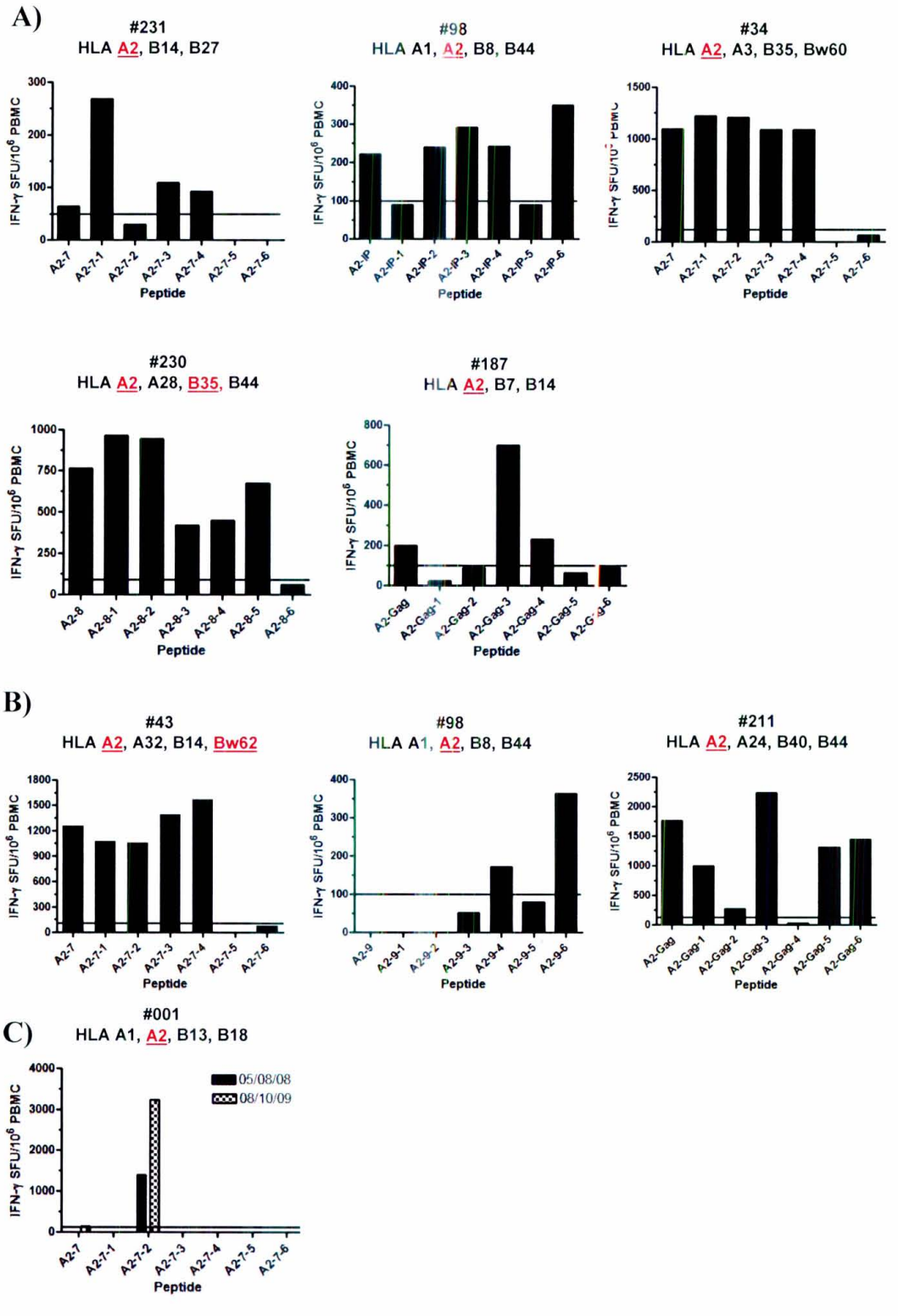

A)
#231
HLA A2, B14, B27
#98
HLA A1, A2, B8, B44
#34
HLA A2, A3, B35, Bw60
#230
HLA A2, A28, B35, B44
#187
HLA A2, B7, B14
B)
#43
HLA A2, A32, B14, Bw62
#98
HLA A1, A2, B8, B44
#211
HLA A2, A24, B40, B44
C)
#001
HLA A1, A2, B13, B18
05/08/08
08/10/09
IFN-γ SFU/10^6 PBMC
Peptide

Figure 3.1.4 Comparison of reference and variant stimulation of IFN-γ production IV. PBMC from HIV-infected and/or HIV-uninfected individuals were tested in duplicate against one or more peptide sets for IFN-γ production by ELISPOT assay. Duplicates were within 20% of each other. Duplicates were averaged and the background was subtracted. A positive response is at least 100 IFN-γ SFU/10^6 PBMC and at least twice background. Representative examples in which variant peptides stimulated A) 100-300 B) 301-700 or C) 701^+ IFN-γ SFU/10^6 PBMC more than reference peptides are shown. The HLA class I allele that presents each peptide set is highlighted in red and underlined.

their own proliferation. As expected, IL-2 responses were much less frequent than IFN-γ responses (Table 3.1.1). IL-2 responses were observed in 15 individuals, however, 1 of these individuals (#131) was tested on two different occasions and his IL-2 response shifted to fit into two categories, therefore, an additional reference-variant comparison was added for a total of 16. Two additional individuals (#34 and #1001) responded by IL-2 production to 2-3 variants, one similarly to the reference and the other(s) superior to the reference, therefore, two additional cases were added for a total of 18 cases. Reference peptides stimulated more IL-2 (> 25 more IL-2 SFU/10^6 PBMC) than variant peptides in 3 of 18 cases (Figure 3.1.5). A third of those individuals who responded by producing IL-2 (6/18) had similar IL-2 responses (within ≤ 25 IL-2 SFU/10^6 PBMC of each other) to the reference peptide and 1 to 2 of the variant peptides within a set (Figure 3.1.6). Variant peptides augmented IL-2 responses relative to reference peptides (> 25 more SFU/10^6 PBMC) in 9/18 cases in which an IL-2 response was elicited (Figure 3.1.7). As can be seen in Figure 3.1.7 A, A2-7 variants stimulated IL-2 responses in 3 individuals, with variant A2-7-2 responsible for this response in two of these three responding individuals. A2-Gag-3 and A2-Gag-6 stimulated IL-2 responses in #211 and #131 respectively (Figure 3.1.7 E) while A2-Flu-1 and A2-Flu-5 enhanced IL-2 production in one HIV-individual (Figure 3.1.7 F). In all other peptide sets (A2-8, A2-9 and A3-2), except B57-1 and A2-IP in which no peptides induced IL-2 production, only 1 variant per set stimulated a stronger IL-2 response relative to reference peptides (Figure 3.1.7 B, C, D). These results demonstrate the ability of variant peptides to enhance IL-2 production relative to reference peptides.

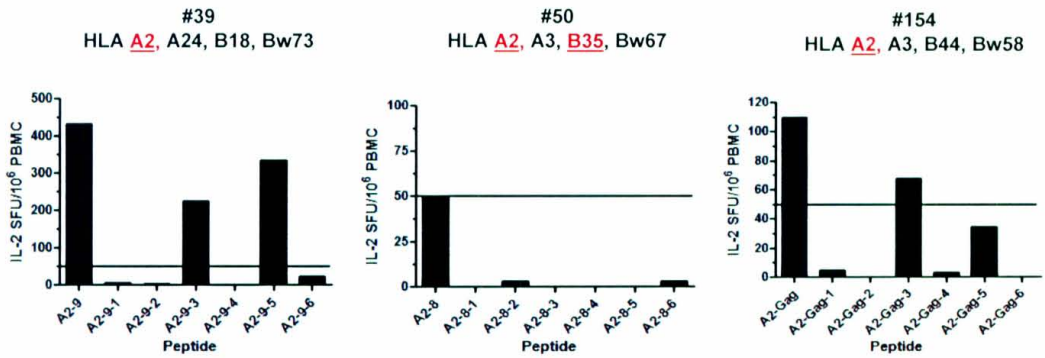


Figure 3.1.5 Comparison of reference and variant peptide stimulation of IL-2 production I. PBMC from HIV-infected and/or HIV-uninfected individuals were tested in a single test against one or more peptide sets for IL-2 production by ELISPOT assay. Examples in which reference peptides stimulated > 25 IL-2 SFU/10^6 PBMC more than variants within their respective peptide sets are shown. A positive response is at least 50 IL-2 SFU/10^6 PBMC and at least twice background. The background was subtracted. The HLA class I allele that presents each peptide set is highlighted in red and underlined.

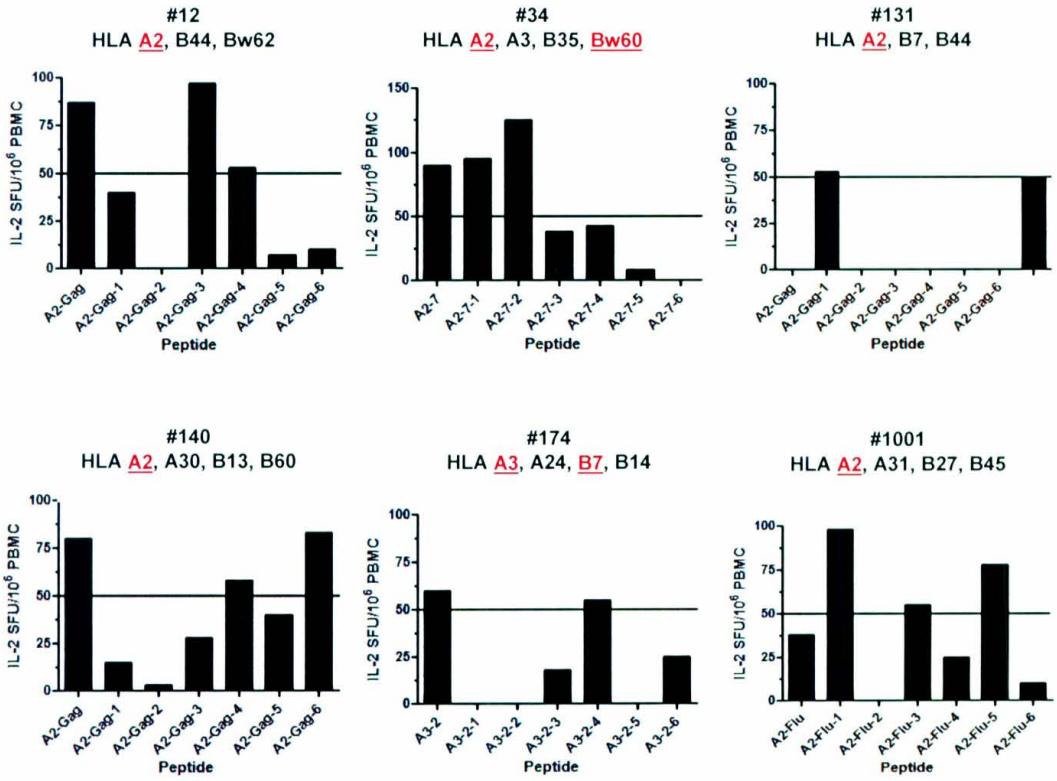


Figure 3.1.6 Comparison of reference and variant peptide stimulation of IL-2 production II. PBMC from HIV-infected and/or HIV-uninfected individuals were tested in a single test against one or more peptide sets for IL-2 production by ELISPOT assay. Representative examples in which reference and one or more variant peptides stimulated within ≤25 IL-2 SFU/10^6 PBMC of each other are shown. The variant peptides that fit this criterion are indicated by arrows. A positive response is at least 50 IL-2 SFU/10^6 PBMC and at least twice background. The background was subtracted. The HLA class I allele that presents each peptide set is highlighted in red and underlined.

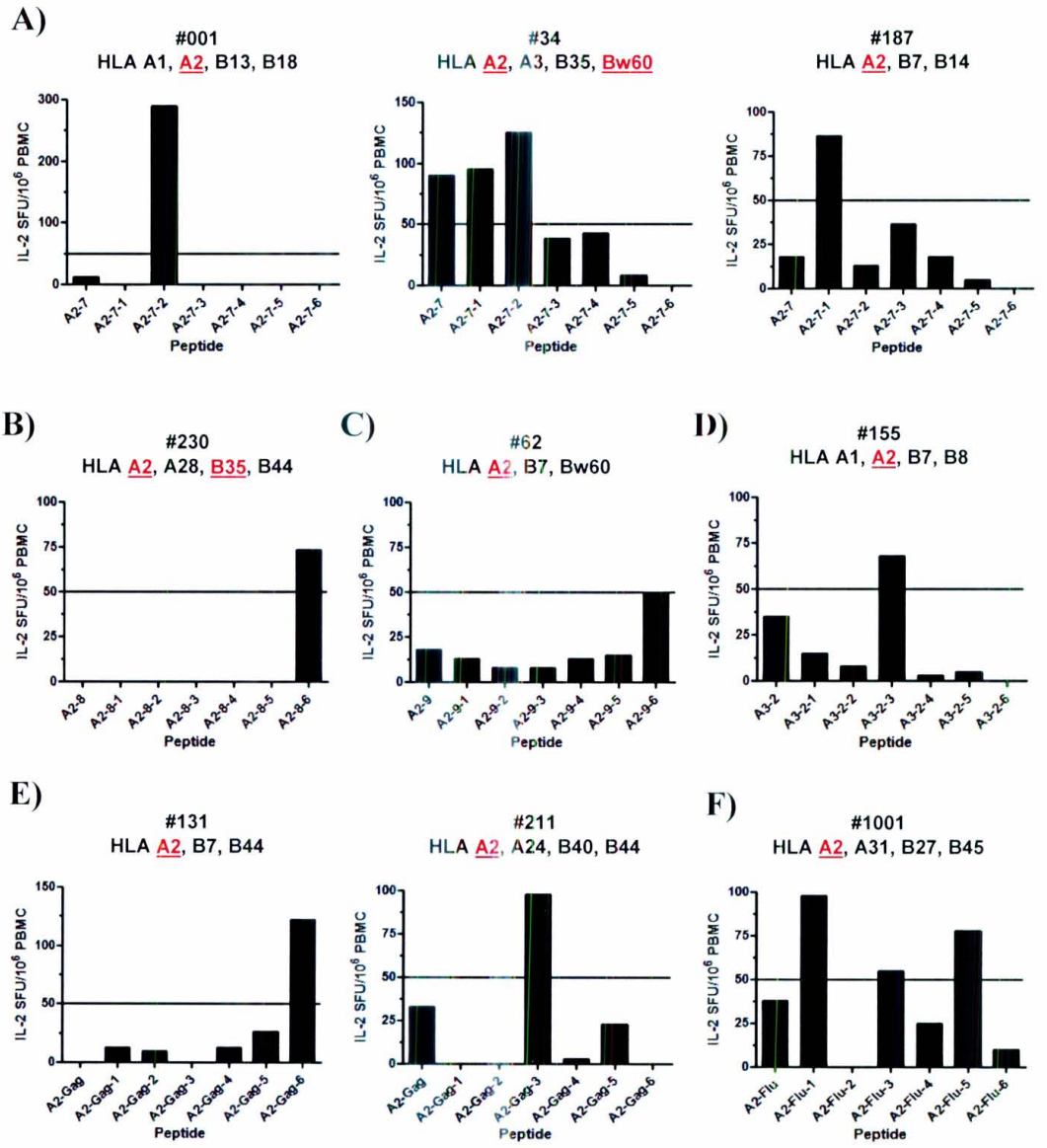

A)
#001
HLA A1, A2, B13, B18
#34
HLA A2, A3, B35, Bw60
#187
HLA A2, B7, B14
B)
#230
HLA A2, A28, B35, B44
C)
#62
HLA A2, B7, Bw60
D)
#155
HLA A1, A2, B7, B8
E)
#131
HLA A2, B7, B44
#211
HLA A2, A24, B40, B44
F)
#1001
HLA A2, A31, B27, B45
IL-2 SFU/10^6 PBMC
Peptide

Figure 3.1.7 Comparison of reference and variant peptide stimulation of IL-2 production III. PBMC from HIV-infected and/or HIV-uninfected individuals were tested in a single test against one or more peptide sets for IL-2 production by ELISPOT assay. Results are shown for peptide sets A) A2-7 B) A2-8 C) A2-9 D) A3-2 E) A2-Gag and F) A2-Flu. A positive response is at least 50 IL-2 SFU/10^6 PBMC and at least twice background. The background was subtracted. The HLA class I allele that presents each peptide set is highlighted in red and underlined.

We next examined the relationship between IFN-γ and IL-2 production regardless of whether the reference or variant peptide was better, worse or similar. In the majority of cases (15/18) IL-2 responses correlated with IFN-γ responses > 150 IFN-γ SFU/10^6 PBMC (Figure 3.1.8). In 3 of 15 cases, IL-2 production was correlated to a low IFN-γ response of 150-300 IFN-γ SFU/10^6 PBMC (Figure 3.1.8 A; Appendix A). In 4 of 15 cases, IL-2 production was correlated with moderate IFN-γ responses (300-700 IFN-γ SFU/10^6 PBMC; Figure 3.1.8 B; Appendix A) and in the majority of cases (8/15), this response was correlated with high IFN-γ responses (> 700 IFN-γ SFU/10^6 PBMC) with 4 of these 8 cases exceeding 1500 IFN-γ SFU/10^6 PBMC (Figure 3.1.8 C; Appendix A). It is important to note, however, that a strong IFN-γ response is not always associated with IL-2 production as there were many cases in which IFN-γ responses were > 300 IFN-γ SFU/10^6 PBMC and no IL-2 response was detected (Example #1001 A2-Flu Figure 3.1.9). In 3/18 cases IL-2 production was not correlated with a high IFN-γ response (Figure 3.1.9). For #230, variant A2-8-6 was the only peptide to induce an IL-2 response. Interestingly, it was also the only peptide that did not induce an IFN-γ response. In the case of #1001, A2-Flu-3 stimulated a positive IL-2 response while this same peptide did not stimulate a positive IFN-γ response. These results demonstrate that IFN-γ production is not always correlated with IL-2 production, however, IL-2 production is generally associated with high IFN-γ responses and, thus, dual functionality of responding $CD8^+$ T cells.

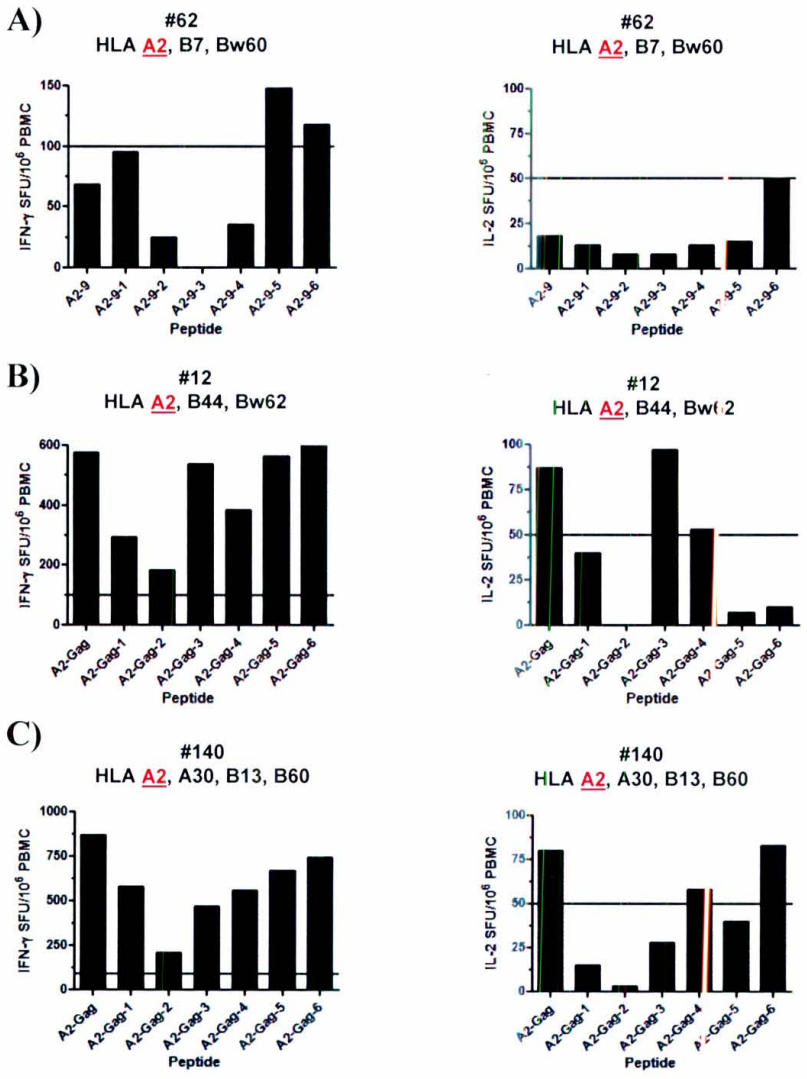
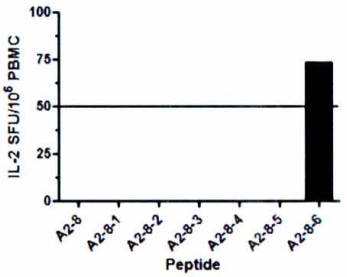
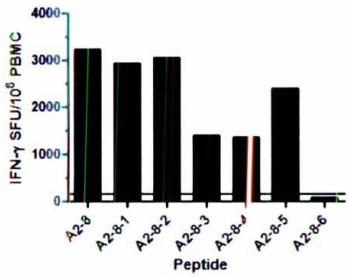

A)
#62
HLA A2, B7, Bw60
IFN-γ SFU/10^6 PBMC
150
100
50
0
A2-9
A2-9-1
A2-9-2
A2-9-3
A2-9-4
A2-9-5
A2-9-6
Peptide
#62
HLA A2, B7, Bw60
IL-2 SFU/10^6 PBMC
100
75
50
25
0
Peptide
B)
#12
HLA A2, B44, Bw62
IFN-γ SFU/10^6 PBMC
600
400
200
0
A2-Gag
A2-Gag-1
A2-Gag-2
A2-Gag-3
A2-Gag-4
A2-Gag-5
A2-Gag-6
Peptide
#12
HLA A2, B44, Bw62
IL-2 SFU/10^6 PBMC
100
75
50
25
0
Peptide
C)
#140
HLA A2, A30, B13, B60
IFN-γ SFU/10^6 PBMC
1000
750
500
250
0
Peptide
#140
HLA A2, A30, B13, B60
IL-2 SFU/10^6 PBMC
100
75
50
25
0
Peptide

Figure 3.1.8 Relationship between IL-2 and IFN-γ responses I. PBMC from HIV-infected and/or HIV-uninfected individuals were tested in duplicate and single test for IFN-γ and IL-2 production, respectively, against one or more peptide sets by ELISPOT assay. Examples in which enhanced IL-2 production is correlated with high IFN-γ responses are shown. Representative examples in which peptides generated A) < 300 IFN-γ SFU/10^6 PBMC B) 300-700 IFN-γ SFU/10^6 PBMC C) > 700 IFN-γ SFU/10^6 PBMC and a positive IL-2 response is observed are shown. A positive response is at least twice background and at least 50 IL-2 SFU/10^6 PBMC or 100 IFN-γ SFU/10^6 PBMC. The background was subtracted from the single test value or from the average of the duplicates. Duplicates were within 20% of each other. The HLA class I allele that presents each peptide set is highlighted in red and underlined.

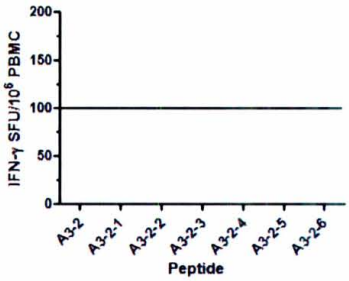
#230
HLA A2, A28, B35, B44



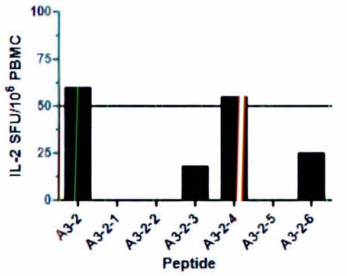
#230
HLA A2, A28, B35, B44



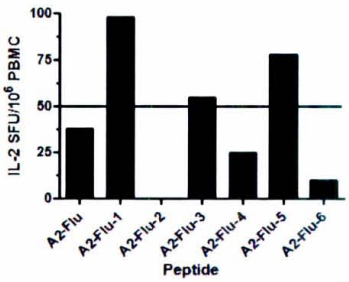
#174
HLA A3, A24, B7, B14



#174
HLA A3, A24, B7, B14



#1001
HLA A2, A31, B27, B45



#1001
HLA A2, A31, B27, B45

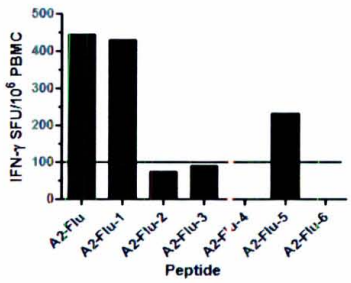


Figure 3.1.9 Relationship between IL-2 and IFN-γ responses II. PBMC from HIV-infected and/or HIV-uninfected individuals were tested in duplicate and single test for IFN-γ and IL-2 production, respectively, against one or more peptide sets by ELISPOT assay. Examples in which enhanced IL-2 production is not correlated with enhanced IFN-γ responses are shown. A positive response is at least twice background and at least 50 IL-2 SFU/10^6 PBMC or 100 IFN-γ SFU/10^6 PBMC. The background was subtracted from the single test value or from the average of the duplicates. The duplicates fell within 20% of each other. The HLA class I allele that presents each peptide set is highlighted in red and underlined.

3.2 Evolution of IFN-γ and IL-2 responses

IFN-γ and IL-2 responses were tested by ELISPOT assay at 2-3 time points for several individuals as shown in Figure 3.2.1. In terms of IFN-γ responses, the overall magnitude changed over time, while the general specificity of these responses was sustained (Figure 3.2.1 A). For subject #34, variants A2-7-1 and A2-7-2 stimulated the same number of IFN-γ SFU/10^6 PBMC on 12/01/10 as the reference peptide. However, at a later time point (21/10/10) these variants surpassed the reference peptide in terms of IFN-γ SFU/10^6 PBMC stimulation. This also occurred for #230 in that variants A2-8-1 and A2-8-2 showed improved antigenicity relative to the reference peptide on 20/08/10 but not on 18/12/09 or 06/01/11. The pattern of IL-2 responses was much less consistent over time (Figure 3.2.1 B). As shown for #001 and #230, IL-2 was detected at just 1 time point. With regard to #131, despite a similar pattern of IFN-γ production at both time points, there was a change in overall magnitude and specificity of IL-2 responses. These results show that $CD8^+$ T cell responses to reference and variant peptides vary over time, primarily in magnitude, but also in fine specificity.

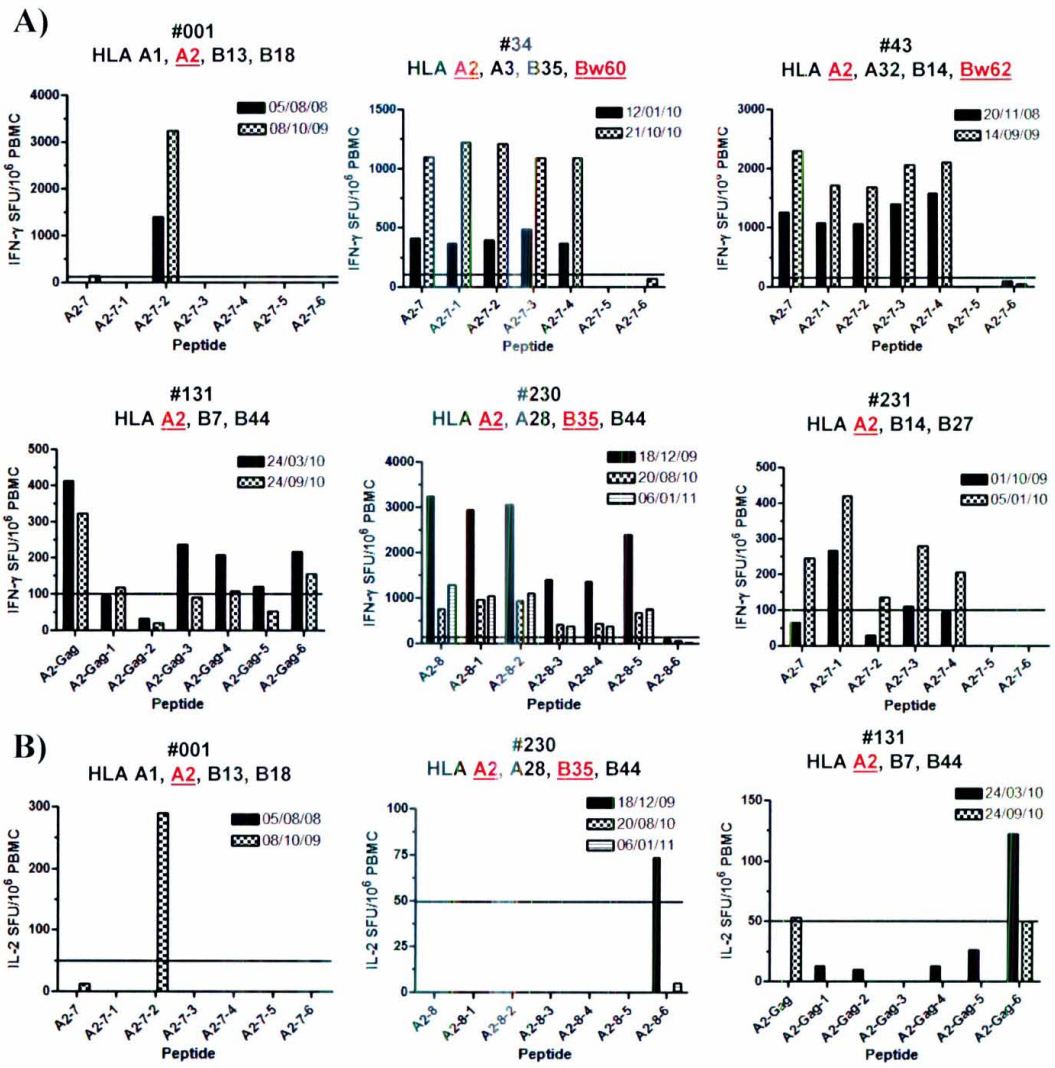

A)
#001
HLA A1, A2, B13, B18
05/08/08
08/10/09
IFN-γ SFU/10^6 PBMC
A2-7
A2-7-1
A2-7-2
A2-7-3
A2-7-4
A2-7-5
A2-7-6
Peptide
#34
HLA A2, A3, B35, Bw60
12/01/10
21/10/10
#43
HLA A2, A32, B14, Bw62
20/11/08
14/09/09
#131
HLA A2, B7, B44
24/03/10
24/09/10
A2-Gag
A2-Gag-1
A2-Gag-2
A2-Gag-3
A2-Gag-4
A2-Gag-5
A2-Gag-6
#230
HLA A2, A28, B35, B44
18/12/09
20/08/10
06/01/11
A2-8
A2-8-1
A2-8-2
A2-8-3
A2-8-4
A2-8-5
A2-8-6
#231
HLA A2, B14, B27
01/10/09
05/01/10
B)
#001
HLA A1, A2, B13, B18
05/08/08
08/10/09
IL-2 SFU/10^6 PBMC
#230
HLA A2, A28, B35, B44
18/12/09
20/08/10
06/01/11
#131
HLA A2, B7, B44
24/03/10
24/09/10

Figure 3.2.1 Evolution of peptide-induced IFN-γ and IL-2 responses. PBMC from HIV-infected and/or HIV-uninfected individuals were tested in duplicate and single test for IFN-γ and IL-2 production, respectively, against one or more peptide sets at one or more time points by ELISPOT assay. Examples of the variation of responses seen within a given individual are shown for A) IFN-γ and B) IL-2. A positive response is at least twice the background and at least 50 IL-2 SFU/10^6 PBMC or 100 IFN-γ SFU/10^6 PBMC. The background was subtracted from the single test value or from the average of the duplicates. The duplicates fell within 20% of each other. The HLA class I allele that presents each peptide set is highlighted in red and underlined. The date of the sample used for each experiment is indicated in the heading for each subject (D/M/Y).

3.3 Reference and variant peptides stimulate overlapping subsets of $CD8^+$ T cells

Based on the differences observed between reference and variant peptides in terms of IFN-γ and IL-2 production, we next asked whether reference and variant peptides stimulated the same or different subsets of $CD8^+$ T cells. To address this question, we performed additive ELISPOT assays in which PBMC from HIV-infected individuals were tested in duplicate for IFN-γ production against reference peptides, variants previously identified that elicited an IFN-γ response and a combination of the reference peptide with each of those IFN-γ-inducing variants previously identified. Due to the rarity and low magnitude of IL-2 responses, we did not perform this experiment for IL-2 production. This was done for 3 peptide sets: A2-7 (Figure 3.3.1 A), A2-Gag (Figure 3.3.1 B) and A2-8 (Figure 3.3.1 C). If different subsets of $CD8^+$ T cells were responding we would expect an additive response in which wells that received a combination of the reference peptide and the variant peptide would show a higher number of IFN-γ SFU/10^6 PBMC equal to the number of IFN-γ SFU/10^6 PBMC induced by the reference peptide + the number of IFN-γ SFU/10^6 PBMC induced by the variant peptide. This, however, was not observed in any of the individuals tested, regardless of peptide set used (Figure 3.3.1). As shown in Figure 3.3.1, the response observed in wells with both reference and variant peptides was no greater than that observed in wells receiving these peptides individually. Although this experiment was only done for one individual per peptide set, it is likely that it is the same subset of $CD8^+$ T cells responding to all peptides within a peptide set for all individuals. Therefore, differences in the ability of reference and variant peptides to

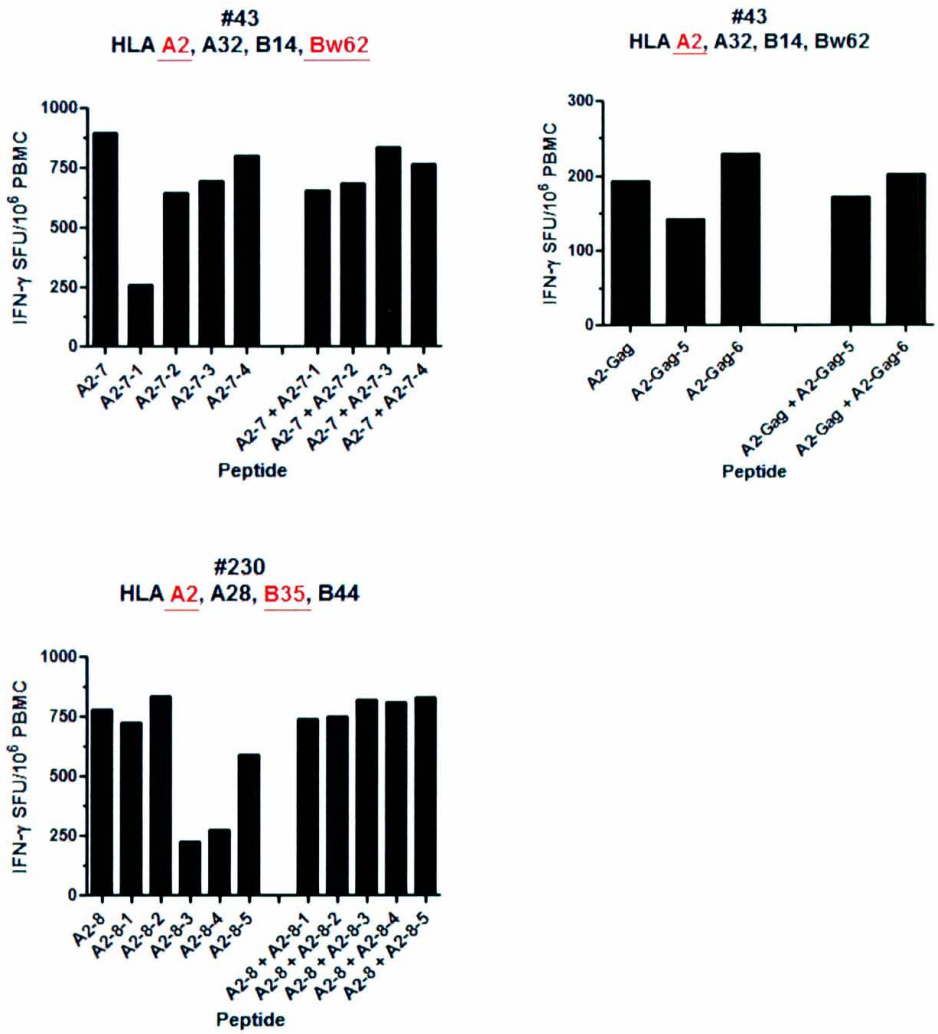


Figure 3.3.1 Responses to combinations of reference and variant peptides. PBMC from HIV-infected individuals were tested in duplicate for IFN-γ production by ELISPOT assay against reference peptides, variants previously identified that elicit a response and a combination of the reference peptide with each of those variants previously identified. Duplicates were within 20% of each other. Duplicates were averaged and the background was subtracted. Results are shown for peptide sets A2-7, A2-Gag and A2-8. The HLA class I allele that presents each peptide set is highlighted in red and underlined.

induce cytokine production by $CD8^+$ T cells are likely due to differences in TCR recognition and signalling.

3.4 A comparison of $CD8^+$ T cell proliferation induced by reference and heteroclitic peptides

The second aim of this project was to investigate whether IL-2-inducing heteroclitic peptides augmented proliferation and differentiation of $CD8^+$ T cells relative to reference peptides. Proliferation was measured by CFSE dilution in which the parental population was designated $CFSE^{high}$ and each subsequent generation was designated $CFSE^{low}$. PBMC from study participants were CFSE labelled, stimulated with reference or heteroclitic peptide, and cultured for 7 days in the absence of exogenous cytokines. On day 7, the cells were stained for CD8 and analyzed by flow cytometry. Fourteen reference and heteroclitic peptide pairs were compared in this manner. Eleven of the heteroclitic peptides stimulated similar or enhanced IL-2 responses by $CD8^+$ T cells compared to reference peptides (ELISPOT) while the remaining 3 had similar or enhanced IFN-γ responses. It is important to note, however, that for 1 of these 3 peptide pairs (A2-7/A2-7-1; #231), IL-2 production was not measured by ELISPOT assay due to a limited number of cells. For another one of these 3 peptide pairs (A2-Gag/A2-Gag-3; #105), IL-2 production was enhanced by the heteroclitic peptide relative to the reference; however, it was less than 50 SFU/10^6 PBMC. In comparing reference and heteroclitic peptide pairs, only pairs in which at least one peptide stimulated $\geq$ 3% proliferation of $CD8^+$ T cells

were considered. Peptides were considered similar if there was ≤ 20% difference in the percent of proliferating $CD8^+$ T cells and were considered better if there was > 20% difference in the percent proliferation of $CD8^+$ T cells. Under these criteria, the reference peptide enhanced proliferation of $CD8^+$ T cells relative to heteroclitic peptides in 5/14 cases (Figure 3.4.1). For #001, A2-7 stimulated more $CD8^+$ T cell proliferation relative to A2-7-2; however, the $CD8^+$ T cells that responded to A2-7-2 underwent additional rounds of division when compared to those who responded to A2-7. Heteroclitic and reference peptides induced similar $CD8^+$ T cell proliferation in 5/14 cases (Figure 3.4.2) while heteroclitic peptides enhanced proliferation relative to reference peptides in 4/14 cases (Figure 3.4.3). These results show that heteroclitic peptides were similar or better than reference peptides at inducing $CD8^+$ T cell proliferation in 9/14 cases.

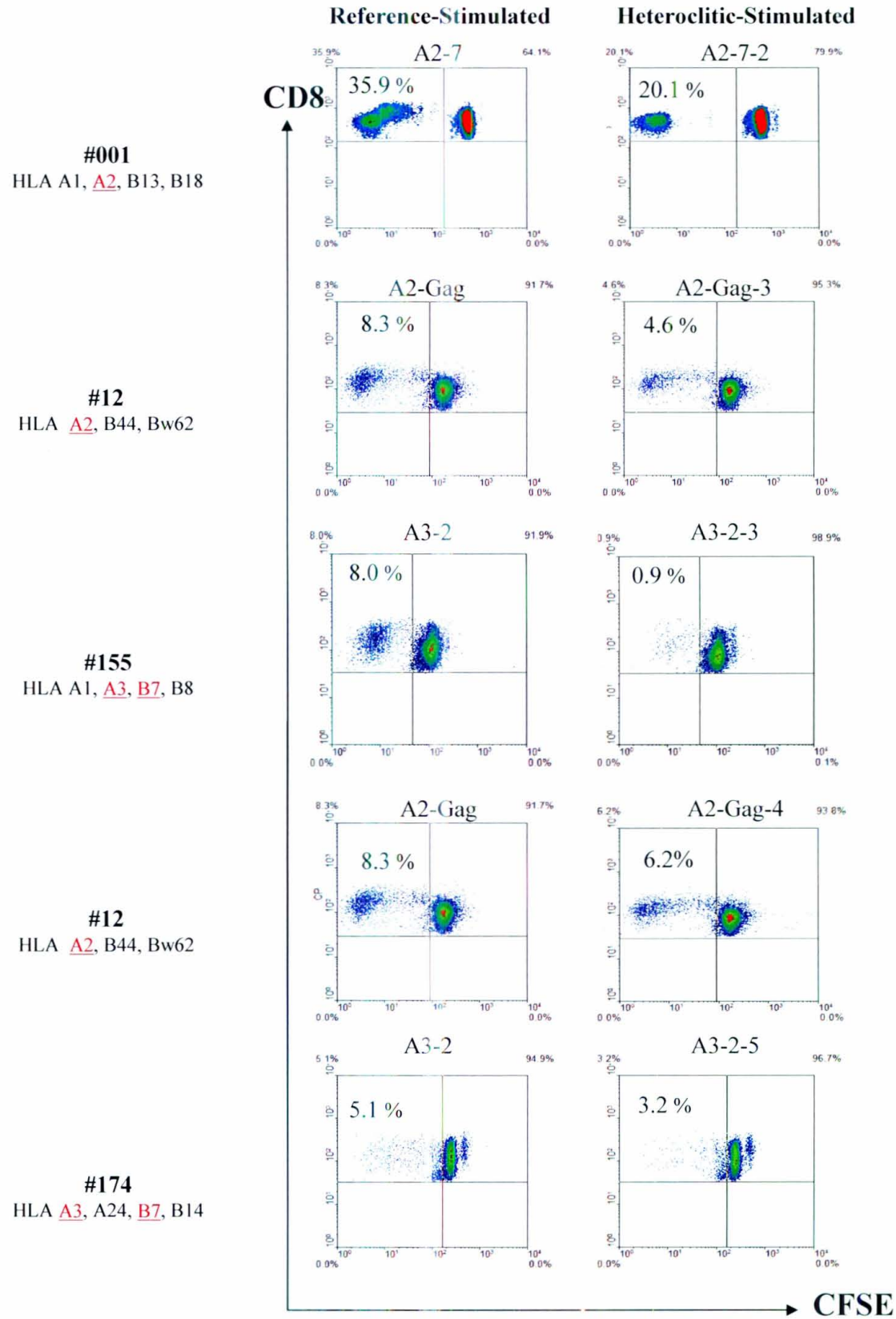

Reference-Stimulated
Heteroclitic-Stimulated
CD8
CFSE
#001
HLA A1, A2, B13, B18
A2-7
35.9 %
A2-7-2
20.1 %
#12
HLA A2, B44, Bw62
A2-Gag
8.3 %
A2-Gag-3
4.6 %
#155
HLA A1, A3, B7, B8
A3-2
8.0 %
A3-2-3
0.9 %
#12
HLA A2, B44, Bw62
A2-Gag
8.3 %
A2-Gag-4
6.2%
#174
HLA A3, A24, B7, B14
A3-2
5.1 %
A3-2-5
3.2 %

Figure 3.4.1 Comparison of $CD8^+$ T cell proliferation induced by reference and heteroclitic peptides I. PBMC from study participants were CFSE labelled and stimulated in vitro with reference or heteroclitic peptide for 7 days and then stained for CD8 and analyzed using flow cytometry. The HLA class I allele that presents each peptide is highlighted in red and underlined. Gated on $CD8^{high}$ cells.

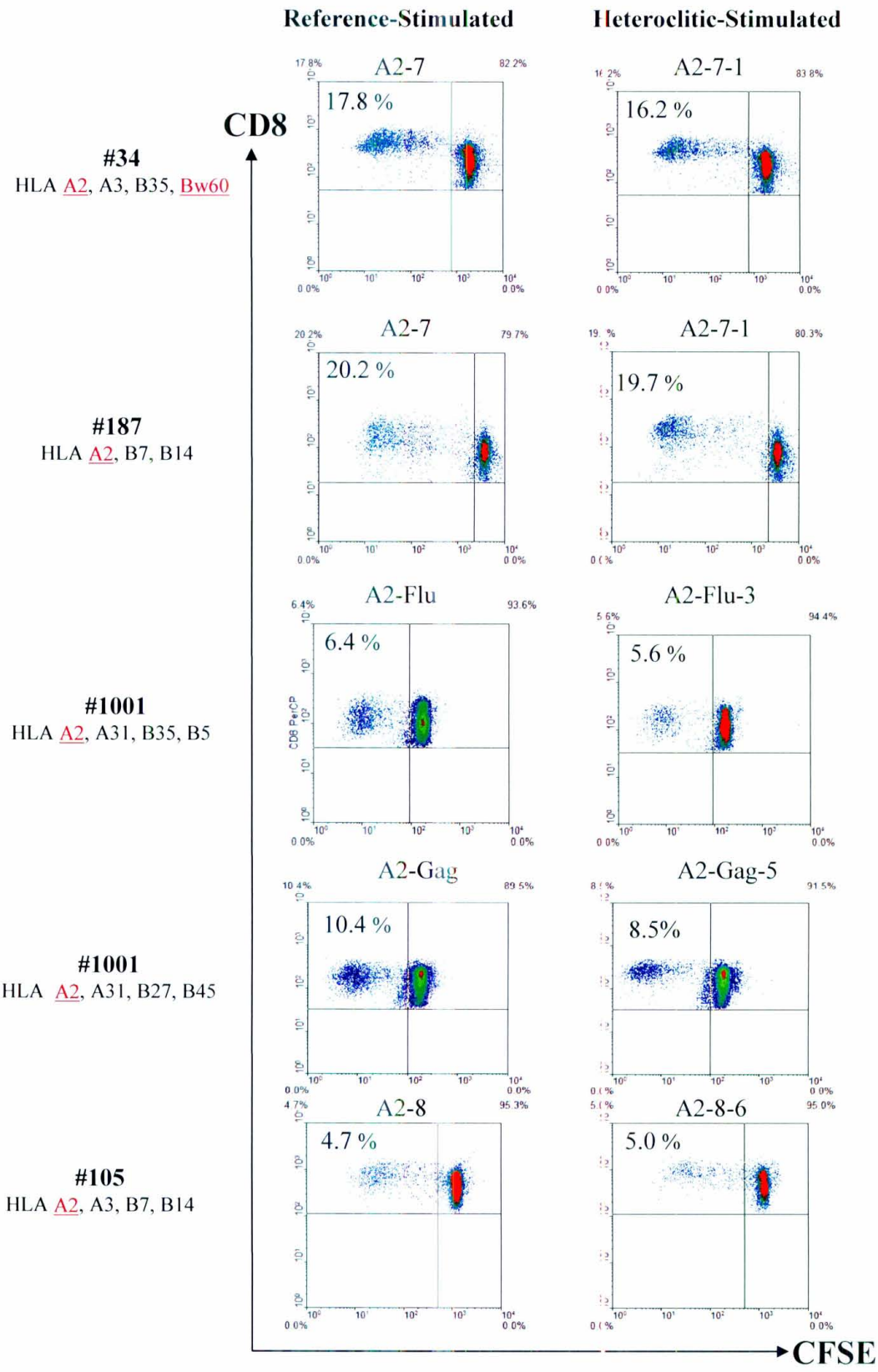

Reference-Stimulated
Heteroclitic-Stimulated
CD8
#34
HLA A2, A3, B35, Bw60
A2-7
17.8 %
A2-7-1
16.2 %
#187
HLA A2, B7, B14
A2-7
20.2 %
A2-7-1
19.7 %
#1001
HLA A2, A31, B35, B5
A2-Flu
6.4 %
A2-Flu-3
5.6 %
#1001
HLA A2, A31, B27, B45
A2-Gag
10.4 %
A2-Gag-5
8.5%
#105
HLA A2, A3, B7, B14
A2-8
4.7 %
A2-8-6
5.0 %
CFSE

Figure 3.4.2 Comparison of $CD8^+$ T cell proliferation induced by reference and heteroclitic peptides II. PBMC from study participants were CFSE labelled and stimulated in vitro with reference or heteroclitic peptide for 7 days and then stained for CD8 and analyzed using flow cytometry. The HLA class I allele that presents each peptide is highlighted in red and underlined. Gated on $CD8^{high}$ cells.

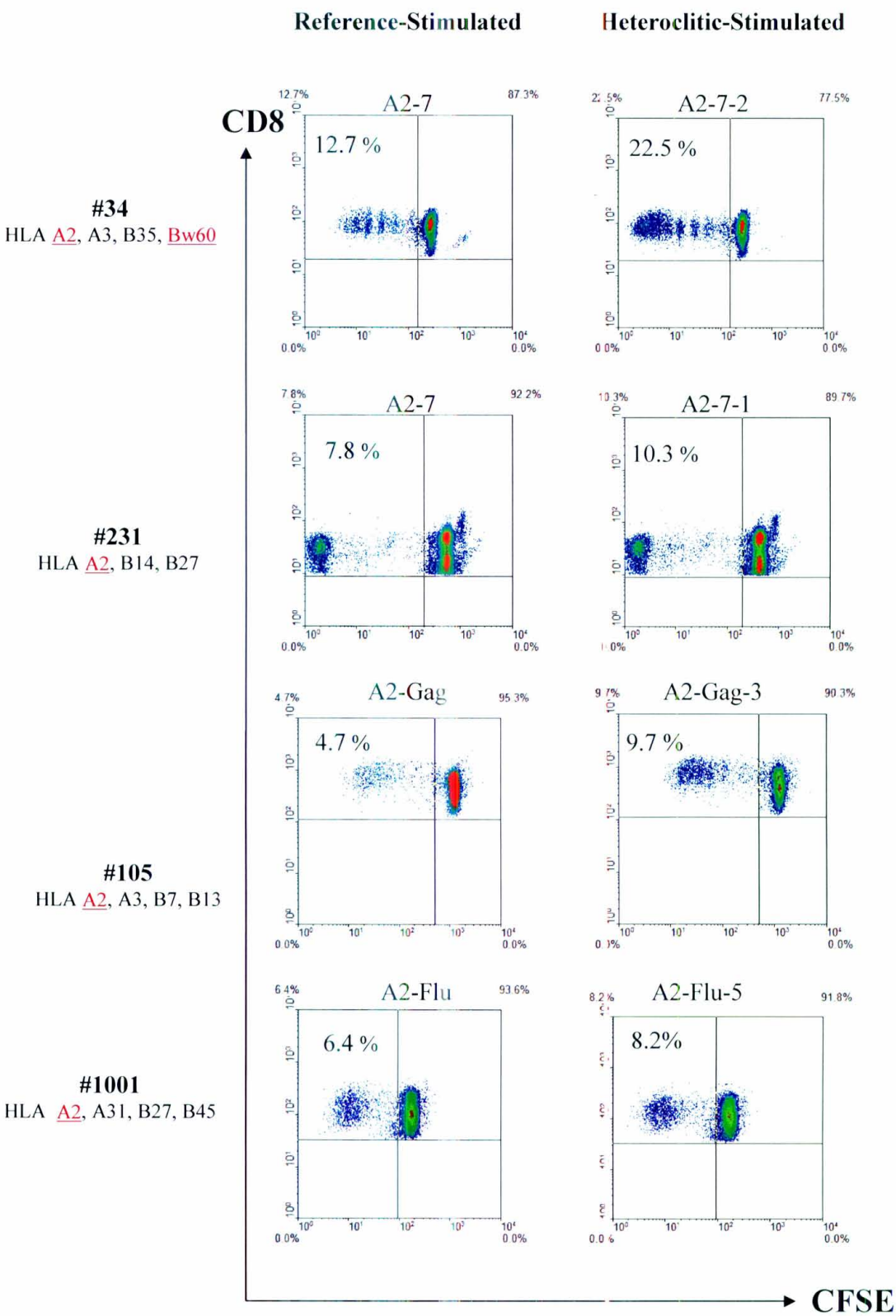

Reference-Stimulated
Heteroclitic-Stimulated
CD8
CFSE
#34
HLA A2, A3, B35, Bw60
#231
HLA A2, B14, B27
#105
HLA A2, A3, B7, B13
#1001
HLA A2, A31, B27, B45
A2-7
12.7 %
A2-7-2
22.5 %
A2-7
7.8 %
A2-7-1
10.3 %
A2-Gag
4.7 %
A2-Gag-3
9.7 %
A2-Flu
6.4 %
A2-Flu-5
8.2%

Figure 3.4.3 Comparison of $CD8^+$ T cell proliferation induced by reference and heteroclitic peptides III. PBMC from study participants were CFSE labelled and stimulated in vitro with reference or heteroclitic peptide for 7 days and then stained for CD8 and analyzed using flow cytometry. The HLA class I allele that presents each peptide is highlighted in red and underlined. Gated on $CD8^{high}$ cells.

3.5 A comparison of $CD8^+$ T cell differentiation and activation induced by reference and heteroclitic peptides

Differentiation and activation of $CD8^+$ T cells induced by reference and heteroclitic peptides was measured by flow cytometry and compared between peptides as well as between $CFSE^{high}$ and $CFSE^{low}$ $CD8^+$ T cell populations. PBMC from study participants were CFSE labelled, stimulated with reference or heteroclitic peptide, and cultured for 7 days in the absence of any exogenous cytokines. On day 7, the cells were stained for CD8, PD-1, CCR7 and/or CD45RA. Staining for intracellular perforin and cytokines (IFN-γ and IL-2) was done after a 4 hour incubation with reference or heteroclitic peptide-pulsed autologous BLCL in the presence of Brefeldin A. As shown in Figure 3.5.1 A, ~50% of $CFSE^{high}$ $CD8^+$ T cells (range 18-71%) expressed CD45RA whereas only ~10% of $CFSE^{low}$ $CD8^+$ T cells (range 0.6-15%) expressed this marker, regardless of which peptide was used for stimulation (Mann-Whitney test, no significant difference). As shown in Figure 3.5.1 B, $\leq$ 5% of $CSFE^{high}$ $CD8^+$ T cells (range 0.6-5%) expressed PD-1, regardless of which peptide was used for stimulation. There was, however, a difference in PD-1 expression between reference and heteroclitic peptide-stimulated $CSFE^{low}$ $CD8^+$ T cell populations, with ~35% of reference-stimulated cells (range 3-52%) expressing PD-1 and ~20% of heteroclitic-stimulated cells (range 5-39%) expressing PD-1. This difference was found to be statistically significant with a p value equal to 0.0262 (one-tailed Student's Paired t test). No CCR7 was detected for $CFSE^{low}$

or $CFSE^{high}$ $CD8^+$ T cell populations when stimulated with either reference or heteroclitic peptides (data not shown).

As shown in Figure 3.5.2, $\leq$ 5% of $CFSE^{high}$ reference and heteroclitic peptide-stimulated $CD8^+$ T cells (range 0.5-5%) expressed perforin or IFN-γ. Perforin expression was detected in 19% of reference-stimulated $CFSE^{low}$ $CD8^+$ T cells (range 2.9-27%) and in 15% of heteroclitic-stimulated $CFSE^{low}$ $CD8^+$ T cells (range 0-46%) (Mann Whitney test: No significant difference; Figure 3.5.2 A). IFN-γ was detected in 62% of reference-stimulated $CSFE^{low}$ $CD8^+$ T cells (range 5-64%) and in 45% of heteroclitic-stimulated $CSFE^{low}$ $CD8^+$ T cells (range 4.4-79%) (Mann Whitney Test: No significant difference; Figure 3.5.2 B). Two individuals were tested for IL-2 production, however, less than 1% proliferation was observed for all but one peptide (data not shown). Given the small sample size, the results do not offer a fair comparison between reference and heteroclitic-stimulated populations. These results show that reference and heteroclitic peptides induce expression of CD45RA, perforin and IFN-γ similarly (< 10% difference). Reference peptides induced higher expression of PD-1 in $CFSE^{high}$ $CD8^+$ T cells than heteroclitic peptides (35% vs 20%; p=0.0262). $CFSE^{low}$ $CD8^+$ T cells expressed more PD-1, perforin and IFN-γ than $CFSE^{high}$ $CD8^+$ T cells while the opposite was observed for CD45RA expression, regardless of which peptide was used for stimulation.

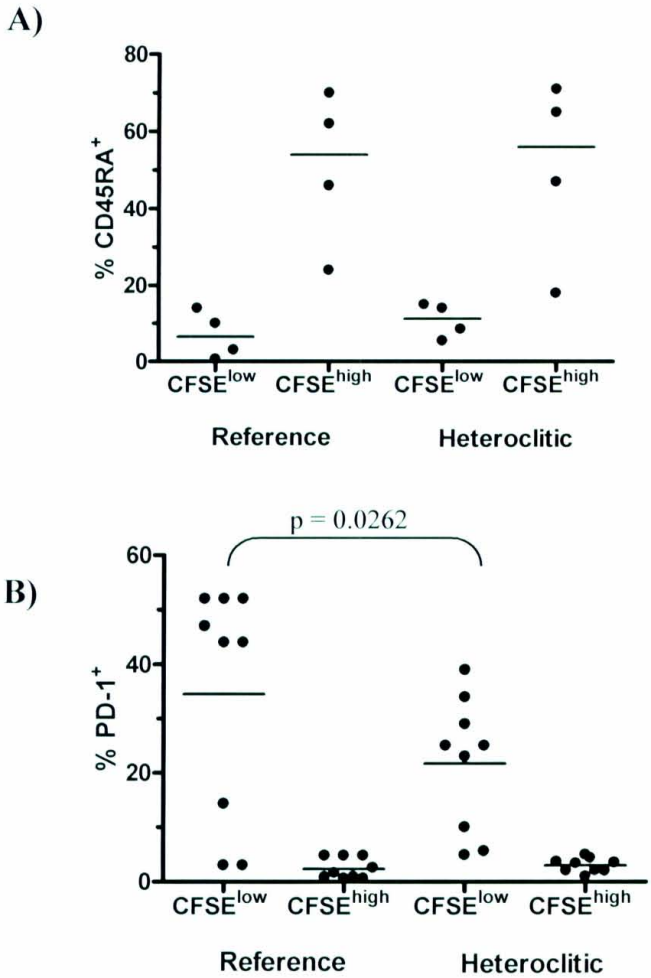


Figure 3.5.1 Comparison of CD45RA and PD-1 expression on reference and heteroclitic peptide-stimulated $CFSE^{low}$ and $CFSE^{high}$ $CD8^+$ T cells. PBMC were stimulated in vitro with reference or heteroclitic peptides in the absence of exogenous cytokines for 7 days and then stained for A) CD45RA or B) PD-1 expression and analyzed using flow cytometry. Gated on $CD8^{high}$ cells. For CD45RA, the horizontal line through the group represents the median frequency. For PD-1, the horizontal line through the group represents the mean frequency.

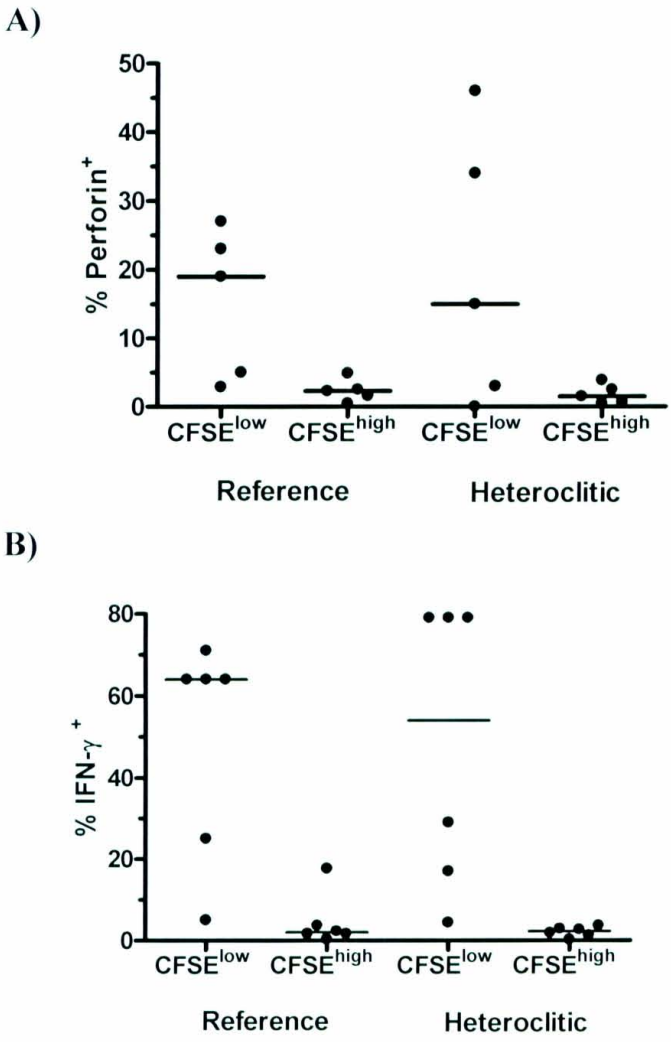
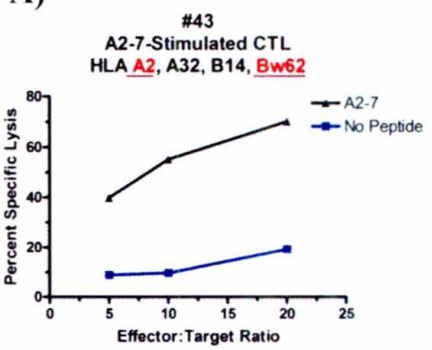


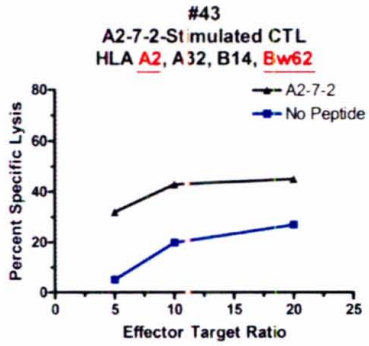
Figure 3.5.2 Comparison of perforin and IFN-γ expression in reference and heteroclitic peptide-stimulated $CFSE^{low}$ and $CFSE^{high}$ $CD8^+$ T cells. PBMC were stimulated in vitro with reference or heteroclitic peptides in the absence of exogenous cytokines for 7 days. On day 7 cells, were incubated with reference or heteroclitic peptide-pulsed autologous BLCL in the presence of brefeldin A then stained for A) perforin or B) IFN-γ expression and analyzed using flow cytometry. Gated on $CD8^{high}$ cells. The horizontal line through the groups represents the median frequency.

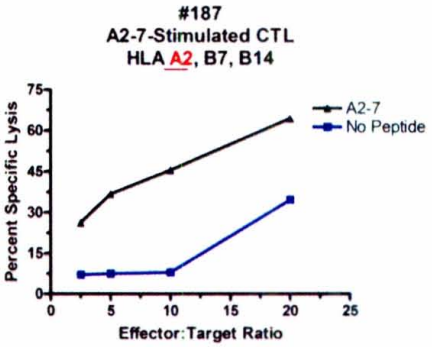
3.6 A comparison of $CD8^+$ T cell cytotoxicity induced by reference and heteroclitic peptides

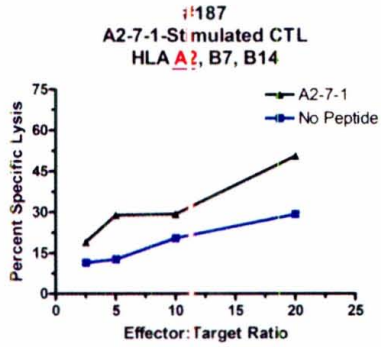
Before proceeding to aims three and four of this project, we first compared the ability of reference and heteroclitic peptides to generate CTL and used this data to determine the appropriate effector to target ratio for subsequent avidity and cross reactivity assays. CTL were generated in vitro in the presence of exogenous IL-7 and IL-2 by stimulating PBMC from study participants with reference peptides, IL-2-inducing heteroclitic peptides and/or variant peptides that enhanced IFN-γ production by $CD8^+$ T cells. CTL activity was assessed between days 8 and 11 by ^{51}Cr release assay, using ^{51}Cr-labelled peptide-pulsed autologous or HLA-matched BLCL as targets. The percent specific lysis was calculated from the amount of ^{51}Cr present in the supernatant of the test wells relative to the amount of ^{51}Cr present in the supernatant of the maximum and minimum release wells. CTL from nine individuals were tested in this manner. One individual (#43) was tested against A2-7 peptides and A2-Gag peptides, therefore, this individual will be counted twice to give 10 cases. Two individuals (#001 and #187) were tested on two or more different occasions and their CTL activity was different each time, therefore, two additional cases were added for a total of 12 cases. In 4/12 cases, reference peptide-stimulated CTL showed greater specific cytotoxicity than variant-stimulated CTL at 3 or more of the 4 effector to target ratios tested (Figure 3.6.1). In 7/12 cases, reference- and variant-stimulated CTL had comparable cytotoxic activity at 3 or more of the effector to target ratios (Figure 3.6.2; Appendix B). In all 7 cases this response was

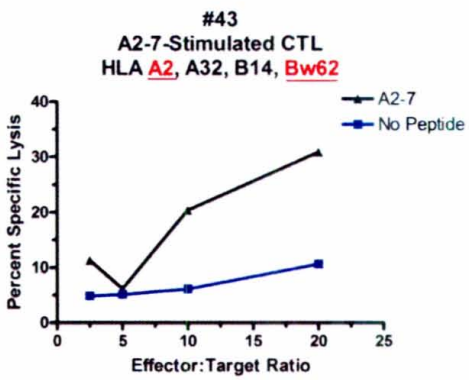
A)

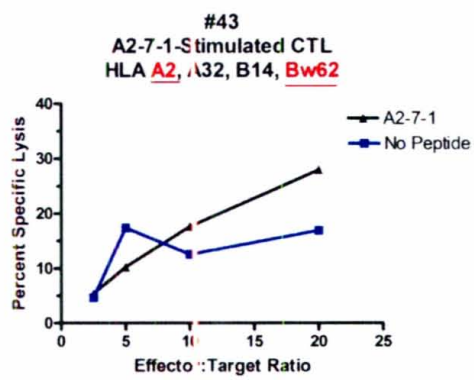

#43
A2-7-Stimulated CTL
HLA A2, A32, B14, Bw62
A2-7
No Peptide
Percent Specific Lysis
Effector:Target Ratio


#43
A2-7-2-Stimulated CTL
HLA A2, A32, B14, Bw62
A2-7-2
No Peptide
Percent Specific Lysis
Effector Target Ratio

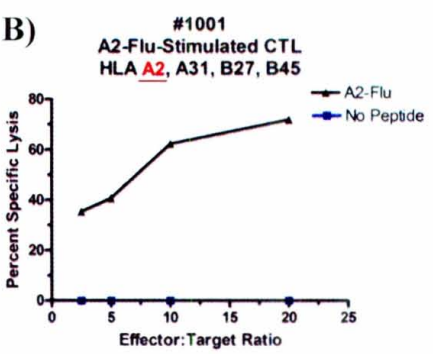

#187
A2-7-Stimulated CTL
HLA A2, B7, B14
A2-7
No Peptide
Percent Specific Lysis
Effector:Target Ratio


#187
A2-7-1-Stimulated CTL
HLA A2, B7, B14
A2-7-1
No Peptide
Percent Specific Lysis
Effector:Target Ratio


#43
A2-7-Stimulated CTL
HLA A2, A32, B14, Bw62
A2-7
No Peptide
Percent Specific Lysis
Effector:Target Ratio


#43
A2-7-1-Stimulated CTL
HLA A2, A32, B14, Bw62
A2-7-1
No Peptide
Percent Specific Lysis
Effecto :Target Ratio

B)


#1001
A2-Flu-Stimulated CTL
HLA A2, A31, B27, B45
A2-Flu
No Peptide
Percent Specific Lysis
Effector:Target Ratio

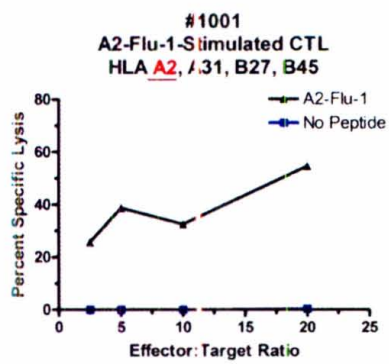
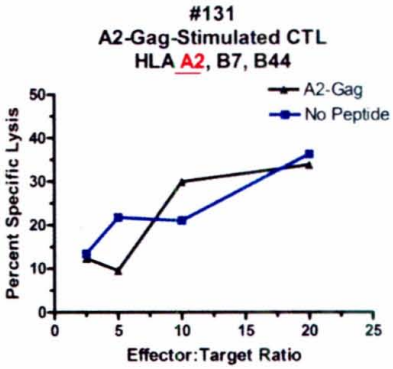
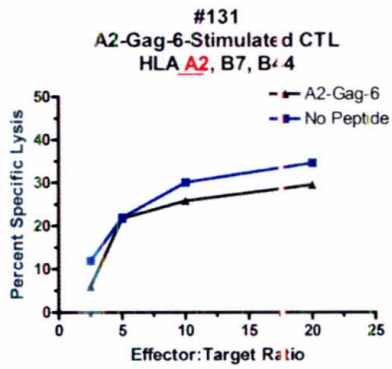
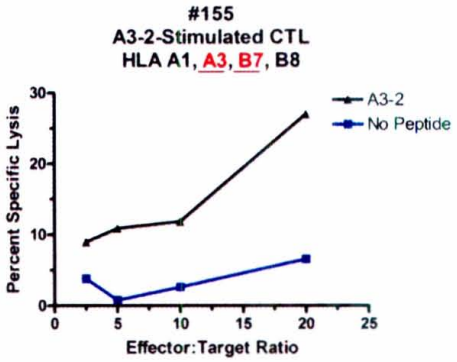
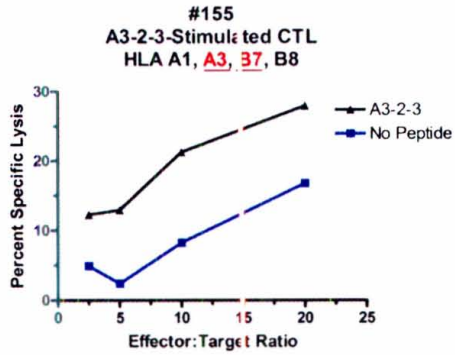

#1001
A2-Flu-1-Stimulated CTL
HLA A2, A31, B27, B45
A2-Flu-1
No Peptide
Percent Specific Lysis
Effector:Target Ratio

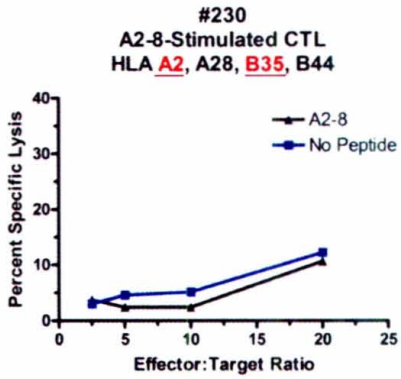
Figure 3.6.1 Comparison of $CD8^+$ T cell cytotoxicity induced by reference and heteroclitic peptides I. Specific cytotoxicity of $CD8^+$ T cells stimulated in vitro with reference or heteroclitic peptides was tested, in duplicate, by ^{51}Cr release assay against reference or heteroclitic peptide-pulsed (A) autologous or (B) HLA-matched BLCL target cells at increasing effector to target ratios. Duplicates fell within 20% of each other. The HLA class I allele that presents each peptide is highlighted in red and underlined. Tests in which peptide-pulsed and non-pulsed BLCL were used as targets are indicated by black triangles and blue squares, respectively.


#131
A2-Gag-Stimulated CTL
HLA A2, B7, B44
A2-Gag
No Peptide
Percent Specific Lysis
Effector:Target Ratio


#131
A2-Gag-6-Stimulated CTL
HLA A2, B7, B44
A2-Gag-6
No Peptide
Percent Specific Lysis
Effector:Target Ratio


#155
A3-2-Stimulated CTL
HLA A1, A3, B7, B8
A3-2
No Peptide
Percent Specific Lysis
Effector:Target Ratio


#155
A3-2-3-Stimulated CTL
HLA A1, A3, B7, B8
A3-2-3
No Peptide
Percent Specific Lysis
Effector:Target Ratio


#230
A2-8-Stimulated CTL
HLA A2, A28, B35, B44
A2-8
No Peptide
Percent Specific Lysis
Effector:Target Ratio

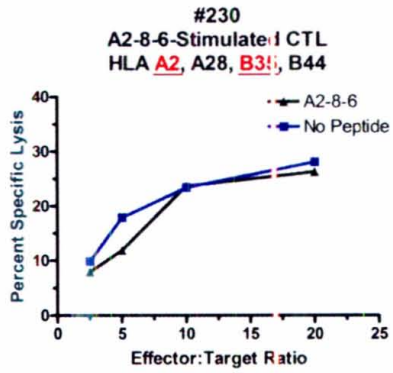

#230
A2-8-6-Stimulated CTL
HLA A2, A28, B35, B44
A2-8-6
No Peptide
Percent Specific Lysis
Effector:Target Ratio

Figure 3.6.2 Comparison of $CD8^+$ T cell cytotoxicity induced by reference and heteroclitic peptides II. Specific cytotoxicity of $CD8^+$ T cells stimulated in vitro with reference or heteroclitic peptides was tested, in duplicate, by ^{51}Cr release assay against reference or heteroclitic peptide-pulsed autologous BLCL target cells at increasing effector to target ratios. Duplicates fell within 20% of each other. The HLA class I allele that presents each peptide is highlighted in red and underlined. Tests in which peptide-pulsed and non-pulsed BLCL were used as targets are indicated by black triangles and blue squares, respectively.

< 12% over background killing regardless of effector to target ratio with the exception of #155 A3-2-stimulated CTL, which showed killing 20% over background at a 20:1 effector to target ratio. In 1/12 cases, heteroclitic peptide-stimulated CTL showed greater specific cytotoxicity than reference-stimulated CTL at all effector to target ratios tested (Figure 3.6.3). Many of these experiments exhibited high background, making it difficult to draw firm conclusions regarding enhanced performance of variant peptides, however, based on these results reference peptides were generally more effective at stimulating peptide-specific CTL than variant peptides.

3.7 Evolution of CTL responses

As shown for cytokine and proliferation responses, CTL responses also varied over time. Reference and variant-stimulated CTL were tested by ^{51}Cr release assay against reference or heteroclitic peptide-pulsed autologous BLCL target cells at 2-3 time points for two individuals (#001 and #187) as shown in Figure 3.7.1. For #001 (Figure 3.7.1 A), IL-2-inducing peptide A2-7-2 stimulated CTL with much stronger peptide-specific cytotoxicity (≥ 30% specific lysis over background at all effector to target ratios) than A2-7-stimulated CTL (no killing over background) on 10/08/09 and on 17/02/10. However, 6 months later on 30/08/10 these responses were reversed with A2-7 stimulating ≥ 20% specific lysis over background and A2-7-2 stimulating no killing over background. For #187 (Figure 3.7.1 B), A2-7-stimulated CTL killed A2-7-pulsed BLCL with an efficiency of ≥ 10% over background on 29/03/10 while A2-7-1-stimulated CTL

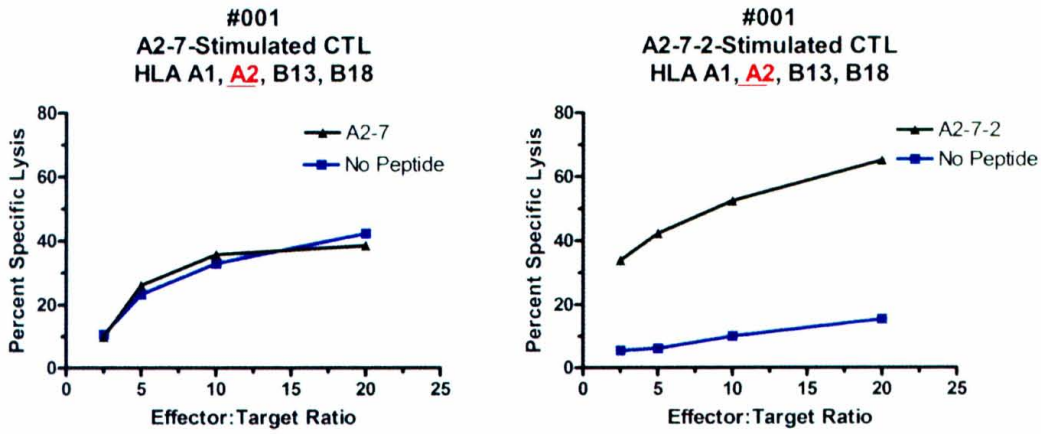
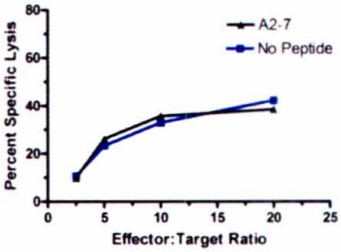
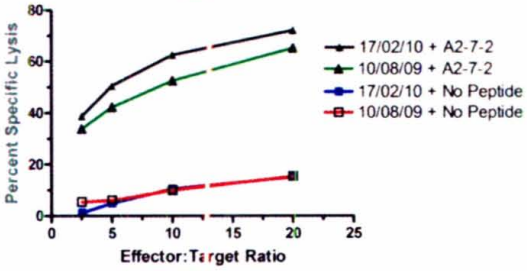
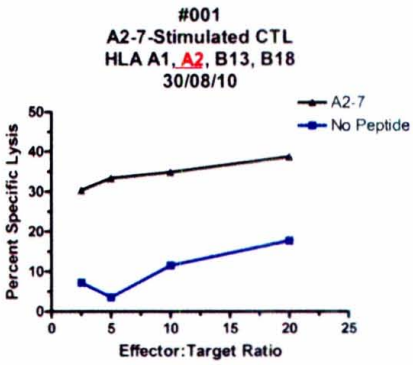


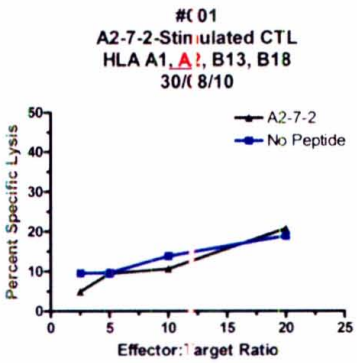
Figure 3.6.3 Comparison of $CD8^+$ T cell cytotoxicity induced by reference and heteroclitic peptides III. Specific cytotoxicity of $CD8^+$ T cells stimulated in vitro with reference or heteroclitic peptides was tested, in duplicate, by ^{51}Cr release assay against reference or heteroclitic peptide-pulsed autologous BLCL target cells at increasing effector to target ratios. Duplicates fell within 20% of each other. The HLA class I allele that presents each peptide is highlighted in red and underlined. Tests in which peptide-pulsed and non-pulsed BLCL were used as targets are indicated by black triangles and blue squares, respectively.

A)

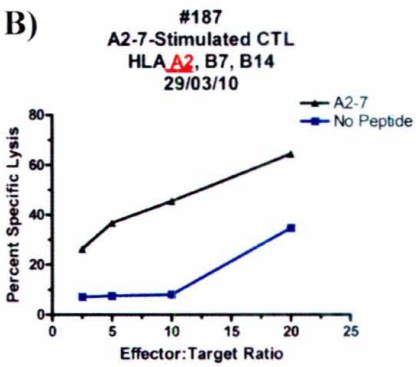

#001
A2-7-Stimulated CTL
HLA A1, A2, B13, B18
17/02/10
A2-7
No Peptide
Percent Specific Lysis
Effector:Target Ratio

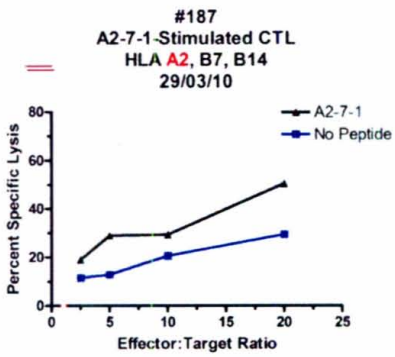

#0(1
A2-7-2-Stimulated CTL
HLA A1, A2 B13, B18
17/02/10 + A2-7-2
10/08/09 + A2-7-2
17/02/10 + No Peptide
10/08/09 + No Peptide
Percent Specific Lysis
Effector:Target Ratio

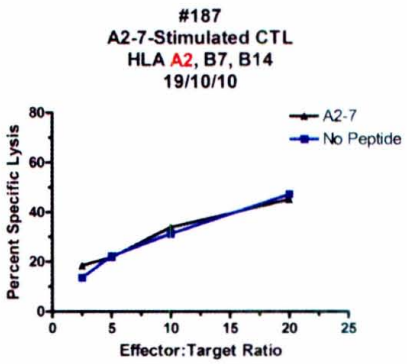

#001
A2-7-Stimulated CTL
HLA A1, A2, B13, B18
30/08/10
A2-7
No Peptide
Percent Specific Lysis
Effector:Target Ratio


#(01
A2-7-2-Stimulated CTL
HLA A1, A2, B13, B18
30/(8/10
A2-7-2
No Peptide
Percent Specific Lysis
Effector:Target Ratio

B)


#187
A2-7-Stimulated CTL
HLA A2, B7, B14
29/03/10
A2-7
No Peptide
Percent Specific Lysis
Effector:Target Ratio


#187
A2-7-1-Stimulated CTL
HLA A2, B7, B14
29/03/10
A2-7-1
No Peptide
Percent Specific Lysis
Effector:Target Ratio


#187
A2-7-Stimulated CTL
HLA A2, B7, B14
19/10/10
A2-7
No Peptide
Percent Specific Lysis
Effector:Target Ratio

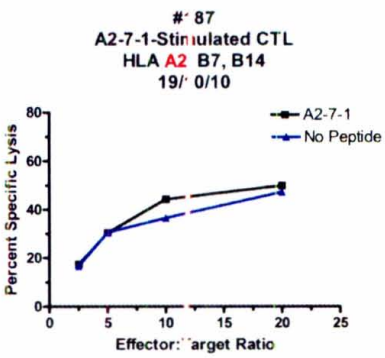

#' 87
A2-7-1-Stimulated CTL
HLA A2 B7, B14
19/' 0/10
A2-7-1
No Peptide
Percent Specific Lysis
Effector:' arget Ratio

Figure 3.7.1 Evolution of peptide-induced CTL responses. Specific cytotoxicity of $CD8^+$ T cells stimulated in vitro with reference or heteroclitic peptides was tested, in duplicate, by ^{51}Cr release assay against reference or heteroclitic peptide-pulsed autologous BLCL target cells at one or more time points. Results are shown for A) #001 and B) #187. Duplicates fell within 20% of each other. The HLA class I allele that presents each peptide is highlighted in red and underlined. Tests in which peptide-pulsed and non-pulsed BLCL were used as targets are indicated by triangles and squares, respectively. The HLA class I allele that presents each peptide set is highlighted in red and underlined. The date of the sample used for each experiment is indicated for each subject (D/M/Y).

killed A2-7-1-pulsed BLCL with an efficiency ≤ 10% over background on the same date. However, on 19/10/10 neither A2-7 nor A2-7-1-stimulated CTL were capable of killing peptide-pulsed BLCL by > 5% over background regardless of effector to target ratio. These results show that $CD8^+$ T cell responses to reference and variant peptides vary over time in both magnitude and general specificity.

3.8 A comparison of TCR/peptide-HLA class I interaction avidity of reference and heteroclitic peptides

The third aim of this project was to measure TCR/peptide-HLA class I interaction avidity of IL-2-inducing heteroclitic HIV peptides relative to index peptides. TCR/peptide-HLA class I avidity was determined by ^{51}Cr release assay in which autologous (or HLA-matched) BLCL were pulsed with diluted concentrations of peptide and then used as targets for reference and heteroclitic peptide-stimulated CTL. The appropriate effector to target ratio, one that allowed the highest killing over background at the lowest ratio, was determined from the previously described cytotoxicity experiments. TCR/peptide-HLA class I avidity was estimated as the concentration of peptide at which the percent specific lysis falls to 50% of the maximum. The avidity of 4 peptides was determined using this method as shown in Figure 3.8.1. As can be seen in Figure 3.8.1 A, A2-7 has ~10 fold higher avidity than A2-7-2. A2-Flu and A2-Flu-1, however, have more comparable avidities at approximately 1 μg/ml each (Figure 3.8.1 B). These results show that heteroclitic peptides are not necessarily higher avidity

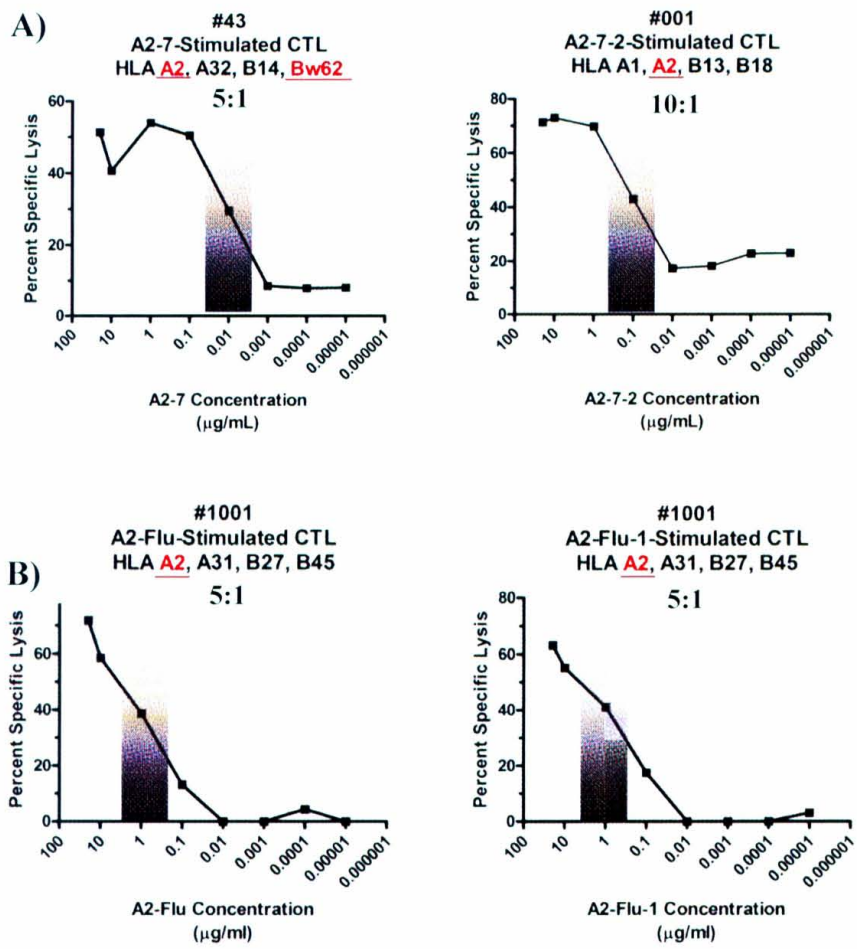
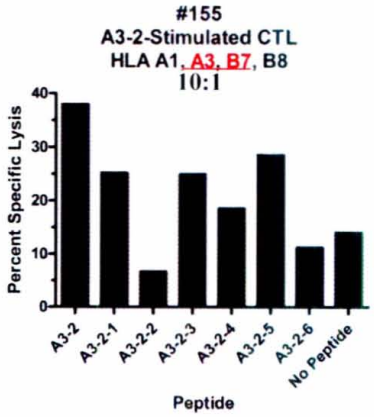


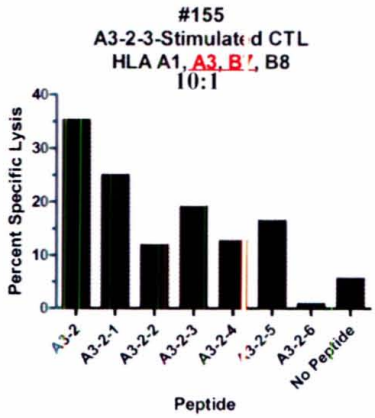
Figure 3.8.1 Comparison of reference and heteroclitic peptide TCR/peptide-HLA class I avidity. CTL were stimulated in vitro with reference or heteroclitic peptides and tested, in duplicate, by ^{51}Cr release assay against diluted concentrations of reference or heteroclitic peptide-pulsed (A) autologous or (B) HLA-matched BLCL target cells. Duplicates fell within 20% of each other. The HLA class I allele that presents each peptide is highlighted in red and underlined. The effector to target ratio used is indicated below each heading. The shaded boxes denote the peptide concentration estimated at 50% of the maximum killing.

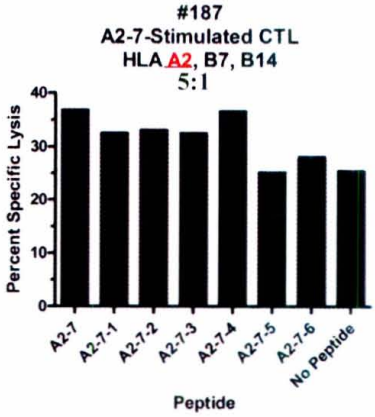
peptides than reference peptides.

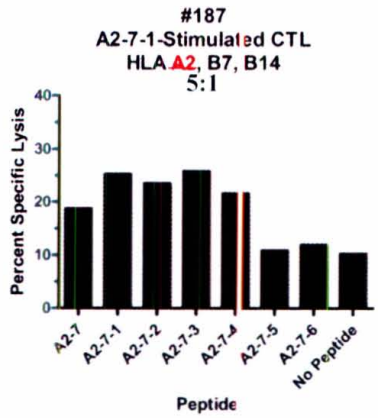
3.9 A comparison of reference and heteroclitic peptide-stimulated-CTL reactivity

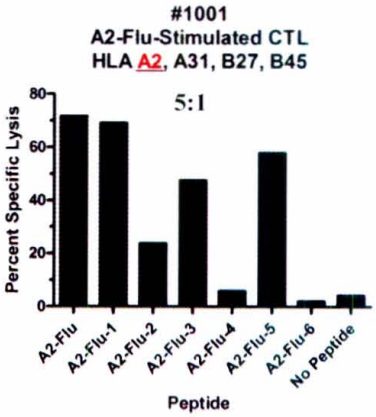
The fourth and final aim of this project was to test the ability of IL-2-inducing heteroclitic HIV peptides to generate broadly reactive CTL. Cross-reactivity was determined by ^{51}Cr release assay in which autologous or HLA-matched BLCL were pulsed with each peptide in the entire peptide set. CTL were added at an appropriate effector to target ratio determined from the previously described cytotoxicity experiments. The ability of reference and heteroclitic peptide-stimulated CTL to kill BLCL pulsed with the entire peptide set was then compared. CTL from 7 individuals, stimulated against the reference and up to 3 other variants within the respective peptide set, were tested in this manner. Three representative examples are shown in Figure 3.9.1 and the remaining are shown in Appendix C. In 6/7 cases, reference and variant peptides showed comparable breadth of reactivity and magnitude of response (< 10% specific lysis in difference). For #1001 (Figure 3.9.1) and #43 (A2-7 set; Appendix C), the reference peptide was marginally better in terms of magnitude of response (< 10% difference from heteroclitic peptide-stimulated CTL). For #155 (Figure 3.9.1), #174 (Appendix C), #187 (Figure 3.9.1) and #43 (A2-Gag set; Appendix C), the heteroclitic peptide was marginally better in terms of magnitude of response (< 10% difference from reference peptide-stimulated CTL). #155 A3-2-3-stimulated CTL were better at killing A3-2-1-pulsed


#155
A3-2-Stimulated CTL
HLA A1, A3, B7, B8
10:1
Percent Specific Lysis
0
10
20
30
40
A3-2
A3-2-1
A3-2-2
A3-2-3
A3-2-4
A3-2-5
A3-2-6
No Peptide
Peptide


#155
A3-2-3-Stimulated CTL
HLA A1, A3, B7, B8
10:1
Percent Specific Lysis
0
10
20
30
40
A3-2
A3-2-1
A3-2-2
A3-2-3
A3-2-4
A3-2-5
A3-2-6
No Peptide
Peptide


#187
A2-7-Stimulated CTL
HLA A2, B7, B14
5:1
Percent Specific Lysis
0
10
20
30
40
A2-7
A2-7-1
A2-7-2
A2-7-3
A2-7-4
A2-7-5
A2-7-6
No Peptide
Peptide


#187
A2-7-1-Stimulated CTL
HLA A2, B7, B14
5:1
Percent Specific Lysis
0
10
20
30
40
A2-7
A2-7-1
A2-7-2
A2-7-3
A2-7-4
A2-7-5
A2-7-6
No Peptide
Peptide


#1001
A2-Flu-Stimulated CTL
HLA A2, A31, B27, B45
5:1
Percent Specific Lysis
0
20
40
60
80
A2-Flu
A2-Flu-1
A2-Flu-2
A2-Flu-3
A2-Flu-4
A2-Flu-5
A2-Flu-6
No Peptide
Peptide

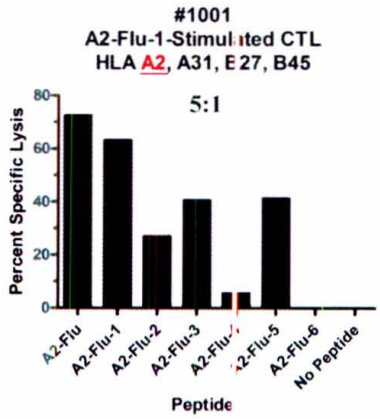

#1001
A2-Flu-1-Stimulated CTL
HLA A2, A31, B27, B45
5:1
Percent Specific Lysis
0
20
40
60
80
A2-Flu
A2-Flu-1
A2-Flu-2
A2-Flu-3
A2-Flu-4
A2-Flu-5
A2-Flu-6
No Peptide
Peptide

Figure 3.9.1 Comparison of reference and heteroclitic peptide-stimulated-CTL reactivity. CTL were stimulated in vitro with reference or heteroclitic peptides and tested, in duplicate, by ^{51}Cr release assay against BLCL pulsed with each peptide within a set. Duplicates fell within 20% of each other. The HLA class I allele that presents each peptide is highlighted in red and underlined. The effector to target ratio used is indicated below each heading.

BLCL than A3-2-stimulated CTL by a 2 fold difference, however, still less than 10% difference overall (20% vs 11% over background, respectively). #001 (Appendix C) was the only individual who showed greater overall killing by heteroclitic peptide-stimulated CTL than reference peptide-stimulated CTL simply because the reference peptide did not stimulate CTL. A2-7-2-stimulated CTL from #001, however, only recognized A2-7-2-pulsed BLCL and A2-7-pulsed B cells. Interestingly, two individuals, #155 (Figure 3.1.9) and #174 (Appendix C), showed stronger killing against A3-2-pulsed BLCL than A3-2-3 and A3-2-4 BLCL, respectively. However, the difference between these two responses was still < 10% specific lysis difference. These results show that reference and heteroclitic peptide-stimulated CTL had similar breadths of reactivity with heteroclitic peptide-stimulated CTL being slightly better in 5/7 cases.

Chapter 4 Discussion

In this study, we identified 9 IL-2-inducing heteroclitic variants of 6 different HLA-A2 and A3-restricted CTL epitopes of HIV and influenza origin. Heteroclitic peptides were created by making conservative and semi-conservative substitutions at amino acid positions 3, 5 and 7 of reference peptides. Consistent with the study by Sette *et al.* [188] we found that heteroclitic peptides are fairly common as heteroclitic variants were identified from 6 of 8 peptide sets tested (Table 2.6.1). Amongst the 9 heteroclitic peptides we identified, no position (3, 5 or 7) or type (conservative or semiconservative) of amino acid substitution was consistently favoured.

4.1 Identification of IL-2-inducing heteroclitic peptides

The first aim of this study was to identify peptides that selectively augmented IL-2 production by HIV-specific $CD8^+$ T cells relative to index peptides. As expected, IL-2 responses were much less frequent than IFN-γ responses (Table 3.1.1). Variant peptides capable of augmenting IL-2 production (by > 25 IL-2 SFU/10^6 PBMC) relative to reference peptides occurred in 50% (9/18) of cases in which an IL-2 response was observed (Figure 3.1.7). Interestingly, variant peptides less frequently augmented IFN-γ responses (by > 100 SFU/10^6 PBMC) relative to reference peptides. This occurred in less than 25% (13/60) of cases in which an IFN-γ response was observed (Figure 3.1.4). In terms of IFN-γ production, the majority of variant peptides produced a lesser response

than that observed with the reference peptide (Figure 3.1.1). This indicates selective enhancement of signaling pathways for the production of IL-2 rather than IFN-γ by heteroclitic peptides. Based on these results, we considered what aspects of a variant peptide might make it better or worse than the reference peptide.

Ultimately, heteroclitic activity is dependent on orientation of peptide side chains that interact with the CDR3 regions of the TCR in the pMHC:TCR complex. Unfortunately, we were not able to model the specific pMHC:TCR interactions and we did not sequence the TCRs of the responding $CD8^+$ T cells, therefore, we can't definitively state why some changes enhanced or abrogated TCR recognition. However, by simply comparing the side chain characteristics of the amino acid substitution in the variants to those of the reference peptides, we can speculate on reasons why certain amino acid enhanced responses and others abrogated responses. For example, comparing peptide A2-7 with its variant A2-7-1, there is a conservative change from valine to leucine at position 3 (Table 2.6.1). Both amino acids possess hydrophobic side chains but the side chain of leucine is one carbon bond longer and less hydrophobic than that of valine based on the Kyte and Doolittle scale of hydrophobicity [205]. It is possible that due to its less hydrophobic nature, the side chain of leucine is oriented more towards the TCR than that of valine, enhancing TCR recognition. Given that A2-7-2 enhanced IL-2 production relative to A2-7 and A2-7-1, and that the change here at position 3 from valine to threonine, is again a change to a less hydrophobic side chain, this effect could be somewhat generalized. Interestingly, changes made at position 7 in A2-7 from histidine to glutamine (A2-7-5) or histidine to arginine (A2-7-6) abrogated the IFN-γ

response observed with A2-7 in all 7 responding individuals. These changes likely abrogated the response because we removed an aromatic ring that may interact in a specific pocket of the TCR. These explanations however, remain speculative. Given that the enhanced responses observed to certain peptides were not universal, we cannot be sure how certain peptides interact with individual TCRs without either sequencing the TCR to properly predict this interaction or purifying the pMHC:TCR complex. The explanations given here may hold true for these particular peptides and responding individuals, but the same changes in other peptides and in other individuals may have opposite effects.

An interesting feature of some of the heteroclitic peptides we identified is their ability to selectively enhance IL-2 production over IFN-γ production by $CD8^+$ T cells. Heteroclitic peptides that enhanced IL-2 production only enhanced IFN-γ production relative to their respective reference peptide in 3/9 cases. Interestingly, however, IL-2 responses were associated with IFN-γ responses > 150 IFN-γ SFU/10^6 PBMC (Figure 3.1.8) in the majority of cases (8/9 heteroclitic peptide cases, 15/18 total cases). In only 1/9 heteroclitic peptide cases (3/18 total cases) the IL-2 response did not correlate with high IFN-γ responses (Figure 3.1.9). The most interesting of these 3 cases occurred in subject #230 in which there was no IFN-γ response to peptide A2-8-6 (observed in 8 other participants as well), but it was the only peptide in the entire set to elicit an IL-2 response in this individual. Unfortunately, we only detected IL-2 at one of three time points tested for this subject (Figure 3.2.1). However, it is worth noting that the overall magnitude of IFN-γ responses was significantly higher at the time point when IL-2 was

detected than at all other time points. This may indicate a decrease in the functional capabilities of the $CD8^+$ T cells that recognize this peptide in subject #230 and explain why IL-2 was only detected at the earliest time point.

Although it is unusual, we are not the first to report such a phenomenon. Using a dual color ELISPOT assay capable of simultaneously detecting IFN-γ and IL-2 secreting cells, Ndongala *et al.* [206] characterized responding $CD8^+$ T cells by their ability to secrete IFN-γ, IL-2 or both in response to the whole HIV proteome. Although the majority of $CD8^+$ T cells produced IFN-γ alone (77.7% acute infection early disease (AIED), 88.9% chronic disease) or IFN-γ and IL-2 (21.1% AIED, 10.8% chronic disease), $CD8^+$ T cells secreting only IL-2 were rarely reported (1.17% AIED, 0.2% chronic disease). For A2-8-6, the change from a tryptophan to arginine at position 7 replaces a large indole functional group with a positively charged side chain of amino groups. It is possible that the TCR that recognizes A2-8-6 has anionic residues at this contact site and that the interaction between this peptide and the TCR was enhanced by the cationic charges and hydrogen bonding properties of the arginine residue. But how changes in peptide structure such as those in our heteroclitic peptides enhance IL-2 production and differentially alter IFN-γ production by $CD8^+$ T cells relative to reference peptides is unknown.

Based on the differences observed between reference and variant peptides in terms of their abilities to stimulate IFN-γ and IL-2 production by $CD8^+$ T cells, we tested whether these peptides stimulated the same or different subsets of $CD8^+$ T cells. Using an additive ELISPOT approach, we determined that the same subset of $CD8^+$ T cells

responded to the reference and variant peptides in peptide sets A2-7, A2-8 and A2-Gag (Figure 3.3.1). Given that we did not alter the HLA-binding properties of the heteroclitic peptides relative to the reference peptides, these results are not surprising. This suggests cross-reactivity of responding $CD8^+$ T cells rather than differential recognition of reference and variant peptides. The ability of variant peptides to stimulate the same subset of $CD8^+$ T cells has important implications in this study. This indicates that differences in IFN-γ and IL-2 induction by reference and heteroclitic peptides are due to differences in intracellular signalling within the responding $CD8^+$ T cells. Salazar *et al.* [207] reported increased tyrosine phosphorylation of TCR signaling proteins ZAP-70 and TCR ζ chains in heteroclitic peptide-stimulated CTL compared to reference peptide-stimulated CTL. But beyond this, little is known about the structural and signaling mechanisms involved in heteroclitic activity.

4.2 Comparison of reference and IL-2-inducing heteroclitic peptides driven proliferation and differentiation

IL-2 secretion by $CD8^+$ T cells is important for proliferation and differentiation into effector $CD8^+$ T cells in the absence of $CD4^+$ T cell help [208]. In this study, we expected that peptides that enhanced IL-2 production by $CD8^+$ T cells would also enhance proliferation and differentiation of these responding cells. Using CFSE dilution and flow cytometry, we compared 14 reference and variant peptide pairs for their ability to stimulate proliferation of $CD8^+$ T cells. Of these 14 peptide pairs, 6 included IL-2-

inducing heteroclitic peptides and 3 included variant peptides that enhanced IFN-γ production relative to their respective reference peptides. Unexpectedly, of the 6 heteroclitic peptides tested, 2 peptides did not stimulate any proliferation, 1 peptide stimulated a similar amount of proliferation as the reference peptide, 1 peptide stimulated less proliferation than the reference, and only 2 peptides enhanced proliferation of $CD8^+$ T cells relative to reference peptides. Interestingly, two of the variant peptides that enhanced IFN-γ production (A2-7-1 in #231 and A2-Gag-3 in #105) also enhanced proliferation of $CD8^+$ T cells relative to reference peptides (Figure 3.4.3). Although we expected all of the IL-2 inducing heteroclitic peptides to enhance proliferation of $CD8^+$ T cells, a complicating factor is that there were often large gaps of time (up to 1 year +) between initial ELISPOT experiments and proliferation assays due to a lack of available PBMC samples for a given subject. Therefore, it is possible that the $CD8^+$ T cells recognizing the heteroclitic peptides became exhausted or lost their ability to produce IL-2 during this time. It is also possible we observed a similar phenomenon as Heath *et al.* [209] who reported that while IL-2 production from the total HIV-specific $CD8^+$ T cell population does correlate with proliferation, routine detection of both IL-2 production and proliferation from the same antigen-specific $CD8^+$ T cells is rare. Their study suggested that distinct populations of $CD8^+$ T cells exist – those capable of proliferation, those with the ability to produce IL-2 and few with dual capacity. We only tested for IL-2 production and proliferation by flow cytometry for 2/14 peptide pairs and in both cases, less than 1% proliferation was observed (data not shown), therefore, we cannot make a fair comparison to the results of Heath *et al.* [209]. However, it is possible that our

heteroclitic peptides still induced IL-2, but the remaining proliferative subpopulation was functionally exhausted and unable to proliferate.

Differentiation and activation of $CD8^+$ T cells induced by reference and heteroclitic peptides was also measured by flow cytometry and compared between peptides as well as between $CFSE^{low}$ and $CFSE^{high}$ populations (Figures 3.5.1 and 3.5.2). $CD8^+$ T cell populations are characterized by the expression of particular cell surface markers such as CCR7, CD45RA and CD28 [197, 210]. In this study, we proposed that heteroclitic peptides selectively stimulating IL-2 producing $CD8^+$ T cells may either be selectively recognized by, or selectively drive the generation of $CD8^+$ T_{CM}. The T_{CM} lineage is characterized by the $CD8^+CD45RA^-CCR7^+CD28^+$ phenotype whereas the T_{EM} lineage is characterized by the $CD8^+CD45RA^+CCR7^-CD28^-$ phenotype [197, 210]. Naive $CD8^+$ T cells are characterized by a $CD8^+CCR7^+CD45RA^+$ phenotype [210]. By flow cytometry, we assessed expression of CD8, CFSE, and appropriate fluorochrome combinations of CD45RA, CCR7, perforin, PD-1, IL-2 and/or IFN-γ. No CCR7 was detected for $CFSE^{low}$ or $CFSE^{high}$ $CD8^+$ T cell populations when stimulated with either reference or heteroclitic peptides. For both reference and heteroclitic peptide-stimulated cultures, ~50% of $CFSE^{high}$ $CD8^+$ T cells expressed CD45RA whereas only ~10% of $CFSE^{low}$ $CD8^+$ T cells expressed this marker (Figure 3.5.1 A). Given that the proliferating $CFSE^{low}$ cells express a $CD8^+CD45RA^{low}CCR7^-$ phenotype and have a higher proportion expressing the effector molecules perforin and IFN-γ (Figure 3.5.2), our peptides (both reference and variant peptides) appear to mainly stimulate T_{EM} populations. This is

consistent with other studies that found a typical phenotype of IFN-γ-producing, proliferating effector cells to be $CD45RA^{-/low}CCR7^{-}$ [155, 208, 211].

Interestingly there was a significant difference in the expression of PD-1 (one tailed paired *t* test, $p = 0.0262$) between the $CFSE^{low}$ reference and heteroclitic-stimulated populations (Figure 3.5.1 B). This phenomenon was observed for peptides A2-7-1, A2-7-2, A2-Gag-3, A2-Gag-5, A2-Flu-1, A2-Flu-3 and A2-Flu-5, with peptides A2-7-1 and A2-Gag-3 each stimulating this effect in two individuals while the remainder stimulated this effect in one individual. PD-1 has been highlighted as a major factor in T cell exhaustion in HIV infection [212-214]. When T cells become exhausted, they can no longer proliferate or secrete cytokines and they lose their cytolytic capability, ultimately leading to ineffective immune responses and an inability to clear virus. Studies in both humans and mice [212, 213, 215] have shown that blocking the PD-1/PD-L1 pathway restores the capacity of exhausted $CD8^{+}$ T cells to undergo proliferation, cytokine production and cytotoxic activity, restoring their protective function and reducing viral load. Our $CFSE^{low}$ heteroclitic peptide-stimulated $CD8^{+}$ T cells showed reduced expression of PD-1 (20%) relative to $CFSE^{low}$ reference peptide-stimulated $CD8^{+}$ T cells (35%) supporting a possible therapeutic role for these peptides in reducing PD-1 expression on stimulated cells and decreasing T cell exhaustion. Other factors besides PD-1 may also contribute to the exhausted phenotype of HIV-specific $CD8^{+}$ T cells. Inhibitory receptors such as 2B4, LAG-3, CD160 and TIM-3 are also upregulated on virus-specific $CD8^{+}$ T cells (reviewed in [216]) and, as one might expect, the more such receptors these cells accumulate, the more exhausted they become. Unfortunately, we did

not compare expression of other markers of exhaustion, but based on the observed downregulation of PD-1 and enhanced ability to produce IL-2 by responding $CD8^+$ T cells, it's possible that heteroclitic peptides also downregulate other inhibitory receptors. This is a novel and potentially significant finding that bears further investigation. Methods to selectively recruit T cells with lesser evidence of functional or phenotypic exhaustion using therapeutic vaccines may be relevant for a number of chronic infections.

4.3 Comparison of reference and heteroclitic peptide:HLA class I:TCR avidity

Aims three and four of this project were to compare reference and IL-2-inducing heteroclitic peptides in terms of pHLA class I:TCR interaction avidity and their ability to generate broadly reactive CTL. In the majority of cases (7/12 total cases tested), reference and variant peptides stimulated similar amounts of cytotoxic activity (Figure 3.6.2), whereas IL-2-inducing heteroclitic peptides stimulated higher cytotoxic activity than reference peptides in only 1 case (Figure 3.6.3). In studies employing heteroclitic cancer peptides, these peptides broke T cell tolerance and dramatically augmented cell-mediated immunity against tumor-associated antigens [188-190]. Enhanced anti-tumor immunity driven by these peptides was attributed to increased stability of the pMHC:TCR complex, which resulted in increased avidity and residence time of the complex [192]. Our heteroclitic peptides, however, did not stimulate stronger cytotoxic responses than reference peptides and were not of higher avidity than reference peptides (Figure 3.8.1). Sette *et al.* [188] reported heteroclitic peptides that produced up to 10^7-

fold increase in T cell sensitivity. Our results do not reflect their observed dramatic increases in avidity. This may be due to differences in methods for measuring avidity – we used a Cr^{51} release assay whereas they measured the level of competition induced by the test peptide for binding of a radiolabeled standard peptide to purified HLA-A2 molecules. Takahashi *et al.* [217] reported a counter-regulation of cytolytic activity and cytokine production in HIV-specific murine $CD8^+$ CTL by free antigenic peptide. They showed that treatment of CTL with their epitopic peptide resulted in a significant reduction of granzyme B activity and mRNA expression of granzyme B and perforin. However, when the level of cytolytic activity was the lowest, they detected maximum amounts of cytokines IL-2, IFN-γ, tumor necrosis factor (TNF)-α, and macrophage inflammatory protein (MIP)-1β in the culture supernatant. Therefore, while our peptides enhance cytokine production, they may have an inhibitory effect on cytolytic activity. Thus ^{51}Cr-release assays may underestimate avidity in some cases.

It is also possible that heteroclitic activity is not as dependent on avidity as others have suggested. One key difference between our study and previous heteroclitic cancer peptide studies [189, 190, 192] that may also explain why avidity does not seem to play as much of a role in our study is the difference in the immunogenicity of the reference peptides. In heteroclitic cancer peptide studies, the reference peptides were poorly immunogenic to begin with as they were often altered forms of self-peptides. The self-reactive CTL that would recognize such peptides are typically deleted during T cell selection resulting in the persistence of only low avidity T cells. However, in our study, reference peptides were initially identified because they were known optimal CTL

epitopes and thus, more immunogenic than the reference peptides of heteroclitic cancer peptide studies. Perhaps our heteroclitic peptides do enhance T cell sensitivity, but at a level undetectable by our methods or simply stabilize and enhance the residence time of the pHLA:TCR interaction in some other way to allow activation of alternate signalling pathways within the T cell. The fact that our study population was suppressed on HAART could have also limited our ability to detect CTL responses. Although our heteroclitic peptides do not stimulate higher-avidity CTL, they may stimulate production of various other cytokines, for which we did not screen, that activate various other cell populations. It is also important to note that CTL responses varied dramatically over time (Figure 3.7.1) as did cytokine (Figure 3.2.1) and proliferation responses (not shown). Had all experiments been done at the same time points, the observed results may have been different.

4.4 Heteroclitic peptides stimulate broadly reactive CTL

Zirlik *et al.* [190] argue that in order for a peptide to be heteroclitic it must not only hyperstimulate T cells and generate high avidity interactions, but must also be able to prime T cells to undergo expansion and further activation driven by the lower avidity, reference peptide. The heteroclitic peptides identified in this study had similar avidities to the reference peptides but interestingly, heteroclitic-peptide stimulated CTL had slightly better breadths of reactivity in 5/7 cases tested (Figure 3.1.9). In two individuals, #155 (Figure 3.1.9) and #174 (Appendix C), heteroclitic peptide-stimulated CTL showed

stronger killing against reference-pulsed BLCL than heteroclitic-pulsed BLCL. As both HIV and Flu exist as many viral quasispecies within a single individual [62, 218], the ability of heteroclitic peptides to generate broadly reactive CTL is a desired feature for therapeutic vaccination. The fact that we identified several peptides that were broadly reactive in this sense is quite promising for this approach as such peptides are capable of activating CTL to react against multiple variations of that peptide within a given host.

4.5 Heteroclitic HIV peptides and their potential in therapeutic HIV vaccines

Consistent with the study by Sette *et al.* [188], we found that variant peptides with heteroclitic activity were fairly common. These results are quite encouraging for the discovery of additional heteroclitic HIV peptides, especially considering that only six variants were made from each reference peptide by substituting only one amino acid at each position. Given the vast array of standard and non-standard amino acids that exist, many more variants could be generated and tested for heteroclitic properties. In addition, heteroclitic peptides could be generated by making substitutions at multiple positions within a peptide, given that the individual substitutions result in enhanced TCR recognition. It is also important to note that the heteroclitic peptides generated in this study were variants of an already immunodominant peptide. We would be more likely to observe differences in pHLA:TCR avidity and differences in protective potential between variants and the reference peptide if the reference peptide was less immunogenic to begin with, as it is with heteroclitic peptide cancer vaccines. Obviously, generating potentially

heteroclitic peptides using peptides that are not normally well presented to the immune system could be a tedious, laborious and costly process. However, this approach could lead to CTL recognition of new epitopes and could stimulate new subsets of T cells that otherwise would not be activated. This approach may be further improved by focusing on conserved antigens, such as those in Gag. Chen *et al.* [145] found that HIV controllers were better able to inhibit viral replication through targeting of the immunodominant epitope of Gag. Future heteroclitic studies should emphasize Gag as HIV control is associated with suppression of Gag. However, such studies should focus on driving immune responses towards less immunodominant regions of Gag.

While the results from our study are promising, they do indicate a challenge for finding a universal therapeutic HIV vaccine. It is likely that therapeutic HIV vaccination using heteroclitic HIV peptides would have to be an individualized therapy as much variation in response exists. Although individualized therapy seems impractical, the type of research required for peptide screening is common in HIV immunology labs and is no more laborious than procedures carried out to optimize ART. Given that the vaccine could induce fewer short- and long-term side effects, provide longer lasting effects following a single administration and reduce dependence on ART, it would be cost effective and highly advantageous for HIV-infected individuals. There is also potential for overlap of peptides for subgroups of individuals given that some heteroclitic peptides (e.g. A2-7-1, A2-Gag-3) did stimulate enhanced responses in more than one individual. Timing of experiments was also an important factor in this study, so in order for this

approach to be used on a practical level, there would have to minimal time between identifying the appropriate peptides for a given individual and administering the vaccine.

Before an effective therapeutic HIV vaccine can be designed using this approach, we need to determine which CTL functions correlate with protection and what magnitude of response in assays correlates to an effective CTL response in vivo. Interestingly, our IL-2-inducing heteroclitic peptides did not stimulate more cytolytic activity than reference peptides, a feature we would expect to be required for viral control. They did, however, stimulate IL-2 production, generate broadly reactive CTL, enhance proliferation in some cases and cause a reduction in PD-1 expression. These peptides drive differential signaling of the same subset of $CD8^+$ T cells and may reverse the exhausted phenotype of these cells. Such features illustrate the potential of heteroclitic peptides to produce dramatic effects and improve therapeutic vaccination. Akinsiku *et al.* [141] showed that IL-2 production by polyfunctional HIV-specific $CD8^+$ T cells is associated with enhanced suppressive capacity of viral replication. Perhaps had we used a similar approach or tested for cytokine production and cytolytic function at the same time point we would have observed similar results. However, IL-2 production by $CD8^+$ T cells often indicates polyfunctionality and since we only screened for production of IL-2 and IFN-γ, it is likely that the responding CTL produce other chemokines and cytokines that stimulate other cell populations. The ability of heteroclitic peptides to stimulate more broadly reactive CTL and to reduce the exhausted phenotype is ideal for use in therapeutic HIV vaccines as this would activate T cells against multiple HIV variants and protect the T cells from exhaustion.

This approach would not be practical for preventative vaccine strategies since hyperstimulating T cells with the potential to be cross-reactive may result in some self-reactivity, especially in a case when the vaccinated individual will never be exposed to HIV. Also, if the individual has never been exposed to HIV, we would not have an indication of which epitopes to base our heteroclitic peptides on for that particular individual. As our study showed, no single heteroclitic variant consistently demonstrated enhanced responses across all patients studied. However, this approach may prove useful in HIV eradication research. Shan *et al.* [219] showed that enhancing CTL responses in chronic HIV infection prior to reactivating latent HIV particles, led to efficient killing of HIV-infected cells. Using heteroclitic peptides to enhance specific CTL responses, combined with efforts to reactivate latent proviruses, may prove useful in eradication efforts.

4.6 Study limitations and suggestions for improvement

This study had several limitations. The most prominent of these was the limited availability of PBMC from HIV-infected participants due to the volumes of blood provided for research purposes. A minimum of 6 million PBMC were needed for screening against a single peptide set to detect IFN-γ and IL-2 production by $CD8^+$ T cells by ELISPOT assay. Cryopreservation of PBMC often resulted in 30-70% cell loss, limiting the number of cells available for these experiments. Because of such cell loss, we often could only test for IL-2 production in single test as 400,000 cells were needed per

well for IL-2 detection. When possible, we used fresh PBMC; however this was not always feasible for several reasons. Some subjects did not donate samples regularly thus waiting for fresh samples would have prolonged the study. Also, since we did not know when we would be receiving specific samples, we would have to coat the ELISPOT plates when the sample arrived meaning the fresh PBMC would have to be stored in the fridge or incubator overnight resulting in some cell loss (~10%). In addition to this, using fresh PBMC often resulted in higher background. One way that we could have improved this initial screening process to use the number of cells we had more effectively would have been to use a dual-color ELISPOT assay. Boulet *et al.* [220] designed and validated the high throughput dual-color ELISPOT assay in 2007. This assay is capable of detecting both IL-2 and IFN-γ secreting cells simultaneously, thus reducing the cell input number. Had we used this assay, we would have been able to screen for both cytokines in duplicate and gain valuable information about the percentage of $CD8^+$ T cells that produced IFN-γ only, IL-2 only or both.

Although the dual-color ELISPOT assay would allow more efficient screening, it would not reduce the number of cells required by much, but would instead allow us to gather more information with the cells we have. Therefore, we would still face the issue of limited cell availability, thus precluding the ability to perform multiple assays. Although this solution would be beneficial given our current facility and equipment, an alternative method relying on multifactor flow cytometry (10^+ colors) would allow simultaneous detection and enumeration of peptide-specific $CD8^+$ T cells and provide additional insight into their specific effector functions. Using this approach, information

on cytokine production (IFN-γ, IL-2, TNF-α), extracellular markers (CD8, CD45RA, CCR7, PD-1), proliferation (CFSE), and cytotoxicity (perforin, granzyme B, CD107a) could be gathered at one time point, using ~$1x10^6$ PBMC per peptide. This approach would also eliminate the disadvantages associated with ^{51}Cr-release assay such as biohazard and disposal problems for the isotope. This comprehensive approach would allow us to better determine the immunological potency associated with each peptide in the context of individual patients and would provide further insight into its use therapeutic vaccines.

Other limitations of this study include caveats associated with the study findings themselves and challenges in moving the heteroclitic approach to therapeutic vaccination forward. In this study, the ELISPOT results are often summarized in terms of the percent of cases where variant peptides induced a "similar or higher" response than the reference peptide. However, given the definition of "heteroclitic", this should be summarized as percent of cases where a variant induced a better response only. In addition to this, IL-2 responses were only detected in a minority of patients and the enhancement of responses elicited by many heteroclitic peptides was relatively modest compared to the reference peptides. These responses also varied drastically over time. The temporal inconsistency of IL-2 responses could be due to sampling bias due to rarity of IL-2 producing cells in vivo, escape in autologous viral sequences and/or HAART-induced suppression of viral load at follow-up timepoints.

As previously mentioned, no single heteroclitic epitope variant consistently demonstrated enhanced responses across all patients studied. This represents a huge

problem to this approach, as universality would be difficult to achieve. Also, the burden of immune escape will be high in individuals with chronic infection. In many cases, the autologous virus may have already escaped by developing mutations that compromise binding to HLA. Thus, stimulating immune responses to epitopes presented by particular HLA alleles will require knowledge of the autologous virus sequence to predict which HLA molecules should be targeted for therapy. Furthermore, in this study, we generated HIV epitope variants by making substitutions at positions 3, 5, and 7 but not 1, 4, 6, or 8. This approach may not be generalizable to all HLA class I alleles nor to all epitopes restricted by a given allele. Finally, there are also potential risks involved using heteroclitic peptide vaccine strategies. One such risk is generating a sequence that stimulates a cross-reactive response to tissue epitopes causing reactivity to self. Although, many self peptides could be tested in vitro to confirm this, it would be difficult to test for them all.

4.7 Conclusion

In conclusion, we identified 9 IL-2-inducing heteroclitic peptides and several non-IL-2-inducing variant peptides with other heteroclitic properties. Although IL-2 responses were rare, heteroclitic peptides enhanced IL-2 production in 50% of cases in which an IL-2 response was observed. The majority of variant peptides produced a lesser IFN-γ response than that observed with the reference peptide, but variant peptides did enhance IFN-γ production in 25% of cases. The high frequency of heteroclitic peptides capable of

enhancing cytokine responses by $CD8^+$ T cells is very promising for future studies as a multitude of possible heteroclitic peptide sequences exist for each of the reference peptides used. Although we have not elucidated the mechanisms by which these peptide act, our results indicate that these peptides stimulate the same subsets of $CD8^+$ T cells as reference peptides, but somehow exert selective signaling effects. They also stimulate broader reactivity of peptide-stimulated $CD8^+$ T cells, reduce the exhausted phenotype of responding $CD8^+$ T cells, and, in some cases, enhance proliferation of responding T cells. These features are all desirable for designing effective therapeutic HIV vaccines. The structural and signaling mechanisms involved in heteroclitic activity remain to be elucidated. Further insight into these mechanisms would provide information on heteroclitic peptide design for HIV $CD8^+$ T cell epitopes and contribute to a basic understanding of the relationship between TCR/peptide interactions and T cell function.

References

1. WHO. *Global Health Observatory: HIV/AIDS*. 2009 [cited 2011 September 9]; Available from: http://www.who.int/gho/hiv/en/index.html.
2. Douek, D.C., M. Roederer, and R.A. Koup, *Emerging concepts in the immunopathogenesis of AIDS.* Annu Rev Med, 2009. **60**: p. 471-84.
3. Kanki, P.J., J.R. Hopper, and M. Essex, *The origins of HIV-1 and HTLV-4/HIV-2.* Ann N Y Acad Sci, 1987. **511**: p. 370-5.
4. Coffin, J.M., S.H. Hughes, and H.E. Varmus, *The Interactions of Retroviruses and their Hosts.* 1997.
5. Zhu, P., et al., *Distribution and three-dimensional structure of AIDS virus envelope spikes.* Nature, 2006. **441**(7095): p. 847-52.
6. Clapham, P.R. and A. McKnight, *Cell surface receptors, virus entry and tropism of primate lentiviruses.* J Gen Virol, 2002. **83**(Pt 8): p. 1809-29.
7. Rizzuto, C.D., et al., *A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding.* Science, 1998. **280**(5371): p. 1949-53.
8. Finzi, A., et al., *Topological layers in the HIV-1 gp120 inner domain regulate gp41 interaction and CD4-triggered conformational transitions.* Mol Cell. **37**(5): p. 656-67.
9. Murphy, K., Travers, P. and M. Walport, *Janeway's Immunobiology*. 7th ed. 2008, New York: Garland Science.
10. Costin, J.M., *Cytopathic mechanisms of HIV-1.* Virol J, 2007. **4**: p. 100.
11. NIAID. *Structure of HIV*. 2012 [cited; Available from: http://www.niaid.nih.gov/topics/HIVAIDS/Understanding/Biology/pages/structure.aspx.
12. Dalgleish, A.G., et al., *The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus.* Nature, 1984. **312**(5996): p. 763-7.
13. Lyerly, H.K., et al., *Human T-cell lymphotropic virus IIIB glycoprotein (gp120) bound to CD4 determinants on normal lymphocytes and expressed by infected cells serves as target for immune attack.* Proc Natl Acad Sci U S A, 1987. **84**(13): p. 4601-5.
14. Deng, H., et al., *Identification of a major co-receptor for primary isolates of HIV-1.* Nature, 1996. **381**(6584): p. 661-6.
15. Dragic, T., et al., *HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5.* Nature, 1996. **381**(6584): p. 667-73.
16. Doranz, B.J., et al., *A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors.* Cell, 1996. **85**(7): p. 1149-58.
17. Philpott, S., et al., *CCR5 genotype and resistance to vertical transmission of HIV-1.* J Acquir Immune Defic Syndr, 1999. **21**(3): p. 189-93.
18. Wilkinson, D.A., et al., *A 32-bp deletion within the CCR5 locus protects against transmission of parenterally acquired human immunodeficiency virus but does not affect progression to AIDS-defining illness.* J Infect Dis, 1998. **178**(4): p. 1163-6.
19. Liu, R., et al., *Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection.* Cell, 1996. **86**(3): p. 367-77.
20. Casper, C., et al., *Coreceptor change appears after immune deficiency is established in children infected with different HIV-1 subtypes.* AIDS Res Hum Retroviruses, 2002. **18**(5): p. 343-52.

21. Clevestig, P., et al., *The X4 phenotype of HIV type 1 evolves from R5 in two children of mothers, carrying X4, and is not linked to transmission.* AIDS Res Hum Retroviruses, 2005. **21**(5): p. 371-8.
22. Arthos, J., et al., *HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells.* Nat Immunol, 2008. **9**(3): p. 301-9.
23. Ganser-Pornillos, B.K., M. Yeager, and O. Pornillos, *Assembly and architecture of HIV.* Adv Exp Med Biol, 2012. **726**: p. 441-65.
24. Yan, N. and J. Lieberman, *Gaining a foothold: how HIV avoids innate immune recognition.* Curr Opin Immunol, 2011. **23**(1): p. 21-8.
25. Sundquist, W.I. and H.G. Krausslich, *HIV-1 Assembly, Budding, and Maturation.* Cold Spring Harb Perspect Med, 2012. **2**(7): p. a006924.
26. Ashkenazi, A. and Y. Shai, *Insights into the mechanism of HIV-1 envelope induced membrane fusion as revealed by its inhibitory peptides.* Eur Biophys J, 2011. **40**(4): p. 349-57.
27. Chan, D.C. and P.S. Kim, *HIV entry and its inhibition.* Cell, 1998. **93**(5): p. 681-4.
28. Starnes, M.C. and Y.C. Cheng, *Human immunodeficiency virus reverse transcriptase-associated RNase H activity.* J Biol Chem, 1989. **264**(12): p. 7073-7.
29. Shaharabany, M. and A. Hizi, *The DNA-dependent and RNA-dependent DNA polymerase activities of the reverse transcriptases of human immunodeficiency viruses types 1 and 2.* AIDS Res Hum Retroviruses, 1991. **7**(11): p. 883-8.
30. Zheng, Y.H., N. Lovsin, and B.M. Peterlin, *Newly identified host factors modulate HIV replication.* Immunol Lett, 2005. **97**(2): p. 225-34.
31. Hiscott, J., H. Kwon, and P. Genin, *Hostile takeovers: viral appropriation of the NF-kappaB pathway.* J Clin Invest, 2001. **107**(2): p. 143-51.
32. Bassuk, A.G., R.T. Anandappa, and J.M. Leiden, *Physical interactions between Ets and NF-kappaB/NFAT proteins play an important role in their cooperative activation of the human immunodeficiency virus enhancer in T cells.* J Virol, 1997. **71**(5): p. 3563-73.
33. Friedrich, B.M., et al., *Host factors mediating HIV-1 replication.* Virus Res, 2011.
34. Stein, B.S. and E.G. Engleman, *Intracellular processing of the gp160 HIV-1 envelope precursor. Endoproteolytic cleavage occurs in a cis or medial compartment of the Golgi complex.* J Biol Chem, 1990. **265**(5): p. 2640-9.
35. Ganser-Pornillos, B.K., M. Yeager, and W.I. Sundquist, *The structural biology of HIV assembly.* Curr Opin Struct Biol, 2008. **18**(2): p. 203-17.
36. O'Brien, M. and M. Markowitz, *Should we treat acute HIV infection?* Curr HIV/AIDS Rep, 2012. **9**(2): p. 101-10.
37. *Understanding Your HIV Test Results*. HIV Info Source 2009 2009 [cited; Available from: http://www.hivinfosource.org/hivis/hivbasics/results/index.html.
38. Langford, S.E., J. Ananworanich, and D.A. Cooper, *Predictors of disease progression in HIV infection: a review.* AIDS Res Ther, 2007. **4**: p. 11.
39. *A Guide to HIV/AIDS Epidemiological and Surveillance Terms* 2006 [cited 2011 October 10]; Available from: http://www.phac-aspc.gc.ca/publicat/haest-tesvs/a-eng.php#AIDS_CASE.
40. Ho, D.D., et al., *Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection.* Nature, 1995. **373**(6510): p. 123-6.

41. Wei, X., et al., *Viral dynamics in human immunodeficiency virus type 1 infection.* Nature, 1995. **373**(6510): p. 117-22.
42. Silvestri, G., et al., *Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia.* Immunity, 2003. **18**(3): p. 441-52.
43. Schindler, M., et al., *Nef-mediated suppression of T cell activation was lost in a lentiviral lineage that gave rise to HIV-1.* Cell, 2006. **125**(6): p. 1055-67.
44. Migueles, S.A., et al., *Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control.* Immunity, 2008. **29**(6): p. 1009-21.
45. Nixon, D.F., et al., *HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides.* Nature, 1988. **336**(6198): p. 484-7.
46. Sacha, J.B., et al., *Gag-specific CD8+ T lymphocytes recognize infected cells before AIDS-virus integration and viral protein expression.* J Immunol, 2007. **178**(5): p. 2746-54.
47. Davenport, M.P., et al., *Understanding the mechanisms and limitations of immune control of HIV.* Immunol Rev, 2007. **216**: p. 164-75.
48. Galati, D. and M. Bocchino, *New insights on the perturbations of T cell cycle during HIV infection.* Curr Med Chem, 2007. **14**(18): p. 1920-4.
49. Wang, L., et al., *A novel mechanism of CD4 lymphocyte depletion involves effects of HIV on resting lymphocytes: induction of lymph node homing and apoptosis upon secondary signaling through homing receptors.* J Immunol, 1999. **162**(1): p. 268-76.
50. *HIV/AIDS*. 2011 [cited 2011 November 21]; Available from: http://www.phac-aspc.gc.ca/tmp-pmv/info/hivaids-eng.php.
51. Fischl, M.A., et al., *The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial.* N Engl J Med, 1987. **317**(4): p. 185-91.
52. Johnson, V.A., *Combination therapy for HIV-1 infection-overview: preclinical and clinical analysis of antiretroviral combinations.* Antiviral Res, 1996. **29**(1): p. 35-9.
53. Palella, F.J., Jr., et al., *Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators.* N Engl J Med, 1998. **338**(13): p. 853-60.
54. *AIDS Info*. 2011 [cited 2011 December 20]; Available from: http://www.aidsinfo.nih.gov.qe2a-proxy.mun.ca/contentfiles/HIVandItsTreatment_cbrochure_en.pdf.
55. Prevelige, P.E., Jr., *New approaches for antiviral targeting of HIV assembly.* J Mol Biol, 2011. **410**(4): p. 634-40.
56. Heath, K.V., et al., *Intentional nonadherence due to adverse symptoms associated with antiretroviral therapy.* J Acquir Immune Defic Syndr, 2002. **31**(2): p. 211-7.
57. Kleeberger, C.A., et al., *Determinants of heterogeneous adherence to HIV-antiretroviral therapies in the Multicenter AIDS Cohort Study.* J Acquir Immune Defic Syndr, 2001. **26**(1): p. 82-92.
58. Gross, R., *Predicting and monitoring antiretroviral adherence.* LDI Issue Brief, 2007. **13**(2): p. 1-4.
59. Das, M., et al., *Decreases in community viral load are accompanied by reductions in new HIV infections in San Francisco.* PLoS One, 2010. **5**(6): p. e11068.

60. Cohen, M.S., et al., *Prevention of HIV-1 infection with early antiretroviral therapy.* N Engl J Med, 2011. **365**(6): p. 493-505.
61. Delva, W., et al., *HIV treatment as prevention: optimising the impact of expanded HIV treatment programmes.* PLoS Med, 2012. **9**(7): p. e1001258.
62. Roberts, J.D., K. Bebenek, and T.A. Kunkel, *The accuracy of reverse transcriptase from HIV-1.* Science, 1988. **242**(4882): p. 1171-3.
63. Schuurman, R., et al., *Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC).* J Infect Dis, 1995. **171**(6): p. 1411-9.
64. Mayers, D.L., *Prevalence and incidence of resistance to zidovudine and other antiretroviral drugs.* Am J Med, 1997. **102**(5B): p. 70-5.
65. Race, E., et al., *Analysis of HIV cross-resistance to protease inhibitors using a rapid single-cycle recombinant virus assay for patients failing on combination therapies.* AIDS, 1999. **13**(15): p. 2061-8.
66. Richman, D.D., *Susceptibility to nucleoside analogues of zidovudine-resistant isolates of human immunodeficiency virus.* Am J Med, 1990. **88**(5B): p. 8S-10S.
67. Reinherz, E.L., et al., *Clonotypic surface structure on human T lymphocytes: functional and biochemical analysis of the antigen receptor complex.* Immunol Rev, 1984. **81**: p. 95-129.
68. Claverie, J.M., A. Prochnicka-Chalufour, and L. Bougueleret, *Implications of a Fab-like structure for the T-cell receptor.* Immunol Today, 1989. **10**(1): p. 10-4.
69. Rudolph, M.G., et al., *Crystal structure of an isolated V(alpha) domain of the 2C T-cell receptor.* J Mol Biol, 2001. **314**(1): p. 1-8.
70. Fields, B.A., et al., *Crystal structure of the V alpha domain of a T cell antigen receptor.* Science, 1995. **270**(5243): p. 1821-4.
71. Fields, B.A., et al., *Crystal structure of a T-cell receptor beta-chain complexed with a superantigen.* Nature, 1996. **384**(6605): p. 188-92.
72. Li, H., et al., *Dual conformations of a T cell receptor V alpha homodimer: implications for variability in V alpha V beta domain association.* J Mol Biol, 1997. **269**(3): p. 385-94.
73. Machius, M., et al., *Crystal structure of a T cell receptor Valpha11 (AV11S5) domain: new canonical forms for the first and second complementarity determining regions.* J Mol Biol, 2001. **310**(4): p. 689-98.
74. von Boehmer, H., *Unique features of the pre-T-cell receptor alpha-chain: not just a surrogate.* Nat Rev Immunol, 2005. **5**(7): p. 571-7.
75. Schubert, U., et al., *Rapid degradation of a large fraction of newly synthesized proteins by proteasomes.* Nature, 2000. **404**(6779): p. 770-4.
76. Reits, E.A., et al., *The major substrates for TAP in vivo are derived from newly synthesized proteins.* Nature, 2000. **404**(6779): p. 774-8.
77. Coux, O., *An interaction map of proteasome subunits.* Biochem Soc Trans, 2003. **31**(2): p. 465-9.
78. Rock, K.L. and A.L. Goldberg, *Degradation of cell proteins and the generation of MHC class I-presented peptides.* Annu Rev Immunol, 1999. **17**: p. 739-79.
79. Sijts, A., et al., *The role of the proteasome activator PA28 in MHC class I antigen processing.* Mol Immunol, 2002. **39**(3-4): p. 165-9.

80. Reits, E.A., A.C. Griekspoor, and J. Neefjes, *How does TAP pump peptides? insights from DNA repair and traffic ATPases.* Immunol Today, 2000. **21**(12): p. 598-600.
81. Serwold, T., et al., *ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum.* Nature, 2002. **419**(6906): p. 480-3.
82. Cresswell, P., *Intracellular surveillance: controlling the assembly of MHC class I-peptide complexes.* Traffic, 2000. **1**(4): p. 301-5.
83. Ortmann, B., et al., *A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes.* Science, 1997. **277**(5330): p. 1306-9.
84. Scholz, C. and R. Tampe, *The peptide-loading complex--antigen translocation and MHC class I loading.* Biol Chem, 2009. **390**(8): p. 783-94.
85. Kostyu, D.D., et al., *HLA class I polymorphism: structure and function and still questions.* Hum Immunol, 1997. **57**(1): p. 1-18.
86. van Bleek, G.M. and S.G. Nathenson, *The structure of the antigen-binding groove of major histocompatibility complex class I molecules determines specific selection of self-peptides.* Proc Natl Acad Sci U S A, 1991. **88**(24): p. 11032-6.
87. Fremont, D.H., et al., *Crystal structures of two viral peptides in complex with murine MHC class I H-2Kb.* Science, 1992. **257**(5072): p. 919-27.
88. Madden, D.R., D.N. Garboczi, and D.C. Wiley, *The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2.* Cell, 1993. **75**(4): p. 693-708.
89. Robey, E. and B.J. Fowlkes, *Selective events in T cell development.* Annu Rev Immunol, 1994. **12**: p. 675-705.
90. Biro, J., et al., *Regulation of T cell receptor (TCR) beta gene expression by CD3 complex signaling in immature thymocytes: implications for TCRbeta allelic exclusion.* Proc Natl Acad Sci U S A, 1999. **96**(7): p. 3882-7.
91. Hall, C., et al., *Requirements for cell surface expression of the human TCR/CD3 complex in non-T cells.* Int Immunol, 1991. **3**(4): p. 359-68.
92. Weiss, A. and D.R. Littman, *Signal transduction by lymphocyte antigen receptors.* Cell, 1994. **76**(2): p. 263-74.
93. Wooldridge, L., et al., *Interaction between the CD8 coreceptor and major histocompatibility complex class I stabilizes T cell receptor-antigen complexes at the cell surface.* J Biol Chem, 2005. **280**(30): p. 27491-501.
94. Artyomov, M.N., et al., *CD4 and CD8 binding to MHC molecules primarily acts to enhance Lck delivery.* Proc Natl Acad Sci U S A, 2010. **107**(39): p. 16916-21.
95. Lin, J. and A. Weiss, *T cell receptor signalling.* J Cell Sci, 2001. **114**(Pt 2): p. 243-4.
96. Okkenhaug, K., et al., *Phosphoinositide 3-kinase in T cell activation and survival.* Biochem Soc Trans, 2004. **32**(Pt 2): p. 332-5.
97. Tuosto, L. and O. Acuto, *CD28 affects the earliest signaling events generated by TCR engagement.* Eur J Immunol, 1998. **28**(7): p. 2131-42.
98. Burbach, B.J., et al., *T-cell receptor signaling to integrins.* Immunol Rev, 2007. **218**: p. 65-81.
99. Russell, J.H. and T.J. Ley, *Lymphocyte-mediated cytotoxicity.* Annu Rev Immunol, 2002. **20**: p. 323-70.
100. Pipkin, M.E. and J. Lieberman, *Delivering the kiss of death: progress on understanding how perforin works.* Curr Opin Immunol, 2007. **19**(3): p. 301-8.

101. Lieberman, J. and Z. Fan, *Nuclear war: the granzyme A-bomb.* Curr Opin Immunol, 2003. **15**(5): p. 553-9.
102. Lord, S.J., et al., *Granzyme B: a natural born killer.* Immunol Rev, 2003. **193**: p. 31-8.
103. Kupfer, A., G. Dennert, and S.J. Singer, *The reorientation of the Golgi apparatus and the microtubule-organizing center in the cytotoxic effector cell is a prerequisite in the lysis of bound target cells.* J Mol Cell Immunol, 1985. **2**(1): p. 37-49.
104. Bodmer, J.L., et al., *TRAIL receptor-2 signals apoptosis through FADD and caspase-8.* Nat Cell Biol, 2000. **2**(4): p. 241-3.
105. Sprick, M.R., et al., *FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2.* Immunity, 2000. **12**(6): p. 599-609.
106. Kagi, D., et al., *Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity.* Science, 1994. **265**(5171): p. 528-30.
107. Rathmell, J.C. and C.B. Thompson, *The central effectors of cell death in the immune system.* Annu Rev Immunol, 1999. **17**: p. 781-828.
108. Schwarz, B.A. and A. Bhandoola, *Trafficking from the bone marrow to the thymus: a prerequisite for thymopoiesis.* Immunol Rev, 2006. **209**: p. 47-57.
109. Griesemer, A.D., E.C. Sorenson, and M.A. Hardy, *The role of the thymus in tolerance.* Transplantation, 2010. **90**(5): p. 465-74.
110. Hernandez, J.B., R.H. Newton, and C.M. Walsh, *Life and death in the thymus--cell death signaling during T cell development.* Curr Opin Cell Biol, 2010. **22**(6): p. 865-71.
111. Baldwin, T.A., et al., *The timing of TCR alpha expression critically influences T cell development and selection.* J Exp Med, 2005. **202**(1): p. 111-21.
112. Starr, T.K., S.C. Jameson, and K.A. Hogquist, *Positive and negative selection of T cells.* Annu Rev Immunol, 2003. **21**: p. 139-76.
113. Surh, C.D. and J. Sprent, *T-cell apoptosis detected in situ during positive and negative selection in the thymus.* Nature, 1994. **372**(6501): p. 100-3.
114. Ueno, T., et al., *CCR7 signals are essential for cortex-medulla migration of developing thymocytes.* J Exp Med, 2004. **200**(4): p. 493-505.
115. Baldwin, K.K., et al., *Negative selection of T cells occurs throughout thymic development.* J Immunol, 1999. **163**(2): p. 689-98.
116. Matzinger, P. and S. Guerder, *Does T-cell tolerance require a dedicated antigen-presenting cell?* Nature, 1989. **338**(6210): p. 74-6.
117. Page, D.M., et al., *Two signals are required for negative selection of CD4+CD8+ thymocytes.* J Immunol, 1993. **151**(4): p. 1868-80.
118. Foy, T.M., et al., *An essential role for gp39, the ligand for CD40, in thymic selection.* J Exp Med, 1995. **182**(5): p. 1377-88.
119. Kishimoto, H. and J. Sprent, *Several different cell surface molecules control negative selection of medullary thymocytes.* J Exp Med, 1999. **190**(1): p. 65-73.
120. Kishimoto, H., C.D. Surh, and J. Sprent, *A role for Fas in negative selection of thymocytes in vivo.* J Exp Med, 1998. **187**(9): p. 1427-38.
121. Anderson, M.S. and M.A. Su, *Aire and T cell development.* Curr Opin Immunol, 2011. **23**(2): p. 198-206.
122. Anderson, M.S., et al., *Projection of an immunological self shadow within the thymus by the aire protein.* Science, 2002. **298**(5597): p. 1395-401.

123. Sette, A. and J. Fikes, *Epitope-based vaccines: an update on epitope identification, vaccine design and delivery.* Curr Opin Immunol, 2003. **15**(4): p. 461-70.
124. Koup, R.A., et al., *Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome.* J Virol, 1994. **68**(7): p. 4650-5.
125. Borrow, P., et al., *Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection.* J Virol, 1994. **68**(9): p. 6103-10.
126. Jin, X., et al., *Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques.* J Exp Med, 1999. **189**(6): p. 991-8.
127. Schmitz, J.E., et al., *Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes.* Science, 1999. **283**(5403): p. 857-60.
128. Couillin, I., et al., *HLA-dependent variations in human immunodeficiency virus Nef protein alter peptide/HLA binding.* Eur J Immunol, 1995. **25**(3): p. 728-32.
129. Borrow, P., et al., *Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus.* Nat Med, 1997. **3**(2): p. 205-11.
130. Goonetilleke, N., et al., *The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection.* J Exp Med, 2009. **206**(6): p. 1253-72.
131. Brumme, Z.L., et al., *Marked epitope- and allele-specific differences in rates of mutation in human immunodeficiency type 1 (HIV-1) Gag, Pol, and Nef cytotoxic T-lymphocyte epitopes in acute/early HIV-1 infection.* J Virol, 2008. **82**(18): p. 9216-27.
132. Salazar-Gonzalez, J.F., et al., *Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection.* J Exp Med, 2009. **206**(6): p. 1273-89.
133. Carrington, M. and S.J. O'Brien, *The influence of HLA genotype on AIDS.* Annu Rev Med, 2003. **54**: p. 535-51.
134. Okulicz, J.F. and O. Lambotte, *Epidemiology and clinical characteristics of elite controllers.* Curr Opin HIV AIDS, 2011. **6**(3): p. 163-8.
135. Rinaldo, C., et al., *High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors.* J Virol, 1995. **69**(9): p. 5838-42.
136. Cao, Y., et al., *Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection.* N Engl J Med, 1995. **332**(4): p. 201-8.
137. Makedonas, G., et al., *HIV-specific CD8 T-cell activity in uninfected injection drug users is associated with maintenance of seronegativity.* AIDS, 2002. **16**(12): p. 1595-602.
138. Rowland-Jones, S., et al., *HIV-specific cytotoxic T-cells in HIV-exposed but uninfected Gambian women.* Nat Med, 1995. **1**(1): p. 59-64.
139. Stranford, S.A., et al., *Lack of infection in HIV-exposed individuals is associated with a strong CD8(+) cell noncytotoxic anti-HIV response.* Proc Natl Acad Sci U S A, 1999. **96**(3): p. 1030-5.
140. Betts, M.R., et al., *HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells.* Blood, 2006. **107**(12): p. 4781-9.

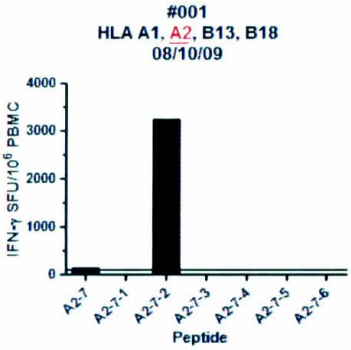
141. Akinsiku, O.T., et al., *Interleukin-2 Production by Polyfunctional HIV-1-Specific CD8 T Cells Is Associated With Enhanced Viral Suppression.* J Acquir Immune Defic Syndr, 2011. **58**(2): p. 132-40.
142. Horton, H., et al., *Preservation of T cell proliferation restricted by protective HLA alleles is critical for immune control of HIV-1 infection.* J Immunol, 2006. **177**(10): p. 7406-15.
143. Lichterfeld, M., et al., *Loss of HIV-1-specific CD8+ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4+ T cells.* J Exp Med, 2004. **200**(6): p. 701-12.
144. Migueles, S.A., et al., *HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors.* Nat Immunol, 2002. **3**(11): p. 1061-8.
145. Chen, H., et al., *TCR clonotypes modulate the protective effect of HLA class I molecules in HIV-1 infection.* Nat Immunol, 2012. **13**(7): p. 691-700.
146. Turnbull, E.L., et al., *HIV-1 epitope-specific CD8+ T cell responses strongly associated with delayed disease progression cross-recognize epitope variants efficiently.* J Immunol, 2006. **176**(10): p. 6130-46.
147. Almeida, J.R., et al., *Antigen sensitivity is a major determinant of CD8+ T-cell polyfunctionality and HIV-suppressive activity.* Blood, 2009. **113**(25): p. 6351-60.
148. Harari, A., et al., *Skewed association of polyfunctional antigen-specific CD8 T cell populations with HLA-B genotype.* Proc Natl Acad Sci U S A, 2007. **104**(41): p. 16233-8.
149. Emu, B., et al., *HLA class I-restricted T-cell responses may contribute to the control of human immunodeficiency virus infection, but such responses are not always necessary for long-term virus control.* J Virol, 2008. **82**(11): p. 5398-407.
150. Chang, J.T., et al., *Asymmetric T lymphocyte division in the initiation of adaptive immune responses.* Science, 2007. **315**(5819): p. 1687-91.
151. Sarkar, S., et al., *Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates.* J Exp Med, 2008. **205**(3): p. 625-40.
152. Murali-Krishna, K., et al., *Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection.* Immunity, 1998. **8**(2): p. 177-87.
153. Ahmed, R. and D. Gray, *Immunological memory and protective immunity: understanding their relation.* Science, 1996. **272**(5258): p. 54-60.
154. Warnock, R.A., et al., *Molecular mechanisms of lymphocyte homing to peripheral lymph nodes.* J Exp Med, 1998. **187**(2): p. 205-16.
155. Sallusto, F., J. Geginat, and A. Lanzavecchia, *Central memory and effector memory T cell subsets: function, generation, and maintenance.* Annu Rev Immunol, 2004. **22**: p. 745-63.
156. Masopust, D., et al., *Preferential localization of effector memory cells in nonlymphoid tissue.* Science, 2001. **291**(5512): p. 2413-7.
157. Freel, S.A., et al., *Phenotypic and functional profile of HIV-inhibitory CD8 T cells elicited by natural infection and heterologous prime/boost vaccination.* J Virol, 2010. **84**(10): p. 4998-5006.
158. Migueles, S.A., et al., *HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors.* Proc Natl Acad Sci U S A, 2000. **97**(6): p. 2709-14.
159. Sun, Y., et al., *Virus-specific cellular immune correlates of survival in vaccinated monkeys after simian immunodeficiency virus challenge.* J Virol, 2006. **80**(22): p. 10950-6.

160. *Public Health Agency of Canada: Immunization Schedules*. 2011 [cited 2011 October 15]; Available from: http://www.phac-aspc.gc.ca/im/ptimprog-progimpt/table-1-eng.php.
161. WHO. *Smallpox Fact Sheet*. 2001 [cited; Available from: http://www.who.int/mediacentre/factsheets/smallpox/en/index.html.
162. WHO. *Poliomyelitis Fact Sheet*. 2010 [cited; Available from: http://www.who.int/mediacentre/factsheets/fs114/en/.
163. Carr, A., et al., *An objective case definition of lipodystrophy in HIV-infected adults: a case-control study*. Lancet, 2003. **361**(9359): p. 726-35.
164. Reust, C.E., *Common Adverse Effects of Antiretroviral Therapy for HIV Disease*. Am Fam Physician, 2011. **83**(12): p. 1443-51.
165. *AIDS Info: Therapeutic HIV Vaccine*. 2006 [cited 2011 October 22]; Available from: http://www.aidsinfo.nih.gov/ContentFiles/Therapeutic_HIV_Vaccines_FS_en.pdf.
166. *HIV/AIDS Clinical Trials: Therapeutic HIV Vaccines*. 2011 [cited 2011 December 19]; Available from: http://aidsinfo.nih.gov/ClinicalTrials/search.aspx?searchType=q&ctID=1&parentLblID=299&childLblID=301&term=(%22therapeutic+vaccine%22+OR+%22therapeutic+HIV+vaccine%22)+%5bALL-FIELDS%5d+AND+HIV+%5bDISEASE%5d+NOT+HERPES+%5bDISEASE%5d+NOT+hepatitis+%5bDISEASE%5d+NOT+%22human+papillomavirus%22+%5bALL-FIELDS%5d+NOT+%22perinatal+sepsis%22+%5bALL-FIELDS%5d.
167. Buchbinder, S.P., et al., *Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial*. Lancet, 2008. **372**(9653): p. 1881-93.
168. Levine, A.M., et al., *Initial studies on active immunization of HIV-infected subjects using a gp120-depleted HIV-1 Immunogen: long-term follow-up*. J Acquir Immune Defic Syndr Hum Retrovirol, 1996. **11**(4): p. 351-64.
169. MacGregor, R.R., et al., *First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response*. J Infect Dis, 1998. **178**(1): p. 92-100.
170. MacGregor, R.R., et al., *Plasmid vaccination of stable HIV-positive subjects on antiviral treatment results in enhanced CD8 T-cell immunity and increased control of viral "blips"*. Vaccine, 2005. **23**(17-18): p. 2066-73.
171. Shimada, M., et al., *Effect of therapeutic immunization using Ad5/35 and MVA vectors on SIV infection of rhesus monkeys undergoing antiretroviral therapy*. Gene Ther, 2009. **16**(2): p. 218-28.
172. Lu, W., et al., *Therapeutic dendritic-cell vaccine for chronic HIV-1 infection*. Nat Med, 2004. **10**(12): p. 1359-65.
173. Garcia, F., et al., *A therapeutic dendritic cell-based vaccine for HIV-1 infection*. J Infect Dis, 2011. **203**(4): p. 473-8.
174. Garcia, F., et al., *Therapeutic immunization with dendritic cells loaded with heat-inactivated autologous HIV-1 in patients with chronic HIV-1 infection*. J Infect Dis, 2005. **191**(10): p. 1680-5.

175. Birx, D.L., et al., *Efficacy testing of recombinant human immunodeficiency virus (HIV) gp160 as a therapeutic vaccine in early-stage HIV-1-infected volunteers. rgp160 Phase II Vaccine Investigators.* J Infect Dis, 2000. **181**(3): p. 881-9.
176. Kran, A.M., et al., *Reduced viral burden amongst high responder patients following HIV-1 p24 peptide-based therapeutic immunization.* Vaccine, 2005. **23**(31): p. 4011-5.
177. Ndhlovu, Z.M., et al., *Mosaic HIV-1 Gag antigens can be processed and presented to human HIV-specific CD8+ T cells.* J Immunol, 2011. **186**(12): p. 6914-24.
178. Singh-Jasuja, H., N.P. Emmerich, and H.G. Rammensee, *The Tubingen approach: identification, selection, and validation of tumor-associated HLA peptides for cancer therapy.* Cancer Immunol Immunother, 2004. **53**(3): p. 187-95.
179. Guichard, G., et al., *Melanoma peptide MART-1(27-35) analogues with enhanced binding capacity to the human class I histocompatibility molecule HLA-A2 by introduction of a beta-amino acid residue: implications for recognition by tumor-infiltrating lymphocytes.* J Med Chem, 2000. **43**(20): p. 3803-8.
180. Webb, A.I., et al., *T cell determinants incorporating beta-amino acid residues are protease resistant and remain immunogenic in vivo.* J Immunol, 2005. **175**(6): p. 3810-8.
181. Marschutz, M.K., et al., *Improvement of the enzymatic stability of a cytotoxic T-lymphocyte-epitope model peptide for its oral administration.* Peptides, 2002. **23**(10): p. 1727-33.
182. Dranoff, G., *GM-CSF-based cancer vaccines.* Immunol Rev, 2002. **188**: p. 147-54.
183. Lee, P., et al., *Effects of interleukin-12 on the immune response to a multipeptide vaccine for resected metastatic melanoma.* J Clin Oncol, 2001. **19**(18): p. 3836-47.
184. Chianese-Bullock, K.A., et al., *MAGE-A1-, MAGE-A10-, and gp100-derived peptides are immunogenic when combined with granulocyte-macrophage colony-stimulating factor and montanide ISA-51 adjuvant and administered as part of a multipeptide vaccine for melanoma.* J Immunol, 2005. **174**(5): p. 3080-6.
185. Tan, M.L., P.F. Choong, and C.R. Dass, *Recent developments in liposomes, microparticles and nanoparticles for protein and peptide drug delivery.* Peptides, 2010. **31**(1): p. 184-93.
186. *Heteroclitic*, in *Webster's Revised Unabridged Dictionary (1913)*, C. and G. Merriam Co.: Springfield, MA.
187. Solinger, A.M., et al., *T-lymphocyte response to cytochrome c. I. Demonstration of a T-cell heteroclitic proliferative response and identification of a topographic antigenic determinant on pigeon cytochrome c whose immune recognition requires two complementing major histocompatibility complex-linked immune response genes.* J Exp Med, 1979. **150**(4): p. 830-48.
188. Tangri, S., et al., *Structural features of peptide analogs of human histocompatibility leukocyte antigen class I epitopes that are more potent and immunogenic than wild-type peptide.* J Exp Med, 2001. **194**(6): p. 833-46.
189. Dyall, R., et al., *Heteroclitic immunization induces tumor immunity.* J Exp Med, 1998. **188**(9): p. 1553-61.
190. Zirlik, K.M., et al., *Cytotoxic T cells generated against heteroclitic peptides kill primary tumor cells independent of the binding affinity of the native tumor antigen peptide.* Blood, 2006. **108**(12): p. 3865-70.

191. Du, A.P., et al., *Structural and immunological characterisation of heteroclitic peptide analogues corresponding to the 600-612 region of the HIV envelope gp41 glycoprotein.* J Mol Biol, 2002. **323**(3): p. 503-21.
192. Slansky, J.E., et al., *Enhanced antigen-specific antitumor immunity with altered peptide ligands that stabilize the MHC-peptide-TCR complex.* Immunity, 2000. **13**(4): p. 529-38.
193. Krug, L.M., et al., *WT1 peptide vaccinations induce CD4 and CD8 T cell immune responses in patients with mesothelioma and non-small cell lung cancer.* Cancer Immunol Immunother, 2010. **59**(10): p. 1467-79.
194. Jain, N., et al., *Synthetic tumor-specific breakpoint peptide vaccine in patients with chronic myeloid leukemia and minimal residual disease: a phase 2 trial.* Cancer, 2009. **115**(17): p. 3924-34.
195. Garboczi, D.N., et al., *Structure of the complex between human T-cell receptor, viral peptide and HLA-A2.* Nature, 1996. **384**(6605): p. 134-41.
196. Doytchinova, I.A. and D.R. Flower, *Modeling the peptide-T cell receptor interaction by the comparative molecular similarity indices analysis-soft independent modeling of class analogy technique.* J Med Chem, 2006. **49**(7): p. 2193-9.
197. Ladell, K., et al., *Central memory CD8+ T cells appear to have a shorter lifespan and reduced abundance as a function of HIV disease progression.* J Immunol, 2008. **180**(12): p. 7907-18.
198. Lefrancois, L. and D. Masopust, *The road not taken: memory T cell fate 'decisions'.* Nat Immunol, 2009. **10**(4): p. 369-70.
199. Shiver, J.W., et al., *Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity.* Nature, 2002. **415**(6869): p. 331-5.
200. Wherry, E.J., et al., *Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment.* J Virol, 2003. **77**(8): p. 4911-27.
201. Almeida, J.R., et al., *Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover.* J Exp Med, 2007. **204**(10): p. 2473-85.
202. Belyakov, I.M., et al., *Impact of vaccine-induced mucosal high-avidity CD8+ CTLs in delay of AIDS viral dissemination from mucosa.* Blood, 2006. **107**(8): p. 3258-64.
203. Lichterfeld, M., et al., *Selective depletion of high-avidity human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T cells after early HIV-1 infection.* J Virol, 2007. **81**(8): p. 4199-214.
204. Pattengale, P.K., R.W. Smith, and P. Gerber, *Selective transformation of B lymphocytes by E.B. virus.* Lancet, 1973. **2**(7820): p. 93-4.
205. Kyte, J. and R.F. Doolittle, *A simple method for displaying the hydropathic character of a protein.* J Mol Biol, 1982. **157**(1): p. 105-32.
206. Ndongala, M.L., et al., *HIV Gag p24 specific responses secreting IFN-gamma and/or IL-2 in treatment-naive individuals in acute infection early disease (AIED) are associated with low viral load.* Clin Immunol, 2009. **131**(2): p. 277-87.
207. Salazar, E., et al., *Agonist peptide from a cytotoxic t-lymphocyte epitope of human carcinoembryonic antigen stimulates production of tc1-type cytokines and increases tyrosine phosphorylation more efficiently than cognate peptide.* Int J Cancer, 2000. **85**(6): p. 829-38.

208. Zimmerli, S.C., et al., *HIV-1-specific IFN-gamma/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells.* Proc Natl Acad Sci U S A, 2005. **102**(20): p. 7239-44.
209. Heath, S.L., et al., *CD8 T-cell proliferative capacity is compromised in primary HIV-1 infection.* J Acquir Immune Defic Syndr, 2011. **56**(3): p. 213-21.
210. Mansoor, N., et al., *Significantly skewed memory CD8+ T cell subsets in HIV-1 infected infants during the first year of life.* Clin Immunol, 2009. **130**(3): p. 280-9.
211. Wherry, E.J. and R. Ahmed, *Memory CD8 T-cell differentiation during viral infection.* J Virol, 2004. **78**(11): p. 5535-45.
212. Trautmann, L., et al., *Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction.* Nat Med, 2006. **12**(10): p. 1198-202.
213. Day, C.L., et al., *PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression.* Nature, 2006. **443**(7109): p. 350-4.
214. Petrovas, C., et al., *PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection.* J Exp Med, 2006. **203**(10): p. 2281-92.
215. Zhang, J.Y., et al., *PD-1 up-regulation is correlated with HIV-specific memory CD8+ T-cell exhaustion in typical progressors but not in long-term nonprogressors.* Blood, 2007. **109**(11): p. 4671-8.
216. Porichis, F. and D.E. Kaufmann, *Role of PD-1 in HIV Pathogenesis and as Target for Therapy.* Curr HIV/AIDS Rep, 2012. **9**(1): p. 81-90.
217. Takahashi, M., et al., *Counter-regulation of cytolytic activity and cytokine production in HIV-1-specific murine CD8+ cytotoxic T lymphocytes by free antigenic peptide.* Int Immunol, 2001. **13**(1): p. 43-51.
218. Roedig, J.V., et al., *Impact of host cell line adaptation on quasispecies composition and glycosylation of influenza A virus hemagglutinin.* PLoS One, 2011. **6**(12): p. e27989.
219. Shan, L., et al., *Stimulation of HIV-1-specific cytolytic T lymphocytes facilitates elimination of latent viral reservoir after virus reactivation.* Immunity, 2012. **36**(3): p. 491-501.
220. Boulet, S., et al., *A dual color ELISPOT method for the simultaneous detection of IL-2 and IFN-gamma HIV-specific immune responses.* J Immunol Methods, 2007. **320**(1-2): p. 18-29.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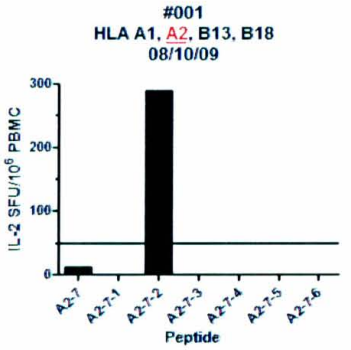
A2-7-2
A2-7-3
A2-7-4
A2-7-5
A2-7-6
Peptide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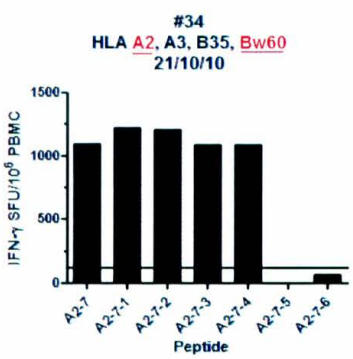
A2-7-2
A2-7-3
A2-7-4
A2-7-5
A2-7-6
Peptide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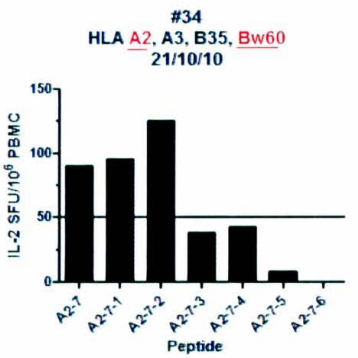
A2-7-2
A2-7-3
A2-7-4
A2-7-5
A2-7-6
Peptide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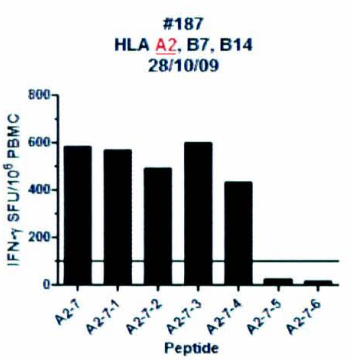
A2-7-2
A2-7-3
A2-7-4
A2-7-5
A2-7-6
Peptide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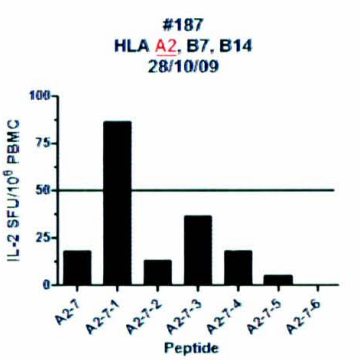
A2-7-3
A2-7-4
A2-7-5
A2-7-6
Peptide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A2-7-2
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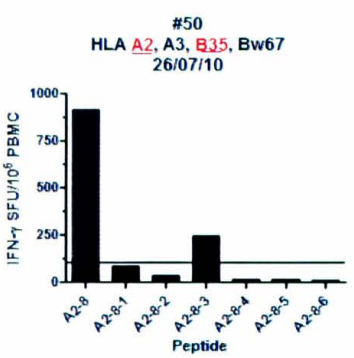
A2-7-4
A2-7-5
A2-7-6
Peptide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250
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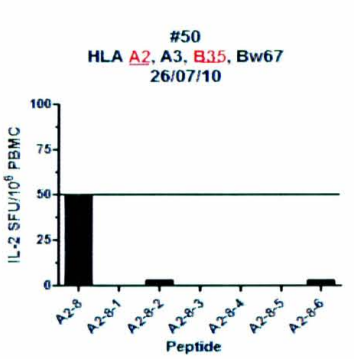
A2-8-6
Peptide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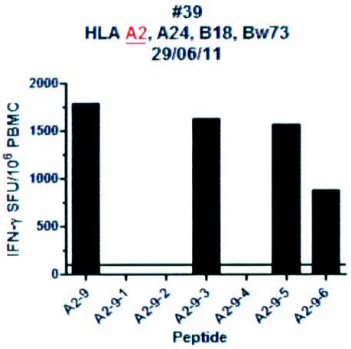
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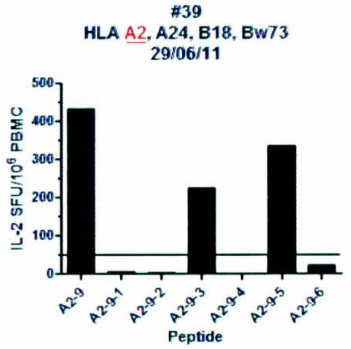
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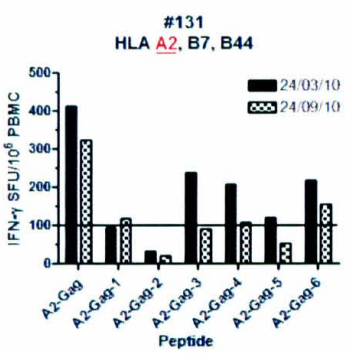
Peptide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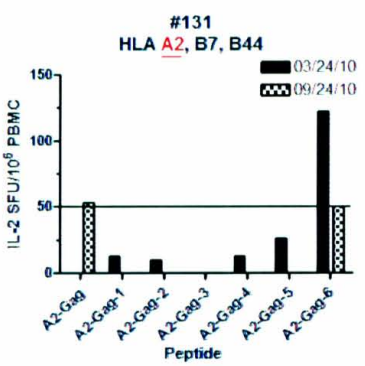
Peptide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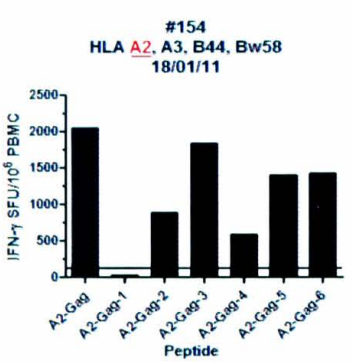
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1000
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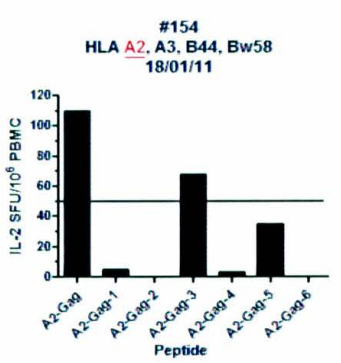
Peptide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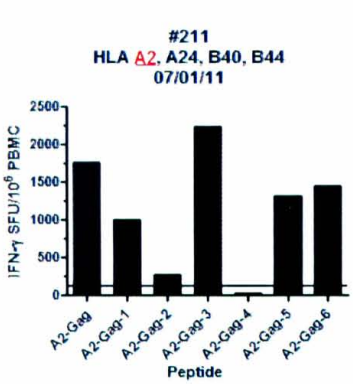
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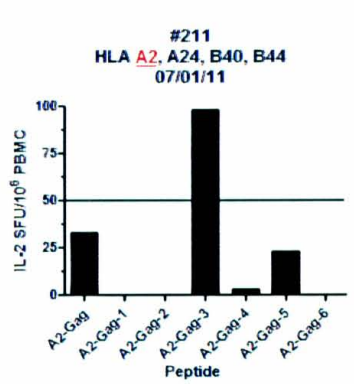
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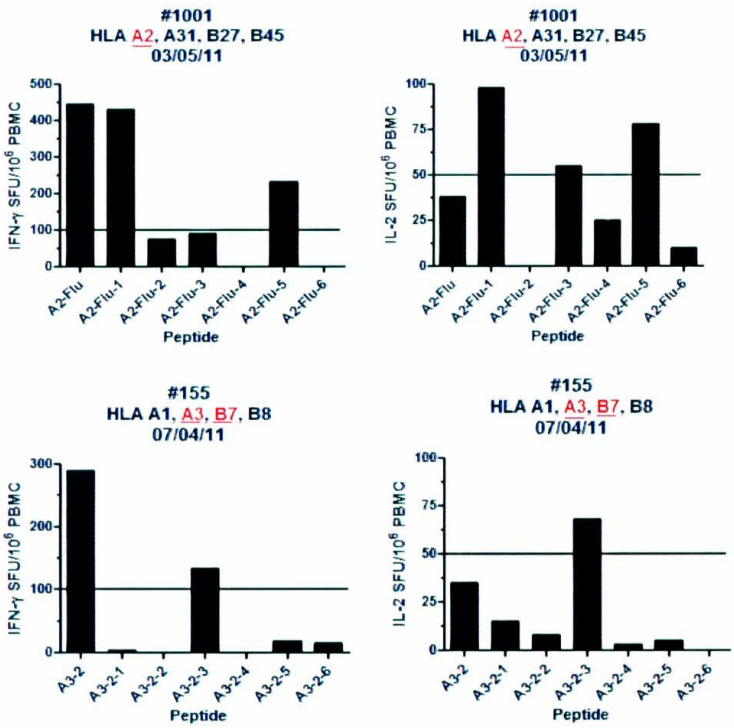
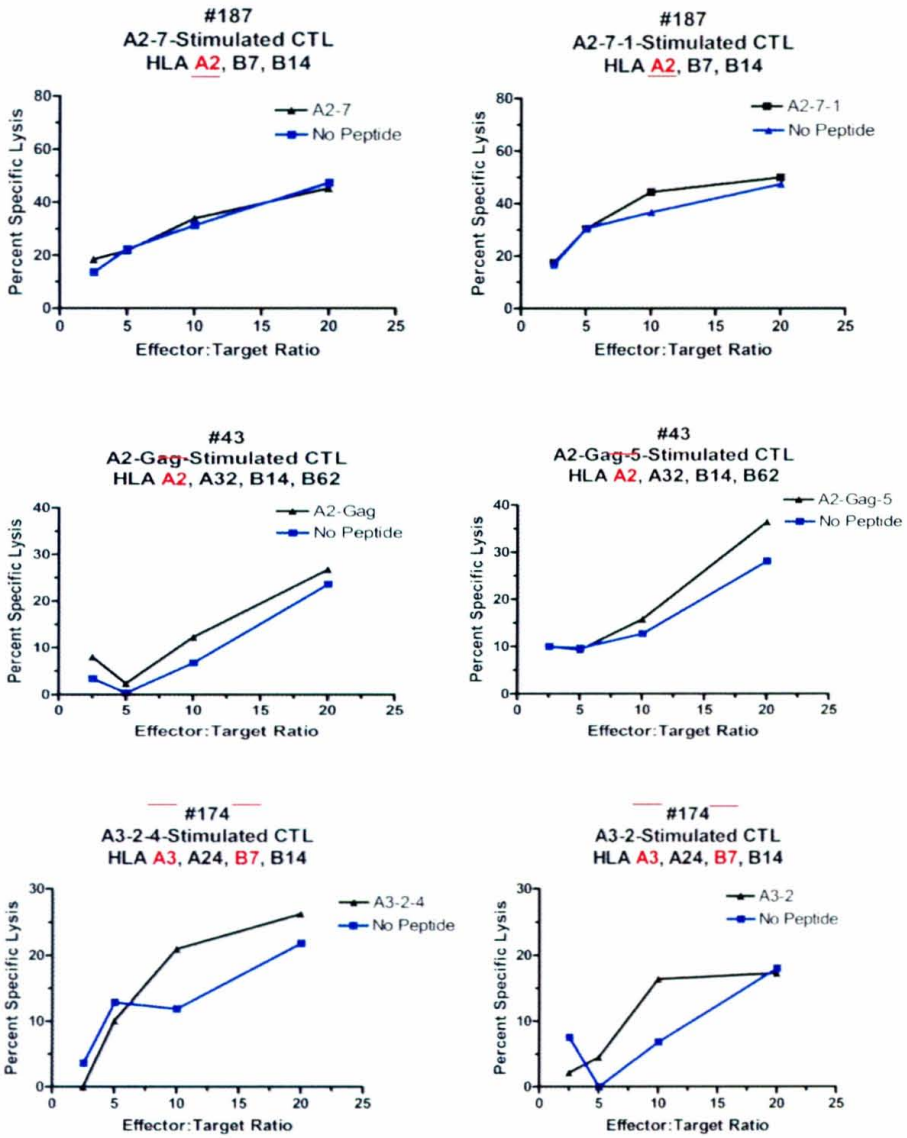
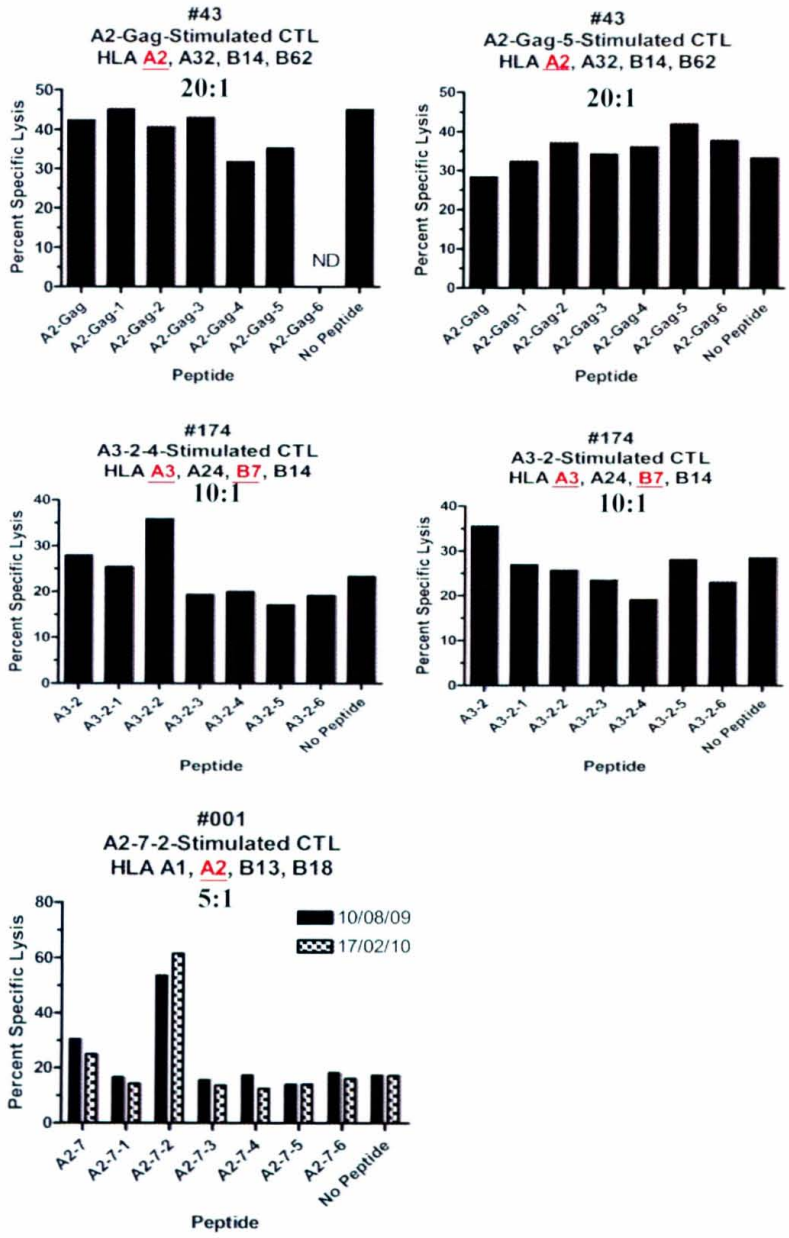


Appendix A: Relationship between IL-2 and IFN-γ responses. PBMC from HIV-infected and/or HIV-uninfected individuals were tested in duplicate and single test for IFN-γ and IL-2 production, respectively, against one or more peptide sets by ELISPOT assay. Examples in which enhanced IL-2 production is correlated with high IFN-γ responses are shown. A positive response is at least twice background and at least 50 IL-2 SFU/10^6 PBMC or 100 IFN-γ SFU/10^6 PBMC. The background was subtracted from the single test value or from the average of the duplicates. Duplicates were within 20% of each other. The HLA class I allele that presents each peptide set is highlighted in red and underlined.



Appendix B: Comparison of $CD8^+$ T cell cytotoxicity induced by reference and heteroclitic peptides II. Specific cytotoxicity of $CD8^+$ T cells stimulated in vitro with reference or heteroclitic peptides was tested, in duplicate, by ^{51}Cr release assay against reference or heteroclitic peptide-pulsed autologous BLCL target cells at increasing effector to target ratios. Duplicates fell within 20% of each other. The HLA class I allele that presents each peptide is highlighted in red and underlined. Tests in which peptide-pulsed and non-pulsed BLCL were used as targets are indicated by black triangles and blue squares, respectively.



Appendix C: Comparison of reference and heteroclitic peptide-stimulated-CTL reactivity. CTL were stimulated in vitro with reference or heteroclitic peptides and tested, in duplicate, by ^{51}Cr release assay against BLCL pulsed with each peptide within a set. For #001, the reference peptide did not induce CTL therefore crossreactivity could not be assessed. Duplicates fell within 20% of each other. The HLA class I allele that presents each peptide is highlighted in red and underlined. The effector to target ratio used is indicated below each heading.