

**Heteroclitic Human Immunodeficiency Virus Peptides
Enhance HIV-specific CD8⁺ T Cell Proliferation and Reduce
PD-1 Expression**

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Abstract

Heteroclitic peptides are sequence variants of native peptide epitopes that stimulate T cell responses superior to the native epitope. Since heteroclitic peptides enhance HIV-specific CD8⁺ T cell cytokine production, we investigated whether they reduce signs of HIV-specific CD8⁺ T cell exhaustion. Twenty-four variant peptides were generated from reference human histocompatibility-linked leukocyte antigen (HLA)-A2-restricted peptide epitopes Nef 83→91, Nef 135→143, Gag 433→440 and Gag 77→85 with conservative and semi-conservative amino acid substitutions at positions 3, 5 and 7 or 3, 5 and 8 of Gag 433→440. Variants that enhanced interferon-gamma (IFN- γ) and/or interleukin-2 (IL-2) production were tested for their effects on proliferation and programmed death-1 (PD-1) expression. Heteroclitic variants enhanced HIV-specific CD8⁺ T cell proliferation by > 20% in 13/29 cases, induced lower PD-1 expression by 15% - 50% in 10 cases, and by \geq 50% in 3 cases. These data indicate unique immunotherapeutic potential for heteroclitic peptides.

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List of Abbreviations

ACD	acid-citrate-dextrose
AIDS	acquired immunodeficiency syndrome
ALP	alkaline phosphatase
AML	acute myeloid leukemia
APC	antigen presenting cell
APOBEC	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
ART	antiretroviral therapy
β_2m	β_2 -microglobulin
cART	combination antiretroviral therapy
CCR5	cc chemokine receptor 5
CD	cluster of differentiation (surface antigen)
CDC	Centers for Disease Control
cDNA	complementary DNA
CDR3	complementarity determining region 3
CFSE	carboxyfluorescein diacetate succinimidyl ester
CLL	chronic lymphocytic leukemia
CMV	cytomegalovirus
CRPRC	California regional primate research center
CRT	calreticulin
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
CXCR4	cxc chemokine receptor 4

DC	dendritic cell
DMSO	dimethyl sulfoxide
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
EDTA	ethylenediaminetetraacetate
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme linked immunospot assay
Env	HIV envelope glycoprotein
ER	endoplasmic reticulum
ERAAP	endoplasmic reticulum aminopeptidase associated with antigen processing
ERAP-1	endoplasmic reticulum aminopeptidase-1
ERK	extracellular signal regulated kinase
FADD	Fas-associated protein with death domain
FasL	Fas ligand
FCS	fetal calf serum
FDA	food and drug administration
FITC	fluorescein isothiocyanate
Gag	HIV group specific antigen
gp	glycoprotein
gp41	envelope glycoprotein 41
gp120	envelope glycoprotein 120

GrB	granzyme B
GRB2	growth factor receptor-bound protein 2
HAART	highly active antiretroviral therapy
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human histocompatibility-linked leukocyte antigen
HTLV-III	human T-cell lymphotropic virus, type 3
HVEM	herpes virus entry mediator
IFN- γ	interferon-gamma
IL-2	interleukin 2
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
ITSM	immunoreceptor tyrosine-based switch motif
JNK	c-Jun N-terminus kinase
LAG3	lymphocyte-activation gene 3
LAT	linker for activation of T cells
LAV	lymphadenopathy-associated virus
LCK	lymphocyte-specific protein tyrosine kinase
LCMV	lymphocytic choriomeningitis virus
LFA	leukocyte function-associated antigen
LTNP	long-term non-progressor
LTR	long terminal repeat

mAb	monoclonal antibody
MAP	mitogen associated protein
MHC	major histocompatibility complex
min	minute
MIP-1 β	macrophage inflammatory protein-1 β
Nef	negative regulation factor
NERPRC	New England regional primate research center
NFAT	nuclear factor of activated T-cells
NF- κ B	nuclear factor-kappa B
NRTIs	nucleoside analog rt inhibitors
NNRTIs	non-nucleoside analog rt inhibitors
OIs	opportunistic infections
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PJP	<i>Pneumocystis jiroveci</i> pneumonia
PD-1	programmed death 1
PD-L1	programmed death protein ligand 1
PerCP	peridinin chlorophyll protein
PHA	phytohemagglutinin
PHAC	Public Health Agency of Canada
PI3K	phosphatidylinositol 3-kinase
PirB	paired-immunoglobulin like receptor B
PKC θ	protein kinase C theta

PLC	peptide-loading complex
PLC γ 1	Phospholipase C, gamma 1
PTK	Protein tyrosine kinase
PTP	protein tyrosine phosphatase
PVDF	polyvinylidene difluoride
RAG	recombination activating gene
Rev	regulator of viral gene expression
RNA	ribonucleic acid
rt	reverse transcriptase
RT	room temperature
SHP-1	SH2-domain containing tyrosine phosphatase 1
SIV	simian immunodeficiency virus
SLP-76	SH2 domain containing leukocyte protein of 76kDa
SMAC	supramolecular adhesion complex
STAT	signal transducer and activator of transcription
Tat	trans-activator of transcription
TAP	Transporter associated with antigen processing
TAR	transactivation response
TCR	T-cell receptor
TIM-3	T-cell immunoglobulin and mucin 3 protein
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAF	TNFR associated factor

TRIM5 α	tripartite motif 5 alpha
TSG101	tumor suppressor gene 101
UNAIDS	United Nations programme on HIV/AIDS
Vif	viral infectivity factor
Vpr	viral protein R
Vpu	viral protein U
ZAP-70	zeta-chain-associated protein kinase 70

1.0 Introduction

1.1 Overview of HIV/AIDS

1.1.1 Discovery

Acquired Immunodeficiency Syndrome (AIDS) was first observed in the United States in the early 1980's among previously healthy young intravenous drug users and gay men who developed *Pneumocystis jiroveci* pneumonia (PJP), opportunistic cryptococcal or cytomegalovirus infections or rare malignancies like Kaposi's sarcoma that normally occur only in immunocompromised patients (1). The rising incidence of PJP and Kaposi's sarcoma in an unusual population prompted the task force team formed at the US Centers for Disease Control and Prevention (CDC) to monitor the outbreak and the term Acquired Immune Deficiency Syndrome was coined to name the fatal disease in 1982 (2,3).

Françoise Barré-Sinoussi and Robert Gallo independently isolated the etiologic agent of AIDS and published their findings in the same issue of *Science* in 1983 (4,5). While Barré-Sinoussi's group named the agent lymphadenopathy-associated virus (LAV), Gallo's group named it human T cell lymphotropic virus, type 3 (HTLV-III) (6). A consensus was reached in May 1986 by the International Committee on the Taxonomy of Viruses, and the name human immunodeficiency virus (HIV) was adopted (7).

Outbreaks of wasting and severe infections were also observed in rhesus macaques (*Macaca mulatta*) by researchers at the California Regional Primate Research Center (CRPRC) and New England Regional Primate Research Center

(NERPRC) shortly after the discovery of human-AIDS (8,9). These symptoms were similar to those observed in AIDS patients and the disease was termed simian-AIDS (10). Western Blot cross-reactivity between HIV-1 antigens and sera from these macaques led to the discovery and naming of the causative agent of simian-AIDS as simian immunodeficiency virus (SIV) (8,11). Sera from Senegalese sex-workers preferentially cross-reacted with SIV antigens on Western Blot assay as opposed to HIV-1 antigens, suggesting exposure to a more SIV-like virus (12). A similar but immunologically distinct strain of HIV was subsequently isolated from West African AIDS patients from Guinea-Bissau and Cape Verde in 1986. This strain was named HIV-2 and it has since remained endemic in West Africa (13).

1.1.2 Epidemiology

The Joint United Nations Programme on HIV/AIDS (UNAIDS) recently reported that approximately 35.3 [32.2 – 38.8] million people were living with HIV worldwide at the end of 2012. There were 2.3 [1.9 – 2.7] million new infections and about 1.6 [1.4 – 1.9] million deaths due to AIDS in 2012 alone. About 95% of the new infections were in low and middle income countries. The sub-Saharan African region appears to be worst affected by this infection, accounting for 57% of people living with HIV worldwide (14).

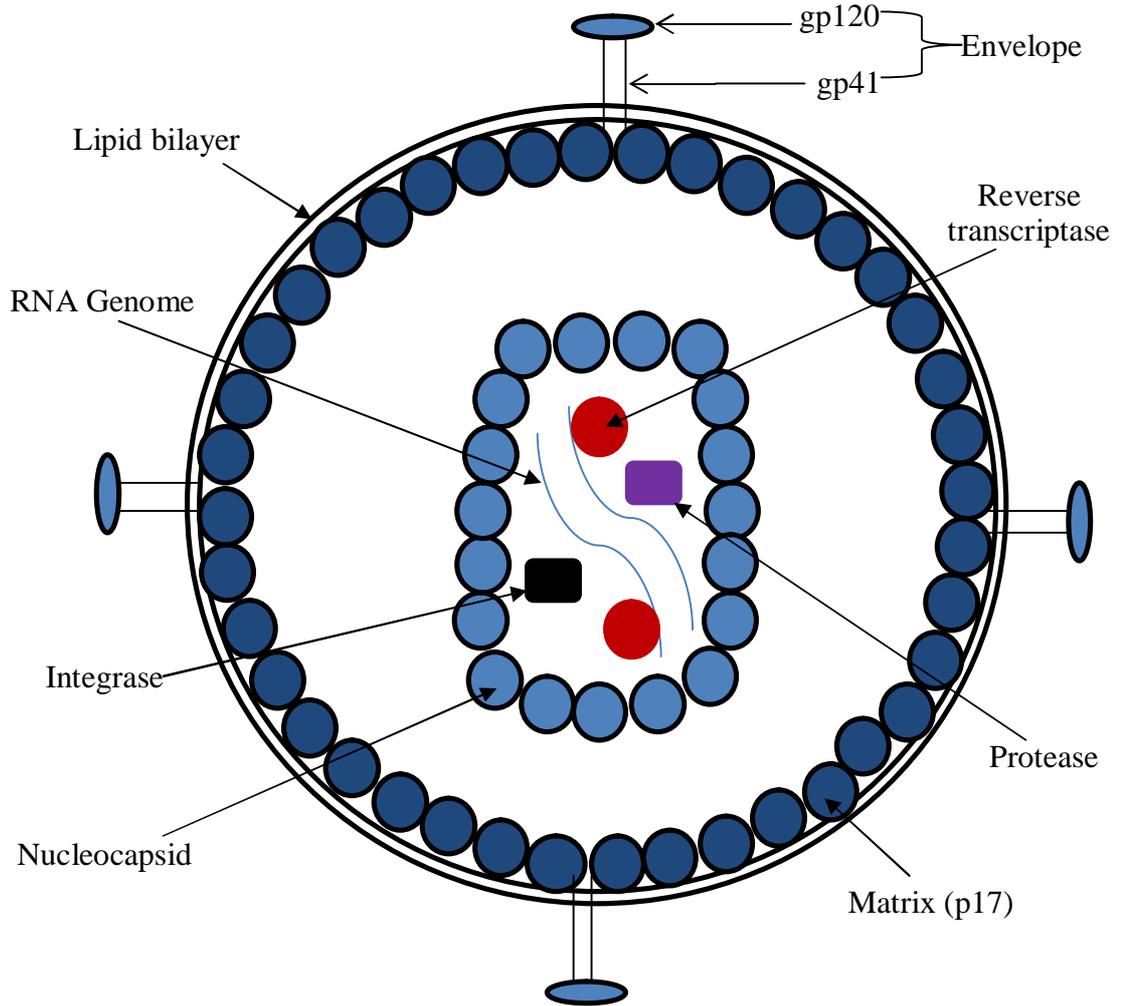
Since the first reported case of HIV in Canada in 1985, a cumulative total of 76,275 positive HIV tests have been reported to the Public Health Agency of Canada (PHAC). A total of 2,062 new infections were reported in 2012, which represents a

7.8% decrease from the 2011 reports (2,237 cases) and is the lowest number of annual HIV cases since incidence was first reported in 1985 (15).

1.1.3 Structure and Genome

HIV is a lentivirus that belongs to the *Retroviridae* family. Viruses belonging to the *lentivirus* genus typically establish infection with a long incubation period (*lente-*, Latin for "slow") before development of disease. The mature HIV particle is spherical, about 100-120 nm in diameter and enveloped with a lipid bi-layer membrane derived from the host cell during budding of a newly formed virion. Within the nucleocapsid are two identical copies of a single-stranded, positive-sense ribonucleic acid (RNA) genome of ~9.7 kb. The nine genes (*gag*, *pol*, *env*, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*) that encode HIV proteins can be found in each copy of the RNA. Flanking both ends of the RNA are long terminal repeat (LTR) sequences, which regulate HIV replication. The structural proteins are encoded by the group-specific core antigen (*gag*) and envelope (*env*) genes. The *Gag* gene codes for the matrix protein (p17), capsid (p24) and nucleocapsid (p7). The *Pol* gene encodes catalytic proteins such as reverse transcriptase, protease and integrase. The *Env* gene encodes the glycoprotein (gp) 160, which is cleaved to the outer envelope protein gp120 and transmembrane protein gp41. Trans-activator of transcription (*tat*) and regulator of viral expression (*rev*) are both regulatory genes. Other genes such as *vpu*, *vif*, *vpr* and *nef* are accessory genes. The function of these genes and their products are summarized in Table 1.1, and discussed in relation to the HIV life cycle below.

(A)



(B)

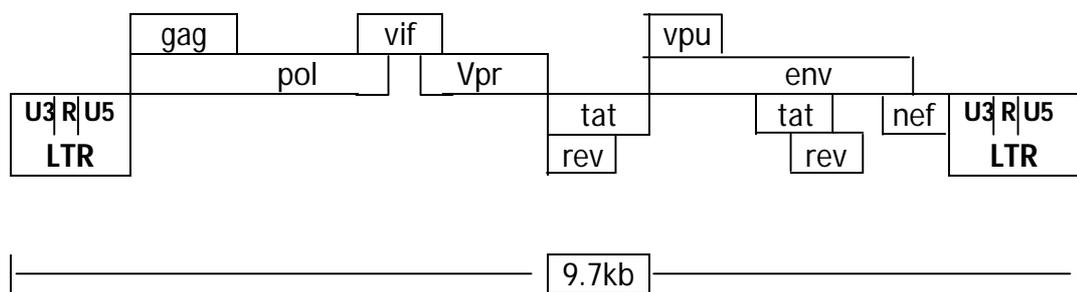


Figure 1.1: HIV structure (A) the envelope proteins (gp120, gp41), RNA genome and other regulatory proteins. (B) The single-stranded HIV RNA genome showing the nine major genes coding for the viral proteins flanked on both sides by the LTR long-terminal repeat.

Table 1.1: The nine major genes of HIV highlighting their products and function.

Adapted by permission from Macmillan Publishers Ltd (16).

Gene	Protein Class	Protein/Function
gag – group specific antigen	Structural	<p>Matrix (p17) *Undergoes myristylation that targets Gag polyprotein to lipid rafts *Implicated in nuclear import of HIV preintegration complex (PIC)</p> <p>Capsid (p24) *forms the inner core-protein layer</p> <p>Nucleocapsid (p7) *binds directly to genomic RNA</p> <p>p6 *Interacts with Vpr *Contains late domain (PTAP) that binds TSG101 and participates in terminal steps of virion budding</p>
pol – polymerase	Enzyme	<p>Protease (p10) *cleaves gag precursor</p> <p>Reverse Transcriptase (p66/51)</p> <p>Integrase (p32)</p>
env – envelope	Envelope	<p>gp160 envelope protein cleaved in endoplasmic reticulum to</p> <p>gp120 *protrudes from envelope and binds CD4 and chemokine receptor</p> <p>gp41 *Transmembrane protein associated with gp120 and required for fusion</p>
tat – transactivator		<p>p14/p16 *Binds transactivation response element *Enhances RNA Pol II elongation on the viral</p>

		DNA template in presence of host cyclin T1 and CDK9
rev – regulator of viral gene expression	Regulatory	p19 *Binds rev responsive element *Allows export of unspliced and singly spliced mRNAs from nucleus
vif – viral infectivity factor		p23 *Promotes infectivity of viral particle *Depletes intracellular stores of the anti-retroviral factors APOBEC3G, thus blocking virion incorporation of this factor
vpr – viral protein R		p15 *Promotes G2 cell cycle arrest *Facilitates HIV infection of macrophages
vpu – viral protein U	Accessory	*Required for efficient viral assembly *Promotes CD4 degradation *Influences budding of virion
nef – negative regulation factor		*Downregulates host-cell CD4 and MHC I expression *Blocks apoptosis *Alters state of cellular activation *Progression to disease slowed significantly in absence of Nef

1.1.4 Life Cycle and Pathogenesis

Viral attachment and entry is initiated when gp120 binds specifically to CD4 molecules expressed on certain T cells, macrophages and dendritic cells (17). The gp120/CD4 binding triggers a structural change, which permits the interaction of gp120 chemokine binding domains with either CC chemokine receptor 5 (CCR5) or CXC chemokine receptor 4 (CXCR4) (18,19). Selective interaction with one or the other of these chemokine receptors determines the virus tropism. M-tropic or R5 strains utilize the CD4 molecule and β -chemokine receptor CCR5 to infect target cells, whereas the T-tropic or X4 strains utilize the CD4 molecule and α -chemokine receptor CXCR4. Viruses that utilize both chemokine receptors are defined as X4/R5 strains (18,20).

The viral gp120 undergoes a conformational change upon binding with the relevant coreceptor. This change exposes the underlying transmembrane gp41, which in turn inserts its hydrophobic NH₂ terminal sequence into the target cell membrane to initiate viral fusion with the target cell (21,22). After successful target cell infection and virus particle uncoating, reverse transcriptase (rt) converts the single-stranded RNA genome into a double-stranded cDNA copy. Approximately one mutation is introduced for every 1000–10,000 nucleotides synthesized due to the error-prone reverse transcription process (23-25). The cDNA forms a pre-integration complex (PIC) with host proteins (such as barrier to autointegration factor 1) and viral proteins (such as vpr, matrix and integrase), is actively transported to the nucleus and integrated into the host genome as a “provirus” by integrase (26,27). The provirus is

replicated as part of the host cell genome or may lie dormant and persist for several years in the latent stage of HIV infection.

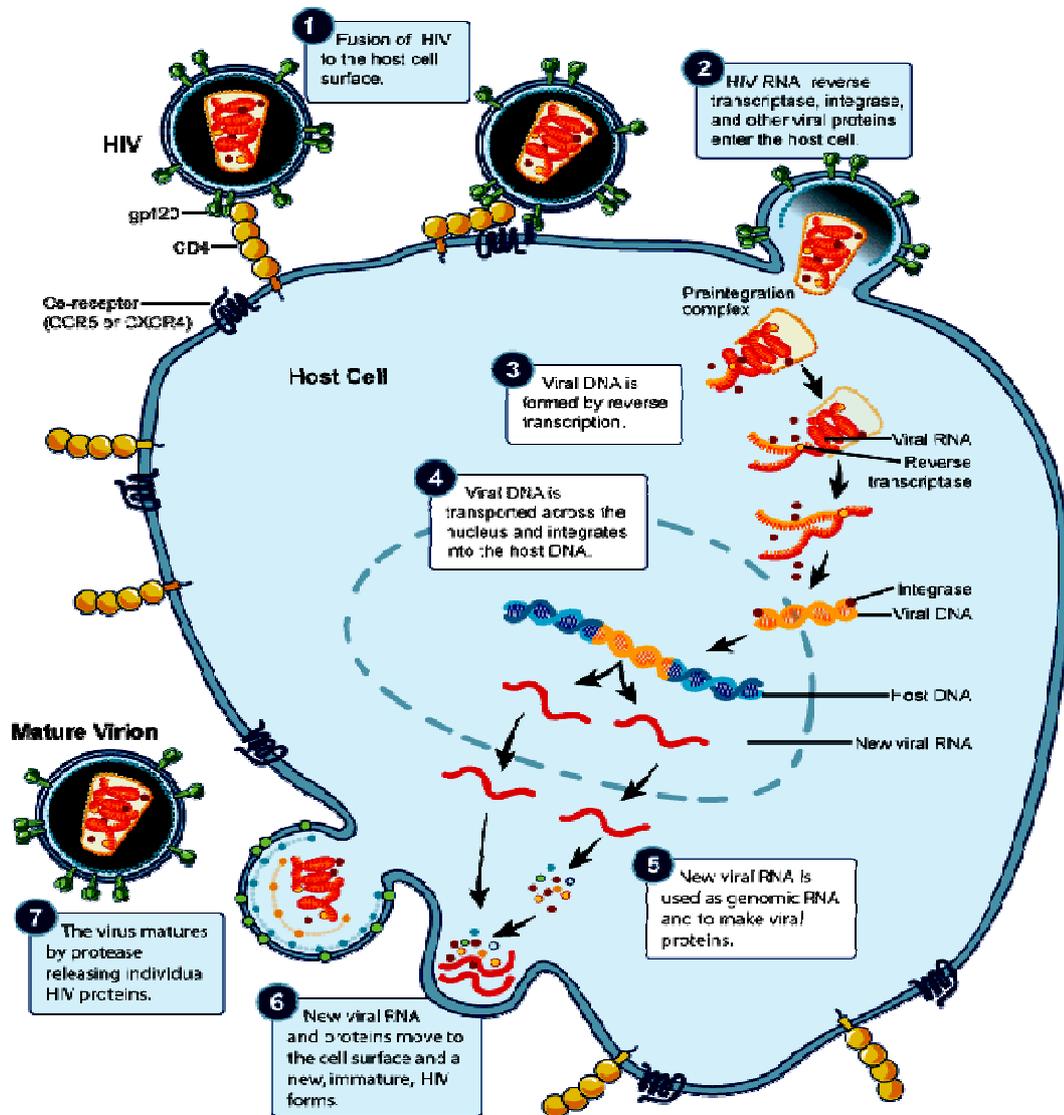


Figure 1.2: The life cycle of HIV. The HIV particle fuses with the host cell membrane through interactions of gp120, gp41, CD4 and CCR5 and/or CXCR4. This is followed by uncoating and release of the viral genomic contents into the host cell cytoplasm. Reverse transcription produces a viral cDNA, which is translocated into the nucleus where it integrates with the host DNA. Copies of viral RNA are made following the activation and gene transcription of the host cell DNA. Viral proteins are made from viral RNAs. In the cytoplasm, the viral proteins mature and assemble into viral particles that bud from the host cell membrane. Figure adapted from (28).

Human tripartite motif 5 alpha (TRIM5 α) is a host protein that acts to inhibit HIV-1 replication soon after uncoating by targeting the capsid for ubiquitination and rapid proteolytic degradation. Viral replication commences postintegration with the production of nascent viral transcripts by cellular RNA polymerases. Transcription is initiated with the binding of cellular transcription factors (such as NF κ B) to the 5' end of the LTR which now serves as the promoter region. Translation of these early transcripts results in the production of regulatory proteins such as tat, rev and nef. Tat induces transcription by binding to transactivation response (TAR) element. Rev facilitates the nuclear export of unspliced RNA transcripts and Nef downregulates expression of CD4 and MHC class I molecules (29).

Accessory proteins such as Vpu enhance CD4 degradation and facilitate virion release by inhibiting the host factor tetherin (30). Vpr enhances cell cycle arrest at the G2/M phase and can disrupt cellular transcription (31). Vif promotes infectivity of the virion and targets host cytidine deaminases (APOBEC3G and APOBEC3F) for ubiquitination and proteosomal degradation (32). Full length viral transcripts are packaged into the immature viral particles assembled into their functional forms by protease. The p6 protein which is present on the c-terminus of Gag interacts with tumor suppressor gene 101 (TSG101) to facilitate budding of the newly assembled viral particles (33).

HIV replication kinetics can raise viremia to more than 100 million HIV-1 RNA copies/ml plasma in the first few weeks of infection. This stage is known as the acute or primary phase of HIV-infection which is often characterized by flu-like symptoms.

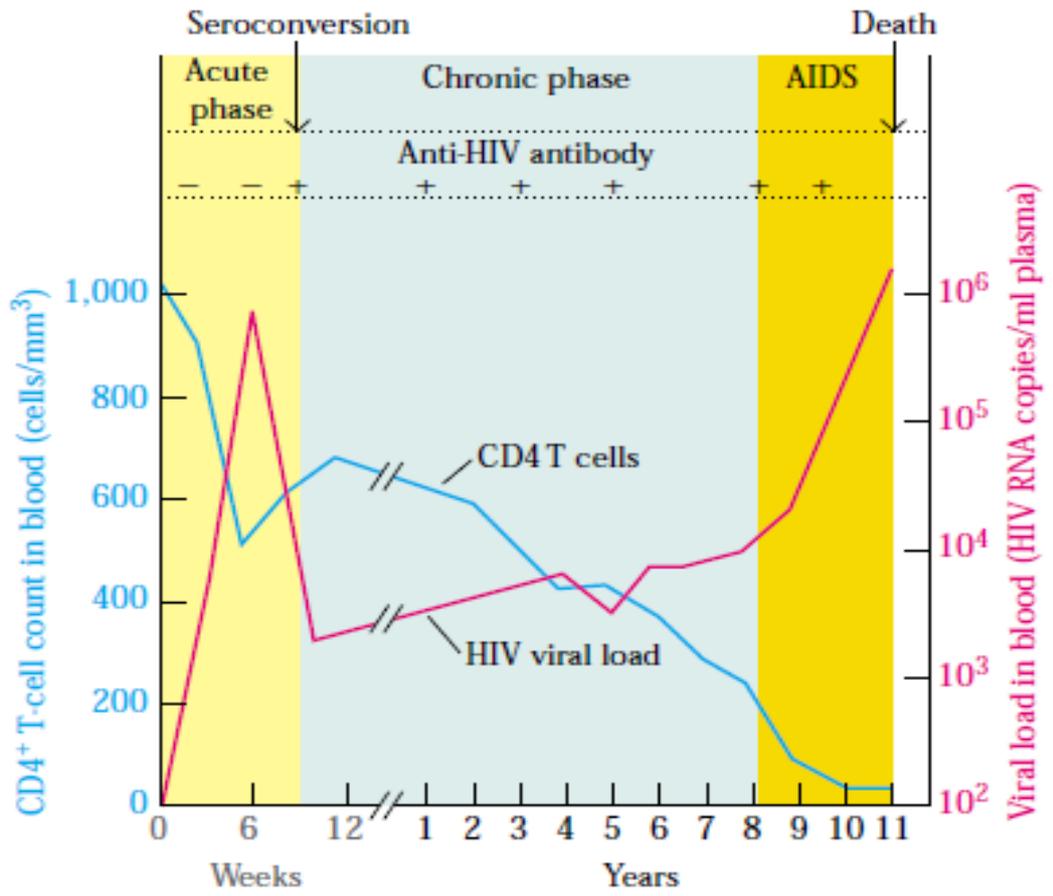


Figure 1.3: Pathogenesis of untreated HIV-1 infection. There is a progressive spike in plasma viremia during acute infection with HIV. This leads to a continuous decline in CD4⁺ T cell count, with no detectable presence of neutralizing HIV-1 antibodies. The viremia is initially controlled by the host immune response (not shown), and the subject then enters a phase of clinical latency (chronic phase). HIV-specific antibodies are detectable at the onset of this stage. Viral replication continues, but the immune system is able to contain it to set-point levels (not illustrated). Total CD4⁺ T cell numbers continue to decline and untreated individuals develop clinical symptoms of full-blown AIDS over time. Reproduced with permission from (34) Copyright Massachusetts Medical Society.

There are no detectable neutralizing antibodies against HIV-1 during primary infection and the first evidence of an HIV-specific CD8⁺ T cell immune response is observed towards the end of this phase (35-37). During the asymptomatic phase, there are no overt clinical symptoms of on-going infection even though the viral replication cycle continues and there is usually a dramatic decline in the systemic CD4⁺ T cell count in untreated individuals (38). The emergence of HIV-specific CD8⁺ T cells is associated with decline of viremia to low levels, but destruction of CD4⁺ T cells continues, especially, within the lymphoid tissues of the gut (39). If left untreated, the CD4⁺ T cells are often reduced below a certain threshold level that permits the development of opportunistic infections (OIs). This phase could last from a few months to more than 15 years (36,40).

AIDS is often defined as the chronic terminal stage of HIV infection. The CD4⁺ T cell count falls to < 200 cells/ μ l, rendering the host susceptible to OIs. These OIs or cancers eventually lead to death (36).

1.1.5 Therapy

Six major classes of antiretroviral therapy (ART) currently exist, and they are designed to interfere with different stages of the HIV life-cycle. Entry inhibitors (CCR5 co-receptor antagonists) prevent the viral particle from binding to host-cell receptors. Fusion inhibitors inhibit the viral particle from fusing with the host-cell membrane. Non-nucleoside rt inhibitors (NNRTIs) inhibit rt from converting HIV-RNA into cDNA, whereas nucleoside/nucleotide rt inhibitors (NRTIs) are faulty DNA building blocks which are inserted into a growing HIV DNA chain, resulting in DNA

chain termination as no additional nucleotides can be attached to them. Integrase inhibitors block HIV DNA integration into host DNA, whereas protease inhibitors prevent the cleavage of polyproteins needed to produce mature infectious virions. Multi-class combination products combine HIV drugs from two or more classes into a single product (41). ART was administered as mono or dual therapy before the advent of combination antiretroviral therapy (cART) (42). cART inhibits viral replication and reduces HIV-1 viremia below the limits of detection by most conventional testing methods (<50 RNA copies/ml) resulting in significant immune reconstitution (43). This also leads to dramatic reduction in the morbidity and mortality associated with HIV infection (44). However, cART is not a curative therapy for HIV infection and drug resistance leading to treatment failure has been documented for all classes of ART (45).

1.1.6 Viral Persistence and the Immune Response

HIV persistence can be attributed to a high mutation rate of $\sim 3 \times 10^{-5}$ mutations per nucleotide base for each cycle of replication, allowing the generation of a genetically diverse population of (10^9 to 10^{10}) virions within an infected host each day (46). A hybrid-virus can be generated through genetic recombination upon infection of a new host cell with multiple strains of HIV, which adds to the overall viral diversity (47). Viral mutations usually generate quasispecies including drug-resistant and immune escape variants (48,49). Most HIV-infected individuals mount effective cellular and humoral immune responses in the first few months of infection.

However, these responses fail to contain viral replication over longer time periods. The scope of this thesis is limited to the role CD8⁺ T cell responses in HIV infection.

1.2 CD8⁺ T Cells

1.2.1 T Cell Selection

T cells are derived from bone marrow hematopoietic stem cells. Committed early immature T cell precursors journey through the bloodstream to the thymus. Thymocytes at this early stage are termed double-negative (DN) thymocytes as they do not express CD4 or CD8 molecules. These DN thymocytes are subdivided into four sequential stages of differentiation based on the surface expression of CD44 and CD25 molecules: DN1, CD44⁺CD25⁻; DN2, CD44⁺CD25⁺; DN3, CD44⁻CD25⁺; and DN4, CD44⁻CD25⁻ (50). DN thymocytes have the potential to differentiate into $\gamma\delta$ or $\alpha\beta$ TCR-expressing cells (51). Cells that differentiate along the $\alpha\beta$ TCR pathway begin to express the surrogate α chain (pre-TCR- α) encoded by a non-rearranging locus (52,53). The surrogate α chain pairs with the TCR β -chain, which was a product of recombination-activating gene (RAG)-mediated gene rearrangement (54,55). This pre-TCR- $\alpha\beta$ -pair associates with CD3/ ζ proteins to form a novel complex called the pre-TCR-complex that is important in proximal signal transduction (56). T cells emerging from the late DN3 and DN4 stages undergo extensive proliferation, while pre-TCR- α expression and TCR β -chain rearrangement ceases. The resultant mature $\alpha\beta$ T cells express CD8 coreceptor proteins first, and then CD4 (51). Cells expressing both CD4 and CD8 molecules, termed double positive (DP) thymocytes, comprise

90% of immature $\alpha\beta$ -TCR-expressing cells populating the lymphoid compartment in the thymus of young individuals.

These small double-positive thymocytes undergo a thymic selection process which is characterized by death by neglect, negative selection, positive selection and lineage-specific development. Due to poor TCR-pMHC interaction, most DP thymocytes die by neglect. Thymocytes bearing TCRs with strong avidity for self-peptides are potentially auto-reactive T cells and they undergo apoptotic cell-death upon TCR ligation with self-pMHC (negative selection). However, cells bearing TCRs that recognize self-peptides and generate signals below a certain threshold of avidity receive a survival signal (positive selection). These processes precede lineage specific differentiation into either CD4⁺ or CD8⁺ mature T cells.

1.2.2 TCR Signaling

Signaling through the TCR and costimulation via receptors such as CD28 are both essential for successful naïve T cell activation (57). TCR signaling is antigen-specific, while costimulatory signals that are usually provided by professional antigen presenting cell (APC) are antigen-independent and serve to modulate the T cell response and survival. Once activated, T cells respond by producing cytokines that modulate other cells, undergo clonal expansion, differentiate into effector and memory T cells, and in the case of cytotoxic T lymphocyte (CTL), mediate killing (cell-mediated cytotoxicity). However, a naïve T cell becomes unresponsive (anergic) and tolerant when exposed to antigen in the absence of a costimulatory (second) signal.

There are two different TCR heterodimers, the $\alpha:\beta$ TCR and $\gamma:\delta$ TCR. About 95% of peripheral T cells bear alpha (α) and beta (β) chain TCRs, while the remaining 5% express gamma and delta (γ/δ) chains. Each chain of the $\alpha\beta$ TCR is made up of a variable (V_α or V_β) and a constant (C_α or C_β) domain. The V_α is encoded by two DNA segments (V_α and J_α), while the V_β is encoded by three segments (V_β , D_β , and J_β). The $\alpha\beta$ -TCR resembles the Fab fragment of the Ig molecule. Upon receptor engagement, the $\alpha\beta$ heterodimer associates with the CD3 molecule (TCR-complex) to initiate signal transduction across the cell membrane and phosphorylation to activate downstream signaling pathways. TCR interaction with the MHC-peptide complex triggers phosphorylation of tyrosine residues on the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 α , δ , ϵ and ζ chains (58). Protein tyrosine kinases (PTKs) important in T cell activation are Lck and Fyn of the Src family and ZAP-70 of the Syk family. These PTKs interact with the ITAMs of the TCR-complex. Activation of Lck kinase is dependent on the protein tyrosine phosphatase (PTP) CD45, which dephosphorylates an inhibitory tyrosine in the cytoplasmic tail of Lck. Activated Lck phosphorylates the ITAMs present in the cytoplasmic tail of the CD3 chains, making it a docking site for ZAP-70. ZAP-70 binds to the phosphorylated ITAMs and is activated (phosphorylated) by Lck. The substrates for ZAP-70 are LAT (linker for activation of T cells), a transmembrane protein with a long cytoplasmic tail and SLP-76 (59,60). Phosphorylated LAT then recruits a number of adaptor proteins such as phospholipase γ 1 (PLC γ 1), growth factor receptor-bound protein 2 (GRB2) and phosphatidylinositol 3-kinase (PI3K), which are crucial to T cell activation. Binding of LAT with these adaptor proteins begins the process of gene transcription through

the NF- κ B, NF-AT and STAT pathway (61-63). Genes coding for the growth factor IL-2, pro-inflammatory cytokines IFN- γ and TNF- α and antiapoptotic protein Bcl-xL are upregulated (64). SLP-76 helps stabilize and maintain the immunological synapse by promoting the activation and clustering of leukocyte function-associated antigen (LFA-1), an integrin molecule (65,66).

1.2.3 T Cell Costimulation

1.2.3.1 CD28/B7 Family

Secondary signals affecting T cells can be stimulatory or inhibitory, and are mainly delivered by molecules belonging to the CD28/B7, tumor necrosis factor/tumor necrosis factor receptor (TNF/TNFR) superfamily and some chemokine receptors (67-69). CD28 and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) are well characterized receptors belonging to the CD28/B7 superfamily. CD28 provides a stimulatory signal to T cells, while CTLA-4 provides an inhibitory signal to activated T cells (70,71). They are both type 1 transmembrane glycoprotein receptors with an MYPPPY motif essentially used for interacting with B7.1 (CD80) and B7.2 (CD86) molecules expressed on APCs (72). CD28 is constitutively expressed on most T cells, and some plasma cells (73,74). CTLA-4 is constitutively expressed on regulatory T cells. CD28 is translocated to the immunological synapse in association with the TCR, SLP-76 and ZAP-70 kinase when ligated with either of B7.1 or B7.2 (75,76). Lck kinase phosphorylates the YXXM motif in the cytoplasmic tail of CD28, which then becomes a docking site for the SH2-domains of Grb2 and PI3K (75). Phosphorylation of PI3K induces the production of phosphatidylinositol (3,4,5)-

triphosphate (PIP3) from its substrate PIP2. This process enhances signal transduction and gene transcription through NF κ B and NFAT pathways (77,78). CTLA-4 has stronger affinity for CD80 and CD86 than CD28, and signaling through this receptor tends to attenuate or terminate T cell responses (71,79), whereas signaling through CD28 prevents anergy in naïve T cells, and enhances IL-2 production, proliferation, and survival (64,70,80). Humans, however, acquire CD28⁻CD8⁺ T cells with age, a situation which is also observable in progressive HIV infection (81-83).

Programmed death-1 [PD-1 (CD279)] is an inhibitory member of the CD28/B7 superfamily that was originally cloned from a T cell hybridoma line exhibiting apoptosis (84). PD-1 is a 288 amino acid type 1 transmembrane protein with a cytoplasmic tail containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). In humans, PD-1 is encoded for by *Pdcd1* gene on chromosome 2, and by *Pdcd1* gene on chromosome 1 in mice (85). Its expression on naïve T cells is extremely low, but it is inducibly expressed on activated T cells, B cells and myeloid cells (85). PD-1 interaction with any of its ligands PD-L1 [B7-H1, or CD274] or PD-L2 [B7-H2, B7-DC or CD273] dampens T cell responses by inhibiting membrane-proximal TCR signaling, downstream T cell proliferation, cytokine production and other effector functions (85-88). However, PD-1-mediated inhibition can be overcome by strong TCR stimulation and CD28 costimulation (86,89). PD-L1 is constitutively expressed on mouse T and B cells, dendritic cells, macrophages, and mesenchymal stem cells, while PD-L2 is inducibly expressed on dendritic cells, macrophages and bone marrow-derived mast cells (90,91). The tyrosine motifs present in the cytoplasmic tail of PD-1 become

phosphorylated upon ligation. SH2-domain containing tyrosine phosphatase 1 (SHP-1) and SHP-2 then binds to the ITIM and ITSM motifs of PD-1. This interaction downregulates TCR signaling and dephosphorylates other signaling intermediates (88). However, a mutation in the ITSM motif of PD-1 abrogates the inhibitory function of PD-1. This indicates that the ITSM alone plays the major role in PD-1-mediated inhibition (88). PD-1 interaction with SHP-2 appears to be stronger than with SHP-1, meaning PD-1 functions by recruiting SHP-2, and perhaps SHP-1 to the TCR complex (92). CTLA-4 prevents Akt activation, while PD-1 inhibits Akt activation as well as PI3K activity. Also, PD-1 signaling inhibits the phosphorylation of ZAP-70, CD3 ζ and PKC θ (93). Upon TCR ligation and T cell activation, CTLA-4 is transported to the immunological synapse from an intracellular store depending on the signaling strength (94). PD-1 molecules are redistributed from uniform cell surface expression to concentrate at the immunological synapse upon TCR ligation (95).

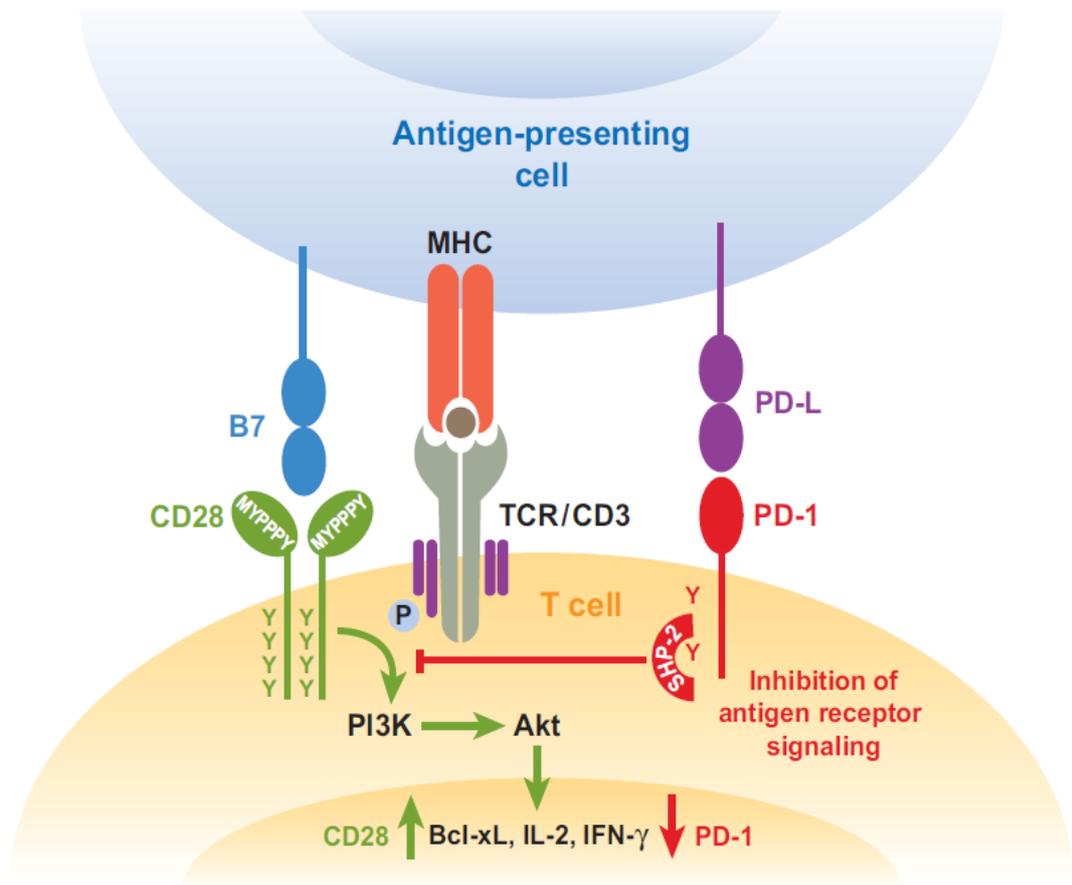


Figure 1.4: PD-1 signaling pathway. PD-1 ligation by PD-L1 or PD-L2 causes the immediate phosphorylation of PD-1 cytoplasmic tyrosine residues ITIM and ITSM. SHP-2 molecules are recruited to the tyrosine residues and their association dephosphorylates molecules associated with signal transduction via the PI3K pathway with resultant inhibition of signaling through the Akt pathway. This process downregulates production of pro-inflammatory cytokines such as IFN- γ , growth cytokines such as IL-2 and antiapoptotic molecules such as Bcl-xL. However, this inhibitory effect can be overcome by strong TCR stimulation and CD28 costimulation with resultant increase in cytokine production and enhanced cell survival. Reprinted by permission from Macmillan Publishers Ltd (85).

1.2.3.2 TNF/TNFR Family

The human homologue of 4-1BB was cloned in 1993 shortly after its discovery in mice (96,97). 4-1BB is a costimulatory molecule that is inducibly expressed on T cells upon activation, and constitutively expressed on APCs (98). Its ligand, 4-1BBL, is expressed on activated professional APCs, neurons, astrocytes, hematopoietic stem cells and myeloid progenitors (98-101). 4-1BBL expression on B cells and DC is mainly regulated by CD40 (99). The 4-1BB/4-1BBL interaction provides a costimulatory signal to T cells, although 4-1BB has a preferential effect on CD8⁺ T cells in antiviral and antitumor immunity in mice (102-104). Its importance in recall CD8⁺ T cell responses in viral infections and in maintenance of effector memory CD8⁺ T cells late in the primary immune response has been elucidated in mouse models (102,105-107). 4-1BB signals independently of CD28 costimulation in the presence of a strong TCR stimulation (107,108). Once bound to its ligand, 4-1BB recruits TNFR-associated factors (TRAFs) - TRAF1, TRAF2 and TRAF3, with downstream activation of NF- κ B, c-Jun N-terminus kinase (JNK), extracellular signal regulated kinase (ERK) and p38 mitogen associated protein (MAP) kinase pathways (109-112). T cell survival is enhanced by 4-1BB through TRAF1 and ERK-dependent downregulation of the pro-apoptotic molecule Bim, and the NF- κ B-dependent upregulation of antiapoptotic genes such as *bcl-X_L* and *bfl-1* (110,111,113,114). CD4⁺, CD8⁺, CD28⁺, and CD28⁻ T cell cytokine production, proliferation and cytolytic effector functions can be enhanced by 4-1BB costimulation (115-119).

1.2.4 Antigen Processing and Presentation

T cells recognize and afford protection against intracellular microbes (cell mediated immunity) that avoid humoral and complement-mediated attack in a two-step process. Firstly, intracellular pathogens are broken down to smaller fragments (peptides) and loaded onto major histocompatibility complex (MHC) molecules (antigen processing). In the second step, the peptide-MHC (pMHC) complexes are transported to the cell surface where they can be easily recognized by an antigen-specific T cell (antigen presentation). $CD8^+$ T cells recognize peptides in association with MHC class I (MHC-I) molecules, whereas $CD4^+$ T cells recognize peptides bound to MHC class II (MHC-II) molecules.

Internalized or endogenous protein antigens in the cytosol are broken down by a large, multicatalytic protease complex called the proteasome. Transporter associated with antigen processing (TAP), a member of the ATP-binding cassette (ABC) family of transporters helps in the translocation of cytosolic generated peptides into the lumen of the endoplasmic reticulum where they are loaded on newly synthesized MHC-I molecules (120,121). The chaperone protein calnexin binds to newly synthesized MHC-I α chains in the ER and assists in protein folding as well as promotes assembly with β_2 -microglobulin (β_2m). The partly folded MHC-I molecule is released from calnexin once it binds to β_2m , and then it binds the peptide-loading complex (PLC) which is made up of TAP, calreticulin (CRT), TAP-associated protein (tapasin) and ERp57. Calreticulin carries out a similar chaperone function to calnexin. Tapasin performs a bridging function between MHC-I and TAP, recruiting MHC-I- β_2m dimers and calreticulin to the PLC (122,123). ERp57 is a thiol oxidoreductase

that assists in the folding of newly synthesized MHC-I α_2 molecules in the ER (124). Endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP), found in mice, is a luminal component that plays a crucial role in the generation of peptide MHC-I complexes (125). Its human homologues are termed endoplasmic reticulum aminopeptidase-1 (ERAP1) and ERAP2 (126,127). Peptides, usually 8-10 amino acids in length, are loaded in the $\alpha_1\alpha_2$ domain of MHC-I, but TAP molecules can transport larger peptides into the ER. The amino termini of these peptides can be trimmed by ERAAP/ERAP1 to yield peptides of an appropriate length for MHC-I binding. Upregulation of ERAAP can also be induced by IFN- γ . Suboptimal, low-affinity peptides do not cause the release of the MHC-I from the PLC. Binding of high-affinity peptide to the partly folded MHC-I may cause conformational changes that complete the folding of the MHC-I molecule and trigger its release from the PLC. The fully folded MHC-I loaded with an appropriate peptide can now be transported to the cell surface. Although hundreds to thousands of potentially generated peptides have appropriate sequence to bind MHC-I, the bulk of responding antiviral CTL population CD8⁺ T cells recognize a tiny fraction of potential epitopes; a phenomenon referred to as immunodominance (128-131).

1.2.5 Peptide Recognition and Activation of CD8⁺ T Cells

CD8⁺ T cells only recognize peptides presented by self-MHC class I molecules. Peptides, usually 8 to 11-mer, that dominate the recognition events with a particular MHC-I allele are termed “immunodominant” epitopes, whereas less-recognized peptides are termed subdominant (129,130). The peptide binding groove of

MHC-I is flanked on both sides by α -helices and has a floor of antiparallel β -strands (132). Anchor residues of peptides interact with allele-specific pockets in the peptide binding groove of MHC class I, making upward pointing amino acid side chains available for interaction with the TCR (133,134). MHC class I molecules constantly present self and pathogen-derived peptides, but most auto-reactive T cells are clonally deleted during thymic selection. Antigen-specific T cells recognize the foreign peptides presented by self-MHC class I and form a stronger supramolecular adhesion complex (SMAC) or immunological complex with the APC in order to activate the T cell. The immunological synapse is further stabilized by the CD8 co-receptor on CD8⁺ T cells (135). Initial T cell priming takes place in the secondary lymphoid organs such as lymph nodes and spleen. The process of signal transduction and activation of CD8⁺ T cells follows the TCR signaling process already discussed in 1.2.2 above.

CD8⁺ T cells can become effector cytotoxic T lymphocytes (CTL) upon activation. CTL mediate the killing of virus-infected and tumor cells through two dominant contact-dependent pathways, the Fas/Fas ligand pathway and perforin/granzyme pathway. These two pathways are not mutually exclusive as an individual CTL may be equipped with both killing options (136). The Fas/Fas ligand and perforin/granzyme pathways result in activation of a family of cytotoxic proteases, the caspases, within the target cell that mediate cell-death. The two pathways differ in how they activate the caspases. Contained in the lytic granules of a CTL are perforin, a Ca²⁺-dependent pore-forming protein similar to the C9 component of complement, and an array of cathepsin-like proteases that are collectively referred to as granzymes. Perforin molecules “perforate” the membrane of the target cell,

thereby, facilitating the entry of granzymes into target cells in a manner that is still much debated (137). Five different granzymes can be found in humans, but granzymes A and B predominate in human CTL and are each capable of proteolytically activating cell death pathways, which ultimately cause fragmentation of the target cell's DNA (138,139). Caspase-8 is recruited to the cytoplasmic tail of the Fas receptor, via the adaptor protein Fas-Associated protein with Death Domain (FADD) upon ligation of Fas on the target cell with FasL on the CTL, moving the cell into apoptosis (140-142). Recruitment of caspase-8 to the receptor complex results in activation of this protease, which in turn amplifies downstream caspase activation, either directly (type I pathway) or indirectly by cleaving Bid and provoking cytochrome c release from mitochondria (type II pathway) that activates the Apaf - 1/caspase- "apoptosome". The apoptosome then promotes activation of downstream effector caspases that kill the cell (136,143). Eventual cell death could take between 1-7 hours and both the perforin-granzyme and Fas-FasL pathways contribute to this. The CTL detaches from the target cell and pursues another target cell as a "serial killer" upon the delivery of the "lethal hit" as the CTL escapes intact. This is achieved by the delivery of the lethal hit in immediate juxtaposition to the target cell membrane.

While immunodominance can be beneficial in that it generates potent immune responses to resolve infections, and contributes to the memory T cell pool to promote rapid immune responses following re-infection; it can be unfavourable in HIV infection due to the high mutation rate of HIV (144). Mutation of the immunodominant epitope in HIV infection favours the emergence of escape variants that evade immune recognition (144).

1.2.6 Importance of Cytotoxic CD8⁺ T Cells (CTL) in Controlling HIV Infection

CTL are potent effector cells of the adaptive immune response that function through direct recognition and killing of malignant cells or virus-infected cells. This is achieved either through the Fas-FasL pathway or the perforin-granzyme pathway, or through secretion of pro-inflammatory cytokines that can raise an antiviral state in neighboring cells (145). The importance of CD8⁺ T cells in controlling HIV infection has been well documented. Emergence of HIV-specific CD8⁺ T cells in primary HIV-1 infection is associated with viremia containment to set-point levels (35,39). This is in turn accompanied by the generation of sequence mutations within immunodominant HIV-1 epitopes, thereby giving rise to CTL escape variants of the virus, which further confirms the selective immune pressure exerted by CTL on HIV-1 replication (37,146,147). The importance of CTL in containment of viremia was also observed in primary simian immunodeficiency virus (SIV) infection in rhesus macaque models (148). Further studies additionally showed that AIDS-related mortality fell and SIV set-point levels were significantly reduced by 2.4 log during vaccination with a T-cell based vaccine that induced potent and broad CTL responses (149). More importantly, enhanced control of viremia has been linked with the expression of particular class I human histocompatibility-linked leukocyte antigens (HLA) such as B*5701, B*51 and B*27 in humans (150,151) and Mamu-B*08 in rhesus monkeys (152).

Although the precise mode of action of these HIV-specific CD8⁺ T cells in containing HIV-1 infection is not fully understood, Wong *et al.* argued that the initial systemic suppression of SIV viremia by SIV-specific CD8⁺ T cells is achieved mainly

through non-cytopathic means (153). Investigators generally believe viral suppression is achieved through their ability to produce pro-inflammatory cytokines such as IFN- γ , TNF- α , growth factor IL-2, chemokine macrophage inflammatory protein-1 β (MIP-1 β) and their ability to undergo clonal expansion upon activation and degranulate upon contact with target cells (154,155). However, progressive loss of CTL effector functions has been reported in HIV-1 infection, a phenomenon often referred to as “exhaustion”.

1.2.7 CD8⁺ T Cell Exhaustion: characteristics and causes

T cell exhaustion is a phenomenon characterized by hierarchical loss of antigen-specific T cell functions, which can ultimately result in clonal deletion. This T cell dysfunction is often reflected in a reduced ability to produce IL-2 and TNF- α , reduced proliferation, a reduction in cytotoxicity, and increased susceptibility to apoptosis relative to polyfunctional T cells (156). Exhausted T cells remain in a monofunctional, IFN- γ -producing state before undergoing clonal deletion (157). Exhaustion was first observed in chronic lymphocytic choriomeningitis virus (LCMV) infection in mice (158). It has since been reported in some cancers, hepatitis B virus (HBV), hepatitis C virus (HCV) and HIV-1 infections (159,160). Accumulation of dysfunctional T cells in progressive HIV-1 infection is also associated with skewed differentiation into an effector memory phenotype. Chemotaxis of effector memory cells is impaired with high viremia, thereby posing another challenge to controlling viral burden (161-163).

Studies revealed that viral persistence and loss of CD4⁺ T cell help in progressive HIV/SIV-infection significantly contribute to CTL exhaustion (164,165). A genome-wide microarray analysis carried out on exhausted CD8⁺ T cells in mice, chronically infected with LCMV, implicated members of the B7/CD28 and TNF/TNFR families, which are exclusively upregulated either individually or in combination, in development of exhaustion. Inhibitory receptors upregulated on exhausted LCMV-specific CD8⁺ T cell in chronic LCMV infection included CTLA-4, PD-1, lymphocyte-activation gene-3 (LAG-3), CD160, 2B4 (CD244), GP49 and paired-immunoglobulin like receptor B (PirB) relative to functional effector or memory cells (166,167).

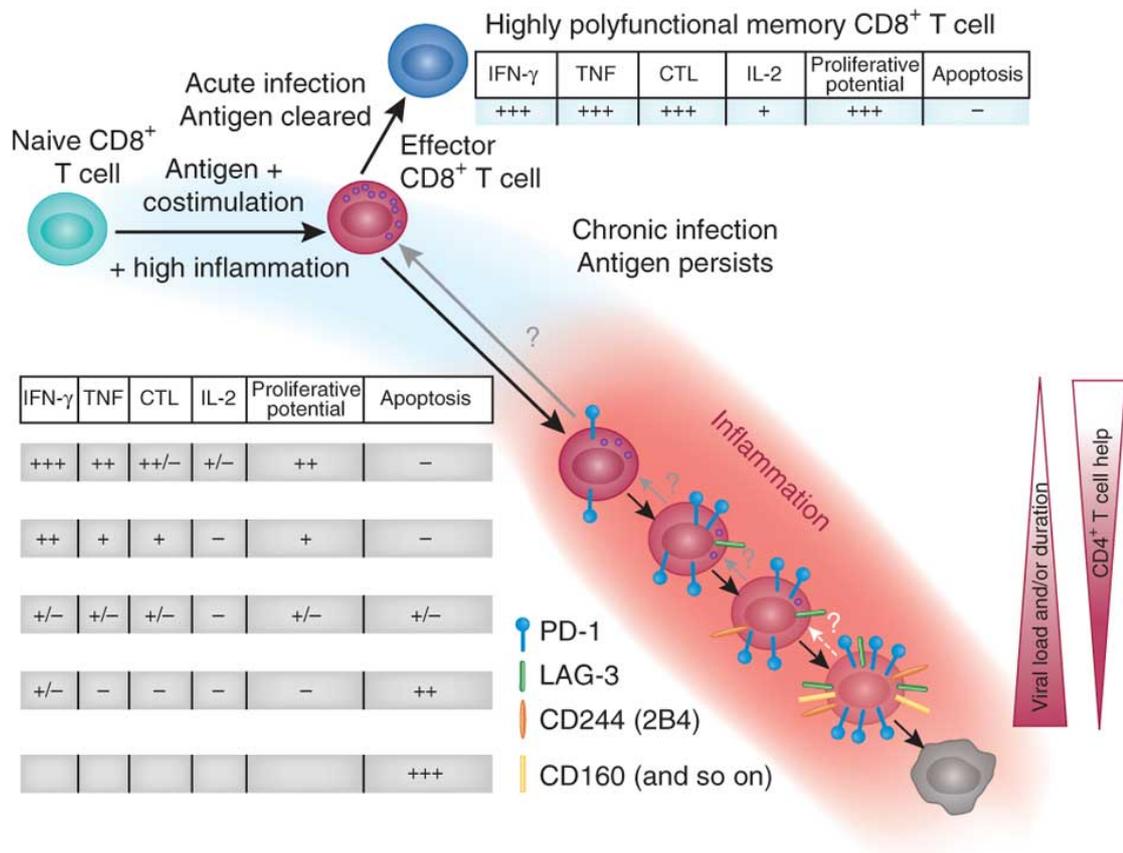


Figure 1.5: T cell exhaustion. A naïve CD8⁺ T cell becomes primed by an antigen with supporting costimulation, and undergoes clonal expansion into effector CD8⁺ T cells which clear viremia. Some clones differentiate into highly polyfunctional memory CD8⁺ T cells which are capable of producing pro-inflammatory cytokines (TNF- α , IFN- γ , and IL-2), proliferate and are resistant to apoptosis. A number of these polyfunctional memory CD8⁺ T cells, however, become functionally impaired in a hierarchical manner as the primary infection progresses to a chronic phase and antigen persists. IL-2 production and proliferation are the first functions lost. Exhaustion become severe with increased viremia and reduced CD4⁺ T cell help and in some cases, these exhausted CD8⁺ T cells die by apoptosis (clonal deletion). Markers associated with T cell exhaustion include PD-1, LAG-3, 2B4, CD160. Excerpt from (159).

Inhibitory signals provided by LAG-3, once bound to its MHC-II ligand, attenuate T cell activation, proliferation, and renewal through cell cycle arrest (168-170). Blockade of LAG-3 alone on LCMV-specific CD8⁺ T cells was not sufficient to reverse exhaustion or reduce viremia (171,172). 2B4 signaling on CD8⁺ T cells can either be activating or inhibitory (173). However, elevated expression of 2B4 has been shown to be inhibitory and contribute to HCV- and LCMV-specific CD8⁺ T cell exhaustion (171,174,175). Expression of CD160 on LCMV-specific CD8⁺ T cells and its coexpression with PD-1 on HIV-specific CD8⁺ T cells is a marker of disease progression, and also defines T cell subsets with advanced impairment (167,171,176). Blocking the interaction of CD160 with its ligand, herpes virus entry mediator (HVEM), enhances proliferation and cytokine production in “exhausted” cytomegalovirus (CMV)- and HIV-specific CD8⁺ T cells (176). Upregulation of PD-1 in chronic HIV-1, HBV and HCV-infections in humans and SIV-infection in rhesus macaques has been linked to antigen-specific T cell dysfunction (164,177). Other negative regulators of T cell activation such as T cell immunoglobulin mucin-3 (Tim-3) and CTLA-4 have also been implicated in HIV and HCV-specific CD4⁺ and CD8⁺ T cell functional impairment as the expression levels of these receptors correlated with failure to control viremia (165,178). Also, coexpression of multiple inhibitory molecules on cancer and HIV-specific CD8⁺ T cells defines subsets with advanced dysfunction (179,180).

A dramatic decline in the expression of these inhibitory molecules was observed in HIV-1 infected subjects treated with cART, although effector functions were not restored in HIV-specific CD8⁺ T cell upon successful treatment

(165,177,181). *In vitro* blockade of PD-1 and LAG-3 interaction with their ligands restored effector functions such as cytokine production, enhanced proliferation and reduced apoptosis (182). Blocking the interaction of PD-L1 with PD-1 *in vivo* also reduced SIV viremia in rhesus macaques and led to clearance of LCMV in mice, though restoration of T cell effector functions was only partial (182,183).

1.3 Immunotherapeutic Vaccines against HIV

Unlike prophylactic vaccines, which provide protection against invading pathogens for uninfected persons, therapeutic (treatment) vaccines are designed to modulate the ongoing immune response in unresolved infections or other chronic conditions. Most therapeutic vaccines are engineered to induce cell-mediated immunity rather than humoral immunity by enhancing existing, or generating new immune responses towards chronic pathogens or tumor antigens.

While most HIV-infected individuals have strong initial CTL responses in the primary phase, chronic infection becomes established partly due to the selective immune pressure exerted on the virus by the HIV-specific CD8⁺ T cell (37). Viral persistence, partly due to inefficient recognition of HIV escape mutants, alters HIV-specific CD8⁺ T cell immunodominance and could precede functional impairment of these HIV-specific T cells (184).

Introduction of cART has dramatically reduced morbidity and mortality among individuals with chronic HIV infection (185). However, HIV-specific CD8⁺ T cell dysfunction persists through successful ART treatment (181). In spite of the improvement in HIV management, there is need for a life-time strict adherence in

order to maintain effective control. Also, the cost of ART, adverse drug effects and the risk of developing resistance are still problematic for many infected individuals. Furthermore, the annual economic burden posed by the HIV epidemic in both the developing and developed world has hindered economic growth. In fact, a recent study shows that for every 10 persons beginning ART, 16 are newly infected with HIV (186). This demonstrates the urgency of exploring other alternatives such as immune-based strategies, which will be economically important and reduce the requirement for “life-time cART adherence”.

The intent of a therapeutic vaccine is to induce a potent anti-HIV immune response or enhance the existing response by deliberate exposure to HIV antigens. Ideally, responses generated through this means should contain viral replication at undetectable levels – a situation found in a small population of HIV-infected ART-naïve individuals (elite controllers) that do not rapidly progress towards AIDS. This phenomenon is termed natural “functional cure” (187). Several lines of evidence suggest that partial control of HIV replication could delay the need for initiation of cART in ART-naïve patients or allow “drug-holidays” in patients on cART. However, partial control of HIV replication might not have maximum clinical benefit because HIV-infected ART-naïve patients with high CD4⁺ cell counts still have higher chances of dying from non-AIDS-related diseases than the general population (188,189). Also, the early initiation of cART significantly improves HIV prognosis in comparison with delayed treatment (190). Furthermore, life expectancy has been greatly reduced in patients placed on “drug-holidays” with the intention of reducing untoward drug effects (191). It is, therefore, desirable for HIV therapeutic vaccines to completely

eradicate infection rather than achieve partial control, although most researchers believe complete viral clearance is impossible due to the virus' ability to integrate into the genome and remain latent for decades (192). Nonetheless, if a therapeutic vaccine is able to partially contain viremia, this will be an important improvement and will justify further investigation into designing new candidate vaccines.

Evidence of functional cure in the Berlin patient suggests that purging the HIV reservoir is possible (193,194). There are on-going clinical trials combining various approaches, such as therapeutic vaccination, gene therapy and cART to drive viral loads down to complete remission (195). There is currently no Food and Drug Administration (FDA) approved therapeutic vaccine for human use (192). Initial HIV vaccine candidates tested such as whole inactivated virus (REMUNE) or recombinant protein (gp120) were administered as prophylactic vaccines, and immune responses generated by these vaccines were disappointing (196,197).

1.3.1 Applying Heteroclitic Peptides to Therapeutic Vaccination

Although specific, TCR are able to interact with multiple peptide antigens (198). If each clone in the T cell repertoire could only recognize one cognate antigen and was non-cross-reactive, there would be limited T cell responses and the repertoire would have to be enormous. The number of T cells specific for a single antigenic determinant is small, and at least one T cell in a few thousand must respond to a foreign peptide in order to generate a substantial immune response (199). There is, therefore, some need for T cell cross-reactivity. Recent studies have suggested the ability of a TCR to recognize more than one peptide (200). We can, therefore,

potentially exploit the “cross-reactivity” of T cells to generate desired immune responses.

Heteroclitic peptides are sequence variants of native peptide epitopes that stimulate T cell responses superior to the native epitope. Heteroclitic peptides contain amino acid substitutions that can enhance peptide-binding affinity for human histocompatibility-linked leukocyte antigens (HLA) and/or improve TCR recognition (201,202). Evidence in several experimental systems suggests that heteroclitic peptides stimulate more potent immune responses against their respective native peptide epitope than the native peptide itself (202). Potentially heteroclitic variants have been previously generated using HLA-A2 and A3-restricted tumor-associated peptides (9mers and 10mers) and HLA-A2-restricted viral peptides including, HBV Pol.455 and HIV Pol.476 (both 9mers). Regardless of the native peptide epitope, variant epitopes showing heteroclitic activity had conservative or semiconservative amino acid (aa) substitutions only at positions 3, 5, or 7, and these heteroclitic epitopes elicited up to a 107-fold increase in T cell responses (203). Evidence from X-ray crystallography-inferred 3-dimensional structure of HLA-A2 and A3 pMHC:TCR complexes show that side chains of aa at positions 3, 5 and 7 in these MHC molecules interact directly with the complementarity determining region 3 (CDR3) of the TCR α and β chains (204,205). A table of aa similarity scores proposed by Sette *et al.* was derived 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 aa side chain volume (measured by H₂O displacement) for each aa pair (203). We propose that candidate heteroclitic

variants of HIV native peptide epitopes can be generated in a similar manner as done by Sette *et al.* tested and incorporated into therapeutic HIV vaccines.

1.4 Rationale for this Study

Chronic HIV-infection often leads to ineffective CD8⁺ T cell responses, in the form of reduced cytokine production, decreased proliferation and ultimately apoptosis of HIV-specific CD8⁺ T cells. Inhibitory signals elicited by some TCR co-receptor molecules such as PD-1 have been implicated in this functional impairment. We previously demonstrated that heteroclitic peptides enhance IFN- γ and IL-2 production by HIV-specific CD8⁺ T cells (206). This finding led to the exploration in this study of whether heteroclitic peptides also improve HIV-specific CD8⁺ T cell proliferation and reduce PD-1 expression. In the second part of this work, we tested the possibility that heteroclitic peptide stimulation in the presence of 4-1BB costimulation or absence of the inhibitory PD-1 pathway may further improve T cell responses elicited by heteroclitic peptides.

Heteroclitic variant epitopes augmenting IL-2 and/or IFN- γ production by HIV-specific CD8⁺ T cells were initially identified by ELISPOT. We then measured heteroclitic peptide-driven proliferation and examined the effect of heteroclitic peptide stimulation on PD-1 expression by HIV-specific CD8⁺ T cells. Also, we investigated the effect of PD-1 signaling blockade or 4-1BBL costimulation on HIV-specific CD8⁺ T cells responding to heteroclitic peptide stimulation.

2.0 Materials and Methods

2.1 Ethics Statement

All study participants gave informed consent for whole blood collection and immunological studies. The study protocol was originally reviewed and approved by the Memorial University of Newfoundland Faculty of Medicine Human Investigation Committee and annual renewal is approved by the Newfoundland and Labrador Provincial Health Research Ethics Authority.

2.2 Study Subjects

Whole blood samples from HIV-1-infected individuals (coded 1 to 288) and HIV-uninfected volunteers (coded 1000 and above) were obtained from attendees of the Newfoundland and Labrador Provincial HIV clinic and Faculty of Medicine personnel, Memorial University of Newfoundland, St. John's, Canada respectively. Sera from the HIV-infected subjects had previously been tested for HIV-1 antibodies by ELISA and serostatus confirmed by Western blot. HIV-infected subjects underwent routine clinical assessment with CD4⁺ T cell counts and viral load performed at least once every six months. Most HIV-infected study subjects had achieved viral suppression with cART below clinical detection limits at the time of participation.

2.3 Peripheral Blood Mononuclear Cell (PBMC) Isolation

PBMC were isolated from 20 ml of fresh whole blood collected by forearm venipuncture into vacutainer tubes containing acid-citrate-dextrose (ACD) anticoagulant. Plasma was collected following centrifugation at 400g for 10 min at room temperature (RT), aliquotted immediately and stored at -80°C. The packed cells and buffy coat were diluted to 2x the original blood volume with phosphate buffered saline (PBS) and 15 ml buffy coat / packed cell suspension was layered over 15 ml Ficoll-Paque™ PLUS (GE Healthcare Biosciences AB, Uppsala, Sweden) gradient separation medium and centrifuged at 400g, for 30 min at RT, with no brake. PBMC, which are suspended between the plasma-Ficoll interface, were carefully transferred into a clean 50 ml tube and washed with PBS containing 1% fetal calf serum (FCS) (Invitrogen). After counting, PBMC were resuspended in complete lymphocyte medium (RPMI 1640 supplemented with 10% FCS, 100 µg/ml streptomycin, 100 IU/ml penicillin, 2 mM L-glutamine, 10 mM HEPES buffer solution and 2×10^{-5} M 2-mercaptoethanol; all from Invitrogen) and either used fresh or cryopreserved until needed.

2.4 PBMC Cryopreservation and Thawing

Freshly isolated PBMC were resuspended in freezing medium [80% complete RPMI 1640 medium, 10% supplemental FCS and 10% dimethyl sulfoxide (DMSO)] at 10×10^6 cells/ml, transferred into 2 ml Nalgene® System 100™ cryovials (Thermo Fisher Scientific, Rochester, NY) and incubated overnight in Thermo Scientific™ Mr. Frosty™ freezing container at -80°C. PBMC were transferred to and maintained in the

liquid nitrogen (LN) tank after overnight incubation until required. To thaw cells, cryopreserved PBMC were immediately immersed in a 37°C water bath and gently agitated until the contents were almost completely thawed and then the contents were immediately transferred into a sterile 15 ml tube containing 10 ml complete medium, and washed three times to remove DMSO. Cells were resuspended in complete medium at 2×10^6 cells/ml and rested overnight at 37°C in a 5% CO₂ incubator to allow for recovery. Cells were counted after overnight recovery and all PBMC used were > 50% viable by trypan blue exclusion.

2.5 HLA Typing

Most participants enrolled in this study before September 2012 were fully typed for HLA class I A and B antigens with the Lambda Monoclonal Typing Tray Second HLA Class I, Lot #6A (One Lambda, Canoga, CA) as per manufacturer's instructions. In order to maximize time while B cells are being transformed, newly enrolled participants expressing HLA-A2 were identified with HLA-A2 specific monoclonal antibody (NFLD.M2) (207), which was a kind gift from Dr. Sheila Drover's Laboratory. Briefly, 1×10^5 PBMC were incubated with the primary A2-specific-antibody at 4°C for 30 mins, washed and labelled with a secondary goat anti-mouse IgG fluorescein isothiocyanate (FITC) fluorochrome-conjugated polyclonal antibody for HLA-A2 antigen detection. PBMC, not incubated with the HLA-A2 specific monoclonal antibody, but stained with FITC-conjugated goat anti-mouse IgG served as a control. PBMC were then analysed by flow cytometry for HLA-A2 expression.

2.6 Peptides

HIV peptide pools stimulating IFN- γ /IL-2 responses (Gag and Nef) by HIV-specific CD8⁺ cells were identified by previous students. Peptides pools consisted of sequential overlapping 15mer peptide sets spanning the major HIV clade B antigens (National Institutes of Health AIDS Research and Reference Reagent Program). Peptide pools were deconvoluted with peptide matrices by ELISPOT as previously described (208) to identify individual 15mers responsible for the cytokine responses. Twenty-four potentially heteroclitic variants were synthesized from reference HLA-A2-restricted, optimally defined 9mer HIV peptide epitopes stimulating IL-2 production by HIV-specific CD8⁺ T cell from our HIV-infected study subjects. Conservative and semi-conservative aa substitutions were made at positions 3, 5 or 7 of Nef 83→91, Nef 135→143, Gag 77→85 and 3, 5 or 8 of Gag 433→440. The choice of aa and position of substitution was made as described in section 1.3.1. The rarity or absence of aa at positions of interest in the Los Alamos HIV sequence database

(http://www.hiv.lanl.gov/content/sequence/QUICK_ALIGN/QuickAlign.html) was also considered in selecting the appropriate aa for substitution. HIV peptides were synthesized by PEPTIDE 2.0. Inc., USA. Individual peptides were dissolved in DMSO at 10 mg/ml, and stock solutions of 1 mg/ml peptides in unsupplemented RPMI 1640 were aliquotted. The sequences of all reference peptides tested and their variants are shown in Table 2.1.

Table 2.1: Reference and corresponding variant peptide sequences

Reference Peptide (name, position: aa sequence)	Variant Peptides (name: aa sequence)
A2-7 (Nef83): AAVDLSHFL	A2-7-1: AA <u>L</u> DLSHFL A2-7-2: AA <u>T</u> DLSHFL A2-7-3: AAVD <u>I</u> SHFL A2-7-4: AAVD <u>V</u> SHFL A2-7-5: AAVDLS <u>Q</u> FL A2-7-6: AAVDLS <u>R</u> FL
A2-8 (Nef135): YPLTFGWCF	A2-8-1: YP <u>I</u> TFGWCF A2-8-2: YP <u>V</u> TFGWCF A2-8-3: YPLT <u>Y</u> GWCF A2-8-4: YPLT <u>W</u> GWCF A2-8-5: YPLTFG <u>F</u> CF A2-8-6: YPLTFG <u>R</u> CF
A2-Gag (Gag77): SLYNTVATL	A2-Gag-1: SL <u>W</u> NTVATL A2-Gag-2: SL <u>L</u> NTVATL A2-Gag-3: SLYN <u>S</u> VATL A2-Gag-4: SLYN <u>W</u> VATL A2-Gag-5: SLYNTV <u>G</u> TL A2-Gag-6: SLYNTV <u>M</u> TL
A2-9 (Gag433): FLGKIWPS	A2-9-1: FL <u>S</u> KIWPS A2-9-2: FL <u>N</u> KIWPS A2-9-3: FLGK <u>T</u> WPS A2-9-4: FLGK <u>K</u> IWPS A2-9-5: FLGKIWP <u>V</u> A2-9-6: FLGKIWP <u>V</u> S

AA substitution made are highlighted in red and underlined

2.7 ELISPOT Assay to Measure Peptide-specific IFN- γ and IL-2 Production by HIV-specific CD8⁺ T Cells

The polyvinylidene difluoride (PVDF) membranes of MultiScreen 96-well microtitre plates were activated by wetting with 15 μ l/well of 35% ethanol, and washed four times with PBS. The plates were coated with 100 μ L 7.5 μ g/ml IFN- γ mAb 1-D1K or 15 μ g/ml IL-2 mAb IL2-1/249 (Mabtech, USA), and incubated overnight at 4°C. Plates were washed four times with PBS and blocked with 150 μ L of PBS containing 1% FCS for 2 hrs at 37°C. Fresh or thawed PBMC rested overnight were counted and their viability determined by trypan blue exclusion. PBMC at a concentration of 2×10^6 cells/ml of complete RPMI 1640 medium were plated at 2×10^5 cells/well in duplicate for IFN- γ or 4×10^5 cells/well singly for IL-2 respectively. Cells in individual wells were stimulated with 4 μ g/ml peptides. Unstimulated PBMC served as a negative control while cells stimulated with 4 μ g/ml phytohemagglutinin (PHA) served as a positive control. The plates were washed four times with PBS after overnight incubation at 37°C in a 5% CO₂ incubator. 100 μ l/well of 1 μ g/ml biotinylated anti-IFN- γ mAb 7-B6-1 or anti-IL-2 mAb IL-2-II (Mabtech) detection antibody were added and incubated for 2 hrs at RT. Plates were washed again four more times with PBS and 100 μ l/well of streptavidin-alkaline phosphatase conjugate (ALP) (Mabtech), diluted 1:1000 in PBS supplemented with 0.5% FCS was added for 1 hr. Wells were washed again four times with PBS, and 100 μ l/well of a 1/100 diluted chromogenic ALP substrate (Bio-Rad Laboratories, Hercules, CA) in colour development solution was added. The plates were incubated with the substrate at RT for 20 mins – 1 hr until the emergence of dark-purple spots. Colour development was

stopped by rinsing the plates with tap water. Plates were air-dried overnight and spots were counted with an ImmunoScan ELISPOT reader (Cellular Technology Ltd., Cleveland, OH). Each spot represents a cytokine-producing cell. Wells producing twice the background spots and at least 50 IL-2 spot forming units (SFU)/10⁶ PBMC or 100 IFN- γ SFU/10⁶ PBMC were considered indicative of a positive response to peptide stimulation.

2.8 Proliferation Assays and Assessment of PD-1 Expression on Dividing CD8⁺ T Cells

CellTraceTM carboxyfluorescein succinimidyl ester (CFSE) Cell Proliferation Kits (Invitrogen, Oregon, USA) were utilized in the proliferation assays. The CFSE dye was purchased from the manufacturer in the diacetylated form, carboxyfluorescein diacetate N-succinimidyl ester (CFDA, SE). The diacetate group of the CFDA, SE makes the dye highly membrane permeant. The intracellular esterases cleave the diacetate group from the CFDA, SE to form CFSE. This makes the CFSE more fluorescent and less membrane permeable. The amino-reactive succinimidyl side chains of the CFSE dye then covalently couple the dye to intracellular proteins, thus making the cells almost permanently fluorescent. CFSE was used to monitor lymphocyte division based on the sequential halving of the fluorescent intensity of the daughter cells. PBMC were either used fresh or thawed and rested overnight at 37°C. PBMC resuspended at 1 x 10⁶ cells/ml in PBS supplemented with 5 mM ethylenediaminetetraacetic acid (EDTA) (Sigma) and 0.25 μ M CFSE were incubated in the dark at 37°C for 10 min. Staining was quenched by

adding 5 volumes of ice-cold lymphocyte media and the cells incubated on ice for 5 min. Cells were pelleted by centrifugation, and washed three times with complete lymphocyte medium. Staining was confirmed with a fluorescent microscope. PBMC were then resuspended at 2×10^6 cells/100 μ l lymphocyte medium, and stimulated for 1 hr at 37°C with 100 μ l of peptide at 200 μ g/ml for a final peptide concentration of 100 μ g/ml, resuspended at 1×10^6 cells/ml in complete lymphocyte medium and maintained in culture for 7 days at 37°C in a 5% CO₂ incubator. For proliferation assays with immunomodulating agents, cells were resuspended in complete lymphocyte medium supplemented with either 500 ng/ml of 4-1BBL fusion protein (R&D Systems, USA) or 10 μ g/ml of anti-PD-1 (PD-1.3.1; Miltenyi Biotec Inc., USA). Cells were harvested on day 7 and stained with a two color panel for surface markers using anti-CD8-PerCP (BW135/80; MiltenyiBiotec) and anti-PD-1-APC (EH12.2H7; BioLegend). Staining was performed according to manufacturer's instructions with the use of appropriate isotype controls. Briefly, cells were washed with PBS supplemented with 5 mM EDTA, 0.5% FCS, 0.2% sodium azide (Sigma), pH adjusted to 7 – 7.2 (flow buffer); and then incubated with anti-CD8-PerCP and anti-PD-1-APC at 4°C for 20 min. Cells were washed again with flow buffer and resuspended in 1% paraformaldehyde. 1×10^5 events (cells) were acquired for analysis within one week of staining with a FACSCalibur™ Cell Analyzer (BD Biosciences). Data was analyzed using WINMDI 2.8 software.

2.9 Statistical Analysis

All statistical analyses were performed using the GraphPad Prism (version 4.0) statistical software package (Graph-Pad Software, San Diego, CA). Normal distribution of data was assessed by the Kolmogorov-Smirnov test and non-parametric testing done for comparisons. Statistical significance (p value) of the effect of heteroclitic peptide stimulation on PD-1 expression was calculated by using Wilcoxon signed-rank test. A p value < 0.05 was considered statistically significant. Correlation between lower PD-1 expression and increased proliferation was assessed by linear regression analysis.

3.0 Results

3.1 Heteroclitic Peptide Identification

Our laboratory previously demonstrated that heteroclitic peptides can enhance IFN- γ and IL-2 production by HIV-specific CD8⁺ T cells (206). In this study, we first identified additional heteroclitic peptides by ELISPOT assay. PBMC from over 80 HIV-infected individuals were screened in duplicate for IFN- γ or in single test for IL-2 production by HIV-specific CD8⁺ T cells responding to one or more of the four peptide sets used in this study (Table 2.1). All peptides were 9mers except A2-9 and variants A2-9-1, A2-9-2, A2-9-3, and A2-9-5, which are 8mers. Heteroclitic peptides were selected based on previously described criteria. Briefly, after background subtraction, variant peptides that stimulated ≥ 100 more IFN- γ SFU/10⁶ PBMC or ≥ 50 more IL-2 SFU/10⁶ PBMC than the corresponding reference peptides were considered heteroclitic, provided that the total SFU/10⁶ PBMC was ≤ 1000 for IFN- γ or ≤ 500 for IL-2. In the case of responses ≥ 1000 IFN- γ SFU/10⁶ PBMC or ≥ 500 IL-2 SFU/10⁶ PBMC, variant peptides were considered heteroclitic when they stimulated $\geq 10\%$ more IFN- γ or IL-2 SFU/10⁶ PBMC than the reference peptide (206). PBMC from 25 subjects with a positive response to one or more of the reference peptides were further tested with their respective variants by ELISPOT assays (Table 3.1). Using the criteria above, we identified 29 heteroclitic peptides with PBMC from 9/25 tested subjects (Table 3.2). Most of the heteroclitic peptides identified augmented IFN- γ production by HIV-specific CD8⁺ T cells, which didn't always correlate with IL-2 responses (representative example is shown in Figure 3.1.A). This was the common trend observed with cytokine responses induced by the variant peptides

tested in our cohort. However, there were instances (A representative example is shown in Figure 3.1.B) where heteroclitic peptides enhanced both IFN- γ and IL-2 responses.

Table 3.1: Number of subjects with positive IFN- γ and/or IL-2 responses against one or more peptides in each set

Peptide Set	A2-7	A2-8	A2-9	A2-Gag
Fraction of tested subjects with IFN- γ responses	10/25	14/25	12/25	13/25
Fraction of tested subjects with IL-2 responses	1/14	3/14	6/14	2/14

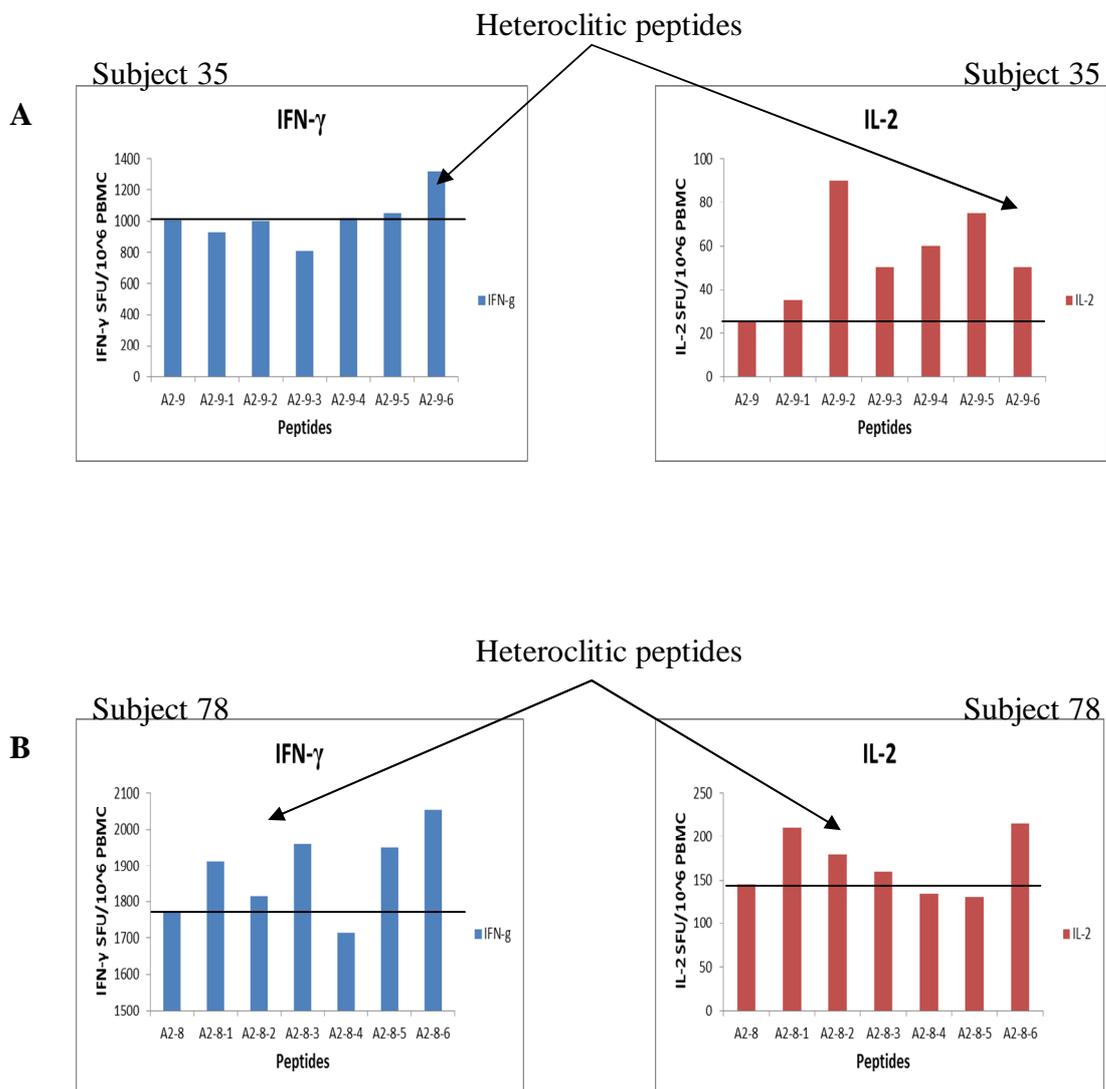
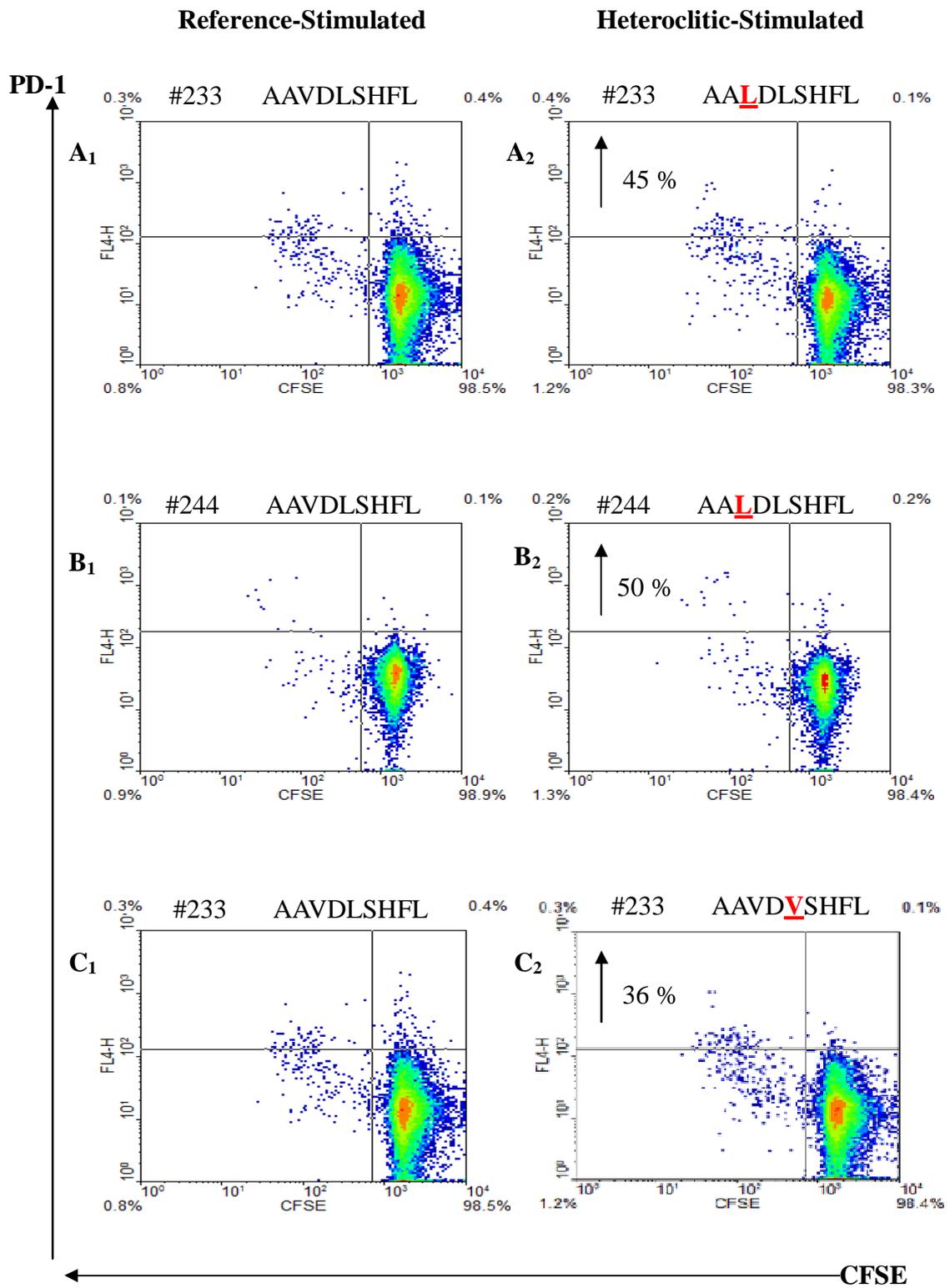


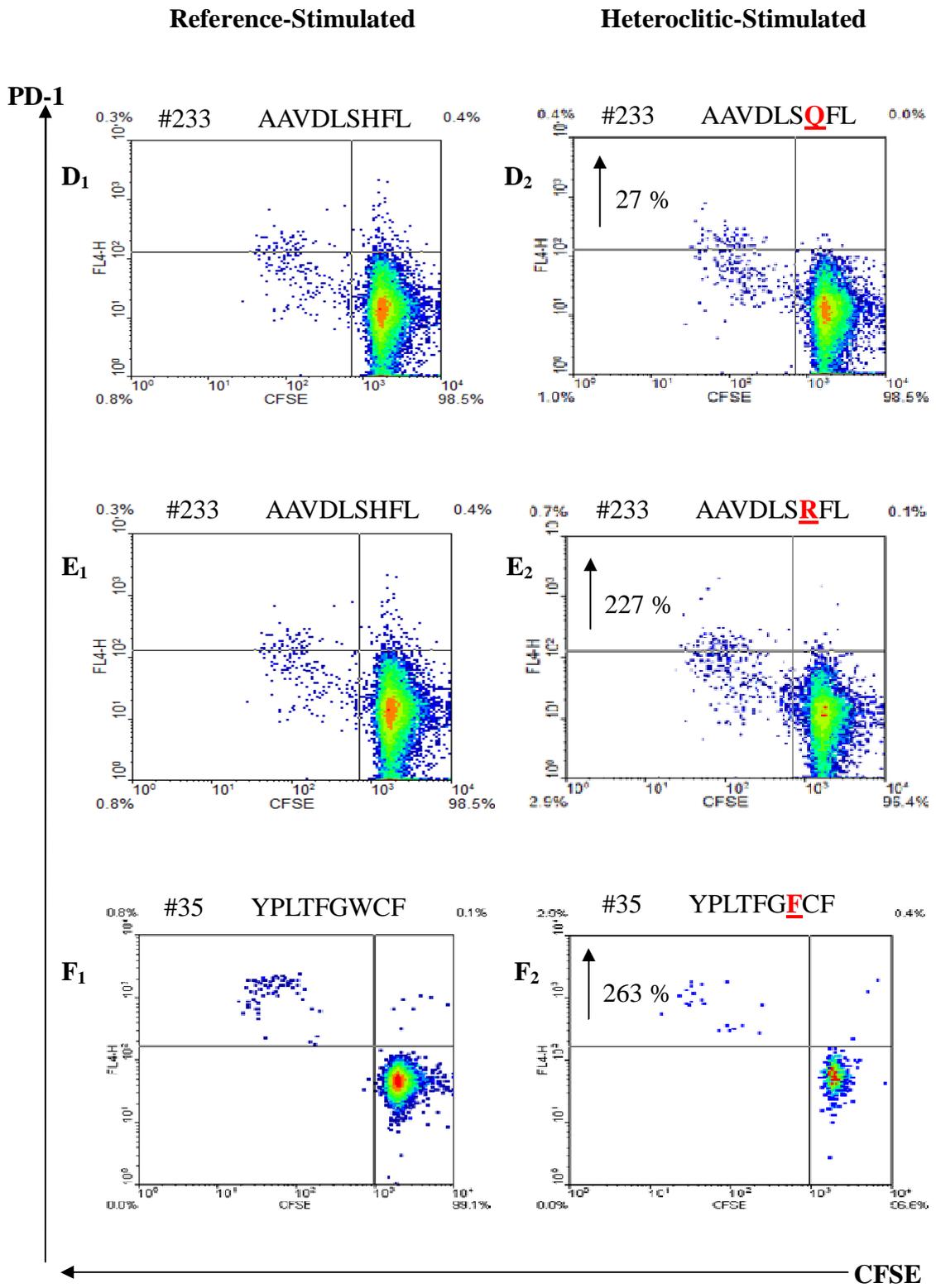
Figure 3.1: Representative cytokine expression patterns of HIV-specific CD8⁺ T cells stimulated with reference or variant peptides in ELISPOT assays. (A) Representative case of no association between IFN- γ production and IL-2 responses. (B) Representative case where heteroclitic peptide enhanced both IFN- γ and IL-2 production.

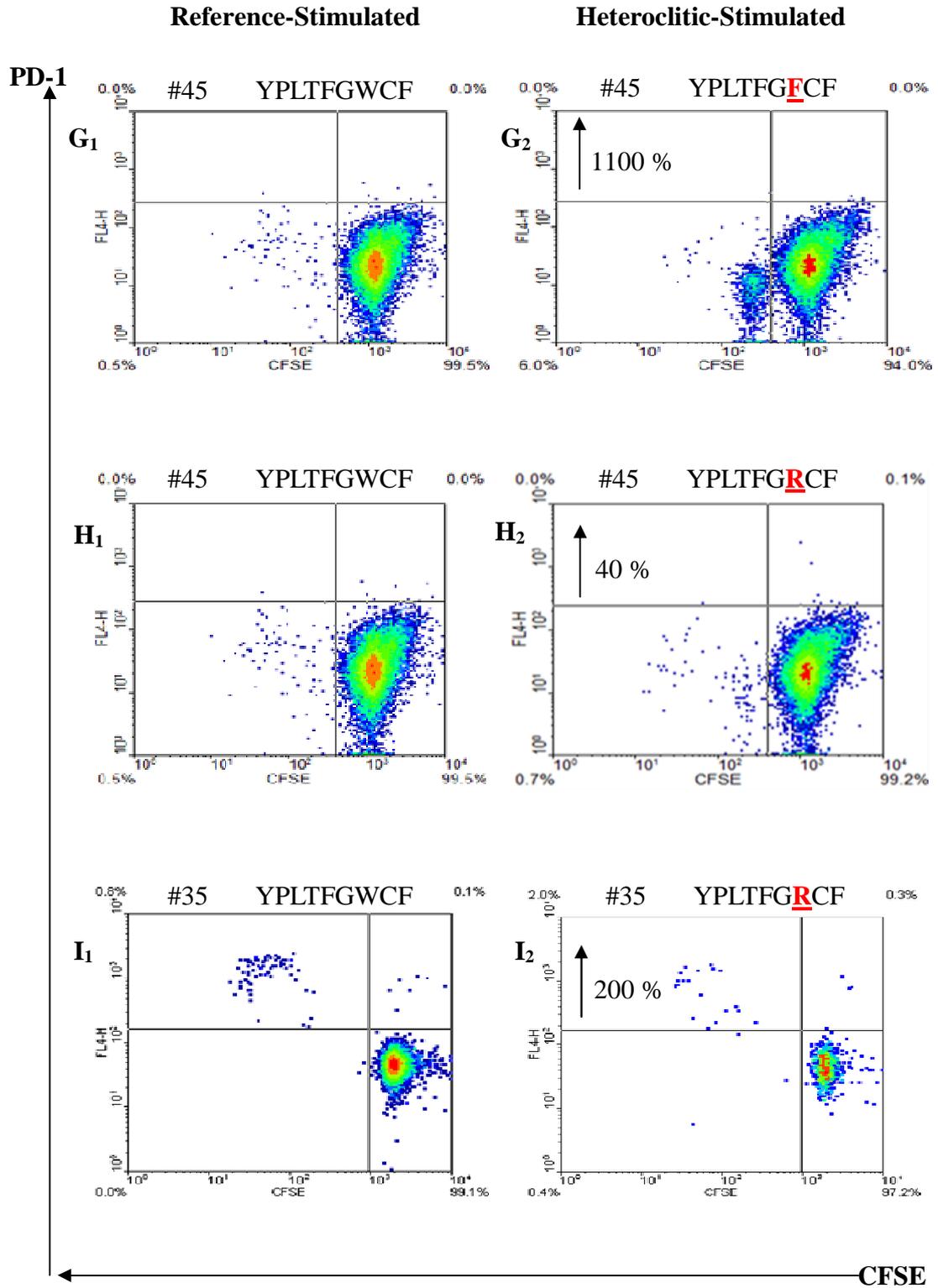
3.2 Heteroclitic Peptide Stimulation Resulted in Enhanced HIV-specific CD8⁺ T Cell Proliferation

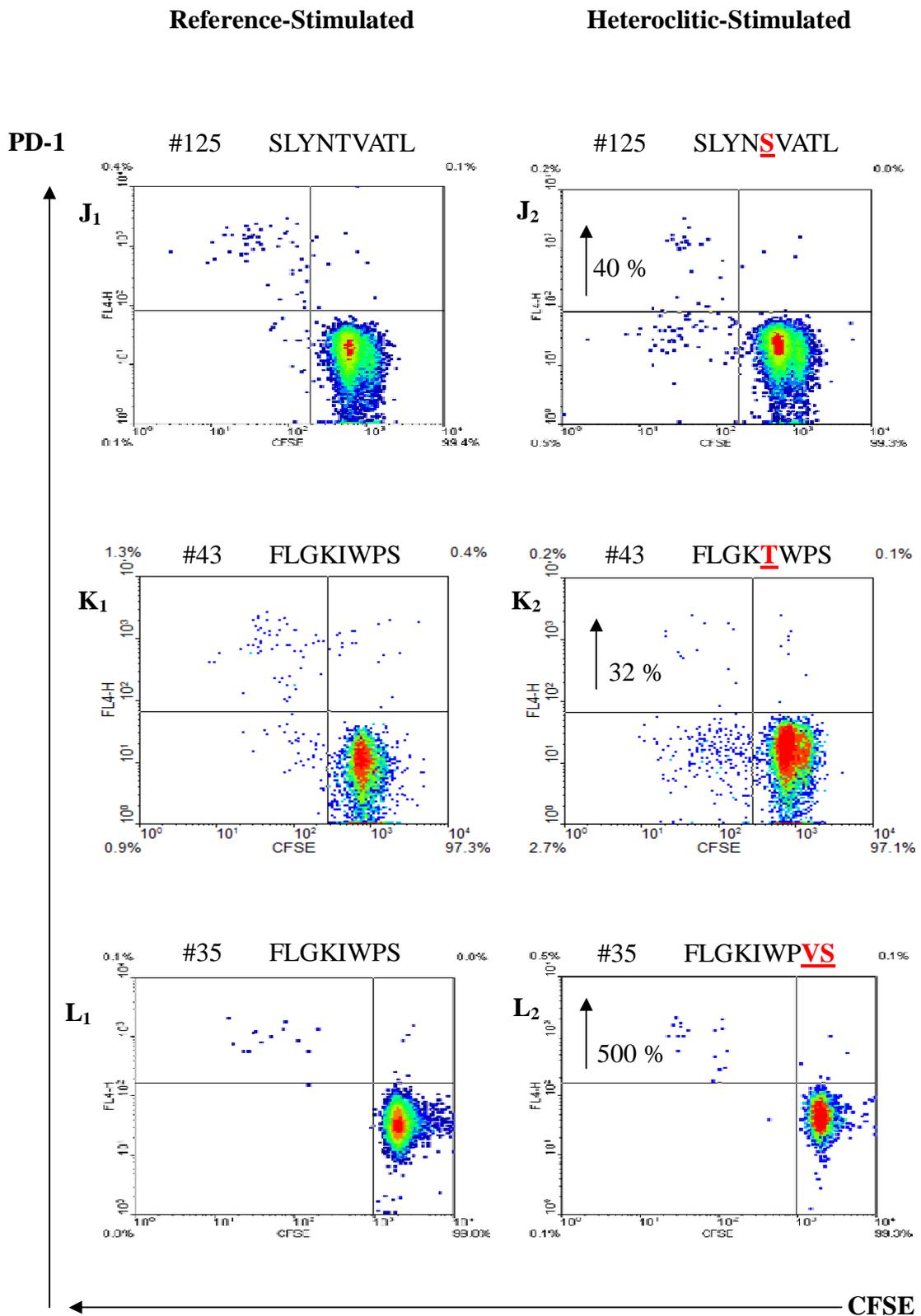
To follow up on our finding that heteroclitic variants of HIV peptides enhance IFN- γ and IL-2 production by HIV-specific CD8⁺ T cells, we investigated whether they also enhance CD8⁺ T cell proliferation. PBMC from nine HIV-infected subjects previously identified with strong IFN- γ and/or IL-2 responses against heteroclitic peptides by ELISPOT assay were labeled with CFSE, stimulated with reference or heteroclitic peptides, and then left in culture for seven days. Cells were harvested on day 7, labeled with anti-CD8 and anti-PD-1, and analysed by flow cytometry. Twenty-nine previously identified heteroclitic peptides were tested in proliferation assays in comparison to their respective reference peptides. Heteroclitic peptides enhanced IFN- γ production in the majority of the cases selected for proliferation testing, except cases A2-9-3; #45, A2-9-6; #45, and A2-8-6; #78, where both IFN- γ and IL-2 production were enhanced (Table 3.2). We measured proliferation based on the fluorescence intensity of the dividing cells as the parental population remains CFSE^{bright}, while fluorescence reduces with each subsequent generation of cells (CFSE^{dim}). Only cases where either reference or heteroclitic peptides drove more than 0.5% absolute CD8⁺ T cell proliferation were considered for comparison. Proliferation was considered enhanced when heteroclitic peptides caused a > 20% increase in the percentage of proliferating CD8⁺ T cells relative to the reference peptides. Heteroclitic peptides enhanced total CD8⁺ T cell proliferation in 13/29 cases (Figure 3.2), and induced similar CD8⁺ T cell proliferation with reference peptides in 7/29 cases (Figure 3.3).

This demonstrates that heteroclitic peptides stimulated equivalent or greater CD8⁺ T cell proliferation than reference peptides in 20/29 cases.









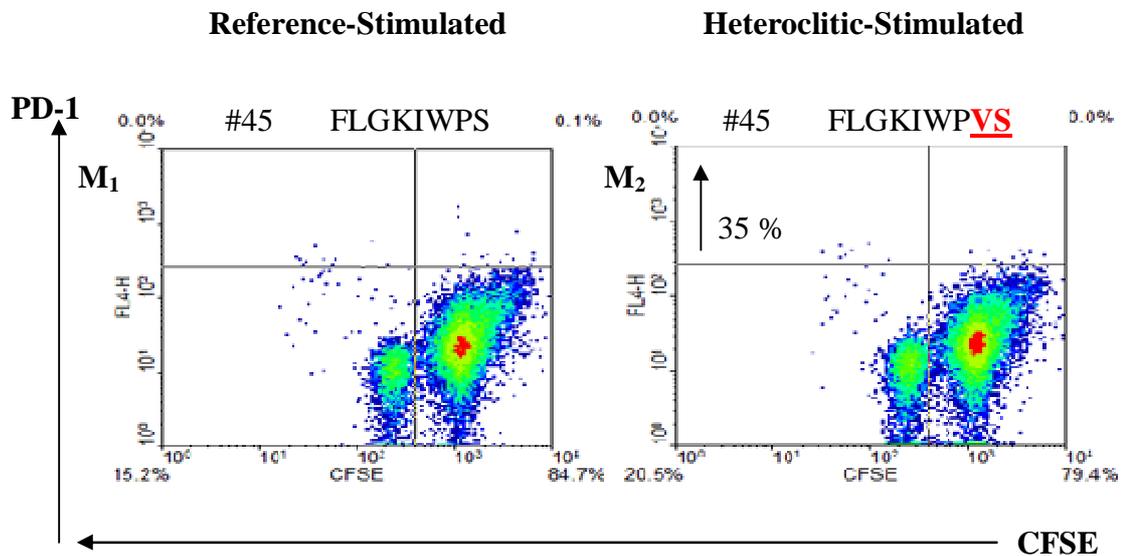
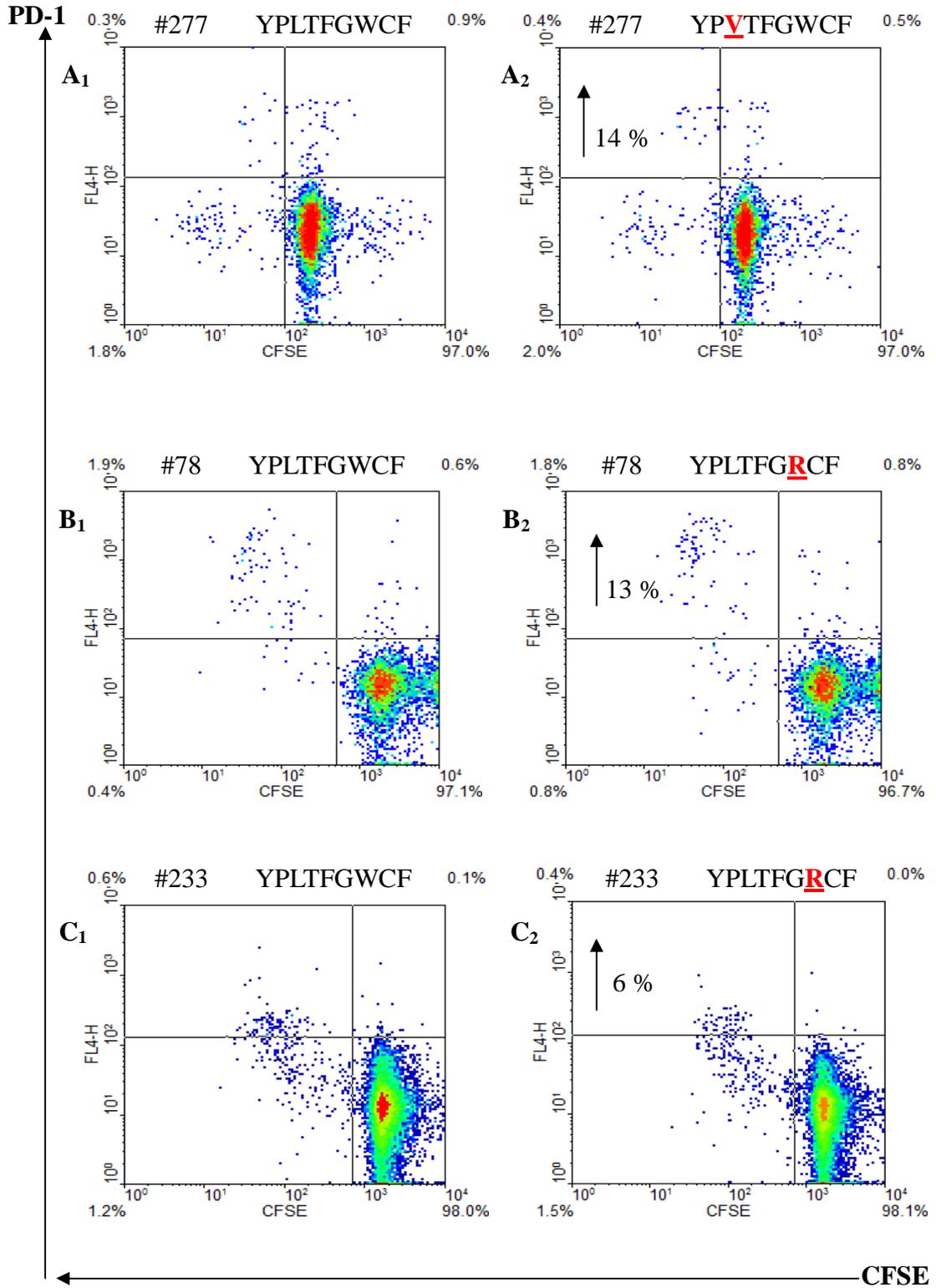
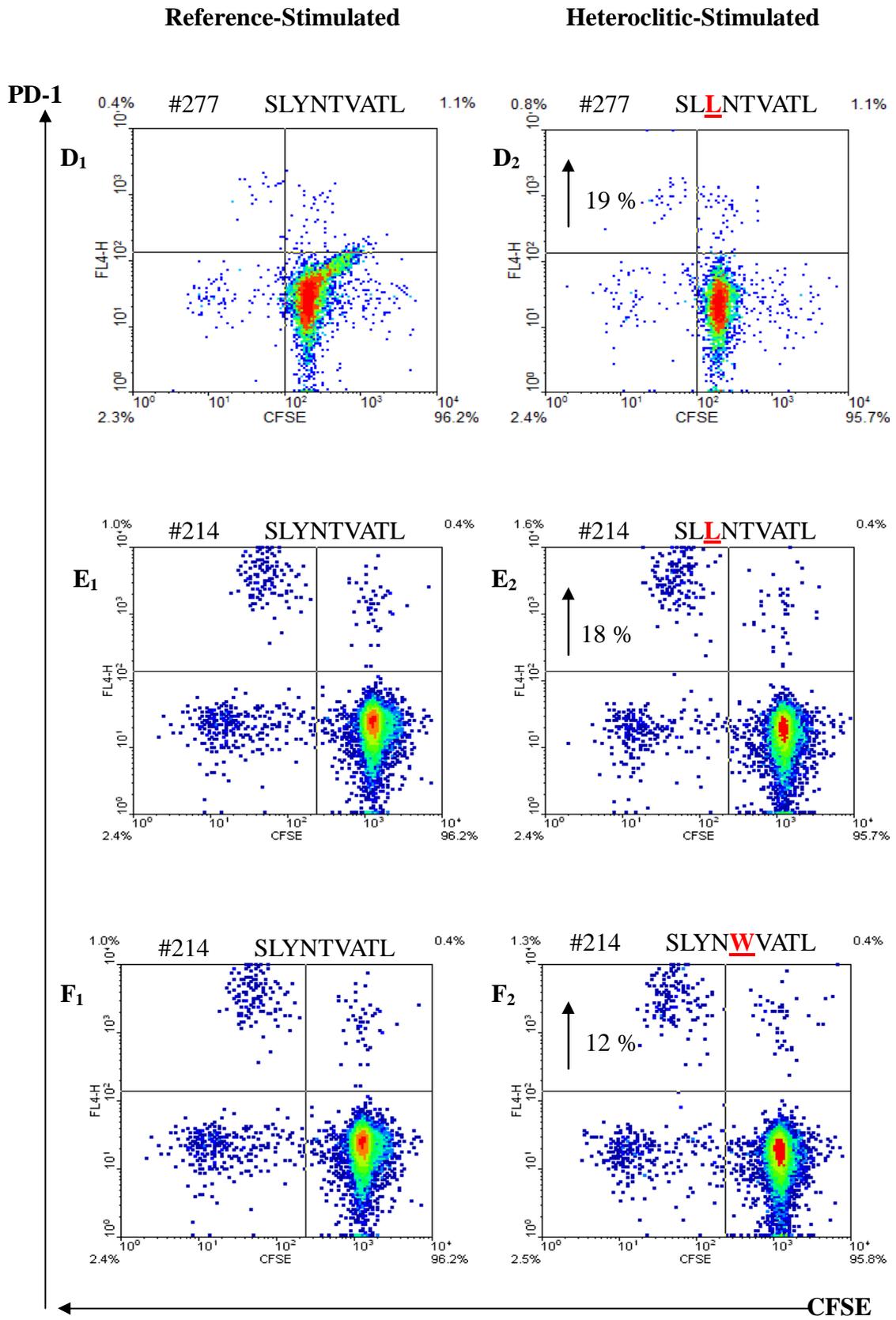


Figure 3.2: Comparison of CD8⁺ T cell proliferation induced by reference or heteroclitic peptides I. PBMC from study participants were CFSE labeled and stimulated with reference or heteroclitic peptides in 7 day culture. CD8⁺ T cells were gated for analysis and results plotted as PD-1 vs. CFSE. Numbers in the quadrants indicate the percentage increase in the proliferating CD8⁺ T cells stimulated with heteroclitic peptides relative to reference peptides. The amino acid substitution made in the heteroclitic peptide sequence is highlighted in red and underlined above the plots. Results on PD-1 expression are discussed in section 3.3.

Reference-Stimulated

Heteroclitic-Stimulated





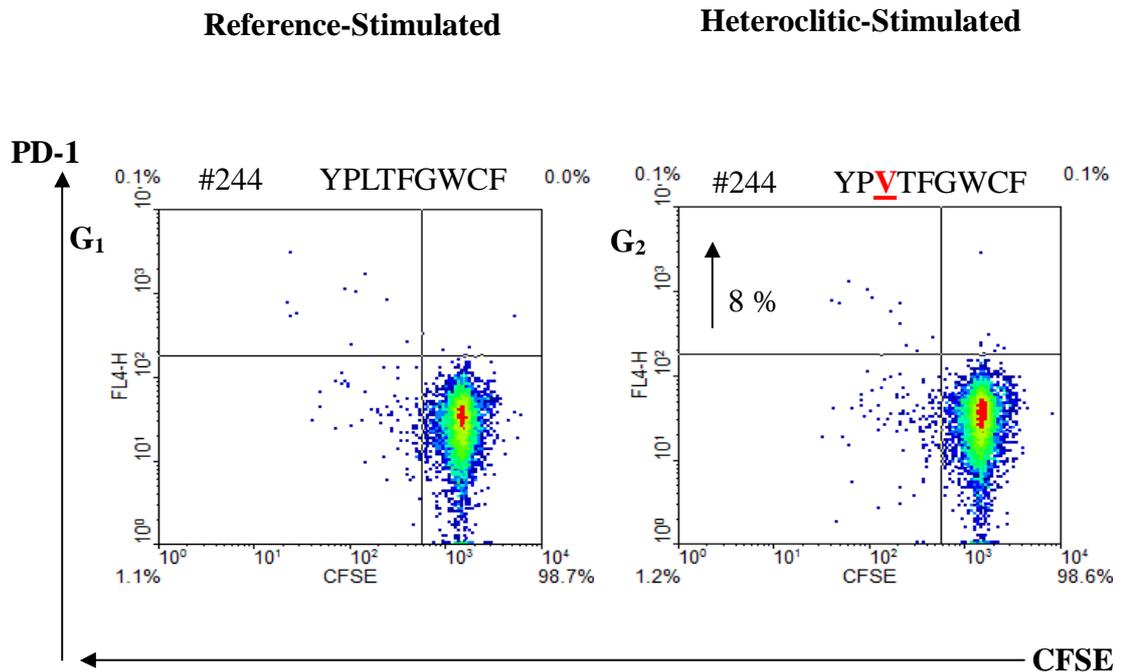


Figure 3.3: Comparison of CD8⁺ T cell proliferation induced by reference or heteroclitic peptides II. PBMC from study participants were CFSE labeled and stimulated with reference or heteroclitic peptides in 7 day culture. CD8⁺ T cells were gated for analysis and results plotted as PD-1 vs. CFSE. Numbers in the quadrants indicate the percentage increase in the proliferating CD8⁺ T cells stimulated with heteroclitic peptides relative to reference peptides. The amino acid substitution made in the heteroclitic peptide sequence is highlighted in red and underlined above the plots. Results on PD-1 expression are discussed in section 3.3.

3.3 Heteroclitic Peptide Induced Lower PD-1 Expression on Proliferating HIV-specific CD8⁺ T Cells

Elevated expression of PD-1 on HIV-specific CD8⁺ T cells in chronic HIV infection has been linked to their dysfunction. This compelled us to investigate the level of PD-1 expression on proliferating CD8⁺ T cells stimulated with reference or heteroclitic peptides. PBMC from nine HIV-infected subjects previously identified with heteroclitic responses to variant peptides in ELISPOT assay were labeled with CFSE, stimulated with reference or heteroclitic peptides and then left in culture for seven days. Cells were harvested on day 7, labeled with anti-CD8 and anti-PD-1 and analysed by flow cytometry. Twenty-nine cases of previously identified heteroclitic peptides were tested in proliferation assays in comparison to their respective reference peptides. The median percentage of proliferating CD8⁺ T cells expressing PD-1 in the group stimulated with reference peptides was significantly higher than in the group stimulated with heteroclitic peptides [Figure 3.4; median PD-1^{hi} CFSE^{dim} CD8⁺ T cells 29% with interquartile range (IQR) 19.5% – 79.5% in the reference peptide stimulated group versus 25%, IQR 17.5% – 64.5% on CD8⁺ T cells stimulated with heteroclitic peptides, n=29, *p* value = 0.005 (Wilcoxon signed-rank test)]. The level of PD-1 expression in the CFSE^{bright} CD8⁺ T cells was < 2% (range 0 – 1.6%) when stimulated with either reference or heteroclitic peptides. There was lower PD-1 expression on proliferating CD8⁺ T cells (15% - 88%) in response to heteroclitic peptide stimulation in 13/29 cases relative to reference peptide stimulation (Figure 3.5).

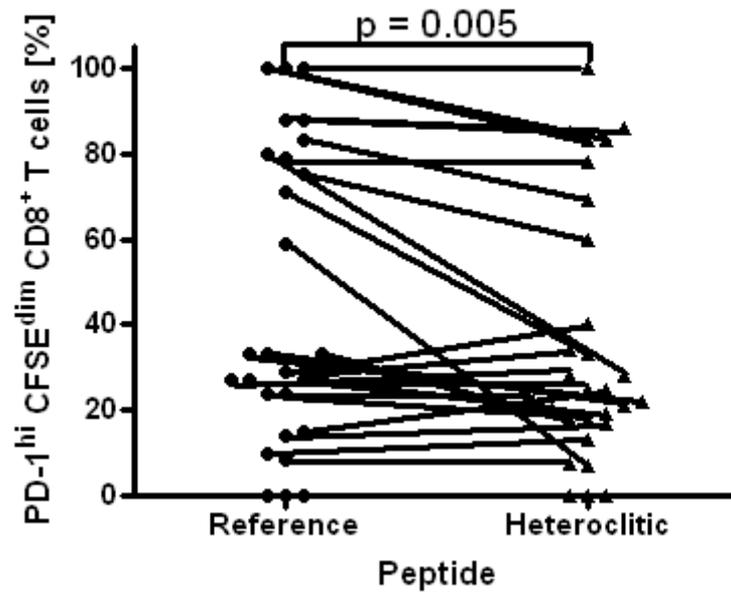
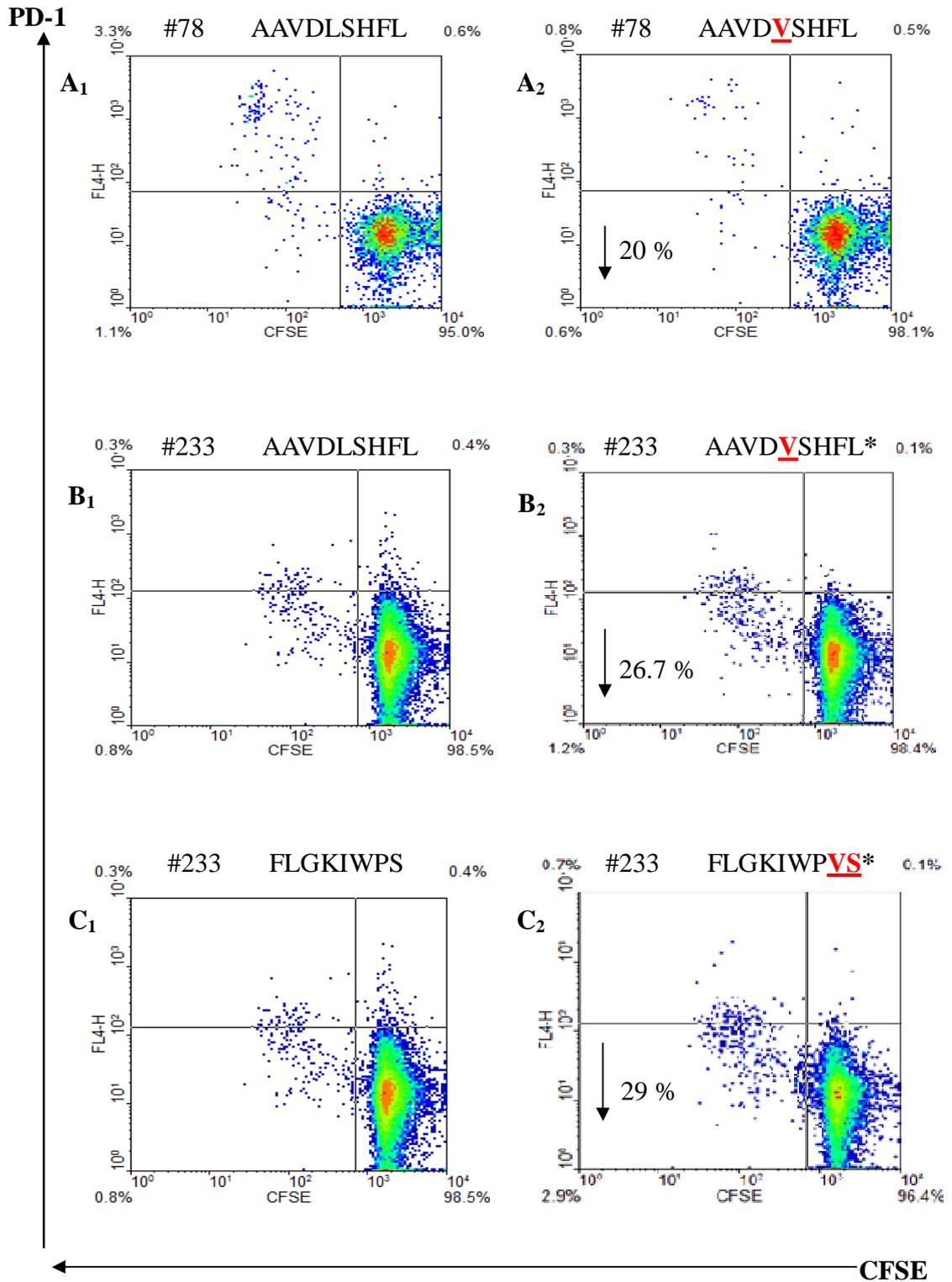
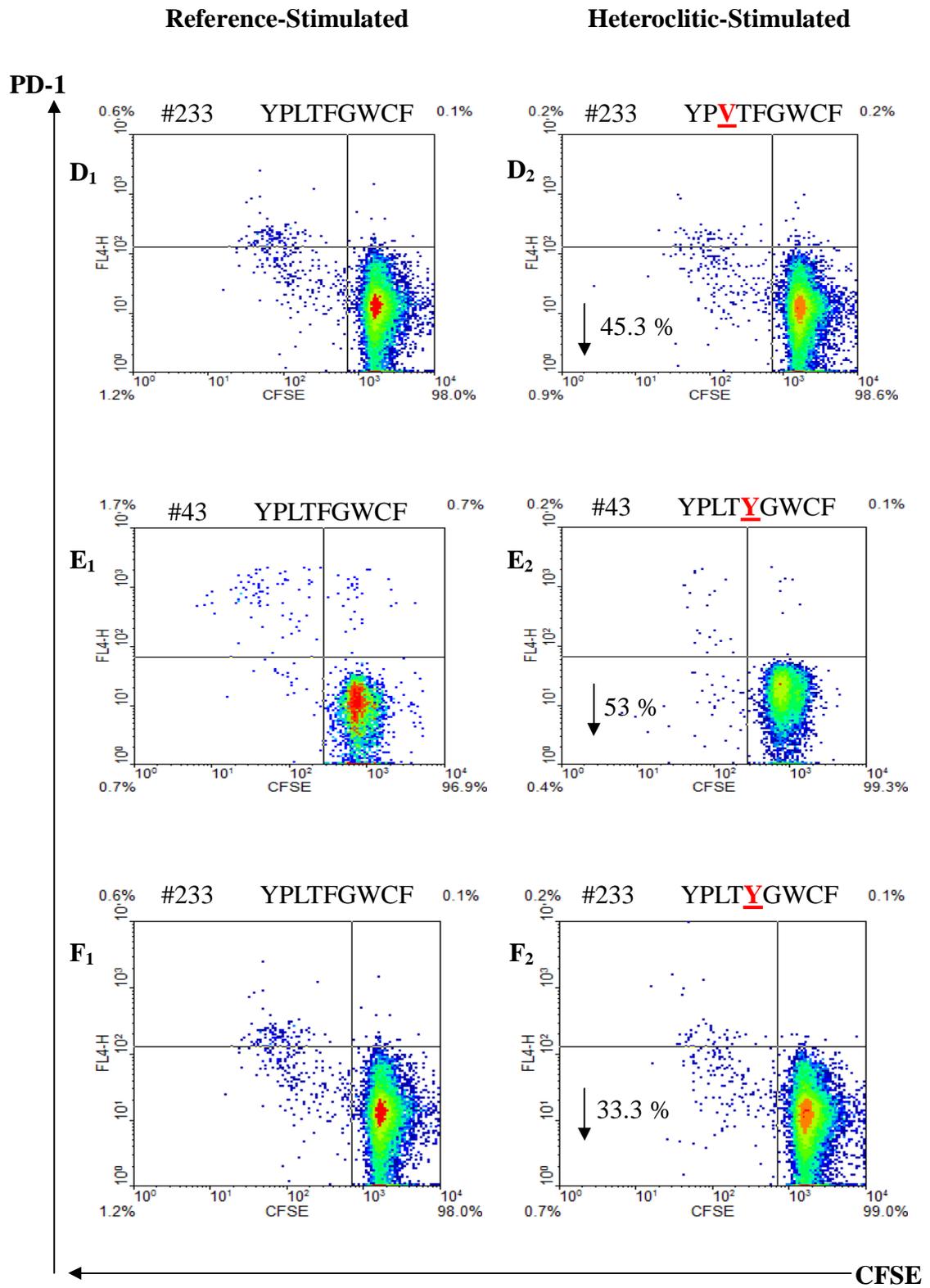


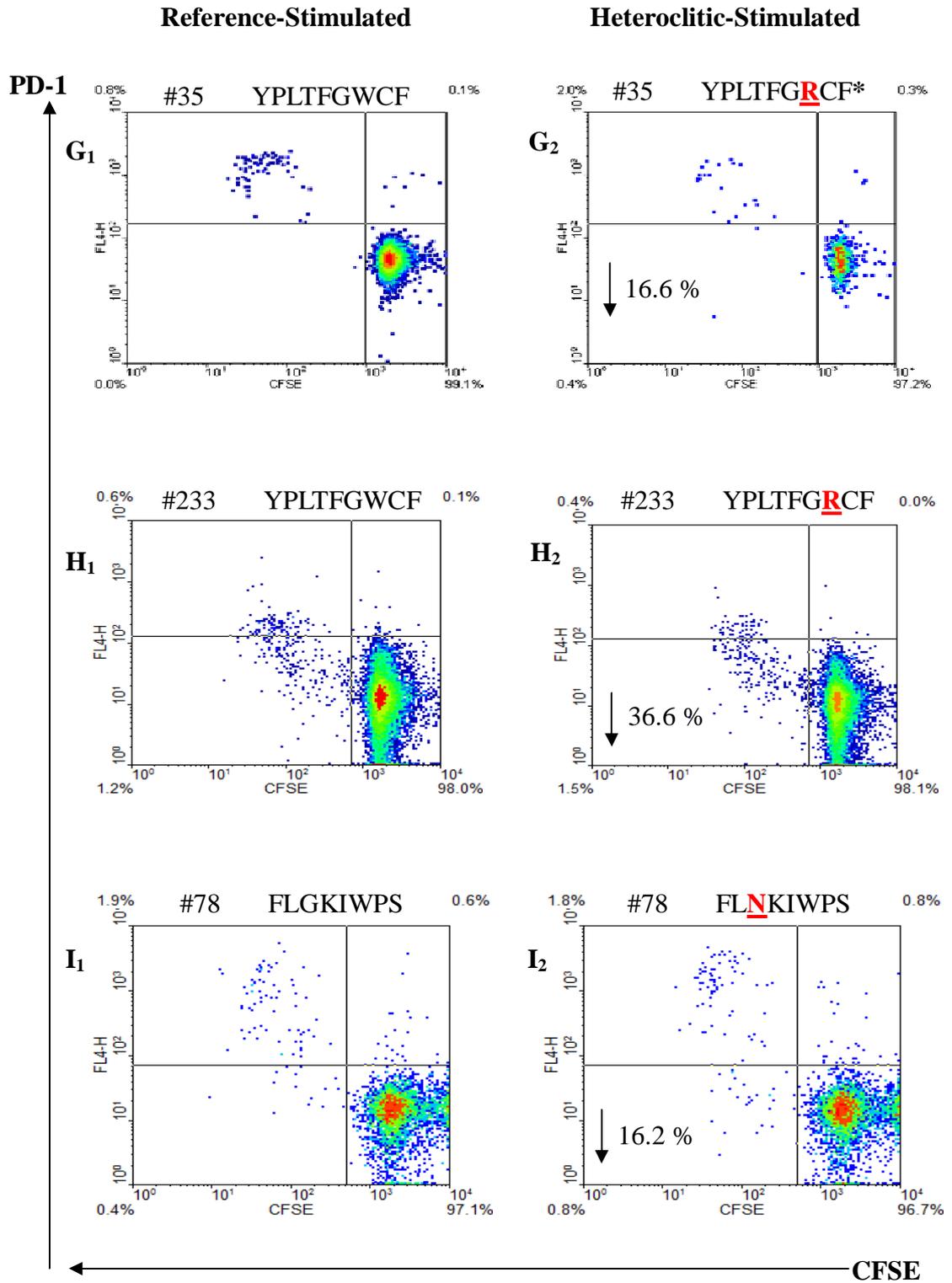
Figure 3.4: The frequency of PD-1^{hi} CFSE^{dim} CD8⁺ T cells responding to reference or heteroclitic peptide stimulation. In paired analysis of 29 cases, stimulation with reference peptides was compared to stimulation with heteroclitic peptides. The median percentage of PD-1^{hi} CFSE^{dim} CD8⁺ T cells was significantly higher with reference peptide stimulation than heteroclitic peptide stimulation (Wilcoxon signed-rank test).

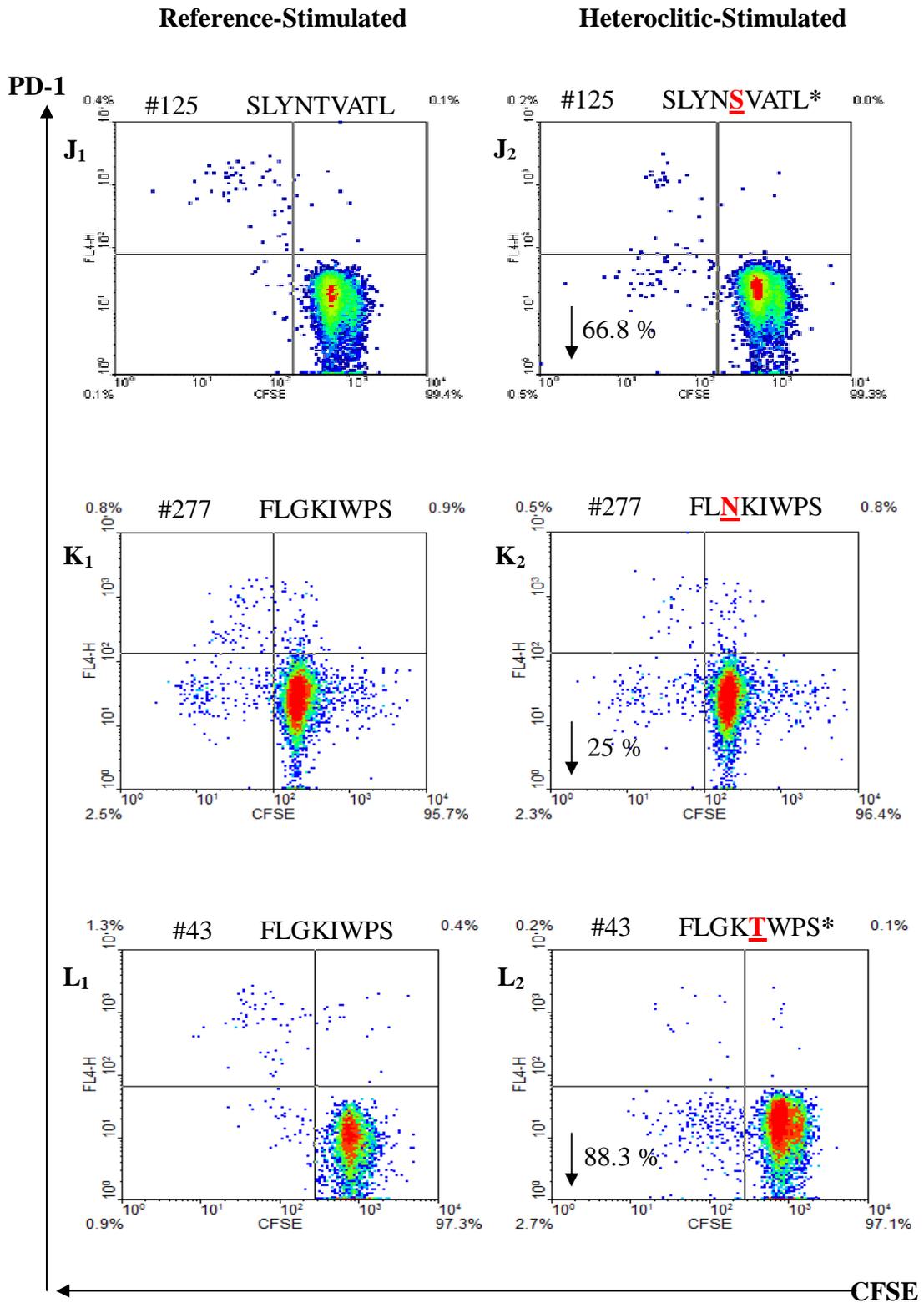
Reference-Stimulated

Heteroclitic-Stimulated









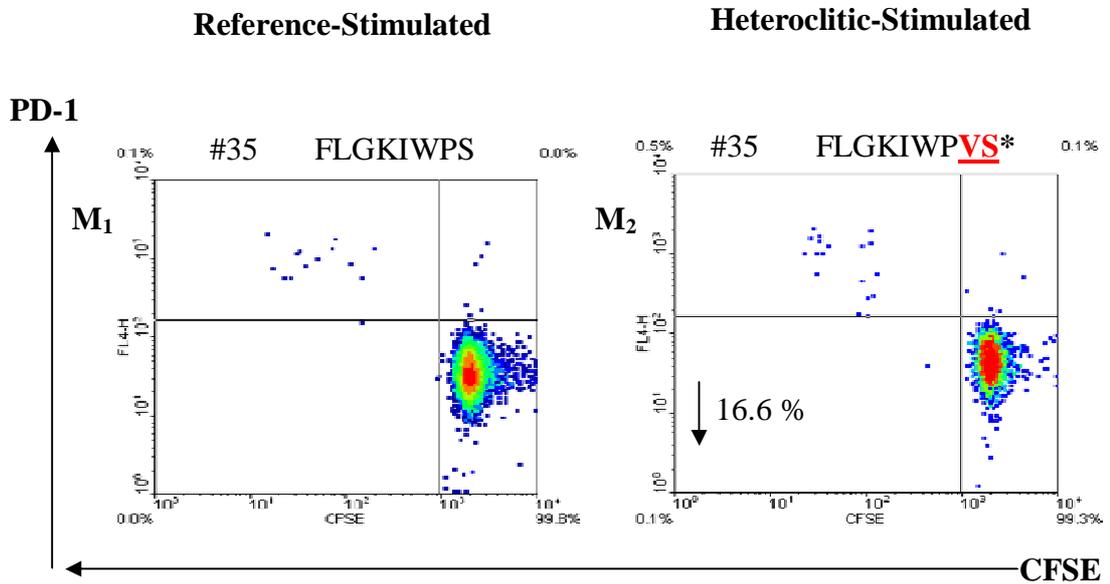


Figure 3.5: Comparison of PD-1 expression on proliferating CD8⁺ T cells stimulated with reference or heteroclitic peptides. PBMC from study participants were CFSE labeled and stimulated with reference or heteroclitic peptide in 7 day culture. CD8⁺ T cells were gated for analysis, and results plotted as PD-1 vs. CFSE. Numbers in the quadrants indicate the percentage reduction in PD-1 expression on proliferating CD8⁺ T cells stimulated with heteroclitic peptides relative to reference peptides. The amino acid substitution made in the heteroclitic peptide sequence is highlighted in red and underlined. Variants enhancing proliferation and lower PD-1 expression are marked with asterisks.

3.4 Correlation between Enhanced CD8⁺ T Cell Proliferation and Lower PD-1 Expression in Response to Heteroclitic Peptides

T cell “exhaustion” is a collective term used to describe one or more different T cell functional impairments, which could range from defective cytokine production or proliferation to clonal deletion. Studies have shown a relationship between sustained upregulation of PD-1 and the reduced capacity of HIV-specific CD8⁺ T cells to proliferate. We investigated whether there was a relationship between lower PD-1 expression and enhanced proliferation in CD8⁺ T cell populations responding to heteroclitic peptide stimulation. CFSE labelled PBMC were stimulated with reference or heteroclitic peptides in a 7 day proliferation assay. Cells were harvested on day 7 and stained with fluorochrome-conjugated antibodies against CD8 and PD-1, and analysed by flow cytometry. We measured the percentage of proliferating CD8⁺ T cells expressing PD-1 in response to reference or heteroclitic peptides and found no significant correlation between enhanced proliferation and lower PD-1 expression on CD8⁺ T cells stimulated with heteroclitic peptides (Figure 3.6.C). However, heteroclitic peptides either induced lower PD-1 expression or enhanced proliferation in 21/29 cases. Also, heteroclitic peptide stimulation enhanced proliferation and lower PD-1 expression in 6/29 cases (Figure 3.5: B₂; C₂; G₂; J₂; L₂ and M₂). Identified heteroclitic peptides and their effects on HIV-specific CD8⁺ T cell proliferation and PD-1 expression is summarised in table 3.2. These data suggest that heteroclitic peptide stimulation exerts differential effects on responding CD8⁺ T cells.

Table 3.2: Effects of heteroclitic peptides on HIV-specific CD8⁺ T cell cytokine production, proliferation and PD-1 expression.

Subject	Peptide	IFN- γ SFU/10 ⁶ PBMC	IL-2 SFU/10 ⁶ PBMC	^a percent proliferation	^b percent PD-1 expression
35	A2-8	843	60	0.8	100
	A2-8-5	938	^c 110	2.9	100
	A2-8-6	1088	15	2.4	83
	A2-9	1005	25	0.1	100
	A2-9-6	1325	50	0.6	83
43	A2-8	1383	ND	2.4	71
	A2-8-3	1563	ND	0.6	33
	A2-9	1460	ND	2.2	59
	A2-9-3	1770	ND	2.9	7
45	A2-8	2123	15	0.5	0
	A2-8-5	2363	30	6.0	0
	A2-8-6	2350	55	0.7	0
	A2-9	1878	15	15.2	88
	A2-9-3	2300	100	2.8	86
	A2-9-6	2258	80	20.5	0
	A2-Gag	2208	95	8.4	88
78	A2-Gag-4	2488	35	4.7	85
	A2-7	1385	100	4.4	75
	A2-7-4	1713	115	1.4	60
	A2-8	1773	145	2.3	83
125	A2-8-6	2053	215	2.6	69
	A2-Gag	58	10	0.5	80
	A2-Gag-3	35	80	0.7	28
214	A2-Gag	250	0	3.4	29
	A2-Gag-2	555	0	4.0	40
	A2-Gag-4	353	0	3.8	34
233	A2-7	500	ND	1.1	27
	A2-7-1	705	ND	1.6	25
	A2-7-4	858	ND	1.5	20
	A2-7-5	813	ND	1.4	28
	A2-7-6	930	ND	3.6	19
	A2-8	998	ND	1.8	33
	A2-8-2	1370	ND	1.1	18
	A2-8-3	1098	ND	0.9	22
244	A2-8-6	1100	ND	1.9	21
	A2-7	868	0	1.0	10
	A2-7-1	1003	15	1.5	13
	A2-9	833	10	1.2	8.4
	A2-9-2	958	5	1.3	7.7

	A2-Gag	698	5	2.4	79
	A2-Gag-2	1400	10	2.3	78
277	A2-8	788	13	2.1	14
	A2-8-2	908	0	2.4	17
	A2-9	465	13	3.3	24
	A2-9-2	583	3	2.8	18
	A2-9-5	618	3	2.3	22
	A2-Gag	638	20	2.7	15
	A2-Gag-2	768	3	3.2	25

^aPercent proliferation represents the percentage of total CD8⁺ T lymphocytes that proliferated over 7 days of stimulation with the indicated peptide.

^bPercent PD-1 expression represents the percentage of the CD8⁺ T cells that proliferated over 7 days of stimulation with the indicated peptide that express PD-1.

^cNumbers in bold text denote cases where either cytokine production or proliferation was increased or PD-1 expression reduced by the indicated (heteroclitic) peptide relative to the reference peptide.

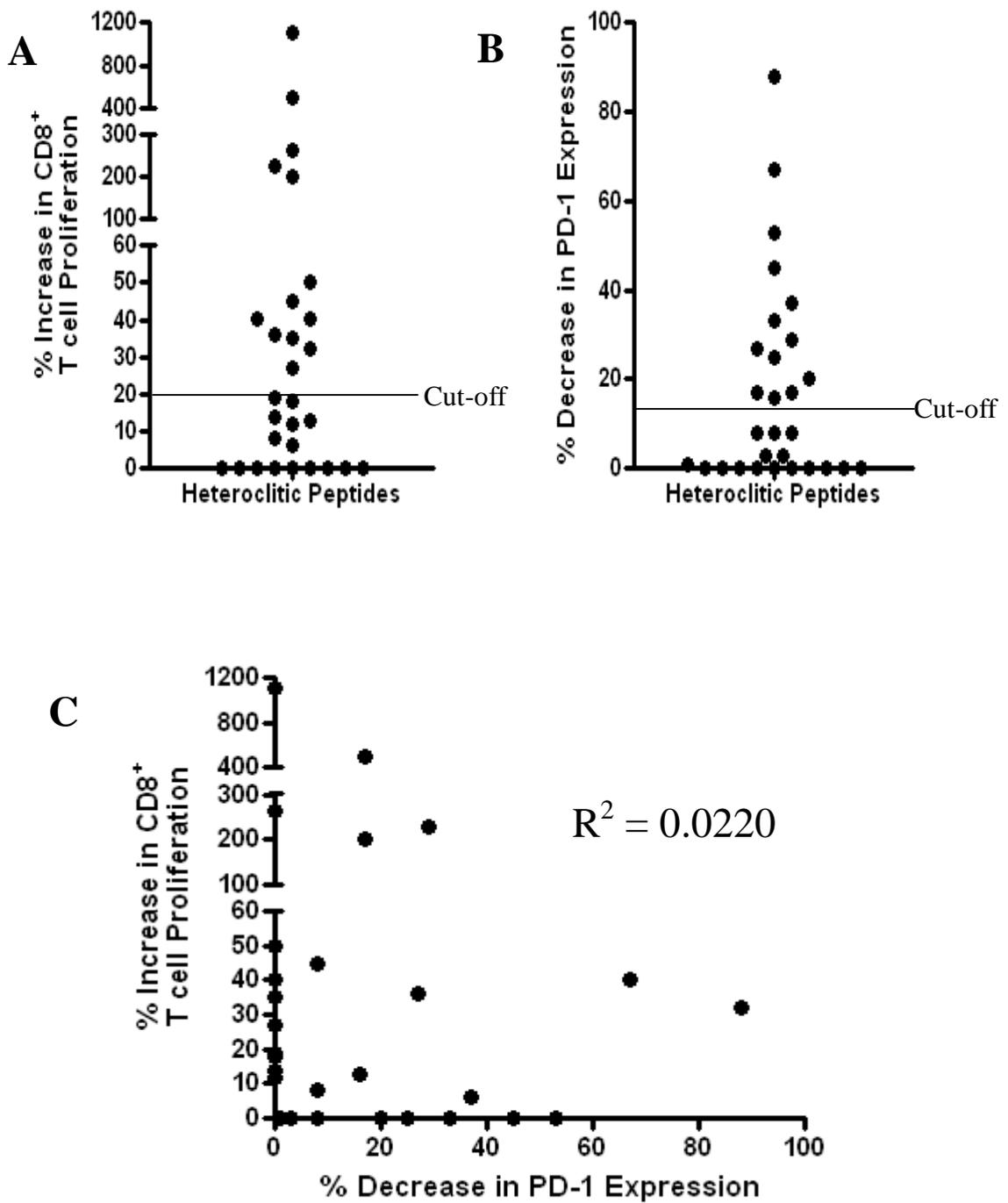


Figure 3.6: Enhanced proliferation is independent of reduced PD-1 expression. (A) Scatter plot showing cases where heteroclitic peptides stimulation enhanced CD8⁺ T cell proliferation by > 20% (n=13). (B) Scatter plot showing cases where heteroclitic peptides stimulation induced lower PD-1 expression on proliferating CD8⁺ T cell by > 15% (n=13). (C) Correlation between enhanced CD8⁺ T cell proliferation and lower PD-1 expression.

3.5 Blockade of PD-1 or Costimulation with 4-1BBL Enhanced HIV-specific CD8⁺ T Cell Responses to Heteroclitic Peptides

Studies have previously shown that blocking the PD-1 inhibitory pathway or providing costimulation via the 4-1BB signaling pathway can improve HIV-specific CD8⁺ T cell responses (177,209). These studies were carried out on memory CD8⁺ T cells using wild-type HIV peptides. We tested whether blocking the PD-1/PD-L1/2 pathway during HIV-specific CD8⁺ T cell stimulation with heteroclitic peptides or delivery of 4-1BB costimulatory signal would further improve responding T cell effector function and reduce exhaustion. CFSE-labeled PBMC from HIV-infected subjects were stimulated with reference or heteroclitic peptides in a 7 day proliferation assay in the presence or absence of anti-PD-1 blocking antibody or 4-1BBL fusion protein. The percentage of CFSE^{dim} CD8⁺ T cells was compared between PBMC populations that were treated with anti-PD-1 blocking antibody or no blocking antibody. We also compared PBMC populations that were cultured with or without 4-1BBL fusion protein. Figure 3.7.A shows representative flow cytometry data where PD-1 inhibition enhanced proliferation of CD8⁺ T cells stimulated with reference peptide by 160%. Alternatively, 4-1BBL costimulation resulted in enhanced proliferation of CD8⁺ T cells by 40% (Figure 3.7.B). It is, however, worthy of note that 4-1BBL costimulation did not induce lower PD-1 expression on proliferating CD8⁺ T cells stimulated with reference peptide. The combined blockade of PD-1 and 4-1BBL costimulation enhanced CD8⁺ T cell proliferation by 280%, which suggests

an additive effect on the responding cells (Figure 3.7.C). On the other hand, blocking the PD-1 pathway enhanced proliferation of CD8⁺ T cells stimulated with heteroclitic peptides by 78% (Figure 3.8.A). Also, 4-1BBL costimulation enhanced proliferation of CD8⁺ T cell responding to heteroclitic peptide by 56%, and lowered PD-1 expression by 36% (Figure 3.8.B). These preliminary results demonstrate that treatment with immunomodulating agents such as anti-PD-1 or 4-1BBL can further boost T cell responses to heteroclitic peptides.

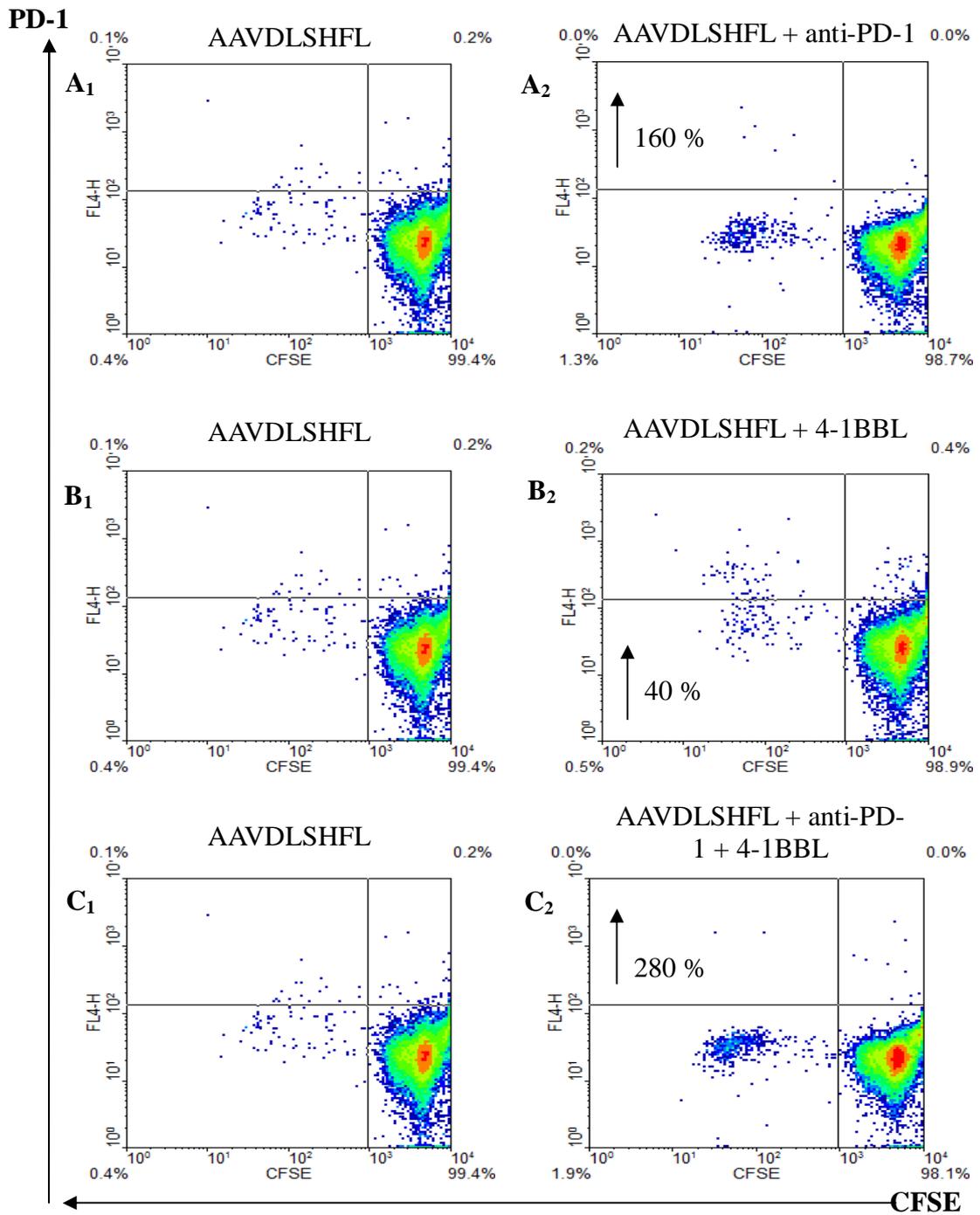


Figure 3.7: PD-1 blockade and / or 4-1BBL costimulation alters HIV-specific CD8⁺ T cell proliferation. (A) Representative flow cytometry data showing the CFSE profile of CD8⁺ T cells responding to reference peptide stimulation in the presence or absence of PD-1 blocking antibody. (B) CFSE profile of CD8⁺ T cells responding to reference peptide stimulation in the presence or absence of 4-1BBL costimulation. (C) CFSE profile of CD8⁺ T cells responding to reference peptide stimulation in the presence or absence of both anti-PD-1 blocking and 4-1BBL fusion protein.

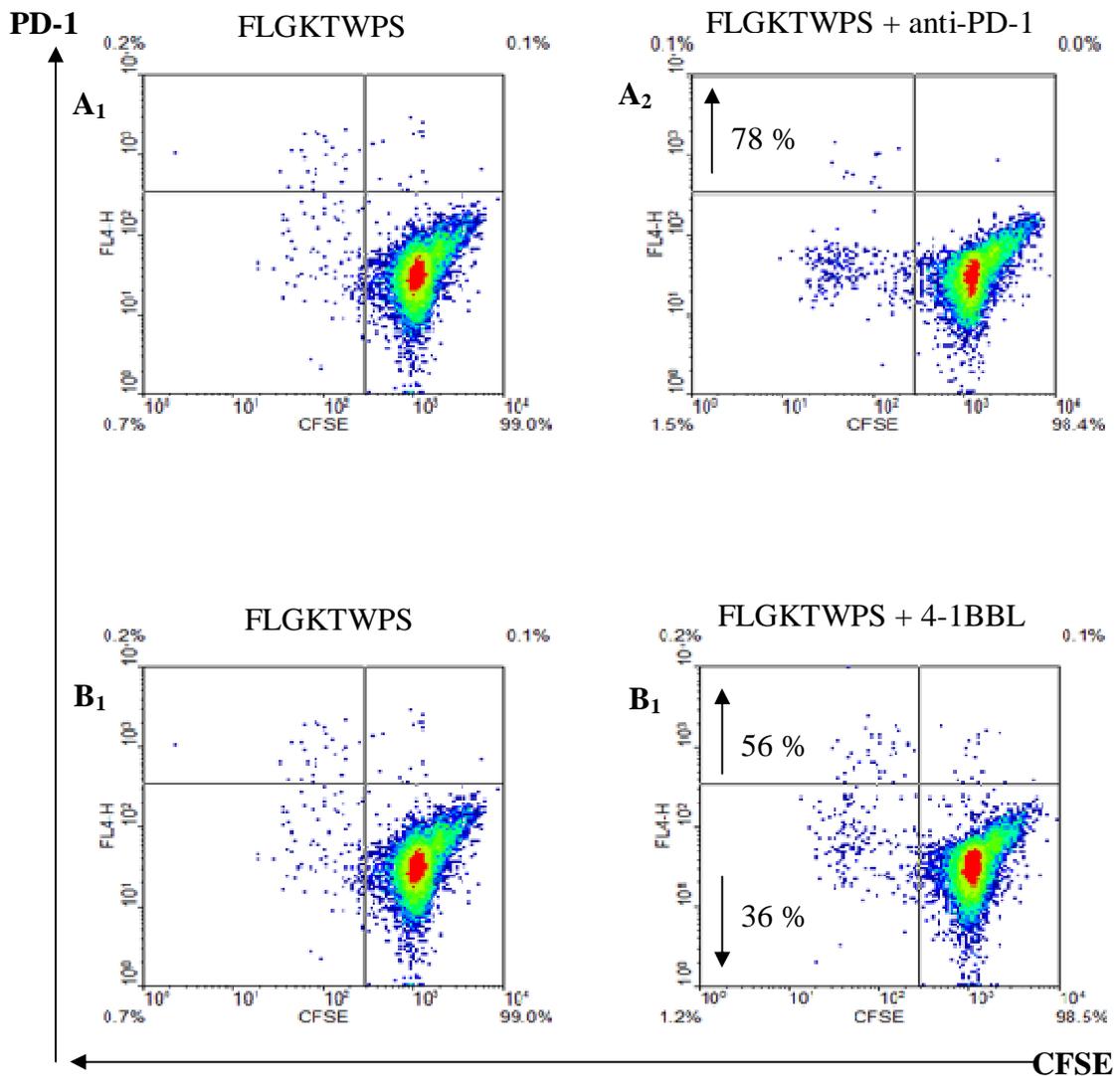


Figure 3.8: PD-1 blockade or 4-1BBL costimulation alters HIV-specific CD8⁺ T cell proliferation. (A) Representative flow cytometry data showing the CFSE profile of CD8⁺ T cells responding to heteroclitic peptide stimulation in the presence or absence of PD-1 blocking antibody. (B) CFSE profile of CD8⁺ T cells responding to heteroclitic peptide stimulation in the presence or absence of 4-1BBL costimulation. Percentage increase in CD8⁺ T cell proliferation and percentage decrease in PD-1 expression are shown in upper-left quadrants and lower-left quadrants respectively.

4.0 Discussion

Different strategies are being explored to develop prophylactic and therapeutic approaches to prevent HIV infection and AIDS. One key strategy is to develop an HIV immunotherapy utilizing HIV-specific CD8⁺ T cells. Activation of HIV-specific CD8⁺ T cells following acute HIV infection contributes to the decline of viremia and delays disease progression (35,39). Also, HIV-specific CD8⁺ CTL persist through disease progression and are resistant to HIV infection since they do not express CD4, which is the main receptor for HIV entry and cellular infection. However, continuous stimulation via their TCR with HIV peptide epitopes during chronic HIV-infection has been implicated in their functional impairment, which persists even through successful ART and antigen withdrawal (181,184). The collection of T cell functional impairments that scientists have termed “T cell exhaustion” ranges from a partial decrease or loss of cytokine production through to clonal deletion (159). We previously demonstrated that heteroclitic peptide stimulation enhances cytokine production by HIV-specific CD8⁺ T cells (206). In this study, we investigated the effect on other T cell functional impairments by stimulating HIV-specific CD8⁺ T cells with heteroclitic variants of native HIV peptides.

The process of T cell functional impairment appears stepwise and progressive in nature with antigen-specific proliferation being an effector function that is extinguished early. This study, therefore, aimed to characterize the effects of heteroclitic peptide stimulation on proliferation of HIV-specific CD8⁺ T cells. We first identified HIV variant peptides that enhanced HIV-specific CD8⁺ T cell cytokine responses by ELISPOT assays, and termed these variants “heteroclitic”. Most of the

identified heteroclitic peptides enhanced IFN- γ production, although some enhanced IL-2 production as well. Similar enhanced IL-2 responses have been reported by stimulation with an heteroclitic influenza virus peptide (A2-Flu-1) relative to the reference peptide (206). However, no systematic comparison of the frequency of heteroclitic peptides related to HIV epitopes versus other viral epitopes has been carried out. In this study, IFN- γ and IL-2 production were independently enhanced as there was no direct correlation between these two cytokine responses when PBMC were stimulated with heteroclitic peptides.

Together with reduced cytokine production, loss of proliferative capacity is one of the major T cell functional impairments that occur in chronic HIV-infection. CD4⁺ T cells often play an important role in the generation of optimal CD8⁺ T cell responses in an ongoing infection. CD4⁺ helper T cells produce IL-2, which is a T cell growth factor that supports HIV-specific CD8⁺ T cell survival and memory T cell formation. Since CD4⁺ T cells are the main target of HIV, there is a dramatic reduction in CD4⁺ T cell counts in untreated HIV-infection. This renders the HIV-specific CD8⁺ T cell “helpless”, and contributes to HIV-specific CD8⁺ T cell exhaustion in progressive HIV infection (210). Zimmerli *et al.* showed that HIV-1-specific IFN- γ /IL-2-producing CD8⁺ T cells are capable of driving their own proliferation in the absence of CD4⁺ T cell help (211). To this end, we followed up to see whether HIV-1-specific IFN- γ /IL-2-producing CD8⁺ T cells activated by heteroclitic peptide stimulation can also drive their clonal expansion. PBMC from HIV-infected subjects that showed higher cytokine responses to heteroclitic peptides in ELISPOT assays were further cultured for 7 days with the heteroclitic peptide that

induced the highest cytokine responses. We found that heteroclitic peptides enhanced HIV-specific CD8⁺ T cell proliferation relative to reference peptides in almost half of the cases where reference / heteroclitic HIV-peptide pairs were compared. Since IL-2 is a T cell growth factor that plays an important role in driving T cell clonal expansion and differentiation, we expected heteroclitic peptides that enhanced IL-2 production to also enhance HIV-specific CD8⁺ T cell proliferation. Surprisingly, HIV-specific CD8⁺ T cell proliferation was not enhanced in at least one case where IL-2 production was increased. Our data suggest that enhanced CD8⁺ T cell proliferation by heteroclitic peptides in many cases is independent of detectable IL-2 production. All the heteroclitic peptides tested in proliferation assays enhanced IFN- γ production. Ahmed *et al.* showed that loss of IL-2 production and proliferation capacity are the early signs of functional impairment observed in LCMV-specific CD8⁺ T cells. IFN- γ production, which is persistent, eventually becomes defective in severe T cell exhaustion (184). This T cell functional impairment has been documented in chronic HIV-infection as well (159,160). Several studies have shown that a significant fraction of HIV-specific CD8⁺ T cells lose the ability to produce IFN- γ in chronic HIV-infection, a phenomenon which indicates severe T cell exhaustion (212-214). Our finding that heteroclitic HIV-peptides enhanced proliferation and cytokine production relative to the native HIV-peptide epitope suggests that heteroclitic peptide stimulation either selectively stimulates non-exhausted T cells or restores “exhausted” HIV-specific CD8⁺ T cells to a functional or even polyfunctional state. Retention of polyfunctional HIV-specific CD8⁺ T cells, which are capable of producing cytokines and proliferating, is an attribute found in only a minority of HIV-infected, ART-naïve

viremic controllers called long-term nonprogressors (LTNP) (215). Hence, our results suggest that heteroclitic peptide stimulation could possibly stimulate activation of the same polyfunctional T cells that help LTNP keep viremia in check.

In progressive HIV infection, chronic expression of PD-1 molecules on HIV-specific CD8⁺ T cells correlates with failure to contain viremia (177). In addition, HIV-specific CD8⁺ T cells in LTNP express a significantly lower amount of PD-1, which is associated with their ability to contain HIV viremia (215). Several interventions aimed at improving exhausted T cell responses employed monoclonal blocking antibodies against PD-1 or its ligands or introduced exogenous costimulatory molecules such as 4-1BB during T cell activation. All of these interventions to a significant extent restored or improved the effector functions of responding T cells (68,216). However, native HIV-peptide epitopes were utilized in stimulating these HIV-specific CD8⁺ T cells in all the interventions. For the first time, we demonstrated that heteroclitic variants of these native HIV-peptide epitopes could improve the character of HIV-specific CD8⁺ T cell responses relative to their reference epitopes, without either introducing exogenous costimulatory molecules or blocking inhibitory receptors. Expression of PD-1 on proliferating HIV-specific CD8⁺ T cells stimulated with heteroclitic peptides was significantly low relative to HIV-specific CD8⁺ T cells stimulated with reference peptides in almost half of the peptide pairs compared. This finding suggests unique features of heteroclitic peptides in reducing T cell “exhaustion” while still enhancing proliferation and cytokine production in responding CD8⁺ T cells. Comparison of cases where PD-1 expression was lower with cases where HIV-specific CD8⁺ T cell proliferation was enhanced showed no

significant correlation between lower PD-1 expression and enhanced proliferation. This could either mean that heteroclitic peptides select for distinct heterogeneous subsets of HIV-specific CD8⁺ T cells, which partly explains the difference in frequency of PD-1 low cells induced by heteroclitic peptides or that activation signals generated by heteroclitic peptide stimulation somehow bypass the PD-1 signaling pathway, thereby allowing enhanced proliferation even of PD-1^{hi} CD8⁺ T cells. However, since elevated PD-1 expression in HIV and other chronic infections is associated with T cell exhaustion and disease progression, lower expression of PD-1 generated by heteroclitic peptides may enhance CD8⁺ T cell effector function and limit disease progression. Normally, inhibitory signals delivered by PD-1 downregulate TCR signaling through direct dephosphorylation of intracellular signaling intermediates. The phosphatases (SHP-1 and SHP-2) associated with PD-1 dephosphorylate CD3 ζ and prevent the phosphorylation of ZAP-70 and PKC θ (85). A possible explanation for the negligible effects of PD-1 signaling on HIV-specific CFSE^{dim} CD8⁺ T cells responding to heteroclitic peptide stimulation as we have observed could be attributed to increased tyrosine phosphorylation of ZAP-70 and TCR ζ chains in CTL stimulated with heteroclitic peptides relative to native peptide-stimulated CTL, as was reported by Salazar *et al.* (202). Again, this supports our suggestion that heteroclitic peptides can potentially bypass exhaustion induced by reference peptides. There was no correlation between the type of cytokine induced by heteroclitic peptides and lowered PD-1 expression (data not shown). Even though IL-2 is a T cell growth factor, studies indicate it can increase the expression of PD-1. A study by Fauci *et al.* showed that culture of purified T cells with common γ -chain

cytokines such as IL-2, IL-7, IL-15, and IL-21 markedly enhanced PD-1 expression *in vitro* (217). This observation was confirmed *in vivo* when they demonstrated that PBMC isolated from HIV-infected subjects expressed higher levels of PD-1 following IL-2 immunotherapy (217). This supports our finding that heteroclitic-HIV peptides induced lower PD-1 expression on HIV-specific CD8⁺ T cells in the absence of detectable IL-2. The goal of heteroclitic HIV-variant peptide stimulation is to generate HIV-specific CD8⁺ T cells with enhanced effector responses towards the native antigen. Studies in our laboratory showed by additive ELISPOT assays that reference peptides A2-7, A2-8, A2-9 and their respective heteroclitic analogs stimulated the same subset of HIV-specific CD8⁺ T cells (206). Since the amino acid substitutions we made in the variant peptide sequences were not at the positions harboring HLA anchor residues, we do not expect any alteration in their HLA-binding affinity relative to the reference peptides. This T cell cross-reactivity, however, suggests that the enhanced responses observed in CD8⁺ T cells stimulated with heteroclitic peptides could be attributed to differential intracellular signaling of the responding CD8⁺ T cells rather than recognition of reference and variant peptides by different T cells.

Even though additive ELISPOT assays suggest that heteroclitic peptides stimulated the same CD8⁺ T cell subsets as the reference peptides, we still cannot rule out the possibility that heteroclitic peptides may also stimulate CD8⁺ T cells that are not specific for the native peptide epitope. The substitution of threonine at position 2 of a well-characterized HLA-A2-restricted melanoma antigen (gp100₂₀₉₋₂₁₇) with methionine (gp100_{2M}) significantly enhanced pMHC binding affinity and antigen presentation (218,219). However, only 25% of T cells from PBMC of melanoma

patients stimulated with melanoma peptide analog gp100_{2M} were able to recognize the native peptide epitope (220). It was also confirmed in an independent study that gp100_{2M}-specific T cell cross-reactivity with the wild-type melanoma antigen is 2-3 orders of magnitude lesser than to the gp100_{2M} peptide (221). Since the aim of heteroclitic peptide stimulation is to enhance responses in HIV-specific CD8⁺ T cells with high avidity for native-HIV proteins, inducing T cells that do not recognize native HIV epitopes or T cells that mask the desired responses would not be beneficial. Tetramer-stained HIV-specific CD8⁺ T cells expanded with reference peptides in proliferation assays can be stimulated with heteroclitic peptides to monitor the frequency of cross-reactive HIV-specific CD8⁺ T cells responding to heteroclitic peptide stimulation. Also, additive proliferation assays in which PBMC from our HIV-infected subjects would be cultured in separate tubes for 7 days with the reference peptides or heteroclitic peptides as explained in the methods section, and a combination of the reference peptide with the heteroclitic peptides in the same tube could be performed in order to investigate heteroclitic peptide-driven T cell cross-reactivity with the native-HIV peptide. An additive response would be expected if different subsets of HIV-specific CD8⁺ T cells were responding to the peptide stimulation. However, if both peptides stimulated the same CD8⁺ T cell subset, the percentage of CFSE^{dim} CD8⁺ T cells in the PBMC population that was stimulated with both reference and heteroclitic peptides would be equal to the percentage of CFSE^{dim} CD8⁺ T cells in the PBMC population that was stimulated with the heteroclitic peptide. We couldn't measure the baseline expression level of PD-1 on CD8⁺ T cells for post-stimulation comparison due to a shortage of PBMC. However, we speculate

that heteroclitic peptides stimulated the same subset of HIV-specific CD8⁺ T cells based on previous ELISPOT results (206).

Finally, we examined the effect of 4-1BBL costimulation or blockade of the PD-1 pathway on CD8⁺ T cells responding to heteroclitic peptide stimulation. The balance of costimulatory and inhibitory signals delivered via the TCR and other cell surface signaling molecules to T cells ultimately determines their effector responses. Several studies have shown that altering both the positive and negative signals delivered to antigen-specific T cells in some cancer and infectious disease models can potentially be therapeutic (183,206,222,223). Also, since flow cytometry was not done before day 7 due to shortage of cells, some dividing cells might be undergoing cell death through activation and it is possible that PD-1 blockade would prevent apoptotic cell death usually induced by PD-1 signaling. One way by which heteroclitic peptides induce better T cell responses is by increasing tyrosine phosphorylation of ZAP-70 and TCR ζ chains (202), which is normally inhibited by PD-1 signaling, elimination of the PD-1 signal should further improve the effect of heteroclitic peptide stimulation on the responding HIV-specific CD8⁺ T cells. Therefore, we blocked the PD-1/PD-L1/2 pathway during peptide stimulation of HIV-specific CD8⁺ T cells. Indeed, blocking the PD-1 signaling pathway further enhanced proliferation of HIV-specific CD8⁺ T cells stimulated with heteroclitic peptides. Since 4-1BB costimulates T cells independently of the major T cell costimulatory molecule CD28 and humans accumulate CD28⁻ T cells with age, we decided to also stimulate the HIV-specific CD8⁺ T cells with peptides in the presence of 4-1BBL. Addition of exogenous 4-1BBL fusion protein as a source of T-cell costimulation also enhanced proliferation

and reduced PD-1 expression on HIV-specific CD8⁺ T cells responding to heteroclitic peptides. A combination of both 4-1BBL costimulation and PD-1 blockade showed an additive effect on HIV-specific CD8⁺ T cells stimulated with the reference peptides but was not tested with heteroclitic peptides.

This study characterized the effects of heteroclitic HIV-peptide stimulation on HIV-specific CD8⁺ T cell effector responses such as cytokine production and clonal expansion, which are impaired with T cell exhaustion. We identified an additional 29 instances of heteroclitic peptide activity, and showed that IFN- γ and IL-2 production were enhanced in HIV-specific CD8⁺ T cells responding to these peptides. We demonstrated that heteroclitic variants of native HIV-peptides are capable of enhancing HIV-specific CD8⁺ T cell proliferation relative to native epitopes, and that improved IL-2 responses are not always associated with enhanced proliferation. The heteroclitic variant peptides employed in this study produced promising results, which could potentially guide the design of other heteroclitic HIV-peptide variants. In this study, we only generated twenty-four variant peptides from A2-7, A2-8, A2-9, and A2-Gag reference peptides (i.e. six variants per peptide set). This was carried out by a single conservative or semiconservative aa substitution at residues distinct from the main MHC anchors. These substitutions often induced better HIV-specific CD8⁺ T cell responses than the reference peptide, consistent with the findings of Sette *et al.* (203). Also, we did not find a significant correlation between enhanced proliferation and lower PD-1 expression with heteroclitic peptide stimulation in this study. Finally, we showed that manipulating T cell costimulatory and/or inhibitory signals could further benefit the T cells responding to heteroclitic peptide stimulation in terms of

proliferation. This study further advances our knowledge on the ability of heteroclitic peptides to enhance effector functions of HIV-specific CD8⁺ T cells.

Analysis of aa substitutions in the reference peptide sequences, that made the variant peptides more antigenic (heteroclitic) could be used to inform the design of more potentially heteroclitic variants with substitutions at multiple positions. It is important to note that heteroclitic peptides employed in this study were variants of immunodominant epitopes. Inter-individual variability in heteroclitic responses could be attributed to the polymorphic nature of HLA presenting the immunodominant HIV epitopes and studies show that HIV immunodominant epitopes, in the context of their HLA-restriction do not often reflect an individual's overall HIV-specific CD8⁺ T cell response (224). This may limit generalizability in terms of vaccine development. However, if heteroclitic peptides enhance antiviral immunity, personalized therapeutic HIV vaccine would not demand inordinate effort or expense relative to genotyping and phenotyping, which is already standard of care for HIV infection. Our findings justify further investigation of the incorporation of heteroclitic peptides into HIV vaccines.

5.0 Conclusions and Future Directions

This study has generated additional knowledge on the potential benefits of incorporating heteroclitic-HIV peptides into HIV therapeutic vaccines to induce stronger and better HIV-specific CD8⁺ T cell responses. We have shown that heteroclitic peptides can alter both the magnitude and character of HIV-specific CD8⁺ T cell responses. Since chronic stimulation with wild-type HIV epitopes is associated with HIV-specific CD8⁺ T cell exhaustion, a longer time-frame would be required to determine whether or not chronic stimulation with heteroclitic HIV-peptides would also lead to T cell functional impairment. Heteroclitic peptide stimulation in this study only lasted 24 hours (ELISPOT) or 7 days (proliferation assay). Since PD-1 expression did not hinder HIV-specific CD8⁺ T cell responses when stimulated with heteroclitic peptides compared to reference peptides, it is worth investigating whether activation signals generated by heteroclitic peptide stimulation bypass or overwhelm the PD-1 signaling pathway in some way. This could be investigated by carrying out a microarray analysis to examine what genes are turned on or repressed in PD-1 expressing CD8⁺ T cells stimulated with heteroclitic peptides compared to those stimulated with reference peptides. This could provide insight into how the TCR/heteroclitic peptide interactions modulate T cell responses. Other T cell inhibitory molecules that have been implicated in T cell exhaustion include TIM-3, LAG-3, CD160, CTLA4, 2B4 and SLAM. We only investigated the effect of heteroclitic peptides on PD-1 expression in this study. Further investigation should determine whether or not heteroclitic peptides modulate the expression of other co-inhibitory molecules and if this contributes to the enhanced T cell responses generated

by heteroclitic peptides. Altogether, this information could be useful for designing heteroclitic peptides that improve effector responses of HIV-specific CD8⁺ T cells with high avidity for native-HIV epitopes.

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Appendix A: General characteristics of study subjects with heteroclitic responses.

^a ID	^b Virus load	^c CD4 ⁺ T cells/ μ l	^d CD4 ⁺ T cell nadir	CD8 ⁺ T cells / μ l	^e Duration of infection	^f HAART
007	3.87	8	8	1239	>17 years	No
028	≤ 1.30	140	0	1180	>17 years	Yes
214	≤ 1.30	189	90	576	>6 years	Yes
078	4.44	228	32	1007	>17 years	Yes
125	≤ 1.60	248	59	464	>14 years	Yes
011	1.78	280	63	2450	>17 years	Yes
283	4.25	352	352	752	>1 year	No
166	≤ 1.30	364	200	663	>11 years	Yes
212	≤ 1.70	378	300	423	>6 years	Yes
244	≤ 1.30	475	51	817	>3 years	Yes
282	≤ 1.30	476	476	532	>1 year	Yes
039	≤ 1.30	483	24	1426	>17 years	Yes
148	≤ 1.30	533	6	468	>13 years	Yes
126	≤ 1.30	620	164	600	>14 years	Yes
001	≤ 1.30	638	51	792	>18 years	Yes
043	≤ 1.30	675	324	420	>17 years	Yes
230	≤ 1.30	696	390	1296	>4 years	Yes
277	≤ 1.30	696	621	986	>1 year	Yes
071	≤ 1.30	768	418	1320	>17 years	Yes
115	≤ 1.30	768	204	1248	>15 years	Yes
134	≤ 1.30	810	96	504	>14 years	Yes
045	≤ 1.30	868	209	1260	>17 years	Yes
237	≤ 1.30	952	480	1836	>4 years	Yes
035	1.48	1020	176	3300	>17 years	Yes
233	1.31	1088	1568	1050	>4 years	Yes

^aSubjects were sequentially coded as they enrolled in the study and are tabulated in order of increasing CD4⁺ T cell counts.

^bVirus loads obtained for the time of testing from clinical charts are expressed as log₁₀ copies HIV RNA/mL plasma.

^cNumber of CD4⁺ and CD8⁺ T cells/ μ L peripheral blood at the time of testing for each subject.

^dCD4⁺ T cell nadir is the lowest recorded CD4⁺ T cell count from each subjects' clinical chart.

^eDuration of infection is based on the earliest record of known HIV-seropositive status.

^fWhether or not subjects were receiving highly active antiretroviral therapy at time of testing is noted.