Title
The Determinants of CD8+ Cytotoxic T Lymphocyte Antiviral Activity Against Human Immunodeficiency Virus Type 1

Author
Chen, Diana Yuhui

Publication Date
2012

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

The Determinants of $CD8^+$ Cytotoxic T Lymphocyte Antiviral Activity Against Human Immunodeficiency Virus Type 1

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Microbiology, Immunology, and Molecular Genetics

by

Diana Yuhui Chen

2012

ABSTRACT OF THE DISSERTATION

The Determinants of $CD8^+$ Cytotoxic T Lymphocyte Antiviral Activity Against Human Immunodeficiency Virus Type 1

by

Diana Yuhui Chen

Doctor of Philosophy in Microbiology, Immunology, and Molecular Genetics

University of California, Los Angeles, 2012

Professor Otto O. Yang, Chair

It is well established that $CD8^+$ cytotoxic T lymphocytes (CTL) are key players in the protective immune response against Human Immunodeficiency Virus Type 1 (HIV-1). However, there are clear differences in the effectiveness with which particular CTL control viral infection. Understanding the factors that contribute to the individual differences in CTL antiviral efficiency will have great implications for vaccine design, as such information will provide important insights on the mechanism of efficacious antiviral activity against HIV-1.

One key factor that affects the CTL-mediated antiviral efficiency is the cell-surface downregulation of human leukocyte antigen class I (HLA-I) mediated by the HIV-1 Nef protein. While the degree of Nef interference on CTL antiviral efficiency has been shown to be epitope specific, the factors that determine the susceptibility of CTL to Nef are not clear. Previous work has indicated that, the timing of epitope expression, functional avidity, and protein targeting as well as HLA-I restriction can have varying degrees of influence on how well a CTL can recognize infected cells and suppress HIV-1 replication. The primary goal of this dissertation is to better define the role these factors play in influencing CTL antiviral activity.

This was addressed using a variety of viral constructs, and HIV-1 specific CTL clones. In Chapter Three, I first examine CTL susceptibility to Nef-mediated HLA-I downregulation using a previously described viral suppression assay, and identify epitope properties that determine susceptibility to Nef. Then in Chapter Four, I focus on the role of protein targeting in CTL antiviral activity. I specifically compare the role of Gag versus Env targeting in viral suppression efficiency, using viral constructs where a Gag epitope is translocated to the Env protein.

The data presented in this dissertation show that the kinetics of epitope presentation are an important determinant of CTL antiviral activity. Individual epitope presentation kinetics vary independently of protein, and the earlier the epitope is presented on the cell surface before Nef-mediated HLA-I downregulation, the more effectively CTL can eliminate virus-infected cells and suppress viral replication. Factors including protein

properties, HLA-I restriction of the epitope, and functional avidity, are poor predictive properties of Nef susceptibility and CTL antiviral efficiency. Together these results suggest that the properties of individual epitopes, such as epitope expression kinetics, most strongly influence CTL antiviral activity. Lastly, future research could potentially focus on the role of host factors, such as T cell receptor functions, in determining CTL antiviral activity against HIV-1. A few proposed ideas are summarized in Chapter Five.

The dissertation of Diana Yuhui Chen is approved.

Christel H. Uittenbogaart

Paul A. Krogstad

Otoniel Martinez-Maza

Otto. O. Yang, Committee Chair

University of California, Los Angeles

2012

To Science

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

CHAPTER 1: INTROUDUCTION

CHAPTER 3: EPITOPE TARGETING AND VIRAL INOCULUM ARE DETERMINANTS OF NEF-MEDIATED IMMUNE EVASION OF HIV-1 FROM CTLS

CHAPTER 4: ANTIVIRAL ACTIVITY OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 GAG-SPECIFIC CYTOTOXIC T LYMPHOCYTE TARGETING IS NOT NECESSARILY INTRINSCIALLY SUPERIOR TO ENV TARGETING

CHAPTER 5: DISCUSSION

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Otto Yang for all his guidance and support over the last four years. He has taught me to think critically, to approach problems creatively, and to design and perform the many experiments and techniques utilized in this thesis. As a result of his knowledge and guidance, I have a much deeper understanding of CTL immunology and HIV-1 immunopathogenesis, and have molded into the scientist that I am today. He has taught me to believe in myself, and encouraged me to follow my passion. I am really grateful for the interest he has shown in helping me with my career search and personal development.

I would also like to thank my committee members, Dr. Christel Uittenbogaart, Dr. Paul Krogstad, and Dr. Otoniel Martinez-Maza, for all their helpful suggestions and advice. I would like to especially acknowledge Dr. Paul Krogstad for his guidance and support during my ACCESS rotation. I would also like to thank Dr. Jonathan Braun, Dr. Linda Baum, and Dr. Nancy Halnon for giving me the opportunity to rotate in their labs during my ACCESS year. I also want to thank Dr. Martha Lewis for all her advice and guidance during my group meeting.

This thesis would not have been possible without the tremendous support provided by my colleagues, especially Dr. Balamurugan Arumugam, who has provided priceless mentorship in my early years of graduate school. My next thanks go to Hwee Ng; not only has she taught me many lab techniques, she has always been a mother figure to

me. I also want to thank Dr. Ayub Ali for all his help in molecular assays and protein biochemistry work, and Justin De la Cruz, Sangeun Park, Dr. Jun Zuo, Angela Yu, and Elizabeth Huang, who have all been comrades in arms in my scientific endeavors.

The dissertation work was supported by NIH grants AI043203 (O.O.Y.) and AI051970 (O.O.Y.), funds from the UCLA AIDS Institute Center for AIDS Research Graduate Student Fellowship (D.Y.C.), and UCLA CFAR grant AI028697 for the infrastructure support for this project. I would like to thank and acknowledge the financial support from the UCLA Graduate Division (Dr. Ursula Mandel Fellowship Award, D.Y.C.), and the Department of Microbiology, Immunology, and Molecular Genetics (Mitsuo Takasugi Award, D.Y.C.). I thank Dr. William Cumberland for his collaborative efforts and discussion, and the National Institutes of Health AIDS Reference and Reagent Program for providing recombinant human interleukin-2, tenofovir, and zidovudine. Also, I would like to thank the individuals from whose blood we derived the CTL clones used in this study.

Finally, I would like to thank my family and friends for all of their love and support. My parents taught me to believe in myself, and gave me the opportunity to receive a better education in the United States. My sister Christiana Chen has always been a never-ending source of support and comfort. My friends Dr. Courtney Kim, Jose Rodriquez, Dr. Shota Yamamoto, and Dr. Justin Lin have always been very kind in helping me with research proposals and essays, and sharing their research ideas with me. My thanks to all of you!

Chapter Three in this thesis is a version of (Chen *et al*, 2012).

Chapter Four in this thesis is a version of (Chen *et al*, 2011).

Chen, D.Y., Balamurugan, A., Ali, A., Ng, H.L., Cumberland, W., and Yang, O.O. Epitope Targeting and Viral Inoculum Are Determinants of Nef-Mediated Immune Evasion of HIV-1 from CTLs. 2012. Submitted.

Chen, D.Y., Balamurugan, A., Ng, H.L., and Yang, O.O. Antiviral Activity of HIV-1 Gag-specific Cytotoxic T Lymphocyte Targeting Is Not Necessarily Intrinsically Superior to Envelope Targeting. 2011. Journal of Virology, 85(5): 2474-2478.

VITA

1984	Born, New Haven, Connecticut, United States of America
2006	B.S. Biology University of Hawaii at Manoa, Honolulu, Hawaii

PUBLICATIONS

1. Halnon, N.J., Cooper, P., **Chen, D.Y.**, Boechat, M., Uittenbogaart, C. Immune Dysregulation after Cardiothoracic Surgery and Incidental Thymectomy: Maintenance of Regulatory T cells despite Impaired Thymopoiesis. 2011. Clinical and Developmental Immunology. Volume 2011: 915864.

2. **Chen, D.Y.**, Balamurugan, A., Ng, H.L., and Yang, O.O. Antiviral Activity of HIV-1 Gag- Specific Cytotoxic T Lymphocyte Targeting Is Not Necessarily Intrinsically Superior to Envelope Targeting. 2011. Journal of Virology, 85(5): 2474-2478.

3. Bo, W., Wingender, G., Fujiwara, D., **Chen D.Y.**, MacPherson, M., Brewer, S., Borneman, J., Kronenberg, M., and Braun, J. Commensal Microbiota and $CD8^+$ T Cells Shape the Formation of Invariant NKT Cells. 2010. Journal of Immunology, 184:1218-1226.

4. Gerschenson, M., Kim, Court., Berzins, B., Taiwo, B., Libutti, D., Choi, J., **Chen, D.Y.**, Weinstein, J., Shore, J., DaSilva, B., Belsey, E., McComsey, G., and Murphy, R. Mitochondrial Function, Morphology and Metabolic Parameters Improve after Switching from Stavudine to a Tenofovir-Containing Regimen. 2009. Journal of Antimicrobial Chemotherapy, 63:1244-1250.

Chapter 1:

Introduction

1.1 The Human Adaptive Immune System

The human immune system involves two major components: the innate immune system and the adaptive immune system (for a detailed description of these systems, see Janeway *et al* [1]). The innate immune system is capable of mounting an immediate, but general response against pathogens. By contrast, the adaptive immune system produces a sustained, pathogen-specific response. Following infection, immunological memory is formed, which can ensure a more rapid and effective response upon a second encounter with a pathogen.

The adaptive immune system is composed of the humoral and the cell-mediated immune components. The extracellular spaces are protected by the humoral response, in which antibodies produced by B cells can prevent the spread of infection by binding to extracellular microorganisms and invoking a directed immune response. The production of these antibodies involves the activation of naïve B lymphocytes and their differentiation into antibody-secreting plasma cells (for reviews, see Pape *et al* [2]; Banchereau *et al* [3]). On the other hand, pathogens which replicate inside cells, and cannot be detected by antibodies are destroyed by the cell-mediated response, which involves the actions of various subpopulations of T lymphocytes. Cell-mediated responses depend on the direct interaction between T lymphocytes and infected cells, which present antigens that the T lymphocytes recognize.

There are two general types of T lymphocytes: $CD4^+$ and $CD8^+$ T lymphocytes. Upon antigen recognition, naïve $CD4^+$ T lymphocytes generally mature into helper T cells, in which they produce various cytokines that can activate and/or regulate either the humoral or the cell-mediated response. On the other hand, naïve $CD8^+$ T lymphocytes mature into cytotoxic T lymphocytes upon activation, which eliminate cells that have been invaded by organisms.

In all cases, T lymphocytes recognize their targets by detecting peptide fragments derived from the foreign proteins of the invading pathogen. These peptides are first captured by major histocompatibility complex (MHC) molecules in the host cells, and are then displayed on the cell surface. There are two classes of MHC molecules, termed MHC class I molecules (for reviews, see Purcell and Elliot [4]; van Endert [5]) and MHC class II molecules (for reviews, see Guermonprez *et al* [6]; van Niel *et al* [7]), each of which are recognized by different functional classes of T cells. MHC class I molecules bearing viral peptides are recognized by CD8-bearing cytotoxic T cells, which then kill the infected cells, while MHC class II molecules bearing peptides derived from pathogens which have been taken up into vesicles by endocytosis are recognized by the CD4-bearing T cells.

Because this dissertation focuses primarily on the $CD8^+$ CTL response to HIV-1 infection, special attention will be paid to the MHC class I molecules and the biology of $CD8^+$ T lymphocytes. The first two sub-sections (1.1.1 and 1.1.2) describe the MHC class I antigen presentation pathway and the human leukocyte antigen class I

molecules. The next sections (1.1.3 and 1.1.4) describe the development of $CD8^+$ CTLs and their effector functions, respectively.

1.1.1 The Major Histocompatibility Complex Class I Antigen Presentation Pathway

The MHC class I (MHC-I) molecules, which are found on the surface of all nucleated cell types, bind short peptides derived from intracellular proteins and present them to $CD8^+$ T cells. Cell surface MHC-I molecules consist of three, non-covalently bound, individual polypeptides: the MHC-encoded heavy chain, β2-microglobulin (β2m), and a cellular protein-derived oligopeptide, typically between 8-10 amino acids in length (see Figure 1.1 for the crystal structure of MHC-I). Because MHC-I complexes are transported to the cell surface, the polypeptide chains of the MHC-I and β2m proteins are translocated into the lumen of the rough endoplasmic reticulum (rER) and are transported to the cell surface once protein folding in the presence of an intracellular peptide is complete (Figure 1.2).

The generation of the peptide –MHC-I complex is a multi-step process (Figure 1.2; for a more detailed description, see Yewdell *et al* [8]; Purcell and Elliot [4]). Proteins translated in the cytosol can be degraded by proteasomes into short peptide fragments. These peptides are then translocated into the rER, where they are loaded onto MHC-I receptors. Peptides generated by proteasomes range in size from 4 to 20 amino acids, but can be further trimmed by aminopeptidases, which reside either in the cytosol or in the rER. Peptide translocation from the cytosol to the rER requires the transporter for antigen processing (TAP), an ER-resident, heterodimeric peptide transporter. Within the rER, peptide binding to the MHC-I molecule is assisted by several rER chaperon proteins, including tapasin, ERp57, and calreticulin. Successful peptide binding releases

the MHC-I molecule from the rER for delivery to the cell surface through the standard secretory pathway. Because all synthesized proteins are potentially subject to this process, many MHC-I peptides are generated from endogenous polypeptides. However, during intracellular infection by invading pathogens, such as viruses, at least some of the MHC-I peptides displayed on the plasma membrane will be derived from viral proteins, and can signal to $CD8^+$ CTLs.

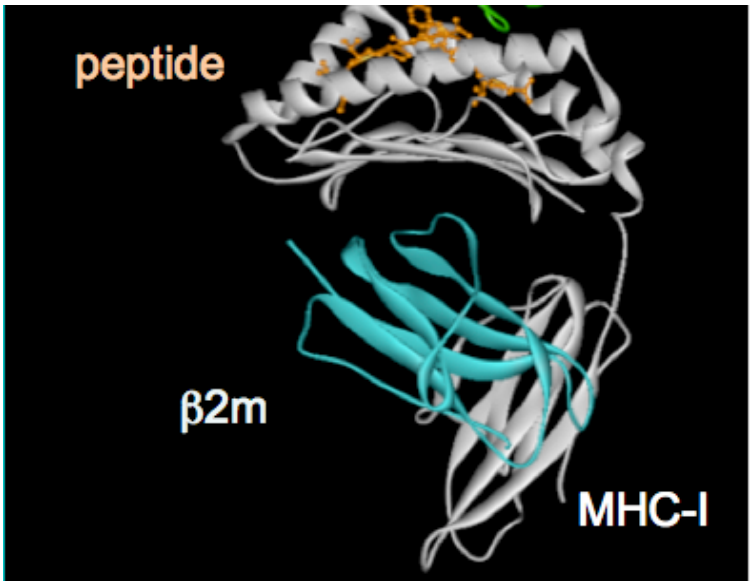


Figure 1.1 The structure of human major histocompatibility complex class I (adapted from Buslepp *et al* [9]). The structure of human leukocyte antigen class I A*02.1 bound to peptide1049 (ALWGFFPVLS) is shown in ribbon display. The A*02 heavy chain is silver, β2-microglobulin is cyan, and the peptide p1049 is gold.

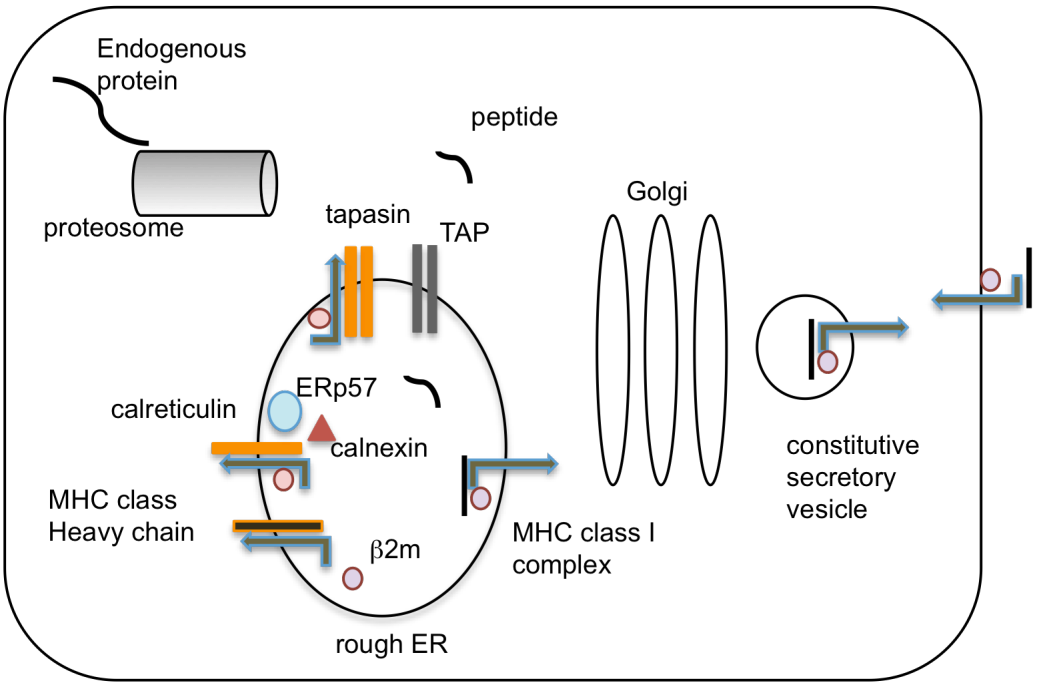


Figure 1.2 A schematic model of the MHC class I antigen processing and presentation pathway. Endogenous antigens are degraded by the proteasome into short peptides. These peptides are transported into the rough endoplasmic reticulum (rER) lumen by TAP. In the rER, a newly synthesized MHC class I heavy chain assembles with a β2m protein and a cellular protein-derived oligopeptide. This assembly process involves the interactions with several rER chaperon proteins, including calnexin, calreticulin, Erp57, and tapasin. Upon peptide binding, the MHC class I heterotrimeric complex is released from the rER and transported to the cell surface via the constitutive secretory pathway.

1.1.2 The Human Leukocyte Antigen Class I

The genes encoding the heavy chain of MHC-I molecules in humans are located on chromosome 6 (see Figure 1.3 for the genetic organization of the major histocompatibility complex in human), and are known as human leukocyte antigen-I or HLA-I genes, while the gene encoding the β2m protein is located on a separate chromosome (chromosome 15). Of the HLA-I heavy chains in humans, there are three distinct haplotypes, called HLA-A, -B, and –C. Furthermore, these HLA-I genes are highly polymorphic; there are approximately 1,757 HLA-A alleles, 1,795 HLA-B alleles, and 946 HLA-C alleles, with each allele being present at relatively high frequencies in the population [10]. Most individuals are likely to be heterozygous at the HLA-I loci, and thus express six different HLA class I alleles. Expression of the HLA alleles is codominant, with the protein products of both the alleles at a locus being expressed in the cell, and both gene products are able to present antigens to T cells. The high polymorphism of HLA loci and three haplotypes of HLA genes increase the number of different HLA-I molecules expressed in an individual, and contribute to the diversity of HLA molecules in the population at large.

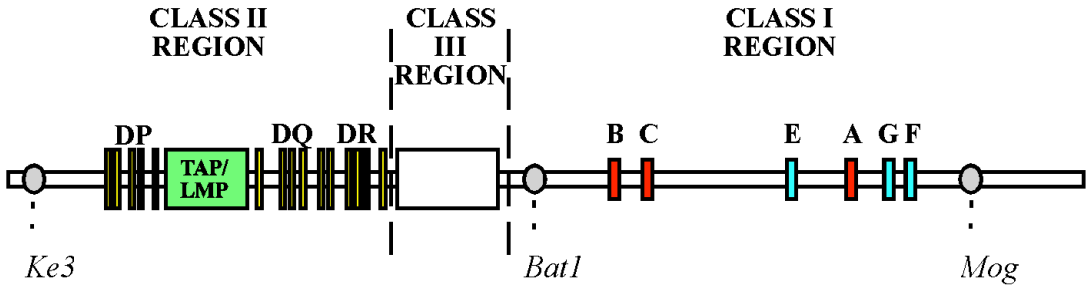


Figure 1.3 The genetic organization of the major histocompatibility complex in human. The organization of the MHC genes is shown for humans, where the MHC gene is termed the HLA and is located on chromosome 6. The class I regions encode for the classical HLA-I genes A, B, and C, and the non-classical HLA-I genes E, F, and G. The class II regions encode for the HLA-II genes. The class III regions encode a large number of genes related to immune system function.

1.1.3 Development of $CD8^+$ Cytotoxic T Lymphocytes

The T lymphocyte precursor cells originate in the bone marrow, and subsequently migrate to the thymus to complete their development into functional T cells. In the thymus, these progenitor T cells interact with the thymic stroma cells to initiate a series of differentiation pathways which consequently result in a change cell-surface molecule composition (see Janeway *et al* [1] for a more detailed description). During the early stage of T cell development, the juxtaposition of various gene segments leads to the generation of T cell receptor (TCR) α and β chains (Figure 1.4). The antigen-recognizing variable domains of TCRs are generated by a somatic recombination of variable (V), diversity (D), and joining (J) gene segments (TCR β chain), or V and J gene segments (TCR α chain). Generally, only one single rearranged Vα and Vβ chain is expressed in the final $CD8^+$ T cells due to allelic exclusion. The random recombination of TCR genes from among many possible configurations (estimated to be $>10^{15}$ configurations) contributes to the uniqueness to each TCR protein. Its uniqueness is further enhanced by the random loss of original nucleotides at the ends where the V and J regions join and the insertion of so-called “N-nucleotides” to patch this gap. Thus, each T cell is endowed with a TCR that has a unique specificity.

In addition to the TCR gene rearrangement, T lymphocytes at this stage of development express several other important surface receptors, including CD4 and CD8 molecules. These cells (often referred to as “double-positive” T cells due to concurrent expression of CD4 and CD8) must undergo both positive and negative selections in the thymus

before they are allowed to enter the peripheral lymphoid organs. These selection processes select for those cells whose receptor can interact, but not too strongly, with self-peptide:self-MHC molecular complexes. This involves selection of T lymphocytes that are functional (positive selection), and elimination of those that are self-reactive. During this process, double-positive T cells lose expression of either CD4 or CD8, depending on their TCRs binding to either MHC-I or MHC-II molecules. Cells that express TCRs which preferentially bind MHC-I express only CD8, while those cells which preferentially bind MHC-II express only CD4. The outcome of this process is the "single-positive" T cells, are exported from the thymus to the periphery, where they can execute their effector function. Because self-reactive T lymphocytes are negatively selected, all remaining TCRs are foreign antigen specific.

As previously mentioned in section 1.1, naïve $CD8^+$ T cells differentiate upon activation into cytotoxic cells, and destroy host cells that have been infected by organisms [11, 12]. These infected cells present peptides derived from foreign proteins on their surface via the MHC-I antigen-presentation pathway, and are thus capable of alerting the immune system. Naïve $CD8^+$ T cell TCRs scan the many different peptide-MHC-complexes throughout the body, and are activated when their TCRs recognize foreign antigens. The TCR-dependent recognition of foreign antigen causes the $CD8^+$ T cell to rapidly proliferate, creating a large pool of daughter cells with the same antigen specificity. These clones mature into CTLs, which then spread systematically and kill cells that display the foreign antigens via direct release of cytolytic enzymes and/or apoptosis induction.

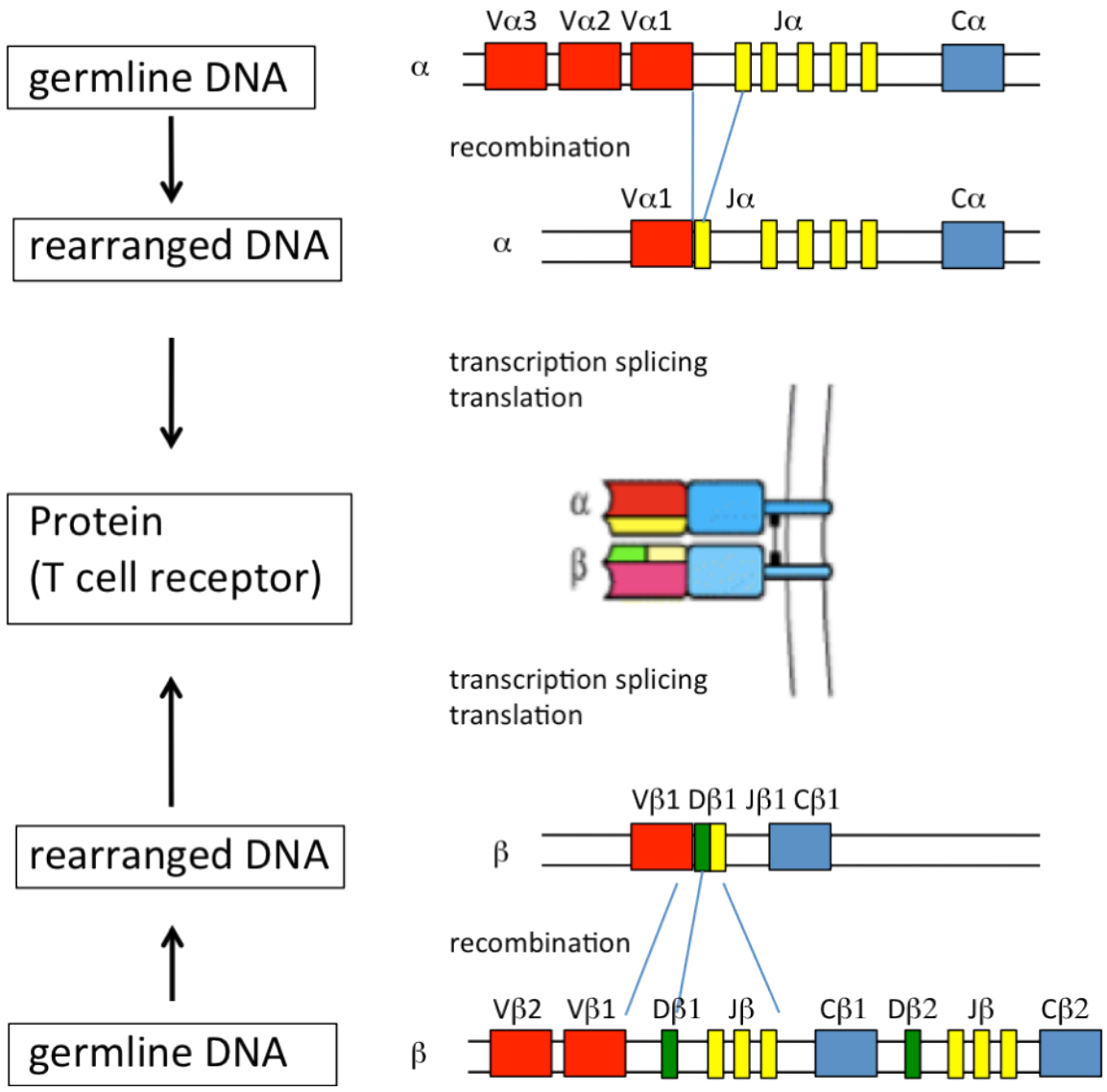


Figure 1.4 Somatic recombination of T cell receptor genes. In the diagram shown above, some TCR genes are omitted for simplicity. T cell receptor α and β chains each consist of a variable (V) amino terminal region and a constant (C) region. The TCRα locus (located on chromosome 14; top) contains variable (Vα) and joining (Jα) gene segments. The TCRα locus consists of 70-80 V gene segments and approximately 61 J genes. TCRβ locus (located on chromosome 7; bottom) contains diversity (D) gene

segment in addition to Vβ and Jβ gene segments, where a cluster of 52 functional Vβ gene segments located distantly from two separate clusters each containing a single D gene segment, together with six or seven J gene segments and a single C gene. In the developing T cell, the first recombination event to occur is between one Dβ and one Jβ segment of the TCRβ chain locus. This process can involve either the joining of the Dβ1 gene segment to one of six Jβ1 segments or the joining of the Dβ2 gene segment to one of seven Jβ2 segments. This DJ gene recombination is followed by the joining of one of the 52 Vβ genes, to form a rearranged VDJ gene segment. The primary transcript then incorporates a Constant gene, either Cβ1 or Cβ2. Any intervening sequences are spliced out during mRNA transcription, followed by the subsequent translation of the full length TCR Vβ protein. The rearrangement of the TCRα chain follows β chain rearrangement, which involves the joining of one Vα gene to one Jα gene, and the subsequent addition of the Constant gene, Cα. Additionally, the VJ region of the TCRαβ chains can undergo random nucleotide deletions, and the resulting gap is then patched with the random insertion of N nucleotides.

1.1.4 Effector Functions of $CD8^+$ Cytotoxic T Lymphocytes

$CD8^+$ cytotoxic T lymphocytes (hereafter referred to as CTL) kill their targets by direct cytolysis and/or inducing the targets to undergo apoptosis [13, 14]. The principal mechanism of this CTL action is the calcium-dependent release of specialized lytic granules upon the recognition of foreign antigen on the surface of the target cell. These lytic granules contain cytotoxic proteins, such as perforin, which polymerizes in target-cell membranes to form transmembrane pores resulting in direct cytolysis [15]. CTLs also release cytotoxic enzymes comprised of at least three serine proteases called granzymes. Granzyme B can direct lyse the cell. It can also cleave cellular proteins and activate a series of the caspase cascades that eventually lead to cellular apoptosis [16]. Both perforin and granzymes are required for effective cell killing [17, 18].

This release of lytic granule contents accounts for vast majority of the CTL cytotoxic activity. Additionally, a second way to induce the apoptosis of target cells involves the binding of the Fas receptor in the target-cell membrane to the Fas ligand (Fas-L), which is present on the activated cytotoxic T cells [19]. Ligation of Fas leads to the activation of specific caspases, which induce apoptosis in the target cell. The mechanisms of infected cell killing by CTLs through either the release of granules or Fas-Fas-L interactions require direct contact between the effector TCRs and target cell peptide:MHC-I complexes. High specificity and selectivity ensure only the targeted abnormal cells are killed in these processes. The narrowly focused action of CTL allows them to kill single infected cell in a tissue without creating widespread tissue damage.

While direct cytolysis of target cells is the main way in which CTLs clear infection, CTLs can also produce cytokines and chemokines that can directly or indirectly suppress viral replication [20-22]. These cytokines and chemokines include gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), RANTES, and macrophage inflammatory protein 1β (MIP-1β) and 1α (MIP-1α). IFN-γ increases the surface expression of HLA-I molecules and activates macrophages to synthesize more MIP-1β [23, 24], while TNF-α has been shown to directly induces apoptosis in infected cells [25]. Chemokines mainly serve to recruit additional immune cells to the site of infection, although it has also been shown that MIP-1α, MIP-1β and RANTES can suppress human immunodeficiency virus type 1 (HIV-1) replication *in vitro* by inhibiting HIV-1 entry to $CD4^+$ target cells [20, 26].

1.2 The Human Immunodeficiency Virus

The Human Immunodeficiency Virus (HIV), first identified in 1983, is the causative agent of acquired immune deficiency syndrome (AIDS) [27-31]. The virus is a member of the genus *Lentivirus* in the *Retroviridae* family [32-34]. The basic pathology in AIDS is a loss of $CD4^+$ T lymphocytes and a variety of disorders in immune cell function. The development of this disease results from a lack of control of HIV replication by the host immune system [35-37]. AIDS is defined by the reduction in $CD4^+$ T lymphocytes (less than 200 cells/μl) or the onset of opportunistic infections and malignancy. Neurologic and gastrointestinal disorders are also commonly found in AIDS patients. There are two types of HIV: HIV-1 and HIV-2. Although both viruses can cause AIDS, HIV-1 has had a greater impact on public health because it is more virulent, widespread, and infectious than HIV-2 [38]. This dissertation, therefore, focuses only on HIV-1.

1.2.1 Origin and Spread of HIV-1

Current literature suggests that HIV-1 epidemic arose by zoonotic transmission of the chimpanzee simian immunodeficiency virus (SIVcpz) from Cameroon to humans [39]. Phylogenetic analyses suggested that there have been three separate cross-species transmissions of SIV cpz from chimps to humans between 1910 and 1950, giving rise to the three major groups of HIV-1, the M, N, and O groups [40, 41]. HIV-1 M group viruses predominate and are responsible for the AIDS pandemic, in which the viruses accounted for 99.6% of the total number of infections worldwide [42]. The M group can further be divided into nine clades, which are designated A to D, F to H, J, K, and circulating recombinant forms (CRFs). Clade B of HIV-1 M group is primarily responsible for the pandemic in North America, although clade C accounts for the majority of infections worldwide.

Researchers believe that SIV may have initially jumped into humans after people came into contact with infected bush meat [43]. Among humans HIV-1 is spread by exposure to contaminated body fluids. This mode of transmission has carried HIV-1 across the globe, hence creating a pandemic. According to the most recent estimates, 33 million people are currently living with HIV, and 25 million persons have died of HIV related AIDS [44]. In the United States 40,000 new cases were reported in 2008 alone, and about 2 million persons are currently infected with HIV [44].

1.2.2 HIV-1 Structure

The HIV-1 virion is about 100 to 120nm in diameter [45]. When HIV-1 is viewed by electron microscopy, it has a cone-shaped core composed of the viral p24 Gag capsid (CA) protein (Figure 1.5A). Infectious (mature) virion contains the envelope (see next paragraph) and three structural Gag proteins: matrix (MA, p17), CA (p24), and nucleocapsid (NC, p7), which result from the proteolytic cleavage of the Gag precursor p55 (for review, see Gomez *et al* [46]) (Figure 1.5B and 1.6). The myristylated MA forms the inner shell in the particle just below the viral membrane, and CA forms the conical core. Inside the Gag capsid, are two usually identical RNA strands with which the viral integrase (IN, p32), reverse transcriptase (RT, p66), and the NC proteins (p9 and p7) are closely associated. Other viral proteins, Vif, and Nef, are also closely associated with the core; Vpr are packaged within the virion but mostly located outside the core. It is estimated that 7 to 20 molecules of Vif [47], 7 molecules of Nef [48], and 200-700 molecules of Vpr are contained per virion [49, 50]. Furthermore, using Cryo-EM and scanning electron microscopy, it has been estimated that an HIV-1 virion contains 5000 structural Gag proteins, and about 1000-1500 Gag CA proteins form the hexameric lattice core [49]. The number of Pol molecules per virion, however, is generally 10 to 20 times less than p24 [51].

The envelope proteins are derived from a 160-kDa precursor glycoprotein, gp160, which is cleaved inside the Golgi apparatus (by a host protease, furin) into a gp120 external surface envelope protein and a gp41 transmembrane protein [52]. The two envelope

proteins interact non-covalently, and are transported to the cell surface, where gp120 and the central and amino-terminal portions of gp41 are expressed on the outside of the virion [53]. The amino-terminal domain of gp41 is important for membrane fusion to host cells upon receptor binding [54], while the long cytoplasmic tail of gp41 appears to be important for HIV-1 envelope glycoprotein incorporations into virions [55]. Several other regions of the envelope, including the V1/V2 and the C2 and V3 domains of gp120, have also been implicated in binding to CD4 and co-receptors (CCR5 or CXCR4) on the target cells [56-58]. The genomic map of HIV-1 Env is depicted in Figure 1.6. In general, the virion has 60 to 100 times more Gag CA protein than envelope gp120 [51, 59, 60].

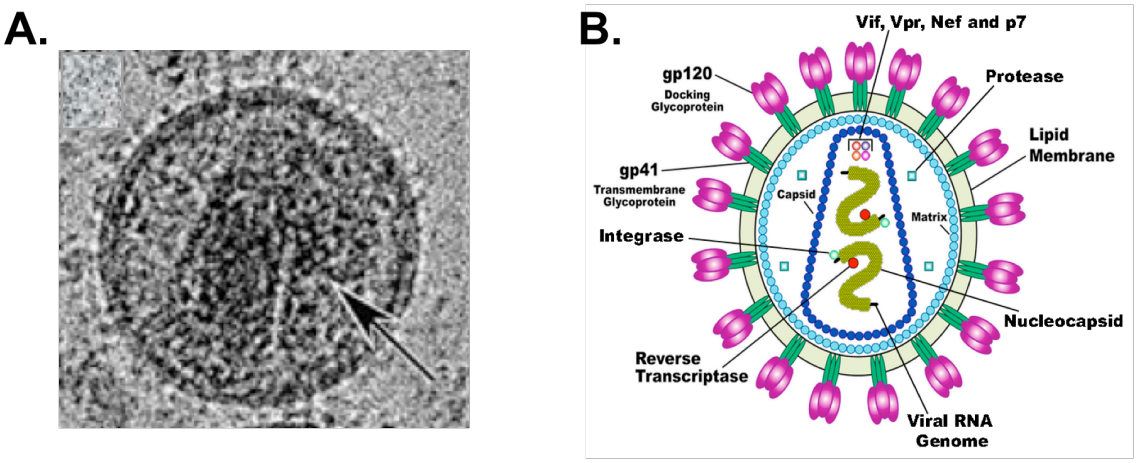


Figure 1.5 The HIV-1 virion. (A) Cryo-electron microscopy analysis of HIV-1 (adapted from Briggs *et al* [61]). (B) A schematic drawing of a single virion is shown (adapted from http://www.cgl.ucsf.edu/chimera/data/hiv09/hiv-demo.html). The surface of HIV-1 virions are covered with gp120-gp41 heterodimers. These heterodimers are embedded in a lipid bilayer and attached to matrix proteins. Within the matrix lies the viral capsid. The capsid contains two copies of the single-stranded RNA genome and various viral proteins, including reverse transcriptase, integrase, Vif, Nef, and Vpr, although most of Vpr proteins are located outside of the core. In the diagram of the virion shown above, some of the structural proteins have been omitted for simplicity.

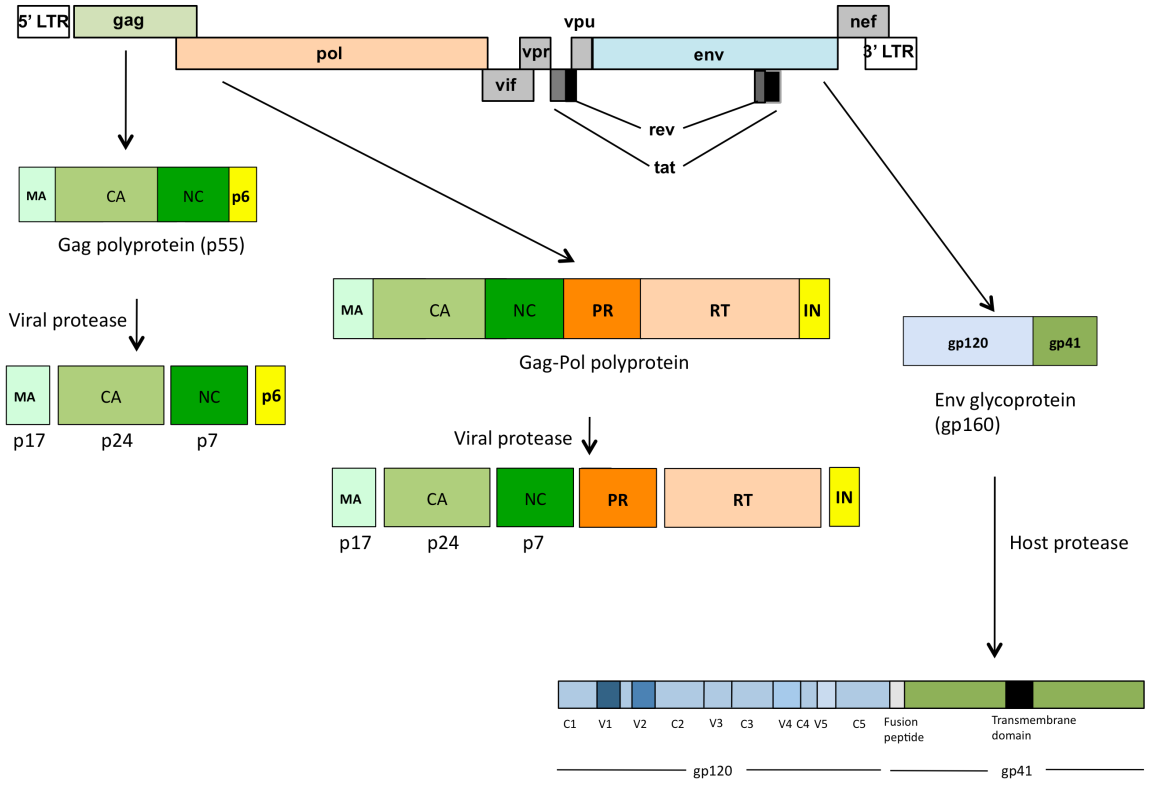


Figure 1.6 Genomic organization of HIV-1. The genome of HIV-1 is flanked by two long terminal repeats (LTR), while the central region contains open reading frames coding for nine proteins. The Gag precursor p55 is further cleaved into MA, CA, NC and p6 proteins by viral protease. The Gag-Pol polyprotein precursor is cleaved into MA, CA, NC, protease, reverse transcriptase, and integrase. Env glycoprotein gp160 is cleaved by the host protease furin into gp120 and gp41, which form the extracellular and the transmembrane domain of Env, respectively.

1.2.3 Transcription of the HIV-1 Genome

The genome size of HIV-1 is approximately 10 kb. Both ends of the genome are flanked by a repeated sequence, known as the long terminal repeat (LTR), while the central region contains open reading frames coding for nine proteins (Figure 1.6). HIV-1 transcription is mediated by a single promoter located in the 5' LTR [62, 63]. The initial activation of the HIV-1 LTR is a consequence of inducible and constitutive cellular transcription factors. Activation of the LTR by constitutive cellular transcription factors leads primarily to the generation of short transcripts; some complete transcripts are generated to allow the production of the Tat protein. The Tat protein then interacts with the trans-activation response (TAR) element in the 5' LTR of the nascent RNA transcript to recruit the transcriptional elongation factor and kinases that activate the elongating polymerase activity of RNA polymerase II. This action of Tat greatly increases the levels of transcription of viral RNAs. The Tat protein thus plays a key role in the activation and maintenance of high levels of transcription (for reviews, see [64] and [65]).

Another important event in the transcription of HIV-1 genes is RNA splicing, which produces many subgenomic mRNAs responsible for the synthesis of other viral proteins [66, 67]. The primary transcript contains multiple splice donors (5' splice sites) and splice acceptors (3' splice sites), which can be spliced into one of more than 30 mRNA species or packaged without further modification into virion particles (to serve as the viral RNA genome) [68]. In general, the HIV-1 mRNAs fall into three size classes (see Figure 1.7):

1. Unspliced full-length RNA. These mRNAs can be translated into the Gag and Gag-Pol precursor polyproteins or be packaged into virions to serve as the genomic RNA.
2. Incompletely spliced RNA. These mRNAs are generally singly spiced and still retain the second intron of HIV-1. These RNAs can express Env, Vif, Vpr, and Vpu.
3. Fully spliced RNA. These mRNAs have spliced out both introns of HIV-1 and have the potential to express Rev, Nef, and Tat. This class of mRNAs does not require the expression of the Rev protein.

In the eukaryotic cells, intron-containing RNAs or incompletely spliced mRNAs cannot exit the nucleus, and are not translated until all introns are removed. This “check point” of RNA splicing prevents the translation of any aberrant protein that contains intronic sequences. To overcome this “check point” for HIV-1, the viral Rev protein binds to incompletely-spliced and unspliced HIV-1 RNAs and directs their export from the nucleus [66, 67, 69]. Fully spliced RNAs, on the other hand, do not require Rev for nuclear transport, and therefore, the protein products of these mRNAs, Nef, Tat, and Rev, can be produced immediately. By contrast, those protein products of unspliced and incompletely spliced RNA will not be translated until the Rev protein is synthesized. Because of this post-transcriptional regulation by Rev, the timing of HIV-1 protein expression can be divided into early and late: the early expressed proteins are Tat, Rev, and Nef, while the late expressed proteins are Gag, Pol, Env, Vpr, Vpu, and Vif [70-72].

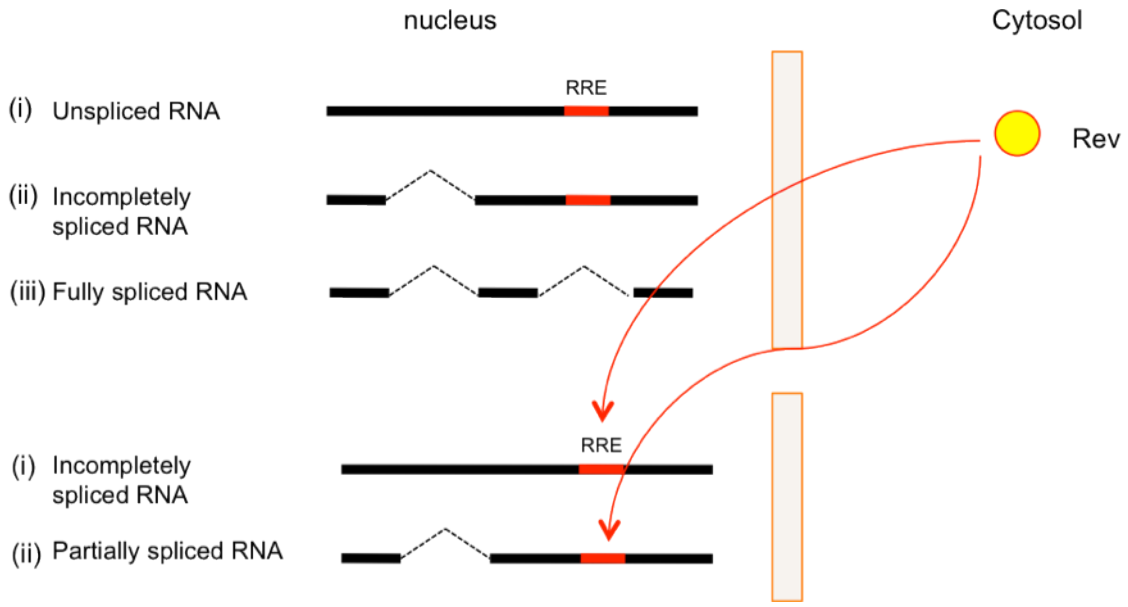


Figure 1.7 Three size classes of HIV-1 mRNA and the role of Rev in exporting incompletely and unspliced RNA for translation. HIV-1 mRNA falls into three major size classes: (i) unspliced RNA, (ii) incompletely spliced RNA, and (iii) fully spliced RNA. In the eukaryotic cells, unspliced and incompletely spliced RNA generally cannot exit the nucleus, and thus are not translated into proteins. Rev is a product of fully spliced RNA, and after its translation, Rev can bind to the Rev responsive element (RRE) on the incompletely spliced and the unspliced RNAs to direct their transport to the cytoplasm for translation.

1.2.4 The HIV-1 Life Cycle

As mentioned above, the surface of the HIV-1 virion is covered with two envelope proteins, gp120 and gp41, which mediate fusion of the virus with a host cell. After membrane fusion, the core of the HIV-1 virion is then released into the cell. Shortly after this process, the viral core proteins disintegrate, allowing the RNA genome, reverse transcriptase, intergrase and other viral proteins to enter the cytoplasm. Single-stranded RNA is then reverse transcribed into double-stranded DNA by reverse transcriptase [73]. The reverse-transcribed DNA is associated with the viral integrase, Vpr, reverse transcriptase, and matrix to form a pre-integration complex [73], and is subsequently transported to the nucleus, where the DNA is inserted in the host cell chromatin by the viral integrase [62, 74, 75]. After viral DNA integration, transcription of the viral genes is dependent on the viral protein Tat, as well as many host cell transcription factors that are commonly expressed in activated T cells [76, 77]. Rev then directs unspliced and incompletely spliced RNAs to the cytoplasm [66, 67, 69], where the Gag polyprotein captures the unspliced RNA and binds to the plasma membrane to form new virions [78]. Nascent virions then bud from the cell surface, taking up the viral envelope proteins. After the virion is released, the viral protease cleaves Gag and Gag-Pol polypeptides into various structural and functional proteins to form mature virions [46]. The entire process, from HIV-1 entry to virus production, takes approximately 24 hours [72].

1.3 $CD8^+$ Cytotoxic T Lymphocyte Responses to HIV-1 Infection

HIV-1 is capable of stimulating strong CTL immune responses in infected patients, despite causing profound immunodeficiency. In the acute phase of infection, the virus load increases exponentially in the blood, reaching ~10^7 HIV-1 RNA copies per ml of plasma [79]. A few weeks after, the virus level falls to a median of ~10^4 HIV RNA copies per ml [80]. This early drop in the blood viral load is temporally associated with the rise of HIV-1 specific CTL responses [81-83] (Figure 1.8), and AIDS is associated with a loss of HIV-1 specific CTLs [84].

The observation of such a temporal relationship between the rise of CTL responses and the fall of HIV-1 viremia during the acute phase of infection suggests that the CTLs can clear HIV-1 to a certain extent. This hypothesis was tested in the SIV/macaque model, where macaques were infused with antibodies specific for the CD8 glycoprotein, thereby depleting cells expressing CD8 molecules, such as CTLs [83, 85]. When these macaques are infected with SIV, the early control of virus fails; if the antibody is instead given during the chronic stage of infection, the virus level increases until the effects of antibody wear off. These data, together with *in vitro* viral suppression experiments and the observations of CTL-mediated escape mutations *in vivo*, support the role of CTLs in the control of HIV-1 infection [86-90].

Another important piece of evidence is the observation that certain HLA-I allele types are associated with the rate of progressing towards AIDS after HIV-1 infection [91-93].

Similar observations have also been made in recent genome wide association studies (GWAS) [94-98]. HLA-I molecules are the key molecules in presenting HIV-1 peptide to the CTLs, and because different HLA types present different peptides, the quality of the immune response generated by individual HLA types will be different. Similarly, heterozygosity at HLA-I loci is associated with a better HIV-1 disease outcome because it offers a more diverse T cell response. Together these studies indicate that CTLs can mediate protection against HIV-1.

Despite the strong induction of CTL responses by HIV-1 during the acute phase of infection (and the observed protective effects conferred by CTL during this phase), containment of HIV-1 by the immune system eventually fails, and without antiviral retroviral therapy most patients will progress to AIDS. However, a small number of infected individuals spontaneously control HIV-1 replication in the absence of treatment and maintain a healthy status in the long term [99]. Many efforts have been aimed at understanding why most host CTL responses are unable to contain HIV-1 replication, but are effective in some individuals. Previous work on this topic had demonstrated that a variety of host and viral factors could affect the quality of CTL response to effectively control HIV-1. Viral factors such as, viral escape mutation, and downregulation of HLA-I molecules by HIV-1 Nef can impair CTL efficiency. Host factors, such as CTL effector functions, antigen specificity, and HLA-I restriction have all been shown to contribute to individual differences in the efficiency of the virus-specific CTL responses (for review, see [100]). While each of these factors greatly impacts the ability of CTLs to control HIV-1 infection, this dissertation will only focus on the viral factor Nef-mediated HLA-I

downregulation, and a few CTL factors. A more detailed background of Nef-mediated CTL immune evasion is provided in next section (1.4), while the determinants of CTL antiviral efficiency pertinent to this dissertation are summarized in section 1.5.

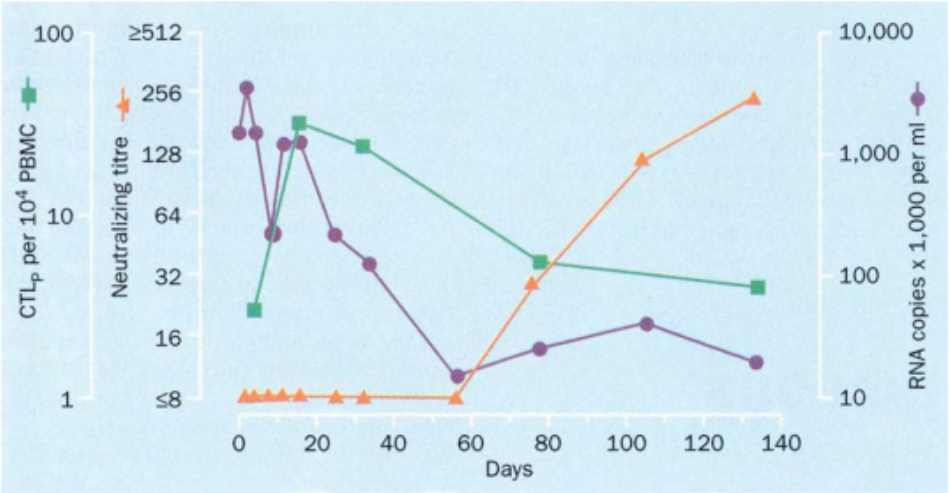


Figure 1.8 Timeline of the immune response to HIV-1 infection (adapted from Koup and Ho [101]). Temporal changes in viremia, frequency of HIV-1 specific CTLs per 10^4 peripheral blood mononuclear cells and neutralizing antibody response in a patient with acute HIV-1 infection. Viral load peaks at 10^6-10^7 RNA copies per ml of plasma two weeks after infection, and then eventually falls to a median of ~10^4 RNA copies per ml plasma. This drop in viral load is temporally associated with the rise of HIV-1 specific CTLs. Note that the rise of HIV-1 specific antibodies lags behind HIV-1 specific CTL responses by about 3-4 weeks.

1.4 HIV-1 Nef

Nef is a regulatory protein present only in the primate lentiviruses HIV-1, HIV-2, and SIV. The *nef* gene is situated at the 3'-end of the viral genome (Figure 1.6). Nef was originally named because it was thought to be a negative factor that inhibited viral replication [102]. However, it has become clear that Nef, in fact contributes "positively" to viral replication [103-106] and plays a central role in the immunopathogenesis of HIV-1 infection [107-109]. The pathogenesis of HIV-1 Nef in the development of AIDS in humans was first established by studies on a particular class of HIV-1 positive patients in the Sydney Blood Bank Cohort (SBBC) [110], who have remained AIDS free for at least 10 years of infection without the aid of antiretroviral therapy [111-114]. These patients are known as HIV long-term non-progressors (LTNP). Further analysis of these LTNP from the SBBC revealed that these patients were infected by the same individual whose HIV-1 strain harbors a large deletion in the Nef open reading frame (ORF) [110]. Similar deletions of the Nef ORF have also been detected among independent LTNP cohorts located in Asia, Europe, and North America [115-117].

Further evidence for a role of Nef in disease progression is provided by studies in rhesus macaques. As with humans, deletion of the Nef ORF reduced the pathogenic potential of SIV, and has dramatically delayed the onset of AIDS in these animals [118]. Furthermore, the virus appeared to be under strong selective pressure to express Nef. When macaques were infected with engineered SIV strains that contained a premature

stop codon or a 12bp deletion in the Nef ORF, the expression of functional Nef was quickly and universally restored [118, 119].

HIV-1 Nef protein interacts with a plethora of cellular proteins to enhance HIV-1 survival in the host (for reviews, see [120-124]). Some pathogenic functions of Nef include the downregulation of CD4 molecules from the plasma membrane to avoid super infection, downregulation of HLA-I molecules to avoid immune surveillance by CTLs, increasing virus infectivity, promoting cell to cell transfer of virions, and including the aberrant activation of T cells to increase the number of target cells for HIV-1. Although a detailed description of each of these functions is beyond the limited scope of this dissertation, special attention will be paid to the HLA-I downregulation function of HIV-1 Nef due to its critical role in immune evasion.

1.4.1 Nef-Mediated HLA-I Downregulation

Antigen presentation by HLA-I molecules provides a mechanism by which our immune system can detect abnormal cells in the body, including cells that have been infected with microorganisms. CTLs circulate throughout the body and survey peptide-loaded HLA-I molecule on the surface of cells via the T-cell receptor. If a foreign peptide-HLA-I complex is detected, CTL will lock on to the target and inject lytic proteins into the target cells, and destroy the target cells.

As with many human pathogens, HIV-1 has developed strategies to subvert host CTL immune surveillance, specifically by encoding the Nef protein that downregulates cell surface expression of HLA-I on infected cells [125-127]. Although HLA-I downregulation by Nef is incomplete (where only 50% of the cell surface HLA-I are downregulated by Nef) [128], this level of downregulation has been shown to have a profound impact on the CTL recognition of HIV-1 infected cells. The first published study to address this effect of Nef was by Collins *et al* [129], who added HIV-1-specific CTLs to reporter-tagged HIV-1 infected cells that ectopically express Nef or without Nef several days after initial infection, then assessing clearance of infected cells by flow cytometry. Nef infected cells persisted whereas cells without expression of Nef disappeared, suggesting a Nef-mediated resistance from these infected cells to clearance by CTLs. Subsequently, Yang *et al* [130] and Tomiyama *et al* [131] confirmed the conclusions of Collins *et al* by demonstrating that Nef interferes with the ability of HIV-1-specific CTL to suppress viral replication, comparing the growth inhibition of Nef-competent and Nef-

defective viruses when CTL were added to acutely infected cells. Furthermore, *in vitro* passaging of HIV-1 in the presence of CTLs revealed that there was a strong selective pressure to maintain this function [132]. When HIV-1 is passaged in the presence of Nef-specific CTL clone alone, mutations in the Nef CTL epitope that abolish HLA downregulation are frequently detected after several rounds of passaging. By contrast, when the same virus is passaged in the presence of Gag and Nef-specific CTL clones, only escape mutations in the Gag epitope are detected. This suggests that in the face of multiple CTL pressures, it is more advantage for the virus to maintain this HLA-I downregulation function of Nef.

In vivo evidence for the preservation of this Nef function came from studies of SIV /macaques models [133, 134] and primary Nef isolates [135]. Macaques infected with SIV containing mutations in Nef that affected the Mamu downregulation function initially experienced low viremia. However, increased viremia in these animals was due to compensatory mutations in SIV Nef that restored the Mamu downregulation function. The fact that SIV quickly restores its Mamu downregulation function suggests that this function is critically important for viral survival *in vivo*. Similarly, in HIV-1 infected patients, the ability of Nef to downregulate HLA-I strongly correlates with the breadth of CTL responses from the host [135]. Altogether these studies indicate that Nef-mediated HLA-I downregulation contributes to HIV-1 persistence *in vivo* by evading CTL immunity.

1.4.2 Mechanisms of Nef-Mediated HLA-I Downregulation

The 27kDa HIV-1 Nef usually exists in the myristoylated form in infected cells. Myristoylation is required for its association with cellular membranes and is critical for all of its biological functions [124, 136-139]. Nef-mediated HLA-I downregulation was first described by Schwartz *et al* in 1996 [125]. However, the mechanisms involved in HLA-I downregulation remain disputable. Of all the models that have been proposed, the most convincing one involves the direct interaction between Nef and the cytoplasmic tail of HLA-I molecules, and the subsequent recruitment of AP-1 complexes that redirect the newly synthesized HLA-I molecules in the early endocytic compartment to the lysosomes [140-144].

The cytoplasmic tail of HLA-A and –B alleles contain the YSQA motif, which resembles the tyrosine-based Yxxϕ sorting motif present in the cytoplasmic tail of numerous cargo proteins and is specifically targeted by Nef [127, 140, 145, 146]. In this model, Nef binding to the YSQA motif of HLA-I cytoplasmic tails cooperatively recruits AP-1 to the complex, and re-direct HLA-I molecules from the trans-Golgi network to the lysosomes where proteolysis occurs [147]. Nef interaction with HLA-I and AP-1 proteins involves various domains of Nef, including the H1 a-helix (aa17-26) containing a methionine residue at position 20, a polyproline motif, as well as the EEEE acidic cluster found in the N-terminal region of Nef.

As aforementioned, the YSQA motif of the HLA-I cytoplasmic tail is required for Nef-binding and the subsequent recruitment of AP-1 complexes; because this motif is only present in the HLA-A and –B cytoplasmic tails and not -C or –E, Nef only reduces cell-surface expression of A and B molecules but not C or E [140, 145, 146]. The selective downregulation of HLA-I molecules by Nef allows HIV-1 to evade cytotoxic T lymphocyte responses which are predominantly A and B-restricted, and to avoid killing by NK cells, whose function is to recognize cells that have reduced cell surface expression of C and E molecules [148, 149].

1.5 The Determinants of CTL Antiviral Activity Against HIV-1

Numerous studies have focused on host and viral genetics, gene expression, and T-cell specificity, phenotype and function, in attempts to identify better correlates of CTL-mediated protection. It has become clear that the “quality” rather the “quantity” of the CTL response is more critical in determining the efficacy of the host response to HIV-1. Importantly, individual differences in the efficacy of the virus-specific CTL response strongly determine the outcome of HIV-1 infection. Some major factors that affect CTL antiviral efficiency are discussed in a more detail below.

1.5.1 The Temporal Relationship Between Epitope Presentation and Nef-mediated HLA-I Downregulation

The kinetics of epitope presentation play an important role in determining the antiviral activity of CTLs [150-152]. The earlier an epitope is presented on the surface of an infected cell, the faster the CTL can eliminate it before progeny virus are released. Because Nef-mediated HLA-I downregulation is not immediate but occurs at 12 hours post-infection [153, 154], it has been hypothesized that CTLs targeting epitopes presented before this time may be more effective in suppressing viral replication. For example, CTLs targeting epitopes derived from the early expressed proteins Tat, Rev, and Nef may be more effective than those directed against epitopes located in the late structural proteins such as Gag, Pol, and Env (see section 1.2.3 for a detailed description on the transcription of HIV-1 genome). Indirect evidence supporting this hypothesis was provided by van Baalen *et al* [151] and Ali *et al* [150] who separately demonstrated that accelerating epitope expression can increase the antiviral efficacy of CTL clones.

Interestingly, although Gag protein is a late expressed HIV-1 protein, recent studies in SIV/macaque models have reported that the incoming virion associated Gag-derived epitopes can be presented on the cell surface of an infected cell as early as 2 hours post-infection [152]. This allows Gag-specific CTLs to eliminate infected cells before SIV Nef-mediated Mamu downregulation and hence, be more effective in suppressing viral replication. Together these studies suggest that the relative timing between epitope

presentation and Nef-mediated HLA-I downregulation is important in determining the antiviral efficacy of CTLs.

1.5.2 Functional Avidity

CTL functional avidity is usually quantified as the antigen concentration that elicits a half-maximal frequency of responses by specific T cells. This assay has been widely used as a marker of the responsiveness or sensitivity of CTL to cognate antigen. High functional avidity has been correlated to efficacious CTL antiviral activity and to superior effector functions. In the study by Alexander-Miller *et al*, CTLs of high functional avidity were more effective in suppressing virus replication *in vivo* than the low avidity CTLs [155]. Similarly, because high avidity CTLs require less antigen densities for recognition, high avidity CTLs lyse virus- infected cells more rapidly than low avidity CTL in the course of a viral infection [156]. High avidity CTLs also proliferate faster than the low avidity ones in response to antigens [157, 158]. Studies of HIV-1 infection have showed that CTL clones specific to HLA-B restricted epitopes had higher functional avidity and hence, were more effective in suppressing HIV-1 replication than those targeting epitopes restricted by HLA-A alleles [157]. Also, the higher functional avidity of CTLs may be more efficient in recognizing infected cells than low avidity CTLs when antigen density is low in the setting of Nef-mediated HLA-I downregulation.

However, not all the studies were able to demonstrate a clear correlation between the functional avidity of virus-specific CTLs and their killing efficiency. Yang *et al* showed that antigen specificity is a more important determinant of CTL antiviral activity than functional avidity [159]. Recent studies by Chen *et al* demonstrated that Gag- and Env-specific cell lines had similar functional avidity but differed by more than 1000 fold in their ability to suppress HIV-1 replication *in vitro* [160]. Moreover, Bennett *et al* found

that a threshold of antigen density must be exceeded in order for CTLs to kill infected target cells, and that additional avidity beyond the required threshold did not increase CTLs antiviral activity [161]. Studies in the SIV/macaque model also failed to show a correlation between functional avidity and CTL antiviral efficacy [162].

The reason for the discrepancy between these studies is not clear. While functional avidity measurements provide a putative correlate of lysis, the assay completely bypasses the factors determining the quantity of viral epitopes presented on the cell surface during natural infection. These factors include levels of viral protein expression, efficiency of epitope processing in the proteasome and transport by the transporter associated with antigen processing [161]. Therefore, for CTL efficiency, it is still more important to measure the actual lysis of infected cells, whenever possible.

1.5.3 Superior Control of HIV-1 by Gag-Specific CTL Responses

There has been considerable interest raised by the observations that the protein-specificity of the CTL response can affect the outcome of HIV-1 control. An early study by Riviere *et al* showed that a Gag-specific CTL response is associated with the successful immune control of HIV-1 [163]. Subsequently, other studies have also observed an inverse relationship between the magnitude and breadth of Gag targeting and the viral load [164-169]. While no association with protection has been observed for non-Gag specific responses, lack of immune control has been observed with Env-specific and Nef-specific CTL responses [163, 164, 166]. These studies suggest that only Gag-specific but not non-Gag-specific CTL responses are important for the long-term control of HIV-1 infection.

One possible reason for the effectiveness of Gag targeting by CTLs is that Gag is highly immunogenic, but also conserved in sequence. Hence escape mutations in Gag can reduce viral fitness. This is supported by the observation that an increasing number of HLA-I associated Gag-specific amino-acid polymorphisms decrease viral replication capacity, and are associated with the long term control of HIV-1 replication [170-172]. These data suggest that the immune control of HIV-1 is mediated in part by the ability of the Gag-specific CTL responses to drive the selection of escape mutations that are detrimental for the virus. Broader Gag targeting may also constrain the virus from escaping.

Another reason why Gag is efficient in controlling HIV-1 replication may be because Gag-specific CTLs can recognize infected cells very early after cellular infection (see section 1.5.1 for more details on the Gag epitopes presentation kinetics). Gag proteins are carried in sufficient quantities by the incoming virion, such that these proteins can be directly processed by proteasomes in the cytoplasm, and the resulting peptides can be presented on the cell surface of an infected cell before *de novo* viral protein synthesis [152]. More recently, a study by Berger *et al* showed that CTLs targeting epitopes located in Gag had on average higher functional avidity than those directed against non-Gag epitopes [173], providing another possible explanation as to why Gag targeting is beneficial *in vivo*.

1.5.4 Host Genetics Associated with the Control of HIV-1 Infection

Functional data and genome wide association studies (GWAS) have emphasized the importance of HLA-I polymorphism in determining viral loads and the outcomes of HIV-1 infection [94-98]. Specifically, certain HLA-B alleles, and a single nucleotide polymorphism (SNP) located 35kb upstream of the HLA-C locus have been linked to variation in viral load.

The HLA-B*57 allele tops all others in terms of its effect on HIV-1, and is represented in 44% of all HIV-1 long-term non-progressors [174, 175]. These individuals have low viral load, and are able to maintain normal $CD4^+$ T cell counts and extremely low or undetectable plasma viral loads for more than 10 years without the aid of antiretroviral therapy [174-177]. Several mechanisms by which the B*57 molecule mediates protection have been suggested. An important feature of B*57 is its ability to mount immunodominant CTL responses during acute infection, with the most frequently targeted epitope being Gag TW10 [178]. Such an acute CTL response has been shown to be important in lowering the viral load and slowing disease progression in these individuals [96]. Furthermore, a T242N escape mutation in the TW10 epitope is frequently detected during the early stage of HIV-1 infection [86, 179, 180]. This mutation significantly reduces viral replicative capacity when tested *in vitro* [179, 180], and quickly reverts back to wild-type upon transmission to a non-B*57 individual [86]. This decrease in viral fitness associated with the T242N mutation may, in part, explain the long-term control of HIV-1 by B*57 positive patients [172, 181].

B*57 also presents three other immunodominant epitopes located in conserved regions of Gag, and escape mutations within these targeted epitopes can impose a fitness cost on the virus as well [171, 182]. Likewise, targeting four different conserved regions of Gag by CTLs also makes it more complicated for the virus to escape. Finally, a recent study has used a mathematical model of T cell selection in the thymus to suggest that the protective effect of B*57 is due to a more diverse B*57 restricted TCR repertoire breadth when compared to other HLA-I allotypes [183]. An increase in TCR breadth implicates a greater cross-reactivity of B*57 restricted CTLs to escaping epitopes.

Other HLA- B alleles that are overrepresented in HIV-1 long-term non-progressors include B*27, which represents 15% of these individuals [175-177, 184]. Similar to B*57, the mechanism of B*27 protection involves the induction of acute immunodominant CTL responses against Gag. However, unlike the B*57 restricted CTL responses, which target up to four Gag epitopes, B*27 restriction is focused on a single, highly conserved epitope, KK10 [88, 185-187]. Under B*27-restricted CTL pressures, the virus undergoes a complex pattern of mutations that eventually result in a fully escaped virus with wild-type levels of replicative capacity [188]. Escape mutations commonly detected in KK10 epitope are S173A, R264K, and L268M, and development of all three escape mutations combined results in rapid disease progression [88, 187-189].

Recently, HLA-C alleles marked by an SNP upstream of the locus was shown to have the second most significant effect on HIV-1 viral loads genome-wide, following an SNP in the HLA- B locus [95, 96, 98]. This variant, located 35kb upstream of the HLA-C gene

(-35C/T) was shown to affect HLA-C gene expression. The -35CC genotype was found to associate with significantly higher HLA-C surface expression than those with the -35TT genotype [96, 190]. The -35CC genotype also associates with better viral control when compared to -35TT individuals [191]. The potential mechanism for the protection conferred by this variant is most likely due to an increase in antigen presentation by HLA-C molecules to CTLs. Additionally, high HLA-C expression may result in more effective education of NK cells during their maturation process through better engagement of inhibitory NK receptors to HLA-C ligands, ultimately leading to stronger NK cell responses against HIV-1 infected targets.

Although specific variants in HLA-I genes are associated with the effective control of HIV-1 replication, the mechanisms of protection conferred by these variants are somewhat different. It is also important to note that similar numbers of infected individuals without “protective alleles” have been identified as long-term non-progressors and having protective alleles does not ensure an HIV-1 long-term control status, per se. To make matters more complicated, detailed analysis of immune parameters between HLA-B*57 non-progressors and progressors were unable to identify any differences [192], suggesting that additional unknown host factors are involved in controlling infection.

Chapter 2:

Materials and Methods

2.1 HIV-1-Permissive Cells and Tissue Culture

$CD4^+$ T1 cells [193] (expressing HLA A*02 and B*40) and the T1/primary $CD4^+$ T lymphocyte hybridoma 1CC4.14 (expressing A*02, B*15, B*40, and B*57) [194] were maintained in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, HEPES, and penicillin-streptomycin (R10) as described [128].

Primary $CD4^+$ T lymphocytes (expressing A*02 and B*57) were expanded from PBMC with a CD3/CD8-bispecific monoclonal antibody [160], confirmed to be >95% $CD3^+/CD4^+$, and maintained in R10 supplemented with 50 U/ml of recombinant human interleukin-2 [160] (R10-50) from the NIH AIDS Reagent Repository. Primary $CD4^+$T lymphocytes were stimulated with 1mg/mL phytohemagglutinin (PHA) for 3 days before viral infection [160].

The 293T cells human renal epithelial cell line is a convenient source of recombinant virus. 293T cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 200mM L-glutamine, 10mM sodium pyruvate, 100U/ml penicillin, 100ug/ml streptomycin, 500ug/ml generation and 5% CO2.

2.2 HIV-1 Specific CTL Clones

CTL clones were derived previously from the peripheral blood mononuclear cells (PBMCs) of HIV-1-infected individuals by limiting-dilution cloning and maintained by periodic re-stimulation with anti-CD3 antibody and irradiated allogeneic PBMCs in R10-50 as described [195].

2.3 Viral Constructs

Replication-competent whole HIV-1: HIV-1 NL4-3.1[196] viruses containing wild type or a methionine-to-alanine mutation at position 20 of Nef (Nef-M20A) were produced and titered as described [197]. For experiments assessing the kinetics of Nef-mediated HLA-I downregulation, the virus also contained the murine CD24/Heat Stable Antigen reporter gene (HSA) in the *vpr* locus [197].

Replication-competent Gag-SL9 translocated to Env HIV-1: Two molecular clones of HIV-1 NL4-3 were modified to alter the endogenous Gag SL9 epitope to a previously described [161] non-recognized sequence (Gag-SL9x, SLYNLVAVL) and to create the SL9 epitope sequence in either of two locations in Env (Table 4. S1). These Env mutations contained amino acid substitutions to create the SL9 sequence in the $gp41_{301-311}$ cytoplasmic domain (Env-SL9-gp41) or the $gp120_{370-381}$ V4 loop (Env-SL9-V4). Additionally, another virus contained the Gag-SL9x mutation, combined a methionine to alanine mutation at position 20 of Nef (M20A) that selectively neutralizes the downregulation of MHC class I by Nef [198].

Single round infectious HIV-1: The NL4-3.1 genome was altered to contain mutations D368R in gp120[199], L26R in gp41 [200], a truncation of 26 amino acids of the gp41 cytoplasmic domain [201], and an additional deletion in the V3 loop (300-329), as well as insertion of the gene for HSA in the *vpr* locus [197]. This replication-defective genome was co-transfected into 293T cells with a vesicular stomatitis virus G glycoprotein (VSV-G) envelope expression vector, and supernatant was harvested after 2 days to produce a stock of single-round infectious HIV-1 pseudotyped with VSV-G envelope (NL4-3-ΔEnv/VSV-G-Env). The virus stock was concentrated by ultracentrifugation and titered by flow cytometry after infection of T1 cells.

Single round infectious GFP-Lentivirus: Viral vectors are derived from the HIV based lentiviral backbones optimized by the laboratory of Dr. Irvin Chen. Green fluorescent protein (GFP) is inserted into the pCCL lentiviral transfer vector under control of the SFV promoter [202]. Lentivirus was produced by transfection of 293T cells with the above lentiviral vector constructs in conjuction with the lentiviral packaging vector pCMVΔR8.2VPR [203] and the vesicular stomatitis virus envelope protein G expression pHCMVG [204] using Fugene HD transfection reagent (Roche). The virus stock was concentrated by ultracentrifugation and titered by flow cytometry after infection of T1 cells.

2.4 Viral Suppression Assay

To compare the susceptibility of viruses to CTLs, a previously described viral suppression assay was performed [161, 194, 195]. Briefly, HIV-1 permissive cells were

infected at a multiplicity of infection of approximately 10^{-2} and cultured with CTL at an effector:target cell ratio of 1:4 in triplicate in 96-well plates. HIV-1 p24 antigen-capture ELISA (Perkin Elmer) was performed on supernatant medium at days three and six after infection to assess viral replication. Raw virus suppression was calculated as the difference in $\log_{10}$ p24 concentration between control wells without CTLs versus those with CTLs at day 6. For normalized comparisons of viral inhibition on day 6, the percent efficiency of log suppression was calculated as raw log units of virus suppression/log units of p24 in the no CTL control, as previously described [161].

2.5 Assessment of Nef Impact on the Antiviral Activities of CTL Clones

The Nef impact assay was previously established to allow standardized comparisons of Nef effects on CTL suppression of HIV-1 replication by comparing wild-type and mutant Nef-M20A viruses [194]. Briefly, cells were acutely infected with NL4-3.1 containing wild-type Nef or Nef-M20A and co-cultured with or without the CTL clone at an effector-to-target ratio of 0.25:1 in triplicate wells. *Inhibition efficiency* was calculated as: ($\log_{10}$ p24 without CTL - $\log_{10}$ p24 with CTL)÷ ($\log_{10}$ p24 without CTL). The " *Nef Effect Ratio*" was then calculated as: (*Inhibition Efficiency* of HIV-1 with wild-type Nef) ÷ (*Inhibition Efficiency* of HIV-1 with Nef-M20A), for which 1 indicates no impact of Nef (same efficiency) and 0 indicates complete ablation of CTL antiviral activity (efficiency of 0 for HIV-1 with wild-type Nef).

2.6 Kinetics of HIV-1 Specific CTL Killing of Virus-Infected Cells

Infected cell killing by CTL clones was assessed by modified chromium release assay [128, 161]. 5 x 10^5 target cells were incubated with virus stock containing 600 femtograms (or the indicated amount) of Gag p24 per target cell for two hours in the presence of 25 mCi ^{51}Cr and 4 mg/ml of polybrene in 100 ml, and washed three times with R10. For cells treated with 500 mM of tenofovir and 500 mM zidovudine (NIH AIDS Research & Reference Reagent Program), the drugs were added to the cells at least 2 hours before infection and maintained throughout the experiment. The target cells were resuspended in R10-50 at a concentration of 6.6 x 10^4 cells/mL and co-cultured with or without the specific CTL clone at an effector-to-target ratio of 3:1 (3 x 10^4 CTL and 10^4 target cells per well in 96 well U-bottom plates) in 250 ml of R10-50 in triplicate wells. At the indicated times, 30 ml supernatant was harvested from each well for measurement of ^{51}Cr release by microscintillation counting (Lumaplate, Perkin Elmer, and Microbeta, Wallac). Controls included target cells with no added CTLs (spontaneous release) and target cells lysed with 2.5% Triton X-100 (maximal release). At each timepoint, the released ^{51}Cr was calculated by multiplying the ^{51}Cr concentration (cpm/ml) by the remaining volume of the media and then adding back the total ^{51}Cr counts removed from each well from earlier time-points. Specific lysis was calculated as: *specific lysis* = (total experimental ^{51}Cr release - total spontaneous ^{51}Cr release) ÷ (total maximal ^{51}Cr release - total spontaneous ^{51}Cr release). An uninfected control was also included in the assay to determine any non-specific killing by CTLs. To define the onset of infected cell killing, the logarithmic regression curve was fitted to the average infected cell lysis over time (with $R^2 > 0.9$) to estimate the time corresponding to 10% specific lysis (K_{10}; the choice of 10% is arbitrary based on the limit of reliable detection).

2.7 CTL Functional Avidity Measurements

Functional avidity of CTL clones was determined by standard peptide titration chromium release assays[128, 161, 195, 196]. Briefly, the ^{51}Cr-labeled target cells were pre-incubated with serial dilutions of the cognate peptide before the chromium release assay. Functional avidity was measured as the sensitizing dose (concentration) of peptide yielding 50% of maximal CTL killing (SD_{50}).

2.8 Measuring Kinetics of Nef-Mediated HLA-I Downregulation

Target cells were infected at excess MOI of 12 with recombinant NL4-3.1 expressing wild-type Nef or Nef-M20A for 4 hours at 37˚C, washed twice, resuspended, and plated at 5 x 10^5 cells/well in a 24 well plate. In some experiments, infection was performed using NL4-3 ΔEnv/VSV-G virus stock at 600 femtograms Gag p24 per cell. Primary $CD4^+$ T lymphocytes were stimulated with 1 mg/mL PHA for three days and infected with the indicated viruses at 600 femtograms Gag p24/cell. To assess HLA-I downregulation, the acutely infected cells were co-stained for the cell surface reporter HSA (mAb M1/69-PE, eBioscience), HLA-A*02 (mAb bb7.2-Alexa Fluor 488, AbD Serotec), and HLA-B*57 (mAb B17-Biotin, One Lambda, with secondary staining using streptavidin-PE/Cy5.5, eBioscience) at the indicated times. Additionally, CD4 expression (mAb OKT4-APC, eBioscience) was monitored on acutely infected primary $CD4^+$ T lymphocytes. Flow cytometry was performed on a FACScan (BD Bioscience) or a FACSCalibur (BD Bioscience), and analyzed using Flowjo software (Tree Star, Inc.). At the indicated time points, infected cells (positive for HSA expression) were gated for

analysis of A*02 and B*57 expression (mean fluorescence intensity, MFI). Productively infected primary $CD4^+$T lymphocytes were identified by gating on low CD4[152] and high HSA reporter expressing cells[197]. The relative expression of HLA-I on cells infected with wild-type Nef virus was then calculated as a fraction of MFI compared to Nef-M20A virus, after subtraction of background MFI (observed using an isotype control). Nef-mediated downregulation of HLA-I on 1CC4.14 cells and primary $CD4^+$ T lymphocytes was assessed in three and six independent experiments respectively.

2.9 Mathematical Modeling of the Kinetics of Nef-Mediated HLA-I Downregulation Kinetics

HLA-I downregulation kinetics were fitted using a modified three parameter Gompertz function: $f(t) = 1 - \beta_1 a^{-a^{-\beta_2(t-\beta_3)}}$, where *a* is any positive constant, and β_1, β_2, β_3 are unknown parameters. The Gompertz curve has two asymptotes: 1 as $t \rightarrow -\infty$ and 1- β_1 as $t \rightarrow \infty$. The left asymptote, 1 as $t \rightarrow -\infty$ was fitted to the downregulation timepoints where the wild-type relative HLA-I expression remained 1 or above. The right asymptote, 1- β_1 as $t \rightarrow \infty$ was fitted to the relative HLA-I expression that had reached a plateau, which represented the maximum HLA-I downregulation observed in infected cells. The Gompertz curve is also asymmetric with a steeper decline from the left-hand asymptote compared to the approach to the right-hand asymptote, which is an appropriate model for fitting HLA-I downregulation by Nef, where downregulation occurs abruptly and then reaches a plateau. We chose $a = 10$ so that the value of the function at β_3 is $f(b_3) = 1 - \beta_1/10$, making β_3 the point at which HLA expression has dropped 10% between asymptotes (the choice of *a* is arbitrary). To compare the fits for A*02 and

B*57, we calculated the natural parameterization and estimates of the parameter and its variance. We also compared the slopes of the fitted curves at the estimated value of β_3: f'(β_3). This required an approximation (using the Delta method) to estimate the variance of the slope. Assuming independence of the estimates, we used the Wald test to compare for each experiment the following: 1) β_3, 2) f'(β_3), and 3) 1- β_1, the right hand asymptote. All statistical calculations were done using the procedure *nl* in Stata version 10.

2.10 Statistical Analyses

The Kruskal-Wallis and Dunn's post-hoc analysis tests were used to compare the functional avidities of CTL responses across different viral proteins, and for the multiple comparison analysis of Nef effects on viral inhibition across different groups of CTL clones; a 2-tailed Student's t-test was performed for comparison of Nef effects on viral inhibition or infected cell killing kinetics (K_{10}) between two groups of CTL clones, using Microsoft Excel 2008. The Pearson test was used to assess the relationship between the functional avidities or infected cell killing kinetic (K_{10}) and Nef effects; p values < 0.05 were considered significant.

Chapter 3:

Epitope Targeting and Viral Inoculum Are Determinants of Nef-Mediated Immune Evasion of HIV-1 from CTLs

3.1 Abstract

The impact of HIV-1 Nef-mediated Human Leukocyte Antigen class I (HLA-I) downregulation on $CD8^+$ cytotoxic T lymphocytes (CTLs) varies by epitope, but the determining factors have not been elucidated. We examine the impact of Nef on the antiviral efficiency of HIV-1 specific CTLs targeting 17 different epitopes to define properties that determine susceptibility to Nef. The impact of Nef did not correlate with CTL HLA-I restriction or functional avidity, but related directly to the kinetics of infected cell clearance. While Gag-specific CTLs generally were less susceptible to Nef than those targeting other proteins, this was determined by the ability to eliminate infected cells before *de novo* synthesis of viral proteins, which was also observed for CTLs targeting a Nef epitope. This very early clearance of infected cells depended on virus inoculum, and the required inoculum varied by epitope. These results suggest that while Gag-specific CTLs are more likely to recognize infected cells before Nef-mediated HLA-I downregulation, this varies depending on the specific epitope and virus inoculum. Generally reduced susceptibility to Nef may contribute to the overall association of Gag-specific CTL responses to better immune control *in vivo*, but this property is not unique to Gag.

3.2 Introduction

Multiple studies have demonstrated a major contribution of $CD8^+$ cytotoxic T lymphocytes (CTLs) to controlling HIV-1 infection (see section 1.3 and 1.5). The antiviral activity of CTLs is mediated by cytolysis of infected cells upon T-cell receptor recognition of viral epitopes that are presented by human leukocyte antigen-class I (HLA-I) molecules on the surface of infected cells [195]. CTLs can mediate cytolysis of infected cells early after infection and therefore can reduce viral replication [128, 195].

HIV-1 Nef is a 27kDa myristoylated protein with a central role in immunopathogenesis. Its downregulation of surface HLA-I molecules on infected cells [125, 126] may facilitate viral persistence through evasion of CTLs (see section 1.4 for detailed descriptions). *In vitro* studies have demonstrated that Nef-mediated HLA-I downregulation impairs the antiviral efficiency of HIV-1-specific CTLs [130, 194, 205], and that CTLs drive the selective pressure to maintain this function [132]. Analogously, *in vivo* studies using the macaque Simian Immunodeficiency Virus (SIV) model have shown that Nef-mediated Mamu downregulation impairs CTL antiviral responses, which exert strong selective pressure to maintain this Nef function [133, 134]. Moreover, the ability of Nef to downregulate HLA-I *in vivo* correlates to the breadth of the HIV-1-specific CTL response [135], further confirming the role of Nef in evasion of CTL antiviral activity.

The impact of HIV-1 Nef on CTL antiviral activity is epitope-specific. HLA-I C-restricted CTLs are unaffected because Nef downregulates cell surface HLA-I A and B but spares

C molecules [194]. Moreover, A- and B- restricted CTLs can vary in their susceptibility [194] or even resist interference by Nef [205], illustrating the epitope-dependent variability of Nef antagonism of CTL antiviral activity.

A proposed factor determining the impact of Nef on CTL antiviral activity is the timing of epitope presentation versus Nef-mediated HLA-I downregulation. Nef is one of the earliest expressed proteins after cell infection [70], but HLA-I downregulation lags by comparison [154]. It has been hypothesized that CTLs targeting epitopes presented before HLA-I downregulation may preempt the antagonistic Nef effect.

Indirect evidence supporting this hypothesis was provided by van Baalen *et al* [151] and Ali *et al* [150], who separately demonstrated that accelerating epitope expression can increase the antiviral efficacy of CTL clones. More direct evidence came from Sacha *et al,* showing that SIV Gag and Pol epitopes from proteins carried by incoming virions can be presented before downregulation of Mamu molecules by Nef, and that CTLs targeting these epitopes can eliminate virus-infected cells before viral protein translation [152, 153]. Overall these studies suggest a crucial role for epitope presentation timing in determining the degree of Nef impact on CTL antiviral activity.

However, the kinetic relationship of HIV-1 epitope presentation versus Nef-mediated HLA-I downregulation is poorly understood. Moreover, whether other factors implicated in the efficacy and shaping of the CTL response (HLA-I restriction, functional avidity, viral protein targeting) [157, 161, 164] affect CTL interaction with Nef is not known.

Given the contribution of Nef-mediated immune evasion to HIV-1 persistence *in vivo*, defining factors determining the ability of Nef to interfere with CTL antiviral activity would shed light on the requirements for optimizing or eliciting efficacious HIV-1 specific CTL responses.

3.3 Results

3.3.1 Gag-Specific CTL Antiviral Activity is Overall Less Susceptible to Nef-Mediated HLA-I Downregulation.

Using a previously described assay [194] (Figure 3.1A), we assessed the impact of Nef on the antiviral activities of CTL clones targeting 17 different epitopes. These epitopes were derived from both structural and accessory proteins, and were presented by various HLA-I types (Table 3. S1). Although most CTLs were affected by wild-type Nef, individual clones differed in their susceptibility to Nef (Figure 3.1B). To determine whether the impact of Nef was determined by the targeted viral protein, Nef effect ratios were compared across epitopes located in different HIV-1 proteins. Gag epitopes in general had higher Nef effect ratios (median 0.73, range 0.56 - 0.93), indicating low CTL susceptibility to Nef-mediated HLA-I downregulation. Nef and Pol epitopes trended towards having lower ratios than Gag (Pol median = 0.29, range 0.24 - 0.34, $p = 0.04$), suggesting greater CTL susceptibility to Nef-mediated HLA-I downregulation (Figure 3.2A). As a whole, Gag epitopes had a higher Nef effect ratio than all non-Gag derived epitopes combined ($p = 0.03$, Figure 3.2B), suggesting that Gag-specific CTLs are overall less susceptible to Nef.

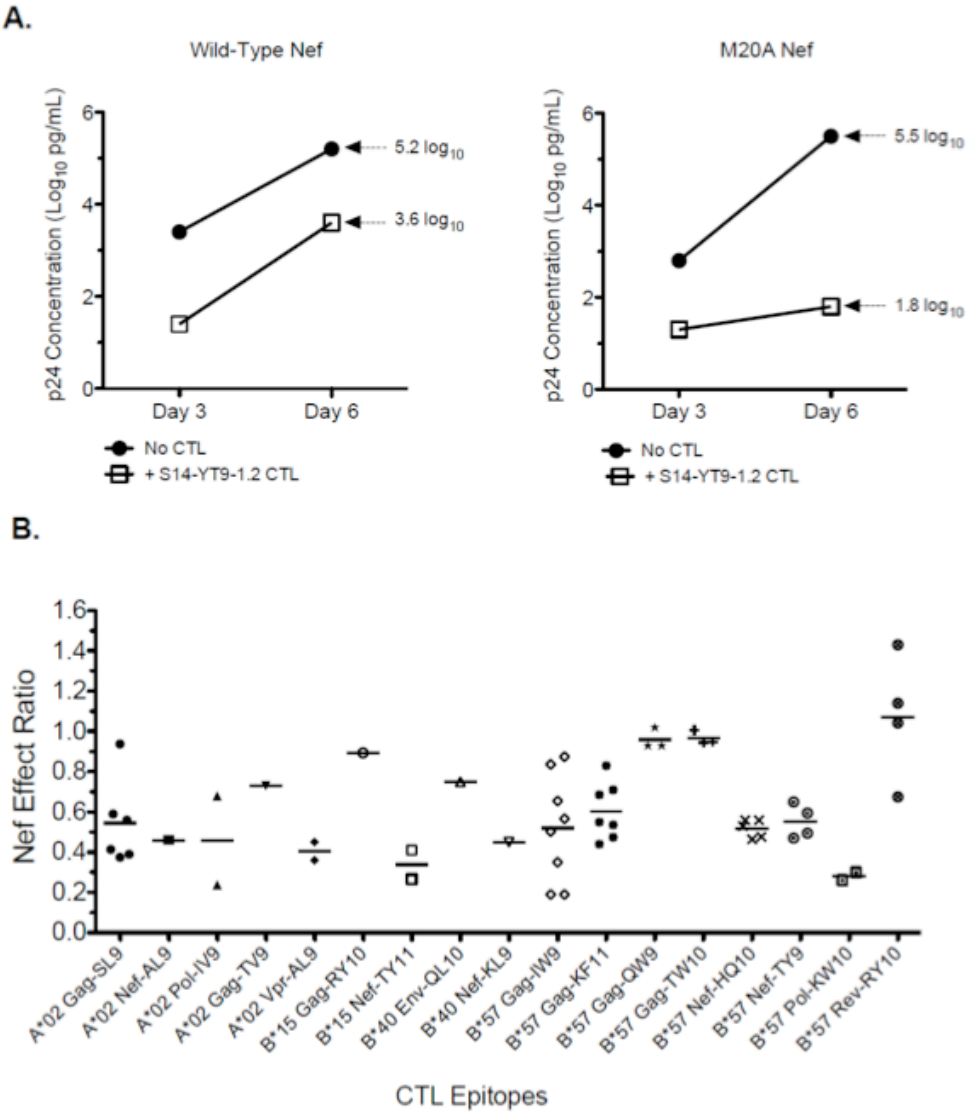


Figure 3.1 Impact of Nef on the antiviral activity of HIV-1 specific CTLs. The susceptibility of HIV-1-specific CTLs to Nef-mediated HLA-I downregulation was measured using a previously described viral suppression assay.

(A) CTL clone S14-YT9-1.2 (B*57 restricted, Nef-specific) was tested for inhibition of NL4-3.1 virus containing wild-type Nef or Nef-M20A (unable to downregulate HLA-I) in parallel. Replication was assessed by measuring supernatant Gag p24 antigen (log_{10} pg/mL) and plotted over time. Inhibition of wild-type virus at day 6 was 1.6 log_{10} units

(5.2 - 3.6 = 1.6) and *Inhibition Efficiency* was 0.3 (1.6 ÷ 5.2 = 0.31). Inhibition of M20A-Nef virus at day 6 was 3.7 $\log_{10}$ units (5.5 - 1.8 = 3.7) and *Inhibition Efficiency* was 0.7 (3.7 ÷ 5.5 =0.67). Thus, the *Nef Effect Ratio* was 0.42 (0.31 ÷ 0.67 = 0.46). A ratio of 0 would therefore indicate complete evasion mediated by Nef, and a ratio of 1 would indicate no effect of Nef.

(B) The Nef effect on viral inhibition is plotted for a panel of HIV-1-specific CTL clones. Each data point represents the average Nef effect ratio of a CTL clone across multiple independent experiments; the horizontal bar represents the mean.

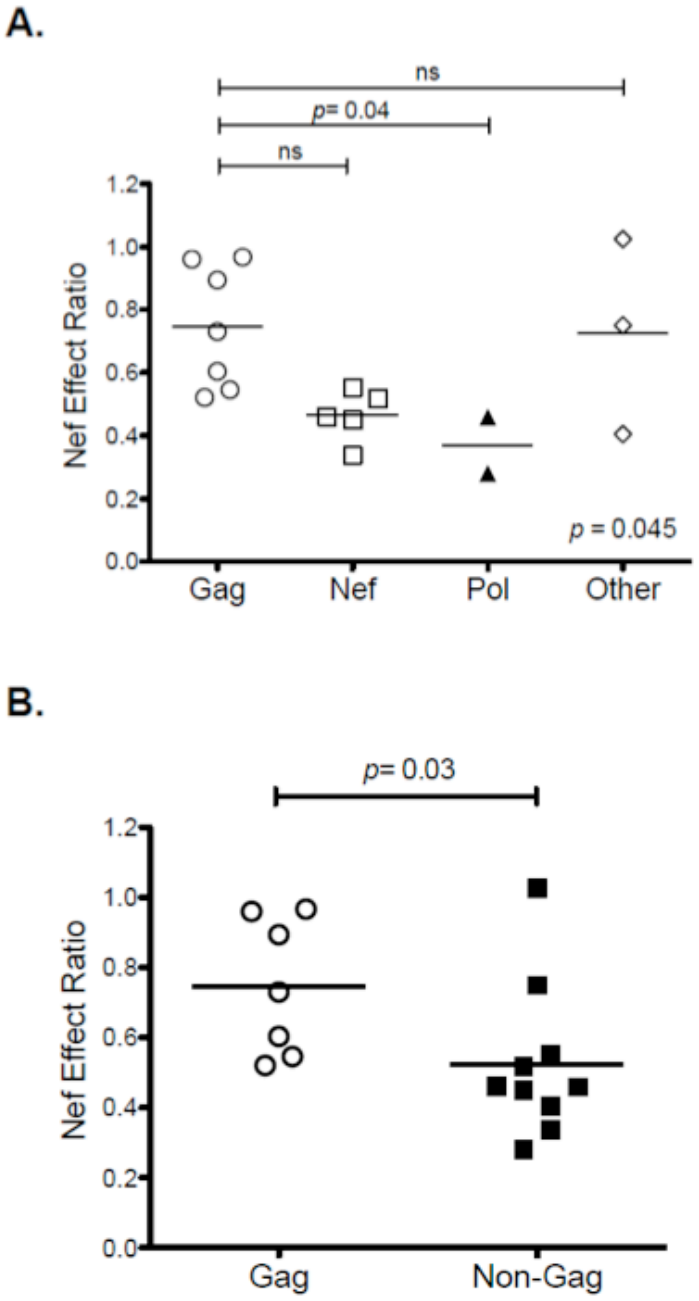


Figure 3.2 Gag-specific CTLs overall are less susceptible to Nef. The effect of Nef on CTL antiviral activity was compared according to the epitope source protein. Gag epitopes in general had higher Nef effect ratios than epitopes located in Pol and Nef. (A) The data are plotted according to proteins. Each dot represents the *Nef Effect Ratio* of an epitope; the horizontal bar represents the mean. Statistical significance was

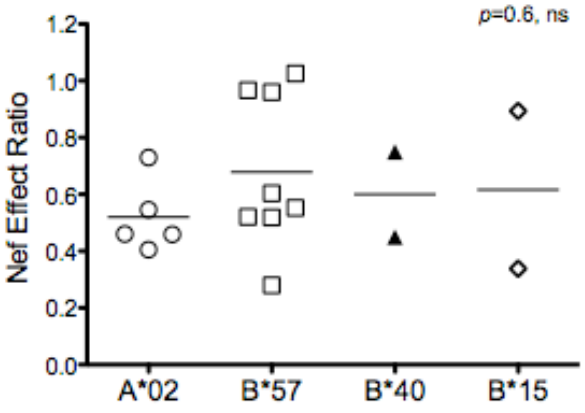
assessed using the Kruskal-Wallis test followed by Dunn's post-test for multiple comparisons.

(B) Gag epitopes are compared to all other epitopes from non-Gag proteins. A 2-tailed Student's t-test was used to compare the two groups.

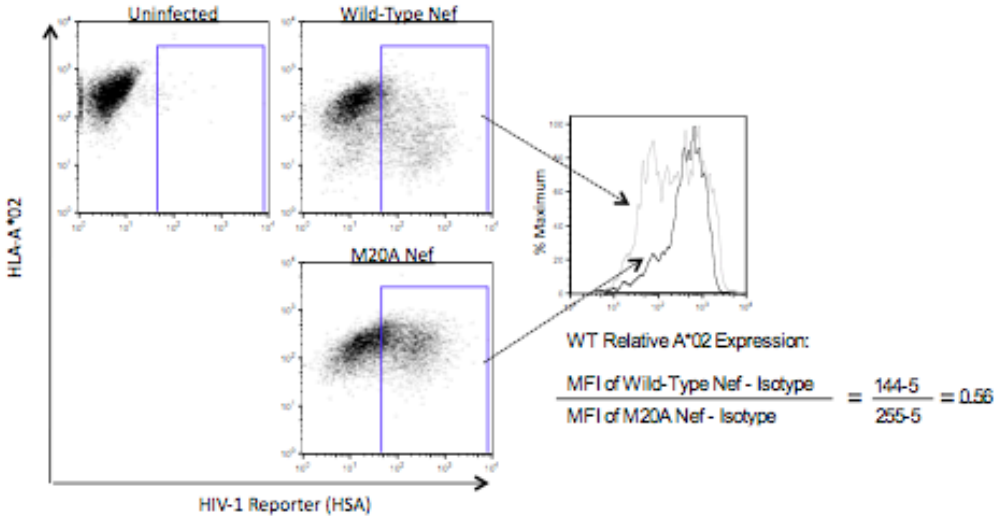
3.3.2 The Influence of Nef on CTL Antiviral Activity is Not Determined by HLA-I Restriction.

Different HLA-I molecules have been associated with better immune control of HIV-1 infection (see section 1.5.4 for detailed descriptions), particularly HLA-B*57, which is overrepresented in persons with low viremia and slow disease progression [91]. We therefore evaluated whether CTL susceptibility to Nef varied according to the presenting HLA-I molecule (Figure 3.3). CTLs targeting epitopes presented by various neutral HLA-I alleles and the protective B*57 allele were compared for their susceptibilities to Nef, revealing no significant difference (p = 0.57, Figure 3.3A). Furthermore, direct comparison of Nef-mediated HLA-I downregulation of A*02 versus B*57 (Figure 3.3B and C) revealed similar magnitudes and kinetics of downregulation (Figure 3.3D and E). Together, these results suggest that the differential Nef effects on HIV-1 specific CTLs are unrelated to the presenting HLA-I type, and that the *in vivo* protective effect of B*57 restricted responses in HIV-1 infection is not due to inefficient downregulation of B*57 by Nef.

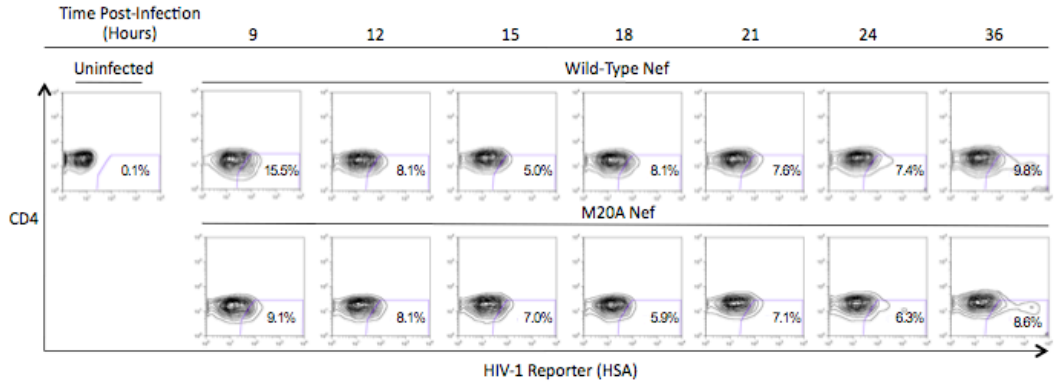
A.


p=0.6, ns
Nef Effect Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A*02
B*57
B*40
B*15

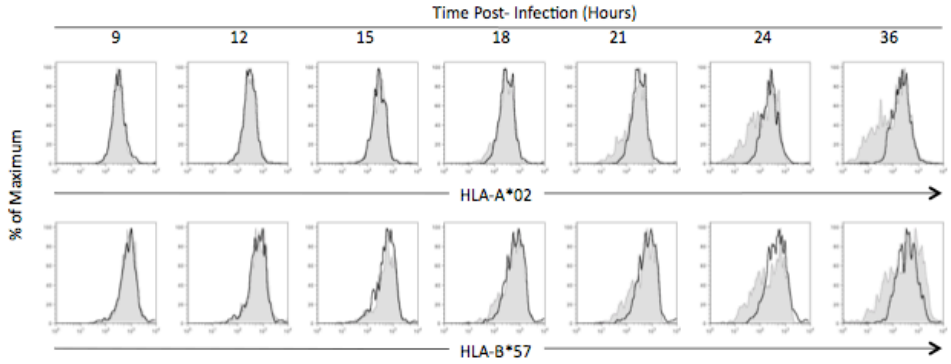
B.


Uninfected
Wild-Type Nef
M20A Nef
HLA-A*02
HIV-1 Reporter (HSA)
% Maximum
WT Relative A*02 Expression:
MFI of Wild-Type Nef - Isotype / MFI of M20A Nef - Isotype = 144-5 / 255-5 = 0.56

C.


Time Post-Infection (Hours)
9
12
15
18
21
24
36
Uninfected
Wild-Type Nef
M20A Nef
0.1%
15.5%
8.1%
5.0%
8.1%
7.6%
7.4%
9.8%
9.1%
8.1%
7.0%
5.9%
7.1%
6.3%
8.6%
CD4
HIV-1 Reporter (HSA)

D.


Time Post- Infection (Hours)
9
12
15
18
21
24
36
% of Maximum
HLA-A*02
HLA-B*57

E.

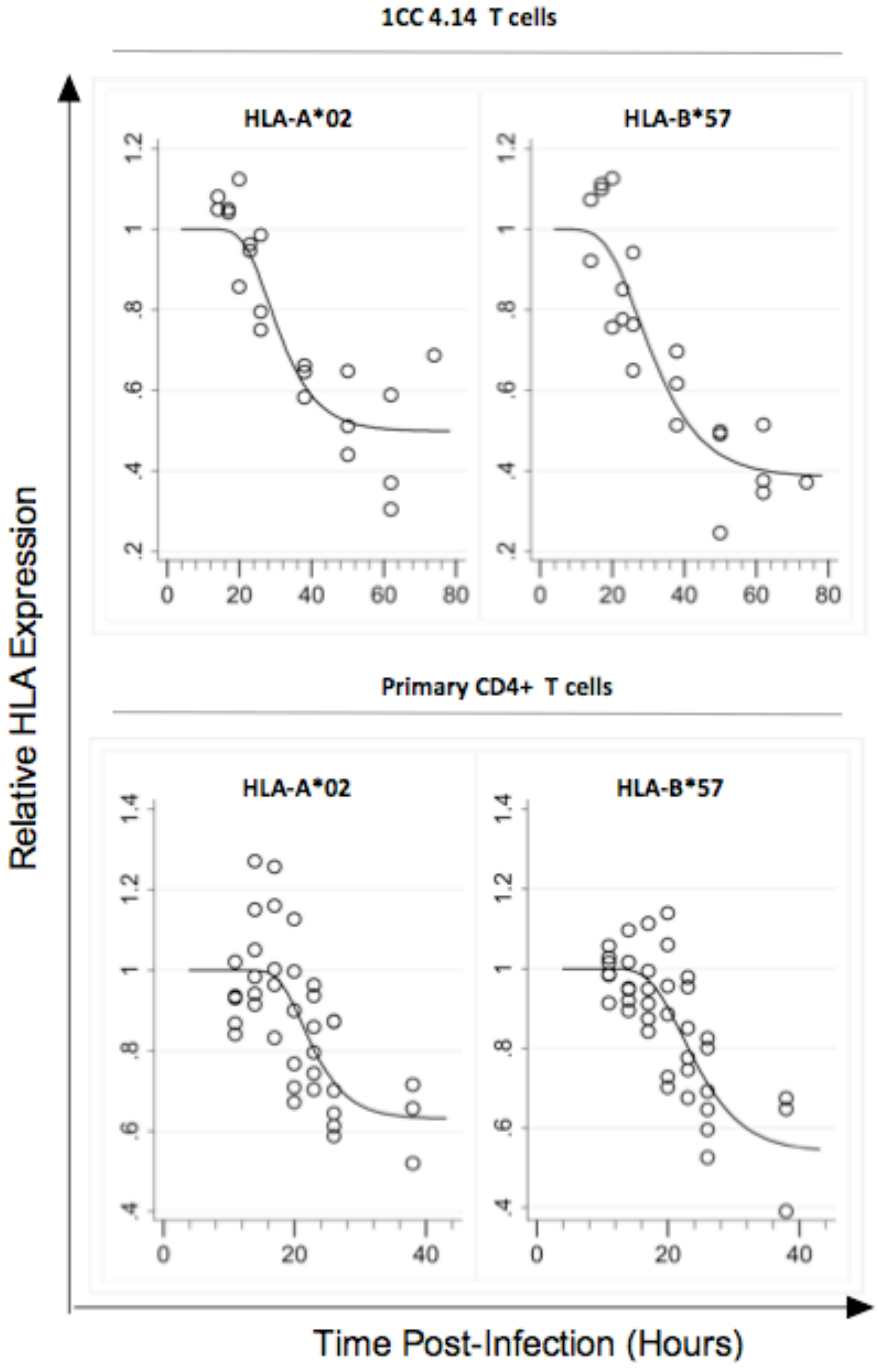

1CC 4.14 T cells
HLA-A*02
HLA-B*57
Primary CD4+ T cells
HLA-A*02
HLA-B*57
Relative HLA Expression
Time Post-Infection (Hours)

Figure 3.3 The effect of Nef on HIV-1-specific CTL antiviral activity is unrelated to HLA-I restriction of the epitope. The impact of Nef was compared across HLA-I restrictions.

(A) *Nef effect ratios* are plotted for the indicated HLA-I types. Each dot represents one epitope; the horizontal bar represents the mean. There was no significant difference between groups (Kruskal-Wallis test).

(B) A flow cytometric approach was used to measure Nef-mediated downregulation of HLA-I A*02 and B*57 on acutely HIV-1-infected cells. A representative experiment shows the analysis of HLA-A*02 downregulation after gating on infected cells (positive for the HSA reporter) and determination of mean fluorescence intensity (MFI) of A*02 staining. The relative expression of A*02 on cells infected with wild-type Nef virus (gray dotted histogram) was then calculated as a fraction of MFI compared to Nef-M20A virus (black solid histogram), after subtraction of background MFI (from an isotype control).

(C) Expression of cell surface CD4 and the HSA reporter over time is demonstrated after acute infection of primary $CD4^+$ T lymphocytes from an HIV-1-infected donor with both A*02 and B*57 (Subject 00036, slow progressor not on treatment).

(D) The infected (HSA^{high+} and $CD4^{dim/-}$, percentages shown) primary $CD4^+$ T lymphocytes were gated and analyzed for A*02 (top panel) and B*57 (bottom panel) expression. HLA-I expression is plotted for HIV-1 with wild-type Nef (gray shaded histograms) versus Nef-M20A (black histograms).

(E) Gompertz plots of Nef-mediated downregulation of A*02 versus B*57 are shown for the laboratory cell line 1CC4.14 T cell line (top panel) or primary $CD4^+$ T lymphocytes* (bottom panel) are shown. In the top panel, the estimates for time to 10% A*02 versus

B*57 downregulation by Nef-mediated are 22.1 and 19.6 hour post-infection, respectively. The slopes of the fitted curves at 10% downregulation of A*02 versus B*57 are -0.016 and -0.015, respectively. The maximum levels of downregulation of A*02 versus B*57 are estimated to be 50% and 40%, respectively. These parameters are not significantly different for A*02 versus B*57. In the bottom panel, the estimates for time to 10% A*02 versus B*57 downregulation by Nef-mediated are 18.1 and 18.0 hours post-infection, respectively. The slopes of the fitted curves at 10% downregulation of A*02 versus B*57 are -0.023 and -0.021, respectively. The maximum levels of downregulation of A*02 versus B*57 are estimated to be 60% and 50%, respectively. These parameters are not significantly different for A*02 versus B*57.

*The efficiency of infection was higher (about 40 to 50% versus the 10% shown in Figure 3C) and Nef-mediated HLA-I downregulation occurred earlier (~12 hours post-infection) in virus-infected primary $CD4^+$ T lymphocytes of HIV-1-uninfected donors (data not shown).

3.3.3 Functional Avidity Does Not Correlate to the Degree of Nef Interference with HIV-1-Specific CTL Antiviral Efficacy.

Previously reported data suggest that functional avidity is a key determinant of CTL antiviral efficacy ([156, 161, 206], and see section 1.5.2 for a more detailed description on this topic). To explore the possibility that higher avidity allows better evasion of Nef, we investigated the relationship between the functional avidity (SD_{50}) and the Nef effect ratios of CTLs. When SD_{50} values were plotted against Nef effect ratios, no significant correlation was apparent (Figure 3.4A). Because Gag-specific CTL responses appeared to be less susceptible to Nef-mediated HLA-I downregulation compared to those directed against all non-Gag epitopes overall, we examined whether Gag-specific CTLs exhibited higher functional avidity than those targeting non-Gag epitopes. The SD_{50} values were not significantly different between CTLs targeting epitopes located in Gag versus other proteins (Figure 3.4B). Together, these data suggest that differential Nef effects on HIV-1 specific CTL antiviral activity are not determined by differences in functional avidity.

3.3.4 The Influence of Nef on CTL Antiviral Activity Is Not Determined by Early Versus Late Transcription of the Gene Coding for the Epitope Source Protein.

It has been hypothesized that CTLs recognizing epitopes derived from the early expressed HIV-1 proteins Rev, Tat and Nef (translated from Rev-independent RNA transcripts; see section 1.2.3 for detailed descriptions) might be less susceptible to Nef because of a kinetic advantage in relation to HLA-I downregulation [150, 151]. Addressing this hypothesis, we compared the effect of Nef on viral inhibition between CTLs targeting epitopes derived from the "late" expressed proteins Gag, Pol, Env, and Vpr (translated from Rev-dependent RNA transcripts) to those targeting epitopes derived from Nef and Rev (Rev-independent). The distributions of Nef effect ratios, however, were not significantly different between the two groups (Figure 3.4C). Despite the "early" expression of Nef, CTLs directed against Nef-derived epitopes remained susceptible to Nef-mediated HLA-I downregulation (Figure 3.2A), in agreement with prior data [194]. By contrast, antiviral activity of CTLs targeting a Rev epitope were functionally unaffected by Nef (Figure 3.1B), confirming that some "early" protein epitopes may have a kinetic advantage, although it has been shown that other CTLs targeting Rev can be sensitive to Nef as well [194]. Overall these findings suggest that "early" versus "late" protein epitopes do not necessarily correspond to early versus late CTL triggering in relation to Nef-mediated HLA-I downregulation.

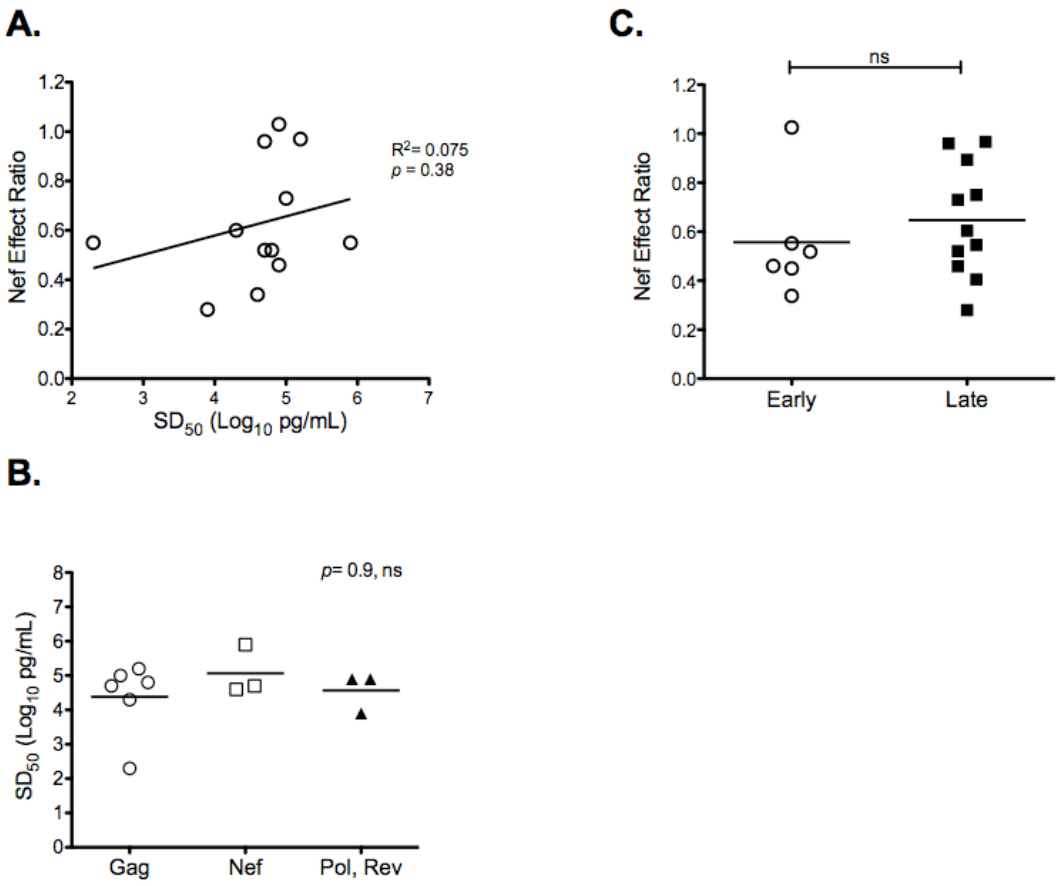


Figure 3.4 The impact of Nef on HIV-1-specific CTL antiviral activity is not influenced by CTL functional avidity or Rev-dependence of the epitope source protein. Nef effect ratios for the tested CTL clones were compared according to functional avidity and Rev-dependence status.

(A) *Nef Effect Ratio* is plotted against functional avidity (SD_{50}) for each tested CTL clone; there was no significant correlation by the Pearson test.

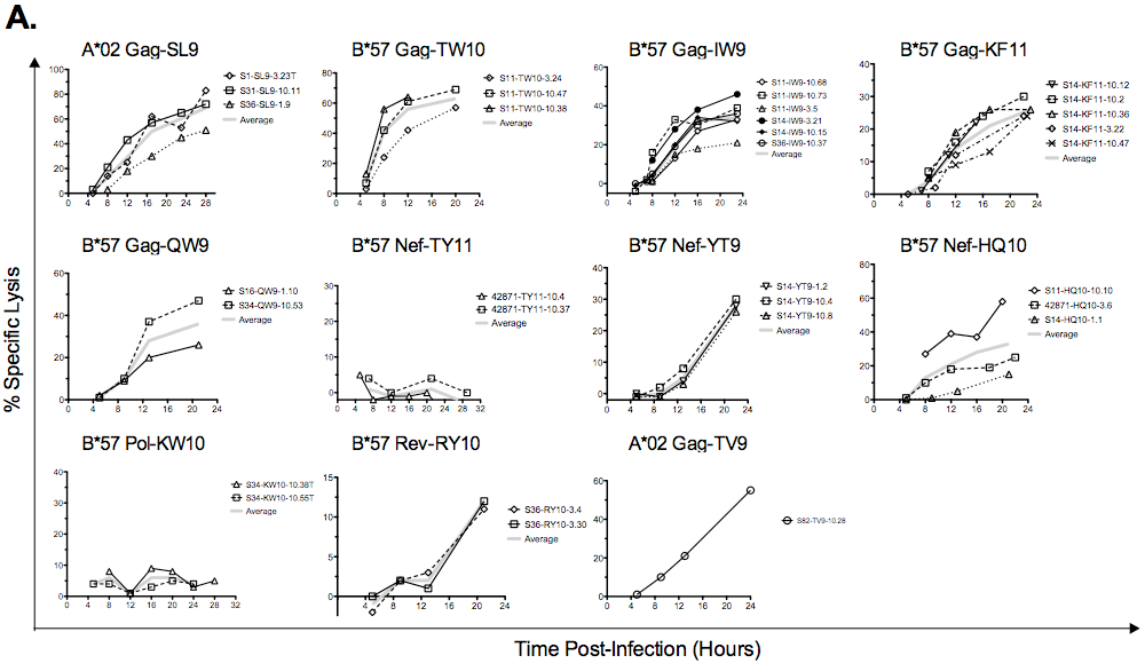
(B) The functional avidities of CTLs targeting Gag-derived epitopes are compared to those directed against epitopes located in non-Gag proteins; the horizontal bars

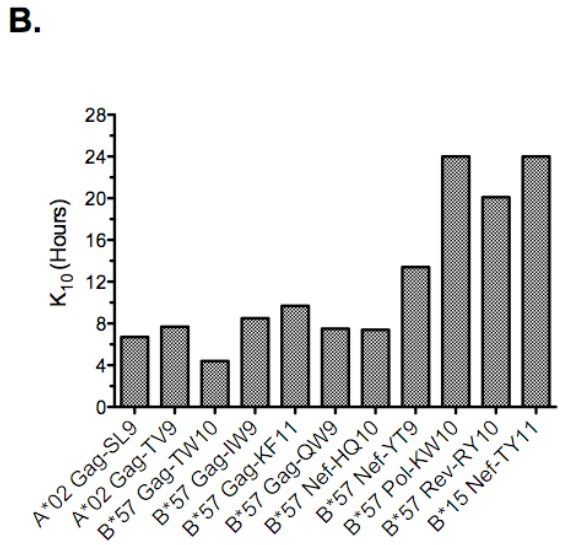
represent the means. There was no significant difference between groups by the Kruskal-Wallis test for multiple comparisons.

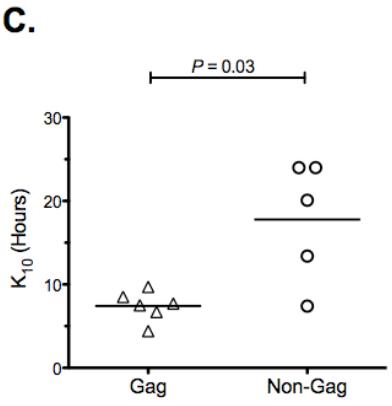
(C) *Nef Effect Ratios* are plotted for CTLs recognizing epitopes from “early” (Rev-independent, Nef and Rev) versus “late” (Rev-dependent, Gag, Pol, Vpr, and Env) proteins. There was no significant difference between groups by a 2-tailed Student’s t-test.

3.3.5 CTL Resistance to Nef-Mediated HLA-I Downregulation Can Be Mediated by Very Early Killing of HIV-1-Infected Cells.

Despite the observations above, clearly the temporal relationship between HIV-1 epitope presentation and Nef-mediated HLA-I downregulation can influence the antiviral efficacy of HIV-1 specific CTLs [150, 151]. We therefore assessed the kinetics of infected cell clearance by HIV-1 specific CTLs to directly investigate these kinetic relationships in a greater detail (Figure 3.5). The onset of infected cell killing (using an arbitrary 10% threshold, K_{10}) was estimated by fitting logarithmic regression curves to the observed specific lysis of acutely infected cells over time (Figure 3.5A). Across epitopes, K_{10} values ranged from as early as 4.4 hours to >24 hours post-infection (Figure 3.5B). Because Gag-specific CTLs were overall less susceptible to Nef than those targeting all non-Gag epitopes combined (Figure 3.2), we compared the killing kinetics of Gag-specific CTLs versus non-Gag-specific CTLs, finding that Gag-derived epitopes as a whole exhibited significantly lower K_{10} values than non-Gag epitopes (p = 0.03, Figure 3.5C), suggesting faster generation of Gag versus non-Gag epitopes, although this was not exclusive to Gag because one Nef epitope exhibited similarly early killing. Comparison of K_{10} values to Nef effect ratios between epitopes suggested an inverse relationship (Figure 3.5D), although this was not statistically significant for this small number of epitopes (R^2 = 0.2, p = 0.16, improved to R^2 = 0.58, p = 0.009 with the removal of one outlier). As a whole, these data indicate that the generally greater resistance of Gag-specific CTL antiviral activity to Nef is due to earlier killing of cells after infection, but that this is not specific to Gag.


A.
A*02 Gag-SL9
B*57 Gag-TW10
B*57 Gag-IW9
B*57 Gag-KF11
B*57 Gag-QW9
B*57 Nef-TY11
B*57 Nef-YT9
B*57 Nef-HQ10
B*57 Pol-KW10
B*57 Rev-RY10
A*02 Gag-TV9
% Specific Lysis
Time Post-Infection (Hours)


B.
K10 (Hours)
A*02 Gag-SL9
A*02 Gag-TV9
B*57 Gag-TW10
B*57 Gag-IW9
B*57 Gag-KF11
B*57 Gag-QW9
B*57 Nef-HQ10
B*57 Nef-YT9
B*57 Pol-KW10
B*57 Rev-RY10
B*15 Nef-TY11


C.
P = 0.03
K10 (Hours)
Gag
Non-Gag

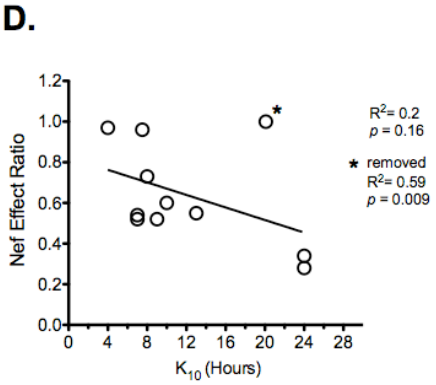

D.
Nef Effect Ratio
K10 (Hours)
R2= 0.2
p = 0.16
* removed
R2= 0.59
p = 0.009

Figure 3.5 CTL resistance to Nef is associated with early killing of HIV-1 infected cells. The timing of CTL killing of 1CC4.14 cells after acute infection with VSV-G Env-pseudotyped NL4-3-ΔEnv was assessed by serial ^{51}Cr measurements over 24 hours.

(A) Specific lysis is plotted over time, by epitope. Results with CTL clones are plotted with symbols, and the average across clones is plotted with a broad gray line.

(B) Estimates of time to reach 10% specific lysis (K_{10}) are indicated. The estimates were obtained by fitting average lysis curves for each epitope with logarithmic regression. Note that CTLs targeting the B*15 restricted Nef TY11 and B*57 restricted Pol KW10 epitopes were unable to recognize virus-infected cells within the first 24 hours of infection, and therefore each epitope was assigned a conservative K_{10} value of 24 hours. The inability of these CTL clones to recognize virus-infected targets was not due to inactivity of the clones, as they efficiently killed cells infected with HIV-1 containing the Nef M20A mutation (data not shown).

(C) K_{10} values of Gag-specific versus non-Gag-specific CTLs are compared. Each point represents the K_{10} value for an epitope; the horizontal bar represents the mean across epitopes. Statistical significance was evaluated with a 2-tailed Student's t-test.

(D) K_{10} values are plotted against *Nef Effect Ratios* for all epitopes. Statistical significance is tested with a Pearson test. *Results after removing an outlier, the B*57-restricted Rev RY10 epitope.

3.3.6 Some Gag- and Nef- Specific CTLs Do Not Require Presentation of de novo Synthesized Viral Proteins for Infected Cell Killing.

To determine the mechanism of early infected cell killing by Gag-specific CTLs, we treated acutely infected cells with the reverse transcriptase inhibitors tenofovir and zidovudine (ART) at concentrations that blocked viral replication and therefore prevented viral protein expression (Figure 3.6A). Treatment had minimal effects on the kinetics and magnitude of infected cell killing by several Gag-specific CTLs (Figure 3.6B). A Nef-specific CTL clone also appeared to be unaffected by drug treatment; in contrast, infected cell killing by a Rev-specific CTL clone was abolished by the treatment, indicating that this phenomenon is epitope-specific. These data were consistent with previous findings that virion-derived proteins can be processed and presented to CTLs before *de novo* protein production by acutely SIV-infected cells [152, 153], and suggest that our observation of early killing and Nef-resistance is explained by this phenomenon.

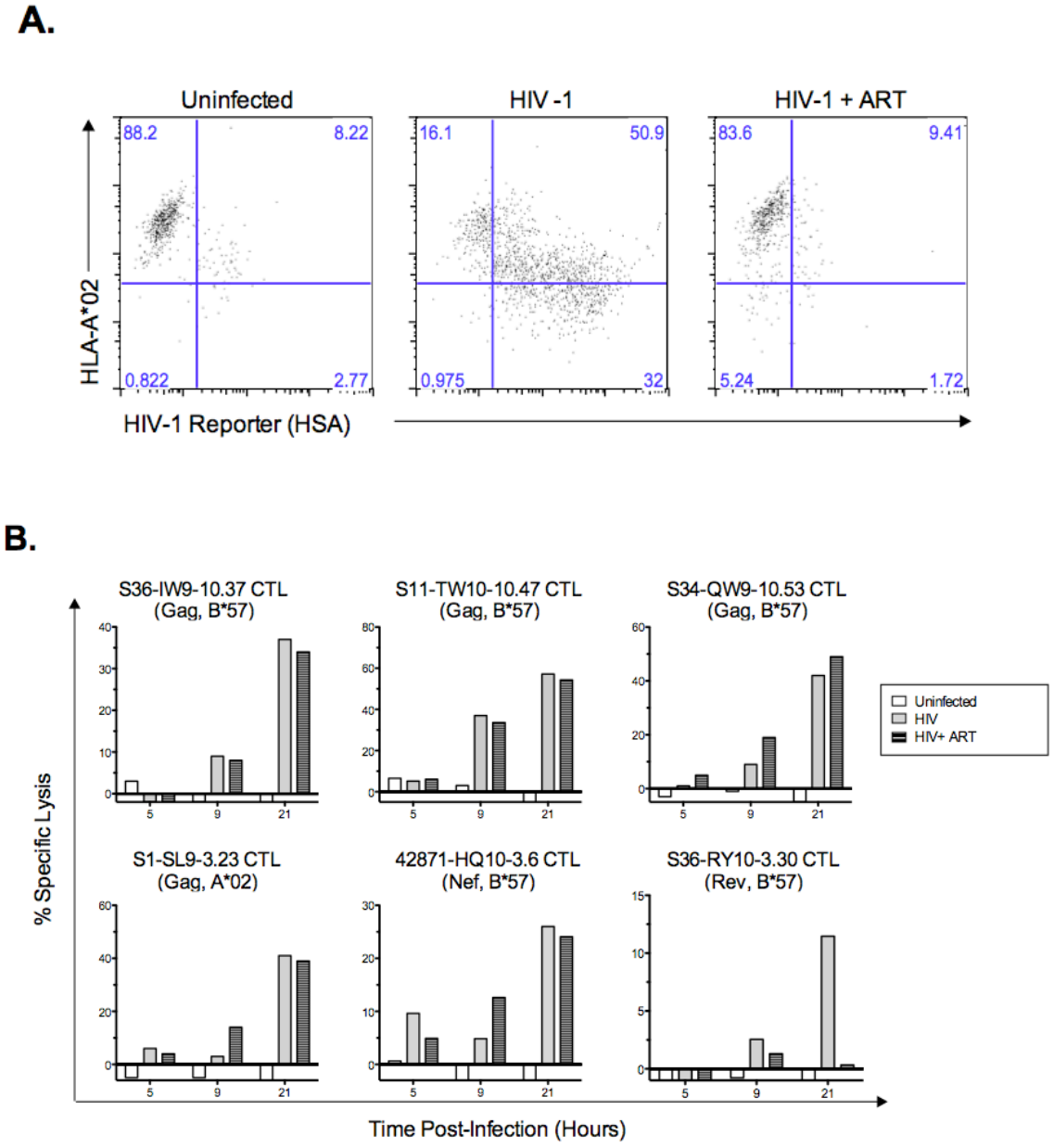


Figure 3.6 Some Gag- and Nef-specific CTLs cells do not require *de novo* viral protein synthesis for early killing of HIV-1 infected cells. 1CC4.14 cells were infected with 600 fg Gag p24/cell of VSV-G Env-pseudotyped NL4-3-ΔEnv-HSA and assessed for killing by CTLs (^{51}Cr release) in the presence and absence of ART.

(A) Uninfected or infected cells with or without ART were examined by flow cytometry for cell surface expression of HLA A*02 and HSA reporter at 48 hours post-infection, confirming that ART blocked the *de novo* viral protein expression seen in untreated cells (HSA expression and A*02 downregulation by Nef).

(B) Specific lysis of uninfected or infected target cells (with or without ART) was assessed by ^{51}Cr release assay over time. The data shown are representative of two independent experiments.

3.3.7 Early CTL Killing of Infected Cells Through Incoming Virion-Derived Epitopes Depends on the Viral Inoculum, and the Required Inoculum Varies by Epitope.

To investigate the amounts of virion-derived proteins that are required to trigger early CTL antiviral activity, we performed a dose response analysis of pseudotyped HIV-1 stocks in the presence and absence of drug treatment, or using GFP- lentivirus lacking HIV-1 genome. Target cells were infected with varying concentrations of viruses corresponding to varying ratios of Gag p24 protein concentration per cell (ranging from 1 to 600 fg p24 per cell) with or without ART, co-cultured with HIV-1-specific CTLs, and assessed for lysis at 12 hours post-infection (Figure 3.7). Each CTL clone that had exhibited early killing was unaffected by ART at a viral inoculum of 400 to 600 fg p24/cell, and that those targeting Gag were able to eliminate GFP-lentivirus infected cells. However, differences were seen between clones with decreasing virus inputs of 60 fg p24/cell or lower. Most clones showed a reduction in activity with dropping inocula, and were unable to kill infected cells at 15 fg p24/cell, although one clone retained efficient killing activity at 15 fg p24/cell (Figure 3.7D). ART treatment of the infected target cells had no effect on killing at a viral inoculum of 400 fg p24/cell or higher, but variably affected the efficiency of killing at lower levels of viral input for different clones, suggesting varying contributions of *de novo* synthesized proteins to killing by 12 hours. The clone that killed infected cells efficiently at an input of 15 fg p24/cell also maintained killing in the presence of ART at that inoculum (Figure 3.7D), suggesting more efficient recognition of incoming virion-derived epitopes. This was further supported by the CTL clone superior killing efficiency against GFP-lentivirus infected cells (Figure 3.7F), which was also observed with a different clone with the same specificity (Figure 3.7G).

Overall, these data indicate that HIV-1-specific CTLs have varying capacity to clear infected cells through recognition of incoming virion-derived epitopes.

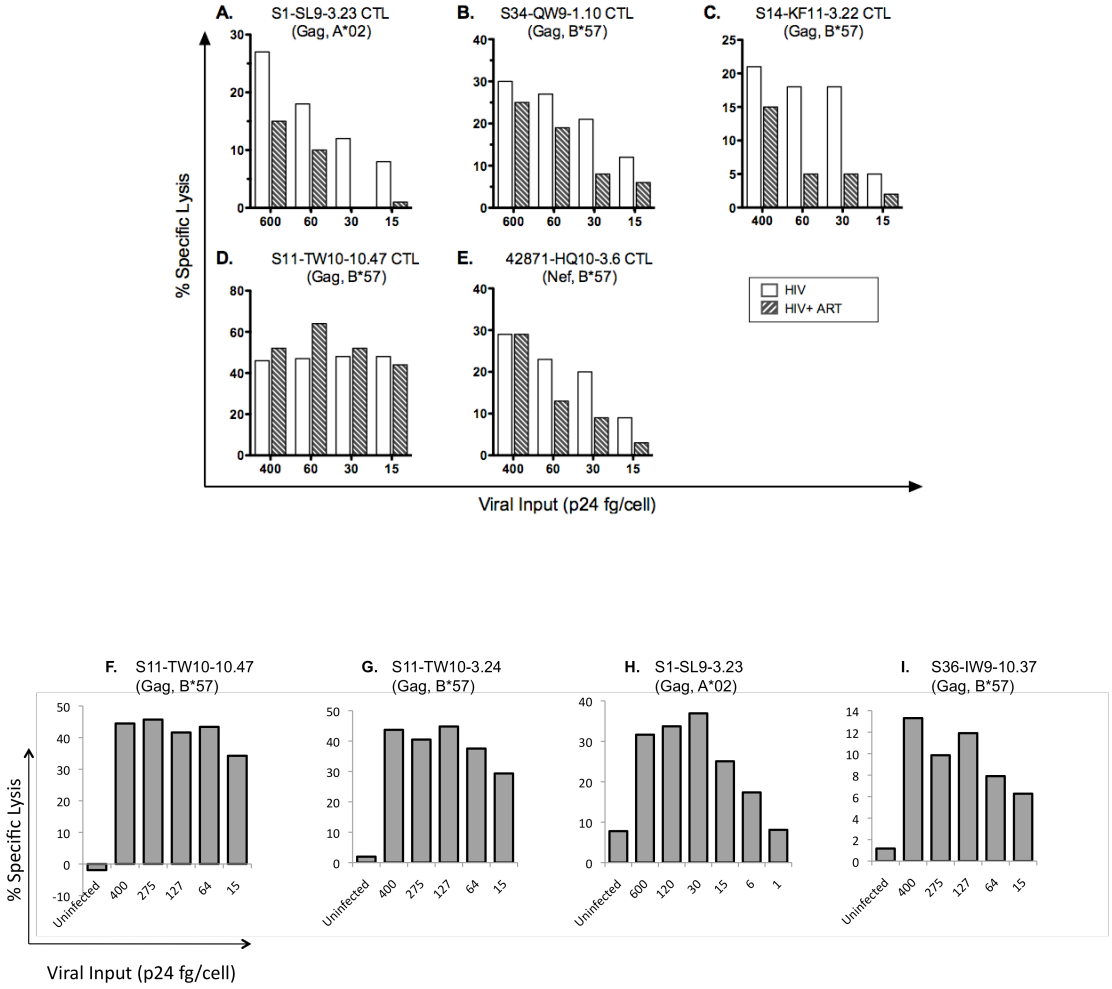


Figure 3.7 Early killing of HIV-1 infected cells by CTLs is viral inoculum-dependent. (A-E) 1CC4.14 cells were infected with varying amounts of VSV-G Env-pseudotyped NL4-3-ΔEnv and assessed for killing by CTLs (^{51}Cr release) in the presence or absence of ART over time at 12 hours post-infection. (F-I) 1CC4.14 cells were infected with varying amounts of VSV-G Env pseudotyped GFP-lentivirius, and assessed for killing by CTLs at 12 hours post-infection. The data are representative of

three independent experiments, except panel *C and I*, which are representative of two independent experiments.

Table 3. S1 Epitopes tested for Nef impact and functional avidity

HLA	Epitope (Location)	Clone (n[a])	Mean Nef Effect Ratio (SD)	Functional Avidity[b](SD)
A*02	SLYNTVATL (Gag p17 77-85)	S1-SL9-1.8 (11) S1-SL9-1.7 (1) S1-SL9-3.23 (18) S36-SL9-1.9 (10) S36-SL9-10.18 (3) S31-SL9-10.11 (4)	0.54 (0.19)	2.3 (0.6)
	TLNAWVKVV (Gag p24 19-27) AAVDLSHFL (Nef 83-91)	S82-TV9-10.28 (2) S58-AL9-10.18 (2)	0.73 (0.09) 0.46	5.0 ND
	ILKEPVHGV (Pol 446-472)	68A62-IV9 (8) S31-IV9-10.4 (1)	0.46 (0.31)	4.9
	AIIRILQQL (Vpr 59-67)	S36-AL9-1.1 (15) S36-AL9-10.10 (1)	0.41 (0.06)	ND
B*15	RLRPGGKKKY (Gag 20-29)	MO471-RY10-1.1 (5)	0.89 (0.24)	
	TQGYFPDWQNY (Nef 117-127)	42871-TY11-10.4 (5) 42871-TY11-10.37 (2)	0.34 (0.10)	4.6 (0.2)
B*40	QELKNSAVNL (Env 805-814)	S82-QL10-1.6 (2)	0.75 (0.12)	ND
	KEKGGLEGL (Nef 92-100)	S16-KL9-4.1 (1)	0.45	ND
B*57	ISPRTLNAW (Gag 147-155)	S11-IW9-10.73 (5) S11-IW9-3.5 (1) S11-IW9-10.68 (2) S11-IW9-10.65 (1) S14-IW9-3.14 (1) S14-IW9-3.21 (3) S14-IW9-10.15 (6) S36-IW9-10.37 (2)	0.52 (0.27)	4.8 (0.3)
	KAFSPEVIPMF (Gag 162-172)	S14-KF11-10.2 (10) S14-KF11-10.12 (2) S14-KF11-10.36 (2) S14-KF11-10.47 (5) S14-KF11-3.22 (9) S14-KF11-1.3 (5) S14-KF11-10.6 (1)	0.60 (0.14)	4.3 (0.2)
	TSTLQEQIGW (Gag 240-249)	S11-TW10-3.24 (2) S11-TW10-10.38 (7) S11-TW10-10.47 (3)	0.97 (0.04)	4.7 (0.7)
	QASQEVKNW (Gag 308-316)	S34-QW9-1.1 (1) S16-QW9-1.10 (1) S34-QW9-10.53 (1)	0.96 (0.05)	5.2 (0.8)
	HTQGYFPDWQ (Nef 116-125)	42871-HQ10-3.6 (4) S14-HQ10-1.1 (2) S14-HQ10-1.3 (3) S11-HQ10-10.10 (1) S11-HQ10-10.31 (2)	0.52 (0.05)	4.7 (1.1)
	YFPDWQNYT (Nef 120-128)	S14-YT9-1.2 (2) S14-YT9-10.4 (2) S14-YT9-10.8 (1) S36-YT9-3.36 (2)	0.55 (0.09)	5.9
	KIATESIVIW (RT 373-383)	S34-KW10-10.38 (6) S34-KW10-10.55 (1)	0.28 (0.03)	3.9
	RTVRLIKLLY (Rev 14-23)	S36-RY10-3.20 (2) S36-RY10-3.3 (4) S36-RY10-3.4 (2) S36-RY10-3.30(1)	1.0 (0.29)	4.9

[a] Number of independent experiments performed with the same clone
[b] Functional avidity (SD_{50}), log_{10} pg/mL
[c] ND, not determined
[d] SD, standard deviation

3.4 Discussion

Nef-mediated HLA-I downregulation impairs the effectiveness of CTL antiviral activity against HIV-1 in an epitope-specific manner [194, 205], but the determining factors have been unclear. Here we examine a panel of 17 viral CTL epitopes varying in sensitivity to Nef, and assess the effects of factors including HLA-I restriction, functional avidity, protein source, and kinetics of CTL recognition. The latter factor is the major determinant of Nef impact, which is consistent with other studies suggesting a role for antigen presentation kinetics and CTL antiviral efficiency in general [150-153, 207].

Although it has been suggested that CTL targeting of the “early” proteins Tat, Rev, and Nef (translated from fully spliced transcripts that are Rev-independent for nuclear exit) yields superior antiviral activity [150, 151], our data demonstrate that “early” versus “late” protein epitope source does not dictate early versus late CTL recognition of infected cells. A Rev- and a Nef-specific CTL clone recognized infected cells relatively late compared to Gag-specific CTLs, although another Nef-specific CTL clone did mediate early recognition. Thus it is likely that epitope processing from incoming virion proteins, and/or other epitope factors (e.g. efficiency of processing) override the role of protein expression kinetics.

Nef-mediated interference of CTL antiviral activity also appears to be unaffected by the HLA-I restriction. Given that some HLA-I types have strong associations with the degree of immune control of HIV-1 [91] and that Nef is a critical protein for HIV-1

virulence [115], varying downregulation of different HLA-I types by Nef would be a potential unifying mechanism. However, two observations in our study make this unlikely. First, the functional impact of Nef on CTL antiviral activity did not differ consistently between CTLs targeting epitopes restricted by B*57 versus those restricted by A*02, B*15, or B*40. Second, the magnitude and timing of B*57 downregulation by HIV-1 Nef was similar to that of A*02. Thus it is likely that the influence of HLA-I type on immune control is unrelated to Nef function, but more likely related to other factors determined by targeting such as epitope sequence constraints for escape [172, 208].

Similarly, functional avidity did not correlate with either the degree of Nef impact or the kinetics of antiviral responses (data not shown). Although it has been suggested that higher avidity CTLs can eliminate infected cells more rapidly and have higher antiviral efficacy [156] and recently reported that CTLs targeting Gag or restricted by B*57 are superior due to higher functional avidity [173], we did not find significant differences in functional avidity of CTLs targeting different proteins or restricted by different HLA-I types. This finding was consistent with prior studies from our group demonstrating no correlation between functional avidity and antiviral efficacy [159], and no greater antiviral activity for additional avidity beyond a required threshold for killing infected cells [161]. Epitope-targeting independent of such factors appears to be the key determinant of Nef impact on CTLs.

There has been considerable interest raised by observations that the magnitude and breadth of Gag targeting by the CTL response correlates with better immune control *in*

vivo [163-166]. While the Gag-specific CTL response appears to have superior antiviral activity compared to the Env-specific CTL response when tested *ex vivo* [160], the mechanism is unclear, and this finding could be related to better match of *in vivo* viral sequences to the test strain or difference in CTL phenotype and function, rather than targeting *per se*. Our data suggest a potential mechanism that is directly related to targeting; in our experimental conditions most Gag-specific CTLs demonstrate early killing of acutely infected cells. Earlier killing of infected cells could translate directly to better clearance of infected cells as well as evasion of Nef-mediated HLA-I downregulation by Gag-specific CTLs on average.

In agreement with similar findings in the SIV-macaque experimental system [152], we find that CTLs against virion-contained proteins other than Gag also can mediate early killing of infected cells. Furthermore, our data further suggest that the efficiency of triggering of this early killing varies by epitope; it is likely that Gag-specific CTLs exhibit efficient earlier killing on average than CTLs targeting other incoming virion-derived epitopes, but that this property is not uniformly dictated by the source protein. This is consistent with the finding that while Gag-specific CTL targeting is statistically significantly associated with better immune control, this is a loose correlation that is not predictive on the individual level.

The contribution of incoming virion-derived epitopes to CTL antiviral activity *in vivo* has been debatable, due to questions about the physiological relevance of the viral inoculum used for *in vitro* studies. Our data examine the dose response of virus

inoculum for early killing, demonstrating that at least 30 fg p24/cell of viral inoculum is required to induce early cytolysis of infected target cells by the tested Gag- and Nef-specific CTLs that were found to mediate early killing. Because each picogram of p24 corresponds to approximately 10^4 virions [49], this corresponds to approximately 300 virions/cell. Recent estimates suggest that an HIV-infected $CD4^+$ T lymphocytes produces between 10^3 to 5 x 10^4 virions [209], and that 10% are infectious [210]. These numbers suggest that the concentration of incoming virus tested experimentally may be achievable *in vivo*, particularly in lymphatic tissues where activated $CD4^+$ T lymphocytes are tightly packed and HIV-1 can spread readily [211]. This concept is further supported by recently published data regarding the role of cell-to-cell spread of HIV-1 in mediating a functionally higher multiplicity of infection [212]. Additionally, killing of acutely infected cells by HIV-1-specific CTLs also would require the immediate access to CTLs; whether these conditions exist to trigger very early killing *in vivo* remains unclear.

Among the evaluated Gag-specific CTLs, those recognizing the B*57-restricted TW10 (Gag 240-249) epitope are particularly efficient in killing infected cells before *de novo* viral protein production. TW10-specific CTLs required an inoculum of about 6 to 15 fg p24/cell input virus (approximately 60 to 150 virions/cell), while the other CTLs required greater than 30 fg p24/cell input virus (approximately 300 virions/cell). Targeting of this immunodominant epitope in acutely-infected persons has been associated with better immune control [213], and it has been demonstrated that this epitope has a particularly long intracellular half-life that contributes to high levels of presentation [214]. This supports our observation regarding the lower inoculum required by TW10-specific CTLs

to recognize epitopes derived from incoming virions, and our findings may further suggest a mechanism for the strong immunodominance of this epitope.

Early infected cell cytolysis through recognition of virion-derived epitopes is not unique to HIV-1 Gag targeting. Studies of SIV-specific CTLs and SIV-infected cells have observed this phenomenon for CTLs targeting non-Gag proteins, including epitopes in Pol [153]-, Vpr [215]-, and Rev- derived epitopes [215] but not Nef[153]. Although we did not observe early infected cell killing by the Pol- or Rev- specific CTLs tested, a Nef-specific CTL clone targeting the B*57 restricted HQ10 epitope (Nef 116-125) exhibited recognition of virion-derived epitopes. Myristoylated Nef associates with the infected cell plasma membrane and is packaged into virions during the process of budding, albeit inefficiently, contained in the virion on the order of 10% of reverse transcriptase and 0.5% of Gag content [48]. The observation of early killing by HQ10-specific CTLs indicates that Nef is carried in sufficient amounts to allow recognition of virion-derived epitopes at viral inocula similar to what is required for several Gag-specific CTLs. Furthermore, our observation of Pol-targeted CTLs that did not mediate early killing again suggests that epitope-specific variability in efficiency of epitope processing and presentation determines the likelihood for CTL recognition of epitopes from incoming virions, emphasizing that the kinetics of epitope presentation and recognition by CTLs are multifactorial and not specific to the targeted viral protein.

One caveat to our data is the use of VSV-G Env-pseudotyped HIV-1 to evaluate CTL recognition of epitopes derived from incoming virions. Whereas HIV-1 Env induces viral

internalization into the cytoplasm through membrane fusion, VSV-G Env induces endocytosis into clathrin-coated pits for early endosomal uptake [216]. However, CTLs recognize epitopes processed through the HLA-I pathway, whereas antigens in endosomes are processed through the HLA-II pathway. Although cross-priming between pathways can occur in professional antigen presenting cells, these pathways are distinct in most cells [217], and HIV-1 entry using VSV Env should be less effective in accessing the HLA-I pathway than normal infection mediated via HIV-1 Env, and thus our results may underestimate the sensitivity of this phenomenon.

In summary, the present study provides evidence for a direct link between the kinetics of CTL recognition of acutely infected cells and susceptibility to Nef-mediated immune evasion. CTLs that can recognize epitopes processed from proteins carried by incoming virions before *de novo* viral protein production temporally bypass the effect of Nef. This capability varies according to individual epitopes and is not determined solely by the source protein, although high copy numbers of Gag in virions makes Gag epitopes the most common triggers of early killing. This may be one factor contributing to the overall beneficial effect of Gag targeting by CTLs derived from proteins carried in virions, but this phenomenon is not unique to Gag and can occur for epitopes from other proteins carried in virions, such as Pol and Nef.

Chapter 4:

Antiviral activity of human immunodeficiency virus type 1 Gag-specific cytotoxic T lymphocyte targeting is not necessarily intrinsically superior to Envelope targeting

4.1 Abstract

Across several cohorts, HIV-1 Gag- and Env-specific $CD8^+$ T lymphocyte (CTL) responses have demonstrated correlation and inverse correlation to viremia respectively, and it has been proposed that the mechanism is superior antiviral activity of Gag-specific CTL through earlier Gag epitope presentation by infected cells. Addressing this hypothesis, we created two HIV-1 constructs with a translocated epitope from Gag (SLYNTVATL) to Env, thereby changing the protein source of the epitope. A virus expressing SL9 in Env was similar to the original virus in susceptibility to SL9-specific CTLs. These results suggest that Env-targeting is not intrinsically inferior to Gag-targeting for CTL antiviral activity.

4.2 Introduction

Several cohort studies have demonstrated a significant correlation between the magnitude and/or breadth of the Gag-specific $CD8^+$ T lymphocyte (CTL) response and viremia [163-166, 169, 218], as well as a negative correlation of viremia to the Env-specific CTL response [163, 164]. Because CTL epitope targeting of HIV-1 is an important determinant of antiviral activity [159, 195], it has been hypothesized that Gag-specific CTLs might be generally superior in suppressing HIV-1 replication. Two observations regarding the virus-neutralizing activity of CTLs *in vitro* suggested that Gag-specific CTLs might be more potent. One study found that simian immunodeficiency virus (SIV) Gag-specific (but not Env-specific) CTLs can kill acutely infected cells very early by recognizing epitopes derived from incoming virions, before *de novo* viral protein translation from infection [152]. Another demonstrated that bulk Gag-specific polyclonal CTL primary cell lines have greater antiviral activity against a laboratory strain of HIV-1 compared to bulk Env-specific cell lines [160]. These data have been interpreted to indicate that Gag-targeted CTL are intrinsically superior to Env-targeted CTL, perhaps due to a specific protein property such as early epitope presentation [160]. Potential caveats to these experimental findings, however, include the high multiplicity of infection of the target cells in the observation of early killing [152], and inability to control for cell function and epitope sequence matching (*in vivo* versus laboratory virus sequences) in the comparisons of virus suppression by Gag- versus Env-specific CTL lines [160].

To explore the role of protein targeting in the antiviral efficiency of CTLs while controlling for lymphocyte function and epitope specificity, we tested the antiviral activity of CTLs targeting the SLYNTVATL (SL9; Gag 77-85 in p17) epitope against that of molecular clones of HIV-1 containing this epitope translocated to Env to alter its protein source. This approach allowed us to hold the effector cells constant and examine whether altering the protein source of the epitope changes the antiviral efficiency of CTL.

4.3 Results

Two molecular clones of HIV-1 NL4-3 were modified to alter the endogenous Gag SL9 epitope to a previously described [161] non-recognized sequence (Gag-SL9x) and to create the SL9 epitope sequence in either of two locations in Env (Table 4.S1). These Env mutations contained amino acid substitutions to create the SL9 sequence in the $gp41_{301-311}$ cytoplasmic domain (Env-SL9-gp41) or the $gp120_{370-381}$ V4 loop (Env-SL9-V4). These viruses (Gag-SL9x/Env-SL9-gp41 and Gag-SL9x/Env-SL9-V4) were compared to the index NL4-3.1 virus (Gag-SL9/Env-WT), which contains the clade B consensus SL9 sequence [196]. Additionally, two control viruses contained either Gag with the clade B consensus B sequence (Gag-SL9) or the Gag-SL9x mutation, combined a methionine to alanine mutation at position 20 of Nef (M20A) that selectively neutralizes the downregulation of MHC class I by Nef [198].

All viruses were compared for their susceptibility to CTLs using a previously described assay for suppression of viral replication ([161, 194, 195] and see section 2.4 for brief descriptions). An SL9-specific CTL clone (S00001-SL9-1.8) demonstrated modest suppression of the index Gag-SL9/Env-WT virus, which was enhanced in the absence of MHC-I downregulation by Nef (Figure 4.1A versus B, and F), in agreement with prior results [129-131]. The control Gag-SL9x/Env-WT/Nef-M20A (SL9 knockout) virus was not inhibited (Figure 4.1C and F), confirming the functional ablation of the native SL9 Gag epitope by the T81L/T84V mutations [161]. The SL9x/Env-SL9-gp41 (SL9 epitope in Env gp41) virus exhibited similar susceptibility to the index virus (Figure 4.1D and F). The Gag-SL9x/Env-SL9-V4 (SL9 in Env V4) virus was also inhibited, although it showed

diminished susceptibility (Figure 4.1E and F). Results from four repeats of this experiment were normalized as a ratio of the efficiency of log suppression versus the index Gag-SL9/Env-WT virus (Figure 4.2A), demonstrating that the similarity between the susceptibility of the index virus and that of the Gag-SL9x/Env-SL9-gp41 (SL9 in Env gp41) virus to the SL9-specific CTLs was consistent. The reason for reduced suppression of the Gag-SL9x/Env-SL9-V4 virus was unclear but may have been due to impaired epitope processing and/or to overall lower expression of Env compared to that of Gag.

To control for CTL susceptibility in general, these viruses were also tested for sensitivity to suppression by an A*02-restricted CTL clone recognizing the AIIRILQQL epitope in Vpr_{59-67}, S00036-AL9-1.1. Across three separate experiments (Figure 4.2B), both Nef-M20A-containing viruses showed increased susceptibility to CTL regardless of SL9 epitope sequence, consistent with the known effect of Nef on CTL susceptibility [129-131], and the SL9 epitope-translocated viruses remained susceptible to this Vpr-specific CTL clone at a level similar to the index virus containing wild type Nef.

Table 4. S1 Virus constructions used in this study

Virus	Gag 76-86 (p17)	Env 399-411 (gp120-V4)	Env 811-823 (gp41)	Nef 20
Gag-SL9/Env-WT (Index NL4-3.1)	RSLYNTVATLY	TWSTEGSNNTEGS	AVNLLNATAIAVA	M
Gag-SL9/Env-WT/Nef-M20A	----------	----------	----------	A
Gag-SL9x/Env-WT/Nef-M20A	-----L--V--	----------	----------	A
GagSL9x/Env-SL9-gp41	-----L--V--	----------	-RS-Y-T--TLY-	-
Gag-SL9x/Env-SL9-V4	-----L--V--	-R-LYNTVA-LY-	----------	-

The SLYNTVATL (SL9) epitope in Gag 77-85 was genetically created in the Env V4 loop of gp120 or the cytoplasmic region of gp41 by substitution mutations within NL4-3, while the native SL9 epitope was altered with T81L/T84V mutations in Gag. Two control viruses also contained the M20A mutation in Nef.

Dashes indicate identity with the index virus.

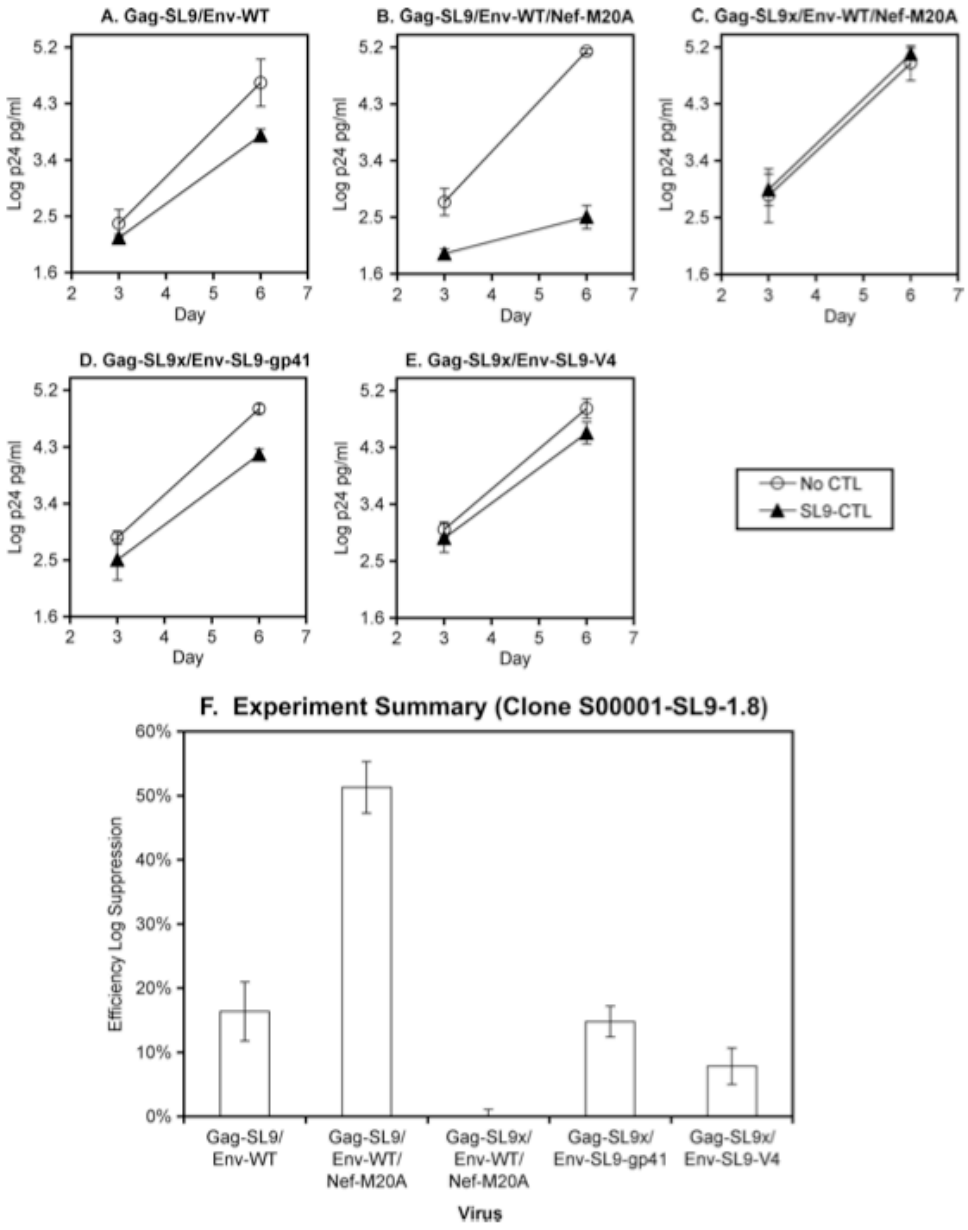


Figure 4.1 SL9-specific CTL suppression of HIV-1 with Env containing an SL9 epitope translocated from Gag. T1 cells were acutely infected with the indicated viruses and cultured in the absence or presence of the SL9-specific CTL clone S00001-SL9-1.8. (A to E) Raw p24 values over time are shown; error bars indicate standard deviations for triplicate experiments. (F) The mean efficiency of virus suppression (log_{10}

units of p24 reduction) is plotted for each virus. Each error bar indicates one standard deviation. These data are representative of four experiments (see Figure 4.2A)

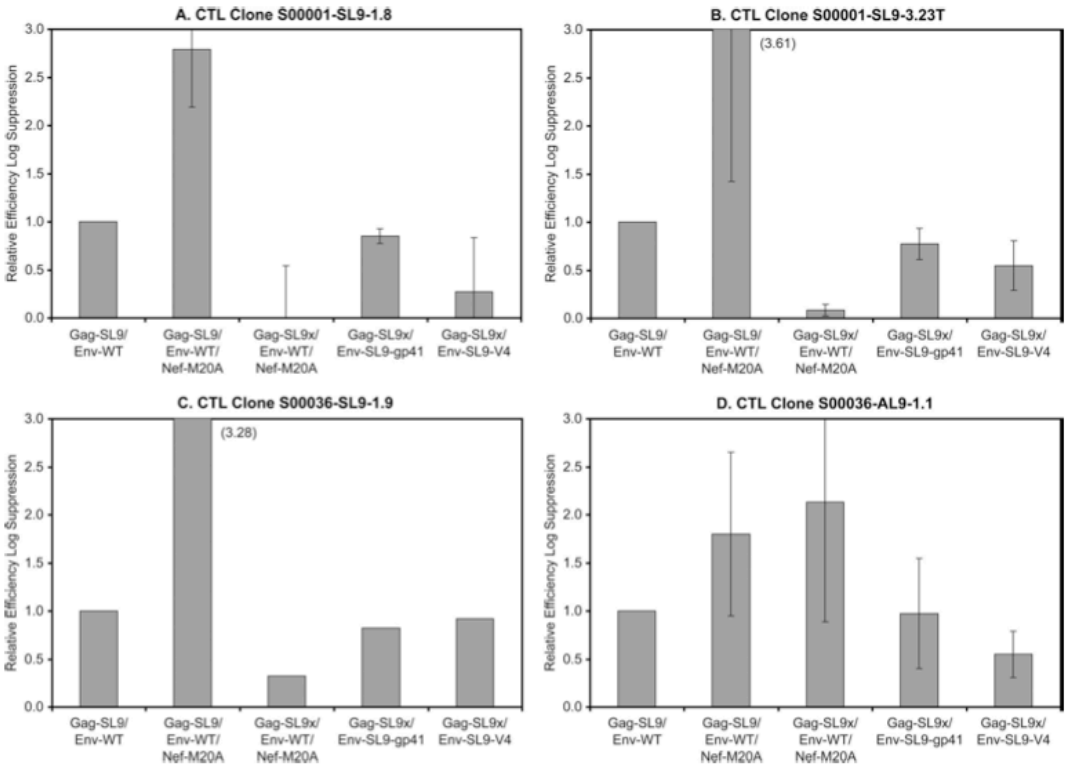


Figure 4.2 Relative susceptibilities of HIV-1 with Env containing an SL9 epitope translocated from Gag to SL9- and AL9-specific CTLs. The antiviral activities of CTL clones targeting SL9 or AL9 (AIIRILQQL; Vpr59–67) were tested against the panel of viruses in Figure 4.1. The efficiency of virus suppression in each experiment was normalized as a ratio versus the efficiency of suppression of the index virus (Gag-SL9/Env-WT). (A) Means and standard deviations for four experiments with the SL9-specific CTL clone S00001-SL9-1.8. (B) Means and standard deviations for six experiments with the SL9-specific CTL clone S00001-SL9-3.23T. Note that the T cell receptor variable-chain usage of this clone was different from that of S00001-SL9-1.8, although it was derived from the same person (data not shown). (C) Means and

standard deviations for three experiments with the SL9-specific CTL clone S00036-SL9-1.9. (D) Means and standard deviations for three experiments with the AL9-specific CTL clone SL00036-AL9-1.1.

4.4 Discussion

These data suggest that the protein origin of the SL9 epitope, whether Gag or Env, does not necessarily have a marked impact on the antiviral efficiency of SL9-specific CTLs. This was observed even though our epitope translocation approach moved the epitope to a less highly expressed protein, and also could have reduced the processing efficiency of this epitope, which is efficiently processed *in situ* [219] and therefore immunodominant in chronic infection. Thus, the similar degree of CTL susceptibility of an SL9 Env translocation mutant suggests that Gag targeting is not necessarily intrinsically superior to Env targeting by CTLs. Note this is consistent with our early studies of Gag- and Env- specific CTL clones, which demonstrated levels of antiviral activity of HLA-B*14-restricted clones recognizing the conserved epitope ERYLKDQQL in qp41 that were equivalent or even superior to those of A*02- and B*14- restricted Gag-specific clones [128, 195].

Our results contrast with the demonstration by Sacha *et al* that SIV Gag-specific CTLs recognize and kill infected cells much earlier than those targeting Tat or Env, within a few hours after acute infection of target cells [152]. It was hypothesized that the plentiful structural Gag protein from incoming virions is processed for epitope presentation on acutely infected cells via the class I pathway, while the Env protein remains on the cell surface after viral membrane fusion to the newly infected cell. In such as case, Gag epitope presentation could occur before *de novo* HIV-1 protein expression, which requires several steps, including reverse transcription, integration, transcription, and translation. While Sacha *et al* demonstrated that Gag-but not Env-specific CTLs could

lyse acutely infected cells at a time after infection that was too early for de novo viral protein expression, in the current study we did not see a functional advantage for Gag versus Env targeting for antiviral activity. This may be explained by methodologic differences; the early-killing observations were with assays performed under conditions of excess infection of target cells, requiring at least 20 to 100 virions per cell and magnetofection, while our virus suppression assays started with low multiplicity of infection. Consistent with our findings, Vojnov *et al* observed that Gag- and Env-specific CTLs can have similar activity against SIV in virus suppression assays [162].

Our results also contrast with those of Chen *et al*, who found that polyclonal Gag- and Env- specific CTL lines markedly differed in antiviral activity [160]. This finding could be due to methodologic caveats acknowledged in that study. First, Gag is generally more conserved than Env, and thus it is likely that the epitopes in the laboratory HIV-1 strains utilized in their virus suppression assays better matched the *in vivo* HIV-1 epitopes for Gag than those for Env. Second, given the key role of antigenic stimulation in proliferation and differentiation of CTLs, the greater variability of Env could affect CTL effector function *in vivo*. Anecdotally, our laboratory has found it difficult to derive viable Env-specific CTL clones from the peripheral blood mononuclear cells (PBMCs) of HIV-1-infected persons despite high frequencies detected by enzyme-linked immunospot (ELISPOT) assays.

Given the clinical associations of Gag-specific CTL responses with better immune control of HIV-1 *in vivo* [163-166, 169, 218], the importance of the inclusion of Gag (and

perhaps exclusion of Env) in CTL-based vaccines has been emphasized. However, our data demonstrate that protein targeting may not be an overriding determinant of CTL efficacy and that Env targeting can be as effective as Gag targeting. This finding underscores the fact that the antigenic unit of CTLs is the epitope, and epitope properties (i.e. level and timing of presentation by an infected cell, which in turn depend on factors such as processing efficiency [220], HLA binding affinity [157], sequence variability [196], etc.) determine CTL function. Thus, while characteristics of the source protein of an epitope (such as expression level and kinetics) influence its properties, ultimately there are many other factors that determine the final profile of epitope presentation.

The benefit of higher levels of Gag targeting by CTLs *in vivo* likely reflects general trends in epitope properties conferred by Gag rather than an intrinsic advantage of Gag targeting. Note that the association of Gag targeting with lower viremia is a statistical correlation seen across large numbers of persons; the predictive value of Gag targeting for viremia in an individual is poor (e.g., note Figure 2a in reference [164]). This suggests that Gag-specific CTLs on average are more effective than but not necessarily superior to other CTLs for any given epitope. Because Gag is highly expressed and relatively conserved in sequence, epitope properties such as high expression levels and resistance to escape are candidates for determinants of antiviral efficacy *in vivo*. Compared to Env, which is expressed at lower levels (60 to 100 times lower than Gag, [51, 59, 60]) and is relatively variable in sequence, Gag epitopes should be more highly

expressed and more resistant to escape on average, but this not always the case for any particular Gag and Env epitopes.

Finally, these data do not exclude a role for better antiviral activity of Gag-specific CTL through early recognition of virion-derived Gag epitopes, as observed by our own group (Figure 3.6 and 3.7) and by Sacha *et al* [152]. As discussed in Chapter Three, the ability of Gag-specific CTLs to recognize infected cells through incoming virion derived epitope varies by epitope and is dependent on the viral inoculum; a multiplicity of infection of 300 is required for most Gag-sepcific CTLs to kill infected cells through this mechanism (Figure 3.7). Conceivably, target $CD4^+$ T lymphocytes in crowded lymphoid tissues could be infected simultaneously with multiple virions, resulting in enough virion-derived Gag for early epitope presentation and recognition. However, Sacha *et al* also reported recently that Vpr- and Rev- specific CTLs can also kill infected cells before viral protein translation [215], yet CTL responses against Vpr and Rev have not been reported to correlate with better immune control [163-166, 169, 218]. If this virion-derived epitope presentation phenomenon does *in vivo*, perhaps factors such as sequence variability offset a benefit of early Vpr and Rev epitope presentation.

Another caveat to our results is the artificial nature of epitope translocation from one protein to another; we cannot exclude that we have altered epitope processing and production levels or that we have interfered with some unforeseen property that affects HIV-1 susceptibility to CTLs (as we noted in an early study of the translocation of SL9 into Nef, where we inadvertently ablated HLA-I downregulation by Nef [150]).

Additionally, the “knockout” of SL9 in Gag could still contribute some degree of recognition, as was seen for clone S00036-SL9-1.9, which showed some suppression of the control SL9x virus (Figure 4.2C).

In summary, we demonstrated that CTLs targeting Gag are not likely intrinsically more effective than those targeting Env in an *in vitro* model of viral suppression. These data underscore the importance of epitope properties rather than just protein properties for the antiviral activity of CTLs. Thus, for immunopathogenesis studies and vaccine design, it is important to consider CTL targeting in terms of epitopes and their properties rather than being constrained by thinking of whole viral proteins as antigenic unit.

Chapter 5

Discussion

5.1 Abstract

This chapter is divided into 3 major sections (5.2-5.4). The first section (5.2) summarizes the major findings and discussions of this dissertation. The next section (5.3) describes potential future work, which focuses on the functions of antigen-specific T cell receptor clonotypes, in CTL efficiency. Some concluding remarks are provided in the final section.

5.2 Summary of Results

The overall aim of this dissertation is to better define the determinants of antiviral activity of HIV-1 specific CTLs. Previous work on this topic has indicated that factors such as Nef-mediated HLA-I downregulation, the timing of epitope expression, functional avidity, protein targeting, and HLA-I restriction can influence how well a CTL can recognize an infected cell and suppress HIV-1 replication (see section 1.5 for a detailed description of each of these factors). Therefore, experiments were carried out to better understand these factors and their relationships as they influence CTL antiviral activity.

One of the well-documented means by which HIV-1 impairs the CTL response is through HLA-I downregulation, which is mediated by the Nef protein (see section 1.4 for a detailed description on this topic). While the impact of Nef on CTL efficiency varies by epitope [194, 205], the determining factors are not well understood. In Chapter Three, we determined the impact of Nef on the antiviral efficiency of HIV-1 specific CTLs targeting 17 different epitopes to define the properties that determine CTL antiviral efficiency and the susceptibility to Nef. We observed variability in the impact of Nef on HIV-1 specific $CD8^+$ T-cell antiviral activity (Figure 3.1). This variability did not correlate with HLA-I restriction or functional avidity (Figure 3.3 and 3.4), but correlated directly with the kinetics of infected cell clearance (Figure 3.5). While CTLs targeting Gag epitopes generally were less affected by Nef than responses directed against all non-Gag-derived epitopes combined (Figure 3.2), this was determined by the ability to

mediate early antiviral activity before Nef-mediated HLA-I downregulation through incoming virion derived epitopes (Figure 3.5, 3.6 and 3.7). Furthermore, the ability to eliminate infected cells by CTLs through virion-derived epitope was dose-dependent on viral inoculum, and varied depending on the specific epitope sequence. Together these results suggest that early infected cell killing by CTL before Nef-mediated HLA-I downregulation is important for viral containment in HIV-1 infected individuals.

The present literature suggests a protective role of Gag targeting in HIV-1 control. One proposed mechanism for the superior antiviral activity of Gag-specific CTLs is that they can eliminate virus-infected cells before Nef-mediated HLA-I downregulation through incoming virion derived epitopes. Although the results in Chapter Three are in agreement with those observations, we also noticed a striking variability in infected cell killing kinetics and antiviral efficiency among Gag-specific CTLs. That is, very early and efficacious infected cell killing by B*57 restricted TW10 specific CTLs was predominantly effected through recognition of the incoming virion-derived epitopes, while infected cell killing by B*57 restricted, Gag-KF11 specific CTLs required the presentation of *de novo* synthesized Gag, which did not occur until 10 hour post-infection (Figure 3.5 and 3.7s). Similarly, comparing the Nef effect ratio on CTL antiviral activity according to the targeted epitope, some Gag-specific CTLs were more susceptible to Nef than some non-Gag-specific CTLs (Figure 3.1B). These results suggest that, although some Gag epitopes are better CTL targets, Gag targeting in general is not necessarily intrinsically superior to other viral protein targeting for CTL antiviral efficiency.

In an attempt to further elucidate the role of Gag targeting in CTL antiviral efficiency, in Chapter Four, we created two viruses with a Gag epitope translocated to Env protein (which has been shown to be positively correlated with viral load) (see section 1.5.3 for a detailed description on this topic; see Table 4.S1 for viral constructs). This allowed switching of the epitope source protein. We then assessed the susceptibility of these two viruses to CTL-mediated lysis compared to the susceptibility of wild-type virus. We found that a virus expressing the SL9 epitope in Env gp41 (Gag-SL9x/Env-SL9-gp41) was equally susceptible to CTL-mediated lysis versus wild-type virus (Figure 4.1), suggesting that epitopes derived from non-Gag proteins are not necessarily inferior to those derived from Gag for CTL antiviral activity.

These findings have great implications for HIV-1 vaccine immunogen design. As mentioned in section 1.5.3, the importance of the inclusion of Gag in a CTL-based vaccine has been emphasized, as the current view of the field is that Gag is the "Achilles heel" for HIV-1. However, our data suggest otherwise; not all the CTLs targeting Gag epitopes were effective in suppressing viral replication. Rather, the efficient antiviral activity of CTL against HIV-1 depends on the individual epitope properties, such as epitope presentation kinetics, which in turn are determined by various factors, including epitope processing efficiency [220], epitope HLA binding affinity [157], and sequence variability [218]. Together these results indicate that the benefit of Gag targeting in HIV-1 control is likely due to its sequence conservation that constrains viral escape rather than its intrinsic superiority in viral suppression, and that

for vaccine design, we need to consider individual epitopes and their properties rather than the whole viral proteins as the basic antigen units in CTL targeting.

5.3 Future Work: The Role of T Cell Receptor Clonotypes in the Antiviral Efficiency of CTLs

The results from Chapter Three show that significant differences in Nef susceptibility can exist among different CTL clones that are directed against the same epitope (Figure 3.1B). Also, while the three TW10-specific CTL clones of one HIV-1 patient (S11-TW10-10.47, S11-TW10-3.24, and S11-TW10-10.38; Table 3. S1) were shown to be resistant to Nef compared to other CTLs, our preliminary results showed that two TW10-specific CTL clones from another patient did not exhibit the same antiviral efficiency (Figure 5.1A, B versus and C, D; Figure 5.2). This finding suggests that, besides specificity, individual physiologic host factors can influence CTL efficiency.

One of the influencing host factors is the T cell receptor (TCR) clonotype. Recent findings on antigen-specific CTL immunity have demonstrated individual differences in TCR clonotypes in antiviral CTL efficacy [221]. TCRs are stochastically-derived through genetic recombination (for a detailed description of TCR rearrangement, see section 1.1.3 and Figure 1.4), and a varying number of distinct TCRs tend to recognize a given epitope. Thus, each antigen-specific T cell population is constituted from several different clonotypes, which can be considered as the fundamental units of T-cell reactivity. Collectively, the properties of these individual clonotypes determine the overall quality of the antiviral response in a given T cell population. The TCR clonotype with the most optimal structural and biophysical features for cognate peptide-MHC interaction will likely exhibit high levels of antigen sensitivity and hence, be more

efficient in clearing viral infection [221]. Moreover, despite the small probability of TCR sharing between individuals (given the vast potential for combinatorial diversity during the process of VDJ gene rearrangement), recently studies have been able to identify public clonotypes in antigen specific T cell populations between individuals [222, 223]. More importantly, their presence can be associated with distinct biological functions [224-226].

Thus, for future studies, I propose to focus on the role of the TCR clonotype functions in the antiviral efficiency of CTLs. Initial studies could focus on the TCR clonotypes specific for the B*57 restricted, p24 TW10 epitope, as this epitope is immunodominant in acute infection, and is linked to slower disease progression rates in B*57 individuals (see section 1.5.4 for a detailed description on this topic). As mentioned in section 1.5.4, a good number of B*57 individuals with TW10-specific CTL responses still progress to AIDS if left untreated. Thus, my hypothesis is that functional differences in TW10-specific TCR clonotypes between B*57 non-progressors versus progressors are responsible for these distinct disease outcomes.

To study TCR functions while controlling for CTL clonal conditions (e.g., effector memory state of the CTLs and degree of functional exhaustion), full-length Vα and Vβ TCR genes of TW10-specific CTL clones from various patients will be sequenced, reconstructed into lentiviral expression vectors, and expressed in $CD8^+$ T lymphocytes of uninfected donors to assess their biological functions. Functional experiments will include testing the antigen sensitivity, functional avidity, the susceptibility to Nef, and

infected cell killing kinetics of these clones. Results from these experiments will allow us to unravel important functional attributes of the TCR clonotypes associated with effective control of HIV-1 replication *in vivo*, and provide clues as to why differences in antiviral efficiency exist between individuals sharing the same antigen-specific CTL response.

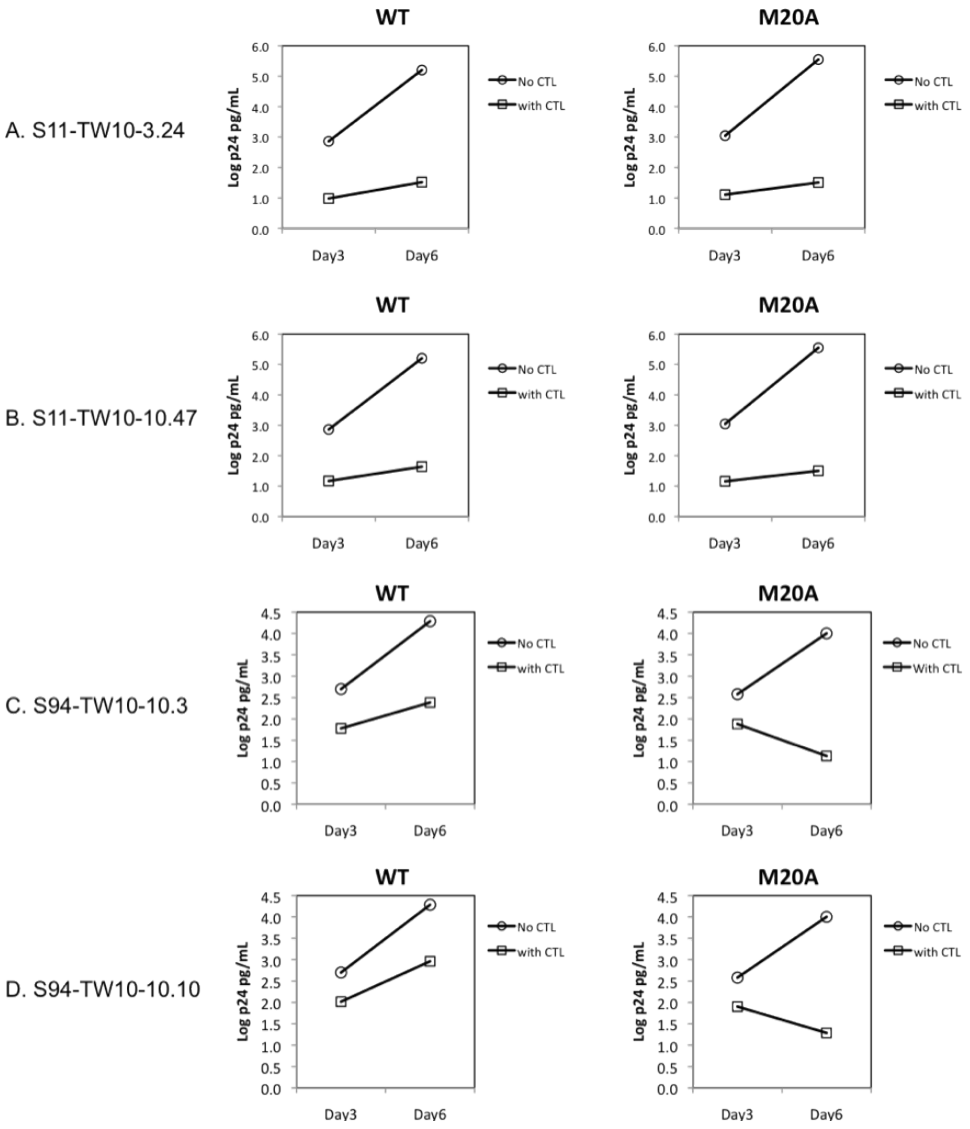


Figure 5.1 Impact of Nef on the antiviral activity of TW10-specific CTL clones. (A-D) Individual CTL clones were tested for inhibition of NL4-3.1 virus containing wild-type Nef or Nef-M20A (unable to downregulate HLA-I) in parallel. Mean raw p24 values of triplicates over time are shown.

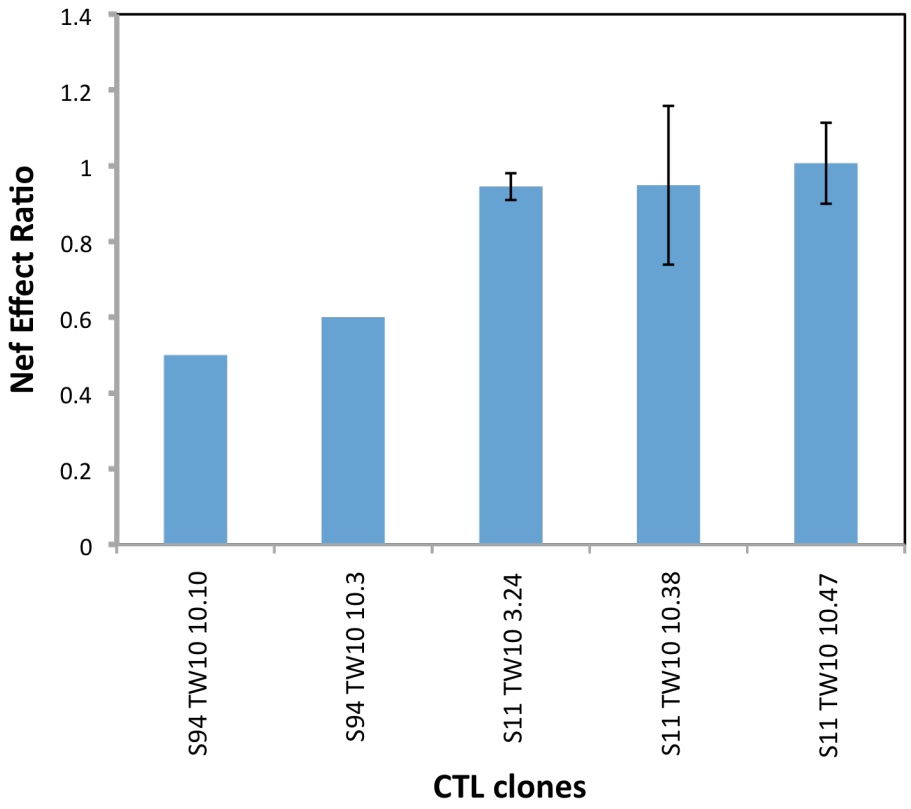


Figure 5.2 Comparison of the Nef effect on viral inhibition between TW10-specific CTL clones. The *Nef effect ratio* for individual CTL clone was determined as described in Chapter 2, section 2.5. Mean Nef effect ratio and standard deviation are plotted for each clone; clone S11-TW10-3.24, S11-TW10-10.38, and S11-TW10-10.47 are results from two, seven, and three independent experiments, respectively, while clone S94-TW10-10.10 and S94-TW10-10.3 are results from one experiment.

5.4 Concluding Remarks

Since the start of the epidemic, more than 60 million people have been infected with HIV and nearly 25 million adults and children have died of AIDS, making it one of the deadliest epidemics in history. As such, the need for an effective HIV vaccine has never been more urgent. While CTL-based vaccines are one of the current vaccine platforms, there is no clear consensus as to which viral proteins should be used as immunogens. The inclusion of early expressed viral proteins Nef, Tat, and Rev, and more recently, the late expressed protein Gag, have all been suggested, in the hope that these proteins would elicit early immune responses needed to effectively control viral replication. The data presented here suggest that antigens associated with efficacious viral control do not necessarily have to be derived from Gag, or any particular viral protein, as long as they are equipped with the properties needed to be targeted by CTLs early in the viral life cycle. Thus, the focus of immunogen design, perhaps, should be on HIV-1 epitopes, such that we have a better understanding of antigen expression kinetics on infected cells, as well as the impact of individual CTL specificities in driving sequence diversity and in controlling virus replication ([150, 227], and see section 1.5.4 for the protective mechanisms associated with Gag specific, B*57 restricted CTL responses). During future HIV vaccine development, it would probably be more beneficial to include stretches of sequences that have the desired properties of eliciting protective CTL responses, rather than considering the protein itself as the basic antigenic unit for CTL responses.

References:

1. Charles A. Janeway, J., Paul Travers, Mark Walport, and Mark J. Shlomchik, *Immunobiology: the immune system in health and disease*. 6th ed. 2005.

2. Pape, K.A., et al., *The humoral immune response is initiated in lymph nodes by B cells that acquire soluble antigen directly in the follicles.* Immunity, 2007. **26**(4): p. 491-502.

3. Ueno, H., et al., *Dendritic cells and humoral immunity in humans.* Immunol Cell Biol. **88**(4): p. 376-80.

4. Purcell, A.W. and T. Elliott, *Molecular machinations of the MHC-I peptide loading complex.* Curr Opin Immunol, 2008. **20**(1): p. 75-81.

5. van Endert, P.M., *Genes regulating MHC class I processing of antigen.* Curr Opin Immunol, 1999. **11**(1): p. 82-8.

6. Guermonprez, P., et al., *Antigen presentation and T cell stimulation by dendritic cells.* Annu Rev Immunol, 2002. **20**: p. 621-67.

7. van Niel, G., R. Wubbolts, and W. Stoorvogel, *Endosomal sorting of MHC class II determines antigen presentation by dendritic cells.* Curr Opin Cell Biol, 2008. **20**(4): p. 437-44.

8. Yewdell, J.W. and J.R. Bennink, *Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses.* Annu Rev Immunol, 1999. **17**: p. 51-88.

9. Buslepp, J., et al., *A correlation between TCR Valpha docking on MHC and CD8 dependence: implications for T cell selection.* Immunity, 2003. **19**(4): p. 595-606.

10. Robinson, J., et al., *The IMGT/HLA database.* Nucleic Acids Res. **39**(Database issue): p. D1171-6.

11. Russell, J.H. and T.J. Ley, *Lymphocyte-mediated cytotoxicity.* Annu Rev Immunol, 2002. **20**: p. 323-70.

12. Nahill, S.R. and R.M. Welsh, *Polyclonality of the cytotoxic T lymphocyte response to virus infection.* Proc Soc Exp Biol Med, 1992. **200**(4): p. 453-7.

13. Razvi, E.S. and R.M. Welsh, *Programmed cell death of T lymphocytes during acute viral infection: a mechanism for virus-induced immune deficiency.* J Virol, 1993. **67**(10): p. 5754-65.

14. Groscurth, P. and L. Filgueira, *Killing Mechanisms of Cytotoxic T Lymphocytes.* News Physiol Sci, 1998. **13**: p. 17-21.

15. Podack, E.R., J.D. Young, and Z.A. Cohn, *Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules.* Proc Natl Acad Sci U S A, 1985. **82**(24): p. 8629-33.

16. Greenberg, A.H. and D.W. Litchfield, *Granzymes and apoptosis: targeting the cell cycle.* Curr Top Microbiol Immunol, 1995. **198**: p. 95-119.

17. Kagi, D., et al., *Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice.* Nature, 1994. **369**(6475): p. 31-7.

18. Lowin, B., et al., *A null mutation in the perforin gene impairs cytolytic T lymphocyte- and natural killer cell-mediated cytotoxicity.* Proc Natl Acad Sci U S A, 1994. **91**(24): p. 11571-5.

19. Baker, S.J. and E.P. Reddy, *Transducers of life and death: TNF receptor superfamily and associated proteins.* Oncogene, 1996. **12**(1): p. 1-9.

20. Cocchi, F., et al., *Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells.* Science, 1995. **270**(5243): p. 1811-5.

21. Guidotti, L.G. and F.V. Chisari, *To kill or to cure: options in host defense against viral infection.* Curr Opin Immunol, 1996. **8**(4): p. 478-83.

22. Kurane, I., A. Meager, and F.A. Ennis, *Dengue virus-specific human T cell clones. Serotype crossreactive proliferation, interferon gamma production, and cytotoxic activity.* J Exp Med, 1989. **170**(3): p. 763-75.

23. Dayton, E.T., et al., *Role of immune interferon in the monocytic differentiation of human promyelocytic cell lines induced by leukocyte conditioned medium.* Blood, 1985. **66**(3): p. 583-94.

24. Tomiyama, H., T. Matsuda, and M. Takiguchi, *Differentiation of human CD8(+) T cells from a memory to memory/effector phenotype.* J Immunol, 2002. **168**(11): p. 5538-50.

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

26. Alkhatib, G., et al., *CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1.* Science, 1996. **272**(5270): p. 1955-8.

27. Barre-Sinoussi, F., et al., *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS).* Science, 1983. **220**(4599): p. 868-71.

28. Barin, F., et al., *Serological evidence for virus related to simian T-lymphotropic retrovirus III in residents of west Africa.* Lancet, 1985. **2**(8469-70): p. 1387-9.

29. Gallo, R.C., et al., *Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS.* Science, 1984. **224**(4648): p. 500-3.

30. Popovic, M., et al., *Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS.* Science, 1984. **224**(4648): p. 497-500.

31. Vilmer, E., et al., *Isolation of new lymphotropic retrovirus from two siblings with haemophilia B, one with AIDS.* Lancet, 1984. **1**(8380): p. 753-7.

32. Chiu, I.M., et al., *Nucleotide sequence evidence for relationship of AIDS retrovirus to lentiviruses.* Nature, 1985. **317**(6035): p. 366-8.

33. Gonda, M.A., et al., *Sequence homology and morphologic similarity of HTLV-III and visna virus, a pathogenic lentivirus.* Science, 1985. **227**(4683): p. 173-7.

34. Levy, J.A., et al., *Infection by the retrovirus associated with the acquired immunodeficiency syndrome. Clinical, biological, and molecular features.* Ann Intern Med, 1985. **103**(5): p. 694-9.

35. Gottlieb, M.S., et al., *Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency.* N Engl J Med, 1981. **305**(24): p. 1425-31.

36. Masur, H., et al., *An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction.* N Engl J Med, 1981. **305**(24): p. 1431-8.

37. Siegal, F.P., et al., *Severe acquired immunodeficiency in male homosexuals, manifested by chronic perianal ulcerative herpes simplex lesions.* N Engl J Med, 1981. **305**(24): p. 1439-44.

38. Rowland-Jones, S.L. and H.C. Whittle, *Out of Africa: what can we learn from HIV-2 about protective immunity to HIV-1?* Nat Immunol, 2007. **8**(4): p. 329-31.

39. Holmes, E.C., *On the origin and evolution of the human immunodeficiency virus (HIV).* Biol Rev Camb Philos Soc, 2001. **76**(2): p. 239-54.

40. Gao, F., et al., *Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes.* Nature, 1999. **397**(6718): p. 436-41.

41. Korber, B., et al., *Timing the ancestor of the HIV-1 pandemic strains.* Science, 2000. **288**(5472): p. 1789-96.

42. Marx, P.A., *Unsolved Questions over the Origin of HIV and AIDS*, in *ASM News*. 2005. p. 5.

43. Sharp, P.M. and B.H. Hahn, *Origins of HIV and the AIDS Pandemic.* Cold Spring Harb Perspect Med. **1**(1): p. a006841.

44. Joint United Nations Programme on HIV/AIDS., *Global report : UNAIDS report on the global AIDS epidemic*, Joint United Nations Programme on HIV/AIDS: Geneva, Switzerland. p. v.

45. Kuznetsov, Y.G., et al., *Atomic force microscopy investigation of human immunodeficiency virus (HIV) and HIV-infected lymphocytes.* J Virol, 2003. **77**(22): p. 11896-909.

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

47. Camaur, D. and D. Trono, *Characterization of human immunodeficiency virus type 1 Vif particle incorporation.* J Virol, 1996. **70**(9): p. 6106-11.

48. Welker, R., et al., *Human immunodeficiency virus type 1 Nef protein is incorporated into virus particles and specifically cleaved by the viral proteinase.* Virology, 1996. **219**(1): p. 228-36.

49. Briggs, J.A., et al., *The stoichiometry of Gag protein in HIV-1.* Nat Struct Mol Biol, 2004. **11**(7): p. 672-5.

50. Muller, B., et al., *Human immunodeficiency virus type 1 Vpr protein is incorporated into the virion in significantly smaller amounts than gag and is phosphorylated in infected cells.* J Virol, 2000. **74**(20): p. 9727-31.

51. Layne, S.P., et al., *Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus.* Virology, 1992. **189**(2): p. 695-714.

52. McCune, J.M., et al., *Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus.* Cell, 1988. **53**(1): p. 55-67.

53. Helseth, E., et al., *Human immunodeficiency virus type 1 gp120 envelope glycoprotein regions important for association with the gp41 transmembrane glycoprotein.* J Virol, 1991. **65**(4): p. 2119-23.

54. Pascual, R., M.R. Moreno, and J. Villalain, *A peptide pertaining to the loop segment of human immunodeficiency virus gp41 binds and interacts with model biomembranes: implications for the fusion mechanism.* J Virol, 2005. **79**(8): p. 5142-52.

55. Murakami, T. and E.O. Freed, *The long cytoplasmic tail of gp41 is required in a cell type-dependent manner for HIV-1 envelope glycoprotein incorporation into virions.* Proc Natl Acad Sci U S A, 2000. **97**(1): p. 343-8.

56. Schulz, T.F., et al., *Conserved structural features in the interaction between retroviral surface and transmembrane glycoproteins?* AIDS Res Hum Retroviruses, 1992. **8**(9): p. 1571-80.

57. Sullivan, N., et al., *Effect of amino acid changes in the V1/V2 region of the human immunodeficiency virus type 1 gp120 glycoprotein on subunit association, syncytium formation, and recognition by a neutralizing antibody.* J Virol, 1993. **67**(6): p. 3674-9.

58. Willey, R.L. and M.A. Martin, *Association of human immunodeficiency virus type 1 envelope glycoprotein with particles depends on interactions between the third variable and conserved regions of gp120.* J Virol, 1993. **67**(6): p. 3639-43.

59. Chertova, E., et al., *Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), Is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus.* J Virol, 2002. **76**(11): p. 5315-25.

60. Moore, J.P., et al., *Direct measurement of soluble CD4 binding to human immunodeficiency virus type 1 virions: gp120 dissociation and its implications for virus-cell binding and fusion reactions and their neutralization by soluble CD4.* J Virol, 1991. **65**(3): p. 1133-40.

61. Briggs, J.A., et al., *Structural organization of authentic, mature HIV-1 virions and cores.* EMBO J, 2003. **22**(7): p. 1707-15.

62. Bushman, F.D., T. Fujiwara, and R. Craigie, *Retroviral DNA integration directed by HIV integration protein in vitro.* Science, 1990. **249**(4976): p. 1555-8.

63. Rosen, C.A., J.G. Sodroski, and W.A. Haseltine, *Location of cis-acting regulatory sequences in the human T-cell leukemia virus type I long terminal repeat.* Proc Natl Acad Sci U S A, 1985. **82**(19): p. 6502-6.

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

65. Hoque, M., et al., *HIV-1 replication and latency are regulated by translational control of cyclin T1.* J Mol Biol. **410**(5): p. 917-32.

66. Feinberg, M.B., et al., *HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA.* Cell, 1986. **46**(6): p. 807-17.

67. Sodroski, J., et al., *A second post-transcriptional trans-activator gene required for HTLV-III replication.* Nature, 1986. **321**(6068): p. 412-7.

68. Schwartz, S., et al., *Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1.* J Virol, 1990. **64**(6): p. 2519-29.

69. Malim, M.H., et al., *The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA.* Nature, 1989. **338**(6212): p. 254-7.

70. Ranki, A., et al., *Expression kinetics and subcellular localization of HIV-1 regulatory proteins Nef, Tat and Rev in acutely and chronically infected lymphoid cell lines.* Arch Virol, 1994. **139**(3-4): p. 365-78.

71. Klotman, M.E., et al., *Kinetics of expression of multiply spliced RNA in early human immunodeficiency virus type 1 infection of lymphocytes and monocytes.* Proc Natl Acad Sci U S A, 1992. **89**(3): p. 1148.

72. Kim, S.Y., et al., *Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression.* J Virol, 1989. **63**(9): p. 3708-13.

73. Harrich, D. and B. Hooker, *Mechanistic aspects of HIV-1 reverse transcription initiation.* Rev Med Virol, 2002. **12**(1): p. 31-45.

74. Clavel, F., et al., *Genetic recombination of human immunodeficiency virus.* J Virol, 1989. **63**(3): p. 1455-9.

75. Roth, M.J., P.L. Schwartzberg, and S.P. Goff, *Structure of the termini of DNA intermediates in the integration of retroviral DNA: dependence on IN function and terminal DNA sequence.* Cell, 1989. **58**(1): p. 47-54.

76. Sen, R. and D. Baltimore, *Multiple nuclear factors interact with the immunoglobulin enhancer sequences.* Cell, 1986. **46**(5): p. 705-16.

77. Sen, R. and D. Baltimore, *Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism.* Cell, 1986. **47**(6): p. 921-8.

78. Gottlinger, H.G., *The HIV-1 assembly machine.* AIDS, 2001. **15 Suppl 5**: p. S13-20.

79. Rosenberg, E.S., et al., *Immune control of HIV-1 after early treatment of acute infection.* Nature, 2000. **407**(6803): p. 523-6.

80. Lyles, R.H., et al., *Natural history of human immunodeficiency virus type 1 viremia after seroconversion and proximal to AIDS in a large cohort of homosexual men. Multicenter AIDS Cohort Study.* J Infect Dis, 2000. **181**(3): p. 872-80.

81. Koup, R.A., et al., *Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome.* J Virol, 1994. **68**(7): p. 4650-5.

82. Borrow, P., et al., *Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection.* J Virol, 1994. **68**(9): p. 6103-10.

83. Schmitz, J.E., et al., *Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes.* Science, 1999. **283**(5403): p. 857-60.

84. Ogg, G.S., et al., *Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA.* Science, 1998. **279**(5359): p. 2103-6.

85. Jin, X., et al., *Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques.* J Exp Med, 1999. **189**(6): p. 991-8.

86. Leslie, A.J., et al., *HIV evolution: CTL escape mutation and reversion after transmission.* Nat Med, 2004. **10**(3): p. 282-9.

87. Geels, M.J., et al., *CTL escape and increased viremia irrespective of HIV-specific CD4+ T-helper responses in two HIV-infected individuals.* Virology, 2006. **345**(1): p. 209-19.

88. Goulder, P.J., et al., *Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS.* Nat Med, 1997. **3**(2): p. 212-7.

89. Karlsson, A.C., et al., *Sequential broadening of CTL responses in early HIV-1 infection is associated with viral escape.* PLoS One, 2007. **2**(2): p. e225.

90. Price, D.A., et al., *Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection.* Proc Natl Acad Sci U S A, 1997. **94**(5): p. 1890-5.

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

92. Carrington, M., et al., *HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage.* Science, 1999. **283**(5408): p. 1748-52.

93. Kiepiela, P., et al., *Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA.* Nature, 2004. **432**(7018): p. 769-75.

94. Dalmasso, C., et al., *Distinct genetic loci control plasma HIV-RNA and cellular HIV-DNA levels in HIV-1 infection: the ANRS Genome Wide Association 01 study.* PLoS One, 2008. **3**(12): p. e3907.

95. Fellay, J., et al., *Common genetic variation and the control of HIV-1 in humans.* PLoS Genet, 2009. **5**(12): p. e1000791.

96. Fellay, J., et al., *A whole-genome association study of major determinants for host control of HIV-1.* Science, 2007. **317**(5840): p. 944-7.

97. Pelak, K., et al., *Host determinants of HIV-1 control in African Americans.* J Infect Dis. **201**(8): p. 1141-9.

98. Limou, S., et al., *Genomewide association study of an AIDS-nonprogression cohort emphasizes the role played by HLA genes (ANRS Genomewide Association Study 02).* J Infect Dis, 2009. **199**(3): p. 419-26.

99. Grabar, S., et al., *Prevalence and comparative characteristics of long-term nonprogressors and HIV controller patients in the French Hospital Database on HIV.* AIDS, 2009. **23**(9): p. 1163-9.

100. Bangham, C.R., *CTL quality and the control of human retroviral infections.* Eur J Immunol, 2009. **39**(7): p. 1700-12.

101. Koup, R.A. and D.D. Ho, *Shutting down HIV.* Nature, 1994. **370**(6489): p. 416.

102. Gallo, R., et al., *HIV/HTLV gene nomenclature.* Nature, 1988. **333**(6173): p. 504.

103. Hammes, S.R., et al., *Nef protein of human immunodeficiency virus type 1: evidence against its role as a transcriptional inhibitor.* Proc Natl Acad Sci U S A, 1989. **86**(23): p. 9549-53.

104. Miller, M.D., M.B. Feinberg, and W.C. Greene, *The HIV-1 nef gene acts as a positive viral infectivity factor.* Trends Microbiol, 1994. **2**(8): p. 294-8.

105. Miller, M.D., et al., *The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages.* J Exp Med, 1994. **179**(1): p. 101-13.

106. Spina, C.A., et al., *The importance of nef in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes.* J Exp Med, 1994. **179**(1): p. 115-23.

107. Cao, Y., et al., *Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection.* N Engl J Med, 1995. **332**(4): p. 201-8.

108. Learmont, J., et al., *Long-term symptomless HIV-1 infection in recipients of blood products from a single donor.* Lancet, 1992. **340**(8824): p. 863-7.

109. Pantaleo, G., et al., *Studies in subjects with long-term nonprogressive human immunodeficiency virus infection.* N Engl J Med, 1995. **332**(4): p. 209-16.

110. Deacon, N.J., et al., *Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients.* Science, 1995. **270**(5238): p. 988-91.

111. Birch, M.R., et al., *An examination of signs of disease progression in survivors of the Sydney Blood Bank Cohort (SBBC).* J Clin Virol, 2001. **22**(3): p. 263-70.

112. Gorry, P.R., et al., *Replication-dependent pathogenicity of attenuated nef-deleted HIV-1 in vivo.* J Acquir Immune Defic Syndr, 2007. **46**(4): p. 390-4.

113. Learmont, J.C., et al., *Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1. A report from the Sydney Blood Bank Cohort.* N Engl J Med, 1999. **340**(22): p. 1715-22.

114. Rhodes, D., et al., *Identification of a new recipient in the Sydney Blood Bank Cohort: a long-term HIV type 1-infected seroindeterminate individual.* AIDS Res Hum Retroviruses, 1999. **15**(16): p. 1433-9.

115. Kirchhoff, F., et al., *Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection.* N Engl J Med, 1995. **332**(4): p. 228-32.

116. Salvi, R., et al., *Grossly defective nef gene sequences in a human immunodeficiency virus type 1-seropositive long-term nonprogressor.* J Virol, 1998. **72**(5): p. 3646-57.

117. Tobiume, M., et al., *Inefficient enhancement of viral infectivity and CD4 downregulation by human immunodeficiency virus type 1 Nef from Japanese long-term nonprogressors.* J Virol, 2002. **76**(12): p. 5959-65.

118. Kestler, H.W., 3rd, et al., *Importance of the nef gene for maintenance of high virus loads and for development of AIDS.* Cell, 1991. **65**(4): p. 651-62.

119. Whatmore, A.M., et al., *Repair and evolution of nef in vivo modulates simian immunodeficiency virus virulence.* J Virol, 1995. **69**(8): p. 5117-23.

120. Foster, J.L. and J.V. Garcia, *HIV-1 Nef: at the crossroads.* Retrovirology, 2008. **5**: p. 84.

121. Joseph, A.M., M. Kumar, and D. Mitra, *Nef: "necessary and enforcing factor" in HIV infection.* Curr HIV Res, 2005. **3**(1): p. 87-94.

122. Roeth, J.F. and K.L. Collins, *Human immunodeficiency virus type 1 Nef: adapting to intracellular trafficking pathways.* Microbiol Mol Biol Rev, 2006. **70**(2): p. 548-63.

123. Kirchhoff, F., et al., *Role of Nef in primate lentiviral immunopathogenesis.* Cell Mol Life Sci, 2008. **65**(17): p. 2621-36.

124. Foster, J.L., et al., *Mechanisms of HIV-1 Nef function and intracellular signaling.* J Neuroimmune Pharmacol. **6**(2): p. 230-46.

125. Schwartz, O., et al., *Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein.* Nat Med, 1996. **2**(3): p. 338-42.

126. Swann, S.A., et al., *HIV-1 Nef blocks transport of MHC class I molecules to the cell surface via a PI 3-kinase-dependent pathway.* Virology, 2001. **282**(2): p. 267-77.

127. Le Gall, S., et al., *Nef interacts with the mu subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules.* Immunity, 1998. **8**(4): p. 483-95.

128. Yang, O.O., et al., *Efficient lysis of human immunodeficiency virus type 1-infected cells by cytotoxic T lymphocytes.* J Virol, 1996. **70**(9): p. 5799-806.

129. Collins, K.L., et al., *HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes.* Nature, 1998. **391**(6665): p. 397-401.

130. Yang, O.O., et al., *Nef-mediated resistance of human immunodeficiency virus type 1 to antiviral cytotoxic T lymphocytes.* J Virol, 2002. **76**(4): p. 1626-31.

131. Tomiyama, H., et al., *Different effects of Nef-mediated HLA class I down-regulation on human immunodeficiency virus type 1-specific CD8(+) T-cell cytolytic activity and cytokine production.* J Virol, 2002. **76**(15): p. 7535-43.

132. Ali, A., et al., *Evasion of cytotoxic T lymphocytes is a functional constraint maintaining HIV-1 Nef expression.* Eur J Immunol, 2005. **35**(11): p. 3221-8.

133. Swigut, T., et al., *Impact of Nef-mediated downregulation of major histocompatibility complex class I on immune response to simian immunodeficiency virus.* J Virol, 2004. **78**(23): p. 13335-44.

134. Munch, J., et al., *Efficient class I major histocompatibility complex down-regulation by simian immunodeficiency virus Nef is associated with a strong selective advantage in infected rhesus macaques.* J Virol, 2001. **75**(21): p. 10532-6.

135. Lewis, M.J., et al., *Functional adaptation of Nef to the immune milieu of HIV-1 infection in vivo.* J Immunol, 2008. **180**(6): p. 4075-81.

136. Hanna, Z., et al., *In vivo mutational analysis of the N-terminal region of HIV-1 Nef reveals critical motifs for the development of an AIDS-like disease in CD4C/HIV transgenic mice.* Virology, 2004. **327**(2): p. 273-86.

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

138. Aldrovandi, G.M., et al., *Regions of human immunodeficiency virus type 1 nef required for function in vivo.* J Virol, 1998. **72**(9): p. 7032-9.

139. Franchini, G., et al., *Expression of the protein encoded by the 3' open reading frame of human T-cell lymphotropic virus type III in bacteria: demonstration of its immunoreactivity with human sera.* Proc Natl Acad Sci U S A, 1986. **83**(14): p. 5282-5.

140. Williams, M., et al., *Direct binding of human immunodeficiency virus type 1 Nef to the major histocompatibility complex class I (MHC-I) cytoplasmic tail disrupts MHC-I trafficking.* J Virol, 2002. **76**(23): p. 12173-84.

141. Kasper, M.R. and K.L. Collins, *Nef-mediated disruption of HLA-A2 transport to the cell surface in T cells.* J Virol, 2003. **77**(5): p. 3041-9.

142. Roeth, J.F., et al., *HIV-1 Nef disrupts MHC-I trafficking by recruiting AP-1 to the MHC-I cytoplasmic tail.* J Cell Biol, 2004. **167**(5): p. 903-13.

143. Kasper, M.R., et al., *HIV-1 Nef disrupts antigen presentation early in the secretory pathway.* J Biol Chem, 2005. **280**(13): p. 12840-8.

144. Lubben, N.B., et al., *HIV-1 Nef-induced down-regulation of MHC class I requires AP-1 and clathrin but not PACS-1 and is impeded by AP-2.* Mol Biol Cell, 2007. **18**(9): p. 3351-65.

145. Cohen, G.B., et al., *The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells.* Immunity, 1999. **10**(6): p. 661-71.

146. Williams, M., et al., *Human immunodeficiency virus type 1 Nef domains required for disruption of major histocompatibility complex class I trafficking are also necessary for coprecipitation of Nef with HLA-A2.* J Virol, 2005. **79**(1): p. 632-6.

147. Schaefer, M.R., et al., *HIV-1 Nef targets MHC-I and CD4 for degradation via a final common beta-COP-dependent pathway in T cells.* PLoS Pathog, 2008. **4**(8): p. e1000131.

148. Lanier, L.L., *NK cell recognition.* Annu Rev Immunol, 2005. **23**: p. 225-74.

149. Yokoyama, W.M., *Natural killer cell receptors.* Curr Opin Immunol, 1995. **7**(1): p. 110-20.

150. Ali, A., et al., *Impacts of epitope expression kinetics and class I downregulation on the antiviral activity of human immunodeficiency virus type 1-specific cytotoxic T lymphocytes.* J Virol, 2004. **78**(2): p. 561-7.

151. van Baalen, C.A., et al., *Impact of antigen expression kinetics on the effectiveness of HIV-specific cytotoxic T lymphocytes.* Eur J Immunol, 2002. **32**(9): p. 2644-52.

152. Sacha, J.B., et al., *Gag-specific CD8+ T lymphocytes recognize infected cells before AIDS-virus integration and viral protein expression.* J Immunol, 2007. **178**(5): p. 2746-54.

153. Sacha, J.B., et al., *Pol-specific CD8+ T cells recognize simian immunodeficiency virus-infected cells prior to Nef-mediated major histocompatibility complex class I downregulation.* J Virol, 2007. **81**(21): p. 11703-12.

154. Walk, S.F., et al., *Design and use of an inducibly activated human immunodeficiency virus type 1 Nef to study immune modulation.* J Virol, 2001. **75**(2): p. 834-43.

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

156. Derby, M., et al., *High-avidity CTL exploit two complementary mechanisms to provide better protection against viral infection than low-avidity CTL.* J Immunol, 2001. **166**(3): p. 1690-7.

157. Bihl, F., et al., *Impact of HLA-B alleles, epitope binding affinity, functional avidity, and viral coinfection on the immunodominance of virus-specific CTL responses.* J Immunol, 2006. **176**(7): p. 4094-101.

158. Dzutsev, A.H., et al., *Avidity of CD8 T cells sharpens immunodominance.* Int Immunol, 2007. **19**(4): p. 497-507.

159. Yang, O.O., et al., *Impacts of avidity and specificity on the antiviral efficiency of HIV-1-specific CTL.* J Immunol, 2003. **171**(7): p. 3718-24.

160. Chen, H., et al., *Differential neutralization of human immunodeficiency virus (HIV) replication in autologous CD4 T cells by HIV-specific cytotoxic T lymphocytes.* J Virol, 2009. **83**(7): p. 3138-49.

161. Bennett, M.S., et al., *Epitope-dependent avidity thresholds for cytotoxic T-lymphocyte clearance of virus-infected cells.* J Virol, 2007. **81**(10): p. 4973-80.

162. Vojnov, L., et al., *Effective simian immunodeficiency virus-specific CD8+ T cells lack an easily detectable, shared characteristic.* J Virol. **84**(2): p. 753-64.

163. Riviere, Y., et al., *Gag-specific cytotoxic responses to HIV type 1 are associated with a decreased risk of progression to AIDS-related complex or AIDS.* AIDS Res Hum Retroviruses, 1995. **11**(8): p. 903-7.

164. Kiepiela, P., et al., *CD8+ T-cell responses to different HIV proteins have discordant associations with viral load.* Nat Med, 2007. **13**(1): p. 46-53.

165. Rolland, M., et al., *Broad and Gag-biased HIV-1 epitope repertoires are associated with lower viral loads.* PLoS ONE, 2008. **3**(1): p. e1424.

166. Masemola, A., et al., *Hierarchical targeting of subtype C human immunodeficiency virus type 1 proteins by CD8+ T cells: correlation with viral load.* J Virol, 2004. **78**(7): p. 3233-43.

167. Klein, M.R., et al., *Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics.* J Exp Med, 1995. **181**(4): p. 1365-72.

168. Edwards, B.H., et al., *Magnitude of functional CD8+ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma.* J Virol, 2002. **76**(5): p. 2298-305.

169. Novitsky, V., et al., *Association between virus-specific T-cell responses and plasma viral load in human immunodeficiency virus type 1 subtype C infection.* J Virol, 2003. **77**(2): p. 882-90.

170. Frater, A.J., et al., *Effective T-cell responses select human immunodeficiency virus mutants and slow disease progression.* J Virol, 2007. **81**(12): p. 6742-51.

171. Matthews, P.C., et al., *Central role of reverting mutations in HLA associations with human immunodeficiency virus set point.* J Virol, 2008. **82**(17): p. 8548-59.

172. Miura, T., et al., *HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte [corrected] recognition.* J Virol, 2009. **83**(6): p. 2743-55.

173. Berger, C.T., et al., *High-functional-avidity cytotoxic T lymphocyte responses to HLA-B-restricted Gag-derived epitopes associated with relative HIV control.* J Virol. **85**(18): p. 9334-45.

174. Migueles, S.A., et al., *HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors.* Proc Natl Acad Sci U S A, 2000. **97**(6): p. 2709-14.

175. Pereyra, F., et al., *Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy.* J Infect Dis, 2008. **197**(4): p. 563-71.

176. Kaslow, R.A., et al., *Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection.* Nat Med, 1996. **2**(4): p. 405-11.

177. Gao, X., et al., *AIDS restriction HLA allotypes target distinct intervals of HIV-1 pathogenesis.* Nat Med, 2005. **11**(12): p. 1290-2.

178. Altfeld, M., et al., *HLA Alleles Associated with Delayed Progression to AIDS Contribute Strongly to the Initial CD8(+) T Cell Response against HIV-1.* PLoS Med, 2006. **3**(10): p. e403.

179. Martinez-Picado, J., et al., *Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1.* J Virol, 2006. **80**(7): p. 3617-23.

180. Brockman, M.A., et al., *Escape and compensation from early HLA-B57-mediated cytotoxic T-lymphocyte pressure on human immunodeficiency virus type 1 Gag alter capsid interactions with cyclophilin A.* J Virol, 2007. **81**(22): p. 12608-18.

181. Navis, M., et al., *Viral replication capacity as a correlate of HLA B57/B5801-associated nonprogressive HIV-1 infection.* J Immunol, 2007. **179**(5): p. 3133-43.

182. Crawford, H., et al., *Compensatory mutation partially restores fitness and delays reversion of escape mutation within the immunodominant HLA-B*5703-restricted Gag epitope in chronic human immunodeficiency virus type 1 infection.* J Virol, 2007. **81**(15): p. 8346-51.

183. Kosmrlj, A., et al., *Effects of thymic selection of the T-cell repertoire on HLA class I-associated control of HIV infection.* Nature. **465**(7296): p. 350-4.

184. Hendel, H., et al., *New class I and II HLA alleles strongly associated with opposite patterns of progression to AIDS.* J Immunol, 1999. **162**(11): p. 6942-6.

185. Ammaranond, P., et al., *A new variant cytotoxic T lymphocyte escape mutation in HLA-B27-positive individuals infected with HIV type 1.* AIDS Res Hum Retroviruses, 2005. **21**(5): p. 395-7.

186. Nixon, D.F., et al., *HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides.* Nature, 1988. **336**(6198): p. 484-7.

187. Feeney, M.E., et al., *Immune escape precedes breakthrough human immunodeficiency virus type 1 viremia and broadening of the cytotoxic T-lymphocyte response in an HLA-B27-positive long-term-nonprogressing child.* J Virol, 2004. **78**(16): p. 8927-30.

188. Schneidewind, A., et al., *Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication.* J Virol, 2007. **81**(22): p. 12382-93.

189. Kelleher, A.D., et al., *Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses.* J Exp Med, 2001. **193**(3): p. 375-86.

190. Stranger, B.E., et al., *Genome-wide associations of gene expression variation in humans.* PLoS Genet, 2005. **1**(6): p. e78.

191. Thomas, R., et al., *HLA-C cell surface expression and control of HIV/AIDS correlate with a variant upstream of HLA-C.* Nat Genet, 2009. **41**(12): p. 1290-4.

192. Migueles, S.A., et al., *The differential ability of HLA B*5701+ long-term nonprogressors and progressors to restrict human immunodeficiency virus replication is not caused by loss of recognition of autologous viral gag sequences.* J Virol, 2003. **77**(12): p. 6889-98.

193. Salter, R.D., D.N. Howell, and P. Cresswell, *Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids.* Immunogenetics, 1985. **21**(3): p. 235-46.

194. Adnan, S., et al., *Nef interference with HIV-1-specific CTL antiviral activity is epitope specific.* Blood, 2006. **108**(10): p. 3414-9.

195. Yang, O.O., et al., *Suppression of human immunodeficiency virus type 1 replication by CD8+ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms.* J Virol, 1997. **71**(4): p. 3120-8.

196. Yang, O.O., et al., *Determinant of HIV-1 mutational escape from cytotoxic T lymphocytes.* J Exp Med, 2003. **197**(10): p. 1365-75.

197. Ali, A., B.D. Jamieson, and O.O. Yang, *Half-genome human immunodeficiency virus type 1 constructs for rapid production of reporter viruses.* J Virol Methods, 2003. **110**(2): p. 137-42.

198. Akari, H., et al., *Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation.* J Virol, 2000. **74**(6): p. 2907-12.

199. Olshevsky, U., et al., *Identification of individual human immunodeficiency virus type 1 gp120 amino acids important for CD4 receptor binding.* J Virol, 1990. **64**(12): p. 5701-7.

200. Freed, E.O., D.J. Myers, and R. Risser, *Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41.* Proc Natl Acad Sci U S A, 1990. **87**(12): p. 4650-4.

201. Freed, E.O. and M.A. Martin, *Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions.* J Virol, 1996. **70**(1): p. 341-51.

202. Follenzi, A. and L. Naldini, *Generation of HIV-1 derived lentiviral vectors.* Methods Enzymol, 2002. **346**: p. 454-65.

203. An, D.S., et al., *An inducible human immunodeficiency virus type 1 (HIV-1) vector which effectively suppresses HIV-1 replication.* J Virol, 1999. **73**(9): p. 7671-7.

204. Burns, J.C., et al., *Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells.* Proc Natl Acad Sci U S A, 1993. **90**(17): p. 8033-7.

205. Tomiyama, H., et al., *Cutting Edge: Epitope-dependent effect of Nef-mediated HLA class I down-regulation on ability of HIV-1-specific CTLs to suppress HIV-1 replication.* J Immunol, 2005. **174**(1): p. 36-40.

206. Almeida, J.R., et al., *Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover.* J Exp Med, 2007. **204**(10): p. 2473-85.

207. Payne, R.P., et al., *Efficacious early antiviral activity of HIV Gag- and Pol-specific HLA-B 2705-restricted CD8+ T cells.* J Virol. **84**(20): p. 10543-57.

208. Boutwell, C.L., C.F. Rowley, and M. Essex, *Reduced viral replication capacity of human immunodeficiency virus type 1 subtype C caused by cytotoxic-T-lymphocyte escape mutations in HLA-B57 epitopes of capsid protein.* J Virol, 2009. **83**(6): p. 2460-8.

209. De Boer, R.J., R.M. Ribeiro, and A.S. Perelson, *Current estimates for HIV-1 production imply rapid viral clearance in lymphoid tissues.* PLoS Comput Biol. **6**(9): p. e1000906.

210. Thomas, J.A., D.E. Ott, and R.J. Gorelick, *Efficiency of human immunodeficiency virus type 1 postentry infection processes: evidence against disproportionate numbers of defective virions.* J Virol, 2007. **81**(8): p. 4367-70.

211. Grossman, Z., et al., *Pathogenesis of HIV infection: what the virus spares is as important as what it destroys.* Nat Med, 2006. **12**(3): p. 289-95.

212. Sigal, A., et al., *Cell-to-cell spread of HIV permits ongoing replication despite antiretroviral therapy.* Nature. **477**(7362): p. 95-8.

213. Altfeld, M., et al., *Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection.* AIDS, 2003. **17**(18): p. 2581-2591.

214. Lazaro, E., et al., *Variable HIV peptide stability in human cytosol is critical to epitope presentation and immune escape.* J Clin Invest. **121**(6): p. 2480-92.

215. Sacha, J.B., et al., *Simian immunodeficiency virus-specific CD8+ T cells recognize Vpr- and Rev-derived epitopes early after infection.* J Virol. **84**(20): p. 10907-12.

216. Sun, X., et al., *Role of clathrin-mediated endocytosis during vesicular stomatitis virus entry into host cells.* Virology, 2005. **338**(1): p. 53-60.

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

218. Zuniga, R., et al., *Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control.* J Virol, 2006. **80**(6): p. 3122-5.

219. Cohen, W.M., et al., *Study of Antigen-Processing Steps Reveals Preferences Explaining Differential Biological Outcomes of Two HLA-A2-Restricted Immunodominant Epitopes from Human Immunodeficiency Virus Type 1.* Journal of Virology, 2002. **76**(20): p. 10219-10225.

220. Tenzer, S., et al., *Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance.* Nat Immunol, 2009. **10**(6): p. 636-46.

221. Appay, V., D.C. Douek, and D.A. Price, *CD8+ T cell efficacy in vaccination and disease.* Nat Med, 2008. **14**(6): p. 623-8.

222. Turner, S.J., et al., *Structural determinants of T-cell receptor bias in immunity.* Nat Rev Immunol, 2006. **6**(12): p. 883-94.

223. Venturi, V., et al., *The molecular basis for public T-cell responses?* Nat Rev Immunol, 2008. **8**(3): p. 231-8.

224. Price, D.A., et al., *T cell receptor recognition motifs govern immune escape patterns in acute SIV infection.* Immunity, 2004. **21**(6): p. 793-803.

225. Price, D.A., et al., *Public clonotype usage identifies protective Gag-specific CD8+ T cell responses in SIV infection.* J Exp Med, 2009. **206**(4): p. 923-36.

226. Menezes, J.S., et al., *A public T cell clonotype within a heterogeneous autoreactive repertoire is dominant in driving EAE.* J Clin Invest, 2007. **117**(8): p. 2176-85.

227. Brumme, Z.L., et al., *HLA-associated immune escape pathways in HIV-1 subtype B Gag, Pol and Nef proteins.* PLoS One, 2009. **4**(8): p. e6687.