DIVERGENCE IN CD8+ T CELL EPITOPES OF HIV-1 AS AN IMMUNE ESCAPE MECHANISM

by

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More than 40 million people are living with human immunodeficiency virus-1 (HIV-1). A prophylactic vaccine inducing a ‘sterilizing immunity’ is desired to prevent further infections, but will require many years to develop. Moreover, prophylactic vaccines will not help the millions of people who are already infected with the virus, and who face life-long treatment with expensive and toxic antiretroviral therapy (ART). This dissertation is based on the proposal that the best strategy for these individuals is a therapeutic vaccine that will attack residual viral reservoirs by expanding HIV-1 specific, primary T cell responses to the person’s own, autologous virus.

Previously, this laboratory demonstrated that mature dendritic cells (DC) loaded with immunodominant HIV-1 peptides or HIV-1 infected apoptotic bodies can activate residual HIV-1 specific memory T cell responses. However, such memory T cells are only partially restored during ART. I hypothesized that targeting naive CD8+ T cells through a DC-based immunotherapy could elicit a robust and broad T cell response to HIV-1. Furthermore, most immunotherapy studies have used consensus strains of HIV-1 antigens that I believe inadequately represent the host’s diverse pool of HIV-1 quasispecies. The current study has provided initial data that support that CD8+ T cells can be primed by \textit{in vitro} engineered DC, even against autologous HIV-1 peptides representing immune escape variants. This study therefore supports the concept of using autologous virus as an antigen in immunotherapy and
demonstrates that the use of autologous viral sequences expands both memory and primary T cell responses \textit{in vitro}. Thus, a potential advantage is that future immunotherapies could use autologous virus representing a large repertoire of the host’s diverse HIV-1 antigen pool. This could elicit primary immune responses specific for each patient’s quasispecies of HIV-1, as well as activation of residual HIV-1 specific memory T cells, giving the broadest immune control of HIV-1 infection during ART. Such an approach has important public health implications by having a strong positive impact on, and improve the control of, HIV-1 infection in persons on ART. It also serves as an \textit{in vitro} priming model for development of prophylactic vaccines against HIV-1 and other infectious agents.
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<th>Description</th>
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<td>Ag</td>
<td>Antigen</td>
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<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<td>APCs</td>
<td>Antigen presenting cells</td>
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<td>APC</td>
<td>Allophycocyanin</td>
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<td>ART</td>
<td>Antiretroviral therapy</td>
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<td>AUC</td>
<td>Area under the curve</td>
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<td>B2</td>
<td>beta 2</td>
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<td>bp</td>
<td>Base pair</td>
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<tr>
<td>BIMAS</td>
<td>Bioinformatics and Molecular Analysis Section</td>
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<tr>
<td>CMV</td>
<td>Cytomeglovirus</td>
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<tr>
<td>CSFE</td>
<td>5,6-carboxyfluorescein diacetate succinimidyl ester</td>
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<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
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<td>DC</td>
<td>Dendritic cells</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>dsRNA</td>
<td>Double stranded ribonucleic acid</td>
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<td>EBV</td>
<td>Epstein Barr virus</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked ImmunoSorbent Assay</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>E:T</td>
<td>Effector to target ratio</td>
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<tr>
<td>FITC</td>
<td>Fluorescin isothiocyanate</td>
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<td>GM-CSF</td>
<td>Granulocyte macophage-colony stimulating factor</td>
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<td>HBV</td>
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<td>HCV</td>
<td>Hepatitis C virus</td>
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<td>HIV-1</td>
<td>Human immunodeficiency virus -1</td>
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<td>Human immunodeficiency virus -2</td>
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<td>HLA</td>
<td>Human leukocyte antigens</td>
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<td>HSV</td>
<td>Herpes simplex virus</td>
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<td>HTLV-III</td>
<td>Human T-cell lymphotropic virus-type III</td>
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<tr>
<td>IC50</td>
<td>Inhibitory concentration 50%</td>
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<td>ICAMs</td>
<td>Intracellular adhesion molecules</td>
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<td>iDC</td>
<td>Immature dendritic cells</td>
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<td>IFNα</td>
<td>Interferon alpha</td>
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<td>Interferon gamma</td>
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<td>IL-1β</td>
<td>Interleukin-1beta</td>
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<td>Int</td>
<td>Integrase</td>
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<td>LAV</td>
<td>Lymphadenopathy-associated virus</td>
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LC  Langerhans cells  
LTNP  Long term nonprogressor  
LTRs  Long terminal repeats  
LTS  Long term survivors  
mAb  Monoclonal antibodies  
MACS  Multicenter AIDS cohort study  
mDC  Mature dendritic cells  
md-DC  monocyte derived- dendritic cells  
MFI  Mean fluorescent intensity  
MHCI  Major histocompatibility complex I  
MHCII  Major histocompatibility complex II  
mRNA  messenger ribonucleic acid  
ORF  Open reading frames  
PBMC  Peripheral blood mononuclear cells  
PCR  Polymerase chain reaction  
PCP  Pneumocystis carinii pneumonia  
PD-1  Programmed death-1  
PD-1L  Programmed death-1 ligand  
PE  R-Phycoerythrin  
PerCP  Peridinin Chlorophyll Protein  
PGE2  Prostaglandin E2  
Poly I:C  Poly inosinic acid + cytidylic acid  
Pro  Protease  
RT  Reverse transcriptase  
RNA  Ribonucleic acid  
SNPs  Single-nucleotide polymorphisms  
SIV  Simian Immunodeficiency Virus  
SE  Standard error  
TAP  Transporter associated with antigen processing  
TCR  T cell receptor  
Th1  T helper cell 1  
Th2  T helper cell 2  
TLR  Toll-like receptor  
TLR3  Toll-like receptor 3  
TNF  Tumor necrosis factor  
WHO  World health organization
II. CHAPTER ONE: INTRODUCTION

A. HIV: HISTORY AND ORIGIN, DISTRIBUTION, GENOME, EPIDEMIOLOGY, TRANSMISSION AND DISEASE PROGRESSION

1. HISTORY AND ORIGIN

For years a new virus silently spread until a glimpse of the future pandemic appeared in 1979-1981 when rare types of pneumonia, cancers (e.g. Kaposi’s Sarcoma), and other illnesses were being reported by doctors in Los Angeles, New York and San Francisco [1]. These patterns of illness were seen predominantly among the homosexual male population and stood out, since individuals with seemingly healthy immune systems, succumbing to these types of conditions was unusual. It was not until 1983 that scientists discovered the virus, first named human T-cell lymphotropic virus-type III / lymphadenopathy-associated virus (HTLV-III/LAV) and later changed to human immunodeficiency virus (HIV) [2,3]. For many years scientists theorized as to the origins of HIV and how it appeared in the human population, most believing that HIV originated in other primates and was introduced into the human population when hunters became exposed to their infected blood. Recently, molecular phylogeny studies revealed that the predominant strain of HIV-1 in the developed world originated in a subspecies of chimpanzees (Pan troglodytes troglodytes) [4-6], and HIV-2 originated in SIVsm of sooty mangabeys (Cercocebus atys) [4,7,8].
2. DISTRIBUTION

HIV is classified as a member of the lentivirus genus of the *Retroviridae* family. Lentivirus isolates from humans are grouped into one of two types, HIV-1 and HIV-2, based on their genomic sequence and pathogenic potential. These types are unevenly distributed throughout the world, HIV-1, the predominant type worldwide, and HIV-2 primarily found in the African continent. HIV-1 has been further divided into two subgroups: the M (major) and O (outlier) groups. The M group (which is divided into clades A-J) is responsible for the majority of infections worldwide whereas the O group is relatively rare and found in Cameroon, Gabon, and France [390].

3. HIV GENOME

Infectious virions of HIV contain two identical copies of positive single-stranded RNA, about 9.2 kb long. The genomes of all replication competent retroviruses contain three prototypic genes (shown in green, Figure 1) flanked by non-coding sequences, termed long terminal repeats (LTRs). The first gene is *Gag*, encoding for structural proteins including the matrix, the capsid, and the nucleocapsid. The second gene is *Pol*, encoding for the viral enzymes reverse transcriptase (RT), protease (Pro), integrase (Int). The third gene, *Env*, encodes the outer glycoprotein that mediates viral entry of permissive cell types. Additionally, there are six open reading frames (ORF) which are composed of (i) two genes that are essential for HIV replication, *tat* and *rev* (shown in red, Figure 1) [9,10], and (ii) four genes of HIV-1, *vif, vpr, vpu* and *nef* (shown in blue) termed accessory genes because they are non-essential for the viral replication in vitro (reviewed in [10]); HIV-2 and SIV differ in an accessory gene, *vpx* [390].
**Figure 1: Genetic organization of lentivirus.** The primate lentivirus RNA proteome is approximately 9.2 kb in size and encodes structural proteins/viral enzymes which include the products of gag, pol, and env genes, which are essential components of the retroviral particle shown in green. Regulatory proteins, Tat and Rev, are shown in red and they modulate transcriptional and posttranscriptional steps of virus gene expression and are essential for virus propagation. The accessory proteins Vif, Vpr, Vpx and Nef are shown in blue and are in general not necessary for viral propagation in tissue culture, but they have been conserved in the different isolates suggesting that their role in vivo is very important.

### 4. EPIDEMIOLOGY

The most recent global statistics [391] estimate 38.6 million (33.4-46.0 million) people are living with HIV-1 and 4.1 million (3.4-6.2 million) became newly infected with HIV-1 in the year. According to the World Health Organization (WHO), 18.8 million people around the world have died of AIDS since the emergence of HIV/AIDS [391]. Focusing specifically in the United States, the cumulative estimated number close to 1 million cases (984,155) and the estimated number of persons living with AIDS is reported as 433,760 as of 2005 [391].
5. TRANSMISSION

Transmission of HIV-1 can occur by several routes: sexual contact (anal, vaginal, or oral), by blood or blood products, and from mother to child (*in utero*, during birth, or through breast feeding) [392]. After it was shown that CD4 was the primary receptor, and CCR5 (for macrophage tropic) and CXCR4 (for T cell tropic) chemokine receptors were identified as coreceptors [11-16] for the virus, several thoughts of the initial entry targets for transmission of HIV-1 emerged. Some researchers believed that CD4⁺ T cells [17-19,20], whereas others put forth APC (e.g. DC, LC and macrophages) [19,21-23], as the likely target of HIV-1 infection. The proposed model of HIV-1 transmission was circumstantially built on a number of studies using several culture models [19,23-25] supported by evidence that cell types (macrophages, DC and CD4 T cells), expressed the CD4 receptor and CCR5 coreceptor [26,27], their location in the mucosa [28] and knowledge of how DC function [29,30]. The proposed sequence of events was that HIV-1 sequentially binds to and/or infects DC, which in turn transported virus to T cells within secondary lymphoid organs [31,32] (e.g., the Trojan horse theory). Recently, Hladik et al. [33] has come the closest to directly proving this through the use of an explant of human vaginal mucosa to demonstrate HIV-1 entry into Langerhans cells (LC) (CD1a⁺ and MHC II⁺) and CD4⁺ T cells (both which express CCR5). These cell targets can be strongly (in the case of T cells) and partially (in case of LC) blocked when tissues are treated with anti-CCR5 antibodies [33].
6. DISEASE PROGRESSION

Although there is a small percentage of individuals including: (i) high risk commercial sex workers (likely repeat exposure to HIV-1 virus) that do not become infected [34-38] (ii) infected individuals that do not exhibit evidence of disease progression over extended period of time, termed long term non-progressors (LTNP) [39,40], and (iii) ‘elite controllers’ that can control virus without any treatment [41-44], the majority of HIV-1 infected individuals follow three basic stages of disease progression: (i) primary or acute infection, (ii) asymptomatic (chronic) and (iii) AIDS, as illustrated in Figure 2 [45].

Figure 2: Schematic of typical course of HIV-1 infection showing changes in CD4 and CD8 T cell counts in peripheral blood and plasma viral load. Figure from Munier et al., Immunol Cell Biol volume 85, 6-15, 2007. Reprinted with permission from Nature Publishing group.
Primary infection consists of establishment of a productive infection with HIV-1 in which approximately 50% of individuals experience flu-like symptoms (e.g. general malaise, fever, lymphoadenopathy) after 3-6 weeks of infection. The persistence and severity of the symptoms vary among individuals and are usually spontaneously resolved after about 1 week. These symptoms are due to viral replication (reaching levels of $10^6$-$10^7$ copies of viral RNA/ml [46] and a decline in numbers of peripheral CD4$^+$ T cells [47,48]. The peak of the HIV-1-specific CTL response occurs shortly after peak viral load [49,50] and helps control viral replication [51,52] resulting in decreases in plasma viral levels (at 3-6 months) that eventually reach a steady state [50]. This steady state or viral set point has been shown to represent a strong predictor for disease progression [53,54]. Second, following this acute stage, an individual enters a phase which is clinically asymptomatic (chronic disease), and can persist for an average of 10 years in humans [55,392]. During this time although clinically asymptomatic, the delicate balance of the host immune system and viral replication dynamics are at constant war. There are high cell turnover levels, leading to a persistent state of activation resulting in chronic progressive immune dysfunction (reviewed in [45]). During this period, CD4$^+$ T cell counts are normally at 600-1,200 cells/mm$^3$, [56] that decrease gradually if infected individuals are left untreated. An untreated individual’s CD4$^+$ T cell counts will eventually drop below 200 cells/mm$^3$ and reach a level in which the individual becomes susceptible to AIDS-defining opportunistic infections [e.g. Pneumocystis carinii pneumonia (PCP)] and neoplasms [45,57,58]. This chronic state of progression can be slowed for a majority of individuals when antiretroviral therapy (ART) is administered. Effective ART treatment reduces the viral replication rate to a degree that is undetectable and partially restores T cells numbers [59-65]. However, it does not
eradicate virus, as viral reservoirs remain and lead to progressive infection if ART is discontinued.

B. IMMUNE RESPONSES: THE PLAYERS INVOLVED IN HIV-1 INFECTION

1. DENDRITIC CELLS

Dendritic cells (DC) are present in essentially every tissue, where they operate at the interface of innate and adaptive immunity by recognizing pathogens and presenting pathogen-derived peptides to T cells. Immature DC (iDC) are professional antigen-presentation cells (APC) that are positioned throughout the peripheral tissue and act as sentinels against invading pathogens [66-69]. Non-specific antigen (Ag) capture is particularly efficient in iDC due to the high level of constitutive macropinocytosis. This Ag uptake of DC of exogenous antigen is not only in the form of peptides, but in the form of virus-infected, apoptotic, or necrotic cells, followed by processing through nonconventional pathways and cross-presentation of antigen in the context of MHC class I molecules (MHCI) to CD8+ T cells [70-77].

In iDC differentiated from blood monocytes, macropinocytosis rates are particularly high, such that the cell takes up its own volume in extracellular material every 60 minutes [78]. In response to immune stimuli, iDC migrate out of damaged or infected tissue and move to secondary lymphoid organs. En route, so-called ‘co-stimulatory’ molecules (e.g. CD80, CD86), adhesion molecules [e.g. CD50, CD54 (ICAMs)] and signaling molecules such as CD40, are upregulated or appear on the DC surface. DC are activated in response to engagement of toll-like receptors (TLR) by natural adjuvants such as bacterial cell wall components [79,80] or by
ligation of CD40 by CD40L expressed by activated CD4 helper T cells [81]. As indicated above, one consequence of activation is increased DC expression of T cell costimulatory ligands. In particular, increased CD80 and CD86 expression levels, above the basal resting state level, are critical for DC induction of CD8⁺ T cell activation. In fact, there is some evidence that it may not always be necessary [82] or sufficient [83,84] to prevent tolerance. Upon arrival in the lymphoid organs, DCs are partially matured, a process that is completed upon subsequent interaction with T cells. At this stage, endocytosis has ceased to be of primary importance to DC function and is virtually shut down.

DC activation stimulates T cells to produce a variety of cytokines which play a central role in Th1/Th2 paradigm. Basically, Th1 cells are considered IFN-γ-producing effector cells that can activate cytotoxic T cells. Th2 lymphocytes secrete IL-4, which induce humoral immune responses dominated by enhanced IgE production such as in allergic diseases. Thus, it is imperative for immunotherapies of HIV-1 infection to induce Th1 cell activity, with resultant enhancement of antiviral CTL activity.

2. CD8⁺ T CELLS

It is known that CD8⁺ T cells recognize peptides of 8-11 amino acids presented within the binding cleft of MHCI molecules. Most cells present peptides derived only from endogenous proteins on MHCI molecules and, by doing so, become targets of the efferent effector function of CD8⁺ T cells. Recognition of infected cells is mediated by interaction of the TCR on the CTL with pathogen derived peptides in the context of MHCI molecules. All nucleated cells, (except
neurons), express MHCI molecules, which allows CTL to CD8\(^+\) T cells to respond. It is these responses that appear to play an important role in viral control. CTL responses are present during primary infection and the onset of this activity correlates in time with peak viral load during primary infection. Depletion of these cells in SIV-infected macaques results in increased viral load and more rapid disease progression [51,52,85]. Many of these characteristics are found in CD8\(^+\) T cells isolated from patients with chronic HIV-1 infection. In contrast to HIV-1-specific cells in patients with naturally controlled infection, CD8\(^+\) T cells from those with chronic progressive infection proliferate poorly in response to cognate antigen, are poor producers of IL-2 and TNF and have an effector rather than central memory phenotype [86,87]. Further studies have suggested a halt in maturational development of these cells with defective expression of CD127 [88-90] and an accumulation of immature effector cells that have apparent deficiencies of effector molecules (granzyme B, perforin) production, increased levels of markers of senescence or terminal differentiation such as CD57 [91] and reduced expression of co-stimulatory molecules such as CD28 and CD27 [92-95]. Flow cytometry has revolutionized the HIV field by allowing researchers, for the first time, the capability of looking at rare events and many cell surface markers at once (11-color, 13 parameters). This has been especially powerful in the definition of CD8\(^+\) and CD4\(^+\) T cells and their classification as naïve, effector, central memory, or effector memory [86,94,95]. The considerable efforts to delineate and characterize the subsets of antigen-specific memory T cells including cell surface markers including CD45RA/RO, CD27, CD28, CD57, CD62L, CD127 and CCR7 have all been used in various combinations to define memory cell populations that are responsible for effective antiviral immunity [86,94,96-99]. This information can be combined with the functional correlates (IFN\(\gamma\), TNF\(\alpha\), IL-2, CD107a and b, perforin, granzyme A and B) which have been termed ‘functional signatures’
Understanding these functional patterns of antigen-specific responses is likely to lead researchers to the crucial missing pieces of viral control in acute and chronic infections.

3. CD4⁺ T CELL HELP

CD4⁺ T cell epitopes are processed by APC in membrane-bound vesicles, where the native proteins are degraded by proteases into the peptide fragments that bind to MHC class II proteins. They are then delivered to the cell surface, where class II-peptide complexes can be recognized by the CD4⁺ T cells [101]. The importance of CD4⁺ T cell was appreciated for another reason, i.e., their help of maintenance of CD8⁺ T cell functions during chronic infections [102,103]. Many chronic infections are more severe in the absence of adequate CD4⁺ T cell help, and the quality of the CD8⁺ T cell response is often substantially worse. Elimination of CD4⁺ T cells also leads to the impaired long-term control of murine gammaherpes virus infection [102,104,105]. For humans, loss of CD4⁺ T cells during HIV-1 infection often precedes or is associated with CD8⁺ T cell dysfunction and AIDS progression [106]. CD4⁺ T cells also appear to play an important role in the optimal priming of CD8⁺ T cells during acute infections [107-109].

The common finding of these recent reports is that secondary expansion of memory CD8⁺ T cells following re-stimulation is dramatically reduced if the CD8⁺ T cells were originally primed in the absence of CD4⁺ T cells [110]. Studies using acute infections of mice suggest that, as long as CD4⁺ T cells were present during the initial priming, then CD4⁺ T cell help was dispensable during secondary challenge. However, recent studies with chimpanzees have
demonstrated that optimal recall CD8+ T cells responses following hepatitis C virus (HCV) infection can depend on the presence of CD4+ T cells at the time of HCV challenge, even when the virus-specific CD8+ T cells were originally primed in the presence of CD4+ T cell help [111]. CD4+ T cell responses (provide CD40 signaling to host APC) are likely important for optimal generation of memory CD8+ T cells following acute infections and for sustained CD8+ T cell responses during chronic infections. However, HCV experiments suggest that CD4+ T cell help may also be critical at the time of challenge with virulent infections. Recently, a new facet to DC communication with CD4+ T cells was shown, where they increase the ability of DC to attract and retain Ag-specific CD8+ T cells [112]. Together, these studies provide evidence that in the absence of signals provided by CD4+ T cells, the differentiation program of CD8+ T cells may be altered. It will be important to determine the impact of CD4+ T cell deficiency on not only the generation of functional effector CD8+ T cells, but also on memory CD8+ T cell differentiation, including the transition from T cell central memory to T cell effector memory [103].

4. CYTOKINE INFLUENCES ON CD8+ T CELLS: IL-7 AND IL-15

During CD8 T cell response to infection, there are three characteristic phases: a period of initial activation and expansion, a contraction or death phase, and the establishment and maintenance of memory [132-134]. The analysis of CD8+ T cell responses has been greatly facilitated by the introduction of MHC tetramer technology that allows accurate enumeration and phenotypic characterization of antigen-specific T cells by flow cytometry [135,136]. IL-15 reduces spontaneous and CD95/Fas-induced apoptosis of HIV-1-specific CD8+ T effector memory populations [119]. An important component of this homeostatic turnover is that there is no net increase in CD8+ T cell numbers, resulting in maintenance of the memory CD8+ T cell
pool at a constant size. The cytokines IL-7 and IL-15 are primarily responsible for this homeostatic turnover of memory CD8$^+$ T cells [137-141]. In particular, in the absence of IL-15 signal, memory CD8$^+$ T cells can be generated, but these cells fail to undergo homeostatic division and their cell numbers decline over time [137]. In comparison, IL-7 signals provided during the memory phase of the immune response appear more important for memory CD8$^+$ T cell survival [138,142].

IL-15 has been reported to have effects on T cell survival and proliferation [113-121], both in vitro [122-124] and in vivo [122,125-128]. This cytokine shares overlapping biologic properties with IL-2, acting through the $\beta$ and $\gamma$ chains of IL-2 receptor [129,130], as well as, through a specific IL-15 receptor $\alpha$ chain. It is thought to possibly mediate anti-apoptotic signals (i.e. Mcl-1 and Bcl-2) [122,131].

Current research by Melchionda [115] has shown that adjuvant IL-7 or IL-15, overcomes immunodominance and improves survival of the CD8$^+$ T cell memory pool. Vaccines that aim to induce and maintain high level T cell responses for chronic infections such as HIV and cancer have had limited success. The challenges of immunization for cancer and chronic infections (e.g. HIV-1, HCV) are multifaceted and include ongoing mutation of target antigens [143], anergy and/or suppression due to chronic antigen overload [144], and the large size of the target pool, which requires dramatic T cell population expansion for therapeutic benefit [145]. Thus, new approaches for increasing in the size and broadening the diversity of effector and memory pools generated after immunization are needed to improve the prospect of generating an effective preventative vaccine for HIV-1 and to enhance the effectiveness of immunotherapy for chronic infections. Currently, paradigms hold that memory CD8$^+$ T cells represent highly “fit” cells
derived from the surviving effector T cell population, thus leading to the prediction that therapies which augment T cell effector pools will also augment T cell memory pools.

C. IMMUNE ESCAPE: GENETIC FACTORS (VIRAL AND HOST)

1. VIRUS HETEROGENEITY

Today we know that due to the infidelity of reverse transcription (RT), HIV-1 rapidly diversifies in the infected individual and, as a consequence, is able to adapt readily to changes in its environment. A possible underlying mechanism for the loss of anti-HIV-1 CTL is development of immunologic escape mutants of HIV-1. Env gp120, and particularly the V1-V5 domains, demonstrates a great deal of heterogeneity during the chronic phase of infection [146,147]. This heterogeneity is due to the infidelity of the RT [148]. Several error mechanisms have been ascribed to viral polymerases [149]. First, direct mis-incorporation of a non-complementary nucleotide produces a single-base substitution error. Second, slippage of the two DNA strands may occur at repetitive sequences to generate either a deletion (unpaired nucleotide(s)) in the template strand or addition (unpaired nucleotide(s) in the primer strand); each of these events can include one or more nucleotides. Third, frame shifts are caused by misincorporations followed by misalignment of the template-primer. Fourth, base substitutions can also result from dislocation mutagenesis, which adheres to the following pathway: slippage, correct incorporation, and realignment. Each of these mechanisms can yield single-nucleotide mutations over large distances to produce changes involving many nucleotides.

The infidelity of RT for a variety of retroviruses has been determined in vitro by measuring misincorporation in reactions with defined RNA or DNA templates. This
misincorporation rate for HIV-1 RT ranges from 1 per 1,700 to 4,000 nucleotides [150,151] whereas, other retroviruses have lower rates (e.g., error rates are 1 per 9,000 to 17,000 nucleotides for avian myeloblastosis virus and 1 per 30,000 nucleotides for murine leukemia virus). Retroviral polymerases do not have 3’-5’ exonuclease proofreading activity for correcting polymerization errors. Therefore, these enzymes as a group have a much higher misincorporation rate than cellular DNA polymerases. The HIV-1 genome is about 9.2 kb with an \textit{in vivo} error rate that is estimated to be about 1 to 3 misincorporations per replication cycle [149].

Through the error mechanisms stated above, as well as recombination events, RT is responsible for the production of viral sequence diversity in infected individuals. HIV-1 infection differs from many other acute and chronic viral infections in the magnitude and duration of viremia, as well as, the high level of genetic variation \textit{in vivo} [152]. This rapid turnover generates $10^9$ to $10^{10}$ virions per day [153,154], which accompanied by a high degree of misincorporation, leads to the generation of a broad range of quasispecies with a heterogeneous pattern of immunogenicity.

\section*{2. GENETIC POLYMORPHISMS AND DISEASE PROGRESSION}

A number of host genetic factors influence the rate of disease progression in HIV-1 infection. The most extensively studied of genetic factors that might affect disease progression are associations between HLA alleles and disease progression not only for HIV-1 but for other pathogens as well. Over many years, it has become clear that host genetic differences between individuals, as well as between species affect the susceptibility or resistance of disease progression. These differences reveal a clinical spectrum of rapid, intermediate, or slow progression or, more rarely, nonprogression to AIDS within infected populations. A range of
distinct genetic host factors, linked to the relative susceptibility or resistance to AIDS, influences disease progression.

The human leukocyte antigens (HLA) loci encode two distinct major MHC I and MHCII, haplotypes of highly polymorphic cell surface glycoproteins that bind and present processed antigenic peptides to T cells of [155,156]. MHC I molecules present endogenous antigen, synthesized and processed in the infected cells, to the CD8⁺ cytotoxic T lymphocytes (CTL) that kill the infected cell. Class II MHC molecules present peptides as well, but is generated in the intracellular vesicles of B cells, macrophages, and other cells to be recognized by CD4⁺ T cells.

The highly polymorphic HLA class I molecule helps to determine the specificity and repertoire of the immune response. The extensive polymorphism at the HLA loci is thought to have arisen through natural selection by infectious diseases, operating on the diversity generated by mutation, gene conversion and recombination [157,158]. At a population level, genetic diversity of the HLA loci is maintained by enhanced antigenic peptide-binding capacity, and therefore resistance to infectious disease. Individuals heterozygous at HLA loci are capable of presenting a broader array of pathogen-derived peptides, resulting in a more diverse CTL repertoire and the ability to resist a greater breadth of infectious pathogens; whereas individuals that are homozygous at HLA loci have been observed as having a more severe disease progression. The MHC alleles that are most consistently reported in the literature of having associations on the impact of HIV-1 disease are: HLA-B27, B57, B58 [44,159-167] nonprogression and, HLA-A1, B8 [168,169], and HLA-B35 rapid progression [170,171]. HLA associations with HIV-1 disease have been somewhat inconsistent [393] depending on the size and population sample studied.
In 2004 instead of looking at a single MHC class I haplotype for disease association, Kiepiela et al. [172] took a more general approach and demonstrated the relative contributions of HLA-A and -B alleles in their inferred impact on disease progression. In studying this cohort (375 HIV-1 infected individuals) it was demonstrated that a 2.5 fold increase in the number of CD8\(^+\) T cell responses were found to HLA-B compared to HLA-A. Furthermore, a greater selection pressure (4.4 fold) is imposed on HIV-1 by HLA-B than HLA-A alleles.

Recently, Fellay et al. [173] used a whole-genome association strategy to try to explain the variations seen among HIV-1 infected individuals. This study looked only at the early phase (characterized by asymptomatic viral set-point) of 30,000 individuals by combining 4 studies in which the authors identified 486 individuals matching their criteria. All the samples were genotyped with Illumina’s HumanHap550 Beadchip with 555,352 single-nucleotide polymorphisms (SNPs). The first association was found in HCP5 (HLA complex P5) which contributed 9.6% of total variation of set point. The authors showed HCP5 and ZNRD1 (zinc ribbon domain-containing 1) contribute to the control of associated HLA-B*5701 (a known association with nonprogression). The second was found in a region of HLA-C (rs9264942) lowered viral load. A third set of polymorphisms was located in a gene upstream that encodes for RNA polymerase I subunit with also contributed to the total variation in disease progression. This important work identified two new mechanisms that were not previously associated with disease progression: HLA-C and RNA polymerase subunit. These new associations give possibilities for new targets in therapeutic interventions.

Other genetic factors affect virus entry and critical processes for the intracellular replication of lentivirions as well as subsequent early innate and highly specific, adaptive host responses [174]. Greater than ten genes and fourteen alleles with polymorphisms in the
chemokines or their receptors have been associated with either a positive or negative effect on infection and disease progression. The most notable, of the chemokine receptors, CCR5 is associated with the resistance to HIV-1 infection [175] by a 32bp deletion--the others include CCR2, CXCR4, CXCL12, CX3CR1 and Rantes (CCL5) (reviewed [176]). Transporter-associations with antigen-presenting (TAP) genes, which are members of the MHC class III family of alleles, also has been observed to play a role in determining the rate of disease progression in HIV-1 infection putatively by their ability of transport efficiency of processed antigen from proteasomal cleavage [177, 178-182].

3. CONSEQUENCES OF HIV-1 GENETIC VARIATION

Sequence variation is thought to affect CTL recognition in at least three ways: (1) blocking correct transport and processing of the antigen, (2) blocking peptide binding to the MHC molecule, and (3) blocking optimal recognition of the peptide MHC complex by the TCR. In the last mechanism, either the peptide/MHC complex will fail to engage the TCR [183], or the TCR may be sub-optimally engaged by the altered peptide/MHC complex, resulting in a decreased ability of that CTL to respond upon encountering a cell that presents that peptide/MHC complex to which the CTL was originally generated (antagonism) [184,185]. The earliest study demonstrating virus escape from a CTL response was in a LCMV model in which mice transgenic for an LCMV specific T cell reported TCR derived from a T cell clone were infected with a high dose of LCMV, and mutant viruses were rapidly selected [186].

Studies have shown that CTL are directed across the proteome and each protein can have multiple T cell epitopes [187,188,189]. In studies reported by Couillin et al. [190], individuals
infected with HIV-1 generated a great diversity of CTL. This study looked at the immunodominant region in the \textit{Nef} protein and suggested that \textit{in vivo} escape from CTL epitopes occurs as a result of alterations in anchor residues (affecting peptide binding to MHC), non-anchor residues (affecting TCR recognition) and flanking residues (affecting transport/processing).

Borrow et al. [191] demonstrated that CTL escape in primary infection on the single epitope level from a HLA-B44 positive subject. The epitope from the CTL clone contained the HLA-B44 binding motif. As disease progressed, the emergence of a mutant virus population was able to escape recognition by the primary HIV-1 specific CTL response. Goulder et al [192] subsequently showed immune evasion for HLA-B27 restricted epitope at stages of high viral replication and turnover. Other studies that included siblings with identical HLA types studied gag by restricted HLA-B8 epitope and were able to demonstrate genetic variation in gag altered CTL recognition.

Brumme et al. [193] analyzed 765 HIV-1 infected individuals that were drug naïve and demonstrated that MHCI-mediated responses within selected functional, accessory and regulatory genes did not target anchor residues for CTL escape within published epitopes. They found an inverse relationship between HLA mutation within these published epitopes and lower CD4$^+$ T cell counts, supporting the link between disease progression and the presence of escape mutations. Many other studies [194-198] looking at HIV-1 escape CTL epitopes across the genome, support this mechanism as one major contribution to the virus’ ability to avoid replication control.
The simian model also supports that CTL escape mutations are associated to disease progression [199-202]. As example, SIV-infected macaques were followed from primary infection to death [203]. Ten CTL epitopes accumulated amino acid replacements and showed evidence of positive selection by the time the macaques died. Many of the amino acid replacements in these epitopes reduced or eliminated MHCI binding and/or CTL recognition.

Interestingly, emerging data suggest that HIV-1 and SIV have adapted to their animal hosts since their introduction into these species, and are likely to continue eliminating important targets of the host cellular immune responses (CTL) [193,194,204,205]. However, in interpreting these studies, one must be cognizant of founder effects which could be responsible for the observation of imprinting (e.g. escape mutations that are selected due to population frequencies of common MHCI alleles) and perpetuate in the population. The escape mutations that can perpetuate in the circulating viral population are capable of frequent reversion of escape CTL epitopes in hosts that do not express the particular allele. These CTL epitopes restricted by common alleles may be eliminated from the circulating viral population, (termed ‘relic’ epitopes) and should be considered in epitope vaccine designs.

For my research, however, having documented that variants of HIV-1 that escape a defined CTL response do exist within patients, it becomes logical to ask if these variants then predominate within the viral quasispecies of the patients. Viruses with variant amino acid sequences in CTL epitopes would have a survival advantage if these sequences escape recognition by the majority of CTL prevalent at the time. The persistence over time of escape mutant sequences that has been documented is strong evidence for this argument. If an epitope region must be conserved to ensure viral replication or survival, such antigens might elicit
sustained CTL responses. If, on the other hand, the virus tolerates amino acid changes in a CTL epitope, then escape mutants might arise. With the help of the Los Alamos Database [393], which compiles sequence differences between various HIV-1 clades and the sequence variability among defined optimal epitopes, we have the capacity to look at autologous virus isolates and CTL epitope changes over disease progression for many subjects.

D. VACCINES AND IMMUNOTHERAPY

The global need for an effective vaccine against HIV-1 is an understatement; growing numbers of individuals living with AIDS is reaching an astounding number. In some countries in Africa the infected individuals that succumb to AIDS leave many orphaned children and the instability of these nations could be leading to unknown levels of economic strife, leaving communities in crisis.

It has been over 200 years since Edward Jenner’s first experimental vaccination—that is, inoculation with the related cow-pox virus to build immunity against smallpox. The history of vaccine development has been largely successful (i.e. influenza, measles, HBV, diphtheria, etc.) however, their development has outpaced our understanding of what surrogate markers correlate with protection.

Over the past decade, major improvements in our understanding of memory T cell generation and differentiation have prompted the design of sophisticated vaccine strategies that induce potent and long-term antigen-specific cellular immunity. In addition to the design, the route of administration, the dose, the timing of the prime/boost regimens, and the processing and
presentation of the immunogens all influence the type and quality of immunity induced by the candidate vaccine.

Many HIV-1 vaccine efforts have emphasized breadth of MHCI restricted CD8\(^+\) CTLs due to previous studies showing a strong correlation with lower viral load. However, the current knowledge of the factors determining HIV-1-specific CTL antiviral efficacy is inadequate to interpret fully their significance when detected. One report provides evidence that broad CD8\(^+\) specificity may not be the answer for vaccination strategies. Kiepiela [206] compared influences of the number of epitopes targeted with HIV-1 proteins (Gag, Pol, Env, and accessory/regulatory) on viral load. The researchers revealed only Gag responses correlated with lower viral loads. Specifically, targeting zero or 1 Gag epitope was not significant, but viral loads were significantly lower when > 1 Gag specific CD8\(^+\) T cells responses were detected, showing an association with disease progression [206].

The functional plasticity of the DC and their unprecedented ability to orchestrate the immune system makes these cells a desirable candidate for manipulation in a vaccine. Their versatile nature, capacity to process and present various forms of antigen (e.g. whole inactivated virus [207-209], live-infected cells [210], apoptotic or necrotic preparations [208] [211], protein and peptides [212-214], transfected DC with HIV-1 plasmids of mRNA or DNA [215-218] and \textit{ex vivo} manipulability, all indicate the seemingly limitless means through which DC may be integrated into vaccine design.

The basic rationale for DC immunotherapy in HIV-1 infected persons is to reinstall T cell functional responses that resemble those observed in the LTS (long term survivors). How best to use DC as a vehicle to overcome immune dysfunction is a difficult challenge. There are many obstacles that must be appreciated to achieve the end result—an immune response that controls
viral replication. Currently, the primary objective is to elicit strong type-1 immunity; and thus, selecting a DC population that engenders such immunity is imperative. There are many cocktails of immunomodulating factors that promote DC maturation, each resulting in different immune outcomes. When GM-CSF and IL-4 are used to culture monocyte derived (md-DC) for 5 days and then compared with IFN$\gamma$ and GM-CSF (to immunomodulate), the stimulation of naïve and memory CD$8^+$T HIV-1-specific responses was superior for the latter. This is most likely because IFN$\alpha$ is a type I IFN and has been shown to be essential for effector T cell generation [103]. If this group (IFN$\alpha$ and GM-CSF) was compared with IFN$\gamma$ and CD40L as T cell immunomodulators, their IL-12 production levels would have been greatly increased and this cytokine contributes prominently to the stimulation capacity of these APC. Current in vitro studies ongoing in our laboratory indicate the requirement of CD40L (normally provided by the CD4$^+$ T cells) for optimal maturation of DC to produce high levels of IL-12 to elicit a Th1 response [219]. Unfortunately, since CD40L is not approved for clinical applications, researchers have to use pro-inflammatory cytokine (IL-1$\beta$, IL-6, TNF$\alpha$ and PGE$_2$) cocktails instead [389].

Another problem with ex vivo manipulation of DC for use in immunotherapy is whether the cultured cells become functionally exhausted prior to administration by a vaccine in vivo. The current standard is to manipulate DC to sustain the production levels of IL-12p70 upon subsequent interaction with activated Th1 cells in vivo [220]. PD-1 (programmed death-1) has recently been shown to be highly expressed on exhausted T cells during chronic viral infections (LCMV, HIV-1 and HCV), and blockade of PD-1 or PD-L1 (programmed death-1 ligand) can revive exhausted T cells, enabling them to proliferate and produce effector cytokines [221-227]. PD-1L interacts specifically with the B7-1 costimulatory molecules to inhibit T cell responses [228]. Therefore, this new parameter should be added to the list of jobs the DC should be able to
perform once manipulated *ex vivo* and re-administered *in vivo*—that is the stimulation should not increase the PD-1 expression levels on CD8+ T cells rendering them ineffective. By artfully priming DC to enhance Th1 over Th2 responses, the deficiency in T cell reactivity during HIV-1 infection could potentially be reversed and immunologic normalcy restored.

Although we remain ignorant of how some individuals are able to control virus replication, LTS (long term survivors) immune response profiles provide researchers a population of individuals in which to study and thereby an opportunity to understand the possible parameters that equate with long term protection [18,229]. As we become more knowledgeable of efficacious CD8+ T cell responses, and what roles they provide in protection; we will be able to develop detection methods as surrogate markers that correlate to protection in evaluating *de novo* vaccination strategies.

**E. METHODS: PREDICTION OF CTL EPITOPES AND PRIMING MODEL**

1. **COMPUTER PREDICTION OF CTL EPITOPES:**

Several computer algorithms have been devised to take advantage of protein sequence information to search for T cell epitopes. A number of CTL epitopes have been predicted for other viruses using this method, including herpes simplex virus (HSV), and HCV. These algorithms test each sub-sequence of a given protein for traits thought to be common to immunogenic peptides, thus locating regions with a higher-than-average likelihood of inducing a cellular immune response *in vitro*. Using these algorithms, the array of sites that would require *in vitro* testing for immunogenicity may be significantly reduced, directing experimental efforts to more promising segments of the protein and thus dramatically reducing the time and effort
needed to locate T cell epitopes. We chose to use such a computer algorithms (BIMAS) (http://thr.cit.nih.gov/molbio/hla_bind) [230-233] in order to scan the vast amount of available autologous viral sequences for each of the patients over the course of their disease progression. This program method was developed by Parker et al. [230] and is based on coefficient tables deduced from the published literature. For HLA-A2, peptide binding data were combined together to generate a table containing 180 coefficients (20 amino acids x 9 positions), each of which represents the contribution of one particular amino acid residue at a specified position within the peptide [230]. This website allows users to locate and rank 8-, 9-, or 10-mer peptides that contain peptide-binding motifs for HLA class I molecules and has been updated over the years from the literature. This program is one that has been shown to be accurate in its predictive value for the haplotypes HLA-A*0201 and B*0702 when compared to other systems [234]. The published literature includes binding motifs for a wide variety of human class I and class II MHC alleles.

However, it remains for the most part unclear whether such motifs hold true predictive value. Only a small fraction of the possible peptides that can be generated from proteins of pathogenic organisms actually generate an immune response. In order to be presented to CD8+ T cells a precursor peptide must be generated by the proteasome. This peptide may be trimmed at the N-terminal by other peptidases in the cytosol. It must then bind to the transporter associated with antigen processing (TAP) in order to be translocated to the endoplasmatic reticulum (ER). Only half the peptides presented on the cell surface are immunogenic probably due to the limited size of the TCR repertoire. The most selective step is binding to the MHCI molecule, since only 1/200 binds with an affinity strong enough to generate an immune response [155,231,235-237]. For comparison the selectivity of TAP binding is reported to be 1/7 [238]. This all happens in
competition with other peptides, so in order for a peptide to be immunogenic, it must go through
the above described process more efficiently than other peptides produced in a given cell
(reviewed in [239]). The CTL response against viruses is often focused on a few epitopes, and
the reason for this selectivity is not obvious. Many peptides were identified in viral proteins by
scanning for MHCI binding motifs that are able to bind to MHCI molecules [240,241], but this
does not ensure antigenicity. Therefore, the use of motif-derived epitopes many not represent the
entire repertoire of peptides from published reports. For this study only two haplotypes: HLA-
A*0201 and –B*0702, were chosen based on their higher frequencies in the Caucasian
population and extensive evaluation, thereby more accurate availability of prediction data. The
prediction motifs for these two haplotypes are shown in Figure 3.

**Figure 3: Motif of HLA-A*0201 and HLA-B*0702.** Primary anchor positions and preferred residues are listed
under the position (Bolded) and the residual or secondary anchor residues are not bold faced. Motifs are based on
2. PRIMING MODEL

Taking what is known about priming of naïve CD8\(^+\) T cells to generate Ag-specific CD8\(^+\) T cells I developed an \textit{in vitro} priming model that was originally based upon previous work in our lab \cite{211,242} and others \cite{243,244}. Naïve CD8\(^+\) T cells egress from the thymus and enter peripheral blood and lymphoid organs where they are engaged by MHCI/peptide complex (signal 1) \cite{245} along with the costimulatory molecules (signal 2) \cite{74,246,247} on the surface of mDC activates the T cells to clonally expand and with the help of CD4\(^+\) T cells \cite{107-109,248}, and IL-12 (signal 3) (Figure 4) \cite{82,249-253}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure4.png}
\caption{Naïve CD8\(^+\) T cell require three signals for full activation and avoidance of tolerance. Stimulation through the T cell receptor (TCR) and CD28 in the absence of a third signal stimulates proliferation, but effector function does not develop and the cells are tolerant long term. The third signal can be provided by interleukin-12 or type I interferons produced by DCs in response to TLR engagement or CD40-dependent interaction with CD4\(^+\) T helper cells. Reprinted with permission from Mescher et al. 2006, Immunological Reviews 211: 81-92.}
\end{figure}
The optimal activation of naïve CD8\(^+\) T cells requires prolonged exposure to all three signals (antigen, costimulatory molecules and IL-12) for 30-60 hours [103,254-256]. However, memory CD8\(^+\) T cells do not require a third signal to develop effector function [82,251]. All these parameters were combined to establish an *in vitro* priming model that used the high efficiency of the APC to process Ag, express high levels of costimulatory molecules (e.g. CD80, CD86) and IL-12 production for long periods of time when they encounter CD8\(^+\) and CD4\(^+\) T cells in culture to evaluate immunogenicity against consensus and autologous HIV-1 viral peptides.
II. CHAPTER TWO: SPECIFIC AIMS

My overall hypothesis is that HIV-1 infected subjects with chronic, progressive infection, have naïve T cells that can be primed against HIV-1 by antigen loaded DC in vitro, and that this can serve as a focus of immunotherapy for HIV-1 infection. Memory CTL derived in vivo from these naïve cells are eventually unable to react to evolving variants of HIV-1, thereby allowing escape mutants to replicate and cause disease. The loss of anti-HIV-1 memory CTL activity could be due to changes in the CTL epitopes that arise in the variant quasispecies, resulting in immune escape. During ART, there is only partial recovery of memory T cell immunity to the virus. The effect of progressive HIV-1 infection, and HIV-1 infection after ART, on the recovery of the naïve T cell population specific for HIV-1 is not known. This presents a major obstacle for development of immunotherapies for HIV-1 infection. To overcome this challenge, I further hypothesize that naïve T cells do recover and are able to be primed by the persons’s own viral antigens, including immune escape variants, after ART. Understanding CD8$^+$ T cell responses in progressive HIV-1 infection and during ART is critical to how researchers deal with antigenic diversity of the virus in vaccine design. Many studies have examined virus-specific CTL responses, but are still unable to correlate their IFN$\gamma$ production to disease progression, which in part, may be due to the use of consensus strain when determining the breadth of responses in different populations. Therefore, the specific aims of this project were:
Specific Aim 1: Identify potential MHC class I CD8$^+$ T cell epitopes in autologous HIV-1 sequences of MACS participant #8.

A. Identify potential HLA-A*0201 MHC class I CTL epitopes in Gag, Nef and Env of HIV-1 autologous sequences of MACS participant #8.

B. Identify potential HLA-B*0702 MHC class I CTL epitopes epitopes in Gag, Nef and Env of HIV-1 autologous sequences of MACS participant #8.

Hypothesis: The loss of anti-HIV-1 CTL activity during progressive HIV-1 infection could be due to changes in the CTL epitopes that arise in the variant quasispecies, specifically in their MHC class I binding capacity and its impact on disease progression (e.g., CD4$^+$ T cell counts and viral load).

Methods: Analyze autologous HIV-1 sequences of Gag (p17 and p24), Nef and Env (C2-V5) regions from the Multicenter AIDS Cohort Study (MACS) participant #8 to determine potential epitopes for binding of peptides to MHCI molecules. The analysis will utilized one of the many computer algorithms (BIMAS) that are available to predict potential MHCI binding to regions of amino acid sequences. Based on the analysis of haplotypes HLA-A*0201 and -B*0702, some 9- and 10-mer epitopes will be further characterized for functional binding to soluble MHCI molecules to access the validity of this model and their impact on disease progression measured by associations with the CD4 T cell counts and viral load.
Specific Aim 2: Develop an *in vitro* priming model to evaluate potential binding of MHC class I CTL epitopes.

A. Determine dendritic cell maturation by phenotyping surface expression of CD80, CD83, CD86, MHC class I (HLA-ABC), MHC class II (HLA-DR).

B. Determine mature dendritic cell cytokine production of IL-10 and IL-12.

C. Determine mature dendritic cells capacity to prime naïve CD8\(^+\) T cells.

**Hypothesis:** HIV-1 peptide antigen loaded, mature dendritic cells (DC) have the capacity to prime naïve CD8\(^+\) T cells *in vitro*.

**Methods:** Define the best methods for maturation and antigen presentation of DC for priming of T cells through monitoring of their phenotype and cytokine production. Different known DC immunomodulation factors will be studied for their capacity to induce primary responses of naïve CD8\(^+\) T cells *in vitro* as assessed by chromium release, IFN\(\gamma\) ELISPOT and tetramer staining of HIV-1 peptides.

Specific Aim 3: Compare primary and memory response in MACS participant #8.

A. Determine memory T cell responses to evolving variants over disease progression in MACS participant #8.

B. Determine naïve T cell responses in preseroconversion samples of MACS participant #8.
**Hypothesis:** The change in HIV-1 epitopes due to CTL selective pressure will result in failure of memory CD8\(^+\) T cell responses to late escape variants. However, on ART it is possible to enhance secondary and primary T cell responses with DC expressing autologous HIV-1 antigens that may potentially help to control viral replication.

**Methods:** The new priming model established in specific aim 2 will be used to compare naïve CD8\(^+\) T cell responses (pre-seroconversion PBMC) to memory (ex vivo PBMC) responses of early, late and ART time points during progressive infection of MACS participant #8. This will be accomplished by utilizing this individual’s autologous ‘founder’ and evolving viral variant sequences over time, and DC derived from his blood monocytes during ART. The immunogenicity of these sequences will be assessed by IFN\(\gamma\) ELISPOT assay.
Preface

This manuscript is in preparation for publication. Bonnie Colleton conducted MHC class I binding experiments using the T2 cell assay and a radioactivity competition assay, and did all the data compilations and analyses. Raj Shankarappa provided the autologous viral sequence data over the course of disease progression for MACS participant #8 and worked with Bonnie Colleton on determining which epitopes should initially be tested for MHC class I binding affinity.
Abstract

A hallmark of HIV-1 infection is its high level of genetic divergence over time, leading to accumulation of genetically distinct quasispecies within an individual. This genetic diversity of HIV-1 can lead to changes in the capacity of viral peptides to bind to MHCI molecules, which in turn could result in altered recognition and response by CD8^+ T cells. To investigate this effect, I identified potential MHCI-binding epitopes of Gag, Nef and Env from evolving viral sequences of untreated, HIV-1 infected individuals using an HLA binding prediction model and derived peptides from these epitopes. I showed that MHCI epitopes of variants evolving from the founder strain have weaker, neutral and stronger patterns of predicted binding. I confirmed these patterns using an MHCI binding assay. These changes in MHCI binding could lead to alterations in the T cell immune responses and represent a potential viral escape strategy.
Introduction

HIV-1, a highly divergent virus, generates genetically variants that are driven by the infidelity of the reverse transcriptase (RT). This high error rate of the RT has been determined in vitro [151,257-259] by measuring misincorporations. The genetically distinct variants that are generated, leads to an accumulation of variants or quasispecies [260] in the host. Assessing the quasispecies within an individual over time is important for our understanding of impact on disease progression and pathogenesis. However, due to the unusually high level of variants, practical approaches dealing with the quasispecies’ impact on disease progression is needed. In this study I propose an approach that uses MHC class I (MHCI) epitope prediction modeling and their correlations to CD4 T cells and HIV-1 copy numbers as a measure of disease progression.

MHCI molecules conventionally bind peptides between 8-10 residues, with N- and C-terminal ends pinned in the binding groove [261]. On average, only 0.1-0.5% of all overlapping 9-mer and 10-mer peptides spanning a protein will bind to an individual HLA class I molecule [262]. Because MHCI binding peptides that bind to different MHCI haplotypes are related by sequence similarity, prediction of MHCI peptide binding has traditionally been accomplished using sequence motif patterns. These sequence patterns are usually extracted from large numbers of known peptides, or from pool sequencing experiments [231,263]. The specific amino acids in the motif are anchor residues, which occur at specific positions [263]. Such sequence patterns, however, have proven to be over simplifications, as the binding ability of a peptide to a given MHC molecule cannot be explained exclusively in terms of the presence or absence of an anchor residue [264,265].

An epitope is defined as the molecular structure recognized by immune receptors, and immunogenicity is defined as the capacity of an epitope to induce a cellular immune response.
Only peptides interacting with MHC molecules above a certain affinity threshold are likely to be recognized by T cells and generate a cellular response [266-268]. Prediction of which peptides can bind MHC molecules is commonly used to assist in the identification of T cell epitopes. In turn, identification of epitopes is a key step toward accurately measuring immune responses, understanding host pathogen interactions, developing diagnostic tools, and the development and evaluation of new vaccines.

Computational prediction and modeling of peptide-MHCI binding is of considerable interest because it can greatly facilitate epitope screening, with tremendous concomitant savings in time and experimental effort [269]. While some of these methods are structure-based [270-274] or make use of structural information [275], the majority of binding prediction methods are sequence-based, including BIMAS [230], SYFPEITHI [231], RANKPEP [276], SVMHC [277], and MULTIPRED [278]. For my analysis of HIV-1 peptide binding to MHCI, I chose BIMAS based on the study by Peters et al. [234] who assembled a large database of experimentally acquired binding energies with a range of MHC molecules. The authors compared 3,089 9-mer and 10-mer peptides binding to HLA-A*0201 to predicted binding scores to measure IC$_{50}$ [nM] (or the disassociation rate) using the BIMAS model, giving an $R^2$ of 0.48 with an area under the curve (AUC) of 0.920 and 0.873, respectively, and an AUC=0.908 for HLA-B*0702 9-mers, supporting a strong correlation with the predictive power of this model. Therefore, this program is one that has been shown to be accurate in its predictive value for the haplotypes HLA-A*0201 and B*0702 when compared to other systems [234] and was used for the present study.

Different in vitro techniques are used to verify these in silico predictions, i.e., pMHC-binding assays using either cell-bound class I molecules (cellular-based assays) or solubilized class I molecules (cell-free assays). I have used three of these binding assays to assess the
BIMAS prediction model for HIV-1 peptides derived from quasispecies derived from Multicenter AIDS Cohort Study subjects with HIV-1 infection. Transporter associated with antigen processing (TAP)-deficient T2 cells [279,280] are HLA-A*0201 human cells that are defective in antigen processing but effectively present exogenous supplied peptides. Class I MHC stabilization assays to estimate peptide binding can be done by incubation of exogenous peptide and subsequent cellular staining with a class I MHC conformation dependent antibody [281]. A second type of cellular-based assay consists of eluting naturally class I MHC-bound peptides from the cell surface of EBV-transformed B cells and of carrying out a competition assay with the test peptide and a fluorescence-labeled reference peptide [282]. A third type of binding assay is cell-free quantitative biochemical binding assays to assess the ability of peptides to associate with purified class MHCI heavy chain and light chain (β2 microglobulin) in vitro [283-286]. Initially, biochemical binding assays used radioactive iodinated peptides in a competition setting [287]. More convenient alternatives to radioactive labeling have been developed by using fluorescent-labeled peptides [288]. Recently, fluorescence polarization was introduced for quantitative affinity measurements [289,290]. The accuracy of affinity determination can be indirectly assessed by correlating the measurements from different methods. Indeed, Buchli et al. showed a good correlation between relative affinities determined by both fluorescence polarization and radioactive methods (R²=0.71-0.86) or between fluorescence polarization and peptide elution-based cellular methods (R²=0.62-0.82) [290]. Thus, fluorescence polarization-based assays feature high-throughput screening capacity and are advantageous over other methods in terms of cost-effectiveness, affinity range, precision and reproducibility.
In this study, I show that MHCI epitopes of variants evolving from the founder strain have weaker, neutral and stronger patterns of predicted binding. I confirmed these patterns using a fluorescence polarization-based assay. These changes in MHCI binding could lead to alterations in the T cell immune responses and represent a potential viral escape strategy.
Materials and Methods:

Study participant

Participant #8 is a homosexual male enrolled in the Multicenter AIDS Cohort Study (MACS) [291] among a group of individuals for which we had characterized the virologic [292-294] [295] and immunologic [296] features. The MACS recruited homosexual/bisexual men in 1983-1984 and has studied them twice yearly by physical examination and laboratory testing. HIV-1 seropositivity was defined as a positive enzyme-linked immunosorbent assay (ELISA) and a Western blot with bands corresponding to at least two of the Gag, Pol, and Env proteins of HIV-1. Multiple Gag (p17, p24), Nef and Env (C2-V5) sequences were derived at 6 month intervals over the course (greater than 10 years) of infection, with the earliest sequences sampled at 4 months following seroconversion [294] were used for this analysis.

Analysis of T cell phenotypes

Lymphocytes are gated on CD45 (PerCP) and then analyzed for CD3\(^+\) (FITC), CD4\(^+\) (PE) or CD3\(^+\) (FITC), CD8\(^+\) (PE) cells were stained according to previously described methods using antibodies obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA). Unstained cells and isotype-matched, control antibodies were used for accurate discrimination of positively staining from negatively staining cells. All analyses were carried out on an EPICS XL flow cytometer (Coulter Electronics, Hialeah, FL) and 5000 lymphocytes were analyzed from each sample.
Measurement of viral load

Levels of plasma HIV-1 RNA were quantitated by an internal control polymerase chain reaction assay [297]. This assay utilizes coreverse transcription and coamplification of the target RNA sequence with a control plasmid HIV-1 RNA containing a deletion in the gag region. The assay is reproducible and can accurately quantitate $10^1$ to $10^4$ copies of HIV-1 RNA within a linear range of amplification, and yields equivalent results on fresh and frozen samples.

Synthetic Peptides

Overlapping 15-mer peptides spanning the entire Gag, Nef, and Env sequences of HIV-1, each overlapped the next by 11 amino acids were analyzed using BIMAS website. Potential epitopes were targeted based on predicted binding scores and previously published as well as trends discovered that support CTL loss of reactivity due to evolving variants. Peptides were made at ResGen Invitrogen Corporation Huntsville, AL or SynBioSci Corporation Livermore CA.

Peptide binding assay

The binding of these peptides was examined using soluble HLA-A*0201 and B*0702 to determined the IC$_{50}$ values (Pure Protein, Oklahoma City, OK). Fluorescently labeled control peptide and sHLA were incubated with each test peptide until equilibration of peptide replacement was reached. Inhibitory concentration (IC$_{50}$) values were calculated using a dose-response curve. A fluorescently labeled control peptide and soluble HLA were incubated with each peptide until equilibration of peptide replacement was reached as read on an Analyst AD plate reader (Molecular Devices, Sunnyvale, CA). Fifty-percent inhibitory concentration (IC$_{50}$) was calculated by using a dose-response curve. The log10 IC$_{50}$ for the peptides used in this study
were compared to those of a panel of reference viral peptides with known binding capacity in order to define their relative binding affinities [298,299]. The fluorescence polarization binding affinity categories were divided based on these cut-offs: high binding affinity < 3.7, medium < 4.7, low < 5.5, and non-binding < 6.0 (calculated to the artificial log IC$_{50}$).

**Statistical Analysis**

Statistical correlations were determined with a nonparametric Pearson’s correlation coefficient and significance (P values) using the standard Student T-Test through SigmaPlot 9.0 software.
Results

MACS participant #8 was selected for this study due to availability of autologous sequences and T lymphocytes data for more than 10 years (Figure 5) and having common haplotypes HLA-A*0201 and HLA-B*0702.

Figure 5: HIV-1 infected participant. T lymphocyte counts and HIV-1 copies over disease progression. Absolute T cell counts over the course of follow-up, CD3 (black diamond), CD4 (green square), CD8 (yellow triangle), and RNA copies (red square) are shown for MACS participant #8.

This analysis was conducted using predictions matrices from the website (http://thr.cit.nih.gov/molbio/hla_bind) that has a free, open-access computer algorithm called BIMAS [231,233,276] through the Los Alamos HIV Database [293]. This prediction model was recently shown to be accurate in its predictive value for the haplotypes chosen for this study [234]. The published literature includes binding motifs for a wide variety of human class I and II MHC alleles, however it remains for the most part unclear whether such motifs hold true predictive value for immunogenicity.
I first identified the ‘founder’ strain of participant #8 for two common haplotypes in the Caucasian population, HLA-A*0201 and HLA-B*0702, including 9- and -10mer analysis to determine the initial number of potentially immunogenic or targeted CTL epitopes spanning the HIV-1 genome (Figure 6A, 6B reveal Gag p17 and Gag p24 respectively, Figure 6C Nef and Figure 6D Env (C2-V5) regions).
Figure 6. Predicted binding of founder strain of HIV-1.
Predicted binding of MACS participant #8 autologous founder strain spanning Gag, Nef, Env. Predicted binding scores are shown for 9-mer A*0201 (blue), 10-mer (green), 9-mer A*0702 (red) 10-mer (black).
While the preponderance of epitopes identified, were present in the Gag (p17 and p24) regions (57%), Nef and Env (C2-V5) did not reveal a significant difference, 21% and 22% respectively. Interestingly, Kiepiela et al. [206] demonstrated that the number of epitopes targeted against Gag were associated with viral control. Although, if you look at the immunologic from a previously published from our lab [296] for this individual, the CTL responses against Gag drop-off precipitously by the second year (Figure 21 page 93). Although many predicted binding epitopes are identified within this region, and therefore would suggest that this individual would control virus, this was not seen. Identification, in itself, of potential epitopes from this region, does not necessarily translate into CD8$^+$ T cell response, determining the IFN$\gamma$ responses (ELISPOT) to these epitopes, along with their variants, would need to be ascertained in order address this critical issue.

I then wanted to determine if there were any differences in the number of potential CTL epitopes identified by HLA alleles (A*0201 or B*0702) because it has been shown that a 2.5 fold increase in the number of CD8$^+$ T cell responses was found to HLA-B when compared to HLA-A [172]. I did not find any significant difference among predicted epitopes by haplotype (HLA-A*0201 = 48%, B*0702 = 52%) or 9-mers versus 10-mers (51%, 49% respectively) of the total potential epitopes identified.

Once the initial epitopes were identified within the founder strain, the task became which potential CD8$^+$ CTL epitopes contributed to this individual’s disease progression. To observe this, I analyzed autologous viral sequences over the course of infection pre- and post-drug visits. A representative analysis of HLA-A*0201 9-mer predicted binding scores is shown in Figure 7 demonstrating evolutionary changes spanning the genome.
Predicted binding to HLA-A*0201 9-mers across the genome, over ~10 years following seroconversion, was mapped for sequences [Gag-p17, Gag-p24, Env (C2-V5), and Nef] in this individual. Each 9-mer amino acid motif in the sequence alignment was colored according to predicted HLA-binding scores indicated in the key. Epitopes that show a pattern of decreasing binding score over time is indicated by red colored numbers at the top (epitopes # 2, 4, 8). Initially, I hypothesized that if a decrease in the predicted binding score of a CTL epitope was determined, this would indicate a decrease in the peptide to bind to the MHCI groove to be...
presented for recognition by the TCR for immune recognition and hence affect disease progression due to the lack of viral replication control.

Through this analysis, however, I found a large number of predicted epitopes that demonstrated a trend to increase the predicted HLA-binding score over time (depicted in blue, epitope #s 1, 3, 5, 6, 7, 9). I then considered the possibility that the accumulation of epitopes demonstrating increasing predicted binding scores could potentially be attributed to a viral escape mechanism. The summation of stronger binding affinities of CTL epitopes that do not efficiently control viral replication in vivo could be associated with disease progression. This illustration represents analysis of HLA-A*0201 9-mers only and the same analysis was repeated for HLA-A*0201 10-mers and HLA-B*0702 9- and 10-mers (supplemental data in Appendix A-C). Scatter plots of each clone were plotted over years post seroconversion, as well as, the average predicted binding score (of the clones) for each visit to determine if a correlation existed with time. If the correlation value was $R^2 \geq 0.5$, the epitope was further analyzed for an association with disease progression via the CD4, CD8 lymphocyte counts and viral load corresponding to that visit. A representative for each of the three trends identified (decreasing, increasing and neutral) is shown in Figure 8 (top row) and correlated with lymphocyte and viral RNA levels (middle row) and then corrected for ART initiation (bottom row) where I did not include visits after the initiation of ART that would have an impact on the immune selective pressure of the viral mutation of epitopes.
Figure 8  Representative predicted MHC class I binding score trends.
The average predicted MHCI binding scores for each visit post seroconversion of participant #8 are depicted in the top row with correlations of the three examples of binding patterns: decreasing, increasing and neutral. Correlations of lymphocytes and HIV copies versus the average predicted binding scores over the course of infection (middle panels), or corrected for ART (bottom panels) in which visits when on drug are excluded.

Average Predicted Binding Scores vs T Lymphocytes and HIV copies

Corrected for ART

Average Predicted Binding Score
Both Gag epitopes displayed, reveal a strong correlation $R^2 > 0.8$ which is further associated with CD4 counts ($R^2 > 0.7$) as compared to a known CTL epitope SL9$_{p17\text{-}77-85\text{LAI}}$ (SLFNTVATL) which did not reveal any associations with the average predicted binding scores over disease progression ($R^2 = 0.0673$), CD4 T cell counts ($R^2 = 0.0042$) or viral RNA levels ($R^2 = 0.0035$) when corrected for ART treatment. All epitopes with decreasing or increasing trends are listed in Table 1.
### Table 1  Summary of MHC class I epitope binding predictions

<table>
<thead>
<tr>
<th>Protein</th>
<th>Haplotype</th>
<th>Sequence</th>
<th>Length</th>
<th>Decreasing Trend</th>
<th>Scatter plot</th>
<th>Avg binding</th>
<th>Avg bind/CD4</th>
<th>Avg bind/CD8</th>
<th>Avg bind/CD4</th>
<th>Avg bind/CD8</th>
<th>Avg bind/RN</th>
<th>Avg bind/CD4</th>
<th>Avg bind/CD8</th>
<th>Avg bind/RN</th>
<th>pvalue</th>
<th>pvalue</th>
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Based on the analysis of eliminating epitopes by $R^2 > 0.5$ correlations, 17 epitopes listed in Table 2 have predicted binding affinities that potentially could be attributing to disease progression. Due to the nature of uncertainty of a predicted value derived from a computer algorithm, I analyzed all the epitopes to see if any associations were neglected by allowing the initial criteria of predicted binding versus time at $R^2 \geq 0.5$ as a cut-off value, which added five epitopes (Table 2 in red) which could be contributing to disease progression based on CD4 and viral load associations.

I next selected some known MHCI epitopes to be accessed for binding affinity analysis as determined by fluorescence polarization [288] which is more convenient than radioactive labeling yet retains the same capacity of detection [290]. The strategy was to
### Table 2. Summary of MHC class I epitope binding predictions

#### Summary of MHC class I epitope binding.

List of all the HLA-A*0201 and B*0702 predicted 9- and 10-mer CD8+ T cell epitopes for Gag, Nef and Env that revealed \( R^2 > 0.5 \) for increasing or decreasing trend over time.
choose known HLA-A*0201 (Table 3) and HLA-B*0702 (Table 4) epitopes and evolving variants that displayed a diverse range of predicted binding scores spanning the viral genome to determine if predicted scores that had low scores could be detected in this assay.

<table>
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<th>Peptide sequence</th>
<th>Peptide origin</th>
<th>Position</th>
<th>Sequence ID</th>
<th>Peptide length</th>
<th>Mol wt.</th>
<th>Inhibition % SE Artificial log (IC50) Affinity Category</th>
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</thead>
<tbody>
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<td></td>
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<td>p17</td>
<td>77-85</td>
<td>ABI97965</td>
<td>9</td>
<td>938 92.6 0.6 3.81 Medium</td>
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**Table 3. Peptide information and sHLA binding data of HLA-A*0201 epitopes**

**List of epitopes studied for binding analysis for HLA-A*0201.**

HLA-A*0201 selected epitopes are listed with their binding affinities. The binding motifs of anchor residues are in **bold** within the sequence.
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Table 4. Peptide information and sHLA binding data of HLA-B*0702 epitopes

List of epitopes studied for binding analysis for HLA-B*0702.

HLA-B*0702 selected epitopes are listed with their binding affinities. The binding motifs of anchor residues are in **bold** within the sequence.
The binding affinity was determined and is displayed in Figure 9 (and Tables 3 and 4), where the log IC₅₀ is plotted versus the predicted binding score. When the binding affinities for the epitopes are grouped by protein (Gag, Nef and Env) only Nef demonstrated a strong correlation ($R^2 = 0.7948$). This strong correlation could be due to the fact that this protein had the greatest number of data points and if Gag and Env had more, possibly they would show a strong correlation as well.

![Figure 9: Binding affinity by protein (Gag, Nef, and Env).](image)

Taking all the binding affinities together and plotting the percent inhibition versus the predicted binding score, there is a low correlation of $R^2 = 0.275$ (Figure 10B). However, if segregating these epitopes by HLA allele, the predictive power for HLA-A*0201 revealed a decent association of $R^2 = 0.6222$ (Figure 10A).
Figure 10: Binding inhibition by prediction scores of HLA-A*0201 and HLA-B*0702. (A) MHC class I binding values for each epitope are grouped by haplotype with an association for the HLA-A*0201 epitopes ($R^2=0.6222$), HLA-B*0702 epitopes ($R^2=0.1537$). (B) Combining binding inhibition values for each epitope and are not grouped according to haplotype the association is only $R^2=0.275$.

Finally, in order to ascertain if this is truly a practical approach to determine epitopes that might contribute to disease, I need to further dissect the remaining predicted epitopes and their variants functional binding affinities and how accurate the model was at predicting these epitopes, and their contribution in disease progression (Table 2).
Discussion

Peptide binding to MHCI molecules is a prerequisite for T cell recognition, but in itself is not sufficient for inducing a CD8+ T cell immune response. Some peptides, although considered strong binders in biochemical assays, are not properly processed for binding and presentation by antigen presenting cells (APC). There are in silico programs that deal with the prediction of proteasomal cleavage sites (e.g. PAProc [300]) FRAGPREDICT [301,302] and NetChop [303]) and TAP binding patterns (Predict [304]); however these types of analyses were not considered in this current study.

Presently, I focused on identifying potential CTL epitopes and their subsequent binding affinities to MHCI molecules. I used the in silico program BIMAS, which is an interactive MHCI binding prediction algorithm, to analyze evolving autologous HIV-1 sequences over the course of disease progression for MACS participant #8. This interactive model was able to identify three consistent patterns of predictive binding capacity: (i) no change (neutral), (ii) decreasing, and (iii) increasing that evolved within the quasispecies.

The analysis of binding affinities of these peptides to MHCI using the cell line and soluble binding assays was deemed too insensitive to reveal differences in binding for our study. I therefore, turned to a soluble MHCI binding using fluorescent polarization methods in competitive binding analysis of these peptides [290,298] which was determined to have a higher throughput potential, as well as, greater sensitivity than our original method (using the TAP deficient cell expressing HLA-A2; T2 cells) [279,280] (data not shown).

Our results for HLA-A*0201 and HLA-B*0702 did show a range from non-binding to a strong binding affinity. However, potential epitopes that were determined to have strong correlative values (R²>0.5) between predicted MHCI binding and CD4 T cell counts or to viral
load need to be further accessed in order to fully appreciate the complete range of scores that were identified within the three predictive binding trends of autologous HIV-1 over time in this individual. Of a special note, it was interesting that an accumulation of strong associations were seen for the predicted binding trend to increase in strength over disease progression. This has been noted with other studies [199] and may, in fact, be an escape mechanism where the higher binding affinity epitopes are selected for, due to their low disassociation rate and ineffective control on viral replication.

Due to the uncertainty of the predicted binding scores in relation to the actual binding affinities, only epitopes with relatively high scores or ones that were previously identified were chosen for the current analysis, which mostly from the neutral category of classification. Thus, there was no significant relationship between predicted and true binding of HIV-1 peptides to MHCI, and disease progression for these chosen epitopes. Although predicting MHCI binding for only the most common HLA variants may provide sufficient population coverage for vaccine design, successful prediction for as many HLA variants as possible is necessary to understand the immune response in transplantation and immunotherapy. Prediction modeling as an approach to evaluating MHCI and II epitopes binding and immunogenicity are desired, in order to reduce the high cost and labor intensive nature of such studies.
IV. CHAPTER FOUR: *IN VITRO* PRIMING OF HIV-1 SPECIFIC CD8⁺ T CELLS

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Preface

This manuscript is in preparation for publication. All work presented here was done by Bonnie Colleton.
Abstract

HIV-1 infected persons currently on antiretroviral therapy (ART) still harbor latent viral reservoirs that can result in resumption of disease progression. Human and monkey studies have shown correlations of viral control and lack of disease progression with levels of HIV-1 or SIV specific cytotoxic T lymphocytes (CTL). Therefore, I propose that a broad and robust primary immune response is important for an effective prophylactic or therapeutic vaccine. In this study, I developed an in vitro priming model to show that a primary CD8+ T cell response to HIV-1 can be activated in vitro by stimulation of naïve T cells with HIV-1 peptide-loaded dendritic cells (DC). The optimal primary T cell response to HIV-1 required maturation of the DC with CD40L and interferon (IFN)-γ, and IL-12 and CD4+ T helper cells. The primed CD8+ T cells recognized multiple regions of Gag, Env and Nef that corresponded to known and predicted MHCI epitopes. This in vitro priming model can be of importance in evaluation of various aspects of HIV-1 vaccines, including the immunogenicity of predicted and known MHCI restricted epitopes.
Introduction

Initiation of antiretroviral therapy (ART) for the majority of human immunodeficiency virus type 1 (HIV-1) infected persons results in a precipitous drop in viral load to undetectable levels and a gradual increase in CD4\(^+\) T cell numbers [305]. This impressive therapy allows host survival, but is ultimately unable to clear the virus from the infected individual. Viral reservoirs persist during the course of ART, and only a partial recovery of anti-HIV-1 T cell immunity is seen [306-308]. It is believed that CD8\(^+\) T cell response are important in host control of HIV-1 infection and prevention of AIDS (reviewed in [309]), so new strategies enhance anti-HIV-1 CD8\(^+\) T cell immune responses during ART [310-312] are being pursued. One such approach is to load antigen (Ag) \textit{ex vivo} to activated dendritic cells (DC) to present peptides representing immunogenic CTL epitopes of HIV-1 [313-316]. These Ag-loaded DC are then re-administered to HIV-1 infected persons to stimulate primary and memory anti-HIV-1 CD8\(^+\) T cell immune responses that could clear the residual viral pools.

In order to determine potential immunogenicity of regions of HIV-1 to use in immunotherapy and potentially for prophylactic vaccine design, I have developed an \textit{in vitro} priming model. Generating and expanding primary immune responses from naïve precursors \textit{in vitro} [71,208,242,243] has been difficult due to the stringent activation and costimulatory requirements. Therefore I examined the three major signals required for priming of naïve CD8\(^+\) T cells, i.e., MHCI/peptide complexes (signal 1) [245], costimulatory molecules (e.g. CD80 and CD86) (signal 2) [74,246,247] on the surface of mDC that activates CD8\(^+\) T cells with the help of CD4\(^+\) T cells [108,109,248,317], and IL-12 (signal 3), which induces IFN\(\gamma\) in Th1 cells that in turn enhances antigen-specific CD8\(^+\) CTL responses [82,103,249-253,255,256,318-321]. The results show that DC from HIV-1 negative subjects that were matured with CD40L and IFN\(\gamma\) and
subsequently loaded with either 9-mer or 15-mer HIV-1 peptides and CD4\(^+\) T cells were required for efficient generation of antigenically broad and robust, primary CD8\(^+\) T cells responses \textit{in vitro}. 
Materials and Methods

Dendritic cells (APC)

To obtain immature DC (iDC), CD14+ monocytes were positively selected from either buffy coats (Central Blood Bank, Pittsburgh, PA) or from peripheral blood mononuclear cells (PBMC) using anti-CD14 monoclonal antibody (mAb)-coated magnetic beads (Miltenyi, Auburn CA) to a purity of >95%, cultured for 5 days in RPMI-1640 (Gibco, Grand Island NY) supplemented with 10% FCS (Cellgro) containing 1000U/ml recombinant granulocyte-monocyte colony-stimulation factor (GM-CSF) (Amgen, Seattle WA) and 1000U/ml of recombinant human interleukin (hIL)-4 (R&D Systems) adding fresh cytokines every other day. On the 5th day, the iDC were harvested and reset at 1x10^6/ml and maturation factors were added for 40 hours. Maturation reagents included: CD40L (0.5ug/ml Amgen), IFNγ (1000U/ml; R&D Systems) IL-1β 25 ng/ml; R&D Systems), IFNα 1000U/ml; Strathmann Biotech), TNF-α (50ng/ml; BD Biosciences), Poly I:C (dsRNA; 20ug/ml, Sigma) for 40 hours to induce DC maturation and immunomodulation.

Synthetic peptides

Synthetic peptides represent dominant HLA-restricted CTL epitopes for CMV_{pp65 (495-503)} and HIV-1 peptides (SL9) SLYNTVATL (p17 77-85LAI), TLNAWVKVV (p24 151-159), KLTSCNTSV (gp120 192-199), RGPGRAFVTI (gp120 311-320), SLLNATIAV (gp41 818-827) prepared by the Protein Research Lab, (University of Illinois, IL) and HIV-1 consensus strain 15-mers overlapping by 11 amino acids were provided by the NIH AIDS Research & Reference Reagent Program (Germantown, MD) were used for priming assays.
Primary stimulations

Normal human HLA-A*0201 PBMC or CD8\(^+\) T cells were used in primary stimulations. CD8\(^+\) T cells were enriched through positive selection using CD8 microbeads and LS columns as described by the manufacturer (Miltenyi Biotec, Auburn, CA). Primary stimulations containing PBMC or CD8\(^+\) T cells/well in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (FCS) (Cellgro) with peptide-loaded DC at responder to stimulator (T/DC) ratios of 10:1. Peptide loading consisted of two methods: (i) pools of 5 immunodominant epitopes were used for establishment of the model and (ii) single peptides were used for tetramer and consensus experiments. Antigen loading of the DC was performed by incubation in medium (RPMI-1640) without supplemental serum containing 50µg/ml peptide for 2 hours in 37\(^0\)C, 5% CO\(_2\) atmosphere. DC were then harvested and re-suspended with PBMC depleted of monocytes. Co-cultures were fed with fresh medium supplemented with recombinant IL-15 (2.5ng/ml; Peprotech) and IL-2 (50 U/ml; Chiron, Emeryville CA) after 5 days and thereafter. Secondary stimulations (boost) with peptide-loaded DC were added directly to cultures and then harvested after 28 days for functional readout assays (Figure 13).
Method of priming of HIV-specific responses in seronegative individuals

**Figure 13 In vitro priming model**
Schematic representing DC generation and two rounds of stimulation to naïve T cells and assays used as readouts of T cell functions (ELISPOT, chromium release or tetramer staining).

**Flow cytometry**

**DC Phenotype:** Untreated iDC and treated DC were stained with multiple maturation markers including CD80, CD83, CD86, MHCI (HLA-ABC), and MHCII (HLA-DR). The DC were stained with either phycoerythrin (PE)-conjugated specific mAb (BD Immunocytometry Systems, San Jose CA) for CD80, CD83, CD86, and MHCII (HLA-DR) or fluorescein isothiocyanate (FITC) for MHCI (HLA-ABC). Appropriate isotype-matched monoclonal antibodies (mAb) were used as controls. The DC were assessed by flow cytometry and gated on large cells with high side scatter (EPICS XL; Coulter, Fullerton CA).
**Tetramer staining:** To analyze specificity of the presence of antigen-specific CD8\(^+\) T cells that were stimulated for primary (SL9\(_{p17\ 77-85LAI}\)) and memory (CMV\(_{pp65\ 495-503}\)) responses after 4 weeks and 2 rounds of stimulation with DC, cells were harvested and stained with MHC Tetramer-Streptavidin-Allophycocyanin (APC) (Immunonomics Beckman Coulter) to either Tet\(^+\)SL9\(_{p17\ 77-85LAI}\), Tet\(^+\)CMV\(_{pp65\ 495-503}\), or Tet\(^-\) all APC conjugated and CD8-conjugated to APC-PC5. The cells were then read on a flow cytometer (EPICS XL; Coulter, Fullerton CA), gating on forward and side scatter for live lymphocytes, and collecting a minimum of 250,000 gated CD8\(^+\) T cells per sample.

**DC Cytokine Production (ELISA)**

Supernatants were collected from untreated and 40 hours of treated iDC with different maturation reagents: (i) CD40L (0.5\(\mu\)g/ml Amgen), (ii) CD40L + IFN\(\gamma\) (CD40L (0.5\(\mu\)g/ml Amgen) (1000U/ml; R&D Systems), (iii) cocktail (IFN\(\gamma\) (1000U/ml; R&D Systems), IL-1\(\beta\) (25ng/ml; R&D Systems), IFN\(\alpha\) 1000U/ml; Strathmann Biotech), TNFa (50ng/ml; BD Biosciences), Poly I:C (dsRNA; 20ng/ml, Sigma) and assayed for production of IL-12p70, IL-12p40, IL-2, IL-15, and IL-10 using ELISA kits (R & D) as per manufacture instructions. Average of 5 normal donors run in triplicate is reported with ± SE.

**T Cell Functional Assays**

**CTL activity:** The cell counts and viability of target cells were monitored by trypan blue dye exclusion. The Cr\(^{51}\) labeled target cells were added to the effector cells in triplicate at effector-to-cell (E:T) ratios (e.g. 40:1, 20:1, 10:1) in 96 well plates. The plates were centrifuged at 50 x g for 3 minutes and then incubated for 4 hours at 37\(^\circ\)C under a 5% CO\(_2\) atmosphere. Afterward, the
radioactivity of the cell-free supernatant was assessed in a gamma counter (Top Count NXT; Perkin-Elmer, Shelton, CT). The percentage of lysis was calculated as $100 \times \frac{\text{experimental counts per minute} - \text{spontaneous counts per minute}}{\text{maximum counts per minute} - \text{spontaneous counts per minute}}$. Specific lysis was expressed as the percentage of lysis in peptide-treated targets minus the percentage of lysis in non-peptide-treated targets.

**ELISPOT:** The 96-well plates (Millipore, MA) were coated overnight at $4^\circ\text{C}$ with $5\mu\text{g/ml}$ anti-interferon $\gamma$ (IFN$\gamma$) monoclonal antibody (Mabtech, Stockholm, Sweden). The antibody-coated plates were washed four times with PBS and blocked with RPMI + 10% FCS for one hour at $37^\circ\text{C}$. Responder cells were stimulated overnight with peptides ($10\mu\text{g/ml}$)-loaded DC at a ratio of 10:1 in $37^\circ\text{C}$, 5% CO$_2$ atmosphere. After development of the spots, the plates were counted with an Elispot reader system (Cell Technology, Columbia, MD). Data were expressed as net spots/10$^6$ cells. The number of spots/10$^6$ responder cells stimulated with HIV-1 antigen expressing APCs minus number of spots/10$^6$ responder cells stimulated with antigen-negative APCs. Values greater than two standard deviations of the mean background were considered to be a positive response.
Results

Properties of DC required for T cell priming. DC are the critical antigen-presenting cell (APC) for inducing primary T cell responses, but they represent a diverse and heterogeneous population of cells. Due to the ease of generation, most clinical studies have used monocyte-derive (md)-DC cultured in GM-CSF and IL-4 to obtain iDC. To assess the most efficient system for priming of CD8\(^+\) T cells with mdDC, I first addressed the role of costimulatory molecules, inflammatory cytokines, toll-like receptor (TLR) 3 ligand and CD4\(^+\) T cells in priming of naïve CD8\(^+\) T cells to HIV-1. The expression of the cell surface markers were assessed for iDC and DC modulated with maturation factors (i) CD40L, (ii) CD40L + IFN\(\gamma\), and (iii) TLR3-cytokine cocktail. Each treatment resulted in significant increases in surface expression of maturation markers CD80, CD83, CD86 and MHCI (HLA-ABC) and MHCII (HLA-DR) in percent positive cells (Figure 11A) and MFI in log scale (Figure 11B).

![Effect of Immune Modulating Factors on DC Phenotypes](image)

Figure 11: DC phenotype

Different maturation factors were added to iDC for 40 hours and surface expression markers were detected for CD80, CD83, CD86, MHCI class I ABC, and MHC class II DR was collected and the average of five donors ± SE is depicted as either (A) percent positive or (B) mean fluorescent intensity (MFI) in log scale.
Thus, surrogates for activated CD4⁺ T cells, i.e., CD40L and IFNγ, and a combination of inflammatory cytokines and the double stranded RNA TLR3 ligand (polyI:C), were both efficient in enhancing expression of DC surface maturation factors (signal 2) that are required for stimulation of T cell reactivity.

The Th1 vs Th2 paradigm is essential in the third signal of CD8⁺ T cell priming. That is, mature DC (mDC) require an additional signal to induce a potent stimuli to produce interleukin 12 (IL-12) and other cytokines that shift or polarize the immune response directed to Th1 cells that in turn lead to induction of CD8⁺ CTL. The production of Th1 polarizing cytokines by untreated or treated DC was therefore determined by ELISA. I found that iDC treated with CD40L + IFNγ produced the most IL-12p40 and p70, supporting their potential for priming of naïve CD8⁺ T cells to the greatest extent (Figure 12).

![Dendritic cell Cytokine Production](image)

**Figure 12 DC production of cytokines**

Average of five seronegative blood donors are shown for DC cytokine (A) IL-12p70, (B) IL-12p40, (C) IL-10, (D) IL-2, and (E) IL-15 for each maturation group (e.g. iDC, CD40L, CD40L + IFNγ, and TLR3-cytokine cocktail).
Interestingly, iDC treated with the maturation cocktail produced high levels of IL-12, but also produced the greatest amount of IL-10, which could down regulate Th1 and CD8 T cell responses. IL-2 and IL-15 were not detected.

*Priming of CD8+ T cells to HIV-1 peptide loaded DC.* I next determined which DC treatment had the best capability to prime naïve CD8+ T cells. For this, DC from a HLA-A*0201 HIVneg CMV+ subject were loaded with either SL9 \(^{(p17 \ 77-85LAI)}\) or CMV\(^{pp65} \ (495-503)\) immunodominant, HLA-A*0201 peptides for 2 hours and then added to PBMC for co-culture at a PBMC:DC ratio of 10:1 for 14 days, followed by a booster (secondary) DC-peptide stimulation. Two weeks after the second stimulation, cells were harvested and stained for CD8 and tetramers for SL9 \(^{(p17 \ 77-85LAI)}\) or CMV\(^{pp65} \ (495-503)\). Cells were analyzed in a flow cytometer and gated on live lymphocytes based on forward and side scatter. A minimum of 250,000 lymphocyte (CD8+ cells) events were collected (Figure 14A) and graphically represented in Figure 14B.
The results demonstrate that iDC matured with CD40L + IFNγ were the most efficient at priming SL9 (p17 77-85LAI)-specific CD8+ T cells, while iDC treated with the maturation cocktail were superior for CMV pp65 (495-503)-specific memory T cell expansion. These studies indicate that the best maturation treatment for md-DC was CD40L + IFNγ based on the high level of signal 2, i.e., co-stimulatory molecules, signal 3 (IL-12) production, and expansion of primary antigen-specific responses to SL9 (p17 77-85LAI).

I then further characterized the functional nature of these primary responses using HLA-A*0201 normal donors and an array of peptides to determine the killing capacity (51Cr release assay) and IFNγ response that could be detected by this in vitro priming system. I treated iDC with CD40L + IFNγ, loaded HIV-1 peptides representing known immunodominant epitopes for 2 hours. I then either co-cultured the mDC with CD8+ T cells or CD8+ and CD4+ T cells, for two
rounds of stimulations. Co-cultures were enriched for CD8$^+$ T cells again to equalize the responses for comparison. Superior primary CTL responses were consistently seen in the group that contained CD4$^+$ T cells in the chromium release assay (Figure 15A) and by IFN$\gamma$ ELISPOT (Figure 15B), supporting a requirement for CD4$^+$ T cells. T cell responses were detected against all five HIV-1 peptides, and were greatest to TLNAWVKV(p24 151-159) and SLLNATDIAV (gp41 818-827).

**Figure 15  CTL activity and IFNg ELISPOT**
Comparison of HIV-1 peptide-loaded autologous DC co-cultured with CD8$^+$T cells alone or with CD4$^+$T cells for CTL (A) or IFN$\gamma$ ELISPOT (B).

**Specificity of primary CD8$^+$ T cell responses for HIV-1.** I next explored the breadth of the immunogenicity of DC matured with CD40L + IFN$\gamma$, loaded with overlapping 15-mer peptides for the Gag, Env and Nef proteins derived from a consensus strain of HIV-1. To validate the MHCI prediction algorithm for HLA-A*0201 CTL epitopes, I used predicted binding to MHCI molecules of potential 9- and 10-mer HLA-A*0201 CTL epitopes to Gag (Figure 16A), Env
(Figure 17A) and Nef (Figure 18A) of the consensus strain of HIV-1. Results are reported as 2 standard deviations over negative control of priming with DC alone (no peptide).
Figure 16 Priming to Consensus Gag

Consensus Gag region of HIV-1 was evaluated for potential CTL epitopes using the algorithm provided by BIMAS (A). Immunogenicity was evaluated by antigen-loading DC matured with CD40L + IFN-γ for 2 rounds of stimulation, and co-cultured with PBMC for 4 weeks and assayed for IFN-γ production in an ELISPOT (B). Results are reported as 2 standard deviations over the negative control (primed with DC and no peptide).
Figure 17 Priming to Consensus Env
Consortium Env region of HIV-1 was evaluated for potential CTL epitopes using the algorithm provided by BIMAS (A). Immunogenicity was evaluated by antigen-loading DC matured with CD40L + IFNγ for 2 rounds of stimulation, and co-cultured with PBMC for 4 weeks and assayed for IFNγ production in an ELISPOT (B). Results are reported as 2 standard deviations over the negative control (primed with DC and no peptide).
Figure 18. Priming to Consensus Nef. Consensus Nef region of HIV-1 was evaluated for potential CTL epitopes using the algorithm provided by BIMAS (A). Immunogenicity was evaluated by antigen-loading DC matured with CD40L + IFN-γ for 2 rounds of stimulation, and co-cultured with PBMC for 4 weeks and assayed for IFN-γ production in an ELISPOT (B). Results are reported as 2 standard deviations over the negative control (primed with DC and no peptide).
The predicted binding scores for peptide with positive ELISPOT responses within these three HIV-1 proteins ranged from 0 to >2000. However, this does not necessarily ensure that the predicted epitopes will actually bind to their respective HLA molecules. In fact, only 0.1-0.5% of peptides representing a whole protein will bind to an individual HLA class I molecule [262]. Therefore, to determine the immune responses to these peptides, the overlapping 15-mers spanning the HIV-1 genome were loaded onto mDC (i.e., CD40L + IFNγ) and co-cultured with PBMC for two weeks, and boosted with a second stimulation. The cells were tested for immunogenicity using the IFNγ response to Gag (Figure 16B), Env (Figure 17B) and Nef (Figure 18B) for T cells from two HLA-A*0201 HIV-1 seronegative subjects. Due to the large amount of peptides and limited number of cells, I was unable to enrich for CD8⁺ T cells and therefore, was unable to definitively exclude IFNγ production by CD4⁺ T cells in some of the responses. The results show that numerous T cell responses were detected to peptides derived from each HIV-1 protein Gag, Env, and Nef. I was able to deduce optimal epitopes from the consensus 15-mer overlappings through the use of BIMAS (an in silico MHCI prediction algorithm model) and previously published epitopes listed in the Los Alamos HIV Database. For Gag (Figure 16B), notably, both HLA-A*0201 matched normal donors were able to mount a response to SL9 (p17 77-85LAI) (SLYNTVATL), which supports that these were truly Ag-specific CD8⁺ T cell responses. I have also determined epitopes ranging in predicted binding scores from < 50 GLLETSEGC to > 700 YMLKHIWVA, which are both predicted 9-mers of HLA-A*0201, as well as 10-mers such as TLQEIQIAWMT. Env (Figure 17B) responses revealed a broad and robust reactivity encompassing, once again, both 9-mer and 10-mer epitopes. My analysis also reveal known epitopes such as ALFYKLDVV (which is also a known HLA-B*0801 epitope) detected in the normal donor with this haplotype. Responses to Nef (Figure 18B) also revealed
these 9-mer (AAVDLSHFL) and 10-mer (WLEAQEEV) CD8+ T cell reactivities. The two donors both targeted all three proteins (Gag, Env, and Nef) comprising of 298 overlapping peptides. Overall, 34% (70 corresponding HLA-A*0201 responses) (Figure 19) of the IFN positive responses (of 208 positive responses) were concordant (e.g. responses to the same epitope) between the two donors. This demonstrates that I am capable of detecting the same HLA-A*0201 responses in different donors to a good degree. These data support that my in vitro priming model is capable of stimulating naïve CD8+ T cells to a broad array of immunogenetic proteins of HIV-1.

Figure 19 Summary of primary T cell responses to consensus Gag, Env, Nef
The two HLA-A*0201 seronegative donors were used to prime naïve CD8+ T cells to 15-mers overlapping Gag, Env, and Nef and these IFNγ ELISPOT results were compiled to determine the number of CTL epitopes that were targeted by both individuals.
Discussion

Priming of naïve CD8⁺ T cells has been shown to require three signals, MHCI/peptide complex (signal 1) [245] along with the costimulatory molecules (e.g. CD80 and CD86) (signal 2) [74,246,247] on the surface of mDC which activates CD8⁺ T cells with the help of CD4⁺ T cells [107-109,248] and IL-12 (signal 3) [82,249,250,252,318,251,253,319-321, 103,255,256]. The present study supports these findings, as DC matured with either (i) CD40L, (ii) CD40L +IFNγ, or (iii) TLR3-cytokine cocktail all expressed high surface marker levels of co-stimulatory ligands, B7.1 (CD80) and B7.2 (CD86) on the DC surface for a strong signal 2 compared to iDC. It has been shown that co-stimulation with signal 1 and 2 can elicit several rounds of cell division, but effector function and memory require a third signal that can be provided by either IL-12 or type I interferons [83,84,322]. In the absence of this signal some cells can survive, but they are immune tolerant [103]. The importance of IL-12 as a third signal is supported by several reports where it augments CTL responses in experimental systems [323,324]. Curtsinger’s group used a clever system employing artificial antigen-presenting cells (aAPCs), where microspheres expressing MHCI/peptide complex (signal 1) and co-stimulatory molecules (signal 2) were used in the presence of IL-12 with naïve or memory cells. The results demonstrated that aAPC were able to stimulate cytolytic activity in memory T cells, but were unable to due so in the naïve CD8⁺ T cells unless IL-12 was present [251]. Other studies have shown that clonal expansion (using carboxyfluorescein-diacetate-succinamidyl-ester (CSFE) dye labeling) can occur, but effector functions are not developed [84,253]. Collectively, this information led us to determine the cytokine profile of the various DC maturation groups in which iDC treated with CD40L + IFNγ produced the most IL-12p40 and p70.
Secondly, I verified that increased levels of Th2 type cytokines (IL-10) were not present, and thus should not skew our desired Th1 priming responses. Indeed, using DC matured with CD40L+IFN\(\gamma\) resulted in priming of CD8\(^+\) SL9\((\text{p}17\text{ 77-85LAI})\) Tet\(^+\) cells. Based on this 3 signal priming model, I then demonstrated the capability of DC treated with CD40L + IFN\(\gamma\) to induce a broad spectrum of primary CD8\(^+\) T cell responses to five immunodominant HLA-A*0201-restricted epitopes. As expected based on the well known helper effects of CD4\(^+\) T cells, I found that CD4\(^+\) T cells were required for efficient priming of HIV-1 specific CD8\(^+\) T cells. The primed CD8\(^+\) T cells exhibited HIV-1 peptide-specific cytolytic activity and IFN\(\gamma\) production that was optimized by the presence of autologous CD4\(^+\) T cells. I postulate that the CD4\(^+\) T cells provided the necessary IL-2 signals when a secondary stimulation is given [325].

I then used this model and naïve CD8\(^+\) T cells from two HIV-1\(^{neg}\) HLA-A*0201 donors in the presence of CD4\(^+\) T cells with CD40L + IFN\(\gamma\) mDC for in vitro priming to consensus sequences of overlapping 15-mers to Gag, Env and Nef regions of HIV-1. I found that the primed CD8\(^+\) T cells from both donors exhibited a broad immunogenicity (IFN\(\gamma\) production) for many well documented epitopes in an ELISPOT as a surrogate of function. Using a stringent cutoff for a positive response, many T cell responses were detected which encompassed HLA-A*0201 9- and 10-mers of HIV-1 known to be epitopes from published reports compiled by the Los Alamos HIV Database [393], as well as unknown, possible epitopes. Moreover, the number of potential T cell epitopes that matched between the two donors was 34%. Using BIMAS prediction modeling with the Los Alamos HIV Database we identified many potential MHCI binding epitopes for Gag, Env and Nef among the peptide regions that were positive for T cell reactivity in our priming system.
Presently, I cannot rule out the possibility that some of these responses are from CD4\(^+\) T cells due to the fact that we were unable to enrich CD8\(^+\) T cells for each of these responses. However, there are many known CTL epitopes within the 15-mers previously detected in functional assays by other groups [393], and predicted in BIMAS by us, thus supporting that the responses are CD8\(^+\) T cell specific. Confirmation that these are indeed CD8\(^+\) T cell epitopes, however, requires that they be mapped to their minimal, 9- and 10-mer MHCI epitopes.

Notably, this is the first study to demonstrate that in vitro priming of CD8\(^+\) T cells to a large library of HIV-1 peptides elicits a broad spectrum of epitope specificities. Because the control of HIV-1 replication is largely dependent on CD8\(^+\) T lymphocyte responses specific for immunodominant viral epitopes, vaccine strategies that increase the breadth of dominant epitope-specific responses by such potent priming effects should contribute to containing HIV-1 spread. However, developing immunotherapy strategies for clinical use to elicit a broad spectrum of CTL responses will require a further understanding of the mechanisms responsible for immunodominance [326]. My data show, nevertheless, that it is feasible to engineer DC to enhance the primary responses of naïve CD8\(^+\) T cells to a broad array of HIV-1 epitopes while on ART. This form of immunotherapy could ultimately improve control of viral replication and disease. Moreover, this in vitro T cell priming model makes it possible to evaluate potential epitopes of many different pathogens and their immunogenicity for vaccine application.
V. CHAPTER FIVE: PRIMARY AND MEMORY CD8+ T CELL REACTIVITY TO AUTOLOGOUS HIV-1 GAG, ENV, AND NEF EPITOPES

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Preface

This manuscript is in preparation. James Mullins, Joseph Margolick, Homayoon Farzadegan, and Raj Shankarappa contributed the autologous virus sequence data for this study. Xiao-Li Huang established and conducted the memory T cell ELISPOT assays. Bonnie Colleton selected and had commercially synthesized all the autologous peptide sequences used in the T cell memory and priming assays, applied her in vitro T cell priming model developed in aim 2. She also conducted all of the T cell priming experiments, setup and helped oversee the memory T cell assays (including the extensive decisions on the choice of HIV-1 peptides to use for each time point, given the low numbers of PBMC available), and analyzed, interpreted, and organized for presentation all of the memory and primary T cell results.
Abstract

Loss of CD8+ T cell reactivity to constantly evolving variants of HIV-1 is associated with progression of HIV-1 infection, termed immune escape. I examined whether immune escape of HIV-1 is related to the inability of HIV-1 variants to prime naive CD8+ T cells to become memory T cells capable of responding to these variants. CD8+ T cells obtained from an HLA A*0201 B*0702 HIV-1 infected subject >1 yr before seroconversion (HIV-1 plasma RNA and antibody negative) were primed with autologous dendritic cells (DC) loaded HIV-1 Gag, Env and Nef peptides derived from autologous HIV-1 sequences over several years after seroconversion to HIV-1. Primary T cell responses (IFNγ ELISPOT) before seroconversion in the MACS subject were compared to memory T cell responses detected during his 15 years of infection, before and after virus-suppressive antiretroviral therapy (ART). The results showed that a robust, broad spectrum, primary CD8+ T cell response was induced by peptide-loaded DC. This included primary T cell reactivity to autologous HIV-1 variants that exhibited immune escape, i.e., failed to activate memory T cell responses several years after seroconversion and during ART. These results show for the first time that a robust, multi-epitope, primary CD8+ T cell response can be induced to autologous HIV-1 strains, including immune escape variants in vitro. This study indicates that the human host has the necessary repertoire of naive CD8+ T cells to respond to HIV-1 variants in vitro, even though memory T cell reactivity is lost over time to these HIV-1 epitope sequences during progressive infection. This supports use of HIV-1 prophylactic and immunotherapeutic vaccines that target DC to prime broad T cell reactivity.
Introduction

The prognosis of HIV-1 infected persons has dramatically improved since the discovery of combination antiretroviral therapy (ART). Although latently infected reservoirs remain in treated individuals, ART has unquestionably been beneficial in reducing HIV-1-associated morbidity and mortality. HIV-1 replication can cause direct cytopathic effects on T cells, as well as disturb the relative contributions of T cell homeostasis and induce apoptosis in subpopulations of T cells. Successful management of HIV-1 disease depends largely on the degree of durability of viral load suppression and on the ability to preserve and restore immune function [60,327-336]. The use of ART is associated with varying degrees of immune reconstitution, especially concerning HIV-1-specific immune responses [337,338].

While recovery of CD4+ T cells in HIV-1 infection during ART has been attributed to the production of naïve T cells, and has been associated with the thymus size in children [339,340] and adults [341-348], the recovery of naïve CD8+ CD45RA+ CD62L+ [335,336] T cells remains unclear. It has been suggested that naïve CD8+ T cells survive without dividing, while memory CD8+ T cells survive by proliferating upon antigen (Ag) recognition [123,349]. The presence of phenotypically naïve T cells in athymic mice and humans suggests that extra-thymic pathways of T cell development exist [350-354]. The bone marrow, liver, intestines, mesenteric lymph nodes and peripheral lymphoid organs [355-358] have all been proposed to provide the signals required for T cell maturation. The level of CD8+ T cells that readily undergo apoptosis in HIV-1 infected persons has been suggested as a predictor of immune restoration [359,360] whereas others [361,362] have determined that the activation levels are independent of viral load and CD4 cell counts. This variability in the literature reflects the different thresholds and T cell markers of subpopulations used in the studies, as well as the time ART was initiated during disease
progression. Indeed, it has been shown that some immunodeficient patients experience increases in immune restoration on ART, regardless of factors such as persistent viral replication [363], age [364], cytokines [365,366] and hormones [367].

I believe that the recovery of naïve CD8+ T cells during ART is critical to restoration of immune control of HIV-1 infection. These naïve CD8+ T cells would serve as precursors for new, central memory and effector memory CD8+ T cells that, if engineered by a potent immunotherapy, could control viral infection together with ART. Furthermore, these CD8+ T cells should have a large magnitude and breadth of responses directed against all HIV-1 proteins as in natural HIV-1 and SIV infection [164,368-372]. However, T cell immunity to HIV-1 during natural infection also shows a clustering of responses to conserved regions of the virus [368-371,381]. Moreover, several studies have failed to demonstrate that anti-HIV-1 CD8 T cell responses correlate with viral load or CD4 T cell counts [368-372]. I believe that the clustering of responses to the conserved regions of HIV-1 could be explained by these studies using overlapping peptides based on consensus HIV-1 strains, and not taking into account the high divergence of HIV-1. I further hypothesized that use of autologous virus, especially for the Env protein, as an immunogen will reveal dominant epitopes that were previously missed.

Our research group has recently administered ex vivo, consensus strain HIV-1 antigen (Ag)-loaded dendritic cells (DC) in a phase I clinical trial to HIV-1 infected persons to enhance anti-HIV-1-specific CTL to further control viral replication during ART [389]. In the present study, I have addressed whether such DC-based immunotherapies have the capacity to prime HIV-1 specific CD8+ T cells. Naïve CD8+ T cell lack unique markers for purification, moreover memory T cells respond and replicate vigorously to antigen in vitro, quickly outcompeting naïve T cells. Therefore I chose not to attempt purifying naïve CD8+ T cells from persons on ART for
use in my priming assays. Instead, I exploited the rare capacity of the Multicenter AIDS Cohort Study (MACS) [291] to access participants for which we had previously characterized the virologic [292,294,306] and immunologic [296] features, to evaluate primary CD8\(^+\) T cells responses to autologous HIV-1 from cryopreserved PBMC obtained pre-seroconversion. These HIV-1 negative samples were stimulated in an \textit{in vitro} priming model (Colleton unpublished) to autologous viral sequences of a MACS participant for comparison to his memory T cell responses after infection, and to an HIV-1 seronegative donor matched at HLA-A*0201 and HLA-B*0702 haplotypes for a comparison of primary CD8\(^+\) T cell responses. I demonstrate a robust, broad spectrum of primary CD8\(^+\) T cell responses that were induced by DC loaded with peptides based on the subject’s ‘founder’ strain and late variants that exhibited immune escape. While the extent of natural immune restoration to autologous HIV-1 antigens on ART remains unresolved, my data suggest that T cell reactivity that has been lost over the course of disease progression can be at least partially recovered during ART by priming with DC loaded with late epitope variants.
Materials and Methods

Study participants

MACS participant #8 is a homosexual male enrolled in the Multicenter AIDS Cohort Study (MACS) [291] among a group of individuals for which had been characterized virologically [292,294,295] and immunologically [296]. The MACS recruited homosexual/bisexual men in 1983-1984 and has studied them twice yearly by physical examination and laboratory testing. HIV-1 seropositivity was defined as a positive enzyme-linked immunosorbent assay (ELISA) and a Western blot with bands corresponding to at least two of the Gag, Pol, and Env proteins of HIV-1. Multiple Gag (p17, p24), Nef and Env (C2-V5) sequences were derived at 6 month intervals over the course (>10 years) of infection, with the earliest sequences sampled at 4 months following seroconversion [294], were used for this analysis. A healthy, HIV-1 seronegative, HLA matched (A*0201 and B*0702) individual was used for comparison.

Analysis of T cell phenotypes

The MACS routinely collects lymphocyte phenotype data at bi-annual visits. Briefly, lymphocytes are gated on CD45 (PerCP) and then analyzed for CD3+(FITC), CD4+(PE) or CD3+(FITC), CD8+(PE) cells were stained according to previously described methods using antibodies obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA). Unstained cells and isotype-matched, control antibodies were used for accurate discrimination of positively staining from negatively staining cells. All analyses were carried out on an EPICS XL flow cytometer (Coulter Electronics, Hialeah, FL) and 5000 lymphocytes were analyzed from each sample.
Synthetic Peptides

Overlapping 15-mer peptides spanning Gag, Nef, and Env (C2-V5) sequences of HIV-1, each overlapped by 11 amino acids representing the ‘founder strain’ [294] and evolving variants (Colleton unpublished, [373]). Potential epitopes were targeted based on predicted binding scores (BIMAS), previously published, as well as, trends discovered that support CTL loss of reactivity due to evolving variants. Peptides were made at ResGen Invitrogen Corporation Huntsville, AL or SynBioSci Corporation Livermore CA).

Dendritic cells (DC)

To obtain immature DC (iDC), CD14+ monocytes were positively selected from cryopreserved peripheral blood mononuclear cells (PBMC) of MACS participant #8 after 3 years on ART using anti-CD14 monoclonal antibody (mAb)-coated magnetic beads (Miltenyi, Auburn CA) for a purity of >95%. The cells were cultured for 5 days in RPMI-1640 (Gibco, Grand Island NY) supplemented with 10% Fetal calf serum (FCS) (Cellgro) containing 1000U/ml recombinant granulocyte-monocyte colony-stimulation factor (GM-CSF) (Amgen, Seattle WA) and 1000U/ml of recombinant human interleukin (hIL)-4 (R&D Systems) adding fresh cytokines every other day. On the 5th day, the iDC were harvested and reset at 1x10⁶/ml and maturation factors were added for 40 hours [CD40L (0.5μg/ml Amgen) for ELISPOT assays and CD40L (0.5μg/ml) + IFNγ (1000U/ml; R&D Systems)] for primary stimulations.

Priming of naïve T cells

For this work, a primary T cell response was defined operationally as the production of IFNγ by T lymphocytes derived from PBMC of an individual who was not infected with HIV-1 (i.e.,
HIV-1 seronegative), in response to four weeks of *in vitro* stimulation with DCs loaded with a specific HIV-1 peptide that was significantly greater than the response of these T cells to medium alone. For these T cell priming studies, I used freshly obtained PBMC from an HLA-A*0201, B*0702 HIV-1 negative subject from the MACS, and cryopreserved PBMC from MACS participant #8 that were originally obtained 2 years prior to seroconversion. The T cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS) (Cellgro) with peptide-loaded DC at a responder-to-stimulator (T/DC) ratio of 10:1. For peptide loading, mDC were incubated in medium (RPMI-1640) without supplemental serum containing 50μg/ml of HIV-1 peptide for 2 hours in 37°C, 5% CO₂ atmosphere. Ag-loaded DC were then harvested and re-suspended with PBMC depleted of monocytes. Co-cultures were fed with fresh medium supplemented with recombinant IL-15 (2.5ng/ml; Peprotech) and IL-2 (50 U/ml; Chiron, Emeryville CA) after the first 5 days and there after. Secondary stimulations (boost) with Ag-loaded DC were added directly to cultures after peptide-pulse (2 hours) and then harvested after 28 days for functional readout (Figure 22).
Method of primary and memory HIV-specific responses

**Primary stimulation**

- **DC Maturation**
- **HIV Peptide Pools Loading**
- **Stimulation**
  - 2 rounds, 2 weeks each

**Memory stimulation**

- **12-16 hrs Overnight**
- **Ex vivo time points**

- **CD8+ T Cell Enrichment**
- **Tetramer**
- **IFN**\(_γ\) ELISPOT
- **Chromium Release Assay**
- **IFN**\(_γ\) ELISPOT

Figure 22. Schematic depiction of the method for primary (in blue across the top of the cartoon) and memory (in purple across the bottom) T cell stimulation. Briefly, iDC are matured with immunomodulating factors for 40 hours, then used as APC for either (i) memory stimulation in which case peptides are loaded onto the mDC for 2 hours and then used in an overnight ELISPOT assay with PBMC, or (ii) primary stimulation in which peptides are loaded onto mDC and co-cultured for 4 weeks and then assayed by ELISPOT, Chromium release, or tetramer staining.

**Stimulation of memory T cells**

Memory T cell responses were defined operationally as the production of IFN\(_γ\) by T lymphocytes derived from PBMC of an individual who was infected with HIV-1 in response to overnight stimulation with DC loaded with a specific HIV-1 peptide that was significantly greater than the response of these T cells to medium alone. To assess memory T cell responses to HIV-1 peptides, cryopreserved PBMC were obtained from the Pittsburgh MACS repository and thawed using the AIDS Clinical Trials Group procedures. The PBMC were routinely >80%
viable by a trypan blue dye exclusion method. The PBMC were suspended to a final concentration of $1 \times 10^5$ cells per microwell in RPMI 1640 cell culture medium with 10% fetal calf serum (FCS) for the overnight ELISPOT assay.

**ELISPOT**

The 96-well plates (Millipore, Billerica, MA) were coated overnight at $4^\circ C$ with 5μg/ml anti-interferon-γ (IFNγ) monoclonal antibody (Mabtech, Stockholm, Sweden). The antibody-coated plates were washed 4 times with PBS and blocked with RPMI supplemented with 10% FCS for 1 hour at $37^\circ C$. Responder cells were stimulated overnight with peptides (10μg/ml)-loaded DC at a ratio of 10:1 in $37^\circ C$, 5% CO₂ atmosphere. After development of the spots, the plates were counted with an Elispot reader system (Cell Technology, Columbia, MD). Data were expressed as net IFNγ spots per $10^6$ cells. The number of spots/$10^6$ responder cells stimulated with HIV-1 antigen expressing APCs minus number of spots/$10^6$ responder cells stimulated with antigen-negative APCs. SEB was used as a positive control and 2 standard deviations over background is shown.
Results

Subject #8 is a Pittsburgh MACS participant whose virus was extensively analyzed for divergence and diversity in our previous investigation [294]. He had documented seroconversion to HIV-1 during our 6 month biannual follow-up approximately 2 years after enrollment in the study. He was chosen for this study because of availability of PBMC from pre-seroconversion through late disease progression and during subsequent suppression of HIV-1 infection on therapy. The natural history of HIV-1 infection in MACS participant #8 is shown in Figure 20, demonstrating a typical early rise in HIV RNA levels and a decline in CD4 counts, while CD8 T cell numbers increased over disease progression until the initiation of ART (>7 years). Viral suppression was achieved on ART while there was a gradual increase in CD4 T cell counts, and concomitantly CD8 T cells decreased.

![Figure 20. Natural history of HIV-1 infection in MACS participant #8. T lymphocyte counts and HIV-1 copies over course of disease progression are shown: CD3 (yellow triangle), CD4 (red circle), CD8 (green diamond), RNA copies (open square).](image-url)
Detection of memory CD8+ T cell responses to autologous virus

Previously, bulk CTL lysis to Gag, Pol and Env was determined for this MACS participant using vaccine vectors expressing proteins of laboratory strain HIV-1 HXB2 [296] (Figure 21).

![Figure 21](image)

**Figure 21.** CTL lysis of previously published work [296] demonstrating reactivity to Gag (red circle), Pol (yellow triangle) and Env (green square) over years of infection. Seroconversion is shown as the leftmost vertical dashed line and time zero (0), and the time of CD3+T cell inflection point is marked by the another dashed line to the right.

The strongest CTL activity was detected against Pol, and Gag, and less so to Env, shortly after seroconversion. These responses decline precipitously by the second year. I hypothesized that the less-than-robust CTL activity directed to Env was due, at least partially, to the recombinant vaccinia virus expressing HXB2 strain of HIV-1 that was used as a target. Hence, it was highly divergent from the individual’s sequences, thus underestimating the CD8+ T cell response.

Therefore, I first determined the IFNγ response of memory CD8+ T cells to autologous ‘founder’ strain Gag (p17 and p24), Nef and Env (C2-V5) and evolving variants to adequately address the host’s response to his quasispecies over disease progression, which our previous studies could not determine. The ‘founder’ strain and evolving variants that were identified from previous analysis of predicted MHCI epitopes corresponding to HLA-A*0201 and B*0702 (Colleton unpublished). For autologous memory T cell responses, I used PBMC that were cryopreserved at sequential years during progressive disease and after the participant went on
therapy. The IFN\(\gamma\) ELISPOT results show a large number (breadth of response) of epitopes targeting to the relatively conserved sequences of Gag (Figure 23A), and Nef (Figure 23B), and a divergent Env (C2-V5) region (Figure 23C). The greatest number of IFN\(\gamma\) responses where directed toward Nef when results are analyzed using the greater than 2 standard deviations from background and having greater than 50 spots per million (Figure 24). Cumulatively, the greatest number of IFN\(\gamma\) spots per million was seen directed towards Gag (Figure 25) when compared to the other two proteins (P<0.001).
Gag (p17 and p24)
Memory T cell responses

Autologous HIV-1 'Founder' and variants

IFN\textgamma Spots/ 10^6
Nef
Memory T cell responses

Autologous HIV-1 ‘Founder’ and variants
Figure 23. Autologous 9- and 10-mer epitopes and 15-mers overlapping by 11 amino acids comprising Gag, Nef and Env regions of HIV-1 were singularly pulsed to DC matured with CD40L and cultured with PBMC from early, late and on ART visits were assayed in a standard overnight ELISPOT assay, to detect Ag-specific CD8+ T cell responses to autologous ‘founder’ and evolving variants of Gag (A), Nef (B) and Env (C) at early (solid black), late (horizontal hatched) and ART (diagonal hatched).
Figure 24  Total number of IFNγ ELISPOTS directed to Gag, Nef and Env displayed in the three time points of Early, Late or on ART.

Figure 25. Total positive CD8+ memory T cell responses at early, late and on ART time points of Gag, Nef and Env were compiled to compare the magnitude of IFNγ spots/10^6 between proteins. P<0.001=***, P<0.01=**, P<0.05=*

The number of total responses at different time points (e.g. early, late and on ART) were directed toward Gag, Nef and Env. I believe that the use of autologous viral peptides allowed for enhanced detection of these T cell responses when compared to chromium release assay data.
Detection of primary CD8\(^+\) T cell responses

Previously, I developed an *in vitro* priming model (Colleton, unpublished) that was modified from our previous model and those of other research groups [71,208,242,243]. This new system can stimulate naïve T cells using immunodominant HIV-1 epitopes and 15-mer consensus strain peptides that had been loaded into DC (Colleton, unpublished). Then I exploited the availability of autologous cryopreserved PBMC (Figure 26) from >1 year prior to HIV-1 detection as our source of naïve CD8\(^+\) T cells.

![Figure 26. Schematic course of disease progression depicting PBMC used to compare primary and memory T cell response. Memory T cell responses were defined as IFN\(\gamma\) production by PBMC obtained at early, late and on ART visits using an overnight ELISPOT assay. The primary responses were defined as IFN\(\gamma\) production by PBMC derived from pre-seroconversion samples, stimulated for over 4 weeks. Monocytes for generating md-DC were obtained from a late ART visit.](image)

Monocyte-derived (md)-DC were generated from autologous CD14\(^+\) monocytes from PBMC at a later visit (on ART for 3 years), and then loaded with founder (infecting virus sequences) and variant peptides representing sequences of MHCI restricted motifs of autologous HIV-1 obtained...
at different times after of disease progression. For each known or predicted MHCI epitope in the founder virus, I assessed the “family” of variants that evolved over time within this viral RNA sequence under immunologic pressure in the absence of ART. After two rounds of stimulation and four weeks of co-culture CD8⁺ T cell responses were determined by the use of a standard ELISPOT assay. The results show that Ag-specific responses were detected against all three regions (Gag (p17 and p24), Nef, and Env (C2-V5)). Significantly greater, (P<0.01), primary CD8⁺ T cell responses were detected compared to memory T cell responses of early, late, and on ART visits (Figure 27).

![Magnitude of primary vs. memory IFNγ response](image)

Figure 27. All positive CD8⁺ T cell memory responses (defined as > 2 SD over background control) comprising early, late and on ART time points to Gag, Nef and Env (black circles) were compiled to compare the magnitude of IFNγ spots/10⁶ and how they compared to primary responses (green circles) between proteins (P<0.001=***, P<0.01= **, P<0.05=*).

As expected, memory T cell responses to a repertoire of early and late HIV-1 epitopes of Gag, Nef and Env were detected at the early visits, but were no longer detected at the late progression visits (representative results, Figure 28). Thus, the latter peptides are
Primary T cells:           Memory T cells:                 Peptides:

Net IFNγ spots/1x10^6

E               L                         E                  L                          E                   L

Gag151-159  Env 298-307 Nef 77-85

Priming Early Late ART

0 200 400 600 800 1000 1200 1400

SPRTLNAWVKVV  TLNAWVKVV  ALNAWVKVV  TLSAWKVV
INCOPNPTRHS  TRNPNNTRKSG  RPNINTRKSL  RPNINTRKSI
RPNINTRKSI  RPSINTRKSI  RPMNTRKSI  RPNINTRKSI
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Figure 28. Representative depiction of memory T cell responses compared to primary T cell responses to evolving variants of three Gag, Nef and Env epitopes detected by ELISPOT. Primary responses are labeled in green, memory responses are labeled as in previous graphs. Brackets are shown to separate the early/founder and late peptides.

considered ‘immune escape’ variants by definition. A limited number of memory T cell responses to early and late HIV-1 epitopes from all three proteins were recovered during ART. However, it is evident that primary T cell responses were detected to some late Gag, Env and Nef immune escape variants that did not stimulate memory T cell responses in the late progression or on ART time periods.

This in vitro study supports the concept that ex vivo engineering of autologous Ag-loaded DC that are re-administered to HIV-1 infected person’s could enhance primary CD8+ T cell responses to immune escape variants of HIV-1 and potentially provide natural viral suppression. To further evaluate the immunogenicity of viral immune escape peptides derived from MACS
subject #8, I used a MACS seronegative HLA-A*0201, B*0702 donor with cryopreserved PBMC and fresh autologous DC matured with CD40L + IFNγ to compare primary responses. I found that the primary responses (Figure 29A-C) were directed at all three proteins (Gag, Nef and Env). MACS participant #8 overall had more responses than the control HLA matched donor (HLA-A*0201 and B*0702). The primary responses were directed to epitopes in the founder strain (early) and to evolving variants (late and on ART time points).
A

Gag (p17 and p24)
memory and primary T cell responses

Autologous HIV-1 ‘Founder’ and variants
B

Nef memory and primary T cell responses

Autologous HIV-1 ‘Founder’ and variants
Figure 29. Autologous 9- and 10-mer epitopes and 15-mers overlapping by 11 amino acids comprising Gag, Nef and Env regions of HIV-1 were singularly pulsed to DC matured with CD40L and cultured with PBMC from early (solid black), late (horizontal hatched) and on ART (diagonal hatched) visits were assayed in a standard overnight ELISPOT assay (IFN-γ spots/10⁶), to detect Ag-specific CD8⁺ T cell responses to autologous 'founder' and evolving variants to Gag (A), Nef (B) and Env (C) for memory responses. These results were added to for either MACS #8 primary response (green bars) or primary response from a HLA matched normal donor (red bars).
However, the magnitude of the primary IFNγ response to Gag was similar between this donor and MACS participant # 8 (Figure 30). In contrast, responses to Nef and Env were significantly lower than for participant #8 (P<0.001). This is in part likely due to differences in priming by peptide specific for non-matched MHCI haplotypes between these persons. Also, individual genetic differences in the TCR repertoires could relate to preferential primary T cell responses to different epitopes. Overall, the data from these two primary T cell responses to HIV-1 Gag, Nef and Env peptides demonstrated showed 11% concordance (same epitope between to priming experiments) (Figure 31) among the reactive epitopes (e.g. IFNγ positive responses).

Figure 30. Total positive IFNγ responses detected for memory (black circles) and primary T cells(green circles) for MACS participant #8. A seronegative HLA-A*0201, B*0702 matched donor was used as a control (red circles). P<0.001=***, P<0.01=**, P <0.05=*.
Figure 31. Summary of concordance in positive T cell responses between the HLA-A*0201, B*0702 matched individual and MACS participant #8.

Discussion

Progressive CD4 T cell depletion, with disproportionate declines in naïve CD4+ and CD8+ cells, characterizes untreated HIV-1 disease [374,375]. The underlying mechanisms of dysfunction can be attributed to direct cytopathic effects of viral replication on T cells, T cell homeostasis, phenotypic alterations of T cell subsets, accelerated apoptosis, and peripheral cytokine-dependent T cell expansion, all which remain poorly defined in HIV-1 disease [376-378]. As our understanding of chronic infections increases, new therapies, such as ART, have allowed HIV-1 infected individuals survival, but persistent viral reservoirs remain. Although management of HIV-1 disease depends largely on the degree of viral load suppression, there are many unanswered problems revolving around to what extent immune function is reconstituted [60,327-336] while on drug.

The recovery of CD4+ T cells in HIV-1 infection during ART has been attributed to the production of naïve T cells associated with the thymus [339-342] while the recovery of naïve CD8 T cells (CD8+ CD45RA+ CD62L+) [335,336] remains controversial [123,349]. Particularly noteworthy, however, is the detection of phenotypically naïve T cells in athymic mice and
humans, suggesting that extra-thymic pathways of T cell development exist [350,351,354]. I hypothesize that residual memory T cells during ART that are specific for HIV-1 inadequately control replication of HIV-1 immune escape variants. Therefore, I further hypothesize that control HIV-1 infection and viral immune escape variants during ART requires priming of naïve CD8+ T cells specific for these variants.

Previously, bulk CTL lysis to Gag, Pol and Env from this MACS participant [296] revealed a strong CTL response to Pol and Gag, with less T cell reactivity to Env. The drop off in reactivity to Env that was seen could be due to the extensive diversity of these viral sequences evolving over disease progression, thereby underestimating the CD8+ T cell responses. In the present study, the loss of memory CD8+ T cell reactivity to evolving quasispecies of HIV-1 was defined as immune escape. With that understanding, I determined the IFNγ response of memory CD8+ T cells to the autologous ‘founder’ strain of Gag (p17 and p24), Nef, and Env (C2-V5), and the evolving variants at different time points during disease progression (e.g. early, late and on ART). I sought to determine if loss of CTL responses that were seen during progression, were recovered on ART.

The IFNγ results show a large number (breadth of response) of epitopes targeting to the relatively conserved sequences of Gag, and Nef, and to the more divergent Env (C2-V5) region. Cumulatively, the greatest number of IFNγ spots per million cells was directed towards Gag compared to Nef and Env (C2-V5) (P<0.001), but the number of total memory T cell responses was greater to Nef and Env (C2-V5). Although definitive comparisons to peptides of laboratory strains of HIV-1 were not possible, the use of autologous viral peptides could have allowed for enhanced detection of T cell responses to these three proteins. As expected, there was a loss of memory T cell responses to the evolving variants of Gag, Nef and Env late in infection. The
basis for this loss of T cell reactivity is unknown. However, the loss of T cell reactivity to these epitope variants was not strongly related to loss of binding affinity for their two HLA haplotypes, HLA A*0201 and HLA B*0702 (Colleton unpublished). Other possible mechanisms for the loss of memory T cell responses that remain to be elucidated in this model include changes in proteolytic processing of the MHCI peptides, anergic T cell reactivity due to poor TCR signaling and post-signaling pathways (e.g., low cytokine or lytic granule production), loss of T cell coreceptor expression, and changes in PD-1/PD1L expression.

Most importantly, my data show that naïve CD8+ T cells from the same individual who lost memory CD8+ T cell reactivity were able to be primed to autologous HIV-1 immune escape variants in vitro. This shows for the first time that it is potentially feasible to induce CTL to immune escape variants. Based on these priming results, it is unlikely that the peptide variants of immunodominant epitopes were unable to bind to their MHCI restriction molecules (Colleton, unpublished). It is also possible that our long-term in vitro priming model is more sensitive at detecting subdominant epitopes than the overnight memory T cell ELISPOT assay. Nevertheless, these data show that viral “immune escape” cannot be generalized across all epitopes, in that loss of memory T cell reactivity to an “immune escape” variant does not necessarily equate with lack of capacity of this variant to prime T cells.

Previously, it was demonstrated that DC matured with CD40L, and subsequently loaded with immunodominant HIV-1 peptides [242] or apoptotic bodies [211] can activate residual HIV-1 specific memory responses while on ART. I propose that targeting immune reconstituted naïve CD8+ T cells through immunotherapy should enhance CD8+ T cell responses, and by exploitation of the DC ability to instruct the T cells to mount a robust and broad response HIV-1 antigens is the target for immune control of this chronic viral infection [164,368-372].
Currently, immunotherapy studies have used consensus strain HIV-1 antigens which inadequately represent the host’s diverse pool of HIV-1 quasispecies (Colleton, unpublished). My study supports the concept of using autologous virus as antigen to represent the host’s diverse pool of quasispecies in immunotherapy to enhance CD8⁺ T cell responses. My data show that use of DC loaded with peptides representing autologous HIV-1 sequences can expand memory and primary T cell responses in vitro. This is also the first study directly comparing memory and primary T cell responses to a virus using cells from the same individuals, and provides a model for future analysis of these basic host immune functions. Finally, my data show a potential advantage for immunotherapies using these approaches with autologous virus representing a large repertoire of the host’s diverse HIV-1 antigen pool. This could elicit the most specific primary immune response for each patient’s quasispecies of HIV-1, giving the broadest immune control of HIV-1 infection during ART. This could allow for ultimate goal of immune control of HIV-1 infection in the absence of ART.
VI. CHAPTER SIX: OVERALL DISCUSSION

Overview of the rationale for this doctoral research

Although it has been almost 25 years since the identification of the causative agent of AIDS [2,3], we still struggle to understand the dynamics of viral-host interactions leading to progressive disease. The HIV-1 epidemic continues, with the latest statistics estimating more than 40 million individuals are living with HIV-1 [391]. These individuals reside chiefly in less fortunate countries where a large portion of the population does not even have access to adequate medical care, let alone ART. The global need for an effective vaccine against HIV-1 is critical. This doctoral research study contributes to the development of our knowledge of viral immunogenicity and enhancing T cell responses towards successful immunotherapy and viral control.

It is known that the infidelity of the reverse transcriptase (RT) of HIV-1 [148] and the rapid turnover [154] quickly diversifies the virus population within an individual, as well as, the circulating clades identified (A-J currently) [390] in geographical populations. Of all the HIV-1 genes, Env, and particularly its V1-V5 domains, demonstrate the greatest amount of heterogeneity [146,147]. One of the hallmarks of HIV-1 infection is its high level of genetic divergence over time, which accumulates into what is termed the quasispecies within an individual. The substantial genetic changes of HIV-1, within a host, are driven by not only the error-rate of the RT, but the host genetic background as well. Mutations within a CTL epitope can have dynamic change in processing, presentation and recognition of that epitope. Viral sequence changes can have an effect in the capacity of viral peptides once processes, to bind to MHCI molecules, which in turn could result in altered recognition and response by CD8+ T cells.
Conclusions and importance of the research findings.

Specific Aim 1: Identify potential MHC class I CD8\(^+\) T cell epitopes in autologous HIV-1 sequences of MACS participant #8.

I hypothesize that the loss of anti-HIV CTL activity could be due to changes in the CTL epitopes that arise in the variant quasispecies, specifically in their MHC class I (MHCI) binding capacity and impacts disease progression (e.g. CD4\(^+\) T cell counts and viral load).

Therefore, the first aim of this project was to examine the MHCI binding affinities of peptides that we had derived from Gag, Nef and Env quasispecies of an HIV-1 infected individual. Notably, there are other parameters that can be considered when assessing affects on CD8\(^+\) T cell recognition such as proteasomal cleavage sites [300-303] and TAP binding patterns (Predict [304]). However, these types of analyses were beyond the scope of this project. Instead, I focused on identifying potential CTL epitopes and their subsequent binding affinities to MHCI molecules, which can be viewed as one of the major factors affecting antigen presentation to CD8\(^+\) T cells that is impacted by genetic diversity of the antigen.

To determine potential CTL epitopes, I used the in silico program BIMAS, which is an interactive MHCI binding prediction algorithm, to analyze the evolving autologous HIV-1 sequences over the course of disease progression for MACS participant #8. Recently this algorithm was compared to many others that are available on-line [234], and BIMAS ranked very high for the two haplotypes, A*0201 and B*0702, that we used in this study. I was able to demonstrate that the predicted binding scores to MHCI epitopes of variants evolving from the founder strain have three basic patterns: (i) weaker (or decreasing), (ii) neutral (or constant), and (iii) stronger (or increasing) that evolved in the quasispecies over disease progression.
I then assessed actual binding of the autologous HIV-1 peptides to MHCI to evaluate the accuracy of the BIMAS prediction method. For this, I first tried to determine the binding affinity of these peptides using T2 cells [279, 280], which are TAP deficient cells expressing HLA-A2, but this method was insensitive. Therefore, I turned to a soluble MHCI binding assay using fluorescent polarization methods in competitive binding analysis [290, 298] for higher throughput potential, as well as greater sensitivity than the T2 cell method. I confirmed some of the predicted binding patterns using this MHCI binding assay for epitopes restricted to HLA-A*0201 and B*0702, and demonstrated a range from non-binding to strong affinity. However, to make definitive conclusions about MHCI binding and HIV-1 disease progression, further assessment of additional potential epitopes that were determined to have strong correlative values ($R^2>0.5$) between predicted MHCI binding and CD4$^+$ T cell counts or to viral load are needed.

It was especially interesting to see an accumulation of strong associations for the predicted binding trend that increased in strength during disease progression. This has been noted in other studies [199] and may be an escape mechanism where the higher binding affinity epitopes are selected due to their low disassociation rate and ineffective control on viral replication. Most of the MHCI binding results on the peptides that were selected for testing were in the neutral category. Thus, there was no significant relationship between predicted and true binding of HIV-1 peptides to MHCI, or to disease progression overall. However, the prediction scores for the Nef epitopes and their variants demonstrated a strong correlation to actual binding affinity ($R^2=0.7948$). Indeed, HLA-A*0201 predicted binding scores ($R^2=0.6222$) correlated much stronger than B*0702 ($R^2=0.1537$) to actual binding affinities.

Numerous putative antigenic and immunogenic peptides have been identified through this method of reverse immunology. The identification of CD8$^+$ T cell defined antigens has improved
over recent years, as our capacity to predict MHCI binding for the most common HLA variants among the population has accumulated more experimental data. However, to use in a vaccine design, successful prediction for as many HLA variants as possible is necessary to increase coverage of the population. Accurate prediction modeling as an approach to evaluating MHCI epitopes binding and immunogenicity are desired to lower the high cost and labor intensive nature of such studies.

Specific Aim 2: Develop an in vitro priming model to evaluate potential binding of MHC class I CTL epitopes.

I hypothesize HIV-1 peptide antigen loaded, mature dendritic cells have the capacity to prime naïve CD8+ T cells in an in vitro system. To address this, I will define the best in vitro priming model for inducing primary responses of naïve CD8+ T cells in HIV-1 seronegative individuals.

Over the past decade, major improvements in our understanding of what constitutes the memory T cell response have revolutionized the field [86,87,91,92]. However, researchers are still trying to ‘spot’ an effective HIV-1 vaccine. As far as the technology has come, reliable surrogate markers of what constitutes “protection” to HIV-1 infection or is critical to impeding HIV-1 disease progression has not been identified. In fact, novel immunotherapeutic vaccines are being developed in the wake of our present inability to develop a preventative HIV vaccine. The ideal vaccine would establish ‘sterilizing immunity’ however, the best expectation presently is a therapeutic vaccine; one that can bolster existing immune responses to keep up with the evolving virus. The basic rationale for DC immunotherapy in HIV-1 infected persons is to instruct the T cell responses to function more effectively to resemble the T cell responses of a long-term
nonprogressor (LTNP) or elite controller. However, utilizing DC as a vehicle to overcome immune dysfunction is a difficult challenge. The primary objective is to elicit a strong Th1 response to enhance CD8\(^+\) T cell responses due to the evidence that supports the importance of virus-specific CD8\(^+\) T cells. The supportive data are multifaceted: (1) peak virus-specific CD8\(^+\) T cell activity coincides with the decline in virus during acute infection [380,381], (2) CD8\(^+\) T cell depletion studies in the SIV model leads to increased virus levels [51,85], (3) infusion of virus-specific CD8\(^+\) T cells leads to decreased viremia [382] and (4) viral escape leads to increasing viral loads [105,143,191,192,383-387].

Based on this literature, I proposed that a broad and robust primary CD8\(^+\) T cell immune response is important for an effective prophylactic or therapeutic vaccine. Priming of naïve CD8\(^+\) T cells has been shown to require three signals involving the MHCI/peptide complex on DC (signal 1) [245] along with the costimulatory molecules (e.g. CD80 and CD86) (signal 2) [74,246,247] on the surface of DC which activate CD8\(^+\) T cells with the help of CD4\(^+\) T cells [107-109,248] and IL-12 (signal 3) [82,103,249-253,255,256,318-321]. Combining this knowledge of how DC work and the correlative value of HIV-1 specific CD8\(^+\) T cells being necessary for viral suppression, I explored the use of an \textit{in vitro} primary stimulation model. I chose to examine CD40L and IFN\(\gamma\), our most potent modulator of memory T cell reactivity to HIV-1 [219,388], compared to a TLR3-cytokine cocktail currently used in DC immunotherapy of cancer [220]. First I phenotyped md-DC to determine their expression for maturation surface markers; all three maturation groups expressed high surface marker levels of co-stimulatory ligands B7.1 (CD80) and B7.2 (CD86) on the DC surface for a strong signal 2, compared to iDC. Second, IL-12, is well known to augment CTL responses in experimental systems [323,324]. Reports have demonstrated that primary expansion can be obtained with signal 1 and 2, but
effector function and memory requires a third signal that can be provided by either IL-12 or type I interferons [83,84,322] and in the absence of this signal some cells can survive, but they are immunotolerant [103]. I therefore determined the cytokine profiles of the various DC maturation groups. Our results showed that all of the DC modulation groups produced IL-12. However, iDC treated with CD40L + IFNγ produced the most IL-12(p40 and p70), which is critical for signal three in this model. Furthermore, I demonstrated that this higher IL-12 production by the DC translated into the superior priming capacity CD8+ SL9_{(p17 77-85LAI)} Tet+ cells. Further work is necessary, such as blocking of IL-12 by neutralizing antibodies, to prove that these high levels of IL-12 produced by the DC are critical to priming of the CD8+ T cells in this in vitro model.

Based on this three signal priming model, I then demonstrated the capability of DC treated with CD40L + IFNγ to induce a broad spectrum of primary CD8+ T cell responses to five immunodominant HLA-A*0201-restricted epitopes. As expected based on the well known helper effects of CD4+ T cells, I found that CD4+ T cells were required for efficient priming of HIV-1 specific CD8+ T cells. The primed CD8+ T cells exhibited HIV-1 peptide-specific cytolytic activity and IFNγ production that was optimized by the presence of autologous CD4+ T cells. I postulate that the CD4+ T cells provided the necessary IL-2 signals when a secondary stimulation is given [325].

Finally, naïve CD8+ T cells from two HIVneg HLA-A*0201 donors in the presence of CD4+ T cells with CD40L + IFNγ mDC for in vitro priming to consensus sequences of overlapping 15-mers to Gag, Env and Nef regions of HIV-1 were evaluated. I determined that both donors exhibited a broad immunogenicity (IFNγ production) for many well documented epitopes in the Los Alamos HIV database [393]. Moreover, the number of potential T cell epitopes that matched between the two donors was 34%. Using BIMAS prediction modeling
with the Los Alamos HIV Database [393], we identified many potential MHC class I binding epitopes for Gag, Env and Nef among the peptide regions that were positive for T cell reactivity in our priming system. Presently, I cannot rule out the possibility that some of these results are from CD4+ T cells due to the fact that I was unable to enrich CD8+ T cells for each of these responses. However, there are many known CTL epitopes within the 15-mers previously detected in functional assays by other groups [381], and predicted in BIMAS by us, thus supporting that the responses are CD8+ T cell specific. Confirmation that these are indeed CD8+ T cell epitopes, however, requires that they be mapped to their minimal, 9- and 10-mer MHC class I epitopes.

In summary, in aim 2 I showed that a primary CD8+ T cell response to HIV-1 can be activated in vitro by stimulation of naïve T cells with HIV-1 peptide-loaded DC. The optimal primary T cell response to HIV-1 required maturation of the DC with CD40L + IFNγ, and IL-12 and CD4+ T helper cells. The primed CD8+ T cells recognized multiple regions of Gag, Env and Nef that corresponded to known and predicted MHC class I epitopes. This in vitro priming model can be of importance in evaluation of various aspects of HIV-1 vaccines, including the immunogenicity of predicted and known MHC class I restricted epitopes.

Specific Aim 3: Compare primary and memory response in MACS participant #8.

I hypothesize that the change in HIV-1 epitopes due to CTL selective pressure will result in failure of priming of CD8+ T cells to late escape variants.

On ART it is possible to enhance secondary and primary responses that may potentially help to control viral replication. Therefore, I applied the priming model established in specific
aim 2, exploiting the unique ability to compare primary naïve CD8+ T cells responses (pre-seroconversion) to memory of early, late and ART time points during MACS participant #8 progressive infection.

Progressive CD4 T cell depletion, with disproportionate declines in naïve CD4+ and CD8+ cells, characterizes untreated HIV-1 disease [374,375]. The underlying mechanisms of dysfunction remain poorly defined in HIV-1 disease [376-378]. Management of HIV disease depends largely on the degree of viral load suppression, but there are many unanswered problems revolving around to what extent immune function is reconstituted [60,327-336] while on drug.

While the recovery of CD4+ T cells in HIV-1 infection during ART has been attributed to the production of naïve T cells that has been associated to the thymus [340-342,344-348], the role of naïve CD8 T cells (CD8+ CD45RA+ CD62L+) [335,336] remains controversial [123,349]. Particularly noteworthy, is the detection of phenotypically naïve T cells in athymic mice and humans suggesting that extra-thymic pathways of T cell development must exist [350,351,354]. Researchers believe that the recovery of naïve CD8+ T cells during ART is critical to restoration of immune control of HIV-1 infection.

Previously, bulk CTL lysis to Gag, Pol and Env from this MACS participant was determined [296], which demonstrated shortly after seroconversion, a strong CTL response was detected to Pol and Gag, while Env was only minimally reactive. I believe the drop off in reactivity to Env that was seen, was due to the diversity of the viral sequences evolving over disease progression, and thereby underestimating the CD8+ T cell responses. Therefore, in my last specific aim, I defined decreasing memory CD8+ T cell reactivity to evolving quasispecies of HIV-1 as immune escape. I determined the IFNγ response of memory CD8+ T cells to
autologous ‘founder’ strain Gag (p17 and p24), Nef, and Env (C2-V5) and the evolving variants at different time points during disease progression (e.g. early, late and on ART) to determine if enhanced CTL response were seen. The IFNγ results show a large number (breadth of response) of epitopes targeting to the relatively conserved sequences of Gag, and Nef, and a divergent Env (C2-V5) region. Cumulatively, the greatest number of IFNγ spots per million cells was seen directed towards Gag when compared to Nef and Env (C2-V5) (P<0.001), but the number of total responses was directed toward Nef and Env (C2-V5). I believe the use of autologous viral peptides did indeed allow for enhanced detection of T cell responses.

Most importantly, I showed that naïve CD8+ T cells from the same individual who lost memory CD8+ T cell reactivity were able to be primed to these autologous HIV-1 immune escape variants from PBMC previous to his seroconversion. This shows for the first time that it is potentially feasible to induce CTL to immune escape variants and although the basis for this reactivity is unknown, the loss of T cell reactivity to these epitope variants was not related to loss of binding affinity for their two HLA haplotypes, HLA A*0201 and HLA B*0702 (Colleton unpublished). It is also possible that our long-term in vitro priming model is more sensitive at detecting subdominant epitopes than the overnight memory T cell ELISPOT assay. Nevertheless, these data show that viral “immune escape” cannot be generalized across all epitopes, in that loss of memory T cell reactivity to an “immune escape” variant does not necessarily equate with lack of capacity of this variant to prime T cells.
Overall conclusions

This research builds directly on 20 years of work on T cell immunity to HIV-1 in this laboratory. Previously, it has been demonstrated that DC matured with CD40L and subsequently loaded with immunodominant HIV-1 peptides [242] or apoptotic bodies [211] can activate residual HIV-1 specific memory responses while on ART. Therefore, I hypothesized that targeting immune reconstituted naive CD8+ T cells through immunotherapy should enhance CD8+ T cell responses, and by exploitation of DC it is possible to instruct the T cells to mount a robust and broad response to the chronic infection [164,368-372]. The current study has provided initial data that support our hypothesis that CD8+ T cells can be primed by in vitro engineered DC, even against HIV-1 peptides representing immune escape variants.

Presently, immunotherapy studies have used consensus strain HIV-1 antigens and I believe that this inadequately represent the host’s diverse pool of HIV-1 quasispecies. This study supports the concept of using autologous virus as an antigen in immunotherapy. These data demonstrate that use of autologous viral sequences expands the memory and primary T cell responses in vitro. Thus, a potential advantage is that immunotherapies can use these approaches for autologous virus representing a large repertoire of the host’s diverse HIV-1 antigen pool. This could elicit the most specific primary immune response for each patient’s quasispecies of HIV-1, giving the broadest immune control of HIV-1 infection during ART. Such an approach has important public health implications in that it could have a strong positive impact on, and improve the control of, HIV-1 infection in HIV-1 infected persons on ART. It also serves as an in vitro model for development of prophylactic vaccines against HIV-1 and other infectious agents.
PUBLIC HEALTH AND SCIENTIFIC SIGNIFICANCE

The most recent global statistics according to the UNAIDS report are that more than 40 million people are living with HIV. These individuals predominantly are in resource deprived countries such as Sub-Saharan Africa where access to health care and medicine is limited. The infection rates have gone down in some of these countries such as Uganda, but remain high in many of the other regions such as Mozambique and Kenya. While preventative measures such as condoms and circumcision can help prevent infection, a prophylactic vaccine is essential to prevent further spread of HIV-1. The ideal T cell response induced by a prophylactic vaccine is one that can eliminate the pathogen before it has a chance to establish an infection, i.e., induce a ‘sterilizing immunity’. Despite researchers best efforts in the past 25 years, however, we are still unable to provide an effective HIV-1 vaccine and are in fact struggling with surrogate markers that are correlative to protection for this virus.

Unfortunately, such prophylactic vaccines will not help the millions of people who are already infected with the virus, and who face life-long treatment with expensive and toxic antiretroviral drugs. Currently, therefore, we believe that the best strategies for these individuals are therapeutic vaccines, in which the goal is to enhance T cell responses within an infected individual while on ART. The objective of this method is to attack residual viral reservoirs by expanding the individual’s HIV-1 specific, primary T cell responses to control virus replication.

Thus the research done in this doctoral program on stimulation of CD8+ T cell responses to HIV-1 by antigen-loaded DC has direct, public health relevance for the development of both prophylactic and immunotherapeutic vaccines. Furthermore, the T cell priming model developed in this doctoral project opens many new possibilities to advance our knowledge of HIV-1
immunity, as well as immunity to other infectious agents. Indeed, this *in vitro* vaccination model for HIV-1 and potentially many other viruses is the only model besides the humanized mouse models that allows intricate dissection of the many variables of induction of T cell immunity in humans. These could include determining the role of many factors in priming of T cells, such as different antigen presenting cells, helper cells and suppressor cells, cell activation states, soluble and membrane-bound factors, and T cell epitope mapping and discovery for HIV-1 and other viruses.
VII. CHAPTER SEVEN: FUTURE DIRECTIONS

While this dissertation has addressed a number of issues pertaining to enhancing T cell immunity, the field still has many questions that need to be addressed. The following sections discuss several questions that still need to be resolved and warrant further investigation.

1. Examining the MHC class I prediction model.

I have demonstrated in one individual that autologous viral sequences change, potentially effecting MHCI binding (e.g., the three binding trends). There was an accumulation of the increasing pattern in binding to MHCI potential, which has also been reported for SIV research. Therefore, this study will be expanded to include other MACS participants where autologous viral sequences are available and who have common haplotypes for at least two alleles and are listed in the Table 5.

Table 5: MACS participants and their haplotypes.

<table>
<thead>
<tr>
<th>MACS Participant #</th>
<th>Status</th>
<th>HLA haplotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Progressor</td>
<td>A2, A2, B27</td>
</tr>
<tr>
<td>2</td>
<td>Progressor</td>
<td>A2, B27</td>
</tr>
<tr>
<td>4</td>
<td>Progressor</td>
<td>A2, A2</td>
</tr>
<tr>
<td>5</td>
<td>Progressor</td>
<td>A2, A2, B27, B7</td>
</tr>
<tr>
<td>7</td>
<td>Progressor</td>
<td>A2, B7</td>
</tr>
<tr>
<td>12</td>
<td>LTNP</td>
<td>A2, B27</td>
</tr>
<tr>
<td>14</td>
<td>Rapid progressor</td>
<td>A2, A2</td>
</tr>
</tbody>
</table>

The Rinaldo laboratory will determine if associations on a larger scale could identify common epitopes amongst HLA matched individuals and whether associations can be seen for disease progression through a measure of these epitopes in relation to viral load and CD4 T cell counts. Due to the limitations of predicting binding epitopes, I was concerned with examining
many sequences that were in fact, not true epitopes. Therefore, binding affinity to soluble MHC was directed at previously published epitopes and their corresponding variants to determine the range of the fluorescent binding assay. Further binding affinities of MHCI epitopes representing the decreasing and increasing patterns need to be determined.

2. **In vitro priming model**

I determined the optimal epitopes that were primed to the consensus strain of Gag, Nef and Env experiments by using synthesized 9- and 10-mer peptides representing the ‘optimal’ epitope deduced by prediction modeling (BIMAS) in dose-response curves in order to determine if these epitopes are truly CD8\(^+\) T cell specific and are not attributed to CD4\(^+\) T cell responses. It is also important to look at the surface expression of primed CD8\(^+\) T cells to determine at which stage they have differentiated into (e.g. CD45RA/RO, CD28, CD27, CD57) as well as the cytokines other than IFN\(\gamma\) they are able to produce (e.g. IL-2, CD107ab, perforin, granzyme AB). Next, under this model the Rianldo laboratory should explore the different dendritic cell immunomodulating factors I used to determine if there are any proliferation (CSFE) differences in the CD8\(^+\) and CD4\(^+\) T cells based on the maturation method. This information is critical as we begin to understand the different subpopulations of memory T cells and their contributions to controlling acute and chronic viral infections. The different dendritic cell immunomodulating factors used in this study need to be further assessed to determine if there are any proliferation (CSFE) differences in the CD8\(^+\) and CD4\(^+\) T cells based on the maturation method. This is an important issue central to the need of enhancing effective T cell responses while on ART for immunotherapy. Having an *in vitro* priming model that is capable of evaluating immunogenicity in the context of proliferation, cytokine proliferation and memory surface markers expression would greatly aid in vaccine strategies.
Finally, it would be of value to expand this model to explore other dendritic cell populations, such as Langerhans cells and plasmacytoid dendritic cells, which could be directly targeted by immunotherapies *in vivo*.

3. **Primary and secondary memory responses.**

In my last specific aim, a novel concept was to compare primary and secondary memory T cell response within the same individual that was subsequently infected with HIV-1. To our knowledge this is the first time this has been done. Although I was able to show loss of anti-HIV-1 CTL reactivity to some evolving viral variants (immune escape) and subsequent priming responses to these variants, more epitopes are needed to confirm and expand my major conclusion. This should include more epitopes and their variants to be tested in MACS participant #8 PBMC from the available specimens left as well as extending this study to examine more of the MACS participants. Implications for supporting the concept of using autologous HIV-1 sequences in immunotherapy to get the most robust, broad T cell responses expanded thereby, potentially controlling viral replication when ART is stopped is a central issue in treatment of HIV-1 infected individuals.
APPENDIX A: Evolutionary changes of 10-mers HLA-A*0201

Viral evolutionary changes in HLA-A*0201 predicted binding scores of 10mers:
subject #8 (HLA-A2, A24, B7, B40)

Figure 32: Scanning the impact of viral evolutionary changes on HLA-binding.
Predicted binding to HLA-A*0201 10-mers across the genome over years following seroconversion, was mapped for sequences (Gag-p17, Gag-p24, Env, Nef) in subject #8. Each 10-mer amino acid motif in the sequence alignment was colored according to predicted HLA-binding score indicated in the score key. Each protein analyzed revealed a trend of three patterns: (i) increasing, (ii) decreasing, (iii) constant.
APPENDIX B: Evolutionary changes of 9-mers HLA-B*0702

Viral evolutionary changes in HLA-B*0702 predicted binding scores of 9-mers:
subject #8 (HLA-A2, A24, B7, B40)

Figure 33: Scanning the impact of viral evolutionary changes on HLA-binding.
Predicted binding to HLA-B*0702 9-mers across the genome over years following seroconversion, was mapped for sequences (Gag-p17, Gag-p24, Env, Nef) in subject #8. Each 9-mer amino acid motif in the sequence alignment was colored according to predicted HLA-binding score indicated in the score key. Each protein analyzed revealed a trend of three patterns: (i) increasing, (ii) decreasing, (iii) constant.
Figure 34: Scanning the impact of viral evolutionary changes on HLA-binding.
Predicted binding to HLA-B*0702 10-mers across the genome over years following seroconversion, was mapped for sequences (Gag-p17, Gag-p24, Env, Nef) in subject #8. Each 10-mer amino acid motif in the sequence alignment was colored according to predicted HLA-binding score indicated in the score key. Each protein analyzed revealed a trend of three patterns: (i) increasing, (ii) decreasing, (iii) constant.
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