Analysis of epitope-specific HIV $CD8^+$ T cell responses elicited during early HIV-1 infection and their association with viral control

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Abstract

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The enormity of global human immunodeficiency virus type 1 (HIV)/acquired immunodeficiency syndrome (AIDS) pandemic underscores the urgency to develop a safe, effective and accessible prophylactic AIDS vaccine. Multiple lines of evidence in humans and animal models have shown that HIV-1-specific $CD8^+$ cytotoxic T lymphocytes (CTL) are important in controlling and preventing HIV infection. However, the precise qualities of effective epitope-specific $CD8^+$ CTL responses that may be responsible for control remain unclear. Several vaccine strategies have been designed to elicit $CD8^+$ T cell responses against HIV. Previous T cell based vaccine candidates that have failed to offer protection and HIV control primarily induced HIV-1-specific T cells that targeted variable regions of HIV-1. Genetic studies have shown an association between specific human leukocyte

antigens (HLAs), (notably HLA-B*27 and -B*57 allele groups) and slower rates of disease progression in the absence of anti-retroviral therapy (ART). HIV-1-specific $CD8^+$ T cells restricted by these HLA alleles are dominant early in infection in individuals expressing these alleles, and predominantly target conserved regions of Gag. These data suggest that an effective T-cell based immunogen should contain conserved regions of HIV-1 as it will increase the likelihood that $CD8^+$ T cells will recognize incoming viral species of diverse clades and decrease the likelihood of rapid escape variants against the recognized epitopes. We extend these observations to comprehensively identify all $CD8^+$ T cell responses that are elicited during early infection. The central goal of this dissertation is to determine if conservation of the epitopes targeted during early HIV-1-infection play an important role on viral control. Here we demonstrate that individuals possessing $CD8^+$ T cells recognizing conserved epitopes of the virus have lower viral load set point than those recognizing only variable epitopes. Collectively, our results imply that the next-generation of T cell based vaccines should focus on strategies that can induce $CD8^+$ T cell responses specifically to conserved regions of HIV-1.

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Abbreviations

AA = amino acid
AIDS = acquired immunodeficiency syndrome
APC = antigen presenting cells
ART = anti-retroviral treatment
ARV = AIDS-associated retrovirus

bCSp = clade-B based prevalence conservation score
bCSe = clade-B based Shannon Entropy conservation score
BLCL = Epstein-Barr Virus (EBV)-transformed B-cell lymphoblastoid cell lines

CD = cluster of differentiation
CTL = cytotoxic T lymphocytes
CRF = circulating recombinant form
CS = conservation score

DPI = days-post-infection
DRiPs = defective ribosomal products

EC50 = half maximal effective concentration
ENV = envelope
ES = entropy score
ELISPOT = Enzyme-linked immunosorbent spot

GRID = Gay Related Immunodeficiency disease
Group-M = group major

HEPS = highly-exposed but persistently seronegative
HIV = human immunodeficiency virus
HLA = human leukocyte antigen
HTLV-III = human T-cell lymphotropic virus type III

IDAV = immunodeficiency-associated virus
IFN = interferon
IL = interleukin

KW = Kruskal-Wallis

LAV = lymphadenopathy-associated virus
LTNP = Long-term nonprogressors
LANL DB = Los Alamos National Laboratory Database

mCSp = group-M based prevalence conservation score
mCSe = group-M based Shannon Entropy conservation score

MHC = major histocompatibility complex
MSM = men who have sex with men
MW = Mann Whitney

NK cell = natural killer cell

PBMC = peripheral blood mononuclear cells
PTE = potential T cell epitope

RM = rhesus macaques

SIV = simian immunodeficiency virus
SNP = single nucleotide polymorphism
TCR = T cell receptor
T_{CM} = central memory
T_{EM} = effector memory
TNF = tumor necrosis factor

VL = viral load

WSR = Wilcoxon matched-pair signed rank

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patience and understanding of the long hours needed to complete my experiments are much appreciated.

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Dedication

This thesis is dedicated to the memory of my late grandfather, Chandra Singh Kunwar, who taught me value of hard work, dedication and good education, which has guided me to come this far. He sowed the seed for love of science in me and raised me with lots of love and care. He always encouraged me to do the best of my abilities and pursue higher studies. I only wish that he had lived to see this day when I have achieved the highest degree. Also, I dedicate this work to my late father, Shiva Sharan Singh Kunwar, whose memories have inspired and guided me during difficult times. I feel eternally blessed for their presence in my life.

Chapter I: Introduction

Overview

As we enter into the third decade since the first documented cases of Acquired Immunodeficiency Syndrome (AIDS) caused by the Human Immunodeficiency Virus (HIV), this disease continues to be a major public health problem today. An estimated 33.3 million people were living with HIV-1 at the end of 2009. There are approximately 2.6 million new infections per year and 1.8 million people die of AIDS every year.

Anti-retroviral therapy (ART) has proven to be successful in controlling the HIV virus, limiting its transmission and elongating life expectancy of HIV-infected individuals; however, ART is only a short term solution due to adherence challenges including high toxicity, the requirement of daily administration and the fact that treatment cannot eradicate the virus from the host. In addition, the cost associated with treatment is so high that in many developing countries, where resources are scarce, the therapy is unreachable to many who need it the most. Therefore, there is an urgent need to develop a safe, effective and accessible prophylactic vaccine. Even though enormous research efforts and funds have been directed towards the development of a safe and effective HIV-1 vaccine for almost three decades, such a vaccine remains elusive. Critical challenges include the enormous global diversity associated with the virus and the subsequent challenges in eliciting an immune response that includes both broadly neutralizing antibody and cellular immune responses.

Despite a considerable research effort in the area, the qualities that define "protective" HIV-1-specific $CD8^+$ cytotoxic T lymphocytes (CTL) are still unknown, making rationale design of vaccines difficult. Multiple lines of evidence in human and animal models suggest that HIV-1-specific CTL play an important role in the control of HIV-1 infection. $CD8^+$ CTLs recognize infected cells via the recognition of small fragments of viral protein (epitopes), presented on infected cell surfaces by MHC class I molecules. After the initial recognition event CTL release perforin and various granzymes, which are responsible for inducing apoptosis of the target cells thereby eliminating the infected cells. While the majority of infected individuals progress to AIDS within 10 years without antiretroviral therapy, the pace of disease progression is highly variable. Certain MHC class I alleles (e.g., HLA-B*35Px allele groups: B*3502, B*3503, B*3504, and B*5301) are associated with rapid progression, while other alleles (e.g., HLA-B*27, -B*57 allele groups) are associated with slow progression to AIDS. While these studies have provided strong evidence that $CD8^+$ CTL play an important role in immune control of HIV, significant numbers of virus-specific $CD8^+$ CTL are also detectable in individuals who progress to AIDS rapidly, suggesting that not all $CD8^+$ CTL response are created equal, rather they differ significantly in their ability to control viral replication. Although the mechanism(s) underlying this difference are not entirely clear, understanding them is critical for development of prophylactic vaccines.

In this work, we conducted a comprehensive analysis of $CD8^+$ CTL epitopes that are recognized during early HIV-1 infection, and found that individuals who mount HIV-specific CTL recognizing conserved regions of the virus have better initial

control of HIV replication. These data have important implications for future design of T-cell-based vaccines.

HIV Biology and Pathogenesis

History of HIV

In the United States, the first case of Acquired Immune Deficiency Syndrome (AIDS) was formally identified in patients in 1981. A growing number of previously healthy men who have sex with men (MSM) in New York and California suddenly began showing symptoms of rare opportunistic infections that seemed stubbornly resistant to any treatment. This new infection, which was eventually called AIDS, was originally referred by diseases that were associated with it (for e.g. lymphadenopathy, Kaposi's Sarcoma, Opportunistic Infections etc). Surprisingly, a common accompanying feature was profound depletion of $CD4^+$ T cells, causing severe immunodeficiency in these patients. Based on this observation, this infection was designated as GRID (Gay Related Immunodeficiency Disease).

In 1983, the etiological agent of AIDS was isolated from the lymph nodes of a patient suffering from generalized lymphadenopathy of unknown origin [1, 2]. Furthermore, it was revealed that this infectious agent displays many characteristics of retroviruses [1]. Further studies characterized the reverse transcriptase activity [3] as well as cytopathic activity on T lymphocytes [4] associated with this new virus. Subsequent studies involved in the cloning of the virus [5] and the sequencing of its genome [6, 7] confirmed that the virus belonged to the *Lentivirus* genus of the

Retrovirus family. While "*Lentivirus*" literally means "slow virus" because of the extended amount of time it takes for these viruses to produce any adverse effect on the host, "*Retrovirus*" literally means 'backward virus' because of the flow of information from RNA back to DNA occurs. Several isolates of the same virus were called by several different names such as lymphadenopathy-associated virus (LAV), human T-cell lymphotropic virus type III (HTLV-III), immunodeficiency-associated virus (IDAV) and AIDS-associated retrovirus (ARV). The virus was officially designated as human immunodeficiency virus in 1986 (HIV) [8].

The Origin and Diversity of HIV

Based on phylogenetic analyses and genomic organization of HIV, the virus is currently divided into two types: HIV-1 and HIV-2, which are believed to have originated from cross-species transmission events from different primate sources [9]. While HIV-1 is thought to have arisen from chimpanzees (SIV_{CPZ}) and gorillas in West Central Africa, HIV-2 is believed to have originated from sooty mangabeys in West Africa (SIV_{SSM}) [10]. When HIV was first discovered as a retrovirus, it was believed that the genome of HIV would be very similar to retroviruses that were already characterized. However, when proviral DNA sequences of viruses from different places around the world were analyzed, the extraordinary genetic heterogeneity of HIV isolates became apparent [11-13]. Further studies uncovered the existence of extensive sequence diversity within an infected individual suggesting that the virus has an extremely fast rate of evolution [14, 15]. The sequence diversity of HIV-1 between and within individuals is largely due to the

error-prone HIV-1 reverse transcriptase enzyme, which on average introduces one mutational error per replication cycle. In addition, the high *in vivo* viral production rate as well the ability of multiple strains to undergo rapid genetic recombination upon co-infection of a single cell further intensifies the rate at which virus diversifies [16] (Figure 1). Astonishingly, the genetic diversity of HIV sequences found within a single infected person appears to be roughly comparable to the annual diversity of influenza sequences worldwide [17, 18] (Figure 2).

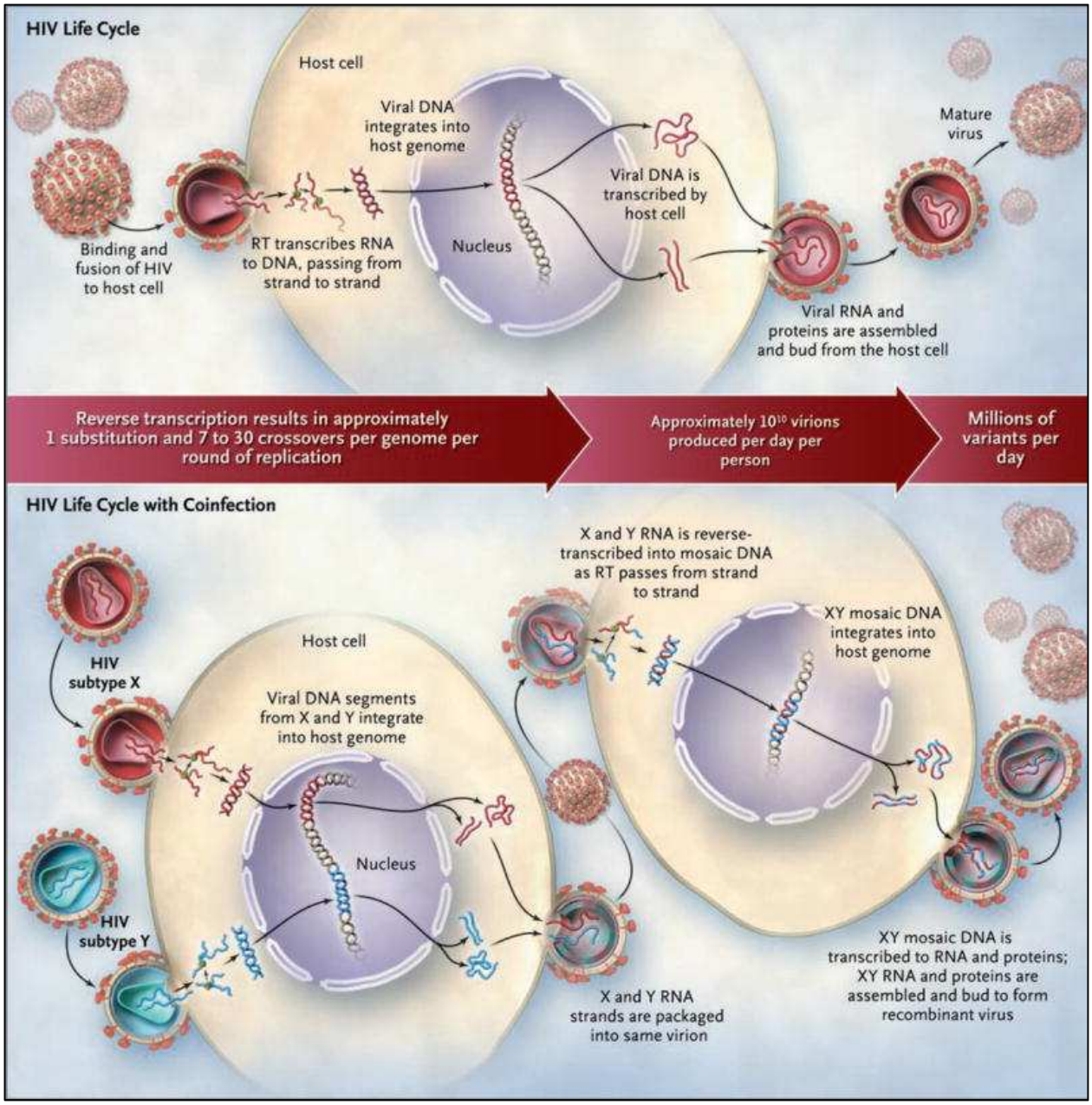


Figure 1: Evolution of genetic diverity of HIV-1 during its typical life cycle. Reproduced with permission from (scientific reference citation), Copyright Massachusetts Medical Society [16].

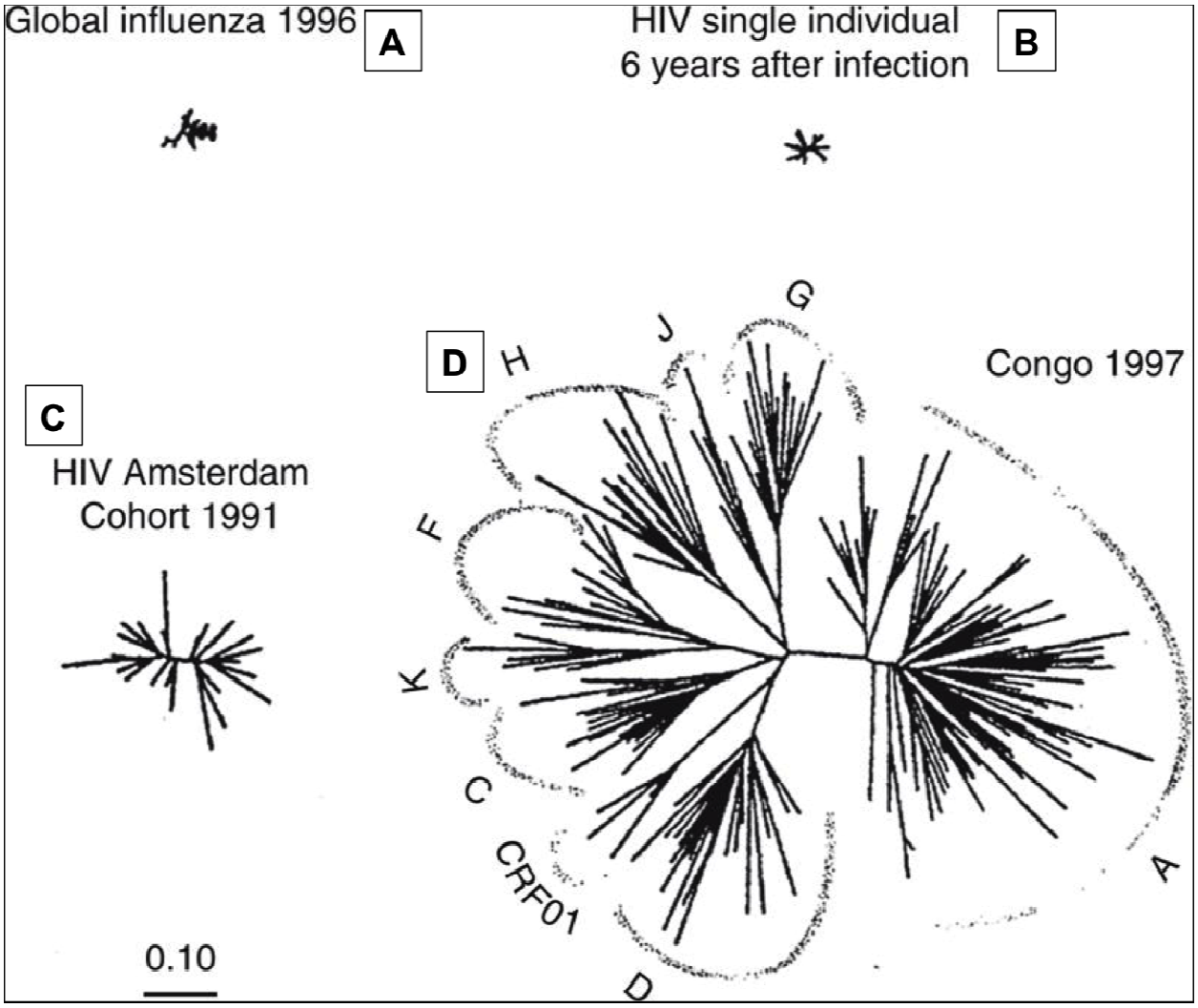


Figure 2: Genetic diversity of HIV-1.
This illustration compares the gentic diversity of (A) the HA gene of seasonal influenza H3N2 in 1 year world wide through phylogenetic analysis to: (B) the gp41 region of HIV-1 envelope gene in a single HIV-infected individuals 6 years after infection, (C) the gp41 region of HIV in an Amsterdam Cohort in 1991, and (D) The HIV-1 V2-C5 sequences sampled in 1997 from individuals infected in the Congo. The scale bar at the bottom shows 10% divergence at the amino acid level. Adapted from AIDS 2012, Thumbi Ndung'u and Robin A. Weiss, On HIV Diversity, Volume No. 26, Page No.1256 Copyright © (2012) Massachusetts Medical Society. Reprinted with permission from Massachusetts Medical Society [18].

Based on HIV-1's remarkable genomic diversity, HIV-1 strains can be divided into four distinct groups which arose from independent cross-species transmission events: M (Major), N (non-M, non-O), O (outlier) and P (putative). While group N is endemic in Cameroon, groups N and P are only described in a handful of cases in Cameroon [19, 20]. However, only group M viruses have spread from Africa, and are responsible for the AIDS pandemic [21, 22]. Figure 3 illustrates the worldwide distribution of HIV-1. Group M are further divided into 9 clades (A-D, F-H and K) and subtypes A and F are subdivided into sub-subtypes, A1-A4, F1 and F2 [23]. Furthermore, recombination between HIV-1 subtypes has resulted in the emergence of numerous circulating recombinant forms (CRFs) [24]. While HIV-1 has spread all over the world, the epidemic of HIV-2 is restricted to West Africa. There are only two main variants of HIV-2, A and B, represented in the HIV-2 epidemic [25-27].

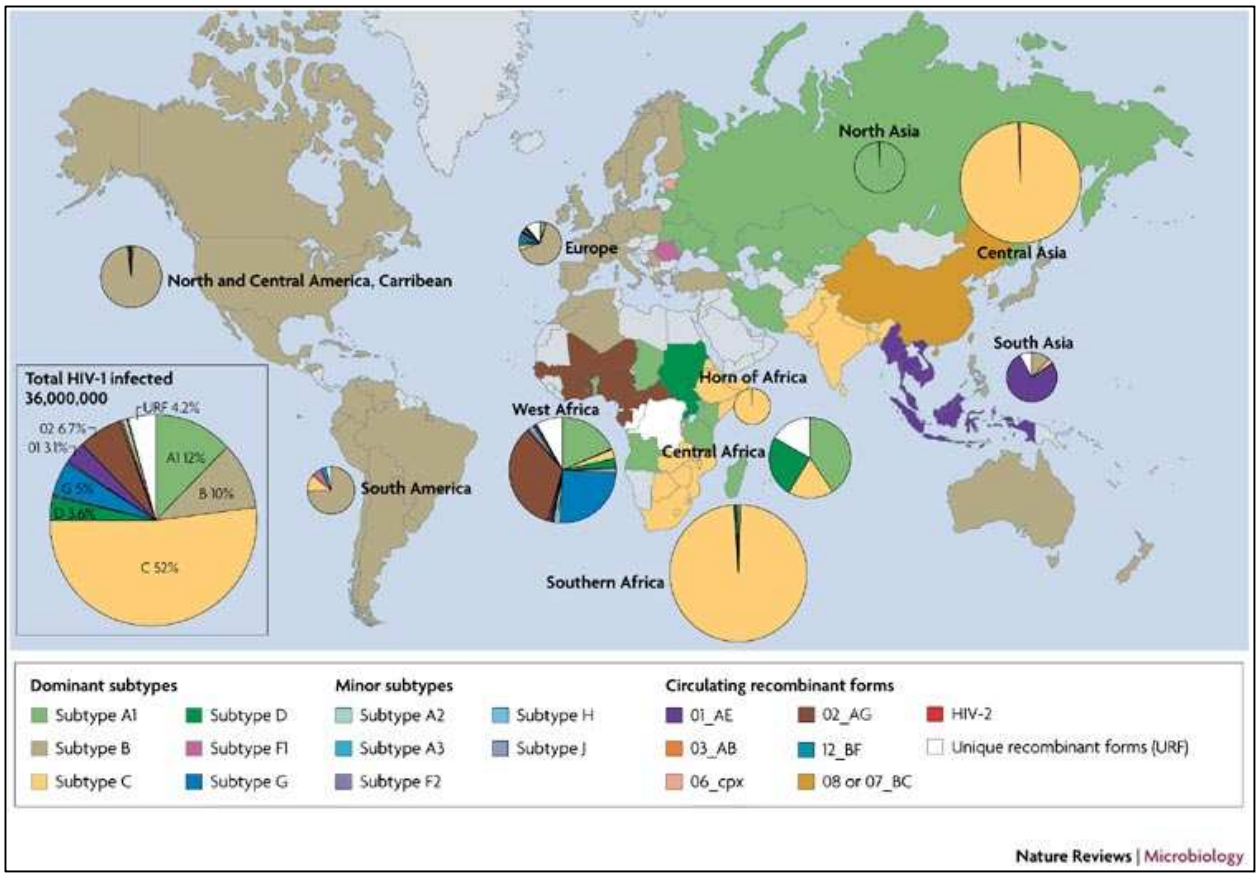


Figure 3: The worldwide diversity and distribution of HIV-1.
The picture depicts the global map based on HIV prevalence per country. The countries are color-coded based on the dominant HIV-1 subtype. The pie charts depict the proportion of each subtype or CRF in each geographical region. The size of the pies is proportional to the number of HIV-1 infected individuals in that particular region. Reprinted by permission from Macmillan Publishers Ltd: Nature, copyright (2007) [23].

HIV-1 Infection and Disease Course

The principal determinant of HIV-1 tropism is the envelope gene of the virus, which encodes the surface glycoprotein-120 and transmembrane glycoprotein-41. Productive HIV-1 infection requires the interaction of glycoprotein-120 with two host membrane proteins, the CD4 glycoprotein as a receptor and CCR5 or CXCR4 as a co-receptor. This interaction triggers a series of conformational changes of glycoprotein-41, leading to fusion of the virus and cell membrane [28, 29]. Both CD4 receptor and CCR5-/CXCR4-co-receptors are expressed on primary $CD4^+$ T cells, macrophages and dendritic cells. Based on co-receptor use, HIV isolates are classified into 3 groups. The isolates that use CCR5 but not CXCR4 are defined as R5-tropic viruses, isolates that use CXCR4 but not CCR5 are defined as X4-tropic viruses, and isolates that can utilize both co-receptors are called R5X4 (dual)-tropic viruses [30, 31]. The differential co-receptor usage is believed to play a critical role in HIV pathogenesis. Viruses generally use CCR5 co-receptor in the majority of primary infections, whereas viruses able to use CXCR4 only emerge at later stages of the HIV-1 infection [32]. The co-receptor CCR5 thus plays a critical role in transmission and early HIV-1 infection. Even after the emergence of X4-tropic viruses, R5-tropic viruses are still present in the majority of individuals [33, 34]. However, the switch from CCR5 to CXCR4 use in HIV-1 infected individuals is associated with increased pathogenicity and rapid progression to AIDS [29, 35]. The relative importance of co-receptors in HIV infection, particularly CCR5, is exemplified by exposed but sero-negative individuals homozygous for a 32-base pair deletion in the CCR5 gene (CCR5Δ32) [36]. CCR5Δ32 is a loss of function mutation that

abrogates the cell surface expression of CCR5 and thereby provides genetic resistance to HIV infection [37]. CCR5Δ32 heterozygosity has been implicated in prolonged AIDS-free survival [38].

In adults, the common route of HIV-1- transmission is through sexual intercourse, use of contaminated needles or syringes, and blood or blood-derived products. In children, transmission is predominantly through mother- to- child (vertical), occurring pre-partum, intra-partum and post-partum. HIV-1 can infect cells both by cell-to-cell and cell-free transmission [39]. The majority of HIV-1-infections are transmitted from a single viral variant [40-42]. Since HIV-1 specifically infects $CD4^+$ T cells, it causes progressive destruction of the immune system, making infected individuals vulnerable to a wide range of opportunistic infections (OI) eventually leading to acquired immunodeficiency syndrome (AIDS). The disease course in the majority of HIV-1 infected individuals can be divided into 3 distinct stages: (1) Acute, (2) Asymptomatic, and (3) AIDS manifestation (Figure 4) [43, 44]. During the acute phase of infection, virus replicates in an uncontrolled manner, reaching a peak of plasma viremia typically over a million particles/mL of plasma in about three weeks. This results in massive depletion of $CD4^+$ T cells in the gut and peripheral blood. The depletion of activated and memory $CCR5^+CD4^+$ T cell located in the gut is more substantial [45, 46]. This depletion never recovers despite years of antiretroviral treatment, which is in contrast to the recovery of peripheral $CD4^+$ T cells under treatment [44]. The initial peak of viremia begins to decline to the set-point viremia (~10^3-10^4 copies/mL of plasma). The level of viremia at the set point has been shown to be inversely correlated to the rate of disease progression[47, 48].

After primary infection, most HIV-1 infected individuals enter a prolonged asymptomatic phase of chronic infection, in which the level of viremia remains relatively stable. The length of the asymptomatic phase is highly variable from patient to patient, on average, it lasts around 10 years. Although, this asymptomatic phase of HIV infection may be clinically latent and patients feel relatively healthy, the virus is continuously replicating, diversifying and destroying the $CD4^+$ T cells directly or indirectly. Eventually, after a decade or more of an untreated HIV infection, the individuals become susceptible to secondary infection, which results from the immune system's failure to fight back other invading pathogens. In this phase, the HIV viral load also appears to spike up. The $CD4^+$ T cells, which play an essential role in immune defenses against the foreign pathogens, are progressively depleted over the course of infection. Over time, HIV-1-infected individuals get susceptible to a variety of opportunistic infections, which ultimately leads to death.

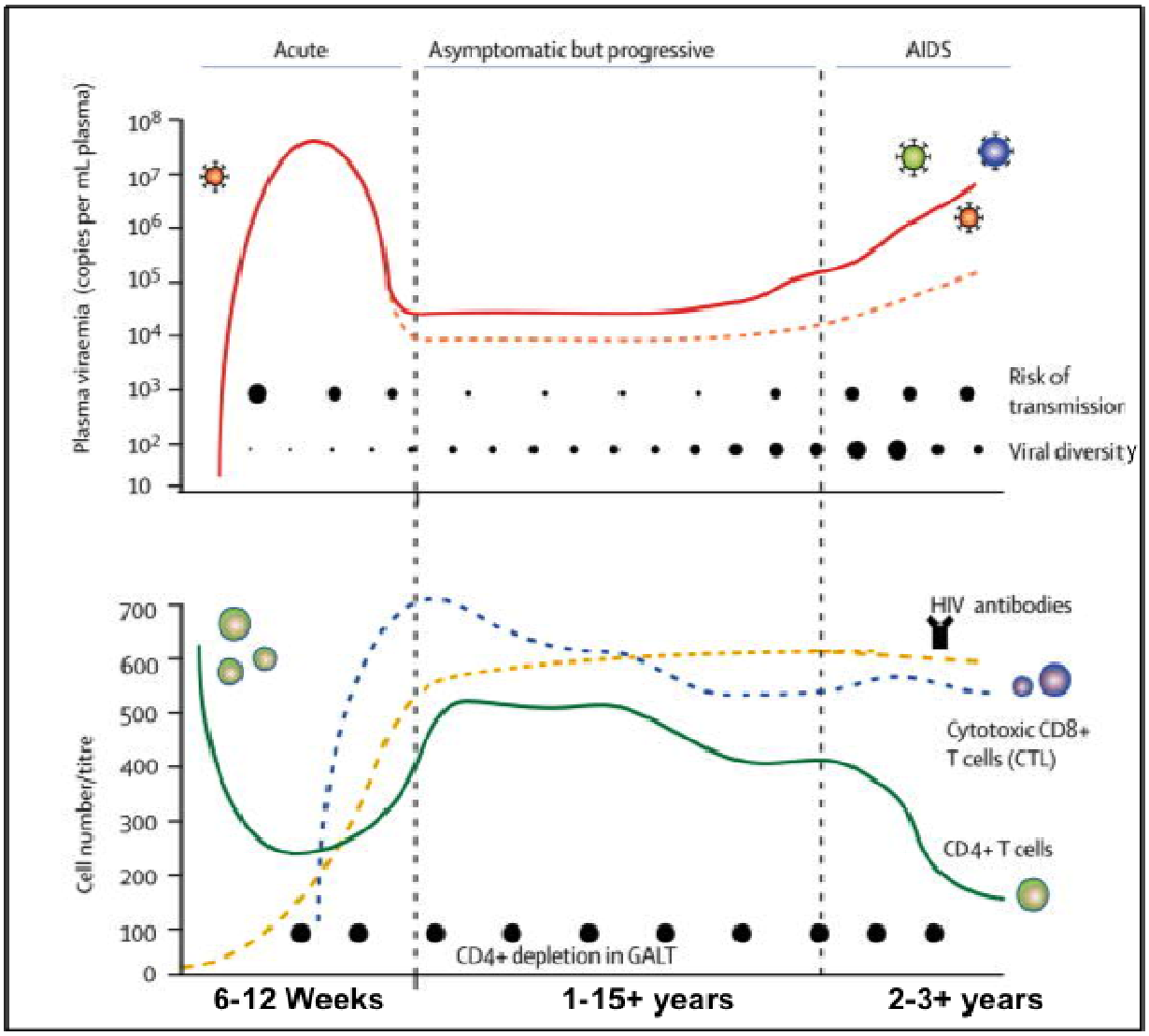


Figure 4: Schematic diagram of course of HIV-1 infection.
The picture depicts the relationship between viral load and CD4+ T cell count over the course of untreated HIV-1 infection, any particular individual's disease course may vary considerabily. The primary HIV-1 infection is characterized by high plasma viremia (red line), low $CD4^+$ T cell count (green). Reprinted by permission from Lancet Publishing Group: Lancet copyright (2006). Adapted from Rowland-Jones, 2003 and Simon et al. 2006 [43, 44].

Biology and Functionality of $CD8^+$ T cells

The CD8 molecule is a transmembrane glycoprotein that serves as a co-receptor primarily for cytotoxic T cells, and as well as for natural killer cells. A functional CD8 is a dimer, either the heterodimer (composed of a CD8-α and CD8-β chain) or the homodimer (composed of a CD8-α and CD8-α chain). $CD8^+$ T cells play an important role in host defense in at least two different ways: they primarily recognize and kill host cells that are either abnormal (e.g. cancer cells) or infected by invading intracellular pathogens (particularly viruses) via their T cell receptor (TCR), and secrete a variety of cytokines and chemokines that directly or indirectly contribute to the suppression of viral replication. All T cells arise from common lymphoid progenitor cells that are originated from a pluripotent hematopoietic stem cell in the bone marrow and migrate through blood to the thymus. In the thymus, developing T cells undergo an extensive selection process of maturation that depends on interactions with thymic cells. During thymic selection T cells that recognize their antigen in context of self-MHC are retained (positive selection) and T cells that recognize self-antigens are deleted (negative selection), allowing for the selection of a functional and self-tolerant mature T-cell repertoire [49, 50].

T Cell Development

Once $CD8^+$ T cells have completed their maturation in the thymus, they migrate through peripheral lymphoid tissues, and sample for host tumor cells or infected cells. These mature recirculating T cells are called naïve T cells, as they have not yet encountered their cognate antigen in the periphery. They are largely quiescent, migrating and non-dividing. The circulation of naïve T cells from the bloodstream into lymph nodes, spleen and mucosa-associated lymphoid tissues and back to blood enables them to make contact with dendritic cells in the lymphoid tissues and sample the peptides presented by MHC complexes on the surfaces of these dendritic cells. The migration of naïve T cells into peripheral lymphoid tissues depends on their binding to high endothelial venules (HEV) through interactions that are non antigen-specific cell-cell interactions governed by cell adhesion molecules (such as selectins, integrins, members of the immunoglobulin superfamily, and mucin-like molecules). Naïve T cells keep circulating through peripheral lymphoid tissues until they recognize their cognate antigen on the surface of mature dendritic cells. Priming of naïve T cells is controlled by three different signals. Signal 1 involves the interaction between the T cell receptor of naïve T cells and their cognate antigen presented by MHC complex (MHC class II for $CD4^+$ and MHC class I for $CD8^+$ T cells respectively) displayed on the surface of activated antigen-presenting cells (APC). Although interaction of the T cell receptor with its cognate antigen is essential for activating naïve T cells, it is not sufficient to induce proliferation and differentiation into effector T cells. Signal 2 (co-stimulatory signal), involves the interaction between CD28 on the T cell and B7 molecules on the

antigen-presenting cells. The net effect of the signal 2 is to increase the survival and proliferation of the T cells that have received signal 1 [50]. Along with signal 1 and 2, the importance of cytokine receptor signaling as a signal 3 for full activation of naïve T cells has been recognized. The requirement of a signal 3 mediated by inflammatory cytokines is considered as a means for CD8 T cells that encounter antigen to determine if there is danger present, and to respond accordingly [51, 52]. Inflammatory cytokines such as IL-12 or type I interferons, which are also transmitted by the APC, are the major sources of signal 3 required for the terminal differentiation of effector $CD8^+$ T cells [53]. The priming of naïve $CD8^+$ T cells generates cytotoxic T cells capable of killing pathogen-infected cells. Once naïve T cells become activated, they stop migrating, start proliferating for several days, and undergo clonal expansion and differentiation that allows for acquisition of effector functionality [54]. This is the first stage towards memory T cell development, which is called the “expansion” phase. These effector cells then recirculate in the bloodstream, through which they migrate to sites of infection. Through the combined abilities of $CD4^+$ and $CD8^+$ effector T cells for their ability to secrete cytokines and kill infected target cells, a typical acute viral infection can be resolved within a days. The main goal of effector T cells is to control the spread of replicating pathogen to uninfected cells and to clear the invading pathogen from the host. During two to three weeks following the peak of $CD8^+$ T cell expansion, the majority (>90%) of the activated effector $CD8^+$ T cells undergoes apoptosis, and this second stage is referred as the “death” phase or “contraction” period. The surviving antigen-specific effector $CD8^+$ T cells enter into the third stage, which is called the “memory” phase

in which a pool of long-lived memory T cells is established and maintained for long periods of time in the absence of antigen [55-57].

Memory T cells are important for future encounter with the same antigen as upon reinfection. Antigen-specific memory $CD8^+$ T cells persist in higher frequencies than naïve T cells and upon reinfection, these cells are able to undergo rapid and robust recall responses generating a large pool of secondary effector T cells [58]. Memory $CD8^+$ T cells can be divided into two subsets with distinct functional and migration properties, central memory (T_{CM}) and effector memory (T_{EM}) [59]. T_{EM} primarily reside in non-lymphoid tissues where they can survive for long periods of time, and upon antigen reencounter, they respond to antigen with immediate effector functions (such as cytokine production and killing), but they are very poor in their ability to proliferate. In contrast, T_{CM} mainly reside in lymphoid organs, and persist for long periods of time and can vigorously proliferate upon antigen reencounter, but exhibit reduced immediate effector function and cytotoxicity [60].

Effector Functions of $CD8^+$ T cell

Effector $CD8^+$ cytotoxic T cells are crucial in host defense against intracellular pathogens. Although these pathogens are susceptible to antibodies before entering into cells, they are not accessible to antibodies once inside cells, therefore they can be only eliminated by the action of $CD8^+$ T cells [50]. Mature $CD8^+$ T cells are very specific for their cognate antigen displayed on professional antigen presenting cells. Their antigen, also known as an epitope, is a 8-11 amino acid long peptides presented by the MHC class I molecule. Most of the peptides that are loaded on

MHC class I molecules are derived from proteins that are degraded in the cytosol by the activity of cytosolic enzyme complex, the proteasomes, which function in normal protein turnover in all cells [61]. A significant proportion of these proteins are derived from defective ribosomal products (DRiPs), which are newly synthesized proteins that fail to fold properly or are defective in some manner and therefore, tagged for degradation [49]. The peptides derived from proteasomal degradation are usually more than 10 amino acids in length and therefore, undergo further trimming before they can fit into an MHC class I molecule. Post-proteasomal trimming occurs with the combined action of a cytosolic resident aminopeptidase or an endoplasmic reticulum (ER) resident peptidase [49, 61].

Antigen-specific cytotoxic $CD8^+$ T cells can induce infected target cells to undergo apoptosis. There are two major mechanisms by which $CD8^+$ T cells kill infected target cells. The efficient way of killing target cells by activated $CD8^+$ T cells is the calcium-dependent release of preformed granules containing granzyme, perforin and granulysin upon recognition of antigen on the surface of a target cell [49, 50]. The second mechanism involves the expression of ligands for death receptors, such as Fas ligand, which is induced on $CD8^+$ T cell upon antigen-specific recognition of an infected target cell and binds to Fas on the infected target cell, inducing the classical caspase-dependent apoptotic pathway [62]. Importantly, inducing apoptosis in target cells prevents the spread of pathogen to uninfected cells, playing a crucial role in controlling intracellular pathogens. Although inducing apoptosis in infected target cells is the main way by which cytotoxic $CD8^+$ T cells eliminate infection, effector and memory $CD8^+$ T cells possess the ability to secrete

various cytokines and chemokines which contribute to host defense in several ways. For example: $CD8^+$ T cells can secrete effector cytokines (like IFN-γ, TNF-α and IL-2), and chemokines (like MIP-1α, MIP-1β and RNATES) that elicit immunomodulatory effects during an active infection. The cytokine IFN-γ is an important cytokine that inhibits viral replication directly, increases antigen presentation of intracellular pathogens by increasing the expression of MHC class I, and stimulates the immunoproteasome and other proteins that are involved in antigen processing [63]. Additionally, IFN-γ signaling has been shown to increase the sensitivity of infected cells to apoptosis [64, 65]. Patients lacking functional IFN-γ receptors (IFN-γR1) demonstrate increased susceptibility to several intracellular pathogens [66]. The cytokine IL-2 is an important growth factor necessary for the proliferation and differentiation of antigen-specific $CD8^+$ T cells [67, 68]. In addition, IL-2 stimulation has been shown to increase perforin expression [67] and promote a robust secondary expansion of memory $CD8^+$ T cells [69]. Finally, TNF-α is a major pro-inflammatory cytokine that amplifies Th1 responses against intracellular invading pathogens by inducing many key cytokines and synergizes with IFN-γ in activating macrophages. TNF-α can also promote pathogen clearance through their interaction with TNF-RI, which induces apoptosis of the infected target cells [50, 70]. It has been shown that mice lacking functional TNF-RI have increased susceptibility to several intracellular pathogens [71]. The secretion of chemokines by T cells guides the migration of other immune cells, such as macrophages or other T cells to the site of infection. The recruitment of many different effector cell types to the site of infection allows the containment and possible clearance of infected cells within the

local microenvironment. Thus, effector $CD8^+$ cytotoxic T cells act in variety of different ways to control the spread of intracellular invading pathogens.

$CD8^+$ CTL Mediated HIV Control

Proposed Correlates of Protection against HIV Infection

Despite an enormous research effort over the last 3 decades, the characteristics that define the correlates of protection to HIV-1 infection are still unknown, making rational design of vaccines difficult. Multiple lines of evidence in human and animal models suggest that $CD8^+$ T cells play an important role in control of HIV-1 infection (reviewed in)[72]. During acute HIV-1 infection, the virus replicates in an uncontrolled manner in activated $CD4^+$ T cells, reaching a peak viremia of over 10^6 particles/mL of blood in about 3 weeks after infection. This initial peak of viremia begins to decline simultaneously with the emergence of HIV-1-specific $CD8^+$ T cells [73], and before the appearance of neutralizing antibodies [74, 75] as shown in Figure 1.5 [76]. HIV-1-specific $CD8^+$ T cells are vigorously expanded during primary HIV-1 infection. These cells can eliminate HIV-1 infected cells, as discussed previously, by cytolysis or through the production of cytokines, chemokines or other soluble factors, thus curtailing the generation of new viral progeny. There was a huge controversy about whether these HIV-specific $CD8^+$ T cells are responsible for the controlling viral load. To address this issue, further studies were carried out in rhesus macaques (RM) using the simian immunodeficiency virus (SIV) model, where $CD8^+$ T cells were depleted using anti-

CD8 monoclonal antibodies. $CD8^+$ depletion in RM led to a dramatic increase in plasma viremia and accelerated clinical disease progression compared to control RM [77, 78], indicating an important role for CTL in control of viremia. Additional evidence of $CD8^+$ T cell mediated control of viremia comes from studies of a small cohort of RM that naturally controlled SIVmac239 to low levels [79] or RM vaccinated with live attenuated SIVmacΔnef and subsequently challenged with a heterologous SIV isolate [80]. The transient depletion of $CD8^+$ T cells in these RM during the chronic phase of infection resulted in 100- to10,000-fold increase in viremia. More importantly, when $CD8^+$ T cells were eventually replenished, virologic control was subsequently re-established [79]. Also, HIV-1-specific $CD8^+$ T cell responses have been identified in highly-exposed but persistently seronegative (HEPS) individuals [81, 82], in particular, HIV-1-specific IFN-γ secreting $CD8^+$ T cells have been detected in cervical mucosa and vaginal washes of HEPS [83, 84]. This suggests that protective $CD8^+$ T cell responses could be induced in individuals who are chronically exposed to HIV-1, yet remain persistently seronegative.

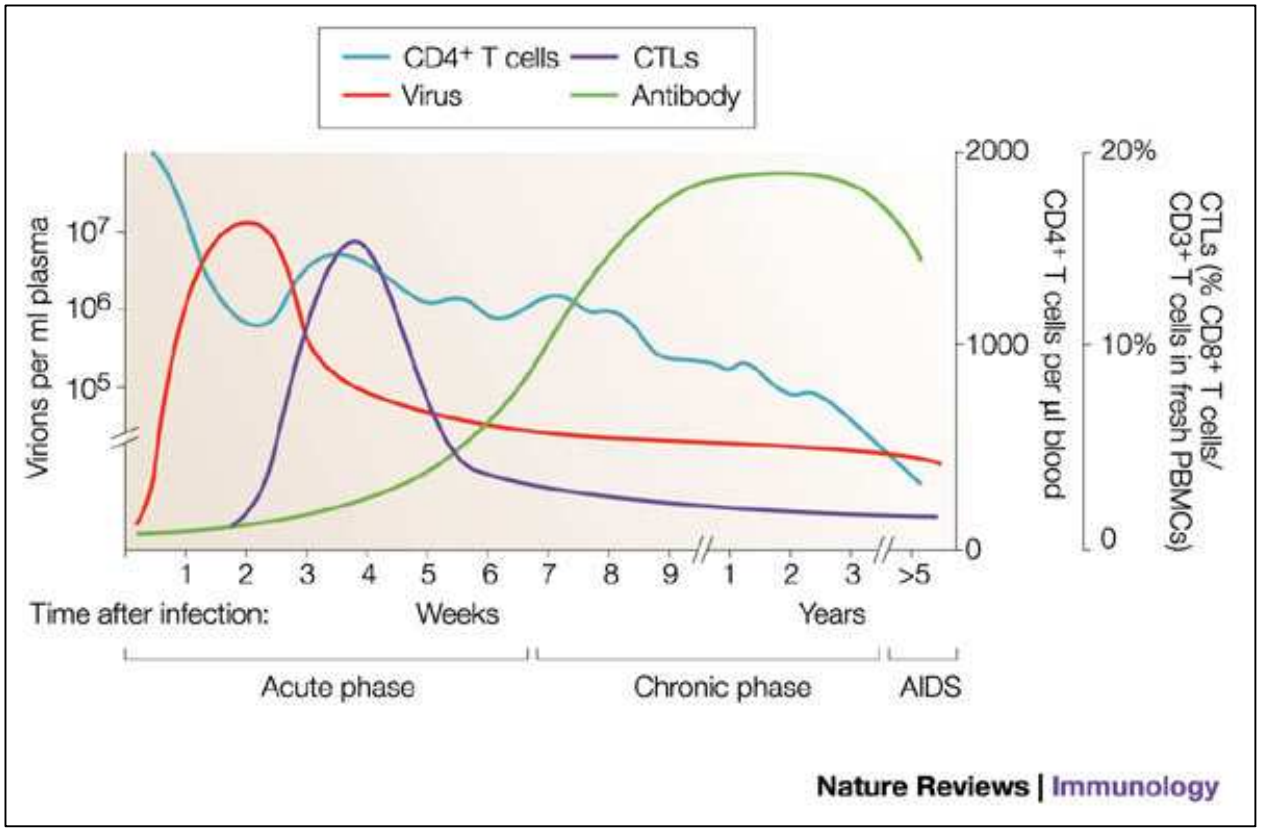


Figure 5: HIV infection and disease course.
This illustration depicts the relationship between HIV-1 plasma viremia (red), emergence of HIV-1 specific $CD8^+$ T cell response (purple) and antibody response (green) in an untreated HIV-1 infected individual. Reprinted by permission from Macmillan Publishers Ltd: Nature (reference citation), copyright (2012) [76].

A single individual can possess up to six different classical MHC class I alleles, which in humans is called Human Leukocyte Antigen (HLA): 2 sets of classical MHC class I loci (1 set comes from mother and another sets comes from father), namely, HLA-A, HLA-B and HLA-C. HIV-positive individuals who are homozygous for one or more HLA loci progress more rapidly to AIDS and death [85]. A hypothesis behind this observed difference is that individuals heterozygous at the HLA loci present a greater breadth of epitopes to $CD8^+$ T cells than individuals who are homozygous, suggesting that breadth of $CD8^+$ T cell responses is important on viral control. As certain HLA alleles are associated with rapid (B*35Px allele groups: B*35:02, B*35:03, B*35:04 and B*53:01) or slow (B*27, B*57 allele groups) progression to AIDS [86, 87] and long-term nonprogressors (LTNP) are consistently enriched with protective alleles (B*27, B*57), further implicating a role of $CD8^+$ T cells in immune control of HIV. Several studies have also reported that single nucleotide polymorphisms (SNPs) within the MHC locus are consistently and prominently associated with lower viral loads [88-90]. As the HLA class I molecule presents the pathogen-derived protein to $CD8^+$ T cells, the association between certain HLA class I and viral control advocates for a role of $CD8^+$ T cells in the context of controlling HIV infection.

Another piece of evidence supporting a role of $CD8^+$ T cells in HIV control comes from a vast number of studies showing the ability of HIV to escape from $CD8^+$ T cell recognition. Multiple studies in 1997 have unequivocally shown that $CD8^+$ T cell escape occurs in natural infection with HIV [91-93]. Subsequently, other studies have shown that mutations in the viral genome during HIV/SIV infection are

concentrated within the $CD8^+$ T cell epitope regions [94](review in)[76], suggesting that escape occurs as a result of $CD8^+$ T cell mediated selection pressure on the virus. Indeed, studies have shown a positive correlation between escape mutations within targeted epitopes (notably B*27 restricted KK10) in HIV-infected individuals and increased viral load [92, 95]. This suggests that $CD8^+$ T cells recognizing this epitope are efficient at controlling viral replication. Interestingly, HIV-1-specific $CD8^+$ T cells begin to put pressure on the virus immediately after their appearance during early infection [91, 96-99]. Many of these escape mutations are associated with a significant fitness cost on the virus [100] and are shown to revert back to wild-type after getting transmitted to individuals not sharing that particular HLA type [101]. Overall, these studies suggest that HIV is continuously attempting to escape from CTL mediated immune pressure, even in the presence of an immense fitness cost, further suggesting a vital role for $CD8^+$ T cell in HIV control.

Is Functionality of HIV-specific T cells Related to Their Ability to Control Viremia?

Despite implications that $CD8^+$ T cell-mediated immunity is important on viral control, the proposed correlation between functionality of $CD8^+$ T cells and viral control remains controversial. Initial studies correlating the functionality of $CD8^+$ T cells and viral control were heavily based on defining the functionality of $CD8^+$ T cells in terms of their ability to secrete a single cytokine, IFN-γ. In these studies the frequency of IFN-γ secreting HIV-1-specific T cells was measured using the ELISPOT assay. Although IFN-γ is an antiviral cytokine, there are no definitive

studies showing that it is the secretion of IFN-γ from HIV-1-specific T cells that contributes to HIV control.

LTNP have been shown to contain a higher proportion of HIV-1-specific $CD8^+$ T cells that are poly-functional for their ability to degranulate (as measured by CD107-α), secrete multiple cytokines (IFN-γ, TNF-α, IL-2) and chemokines (MIP-1β) upon HIV peptide stimulation compared to progressors [102], suggesting that T cells with poly-functionality are correlated with non-progression and HIV control. However, $CD8^+$ T cells, regardless of proliferative ability, have been shown to secrete IFN-γ, TNF-α and degranulate [103]. In addition, T cells induced by the Merck vaccination in the STEP trial secreted two or more antiviral cytokines (IFN-γ, TNF-α, IL-2), but they were not effective at controlling infection [104]. These data suggest that measurement of multiple cytokines alone is not sufficient to determine the effectiveness of HIV-specific T cells to control HIV-1 replication.

Additionally, studies have also shown that HIV-1-specific $CD8^+$ T cells from controllers demonstrate superior functional avidity compared to those from progressors: $CD8^+$ T cells from controllers can get activated even at low concentration of the antigen [105, 106]. Indeed B*27-KK-specific $CD8^+$ T cells have been shown to possess superior functional avidity [107]. Moreover, multiple studies have shown that HIV-1-specific $CD8^+$ T cells from controllers have enhanced cytotoxic functionality compared to progressors. $CD8^+$ T cells from controllers have a superior ability to suppress viral replication in autologous infected $CD4^+$ T cells *ex vivo* [108]. To date, the only functional characteristic of HIV-1 specific $CD8^+$ T cells that inversely correlates with viral load is the proliferative ability. In turn, the ability of

$CD8^+$ T cells to proliferate is directly coupled to their perforin expression [109], which presumably contributes to their ability to kill infected cells. $CD8^+$ T cells from LTNP have been shown to possess extraordinary cytotoxic capacity on a per-cell basis, and to eliminate primary autologous HIV-1-infected $CD4^+$ T cells more efficiently than $CD8^+$ CTL from progressors, which was directly mediated by delivery of granzyme B to target cells [110]. Furthermore, $CD8^+$ T cells from controllers express higher levels of perforin directly *ex vivo* [111]. Both the proliferative and cytolytic capabilities of $CD8^+$ T cells are impaired in individuals who are progressing to AIDS but are maintained in LTNP [109, 112]. While the majority of HIV-1-specific $CD8^+$T cells become tolerant during chronic infection, studies have shown that $CD8^+$ T cells restricted by B*27 and B*57 are resistant to peripheral tolerance allowing them to proliferate throughout the chronic infection [103, 113].

While these studies suggest that functionality of $CD8^+$ T cells that are induced by HIV-1 infection play a critical role in HIV control and non-progression, most of these studies have looked at functionality of bulk $CD8^+$ T cells and studied samples from chronic infection. There is a marked diversity in functionality of epitope specific T cells elicited even in a single individual [113]. Therefore, more work needs to be carried out to identify the functionality of CD8+ T cells on samples from early HIV-1 infection, which can be followed longitudinally, teasing out the differences in the functionality of epitope-specific $CD8^+$ T cells. The qualities of $CD8^+$ T cells that are elicited during early infection most likely play a pivotal role in determining overall clinical outcome.

Is Magnitude of the CD8$^+$ T cell Response Correlated to Their Ability to Control Viremia?

Although our understanding of the role of CD8$^+$ T cell-mediated immunity in HIV control has greatly advanced in the past decade, the proposed correlation between magnitude of CD8$^+$ T cell responses and viral control remains largely unknown. Initial HLA-tetramer based studies have demonstrated an inverse correlation between the frequency of A*0201-restricted HIV-specific CD8$^+$ T cells and plasma viral load [114]. However, subsequent IFN-γ ELISPOT-based studies examining the magnitude of overall IFN-γ-secreting HIV-specific CD8$^+$ T cell responses that are induced during HIV infection did not find an impact of magnitude of the CD8$^+$ T cells responses on viral control [115-118]. However, significant numbers of virus-specific CD8$^+$ T cells are also detectable in individuals who progress to AIDS rapidly [117, 119]. Indeed, other studies have found a positive correlation between magnitude of CD8$^+$ T cell responses and viral load [120, 121]. While studies examining a total magnitude of CD8$^+$ T cell responses on viral control have not shown a consistent outcome on the role of CD8$^+$ T cell responses on viral control, the magnitude of CD8$^+$ T cell responses directed against Gag epitopes have been shown to play an important role on the containment of HIV [122]. Overall, it suggests that there is either the lack of a true correlation between magnitude of CD8$^+$ T cell responses for the observed differences in disease outcome in controllers compared to progressors or limitations in the currently used assays to assess the magnitude of CD8$^+$ T cell responses.

Is Breadth of $CD8^+$ T cell Response Correlated to Their Ability to Control Viremia?

Earlier studies showing the advantage of HLA class I heterozygosity on the delay of disease progression [85, 87], implicate breadth of the $CD8^+$ T cell epitopes in the containment of HIV, however, this is controversial. A series of studies have shown that breadth of HIV-1 specific $CD8^+$ T cell responses does not correlate with viral load [115, 116]. However, Altfeld *et al*. showed that the breadth of $CD8^+$ T cells responses are inversely correlated with viral load at presentation in treated individuals before HIV-1 seroconversion [123]. While all these studies examined the total breadth of all $CD8^+$ T cell epitopes that are recognized, studies comparing the breadth of protein-specific $CD8^+$ T cell responses have shown that breadth of Gag-specific $CD8^+$ T cell responses correlate consistently with reduced viremia [122, 124]. Therefore, it is becoming clear that the total breadth of HIV-1-specific $CD8^+$ T cell responses does not always explain the discrepancy seen in HIV infected individuals to control viral replication compared to individuals who cannot. But rather, the breadth of $CD8^+$ T cell responses directed against conserved proteins like Gag is important in the containment of the virus.

Caveats of Studies Associating Magnitude/Breadth with Viral Control

Direct comparison of data from the various studies on the impact of magnitude and breadth of $CD8^+$ T cell responses in HIV control is limited as many factors involved in mapping of $CD8^+$ T cell responses can influence the overall outcome of the correlation. First, the type of peptide sets used to map T cell

responses could potentially influence overall outcome of the correlation. For example, the most comprehensive mapping of $CD8^+$ T cell responses uses autologous peptide sets based on pool of infecting viral sequences [125]. However, synthesis of autologous peptide sets for all individuals within the study is prohibitively expensive. For this reason, many studies use global peptide sets like Potential T cell Epitope (PTE) peptide sets, which is relatively comprehensive compared to consensus B peptide sets to map $CD8^+$ T cell responses. PTE peptides are 15mers that are designed to encompass all potential 10-mer epitopes that are present at 15% or greater in current circulating M clade strains [126]. Although they are not autologous peptides, they will still contain multiple variants of each potential $CD8^+$ T cell epitope. Autologous and PTE peptide sets have a greater coverage of circulating viral sequences compared to consensus B peptide sets, and shown to increase the number of responses detected in infected individuals over consensus B peptide sets [125, 127]. Since consensus B peptide sets are designed based on the most frequent amino acid found at each position in a clade-B HIV sequence alignment, the use of these peptide sets would bias the detection of responses towards more conserved epitopes, and therefore would miss the unique responses directed against less conserved epitopes between the isolates [116].

Second, studies identifying the total magnitude and breadth of $CD8^+$ T cell responses in therapy treated individuals would miss the true $CD8^+$ T cell responses that are induced during untreated HIV infection [115, 123]. Third, the overall outcome of a correlation study comparing magnitude and breadth of $CD8^+$ T cells elicited during early HIV-1 infection to viral load will be unreliable if the concurrent

viral load [115, 116, 123] rather than set point viremia is used for analyses. This is because viral load undergoes intense dynamic changes during this short period of time. It is also possible that the role of $CD8^+$ T cells may be more pronounced in early infection, as virus has not yet diversified, compared to chronic infection. Moreover, the outcome of the role of $CD8^+$ T cells may be inaccurate when $CD8^+$ T cell responses elicited during both early and chronic HIV-1-infection are assessed in the same study and correlated together with concurrent viral load [115].

Is Specificity of T cell Response Correlated to Their Ability to Control Virus?

As mentioned above, functionality, magnitude and breadth of bulk $CD8^+$ T cell responses elicited in response to HIV infection does not consistently correlate with viral load, suggesting that not all $CD8^+$ T cell responses are created equally and differ significantly in their ability to control viral replication. While the ability of the virus to escape from immune mediated pressure is a major mechanism of the virus to evade the immune system, escape occurs at very different rates in different epitopes recognized by HIV-1-specific $CD8^+$ T cells suggesting that the virus may find it difficult to mutate certain epitopes [128].

The presence of broadly cross-reactive HIV-specific $CD8^+$ T cell responses to multiple conserved HIV epitopes in highly-exposed but persistently uninfected women [129-132] suggests that $CD8^+$ T cells recognizing conserved epitopes of the virus potentially provides protection against HIV-1. Studies have shown an inverse correlation between breadth of Gag-specific $CD8^+$ T cell responses with HIV-1 RNA levels during chronic infection [124]; and $CD8^+$ T cells that target Gag are more

efficient at controlling SIV replication than those that target Envelope due to the lack of requirement for integration and transcription to get sufficient Gag epitopes to the cell surface [133]. Furthermore, HIV-specific $CD8^+$ T cell responses restricted by protective alleles target highly conserved areas of the virus (Gag region), and viral escape from these Gag-specific $CD8^+$ T cell responses is either not possible or occurs with a viral fitness cost [101, 134]. Indeed studies have shown a positive correlation between escape mutation within targeted epitopes (notably B*27 restricted KK10) in HIV-infected individuals and increased viral load [92, 95]. This suggests that $CD8^+$ T cells recognizing this epitope are efficient at controlling viral replication. This KK10 epitope, recognized in LTNP possessing B*27 allele is highly conserved with a conservation score of 0.87 (i.e. 87% of all M clade sequences in Los Alamos National Laboratory Database (LANL DB) possess this exact epitope match). Interestingly, we have observed that $CD8^+$ T cells restricted by unfavorable allele groups (notably B*35Px) target epitopes with lower conservation score (DL9 0.12; IL9 0.09). Recently Li et al. has shown that majority of epitopes that are targeted by Merck Ad5 vaccine are variable [135]. These observations suggest that $CD8^+$ T cells that target conserved HIV-1 epitopes early in infection are superior compared to T cells targeting variable epitopes, and these responses are critical for the subsequent control of viral replication.

HIV Vaccines

The isolation of HIV as the causative agent of AIDS in 1983 engendered the phase of hopefulness and faith that an HIV-1 vaccine would be available within the next 10 years, but the real complexity of the virus was not known at that time. Despite three decades of intensive research effort in the field of HIV-1, such a vaccine is still elusive. Although non-vaccine combination strategies such as behavioral interventions, topical microbicides, and pre-exposure prophylaxis have proven to be effective as prevention measures [136-141], the success of these strategies are highly dependent on patient adherence, time of detection after infection and time of treatment. Therefore, most of the research in the field has heavily focused in development of a safe and highly effective vaccine with the hope that such a vaccine would avoid the need for multiple preventative strategies, as these strategies are not practical in the developing countries where the disease burden is the highest. But unfortunately, the development of an HIV vaccine has presented a daunting challenge so far.

The HIV Vaccine Challenge

The quest for developing an efficacious HIV vaccine has been faced with several major challenges (reviewed in)[142]. The first obstacle to HIV-1 vaccine development is associated with the extreme genetic diversity of circulating strains of HIV-1 [143]. The genetic diversity is larger than any other agent [17, 18]. Driven by highly error-prone viral reverse transcriptase, and the capacity to recombine different strands of genomic RNA during transcription, HIV has the ability to mutate rapidly

such that amino acids sequences of Env proteins can differ up to 20% within a particular clade and over 35% between clades [143, 144]. A successful vaccine will therefore need to tackle this immense diversity of the virus and provide protection across all circulating clades and circulating recombinant forms of the virus. Due to the massive antigenic diversity of the virus, HIV-1 evades effectively both neutralizing antibodies as well as T cell responses elicited during natural HIV-1-infection. Furthermore, Env heterogeneity, Env glycosylation and conformational shielding of conserved epitopes make it difficult to induce neutralizing antibodies (reviewed in)[145]. The second key challenge is the lack of a definitive immune correlate of protection, because HIV-1 infected patients even LTNP are unable to eradicate the virus. Third, HIV uses multiple immune evasion mechanisms including down-regulating T cell immune responses to the virus. Fourth, HIV has multiple transmission routes. HIV can be transmitted as both a cell-free virus as well as a cell associated virus. Although, the most common route of transmission of HIV is through a sexual contact, it can also be transmitted from mother-to-child or though reaching the blood stream directly. Fifth, HIV integrates into the host genome and integrated viral DNA may remain dormant. Finally, the primary cellular target of HIV-1 is $CD4^+$ T cells which play an essential role in immune defenses against pathogens.

A successful HIV-1 vaccine will need to elicit robust humoral and cellular immune responses despite these numerous challenges. While induction of HIV-1-specific antibodies would function to block HIV-acquisition at the entry sites at mucosal surfaces, the induction of HIV-1-specific cellular immunity would control the

spread and amplification of the breakthrough virus by reducing steady-state VL and $CD4^+$ loss thereby slowing or preventing disease progression [146].

HIV-1 Vaccine Efficacy Trials

HIV vaccine development has focused on either induction of antibody responses or T cell responses, rarely on both. The first HIV-1 vaccine trial was conducted in 1987 [147], since then there have been more than 200 vaccine trials, and only four of these have been tested in clinical efficacy trials as depicted in Figure 6 [145].

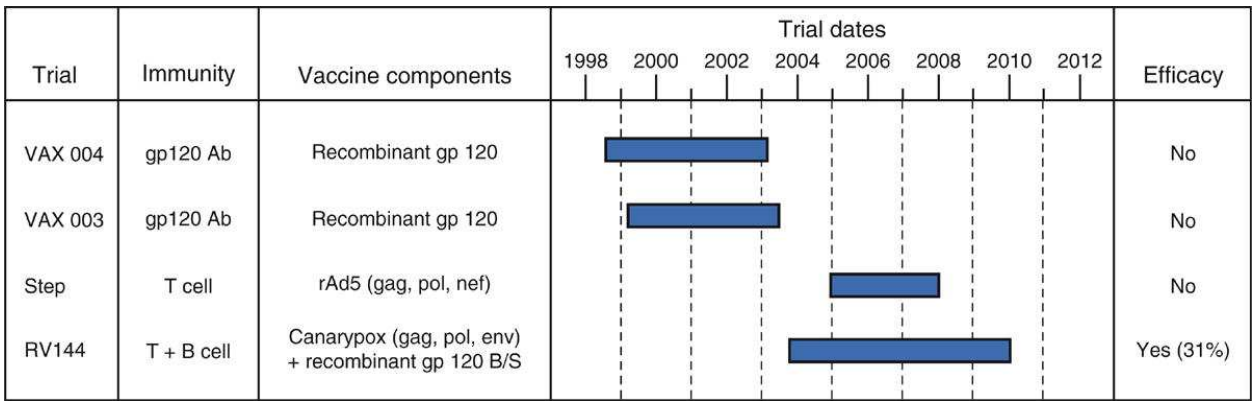


Figure 6: Vaccine concepts, timelines and outcomes of HIV-1 vaccine efficacy trials.
Four vaccine efficacy trails have been performed to date (VAX004, VAX004, Step and RV144). The type of vaccine-induced immunity, the vaccine components, the timeline of the trial and the resulting efficacy are shown. Reprinted by permission from Macmillan Publishers Ltd: AIDS (reference citation), copyright (2012)

VAX003 and VAX004

Initial two phase III clinical trials, VAX003 and VAX004, were aiming at induction of Env-specific anti-HIV neutralizing antibodies by vaccination with recombinant gp120. VAX003 (AIDSVAX B/E), contained one clade B and one clade E gp120, conducted in Thailand in a high-risk study population of injection drug users [148]. VAX004 (AIDSVAX B/B), comprised of two clade B gp120s, conducted in the USA, Canada and Netherlands in high-risk MSM individuals [149]. Neither of these vaccination trials reduced the number of HIV infections, delayed disease progression or lowered the viral load in vaccinated individuals who got infected [148-152]. While these Env-based vaccination strategies were known to be immunogenic and can elicit anti-HIV antibody responses that can neutralize laboratory-adapted strains of HIV-1, later analyses showed that these vaccine-induced antibodies could not neutralize most naturally circulating strains of HIV-1 (reviewed in)[153]. It was then believed that neutralizing antibodies are rarely developed even in the natural context of infection, so it would be hard to elicit such a response by vaccination. Consequently, the HIV-1 vaccine field shifted to focus on development of T cell based vaccines as multiple lines of evidence in humans and animal models suggested that cytotoxic $CD8^+$ T cell play an important role in the containment of the virus (reviewed in) [72].

The STEP Study

The other two phase IIb proof-of-concept trials, the STEP (HVTN 502) and the Phambili (HVTN 503) studies were designed to induce $CD8^+$ T cell responses by vaccination to lower viral load and slow disease progression. The T cell based immunization approach was encouraged by the failure of B-cell based vaccination approach as well as the result of the preclinical studies in non-human primate (NHP) immunized with an Ad5 vector carrying the Gag gene demonstrating more effective control of viral load after primary infection [154]. High-risk volunteers were vaccinated with MRKAd5 gag/pol/nef vaccine, formulated as a trivalent mixture of replication-incompetent recombinant Ad5 vectors expressing HIV-1 clade B Gag, Pol and Nef antigens, delivered as a high-titer homologous prime-boost regimen. While STEP study was conducted in North America, South America, the Caribbean and Australia, the sister Phambili study was designed in South Africa. HVTN 502 was unexpectedly terminated in September of 2007 at the first interim review of the Data and Safety Monitoring Board [155]. Because of this, the Phambili study, which had used the same vaccine regimen, was also terminated. MRKAd5 vaccine was able to elicit HIV-1 specific $CD8^+$ T cell responses targeting Gag, Pol and Nef in 77% of vaccinated individuals. These $CD8^+$ T cells expressed two or more antiviral cytokines (IFN-γ, TNF-α, IL-2) [104], but it did not confer the protection against infection or reduce in viral load on vaccinated individuals. Although, T cell based vaccines were not usually expected to impact on the acquisition of infection, vaccinated individuals with pre-existing immunity to Ad5-specific neutralizing antibodies titers had increased rates of HIV acquisition compared to the placebo

group. Moreover, post-hoc multivariate analysis suggested that the greatest risk was in men who were uncircumcised and had pre-existing Ad5-specifc neutralizing antibodies[155]. Furthermore, a recent study analyzing $CD8^+$ T cell responses elicited by MRKAd5 vaccination showed that the majority of epitopes recognized by CTL induced by vaccination were variable [135], making it likely that these vaccine-induced T cell responses were not directed to infecting HIV-1 virus isolates.

The failure of the MRKAd5 clinical trial had a profound impact on the vaccine field. First, it has raised the question on both development of T cell based vaccines and the assays used to measure T cell immunogenicity. Because of this, the field has moved away from traditional ELISPOT assays to measure the number of IFN-γ secreting cells in response to HIV-1 antigen and investigated more suitable immunogenicity assays such as viral inhibition assays for the in vitro assessment of antiviral activity [156, 157]. Second, the lack of consistency between Ad5 vaccines in reducing viral load in the NHP study and the lack of this effect in the human clinical trials has raised concerns over the appropriateness of NHP models to predict human efficacy [154, 158, 159]. However, finding the optimal alternatives for challenge experiments is not a viable task at present. Third, the Step study emphasized the potential detrimental impact of pre-existing immunity to Ad5 viral vectors. Efforts are still ongoing to identify other suitable viral vectors.

RV144 Study

The RV144 trial was a phase III trial designed to potentially induce both antibody and $CD8^+$ T cell responses. The Rv144 vaccine contained a live, recombinant, non-replicating canarypox vector expressing clade B gag/pol and a membrane-anchored version of a clade E Env (ALVAC-HIV) as prime followed by a recombinant gp120 from clades B and E (AIDSVAX B/E) protein boost. The trial was conducted in Thailand, in individuals at low risk of infection. The vaccination provided modest protection from infection (31% efficacy in preventing acquisition of HIV-1 infection) [160]. There were no differences in terms of $CD4^+$ T-cell counts and viral load in vaccinated individuals who got infected compared to placebo group [160]. The antibody responses induced by RV144 vaccination were also unable to neutralize primary HIV-1 isolates. Initial analysis of immune responses induced by RV144 vaccination suggested that the protection was mediated by the presence of non-neutralizing antibodies to V1/V2 region of Env, and high levels of Env-specific IgA antibodies may have inhibited the protective effects observed with anti-V1/V2 IgG antibody against HIV-1 infection [161], but the mechanism underlying the role of these antibodies in protection are not yet fully revealed. However, the interpretation of the significance of immunologic responses induced by RV144 vaccination is limited by sample availability. Due to the lack of efficacy of HIV-1 vaccines aiming to induce humoral or cellular immunity, presently the HIV vaccine field believes that in order to improve on the efficacy of protection offered by RV144 vaccination, the next generation vaccine should be designed to induce both antibodies and T cell responses.

Current Approaches for T cell Based HIV Immunogen Design

To overcome the problem of incredible diversity of HIV-1, T cell based vaccine approaches have focused on two main different strategies: mosaic and conserved immunogens.

Mosaic Immunogen

This immunogen strategy focuses on maximizing the breadth of the vaccine-induced T cell responses to confer protection against incredibly diverse strains of HIV-1. The approach uses a computational optimization method to generate polyvalent artificial mosaic proteins that maximize coverage of sequences of natural HIV strains worldwide [162-164]. The approach is based upon the theory that inclusion of multiple variants in a polyvalent vaccine would enable responses to a broader range of circulating strains and could potentially prime the immune system against common escape mutants. The mosaic immunogens are cocktails containing a small number of composite proteins that have been optimized to include the maximum number of potential T cell epitopes from a set of viral proteins. They resemble natural protein sequences, but systematically include common (and exclude rare) potential epitopes. Thus the immunogens should be processed the same as the natural full-length sequences are processed but they exclude low-frequency epitopes that are irrelevant to circulating strains. Indeed, these mosaic antigens have been shown to be processed and presented to human HIV-specific $CD8^+$ T cells [165]. Moreover, mosaic vaccines have been shown to induce increased breadth and depth of HIV-1-specific cellular immune responses compared

to natural and consensus HIV-1 antigens in RM [166-168]. A recent study has shown that full-length mosaic immunogens induce greater magnitude and comparable breadth of HIV-1-specific cellular immune responses to conserved HIV-1 regions compared with conserved-region-only HIV-1 immunogens in RM [169]. Although, overall data from these studies suggest that mosaic-immunogen strategy can increase the breadth of T cell responses, none of the studies have shown whether or not mosaic-immunogen-induced responses can confer protection from viral challenge.

Conserved Immunogen

The conserved immunogen strategy focuses the vaccine-induced T cell responses specifically to highly conserved regions of the virus to contend with profound genetic diversity of HIV-1[170, 171]. A primary motivating factor for working towards a conserved immunogen approach comes from the observation that the alleles that are associated with HIV control and long-term non-progression mount $CD8^+$ T cell responses against highly conserved regions of the virus[92]. Mutation of these regions has severe fitness costs for the virus. This immunogen strategy is based on the inclusion of conserved-only regions of the HIV-1 proteome (excluding variable region of HIV-1 proteome) that are present on diverse clades of HIV-1 isolates. Conserved region-only vaccine induced responses will thus increase the likelihood that T cells induced by vaccination will be able to recognize incoming viral species of diverse clades and decrease the likelihood of rapid escape variants against the recognized epitopes. However, the potential concern in the field is that

these conserved epitopes may not be processed and presented to T cells during natural HIV infection. Despite this concern, the conserved-region vaccine approach (HIV_{consv}) has been shown to generate T cell responses to vaccine antigens expressed as DNA, in an MVA vector, and in a human Ad5 vector in HLA-A*0201 transgenic mice [170]. Pre-clinical studies of HIV_{consv} immunogens in combination with different vector regimen/delivery method in RM have shown that HIV_{consv} vaccines, and particularly when adjuvanted with synthetic long peptides ($SLP.HIV_{consv}$) boost induces polyfuncional and broad T cell responses against conserved regions of HIV-1 [172]. Stephenson et al. demonstrated that the conserved-region-only immunogens induced similar breadth of $CD8^+$ T cell responses against conserved HIV-1 epitopes as compared to full-length mosaic Gag/Pol/Env immunogens suggesting that the full-length mosaic immunogens offer no clear benefit in terms of expanding breadth against conserved epitopes compared with the conserved-region-only immunogens [169]. Despite the fact that these data suggest that the conserved-region-only immunogen strategy can be immunogenic in RM, none of the studies have shown whether these vaccine-induced responses confer protection from viral challenge so far. Based on these encouraging data from pre-clinical studies, both the HIV_{consv} vaccine and mosaic vaccine approaches have moved forward into human safety and immunogenicity trials.

Conclusion and Project Aims

The global diversity of the virus creates the greatest hurdle for developing a prophylactic HIV vaccine. Although, the HIV virus is the most genetically diverse virus, there are certain regions that the virus keeps relatively stable. Perhaps these regions are associated with a fitness cost, and they provide hope that vaccine-induced responses against these regions can provide durable immune-mediated control of HIV viremia. A growing body of evidence in human and animal models suggests a role for $CD8^+$ T cells in controlling or preventing HIV infection providing a strong rational for renewed efforts to optimize T cell based vaccines specifically targeting conserved sites of vulnerability in HIV. While only small proportions of HIV infected people control viremia, they do provide the hope that durable immune-mediated control of HIV viremia can be achieved through vaccination. Previous studies in our lab provide data showing that individuals who mount HIV-specific $CD8^+$ T cell responses early in infection against conserved epitopes of HIV have better clinical outcomes than individuals who mount HIV-specific T cell responses against variable epitopes. However, this study was limited to only a few epitope specificities. There are no studies looking at whether conserved or variable epitopes are preferentially recognized by $CD8^+$ CTL during early HIV-1 infection, and how this correlates with initial viral control. So the primary goal of this project was to characterize epitope specificity of all $CD8^+$ T cell responses that are induced during early HIV infection in therapy naïve HIV infected individuals, and assess the impact of the epitope specificities and breadth of $CD8^+$ T cell epitopes targeted during early HIV-1-infection on initial viral control. The secondary aim of this work was to further

evaluate of the impact of the avidity of $CD8^+$ T cells on viral control. The hope is that the results from this work will further increase our understanding of $CD8^+$ T cell responses that are effective versus ineffective, which would eventually guide the field to design an efficacious HIV vaccine.

Chapter II: Materials and Methods

Study Subjects.

HIV-specific $CD8^+$ CTL responses were characterized in 23 HIV-1-infected therapy naïve subjects from Seattle, Washington. These subjects were selected from the University of Washington Primary Infection Cohort (PIC) based on availability of specimens from ART naïve subjects within 6 months post infection (except one 7.5 months post infection). The mean days-post-infection (dpi) was 84 (range 16-226 days). The PIC cohort's estimated HIV-1 infection date was used as the date of infection; this was the symptom onset date for symptomatic subjects or the midpoint between the last negative and first positive HIV tests in those lacking symptoms. Before July 2003, blood HIV-1 viral-load testing employed a branched-chain DNA (bDNA) assay (Chiron Diagnostics), which had a lower limit of detection of 500 HIV-1 RNA copies per mL [173]. After 2002, an in-house real-time reverse transcription (RT)-PCR method was used, with a lower limit of detection of 50 copies per ml [174]. Most of these subjects were Caucasian men who have sex with men (MSM). Clinical information and HLA genotype of these subjects is summarized in Table S1. The appropriate institutional review boards approved the studies, and all participants provided written informed consent.

Longitudinal clinical data were collected from all study participants. VL set point was defined as the mean of available viral load results from 91-426 days (3-15 months) dpi prior use of ART (Table 1). Of the 23 subjects, 15 subjects reached set point viremia (mean 5.3, range 1-13 values) during this period.

Table 1: Assessment of VL set point of subjects with primary HIV-1 infection

PICID	Est HIV date	SampleDPI [a]	SetpointVL	SPVL# [b]	SPVL-DPIRange
1545	10/6/05	35	17,424	6	91-375
1577	6/2/01	177	340	8	107-415
1585	5/26/01	150	46	13	124-417
1693	5/19/04	16	136,253	5	96-321
1706	8/18/04	44	5,554	4	202-426
1735	10/13/05	34	59,540	5	109-333
1745	12/30/05	96	1,604	9	96-396
1767	7/9/06	22	48,069	6	94-346
1778	1/25/07	36	21,525	1	151
1785	4/20/07	47	225,585	3	139-243
1793	6/23/07	65	77,654	4	108-352
1815	6/4/08	226	1,314	4	103-217
1853	4/7/10	177	965	5	128-350
1865	7/25/10	108	6,011	5	108-365
1871	9/24/10	194	1,712	2	173-194
9148	1/14/09	69	none		
9150	2/23/09	16	none		
9166	7/23/09	83	none		
9167	7/28/09	79	none		
9170	8/22/09	67	none		
9176	2/10/10	69	none		
9187	3/15/10	44	none		
9195	5/22/10	81	none		

[a] DPI, days post infection

[b] SPVL#, number of longitudinal viral load data points used to identify viral load set point

Epitope Mapping of T cell response (IFN-γ ELISPOT and ICS).

Cryopreserved peripheral blood mononuclear cells (PBMC) were thawed and rested overnight at 37^{o}C before plating 100,000-200,000 PBMC per well in IFN-γ ELISPOT assays (Millipore), as previously described [103, 175]. Briefly, PBMC were stimulated with master pools of up to 100 peptides. These peptides were 15-mers overlapping by 11 amino acids spanning the entire coding sequence of HIV-1 (PTE peptide sets, provided by NIH AIDS Research & Reagent Program). Once a positive

response to a master pool was observed, PBMC were stimulated with sub pools in a matrix system of 8-15 15mers per pool to determine the individual 15mer giving the T cell response (Figure 7) [176, 177]. These 15mers were then tested individually to confirm recognition. Unstimulated cells served as a negative control, and phytohemagglutinin (PHA; Remel) stimulated cells served as a positive control. Biosyn Corp., and Sigma-Aldrich synthesized all HLA class I restricted peptides (8- to 11-mer). Peptides were used at a final concentration of 2 μg/mL. The number of spot forming cells (SFC) was calculated by subtracting the mean number of spots in the negative control wells from the mean number of spots for each stimulation condition. An IFN-γ result was considered positive when the background-subtracted number of SFC was twice the background (negative control) and at least 50 SFC per million PBMC.

Intracellular cytokine staining was performed as previously described [175, 178] with 15mer peptides that were positive in the ELISPOT assay, to differentiate the phenotype of T cells ($CD8^+$ versus $CD4^+$ T cells). Only MHC class I-restricted $CD8^+$ T cell responses were further mapped. For each of the 15mers mediating MHC class I-restricted $CD8^+$ T cell response, optimal epitopes were predicted based on an individual's HLA type from LANL DB and tested for reactivity by IFN-γ ELISPOT. If no known epitopes were predicted, overlapping 9-mers encompassing the 15-mer were synthesized and tested for reactivity by IFN-γ ELISPOT to identify the optimal epitope sequence. Epitopes were defined as novel epitopes (i) if the epitope had not been previously defined in the LANL DB and (ii) if the subject did not possess the known restricting allele of the previously defined epitopes.

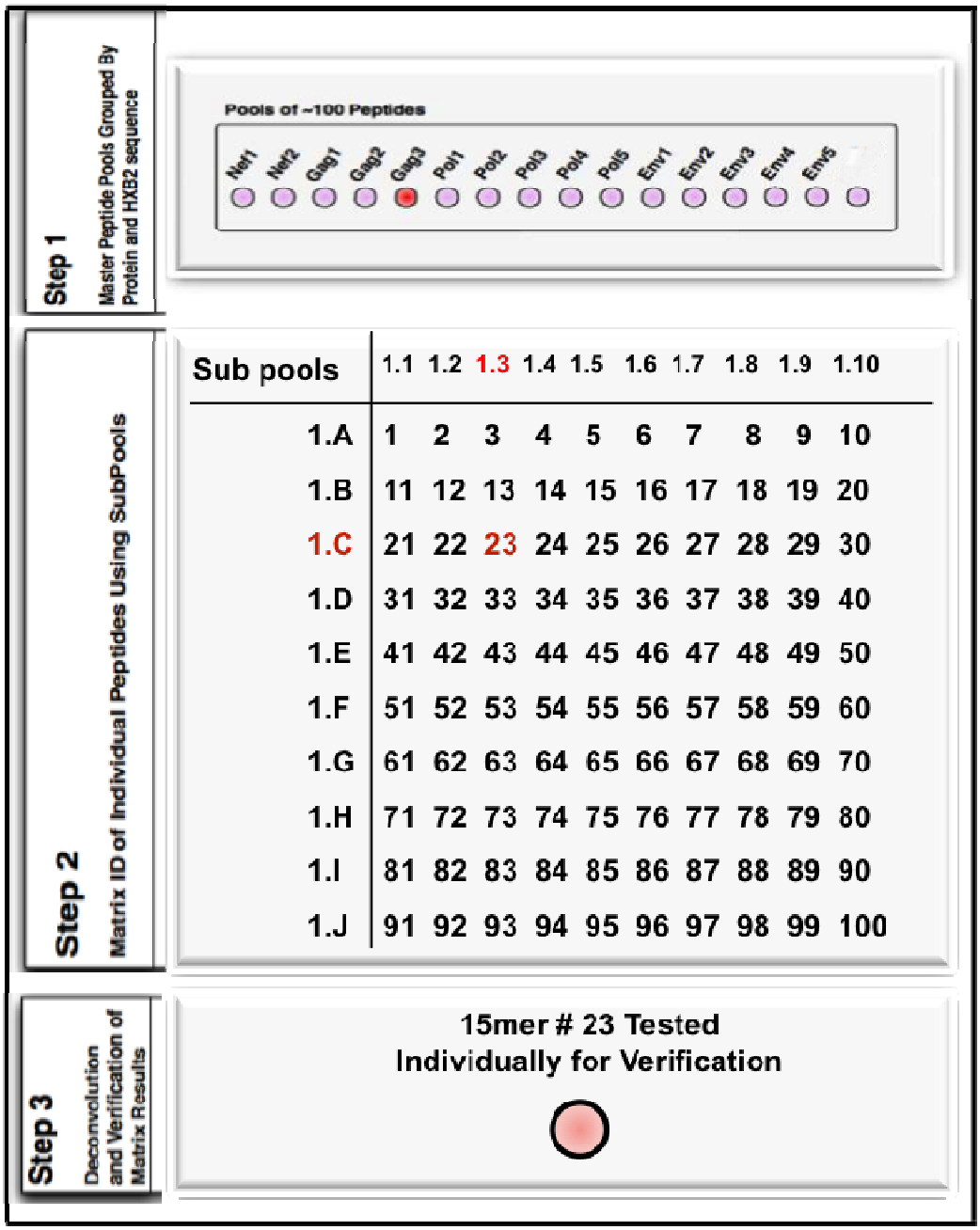


Figure 7: Epitope mapping of T cell responses in early HIV-1 infection. Cryopreserved PBMCs were stimulated with pools up to 100 peptides (15mers overlapping by 11aa, pools were made sequentially based on HXB2 reference sequence) covering the entire coding sequence of HIV-1 genome (Step 1). Once a positive response to a peptide pool was observed, the single 15-mer peptide recognized by the $CD4^+$ or $CD8^+$ T cells was identified using a matrix approach (Step 2). The 15mers were then tested individually to confirm recognition (Step 3).

Class I MHC restriction.

IFN-γ ELISPOT assay was used to determine MHC restriction of each of the epitopes with no known restriction as previously described [116]. Briefly, a panel of Epstein-Barr Virus (EBV)-transformed B-cell lymphoblastoid cell lines (BLCL) was mismatched with the initial responder except for one HLA allele. Each BLCL was incubated with or without the epitope (2 µg/mL) at 37°C for 3 hours, washed four times in phosphate-buffered saline (PBS), and incubated with reactive PBMC for 20 hours (Figure 8). The remainder of the ELISPOT assay was performed as described above. Wells containing BLCLs (without peptide-pulsed) and the same subject's PBMCs served as negative control.

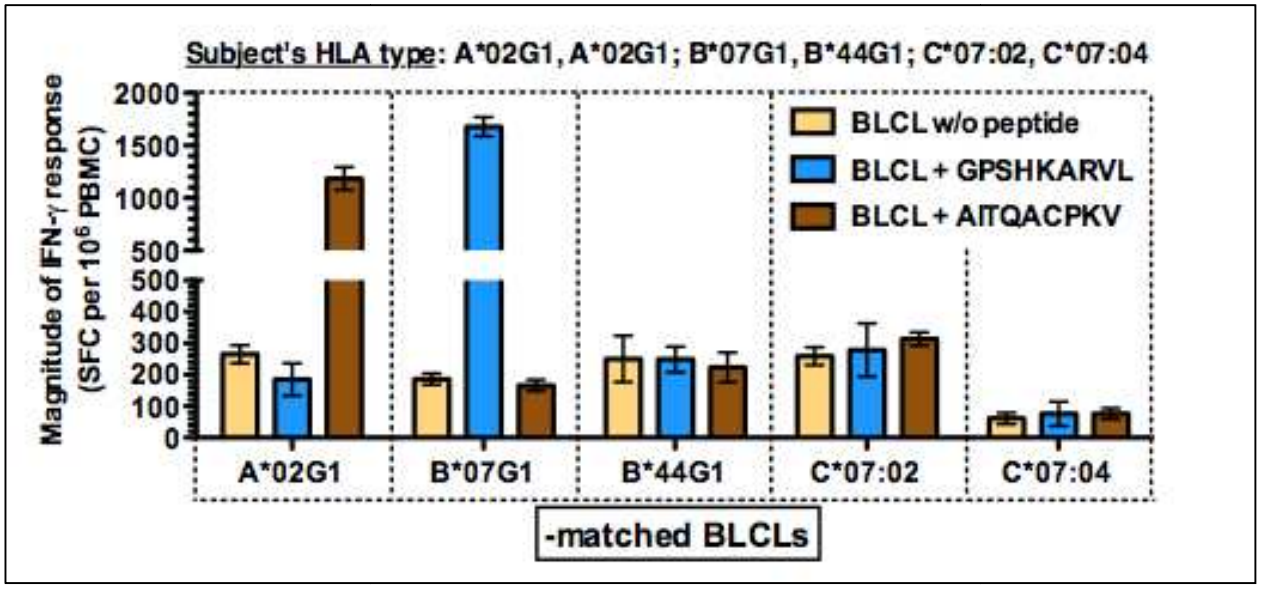


Figure 8: Example of HLA class I restriction of the new peptides.
Five mismatched BLCLs were pulsed with (blue or brown) or without (yellow) the GPSHKARV and AITQACPKV peptides. The matched BLCLs for each of the HLA class I of the subject (A*02**G**1; B*07**G**1, B*44**G**1; C*07:02 and C*07:04) are indicated. (**G** codes for reporting of ambiguous HLA alleles that have identical nucleotide sequences across the exons encoding the peptide binding domains). Standard IFN-γ ELISPOT assay was carried out after incubating subject's PBMC with or without peptide-pulsed BLCLs.

TCR Functional Avidity.

We used a previously described method [116], with modifications, to identify functional avidities of epitope-specific $CD8^+$ CTL using standard IFN-γ ELISPOT with 5-fold serial dilutions of their cognate epitopes ranging from $1x10^2$ µg/mL to 5.12×10^{-6} µg/mL. The molar peptide concentration was calculated based on the molecular weight of each peptide and plotted as a peptide concentration. The SFC per million of PBMC were plotted against the $\log_{10}$ peptide concentration. The peptide concentration (nM) that resulted in 50% of the maximum response (50% effective concentration [EC50]) was calculated with Graphpad Prism software (version 5.0d; GraphPad Software) (Figure 9).

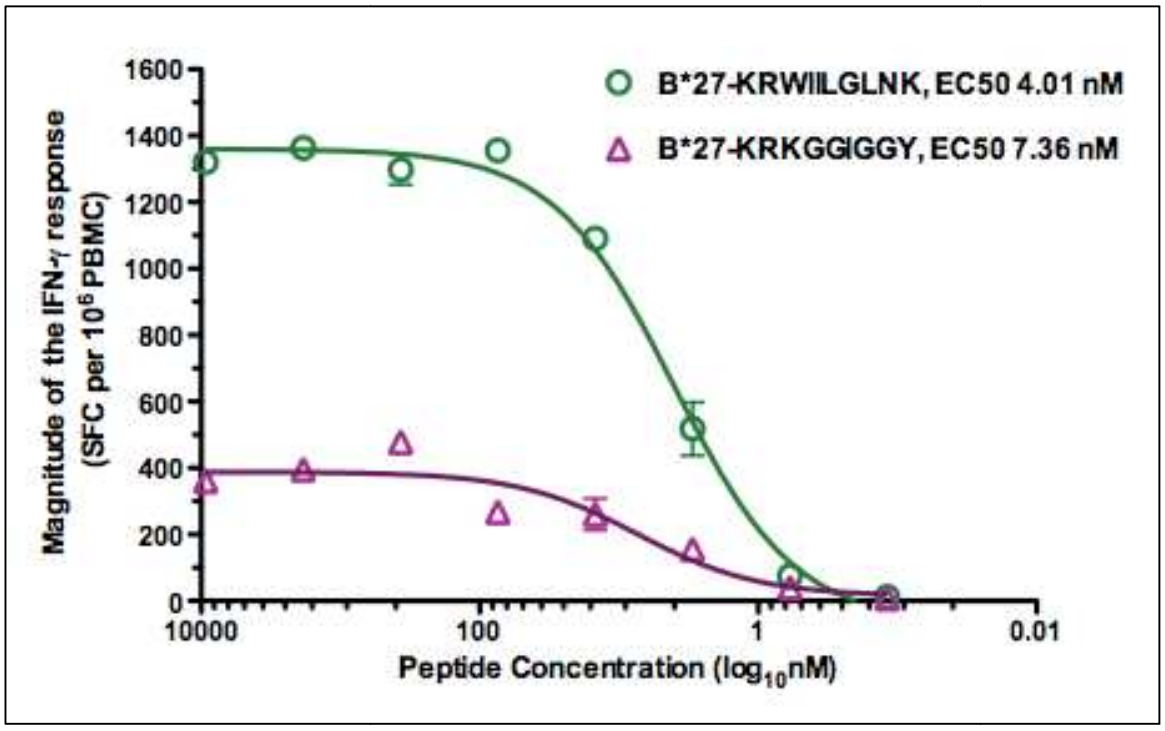


Figure 9: Example of TCR functional avidity experiment.
Standard IFN-γ ELISPOT assay was carried out with the indicated peptides at 100 µg/mL to 5.12×10^{-6} µg/mL. The regression curve is drawn with the Sigmoidal Fit tool in Graphpad Prism.

Definition of Conserved versus Variable Epitopes.

Conservation scores were computed using two common different approaches which have been previously used in the HIV field: (1) Epitope prevalence scores [135, 179] and (2) Shannon entropy [180]. All of these scores use a set of aligned HIV sequences as input. In this work we considered both a clade-B and a clade-M alignments [171] downloaded from the LANL DB of 2005, and all scores were computed using the same alignments. By using both a clade-B and clade-M alignments, we obtained conservation estimates based on sequences that were clade matched to our clinical cohort (clade-B) to those based on using a more diverse set of sequences from all clades.

Epitope Prevalence Scores – this score is based on the frequency of a given epitope in a set of circulating clade-B (bCSp) or group-M (mCSp) sequence alignments. Epitopes were classified into two groups as described previously with a slight modification [135]: *Conserved* epitopes were defined as those that were ≥80% prevalent in a given sequence set, and *relatively conserved* epitopes were ones that occurred between 50-80% in the data set. All epitopes with prevalence scores ≤50% were considered *variable*.

Shannon Entropy Scores – entropy is a common measure for conservation that measures the level of uncertainty in a random variable [180]. This measure is computed independently for each position in an aligned set of circulating clade-B

(bCSe) or group-M (mCSe) sequences. Positions with low entropy are more conserved than those with high entropy. The entropy CS of an epitope is the average entropy scores of all positions along the epitope [181-183]. In order to define thresholds for conserved vs. variable epitopes using this score, we used the distribution of 9mer scores over all HIV proteins. Conserved epitopes were defined as epitopes with entropy CS $\leq 20^{th}$ percentile of this distribution (i.e., were more conserved than 80% of all potential 9mers). Different cutoffs were computed for entropy CS computed on the clade-B or clade-M alignments.

Statistical Analysis.

Subjects' VL set points were log10 transformed for analysis to create a more symmetrical distribution. The distributions of a continuous variable, such as VL set point, were compared between two groups using a Mann Whitney (MW) test or paired data in two groups using a Wilcoxon matched-pair signed rank (WSR) test, among three or more groups using a Kruskal-Wallis Rank Sum (KW) test or paired data among three or more groups using a Friedman test. The differences in mean magnitude of $CD8^+$ T cell responses by protein types, estimated by a generalized estimating equation, was compared using a Wald test. Correlations between two continuous variables, such as impact of breadth or magnitude of $CD8^+$ T cell responses on VL set point, were computed using the Spearman Rank Correlation, denoted r. The association between two categorical variables was assessed using a Fisher's exact test. A univariate linear model was used to assess the effect of each variable, such as conserved response, on VL set point. A multivariate model was

used to examine the relationship between CS or breadth or interaction effect of CS and breadth on VL set point. P values are not adjusted for multiple comparisons. P values less than 0.05 are considered significant. All analyses were performed using Prism software (version 5.0d) and R statistical software (version 2.13.0).

Chapter III: Superior Control of HIV-1 Replication by $CD8^+$ T cells Targeting Conserved Epitopes: Implications for HIV Vaccine Design

Abstract

A successful HIV vaccine will likely induce both humoral and cell-mediated immunity, however the enormous diversity of HIV has hampered the development of a vaccine that effectively elicits both arms of the adaptive immune response. To tackle the problem of viral diversity, T cell-based vaccine approaches have focused on two main strategies (i) increasing the breadth of vaccine-induced responses or (ii) increasing vaccine-induced responses targeting only conserved regions of the virus. The relative extent to which set-point viremia is impacted by breadth or epitope-conservation of $CD8^+$ T cell responses elicited during early HIV-infection is unknown but has important implications for vaccine design. To address this question, we comprehensively mapped HIV-1 $CD8^+$ T cell epitope-specificities in 23 ART-naïve individuals during early infection and computed their conservation score (CS) by two different methods (prevalence and entropy) on clade-B and group-M sequence alignments. The majority of $CD8^+$ T cell responses were directed against variable epitopes ($p < 0.01$). Interestingly, increasing breadth of $CD8^+$ T cell responses specifically recognizing conserved epitopes was associated with lower viremia ($r = -0.65$, $p = 0.009$). Moreover, subjects possessing $CD8^+$ T cells recognizing at least one conserved epitope had 1.4 $\log_{10}$ lower viremia compared to those recognizing only variable epitopes ($p = 0.021$). The inverse association between viral control and the breadth of conserved $CD8^+$ T cell responses were independent of method of CS

definition and sequences used to determine conservation levels. Strikingly, targeting variable versus conserved epitopes was independent of HLA type ($p = 0.215$). The associations with viral control were independent of the functional avidity of $CD8^+$ T cell responses elicited during early infection. Taken together, these data suggest that the next-generation of T-cell based HIV-1 vaccines should focus on strategies that can elicit broad $CD8^+$ CTL responses to conserved epitopes of HIV-1.

Author's Summary

As we enter the 3rd decade since the first documented case of AIDS, this disease continues to be a major public health problem. An estimated 34.2million people living with HIV-1 at the end of 2011, 2.7 million new infections per year and 1.8 million deaths per year underscore the urgency to develop safe and effective vaccines [184]. Critical challenges include the enormous global diversity associated with the virus and subsequent challenges in eliciting effective immune responses. In the absence of intervention, only a small fraction of HIV infected individuals control viremia for prolonged periods of time [185]. Identifying and understanding factors associated with viral control should improve our understanding of the correlates of protection and inform future vaccine design. Here we performed a comprehensive analysis of $CD8^+$ T cell responses generated during early HIV-1 infection in a group of untreated HIV-infected individuals. We show that people who control virus replication elicit $CD8^+$ T cell responses against multiple conserved regions of the virus. Thus, future T-cell based vaccines should aim to elicit broad $CD8^+$ T cell responses recognizing conserved regions of HIV-1.

Introduction

An efficacious prophylactic HIV-1 vaccine will likely need to elicit both HIV-1-specific antibodies and T cell responses, as there is evidence that both arms of the adaptive immune system play an important role in viral control (reviewed in refs.[72, 186, 187]). Most previous candidate HIV-1 vaccines designed to induce protective antibody or $CD8^+$ CTL responses have failed to prevent infection or reduce viral load (reviewed in ref [188]). The recent RV144 trial has only shown a marginal protection in preventing infection without an effect on viral load [160] and this modest protection appears to be mediated by antibody responses [161]. However, the RV144 trial lacked proper sample acquisition to optimally correlate T cell responses with protection. Furthermore, the immunogen included in the RV144 vaccine may not be optimal for eliciting protective T cell responses. Indeed the most effective prophylactic vaccines tested to date in non-human primates (NHP) have all induced robust $CD8^+$ T cell responses that correlate with protection [189, 190]. These studies underscore the necessity to optimize immunogens to induce both humoral and cell-mediated arms of the adaptive immune system.

Several lines of evidence demonstrate the role of $CD8^+$ T responses in controlling or preventing HIV infection providing a strong rationale for renewed efforts to optimize T-cell-based immunogens [72, 77]. Evidence for control of established infection is emphasized by studies showing the link between HLA types and viral control [88-90]. Although the majority of infected people progress to AIDS within 10 years without antiretroviral therapy, the pace of clinical progression is highly variable. Certain MHC class I alleles are associated with rapid (B*35Px allele

groups: B*35:02, B*35:03, B*35:04, and B*53:01) vs. slow (B*27 and B*57 allele groups) progression to AIDS [85, 87] implicating the role for $CD8^+$ T cells in HIV control. In addition, the extensive literature on viral escape from $CD8^+$ T cells [91-93] supports the view that this arm of immune system is applying pressure on the virus. While it has been repeatedly shown that T cells can control established infection, there is also convincing evidence that $CD8^+$ T cells can prevent infection. The presence of cross-reactive HIV-specific $CD8^+$ T cell responses in highly exposed but persistently uninfected women [129-132] suggest that $CD8^+$ T cells can prevent HIV infection. Previous studies have shown that a live attenuated SIV vaccine (SIVmac239Δnef) confer a significant level of protection against heterologous SIV challenge [80, 191]. $CD8^+$ T cells depletion studies further demonstrated that this protection was indeed mediated by cellular immune responses [80]. Moreover, other studies by this group have shown that induction of SIV-specific effector memory T cells using a CMV vector provides protection against SIV infection [190].

While data on HIV control and prevention do support efforts to design T cell based HIV vaccines, the extraordinary worldwide diversity of HIV presents a huge challenge. To tackle this problem, T cell-based vaccine approaches have come up with two main strategies: the Mosaic Immunogen, which emphasizes increasing the breadth of vaccine-induced responses [166, 167], and the conserved immunogens, which emphasizes increasing the breadth of vaccine-induced T-cell responses only to highly conserved viral regions [170, 171, 192, 193]. While both approaches are theoretically sound, there are no current data suggesting that either approach will be successful in inducing T cells with superior antiviral efficacy.

Despite a considerable research effort in this area, the qualities that define "protective" HIV-specific $CD8^+$ CTL are still unknown, making rationale design of vaccines difficult. While numerous studies provide strong evidence that $CD8^+$ CTL play an important role in immune control of HIV, a significant number of virus-specific $CD8^+$ CTL are also detectable in individuals who fail to control viremia [117, 119], suggesting that not all $CD8^+$ CTL responses are created equally but that they differ significantly in their ability to control viral replication. Although the mechanism(s) underlying these differences are not entirely clear, understanding them is critical for development of prophylactic vaccines. Following four qualities of HIV-1-specific $CD8^+$ CTL have been previously suggested to play an important role in controlling HIV-1 replication: frequency, breadth, functionality and specificity. HLA-tetramer based studies initially demonstrated an inverse correlation between frequency of A*0201-restricted HIV-specific $CD8^+$ CTL and plasma viral load [114]. However, subsequent IFN-γ ELISPOT-based studies examining the frequency of IFN-γ-secreting HIV-specific $CD8^+$ CTL did not show an inverse correlation with viral load [115, 116, 118, 119, 121]. Similarly, the breadth of HIV-specific $CD8^+$ CTL responses has not shown an inverse correlation with plasma viral load [115, 116]. Increased poly-functionality and proliferative capacity of $CD8^+$ CTL has been associated with slow HIV disease progression [102, 103, 194, 195], although a recent study showed no association between antiviral efficacy and poly-functionality, or proliferative capacity of $CD8^+$ CTL [196]. Thus, to date, the precise qualities of effective epitope-specific $CD8^+$ CTL responses that may be responsible for immune control of HIV remain unclear. Multiple studies have shown an inverse correlation

between Gag-specific $CD8^+$ CTL responses and viral load [124, 133] and an association between $CD8^+$ T cell responses targeting conserved regions with improved disease outcome [197, 198]. Studies showing a positive correlation between escape in the well-defined B*27-restricted KK10 epitope and increased viral load [92, 95] suggest that $CD8^+$ CTL recognizing certain epitopes are efficient at controlling viral replication. In addition, $CD8^+$ CTL responses restricted by protective alleles have been shown to target highly conserved regions (Gag p24) of the virus and escape from these Gag-specific $CD8^+$ CTL responses was either not possible or occurred with a coincident viral fitness cost [101, 134]. Furthermore, we have shown that the association between HIV-1 disease progression and distinct MHC class I alleles is linked to $CD8^+$ CTL recognition of conserved HIV-1 epitopes early in infection [199]. Although, these studies suggest that the epitope-conservation of $CD8^+$ T cells plays an important role in mediating control of HIV infection, a comprehensive study that examines the conservation of the complete $CD8^+$ T cell repertoire in an acutely infected therapy naïve individuals, and how conservation score (CS) of the recognized epitopes correlates with viral control has not been conducted to date.

In the present study, we comprehensively mapped the $CD8^+$ T cell response in 23 therapy naïve individuals during early HIV-1 infection to address the question of whether CS of $CD8^+$ CTL epitopes play an important role in viral control. Additionally, we assessed the impact of breadth, magnitude and functional avidity of $CD8^+$ CTL responses elicited in early HIV-1 infection with viral control. Our data provide the first clear evidence that the majority of $CD8^+$ CTL responses elicited

during early HIV-1 infection are directed against variable epitopes. The data show that the breadth of initial $CD8^+$ CTL responses to HIV is important for the subsequent control of viremia. Furthermore, we show that individuals possessing $CD8^+$ CTL directed against at least one conserved epitope early in infection have lower viral load (VL) set point than those individuals possessing CTL directed against variable epitopes. These findings suggest that broadly directed HIV-specific $CD8^+$ CTL responses specifically recognizing conserved epitopes elicited during early infection are superior at controlling viral replication *in vivo*, providing important implications for rationale design of future T cell-based immunogens.

Results

Assessment of HIV-1-specific T-cell responses using a Potential T Cell Epitope [PTE] peptide set-based IFN-γ ELISPOT Assay.

Twenty-three HIV-infected individuals from the Seattle Primary Infection Cohort were evaluated for the epitope specificity, breadth, magnitude and functional avidity of HIV-1-specific $CD8^+$ CTL responses elicited during early infection. The median time to evaluation was 69 days post infection. To maximally increase the probability of identifying responses to novel epitopes as well as increase the ability to accurately assess the breadth of $CD8^+$ T cell responses, we used PTE peptide sets [126] to map the T cell responses against the entire HIV-1 proteome. The breadth and magnitude of virus-specific T-cell responses differed significantly among individuals at different stages of HIV-1 infection. We found broader $CD8^+$ CTL responses in people who have been infected for a longer period of time (Spearman Rank Correlation, $r = 0.63$, $p < 0.002$, Figure 10). The median number of $CD8^+$ T cell epitopes detected was 7 (range, 3-13), mean magnitude of the epitope-specific T cell response was 779 SFC/10^6 PBMC (median, 516; range, 57-3836) and the mean total magnitude was 5838 SFC/10^6 PBMC (median, 5069; range 970-14906). The HIV-1-specific $CD8^+$ T cells induced during early infection in the 23 individuals recognized a total of 123 distinct optimal epitopes (8-11mers) spanning all HIV-1 proteins except Vpu and were restricted by 27 distinct class I alleles. Responses were detected to Gag 19 (83%), Pol 17 (74%), Env 17 (74%), Nef 15 (65%) and other accessory proteins 11 (48%) in subjects. Interestingly, 39 (32%) were novel epitopes. The MHC restrictions of 28% of these novel epitopes were identified

(Table 2). Novel epitopes were discovered in Env (n = 13), Pol (n = 11), Gag (n = 8), Accessory proteins (n = 6) and Nef (n = 1). Taken together, these data indicate that the use of only known HLA restricted epitopes would lead to an under-estimation of the true $CD8^+$ CTL response in HIV-1-infected individuals.

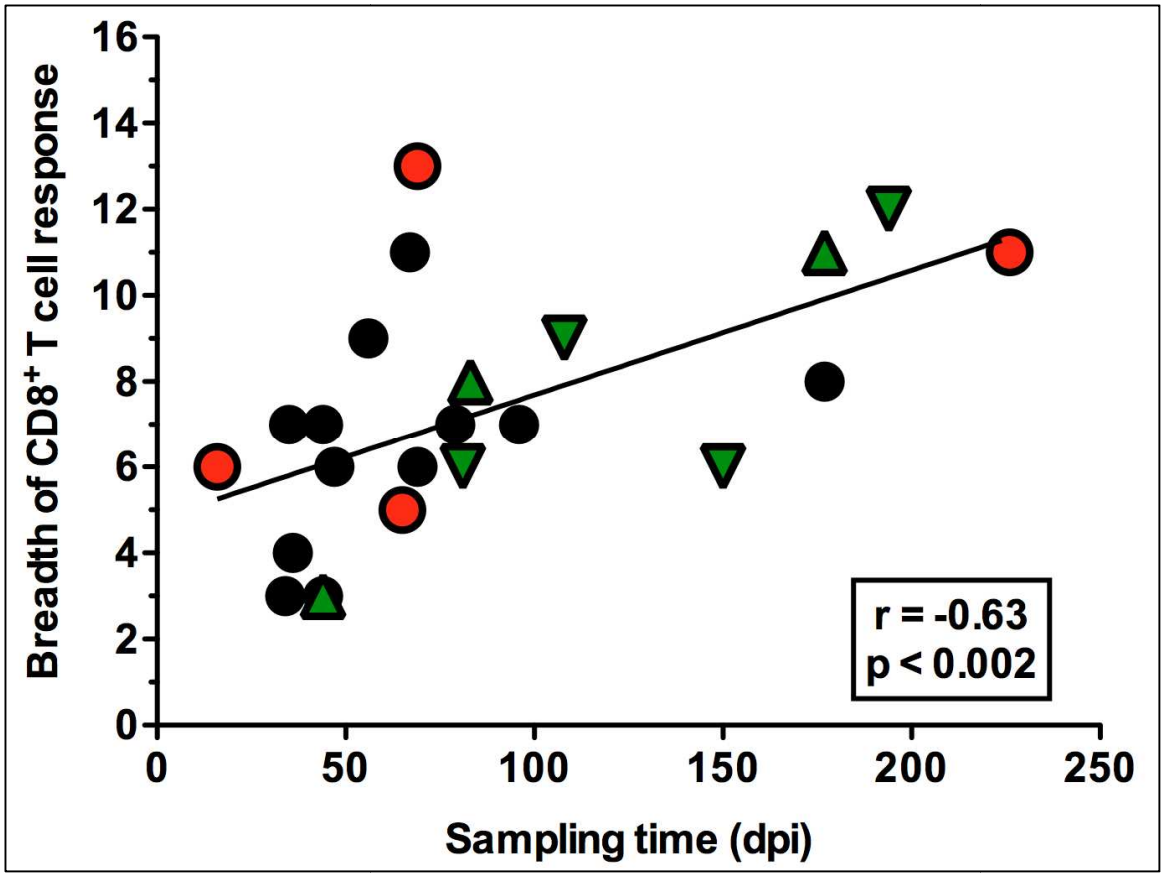


Figure 10: Breadth of $CD8^+$ T cell responses in relation to duration of infection. The number of $CD8^+$ T cell epitopes recognized in each individual was plotted against the duration of infection (dpi). Subjects possessing B*35Px, B*27 and B*57 alleles are represented by red circles, green triangles and inverted green triangles respectively. The solid line represents a regression line.

Table 2: HIV-1 specific CD8$^+$ T cell responses against novel epitopes in early infection: epitope specificity, MHC restriction, and frequency

PTIDs	HLA Class I type	HIV protein	HXB2 site[a]		Epitope Sequence[b]	HLA restriction[c]	SFC/10^6 PBMC
1545	A*0101, 29; B*3801, 4403; Cw*1203, 1601	gp160	620	628	**NEIWDNMTW**	**B*4403**	3144
		Integrase	227	235	**YRDSRDPLW**	**B*3801**	1351
1577	A*0201, 0205; B*2705, 4901; Cw*0202, 0701	p17	11	19	**GELDRWEKI**	**B*4901**	1033
		p17	15	23	**RWEKIRLRP**	**A*0205**	1013
		gp160	777	785	**IVTRIVELL**	**A*0205**	278
		Rnase	60	68	**QYALGIIQA**	**A*0201**	175
		Rnase	77	85	**LVSQIIEQL**	**A*0205**	1086
		Integrase	165	173	**VRDQAEHLK**	**A*0205**	607
1585	A*0101, 4801; B*4001, 5701; Cw*0304, 0602	p2p7p1p6	119	127	ELYPLASLR		278
1706	A*0201, 3201; B*0801, 5501; Cw*0303, 0701	gp160	704	712	VINRVRQGY		693
1735	A*2902, 3201; B*35G1, 4403; Cw*04G1, 1601	gp160	704	712	VINRVRQGY	B*4403	77
1785	A*0101, 03G1; B*07G1, 3701; Cw*0602, 0702	p17	87	95	CVHQRIEVK	A*0301	270
		gp160	32	40	EKLWVTVYY	Cw*0602,0702	72
		RT	206	214	REHLLRWGF	Cw*0702	938
1793	A*24G1, 24G1; B*3502, 3801; Cw*04G1, 1203	Vif	94	102	YSTQVDPDL	B*3801	725
		gp160	44	52	VWKDAETTL	B*3801	458
		gp160	621	629	EIWDNMTWL	B*3801	148
1815	A*03G1, 1101; B*3503, 5301; Cw*04G1, 04G1	gp160	621	629	DIWDNMTWM	B*3503	516
		Nef	175	183	DPEKEVLVW	B*5301	366
		p24	176	184	QASQDVKNW	B*5301	183
1865	A*2301, 68G1; B*3543, 5703; Cw*0102, 0210	Vif	61	69	DARLVITTY	B*3501	278
		Pol	23	31	ANSPTSREL		286
1871	A*0101, 24G1; B*0801, 5701; Cw*0602, 07G1	gp160	805	814	QELKNSAVSL	B*0801	143
		p17	87	95	CVHQRIEVK	A*02	633
9148	A*2301, 2601; B*3801, 3801; Cw*04G1, 1203	gp160	53	61	**FCASDAKSY**	**B*3801**	650
		Integrase	11	19	**EHEKYHNNW**	**B*3801**	973
		Integrase	95	103	QETAYFILK		375
		Integrase	227	235	**YRDSRDPLW**	**B*3801**	1020
9150	A*02G1, 02G1; B*07G1, 44G1; Cw*0702, 0704	gp160	200	208	**AITQACPKV**	**A*02G1**	1608
9166	A*0101, 0101; B*2705, 5201; Cw*0201, 1202	Tat	39	47	ITKGLGISY		305
		gp160	836	845	IGRAILHIPR	B*2705	638
		p17	81	89	TVATLYCVH		345
9167	A*02G1, 02G1; B*1302, 35G1; Cw*0419, 0602	Vif	61	69	DARLVITTY	B*35	173
		p24	8	17	GQMVHQAISP		2090
9170	A*02G1, 02G1; B*40G1, 44G1; Cw*0304, 0501	Vif	102	111	LADQLIHLYY		493
		Vpr	12	20	REPYNEWTL	B*4001	731
		p17	119	128	AADTGNSSQV		1186
9176	A*0101, 02G1; B*0801, 3503; Cw*04G1, 07G1	p2	6	14	QVTNSATIM	B*3503	76
		Rnase	21	29	RGRQKVVSL	B*0801	111
		gp160	848	856	RQGFERALL	A*02	1423
		gp160	814	822	LLNTTAIVV	A*0201	362
9187	A*24G1, 24G1; B*3505, 40G1; Cw*0304, 04G1	Vif	116	124	SESAIRNAI	B*4002	367
		Vpr	12	20	REPYNEWTL	B*4002	187
9195	A*02G1, 24G1; B*44G1, 5701; Cw*0501, 6002	Integrase	114	123	HTDNGSNFTS		1146

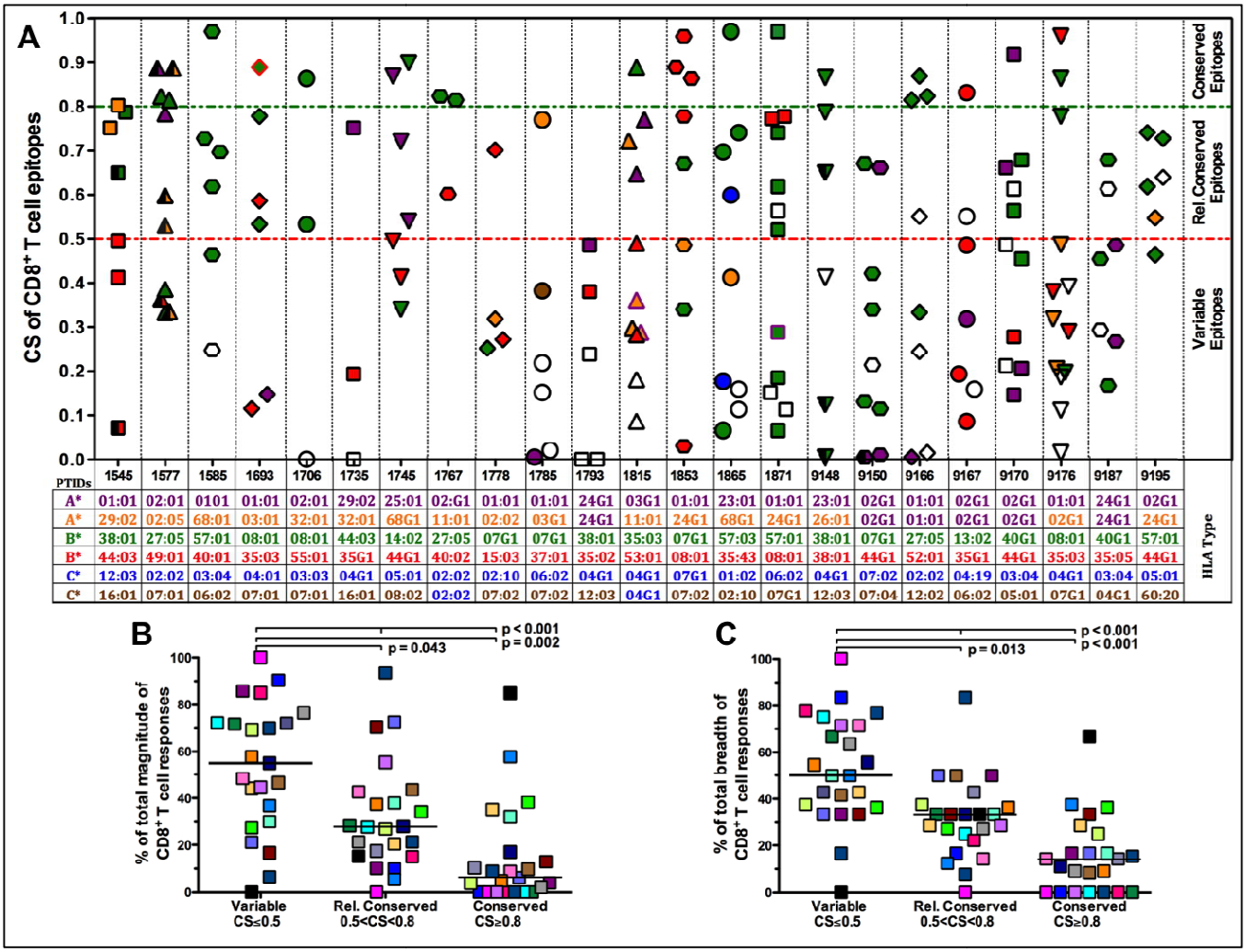
[a] Epitope position (based on HXB2 amino acid sequence) in HIV-1 proteins

[b] Amino acid sequence of identified new T-cell epitopes, with newly defined HLA restriction shown in bold

[c] Predicted Restricting HLA allele of the novel epitope, with the newly confirmed HLA restriction shown in bold

Early CD8$^+$ T cell responses are preferentially directed against variable epitopes.

The CS of targeted epitopes was defined as the proportion of HIV-1 clade-B amino acid sequences in the Los Alamos HIV-1 sequence database (LANL DB) that include the epitope. *Conserved* and *variable* epitopes were defined as those that were prevalent in ≥80% and ≤50% in a given sequence alignment, respectively. The majority of epitopes targeted early in HIV infection were variable, 60% (73 out of 123), and only 13% (16 out of 123) were conserved (Figure 11A). Most of the conserved responses were directed to Gag and Pol, with a median CS of targeted Gag and Pol epitopes being higher than the median CS of Env epitopes (MW, $p = 0.033$, and $p = 0.001$, respectively, Figure 12A). However, the magnitude of responses elicited by Gag and Pol epitopes was not significantly different than those elicited by Nef, Env and Acc epitopes (Wald, $p = 0.930$, Figure 12B). Twenty-five percent (4/16) of conserved epitopes identified in our study were restricted by favorable alleles. Three of these conserved epitopes KRWIILGLNK (B*27-KK10), KRKGGIGGY (B*27-KY9) and KAFSPEVIPMF (B*57-KF11) were recognized in 100% of individuals who possessed these alleles. In contrast, only one conserved epitopes (TVLDVGDAY) was restricted by an unfavorable allele (B*35:01). The remaining 69% (11/16) of conserved epitopes that were identified in our study were restricted by neutral alleles. Most of the novel epitopes, 74% (29/39) were variable and only 5% (2/39) were conserved.



PTIDs	1545	1577	1585	1693	1706	1735	1745	1767	1778	1785	1793	1815	1853	1865	1871	9148	9150	9166	9167	9170	9176	9187	9195
A*	01:01	02:01	0101	01:01	02:01	29:02	25:01	02:G1	01:01	01:01	24G1	03G1	01:01	23:01	01:01	23:01	02G1	01:01	02G1	02G1	01:01	24G1	02G1
A*	29:02	02:05	68:01	03:01	32:01	32:01	68G1	11:01	02:02	03G1	24G1	11:01	24G1	68G1	24G1	26:01	02G1	01:01	02G1	02G1	02G1	24G1	24G1
B*	38:01	27:05	57:01	08:01	08:01	44:03	14:02	27:05	07G1	07G1	38:01	35:03	07G1	57:03	57:01	38:01	07G1	27:05	13:02	40G1	08:01	40G1	57:01
B*	44:03	49:01	40:01	35:03	55:01	35G1	44G1	40:02	15:03	37:01	35:02	53:01	08:01	35:43	08:01	38:01	44G1	52:01	35G1	44G1	35:03	35:05	44G1
C*	12:03	02:02	03:04	04:01	03:03	04G1	05:01	02:02	02:10	06:02	04G1	04G1	07G1	01:02	06:02	04G1	07:02	02:02	04:19	03:04	04G1	03:04	05:01
C*	16:01	07:01	06:02	07:01	07:01	16:01	08:02	02:02	07:02	07:02	12:03	04G1	07:02	02:10	07G1	12:03	07:04	12:02	06:02	05:01	07G1	04G1	60:20

Figure 11. Variable epitopes are preferentially recognized in early HIV-1-infection.

(A) A total of 123 epitopes identified in 23 subjects with early HIV-1-infection were plotted against their CS. The HLA information of each subject is also shown. CS of each epitope was calculated as the frequency of an exact epitope match in aligned clade-B sequences. Each symbol represents an epitope recognized by HIV-specific $CD8^+$ T cells in an individual. Symbols are colored to denote the HLA restriction of the epitope. (B) The percentage of total magnitude of IFN-γ responses in each individual directed against epitopes by CS grouping (Friedman, $p < 0.001$). (C) The percentage of total breadth of $CD8^+$ CTL responses in each individual directed against epitopes by CS grouping (Friedman, $p < 0.0001$). (B–C) The % of the total magnitude and breadth of $CD8^+$ CTL responses targeting "Variable", "Relatively Conserved" and "Conserved" epitopes. Individual subjects are denoted by a specific color. Horizontal lines indicate median values; statistical significance was assessed by matched test for 2 groups (Wilcoxon matched-pairs signed rank test) or multiple groups (Friedman test).

Furthermore, we assessed the magnitude and breadth of $CD8^+$ CTL responses directed against epitopes with different CS in each individual. Interestingly, we found that in any individual subject, significantly higher proportions of $CD8^+$ CTL responses were directed against variable epitopes than those directed against conserved epitopes (WSR, $p = 0.002$, Figure 11B and $p < 0.001$, Figure 11C respectively). A median of around 50% of the total magnitude and breadth of responses were directed against variable epitopes (Figure 11B and Figure 11C respectively). Collectively, This emphasizes the fact that the most of the early $CD8^+$ CTL responses to HIV-1 are directed against variable epitopes.

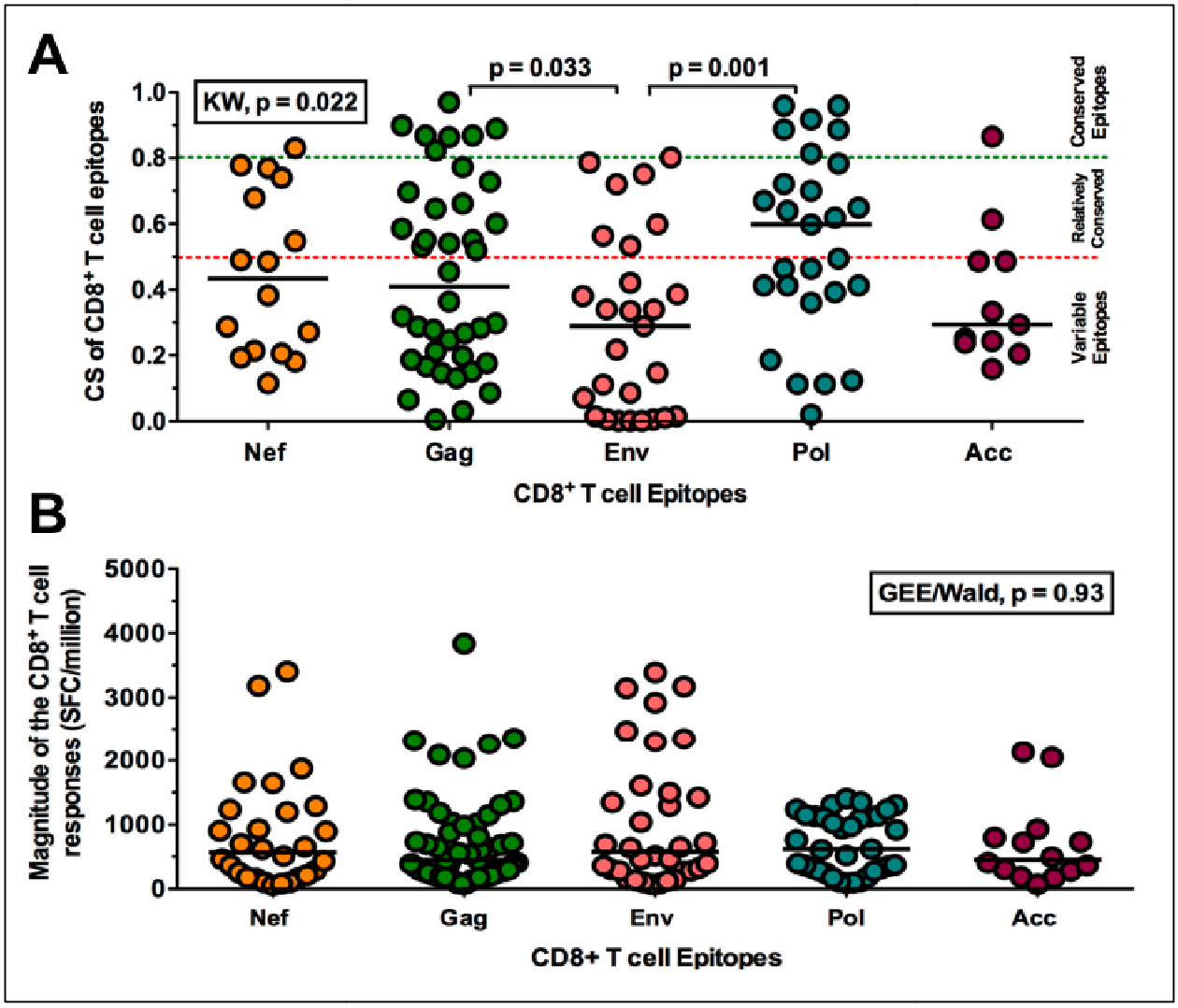


Figure 12. Majority of conserved epitopes targeted in early HIV-1 infections are Gag and Pol.

The CS and magnitude of $CD8^+$ T cell responses of total of 123 epitopes identified in 23 subjects were analyzed based on their protein types. (A) The median CS of $CD8^+$ T cell epitopes by HIV protein types (Kruskal-Wallis, p = 0.022). (B) The median magnitude of responses (SFC/M) by protein types (Kruskal-Wallis, p = 0.873). Horizontal lines indicate median.

Lower viral load is associated with $CD8^+$ CTL responses directed to conserved epitopes.

To evaluate the impact of $CD8^+$ T cells targeting epitopes with higher CS on the control of viral replication, we compared the plasma VL set point of individuals who mounted responses against at least one conserved epitope to individuals who did not mount such responses. Interestingly, increasing breadth of $CD8^+$ T cell responses recognizing conserved epitopes was associated with lower viremia ($r = -0.65$, $p = 0.009$, Figure 13A). Next, we compared the plasma VL set point of individuals who mounted responses against at least one conserved epitope to individuals who did not mount such a response. Individuals possessing $CD8^+$ CTL recognizing even one conserved epitope had lower VL set point than those who did not recognize any conserved epitope (MW, $p = 0.018$, Figure 13B). In a univariate linear model, the mean VL set point in subjects who mounted $CD8^+$ CTL responses against at least one conserved epitope was 1.4 $\log_{10}$ lower than those who failed to target at least one conserved epitope (95% CI: -2.5, -0.24, $p = 0.021$). Collectively, these data suggest that possessing $CD8^+$ CTL recognizing conserved epitopes is associated with lower VL set point.

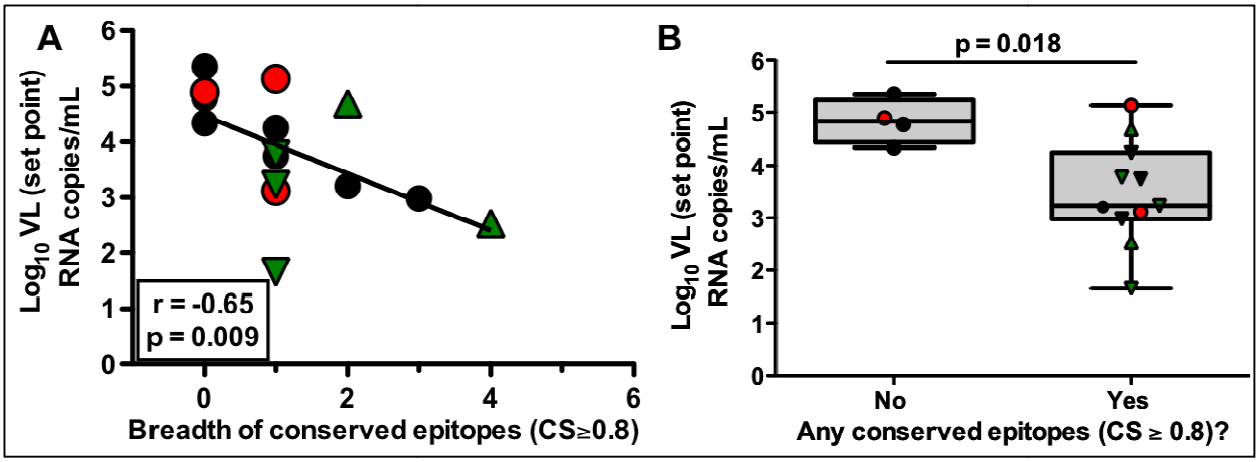


Figure 13. CD8$^+$ CTL responses against conserved epitopes are associated with viral control.
(A) The plasma VL set point was compared to breadth of CD8$^+$ T cell responses directed against conserved epitopes (Spearman Rank Correlation, r = -0.65, p = 0.009). The solid line represents a regression line. (B) The median plasma viral set point in individuals who mounted CD8$^+$ CTL responses against at least one conserved epitope (Mann Whitney, p = 0.018). (A to B) Subjects possessing B*35Px, B*27 and B*57 alleles are represented by red circles, green triangles and inverted green triangles respectively.

Targeting conserved epitopes was independent of possession of favorable alleles.

Given that, five of the eleven individuals who mounted CD8$^+$ CTL responses against conserved epitopes had a protective HLA allele, we next assessed whether the association between CD8$^+$ T cells recognizing conserved epitopes and lower VL set point may have been due to the fact that these conserved epitopes were recognized by CD8$^+$ CTLs restricted by protective alleles. CS of CD8$^+$ T cells epitopes restricted by protective allele groups (such as HLA-B*27, HLA-B*57), neutral alleles and unfavorable allele groups (such as HLA-B*35Px alleles) was compared. Surprisingly, the median CS of CD8$^+$ T cell epitopes restricted by

protective alleles was not significantly different from those restricted by neutral or unfavorable alleles (KW, p = 0.215, Figure 14A).

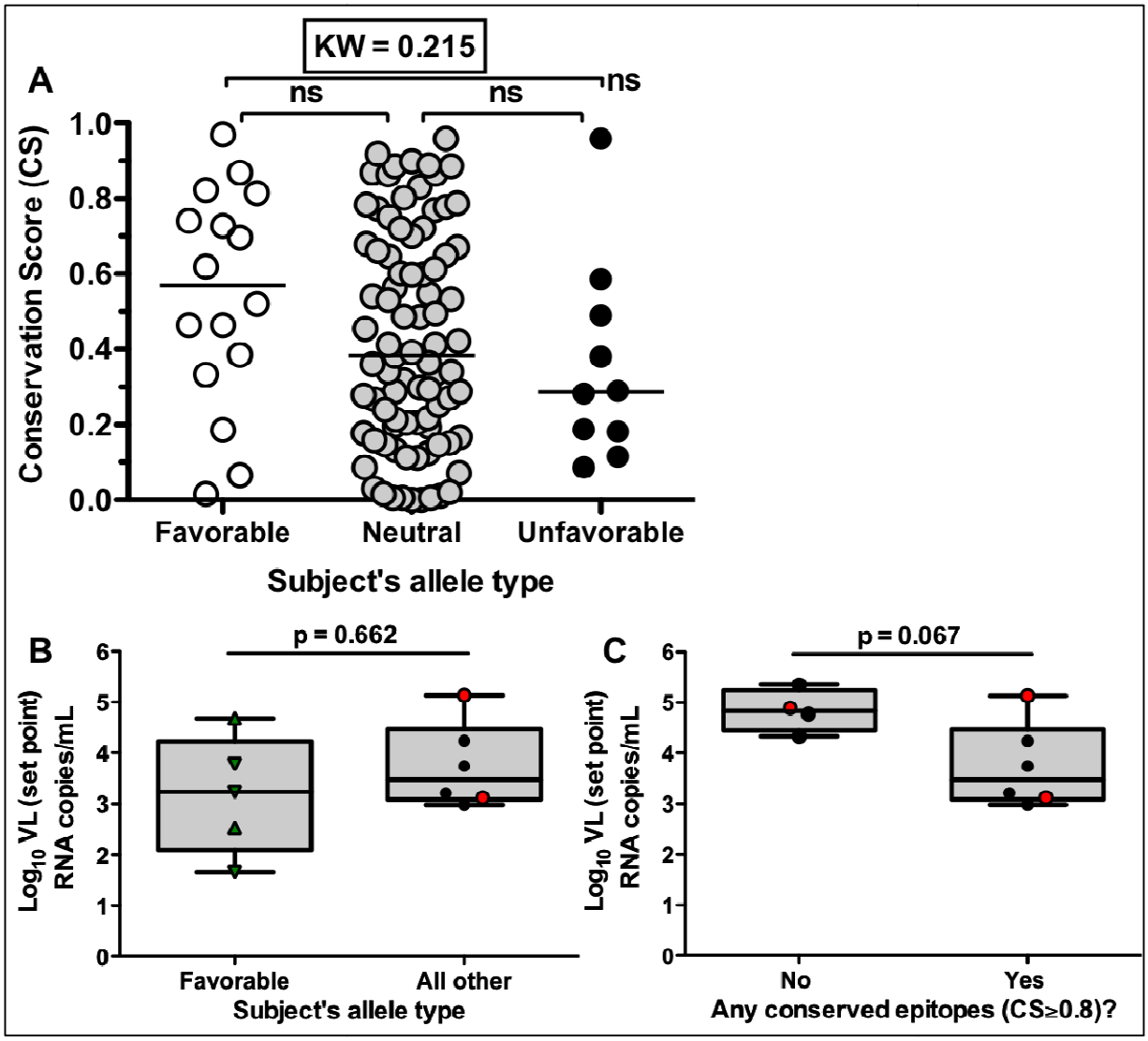


Figure 14. Targeting conserved epitope was independent of possession of favorable alleles.
(A) The median CS of epitopes by HLA group (Kruskal-Wallis, p = 0.215). Horizontal lines indicate median value. (B) The median plasma VL set point in individuals (subjects not recognizing at least one conserved epitopes were excluded on this analysis) recognizing at least one conserved epitope by allele types (Mann Whitney, p = 0.662). (C) The median plasma VL set point in individuals (not possessing favorable alleles, subjects possessing favorable alleles were excluded) who elicited $CD8^+$ CTL responses against at least one conserved epitope (Mann Whitney, p = 0.067). (B to C) Subjects possessing B*35Px, B*27 and B*57 alleles are represented by red circles, green triangles and inverted green triangles respectively.

To assess whether the association between $CD8^+$ T cells recognizing conserved epitopes with lower VL set point may have been due to the possession of favorable alleles, we compared average plasma VL set point in individuals with their HLA types. Unexpectedly, the median plasma VL set points were not significantly different by HLA grouping although there was a trend for lower VL set point in individuals possessing favorable alleles (KW, $p = 0.296$, Figure 15A-C). Next, we performed a subset analysis excluding all the subjects who did not recognize any conserved epitopes in order to compare the VL set points in individuals possessing favorable alleles versus those who did not. There was no statistically significant difference in VL set points (MW, $p = 0.662$, Figure 14B). In another subset analysis, we excluded subjects possessing favorable alleles in order to specifically compare VL set points in subjects who recognized at least one conserved epitope versus those who did not recognize any conserved epitopes. Although, we did not have enough power to detect differences, there was a trend of lower VL set point in subjects who mounted $CD8^+$ CTL responses against at least one conserved epitope (MW, $p = 0.067$, Figure 14C). Collectively, these data suggest that possessing $CD8^+$ CTL recognizing conserved epitopes is associated with lower VL set point.

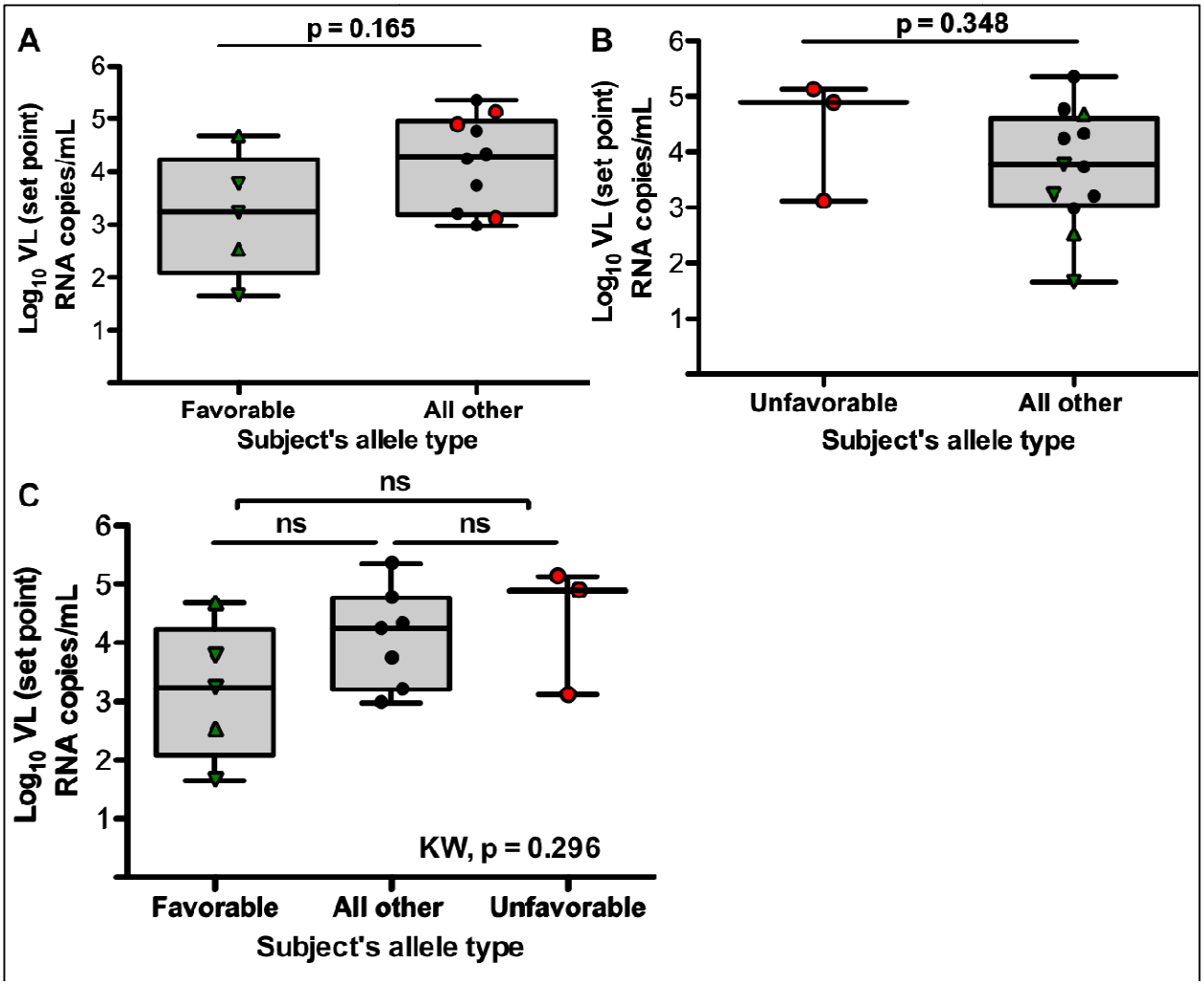


Figure 15. HIV-1 VL set points are not significantly different by HLA types. (A-C) The median plasma VL set point in individuals possessing favorable, unfavorable or all other alleles (Kruskal-Wallis, p = 0.296). Horizontal lines indicate median. Subject possessing B*35Px, B*27 and B*57 allele are represented by red circles, green triangles and inverted green triangles respectively.

Association between total magnitude of $CD8^+$ CTL responses with viral control.

We next evaluated the impact of total magnitude of $CD8^+$ CTL responses on the control of viral replication. For this purpose, we assessed the magnitude (SFC/M of PBMC) of all epitope-specific $CD8^+$ CTL responses. First, we attempted to tease out the relationship between CS of epitopes and the magnitude of the response elicited by corresponding epitopes. There was no correlation between magnitude of $CD8^+$ CTL responses and the CS of CTL epitopes ($r = -0.02$, $p = 0.820$, Figure 16A). We then examined the plasma VL set point of an individual with total magnitude of $CD8^+$ CTL responses. Interestingly, the total magnitude of $CD8^+$ CTL responses correlated inversely to the plasma VL set point ($r = -0.56$, $p = 0.031$, Figure 16B). We next performed subset analysis excluding subjects who mounted responses against conserved epitopes in order to avoid a bias introduced from subjects mounting $CD8^+$ CTL responses against conserved epitopes. This association between total magnitude of $CD8^+$ CTL responses and VL set point was not significant in the absence of individuals mounting responses to conserved epitopes ($r = -0.40$, $p = 0.75$, data not shown). As expected, total magnitude of Gag-specific $CD8^+$ CTL responses correlated inversely to the plasma VL set point ($r = -0.82$, $p < 0.001$, Figure 16C). We also observed a trend of inverse correlation between total magnitude of Pol-specific responses and VL set point ($r = -0.49$, $p = 0.066$, Figure 16D). In univariate analysis, the estimated difference in mean $\log_{10}$ VL set point between subjects who mounted responses against Pol epitopes compared to those who did not was -0.57 (95% CI: -1.1, -0.026; $p = 0.041$). However, there was no

significant correlation between total magnitude of Nef-specific, Env-specific and other Accessory protein-specific $CD8^+$ CTL response with VL set point (data not shown).

In order to identify the role of magnitude of $CD8^+$ CTL responses directed against conserved epitopes in viral control, we next compared total magnitude of $CD8^+$ CTL responses recognizing conserved epitopes with VL set point. We observed a trend of inverse correlation between magnitude of conserved responses and VL set point ($r = -0.48$, $p = 0.068$, Figure 16E). We further compared the magnitude of responses against conserved Gag or Pol epitopes with VL set point. We found a trend for an inverse correlation between magnitude of conserved Gag epitopes and VL set point ($r = -0.45$, $p = 0.065$, Figure 16F). The magnitude of conserved Pol epitopes did not correlate with VL set point ($r = -0.35$, $p = 0.198$, data not shown). Taken together, these data suggest that the magnitude of $CD8^+$ CTL responses directed against Gag and a trend in other conserved epitopes in early HIV-1-infection contribute to viral control.

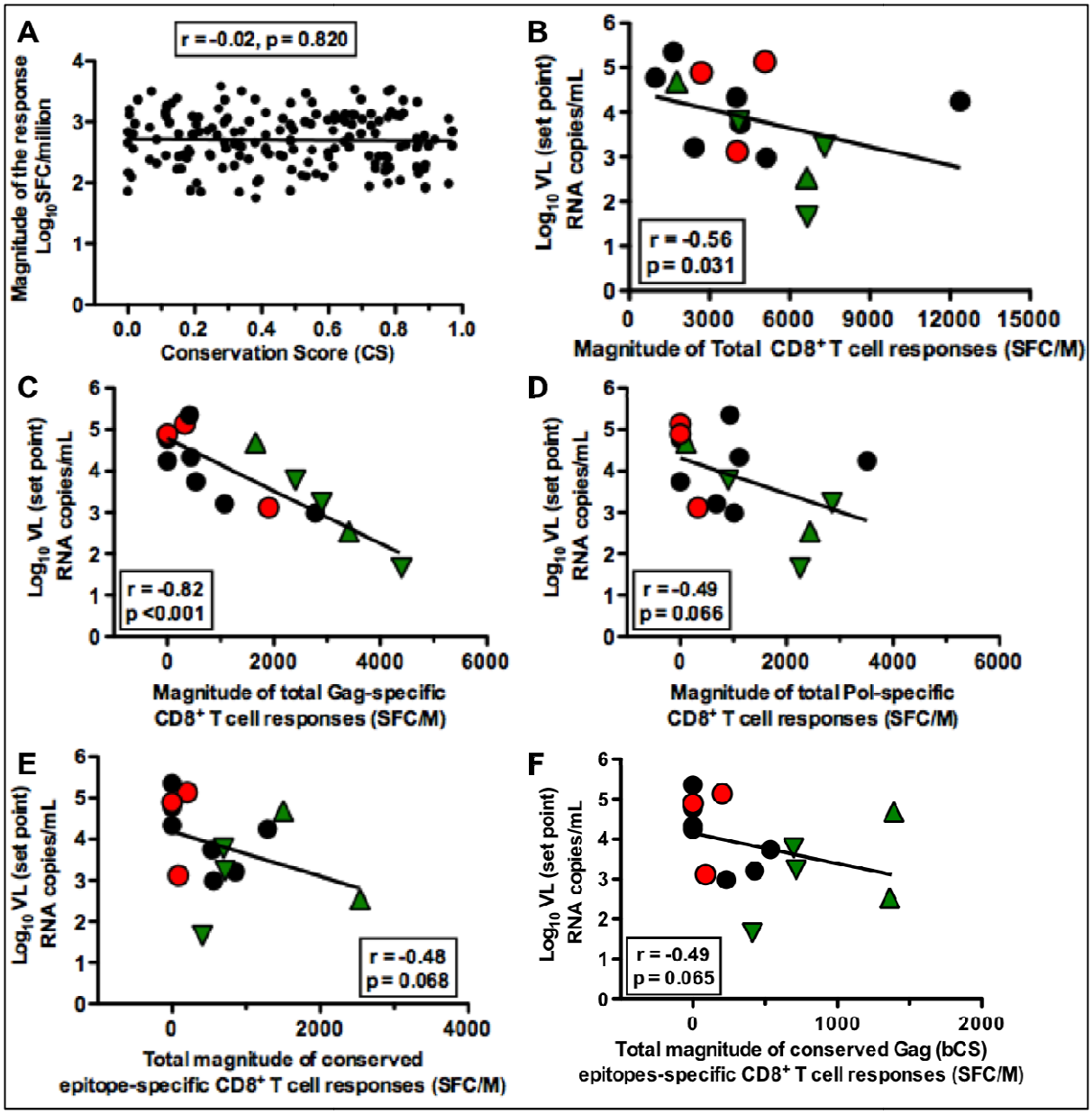


Figure 16. Correlation between magnitude of HIV-1-specific CD8$^+$ T cell responses and viremia.

VL set point was compared to magnitude of CD8$^+$ T cell response. (A) Correlation between magnitude and CS of CD8$^+$ T cells. (B) Average plasma VL set point was compared with total magnitude of the CD8$^+$ T cell response (SFC/M) elicited in each individual (r = -0.56, p = 0.031). (C-D) Correlation between total magnitude of CD8$^+$ T cell responses elicited by Gag or Pol epitopes with plasma VL set point. (E-F) Correlation between total magnitude of CD8$^+$ T cell responses elicited by conserved epitopes (E), and Gag conserved epitopes (F) with plasma VL set points. (B to F) Correlation statistics were analyzed using Spearman Rank Correlation. The solid line represents a regression line. Subject possessing B*35Px, B*27 and B*57 allele are represented by red circles, green triangles and inverted green triangles respectively.

Breadth of $CD8^+$ T cell responses recognizing conserved Gag epitopes is associated with lower VL set point.

We next investigated the contribution of breadth of $CD8^+$ CTL responses to the control of viral replication. The total breadth of $CD8^+$ CTL responses elicited during early HIV-1 infection was significantly inversely correlated with the VL set point ($r = - 0.55$, $p = 0.035$, Figure 17A). In order to avoid a bias introduced from subjects mounting $CD8^+$ CTL responses against conserved epitopes, we performed further subset analysis excluding subjects who mounted responses against conserved epitopes. Interestingly, in the absence of individuals mounting responses against conserved epitope, the breadth of $CD8^+$ CTL responses no longer remained significantly inversely correlated with the VL set point (Spearman Rank Correlation, $r = 0.80$, $p = 0.333$, data not shown). We also performed a multivariate model to examine the joint effects of CS and breadth of $CD8^+$ CTL responses on plasma VL set point and found that main effects of both CS and breadth and interaction between them are not significant (data not shown); however, the direction of individual main effect of CS and breadth is to lower VL set point. Similar to the subset analysis above, VL set point is lowered when targeting conserved epitopes in addition to increasing breadth.

We further assessed the relationship between the breadth of $CD8^+$ CTL responses elicited by Gag epitopes and plasma VL set point. Similar to the contribution of magnitude of $CD8^+$ CTL responses elicited by Gag epitopes on control of viral replication, the breadth of Gag and Pol epitopes targeted during early HIV-1 infection was correlated inversely with VL set point ($r = - 0.64$, $p = 0.010$, and r

= -0.69, p = 0.005, Figure 17B and 17C respectively). In contrast, there was no significant correlation between breadth of Nef-specific, Env-specific and other Accessory protein-specific $CD8^+$ CTL response with VL set point (data not shown).

We next compared breadth of conserved Gag epitopes or conserved Pol epitopes with VL set point in order to identify the contribution of $CD8^+$ CTL responses recognizing conserved Gag or Pol epitopes in viral control. We found a statistically significant inverse correlation between breadth of conserved Gag epitopes and VL set point (r = -0.65, p = 0.009, Figure 17D). Strikingly, the breadth of $CD8^+$ CTL responses to variable Gag epitopes did not significantly inversely correlate with the VL set point (Spearman Rank Correlation, r = 0.32, p = 0.250, Figure 17E). In contrast, the breadth of conserved Pol epitopes did not significantly correlate with VL set point (r = -0.38, p = 0.165, Figure 17F). We subsequently compared the VL set point in subjects who mounted responses against at least one conserved Gag or Pol epitope compared to subjects who did not mount such responses. Interestingly, there was lower VL set point in individuals who mounted at least one conserved Gag response versus those who did not (MW, p = 0.050, Figure 17FG. In contrast, subjects mounting $CD8^+$ CTL responses against at least one conserved Pol epitope did not have significantly lower VL set point than those who did not mount such responses (p = 0.215, Figure 17H). The estimated difference between subjects mounting responses to at least one conserved Gag epitope and those who did not was -1.0 $\log_{10}$ (95% CI: -2.1, 0.15; p = 0.082). Overall, these data suggest that the previous associations between breadth of Gag responses and lower

viral load may have been driven exclusively by only the conserved Gag epitopes that were targeted.

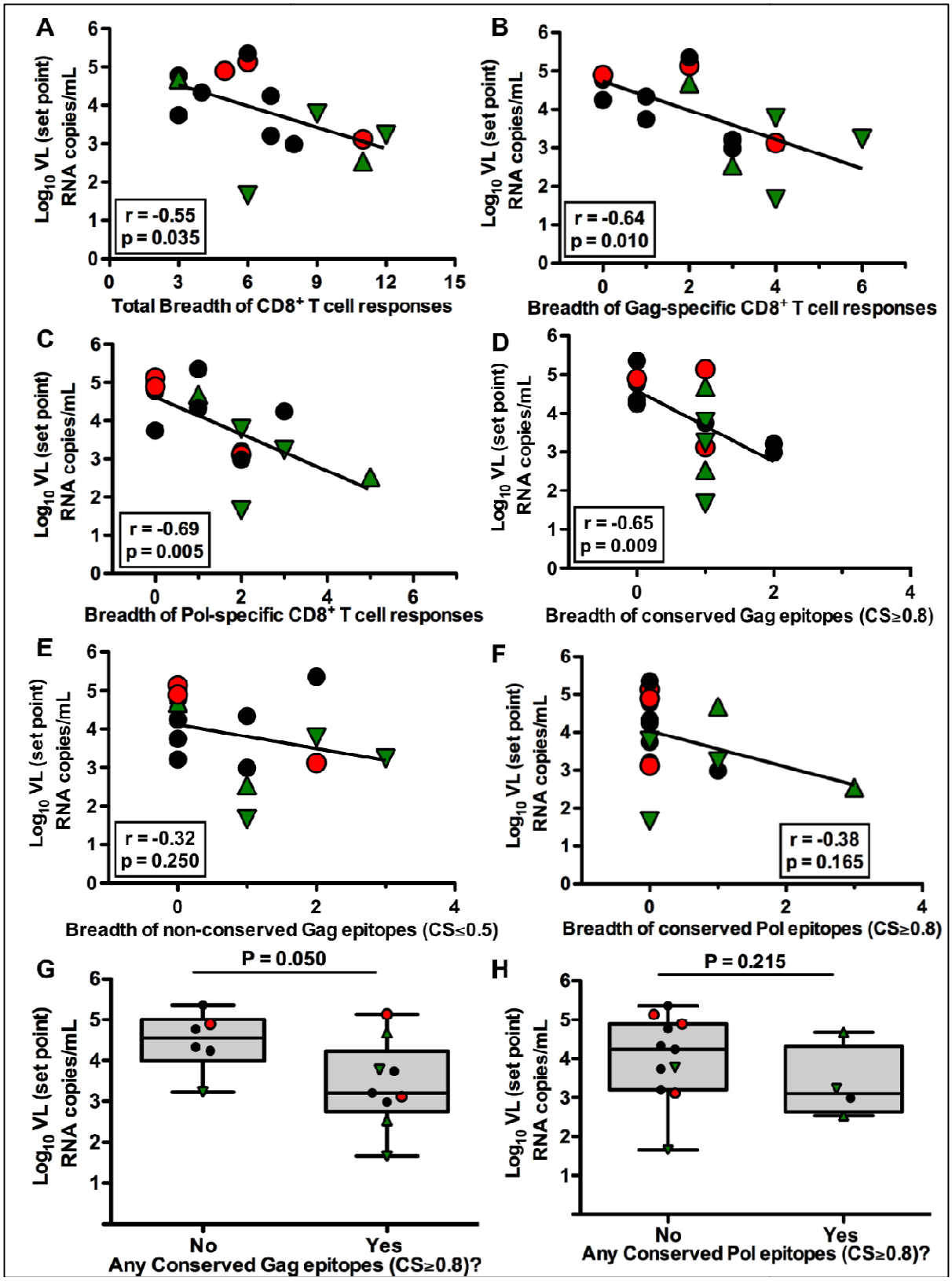

A
Log10 VL (set point) RNA copies/mL
r = -0.55
p = 0.035
Total Breadth of CD8+ T cell responses
B
Log10 VL (set point) RNA copies/mL
r = -0.64
p = 0.010
Breadth of Gag-specific CD8+ T cell responses
C
Log10 VL (set point) RNA copies/mL
r = -0.69
p = 0.005
Breadth of Pol-specific CD8+ T cell responses
D
Log10 VL (set point) RNA copies/mL
r = -0.65
p = 0.009
Breadth of conserved Gag epitopes (CS≥0.8)
E
Log10 VL (set point) RNA copies/mL
r = -0.32
p = 0.250
Breadth of non-conserved Gag epitopes (CS≤0.5)
F
Log10 VL (set point) RNA copies/mL
r = -0.38
p = 0.165
Breadth of conserved Pol epitopes (CS≥0.8)
G
P = 0.050
Log10 VL (set point) RNA copies/mL
No
Yes
Any Conserved Gag epitopes (CS≥0.8)?
H
P = 0.215
Log10 VL (set point) RNA copies/mL
No
Yes
Any Conserved Pol epitopes (CS≥0.8)?

Figure 17. Breadth of HIV-1-specific $CD8^+$ T cell responses correlate with lower viral set point.

VL set point was compared to breadth of $CD8^+$ T cell responses. (A) Correlation between total breadth of $CD8^+$ T cell responses and average plasma VL set point (Spearman Rank Correlation, r = -0.55, p = 0.035). (B and C) Correlation between breadth of $CD8^+$ T cell responses against Gag or Pol epitopes with plasma VL set point (Spearman Rank Correlation, r = -0.64, p = 0.010 and r = -0.69, p = 0.005 respectively). (D to F) Correlation between breadth of $CD8^+$ T cell responses against conserved Gag or non-conserved Gag or conserved Pol epitopes with plasma VL set point (Spearman Rank Correlation, r = -0.65, p = 0.009; r = -0.32, p = 0.25; and r = -0.38, p = 0.165 respectively). (A to F) The solid line represents a regression line. Subject possessing B*35Px, B*27 and B*57 allele are represented by red circles, green triangles and inverted green triangles respectively. (G and H) The median plasma VL set point in individuals who mounted $CD8^+$ CTL responses against at least one conserved Gag or Pol epitope (Mann Whitney, p = 0.05 and p = 0.215 respectively).

The association between $CD8^+$ CTL responses directed against conserved epitopes and viremia is independent of functional avidity.

We next assessed whether $CD8^+$ CTL responses with high functional avidity have an impact on the VL set point. For this purpose, we assessed the log_{10}-transformed EC_{50} of all $CD8^+$ CTL responses. There was no correlation between functional avidity of $CD8^+$ CTL responses and the CS of CTL epitopes ($r = -0.05$, $p = 0.553$, Figure 18A). We then compared the functional avidities of $CD8^+$ CTL responses elicited by epitopes that were variable, relatively conserved and conserved. The median functional avidities of $CD8^+$ CTL responses were no different by CS grouping (KW, $p = 0.681$, Figure 18B). Interestingly, there was no significant difference between median functional avidities of $CD8^+$ CTL responses elicited by variable epitopes compared to those elicited by conserved epitopes. To test whether high functional avidities of $CD8^+$ CTL responses play an important role in viral control, we compared the functional avidities of average, maximum and immunodominant epitope-specific $CD8^+$ CTL responses with plasma VL set point. In line with the above findings, there was no significant correlation between functional avidities of average, maximum or immunodominant $CD8^+$ CTL responses with VL set point ($r = -0.07$, $p = 0.800$, Figure 18C; $r = 0.18$, $p = 0.516$, Figure 18D and $r = -0.44$, $p = 0.105$ Figure 18E respectively). Taken together, these data suggest that the association between viral control and breadth of $CD8^+$ CTL specifically targeting conserved epitopes is not due to the fact that these $CD8^+$ CTLs have higher functional avidities.

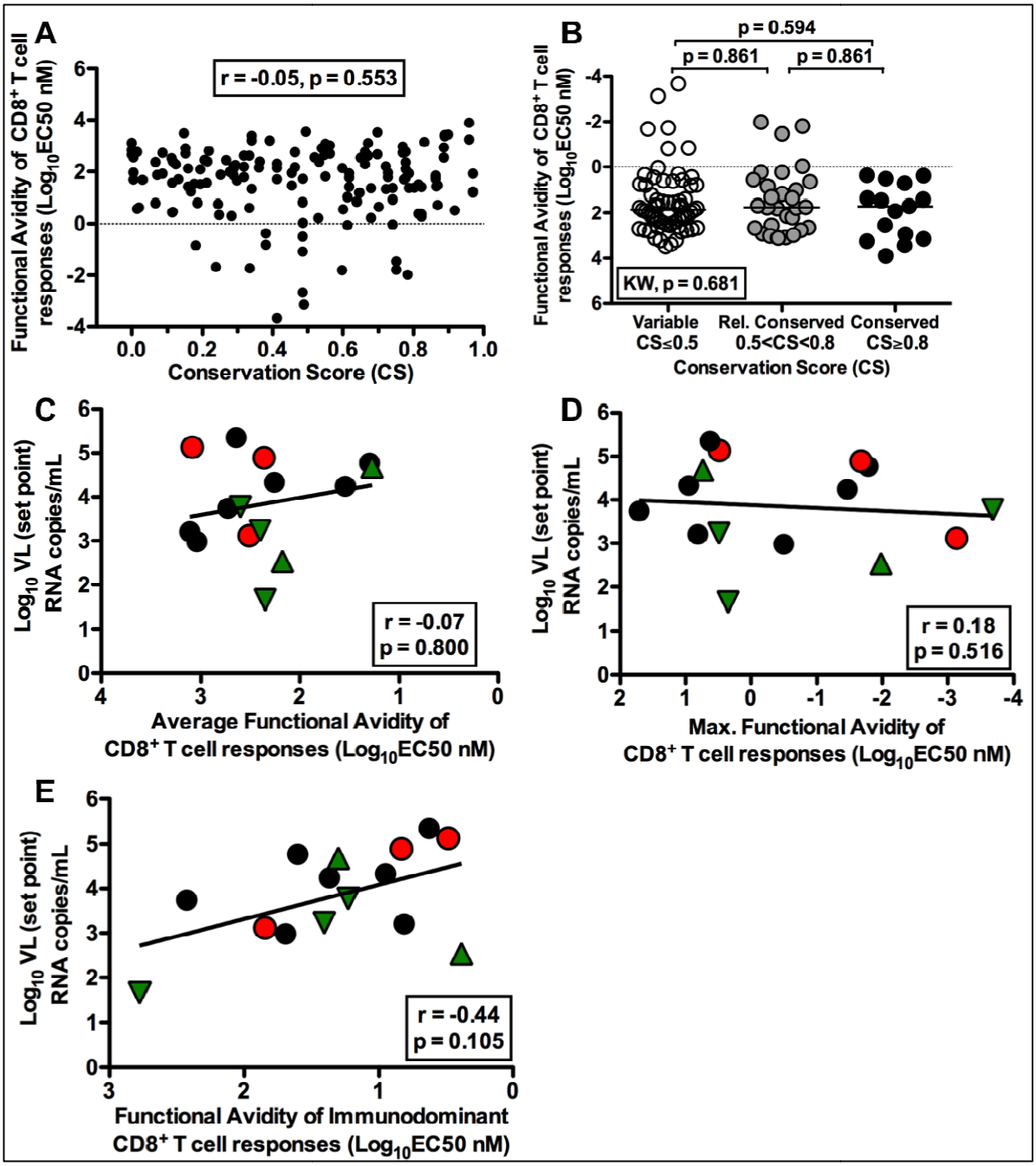


Figure 18. CD8$^+$ T cell functional avidity and control of viral replication. Correlation between functional avidity and CS of CD8$^+$ T cell epitopes. (B) The functional avidity of CD8$^+$ T cells by CS grouping (Kruskal-Wallis, p = 0.681). Horizontal lines indicate median values. (C-E) Correlation between average (C), maximum (D) and immunodominant (E) functional avidity of CD8$^+$ T cells in each subject with average plasma viral set point (Spearman Rank Correlation, r = -0.07, p = 0.800; r = -0.18, p = 0.516 and r = -0.44, p = 0.105 respectively). (C) to (E) The solid line represents a regression line. Subject possessing B*35Px, B*27 and B*57 allele are represented by red circles, green triangles and inverted green triangles respectively.

Method of determining CS influences significance of T cell association with viral control.

Since we observed a significant association of viral control and $CD8^+$ T cells targeting conserved epitopes based on clade-B alignments, we further wanted to investigate if the same pattern exists if epitope-conservation of $CD8^+$ T cell epitopes were to define on different sequence alignments with different methods. For this purpose, the epitope-conservation of $CD8^+$ T cell epitopes was defined based on clade-B or more diverse group-M alignments with two different methods, (i) clade-B prevalence CS (bCSp) or group-M prevalence CS (mCSp): the proportion of clade-B (as defined on Figure 13) or group-M amino acid sequences in the LANL database that include the epitope. *Conserved* epitopes were defined as those that were more than 80% prevalent in a given sequence set. (ii) bCSe or mCSe: defined based on Shannon entropy score calculated for each position in all circulating clade-B or group-M alignments. Conserved epitopes were defined as epitopes with average entropy score below the 20^{th} percentile of this distribution (i.e. were more conserved than 80% of all potential 9mers). The CS of epitopes defined by different methods is shown in Table 3.

We compared the breadth of $CD8^+$ T cells recognizing conserved epitopes using the different methods with plasma VL set point. Interestingly, the inverse correlation observed for bCSp did not reach statistical significance (13A) when epitope-conservation was defined as mCSp ($r = -0.40$, $p = 0.139$, Figure 19A), although the data showed a trend in the same direction as was observed for the bCSp analysis. Remarkably, the breadth of $CD8^+$ T cells recognizing conserved

epitopes based on entropy inversely correlated with VL set point whether entropy was defined based on clade-B or group-M alignments ($r = -0.52$, $p = 0.048$, Figure 19B and $r = -0.52$, $p = 0.043$, Figure 19C, respectively).

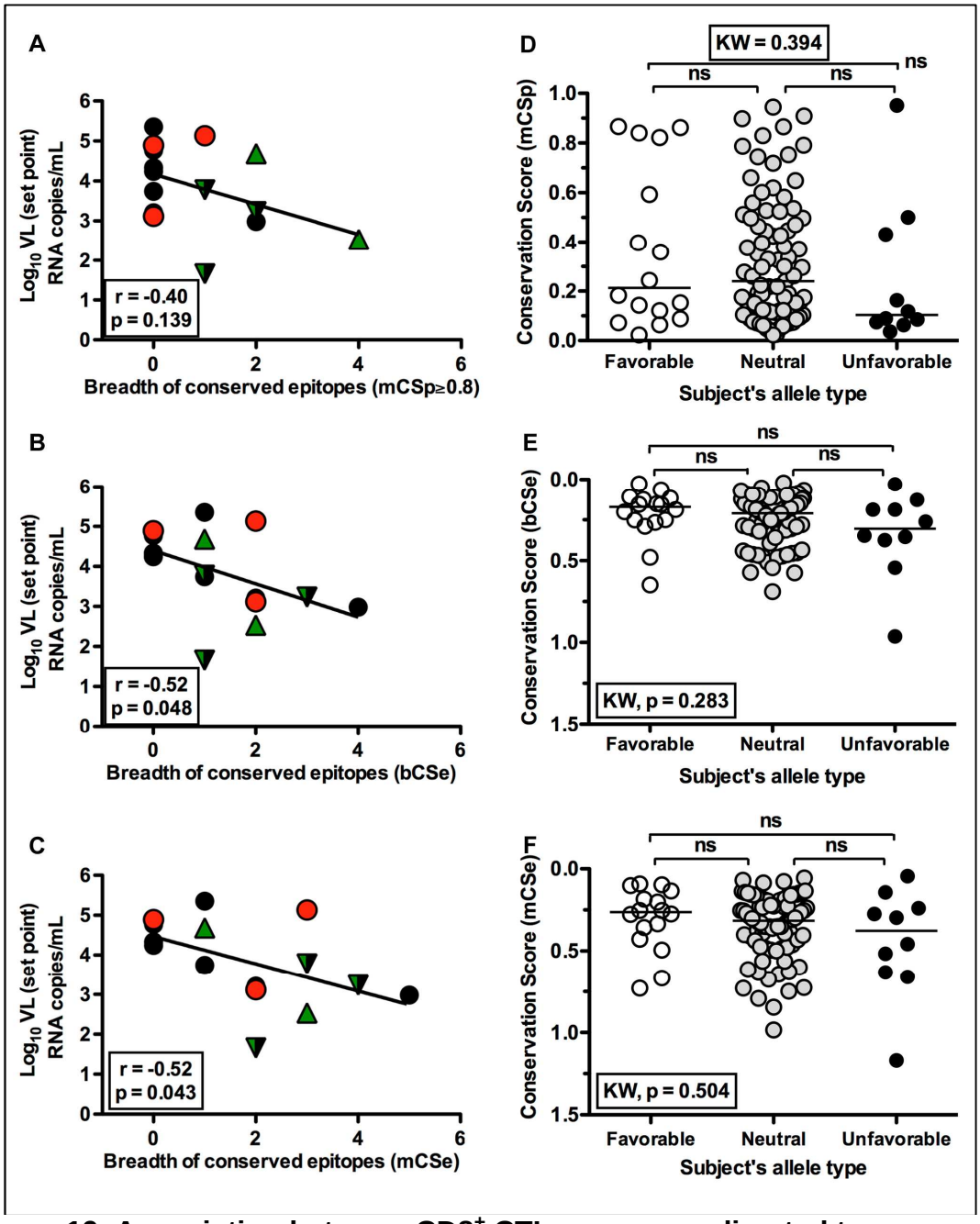


Figure 19. Association between CD8$^+$ CTL responses directed to conserved epitopes (computed by other methods) and viral control.

(A) The plasma VL set point was compared to breadth of conserved epitopes based on mCSp (Spearman Rank Correlation, r = -0.40, p = 0.139). (B, C) The plasma VL set point was compared to breadth of conserved epitopes based on bCSe (Spearman Rank Correlation, r = -0.52, p = 0.048) and mCSe (Spearman Rank Correlation, r = -0.52, p = 0.043) respectively. Subject possessing B*35Px, B*27 and B*57 allele are represented by red circles, green triangles and inverted green triangles respectively. (D-F) The median mCSp, bCSe, and mCSe of epitopes by HLA group (Kruskal-Wallis, p = 0.394, p = 0.283 and p = 0.504 respectively). Horizontal lines indicate median.

We next assessed whether the association between $CD8^+$ T cells targeting conserved epitopes and lower VL set point may have been due to the fact that these conserved epitopes were recognized by $CD8^+$ T cells restricted by protective alleles. For this, epitope-conservation of $CD8^+$ T cells epitopes restricted by protective alleles (such as HLA-B*27, HLA-B*57), neutral alleles and unfavorable alleles (such as HLA-B*35Px alleles) was compared. Surprisingly, the median epitope-conservation of $CD8^+$ T cell epitopes restricted by protective alleles was not significantly different from those restricted by neutral or unfavorable alleles regardless of method of definition epitope-conservation or type of alignment used to define epitope-conservation (KW, $p = 0.395$, Figure 19D for mCSp; KW, $p = 0.283$, Figure 5G for bCSe and KW, $p = 0.215$, Figure 5H for mCSe). Overall, these data suggest that epitope-conservation of $CD8^+$ T cell epitopes play important role on viral control and targeting epitope with high conservation is independent of HLA type.

Table 3. HIV-1 spepcific CD8$^+$ T cell responses in early infection: comparison of CD8$^+$ T cell epitope-conservation by different methods.

Epitope Sequence[a]	HXB2 site[b]		HIV protein	bCS[c]	mCS[d]	bES[e]	mES[f]
DPNPQEVVL	78	86	Env	0.38	0.12	0.35	0.52
TAVPWNASW	606	614	Env	0.29	0.07	0.37	0.46
DIWDNMTWM	621	629	Env	0.09	0.09	0.54	0.63
GRRGWEALK	786	794	Env	0.39	0.15	0.48	0.67
IGRAILHIPR	836	845	Env	0.02	0.02	0.65	0.73
VINRVRQGY	704	712	Env	0	0.04	0.34	0.44
IVTRIVELL	777	785	Env	0.34	0.1	0.58	0.79
RPNNNTRKSI	298	307	Env	0.42	0.28	0.44	0.6
IPRRIRQGL	843	851	Env	0.34	0.3	0.47	0.49
QELKNSAVSL	805	814	Env	0.56	0.16	0.45	0.64
SFEPIPIHY	209	217	Env	0.75	0.35	0.12	0.25
RQGLERALL	848	856	Env	0.53	0.33	0.46	0.56
YCAPAGFAIL	217	226	Env	0.6	0.46	0.18	0.2
EKLWVTVYY	32	40	Env	0.22	0.07	0.31	0.39
NVTENFNMW	88	96	Env	0.72	0.66	0.17	0.18
MHEDIISLW	104	112	Env	0.79	0.52	0.12	0.23
FNCGGEFFY	376	384	Env	0.8	0.51	0.15	0.23
AVLSVVNRV	700	708	Env	0.01	0.03	0.51	0.85
RRGWEVLKY	787	795	Env	0.15	0.05	0.48	0.98
EIWDNMTWL	621	629	Env	0	0.05	0.54	0.63
VWKDAETTL	44	52	Env	0	0.08	0.15	0.43
NEIWDNMTW	620	628	Env	0.07	0.04	0.69	0.73
FCASDAKSY	53	61	Env	0.01	0.09	0.1	0.16
AITQACPKV	200	208	Env	0.01	0.26	0.21	0.35
ERYLRDQQL	584	592	Env	0.34	0.18	0.22	0.35
LLNTTAIVV	814	822	Env	0.02	0.06	0.57	0.73
RQGFERALL	848	856	Env	0.11	0.15	0.46	0.56
HPVHAGPI	84	91	Gag	0.59	0.5	0.26	0.3
QVTNSATIM	6	14	Gag	0.19	0.06	0.96	1.17
QASQDVKNW	176	184	Gag	0.28	0.16	0.19	0.27
IRLRPGGKK	19	27	Gag	0.87	0.82	0.06	0.1
KRWIILGLNK	131	140	Gag	0.82	0.87	0.11	0.09
QASQEVKNW	176	184	Gag	0.52	0.18	0.19	0.27
ISPRTLNAW	15	23	Gag	0.7	0.59	0.12	0.13
KAFSPEVIPMF	30	40	Gag	0.97	0.84	0.03	0.1
TSTLQEQIGW	108	117	Gag	0.73	0.4	0.15	0.25
TSTLQEQIAW	108	117	Gag	0.07	0.24	0.15	0.25
GQMVHQAISP	8	17	Gag	0.55	0.42	0.15	0.28
ELYPLASLR	119	127	Gag	0.25	0.08	0.51	0.7

TVATLYCVH	81	89	Gag	0.55	0.6	0.18	0.25
AADTGNSSQV	119	128	Gag	0.21	0.08	0.86	1.46
SLYNTVATL	77	85	Gag	0.32	0.33	0.29	0.29
RLRPGGKKK	20	28	Gag	0.65	0.45	0.16	0.25
KYKLKHIVW	28	36	Gag	0.27	0.12	0.31	0.48
QAISPRTLNAW	13	23	Gag	0.54	0.42	0.15	0.21
ETINEEAAEW	71	80	Gag	0.87	0.37	0.07	0.14
ELRSLYNTV	74	82	Gag	0.2	0.19	0.32	0.34
PPIPVGDIY	122	130	Gag	0.09	0.24	0.11	0.19
IEIKDTKEAL	92	101	Gag	0.17	0.1	0.2	0.32
AEQASQDVKNW	174	184	Gag	0.28	0.16	0.16	0.23
FLGKIWPS	70	77	Gag	0.66	0.75	0.17	0.19
EIYKRWII	128	135	Gag	0.86	0.56	0.1	0.15
TERQANFL	64	71	Gag	0.6	0.79	0.25	0.19
EIYKRWIIL	128	136	Gag	0.77	0.53	0.14	0.17
GELDRWEKI	11	19	Gag	0.36	0.13	0.28	0.39
GPSHKARVL	223	231	Gag	0.13	0.38	0.15	0.16
CVHQRIEVK	87	95	Gag	0.15	0.09	0.36	0.67
ATLYCVHQR	83	91	Gag	0.3	0.19	0.26	0.49
KELYPLASL	33	41	Gag	0.45	0.17	0.46	0.75
RWEKIRLRP	15	23	Gag	0.53	0.22	0.17	0.28
GTEELRSLY	71	79	Gag	0.01	0.12	0.25	0.35
MTSNPPIPV	118	126	Gag	0.15	0.3	0.2	0.26
YSPVSILDI	145	153	Gag	0.18	0.6	0.19	0.16
DRFYKTLRAEQ	166	176	Gag	0.9	0.4	0.07	0.14
DIKDTKEAL	93	101	Gag	0.03	0.02	0.21	0.33
NPDCKTIL	195	202	Gag	0.89	0.79	0.1	0.13
GSEELRSLY	71	79	Gag	0.29	0.12	0.25	0.35
YPLTFGWCF	135	143	Nef	0.49	0.43	0.19	0.24
DPEKEVLVW	175	183	Nef	0.18	0.04	0.35	0.66
FPVKPQVPL	68	76	Nef	0.12	0.09	0.13	0.14
HTQGYFPDW	116	124	Nef	0.74	0.36	0.12	0.18
QVPLRPMTYK	73	82	Nef	0.77	0.72	0.1	0.14
AVDLSHFLK	84	92	Nef	0.29	0.14	0.26	0.33
TPGPGVRYPL	128	137	Nef	0.19	0.34	0.27	0.23
VPLRPMTY	74	81	Nef	0.83	0.74	0.08	0.15
KEKGGLEGL	92	100	Nef	0.68	0.5	0.16	0.17
AAVDLSHFL	83	91	Nef	0.21	0.09	0.3	0.41
FLKEKGGL	90	97	Nef	0.78	0.91	0.09	0.08
RPMTYKAAV	77	85	Nef	0.21	0.1	0.32	0.24
RYPLTFGW	134	141	Nef	0.55	0.58	0.2	0.19
RYPLTFGWCF	134	143	Nef	0.49	0.43	0.18	0.22
WRFDSRLAF	183	191	Nef	0.27	0.07	0.25	0.46

KRQDILDLWVY	105	115	Nef	0.38	0.22	0.21	0.23
TVLDVGDAY	107	115	Pol	0.96	0.95	0.03	0.04
IVLPEKDSW	244	252	Pol	0.62	0.14	0.15	0.43
STTVKAACWW	123	132	Pol	0.46	0.09	0.25	0.33
ITTESIVIW	375	383	Pol	0.19	0.06	0.25	0.49
KRKGGIGGY	186	194	Pol	0.81	0.86	0.2	0.2
STTVKAACW	838	846	Pol	0.46	0.07	0.27	0.36
QETAYFILK	95	103	Pol	0.41	0.38	0.19	0.32
HTDNGSNFTS	114	123	Pol	0.64	0.77	0.21	0.22
ANSPTSREL	23	31	Pol	0.11	0.31	0.33	0.46
GPKVKQWPL	18	26	Pol	0.96	0.87	0.02	0.07
QIIEQLIKK	520	528	Pol	0.72	0.44	0.19	0.4
QIYAGIKVK	269	277	Pol	0.36	0.22	0.19	0.32
LVGPTPVNI	76	84	Pol	0.78	0.83	0.09	0.09
EEMNLPGRW	34	42	Pol	0.41	0.07	0.28	0.37
RKAKIIRDY	263	271	Pol	0.7	0.54	0.21	0.32
QYALGIIQA	60	68	Pol	0.89	0.9	0.11	0.15
SPAIFQSSM	156	164	Pol	0.67	0.65	0.12	0.16
YQYMDDLYV	181	189	Pol	0.92	0.94	0.05	0.06
EEHEKYHSNW	10	19	Pol	0.49	0.26	0.23	0.35
YRDSRDPLW	227	235	Pol	0.65	0.15	0.29	0.31
EHEKYHNNW	11	19	Pol	0.12	0.18	0.21	0.35
LVSQIIEQL	77	85	Pol	0.6	0.19	0.25	0.4
VRDQAEHLK	165	173	Pol	0.89	0.62	0.16	0.27
REHLLRWGF	206	214	Pol	0.02	0.13	0.23	0.39
RGRQKVVSL	21	29	Pol	0.39	0.3	0.32	0.47
QETAYFILKL	95	104	Pol	0.41	0.38	0.18	0.3
YTAFTIPSV	127	135	Pol	0.11	0.12	0.09	0.15
RPAEPVPLQL	66	75	Rev	0.25	0.16	0.39	0.44
ITKGLGISY	39	47	Tat	0.24	0.12	0.35	0.4
LADQLIHLYY	102	111	Vif	0.49	0.21	0.32	0.41
ALAALITPK	149	157	Vif	0.2	0.07	0.48	0.63
WHLGQGVSI	79	87	Vif	0.87	0.47	0.17	0.26
YSTQVDPDL	94	102	Vif	0.24	0.11	0.43	0.48
GLADQLIHL	101	109	Vif	0.49	0.06	0.29	0.36
SESAIRNAI	116	124	Vif	0.29	0.12	0.46	0.5
DARLVITTY	61	69	Vif	0.16	0.07	0.47	0.61
VRHFPRIWL	31	39	Vpr	0.33	0.12	0.29	0.28
REPYNEWTL	12	20	Vpr	0.61	0.5	0.21	0.3

[a] Amino acid sequence of identified new T-cell epitopes, with newly defined HLA restriction shown in bold

[b] Epitope position (based on HXB2 amino acid sequence) in HIV-1 proteins

[c] CS was calculated as the frequency of the exact epitope matches in aligned clade-B sequences

[d] CS was calculated as the frequency of the exact epitope matches in aligned group-M sequences
[e] ES was calculated as the frequency of the exact epitope matches in aligned clade-B sequences
[f] ES was calculated as the frequency of the exact epitope matches in aligned group-M sequences

Discussion

Understanding the characteristics that define the correlates of protection against HIV-1 infection would guide rational design of effective vaccines. Although, $CD8^+$ CTL responses elicited during early HIV-1 infection are thought to be important in containment of HIV-1 (reviewed in ref.[72, 73, 91]), the qualities that define effective versus ineffective CTLs are still not known. To date, studies correlating early HIV-1-specific $CD8^+$ CTL responses and viral control have been restricted to the breadth and magnitude of IFN-γ secreting HIV-1-specific $CD8^+$ CTLs [115, 116, 123, 200]. Thus relative contributions of both "breadth" and "epitope-conservation", as related to CS of the overall epitope-specific $CD8^+$ CTL responses elicited during early HIV-1 infection and their relationship to HIV control are largely unclear. Furthermore, most studies investigating the breadth of $CD8^+$ T cell responses during early HIV-1 infection have focused on samples from treated individuals during early HIV-1-infection [115, 123] and a panel of HLA-restricted epitopes [123, 200] or a limited selection of HIV-1 proteins [123] rather than using samples from therapy naïve individuals and peptide sets spanning the whole HIV-1 proteome. To our knowledge, there is only one published study analyzing the comprehensive $CD8^+$ CTL responses elicited during early HIV-1 infection in therapy naïve individuals using overlapping peptide sets spanning the HIV-1 proteome based on clade-B consensus sequences [116]. However, this study did not look at the relative contribution of specificity of $CD8^+$ CTL epitopes on viremia control. We therefore investigated the relative contribution of "breadth" compared with "epitope-conservation" of $CD8^+$ T cell responses elicited during early HIV-1 infection on viral

control. As a secondary objective, we also assessed magnitude, functional avidity and MHC utilized to mount HIV-1-specific $CD8^+$ CTL responses in early HIV-1 infection on viral control. For this, we performed a comprehensive mapping of $CD8^+$ T cell responses directed towards all HIV-1 proteins in 23 therapy naïve subjects using PTE peptide sets. We compared the qualities of early $CD8^+$ CTL responses to VL set point. To our knowledge, no studies have determined whether conserved or variable epitopes are preferentially targeted during early HIV-1-infection and how this is related to subsequent viral control.

PTE peptides are 15mers that are designed to encompass all potential 10-mer epitopes present at 15% or greater in current circulating group-M strains [126]. These peptide reagents have been shown to increase the number of responses detected in infected individuals over consensus B reagents [127]. Data from other investigators have demonstrated that use of autologous peptide sets enhances the ability to detect responses in HIV-1-infected individuals over consensus clade-B peptide sequences [125]. It is possible that the use of autologous peptide sets would have detected more responses against variable epitopes than PTE peptide sets, however, since our study already shows that the majority of responses elicited during early infection target variable epitopes, it is not clear that any additional data would have been gained using autologous peptide sets. Although PTE peptides are not autologous peptides, they still contain multiple variants of each potential epitope, and should enhance the probability of detecting all the T cell responses elicited within an individual. Indeed, 60% (74/123) of epitopes recognized during early HIV infection were variable, 24% (29/123) of epitopes identified were novel epitopes and

74% (29/39) of those had a CS ≤0.5 (based on clade-B alignments). Similarly, when CS were defined by more stringent method based on group-M alignments, 76% (94/123) of epitopes targeted early in HIV infection were variable, 28% (35/123) of epitopes identified were novel epitopes and 90% (35/39) of those had a CS of ≤0.5. These results suggest that the use of only known HLA restricted epitopes would lead to an underestimation of $CD8^+$ CTL response in HIV-1-infeciton. Because of the comprehensive analysis of $CD8^+$ CTL responses with PTE peptides in patients with diverse HLA types, it is not surprising that so many new epitopes in variable regions were identified. However, we anticipate that $CD8^+$ CTL responses that are elicited in early infection to variable epitopes could still be underrepresented in our analysis because the peptide sets we used were not autologous.

In our study, the median number of $CD8^+$ CTL responses elicited during early HIV-1 infection was 7 (range, 3-13), which is higher compared to previous studies (median, 2-4; range, 0-7) [115, 116, 123]. The observed differences could be at least partially attributable to human genetic variation, sampling time after infection, treatment status [115, 123], and use of a more comprehensive peptide set since the previous studies used peptides based on clade-B consensus sequences [116]. Our results demonstrate that the majority of $CD8^+$ CTL responses are directed against variable epitopes. However, this contrasts with findings made in a previous study showing that both conserved and variable epitopes are recognized with a similar probability by $CD8^+$ T cells [179]. The observed differences could be due to the approach used to identify the probability of recognition of conserved epitopes. Our finding is in agreement with a study [135] that looked at whether the T cell responses

elicited by Merck (Ad5 containing HIV-1 Gag, Pol and Nef) vaccination targeted conserved or variable epitopes. Furthermore, this finding is also in line with a previous study showing that $CD8^+$ T cell responses in early infection are directed towards high entropy 15-20mer peptides (i.e., lower amino acid conservation) compared to chronic infection [181], however, they only studied specificity of $CD8^+$ CTL responses at the15mer peptide level based on entropy alone. In addition, this former study did not look at whether $CD8^+$ CTL responses mounted against lower entropy epitopes were associated with viral control.

The potential impact of epitope-conservation of $CD8^+$ T cell response has not been studied. Therefore we attempted to define conservation of recognized epitopes by three different methods. We found a significantly lower VL set point in subjects possessing $CD8^+$ CTLs targeting conserved epitopes compared to those not targeting conserved epitopes when epitope-conservation was defined based on bCS, however, this association was less significant when the epitope-conservation was defined based on mCS. Interestingly, the inverse association between number of conserved epitopes with VL set point persists whether epitope-conservation was defined as bES or mES. Collectively, these data suggest that $CD8^+$ CTL responses targeting conserved epitopes of HIV would confer protection as these regions have a fitness cost for the virus, so they can't escape easily. Although certain MHC class I alleles notably B*27 and B*57 are associated with slow HIV disease progression [87], the relative contribution of the restricting allele versus the restricted epitope towards this protection is not entirely clear. In this study, we found that $CD8^+$ CTLs restricted by protective alleles do not always mount responses against conserved

epitopes. Although, our study was limited by sample size, the lack of significant association between particular HLA type and viral control was consistent with previous finding by Kiepiela *et al*.[124], who also did not see this association in chronic HIV-1-infection. This is surprising and contrasts with findings made in previous studies [87, 90], where there is a strong association between protective alleles and HIV control during chronic infection in LTNPs, and more recently one mechanism of this HIV control has been elucidated [113]. This association may be less relevant during early HIV-1-infection, however, these observed differences could also be attributable to human genetic variation, as well as to the smaller sample size in our study. The lack of association between HLA type and viral control during early HIV infection does not rule out the well-defined relationship established during chronic infection. Overall, this finding may suggest that the correlation between $CD8^+$ CTL responses targeting conserved epitope with viral control is potentially not due to the fact that these responses are always restricted by protective alleles.

In contrast to a previous report [116], our study demonstrated that breadth and magnitude of $CD8^+$ CTL responses in early HIV-1 infection inversely correlated with plasma VL set point. The reasons that our study demonstrated correlations between $CD8^+$ CTL responses and VL set point could be due to a number of factors. Firstly, in this study, we used PTE peptide sets, thereby increasing the chances of comprehensively detecting the accurate total $CD8^+$ CTL responses. Secondly, we used plasma VL set point not the concurrent VL from each subject to avoid the chances of correlating VL from the peak as we studied $CD8^+$ CTL responses in early

HIV-1 infection. This finding is in agreement with a study [123] that reported an inverse correlation between the breadth of $CD8^+$ CTL responses and VL at presentation in treated individuals before HIV-1 seroconversion. In agreement with previous studies, we found that the breadth of Gag-specific $CD8^+$ CTL responses [122, 124] and magnitude of Gag-specific $CD8^+$ CTL responses [122] contributes to viral control. However, these studies looked at association only in chronic HIV infection. Our data showed, for the first time, an inverse correlation of breadth of early Gag-specific $CD8^+$ CTL responses directed against conserved epitopes with VL set point. Taken together, these findings support the hypothesis that the early and strong $CD8^+$ CTL recognition of conserved Gag-epitopes during the initial phase of HIV-1-infection is an effective contributor of viral control.

Several studies have demonstrated the potential importance of functional avidity of $CD8^+$ T cells in HIV control [105-107]. In contrast to earlier reports [105-107], we did not see any correlation between functional avidity of $CD8^+$ T cells and viral control. The observed differences could be attributable to the methodology to determine the functional avidity of $CD8^+$ T cells [105, 106] or in the methodology to calculate VL (plasma VL set point versus cell associated VL) [107]. Perhaps more importantly, the previous studies assessed $CD8^+$ CTL responses in chronic infection, whereas our study assessed $CD8^+$ CTL responses in early HIV-1-infection, and there has been a study showing that functional avidities change over time during infection [201]. Our finding indicates that there is no correlation between CS of $CD8^+$ T cell epitopes and functional avidity of $CD8^+$ T cells. Taken together, these findings

suggest that functional avidity of $CD8^+$ T cells may not be a crucial feature of immune control, at least during early HIV-1-infection.

A few caveats of our study must be considered. It is possible that the contribution of overall $CD8^+$ CTL responses on viral control cannot be elucidated completely by the cross-sectional nature of our study. Second, our calculation of conservation scores weighted equally all positions in the epitope sequence based on clade-B or group-M sequences. Extending these analyses with CS of $CD8^+$ T cell epitopes calculated by different approaches may further inform the effects of epitope conservation on viral control. An approach weighting amino acids based on their known interactions with major histocompatibility complex (MHC) and T cell receptor (TCR) could provide different results as mutations in these amino acids alter peptide binding to MHC and T cell recognition. Third, the definition of conserved epitope (conserved if CS≥0.8) was arbitrary since the B*27 restricted KRWIILGLNK epitope (which has been shown to be associated with viral control [103]) has a CS of 0.82 and 0.87 (based on clade-B and group-M alignments respectively). Finally, due to the limited sample size of subjects who reached VL set point, our study has lack of power to investigate the interaction between breadth, CS and allele types. Larger studies will be needed to address these issues, although this may be difficult due to the current treatment guidelines of early initiation of therapy.

In summary, magnitude, breadth and epitope-conservation of HIV-specific $CD8^+$ T cells elicited during early infection are important for controlling viral replication *in vivo.* From our limited sample size, it appears that these associations may be independent of possession of particular HLA types. From a vaccine design

perspective, our study has profound implications. Given limited feasibility to design vaccines based on an individual's HLA haplotype, our data suggest that a T cell based vaccine should be designed using conserved regions of HIV [171], regardless of the vaccinee's HLA type. Overall, our results provide information that may guide the selection of immunogens for development of effective HIV-1 vaccines. Rationale design of immunization approaches should aim at induction of greater breadth and magnitude of $CD8^+$ T cell responses precisely recognizing only conserved epitopes of HIV.

Chapter IV: Discussion

Implications and Considerations

The vision of living in a world free of HIV has faced many challenges. However, this vision can only be accomplished with the development of a safe, efficacious and affordable HIV-1 vaccine. Most currently available vaccines were designed without knowledge of correlates of protection from the pathogens they protect against. They predominantly incorporate protein subunits or live attenuated versions of the pathogens. But these approaches have not proved effective for HIV vaccines. An ideal prophylactic HIV-1 vaccine should completely block infection and provide sterilizing immunity, however, this is probably a difficult goal for the HIV because of the enormous diversity associated with the virus. To achieve the protection, successful prophylactic HIV-1 vaccines will likely need to induce multiple arms of the host immune response, which include induction of broadly cross-reactive neutralizing antibodies, effective blocking antibodies and potent cell-mediated responses. Although several studies in human and animal models suggest that the cellular immune response plays a critical role in HIV control, other studies, including studies from our laboratory, show that CTL differ in their effectiveness at controlling HIV.

Our laboratory expends considerable effort on defining what characteristics make the most effective anti-HIV CTL with the aim of designing better T cell based immunogens. Vaccine development for many viruses has been moderately easy to achieve, at least relative to HIV, because of less viral diversity. However, as described in Chapter I, HIV is enormously diverse and presents many incredible

challenges that are not easily overcome with current vaccination strategies. The primary barrier to generate an effective cell-mediated immune response by vaccination is our limited understanding of how to induce these protective cross-reactive immune responses against an enormously diverse HIV-1 by vaccination. The research described in this dissertation focuses on a detailed analysis of $CD8^+$ T cell responses elicited during early HIV-1 infection in the context of vaccine development. The hope is that the work presented within this dissertation will add to our understanding of $CD8^+$ T cell responses that are effective versus ineffective and will guide rational vaccine development.

By examining the comprehensive $CD8^+$ T cell responses elicited during early HIV infection, this thesis has revealed a correlation between the conservation score of targeted $CD8^+$ T cell epitopes and viral control (Chapter III), which is something that has always been assumed but never been demonstrated formally and comprehensively in the field. In this study we did not show that $CD8^+$ T cell responses targeting conserved epitopes are superior in the context of controlling viral replication *in vitro* than those targeting variable epitopes. However, future studies will address this. Furthermore, the impact of breadth, magnitude and avidity of $CD8^+$ T cell responses on viral control were assessed. Below, the principal findings of these studies and their implication for future vaccine design are described.

In Chapter III, we sought to better understand how $CD8^+$ T cells exert pressure on the virus. Previously, our group demonstrated, for the first time, that individuals who mount HIV-specific $CD8^+$ T cell responses early in infection against

conserved epitopes of HIV have better clinical outcomes than individuals who mount HIV-specific $CD8^+$ T cell responses against variable epitopes [199]. Although, these data suggest that the epitope-conservation of $CD8^+$ T cells plays an important role in mediating control of HIV, the study only measured responses to a limited number of epitopes restricted by a few HLA alleles. A comprehensive study that determined the complete $CD8^+$ T cell repertoire in acutely infected, therapy naïve individuals and examined how conservation score of the recognized epitopes correlated with viral control had not been conducted previously. Therefore, the primary aim of this study was to determine if conservation score of the epitopes targeted by $CD8^+$ T cells during natural infection correlated with VL set point. The principal finding of this study was that individuals who mounted $CD8^+$ T cell responses against multiple conserved epitopes during early infection had better control of viral replication. Additionally, we found that early $CD8^+$ T cell responses elicited in the majority of individuals were preferentially directed against variable epitopes of the virus. Moreover, targeting variable versus conserved epitopes was independent of possession of a particular HLA type. These findings have several implications. First, the data suggest that future T cell based vaccines should be designed to predominantly incorporate conserved epitopes, which are potentially vulnerable regions of the virus. Second, our finding that most individuals mount $CD8^+$ T cell responses in early infection directed against variable epitopes provides an explanation for why most of individuals progress to AIDS rapidly. Additionally, it suggests that HIV-1 steers $CD8^+$ T cell responses to variable regions (which are potentially not associated with fitness cost for the virus), therefore designing an

immunogen that would counteract this pattern would be advantageous to the host. Due to the HLA diversity in the human population, $CD8^+$ T cell responses to HIV will differ greatly among different individuals. Thus, each individual recognizes a different set of HIV epitopes, which in turn impacts the effectiveness of the $CD8^+$ T cell mediated immune response. Thus, for vaccine approaches based on the whole HIV genome, the human immune system will preferentially mount responses to variable regions of HIV, responses that may not be protective as these regions can easily be mutated and may be different in circulating strains. Thus, the likelihood of eliciting cross-reactive $CD8^+$ T cell responses against epitopes that are present across diverse isolates becomes less likely, especially considering that HIV has a lot more variable regions than conserved regions. Our result demonstrating the association between $CD8^+$ T cells recognizing conserved epitopes and lower VL set point, therefore, warrants careful consideration on selection of vaccine antigens containing only conserved regions of HIV-1, so that the immune system will be forced to mount T cell responses against the regions that are potentially associated with a fitness cost for the virus.

In the secondary aim of this project, we assessed the breadth of $CD8^+$ T cell responses on viral control. The “mosaic” immunogen strategy based on observations from multiple correlation studies showing total breadth [123] and especially Gag-breadth of $CD8^+$ T cell responses [122, 124] elicited in HIV infection correlates with viral control, focuses on maximizing the breadth of the vaccine-induced T cell responses to confer protection against incredibly diverse strains of HIV-1. In our study, not only the total breadth and Gag breadth, but also, more specifically breadth

of conserved Gag epitopes inversely correlated with viral control. These findings have several implications, along the similar line of the primary findings of this study, and further confirm that the breadth of $CD8^+$ CTL responses elicited during early HIV-1 infection directed against conserved Gag epitopes responses contribute to enhanced viral control.

Collectively, the results reported in this thesis – together with a growing amount of data from other groups [92, 95, 101, 134, 199] - implicate that the epitope specificity of $CD8^+$ T cells is crucial for the containment of viral replication. Interestingly, the VL set point in subjects who mounted $CD8^+$ CTL responses against at least one conserved epitope was lower than those who failed to target at least one conserved epitope. We found that targeting conserved epitopes was independent of possession of favorable alleles and functional avidity of $CD8^+$ T cells. Recent data suggest that the differences in protective effect between HLA molecules are due to the specific HIV-1 peptides that are being presented by different HLA molecules [199]. To date, the importance of $CD8^+$ T cell responses against conserved epitopes in the control of HIV infection has been supported by observations from multiple groups who found that a mutation on conserved B*27-restricted KK10 epitope leads to increase in plasma viremia [92, 95]. Along these lines, our results support the notion that early $CD8^+$ T cell responses against multiple conserved epitopes of the virus can be vital to the containments of viral replication.

Caveats of Study

Due to the limited sample size of subjects who reached VL set point (n = 15), our study lacked of power to investigate the interaction between breadth, conservation score and allele types. Larger studies will be needed to address these issues, although this may be difficult due to the current treatment guidelines of early initiation of therapy. This study had originally intended to assess the comprehensive mapping of $CD8^+$ T cell responses in sample size of 40 therapy naïve individuals from early HIV-1 infection. However, due to the lack of therapy naïve samples from early HIV-1 infection that also reached the set point viremia, we were only able to do correlation studies on 15 individuals. Other samples we mapped were also from a therapy naïve early time point, but these individuals did not reach the set point viremia before initiating ART.

The definition of “conserved” vs. “variable” epitopes of HIV-1 is biased by the sequences that are present in the LANL DB. It is conceivable that not all the conserved epitopes we have identified in our study are conserved because mutation in these regions would have severe fitness cost to the virus. There is a possibility that these epitopes are identified to be conserved simply because they may not have mutated because they are subdominant epitopes in infected individuals, which are infrequently targeted by the immune system. Thus they have not been under pressure to mutate. Future studies should be able to test this hypothesis in *in vitro* viral suppression assays [202] by adding these epitope-specific $CD8^+$ T cells to autologous infected $CD4^+$ target cells. The areas of the virus that encompass the

epitopes targeted by the $CD8^+$ T cell can be sequenced to determine if escape occurs over the course of the assay.

Summary and Future Directions

Throughout this thesis, we attempted to stress that fact that we observed a strong correlation between $CD8^+$ T cell responses against multiple conserved epitopes and viral control. While we focused solely on comprehensive mapping of $CD8^+$ T cell responses targeted during early HIV-1-infection, future longitudinal studies could address the potential contribution of early versus late appearance of $CD8^+$ T cells targeting conserved epitopes disease outcome. While we focused solely on conservation score of $CD8^+$ T cell epitopes in HIV control in this study, future studies should address the potential contribution of other antiviral factors to the superiority of certain $CD8^+$ T cells in the context of HIV control. For example, several groups have reported that poly-functionality of T cells, which is described as the ability of a cell to secrete at least three measured markers such as IFN-γ, TNF-α, IL-2, CD107-α, MIP-1β and perforin, are associated with $CD8^+$ T cell mediated viral control [102, 203], as we previously mentioned. Currently it is not known whether $CD8^+$ T cells recognizing conserved epitopes are more poly-functional than those targeting variable epitopes. Migueles *et al*. have shown that the lytic granule content of $CD8^+$ T cells correlates with HIV control, and this led to more effective killing of HIV-1 infected target cells [110]. Future studies could determine if $CD8^+$ T cells recognizing conserved epitopes have higher contents of lytic granules compared to $CD8^+$ T cell targeting variable epitopes. Additionally, controllers have been shown to

possess a higher proportion of $CD8^+$ T cells that can proliferate than $CD8^+$ T cells from progressors, which is directly coupled to their perforin expression [109], and which presumably is necessary for efficient granzyme-B mediated killing of infected cells [110]. It is not yet clear whether $CD8^+$ T cell recognizing conserved epitopes can directly contribute to HIV control because these cells have a higher proliferative capacity compared to $CD8^+$ T cells recognizing variable epitopes. Studies have suggested that immunodominant $CD8^+$ T cells restricted by protective alleles are activated at very low concentration of peptides [107, 204], implicating that a high functional avidity of $CD8^+$ T cells contributes to the control of viral replication [105-107]. However, our data shows that antiviral properties of $CD8^+$ T cells targeting conserved epitopes are not associated with functional avidity of $CD8^+$ T cells, at least during early HIV-1 infection. As functional avidities change over time during the infection [201], future studies need to be done to assess if the functional avidity of $CD8^+$ T cells targeting conserved versus variable epitopes change in longitudinal samples.

Given the association of alleles in HIV control [85, 87], future studies should address if the functionality of $CD8^+$ T cells targeting conserved epitopes is different than those targeting variable epitopes regardless of the MHC restriction. Future studies need to uncover if this phenomenon of superiority of $CD8^+$ T cells recognizing conserved epitopes at controlling replication of diverse clades of HIV-1 potentially in an *in vitro* viral inhibition assay and cytotoxic assays compared to $CD8^+$ T cells recognizing variable epitopes regardless of the MHC restriction. Additional studies could look at the ability of viral escape from $CD8^+$ T cell responses to

conserved versus variable epitopes by following therapy naïve individuals longitudinally, and assess formally that it is the CD8+ T cell responses targeting conserved epitopes contribute to the viral control. Longitudinal studies in a group of individuals who eventually progress to disease would allow us to identify if escape in conserved epitopes leads to disease progression. Although LTNPs are enriched with protective alleles such as B*27/*57, an equal number of infected individuals without a protective allele have been identified as LTNPs, and possession of protective alleles does not ensure non-progressor or controller status. In our Seattle LTNP cohort, only 56% (24/43) of the individuals possess protective alleles. Interestingly, all individuals (n = 11, n = 4 not possessing protective alleles) our lab has mapped mount $CD8^+$ T cell responses against conserved epitopes, further implying that conservation score of $CD8^+$ T cell epitopes play an important role in HIV control.

Concluding Statement

The journey toward an HIV-free world faces enormous scientific challenges. Despite the challenges, multiple studies have highlighted the key role of cellular immunity, encouraging the design of T cell-based HIV-vaccines.

For instance, we know from a recent study that individuals possessing $CD8^+$ T cell responses recognizing conserved epitopes during early in infection survived longer [199]. We also know from this study that individuals possessing $CD8^+$ T cell responses against conserved epitopes control viral replication compared to those recognizing variable epitopes. Although the failure of first T cell based vaccine trial (Step Trial) was very disappointing, it was, perhaps, not surprising given that vaccine-induced $CD8^+$ T cells primarily recognized variable epitopes of HIV-1 [135]. The failure of the Step trial has called into question the validity of using T-cell based vaccine approaches. However, the data generated in this study, together with previous studies, suggest that efficacious T cell based vaccines can be developed if properly designed. To overcome the enormous diversity of HIV as well as diversity of HLA haplotype in the human population, our data suggest that the next-generation of T cell based vaccines should be designed to remove variable regions of the HIV-1 proteome so that the host immune response has only the option of mounting responses to conserved regions of HIV-1.

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