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

by

Bonnie Colleton

B.A., Wheaton College, 1993

Submitted to the Graduate Faculty of

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2007

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This dissertation was presented

by

Bonnie Colleton

It was defended on

August 17, 2007

and approved by

Simon Barratt-Boyes, BVSc, PhD
Associate Professor
Department of Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

Phalguni Gupta, PhD
Professor
Department of Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

Russell Salter, PhD
Professor
Department of Immunology
School of Medicine, University of Pittsburgh

Walter Storkus, PhD
Professor
Department of Dermatology
School of Medicine, University of Pittsburgh

Dissertation Advisor: Charles Rinaldo Jr., PhD
Chairman and ProfessorDepartment of Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

Charles R. Rinaldo, Jr. PhD

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

Bonnie Colleton, PhD

University of Pittsburgh, 2007

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 persons'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 *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 *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 *in vitro* priming model for development of prophylactic vaccines against HIV-1 and other infectious agents.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to express my appreciation to my mentor Dr. Charles Rinaldo, Jr. for his support and encouragement over the years and the opportunities he has afforded me. I would also like to thank my committee members Drs. Phalguni Gupta, Russell Salter, Walter Storkus, Simon Barratt-Boyes (and Raj Shankarappa) for their invaluable expertise, time and efforts towards the completion of this dissertation.

I would like to extend my appreciations to my co-workers who provided me with support over the years; including the Flow laboratory: LuAnn, Kim, Edwin; Clinical laboratory: Chris, Susan, Alycia and Hope; Research laboratories: Drs. Fan, Piazza, Rappocciolo, as well as, Mariel and Ping. I am especially thankful to Dr. Huang and Weimin over the last couple of years for all their help and support. I would like to thank the hard working administration staff: Judy, Cheryl, Nancy, Debbie, Robin, and Joe that has helped with many things over the years.

Deena, Kathy and Bill for all their help in scheduling all the buffy coats I have used over the years as well as, all the blood donors used for this project including normal and MACS participants for which this project could not have been completed without.

I am forever grateful of my fellow graduate students for their friendship and scientific discussions any help in their areas of research they were able to provide over the years. I would also like to thank my friends outside the laboratory for all their tremendous belief in me.

I am forever grateful to my parents, Norman and Barbara for their love and constant encouragement leading up to this achievement, and for their understanding for all the missed holidays and family events over the years.

Lastly, I would like to thank my wonderful partner in life, Tom, for all his understanding, love and support through the years and appreciating the few moments we have been able to coexist as a family with Percie and Schenley. I love you and here is to our future together.

LIST OF ABBREVIATIONS

Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
APCs	Antigen presenting cells
APC	Allophycocyanin
ART	Antiretroviral therapy
AUC	Area under the curve
B2	beta 2
bp	Base pair
BIMAS	Bioinformatics and Molecular Analysis Section
CMV	Cytomeglovirus
CSFE	5-,6-carboxyfluorescein diacetate succinimidyl ester
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
EBV	Epstein Barr virus
ELISA	Enzyme Linked ImmunoSorbent Assay
ER	Endoplasmic reticulum
E:T	Effector to target ratio
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage-colony stimulating factor
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV-1	Human immunodeficiency virus -1
HIV-2	Human immunodeficiency virus -2
HLA	Human leukocyte antigens
HSV	Herpes simplex virus
HTLV-III	Human T-cell lymphotropic virus-type III
IC_{50}	Inhibitory concentration 50%
ICAMs	Intracellular adhesion molecules
iDC	Immature dendritic cells
IFNα	Inferon alpha
IFNβ	Interferon beta
IFNγ	Interferon gamma
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-15	Interleukin-15
IL-1β	Interleukin-1beta
Int	Integrase
LAV	Lymphadenopathy-associated virus

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].

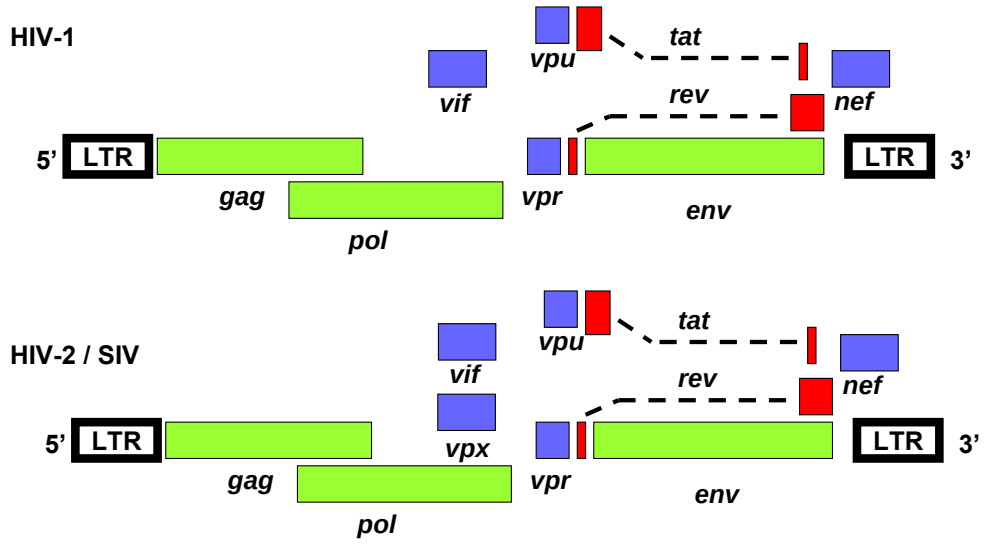


Figure 1: Genetic organization of lentivirus. The primate lentivirus RNA proteome is approximately 9.2 kb in size and encodes structural proteins/viral enzymes which include the products of gag, pol, and env genes, which are essential components of the retroviral particle shown in green. Regulatory proteins, 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 co-receptors [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 lymphnoid 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].

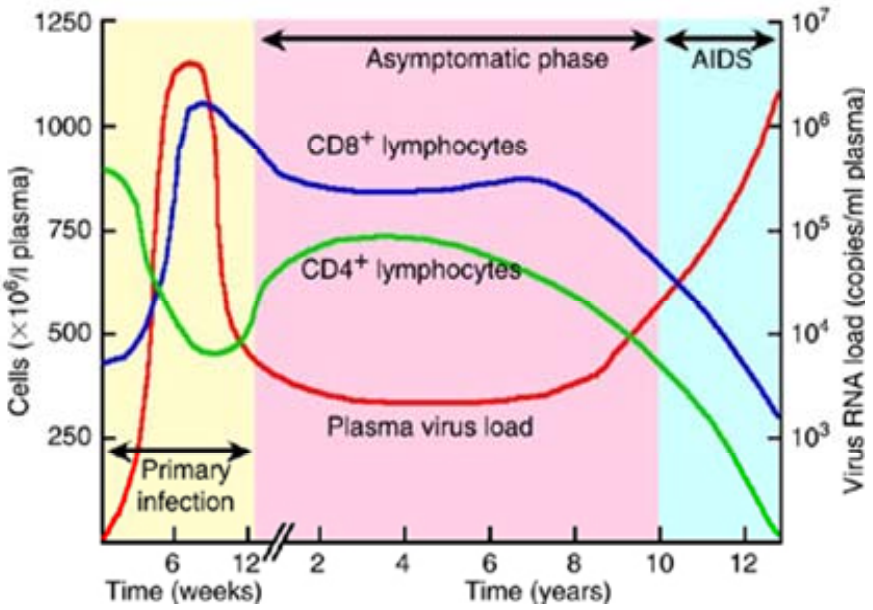


Figure 2: Schematic of typical course of HIV-1 infection showing changes in CD4 and CD8 T cell counts in peripheral blood and plasma viral load. Figure from Munier et al., Immunol Cell Biol volume 85, 6-15, 2007. Reprinted with permission from Nature Publishing group.

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 IFNγ-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 (IFNγ, TNFα, IL-2, CD107a and b, perforin, granzyme A and B) which have been termed ‘functional signatures’

[87,100]. 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 β and γ chains of IL-2 receptor [129,130], as well as, through a specific IL-15 receptor α 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 *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 *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.

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 MHCI and MHCII, haplotypes of highly polymorphic cell surface glycoproteins that bind and present processed antigenic peptides to T cells of [155,156]. MHCI 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 $CD8^+$ T cell responses were found to HLA-B compared to HLA-A. Futhermore, 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, CX_3CR1 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 *Nef* protein and suggested that *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 turn over. 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 interpretating 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 *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 IFNγ and GM-CSF (to immunomodulate), the stimulation of naïve and memory $CD8^+$T HIV-1-specific responses was superior for the latter. This is most likely because IFNα is a type I IFN and has been shown to be essential for effector T cell generation [103]. If this group (IFNα and GM-CSF) was compared with IFNγ 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-1β, IL-6, TNFα 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 acurate availability of prediction data. The prediction motifs for these two haplotypes are shown in Figure 3.

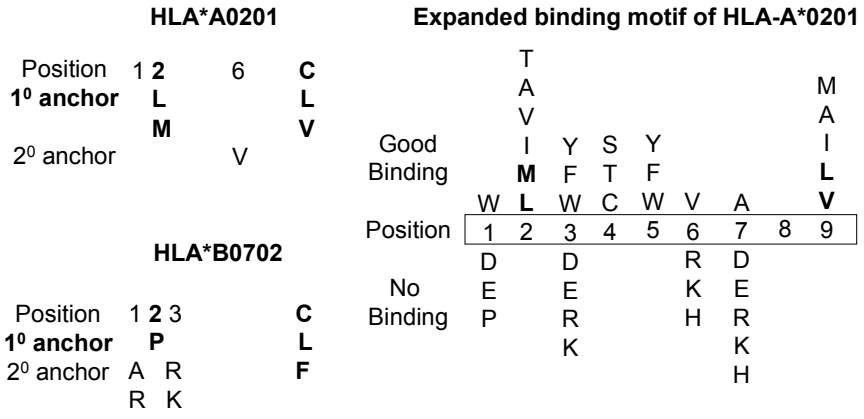


Figure 3: Motif of HLA-A*0201 and HLA-B*0702. Primary anchor positions and preferred residues are listed under the position (**Bolded**) and the residual or secondary anchor residues are not bold faced. Motifs are based on Falk et al. 1991, Barouch et al. 1995, Englehard et al. 1993 Rammensee et al. 1999 and Brusic et al. 2004.

2. PRIMING MODEL

Taking what is known about priming of naïve $CD8^+$ T cells to generate Ag-specific $CD8^+$ T cells I developed an *in vitro* priming model that was originally based upon previous work in our lab [211,242] and others [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) [245] along with the costimulatory molecules (signal 2) [74,246,247] on the surface of mDC activates the T cells to clonally expand and with the help of $CD4^+$ T cells [107-109,248], and IL-12 (signal 3) (Figure 4) [82,249-253].

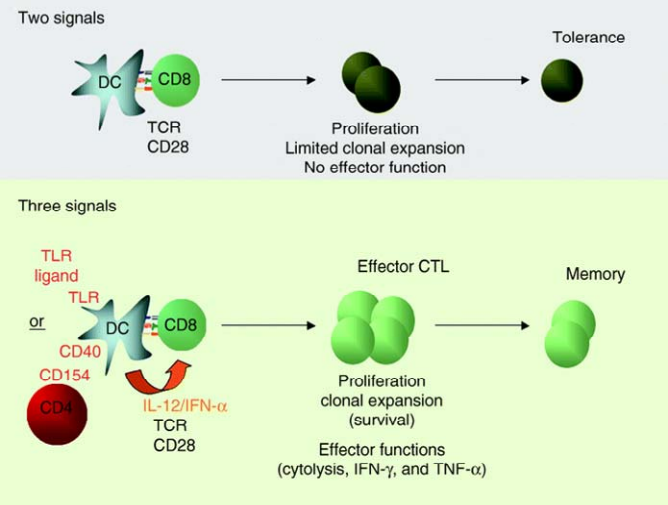


Figure 4: Naïve $CD8^+$ T cell require three signals for full activation and avoidance of tolerance. Stimulation through the T cell receptor (TCR) and CD28 in the absence of a third signal stimulates proliferation, but effector function does not develop and the cells are tolerant long term. The third 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.

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 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 IFNγ 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, IFNγ 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 IFNγ ELISPOT assay.

III. CHAPTER THREE: MHC CLASS I BINDING OF HIV-1 PEPTIDES DERIVED FROM EVOLVING VIRAL SEQUENCES OF GAG, ENV AND NEF

Bonnie Colleton[1], Raj Shankarappa[1], Charles R. Rinaldo[1]

Graduate School of Public Health, Infectious Diseases and Microbiology, University of Pittsburgh[1], Pittsburgh, Pennsylvania, 15261

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 (R^2=0.71-0.86) or between fluorescence polarization and peptide elution-based cellular methods (R^2=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 artifical 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.

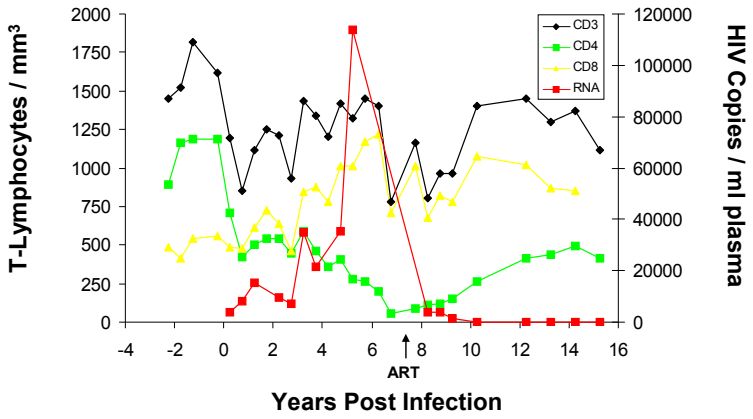


Figure 5: HIV-1 infected participant. T lymphocyte counts and HIV-1 copies over disease progression. Absolute T cell counts over the course of follow-up, CD3 (black diamond), CD4 (green square), CD8 (yellow triangle), and RNA copies (red square) are shown for MACS participant #8.

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).

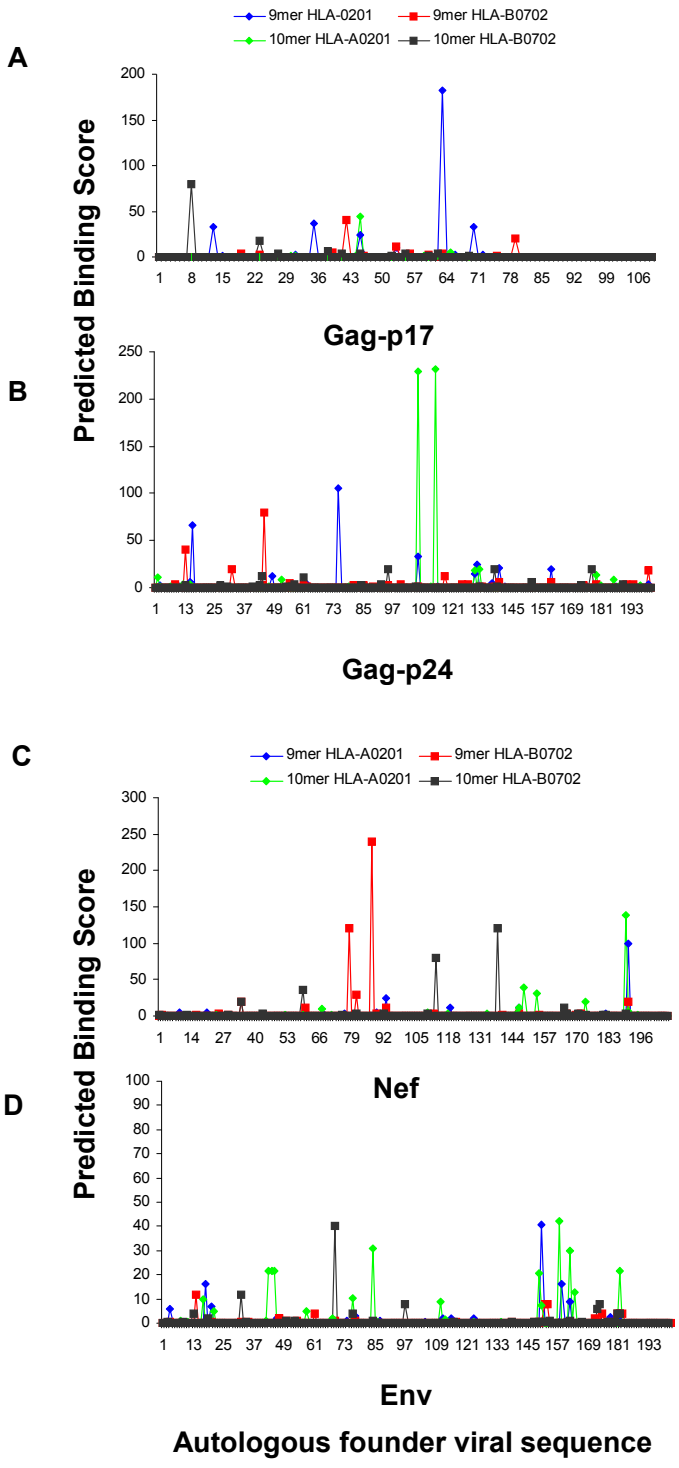


Figure 6. Predicted binding of founder strain of HIV-1.
Predicted binding of MACS participant #8 autologous founder strain spanning Gag, Nef, Env. Predicted binding scores are shown for 9-mer A*0201 (blue), 10-mer (green), 9-mer A*0702 (red) 10-mer (black).

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 IFNγ 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.

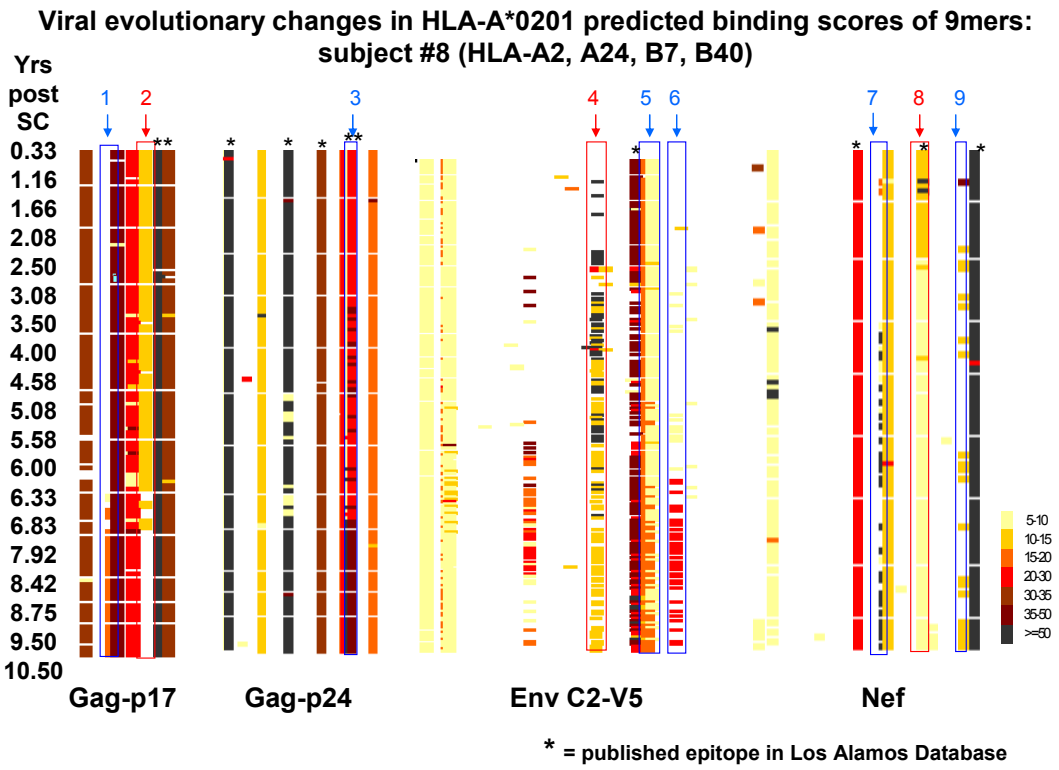


Figure 7: Scanning the impact of viral evolutionary changes on HLA-binding.
Predicted binding to HLA-A*0201 9mers across the genome over years following seroconversion, was mapped for sequences (Gag-p17, Gag-p24, Env, Nef) in subject #8. Each 9-mer amino acid motif in the sequence alignment was colored according to predicted HLA-binding score indicated in the score key. Each protein analyzed revealed a trend of three patterns: (i) increasing (shown in blue: #'s 1, 3, 5, 6, 7, 9), (ii) decreasing (shown in red: #'s 2, 4, 8) (iii) constant. CTL epitopes that are denoted with an asterisk (*) at the top are HLA-A*0201 epitopes published in the Los Alamos database.

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 effect 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.

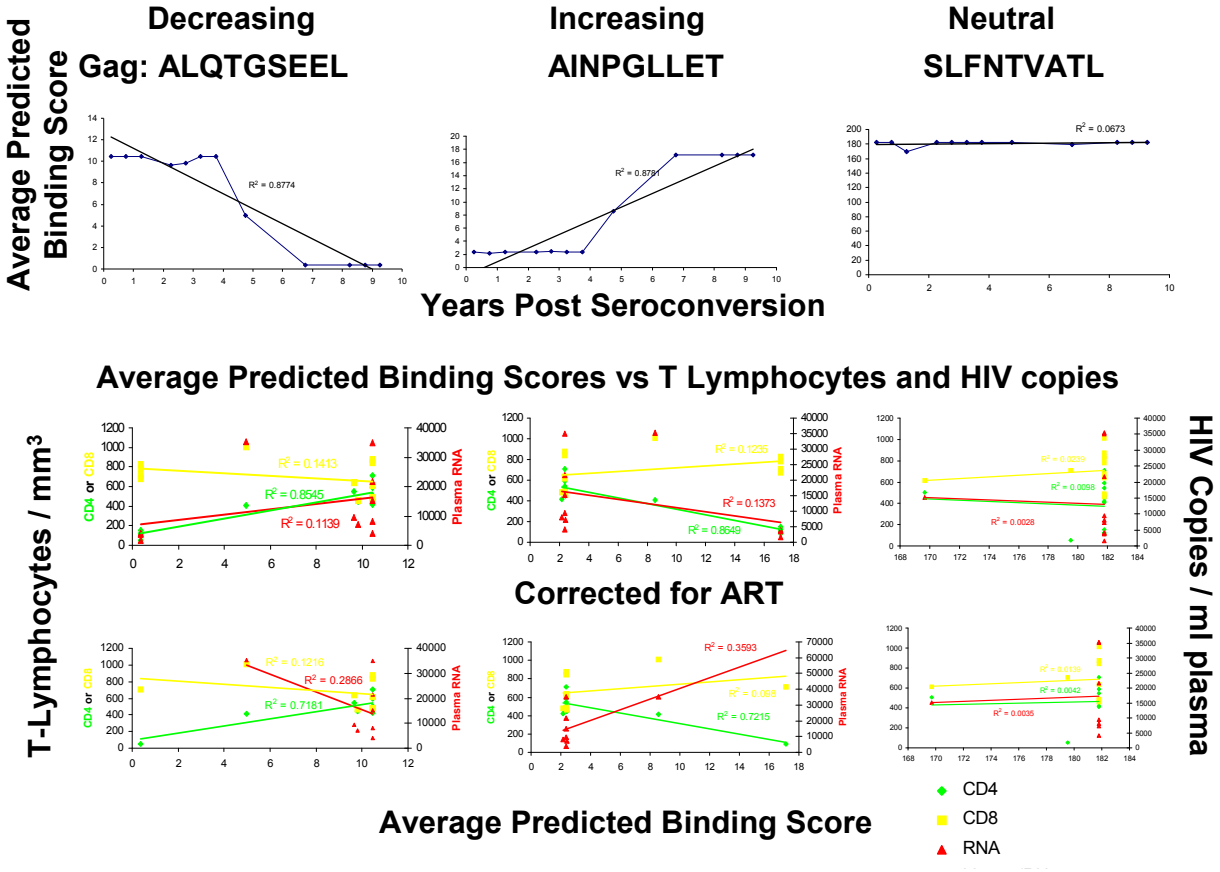


Figure 8 Representative predicted MHC class I binding score trends.
The average predicted MHCI binding scores for each visit post seroconversion of participant #8 are depicted in the top row with correlations of the three examples of binding patterns: decreasing, increasing and neutral. Correlations 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.

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 $SL9_{(p17\ 77\text{-}85LAI)}$ (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 **pre drug**

Decreasing Trend Protein	Haplotype	Sequence	Length	Scatter plot R^2 value	Avg binding R^2 value	pvalue	Avg bind/CD4 R^2 value	pvalue	Avg bind/CD8 R^2 value	pvalue	Avg bind/RNA R^2 value	pvalue	Avg bind/CD4 R^2 value	pvalue	Avg bind/CD8 R^2 value	pvalue	Avg bind/RNA R^2 value	pvalue
Gag	A*0201	ALQTGSEEL	9mer	0.5577	0.8774	4.904E-06	0.8545	4.9E-06	0.1413	2.47E-12	0.1139	0.00125	0.7181	1.12E-06	0.1216	1.8E-08	0.2866	0.0009
Gag	B*0702	GCRQILEQL	9mer	0.0226	0.2688	2.753E-11	0.162	1.45E-05	0.0077	5.69E-12	0.1983	0.00128	0.1461	2.59E-06	0.2997	3.35E-08	0.1818	0.001
Gag	A*0201	NLQGQMVHQA	10mer	0.5622	0.4887	0.4028001	0.2274	4.24E-06	0.377	2.21E-12	0.0632	0.00125	0.1756	9.77E-07	0.391	1.62E-08	0.1754	0.0009
Gag	A*0201	AEWDRLHPV	9mer	0.0154	0.0072	4.241E-14	0.0434	0.000163	0.4815	4.05E-11	0.7801	0.00133	0.0073	1.52E-05	0.6046	1.26E-07	0.8466	0.001
Nef	A*0201	LTFGWCFKL	9mer	0.5013	0.4066	0.0202811	0.1603	1.26E-06	0.6453	2.58E-09	0.1381	0.04793	0.1359	9.9E-08	0.6326	5.43E-08	0.2079	0.048
Nef	A*0201	LTFGWCFKLV	10mer	0.5358	0.4268	0.0118598	0.1797	1.27E-06	0.6415	2.6E-09	0.1383	0.04793	0.1544	1E-07	0.6281	5.48E-08	0.2124	0.048
Nef	B*0702	SPKRQEILDL	10mer	0.0442	0.1628	0.0675555	0.0684	5.1E-05	0.1232	9.29E-08	0.0207	0.04844	0.0146	1.15E-05	0.1012	1.39E-06	0.0494	0.0485
Nef	B*0702	TPGPTRYPL	9mer	0.0411	0.0085	3.705E-09	0.0211	5.25E-05	0.0226	3.59E-08	0.4392	0.04878	0.5837	3.8E-06	0.2048	5.86E-07	0.7037	0.0488
Env	A*0201	NIGPGRAFYA	10mer	0.4108	0.5021	0.9928496	0.2725	7.05E-07	0.4001	7.28E-14	0.07	0.01279	0.2208	1.7E-07	0.4472	2.47E-09	0.1965	0.0147
Env	A*0201	NTLEQVVKKL	10mer	0.006	0.0499	0.0461108	0.0639	1.19E-06	0.0468	1.05E-13	0.0376	0.01283	0.0345	2.62E-07	0.0548	3.21E-09	0.083	0.0148

Increasing Trend Protein	Haplotype	Sequence	Length	Scatter plot R^2 value	Avg binding R^2 value	pvalue	Avg bind/CD4 R^2 value	pvalue	Avg bind/CD8 R^2 value	pvalue	Avg bind/RNA R^2 value	pvalue	Avg bind/CD4 R^2 value	pvalue	Avg bind/CD8 R^2 value	pvalue	Avg bind/RNA R^2 value	pvalue
Gag	A*0201	AINPGLLET	9mer	0.5964	0.8781	0.1394641	0.8649	3.53E-06	0.1235	2.58E-12	0.1373	0.00125	0.7215	4.71E-07	0.098	1.66E-08	0.3593	0.0009
Gag	A*0201	KIVRMYSPT	9mer	0.59	0.897	0.0079637	0.8828	6.54E-06	0.1247	4.46E-12	0.1556	0.00127	0.7631	6.23E-07	0.1015	2.05E-08	0.4077	0.0009
Gag	A*0201	RMYSPTSIL	9mer	0.4473	0.8999	3.597E-08	0.8331	9.1E-06	0.0945	5.36E-12	0.1633	0.00127	0.6544	9.1E-07	0.0367	2.7E-08	0.1167	0.001
Gag	B*0702	SNPDCKTIL	9mer	0.507	0.8418	0.1247798	0.8368	3.35E-06	0.0362	2.47E-12	0.2928	0.00125	0.7082	4.7E-07	0.0002	1.66E-08	0.1008	0.0009
Gag	B*0702	ASRELERFAI	10mer	0.5936	0.8748	0.0034392	0.856	0.000161	0.1414	1.24E-10	0.1153	0.00132	0.7223	5.73E-06	0.1232	6.87E-08	0.3463	0.001
Nef	A*0201	RQEILDWI	9mer	0.1965	0.7325	0.0112086	0.375	1.99E-06	0.5431	3.88E-09	0.0939	0.0386	0.1074	1.45E-07	0.6474	7.25E-08	0.3603	0.0396
Nef	B*0702	GALDSHFL	9mer	0.4821	0.365	3.301E-07	0.1893	2.77E-06	0.5552	4.82E-09	0.1076	0.03868	0.1834	2.11E-07	0.5443	9.36E-08	0.1535	0.0397
Nef	B*0702	LVYSPKRQEI	10mer	0.3736	0.2934	0.0304435	0.3309	1.52E-06	0.3492	3.19E-09	0.1383	0.03855	0.5102	1.24E-07	0.3456	6.56E-08	0.2383	0.0396
Nef	A*0201	KLVPVEPGQV	10mer	0.1138	0.6122	4.84E-05	0.494	3.57E-06	0.041	5.85E-09	0.0463	0.03872	0.4089	2.11E-07	0.1197	9.4E-08	0.4888	0.0397
Env	A*0201	GQIKCLSNI	9mer	0.2232	0.5703	0.4823079	0.5396	1.88E-07	0.0237	3.22E-15	0.0119	0.01332	0.4964	3.46E-08	0.1305	1.25E-10	0.7922	0.016
Env	A*0201	TLEQVVKKL	9mer	0.055	0.2921	0.0831713	0.4228	2.06E-07	0.0704	3.41E-15	0.0139	0.01332	0.6039	3.96E-08	0.1594	1.36E-10	0.2717	0.016
Env	B*0702	CVSNITGLL	9mer	0.3888	0.5836	9.233E-07	0.5188	1.71E-06	0.3211	5.13E-15	0.0494	0.0151	0.26	4.8E-07	0.6172	2.9E-10	0.2103	0.0176
Env	B*0702	LPCRIKQII	9mer	0.0344	0.0902	0.0002767	0.1879	1.14E-06	0.0795	3.92E-15	0.1261	0.01507	0.1206	3.75E-07	0.1063	2.49E-10	0.0609	0.0176
Env	A*0201	QLNESVEINS	10mer	0.0044	0.0057	1.083E-05	0.0022	1.42E-06	0.0505	4.53E-15	0.0442	0.01509	0.018	4.36E-07	0.0819	2.73E-10	0.0847	0.0176
Env	A*0201	IIGDIRQAYC	10mer	0.5467	0.7083	3.511E-06	0.5831	2.05E-06	0.5499	5.81E-15	0.0679	0.01512	0.5354	5.31E-07	0.6966	3.1E-10	0.2688	0.0176
Env	A*0201	AMYAPPISGL	10mer	0.1222	0.0057	1.083E-05	0.002	1.18E-06	0.0513	5.74E-15	0.0227	0.00823	0.0179	3.05E-07	0.0827	3.29E-10	0.0527	0.009
Env	A*0201	NMWQSVGKAM	10mer	0.0211	0.0753	1.194E-07	0.0728	3.5E-06	0.1878	1.19E-14	0.6002	0.00828	0.0007	8.32E-07	0.2116	6.1E-10	0.667	0.009
Env	B*0702	CVSNITGLLL	10mer	0.4548	0.653	2.488E-06	0.415	1.41E-06	0.4592	6.43E-15	0.1006	0.00824	0.3403	3.29E-07	0.5799	3.45E-10	0.3269	0.009
Env	B*0702	AMYAPPIRGQ	10mer	0.2252	0.4977	0.1060592	0.456	1.29E-07	0.0115	3.56E-15	0.0929	0.00732	0.3336	2.06E-08	0.0808	1.38E-10	8.00E-18	0.0084

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

													pre-drug					
Decreasing Trend				Scatter plot	Avg binding		Avg bind/CD4		Avg bind/CD8		Avg bind/RNA		Avg bind/CD4		Avg bind/CD8		Avg bind/RNA	
Protein	Haplotype	Sequence	Length	R^2 value	R^2 value	pvalue	R^2 value	pvalue	R^2 value	pvalue	R^2 value	pvalue	R^2 value	pvalue	R^2 value	pvalue	R^2 value	pvalue
Gag	A*0201	ALQTGSEEL	9mer	0.5577	0.8774	4.9E-06	0.8545	4.90436E-06	0.1413	2.47E-12	0.1139	0.001253	0.7181	1.12215E-06	0.1216	1.79995E-08	0.2866	0.00094142
Gag	A*0201	NLQGQMVHQA	10mer	0.5622	0.4887	0.4028	0.2274	4.23754E-06	0.377	2.20816E-12	0.0632	0.00125	0.1756	9.76654E-07	0.391	1.62411E-08	0.1754	0.00093909
Nef	A*0201	LTFGWCFKL	9mer	0.5013	0.4066	0.020281	0.1603	1.25573E-06	0.6453	2.57558E-09	0.1381	0.047928	0.1359	9.8985E-08	0.6326	5.43425E-08	0.2079	0.04801021
Nef	A*0201	LTFGWCFKLV	10mer	0.5358	0.4268	0.01186	0.1797	1.27342E-06	0.6415	2.60073E-09	0.1383	0.047931	0.1544	1.00291E-07	0.6281	5.4826E-08	0.2124	0.04801343
Env	A*0201	NIGPGRAFYA	10mer	0.4108	0.5021	0.99285	0.2725	7.05155E-07	0.4001	7.28279E-14	0.07	0.012792	0.2208	1.70253E-07	0.4472	2.46661E-09	0.1965	0.01473344
Gag	A*0201	AEWDRLHPV	9mer	0.0154	0.0072	4.24E-14	0.0434	0.000162888	0.4815	4.05428E-11	0.7801	0.00133	0.0073	1.52057E-05	0.6046	1.25888E-07	0.8466	0.00098154
Nef	B*0702	TPGPTRYPL	9mer	0.0411	0.0085	3.7E-09	0.0211	5.25086E-05	0.0226	3.59148E-08	0.4392	0.048784	0.5837	3.79539E-06	0.2048	5.85535E-07	0.7037	0.04880687
Increasing Trend				Scatter plot	Avg binding		Avg bind/CD4		Avg bind/CD8		Avg bind/RNA		Avg bind/CD4		Avg bind/CD8		Avg bind/RNA	
Protein	Haplotype	Sequence	Length	R^2 value	R^2 value	pvalue	R^2 value	pvalue	R^2 value	pvalue	R^2 value	pvalue	R^2 value	pvalue	R^2 value	pvalue	R^2 value	pvalue
Gag	A*0201	AINPGLLET	9mer	0.5964	0.8781	0.139464	0.8649	3.52905E-06	0.1235	2.58189E-12	0.1373	0.001254	0.7215	4.70672E-07	0.098	1.66346E-08	0.3593	0.00093963
Gag	A*0201	KIVRMYSPT	9mer	0.59	0.897	0.007964	0.8828	6.54312E-06	0.1247	4.4576E-12	0.1556	0.001266	0.7631	6.22675E-07	0.1015	2.0498E-08	0.4077	0.00094345
Gag	A*0201	RMYSPTSIL	9mer	0.4473	0.8999	3.6E-08	0.8331	9.0993E-06	0.0945	5.35599E-12	0.1633	0.001275	0.6544	9.10258E-07	0.0367	2.69682E-08	0.1167	0.00095007
Gag	B*0702	SNPDCKTIL	9mer	0.507	0.8418	0.12478	0.8368	3.35262E-06	0.0362	2.46553E-12	0.2928	0.001253	0.7082	4.70118E-07	0.0002	1.66135E-08	0.1008	0.00093968
Gag	B*0702	ASRELERFAI	10mer	0.5936	0.8748	0.003439	0.856	0.000161346	0.1414	1.24307E-10	0.1153	0.001315	0.7223	5.73354E-06	0.1232	6.87024E-08	0.3463	0.00095833
Nef	A*0201	RQEILDWI	9mer	0.1965	0.7325	0.011209	0.375	1.98584E-06	0.5431	3.88019E-09	0.0939	0.038598	0.1074	1.4501E-07	0.6474	7.2478E-08	0.3603	0.03963846
Nef	A*0201	KLVPVEPGQV	10mer	0.1138	0.6122	4.84E-05	0.494	3.57431E-06	0.041	5.84713E-09	0.0463	0.038723	0.4089	2.10628E-07	0.1197	9.40365E-08	0.4888	0.03973451
Env	A*0201	GQIKCLSNI	9mer	0.2232	0.5703	0.482308	0.5396	1.87766E-07	0.0237	3.21804E-15	0.0119	0.013316	0.4964	3.46476E-08	0.1305	1.25259E-10	0.7922	0.0160369
Env	B*0702	CVSNITGLL	9mer	0.3888	0.5836	9.23E-07	0.5188	1.71459E-06	0.3211	5.13083E-15	0.0494	0.015103	0.26	4.8047E-07	0.6172	2.9047E-10	0.2103	0.017638
Env	A*0201	IIGDIRQAYC	10mer	0.5467	0.7083	3.51E-06	0.5831	2.05303E-06	0.5499	5.80673E-15	0.0679	0.015117	0.5354	5.31463E-07	0.6966	3.09714E-10	0.2688	0.01764657
Env	B*0702	CVSNITGLLL	10mer	0.4548	0.653	2.49E-06	0.415	1.40665E-06	0.4592	6.43101E-15	0.1006	0.008242	0.3403	3.29295E-07	0.5799	3.45127E-10	0.3269	0.00896586
Env	B*0702	AMYAPPIRGQ	10mer	0.2252	0.4977	0.106059	0.456	1.28875E-07	0.0115	3.55578E-15	0.0929	0.007317	0.3336	2.06111E-08	0.0808	1.37841E-10	8.00E-18	0.00835818
Nef	B*0702	LVYSPKRQEI	10mer	0.3736	0.2934	0.030444	0.3309	1.51968E-06	0.3492	3.18953E-09	0.1383	0.038545	0.5102	1.24073E-07	0.3456	6.55583E-08	0.2383	0.03960952
Env	A*0201	TLEQVVKKL	9mer	0.055	0.2921	0.083171	0.4228	2.05771E-07	0.0704	3.41002E-15	0.0139	0.013322	0.6039	3.96138E-08	0.1594	1.36029E-10	0.2717	0.01604834
Env	A*0201	NMWQSVGKAM	10mer	0.0211	0.0753	1.19E-07	0.0728	3.50343E-06	0.1878	1.18955E-14	0.6002	0.008284	0.0007	8.31904E-07	0.2116	6.09592E-10	0.667	0.00901205

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.

	Peptide sequence	Peptide origin	Position	Sequence ID	Peptide length	Mol wt.	Inhibition %	SE	Artificial log (IC50)	Affinity Category
Gag										
1	SLFNTVAT**L**	p17	77-85	ABI97965	9	938	92.6	0.6	3.81	Medium
2	SL**F**NTVAA**L**	p17	77-85	ABC94954	9	965	91.9	0.8	3.84	Medium
3	SLFNTVAT**P**	p17	77-85	AAK31062	9	935	31.4	1.1	5.24	Low
4	SLFSTVAT**L**	p17	67-75	CAB00235	9	981	94.5	0.8	3.66	High
5	SLFNTIAT**L**	p17	77-85	AAQ73334	9	979	90.6	0.9	3.92	Medium
6	SLYNTVAT**L**	p17	77-85	AAQ84410	9	949	91.6	0.8	3.86	Medium
7	P**L**NAWVKV**V**	p24	16-24	ABI80476	9	999	55.2	1.5	4.81	Low
8	A**L**NAWVKV**V**	p24	19-27	AAQ10844	9	1029	90.2	0.8	3.93	Medium
9	T**L**NAWVKV**V**	p24	13-21	CAF74725	9	1002	84.7	0.7	4.16	Medium
10	T**L**SAWVKV**V**	p24	19-27	BAA93825	9	1030	84.1	0.8	4.18	Medium
11	T**L**DAWVKV**V**	p24	151-159	AAK66106	9	1025	79.8	1	4.3	Medium
gp120										
12	IGPGRAFYAT	Env	40-49	AAW67700	10	1022	89.4	0.9	3.96	Medium
13	IGSGRAFYAT	Env	83-92	AAY67139	10	1052	8.7	1	5.91	Very Low
14	IGPGRAFYAA	Env	83-92	AAY67369	10	1009	15.5	0.9	5.64	Very Low
15	IGPGRASYAT	Env	40-49	CAA87577	10	1124	72.4	0.8	4.48	Medium
16	IGPGIAGYAT	Env	43-52	AAF16166	10	992	0	0.6	6.52	No binder
17	IGPERAFYAT	Env	43-53	AAF16391	10	1042	8.6	0.7	5.92	Very Low
Nef										
18	P**L**TFGWCFK**L**	Nef	142-151	AAW83599	10	1212	0	1.5	6.35	No Binder
19	P**L**TLGWCFK**L**	Nef	141-150	ABC94978	10	1214	5.5	1	6.1	No Binder
20	P**I**TFGWCFK**L**	Nef	-	-	10	1232	1.2	1.6	6.35	No Binder
21	P**V**TFGWCFK**L**	Nef	136-145	CAA13504	10	1200	0	1.5	6.48	No Binder
22	P**L**CFGWCFK**L**	Nef	136-146	ABF47497	10	1212	52.4	1.2	4.86	Low
23	P**V**CFGWCFK**L**	Nef	136-147	ABI15291	10	1177	23.7	1.8	5.41	Low
24	P**L**CFGWCFK**P**	Nef	126-135	AAD17151	10	1200	26.5	0.4	5.34	Low
25	P**M**CFGWCFK**L**	Nef	134-142	ABC94970	10	1198	28.3	1.7	5.3	Low
26	V**L**VWRFDSS**L**	Nef	222-231	ABG91352	10	1221	75.9	1.4	4.4	Medium
27	V**L**VWKFDSS**L**	Nef	181-190	ABG90487	10	1193	77.4	1.2	4.37	Medium
28	VLVWKSDSS**L**	Nef	185-194	AAW80944	10	1235	58	1.7	4.76	Low
29	V**L**VWKFDSN**L**	Nef	183-192	AAG44220	10	1263	79.4	0.8	4.31	Medium
30	V**L**VWKFDSK**L**	Nef	-	-	10	1220	54.6	5	4.82	Medium
31	V**L**VWKFDSH**L**	Nef	-	-	10	1133	82.9	1	4.22	Medium
32	V**L**VWKFDSR**L**	Nef	181-190	ABI81102	10	1243	74.3	1.4	4.44	Medium
33	AFHHVAREK	Nef	194-202	BAF31431	9	1094	0.0	1.4	6.60	No binder
34	AFHHVAKEK	Nef	191-199	ABD78408	9	1066	0.0	1.5	6.65	No binder
35	AFHHVAREM	Nef	197-205	AAL65487	9	1097	0.0	1.1	6.62	No binder
36	AFRHVAREI	Nef	189-197	AAL65487	9	1098	0.0	0.7	6.62	No binder
37	AFHHMAKEK	Nef	189-197	CAD23397	9	1098	0.0	1.2	6.64	No binder
38	AFHHVAREI	Nef	190-198	AAD31234	9	1079	0.0	1.5	6.53	No binder
39	ASHHVAREK	Nef	188-196	ABD78411	9	1034	0.0	1.1	6.54	No binder
40	ALHHVAREK	Nef	190-198	AAL65568	9	1060	0.0	0.7	6.66	No binder
41	AFHHMAREM	Nef	188-196	AAP74168	9	1129	0.0	1.1	6.62	No binder
42	AFHHLAREK	Nef	193-201	AAQ73887	9	1108	0.0	1.8	6.64	No binder
43	AFHHAAREK	Nef	188-196	AAZ41643	9	1066	0.0	1.7	6.56	No binder
44	AFHHVAKGK	Nef	-	-	9	994	0.0	1.6	6.51	No binder

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.

	Peptide sequence	Peptide origin	Position	Sequence ID	Peptide length	Mol wt.	Inhibition %	SE	Artificial log (IC50)	Affinity Category
Gag										
45	S**P**RALNAWV	**p24**	**145-153**	**AAZ91801.1**	**9**	**1013**	**87.2**	**1.2**	**4.07**	**Medium**
46	S**P**RTLNAWV	**p24**	**146-154**	**AA047111.2**	**9**	**1043**	**83.9**	**1.4**	**4.18**	**Medium**
47	S**P**RPLNAWV	**p24**	**10-18**	**CAF74725**	**9**	**1040**	**83.9**	**1.7**	**4.18**	**Medium**
48	**P**PRTLNAWV	**p24**	**146-154**	**AAN73737.1**	**9**	**1053**	**82.8**	**1.5**	**4.22**	**Medium**
49	S**P**RTLDAWV	**p24**	**148-156**	**AAK66106.1**	**9**	**1044**	**76.9**	**1.8**	**4.38**	**Medium**
50	T**P**QGLNTML	**p24**	**180-188**	**AAK66135.1**	**9**	**974**	**94**	**0.9**	**3.71**	**Medium**
51	T**P**QDLNTML	**p24**	**181-189**	**ABK63986.1**	**9**	**1032**	**91.1**	**1**	**3.89**	**Medium**
52	T**P**QDLNTVL	**p24**	**180-188**	**AAK66109.1**	**9**	**1000**	**82.9**	**1.6**	**4.22**	**Medium**
53	T**P**QDLSTVL	**p24**	**180-188**	**AAV53316.1**	**9**	**1005**	**80.5**	**1.9**	**4.28**	**Medium**
gp120										
54	R**P**NNNTRKSI	**Env**	**17-26**	**ABL61570.1**	**10**	**1199**	**95.6**	**1.0**	**3.56**	**High**
55	R**P**NNNTRKS**L**	**Env**	**17-26**	**AAR90645.1**	**10**	**1199**	**94.2**	**1.0**	**3.69**	**High**
56	R**P**TNNTRKSI	**Env**	**3-12**	**AAA44380.1**	**10**	**1186**	**93.3**	**0.8**	**3.75**	**Medium**
57	R**P**NNDTRKSI	**Env**	**3-12**	**AAN63831.1**	**10**	**1200**	**91.8**	**0.7**	**3.85**	**Medium**
58	R**P**NNSTRKSI	**Env**	**3-12**	**ABF49652.1**	**10**	**1172**	**91.6**	**1.3**	**3.87**	**Medium**
59	R**P**NNNTRRSI	**Env**	**3-12**	**ABJ09741.1**	**10**	**1227**	**89.1**	**1.1**	**3.99**	**Medium**
60	R**P**NNNTRKST	**Env**	**3-12**	**AAC04009.1**	**10**	**1187**	**87.8**	**1.3**	**4.04**	**Medium**
61	R**P**SNNTRKRI	**Env**	**15-24**	**CAA10604.1**	**10**	**1241**	**84.3**	**1.5**	**4.17**	**Medium**
62	R**P**NNNTRKRI	**Env**	**3-12**	**ABK33582.1**	**10**	**1268**	**79.3**	**1.8**	**4.32**	**Medium**
63	RSNNNTRKSI	**Env**	**24-33**	**AAC97418.1**	**10**	**1189**	**78.6**	**1.5**	**4.33**	**Medium**
64	R**P**NNNTGKRI	**Env**	**3-12**	**AAC04032.1**	**10**	**1169**	**77.4**	**1.8**	**4.36**	**Medium**
65	R**P**NNNTRKCI	**Env**	**14-23**	**AAY17456.1**	**10**	**1215**	**65.2**	**2.0**	**4.63**	**Medium**
Nef										
66	F**P**IRPQVP**L**	**Nef**	**68-76**	**ABF30477.1**	**9**	**1066**	**95.0**	**1.0**	**3.62**	**High**
67	F**P**ARPQVP**L**	**Nef**	**68-76**	**AAL65373.1**	**9**	**1024**	**94.6**	**0.8**	**3.66**	**High**
68	S**P**VRPQVP**L**	**Nef**	**68-76**	**AAL65364.1**	**9**	**1052**	**94.4**	**0.7**	**3.67**	**High**
69	FSVRPQVP**L**	**Nef**	**68-76**	**AAA44965.1**	**9**	**992**	**53**	**2.7**	**4.85**	**Low**
70	F**P**VRPQVP**L**	**Nef**	**-**	**-**	**9**	**1042**	**92.1**	**1.1**	**3.83**	**Medium**
71	R**P**MTCKGA**L**	**Nef**	**-**	**-**	**9**	**976**	**93.5**	**0.8**	**3.74**	**Medium**
72	R**P**MTRKAA**L**	**Nef**	**-**	**-**	**9**	**1043**	**92.4**	**1.1**	**3.82**	**Medium**
73	R**P**MTWKGA**L**	**Nef**	**75-83**	**ABF30198.1**	**9**	**1059**	**91.9**	**0.9**	**3.85**	**Medium**
74	R**P**MTWKAA**L**	**Nef**	**80-88**	**AAG44218.1**	**9**	**1073**	**91.7**	**1.2**	**3.86**	**Medium**
75	R**P**ITYKAA**L**	**Nef**	**77-85**	**AAD31205.1**	**9**	**1032**	**91.4**	**1.2**	**3.87**	**Medium**
76	R**P**MTYKGA**L**	**Nef**	**77-85**	**ABF30661.2**	**9**	**1036**	**91.1**	**1.2**	**3.89**	**Medium**
77	R**P**MTYKAA**L**	**Nef**	**80-88**	**ABF30746.1**	**9**	**1050**	**88.0**	**1.8**	**4.04**	**Medium**
78	T**P**GPGIRFP**L**	**Nef**	**128-137**	**ABF30746.1**	**10**	**1054**	**96.9**	**0.7**	**3.4**	**High**
79	T**P**GPGTRFP**L**	**Nef**	**133-142**	**ABK6398.1**	**10**	**1042**	**96.5**	**0.7**	**3.46**	**High**
80	T**P**GPGIRYPM	**Nef**	**-**	**-**	**10**	**1088**	**96.2**	**0.8**	**3.5**	**High**
81	T**P**GPGTRYP**L**	**Nef**	**133-142**	**AAO47198.2**	**10**	**1056**	**96.1**	**0.6**	**3.51**	**High**
82	T**P**GPGPRYPM	**Nef**	**-**	**-**	**10**	**1072**	**95.5**	**1.0**	**3.57**	**High**
83	T**P**GPGTRYP**L**	**Nef**	**133-142**	**AAO47198.2**	**10**	**1058**	**95.5**	**0.7**	**3.57**	**High**
84	T**P**GPGVRYP**L**	**Nef**	**133-142**	**ABF30533.1**	**10**	**1056**	**94.8**	**0.9**	**3.64**	**High**
85	T**P**GPGPRFP**L**	**Nef**	**133-142**	**ABF30123.1**	**10**	**1038**	**94.6**	**1.0**	**3.66**	**High**
86	T**P**GPGIRYPV	**Nef**	**128-137**	**AAN03318.1**	**10**	**1056**	**93.5**	**1.0**	**3.74**	**Medium**
87	T**P**GPGPRYP**L**	**Nef**	**133-142**	**ABF30162.1**	**10**	**1054**	**93.1**	**0.8**	**3.77**	**Medium**
88	T**P**GPGIRFPI	**Nef**	**-**	**-**	**10**	**1054**	**93**	**0.9**	**3.77**	**Medium**
89	T**P**GPGIRYP**L**	**Nef**	**133-142**	**ABF30613.1**	**10**	**1070**	**93**	**1.0**	**3.78**	**Medium**
90	T**P**GPGPRYPV	**Nef**	**-**	**-**	**10**	**1040**	**92.3**	**1.0**	**3.82**	**Medium**
91	TSGPGTRFP**L**	**Nef**	**128-137**	**ABD34935.1**	**10**	**1032**	**52.9**	**1.6**	**4.85**	**Low**
92	TKGPGIRFP**L**	**Nef**	**-**	**-**	**10**	**1085**	**23.7**	**1.9**	**5.41**	**Low**

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 IC_{50} 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.

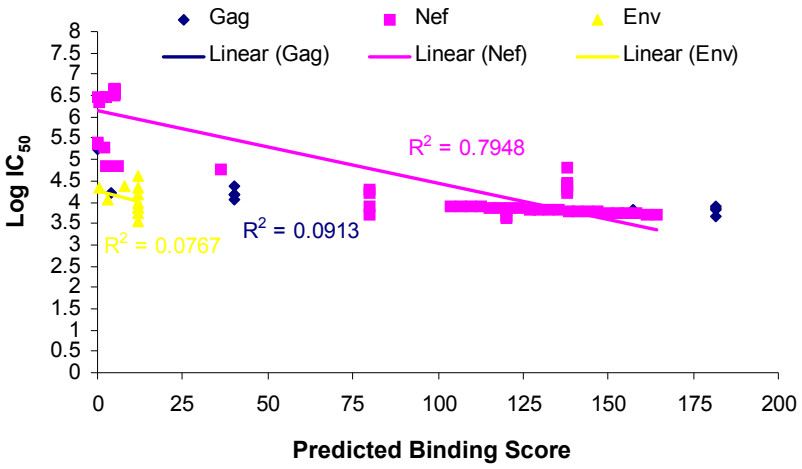


Figure 9: Binding affinity by protein (Gag, Nef, and Env). The logIC_{50} binding affinity was plotted against the predicted binding score for each epitope and subdivided by HIV-1 protein (Gag, Nef, and Env). Pearson's product was calculated for each protein. Nef values demonstrate association (R^2=0.7948).

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).

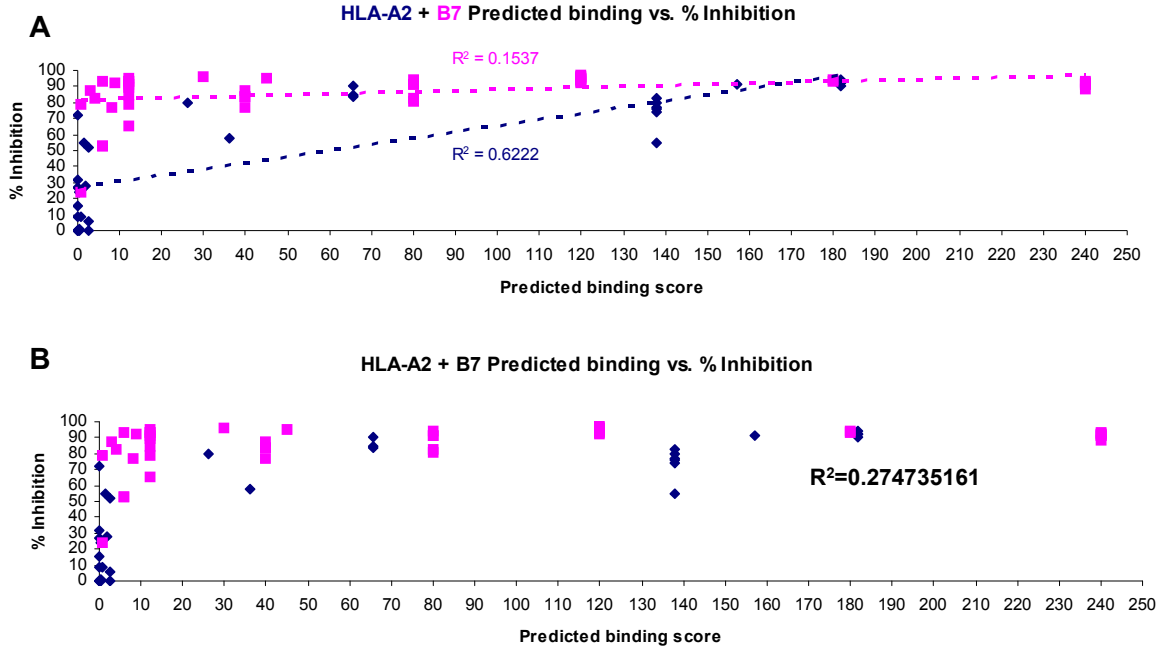


Figure 10: Binding inhibition by prediction scores of HLA-A*0201 and HLA-B*0702. (A) MHC class I binding values for each epitope are grouped by haplotype with an association for the HLA-A*0201 epitopes (R^2=0.6222), HLA-B*0702 epitopes (R^2=0.1537). (B) Combining binding inhibition values for each epitope 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 afftinities 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 (R^2>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

Bonnie A Colleton[1], Xiao-Li Huang[1] and Charles R Rinaldo[1]

Graduate School of Public Health, Infectious Disease and Microbiology, University of Pittsburgh, Pittsburgh[1], Pennsylvania 15261

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) *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 *in vitro* priming model. Generating and expanding primary immune responses from naïve precursors *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 IFNγ 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 IFNγ 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 *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 5^{th} 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 100̃0U/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 $CMV_{pp65\ (495\text{-}503)}$ and HIV-1 peptides (SL9) SLYNTVATL $_{(p17\ 77\text{-}85LAI)}$, $TLNAWVKVV_{(p24\ 151\text{-}159)}$, $KLTSCNTSV_{(gp120\ 192\text{-}199)}$, $RGPGRAFVTI_{(gp120\ 311\text{-}320)}$, $SLLNATIAV_{(gp41\ 818\text{-}827)}$ 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 37^0C, 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; Peptrotech) 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).

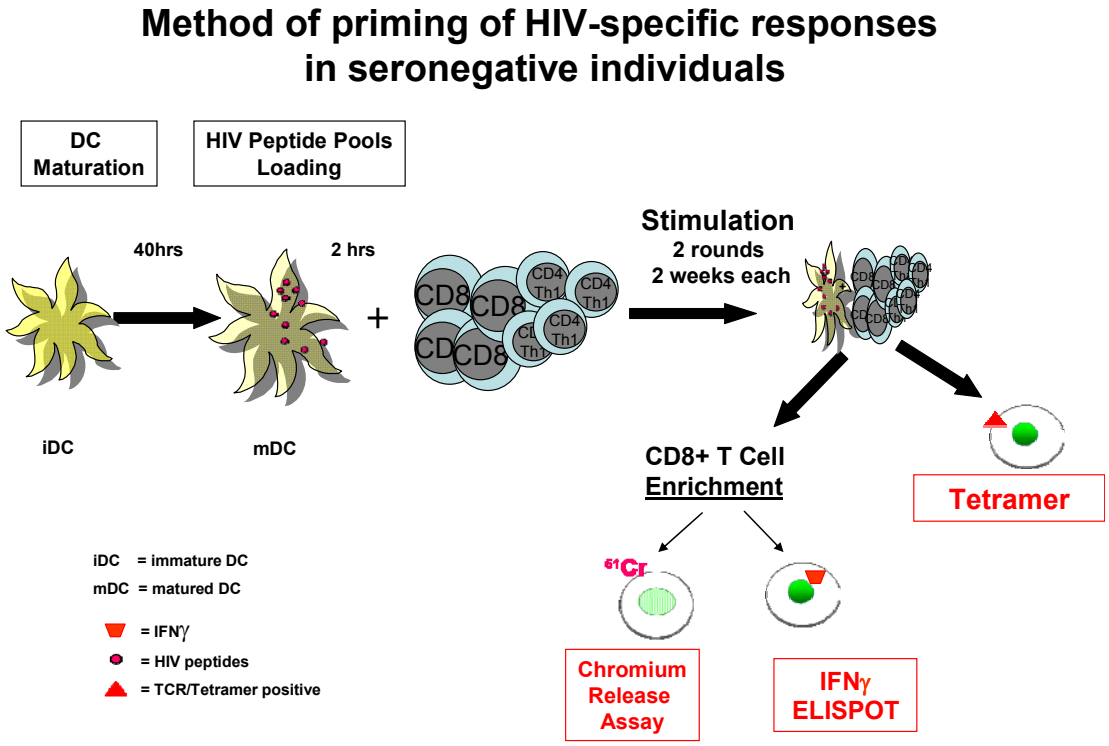


Figure 13 *In vitro* priming model
Schematic representing DC generation and two rounds of stimulation to naïve T cells and assays used as readouts of T cell functions (ELISPOT, chromium release or tetramer staining).

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 ($SL9_{(p17\ 77\text{-}85LAI)}$) and memory ($CMV_{pp65\ (495\text{-}503)}$) 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 $Tet^+SL9_{(p17\ 77\text{-}85LAI)}$, $Tet^+CMV_{pp65\ (495\text{-}503)}$, or Tet^{neg} 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 (0.5μg/ml Amgen), (ii) CD40L + IFNγ (CD40L (0.5μg/ml Amgen) (1000U/ml; R&D Systems), (iii) cocktail (IFNγ (1000U/ml; R&D Systems), IL-1β (25ng/ml; R&D Systems), IFNα 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 $\pm$ 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 37^0C 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 X (experimental counts per minute – spontaneous counts per minute) / (maximum counts per minute – 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 4^0C with 5μg/ml anti-interferon γ (IFNγ) 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 37^0C. Responder cells were stimulated overnight with peptides (10μg/ml)-loaded DC at a ratio of 10:1 in 37^0C, 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 + IFNγ, 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).

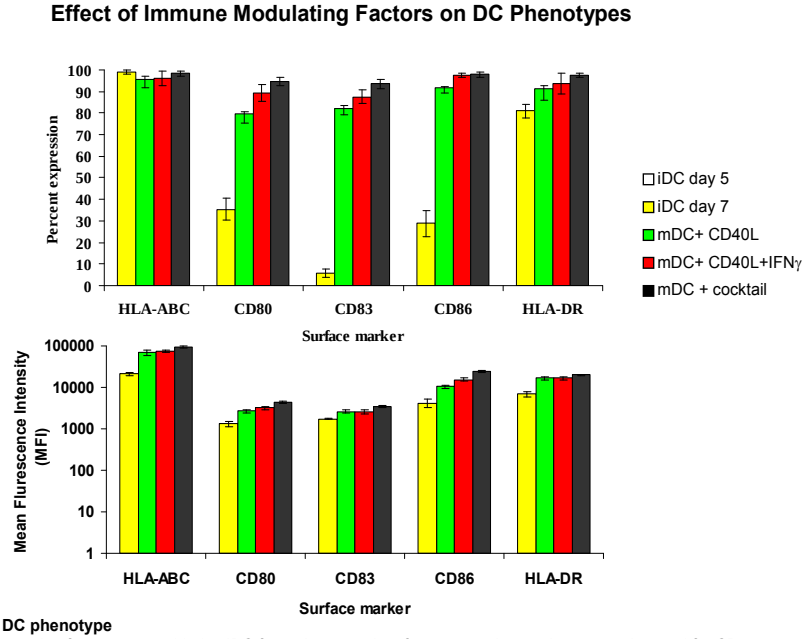


Figure 11: DC phenotype
Different maturation factors were added to iDC for 40 hours and surface expression markers were detected for CD80, CD83, CD86, MHC class I ABC, and MHC class II DR was collected and the average of five donors ± SE is depicted as either (A) percent positive or (B) mean fluorescent 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).

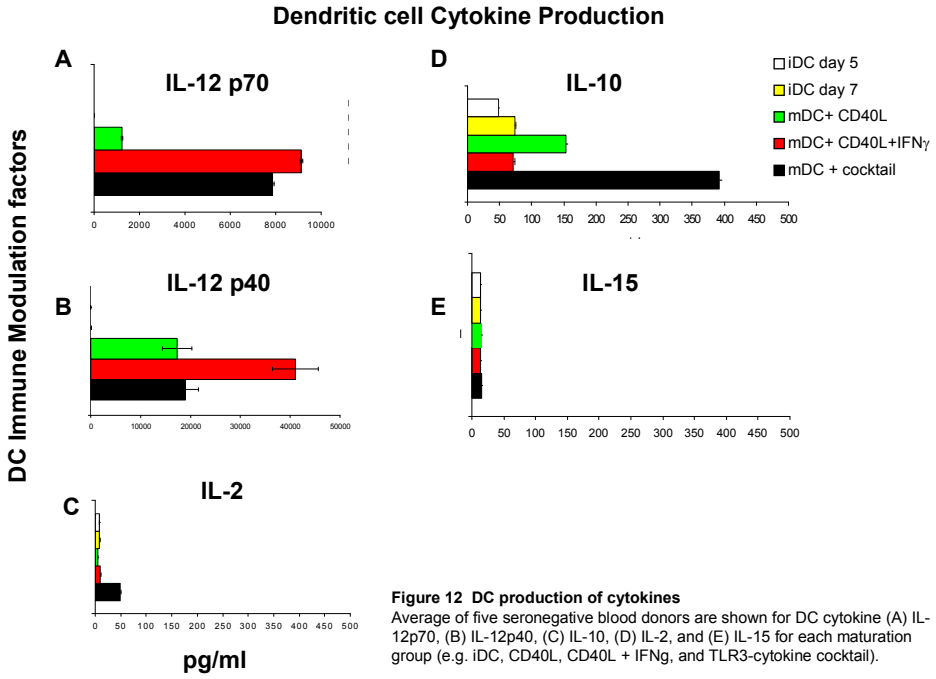


Figure 12 DC production of cytokines
Average of five seronegative blood donors are shown for DC cytokine (A) IL-12p70, (B) IL-12p40, (C) IL-10, (D) IL-2, and (E) IL-15 for each maturation group (e.g. iDC, CD40L, CD40L + IFNg, and TLR3-cytokine cocktail).

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 HIV^{neg} CMV^+ subject were loaded with either SL9 $_{(p17\ 77\text{-}85LAI)}$ or $CMV_{pp65\ (495\text{-}503)}$ 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 $_{(p17\ 77\text{-}85LAI)}$ or $CMV_{pp65\ (495\text{-}503)}$. 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.

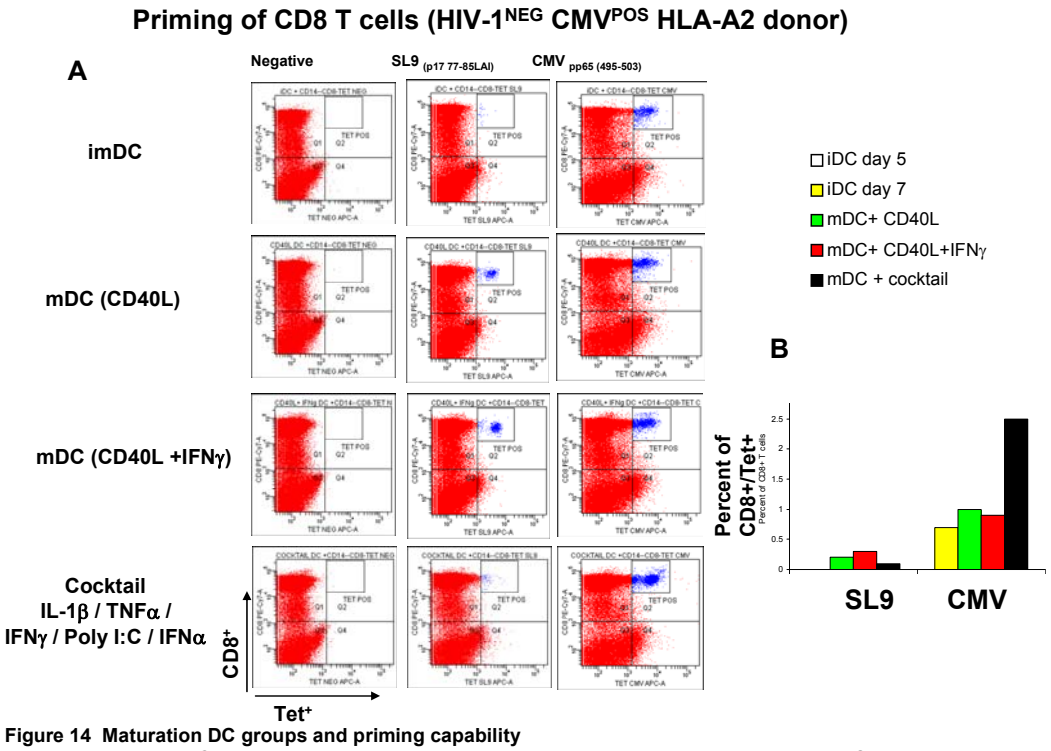


Figure 14 Maturation DC groups and priming capability
A HLA-A*0201 HIV^{neg} CMV+ donor was used to prime the well documented HLA-A*0201 epitopes SL9 $_{(p17\ 77\text{-}85LAI)}$ and CMV$_{pp65\ (495\text{-}503)}$. Differences in priming and memory expansion can be seen (A) and graphically represented in (B).

The results demonstrate that iDC matured with CD40L + IFNγ were the most efficient at priming SL9 $_{(p17\ 77\text{-}85LAI)}$-specific CD8^{+}T cells, while iDC treated with the maturation cocktail were superior for CMV$_{pp65\ (495\text{-}503)}$-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 $_{(p17\ 77\text{-}85LAI)}$.

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 (^{51}Cr 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 IFNγ 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 TLNAWVKVV$_{(p24\ 151\text{-}159)}$ and SLLNATDIAV $_{(gp41\ 818\text{-}827)}$.

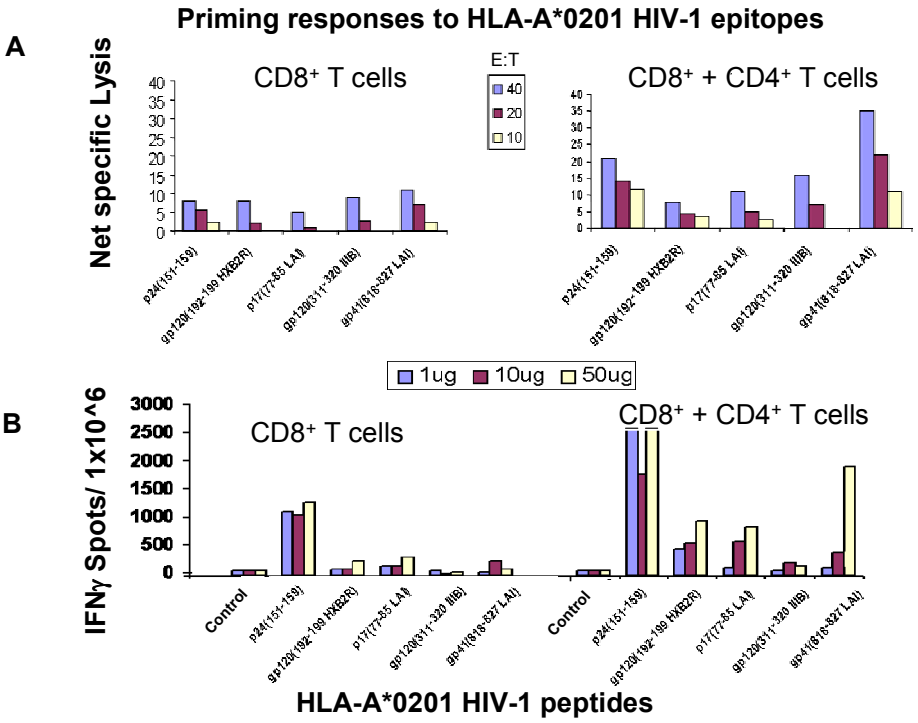


Figure 15 CTL activity and IFNg ELISPOT
Comparison of HIV-1 peptide-loaded autologous DC co-cultured with $CD8^+$T cells alone or with $CD4^+$T cells for CTL (A) or IFNγ ELISPOT (B).

Specificity of primary $CD8^+$ T cell responses for HIV-1. I next explored the breadth of the immunogenicity of DC matured with CD40L + IFNγ, 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).

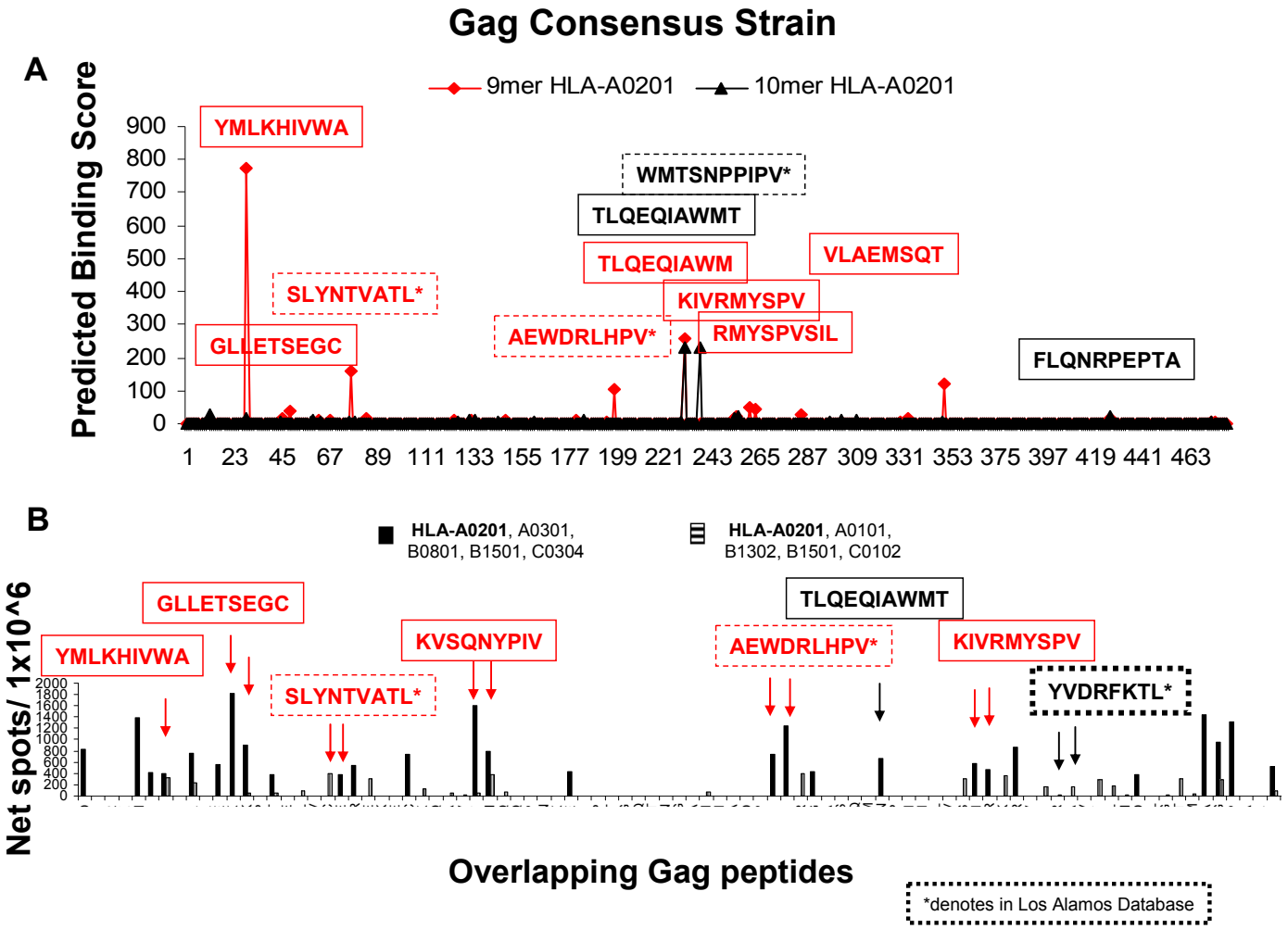


Figure 16 Priming to Consensus Gag

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 + 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).

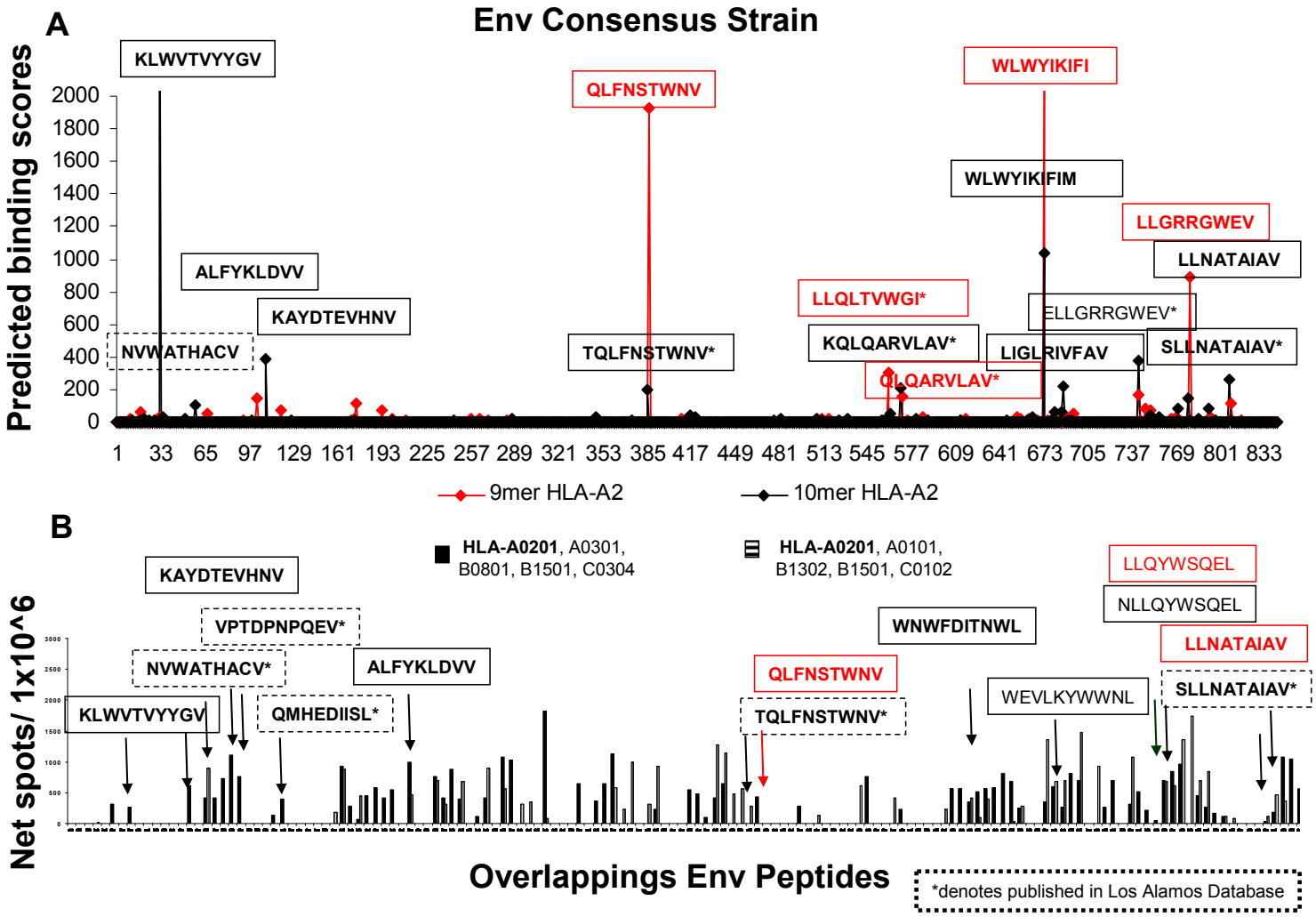


Figure 17 Priming to Consensus Env
Consensus Env region of HIV-1 was evaluated for potential CTL epitopes using the algorithm provided by BIMAS (A). Immunogenicity was evaluated by antigen-loading DC matured with CD40L + IFNγ for 2 rounds of stimulation, and co-cultured with PBMC for 4 weeks and assayed for IFNγ production in an ELISPOT (B). Results are reported as 2 standard deviations over the negative control (primed with DC and no peptide).

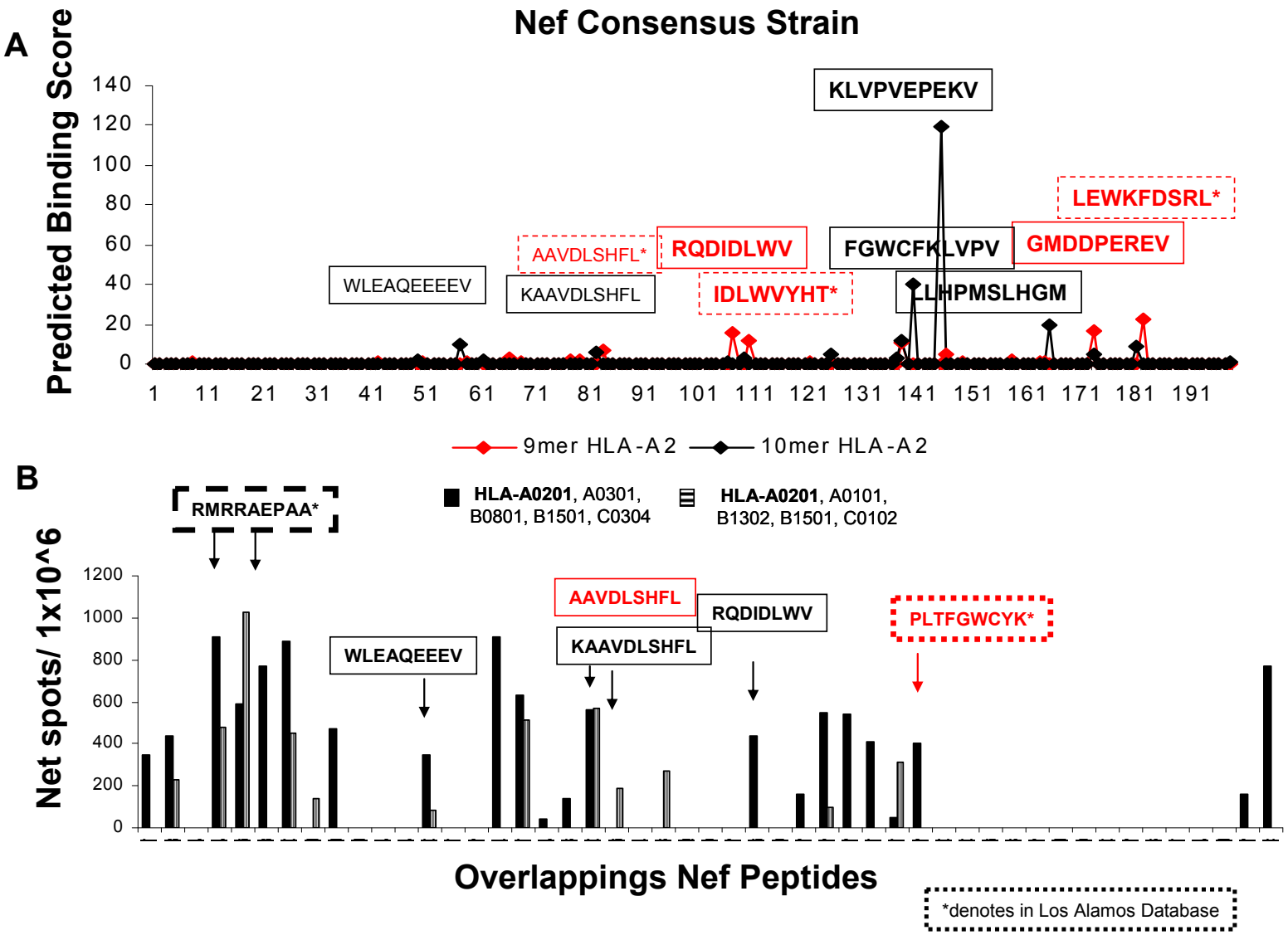


Figure 18. Priming to Consensus Nef. Consensus Nef region of HIV-1 was evaluated for potential CTL epitopes using the algorithm provided by BIMAS (A). Immunogenicity was evaluated by antigen-loading DC matured with CD40L + IFNγ for 2 rounds of stimulation, and co-cultured with PBMC for 4 weeks and assayed for IFNγ production in an ELISPOT (B). Results are reported as 2 standard deviations over the negative control (primed with DC and no peptide).

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 $_{(p17\ 77\text{-}85LAI)}$ (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 YMLKHIVWA, which are both predicted 9-mers of HLA-A*0201, as well as 10-mers such as TLQEQIAWMT. 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.

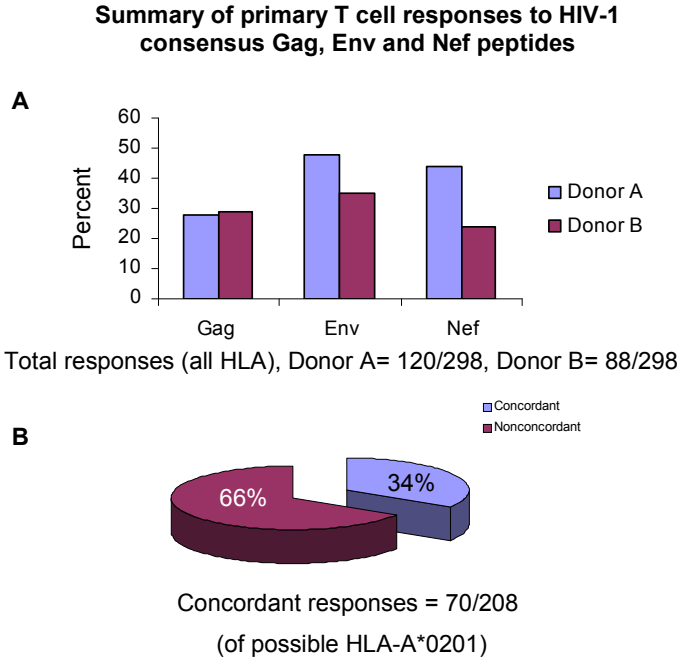


Figure 19 Summary of primary T cell responses to concensus Gag, Env, Nef
The two HLA-A*0201 seronegative donors were used to prime naïve $CD8^+$T cells to 15-mers overlapping Gag, Env, and Nef and these IFNγ ELISPOT results were compiled to determine the number of CTL epitopes that were targeted by both 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+IFNγ resulted in priming of $CD8^+$ $SL9_{(p17\ 77\text{-}85LAI)}$ Tet^+cells. Based on this 3 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 IFN□ 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 + IFNγ 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 (IFNγ 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

Bonnie A Colleton[1], Xiao-Li Huang[1], Zheng Fan[1], Raj Shankarappa[1], Charles R. Rinaldo[1]

Multicenter AIDS Cohort Study (MACS), Graduate School of Public Health, Infectious Disease and Microbiology, University of Pittsburgh,[1] Pittsburgh, Pennsylvania 15261

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 *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 5^{th} day, the iDC were harvested and reset at $1x10^6$/ml and maturation factors were added for 40 hours [CD40L (0.5ug/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 37^0C, 5% CO_2 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).

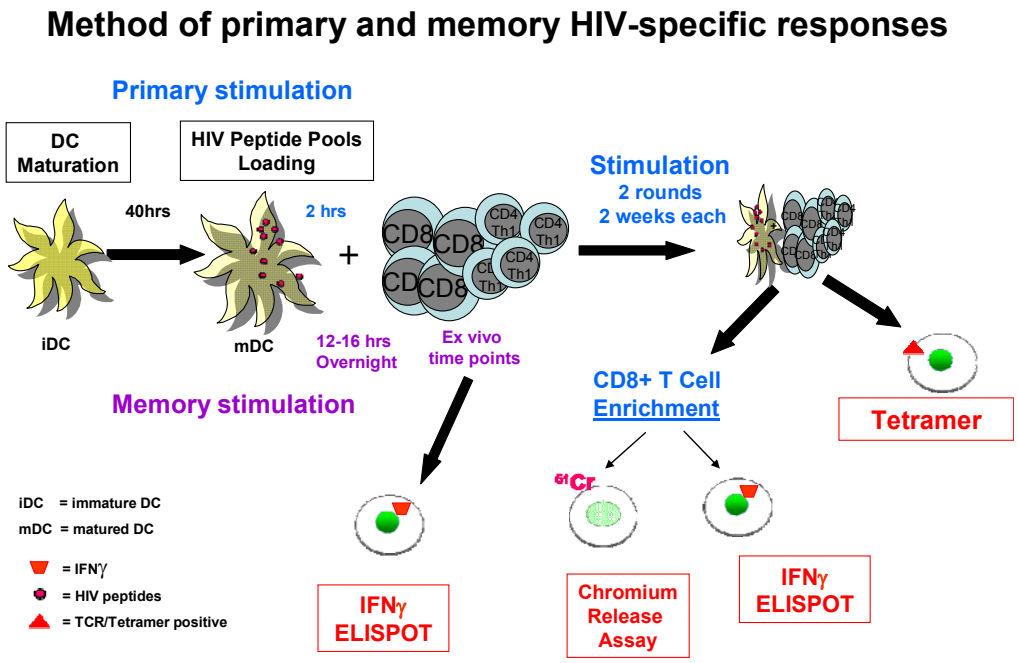


Figure **22. Schematic depiction of the method for primary (in blue across the top of the cartoon) and memory (in purple across the bottom) T cell stimulation.** Briefly, iDC are matured with immunomodulating factors for 40 hours, then used as APC for either (i) memory stimulation in which case peptides 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 IFNγ 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 $1x10^5$ 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 4^0C 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 37^0C. Responder cells were stimulated overnight with peptides (10μg/ml)-loaded DC at a ratio of 10:1 in 37^0C, 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 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.

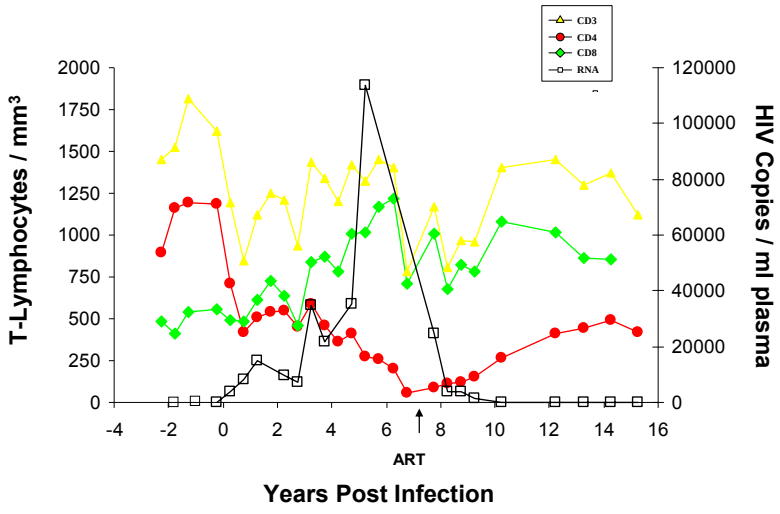


Figure 20. Natural history of HIV-1 infection in MACS participant #8. T lymphocyte counts and HIV-1 copies over course of disease progression are shown: CD3 (yellow triangle), CD4 (red circle), CD8 (green diamond), RNA copies (open square).

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).

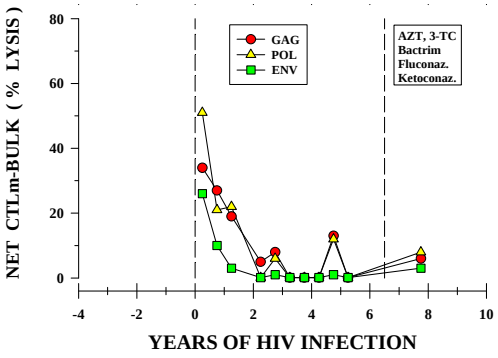
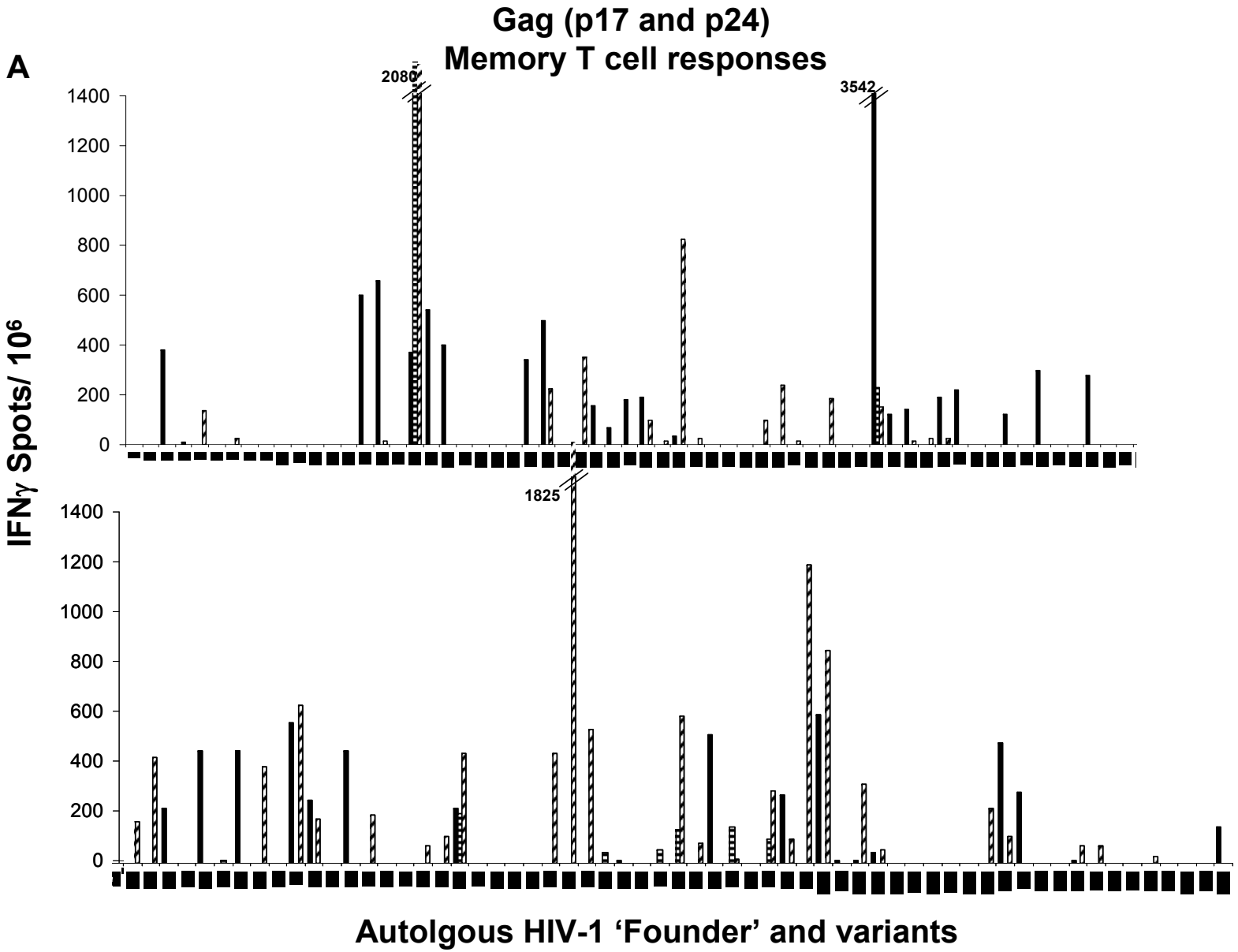


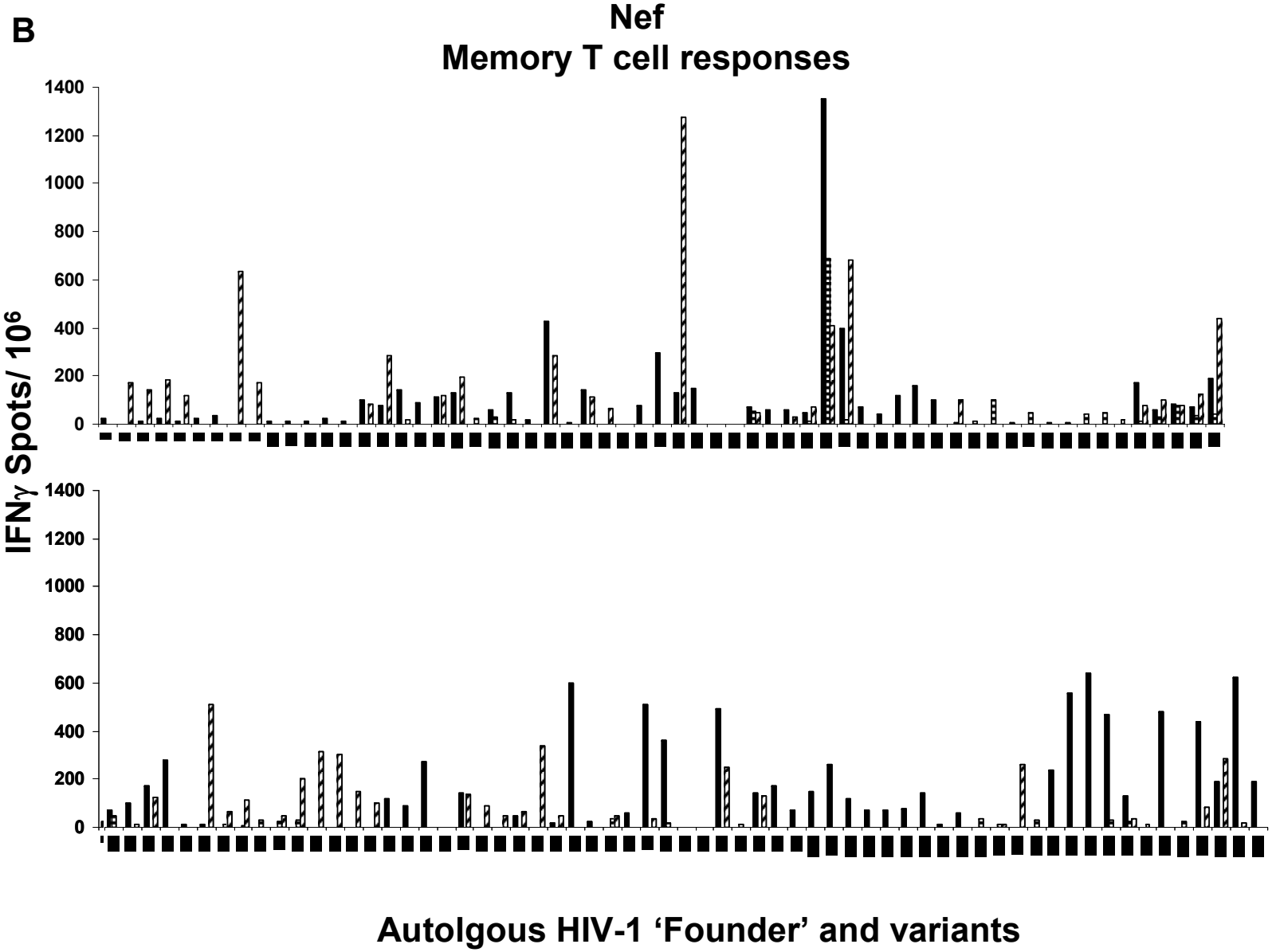
Figure 21. CTL lysis of previously published work [296] demonstrating reactivity to Gag (red circle), Pol (yellow triangle) and Env (green square) over years of infection. Seroconversion is shown as the leftmost vertical dashed line and time zero (0), and the time of CD3$^+$T cell inflection point is 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 IFNγ. 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 IFNγ 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 IFNγ spots per million was seen directed towards Gag (Figure 25) when compared to the other two proteins ($P<0.001$).


A
Gag (p17 and p24)
Memory T cell responses
2080
3542
1825
1400
1200
1000
800
600
400
200
0
IFNγ Spots/ 10⁶
Autolgous HIV-1 'Founder' and variants


B
Nef
Memory T cell responses
IFNγ Spots/ 10^6
1400
1200
1000
800
600
400
200
0
Autolgous HIV-1 'Founder' and variants

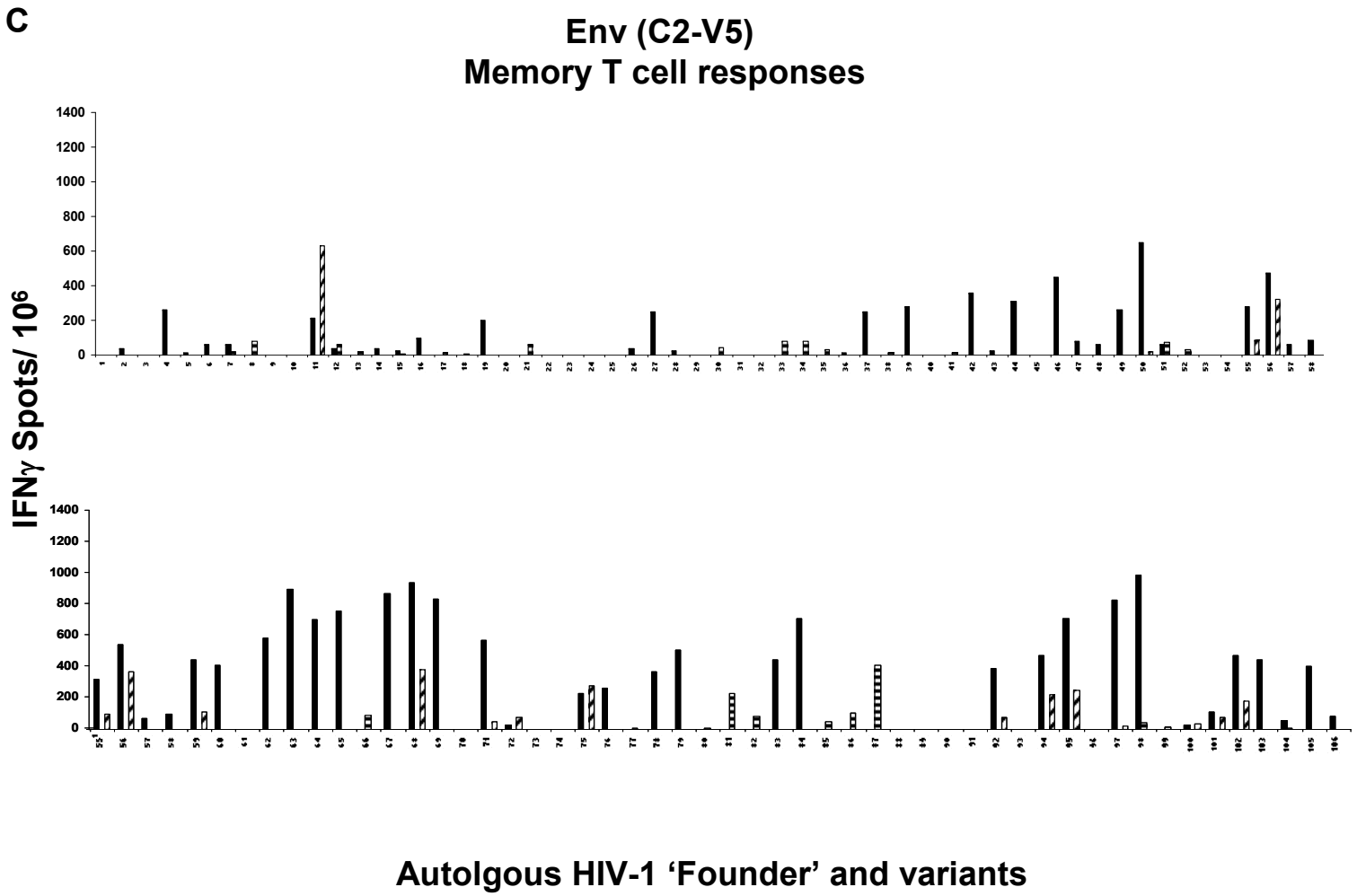


Figure 23. Autologous 9- and 10-mer epitopes and 15-mers overlapping by 11 amino acids comprising Gag, Nef and Env regions of HIV-1 were singularly pulsed to DC matured with CD40L and cultured with PBMC from early, late and on ART visits were assayed in a standard overnight ELISPOT assay, to detect Ag-specific 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).

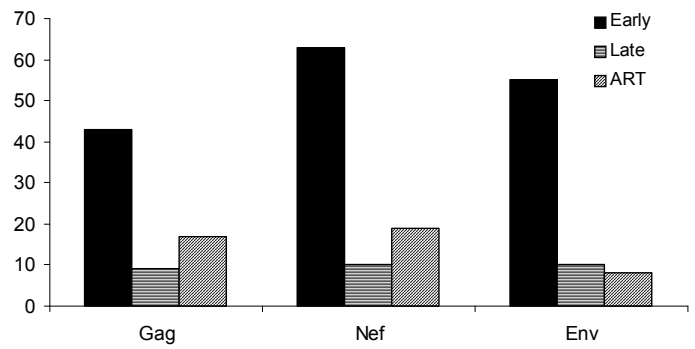


Figure 24 Total number of IFNγ ELISPOTS directed to Gag, Nef and Env displayed in the three time points of Early, Late or on ART.

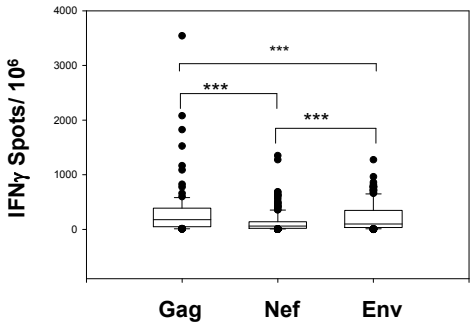


Figure 25. Total positive $CD8^+$ memory T cell responses at early, late and on ART time points of Gag, Nef and Env were compiled to compare the magnitude of IFNγ spots/10^6 between proteins. P<0.001=***, P<0.01=**, P<0.05=*.

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.

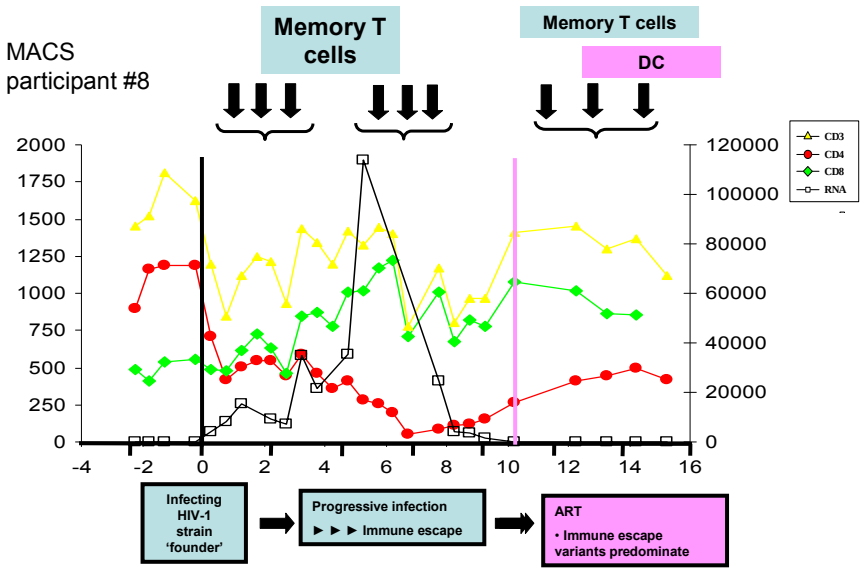


Figure 26. Schematic course of disease progression depicting PBMC used to compare primary and memory T cell response. Memory T cell responses were defined as IFNγ production by PBMC obtained at early, late and on ART visits using an overnight ELISPOT assay. The primary responses were defined as IFNγ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

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).

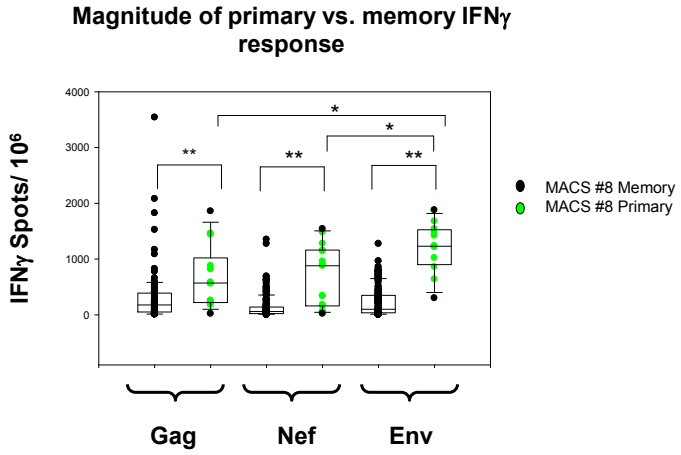


Figure 27. All positive $CD8^+$ T cell memory responses (defined as > 2 SD over background control) comprising early, late and on ART time points to Gag, Nef and Env (black circles) were compiled to compare the magnitude of IFNγ spots/10^6 and how they compared to primary responses (green circles) between 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

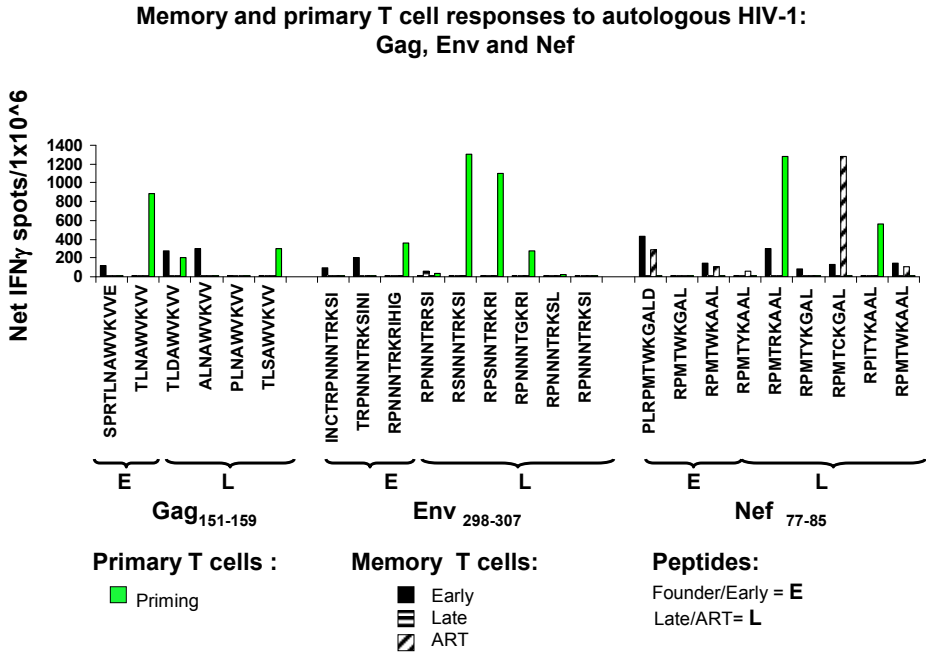
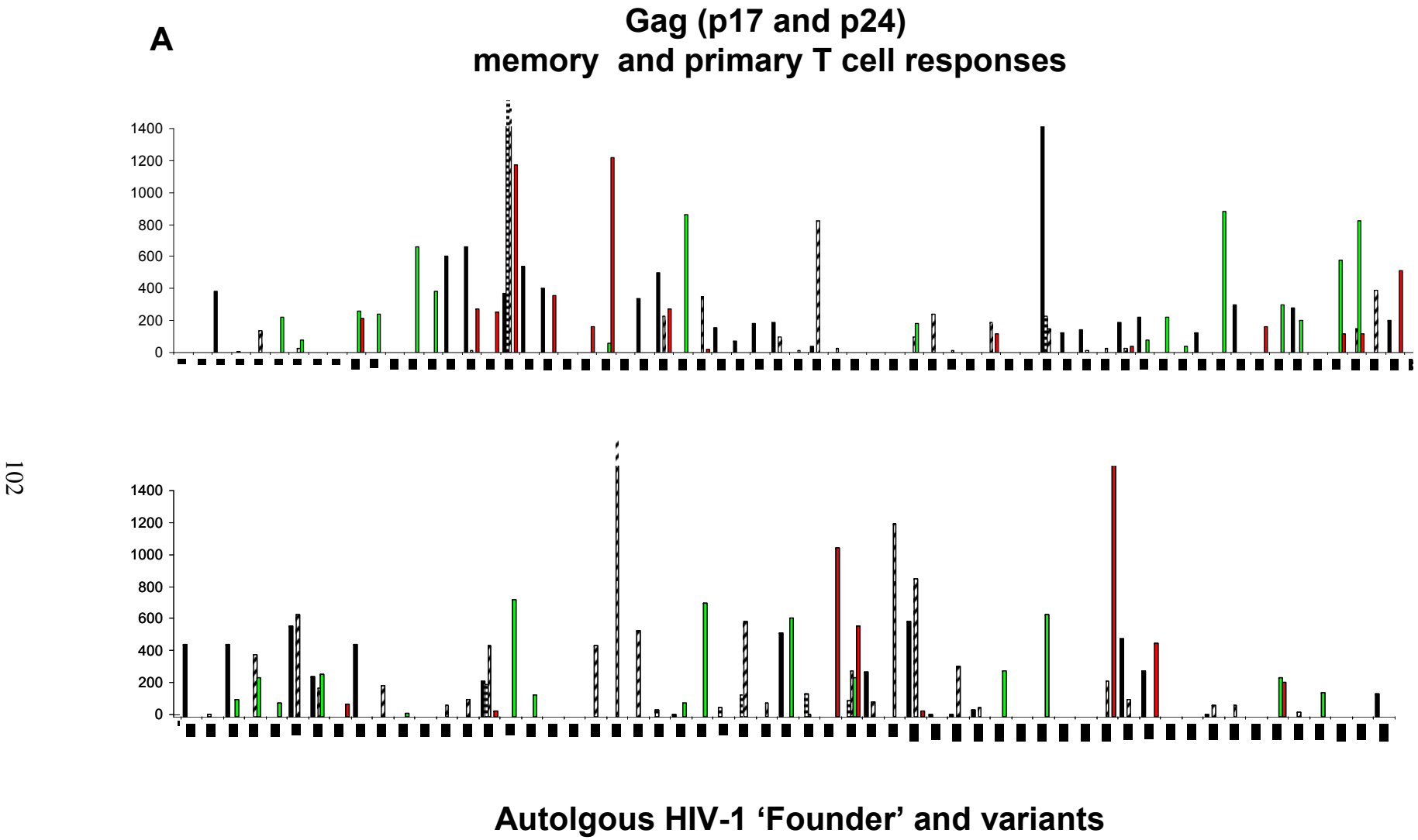


Figure 28. Representative depiction of memory T cell responses compared to primary T cell responses to evolving variants of three Gag, Nef and Env epitopes detected by ELISPOT. Primary responses are labeled in green, memory responses are labeled as in previous graphs. Brackets are shown to separate the 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.

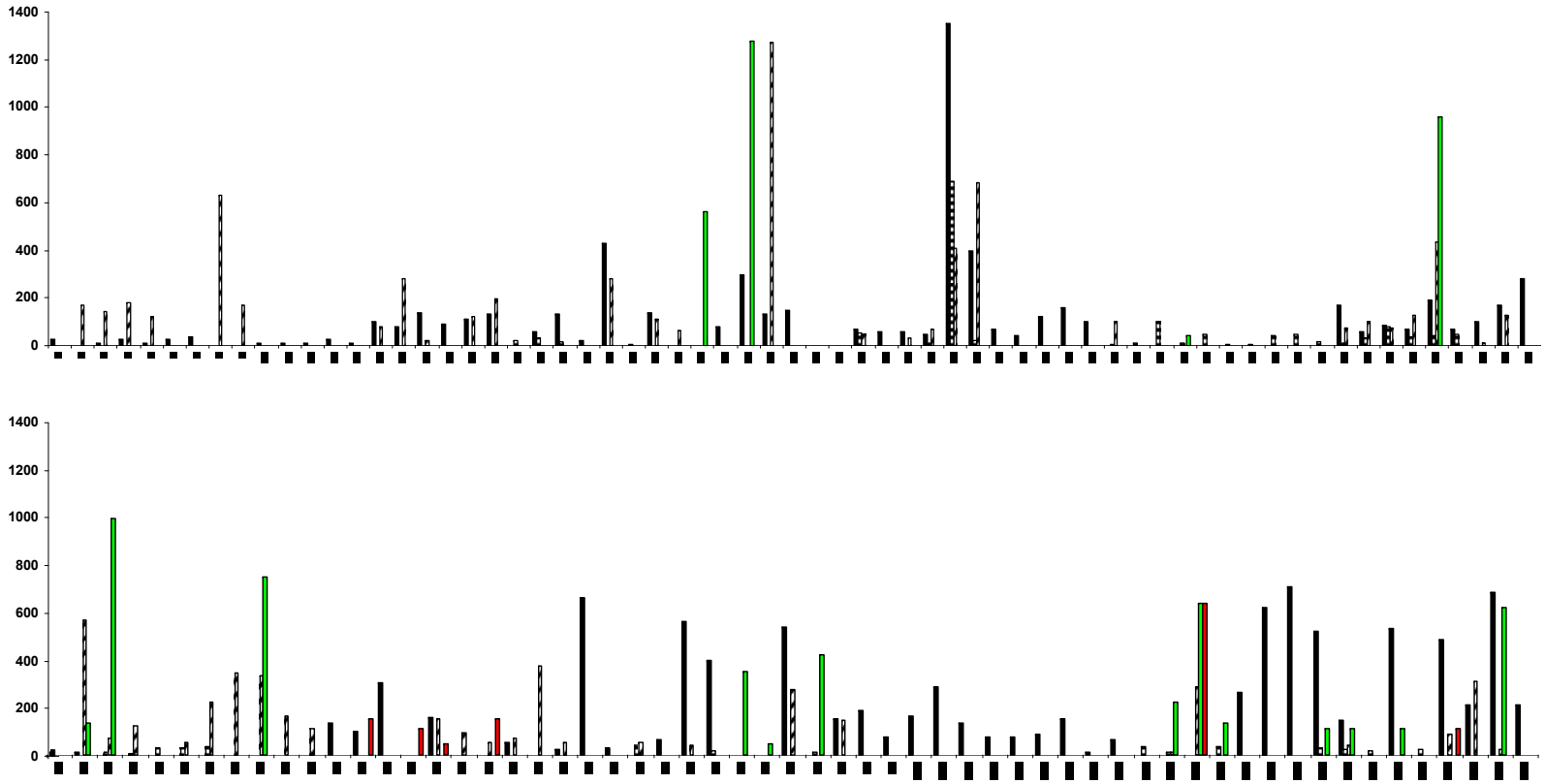
This *in vitro* study supports the concept that *ex vivo* engineering of autologous Ag-loaded DC that are re-administered to HIV-1 infected person's could enhance primary $CD8^+$ T cell responses to immune escape variants of HIV-1 and potentially provide natural viral suppression. To further evaluate 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
1400
1200
1000
800
600
400
200
0
1400
1200
1000
800
600
400
200
0
Autolgous HIV-1 'Founder' and variants

B

Nef
memory and primary T cell responses



Autolgous HIV-1 'Founder' and variants

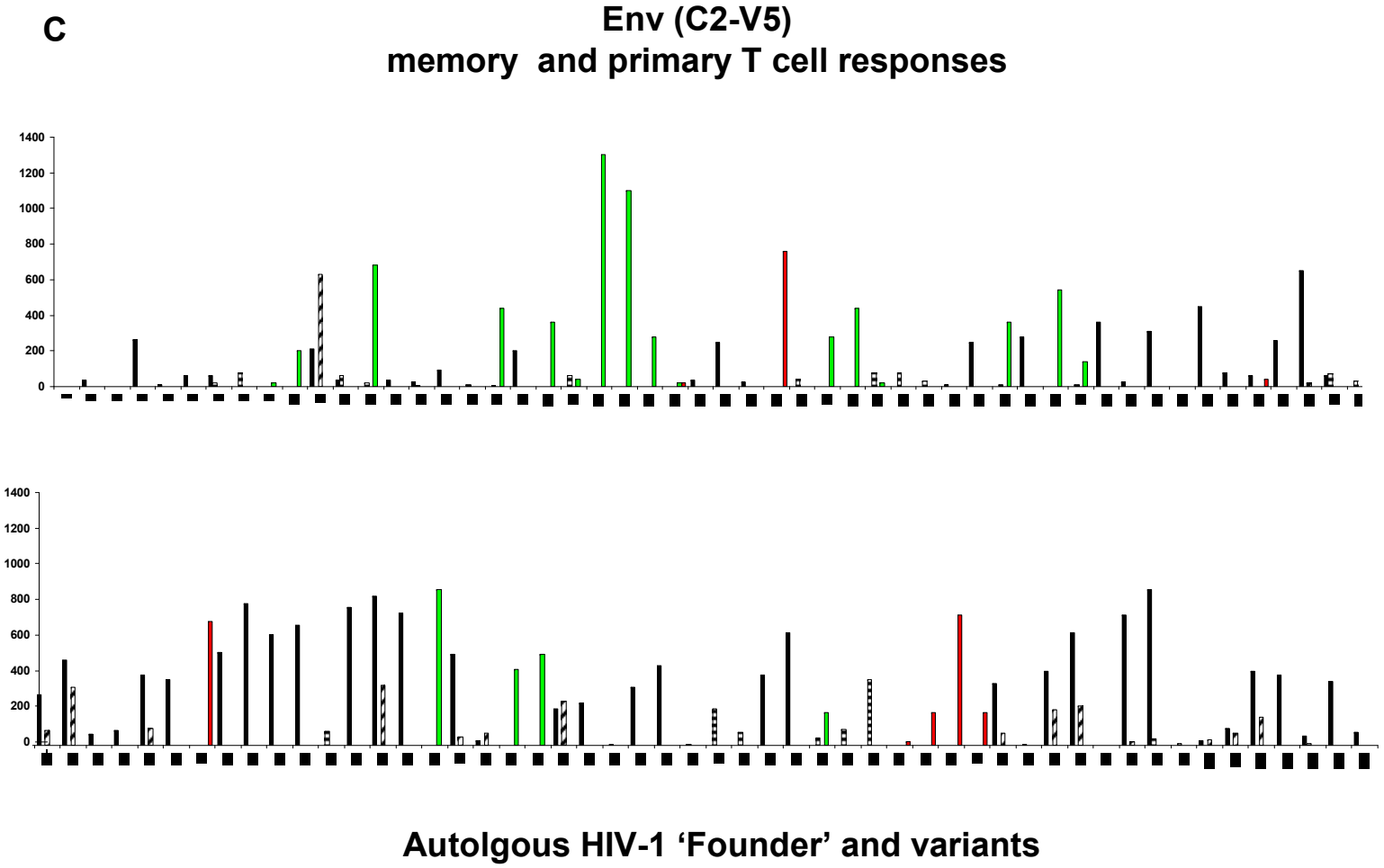


Figure 29. Autologous 9- and 10-mer epitopes and 15-mers overlapping by 11 amino acids comprising Gag, Nef and Env regions of HIV-1 were singularly pulsed to DC matured with CD40L and cultured with PBMC from early (solid black), late (horizontal hatched) and on ART(diagonal hatched) visits were assayed in a standard overnight ELISPOT assay (IFNγ spots/10^6), 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

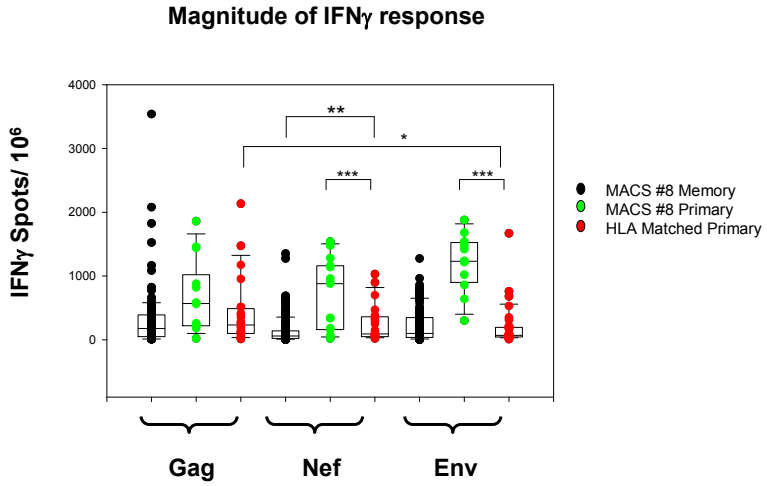


Figure 30. Total positive IFNγ responses detected for memory (black circles) and primary T cells(green circles) for MACS participant #8. A seronegative HLA-A*0201, B*0702 matched donor was used as a control (red circles). P<0.001=***, P<0.01=**, P <0.05=*.

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).

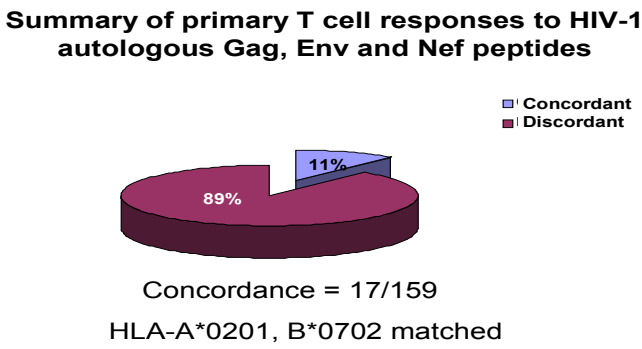


Figure 31. Summary of concordance in positive T cell responses between the HLA-A*0201, B*0702 matched individual and MACS participant #8.

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 naive $CD8^+$ 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 predicting 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 *in vitro* primary stimulation model. I chose to examine CD40L and IFNγ, 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 $CD8^+$ $SL9_{(p17\ 77\text{-}85LAI)}$ 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 HIV^{neg} 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.

MACS Participant #	Status	HLA haplotypes
1	Progressor	A2, A2, B27
2	Progressor	A2, B27
4	Progressor	A2, A2
5	Progressor	A2, A2, B27, B7
7	Progressor	A2, B7
12	LTNP	A2, B27
14	Rapid progressor	A2, A2

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 IFNγ 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

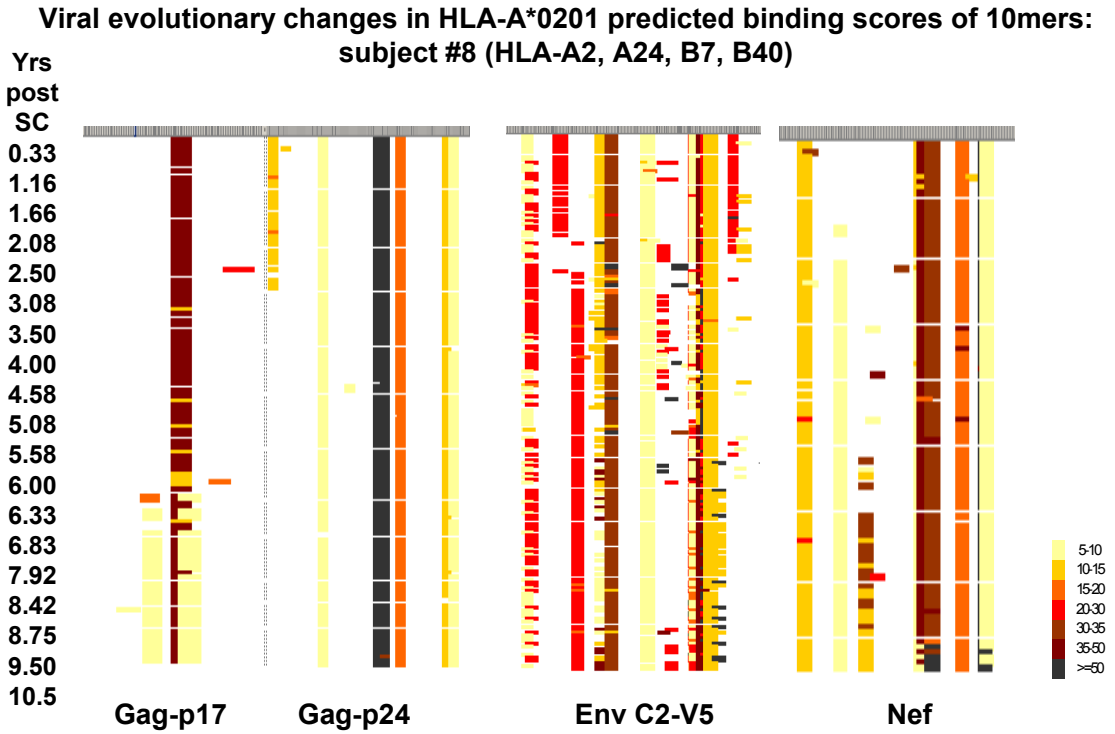


Figure 32: Scanning the impact of viral evolutionary changes on HLA-binding.
Predicted binding to HLA-A*0201 10-mers across the genome over years following seroconversion, was mapped for sequences (Gag-p17, Gag-p24, Env, Nef) in subject #8. Each 10-mer amino acid motif in the sequence alignment was colored 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

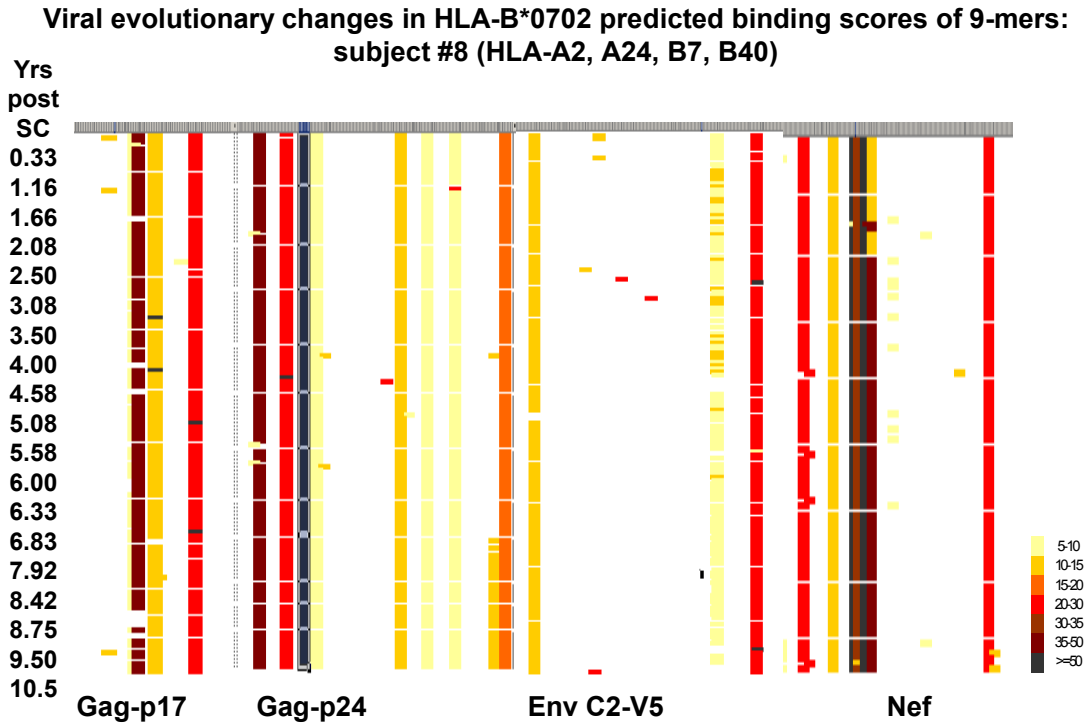


Figure 33: Scanning the impact of viral evolutionary changes on HLA-binding.
Predicted binding to HLA-B*0702 9-mers across the genome over years following seroconversion, was mapped for sequences (Gag-p17, Gag-p24, Env, Nef) in subject #8. Each 9-mer amino acid motif in the sequence alignment was colored 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.

APPENDIX C: Evolutionary changes of 10-mers HLA-B*0702

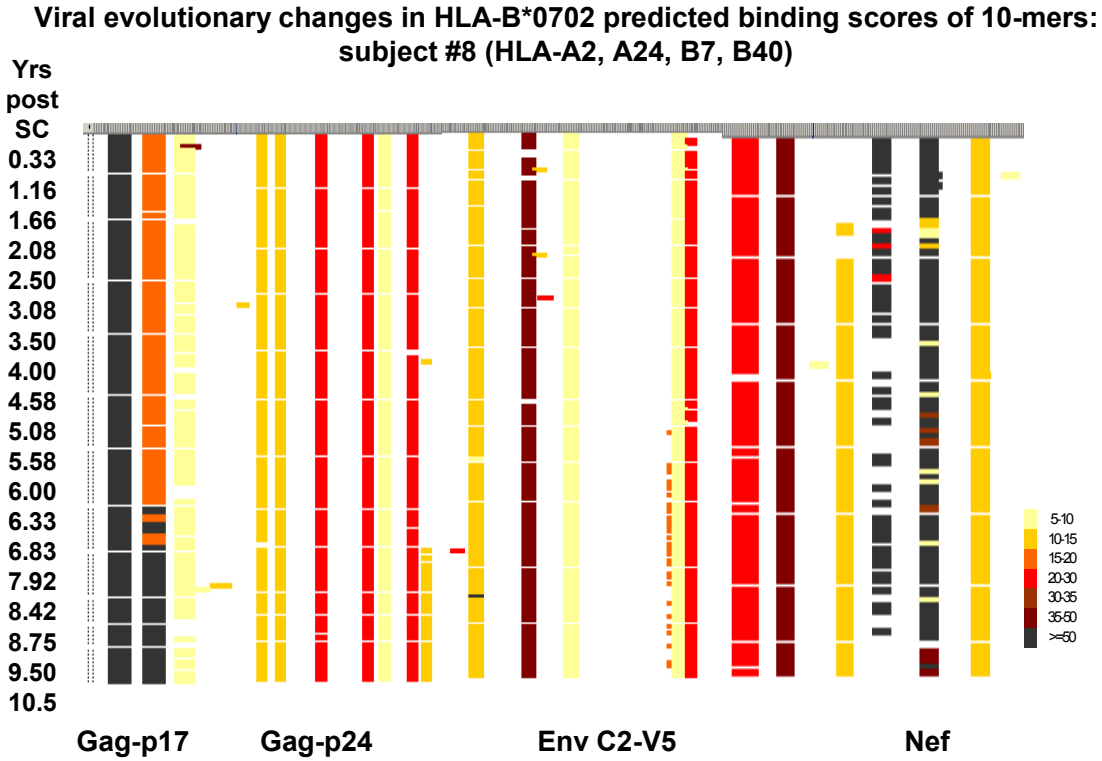


Figure 34: Scanning the impact of viral evolutionary changes on HLA-binding.
Predicted binding to HLA-B*0702 10-mers across the genome over years following seroconversion, was mapped for sequences (Gag-p17, Gag-p24, Env, Nef) in subject #8. Each 10-mer amino acid motif in the sequence alignment was colored 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.

BIBLIOGRAPHY

1. Gottlieb GJ, Ragaz A, Vogel JV, Friedman-Kien A, Rywlin AM, Weiner EA, Ackerman AB: **A preliminary communication on extensively disseminated Kaposi's sarcoma in young homosexual men**. *Am J Dermatopathol* 1981, **3**:111-114.

2. Gallo RC, Sarin PS, Gelmann EP, Robert-Guroff M, Richardson E, Kalyanaraman VS, Mann D, Sidhu GD, Stahl RE, Zolla-Pazner S, et al.: **Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS)**. *Science* 1983, **220**:865-867.

3. Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, et al.: **Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS)**. *Science* 1983, **220**:868-871.

4. Keele BF, Van Heuverswyn F, Li Y, Bailes E, Takehisa J, Santiago ML, Bibollet-Ruche F, Chen Y, Wain LV, Liegeois F, et al.: **Chimpanzee reservoirs of pandemic and nonpandemic HIV-1**. *Science* 2006, **313**:523-526.

5. Heeney JL, Dalgleish AG, Weiss RA: **Origins of HIV and the evolution of resistance to AIDS**. *Science* 2006, **313**:462-466.

6. Heeney JL, Rutjens E, Verschoor EJ, Niphuis H, ten Haaft P, Rouse S, McClure H, Balla-Jhagjhoorsingh S, Bogers W, Salas M, et al.: **Transmission of simian immunodeficiency virus SIVcpz and the evolution of infection in the presence and absence of concurrent human immunodeficiency virus type 1 infection in chimpanzees**. *J Virol* 2006, **80**:7208-7218.

7. Damond F, Worobey M, Campa P, Farfara I, Colin G, Matheron S, Brun-Vezinet F, Robertson DL, Simon F: **Identification of a highly divergent HIV type 2 and proposal for a change in HIV type 2 classification**. *AIDS Res Hum Retroviruses* 2004, **20**:666-672.

8. Santiago ML, Bibollet-Ruche F, Gross-Camp N, Majewski AC, Masozera M, Munanura I, Kaplin BA, Sharp PM, Shaw GM, Hahn BH: **Noninvasive detection of Simian immunodeficiency virus infection in a wild-living L'Hoest's monkey (Cercopithecus Ihoesti)**. *AIDS Res Hum Retroviruses* 2003, **19**:1163-1166.

9. Kao SY, Calman AF, Luciw PA, Peterlin BM: **Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product**. *Nature* 1987, **330**:489-493.

10. Cullen BR, Hauber J, Campbell K, Sodroski JG, Haseltine WA, Rosen CA: **Subcellular localization of the human immunodeficiency virus trans-acting art gene product**. *J Virol* 1988, **62**:2498-2501.

11. Alkhatib G, Broder CC, Berger EA: **Cell type-specific fusion cofactors determine human immunodeficiency virus type 1 tropism for T-cell lines versus primary macrophages**. *J Virol* 1996, **70**:5487-5494.

12. Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA: **CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1**. *Science* 1996, **272**:1955-1958.

13. Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, et al.: **The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates**. *Cell* 1996, **85**:1135-1148.

14. Feng Y, Broder CC, Kennedy PE, Berger EA: **HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor**. *Science* 1996, **272**:872-877.

15. Clapham PR, McKnight A: **Cell surface receptors, virus entry and tropism of primate lentiviruses**. *J Gen Virol* 2002, **83**:1809-1829.

16. Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, et al.: **Identification of a major co-receptor for primary isolates of HIV-1**. *Nature* 1996, **381**:661-666.

17. Veazey R, Lackner A: **The mucosal immune system and HIV-1 infection**. *AIDS Rev* 2003, **5**:245-252.

18. Veazey RS, Shattock RJ, Pope M, Kirijan JC, Jones J, Hu Q, Ketas T, Marx PA, Klasse PJ, Burton DR, et al.: **Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120**. *Nat Med* 2003, **9**:343-346.

19. Hu Q, Frank I, Williams V, Santos JJ, Watts P, Griffin GE, Moore JP, Pope M, Shattock RJ: **Blockade of attachment and fusion receptors inhibits HIV-1 infection of human cervical tissue**. *J Exp Med* 2004, **199**:1065-1075.

20. Gupta P, Collins KB, Ratner D, Watkins S, Naus GJ, Landers DV, Patterson BK: **Memory CD4(+) T cells are the earliest detectable human immunodeficiency virus type 1 (HIV-1)-infected cells in the female genital mucosal tissue during HIV-1 transmission in an organ culture system**. *J Virol* 2002, **76**:9868-9876.

21. Hu J, Gardner MB, Miller CJ: **Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells**. *J Virol* 2000, **74**:6087-6095.

22. Spira AI, Marx PA, Patterson BK, Mahoney J, Koup RA, Wolinsky SM, Ho DD: **Cellular targets of infection and route of viral dissemination after an intravaginal inoculation**

of simian immunodeficiency virus into rhesus macaques. *J Exp Med* 1996, **183**:215-225.

23. Collins KB, Patterson BK, Naus GJ, Landers DV, Gupta P: **Development of an in vitro organ culture model to study transmission of HIV-1 in the female genital tract**. *Nat Med* 2000, **6**:475-479.

24. Greenhead P, Hayes P, Watts PS, Laing KG, Griffin GE, Shattock RJ: **Parameters of human immunodeficiency virus infection of human cervical tissue and inhibition by vaginal virucides**. *J Virol* 2000, **74**:5577-5586.

25. Maher D, Wu X, Schacker T, Horbul J, Southern P: **HIV binding, penetration, and primary infection in human cervicovaginal tissue**. *Proc Natl Acad Sci U S A* 2005, **102**:11504-11509.

26. Zaitseva M, Blauvelt A, Lee S, Lapham CK, Klaus-Kovtun V, Mostowski H, Manischewitz J, Golding H: **Expression and function of CCR5 and CXCR4 on human Langerhans cells and macrophages: implications for HIV primary infection**. *Nat Med* 1997, **3**:1369-1375.

27. Weissman D, Rabin RL, Arthos J, Rubbert A, Dybul M, Swofford R, Venkatesan S, Farber JM, Fauci AS: **Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor**. *Nature* 1997, **389**:981-985.

28. Hackemann M, Grubb C, Hill KR: **The ultrastructure of normal squamous epithelium of the human cervix uteri**. *J Ultrastruct Res* 1968, **22**:443-457.

29. Miller CJ, Alexander NJ, Vogel P, Anderson J, Marx PA: **Mechanism of genital transmission of SIV: a hypothesis based on transmission studies and the location of SIV in the genital tract of chronically infected female rhesus macaques**. *J Med Primatol* 1992, **21**:64-68.

30. Parr R: **Reflex labelling of high-risk individuals does not limit HIV**. *Nurs Times* 1990, **86**:12.

31. Gurney KB, Elliott J, Nassanian H, Song C, Soilleux E, McGowan I, Anton PA, Lee B: **Binding and transfer of human immunodeficiency virus by DC-SIGN+ cells in human rectal mucosa**. *J Virol* 2005, **79**:5762-5773.

32. Geijtenbeek TB, Kwon DS, Torensma R, van Vliet SJ, van Duijnhoven GC, Middel J, Cornelissen IL, Nottet HS, KewalRamani VN, Littman DR, et al.: **DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells**. *Cell* 2000, **100**:587-597.

33. Hladik F, Sakchalathorn P, Ballweber L, Lentz G, Fialkow M, Eschenbach D, McElrath MJ: **Initial events in establishing vaginal entry and infection by human immunodeficiency virus type-1**. *Immunity* 2007, **26**:257-270.

34. Clerici M, Berzofsky JA, Shearer GM, Giorgi JV, Tacket C: **HIV-1 from a seronegative transplant donor**. *N Engl J Med* 1992, **327**:564-565.

35. Plummer FA, Ball TB, Kimani J, Fowke KR: **Resistance to HIV-1 infection among highly exposed sex workers in Nairobi: what mediates protection and why does it develop?** *Immunol Lett* 1999, **66**:27-34.

36. Fowke KR, Nagelkerke NJ, Kimani J, Simonsen JN, Anzala AO, Bwayo JJ, MacDonald KS, Ngugi EN, Plummer FA: **Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya**. *Lancet* 1996, **348**:1347-1351.

37. Barcellini W, Rizzardi GP, Velati C, Borghi MO, Fain C, Lazzarin A, Meroni PL: **In vitro production of type 1 and type 2 cytokines by peripheral blood mononuclear cells from high-risk HIV-negative intravenous drug users**. *Aids* 1995, **9**:691-694.

38. Alimonti JB, Kimani J, Matu L, Wachihi C, Kaul R, Plummer FA, Fowke KR: **Characterization of CD8 T-cell responses in HIV-1-exposed seronegative commercial sex workers from Nairobi, Kenya**. *Immunol Cell Biol* 2006, **84**:482-485.

39. Pantaleo G, Menzo S, Vaccarezza M, Graziosi C, Cohen OJ, Demarest JF, Montefiori D, Orenstein JM, Fox C, Schrager LK, et al.: **Studies in subjects with long-term nonprogressive human immunodeficiency virus infection**. *N Engl J Med* 1995, **332**:209-216.

40. Cao Y, Qin L, Zhang L, Safrit J, Ho DD: **Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection**. *N Engl J Med* 1995, **332**:201-208.

41. Friedrich TC, Valentine LE, Yant LJ, Rakasz EG, Piaskowski SM, Furlott JR, Weisgrau KL, Burwitz B, May GE, Leon EJ, et al.: **Subdominant CD8+ T-cell responses are involved in durable control of AIDS virus replication**. *J Virol* 2007, **81**:3465-3476.

42. Huff B: **Who are the elite controllers?** *GMHC Treat Issues* 2005, **19**:12.

43. Shacklett BL: **Understanding the "lucky few": the conundrum of HIV-exposed, seronegative individuals**. *Curr HIV/AIDS Rep* 2006, **3**:26-31.

44. Bailey JR, Williams TM, Siliciano RF, Blankson JN: **Maintenance of viral suppression in HIV-1-infected HLA-B*57+ elite suppressors despite CTL escape mutations**. *J Exp Med* 2006, **203**:1357-1369.

45. Munier ML, Kelleher AD: **Acutely dysregulated, chronically disabled by the enemy within: T-cell responses to HIV-1 infection**. *Immunol Cell Biol* 2007, **85**:6-15.

46. Clark SJ, Saag MS, Decker WD, Campbell-Hill S, Roberson JL, Veldkamp PJ, Kappes JC, Hahn BH, Shaw GM: **High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection**. *N Engl J Med* 1991, **324**:954-960.

47. Cooper DA, Tindall B, Wilson EJ, Imrie AA, Penny R: **Characterization of T lymphocyte responses during primary infection with human immunodeficiency virus**. *J Infect Dis* 1988, **157**:889-896.

48. Gaines H, von Sydow MA, von Stedingk LV, Biberfeld G, Bottiger B, Hansson LO, Lundbergh P, Sonnerborg AB, Wasserman J, Strannegaard OO: **Immunological changes in primary HIV-1 infection**. *Aids* 1990, **4**:995-999.

49. Kaufmann D, Pantaleo G, Sudre P, Telenti A: **CD4-cell count in HIV-1-infected individuals remaining viraemic with highly active antiretroviral therapy (HAART). Swiss HIV Cohort Study**. *Lancet* 1998, **351**:723-724.

50. Kaufmann GR, Duncombe C, Zaunders J, Cunningham P, Cooper D: **Primary HIV-1 infection: a review of clinical manifestations, immunologic and virologic changes**. *AIDS Patient Care STDS* 1998, **12**:759-767.

51. Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, Irwin CE, Safrit JT, Mittler J, Weinberger L, et al.: **Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques**. *J Exp Med* 1999, **189**:991-998.

52. Matano T, Shibata R, Siemon C, Connors M, Lane HC, Martin MA: **Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques**. *J Virol* 1998, **72**:164-169.

53. Mellors JW, Kingsley LA, Rinaldo CR, Jr., Todd JA, Hoo BS, Kokka RP, Gupta P: **Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion**. *Ann Intern Med* 1995, **122**:573-579.

54. Mellors JW, Rinaldo CR, Jr., Gupta P, White RM, Todd JA, Kingsley LA: **Prognosis in HIV-1 infection predicted by the quantity of virus in plasma**. *Science* 1996, **272**:1167-1170.

55. Moss R: **Rebuilding the immune system: promising new therapy uses the HIV virus itself to awaken the damaged immune system. Interview by Brian Coppedge and Dan Dawson**. *STEP Perspect* 1998, **98**:13-16.

56. Highleyman L: **HIV and hepatitis C coinfection**. *Beta* 2003, **15**:32-44.

57. Masur H: **Opportunistic infections are major factor in HIV mortality**. *Am Fam Physician* 1989, **40**:149.

58. Masur H: **Clinical studies of Pneumocystis carinii and relationships to AIDS**. *J Protozool* 1989, **36**:70-74.

59. Kaufmann D, Munoz M, Bleiber G, Fleury S, Lotti B, Martinez R, Pichler W, Meylan P, Telenti A: **Virological and immunological characteristics of HIV treatment failure**. *Aids* 2000, **14**:1767-1774.

60. Kaufmann GR, Bloch M, Zaunders JJ, Smith D, Cooper DA: **Long-term immunological response in HIV-1-infected subjects receiving potent antiretroviral therapy**. *Aids* 2000, **14**:959-969.

61. Kaufmann GR, Cooper DA: **Antiretroviral therapy of HIV-1 infection: established treatment strategies and new therapeutic options**. *Curr Opin Microbiol* 2000, **3**:508-514.

62. Kaufmann GR, Zaunders JJ, Cunningham P, Kelleher AD, Grey P, Smith D, Carr A, Cooper DA: **Rapid restoration of CD4 T cell subsets in subjects receiving antiretroviral therapy during primary HIV-1 infection**. *Aids* 2000, **14**:2643-2651.

63. Kaufmann GR, Furrer H, Ledergerber B, Perrin L, Opravil M, Vernazza P, Cavassini M, Bernasconi E, Rickenbach M, Hirschel B, et al.: **Characteristics, determinants, and clinical relevance of CD4 T cell recovery to <500 cells/microL in HIV type 1-infected individuals receiving potent antiretroviral therapy**. *Clin Infect Dis* 2005, **41**:361-372.

64. Zaunders JJ, Cunningham PH, Kelleher AD, Kaufmann GR, Jaramillo AB, Wright R, Smith D, Grey P, Vizzard J, Carr A, et al.: **Potent antiretroviral therapy of primary human immunodeficiency virus type 1 (HIV-1) infection: partial normalization of T lymphocyte subsets and limited reduction of HIV-1 DNA despite clearance of plasma viremia**. *J Infect Dis* 1999, **180**:320-329.

65. Aiuti F, Mezzaroma I: **Failure to reconstitute CD4+ T-cells despite suppression of HIV replication under HAART**. *AIDS Rev* 2006, **8**:88-97.

66. Banchereau J, Steinman RM: **Dendritic cells and the control of immunity**. *Nature* 1998, **392**:245-252.

67. Wu L, KewalRamani VN: **Dendritic-cell interactions with HIV: infection and viral dissemination**. *Nat Rev Immunol* 2006, **6**:859-868.

68. Sabatte J, Maggini J, Nahmod K, Amaral MM, Martinez D, Salamone G, Ceballos A, Giordano M, Vermeulen M, Geffner J: **Interplay of pathogens, cytokines and other stress signals in the regulation of dendritic cell function**. *Cytokine Growth Factor Rev* 2007, **18**:5-17.

69. Pohl C, Shishkova J, Schneider-Schaulies S: **Viruses and dendritic cells: enemy mine**. *Cell Microbiol* 2007, **9**:279-289.

70. Larsson M, Fonteneau JF, Bhardwaj N: **Dendritic cells resurrect antigens from dead cells**. *Trends Immunol* 2001, **22**:141-148.

71. Larsson M, Fonteneau JF, Somersan S, Sanders C, Bickham K, Thomas EK, Mahnke K, Bhardwaj N: **Efficiency of cross presentation of vaccinia virus-derived antigens by human dendritic cells**. *Eur J Immunol* 2001, **31**:3432-3442.

72. Albert ML, Sauter B, Bhardwaj N: **Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs**. *Nature* 1998, **392**:86-89.

73. Heath WR, Carbone FR: **Cross-presentation in viral immunity and self-tolerance**. *Nat Rev Immunol* 2001, **1**:126-134.

74. Heath WR, Carbone FR: **Cross-presentation, dendritic cells, tolerance and immunity**. *Annu Rev Immunol* 2001, **19**:47-64.

75. Herr W, Ranieri E, Olson W, Zarour H, Gesualdo L, Storkus WJ: **Mature dendritic cells pulsed with freeze-thaw cell lysates define an effective in vitro vaccine designed to elicit EBV-specific CD4(+) and CD8(+) T lymphocyte responses**. *Blood* 2000, **96**:1857-1864.

76. Subklewe M, Paludan C, Tsang ML, Mahnke K, Steinman RM, Munz C: **Dendritic cells cross-present latency gene products from Epstein-Barr virus-transformed B cells and expand tumor-reactive CD8(+) killer T cells**. *J Exp Med* 2001, **193**:405-411.

77. Tabi Z, Moutaftsi M, Borysiewicz LK: **Human cytomegalovirus pp65- and immediate early 1 antigen-specific HLA class I-restricted cytotoxic T cell responses induced by cross-presentation of viral antigens**. *J Immunol* 2001, **166**:5695-5703.

78. Sallusto F, Cella M, Danieli C, Lanzavecchia A: **Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products**. *J Exp Med* 1995, **182**:389-400.

79. Aderem A, Ulevitch RJ: **Toll-like receptors in the induction of the innate immune response**. *Nature* 2000, **406**:782-787.

80. Medzhitov R: **Toll-like receptors and innate immunity**. *Nat Rev Immunol* 2001, **1**:135-145.

81. Grewal IS, Flavell RA: **CD40 and CD154 in cell-mediated immunity**. *Annu Rev Immunol* 1998, **16**:111-135.

82. Schmidt CS, Mescher MF: **Peptide antigen priming of naive, but not memory, CD8 T cells requires a third signal that can be provided by IL-12**. *J Immunol* 2002, **168**:5521-5529.

83. Albert ML, Jegathesan M, Darnell RB: **Dendritic cell maturation is required for the cross-tolerization of CD8+ T cells**. *Nat Immunol* 2001, **2**:1010-1017.

84. Hernandez J, Aung S, Marquardt K, Sherman LA: **Uncoupling of proliferative potential and gain of effector function by CD8(+) T cells responding to self-antigens**. *J Exp Med* 2002, **196**:323-333.

85. Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, Racz P, Tenner-Racz K, Dalesandro M, Scallon BJ, et al.: **Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes**. *Science* 1999, **283**:857-860.

86. Takata H, Takiguchi M: **Three memory subsets of human CD8+ T cells differently expressing three cytolytic effector molecules**. *J Immunol* 2006, **177**:4330-4340.

87. Pantaleo G, Harari A: **Functional signatures in antiviral T-cell immunity for monitoring virus-associated diseases**. *Nat Rev Immunol* 2006, **6**:417-423.

88. Colle JH, Moreau JL, Fontanet A, Lambotte O, Joussemet M, Delfraissy JF, Theze J: **CD127 expression and regulation are altered in the memory CD8 T cells of HIV-infected patients--reversal by highly active anti-retroviral therapy (HAART)**. *Clin Exp Immunol* 2006, **143**:398-403.

89. Read SW, Higgins J, Metcalf JA, Stevens RA, Rupert A, Nason MC, Lane HC, Sereti I: **Decreased CD127 expression on T Cells in HIV-1-infected adults receiving antiretroviral therapy with or without intermittent IL-2 therapy**. *J Acquir Immune Defic Syndr* 2006, **42**:537-544.

90. Fuller MJ, Hildeman DA, Sabbaj S, Gaddis DE, Tebo AE, Shang L, Goepfert PA, Zajac AJ: **Cutting edge: emergence of CD127high functionally competent memory T cells is compromised by high viral loads and inadequate T cell help**. *J Immunol* 2005, **174**:5926-5930.

91. Appay V, Almeida JR, Sauce D, Autran B, Papagno L: **Accelerated immune senescence and HIV-1 infection**. *Exp Gerontol* 2007, **42**:432-437.

92. Romero P, Zippelius A, Kurth I, Pittet MJ, Touvrey C, Iancu EM, Corthesy P, Devevre E, Speiser DE, Rufer N: **Four functionally distinct populations of human effector-memory CD8+ T lymphocytes**. *J Immunol* 2007, **178**:4112-4119.

93. Hamann D, Kostense S, Wolthers KC, Otto SA, Baars PA, Miedema F, van Lier RA: **Evidence that human CD8+CD45RA+CD27- cells are induced by antigen and evolve through extensive rounds of division**. *Int Immunol* 1999, **11**:1027-1033.

94. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, Ogg GS, King A, Lechner F, Spina CA, et al.: **Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections**. *Nat Med* 2002, **8**:379-385.

95. Papagno L, Spina CA, Marchant A, Salio M, Rufer N, Little S, Dong T, Chesney G, Waters A, Easterbrook P, et al.: **Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection**. *PLoS Biol* 2004, **2**:E20.

96. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A: **Two subsets of memory T lymphocytes with distinct homing potentials and effector functions**. *Nature* 1999, **401**:708-712.

97. Unsoeld H, Krautwald S, Voehringer D, Kunzendorf U, Pircher H: **Cutting edge: CCR7+ and CCR7- memory T cells do not differ in immediate effector cell function**. *J Immunol* 2002, **169**:638-641.

98. Ravkov EV, Myrick CM, Altman JD: **Immediate early effector functions of virus-specific CD8+CCR7+ memory cells in humans defined by HLA and CC chemokine ligand 19 tetramers**. *J Immunol* 2003, **170**:2461-2468.

99. Ellefsen K, Harari A, Champagne P, Bart PA, Sekaly RP, Pantaleo G: **Distribution and functional analysis of memory antiviral CD8 T cell responses in HIV-1 and cytomegalovirus infections**. *Eur J Immunol* 2002, **32**:3756-3764.

100. Harari A, Dutoit V, Cellerai C, Bart PA, Du Pasquier RA, Pantaleo G: **Functional signatures of protective antiviral T-cell immunity in human virus infections**. *Immunol Rev* 2006, **211**:236-254.

101. Rudolph MG, Stanfield RL, Wilson IA: **How TCRs bind MHCs, peptides, and coreceptors**. *Annu Rev Immunol* 2006, **24**:419-466.

102. Matloubian M, Concepcion RJ, Ahmed R: **CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection**. *J Virol* 1994, **68**:8056-8063.

103. Mescher MF, Curtsinger JM, Agarwal P, Casey KA, Gerner M, Hammerbeck CD, Popescu F, Xiao Z: **Signals required for programming effector and memory development by CD8+ T cells**. *Immunol Rev* 2006, **211**:81-92.

104. Cardin RD, Brooks JW, Sarawar SR, Doherty PC: **Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells**. *J Exp Med* 1996, **184**:863-871.

105. Altfeld M, Rosenberg ES: **The role of CD4(+) T helper cells in the cytotoxic T lymphocyte response to HIV-1**. *Curr Opin Immunol* 2000, **12**:375-380.

106. Edwards BH, Bansal A, Sabbaj S, Bakari J, Mulligan MJ, Goepfert PA: **Magnitude of functional CD8+ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma**. *J Virol* 2002, **76**:2298-2305.

107. Bourgeois C, Veiga-Fernandes H, Joret AM, Rocha B, Tanchot C: **CD8 lethargy in the absence of CD4 help**. *Eur J Immunol* 2002, **32**:2199-2207.

108. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP: **CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes**. *Nature* 2003, **421**:852-856.

109. Shedlock DJ, Shen H: **Requirement for CD4 T cell help in generating functional CD8 T cell memory**. *Science* 2003, **300**:337-339.

110. Krawczyk CM, Shen H, Pearce EJ: **Memory CD4 T cells enhance primary CD8 T-cell responses**. *Infect Immun* 2007, **75**:3556-3560.

111. Grakoui A, Shoukry NH, Woollard DJ, Han JH, Hanson HL, Ghrayeb J, Murthy KK, Rice CM, Walker CM: **HCV persistence and immune evasion in the absence of memory T cell help**. *Science* 2003, **302**:659-662.

112. Beuneu H, Garcia Z, Bousso P: **Cutting edge: cognate CD4 help promotes recruitment of antigen-specific CD8 T cells around dendritic cells**. *J Immunol* 2006, **177**:1406-1410.

113. Alpdogan O, van den Brink MR: **IL-7 and IL-15: therapeutic cytokines for immunodeficiency**. *Trends Immunol* 2005, **26**:56-64.

114. Mastroianni CM, d'Ettorre G, Forcina G, Vullo V: **Teaching tired T cells to fight HIV: time to test IL-15 for immunotherapy?** *Trends Immunol* 2004, **25**:121-125.

115. Melchionda F, Fry TJ, Milliron MJ, McKirdy MA, Tagaya Y, Mackall CL: **Adjuvant IL-7 or IL-15 overcomes immunodominance and improves survival of the CD8+ memory cell pool**. *J Clin Invest* 2005, **115**:1177-1187.

116. Lubong R, Ng HL, Uittenbogaart CH, Yang OO: **Culturing of HIV-1-specific cytotoxic T lymphocytes with interleukin-7 and interleukin-15**. *Virology* 2004, **325**:175-180.

117. Fonseca SG, Reis MM, Coelho V, Nogueira LG, Monteiro SM, Mairena EC, Bacal F, Bocchi E, Guilherme L, Zheng XX, et al.: **Locally Produced Survival Cytokines IL-15 and IL-7 may be Associated to the Predominance of CD8(+) T cells at Heart Lesions of Human Chronic Chagas Disease Cardiomyopathy**. *Scand J Immunol* 2007, **66**:362-371.

118. Mueller YM, Bojczuk PM, Halstead ES, Kim AH, Witek J, Altman JD, Katsikis PD: **IL-15 enhances survival and function of HIV-specific CD8+ T cells**. *Blood* 2003, **101**:1024-1029.

119. Mueller YM, Makar V, Bojczuk PM, Witek J, Katsikis PD: **IL-15 enhances the function and inhibits CD95/Fas-induced apoptosis of human CD4+ and CD8+ effector-memory T cells**. *Int Immunol* 2003, **15**:49-58.

120. Dubsky P, Saito H, Leogier M, Dantin C, Connolly JE, Banchereau J, Palucka AK: **IL-15-induced human DC efficiently prime melanoma-specific naive CD8+ T cells to differentiate into CTL**. *Eur J Immunol* 2007, **37**:1678-1690.

121. Sato N, Patel HJ, Waldmann TA, Tagaya Y: **The IL-15/IL-15Ralpha on cell surfaces enables sustained IL-15 activity and contributes to the long survival of CD8 memory T cells**. *Proc Natl Acad Sci U S A* 2007, **104**:588-593.

122. Zhang X, Sun S, Hwang I, Tough DF, Sprent J: **Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15**. *Immunity* 1998, **8**:591-599.

123. Tanchot C, Lemonnier FA, Perarnau B, Freitas AA, Rocha B: **Differential requirements for survival and proliferation of CD8 naive or memory T cells**. *Science* 1997, **276**:2057-2062.

124. Smeltz RB: **Profound enhancement of the IL-12/IL-18 pathway of IFN-gamma secretion in human CD8+ memory T cell subsets via IL-15**. *J Immunol* 2007, **178**:4786-4792.

125. Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, Matsuki N, Charrier K, Sedger L, Willis CR, et al.: **Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice**. *J Exp Med* 2000, **191**:771-780.

126. Lodolce JP, Boone DL, Chai S, Swain RE, Dassopoulos T, Trettin S, Ma A: **IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation**. *Immunity* 1998, **9**:669-676.

127. Ku CC, Murakami M, Sakamoto A, Kappler J, Marrack P: **Control of homeostasis of CD8+ memory T cells by opposing cytokines**. *Science* 2000, **288**:675-678.

128. Stoklasek TA, Schluns KS, Lefrancois L: **Combined IL-15/IL-15Ralpha immunotherapy maximizes IL-15 activity in vivo**. *J Immunol* 2006, **177**:6072-6080.

129. Grabstein KH, Eisenman J, Shanebeck K, Rauch C, Srinivasan S, Fung V, Beers C, Richardson J, Schoenborn MA, Ahdieh M, et al.: **Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor**. *Science* 1994, **264**:965-968.

130. Giri JG, Anderson DM, Kumaki S, Park LS, Grabstein KH, Cosman D: **IL-15, a novel T cell growth factor that shares activities and receptor components with IL-2**. *J Leukoc Biol* 1995, **57**:763-766.

131. Berard M, Brandt K, Bulfone-Paus S, Tough DF: **IL-15 promotes the survival of naive and memory phenotype CD8+ T cells**. *J Immunol* 2003, **170**:5018-5026.

132. Kaech SM, Hemby S, Kersh E, Ahmed R: **Molecular and functional profiling of memory CD8 T cell differentiation**. *Cell* 2002, **111**:837-851.

133. Kaech SM, Wherry EJ, Ahmed R: **Effector and memory T-cell differentiation: implications for vaccine development**. *Nat Rev Immunol* 2002, **2**:251-262.

134. Homann D, Teyton L, Oldstone MB: **Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory**. *Nat Med* 2001, **7**:913-919.

135. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM: **Phenotypic analysis of antigen-specific T lymphocytes**. *Science* 1996, **274**:94-96.

136. Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, Segal JP, Cao Y, Rowland-Jones SL, Cerundolo V, et al.: **Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA**. *Science* 1998, **279**:2103-2106.

137. Becker TC, Wherry EJ, Boone D, Murali-Krishna K, Antia R, Ma A, Ahmed R: **Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells**. *J Exp Med* 2002, **195**:1541-1548.

138. Goldrath AW, Sivakumar PV, Glaccum M, Kennedy MK, Bevan MJ, Benoist C, Mathis D, Butz EA: **Cytokine requirements for acute and Basal homeostatic proliferation of naive and memory CD8+ T cells**. *J Exp Med* 2002, **195**:1515-1522.

139. Kieper WC, Tan JT, Bondi-Boyd B, Gapin L, Sprent J, Ceredig R, Surh CD: **Overexpression of interleukin (IL)-7 leads to IL-15-independent generation of memory phenotype CD8+ T cells**. *J Exp Med* 2002, **195**:1533-1539.

140. Tan JT, Ernst B, Kieper WC, LeRoy E, Sprent J, Surh CD: **Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells**. *J Exp Med* 2002, **195**:1523-1532.

141. Schluns KS, Williams K, Ma A, Zheng XX, Lefrancois L: **Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells**. *J Immunol* 2002, **168**:4827-4831.

142. Schluns KS, Kieper WC, Jameson SC, Lefrancois L: **Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo**. *Nat Immunol* 2000, **1**:426-432.

143. Gaschen B, Taylor J, Yusim K, Foley B, Gao F, Lang D, Novitsky V, Haynes B, Hahn BH, Bhattacharya T, et al.: **Diversity considerations in HIV-1 vaccine selection**. *Science* 2002, **296**:2354-2360.

144. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R: **Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment**. *J Virol* 2003, **77**:4911-4927.

145. Dudley ME: **A stimulating presentation**. *Nat Biotechnol* 2002, **20**:125-126.

146. Starcich BR, Hahn BH, Shaw GM, McNeely PD, Modrow S, Wolf H, Parks ES, Parks WP, Josephs SF, Gallo RC, et al.: **Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS**. *Cell* 1986, **45**:637-648.

147. Modrow S, Hahn BH, Shaw GM, Gallo RC, Wong-Staal F, Wolf H: **Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions**. *J Virol* 1987, **61**:570-578.

148. Coffin JM: **Genetic diversity and evolution of retroviruses**. *Curr Top Microbiol Immunol* 1992, **176**:143-164.

149. Bebenek K, Abbotts J, Wilson SH, Kunkel TA: **Error-prone polymerization by HIV-1 reverse transcriptase. Contribution of template-primer misalignment, miscoding, and termination probability to mutational hot spots**. *J Biol Chem* 1993, **268**:10324-10334.

150. Preston BD, Poiesz BJ, Loeb LA: **Fidelity of HIV-1 reverse transcriptase**. *Science* 1988, **242**:1168-1171.

151. Roberts JD, Bebenek K, Kunkel TA: **The accuracy of reverse transcriptase from HIV-1**. *Science* 1988, **242**:1171-1173.

152. Wain-Hobson S: **The fastest genome evolution ever described: HIV variation in situ**. *Curr Opin Genet Dev* 1993, **3**:878-883.

153. Perelson AS, Hightower R, Forrest S: **Evolution and somatic learning in V-region genes**. *Res Immunol* 1996, **147**:202-208.

154. Perelson AS, Neumann AU, Markowitz M, Leonard JM, Ho DD: **HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time**. *Science* 1996, **271**:1582-1586.

155. Yewdell J, Anton LC, Bacik I, Schubert U, Snyder HL, Bennink JR: **Generating MHC class I ligands from viral gene products**. *Immunol Rev* 1999, **172**:97-108.

156. Yewdell JW, Bennink JR: **Mechanisms of viral interference with MHC class I antigen processing and presentation**. *Annu Rev Cell Dev Biol* 1999, **15**:579-606.

157. Hillis DM, Moritz C, Porter CA, Baker RJ: **Evidence for biased gene conversion in concerted evolution of ribosomal DNA**. *Science* 1991, **251**:308-310.

158. Little JB, Benjamin MB: **Molecular structure of mutations at an autosomal locus in human cells: evidence for interallelic homologous recombination**. *Ann Genet* 1991, **34**:161-166.

159. Carrington M, Nelson GW, Martin MP, Kissner T, Vlahov D, Goedert JJ, Kaslow R, Buchbinder S, Hoots K, O'Brien SJ: **HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage**. *Science* 1999, **283**:1748-1752.

160. Goulder PJ, Bunce M, Krausa P, McIntyre K, Crowley S, Morgan B, Edwards A, Giangrande P, Phillips RE, McMichael AJ: **Novel, cross-restricted, conserved, and immunodominant cytotoxic T lymphocyte epitopes in slow progressors in HIV type 1 infection**. *AIDS Res Hum Retroviruses* 1996, **12**:1691-1698.

161. Tang J, Wilson CM, Meleth S, Myracle A, Lobashevsky E, Mulligan MJ, Douglas SD, Korber B, Vermund SH, Kaslow RA: **Host genetic profiles predict virological and immunological control of HIV-1 infection in adolescents**. *Aids* 2002, **16**:2275-2284.

162. Gao X, Nelson GW, Karacki P, Martin MP, Phair J, Kaslow R, Goedert JJ, Buchbinder S, Hoots K, Vlahov D, et al.: **Effect of a single amino acid change in MHC class I molecules on the rate of progression to AIDS**. *N Engl J Med* 2001, **344**:1668-1675.

163. Kaslow RA, Rivers C, Tang J, Bender TJ, Goepfert PA, El Habib R, Weinhold K, Mulligan MJ: **Polymorphisms in HLA class I genes associated with both favorable prognosis of human immunodeficiency virus (HIV) type 1 infection and positive cytotoxic T-lymphocyte responses to ALVAC-HIV recombinant canarypox vaccines**. *J Virol* 2001, **75**:8681-8689.

164. Altfeld M, Addo MM, Rosenberg ES, Hecht FM, Lee PK, Vogel M, Yu XG, Draenert R, Johnston MN, Strick D, et al.: **Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection**. *Aids* 2003, **17**:2581-2591.

165. Jansen CA, Kostense S, Vandenberghe K, Nanlohy NM, De Cuyper IM, Piriou E, Manting EH, Miedema F, van Baarle D: **High responsiveness of HLA-B57-restricted Gag-specific CD8+ T cells in vitro may contribute to the protective effect of HLA-B57 in HIV-infection**. *Eur J Immunol* 2005, **35**:150-158.

166. Lopez-Larrea C, Njobvu PD, Gonzalez S, Blanco-Gelaz MA, Martinez-Borra J, Lopez-Vazquez A: **The HLA-B*5703 allele confers susceptibility to the development of spondylarthropathies in Zambian human immunodeficiency virus-infected patients**

with slow progression to acquired immunodeficiency syndrome. *Arthritis Rheum* 2005, **52**:275-279.

167. Frahm N, Adams S, Kiepiela P, Linde CH, Hewitt HS, Lichterfeld M, Sango K, Brown NV, Pae E, Wurcel AG, et al.: **HLA-B63 presents HLA-B57/B58-restricted cytotoxic T-lymphocyte epitopes and is associated with low human immunodeficiency virus load**. *J Virol* 2005, **79**:10218-10225.

168. McNeil AJ, Yap PL, Gore SM, Brettle RP, McColl M, Wyld R, Davidson S, Weightman R, Richardson AM, Robertson JR: **Association of HLA types A1-B8-DR3 and B27 with rapid and slow progression of HIV disease**. *Qjm* 1996, **89**:177-185.

169. Reid SW, McAdam S, Smith KJ, Klenerman P, O'Callaghan CA, Harlos K, Jakobsen BK, McMichael AJ, Bell JI, Stuart DI, et al.: **Antagonist HIV-1 Gag peptides induce structural changes in HLA B8**. *J Exp Med* 1996, **184**:2279-2286.

170. Jin X, Gao X, Ramanathan M, Jr., Deschenes GR, Nelson GW, O'Brien SJ, Goedert JJ, Ho DD, O'Brien TR, Carrington M: **Human immunodeficiency virus type 1 (HIV-1)-specific CD8+-T-cell responses for groups of HIV-1-infected individuals with different HLA-B*35 genotypes**. *J Virol* 2002, **76**:12603-12610.

171. Carrington CV, Kondeatis E, Ramdath DD, Norman PJ, Vaughan RW, Stephens HA: **A comparison of HLA-DR and -DQ allele and haplotype frequencies in Trinidadian populations of African, South Asian, and mixed ancestry**. *Hum Immunol* 2002, **63**:1045-1054.

172. Kiepiela P, Leslie AJ, Honeyborne I, Ramduth D, Thobakgale C, Chetty S, Rathnavalu P, Moore C, Pfafferott KJ, Hilton L, et al.: **Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA**. *Nature* 2004, **432**:769-775.

173. Fellay J, Shianna KV, Ge D, Colombo S, Ledergerber B, Weale M, Zhang K, Gumbs C, Castagna A, Cossarizza A, et al.: **A whole-genome association study of major determinants for host control of HIV-1**. *Science* 2007, **317**:944-947.

174. O'Brien SJ, Nelson GW: **Human genes that limit AIDS**. *Nat Genet* 2004, **36**:565-574.

175. Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R, Goedert JJ, Buchbinder SP, Vittinghoff E, Gomperts E, et al.: **Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study**. *Science* 1996, **273**:1856-1862.

176. Reiche EM, Bonametti AM, Voltarelli JC, Morimoto HK, Watanabe MA: **Genetic polymorphisms in the chemokine and chemokine receptors: impact on clinical**

course and therapy of the human immunodeficiency virus type 1 infection (HIV-1). *Curr Med Chem* 2007, **14**:1325-1334.

177. Mann DL, Garner RP, Dayhoff DE, Cao K, Fernandez-Vina MA, Davis C, Aronson N, Ruiz N, Birx DL, Michael NL: **Major histocompatibility complex genotype is associated with disease progression and virus load levels in a cohort of human immunodeficiency virus type 1-infected Caucasians and African Americans**. *J Infect Dis* 1998, **178**:1799-1802.

178. Carrington M, O'Brien SJ: **The influence of HLA genotype on AIDS**. *Annu Rev Med* 2003, **54**:535-551.

179. Kaslow RA, Carrington M, Apple R, Park L, Munoz A, Saah AJ, Goedert JJ, Winkler C, O'Brien SJ, Rinaldo C, et al.: **Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection**. *Nat Med* 1996, **2**:405-411.

180. Kutsch O, Vey T, Kerkau T, Hunig T, Schimpl A: **HIV type 1 abrogates TAP-mediated transport of antigenic peptides presented by MHC class I. Transporter associated with antigen presentation**. *AIDS Res Hum Retroviruses* 2002, **18**:1319-1325.

181. Obst R, Armandola EA, Nijenhuis M, Momburg F, Hammerling GJ: **TAP polymorphism does not influence transport of peptide variants in mice and humans**. *Eur J Immunol* 1995, **25**:2170-2176.

182. Powis SJ, Deverson EV, Coadwell WJ, Ciruela A, Huskisson NS, Smith H, Butcher GW, Howard JC: **Effect of polymorphism of an MHC-linked transporter on the peptides assembled in a class I molecule**. *Nature* 1992, **357**:211-215.

183. Walker BD, Chakrabarti S, Moss B, Paradis TJ, Flynn T, Durno AG, Blumberg RS, Kaplan JC, Hirsch MS, Schooley RT: **HIV-specific cytotoxic T lymphocytes in seropositive individuals**. *Nature* 1987, **328**:345-348.

184. Jameson SC, Carbone FR, Bevan MJ: **Clone-specific T cell receptor antagonists of major histocompatibility complex class I-restricted cytotoxic T cells**. *J Exp Med* 1993, **177**:1541-1550.

185. Sloan-Lancaster J, Evavold BD, Allen PM: **Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells**. *Nature* 1993, **363**:156-159.

186. Pircher H, Moskophidis D, Rohrer U, Burki K, Hengartner H, Zinkernagel RM: **Viral escape by selection of cytotoxic T cell-resistant virus variants in vivo**. *Nature* 1990, **346**:629-633.

187. Culmann B, Gomard E, Kieny MP, Guy B, Dreyfus F, Saimot AG, Sereni D, Levy JP: **An antigenic peptide of the HIV-1 NEF protein recognized by cytotoxic T lymphocytes**

of seropositive individuals in association with different HLA-B molecules. *Eur J Immunol* 1989, **19**:2383-2386.

188. Culmann B, Gomard E, Kieny MP, Guy B, Dreyfus F, Saimot AG, Sereni D, Sicard D, Levy JP: **Six epitopes reacting with human cytotoxic CD8+ T cells in the central region of the HIV-1 NEF protein**. *J Immunol* 1991, **146**:1560-1565.

189. Hadida F, Parrot A, Kieny MP, Sadat-Sowti B, Mayaud C, Debre P, Autran B: **Carboxyl-terminal and central regions of human immunodeficiency virus-1 NEF recognized by cytotoxic T lymphocytes from lymphoid organs. An in vitro limiting dilution analysis**. *J Clin Invest* 1992, **89**:53-60.

190. Couillin I, Connan F, Culmann-Penciolelli B, Gomard E, Guillet JG, Choppin J: **HLA-dependent variations in human immunodeficiency virus Nef protein alter peptide/HLA binding**. *Eur J Immunol* 1995, **25**:728-732.

191. Borrow P, Lewicki H, Wei X, Horwitz MS, Peffer N, Meyers H, Nelson JA, Gairin JE, Hahn BH, Oldstone MB, et al.: **Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus**. *Nat Med* 1997, **3**:205-211.

192. Goulder PJ, Edwards A, Phillips RE, McMichael AJ: **Identification of a novel HLA-B*2705-restricted cytotoxic T-lymphocyte epitope within a conserved region of HIV-1 Nef**. *Aids* 1997, **11**:536-538.

193. Brumme ZL, Brumme CJ, Heckerman D, Korber BT, Daniels M, Carlson J, Kadie C, Bhattacharya T, Chui C, Szinger J, et al.: **Evidence of Differential HLA Class I-Mediated Viral Evolution in Functional and Accessory/Regulatory Genes of HIV-1**. *PLoS Pathog* 2007, **3**:e94.

194. Leslie A, Kavanagh D, Honeyborne I, Pfafferott K, Edwards C, Pillay T, Hilton L, Thobakgale C, Ramduth D, Draenert R, et al.: **Transmission and accumulation of CTL escape variants drive negative associations between HIV polymorphisms and HLA**. *J Exp Med* 2005, **201**:891-902.

195. Leslie A, Price DA, Mkhize P, Bishop K, Rathod A, Day C, Crawford H, Honeyborne I, Asher TE, Luzzi G, et al.: **Differential selection pressure exerted on HIV by CTL targeting identical epitopes but restricted by distinct HLA alleles from the same HLA supertype**. *J Immunol* 2006, **177**:4699-4708.

196. Martinez-Picado J, Prado JG, Fry EE, Pfafferott K, Leslie A, Chetty S, Thobakgale C, Honeyborne I, Crawford H, Matthews P, et al.: **Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1**. *J Virol* 2006, **80**:3617-3623.

197. Feeney ME, Tang Y, Roosevelt KA, Leslie AJ, McIntosh K, Karthas N, Walker BD, Goulder PJ: **Immune escape precedes breakthrough human immunodeficiency virus type 1 viremia and broadening of the cytotoxic T-lymphocyte response in an HLA-B27-positive long-term-nonprogressing child**. *J Virol* 2004, **78**:8927-8930.

198. Draenert R, Verrill CL, Tang Y, Allen TM, Wurcel AG, Boczanowski M, Lechner A, Kim AY, Suscovich T, Brown NV, et al.: **Persistent recognition of autologous virus by high-avidity CD8 T cells in chronic, progressive human immunodeficiency virus type 1 infection**. *J Virol* 2004, **78**:630-641.

199. Loffredo JT, Sidney J, Wojewoda C, Dodds E, Reynolds MR, Napoe G, Mothe BR, O'Connor DH, Wilson NA, Watkins DI, et al.: **Identification of seventeen new simian immunodeficiency virus-derived CD8+ T cell epitopes restricted by the high frequency molecule, Mamu-A*02, and potential escape from CTL recognition**. *J Immunol* 2004, **173**:5064-5076.

200. Friedrich TC, Dodds EJ, Yant LJ, Vojnov L, Rudersdorf R, Cullen C, Evans DT, Desrosiers RC, Mothe BR, Sidney J, et al.: **Reversion of CTL escape-variant immunodeficiency viruses in vivo**. *Nat Med* 2004, **10**:275-281.

201. Friedrich TC, McDermott AB, Reynolds MR, Piaskowski S, Fuenger S, De Souza IP, Rudersdorf R, Cullen C, Yant LJ, Vojnov L, et al.: **Consequences of cytotoxic T-lymphocyte escape: common escape mutations in simian immunodeficiency virus are poorly recognized in naive hosts**. *J Virol* 2004, **78**:10064-10073.

202. Allen TM, O'Connor DH, Jing P, Dzuris JL, Mothe BR, Vogel TU, Dunphy E, Liebl ME, Emerson C, Wilson N, et al.: **Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia**. *Nature* 2000, **407**:386-390.

203. Evans DT, Knapp LA, Jing P, Mitchen JL, Dykhuizen M, Montefiori DC, Pauza CD, Watkins DI: **Rapid and slow progressors differ by a single MHC class I haplotype in a family of MHC-defined rhesus macaques infected with SIV**. *Immunol Lett* 1999, **66**:53-59.

204. Gaudieri S, Rauch A, Park LP, Freitas E, Herrmann S, Jeffrey G, Cheng W, Pfafferott K, Naidoo K, Chapman R, et al.: **Evidence of viral adaptation to HLA class I-restricted immune pressure in chronic hepatitis C virus infection**. *J Virol* 2006, **80**:11094-11104.

205. Brander C, Walker BD: **Gradual adaptation of HIV to human host populations: good or bad news?** *Nat Med* 2003, **9**:1359-1362.

206. Kiepiela P, Ngumbela K, Thobakgale C, Ramduth D, Honeyborne I, Moodley E, Reddy S, de Pierres C, Mncube Z, Mkhwanazi N, et al.: **CD8+ T-cell responses to different HIV proteins have discordant associations with viral load**. *Nat Med* 2007, **13**:46-53.

207. Lu W, Andrieu JM: **In vitro human immunodeficiency virus eradication by autologous CD8(+) T cells expanded with inactivated-virus-pulsed dendritic cells**. *J Virol* 2001, **75**:8949-8956.

208. Larsson M, Fonteneau JF, Lirvall M, Haslett P, Lifson JD, Bhardwaj N: **Activation of HIV-1 specific CD4 and CD8 T cells by human dendritic cells: roles for cross-presentation and non-infectious HIV-1 virus**. *Aids* 2002, **16**:1319-1329.

209. Fonteneau JF, Gilliet M, Larsson M, Dasilva I, Munz C, Liu YJ, Bhardwaj N: **Activation of influenza virus-specific CD4+ and CD8+ T cells: a new role for plasmacytoid dendritic cells in adaptive immunity**. *Blood* 2003, **101**:3520-3526.

210. Harshyne LA, Watkins SC, Gambotto A, Barratt-Boyes SM: **Dendritic cells acquire antigens from live cells for cross-presentation to CTL**. *J Immunol* 2001, **166**:3717-3723.

211. Zhao XQ, Huang XL, Gupta P, Borowski L, Fan Z, Watkins SC, Thomas EK, Rinaldo CR, Jr.: **Induction of anti-human immunodeficiency virus type 1 (HIV-1) CD8(+) and CD4(+) T-cell reactivity by dendritic cells loaded with HIV-1 X4-infected apoptotic cells**. *J Virol* 2002, **76**:3007-3014.

212. Fan Z, Huang XL, Borowski L, Mellors JW, Rinaldo CR, Jr.: **Restoration of anti-human immunodeficiency virus type 1 (HIV-1) responses in CD8+ T cells from late-stage patients on prolonged antiretroviral therapy by stimulation in vitro with HIV-1 protein-loaded dendritic cells**. *J Virol* 2001, **75**:4413-4419.

213. Tanaka Y, Dowdy SF, Linehan DC, Eberlein TJ, Goedegebuure PS: **Induction of antigen-specific CTL by recombinant HIV trans-activating fusion protein-pulsed human monocyte-derived dendritic cells**. *J Immunol* 2003, **170**:1291-1298.

214. Andrieu M, Desoutter JF, Loing E, Gaston J, Hanau D, Guillet JG, Hosmalin A: **Two human immunodeficiency virus vaccinal lipopeptides follow different cross-presentation pathways in human dendritic cells**. *J Virol* 2003, **77**:1564-1570.

215. Weissman D, Ni H, Scales D, Dude A, Capodici J, McGibney K, Abdool A, Isaacs SN, Cannon G, Kariko K: **HIV gag mRNA transfection of dendritic cells (DC) delivers encoded antigen to MHC class I and II molecules, causes DC maturation, and induces a potent human in vitro primary immune response**. *J Immunol* 2000, **165**:4710-4717.

216. Hokey DA, Weiner DB: **DNA vaccines for HIV: challenges and opportunities**. *Springer Semin Immunopathol* 2006, **28**:267-279.

217. Lori F, Kelly LM, Lisziewicz J: **APC-targeted immunization for the treatment of HIV-1**. *Expert Rev Vaccines* 2004, **3**:S189-198.

218. Kavanagh DG, Kaufmann DE, Sunderji S, Frahm N, Le Gall S, Boczkowski D, Rosenberg ES, Stone DR, Johnston MN, Wagner BS, et al.: **Expansion of HIV-specific CD4+ and CD8+ T cells by dendritic cells transfected with mRNA encoding cytoplasm- or lysosome-targeted Nef**. *Blood* 2006, **107**:1963-1969.

219. Fan Z, Huang XL, Kalinski P, Young S, Rinaldo CR, Jr.: **Dendritic cell function during chronic hepatitis C virus and HIV-1 infection**. *Clin Vaccine Immunol* 2007.

220. Mailliard RB, Wankowicz-Kalinska A, Cai Q, Wesa A, Hilkens CM, Kapsenberg ML, Kirkwood JM, Storkus WJ, Kalinski P: **alpha-type-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity**. *Cancer Res* 2004, **64**:5934-5937.

221. Barber LD, Jordan S, Whitelegg AM, Madrigal JA, Savage P: **HLA class I mono-specific APCs and target cells: a method to standardise in vitro CD8+ T cell expansion and functional assays**. *J Immunol Methods* 2006, **314**:147-152.

222. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, et al.: **PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression**. *Nature* 2006, **443**:350-354.

223. Petrovas C, Casazza JP, Brenchley JM, Price DA, Gostick E, Adams WC, Precopio ML, Schacker T, Roederer M, Douek DC, et al.: **PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection**. *J Exp Med* 2006, **203**:2281-2292.

224. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, Boulassel MR, Delwart E, Sepulveda H, Balderas RS, et al.: **Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction**. *Nat Med* 2006, **12**:1198-1202.

225. Zhang Y, Chung Y, Bishop C, Daugherty B, Chute H, Holst P, Kurahara C, Lott F, Sun N, Welcher AA, et al.: **Regulation of T cell activation and tolerance by PDL2**. *Proc Natl Acad Sci U S A* 2006, **103**:11695-11700.

226. Freeman GJ, Wherry EJ, Ahmed R, Sharpe AH: **Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade**. *J Exp Med* 2006, **203**:2223-2227.

227. Keir ME, Francisco LM, Sharpe AH: **PD-1 and its ligands in T-cell immunity**. *Curr Opin Immunol* 2007, **19**:309-314.

228. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ: **Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses**. *Immunity* 2007, **27**:111-122.

229. Sankaran S, Guadalupe M, Reay E, George MD, Flamm J, Prindiville T, Dandekar S: **Gut mucosal T cell responses and gene expression correlate with protection against**

disease in long-term HIV-1-infected nonprogressors. *Proc Natl Acad Sci U S A* 2005, **102**:9860-9865.

230. Parker KC, Bednarek MA, Coligan JE: **Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains**. *J Immunol* 1994, **152**:163-175.

231. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S: **SYFPEITHI: database for MHC ligands and peptide motifs**. *Immunogenetics* 1999, **50**:213-219.

232. Reche PA, Glutting JP, Zhang H, Reinherz EL: **Enhancement to the RANKPEP resource for the prediction of peptide binding to MHC molecules using profiles**. *Immunogenetics* 2004, **56**:405-419.

233. Korber B, LaBute M, Yusim K: **Immunoinformatics comes of age**. *PLoS Comput Biol* 2006, **2**:e71.

234. Peters B, Bui HH, Frankild S, Nielson M, Lundegaard C, Kostem E, Basch D, Lamberth K, Harndahl M, Fleri W, et al.: **A community resource benchmarking predictions of peptide binding to MHC-I molecules**. *PLoS Comput Biol* 2006, **2**:e65.

235. Lipford GB, Hoffman M, Wagner H, Heeg K: **Primary in vivo responses to ovalbumin. Probing the predictive value of the Kb binding motif**. *J Immunol* 1993, **150**:1212-1222.

236. Nijman HW, Houbiers JG, Vierboom MP, van der Burg SH, Drijfhout JW, D'Amaro J, Kenemans P, Melief CJ, Kast WM: **Identification of peptide sequences that potentially trigger HLA-A2.1-restricted cytotoxic T lymphocytes**. *Eur J Immunol* 1993, **23**:1215-1219.

237. Calin-Laurens V, Trescol-Biemont MC, Gerlier D, Rabourdin-Combe C: **Can one predict antigenic peptides for MHC class I-restricted cytotoxic T lymphocytes useful for vaccination?** *Vaccine* 1993, **11**:974-978.

238. Uebel S, Kraas W, Kienle S, Wiesmuller KH, Jung G, Tampe R: **Recognition principle of the TAP transporter disclosed by combinatorial peptide libraries**. *Proc Natl Acad Sci U S A* 1997, **94**:8976-8981.

239. Yewdell JW, Hill AB: **Viral interference with antigen presentation**. *Nat Immunol* 2002, **3**:1019-1025.

240. Kast WM, Brandt RM, Sidney J, Drijfhout JW, Kubo RT, Grey HM, Melief CJ, Sette A: **Role of HLA-A motifs in identification of potential CTL epitopes in human papillomavirus type 16 E6 and E7 proteins**. *J Immunol* 1994, **152**:3904-3912.

241. Oldstone MB: **Viral persistence: mechanisms and consequences**. *Curr Opin Microbiol* 1998, **1**:436-441.

242. Huang XL, Fan Z, Zheng L, Borowski L, Li H, Thomas EK, Hildebrand WH, Zhao XQ, Rinaldo CR, Jr.: **Priming of human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T cell responses by dendritic cells loaded with HIV-1 proteins**. *J Infect Dis* 2003, **187**:315-319.

243. Zarling AL, Johnson JG, Hoffman RW, Lee DR: **Induction of primary human CD8+ T lymphocyte responses in vitro using dendritic cells**. *J Immunol* 1999, **162**:5197-5204.

244. Fonteneau JF, Larsson M, Somersan S, Sanders C, Munz C, Kwok WW, Bhardwaj N, Jotereau F: **Generation of high quantities of viral and tumor-specific human CD4+ and CD8+ T-cell clones using peptide pulsed mature dendritic cells**. *J Immunol Methods* 2001, **258**:111-126.

245. Bevan MJ: **Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming**. *J Immunol* 1976, **117**:2233-2238.

246. Steinman RM, Pope M: **Exploiting dendritic cells to improve vaccine efficacy**. *J Clin Invest* 2002, **109**:1519-1526.

247. Moser M: **Dendritic cells in immunity and tolerance-do they display opposite functions?** *Immunity* 2003, **19**:5-8.

248. Sun JC, Bevan MJ: **Defective CD8 T cell memory following acute infection without CD4 T cell help**. *Science* 2003, **300**:339-342.

249. Hochrein H, Shortman K, Vremec D, Scott B, Hertzog P, O'Keeffe M: **Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets**. *J Immunol* 2001, **166**:5448-5455.

250. Schmidt CS, Mescher MF: **Adjuvant effect of IL-12: conversion of peptide antigen administration from tolerizing to immunizing for CD8+ T cells in vivo**. *J Immunol* 1999, **163**:2561-2567.

251. Curtsinger JM, Schmidt CS, Mondino A, Lins DC, Kedl RM, Jenkins MK, Mescher MF: **Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells**. *J Immunol* 1999, **162**:3256-3262.

252. Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K: **Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection**. *J Exp Med* 2005, **202**:637-650.

253. Curtsinger JM, Lins DC, Mescher MF: **Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function**. *J Exp Med* 2003, **197**:1141-1151.

254. Bevan MJ, Fink PJ: **The CD8 response on autopilot**. *Nat Immunol* 2001, **2**:381-382.

255. Hugues S, Fetler L, Bonifaz L, Helft J, Amblard F, Amigorena S: **Distinct T cell dynamics in lymph nodes during the induction of tolerance and immunity**. *Nat Immunol* 2004, **5**:1235-1242.

256. Mempel TR, Henrickson SE, Von Andrian UH: **T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases**. *Nature* 2004, **427**:154-159.

257. Mansky LM, Temin HM: **Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase**. *J Virol* 1995, **69**:5087-5094.

258. Pathak VK, Temin HM: **Broad spectrum of in vivo forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: substitutions, frameshifts, and hypermutations**. *Proc Natl Acad Sci U S A* 1990, **87**:6019-6023.

259. Williams KJ, Loeb LA: **Retroviral reverse transcriptases: error frequencies and mutagenesis**. *Curr Top Microbiol Immunol* 1992, **176**:165-180.

260. Eigen M: **The origin of genetic information: viruses as models**. *Gene* 1993, **135**:37-47.

261. Madden DR: **The three-dimensional structure of peptide-MHC complexes**. *Annu Rev Immunol* 1995, **13**:587-622.

262. Brusic V, Bajic VB, Petrovsky N: **Computational methods for prediction of T-cell epitopes--a framework for modelling, testing, and applications**. *Methods* 2004, **34**:436-443.

263. Falk K, Rotzschke O, Deres K, Metzger J, Jung G, Rammensee HG: **Identification of naturally processed viral nonapeptides allows their quantification in infected cells and suggests an allele-specific T cell epitope forecast**. *J Exp Med* 1991, **174**:425-434.

264. Bouvier M, Wiley DC: **Importance of peptide amino and carboxyl termini to the stability of MHC class I molecules**. *Science* 1994, **265**:398-402.

265. Ruppert J, Sidney J, Celis E, Kubo RT, Grey HM, Sette A: **Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules**. *Cell* 1993, **74**:929-937.

266. Sette A, Alexander J, Ruppert J, Snoke K, Franco A, Ishioka G, Grey HM: **Antigen analogs/MHC complexes as specific T cell receptor antagonists**. *Annu Rev Immunol* 1994, **12**:413-431.

267. Sette A, Sidney J, del Guercio MF, Southwood S, Ruppert J, Dahlberg C, Grey HM, Kubo RT: **Peptide binding to the most frequent HLA-A class I alleles measured by quantitative molecular binding assays**. *Mol Immunol* 1994, **31**:813-822.

268. Sette A, Vitiello A, Reherman B, Fowler P, Nayersina R, Kast WM, Melief CJ, Oseroff C, Yuan L, Ruppert J, et al.: **The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes**. *J Immunol* 1994, **153**:5586-5592.

269. Flower DR, Doytchinova IA: **Immunoinformatics and the prediction of immunogenicity**. *Appl Bioinformatics* 2002, **1**:167-176.

270. Rosenfeld R, Vajda S, DeLisi C: **Flexible docking and design**. *Annu Rev Biophys Biomol Struct* 1995, **24**:677-700.

271. Rosenfeld R, Zheng Q, Vajda S, DeLisi C: **Flexible docking of peptides to class I major-histocompatibility-complex receptors**. *Genet Anal* 1995, **12**:1-21.

272. Tong JC, Tan TW, Ranganathan S: **Methods and protocols for prediction of immunogenic epitopes**. *Brief Bioinform* 2007, **8**:96-108.

273. Bui HH, Sidney J, Dinh K, Southwood S, Newman MJ, Sette A: **Predicting population coverage of T-cell epitope-based diagnostics and vaccines**. *BMC Bioinformatics* 2006, **7**:153.

274. Antes I, Siu SW, Lengauer T: **DynaPred: a structure and sequence based method for the prediction of MHC class I binding peptide sequences and conformations**. *Bioinformatics* 2006, **22**:e16-24.

275. Jojic N, Reyes-Gomez M, Heckerman D, Kadie C, Schueler-Furman O: **Learning MHC I--peptide binding**. *Bioinformatics* 2006, **22**:e227-235.

276. Reche PA, Glutting JP, Reinherz EL: **Prediction of MHC class I binding peptides using profile motifs**. *Hum Immunol* 2002, **63**:701-709.

277. Donnes P, Elofsson A: **Prediction of MHC class I binding peptides, using SVMHC**. *BMC Bioinformatics* 2002, **3**:25.

278. Zhang GL, Khan AM, Srinivasan KN, August JT, Brusic V: **MULTIPRED: a computational system for prediction of promiscuous HLA binding peptides**. *Nucleic Acids Res* 2005, **33**:W172-179.

279. Salter RD: **Mutant HLA-A201 heavy chains with lowered affinity for beta 2m are transported after growth at reduced temperatures**. *Hum Immunol* 1992, **35**:40-49.

280. Salter RD, Cresswell P: **Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid**. *Embo J* 1986, **5**:943-949.

281. Pogue RR, Eron J, Frelinger JA, Matsui M: **Amino-terminal alteration of the HLA-A*0201-restricted human immunodeficiency virus pol peptide increases complex stability and in vitro immunogenicity**. *Proc Natl Acad Sci U S A* 1995, **92**:8166-8170.

282. Kessler JH, Mommaas B, Mutis T, Huijbers I, Vissers D, Benckhuijsen WE, Schreuder GM, Offringa R, Goulmy E, Melief CJ, et al.: **Competition-based cellular peptide binding assays for 13 prevalent HLA class I alleles using fluorescein-labeled synthetic peptides**. *Hum Immunol* 2003, **64**:245-255.

283. Townsend A, Elliott T, Cerundolo V, Foster L, Barber B, Tse A: **Assembly of MHC class I molecules analyzed in vitro**. *Cell* 1990, **62**:285-295.

284. Elvin J, Potter C, Elliott T, Cerundolo V, Townsend A: **A method to quantify binding of unlabeled peptides to class I MHC molecules and detect their allele specificity**. *J Immunol Methods* 1993, **158**:161-171.

285. Fahnestock ML, Tamir I, Narhi L, Bjorkman PJ: **Thermal stability comparison of purified empty and peptide-filled forms of a class I MHC molecule**. *Science* 1992, **258**:1658-1662.

286. Ottenhoff TH, Geluk A, Toebes M, Benckhuijsen WE, van Meijgaarden KE, Drijfhout JW: **A sensitive fluorometric assay for quantitatively measuring specific peptide binding to HLA class I and class II molecules**. *J Immunol Methods* 1997, **200**:89-97.

287. Olsen AC, Pedersen LO, Hansen AS, Nissen MH, Olsen M, Hansen PR, Holm A, Buus S: **A quantitative assay to measure the interaction between immunogenic peptides and purified class I major histocompatibility complex molecules**. *Eur J Immunol* 1994, **24**:385-392.

288. Tan TL, Geluk A, Toebes M, Ottenhoff TH, Drijfhout JW: **A novel, highly efficient peptide-HLA class I binding assay using unfolded heavy chain molecules: identification of HIV-1 derived peptides that bind to HLA-A*0201 and HLA-A*0301**. *J Immunol Methods* 1997, **205**:201-209.

289. Dedier S, Reinelt S, Rion S, Folkers G, Rognan D: **Use of fluorescence polarization to monitor MHC-peptide interactions in solution**. *J Immunol Methods* 2001, **255**:57-66.

290. Buchli R, VanGundy RS, Hickman-Miller HD, Giberson CF, Bardet W, Hildebrand WH: **Development and validation of a fluorescence polarization-based competitive**

peptide-binding assay for HLA-A*0201--a new tool for epitope discovery. *Biochemistry* 2005, **44**:12491-12507.

291. Kaslow RA, Ostrow DG, Detels R, Phair JP, Polk BF, Rinaldo CR, Jr.: **The Multicenter AIDS Cohort Study: rationale, organization, and selected characteristics of the participants**. *Am J Epidemiol* 1987, **126**:310-318.

292. Jensen MA, Li FS, van 't Wout AB, Nickle DC, Shriner D, He HX, McLaughlin S, Shankarappa R, Margolick JB, Mullins JI: **Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 env V3 loop sequences**. *J Virol* 2003, **77**:13376-13388.

293. Jensen MA, van 't Wout AB: **Predicting HIV-1 coreceptor usage with sequence analysis**. *AIDS Rev* 2003, **5**:104-112.

294. Shankarappa R, Margolick JB, Gange SJ, Rodrigo AG, Upchurch D, Farzadegan H, Gupta P, Rinaldo CR, Learn GH, He X, et al.: **Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection**. *J Virol* 1999, **73**:10489-10502.

295. Shriner D, Rodrigo AG, Nickle DC, Mullins JI: **Pervasive genomic recombination of HIV-1 in vivo**. *Genetics* 2004, **167**:1573-1583.

296. Rinaldo CR, Jr., Gupta P, Huang XL, Fan Z, Mullins JI, Gange S, Farzadegan H, Shankarappa R, Munoz A, Margolick JB: **Anti-HIV type 1 memory cytotoxic T lymphocyte responses associated with changes in CD4+ T cell numbers in progression of HIV type 1 infection**. *AIDS Res Hum Retroviruses* 1998, **14**:1423-1433.

297. Gupta P, Ding M, Cottrill M, Rinaldo C, Kingsley L, Wolinsky S, Mellors J: **Quantitation of human immunodeficiency virus type 1 DNA and RNA by a novel internally controlled PCR assay**. *J Clin Microbiol* 1995, **33**:1670-1673.

298. Buchli R, VanGundy RS, Hickman-Miller HD, Giberson CF, Bardet W, Hildebrand WH: **Real-time measurement of in vitro peptide binding to soluble HLA-A*0201 by fluorescence polarization**. *Biochemistry* 2004, **43**:14852-14863.

299. Buchli R, Vangundy RS, Giberson CF, Hildebrand WH: **Critical factors in the development of fluorescence polarization-based peptide binding assays: an equilibrium study monitoring specific peptide binding to soluble HLA-A*0201**. *J Immunol Methods* 2006, **314**:38-53.

300. Kuttler C, Nussbaum AK, Dick TP, Rammensee HG, Schild H, Hadeler KP: **An algorithm for the prediction of proteasomal cleavages**. *J Mol Biol* 2000, **298**:417-429.

301. Holzhutter HG, Kloetzel PM: **A kinetic model of vertebrate 20S proteasome accounting for the generation of major proteolytic fragments from oligomeric peptide substrates**. *Biophys J* 2000, **79**:1196-1205.

302. Holzhutter HG, Frommel C, Kloetzel PM: **A theoretical approach towards the identification of cleavage-determining amino acid motifs of the 20 S proteasome**. *J Mol Biol* 1999, **286**:1251-1265.

303. Kesmir C, Nussbaum AK, Schild H, Detours V, Brunak S: **Prediction of proteasome cleavage motifs by neural networks**. *Protein Eng* 2002, **15**:287-296.

304. Yu K, Petrovsky N, Schonbach C, Koh JY, Brusic V: **Methods for prediction of peptide binding to MHC molecules: a comparative study**. *Mol Med* 2002, **8**:137-148.

305. Palella FJ, Jr., Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, Holmberg SD: **Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators**. *N Engl J Med* 1998, **338**:853-860.

306. Rinaldo CR, Jr., Huang XL, Fan Z, Margolick JB, Borowski L, Hoji A, Kalinyak C, McMahon DK, Riddler SA, Hildebrand WH, et al.: **Anti-human immunodeficiency virus type 1 (HIV-1) CD8(+) T-lymphocyte reactivity during combination antiretroviral therapy in HIV-1-infected patients with advanced immunodeficiency**. *J Virol* 2000, **74**:4127-4138.

307. Persaud D, Zhou Y, Siliciano JM, Siliciano RF: **Latency in human immunodeficiency virus type 1 infection: no easy answers**. *J Virol* 2003, **77**:1659-1665.

308. Cohen DE, Walker BD: **Human immunodeficiency virus pathogenesis and prospects for immune control in patients with established infection**. *Clin Infect Dis* 2001, **32**:1756-1768.

309. Piazza P, Fan Z, Rinaldo CR, Jr.: **CD8+ T-cell immunity to HIV infection**. *Clin Lab Med* 2002, **22**:773-797.

310. Kulkosky J, Nunnari G, Otero M, Calarota S, Dornadula G, Zhang H, Malin A, Sullivan J, Xu Y, DeSimone J, et al.: **Intensification and stimulation therapy for human immunodeficiency virus type 1 reservoirs in infected persons receiving virally suppressive highly active antiretroviral therapy**. *J Infect Dis* 2002, **186**:1403-1411.

311. Kulkosky J, Pomerantz RJ: **Approaching eradication of highly active antiretroviral therapy-persistent human immunodeficiency virus type 1 reservoirs with immune activation therapy**. *Clin Infect Dis* 2002, **35**:1520-1526.

312. Cohen OJ, Fauci AS: **Current strategies in the treatment of HIV infection**. *Adv Intern Med* 2001, **46**:207-246.

313. Banchereau J, Schuler-Thurner B, Palucka AK, Schuler G: **Dendritic cells as vectors for therapy**. *Cell* 2001, **106**:271-274.

314. Buchler T, Hajek R: **Dendritic cell vaccines in the treatment of multiple myeloma: advances and limitations**. *Med Oncol* 2002, **19**:213-218.

315. Parmiani G: **Vaccine therapy of cancer**. *Suppl Tumori* 2002, **1**:S28.

316. Parmiani G, Castelli C, Dalerba P, Mortarini R, Rivoltini L, Marincola FM, Anichini A: **Cancer immunotherapy with peptide-based vaccines: what have we achieved? Where are we going?** *J Natl Cancer Inst* 2002, **94**:805-818.

317. Bourgeois C, Rocha B, Tanchot C: **A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory**. *Science* 2002, **297**:2060-2063.

318. Filatenkov AA, Jacovetty EL, Fischer UB, Curtsinger JM, Mescher MF, Ingulli E: **CD4 T cell-dependent conditioning of dendritic cells to produce IL-12 results in CD8-mediated graft rejection and avoidance of tolerance**. *J Immunol* 2005, **174**:6909-6917.

319. Curtsinger JM, Gerner MY, Lins DC, Mescher MF: **Signal 3 availability limits the CD8 T cell response to a solid tumor**. *J Immunol* 2007, **178**:6752-6760.

320. Valenzuela J, Schmidt C, Mescher M: **The roles of IL-12 in providing a third signal for clonal expansion of naive CD8 T cells**. *J Immunol* 2002, **169**:6842-6849.

321. Chang J, Cho JH, Lee SW, Choi SY, Ha SJ, Sung YC: **IL-12 priming during in vitro antigenic stimulation changes properties of CD8 T cells and increases generation of effector and memory cells**. *J Immunol* 2004, **172**:2818-2826.

322. Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM: **The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation**. *J Exp Med* 2004, **199**:1607-1618.

323. Gately MK, Wolitzky AG, Quinn PM, Chizzonite R: **Regulation of human cytolytic lymphocyte responses by interleukin-12**. *Cell Immunol* 1992, **143**:127-142.

324. Trinchieri G: **Interleukin-12: a cytokine at the interface of inflammation and immunity**. *Adv Immunol* 1998, **70**:83-243.

325. Williams MA, Tyznik AJ, Bevan MJ: **Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells**. *Nature* 2006, **441**:890-893.

326. Newberg MH, McEvers KJ, Gorgone DA, Lifton MA, Baumeister SH, Veazey RS, Schmitz JE, Letvin NL: **Immunodomination in the evolution of dominant epitope-specific CD8+ T lymphocyte responses in simian immunodeficiency virus-infected rhesus monkeys**. *J Immunol* 2006, **176**:319-328.

327. Hammer SM, Squires KE, Hughes MD, Grimes JM, Demeter LM, Currier JS, Eron JJ, Jr., Feinberg JE, Balfour HH, Jr., Deyton LR, et al.: **A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team**. *N Engl J Med* 1997, **337**:725-733.

328. Gulick RM: **Current antiretroviral therapy: an overview**. *Qual Life Res* 1997, **6**:471-474.

329. Deeks SG, Hecht FM, Swanson M, Elbeik T, Loftus R, Cohen PT, Grant RM: **HIV RNA and CD4 cell count response to protease inhibitor therapy in an urban AIDS clinic: response to both initial and salvage therapy**. *Aids* 1999, **13**:F35-43.

330. Mezzaroma I, Carlesimo M, Pinter E, Alario C, Sacco G, Muratori DS, Bernardi ML, Paganelli R, Aiuti F: **Long-term evaluation of T-cell subsets and T-cell function after HAART in advanced stage HIV-1 disease**. *Aids* 1999, **13**:1187-1193.

331. Notermans DW, Pakker NG, Hamann D, Foudraine NA, Kauffmann RH, Meenhorst PL, Goudsmit J, Roos MT, Schellekens PT, Miedema F, et al.: **Immune reconstitution after 2 years of successful potent antiretroviral therapy in previously untreated human immunodeficiency virus type 1-infected adults**. *J Infect Dis* 1999, **180**:1050-1056.

332. Renaud M, Katlama C, Mallet A, Calvez V, Carcelain G, Tubiana R, Jouan M, Caumes E, Agut H, Bricaire F, et al.: **Determinants of paradoxical CD4 cell reconstitution after protease inhibitor-containing antiretroviral regimen**. *Aids* 1999, **13**:669-676.

333. Connick E, Lederman MM, Kotzin BL, Spritzler J, Kuritzkes DR, St Clair M, Sevin AD, Fox L, Chiozzi MH, Leonard JM, et al.: **Immune reconstitution in the first year of potent antiretroviral therapy and its relationship to virologic response**. *J Infect Dis* 2000, **181**:358-363.

334. Wood E, Yip B, Hogg RS, Sherlock CH, Jahnke N, Harrigan RP, O'Shaughnessy MV, Montaner JS: **Full suppression of viral load is needed to achieve an optimal CD4 cell count response among patients on triple drug antiretroviral therapy**. *Aids* 2000, **14**:1955-1960.

335. Teixeira L, Valdez H, McCune JM, Koup RA, Badley AD, Hellerstein MK, Napolitano LA, Douek DC, Mbisa G, Deeks S, et al.: **Poor CD4 T cell restoration after suppression of HIV-1 replication may reflect lower thymic function**. *Aids* 2001, **15**:1749-1756.

336. Choremi-Papadopoulou H, Tsalimalma K, Dafni U, Dimitracopoulou A, Kordossis T: **Limited long-term naive CD4+ T cell reconstitution in patients experiencing viral load rebounds during HAART**. *J Med Virol* 2004, **73**:235-243.

337. Pontesilli O, Kerkhof-Garde S, Notermans DW, Foudraine NA, Roos MT, Klein MR, Danner SA, Lange JM, Miedema F: **Functional T cell reconstitution and human**

immunodeficiency virus-1-specific cell-mediated immunity during highly active antiretroviral therapy. *J Infect Dis* 1999, **180**:76-86.

338. Valdez H: **Immune restoration after treatment of HIV-1 infection with highly active antiretroviral therapy (HAART)**. *AIDS Rev* 2002, **4**:157-164.

339. Vigano A, Vella S, Saresella M, Vanzulli A, Bricalli D, Di Fabio S, Ferrante P, Andreotti M, Pirillo M, Dally LG, et al.: **Early immune reconstitution after potent antiretroviral therapy in HIV-infected children correlates with the increase in thymus volume**. *Aids* 2000, **14**:251-261.

340. Clerici M, Saresella M, Trabattoni D, Ferrante P, Vanzulli A, Vigano A: **Thymic volume predicts long-term immune reconstitution in HIV-infected children treated with highly active antiretroviral therapy**. *Aids* 2002, **16**:2219-2221.

341. Smith KY, Valdez H, Landay A, Spritzler J, Kessler HA, Connick E, Kuritzkes D, Gross B, Francis I, McCune JM, et al.: **Thymic size and lymphocyte restoration in patients with human immunodeficiency virus infection after 48 weeks of zidovudine, lamivudine, and ritonavir therapy**. *J Infect Dis* 2000, **181**:141-147.

342. Franco JM, Rubio A, Martinez-Moya M, Leal M, Merchante E, Sanchez-Quijano A, Lissen E: **T-cell repopulation and thymic volume in HIV-1-infected adult patients after highly active antiretroviral therapy**. *Blood* 2002, **99**:3702-3706.

343. Kolte L, Dreves AM, Ersboll AK, Strandberg C, Jeppesen DL, Nielsen JO, Ryder LP, Nielsen SD: **Association between larger thymic size and higher thymic output in human immunodeficiency virus-infected patients receiving highly active antiretroviral therapy**. *J Infect Dis* 2002, **185**:1578-1585.

344. Kolte L, Strandberg C, Dreves AM, Ersboll AK, Jeppesen DL, Ryder LP, Nielsen SD: **Thymic involvement in immune recovery during antiretroviral treatment of HIV infection in adults; comparison of CT and sonographic findings**. *Scand J Infect Dis* 2002, **34**:668-672.

345. de la Rosa R, Leal M, Rubio A, Martinez-Moya M, Delgado J, Ruiz-Mateos E, Merchante E, Sanchez-Quijano A, Eduordo L: **Baseline thymic volume is a predictor for CD4 T cell repopulation in adult HIV-infected patients under highly active antiretroviral therapy**. *Antivir Ther* 2002, **7**:159-163.

346. Rubio A, Martinez-Moya M, Leal M, Franco JM, Ruiz-Mateos E, Merchante E, Sanchez-Quijano A, Lissen E: **Changes in thymus volume in adult HIV-infected patients under HAART: correlation with the T-cell repopulation**. *Clin Exp Immunol* 2002, **130**:121-126.

347. Ruiz-Mateos E, Rubio A, Vallejo A, De la Rosa R, Sanchez-Quijano A, Lissen E, Leal M: **Thymic volume is associated independently with the magnitude of short- and long-**

term repopulation of CD4+ T cells in HIV-infected adults after highly active antiretroviral therapy (HAART). *Clin Exp Immunol* 2004, **136**:501-506.

348. Landay A, da Silva BA, King MS, Albrecht M, Benson C, Eron J, Glesby M, Gulick R, Hicks C, Kessler H, et al.: **Evidence of ongoing immune reconstitution in subjects with sustained viral suppression following 6 years of lopinavir-ritonavir treatment**. *Clin Infect Dis* 2007, **44**:749-754.

349. Hainaut M, Ducarme M, Schandene L, Peltier CA, Marissens D, Zissis G, Mascart F, Levy J: **Age-related immune reconstitution during highly active antiretroviral therapy in human immunodeficiency virus type 1-infected children**. *Pediatr Infect Dis J* 2003, **22**:62-69.

350. Eysteinsdottir JH, Freysdottir J, Haraldsson A, Stefansdottir J, Skaftadottir I, Helgason H, Ogmundsdottir HM: **The influence of partial or total thymectomy during open heart surgery in infants on the immune function later in life**. *Clin Exp Immunol* 2004, **136**:349-355.

351. Walker RE, Carter CS, Muul L, Natarajan V, Herpin BR, Leitman SF, Klein HG, Mullen CA, Metcalf JA, Baseler M, et al.: **Peripheral expansion of pre-existing mature T cells is an important means of CD4+ T-cell regeneration HIV-infected adults**. *Nat Med* 1998, **4**:852-856.

352. Haynes BF: **HIV infection and the dynamic interplay between the thymus and the peripheral T cell pool**. *Clin Immunol* 1999, **92**:3-5.

353. Haynes BF, Hale LP: **Thymic function, aging, and AIDS**. *Hosp Pract (Minneap)* 1999, **34**:59-60, 63-55, 69-70, passim.

354. Haynes BF, Hale LP, Weinhold KJ, Patel DD, Liao HX, Bressler PB, Jones DM, Demarest JF, Gebhard-Mitchell K, Haase AT, et al.: **Analysis of the adult thymus in reconstitution of T lymphocytes in HIV-1 infection**. *J Clin Invest* 1999, **103**:921.

355. Klein M: **Prospects and challenges for prophylactic and therapeutic HIV vaccines**. *Vaccine* 2003, **21**:616-619.

356. Guy-Grand D, Azogui O, Celli S, Darche S, Nussenzweig MC, Kourilsky P, Vassalli P: **Extrathymic T cell lymphopoiesis: ontogeny and contribution to gut intraepithelial lymphocytes in athymic and euthymic mice**. *J Exp Med* 2003, **197**:333-341.

357. Collins C, Norris S, McEntee G, Traynor O, Bruno L, von Boehmer H, Hegarty J, O'Farrelly C: **RAG1, RAG2 and pre-T cell receptor alpha chain expression by adult human hepatic T cells: evidence for extrathymic T cell maturation**. *Eur J Immunol* 1996, **26**:3114-3118.

358. Bas A, Hammarstrom SG, Hammarstrom ML: **Extrathymic TCR gene rearrangement in human small intestine: identification of new splice forms of recombination activating gene-1 mRNA with selective tissue expression**. *J Immunol* 2003, **171**:3359-3371.

359. Lewis DE, Yang L, Luo W, Wang X, Rodgers JR: **HIV-specific cytotoxic T lymphocyte precursors exist in a CD28-CD8+ T cell subset and increase with loss of CD4 T cells**. *Aids* 1999, **13**:1029-1033.

360. Lewis DE: **HIV vaccines: the future looks promising**. *Res Initiat Treat Action* 2007, **12**:29-32.

361. Mildvan D, Bosch RJ, Kim RS, Spritzler J, Haas DW, Kuritzkes D, Kagan J, Nokta M, DeGruttola V, Moreno M, et al.: **Immunophenotypic markers and antiretroviral therapy (IMART): T cell activation and maturation help predict treatment response**. *J Infect Dis* 2004, **189**:1811-1820.

362. Grelli S, d'Ettorre G, Lauria F, Montella F, Di Traglia L, Lichtner M, Vullo V, Favalli C, Vella S, Macchi B, et al.: **Inverse correlation between CD8+ lymphocyte apoptosis and CD4+ cell counts during potent antiretroviral therapy in HIV patients**. *J Antimicrob Chemother* 2004, **53**:494-500.

363. Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, Haynes BF, Polis MA, Haase AT, Feinberg MB, Sullivan JL, et al.: **Changes in thymic function with age and during the treatment of HIV infection**. *Nature* 1998, **396**:690-695.

364. Kalayjian RC, Landay A, Pollard RB, Taub DD, Gross BH, Francis IR, Sevin A, Pu M, Spritzler J, Chernoff M, et al.: **Age-related immune dysfunction in health and in human immunodeficiency virus (HIV) disease: association of age and HIV infection with naive CD8+ cell depletion, reduced expression of CD28 on CD8+ cells, and reduced thymic volumes**. *J Infect Dis* 2003, **187**:1924-1933.

365. Ruiz-Mateos E, de la Rosa R, Franco JM, Martinez-Moya M, Rubio A, Soriano N, Sanchez-Quijano A, Lissen E, Leal M: **Endogenous IL-7 is associated with increased thymic volume in adult HIV-infected patients under highly active antiretroviral therapy**. *Aids* 2003, **17**:947-954.

366. Napolitano LA, Grant RM, Deeks SG, Schmidt D, De Rosa SC, Herzenberg LA, Herndier BG, Andersson J, McCune JM: **Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis**. *Nat Med* 2001, **7**:73-79.

367. Napolitano LA, Lo JC, Gotway MB, Mulligan K, Barbour JD, Schmidt D, Grant RM, Halvorsen RA, Schambelan M, McCune JM: **Increased thymic mass and circulating naive CD4 T cells in HIV-1-infected adults treated with growth hormone**. *Aids* 2002, **16**:1103-1111.

368. Addo MM, Altfeld M, Rosenberg ES, Eldridge RL, Philips MN, Habeeb K, Khatri A, Brander C, Robbins GK, Mazzara GP, et al.: **The HIV-1 regulatory proteins Tat and Rev are frequently targeted by cytotoxic T lymphocytes derived from HIV-1-infected individuals**. *Proc Natl Acad Sci U S A* 2001, **98**:1781-1786.

369. Betts MR, Casazza JP, Koup RA: **Monitoring HIV-specific CD8+ T cell responses by intracellular cytokine production**. *Immunol Lett* 2001, **79**:117-125.

370. Novitsky V, Cao H, Rybak N, Gilbert P, McLane MF, Gaolekwe S, Peter T, Thior I, Ndung'u T, Marlink R, et al.: **Magnitude and frequency of cytotoxic T-lymphocyte responses: identification of immunodominant regions of human immunodeficiency virus type 1 subtype C**. *J Virol* 2002, **76**:10155-10168.

371. Novitsky V, Gilbert P, Peter T, McLane MF, Gaolekwe S, Rybak N, Thior I, Ndung'u T, Marlink R, Lee TH, et al.: **Association between virus-specific T-cell responses and plasma viral load in human immunodeficiency virus type 1 subtype C infection**. *J Virol* 2003, **77**:882-890.

372. Yu XG, Addo MM, Rosenberg ES, Rodriguez WR, Lee PK, Fitzpatrick CA, Johnston MN, Strick D, Goulder PJ, Walker BD, et al.: **Consistent patterns in the development and immunodominance of human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T-cell responses following acute HIV-1 infection**. *J Virol* 2002, **76**:8690-8701.

373. Kesturu GS, Colleton BA, Liu Y, Heath L, Shaikh OS, Rinaldo CR, Jr., Shankarappa R: **Minimization of genetic distances by the consensus, ancestral, and center-of-tree (COT) sequences for HIV-1 variants within an infected individual and the design of reagents to test immune reactivity**. *Virology* 2006, **348**:437-448.

374. Rabin RL, Roederer M, Maldonado Y, Petru A, Herzenberg LA, Herzenberg LA: **Altered representation of naive and memory CD8 T cell subsets in HIV-infected children**. *J Clin Invest* 1995, **95**:2054-2060.

375. Roederer M, Dubs JG, Anderson MT, Raju PA, Herzenberg LA, Herzenberg LA: **CD8 naive T cell counts decrease progressively in HIV-infected adults**. *J Clin Invest* 1995, **95**:2061-2066.

376. Correa R, Munoz-Fernandez MA: **Viral phenotype affects the thymic production of new T cells in HIV-1-infected children**. *Aids* 2001, **15**:1959-1963.

377. Clerici M, Stocks NI, Zajac RA, Boswell RN, Bernstein DC, Mann DL, Shearer GM, Berzofsky JA: **Interleukin-2 production used to detect antigenic peptide recognition by T-helper lymphocytes from asymptomatic HIV-seropositive individuals**. *Nature* 1989, **339**:383-385.

378. Sleasman JW, Aleixo LF, Morton A, Skoda-Smith S, Goodenow MM: **CD4+ memory T cells are the predominant population of HIV-1-infected lymphocytes in neonates and children**. *Aids* 1996, **10**:1477-1484.

379. Ljunggren HG, Stam NJ, Ohlen C, Neefjes JJ, Hoglund P, Heemels MT, Bastin J, Schumacher TN, Townsend A, Karre K, et al.: **Empty MHC class I molecules come out in the cold**. *Nature* 1990, **346**:476-480.

380. Koup RA, Ho DD: **Shutting down HIV**. *Nature* 1994, **370**:416.

381. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB: **Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection**. *J Virol* 1994, **68**:6103-6110.

382. Lieberman J, Skolnik PR, Parkerson GR, 3rd, Fabry JA, Landry B, Bethel J, Kagan J: **Safety of autologous, ex vivo-expanded human immunodeficiency virus (HIV)-specific cytotoxic T-lymphocyte infusion in HIV-infected patients**. *Blood* 1997, **90**:2196-2206.

383. Phillips RE, Rowland-Jones S, Nixon DF, Gotch FM, Edwards JP, Ogunlesi AO, Elvin JG, Rothbard JA, Bangham CR, Rizza CR, et al.: **Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition**. *Nature* 1991, **354**:453-459.

384. Koenig S, Conley AJ, Brewah YA, Jones GM, Leath S, Boots LJ, Davey V, Pantaleo G, Demarest JF, Carter C, et al.: **Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression**. *Nat Med* 1995, **1**:330-336.

385. Barouch DH, Letvin NL: **Viral evolution and challenges in the development of HIV vaccines**. *Vaccine* 2002, **20 Suppl 4**:A66-68.

386. Goulder PJ, Tang Y, Brander C, Betts MR, Altfeld M, Annamalai K, Trocha A, He S, Rosenberg ES, Ogg G, et al.: **Functionally inert HIV-specific cytotoxic T lymphocytes do not play a major role in chronically infected adults and children**. *J Exp Med* 2000, **192**:1819-1832.

387. Goulder PJ, Brander C, Annamalai K, Mngqundaniso N, Govender U, Tang Y, He S, Hartman KE, O'Callaghan CA, Ogg GS, et al.: **Differential narrow focusing of immunodominant human immunodeficiency virus gag-specific cytotoxic T-lymphocyte responses in infected African and caucasoid adults and children**. *J Virol* 2000, **74**:5679-5690.

388. Huang XL, Fan Z, Colleton BA, Buchli R, Li H, Hildebrand WH, Rinaldo CR, Jr.: **Processing and presentation of exogenous HLA class I peptides by dendritic cells from human immunodeficiency virus type 1-infected persons**. *J Virol* 2005, **79**:3052-3062.

389. Connolly NC, Whiteside TL, Wilson CC, Kondragunta V, Rinaldo CR, Riddler SA. **Therapeutic immunization with HIV-1 peptide-loaded dendritic cells is safe and immunogenic in HIV-1-infected individuals. Clin Vacc Immunol, in revision.**

390. Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA: **Fields Virology; 2006 Lippincott Williams & Wilkins.**

391. **HIV/AIDS Surveillance Report; http://www.cdc.gov/hiv/topics/surveillance/ resources/reports/. Ref Type: Electronic citation.**

392. **CDC; http://www.cdc.gov/hiv/resources/factsheets/index.htm. Ref Type: Electronic citation.**

393. HIV Molecular Immunology 2006/2007, Editors: Bette TM Korber, Christian Brander, Barton F Haynes, Richard Koup, John P Moore, Bruce D Walker, and David I Watkins. Publisher: Los Alamos National Laboratory. **Theoretical Biology and Biophysics, Los Alamos, New Mexico. LA-UR 07-4752. (http://www.hiv.lanl.gov/).**