Microbiology and Immunology

STRUCTURAL AND FUNCTIONAL DETERMINANTS OF EFFECTIVE $CD8^+$ T CELL SUPPRESSION OF HIV-1 REPLICATION

Brenna Colleen Simons

Dissertation under the direction of Dr. Spyros Kalams

$CD8^+$ T cells are a critical component of an HIV-specific immune response. Our studies aimed to define the T cell receptor (TCR) structural determinants as well as effector functions associated with $CD8^+$ T cell suppression of HIV replication. We demonstrate biased gene usage in dominant HIV epitope-specific TCR repertoires during chronic HIV infection. Despite this evidence for convergent evolution to a highly conserved viral epitope, our results indicate TCR diversity can still provide the structural ability to recognize viral variants. To better understand the role of proliferative capacity in $CD8^+$ T cell-mediated suppression of HIV replication, we directly assessed proliferation and suppression simultaneously in vitro. We found that low proliferating $CD8^+$ T cells suppressed HIV replication in vitro similar to levels of suppression of high-proliferating $CD8^+$ T cells. Our data also revealed low-proliferating cells to have higher frequencies of HIV-specific $IFN\gamma^+TNF\alpha^+$ T cells. These results suggest a critical role for remaining effector functions in the absence of proliferation. Together our findings have implications for improved assessment of candidate HIV vaccine elicited immune responses and further investigations into the correlates of control of HIV-1 viremia.

Approved _____ Date _____

STRUCTURAL AND FUNCTIONAL DETERMINANTS OF EFFECTIVE $CD8^+$ T CELL SUPPRESSION OF HIV-1 REPLICATION

By

Brenna Colleen Simons

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

In

Microbiology and Immunology

May, 2009

Nashville, Tennessee

Approved:

Professor Spyros Kalams

Professor Luc Van Kaer

Professor Claudio Mosse

Professor Christopher Aiken

Professor Richard D'Aquila

To my loving parents, Pat and Chuck, for their everlasting love and support.

I love you both very much, and this could not have been possible without you.

ACKNOWLEDGEMENTS

I think any graduate student would tell you that there is as much to learn out of the laboratory as there is at the bench during this experience. I am grateful for my time spent at Vanderbilt and for the incredible mentorship and friendship I have received during this experience. I would like to first thank my mentor, Dr. Spyros Kalams. Over the past 5 years since I first joined the Kalams lab, we have navigated the graduate school experience together. As your first student here at Vanderbilt, our journey was not always easy, but I am grateful for being allowed the independence and self-direction that I believe helped develop my confidence and self-reliance as a scientist. You are an excellent writer and a creative thinker, and I hope to continue to build upon these strong attributes I have learned from you. I probably did not say this enough, but thank you for all of your support over the years.

I would like to thank all of the past and present members of the Kalams laboratory for continued technical and scientific input as well as their personal support. I want to especially thank Dr. Lakshmi Sadagopal and Dr. Dirk Meyer-Olson for their additional mentorship, as you were always willing to read a manuscript, or a thesis research proposal, with no questions asked, thank you again. My gratitude also extends to members of the D'Aquila lab as well as the Vanderbilt-Meharry Center for AIDS Research for further laboratory support.

In addition to the guidance I received from my mentor and fellow laboratory members, I am also very grateful for all of the helpful efforts and input given to

me by the members of my thesis committee; Dr. Richard D'Aquila, Dr. Claudio Mosse, Dr. Luc Van Kaer, Dr. Chris Aiken, and former member Dr. Derya Unutmaz (NYU). I truly appreciate your commitment to my continual progress and development as a scientist. Thank you for always keeping your doors open and finding the time to meet with me despite your busy schedules.

My final extension of mentorship lies in two strong and intelligent women, whom I respect and appreciate very much. Dr. Kyra Richter has been a friend and advisor to me every since we were both students in the Microbiology and Immunology Department. Kyra, thank you for your unwavering support, you never hesitated once to help me or just to listen, and I have learned so much from you. Dr. Amanda Antons, has been my friend and colleague since the beginning of our graduate school journey. Whether it was studying months ahead of time for our qualifying exams despite being ridiculed, volunteering on the weekend, or just discussing our world and enjoying the similarity of our views, Amanda, you have always been there for me as an example of a woman who is brave enough to be assertive in her beliefs and convictions.

Throughout the years I have always felt supported by the Department of Microbiology and Immunology, thanks to the administrative staff and direction of Dr. Jacek Hawiger. Thank you all for your willingness to listen to any question or concern, no matter how small. I would like to thank Dr. Chris Aiken and the Department of Microbiology and Immunology for my continuous financial support by the NIH-funded HIV and AIDS Research Training Program.

Until this experience, I would never have believed 6 years to go by so quickly. Although gratifying, graduate school can be tough. I believe my experience here at Vanderbilt was improved dramatically thanks to my fellow students and friends. The Microbiology and Immunology GSA was very supportive, by planning qualifying exam practices and advocating on my behalf to the faculty of the department. Older, and often times wiser, students were a great asset to me in my early years in the lab. I want to thank Doctors Melody Davis, Kristen Guglielmi and Rachel Henry who were always willing to give advice and share their experiences to perhaps make mine a little easier. And of course, my every day experience was enhanced by my lab partners in crime, Joseph Conrad and Michael Vetter who were always there with humor to commiserate or just to laugh with me during the day-to-day grind in lab.

My friends here at Vanderbilt have been my extended family. I want to thank Sarah, Jenn, Mike, Amanda, Kyra, and Karen for always being by my side through thick and thin, or as I like to say through sane and crazy, and doing so while all the time having wonderful senses of humor and making me smile. Working weekends and destroying 2 days worth of laboratory set up in a single goof is much easier to swallow when you have good friends to share that experience with. My time spent here at Vanderbilt, and in Nashville has been a special one, thanks to all of you.

The people in my life, with whom I have grown up and shared my experiences from an early age, are very special to me. Through marriage, babies, promotions, graduations and cross-country moves, I have been fortunate enough

to have a home base for me to return to, with friends who are caring and loyal. I want to thank Matt, Joe, John, Sue, and Laura for always being by my side, no questions asked. Thank you for always being there, to remind me of where I come from, and being willing to ground me back to what is important when I needed it.

Besides being blessed with a wonderful community of friendship and support, I am lucky enough to have an incredibly loving and supportive family. My brother Seth was throughout our moves and adventures as kids, my best friend, and sometimes my only friend. Thank you Seth for your support through my graduate school endeavors and beyond.

My final, but most important, acknowledgment is to my parents Pat and Chuck. My parents are the example of living life to the fullest, taking every opportunity offered with no hesitation and no regrets. From moving up to Alaska with a young baby girl, to taking advantage of living in Europe for two years with young kids, my parents have never asked 'how', but rather 'when'. So of course, the sense of adventure runs in the family, and when I told my parents I was applying for doctorate programs, again, with no hesitation, the question was when do you start? My parents' steadfast belief in my own dreams, and their encouragement to do whatever I set my mind to has been the platform for my success. Mom, when I think of you at home with two young kids being an amazing and active mother during the day, and going to college at night, I truly appreciate what you have sacrificed for us. I learned from you that nothing is impossible, and I hope to some day have your passion to always do and say what is right, you are truly

an inspiration to me. Dad, I consider myself lucky to have a strong supportive father like you. Whether it was going to the batting cages, rooting for me on the field hockey field or supporting me through these past 6 years of school, you were always my number one fan. I want to thank both of you for providing every opportunity to me and for always being my strongest supporters.

I love you both very much and this could not have been possible without you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AICD	=	Activation induced cell death
AIDS	=	Acquired immunodeficiency syndrome
APC	=	Allophycocyanin
ART	=	Anti-retroviral therapy
CDR	=	Complementarity determining region
CFSE	=	Carboxyfluorescein diacetate succinimidyl ester
CTL	=	Cytotoxic T Lymphocyte
DC	=	Dendritic cell
DNA	=	Deoxyribonucleic acid
EBV	=	Epstein-Barr virus
ELISA	=	Enzyme-linked immunosorbent assay
ELISpot	=	Enzyme-linked immunosorbent spot assay
E: T	=	Effector to Target
FACS	=	Fluorescence-activated cell sorting
GFP	=	Green fluorescent protein
gp	=	glycoprotein
HCV	=	Hepatitis C virus
HIV	=	Human immunodeficiency syndrome
HLA	=	Human Leukocyte Antigen
ICS	=	Intracellular Cytokine Secretion
IFN	=	Interferon

IFU	=	Infectious Units
IL	=	Interleukin
LCMV	=	Lymphocytic choriomeningitis virus
MFI	=	Mean Fluorescence Intensity
MHC	=	Major Histocompatibility Complex
MOI	=	Multiplicity of infection
NEF	=	Negative factor
NFAT	=	Nuclear factor of activated T cells
PBMC	=	Peripheral blood mononuclear cells
PBS	=	Phosphate-buffered saline
PCR	=	Polymerase chain reaction
PD-1	=	Programmed Death -1
PE	=	Phycoerythrin
PHA	=	Phytohemagglutinin
RNA	=	Ribonucleic acid
SEB	=	Staphylococcal Enterotoxin B
SFC	=	Spot Forming Cells
SIV	=	Simian immunodeficiency virus
TCR	=	T cell receptor
TdT	=	Terminal Deoxynucleotidyl Transferase
T_E	=	Effector T cell
Th	=	T helper
T_M	=	Memory T cell

T_N	=	Naïve T cell
TNF	=	Tumor necrosis factor
TRBV	=	T cell receptor β-chain V region
$T_{termdiff}$	=	Terminally differentiated T cell
VSV-G	=	Vesicular stomatitis virus glycoprotei

CHAPTER I

BACKGROUND AND RESEARCH OBJECTIVES

The Human Immunodeficiency Virus (HIV) has created a large impact on the global population. In 2007, the World Health Organization (WHO) reported an estimated 33 million people to be infected worldwide. The impact of a pandemic this size extends past the issue of mortality and has dire financial and cultural implications as well. Prevention and treatment of HIV infection are critical for resolving this pandemic. Understanding the mechanisms of HIV infection and subsequent host immune responses will allow new options for treatment and prevention of HIV.

Human Immunodeficiency Virus

HIV is an enveloped lentivirus (1, 2) containing a characteristic conical core structure composed of multiple subunits of capsid protein (p24) (Figure 1-1**)**. This viral genome, consisting of two positive single strands of RNA (2), is held within the conical core. There are four main proteins that provide the general means for HIV replication; Gag, Pol, Env, Nef, and several accessory proteins that play a role in HIV infectivity (2). HIV primarily targets activated $CD4^+$ T cells (2). The viral receptors for HIV attachment to the host target cell are gp120 and gp41 (Figure 1-1) (2). These viral receptors bind CD4 in combination with either

CCR5 or CXCR4 chemokine receptors on the cell surface of $CD4^+$ T cells. Host CD4 is the main receptor for HIV binding and fusion (1, 2). Upon HIV binding and fusion with the host target cell (Figure 1-1, Step 1), the conical core is released into the host cell cytosol (Figure 1-1, Step 2), and uncoating occurs, depositing the viral genome (1, 2). Maintenance of the conical core structure is critical for HIV infectivity (3-6). Concordantly, many amino acids within the p24 capsid protein are highly conserved despite high rates of viral mutation in HIV (7-10). The viral genome, which is RNA, must first be reverse transcribed into DNA, using viral proteins (Figure 1-1, Step 3). These reverse transcripts, which are then DNA, are integrated into the host genome (Figure 1-1, Step 4), where the virus takes advantage of host machinery to complete the life cycle (Figure 1-1, Steps 5-8) (2). HIV-specific mRNA transcribed from the integrated HIV DNA in the host genome, is translated into viral proteins which gather at the host cell membrane to form new virions (2). $CD4^+$ T cells are key helper cells in the formation of an adaptive cellular immune response. Loss of these cells disables coordination of the immune system, thus impeding a proper and rapid response to an invading pathogen (11). By infecting and subsequently killing these $CD4^+$ T cell targets, HIV disables a critical arm of the host immune response, leaving the host with increased susceptibility to opportunistic infections (1, 11, 12). There are two main types of HIV, HIV type 1 (HIV-1) and HIV type 2 (HIV-2). HIV-1 is prominent worldwide, whereas HIV-2 has spread more slowly beyond western Africa (13). HIV-1 and HIV-2 are highly related, but HIV-2 is reported to be less virulent and the mechanism behind these findings is not clear (13). Because of

the significantly increased prevalence of HIV-1, our studies and findings will focus on examining HIV-1 infection.

In the past 25 years since HIV was first identified, significant advances have been made in understanding the mechanisms by which HIV infects and affects the human host. These data have led to several candidates for vaccines, microbicides and medications that could possibly prevent or delay HIV infection. Thus far, several modalities of HIV suppression and prevention have shown promising results, but none has been proven to be a complete cure for HIV infection. The resolution of the HIV pandemic is long from over, maintaining the importance in efforts for advancements in HIV treatment to continue a broad scope of HIV research. By understanding the mechanisms by which the host immune response thrives and fails in the control of HIV-1 viremia we may provide critical insight into future design and assessment of HIV vaccines.

$CD8^+$ T cells

The purpose of the immune system is to distinguish self from non-self. The immune system has both an early, less specific response (innate), and a later response with greater specificity that includes immunologic memory (adaptive). Both the innate and adaptive immune responses work together to detect, control and clear viral infections. During a viral infection, cells of the innate immune response first detect the viral pathogen breech and conduct an initial attack, while simultaneously alerting the immune cells of the more specific adaptive immune response (1, 14, 15)

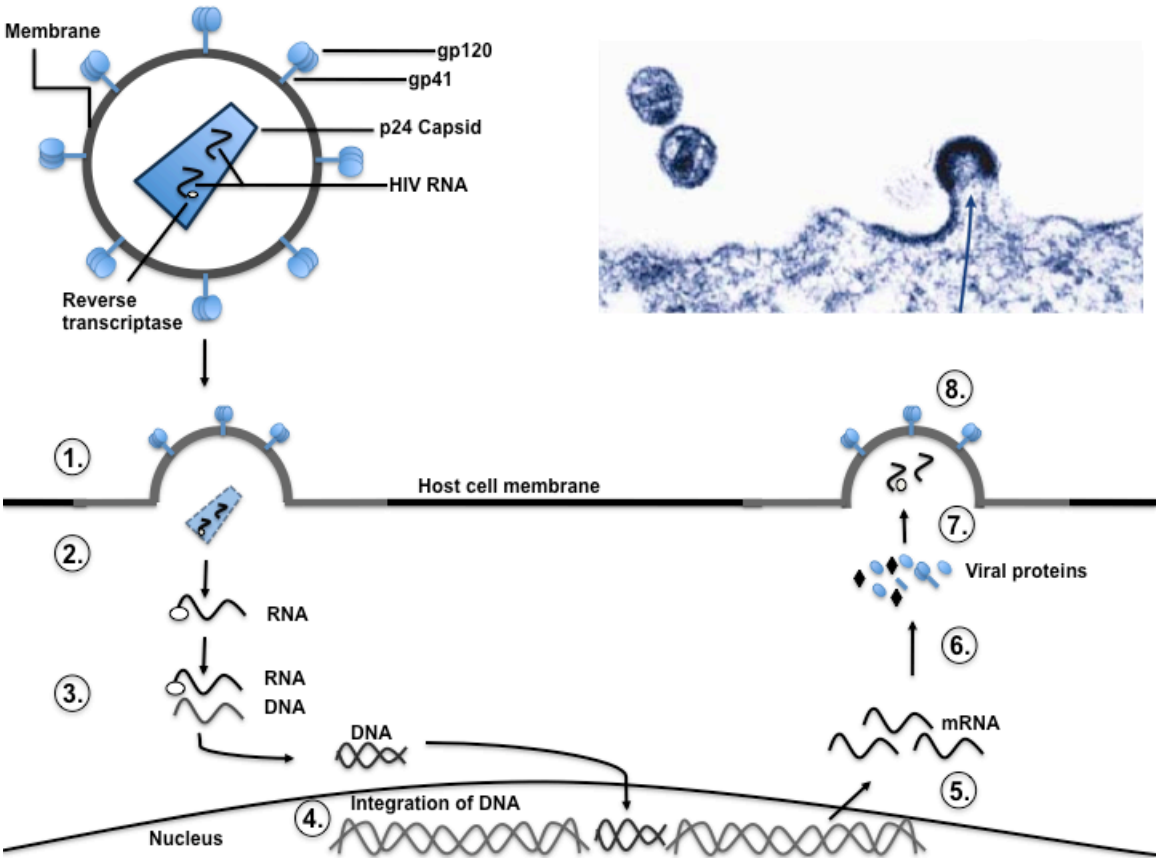


Figure 1-1. Human Immunodeficiency Virus (HIV-1) structure and life cycle. Basic structure of HIV-1 virion diagrammed top left. 9 main steps of HIV-1 life cycle are illustrated here.

Image and figure adapted from Simon V, and Ho D. Nature Rev. Micro. 2003. 1:181-190 (2).

There are two main branches of the adaptive immune response; humoral and cellular. The humoral response to viral infection is primarily focused on opsonizing the virus by detection of viral surface proteins via antibodies, and clearing circulating virus by phagocytosis (11). The cellular response to viral infection consists of effector lymphocytes that include $CD8^+$ T (11). $CD8^+$ T cells are selected in the thymus, derived from double positive ($CD4^+CD8^+$) T lymphocytes, and enter the lymphatic system as $T_{naïve}$ (T_N) cells (Figure 1-2) (11, 15). A naïve cell is a cell that has not yet been presented antigen in the presence of costimulation. $CD8^+$ T cells can be distinguished by the expression of CD8 as well as other physiologic and structural features. CD8 is most often a hetero-dimer consisting of an alpha and beta subunit (Figure 1-2) (16). The subunits have a similar structure, with a long trans-membrane tail and a globular head. CD8 is a co-receptor that synergizes with the TCR in $CD8^+$ T cell activation and subsequent signaling after antigen-specific interactions (16). This activation results in further development of the $CD8^+$ T cell, in which the $CD8^+$ T cell progresses from a T_N cell to one that is defined to be a $T_{Effector}$ (T_E) cell (Figure 1-2) (15). $CD8^+$ T_E cells have been presented antigen and produce the appropriate immune response. These effector cells continue to expand and mount an immune response until the pathogen begins to be cleared. As the pathogen clears, lower levels of antigen are presented. As the levels of antigen presentation decreases, the numbers of effector $CD8^+$ T cells decrease. At this stage there is a significant amount of cell death, and the immune response only maintains a small number of these effector $CD8^+$ T cells as a memory pool of

effector $CD8^+$ T cells. In this pool, these cells are now primed for a secondary infection of the same pathogen, which is defined as immunologic memory (Figure 1-2) (1, 15). Several markers are associated with this differentiation process and identify the different stages of T cell activation and differentiation (Figure 1-2). During HIV infection, as well as other chronic viral infections, as the pathogen persists, levels of antigen presentation do not diminish, leading to chronic activation of $CD8^+$ T cells as well as dysfunctional $CD8^+$ T cell memory differentiation (Figure 1-2) (14, 15, 17).

$CD8^+$ T cells are specifically designed to clear viral infections. These cells have the specific ability to lyse infected targets (17, 18). Once a $CD8^+$ T cell recognizes a virally infected target cell via the T cell receptor (TCR), an immediate release of cytotoxic granules from the cell is triggered (18-20). $CD8^+$ T cells contain many of these granules, which are located proximal to each TCR and are pre-formed and poised directly underneath the cell membrane, ready for rapid release (Figure 1-2) (18). These granules contain the cytotoxic proteins perforin and granzyme B. Perforin disrupts the target cell plasma membrane and allows for excess water to enter the cell causing lysis. Granzyme B is a serine protease that is highly effective in activating caspase-signaling pathways, which are involved in cell apoptosis (18-20). $CD8^+$ T cells also express Fas Ligand, which when bound to Fas on an infected cell, triggers apoptosis of the infected target. In HIV immunopathogenesis, Fas has been shown to be upregulated on $CD4^+$ T cells, and the FasL-Fas pathway is thought to be a prominent mechanism of CD4-depletion (21-23).

$CD8^+$ T cells have the ability to bind to several infected targets at once, releasing cytotoxic granules simultaneously at each immunologic synapse (19, 20). This ability for multiple binding allows $CD8^+$ T cells to be effective at low E:T (effector:target) ratios. With this massive release of lytic and toxic proteins from granule stores, $CD8^+$ T cells could also be at risk for cytotoxicity and apoptosis, however, $CD8^+$ T cell granules also contain Cathepsin B, a lysosomal protease that locates to the membrane immediately following granule release and is able to inactivate perforin (18, 24).

In addition to cytolytic function of $CD8^+$ T cells, these effector cells also produce and secrete anti-viral cytokines including IFNγ and TNFα. Cytokine secretion by $CD8^+$ T cells is initiated upon recognition of antigen presented by infected target cells. The TNFα cytokine induces apoptosis, cell death, of viral-infected target cells, and both IFNγ and TNFα specifically interfere with viral replication within infected targets while simultaneously activating and recruiting a robust immune response (25).

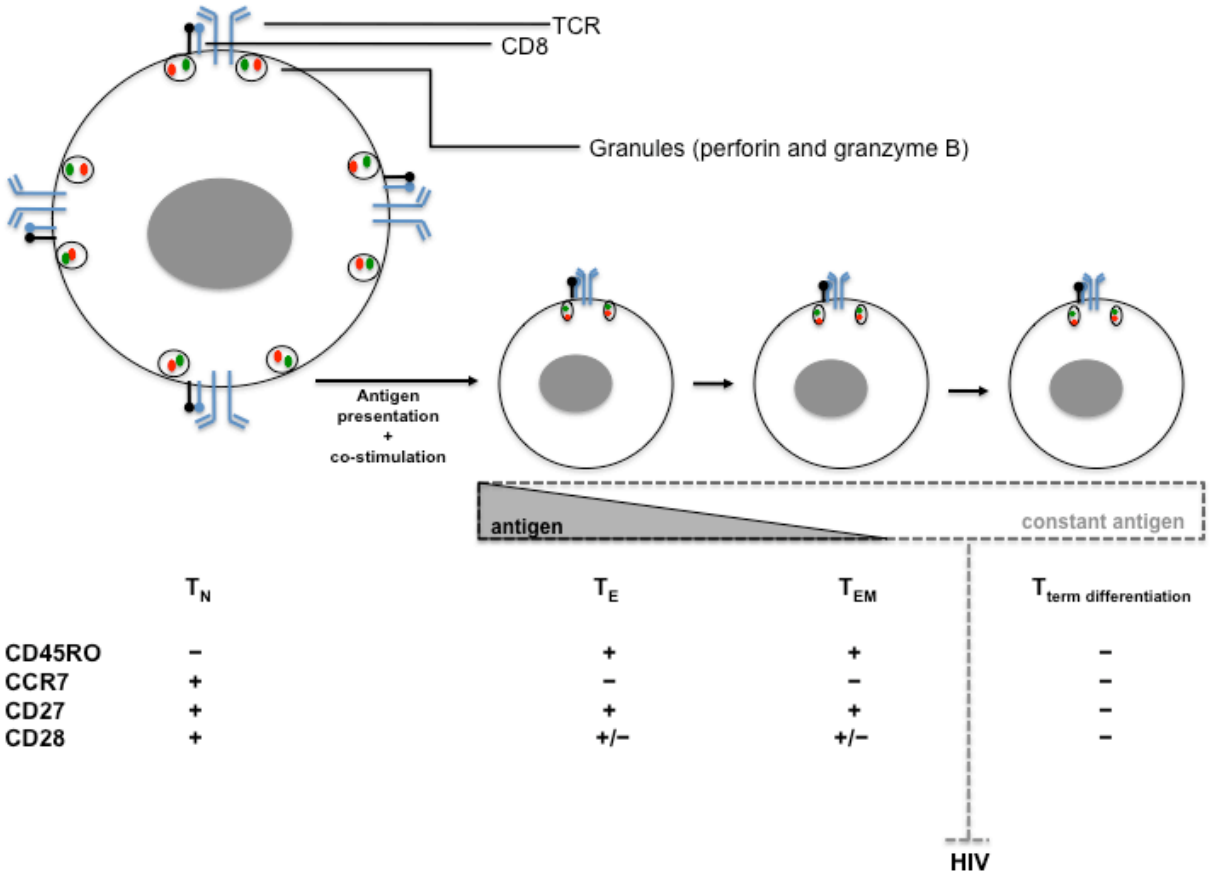


Figure 1-2. CD8$^+$ T cell differentiation. CD8$^+$ T_N cells begin differentiating upon antigen presentation and co-stimulation signaling. Distinct immunologic marker expression is associated with differed states of CD8$^+$ T cell differentiation. $T_{effector}$ (T_E) cells are activated, producing anti-viral response. $T_{effector\ memory}$ (T_{EM}) cells are activated in the presence of diminishing levels of antigen. Terminally differentiated T cells ($T_{term\ differentiation}$) have proliferated extensively, and although proliferative capacity may be diminished, effector functions that include cytokine production and direct cell lysis remain in tact. The linear differentiation of CD8$^+$ T cells progresses through the clearance of a pathogen, as the levels of antigen presentation diminish, reaching a final state of terminal differentiation. However, during chronic viral infections such as HIV-1, the level of antigen presentation does not diminish, and blocks in full terminal differentiation in HIV$^+$ subjects are observed.

Figure adapted from Wherry J. Nature Rev. Immunol. 2002. 2: 251-262.

HIV-1 disease progression

Generally in viral infections, once activated, $CD8^+$ T cells identify infected targets and function successfully in the suppression of viral replication. The anti-viral immune response, in which $CD8^+$ T cells play a major role, eventually gains control, clearing the viral infection and developing a memory response for future attacks (11). However, due to the chronic nature of HIV-1 infection, normal immune function and development are altered.

As in most viral infections, HIV-1 begins with an acute phase, but the infection is not cleared, leaving HIV-1 to develop into a chronic state of infection. There are three phases of HIV-1 disease progression; Acute, Chronic, and Advanced HIV-1 disease which will progress to what is clinically defined as AIDS (Figure 1-3) (1). During acute infection, dramatically high viral loads are present in plasma, at levels of several million viral copies per milliliter (copies/mL) (1). These high viral loads correspond with an initial $CD8^+$ T cell expansion and response (Figure 1-3). The end of the acute phase of infection is marked by a decrease in viral replication, and leads to the establishment of the viral set point in plasma(Figure 1-3). The viral set point is defined as the level of viral replication that plateaus to a consistent level of viral replication following acute phase of HIV infection and marks the chronic phase of infection (Figure 1-3) (1). Several studies have indicated that the viral set point is directly related to the rate of HIV-1 immunodeficiency (i.e. a lower the viral set point is associated with longer AIDS-free survival) (1). During the chronic phase of HIV-1 infection, the viral load remains relatively constant, with correspondingly maintained elevation of $CD8^+$ T

cell activity. The duration of the chronic phase in HIV^+ subjects that remain off anti-retroviral therapy (ART), or therapy naïve, is what defines HIV^+ subjects into categories of HIV -1 progression. Rapid progressors are subjects that have very short phases of clinical latency, on average 1-3 years, before $CD4^+$ T cell counts decrease to less than 200 cells/mm^3 and viral loads begin to increase, signifying advanced HIV-1 disease (Figure 1-3) (1). On average, most subjects have a clinical latency period of 8-10 years before $CD4^+$ T cell counts begin to drop and viral loads begin to increase. Subjects with longer phases of clinical latency, greater than 10 years, are categorized as slow progressors. Several subcategories of these slow progressor subjects have been defined over the years, to help differentiate these groups even further. Long Term Non Progressors, maintain clinical latency with a HIV-1 viral load below 500 viral copies/mL for greater than 10 years (1). Elite controllers are those that maintain a viral load below the current conventional detection limit of 50 copies/mL (26). This indicates a markedly lower degree of ongoing HIV replication, but does not mean that replication has stopped.

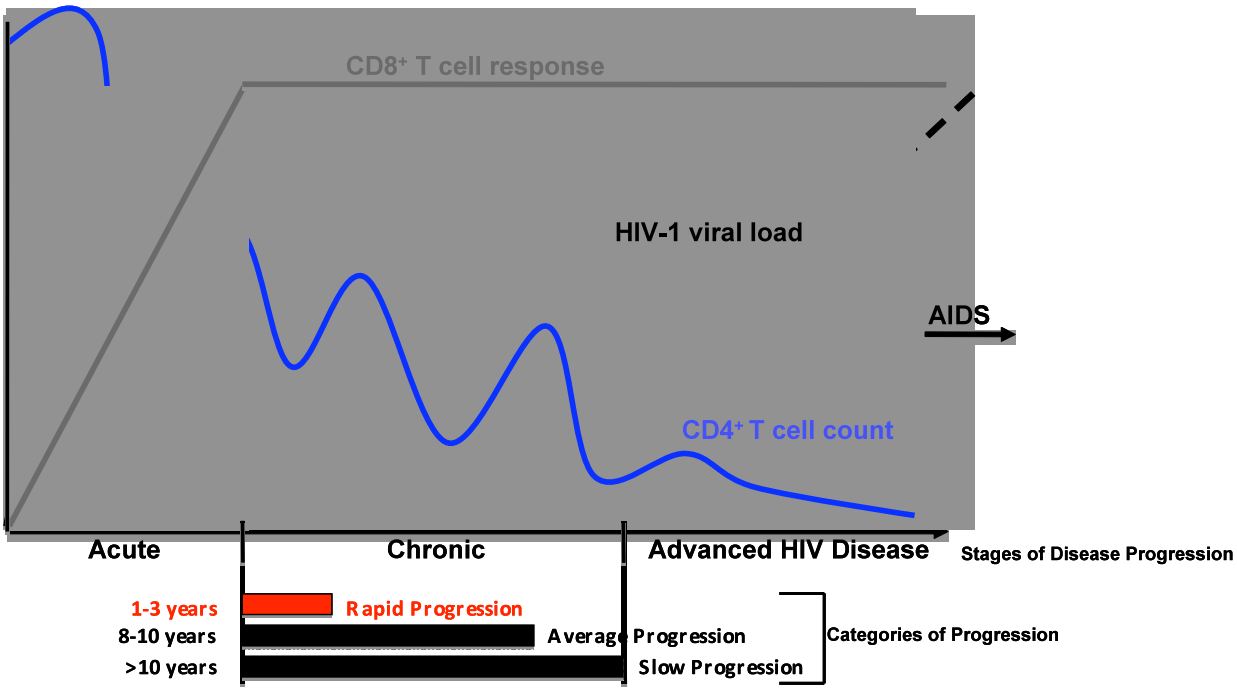


Figure 1-3. HIV-1 Disease Progression. Characteristics of the three phases of HIV-1 disease progression. The three phases are defined by; $CD8^+$ T cell responses (gray), HIV viral load (dashed line) and $CD4^+$ T cell counts (blue line). HIV-1 disease progression categories in therapy naïve subjects are defined by length of chronic, or clinical latency, phase of HIV-1 infection; (red) rapid progression 1-3 years, (black) average length of progression 8-10 years, (blue) slow progression greater than 10 years.

$CD8^+$ T cells are critical for long-term control of HIV-1 viremia

The host $CD8^+$ T cell immune response has been implicated as a critical component in the establishment of the viral set point, and the subsequent pace of progression of HIV-1 disease (27-30). Several studies in both HIV^+ subjects as well as SIV non-human primates have shown the critical need for $CD8^+$ T cells. Observational studies of HIV^+ subjects during acute infection revealed a direct correlation between the expansion of $CD8^+$ T cells and the decrease in HIV-1 viremia during acute phase of HIV-1 disease (27, 28). $CD8^+$ T cells were observed to retain increased cytotoxic activity past the establishment of the viral set point (31, 32). These observational studies in HIV^+ subjects correspond with data from the SIV non-human primate model (27, 28).

A more direct role of $CD8^+$ T cells for control of viremia was confirmed in studies of SIV-infected Rhesus Macaques, where $CD8^+$ T cell depletion resulted in dramatic increases in SIV viremia (33). Further evidence for the importance of $CD8^+$ T cells was illustrated by reconstitution of $CD8^+$ T cells in the study animals resulting in decreased SIV viremia (33). Although in neither the SIV nor HIV studies, were $CD8^+$ T cells capable of complete clearance of viremia, these studies suggest that the cellular arm of the adaptive immune response is playing some critical role in any possible suppression of both SIV and HIV infection. A small group of individual antibodies have been identified to recognize HIV with high affinity, however, humoral responses in HIV^+ subjects are rarely detected until after the establishment of the HIV-1 viral set point (28). Currently, there is

no convincing evidence for a direct relationship between humoral response expansion and HIV-1 viremia suppression.

Although the presence of $CD8^+$ T cells has been shown to be critical for suppression of HIV-1 viremia, even to undetectable levels, the virus is not cleared by the host. Despite advancements in HIV-1 treatment, two specific characteristics of HIV-1 infection may make successful clearance of the virus impossible. The first barrier is the rapid nature of HIV genetic mutation. HIV has been shown to have a mutation rate of approximately 10^{-4} base pairs (1 mutation/ 10,000 base pairs) (2). The total genome of HIV is 9700 base pairs, making the rate of mutation approximately 1 mutation for every round of HIV replication. Coinciding with HIV accumulation of mutations, the environment that surrounds HIV during infection, including the use of ART and the host immune response, applies pressures on the virus. This environment of rapid viral mutation and external environmental pressures is conducive for rapid adaptation of the virus and eventual viral evasion of both medications and immune responses (2, 34-37). The second barrier in clearance of HIV-1 infection is the aspect of 'latent' infection. HIV integrates its genome into the host genome (provirus), and remains a permanent fixture of the $CD4^+$ T cell target that has cycled back to rest (1) (Figure 1-1). Several studies have shown drastic decreases in viral loads in subjects on ART (1, 2). However, HIV-1 remains in these 'reservoirs' of infected but resting target cells, and if these cells proliferate, the viral replication can reactivate from these reservoirs, making HIV-1 seemingly impossible to clear from the host (1, 2).

These barriers of overcoming HIV infection have altered the focus of HIV vaccine design. Traditionally, a preventative vaccine would be designed to protect an HIV seronegative population from HIV infection upon exposure (Figure 1-4. However, with the high mutation rate of HIV and the virus' ability to establish latent reservoirs of infection, traditional vaccine designs have failed. $CD8^+$ T cell-mediated vaccine research is now predominantly focused on a therapeutic approach (Figure 1-4). The basic goal of a therapeutic vaccine would be to boost the immune responses of HIV^+ people in a way that would directly improve host immune ability to suppress HIV replication. This would alleviate the damage done to the host immune response and delay HIV-1 disease progression, as well as decrease the incidence of new HIV infections by HIV^+ people having unprotected contact with HIV- people (Figure 1-4).

Failure to control HIV-1 infection begins with the inability of the host immune response to clear the infection during the acute phase of HIV-1 disease, and continues into the chronic phase of HIV-1 disease. Understanding mechanisms that moderate control of HIV-1 viremia, will contribute to better control and possibly clearance.

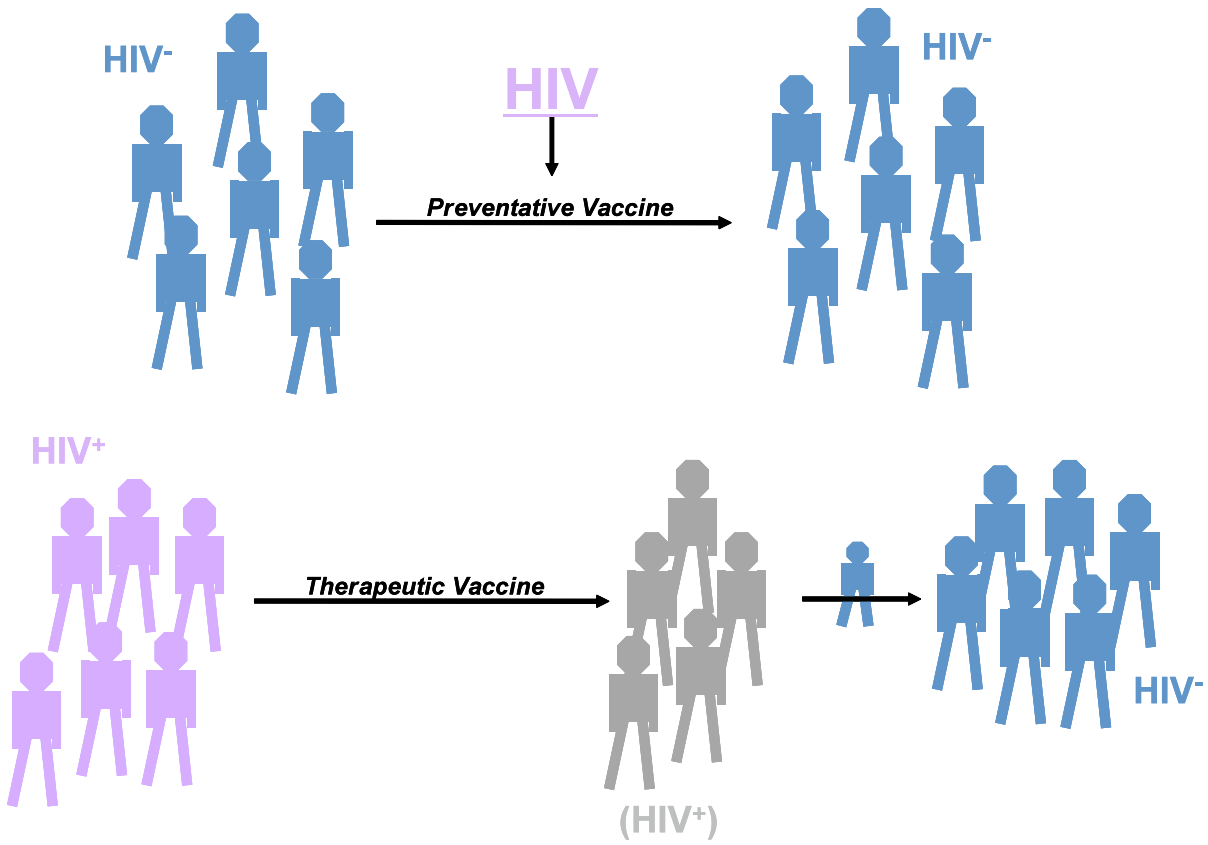


Figure 1-4. Strategy for HIV treatment targets the design of a therapeutic vaccine. Traditional preventative vaccine designs have failed due to high mutation rates of HIV-1, HIV-specific immune escape and failure for HIV-specific antibodies to recognize and bind highly glycosylated HIV envelope. Therapeutic designs will include the boost of the HIV-specific $CD8^+$ T cell response and benefit both HIV^+ people by decreasing viral loads and minimizing immune damage a well as HIV^- people by decreasing probability of infection.

The $CD8^+$ T cell receptor

The TCR of $CD8^+$ T cells is critical for $CD8^+$ T cell anti-viral functions, as it is at the crux of $CD8^+$ T viral recognition and subsequent anti-viral activity. The TCR is a heterodimer consisting of a beta chain and an alpha chain, each consisting of a variable region (V), and a constant region encoded by germ line segments (Figure 1-4) (38, 39). The beta chain is encoded by V, diversity (D) and a joining region (J) whereas the alpha chain is encoded by V and J segments only (Figure 1-5) (38, 39). The variable region contains three complementarity determining regions (CDR) that come in to contact with the peptide-MHC complex (39). CDR1 and CDR2 are encoded within the variable region (Figure 1-5), whereas CDR3 region is composed of the junction between the variable region and joining regions (Figure 1-5) (and for TCR beta this includes the D segment) (38, 39). CDR3 diversity is determined by recombination and TdT nucleotide insertions, and it is the nucleotide sequence of the CDR3 that defines individual TCR clonotypes (39). Several factors shape the development of an epitope-specific TCR repertoire, among them being the initial development of the naïve TCR repertoire. The structure of the peptide MHC, antigen processing and presentation, and the influence of TRBV usage and clonotype expansion within an epitope-specific repertoire (39).

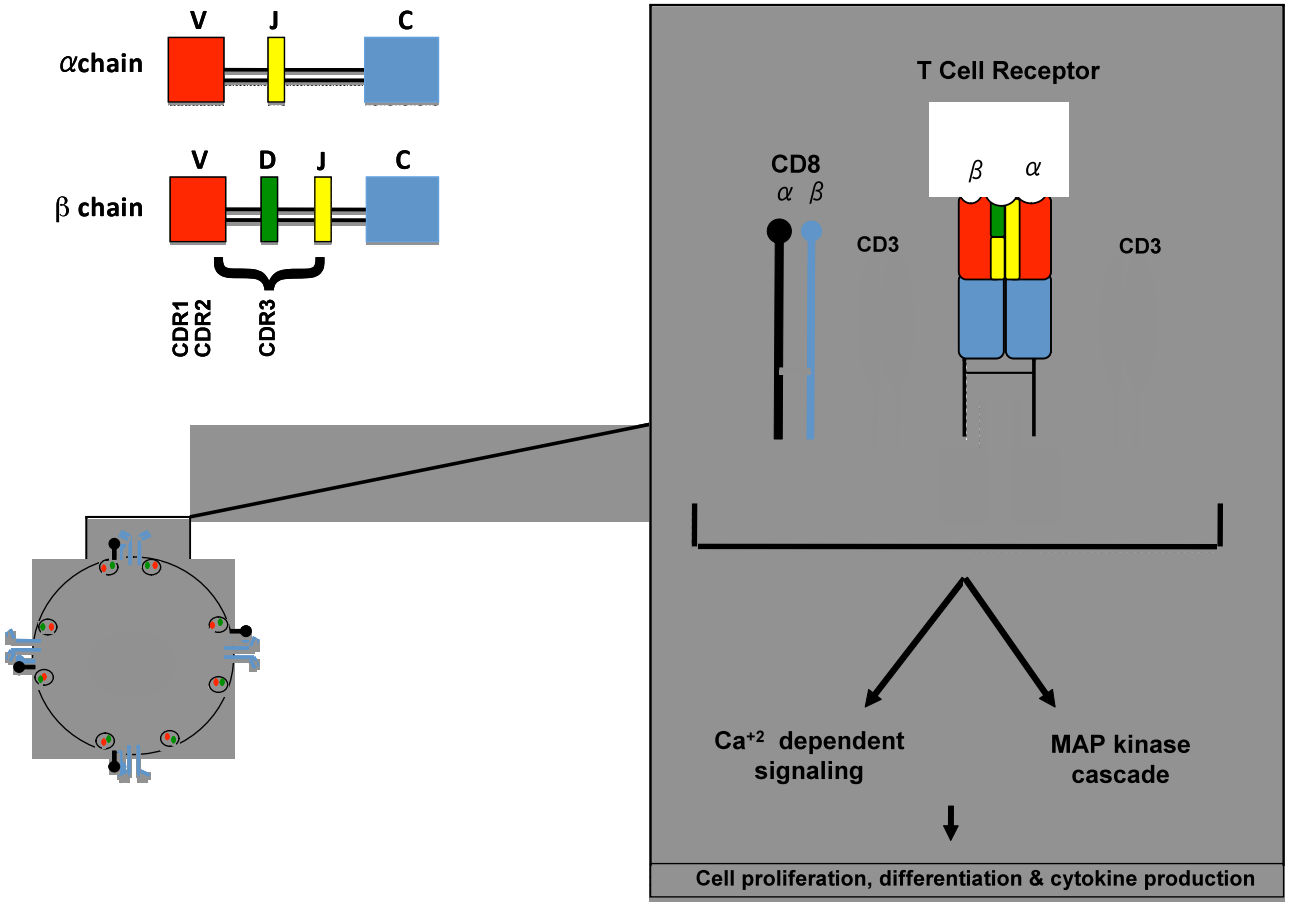


Figure 1-5. T cell receptor formation and diversity. All TCR structures are composed of an alpha and beta subunit. CDR3 is the most variable CDR due to junctional diversity**.**

Figure adapted from Janeway Immunobiology. 5th Edition.

The formation of a T Cell Receptor repertoire

A significant factor in the formation of a $CD8^+$ TCR repertoire is antigen presentation by MHC class I (39, 40). MHC is the most polymorphic gene locus in the human genome. The MHC genes in humans are also referred to as Human Leukocyte Antigen (HLA) and are co-dominantly expressed, meaning both alleles for each gene are expressed simultaneously, leading to further breadth of individual diversity (39). MHC class I is involved in two significant roles of $CD8^+$ T cell selection. First, MHC play a role in early thymic selection of naïve $CD8^+$ T cells, negatively selecting $CD8^+$ T cells that bind too tightly to the MHC class I: self peptide complex, and positively selecting $CD8^+$ T cells that bind sufficiently for activation by the MHC class I: self peptide complex, allowing for the propagation of $CD8^+$ T cells (39). Second, MHC I present processed peptides on the surface of an APC during a viral infection. This peptide-MHC I complex is recognized by $CD8^+$ T_N cells, with the appropriate TCR, and during viral infections, this recognition interaction triggers $CD8^+$ T cell anti-viral activity via the TCR (39).

An over-representation of certain HLA B alleles was noted in HIV^+ subjects with increased duration of chronic HIV-1 disease progression and consistently lower levels viremia. The two strongest associating HLA B alleles with decreased viral loads are HLA B*57 and HLA B*27 (41-44). In the general population these alleles are rare, with a prevalence of approximately 10%, whereas in populations of slow progressors, the prevalence of these HLA B alleles has been found to be as high as 60% (42, 43). Numerous studies have confirmed this over-

representation, but the mechanism behind why this genetic factor is found to be associated with improved control of HIV viremia is unclear. It has been proposed that HLA allele expression influences both TCR structural selection, and subsequent formation of the TCR repertoire (39). Because the MHC class I will select both what naïve TCR will be in circulation as well as epitope-specific TCR repertoires, more scientific study is needed to investigate the potential connection between HLA, TCR diversity, and subsequent observed control of HIV-1 viremia.

In Chapter II of this dissertation, I demonstrate a common TCR structure within a dominant HIV epitope-specific $CD8^+$ T cell population amongst HIV^+ subjects that share a common MHC class I. Although this common TCR did not have stronger structural avidity or improved cross-reactivity to HIV variants, I did observe loss of recognition of an autologous HIV variant on the clonotype level. These studies demonstrate that TCR repertoire diversity can provide recognition of autologous HIV variants, which would be important during continuous control of HIV, a viral infection with rapid mutation and variation, by $CD8^+$ T cells in vivo.

The role of TCR diversity in the control of viral infection

Viral immune escape from $CD8^+$ T cell responses is common over the course of HIV-1 infection, and is likely to be an important factor for HIV-1 disease progression (34, 36, 45-49). The technology for evaluating epitope-specific responses has evolved to sorting epitope-specific $CD8^+$ T cells directly ex vivo,

reducing sequencing bias and providing a more comprehensive analyses of TCR usage to dominant recognized epitopes. Since the early identification of viral epitopes recognized by HIV-specific $CD8^+$ T cells, there has been speculation that the circulating TCR repertoire is critical to the recognition of potential HIV-1 variants. The immune TCR repertoire directed against a peptide-MHC complex is the sum of individually rearranged TCRs. The impact of $CD8^+$ T cell TCR diversity on the recognition and control of viral infections, including HIV-1, is not completely understood.

Many pathogens evolve and adapt to immune recognition, altering structures and functions to evade immune recognition. Because of this evolution, the immune system generates diversity of TCR structure in naïve cells of the adaptive immune response in order to control these evolving pathogens. Although naïve T cell receptor repertoires are generated to be broad, a significant amount of narrowing in repertoire occurs during the development of an epitope-specific TCR repertoire.

Several models of chronic viral infection have been explored to determine the role of TCR diversity in the control of viral replication. The mouse LCMV model can be manipulated to demonstrate viral clearance or chronic infection (50, 51). Early studies demonstrated heterogeneous TCR usage at the clonal level for $CD8^+$ T cells able to recognize the same epitope, and the hypothesis was put forth that diverse TCR usage could play a role in the recognition of potential immune escape variants (52, 53). In the LCMV model, it has recently been shown that narrowed epitope-specific TCR usage leads to immune escape (54).

Chronic viral infections with extensive variation, such as Hepatitis C (HCV) and HIV-1, pose a far greater challenge for cellular immune responses, as well as for the interpretation of the role of TCR diversity for immune recognition. In these infections, both the magnitude and quality of the host response, in this case defined by TCR repertoire diversity, may be critical to the control of viral replication. In the HCV chimpanzee model, our laboratory found that narrow TCR diversity during the early phase of infection was associated with subsequent immune escape and the establishment of chronic viral infection, whereas broader epitope-specific TCR diversity was associated with a lack of escape or resolution of infection (55).

In the SIV model, $CD8^+$ T cells are critical for the control of viremia (33). Viral escape during early infection has been associated with mutations in several MHC class I-restricted epitopes and accelerated disease progression (56-66). Early studies found the TCR repertoire of the dominantly recognized Gag epitope CM9 to be clonally diverse and able to recognize potential escape variants (67, 68). Tat (transactivator) is a regulatory gene that is involved in the acceleration of HIV replication (2). When comparing this dominant gag-specific TCR repertoire with that of a tat-specific repertoire, a drastic difference in TCR repertoire diversity was measured. The epitope-specific TCR repertoire of one dominant epitope remained much more diverse than that of the other co-dominant epitope specific TCR repertoire. In the narrower epitope-specific TCR repertoire, specific TCR clones that recognized this epitope lost the ability to recognize and respond to HIV viral epitope variants (64, 69-71). These studies

emphasize that a limited clonotype repertoire may be quite effective at suppressing viral replication, but given the extensive variation and high-level viral replication in vivo, immune escape is a likely possibility, especially if such a variant is without a fitness cost to the virus.

HIV-1 is known to modulate the TCR repertoire. Using the technique of CDR3 length 'spectratyping', earlier studies demonstrated skewing of the TCR repertoire during acute HIV-1 infection (72-74). In a study of chronic HIV-1 infected subjects, ART afforded some correction of this repertoire skewing (75). Whereas these studies showed some degree of perturbation of the TCR repertoire as a consequence of HIV infection, they did not address the direct contribution of the virus-specific TCR repertoire to the recognition of circulating virus.

The role of HIV-specific TCR repertoire diversity in the control of HIV-1 viremia remains unclear. Our laboratory longitudinally followed several individuals with dominant recognition of the same epitope located within HIV nef; HLA B*8 restricted FL8 (FLKEKGGL). We documented diverse TCR usage in these subjects that had been taken off ART, as TCR usage fluctuated over time (76). This was consistent with a previous study that found fluctuations in TCR clonotype frequency within an HLA A2 restricted HIV Gag epitope-specific response (77). Furthermore, in our studies, individual clonotypes demonstrated differential ability to recognize and respond to their cognate epitope, suggesting the importance of the clonotypic repertoire in the recognition of circulating

epitope variants (76). These studies suggest that heterogeneous TCR usage can benefit the host.

Several epitopes restricted by the HLA B57 allele have been identified, and some dominantly recognized epitopes show little evidence of epitope variation (10, 78). A more recent study found highly conserved TCR usage within the B57-restricted KF11-specific response. Three subjects out of five studied were found to use one structurally similar TCR. In which, two subjects this particular TCR clonotype was dominantly represented in the total KF11-specific $CD8^+$ T cell populations within these individuals (79). An additional study indicated narrowed TCR repertoires associated with decreased epitope diversity in HLA B57 HIV^+ subjects compared to HLA B57 HIV^+ subjects with increased diversity of the same HIV-specific HLA B57 restricted epitope (80).

These observations suggest that structural components of the $CD8^+$ TCR repertoire may have profound effects on the dynamics of viral replication. The ability to generate and maintain a diverse TCR repertoire can allow the recognition of epitope variants. In the setting of less variable epitopes, the generation of a limited repertoire of TCRs able to control viremia quickly may limit subsequent virus variability. Regardless of whether TCR repertoire diversity is required for complete control of viral infections, the structure of the TCR is critical for recognizing the peptide MHC complex and subsequent $CD8^+$ T cell anti-viral activity. Further studies will allow us to determine whether TCR repertoire analyses will provide an additional measure of the 'quality' of immune responses coincident with $CD8^+$ T cell functions associated with the control of HIV viremia.

$CD8^+$ T cell function and suppression of HIV-1 replication

Despite numerous studies indicating the importance of $CD8^+$ T cells in the control of HIV-1 viremia, $CD8^+$ T cells are not sufficient for the successful suppression of HIV-1 infection (27, 29). HIV^+ subjects are observed to have significant expansions of $CD8^+$ T cells (CD4:CD8 is 1:2 rather than 2:1) (81), but these cells fail to mount an appropriate and effective response that would suppress HIV-1 infection. In corroboration with these findings, it has also been observed that large $CD8^+$ T cell expansions are documented in both high and low viremic HIV^+ subjects (43, 81). Early in the epidemic, investigators found that these $CD8^+$ T cell expansions contained a large fraction of HIV-specific $CD8^+$ T cells with lytic function (82). Later studies using superior assays able to measure the frequency of cytokine-producing cells similarly found very high frequencies of circulating IFNγ producing cells in HIV infected subjects. However, the magnitude of IFNγ producing cells does not correlate with control of viremia (83-85). This leads one to the conclusion that the effectiveness of $CD8^+$ T cell suppression of HIV-1 viremia seems to lie in the quality rather than that quantity of the immune response.

Observational studies in HIV^+ slow progressors have provided evidence for the critical role of several functions of $CD8^+$ T cells that correspond with control of HIV-1 viremia. Diversity of anti-viral cytokine production has been shown to increase in HIV-1 controllers (86-88). $CD8^+$ T cells that produce a variety of cytokines are categorized as 'poly-functional' effector T cells. In addition to a poly-functional response, several viral infection models have provided evidence

for the importance of maintaining a response to a broad range of viral variants. In a response to a highly mutagenic virus such as HIV, effective cytokine secretion in response to viral variants could be an important factor.

In animal models of chronic viral infection, the proliferative capacity of virus-specific T cells represents the best correlate of control of viremia (15, 89, 90). Proliferative capacity is defined as the ability of a cell to proliferate, or duplicate, several times, expanding identical sister cells from, in this example, a single epitope-specific $CD8^+$ T cell. The advantage of proliferation is considered to be the ability to rapidly expand populations of dominant effector cells, increasing E:T ratios, and thus effector efficiency in suppressing HIV replication. As T cells expand in response to antigenic stimulation, they mature to T_E, and become more efficient at suppressing HIV replication (1, 15, 74, 91-95). IL-2 is an auto-regulatory cytokine that is produced as well as bound by the IL-2 receptor of an individual cell. This interaction causes cellular signaling that promotes proliferation of the cell (96). Early studies assessed the ability to boost the proliferative capacity of the overall immune response by administering recombinant IL-2 therapy to recently infected HIV^+ subjects (97). No increase in HIV-specific $CD8^+$ T cell responses was observed, thus suggesting that any benefit of increased proliferation may require a more refined HIV-specific stimulation.

A recent study has reported observations of increased proliferation (in vitro) to optimal HIV-specific peptides in HIV-1 controllers (viral loads less than 2000 copies/mL) as compared to HIV-1 non-controllers (viral loads greater than 2000

copies/mL) (89). The increase in specific proliferation to cognate peptide was also associated with increased production and release of perforin, the lytic protein that is secreted through $CD8^+$ T cell granules. These data indicating the association of high HIV-specific proliferation with lower levels of viremia have led to the hypothesis that the proliferative capacity of dominant HIV-specific $CD8^+$ T cells influences their overall ability to suppress HIV replication. Recent studies have associated the expression of cellular markers such as CD38 and HLA-DR, which are markers that indicate a heightened proliferative state, directly with $CD8^+$ T cell ability to suppress HIV-1 replication in vitro (98). However, further studies are required to directly assess defined proliferative cell markers with $CD8^+$ T cell proliferative capacity and suppression of HIV-1 replication.

Since proliferative potential appears to be important for control of viremia, surface markers that are able to predict proliferative capacity might be useful in the identification and assessment of cells that are qualitatively better at suppressing viral replication. Characteristic of chronic infection and constant antigen presentation, HIV-1 causes characteristics of cellular aging (such as decreased telomere length) and dysfunction of the immune response (1, 99, 100). Functional exhaustion was first observed in the murine LCMV chronic model of viral infection (51), in which virus-specific $CD8^+$ T cells that were present during LCMV infection were unable to produce effector cytokines during in vitro antigen stimulation (51). Additional studies in the LCMV model revealed that this state of functional exhaustion further impacted several anti-viral properties of $CD8^+$ T cells including IL-2 production and proliferation, cytolyses,

and eventual loss of anti-viral cytokine production (101). The Programmed Death-1 (PD-1) receptor was first identified in the chronic LCMV model as a marker for this state of functional exhaustion (102, 103), as studies indicated increased expression of PD-1 on functionally exhausted $CD8^+$ T cells. Studies were extended to find similar observations of increased PD-1 expression on functionally exhausted HCV and HIV-specific $CD8^+$ T cells (100, 104-107). In addition, increased expression levels were higher on HIV-specific $CD8^+$ T cells in HIV-1 progressors as compared to HIV-1 long-term non-progressors. The mechanism of how PD-1 signaling directly influences $CD8^+$ T cell suppression of HIV replication is unknown, as PD-1 expression is associated with varying degrees of $CD8^+$ T cell proliferative capacity, cytokine secretion and direct cytotoxicity. Examining the role of proliferative capacity in suppression of HIV replication requires a more definitive marker for assessing the proliferative potential of a $CD8^+$ T cell.

CD57 has been identified as a marker for immunologic senescence and is increased in chronic infection as well as a consequence of general host aging (99). Several studies have demonstrated that $CD57^+$ cells have significant decreases in proliferative capacity in response to αTCR and cognate peptide stimulation (98, 99, 108). Despite their decreased proliferation, $CD8^+CD57^+$ T cells maintain strong effector functions of direct cytotoxicity and anti-viral cytokine production (98, 99, 108). Furthermore, analysis revealed that $CD8^+CD57^+$ T cells have increased upregulation of genes involved in all effector functions as compared to their $CD57^-$ counterparts (108). Although CD57 expression on

$CD8^+$ T cells is increased in HIV^+ individuals, these microarray analyses indicated that transcriptional profiles of $CD8^+CD57^+$ T cells in both HIV^+ and HIV^- subjects did not vary (108), suggesting that HIV influence on the immune response is specifically in the differentiation to this state of deficient proliferative capacity, and not the remaining effector functions of these $CD8^+CD57^+$ T cells. Distinguishing $CD8^+$ T cells by the expression of CD57 allows for a unique opportunity to assess the role of proliferative capacity in an environment of maintained effector functions that remain despite a lack in proliferative capacity. By distinguishing $CD8^+$ T cells based on the expression of CD57, the role of proliferative capacity in the suppression of HIV replication can be solely examined.

In Chapter III of this dissertation, I developed an in vitro assay that allowed for the simultaneous assessment of proliferative capacity and $CD8^+$ T cell-mediated suppression of HIV replication. I differentiated $CD8^+$ T cells, which were derived directly ex vivo, by the ability to proliferate based on CD57 expression. By using this assay I was able to demonstrate that proliferative capacity did not play a direct role in $CD8^+$ T cell-mediated suppression of HIV replication in vitro.

HIV provides a unique environment in which to study the effects of immune system breakdown and exhaustion. By elucidating the mechanisms by which $CD8^+$ T cells sustain and function in successful suppression of viral replication despite the hurdles HIV places in front of the immune response, improved understanding and development of preventative and therapeutic vaccines can be attained for not only HIV, but perhaps other chronic viral infections with increased viral variation.

Research Objectives

The main objectives of my research were: (1) to investigate the role of TCR repertoire diversity in the recognition of dominant HIV epitopes (2) to define autologous HIV variants *in vivo* and evaluate the ability of recognition of these variants in vitro on a clonotype level, and (3) to directly assess the role of $CD8^+$ T cell proliferative capacity in the suppression of HIV replication in vitro. In Chapter II, I demonstrate that although TCR repertoire development to a highly conserved epitope consists of biased gene usage, maintenance of a diverse TCR repertoire plays a role in the recognition of HIV-1 variants. In Chapter III, my studies indicate that proliferative capacity does not play a direct role in $CD8^+$ T cell suppression of HIV-1 replication in vitro. I further demonstrate that remaining $CD8^+$ T cell effecter functions such as cytokine secretion and cytolytic properties are what determine $CD8^+$ T cell abilities of HIV suppression.

Collectively my research provides the scientific community with a thorough examination of the relationship between HIV-1 epitope-specific TCR diversity and $CD8^+$ T cell recognition of HIV variants as well as gives insight into the role of proliferative capacity in $CD8^+$ T cell suppression of HIV-1 replication. These findings have important implications for assessing vaccine-induced immune responses and the role of immune exhaustion in the ability to control chronic viral infections.

CHAPTER II

TCR DIVERSITY CAN PROVIDE RECOGNITION OF CIRCULATING EPITOPE VARIANTS

Abstract

The role of epitope-specific T cell receptor (TCR) repertoire diversity in the control of HIV-1 viremia is unknown. In collaboration with Dr. Dirk Meyer-Olson, I performed in depth analyses of T cell clonotypes directed against a dominantly recognized HLA B57-restricted epitope (KAFSPEVIPMF; KF11) and identified common usage of the TCR beta chain TRBV7 in 8 of 9 HLA B57 subjects examined, regardless of HLA B57 subtype. Despite this convergent TCR gene usage, structural and functional assays demonstrated no substantial difference in functional or structural avidity between TRBV7 and non-TRBV7 clonotypes and this epitopic peptide. In a subject where TRBV7-usage did not confer cross-reactivity against the dominant autologous sequence variant, another circulating TCR clonotype was able to preferentially recognize the variant peptide. These data demonstrate that despite selective recruitment of TCR for a conserved epitope over the course of chronic HIV-1 infection, TCR repertoire diversity may benefit the host through the ability to recognize circulating epitope variants.

Introduction

Chronic viral infections with extensive variation, such as Hepatitis C and HIV, pose a great challenge for the cellular immune response (37, 109-111). Several studies have attempted to link the quantity, typically measured by the frequency of virus-specific cytokine-producing cells, and quality of host responses, assessed by either the ability to secrete a diverse array of cytokines or by the proliferative capacity of virus-specific T cells, to the level of control of viremia (88, 89, 112, 113). The Kalams laboratory has recently demonstrated that the level of epitope-specific TCR repertoire diversity during acute HCV infection may be critical for limiting immune escape and for subsequent control of viral replication (55). These findings have been replicated in the SIV acute infection model (55, 58, 114). However, the level of TCR diversity in subjects with long-term control of HIV-1 viremia, and its potential role in the control of viremia has not been completely defined.

Efforts to understand TCR repertoire diversity have focused on epitopes frequently recognized by subjects with a particular HLA allele. HLA B57 has shown the strongest association with control of viremia, and subjects with this allele typically have robust HLA B57-restricted $CD8^+$ T cell responses (10, 42, 115, 116). Furthermore, several HLA B57 epitopes have been fine-mapped, including a dominantly recognized, highly conserved epitope located in p24 Gag, KF11 (10, 36). Patterns of TCR usage directed against this epitope may shed light on how TCR recruitment over the course of infection mediates control of viremia.

The TCR gene usage of KF11-specific immune responses has been assessed for shared motifs. In previous work Gillespie et al. found conserved TRBV usage (TRBV19) and highly conserved CDR3 region motifs among isolated CD8+ T CELLS clones from 3 out of 5 subjects recognizing the KF11 epitope (79). Yu et al. more recently studied TCR usage of KF11-specific T cells and found that HLA B*5701 subjects had striking usage of TRBV19 with shared CDR3 motifs, very little variation of the circulating KF11 epitope, and these TCRs displayed cross reactivity to published KF11 variants (80). In contrast, HLA B*5703-restricted responses had more diverse TCR usage and more in vivo variation of the KF11 epitope, yet were unable to recognize in-vivo HIV variants. These researchers concluded that a 2 amino acid difference between the HLA B*5701 and B*5703 alleles was likely responsible for the HIV epitope variation seen in HLA B*5703 subjects, and consequently these subjects had more diverse TCR repertoires (80). While it remains unclear whether TCR diversity is a prerequisite for control of HIV-1 viremia, the structure of the TCR repertoire is the driving force behind the immune system's ability to recognize and respond to virus variants. Therefore, understanding how the selection of TCR repertoires influences the host's ability to contend with HIV variation is important for understanding the correlates of control of viremia.

With assistance from Dr. Dirk Meyer-Olson, I evaluated the TCR repertoires of HLA B*57-KF11 specific $CD8^+$ T cells in subjects with either the HLA B*5701 or the HLA B*5703 allele. My goal was to determine whether the diversity of the TCR repertoire specific for this immunodominant epitope was directly related to

control of HIV-1 viremia. In addition to a detailed TCR repertoire analyses of directly sorted T cells, I sequenced autologous virus, performed detailed tetramer off-rate analyses, and functional avidity assays by ELISpot. I identified common usage of the TCR beta chain TRBV7 (IMGT) within the KF11-specific repertoires of 8 of the 9 HLA B*57 subjects examined, regardless of whether these subjects possessed the HLA B*5701 or HLA B*5703 allele. I found a wide range in the number of epitope-specific TCR clonotypes within each subject, and no apparent structural or functional advantage to this TRBV7 usage. However, analyses of the functional avidity for a KF11 variant I observed in our cohort, K162R, revealed a potential role for clonotype diversity in the recognition of viral variants during chronic infection. These data suggest some degree of selective recruitment of TCR for a conserved epitope and highlight convergent TCR usage in subjects with a favorable disease course.

Materials and Methods

Study subjects

The Vanderbilt-Meharry CFAR Cohort was comprised of subjects recruited through the Comprehensive Care Center (Nashville, TN) and all subjects were HLA class I typed (4 digit resolution) (DCI, Nashville, TN). Based on HLA typing results, 17 HLA B57 subjects were selected for further study. All subjects were antiretroviral therapy naïve at the time of study with a range of $CD4^+$T cell numbers from 144 to 1260/mm^3 and log viral load measurements from 1.7 to

4.25 copies/ml. This study was approved by the Vanderbilt University Medical Institutional Review Board, and all subjects provided informed consent.

Sequencing of autologous virus

Autologous virus was population sequenced from plasma RNA. Viral RNA was isolated from plasma and reverse transcribed as described (76). Gag DNA was amplified by PCR with the following primers: 5gag7-28 5'-GCG AGA GCG TCA GTA TTA AGC G – 3' and 3gag1668-1693 5' TCT GAG GGA AGC TAA AGG ATA CAG TT – 3'. PCR fragments were then gel purified and sequenced bi-directionally on an ABI 3100 PRISM automated sequencer. Sequencher (Gene Codes Corp., Ann Arbor, MI) was used to edit and align sequences.

Sorting of tetramer-positive $CD8^+$ T cell populations

Fresh or cryopreserved PBMC samples were first $CD8^+$ T cell enriched by magnetic separation (Robosep, Stem Cell Technologies, Vancouver, British Columbia, Canada), and then stained with phycoerythrin-labeled B*5701/KF11 tetramer, which has been previously shown to bind equally to B5701- and B5703-restricted KF11-specific $CD8^+$ T cells (117). Tetramer-positive $CD8^+$ T cells, as well as an equal number of tetramer-negative $CD8^+$ T cells (as a negative control) were sorted by a FACS Aria instrument (BD Biosciences) under BSL3 conditions direCD8+ T cells into STAT 60. Electronic compensation was performed with PBMC from the same subject stained separately with individual

antibodies used in the test samples. The purity of sorted cell populations was consistently greater than 95%.

cDNA synthesis and TRBV sequencing

RNA was extracted from purified T cells using STAT-60 (Tel-Test B, Friendswood, TX). A modified anchored RT-PCR was performed with Powerscript Reverse transcriptase (Clontech, Palo Alto, CA) from total RNA as previously described (55) using a gene-specific primer for the beta constant region with a modified cDNA anchor primer (Clontech, Palo Alto, CA). Negative controls were included at all amplification steps. Amplification of cDNA by PCR was performed using TCR constant region based primers and an anchor-specific primer 5' – AAT CCT TTC TCT TGA CCA TG-3'. PCR products of 600 to 700 base pairs were gel purified and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Selected colonies were sequenced using Taq DyeDeoxy Terminator cycle sequencing Kit (PE Applied Biosystems, Norwalk, CT) and capillary electrophoresis on an ABI 3700 PRISM automated sequencer (PE Applied Biosystems, Norwalk, CT). Sequences were edited and aligned using Sequencher (Gene Codes Corp., Ann Arbor, MI) and compared to the human TRBV genes database (http://imgt.cines.fr). To accommodate for different CDR3 regions length, an alignment using clustalW was performed. Positions with >50% gaps were excluded from analyses to prevent any substantial bias introduced by minor populations. The TRBV classification system is that of the international ImMunoGeneTics database.

Statistical Analyses

Amino acid variability in TCR CDR3 regions was determined using the Shannon entropy (H) calculation for protein sites as described previously (76) by the formula $H = -\sum pi \log2\ pi$ where pi is the fraction of residues at a site that is amino acid type i. For the 20 amino acids, H can range from 0 (site contains only one amino acid in all sequences) to 4.32 (all amino acids are represented equally at this site). Positions that contained >50% gaps were excluded from analyses. In the comparative analyses of TRBV population off rates, the Mann-Whitney unpaired T test was used to calculate the difference in means and the Fligner-Kileen test was used to compare the difference in variance between the TRBV populations off rates.

Tetramer off-rate

Cryopreserved PBMC were thawed and resuspended at a concentration of 10^7/mL in R10 medium and stained with PE-KF11 Tetramer (Beckman Coulter) at a concentration of 1:100 in FACS buffer + Sodium Azide (1%). Remaining surface antibodies, including the appropriate Vß antibody, were added 10 minutes after the initial tetramer stain. Vß antibody (Beckman Coulter, USA) selection was determined by sequencing data obtained for KF11-specific CD8+ T cells (Figure 2-5, Figure 2-7). An individual well was set up for each Vß population in order to determine Vß off rates. In order to determine the TRBV7 off rate, all antibodies corresponding to the identified Vß populations by sequencing were including in one condition, and the 'non- Vß' population was

gated in order to measure loss of fluorescence (Table 2-1). Preliminary experiments performed to assess the effects of Vß staining on tetramer off-rate kinetics indicated no effect on tetramer off-rate values. Due to feasibility every Vß and tetramer combination were not tested. PBMC were then washed and resuspended in 100 µl of a 1:4 concentration of APC KF11 Tetramer (Beckman Coulter) in FACS buffer (PBS/2% FCS/0.1% Sodium Azide). PBMC were then incubated at 37°C and at each time point (0, 10, 20, 40, 80 minutes) 20 µl of the mixture was removed and placed into 180 µl of 1% Para formaldehyde. Samples were analyzed on a FACS Aria using FACS Diva software. $CD8^+$/$tetramer^+$/$Vß^+$ cells were gated and this gate extended over the entire range of PE expression levels. The geometric mean of PE fluorescence was measured over time and normalized to time 0. Tetramer off-rates were calculated with Graph Prism software, 1^{st} rate kinetics equations. Individual data sets were normalized to compare off rates amongst subjects and individual cell populations.

Functional Avidity ELISpot

96-well MultiScreen filtration plates (MILLIPORE) were coated with 0.1ug/mL of an anti-human gamma interferon (IFNγ) monoclonal antibody (Mabtech, Stockholm, Sweden). $CD8^+$-depleted PBMC were added at a concentration

Table 2-1. Corresponding Vβ antibodies to TRBV sequences. KF11-specific TCR Vβ sequences listed in IMGT nomenclature and corresponding commercially available antibodies for surface staining in the Arden nomenclature.

Subject	IMGT	ARDEN (Beckman Coulter)
10004		
	TRBV19	Vß 17
	TRBV24	(Vß 15)
	TRBV7-9	(Vß 6)
10071		
	TRBV5-1	Vß 5.1
	TRBV7-6	(Vß 6)
10067		
	TRBV7-6	(Vß 6)
10027		
	TRBV10-3	Vß 12
	TRBV6-5	Vß 13.1
	TRBV20-1	Vß 2
10002		
	TRBV6-5	Vß 13.1
	TRBV28	Vß 3
	TRBV12-4	Vß 8
	TRBV13	Vß 23
	TRBV7-9	(Vß 6)
10024		
	TRBV3-1	Vß 9
	TRBV7-9	(Vß 6)
20018		
	TRBV24-1	(Vß 15)
	TRBV5-6	Vß 5.2
	TRBV28-1	Vß 3
	TRBV7-9	(Vß 6)
	TRBV7-6	(Vß 6)
10070		
	TRBV11-2	Vß 21.3
	TRBV14	Vß 16
	TRBV7-9	(Vß 6)
	TRBV7-8	(Vß 6)
	TRBV7-6	(Vß 6)
10076		
	TRBV2	Vß 22
	TRBV7-9	(Vß 6)
(commercially unavailable)		

of 100,000 cells per well in a volume of 100 µl of RPMI 1640 medium supplemented with fetal calf serum (10%), HEPES Buffer (10mM), L-glutamine (2mM) and penicillin-streptomycin (50U/mL) (R10 medium). Cryopreserved PBMC were $CD8^+$ enriched (Stem Cell, Vancouver CA) by magnetic separation, sorted on a FACSAria to >98% purity by Vβ specificity (Figure 2-1), and added back to the ELISpot plate in the appropriate wells. Vβ specificity and selection were based on the individual Vβ populations identified by sequencing of the KF11- tetramer specific populations (Table 2-1). In order to insure a positive and interpretable response, the number of selected $CD8^+$ T cells added back was individually calculated based on tetramer percentage as well as clonotype frequency within that tetramer population. A positive response was defined as a minimum of 50 SFC/10^6 cells at all concentrations and 3 times above background. Peptides were serially diluted in R10 medium and added to each well in a volume of 10 µl. Plates were incubated over night at 37^{o}C in 5% CO_2 and developed the following day (118). Wells containing PBMC and medium with SEB or without any peptide were used as positive and negative controls respectively, and run in duplicate on each plate. Peptide-stimulated, CD8 - depleted PBMC were also included as a negative control. To calculate the number of specific T cells, the number of spots in the negative control wells was subtracted from the counted number of spots in each well. All negative controls were less than 30 SFC/10^6 cells.

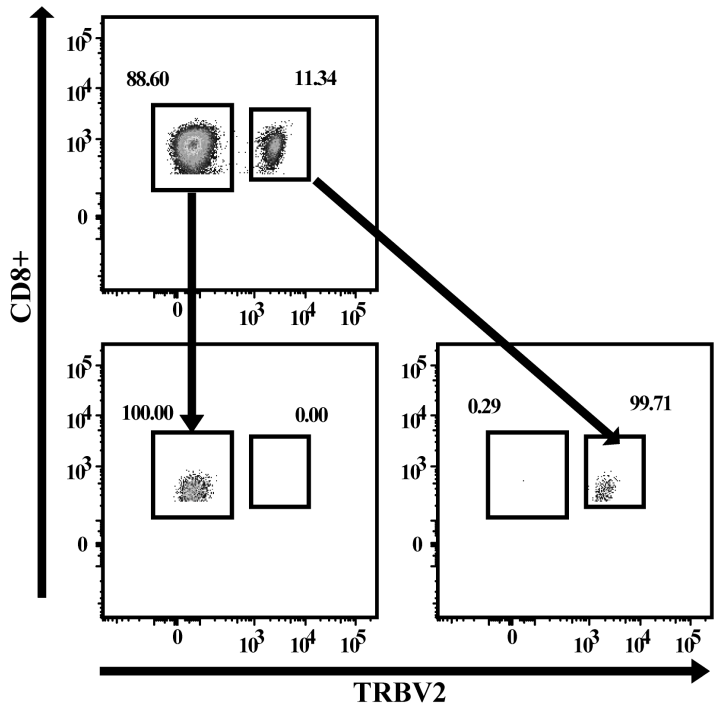


Figure 2-1. Schematic of Vβ sorting from enriched CD8$^+$ T cells. Numbers indicate log scale of magnitude of fluorescence.

Results

KF11-specific CD8$^+$ T cell responses remain stably dominant over time

In order to examine the KF11 specific response, Shelly Lorey identified 17 subjects with expression of the HLA B*57 allele from a cohort of 146 therapy-naïve subjects and I evaluated their response to HIV-1 HLA class I-restricted epitopes. I assessed these subjects' abilities to recognize HIV-1 peptides by IFNγ ELISpot, and compared the sum of all HLA B*57 restricted responses to the total IFNγ response directed against all HLA restricted peptides. The total B*57-restricted responses typically made up the majority of the overall HLA-restricted responses (average 74%) (Figure 2-2). This confirms recent findings describing the immunodominance of HLA B*57-restricted responses (116). I was able to longitudinally follow 8 of these subjects. In 7 of the 8 subjects, this HLA B*57 dominance remained stable over a 10-21 month follow-up period (Figure 2-3). At the epitope level, these subjects had dominant recognition of the HLA B*57-restricted KF11 peptide. Fourteen of the 17 subjects tested recognized this epitope, and in 10 of these subjects, it was the highest magnitude response. Of the 8 longitudinally followed subjects, 6 subjects that recognized KF11 maintained dominant KF11 responses over time (Figure 2-4).

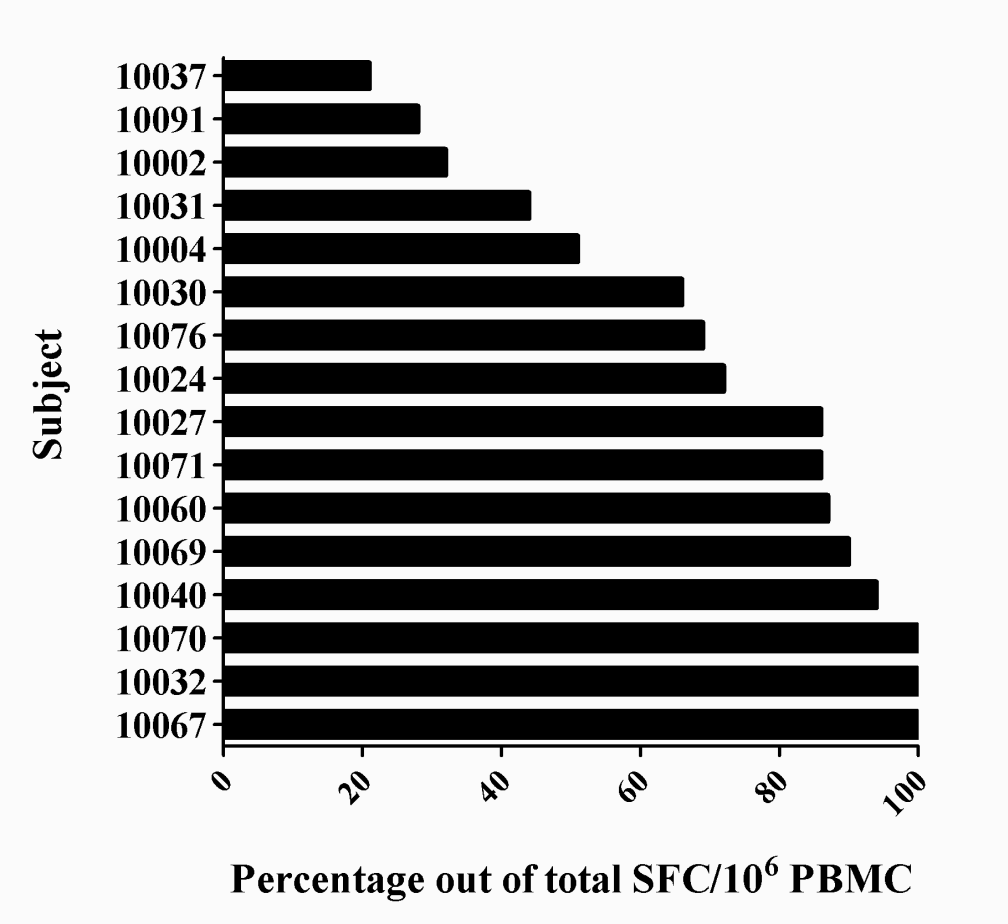


Figure 2-2. HLA B*57-restricted responses are dominant. Black bars represent the B*57 restricted response as a percentage of total HLA-restricted responses.

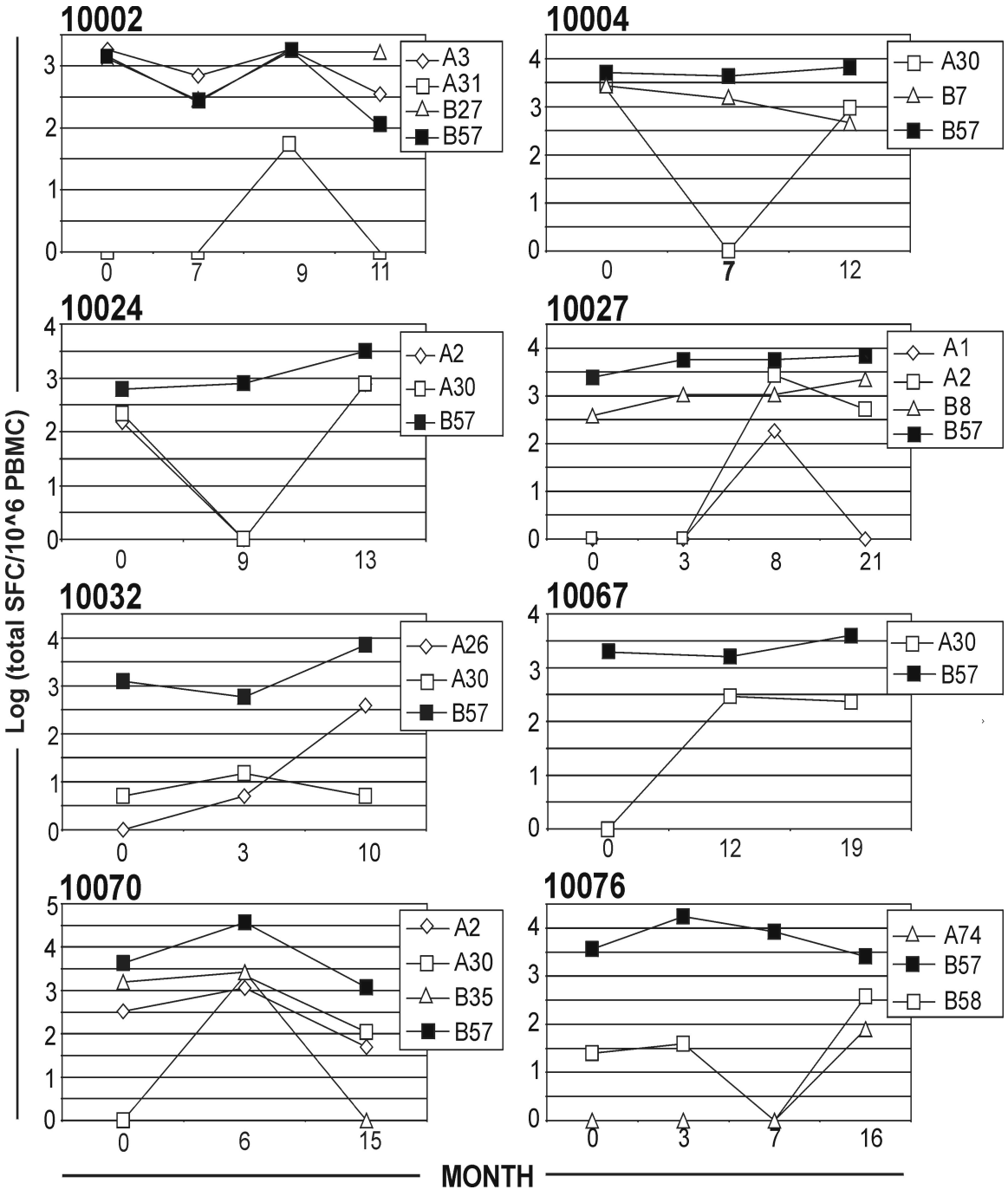


Figure 2-3. HLA B*57-restricted responses remain dominant over time.
Black squares represent total HLA B*57-restricted responses at each time point.

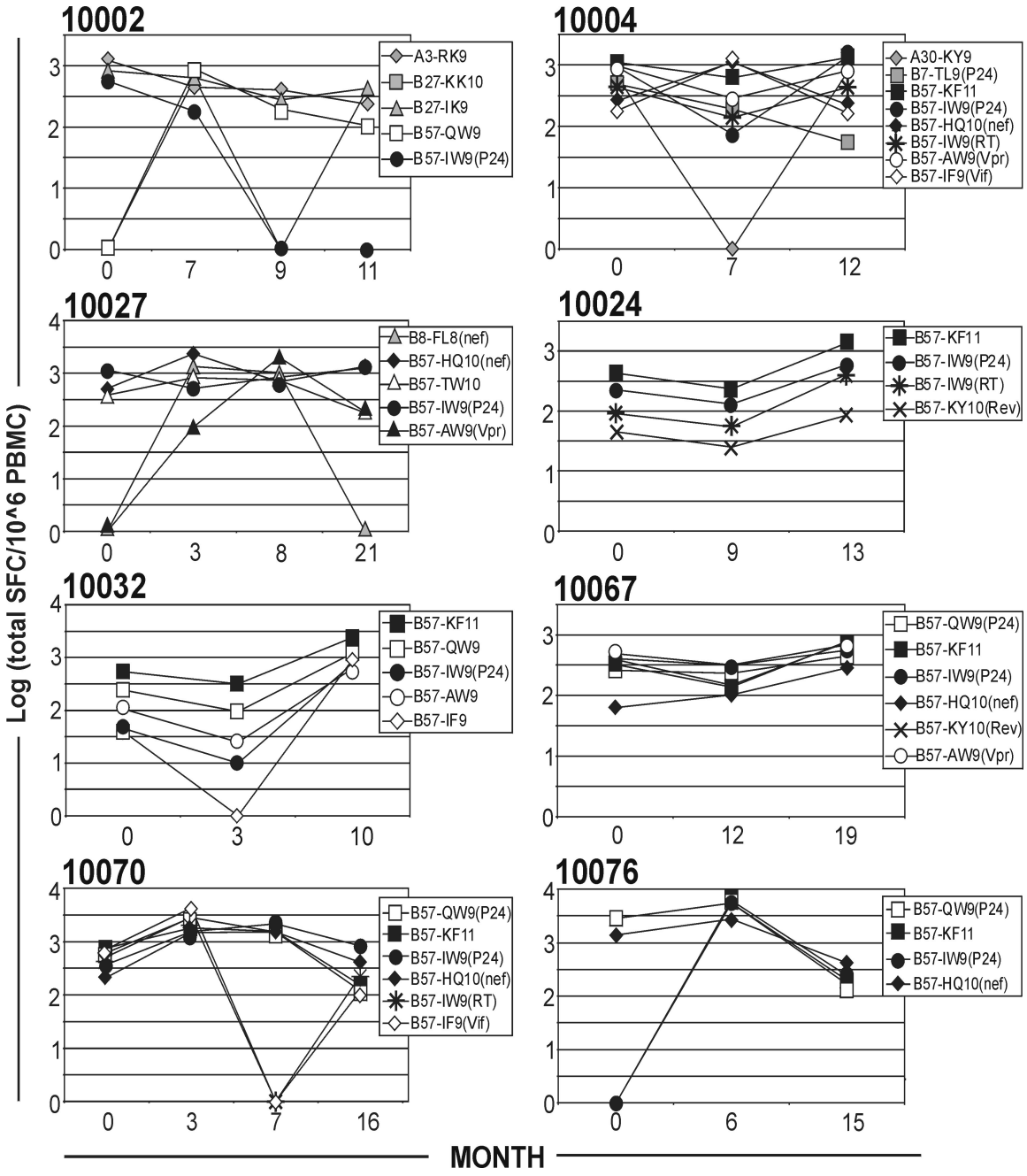


Figure 2-4. KF11-specific CD8$^+$ T cell responses remain dominant over time. Black boxes represent magnitude of KF11-specific response at each time point.

Analyses of epitope-specific TCR repertoire diversity

Having identified subjects with robust recognition of the KF11 epitope, I next evaluated the diversity of these immune responses at the clonotype level. With assistance from Louise Barnett in the Center For AIDS Research Immunopathogenesis Flow Cytometry core, I directly sorted KF11-specific T cells from 9 chronically infected HIV^+ B57 subjects (Table 2-2 and Figure 2-5). In parallel, an equivalent number of KF11 tetramer-depleted $CD8^+$ T cells were sorted and also subjected to TCR sequence analyses. At least 45 sequences from each sorted population of cells were analyzed for each individual subject. Sequences were assessed for diversity by calculating the entropy of aligned Vβ CDR3 sequences and categorized as individual clonotypes by identification of CDR3 nucleotide sequences. The mean (and median) number of TCR clonotypes utilized was 5, with a range of 1 (subject 10067) to 10 clonotypes (10070 and 20018) within each KF11-specific repertoire (Figure 2-6). Despite a previous report suggesting differences in TRBV usage between the KF11 specific TCR repertoires of B*5701 and B*5703 subjects (80), I found no such differences in our subject cohort. In 8 of the 9 subjects, regardless of the level of clonotype diversity as defined by distinct CDR3 gene usage, clonotypes tended to use the TRBV7 gene. Only one individual, subject 10027 did not use TRBV7 for this response. We could not identify common motifs within the CDR3 regions of the KF11 specific TCR repertoires in our subject group (Figure 2-6). However, when compared to the B*5703 subject group published by Yu et al (80) I found four CDR3 motif similarities, and shared TRBJ usage amongst our subjects, and the majority of the clonotypes utilized TRBV7.

Table 2-2. Therapy naïve HIV+ cohort. (*) Virus could not be sequences from plasma nor from proviral DNA. CD4 and CD8 T cell data reflects the number of cells per cubic millimeter of blood.

Subject	Log Viral Load	HLA Type	Year Infected	CD4	CD8	Frequency of KF11+ CD8+ T cells	KF11 Sequence KAFSPEVIPMF
10004	1.7	A3 A30 B7 **B5701**	1983	144	496	1.78%	*
10071	1.7	A1 A66 B8 **B5701**	1992	658	350	1.92%	*
10067	2.1	A30 A33 B13 **B5703**	1991	988	456	2.81%	*
10027	2.5	A1 A2 B8 **B5701**	1992	544	782	1.01%	----------
10002	2.59	A3 A31 B27 **B5701**	1984	693	1071	6.83%	----------
10024	2.71	A2 A30 B58 **B5703**	1998	783	1161	3.11%	R---------
20018	3.28	A2 A26 B40 **B5701**	2004	1374	1035	9.38%	----------
10070	3.95	A23 A74 B58 **B5703**	1996	846	414	11.76%	----------
10076	4.25	A2 A30 B35 **B5701**	1999	832	1952	9.38%	R---------

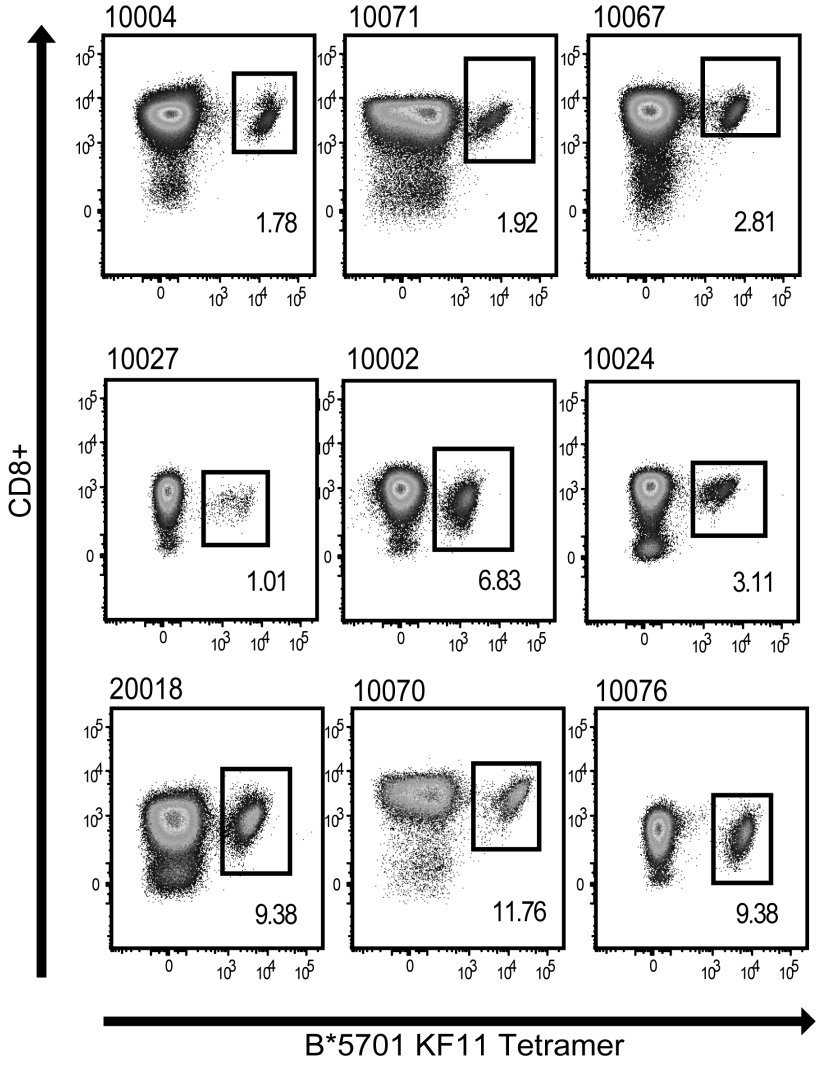


Figure 2-5. Frequency of KF11-specific CD8$^+$ T cells. Numbers along axes reflect the log scale for the magnitude of fluorescence.

10004

TRBV	CDR3 (aa)	TRBJ	Frequency
TRBV19	ASSLTYGYT	TRBJ1-2	1/50
TRBV19	ASSSRTGGYGYT	TRBJ1-2	1/50
TRBV24	ATSDRMDNEQF	TRBJ2-1	1/50
TRBV7-9	ASGGEFYGYT	TRBJ1-2	8/50
TRBV7-9	ASPHPDRPNYGYT	TRBJ1-2	39/50

10071

TRBV	CDR3 (aa)	TRBJ	Frequency
TRBV5-1	ASYNFGQYGYT	TRBJ1-2	39/52
TRBV7-6	ASSPMDLLDEQY	TRBJ2-7	13/52

10067

TRBV	CDR3 (aa)	TRBJ	Frequency
TRBV7-6	ASSFWGQGTDTQY	TRBJ2-3	54/54

10027

TRBV	CDR3 (aa)	TRBJ	Frequency
TRBV10-3	AIGGHDYGYT	TRBJ1-2	40/88
TRBV6-5	ASSSLVNTGELF	TRBJ2-1	20/88
TRBV6-5	ALTGGDYGYT	TRBJ1-2	6/88
TRBV20-1	SARGWVSNNRETQY	TRBJ2-5	12/88
TRBV20-1	AASTSAVLGKKGSQETQY	TRBJ2-5	2/88
TRBV20-1	SAREKGSQETQY	TRBJ2-5	7/88
TRBV28-1	ASSGPGGEQY	TRBJ2-7	1/88

10002

TRBV	CDR3 (aa)	TRBJ	Frequency
TRBV6-5	ASRKGQGDWEAFF	TRBJ1-1	34/63
TRBV28	ASSLNSKVSSSYEQYF	TRBJ2-7	1/63
TRBV12-4	ASSLIAGGGEDTQYF	TRBJ2-3	4/63
TRBV13	ASSLGLDETQYF	TRBJ2-5	1/63
TRBV7-9	ASGGANYGYTF	TRBJ1-2	23/63

10024

TRBV	CDR3 (aa)	TRBJ	Frequency
TRBV3-1	ASSQGVTTGGFLNYGYT	TRBJ1-2	16/50
TRBV3-1	ASMGLAQETQY	TRBJ1-2	1/50
TRBV7-9	ASTDTGYYGYT	TRBJ1-2	32/50
TRBV7-9	ASTDTGDYGYT	TRBJ1-2	1/50

20018

TRBV	CDR3 (aa)	TRBJ	Frequency
TRBV24-1	ATSDLMDNEQF	TRBJ2-1	1'51
TRBV5-6	ASILTSGRNEQF	TRBJ2-1	1/51
TRBV28-1	ATSDLMDNEQF	TRBJ2-1	2/51
TRBV7-9	ASELSGNTIY	TRBJ1-3	35/51
TRBV7-9	ASSYLNTIY	TRBJ1-3	1/51
TRBV7-9	ASEGGNTIY	TRBJ1-3	1/51
TRBV7-9	ATEASGNTIY	TRBJ1-3	6/51
TRBV7-9	ASEITRDRRNTIY	TRBJ1-3	1/51
TRBV7-6	ASSSWTGQDEQF	TRBJ2-1	1/51
TRBV7-9	ASSGFTGFANEAF	TRBJ2-6	1/51

10070

TRBV	CDR3 (aa)	TRBJ	Frequency
TRBV11-2	ASSDGTGVGLGYT	TRBJ1-2	2/44
TRBV11-2	ASSDGQGRLGYT	TRBJ1-2	1/44
TRBV14	ASSPRDSQETQY	TRBJ2-5	1/44
TRBV7-8	ASEDFKNIQY	TRBJ2-4	7/44
TRBV7-9	ASSLGGGYT	TRBJ1-2	23/44
TRBV7-9	ATPGEVLSPNYGYT	TRBJ1-2	1/44
TRBV7-9	ASSLGGGQNGYT	TRBJ1-2	1/44
TRBV7-9	ASSPGQTNYGYT	TRBJ1-2	6/44
TRBV7-6	ASSSMGGGTDTQY	TRBJ2-3	1/44
TRBV7-9	ASSLAGGYT	TRBJ1-2	1/44

10076

TRBV	CDR3 (aa)	TRBJ	Frequency
TRBV2	ASRGGSGELF	TRBJ2-2	34/54
TRBV7-9	ASSGFRDRVNEQY	TRBJ2-7	18/54

Figure 2-6. KF11-specific clonotypes. TRBV7 usage highlighted in gray; subjects are listed from low to high viral load beginning with the top left.

Only subjects 20018 and 10070 shared some of the following CDR3 motifs with the previously published B*5703 cohort (80): TRBV7-6 -ASSSW-X-G-X-D-X-Q-X (TRBJ 2-1): TRBV7-9 -ASS-XX-GGYT (TRBJ 1.2): TRBV24-1 - ATSDL-XXX-QF (TRBJ 2.1): TRBV7-9 - ASE-X-GNTIY (TRBJ 1.3) (80). Despite this occasional presence of shared motifs, these clonotypes did not make up the majority of sequences within each KF11-specific population.

Of subjects with TRBV7 clonotypes within KF11 specific repertoires, the frequency of TRBV7 usage amongst TCR sequences ranged from 25% (subject 10071) to 100% (subject 10067). I found no relationship between the diversity of KF11-specific TRBV7-clonotypes or the percentage of TRBV7-using tetramer$^+$ cells and markers of HIV-1 disease progression such as concurrent CD4$^+$ T cell number or viral load (Table 2-2). In order to determine whether the high frequency of TRBV7 within KF11 specific repertoires was a true usage bias, 12 other MHC class I-restricted epitope specific repertoires were analyzed (3 non KF11-specific HLA B57-restricted responses, and 9 non HLA B57-restricted responses). The TCR sequences derived from these responses demonstrated TRBV7 usage in only 2 cases compared to 8 of the 9 KF11 specific repertoires ($p = 0.001$ Fishers Exact, data not shown), suggesting that TRBV7 usage bias is a feature of the KF11-specific immune response regardless of the HLA B57 subtype.

I next evaluated the entropy of TCR sequences specific for KF11. Prior studies from our laboratory and others (55, 71, 77) suggested diverse TCR usage during acute infection could limit epitope escape and potentially contribute to control of

viremia, but there are limited data evaluating TCR repertoires during HIV-1 chronic infection (79, 119, 120). The range in mean entropy of KF11 specific sequences from the 9 subjects was 0 to 1.04 (Figure 2-7) and there was a strong correlation ($p = 0.0150$) between the entropy values and the number of clonotypes within each individual repertoire (Figure 2-8). KF11 specific TCR entropy values were substantially lower than those of equivalent numbers of tetramer-depleted $CD8^+$ T cells ($p < 0.001$ Mann-Whitney) derived from the same sort, and ruled against PCR bias (Figure 2-9).

In order to determine the definition of narrow and broad repertoires defined by mean entropy calculations, I compared the entropies of KF11 specific repertoires to the entropies of 12 other epitope specific TCR repertoires (3 HLA B57-restricted and 9 non-HLA B57-restricted responses), as well as to entropies I calculated based on recently published results by Yu et al (80). The range in entropy was similar between the two B57 cohorts (Yu et al 0.00-1.37). I found no difference in mean entropy values between the KF11 specific repertoires and the other epitope specific repertoires ($p = 0.28$ Mann-Whitney), indicating that KF11 specific TCR repertoires have similar diversity to that of other epitope-specific repertoires. I also found no relationship between the degree of KF11-specific TCR repertoire diversity and markers for disease progression such as CD4 count or viral load in the 9 subjects. Although the level of KF11 specific CDR3 diversity was not distinct from other epitope specific sequences, our observation of TRBV7 usage bias prompted a more detailed structural and functional analyses of KF11-specific cells at the clonotype level.

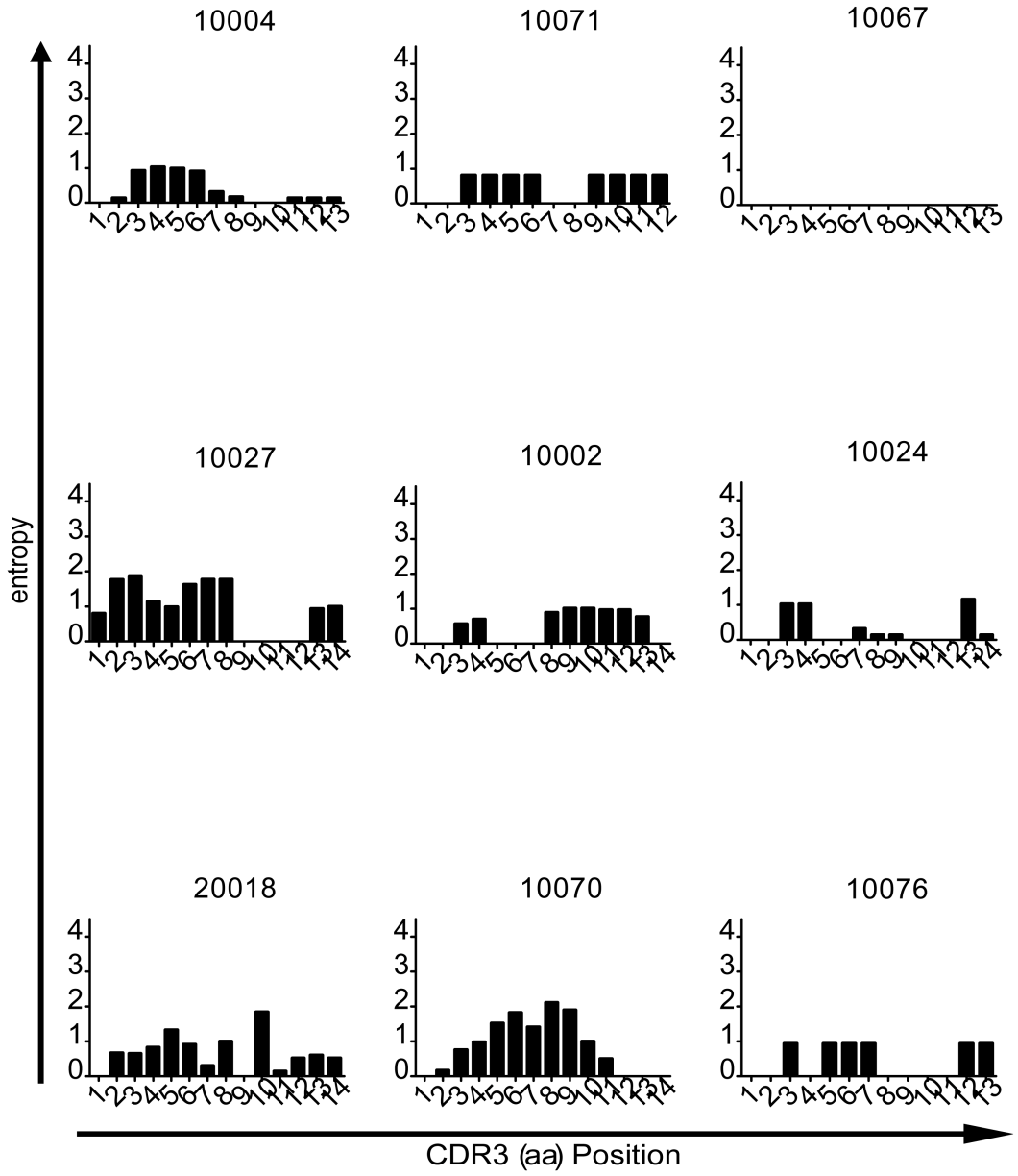


Figure 2-7. Vβ CDR3 region entropy of KF11-specific CD8$^+$ TCR repertoires. Amino acids of the Vβ CDR3 region are depicted along the x-axis while the range in calculated entropy (0-4.3) is depicted on the y-axis.

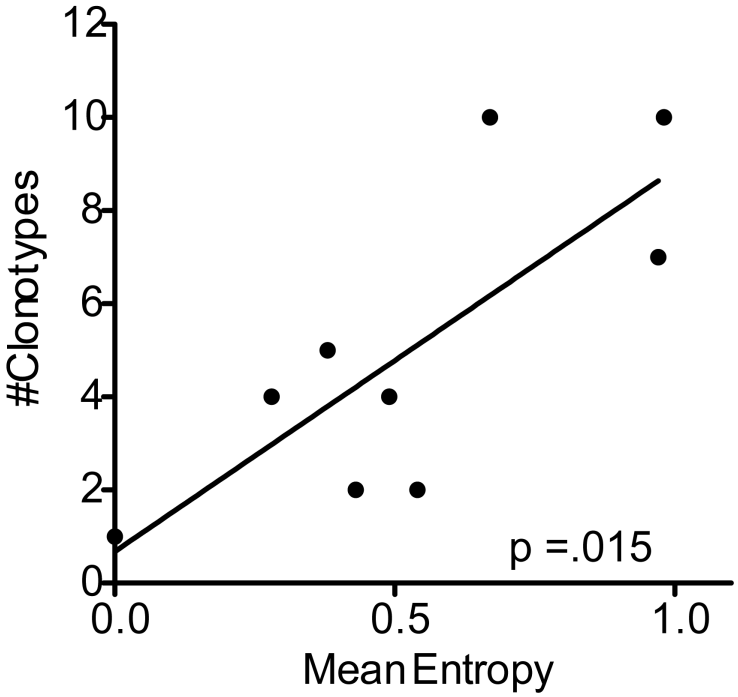


Figure 2-8. Association between the number of clonotypes and mean entropy values within KF11-specfic responses. Mean entropies are represented on the x-axis. The number of clonotypes within each of the nine KF11-specific repertoires is represented on the y-axis.

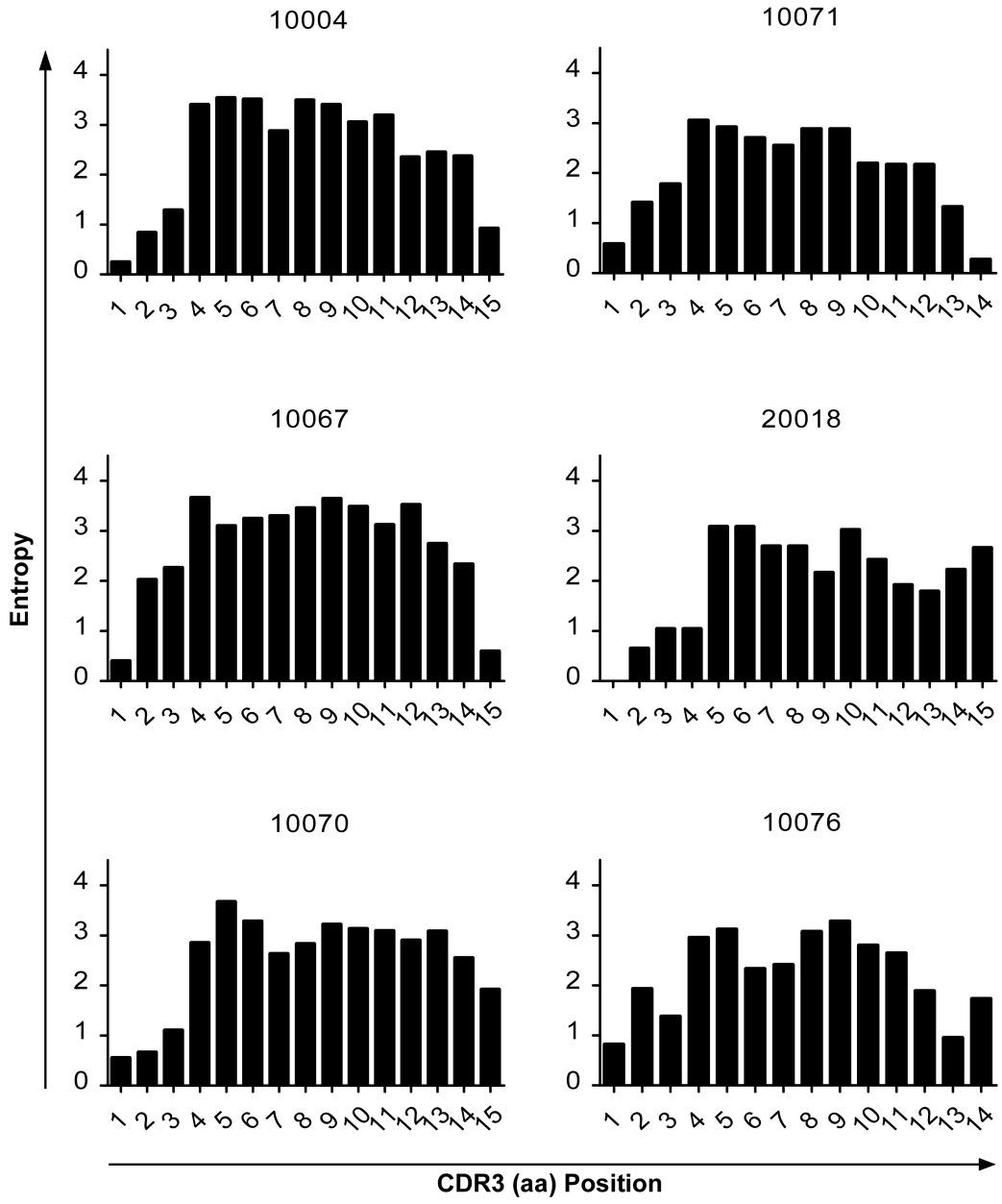


Figure 2-9. VβCDR3 region entropy values of KF11 tetramer-depleted CD8$^+$ T cells. The amino acid position of the CDR3 region is on the x-axis and entropy values (0-4.3) are on the y-axis.

Structural avidity of KF11 specific TCR repertoires

To determine whether shared TRBV7 usage corresponds to distinct structural or functional characteristics of subpopulations of tetramer positive cells, in collaboration with Dr. Scott VanCompernolle, I conducted tetramer off-rate experiments that included TCR Vβ staining and analyses by flow cytometry. Although antibodies to TRBV7 are not currently available, in each case TRBV antibodies were available for the corresponding non-TRBV7 TCR sequence derived from directly sorted tetramer$^+$ cells (Table 2-1). Briefly, our gating scheme encompassed the entire breadth of PE fluorescence of tetramer$^+$ Vß$^+$ populations (Figure 2-10C), and the geometric mean fluorescence was used as a raw measurement of tetramer decay. Raw measurements were normalized to time 0 and off rates were calculated using 1st rate kinetics (GraphPrizm – GraphPad Software).

In general, the tetramer off-rates of KF11-specific CD8$^+$ T cells with TRBV7 usage were faster than non-TRBV-using cells (TRBV7 half life: 20 minutes vs. nonTRBV7 half life: 35 minutes, p = 0.17 Mann-Whitney) (Figure 2-10A and B). Therefore, although this observation only trended towards significance, TRBV7 clonotypes appeared to have lower structural avidity for the HLA B57: KF11 complex. In addition, the variance of off-rates amongst TRBV7-using KF11-specific populations was low as compared to the non-TRBV7 populations (p = 0.015 Fligner-Killeen test of homogeneity of variances), indicating structural similarity amongst TRBV7 clonotypes. I found no relationship between TRBV7 structural avidity (half-life) and HIV viral load.

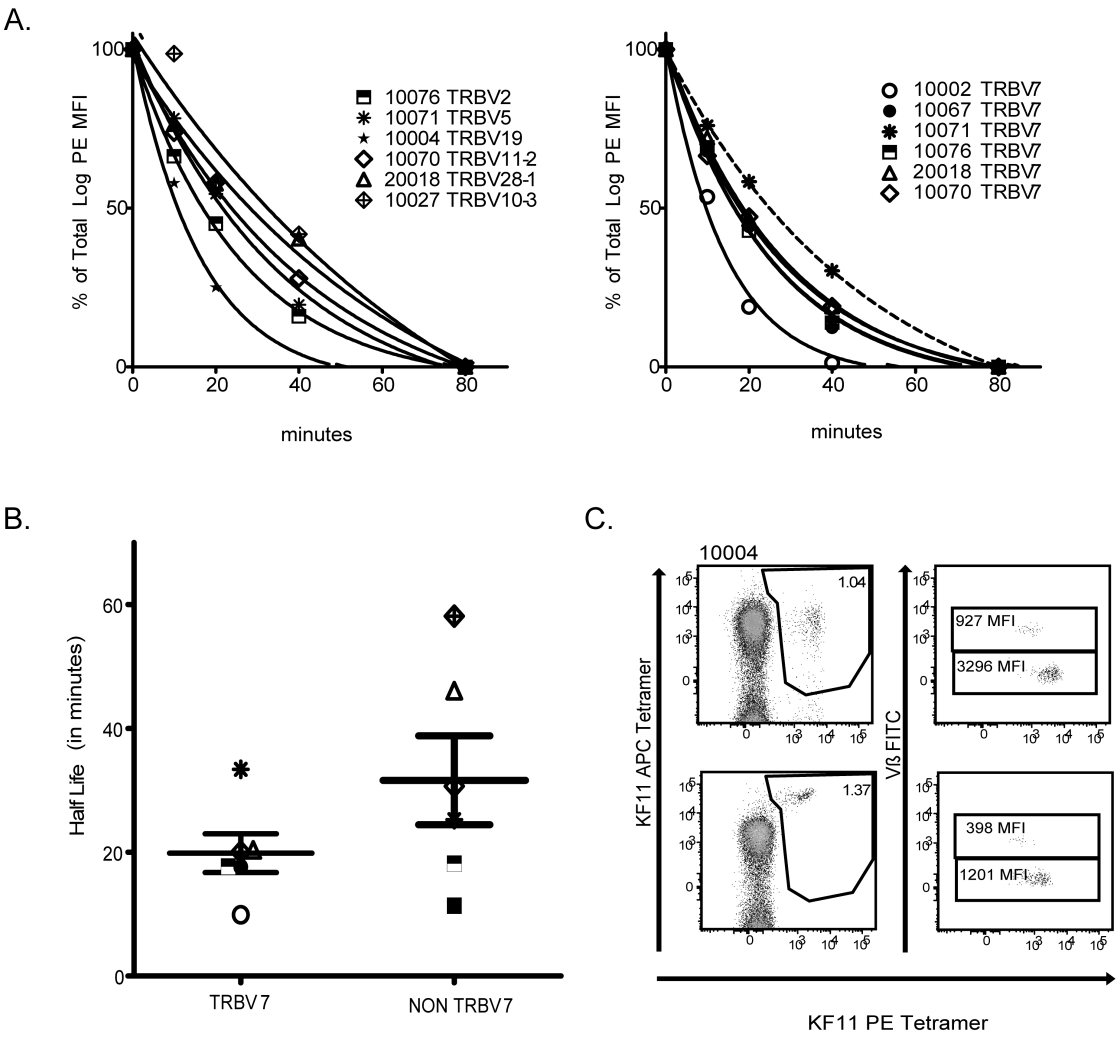


Figure 2-10. Tetramer off-rate analyses of KF11-specific clonotypes. (A) TRBV7 clonotype off rates (right) and non-TRBV7 clonotype rates (left). (B) Mean half lives compared between TRBV7 clonotypes and non-TRBV7 clonotypes. Each symbol represents a separate subject. (C) Gating scheme for measuring individual geometric mean values of each population. Numbers on axes reflect the scale of magnitude of fluorescence.

Clonotypic recognition of circulating epitope variants

With the assistance of Rita Smith, I next assessed whether T cell clonotype structural avidity was related to functional avidity, which was assessed by IFNγ ELISpot. HIV Gag was sequenced from the plasma from 6 of the 9 study subjects. We were unable to obtain Gag sequences from subjects with the lowest viral loads in our study cohort (10004, 10071 and 10067); despite efforts that included large volume plasma concentration and nested PCR specific for proviral gag DNA. KF11 has been described as a highly conserved epitope, and we found the majority of subjects harbored the consensus HIV clade B sequence. However, 2 subjects (subjects 10024 and 10076) harbored a K162R mutation (Table 2-2). Due to the detailed nature of the sorting experiments, and the relatively large cell requirements, I focused my subsequent experiments on these two peptide sequences.

My initial studies included whole PBMC IFNγ ELISpot with both the consensus KF11 (KAFSPEVIPMF) and the K162R variant (RAFSPEVIPMF). Results from whole PBMC IFNγ ELISpot indicated universal recognition of both the consensus KF11 and the K162R variant in all subjects. The magnitude of the responses to KF11 consensus peptide in subjects tended to be equal or higher than that of responses directed against the K162R variant (Figure 2-11). Of the 2 subjects with a dominant circulating K162R variant, subject 10024 had a higher magnitude of response to R162K (maximal response 1,450 SFC/10^6 PBMC) as compared to KF11 consensus peptide (maximal response 495 SFC/10^6 PBMC), but the functional avidity of the consensus KF11 peptide (SD50 20 ng/mL) was higher

than that of the R162K variant (SD50 2500 ng/mL). Conversely, subject 10076 exhibited preferential recognition of the consensus KF11 peptide (Figure 2-11), with a higher magnitude of response (maximal response 7750 SFC/10^6 PBMC), however functional avidity (SD50 4 ng/mL) was similar when compared to R162K recognition (maximal response 2500 SFC/10^6 PBMC, SD50 5 ng/mL). With such a high incidence of cross-recognition of K162R, I wanted to determine whether cross-reactivity to this variant was mediated by the TCR using the dominant TRBV7 gene.

To evaluate differences in peptide recognition by T cell clonotypes, with the help of Louise Barnett, I performed a series of flow sorting experiments in which $CD8^+$ T cells were separated and evaluated based on their TCR Vβ expression. Once I determined the TCR gene usage of the $tetramer^+$ cell populations in each individual, I used this information to either positively select or deplete $CD8^+$ T cells of the corresponding TRBV populations. For this series of experiments I did not simultaneously stain with the KF11 tetramer since tetramer binding led to activation and interferon gamma secretion from the tetramer positive cells (data not shown). Since a commercial TRBV7 (Vβ6) antibody is not available, for each subject I depleted $CD8^+$ T cells of the entire TRBV population corresponding to the "non-TRBV7" population of tetramer positive cells. For example, total $CD8^+$ T cells from subject 10071 were depleted of all TRBV5-1 using T cells, thereby leaving behind all KF11-specific TRBV7 expressing cells. Negative controls were set up to insure that responses were only elicited from KF11 specific $CD8^+$ T

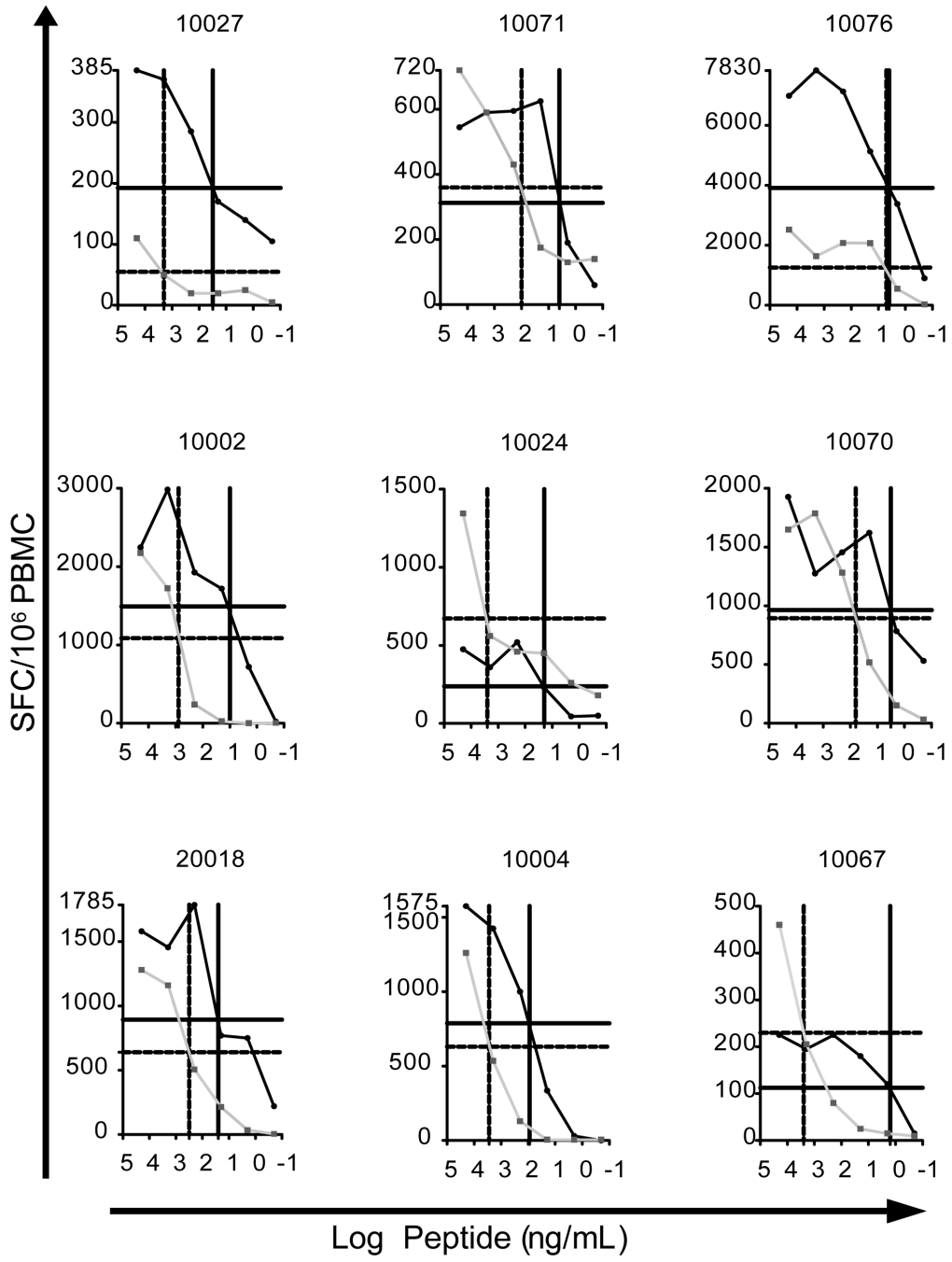


Figure 2-11. Dominant recognition of KF11 consensus compared with variant peptide. KF11 responses (black) and K162R responses (gray). SD50 values: KF11 (solid) and K162R (dotted). Subject data are listed from low to high frequency of TRBV7 clonotypes.

cells (described in methods). These two populations of $CD8^+$ T cells (in this case the TRBV5-1 $CD8^+$ sorted population as well as the $CD8^+$ TRBV5-1 depleted population) were added back to CD8-depleted PBMC pulsed with serially diluted peptides, and evaluated by IFNγ ELISpot assay.

The consensus KF11 peptide was recognized by all identified KF11-specific clonotypes. In subject 10071, both TRBV5-1 and TRBV7-utilizing $CD8^+$ T cells recognized the KF11 peptide with similar functional avidities with SD50 values of 3 ng/mL (TRBV5-1 clonotype) and 6 ng/mL (TRBV7 clonotype) (Figure 2-12A). In contrast, the circulating clonotypes in subjects10024 (Figure 2-12B) and 10076 (Figure 2-12C) had different SD50 values. In subject 10024 the TRBV7 clonotype exhibited higher avidity (SD50 0.6 ng/mL) than TRBV3 (SD50 7.9 ng/mL). In subject 10076, the TRBV7 clonotype exhibited lower avidity (SD50 50 ng/mL) than TRBV2 (SD50 5 ng/mL). Since a prior study suggested that dominant TRBV usage may imply cross-reactivity against epitope variants (121), I next evaluated recognition of the only epitope variant present in our study subject population, K162R.

Although all clonotypes were able to recognize the KF11 consensus peptide, not all were able to recognize epitope variants. In subject 10071, both clonotypes were able to recognize the K162R variant peptide. The TRBV5-1 clonotype maintained higher functional avidity (SD50 0.6ng/mL) than the TRBV7 clonotype (SD50 8 ng/mL) (Figure 2-12A). Analyses of subject 10024 demonstrated

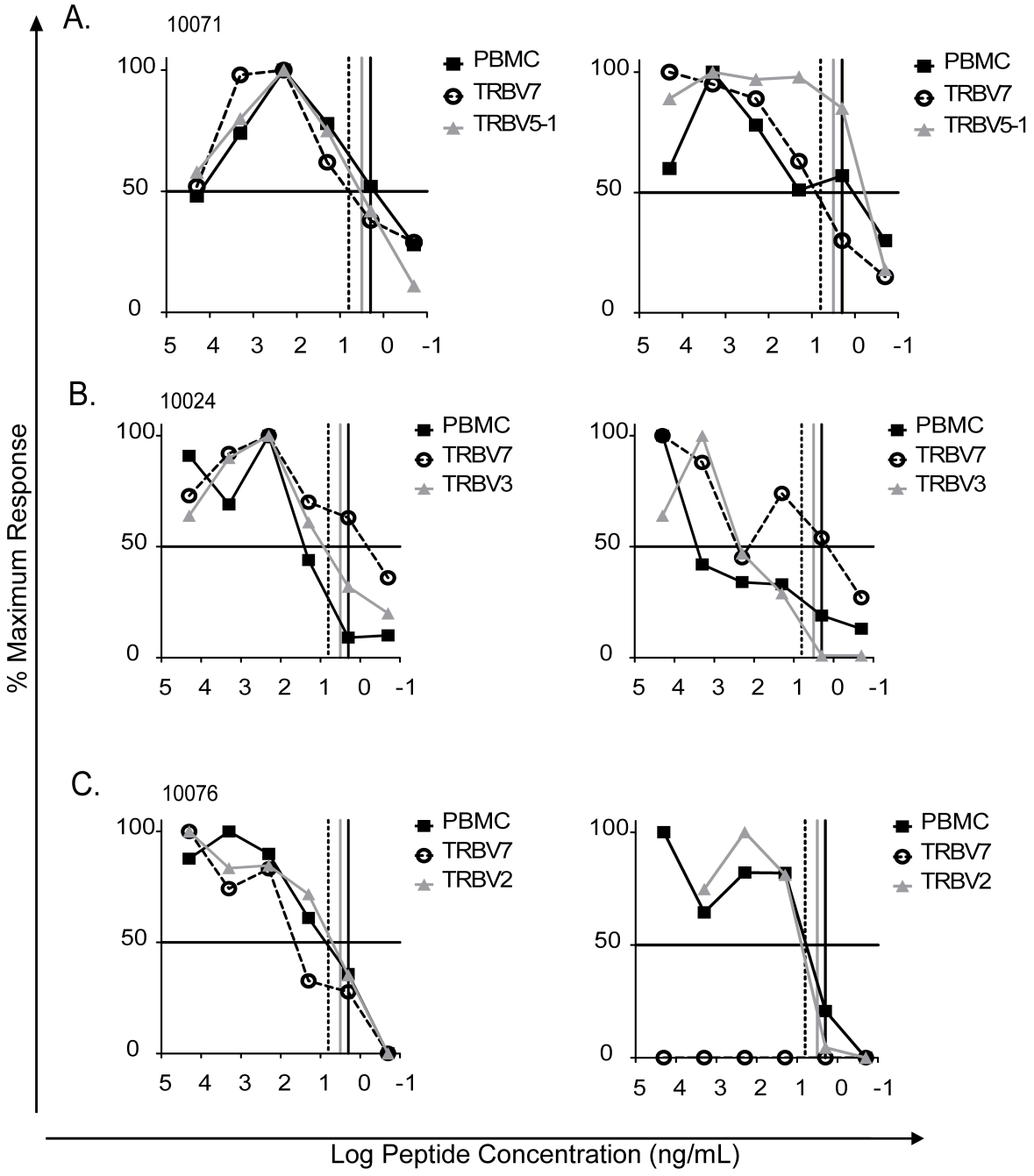


Figure 2-12. Functional avidity varies on the clonotype level. Responses were normalized to the maximum response (SFC/10^6 cells). (A) Subject 10071 (B) Subject 10024 (C) Subject 10076.

that the TRBV7 clonotype had a higher K162R avidity, with a SD50 of 1.25 ng/mL, as compared to the TRBV3 clonotype with a SD50 of 251 ng/mL (Figure 2-12B). Results from subject 10076 indicated that only the TRBV2 clonotype recognized the variant with an SD50 of 8 ng/mL (Figure 2-12C). The TRBV7 population was unable to recognize the variant, even at the highest concentration despite recognition of the consensus KF11 peptide (Figure 2-12C). This series of experiments demonstrates that functional avidity and cross-reactivity of an epitope-specific response can vary at the clonotype level.

Discussion

Epitope-specific TCR diversity has been shown to influence the course of chronic viral infections. In the HCV chimpanzee model, our laboratory found that narrow TCR diversity during the early phase of infection was associated with subsequent immune escape and the establishment of chronic viral infection, whereas broader epitope-specific TCR diversity was associated with a lack of escape or resolution of infection (55). In the SIV model, the level of epitope-specific TCR diversity during early infection is linked to mutations within dominant MHC class I-restricted epitopes (64, 69, 114). The TCR repertoires of two dominantly recognized Mamu A*01-restricted epitopes CM9 (Gag) and TL8 (Tat) differ in their level of TCR diversity, as the CM9 specific repertoire is diverse in comparison to the TL8 specific repertoire (114). In-vitro studies suggested that TL8-specific $CD8^+$ T cells were more effective suppressing viral replication; however, the TL8 epitope mutation is known to occur very early in the course of

SIV infection, providing escape with little fitness cost to the virus (64). This emphasizes that a limited TCR repertoire may be quite effective at suppressing viral replication, but in the environment of extensive variation and high-level viral replication in vivo, the limited repertoire is more susceptible to immune escape. It is unknown whether broad TCR repertoires directed against conserved epitopes are maintained throughout the course of chronic infection in this model.

The HLA B*57-restricted epitope KF11 is both dominantly recognized and highly conserved (10). Here I demonstrate a wide range of clonotypic diversity among subjects able to recognize this epitope. However, despite this clonotypic diversity, I found dominant TRBV7 usage among KF11-specific clonotypes. HLA B*57- restricted responses, and responses to the KF11 epitope in particular, are dominant in HIV-1^{+} individuals with chronic infection (116). Migueles et al observed conservation of HLA B*5701-restricted epitopes and the responses directed against these epitopes during chronic infection in both HLA B*5701 long term non progressors and progressors, suggesting that B*5701 epitope variation does not contribute to disease progression in these subjects (10). Even in the case of dominant circulating epitope variants, immune responses against a consensus peptide can remain dominant during chronic infection, as Koibuchi et al demonstrated in 2 non HLA B*57 subjects followed longitudinally over 6 years (122). I likewise observed stably dominant KF11 responses in all 6 subjects that recognized this epitope, despite mutations within KF11 in the circulating plasma viral populations of 2 of these subjects (Table 2-2).

I found no difference in TRBV usage between HLA B*5701 and HLA B*5703 subtypes. This observation is in contrast to recent findings suggesting the dominant usage of TRBV19 (79, 80) and structural differences in TCR repertoires among HLA B*5701 controllers when compared to subjects with the B*5703 allele (80). I only found TRBV19 usage in one HLA B*5701 subject, and in this case it made up a very small proportion of the KF11-specific TCR repertoire (2 out of 50 TCR sequences in subject 10004). Instead, I found dominant TRBV7 usage in our subjects regardless of HLA B*57 subtype, results which are similar to the TCR usage previously described in B*5703 subjects (80). The reason for these discordant results is not clear, but may be due to the shorter duration of infection, or early initiation of anti-retroviral therapy during acute HIV infection in some cohorts (80, 123-125), which may influence the development of the HIV specific $CD8^+$ TCR repertoire (126, 127). Our subjects were completely anti-retroviral therapy naïve, and were infected a mean of 13 years (range 3-24). In this regard the natural infection history of our study subjects may more closely resemble the B*5703 African subjects examined by Yu et al (80). It is therefore possible that the duration of chronic infection in the absence of early anti-retroviral therapy has a greater influence on the make-up of the TCR repertoire than HLA subtype or the clade of HIV infection.

Common TRBV19 usage among KF11 specific T cell clones was demonstrated by Gillespie et al (79). In that study TRBV usage was first analyzed by Vβ staining. Based on the Vβ staining results, appropriate TRBV specific primers were subsequently used for TRBV sequencing (79). There is currently no

commercially available TRBV7 (Vβ6) antibody, and the current panels of TRBV antibodies only cover approximately 70% of expressed TRBV chains. Thus, antibody screening would overlook TRBV7 usage. Until more antibodies are available, direct sorting of epitope-specific cells is more reliable for TCR repertoire analyses.

The duration of HIV infection prior to study initiation may also determine the level of TCR diversity. I found no difference in TCR CDR3 variability (entropy analyses) between responses in subjects with HLA B*5701 and B*5703 subtypes. I performed a similar analyses on the data published by Yu et al and likewise found no difference in entropies between responses restricted by the different HLA subtypes ($p = 0.28$ Mann-Whitney). Yu et al also demonstrated that 6 of 10 HLA B*5703 subjects used the TRBV7 genes without evidence of CDR3 motifs for this response. The non-TRBV7 sequences showed a wide variety of TRBV genes, likewise without evidence of CDR3 motifs, which is concordant with our results. However, when I compared cohorts we found several shared CDR3 motifs as well as corresponding TRBJ regions amongst 3 TRBV7 clonotypes and 1 TRBV24-1 clonotype between subjects 10070 (B*5703) and 20018 (B*5701) from my study and subjects within the cohort published by Yu et al, despite differences in B*57 subtypes. This provides evidence for convergent TCR usage beyond the TRBV portion of the epitope-specific TCR.

In my study, I found KF11 specific TCR diversity not to correspond with viral load, CD4 count, or duration of infection. Yet, since the HIV specific TCR repertoire shapes and directs the HIV-specific $CD8^+$ T cell immune response, I extended

my analyses to the structural and functional aspects of the KF11-specific TCR repertoire at the clonotype level. TRBV7 structural avidity was assessed by a tetramer off-rate assay similar to recently published methods (128). Recent findings have indicated high structural avidity as a beneficial characteristic of HIV specific $CD8^+$ T cells (88, 128-131), as well as convergent evolution of epitope-specific responses in murine models and human influenza studies (132-134). These data suggest a beneficial role of common TRBV usage against conserved epitopes, perhaps as a result of greater functional capacity. However, I found TRBV7 clonotypes generally had a lower structural avidity than other KF11-specific TRBV clonotypes. The variance in tetramer off-rates among TRBV7 clonotypes was significantly narrower than those of the non-TRBV7 clonotypes ($p = 0.015$ Fligner-Kileen) indicating a direct influence of TRBV structure on TCR avidity. This does not necessarily mean that strong structural avidity is not important for a particular immune response, but raises the possibility that an avidity threshold for optimal TCR activation exists, as has been described (135). One caveat of our off rate experiments was the potential for Vß antibodies to influence the binding of the KF11 tetramer, and subsequently the possible change in off rate kinetics. Previous experiments in our lab in a subject that recognized a B*1501 tetramer indicated no effect on tetramer off rates of Vß populations with our without the presence of a Vß antibody (data not shown). However, to truly account for any influence of the Vß antibodies on tetramer off rate kinetics, one would need to analyze all Vß antibodies used. With only 70% of Vß commercially available antibodies it would be difficult to conduct a thorough

examination, especially in the case of TRBV7, and thus, this remains a limitation of our tetramer off rate findings. HIV-1 infection has been associated with immune exhaustion, as measured by the expression of surface markers such as CD57 and PD-1 (99, 100, 136). When antigen is persistently present it is possible that high structural avidity is disadvantageous, leading to constant activation and eventual exhaustion of the high avidity clonotypes (128).

Despite the frequent TRBV7 usage of KF11-specific $CD8^+$ T cells in our study cohort, TRBV7 usage does not necessarily correspond to recognition of epitope-variants. Prior studies have described narrow KF11-specific TCR repertoires in some individuals (80), as well as the ability of KF11-specific $CD8^+$ T cells to recognize in vivo epitope variants (79, 80). It has therefore been suggested that the degree of epitope variant cross recognition is more important than the overall diversity of the KF11-specific TCR repertoire for control of viral replication. To further explore the epitope recognition of TRBV7 clonotypes, I assessed functional avidity of sorted subpopulations of $tetramer^+$ cells to the epitope variants identified in our subject cohort. In the overall PBMC peptide titrations all subjects recognized the KF11 consensus peptide as well as the K162R variant peptide, although KF11 recognition was generally higher than K162R in magnitude at maximal response (Figure 2-11). However, at the clonotype level, differential recognition and functional avidity was observed. Subject 10071 recognized both KF11 and K162R, and both KF11-specific clonotypes (TRBV7 and TRBV5-1) demonstrated cross-recognition of the K162R variant. Results for subject 10024 were similar. In contrast, although both KF11-specific clonotypes

from subject 10076 recognized the consensus KF11 peptide, the TRBV7 clonotype did not recognize the R162K variant even at the highest tested concentration. Both subjects 10076 and 10024 were identified to possess the K162R variant in circulating viral populations and subject 10076 has the highest viral load in our subject group (Table 2-2). Due to sample availability and the extensive sorts that require large numbers of PBMC, I was only able to focus on the consensus KF11 and one KF11 variant we were able to identify in our cohort, thus our findings are limited to the two peptides analyzed. These data demonstrate that dominant clonotypes do not necessarily cross-react with epitope variants, which we have previously described at the clonal level (137), and suggest a potentially beneficial role for maintenance of TCR diversity even in the setting of restricted TRBV usage.

While I could not find a relationship between TRBV7 usage within the KF11 specific repertoire and overall disease outcome it appears to be a reproducible feature among chronically infected HLA B57 subjects (80). My finding that TRBV7 clonotype avidity tended to be lower than that of the other KF11 specific clonotypes is consistent with the early deletion of high avidity T cell clonotypes after acute infection (128). Dominant TRBV7 usage may therefore represent convergent evolution toward populations of epitope-specific T cells with sufficient avidity to mediate control of viremia during chronic infection.

These studies highlight the importance of continuing analyses of the HIV-specific immune response at the clonotype level, which will help define how the development and maintenance of epitope-specific TCR repertoires influences

HIV disease progression. However, the true mechanism by which TCR repertoire diversity plays a mechanistic role in the control of HIV-1 replication will need to be elucidated by directly measuring $CD8^+$ T cell-mediated suppression on an epitope-specific and clonotype level. As new assays are developed for the evaluation of vaccine-induced immune responses it will be important to maintain incorporation of clonotype analyses as a bridge between structure, phenotype, and the ability to control HIV-1 replication.

CHAPTER III

PROLIFERATIVE CAPACITY PLAYS A MINIMAL ROLE IN DIRECT $CD8^+$ T-MEDIATED SUPPRESSION OF HIV REPLICATION IN VITRO

Abstract

Proliferative capacity of HIV-specific $CD8^+$ T cells has been associated with control of HIV viremia in vivo. It has been hypothesized that high proliferative capacity provides continuous supply of antiviral effector cells, which then mediate immediate antiviral effector functions. Here, we describe significant differences in proliferative capacity between sorted $CD8^+CD57^-$ and $CD8^+CD57^+$ T cell subsets from HIV-positive subjects in response to HIV super-infection in vitro. Although CD57 proved a reliable marker to gauge proliferative capacity, superior expansion potential did not translate to dramatic increases in virus suppression by $CD8^+CD57^-$ T cells. Further analyses revealed $CD8^+CD57^+$ T cells to have increased suppression on a gag-specific per-cell basis, and with a trend toward increased frequencies of HIV-specific $IFN\gamma^+TNF\alpha^+$ $CD8^+$ T cells. These data highlight the superior ability of differentiated $CD8^+$ effector cells with minimal proliferative capacity to suppress HIV replication.

Introduction

$CD8^+$ T cells are a critical component of the immune response to viral infections, with capabilities of direct cell lysis and secretion of anti-viral cytokines and chemokines (86, 113, 138), and have been indicated as an important immunological determinant in the progression of HIV-1 disease (27, 33). HIV-specific $CD8^+$ T cells have been shown both in observational human studies as well as direct CD8-depletion experiments in non-human primate models to be critical for maximum control of HIV-1 and SIV replication respectively (27, 33, 69, 73, 139). To further understand the effectiveness of $CD8^+$ T cell-mediated immune responses, we need improved methods to assess the ability of defined $CD8^+$ T cell subpopulations to suppress HIV replication.

In animal studies of chronic viral infection, the expansion potential of T cells after adoptive transfer mediates control of viremia (140). Several studies have shown a strong relationship between the ability of $CD4^+$ (124) and $CD8^+$ T cells (30, 89, 90, 138, 141, 142) to proliferate in response to cognate antigens and control of HIV-1 viremia. The expansion of HIV-specific $CD8^+$ cells in-vitro is also associated with increased effector function (30, 89, 99, 100, 124, 138, 143). These observations suggest that while the exact effector functions that mediate suppression of viral replication are unknown, the ability of cells to expand in response to cognate antigen and acquire these functions may be the most important predictor of a successful response. It is unknown whether proliferating $CD8^+$ T cells provide immediate antiviral effector functions themselves or,

alternatively, provide a constant supply of short-lived effector cells with strong antiviral properties.

Despite extensive data correlating $CD8^+$ T cell function and overall control of HIV-1 viremia, the mechanisms by which these effector functions execute suppression of HIV replication are not completely understood. Recent studies have employed assays to directly measure $CD8^+$ T cell ability to suppress HIV replication in vitro (70, 90, 98, 144, 145). Saez-Cirion *et. al.* evaluated the ability of directly isolated $CD8^+$ T cells to suppress HIV replication in target cells. They found enhanced ability of $CD8^+$ T cells from HIV controllers to suppress viral replication, and this ability was associated with an enrichment of a particular cellular phenotype with low levels of CD38 and high levels of HLA-DR (98). In a more recent study, Migueles et al demonstrated the ability of in-vitro expanded $CD8^+$ T cells to acquire cytotoxic function and suppress HIV replication (90). Although these data provide insight into the relationship between $CD8^+$ T cell phenotype, proliferative potential and suppression of viral replication, a more direct assessment of $CD8^+$ T cells derived directly ex vivo and sorted by phenotype offers a more precise way to determine mechanisms of viral suppression in vivo.

In this study I evaluated the ability of $CD8^+$ T cells with high and low proliferative capacity to suppress HIV super-infection in vitro. CD57 is an immunological marker that identifies $CD8^+$ T cells that are senescent (99, 108). CD57, also known as HNK-1 (Human Natural Killer-1), is expressed on several different types of cells, including Natural Killer (NK) cells and T lymphocytes (146).

Several studies indicate $CD8^+CD57^+$ T cells have limited ability to proliferate to cognate peptide, have decreased telomere length, and are more prone to AICD (98, 99, 108). However, $CD8^+$ T cells expressing CD57 are capable of producing IFNγ and TNFα (99, 108), and have been shown to have extensive effector cytotoxic capabilities (108). Thus, the ability to isolate T cells based on CD57 expression allowed me to perform a focused analysis of the role of proliferation in the control of viral replication.

I simultaneously assessed $CD8^+$ T cell proliferation and $CD8^+$ T cell suppression of viral replication in vitro. $CD8^+$ T cells derived ex vivo from HIV^+ subjects were able to suppress viral replication in vitro in a dose-dependent manner, but not those from HIV^- subjects. I observed dramatic differences in proliferative capacity between sorted $CD8^+CD57^+$ and $CD8^+CD57^-$ T cells in the presence of in vitro HIV-1 super infection. Despite the superior ability of $CD8^+CD57^-$ T cells to proliferate in vitro, this ability did not correlate with suppression of HIV-1 replication. When I analyzed the ability of Gag-specific $CD8^+$ T cells to suppress viral replication on a per-cell basis, $CD8^+CD57^+$ T cells had higher suppressive ability. These data demonstrate a minimal ability of CD57 expression (as a surrogate for a lack of proliferative capacity) to predict suppression of HIV in vitro and perhaps suggest a higher degree of significance for $CD8^+CD57^+$ T cell effector functions such as cytolysis and anti-viral cytokine production.

Materials and Methods

Study Subjects

The Vanderbilt-Meharry CFAR Cohort is comprised of subjects recruited through the Comprehensive Care Center (Nashville, TN) and all subjects were HLA class I typed (4 digit resolution) (DCI, Nashville, TN). Based on accessibility to aphaeresis samples, 12 subjects were selected for further study. All subjects were antiretroviral therapy naïve at the time of study with a range of $CD4^+$T cell numbers from 144 to 1374/mm^3 and log viral load measurements from 1.7 to 5.33 log viral load (copies/mL). This study was approved by the Vanderbilt University Medical Institutional Review Board, and all subjects provided informed consent.

In vitro CD8 Depletion-Add Back-Infection Assay

Fresh or Frozen PBMC were CD8-depleted by magnetic separation (Robosep, Stem cell Technologies, Vancouver, Canada) to a minimum of 95% purity. Total CD8-depleted PBMC, as well as complete PBMC, were then incubated at $5x10^6$ cells/mL for approximately 18 hours at 37^{o}C in 5% CO_2, in RPMI 1640 medium supplemented with fetal calf serum (10%), HEPES Buffer (10mM), L-glutamine (2mM) and penicillin-streptomycin (50U/mL) (R10 medium) and Phytoglutinin A (PHA) (5µg/mL). Following the 18 hour incubation, both the PBMC and CD8-depleted PBMC cultures were spun down and washed twice with PBS supplemented with 1% fetal calf serum and resuspended in R10 medium supplemented with 100 units of IL-2 (R10-100 medium). $CD8^-$ PBMC were resuspended at 10^6/mL, and PBMC were resuspended to a concentration adjusted to 10^6 $CD4^+$ T cells/mL. Once resuspended, ($CD8^-$) PBMC and PBMC cultures were placed in a 96 well plate, 100µl/well, and then infected, 4 hours

prior to the add back of $CD8^+$ T cells, or $CD8^+$ T cell subsets, at an MOI of 2. During the 4-hour infection, more PBMC from the same subject were $CD8^+$ T cell enriched by magnetic separation (Robosep, Stem Cell Technologies) at a minimum 95% purity. For general $CD8^+$ T cell add back experiments, purified $CD8^+$ T cells from the magnetic separation were resuspended in R10-100 medium and added back to the ($CD8^-$) PBMC cultures in a 50µl volume, 4 hours post infection, at the appropriate effecter to target (E:T) ratio.

Magnetic bead-enriched $CD8^+$ T cells were stained with anti -CD3, -CD8, -CD57, a dump channel that included anti –CD14, CD16, and –CD56 (BD Bioscience, California) and a viability dye (BD Bioscience, California). Purity yields were a minimum of 95%. Each CD8 population was first CFSE stained as described previously (147). Briefly, cells were resuspended in PBS alone at $20x10^6$ cells/mL. CFSE was first diluted 1:6000 in PBS, and then added 1:1 to resuspended effector cells. Cells were then incubated in a 37°C water bath for 3 minutes in the dark. The CFSE stain was quenched by adding 50% fetal calf serum at 1:1 and incubated at room temperature for 5 minutes. Samples were then spun down and washed once more with 50% fetal calf serum, and then washed twice with R10 medium. Effector cells were re-suspended at a concentration of $4x10^6$ cells/mL in R10-100 and added back at several E: T ratios, 4 hours post infection. After the addition of effector cells, cultures were briefly spun down for 1 minute at 1800 rpm and then incubated at 37°C in 5% CO_2 for 3-7 days depending on experimental conditions. 24 hours post infection, all cultures were spun down, washed twice with PBS supplemented with 1% fetal

calf serum, and resuspended in 100µl/well of R10-100 medium. Experiments were set up in triplicate, for each condition.

Flow Cytometry Analyses of Infection

For the measurement of p24 antigen, 25ul of supernatant was spun down for 5 minutes at 1700 rpm and then transferred to a 96 well plate with 10µl of PBS supplemented with .05% Triton-X 100 and 10% fetal calf serum (p24 sample diluent), samples were stored at -80^{o}C for later use in the p24 ELISA assay. Staining of cell cultures included antibodies for CD3, CD4, CD8 (BD Biosciences, California), and if necessary, Human anti-Mouse CD24 (hsa) (BD Biosciences, California). Samples were run either on a LSR II, high throughput system (BD Biosciences, California), or a FACS ARIA (BD Biosciences, California). $CD3^{+}CD4^{+}$ T cells were gated on GFP, and GFP expression was interpreted as a positive infection. Additional p24 ELISA assays were used to corroborate the flow cytometry data.

p24 ELISA

Collected supernatants from each experiment were tested for viral production of p24 by ELISA, using methods previously described (5). Briefly, 96 well flat bottom plates were coated with Coating Antibody-183 in PBS, and incubated overnight at 37^{o}C. Plates were washed and then blocked with PBS supplemented with 10% fetal calf serum. After blocking, plates were washed and samples were serially diluted in p24 sample diluent and added to the plate in a total volume of 100ul. A p24 standard was included (NIH HIV repository) and

was serially diluted for a standard curve. In addition, a blank consisting of only p24 ELISA Diluent was added as a negative control.

Viral Constructs

HIV_{R5_GFP} was generated and purified as described previously (148). Briefly, replication competent CCR5-tropic HIV (R5.HIV) was prepared by transfecting 293T cells with HIV that encodes R5-tropic (BAL) envelop and eGFP (Clontech) in place of the *nef* gene. Viral titers were determined by serial dilution on HUT 78 cell lines (NIH AIDS Research and Reference Reagent Program); viral titer was measured 3 days post infection by GFP expression on a FACSAria (BD Biosciences).

Viral Concentration

In order to infect culture at a MOI of 2, while maintaining small volumes, all viruses were concentrated by centrifugation. Samples were first filtered (45µm, Nalgene) and then placed in a Centrifugal Filter Device (Amicon Ultra, Millipore, Ireland) and spun for 15 minutes at 2000 rpm. Viral samples were then aliquoted and stored at -80^{o}C. Typically final viral titers ranged from 7-12x10^{6} ifu/mL.

Intracellular Cytokine Assay (ICS)

The ICS assay was preformed as described previously (76). Briefly, PBMC were suspended at 10^{7}/mL in R10 medium containing soluble anti-CD28 (1μg/mL, BD Biosciences) and anti-CD49d (1μg/mL, BD Biosciences), and then aliquoted at 2x10^6/well in a 96 well round bottom plate. PBMC were stimulated individually with the following; SEB (2.5 μg/mL), Gag-pool of 18mer peptides (NIH) (20

μg/mL), Nef-pool of 18mer peptides (NIH) (20 μg/mL), and optimal MHC Class I-restricted peptides (200 μg/mL), for 1.5 hours at 37°C in a total volume of 100ul. Then, to prevent protein transport from the Golgi apparatus, 1 μg/mL of Brefeldin A (Beckman Coulter) was added to the wells after initial 1.5 h incubation in a 100 μl volume, final volume within each well was 200 μl. PBMC were then incubated for an additional 5 hours at 37°C. Cells were washed and stained for surface expression of CD3, CD8 and CD57 (BD Biosciences), then fixed and permeabilized (BD Biosciences, Cyto FixPerm) at 4°C. Cells were then stained for IFNγ and TNFα (BD Biosciences) and resuspended in FACS buffer (BD Biosciences). Events were collected on a FACSAria flow cytometer (BD Biosciences) and analyzed with FACSDiva software (BD Biosciences). All background, determined by the negative control (Media only), was subtracted from the data.

Statistics

Paired analyses were compared using the non-parametric Wilcoxon paired t test, and unpaired analyses were compared using the non-parametric Mann-Whitney unpaired t test. Correlation values were calculated using the non-parametric Spearman correlation calculation. Statistical assistance was given by Cathy Jenkins from the Department of Biostatistics (Vanderbilt University).

RESULTS

HIV therapy naïve subjects from the Vanderbilt-Meharry CFAR Cohort were recruited with a wide range of viral loads from less than 50 copies/mL (Log 1.7) to 213,500 copies/mL (Log 5.33). The median duration of infection was 12 years (range 5 – 26 years). Although HLA type was not used as a selection criterion, the majority of subjects with low viral loads had the HLA B57 allele, in accordance with prior studies (34, 42, 43, 116, 149) (Table 3-1).

$CD8^+$ T cells derived from HIV $^+$ subjects are able to suppress viral replication in vitro

I developed an in vitro assay that allows for the direct simultaneous assessment of ex vivo derived $CD8^+$ T cell proliferation and suppression of HIV-1 replication in autologous $CD4^+$ T cell targets. I first assessed the overall effect of $CD8^+$ lymphocyte presence in culture on viral replication at day-three post infection. Subject PBMC were depleted of total $CD8^+$ lymphocytes and infected with HIV_{R5_GFP} at an MOI of 2. Infection was assessed by GFP expression within $CD4^+$ T cells and confirmed by p24 ELISA. In HIV sero-positive donors, the presence of $CD8^+$ lymphocytes was critical for maximum suppression of HIV-1 replication ($p = .005$, Wilcoxon paired t test) (Figure 3-1A). Although $CD8^+$ lymphocytes provided some degree of viral suppression in HIV^- donors ($p = .008$, Mann-Whitney unpaired t test) (Figure 1A), infection percentages within CD8-depleted PBMC cultures were significantly higher in these HIV sero-negative donors (average 26%) when compared to that of the HIV sero-positive (average 6%) donors ($p = 0.0047$, Mann-Whitney).

Table 3-1. Therapy naïve chronic HIV^{+} cohort.

Subject	HLA -B	Year Infected	CD4	CD8	Log Viral Load	Symbol
10004	B57, B7	1983	144	496	1.70	✳
10031	B14, B57	1997	612	731	1.70	★
10071	B8, B57	1992	658	350	1.70	△
10067	B13, B57	1991	988	456	2.10	▼
10027	B8, B57	1992	544	782	2.50	⬓
10002	B27, B57	1984	693	1071	2.59	▲
10065	B7, B45	1998	565	366	2.79	◇
10060	B42, B57	2002	688	1042	2.86	○
20018	B40, B57	2004	1374	1035	3.28	✕
20011	B8, B8	2004	512	640	3.42	□
10070	B57, B58	1996	846	414	3.95	●
10076	B35, B57	1999	832	1952	4.25	■
10117	B18, B58	2003	355	498	4.50	◆
10098	B7, B51	1995	778	643	5.33	▽

I designed my experiments to isolate the suppressive effect of $CD8^+$ T cells. Previous studies have indicated the important role that Natural Killer (NK) lymphocytes, which include $CD8^+$ subpopulations, may play in HIV infection (150-152). By using magnetic CD8 depletion, all $CD8^+$ lymphocytes are removed from the PBMC cultures in these experiments, including a substantial fraction of NK cells. Although the presence of total $CD8^+$ lymphocytes had a measurable suppressive ability in both HIV sero-positive and sero-negative donors, I hypothesized $CD8^+$ T cells were actively suppressing super infection in only the HIV^+ donors. In order to differentiate $CD8^+$ T cell suppression of HIV-1 replication from that of NK cells, we examined the effect of adding sorted $CD8^+$ T cells back into CD8-depleted PBMC cultures at 1:10 (CD8:CD4) Effector-to-Target (E:T) ratios, in both HIV^+ and HIV^- donors. Infection was normalized to the maximum infection obtained in the experiment. The highest rate of infection was observed in the CD8-depleted PBMC cultures for each individual subject. In HIV^+ subjects, autologous $CD8^+$ T lymphocytes were found to have a dose-dependent effect on the suppression of HIV replication as shown in Figure 3-1B, whereas $CD8^+$ T cells from HIV- donors were not (Figure 3-1B).

To compare the degree of $CD8^+$ T cell suppression amongst a variable subject cohort, data was normalized to the maximum infection for each individual subject (Figure 3-1C). HIV^+ donors had significantly higher suppression (mean 50.61% $^+/_-$ 24.68%)by the addition of autologous $CD8^+$ T cells at day 3 post infection, as compared to HIV^- donors (mean 14.83% $^+/_-$ 12.38%) at equivalent 1:10 E:T ratios (Figure 3-1D, $p = .005$ Mann Whitney). Although I observed suppression by HIV^-

donor $CD8^+$ T cells as high as 32%, suppression in the 1:10 E:T condition (Figure 3-1D), suppression by HIV^- donors did not improve three days post-infection at increased E:T ratios of 4:1 and a decreased MOI of 0.3 (data not shown). Although overall $CD8^+$ T cell suppression of in vitro HIV-1 super infection was observed in HIV^+ donors, a range in suppression was evident (Figure 3-1D), suggesting differences in effectiveness among individual $CD8^+$ T cell populations.

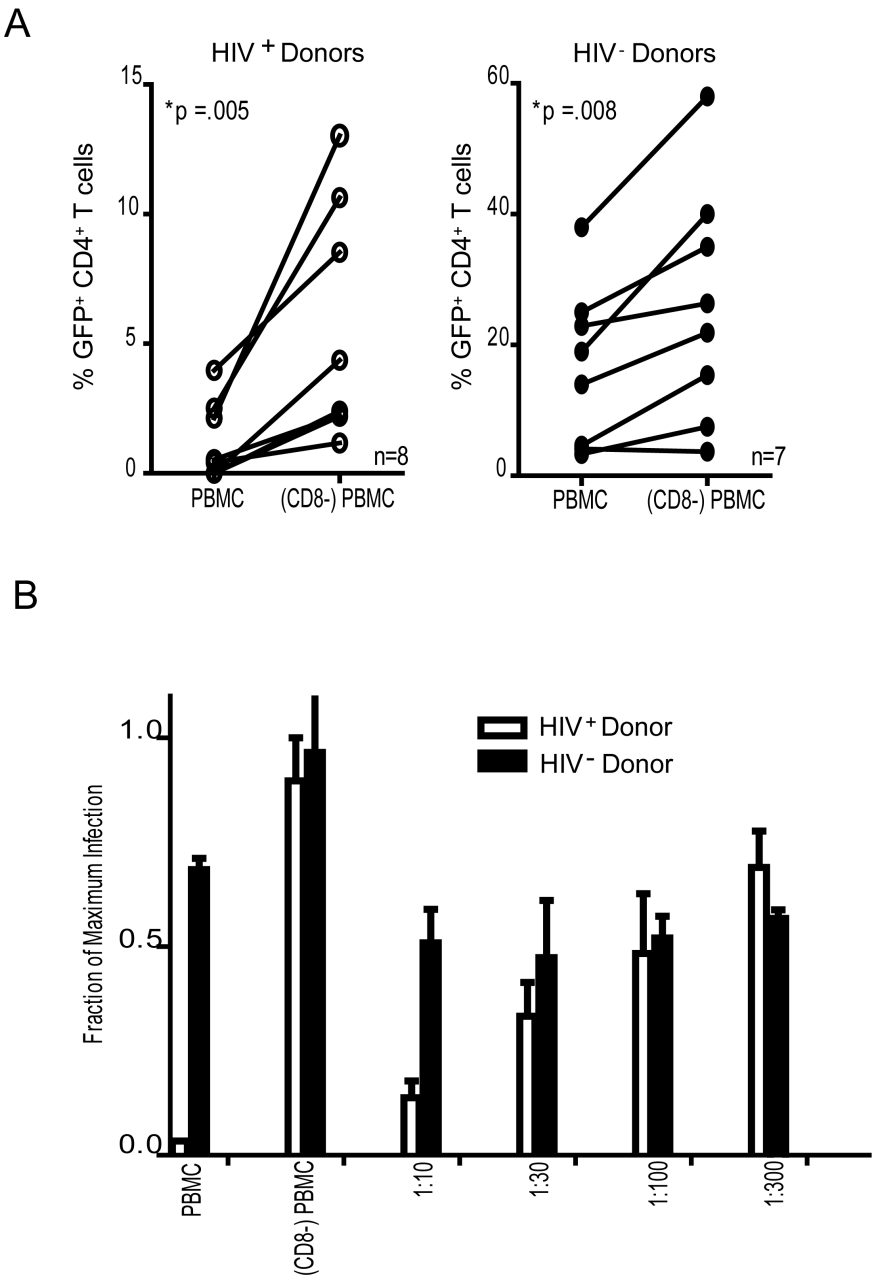


Figure 3-1A and B. The presence of CD8+ T lymphocytes is critical for maximum suppression of HIV replication in vitro. PBMC targets were CD8-depleted and both PBMC and CD8-depleted PBMC were infected prior to addition of $CD8^+$ T cells. (A) $CD8^+$ lymphocyte depletion in HIV^+ ($p = .005$, Wilcoxon paired t-test) and HIV^- ($p = .008$, Wilcoxon paired t-test) donors results in increase in total CD4 infection. (B). Representative plot of total $CD8^+$ T lymphocyte addition to infected cultures. HIV^+ donor shows titratable effect of $CD8^+$ T cell addition, whereas HIV^- donor does not.

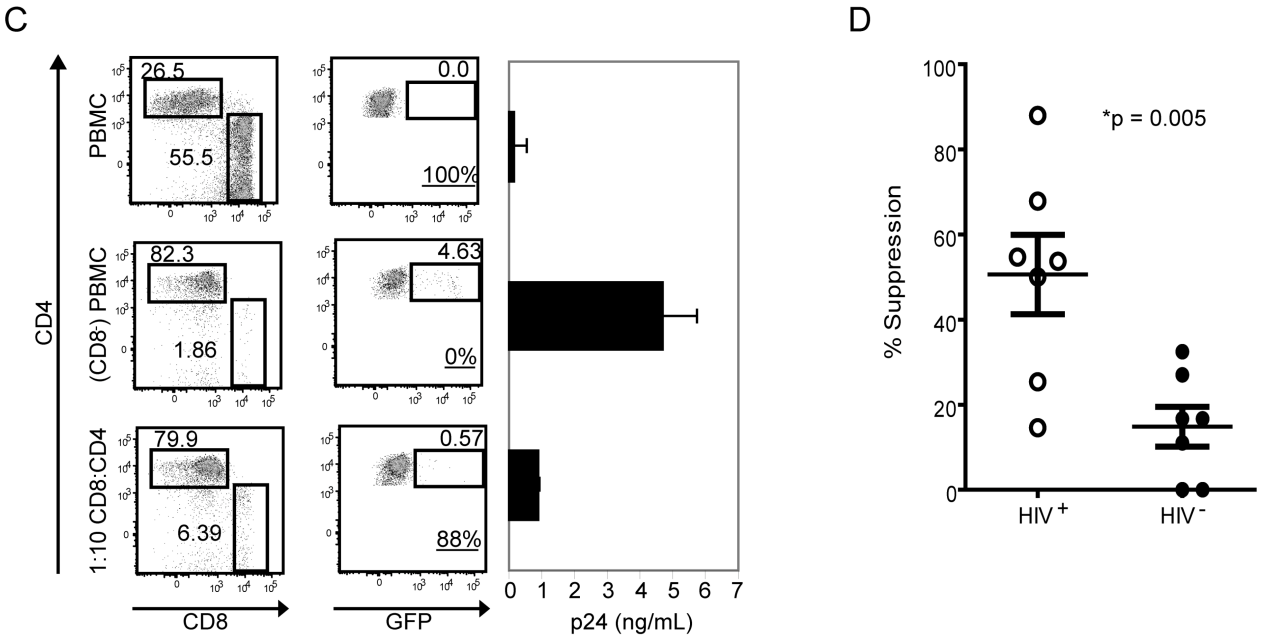


Figure 3-1C and D. Presence of CD8+ T cells is critical for maximum suppression of HIV replication in vitro. (C). Representative plots for calculation of suppression (subject 10002). Suppression percentage underlined values (D). HIV^+ donors have greater suppression by the addition of $CD8^+$ T lymphocytes 1:10 E: T ($p = .01$, Mann-Whitney).

$CD8^+CD57^+$ T cells demonstrate diminished proliferative capacity

The proliferative capacity of $CD8^+$ T cells has been correlated with control of HIV-1 viremia (89, 153). Previous studies have shown $CD57^+$ $CD8^+$ T cells to have decreased proliferation in response to in vitro TCR and cognate peptide stimulation (98, 99, 108), establishing CD57 as a marker for immunologic senescence. To examine $CD8^+$ T cell proliferation simultaneously with $CD8^+$ T cell suppression of viral replication, $CD8^+$ T cells were sorted into $CD57^+$ and $CD57^-$ populations at purities of 95% or greater (Figure 3-2A, subject 10076). $CD8^+CD57^-$ and $CD8^+CD57^+$ lymphocytes were CFSE-stained post-sort and co incubated with super-infected CD8-depleted PBMC cultures at equivalent 1:1 E:T ratios, with the exception of two subjects, 10031 and 10070, where due to cell limitation, effectors were co incubated at 1:3 E:T ratios . The percentage of $CFSE^{lo}$ was a direct measurement of the total percentage of $CD8^+$ T cells that divided in culture (Figure 3-2B).

There were significant differences in the extent of proliferation between the $CD8^+CD57^+$ and $CD8^+CD57^-$ T cell populations (Figure 3-2C). The median of individual $CFSE^{lo}$ percentages within $CD8^+CD57^+$ T cell populations was 20.9% whereas in the $CD8^+CD57^-$ populations, the median $CFSE^{lo}$ percentage was 61.9% ($p = .002$, Wilcoxon paired t-test). These data are consistent with previously published data contrasting the proliferative phenotype between these two populations in the context of in vitro cognate peptide and general TCR stimulation (99, 108). Subjects 10117 and 20018 had to be excluded from the data set due to overwhelming HIV-1 infection in culture, and complete cell death

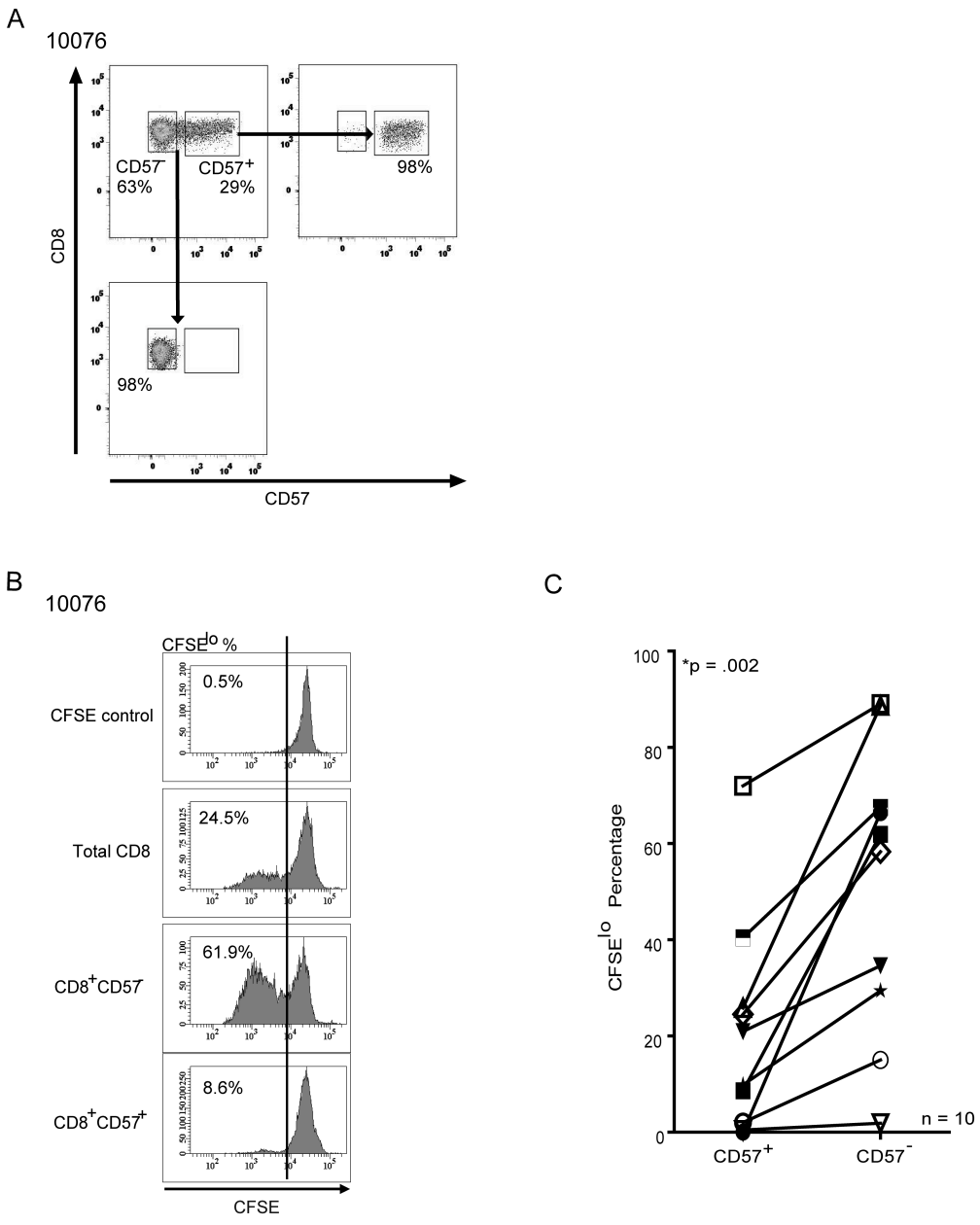


Figure 3-2. Impairment of proliferative capacity in $CD57^+CD8^+$ T lymphocytes. (A). Gating scheme for CD57-specific $CD8^+$ T cell sorts. Sorts were performed at a minimum of 95% purity. (B). Representative plot of proliferation measurement. The percentage of $CFSE^{lo}$ was used as the measurement for proliferation. (C). Proliferation was significantly increased in$CD8^+CD57^-$ T cell populations (p = 0.002, Wilcoxon paired t test).

of the $CD8^+$ T cells that were in culture, making assessment of proliferation not possible.

Proliferative potential of $CD8^+CD57^-$ T cells plays a minimal role in suppression of viral replication in vitro

I next compared the ability of sorted $CD8^+CD57^+$ and $CD8^+CD57^-$ T cells to suppress viral replication at the 1:1 E: T ratio. Over the course of each assay, I evaluated the ability of $CD8^+$ T cells to proliferate simultaneously with the percentage of GFP^+ $CD4^+$ T cell population as a measurement of infection. $CD8^+CD57^-$ T cell populations demonstrated a subtle increase in suppression as compared to corresponding $CD8^+CD57^+$ T cell subsets (median fold difference 1.29) at equivalent E:T ratios within each subject (Figure 3-3A, p = .0273, Wilcoxon paired t-test). Subject 10076 was the one outlier in this fold difference, as $CD8^+CD57^-$ T cells suppressed 22-fold higher than the corresponding $CD8^+CD57^+$ T cell population, however, maximum suppression was only 22% by the $CD8^+CD57^-$ T cells of that subject. Three subjects produced greater than 50% suppression in both $CD8^+$ T cell CD57 subsets (10031, 20011, 10067), and a total of 5 subjects exhibited at least 50% suppression within $CD8^+CD57^-$ T cell populations (10031, 10065, 10067, 10070, 20011) (Figure 3-3A).

Although the expression of CD57 was a reliable predictor of proliferative capacity there was considerable variation in the proliferation among study subjects. These large differences in proliferation between $CD57^+$ (black) and $CD57^-$ (red) subsets did not translate directly to differences in the ability to suppress HIV-1 replication (p = 0.17, Spearman Rank) (Figure 3B), and even some low proliferating populations had over 40% suppression (subjects 10071 and 20018, Figure 3-3B).

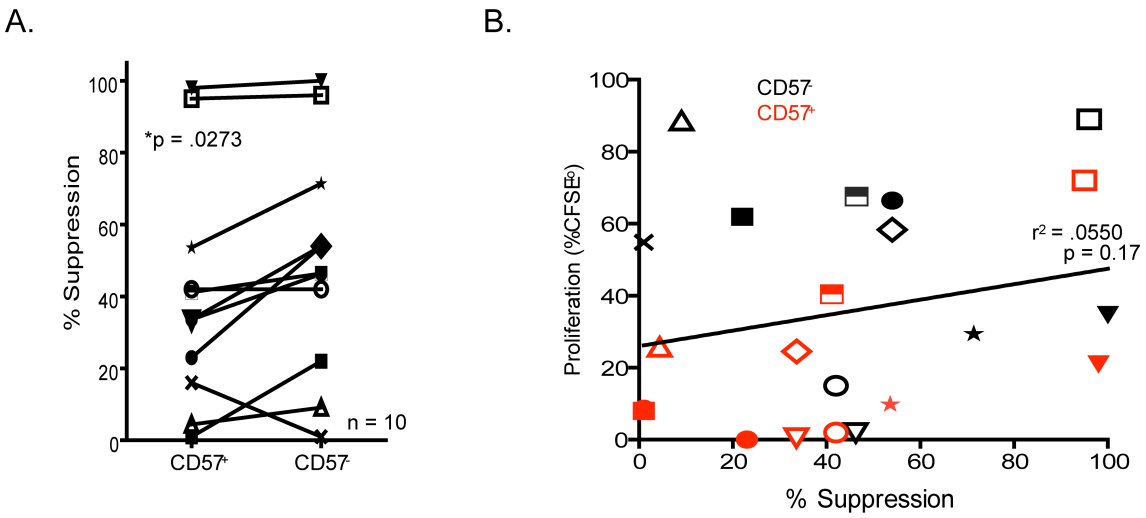


Figure 3-3. Proliferative potential plays a minor role in CD8$^+$ T cell suppression of HIV-1 replication. (A) CD8$^+$CD57$^-$ T cells exhibit higher levels of suppression (p = 0.0273, Wilcoxon paired t test) (B) Overall suppression did not directly correspond with proliferative capacity of individual CD8$^+$CD57$^+$ (black) and CD8$^+$CD57$^-$(grey) T cell subsets (p = 0.4, Spearman rank).

We next determined whether the failure of direct translation between increased proliferation and suppression of viral replication was due to differences in frequency of HIV-specific $CD8^+$ T cells within $CD8^+CD57^+$ and $CD8^+CD57^-$ cell populations, and the preservation of effector functions in $CD8^+CD57^+$ T cells.

Evaluating frequencies of HIV-specific T cells in $CD8^+CD57^+$ and $CD8^+CD57^-$ subsets.

Previous studies have indicated that despite loss of proliferation by $CD8^+CD57^+$ T cells, remaining $CD8^+$ T cell effector functions, such as polyfunctional cytokine production and cell lysis remain intact (98, 99, 108). With the assistance of Shelly Lorey, I first assessed the distribution of known immunodominant and subdominant HIV-specific responses (43) within $CD8^+CD57^-$ and $CD8^+CD57^+$ T cell populations by tetramer staining. Gating schemes were based on those established during the live cell sorting for each individual subject (representative plot, subject 10071, Figure 3-4A).

The frequency of $tetramer^+$ $CD8^+$ T cells within each $CD57^+$ population (median 0.7%, 0.1-8.5) was consistently lower than the frequencies in corresponding $CD57^-$ populations (median 3.4%, 0.1-13.3) ($p = .0238$, Wilcoxon paired t test) (Figure 3-4B) suggesting that a greater number of immunodominant and subdominant epitope-specific $CD8^+$ T cells were present in $CD8^+CD57^-$ T cells at the time of co-incubation with autologous infected targets. Because $CD8^+$ T cell identification by tetramer only measures the frequency of individual epitopes, a much broader assessment was needed to accurately measure the total frequency of HIV-specific $CD8^+$ T cells in each cell population.

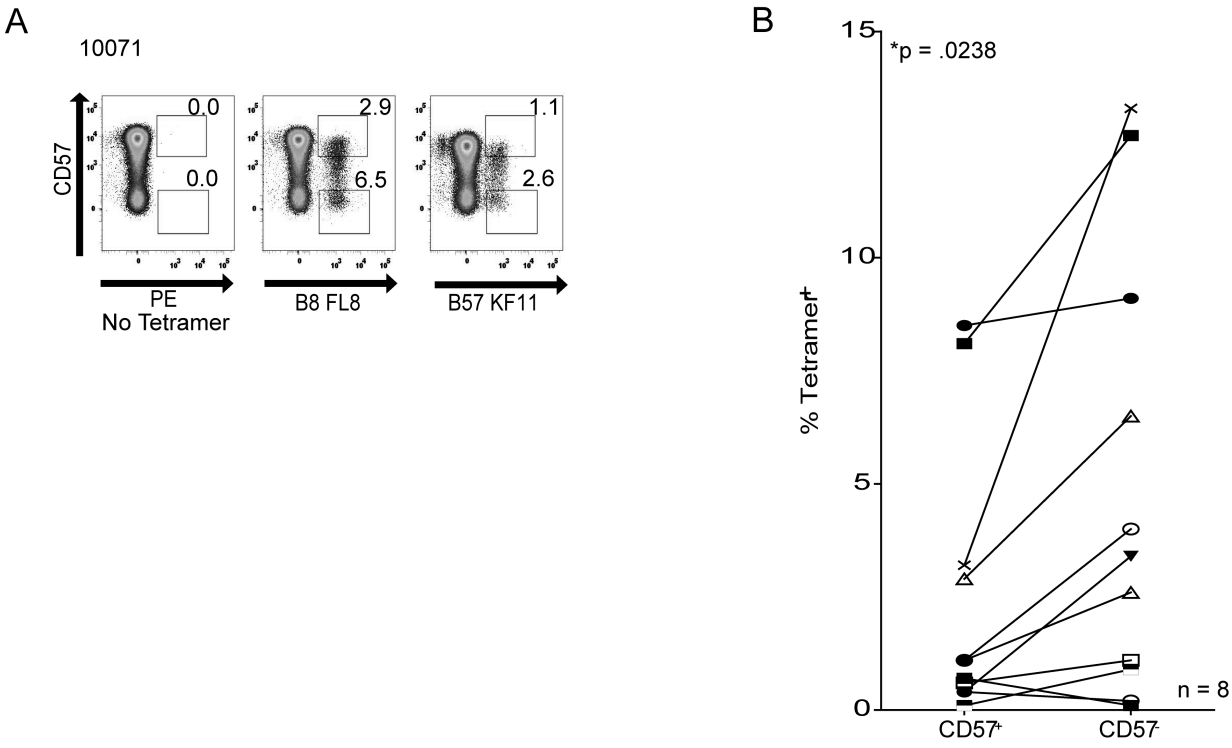
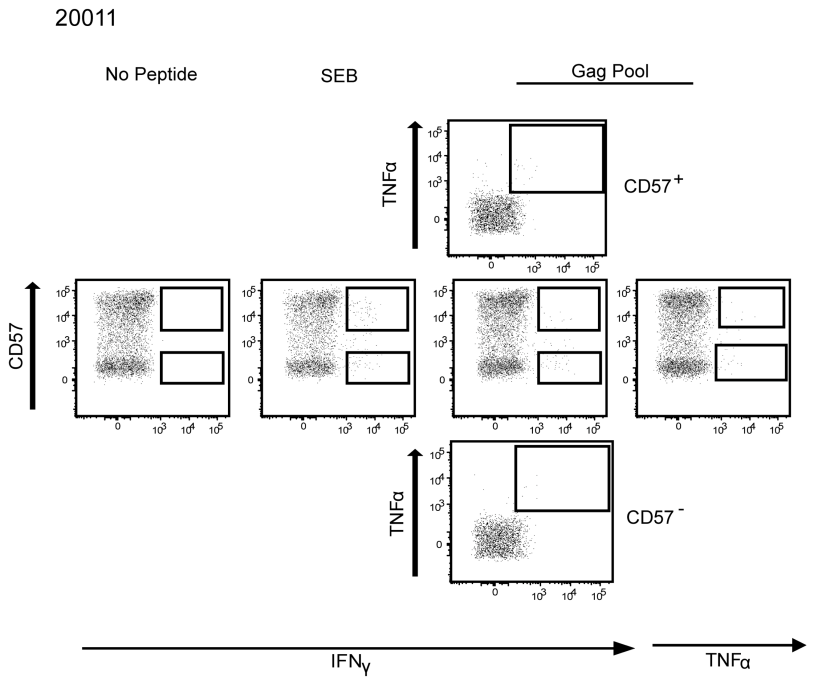


Figure 3-4. Skewing of $CD8^{+}CD57^{-}$ T cells within $tetramer^{+}CD8^{+}$ T cells. (A). Representative plot for dominant epitope tetramer analyses (subject 10071). Numbers reflect the percentage of $tetramer^{+}$ $CD8^{+}$ T cells within each CD57 population. (B). Frequency of $tetramer^{+}$ $CD8^{+}$ T cells is increased in $CD8^{+}CD57^{-}$ T cells ($p = .0238$, Wilcoxon paired t-test).

I next examined the frequency of Gag-specific cells within $CD8^+CD57^+$ and $CD8^+CD57^-$ T cell populations by gag peptide pool stimulation and intracellular cytokine secretion (ICS) assays. Several studies have shown Gag-specific epitopes to be preferentially targeted by l HIV-specific $CD8^+$ T cells, as well as a positive association between Gag-specific immune responses and control of HIV-1 viremia (10, 43, 116, 144, 154, 155). In a recent study by Chen et al, Gag-specific $CD8^+$ T cells demonstrated superior ability to suppress viral replication in vitro as compared to Env-specific $CD8^+$ T cells (156). I measured the frequency of $IFN\gamma^+$ and $TNF\alpha^+$ cells individually, as well as the frequency of dual positive cells within $CD8^+CD57^+$ or $CD8^+CD57^-$ T cell populations (Figure 3-5A, subject 20011).

In concordance with our measurement of tetramer frequencies (Figure 4B), I found higher frequencies of Gag-specific $IFN\gamma^+CD8^+$ T cells in $CD8^+CD57^-$ T cell populations ($p = .03$, Wilcoxon paired t test) (Figure 3-5B) with a median 2.21 fold higher frequency than $CD8^+CD57^+$ T cell subsets. The frequencies of $TNF\alpha^+$ cells were equally distributed between $CD8^+CD57^+$ and $CD8^+CD57^-$ subjects, but there was a trend toward a higher frequency of dual $CD8^+IFN\gamma^+TNF\alpha^+$ cells within $CD57^+$ subsets (Figure 3-5B, $p=.08$). In these studies, subject 10031 did not have an $IFN\gamma^+TNF\alpha^+$ response in either of the $CD8^+$ T cell CD57 subsets, despite both cellular subsets exhibiting suppression greater than 50%.

A.



B.

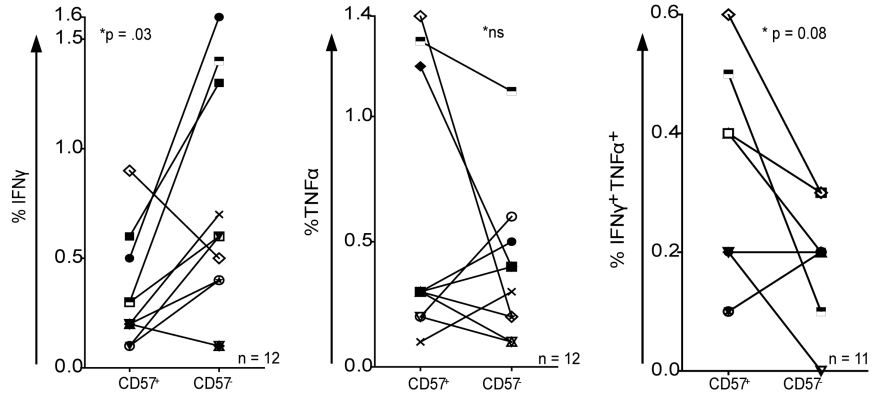


Figure 3-5. Skewing of CD8$^+$CD57$^-$ T cells within tetramer$^+$CD8$^+$ T cells. (A). Representative plot for dominant epitope tetramer analyses (subject 10071). Numbers reflect the percentage of tetramer$^+$ CD8$^+$ T cells within each CD57 population. (B). Frequency of tetramer$^+$ CD8$^+$ T cells is increased in CD8$^+$CD57$^-$ T cells (p = .0238, Wilcoxon paired t-test). (C). Suppression on an epitope-specific CD8$^+$ T per-cell basis shows no role for proliferative capacity in tetramer$^+$CD8$^+$ T cell suppression of HIV-1 replication in vitro.

$CD8^+CD57^+$ T cells demonstrate greater suppression of HIV replication in vitro on an overall Gag-specific per-cell basis.

To determine whether suppression of HIV replication in vitro on a gag-specific per-cell basis varied based on CD57 expression, I adjusted the degree of suppression (Figure 3a) for the total number of gag-specific $CD8^+$ T cells that were present during the co-incubation of effector cells with super-infected autologous targets in each culture. In this examination, I excluded subject data that exhibited suppression less than 32% suppression by both CD57 cellular subsets, as this was the maximum level of non-specific $CD8^+$ T cell suppression from HIV^- donor samples I observed (**Figure 1D).** In this analysis, Gag-specific $CD8^+CD57^+$ T cells demonstrated increased suppression on a per-cell basis than their $CD8^+CD57^-$ T cell counterparts (**Figure 6,** $p = 0.039$ Wilcoxon paired t test).

.

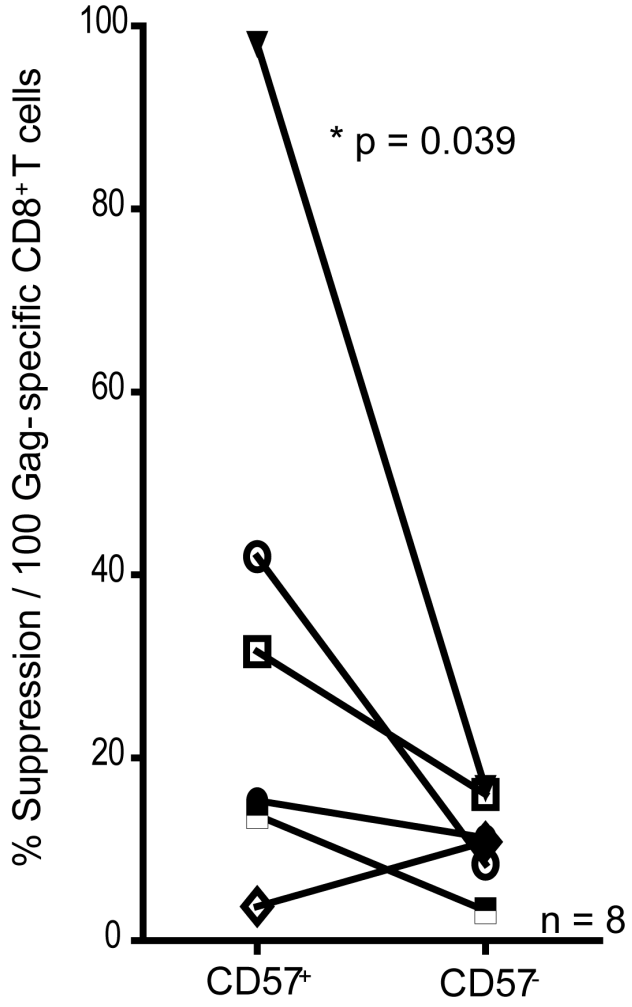


Figure 3-6. Increased suppression of CD8+CD57+ T cells on a per Gag-specific cell basis. Suppression normalized to Gag-specific CD8+ T cells (p = 0.039, Wilcoxon paired t test).

DISCUSSION

The ability to identify subpopulations of antigen-specific $CD8^+$ T cell subpopulations able to suppress HIV-1 replication is essential for our understanding of viral pathogenesis. We have developed a method to assess both $CD8^+$ T cell proliferation and $CD8^+$ T cell suppression of viral replication simultaneously in vitro. Significant differences in proliferative capacity between $CD57^+CD8^+$ and $CD57^-CD8^+$ T cells were observed in the context of in vitro HIV-1 super infection. Despite significant differences in proliferation, suppression of viral replication on a HIV-specific per cell basis was in fact higher in $CD8^+CD57^+$ T cell subsets. This study suggests that while proliferative ability may be important for control of viremia, direct effector function mediated by cells with limited ability to divide may also play an important role in suppression of HIV replication.

We have corroborated previous studies by demonstrating the importance of the presence of $CD8^+$ T cells in the suppression of HIV replication in vitro (90, 98, 138, 156). The high degree of infection of HIV^- donor target cells highlights cellular differences that may lead to drastic differences in infection rates. To address this issue, normalization of data was necessary to adjust for the large range in variation of infection amongst subjects. By using a *nef*-deleted virus (HIV_{R5_GFP}), we acknowledge that the potency of the virus is somewhat diminished without the nef-mediated downregulation of MHC Class I. However, preliminary experiments in our lab also included an X4-tropic HIV strain with murine heat shock protein (CD24, BD Biosciences) inserted into vpr (HIV_{X4_HSA}).

Although we observed higher infectivity by the HIV_{X4_HSA}, normalization of our experimental data produced similar results of suppression (data not shown), and these data are now supported by the recent studies by Chen *et al* (156). Although total depletion of $CD8^+$ lymphocytes resulted in increases in viral replication in both HIV^+ and HIV^- donors, $CD8^+$ T lymphocytes were able to significantly suppress viral replication in only HIV^+ donors at the 1;10 E:T ratio. Natural Killer (NK) lymphocytes, which also express CD8, have been shown to play a role in the innate immune response to HIV-1 (150-152). Since the presence of $CD8^+$ lymphocytes was found to be critical for suppression in both HIV^- and HIV^+ donors, this may reflect the role NK lymphocytes play in the innate HIV-1 specific response.

Previous studies have described in vitro assays to assess the ability of $CD8^+$ T cells to suppress either HIV-1 or SIV replication (70, 90, 98, 144, 145, 156-159). Several of these studies examined $CD8^+$ T cells expanded in vitro by TCR or peptide stimulation when cell numbers were limiting (64, 90, 144, 156). In contrast, studies by Saez-Cirion et al utilized ex-vivo derived $CD8^+$ T cells (98) and further correlated the frequency of the $CD38^{lo}$ HLA-DR^{hi} $CD8^+$ T cell phenotype, which has substantial proliferative ability, with superior ability to suppress HIV-1 super infection in vitro. We have expanded this assay to include direct assessment of $CD8^+$ T cell proliferation in the context of in vitro HIV-1 super-infection.

In this study we confirmed distinct differences in proliferation between $CD8^+$ T cell populations expressing CD57. Prior studies measured proliferative potential of

$CD8^+$ T cell CD57 subsets in the context of in vitro cognate peptide stimulation or in vitro TCR stimulation (98, 99). In addition to demonstrating differences in proliferative potential between $CD8^+$ $CD57^+$ and $CD8^+CD57^-$ T cells, these studies also indicated the failure to rescue $CD8^+CD57^+$ T cells from a senescent state in the presence of soluble IL-2 or IL-15 (98, 99). We also demonstrate that despite the presence of PHA-blast target cells and IL-2 in culture, $CD8^+CD57^+$ T cells had limited ability to proliferate. Although we observed a deficiency in proliferation in $CD8^+CD57^+$ T cells, we did not see consistent survival defects in these cells over the culture period, with the exception of subjects 10117 and 20018, where $CD8^+CD57^+$ cells declined to undetectable levels after 5 days. These data are consistent with a recent study suggesting that $CD8^+CD57^+$ T cells derived directly ex vivo are not as sensitive to Activation-Induced Cell Death (AICD) as $CD8^+CD57^+$ T cells that are stimulated in vitro by α-CD3 or cognate peptide (108) .

We hypothesized that the differences in proliferation we measured between $CD57^+$ and $CD57^-$ $CD8^+$ T cells, would translate to significant differences in suppression between the two $CD8^+$ T cell populations (90, 98). However, we observed only a subtle difference in suppression between the two cellular subsets, as $CD57^-CD8^+$ T cells had a median 1.29 fold higher percentages of suppression (Figure 3-3B) ($p = .0238$, Wilcoxon paired t test). This was despite significantly higher frequencies of tetramer positive $CD8^+$ T cells (Figure 3 4) and interferon-g producing T cells were higher within $CD8^+CD57^-$ T cell subsets (Figure 3-5). ICS after stimulation with overlapping Gag peptide pools confirmed

the maintenance of $CD8^+CD57^+$ T cell effector functions, despite loss of proliferative capacity to cognate peptide (99, 108). When adjusted for the number of antigen-specific cells within each population, $CD57^+$ cells had superior ability to inhibit HIV replication. While our results may appear to be in contrast to a recent study describing the association between increased proliferation, cytotoxicity, and increased elimination of HIV super-infected autologous targets (90), Migueles et. al. required prior expansion of T cells in vitro prior to measuring their suppressive ability. Studies that rely on prior in-vitro expansion of T cells provide an excellent platform for understanding the mechanism by which effector functions may be enhanced; however, direct ex-vivo examination of $CD8^+$ T cells requires less manipulation, and may better reflect cellular function in vivo. Although proliferative capacity in our studies played a minimal role in the overall ability to suppress HIV super-infection, studies have shown that despite deficiencies in proliferation, effector functions of $CD8^+CD57^+$ T cells remain intact (99, 108). Polyfunctional HIV-specific $CD8^+$ T cells have been correlated with control of HIV-1 viremia (77, 86, 88, 160) and although only trending toward significance, we observed a higher frequency of dual $IFN\gamma^+$ $TNF\alpha^+$ cells within the $CD8^+CD57^+$ subsets. This was despite overall higher frequencies of Gag-specific cells when only interferon-g production was measured.

One aspect of $CD8^+$ T cell effector function that remains unexplored is effector migration. Several studies involving either primary $CD8^+$ T cells or $CD8^+$ T cell lines have shown the critical role that cell contact plays in overall $CD8^+$ T cell cytotoxicity (90, 98, 130, 137, 145). Le Priol et al indicated differences in homing

marker expression between $CD8^+CD57^+$ and $CD8^+CD57^-$ T cells as $CD8^+CD57^+$ T cells were found to have higher expression of CX3CR1 and a deficiency in CD62 levels, a critical marker for lymphoid homing. Clinical observations of diffuse infiltrative CD8 lymphocytosis syndrome in HIV^+ subjects, characterized by very high levels of $CD8^+CD57^+CX3CR1^+$ T cells, supports this idea of misdirection of otherwise potent and effective $CD8^+$ T cells (108, 161). This suggests the lack of correlation between cytokine activity of $CD8^+CD57^+$ T cells and control of viremia could be due to misdirection of these cells to non-lymphoid tissues rather than to the site of infection. This block is eliminated in our in vitro assay, and perhaps suggests that if these cells migrate properly to the site of HIV-1 infection, despite impairment in proliferative capacity, they would effectively suppress HIV-1 replication.

PD-1 has also been shown to be a marker of immune exhaustion. T cells with high PD-1 expression also demonstrate diminished replicative capacity as well as decreased cytokine production (100, 103, 105, 106, 162-164). We chose CD57 as a marker since it afforded us the ability to focus on the role of proliferative capacity alone. However future studies with this system will allow us to evaluate the role of PD-1 expression, or combinations of surface markers that potentially predict HIV-suppressive ability.

Using CD57 as a marker for $CD8^+$ T cell proliferative capacity, we have assessed the role of proliferation in $CD8^+$ T cell ability to suppress HIV-1 replication in vitro. Despite the fact that proliferative capacity is likely to be important to replenish effector cells, our study demonstrates that $CD57^+CD8^+$ T cells are capable of

efficiently suppressing HIV-1 replication. We believe ongoing studies with directly isolated ex vivo $CD8^+$ T cells, which would include super-infection challenges in vitro with autologous virus (165, 166) as well as the examination of anti-viral effector functions such as cytolysis and cytokine secretion, will enable further examination of individual effector functions that contribute to suppression of HIV-1 replication in vivo.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Vaccines have an established history of efficacy in the prevention of viral infections. By eliciting immunological memory from the adaptive immune response, vaccines can provide protection as well as therapeutic boosts of the immune response to the host. Cellular immune responses during HIV-1 infection have been shown to be critical in any hope of control of HIV-1 viremia (27, 28, 30). The ability to manipulate $CD8^+$ T cell anti-viral functions as well as accurately assess HIV vaccine-elicited immune responses may provide novel therapeutics as well as more transparency between clinical trials and effective HIV vaccines. Therefore it is essential to understand the relationships and mechanisms that mediate $CD8^+$ T cell suppression of HIV-1 replication.

TCR repertoire diversity is required during chronic HIV infection

Chronic viral infections with extensive variation, such as HCV and HIV, pose a great challenge for the cellular immune response. The Kalams laboratory recently demonstrated that the level of epitope-specific TCR repertoire diversity during acute HCV infection may be critical for limited immune escape and subsequent control of viral replication (37, 109, 110). In addition, similar findings

have been replicated in the SIV acute infection model (55, 58, 114). During acute infection, cellular responses that would be able to suppress viral replication quickly would also be able to block the development and production of viral variants that have the potential to escape the cellular immune response. In this model, TCR diversity would perhaps not be as essential, if a strong narrow TCR-mediated response would be able to suppress infection quickly and successfully and then maintain suppression in the host. However, during HIV infection, several studies have indicated the deletion of highly active $CD8^+$ T cells during acute HIV infection, as the virus leaves a permanent impression on the overall $CD8^+$ T cell response (11, 167). The resolution of HIV infection proves difficult for the host, and after the original damage during acute infection, the immune response that is left to be active during chronic HIV infection is damaged. However, there are groups of subjects who maintain a long duration of low viral loads and delayed HIV disease progression.

Although there are several possible factors that may delay disease progression, HLA B allele expression has been shown to strongly correlate with the control of HIV-1 viremia. The HLA B allele B*57 is found in higher frequencies in HIV^+ subjects with control of viremia (42), and HLA B*57-restricted $CD8^+$ T cell responses have been indicated to dominate total HIV-specific $CD8^+$ T cell responses (43, 116). Antigen presentation by MHC class I is a strong factor in the determination of TCR selection and the development of epitope-specific TCR repertoires (40). Patterns of TCR usage directed against this strong epitope may

shed light on how TCR recruitment over the course of infection mediates control of viremia.

Several HLA B*57 epitopes have been fine-mapped, including dominantly recognized, highly conserved epitope located in p24 Gag, KF11 (KAFSPEVIPMF) (10, 36). The TCR gene usage of KF11-specific immune responses has been assessed for shared motifs. Previous studies implicated a model in which HIV^+ controllers exhibited strong usage bias within individual KF11-specific TCR repertoires, and subsequently narrower repertoires if the KF11 epitope was conserved, thus concluding that TCR diversity is not a prerequisite for control of HIV viremia. While it remains unclear whether TCR diversity is the driving force of $CD8^+$ T cell control of HIV, the structure of a TCR repertoire is the driving force behind the immune system's ability to recognize and respond to viral variants. Therefore, in order to understand how the selection of TCR repertoires influenced the host's ability to contend with HIV variation, I examined KF11-specific TCR clonotype ability to recognize autologous viral variants.

Based on previous data from the Kalams laboratory that indicated TCR diversity to be an immunologic factor in clearance of a chronic viral infection with extensive variation (in this case, HCV), I originally hypothesized HIV^+ controllers, whom were therapy naïve would indicate broader KF11-specific TCR repertoire diversity. The model behind this hypothesis indicates a broad TCR repertoire was maintaining recognition of dominant HIV epitopes, including variant epitopes derived from circulating HIV variation in vivo (Figure 4-1).

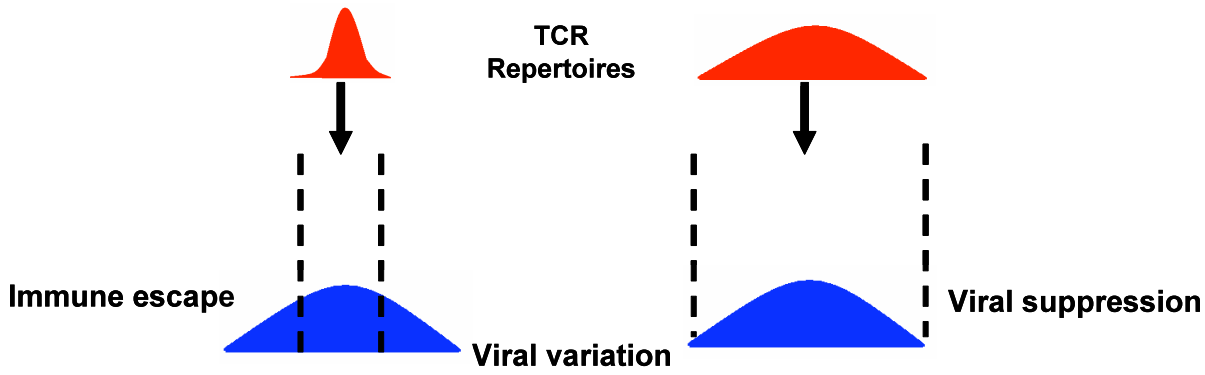


Figure 4-1. Model of the hypothesis that a broad T Cell Receptor (TCR) repertoires provides for recognition of viral variation in vivo. TCR repertoire breadth represented on top (red), viral variation in vivo represented on bottom (blue). The model indicates a narrow TCR repertoire is unable to recognize viral variants, and therefore loses the ability to control HIV replication.

Results indicated that KF11-specific diversity did not correspond with any clinical marker of HIV disease progression. I also found that KF11-specific TCR repertoire diversity was within the same range of entropy as other epitope-specific TCR repertoires, but much lower than total $CD8^+$ TCR repertoire diversity. I observed a dominant usage of TRBV7 among KF11-specific clonotypes. Previous studies indicated clonal hierarchy by TCR avidity and dominance. I believed these results suggested an immune preference for TRBV7 in KF11-specific $CD8^+$ T cells and hypothesized that TRBV7 clonotypes would have greater structural avidity and greater ability to recognize viral variants.

Structural avidity of TRBV7 clonotypes, assessed by tetramer off rate assays, was found to be collectively lower than the remaining KF11-specific clonotypes. The variance in tetramer off-rates among TRBV7 clonotypes was significantly lower than those of the non-TRBV7 clonotypes, suggesting a direct influence of TRBV structure on TCR avidity. I believe these data provide evidence for the model where high avidity clones are deleted during acute HIV infection, as well as suggest a threshold for optimal TCR activation exists, as has been described (135). HIV infection has been associated with immune exhaustion, as measured by the expression of surface markers such as CD57 and PD-1 (98-100, 104-106). I believe that in the model of chronic infection, in which antigen is persistently presented, it is possible that high structural avidity is disadvantageous, and would perhaps lead to constant activation and eventual exhaustion of the high avidity clonotypes (88).

I hypothesized that if lower structural avidity was advantageous in the context of KF11-specific TRBV7 usage, that the TRBV7 clonotypes would exhibit increased ability to recognize viral variants. Despite the frequent TRBV7 usage of KF11-specific $CD8^+$ T cells in our study cohort, TRBV7 usage did not necessarily correspond to recognition of epitope-variants. Earlier studies had described narrow KF11-specific TCR repertoires in HIV^+ HLA B*57 individuals (79, 80), and continual recognition of epitope variants (79, 80). It was therefore suggested that the degree of epitope variant cross recognition is more important than the overall diversity of the KF11-specific TCR repertoire of control of viral replication.

I next wanted to assess the ability of TRBV7 clonotypes to recognize autologous HIV variants on an individual basis. I hypothesized that if TRBV7 clonotypes were able to recognize all viral variants presented, perhaps the data would correspond to the model in which cross recognition rather than TCR diversity determined overall HIV-specific $CD8^+$ T cell responses to recognize HIV and its variants. However, at the clonotype level, differential recognition and functional avidity was observed amongst TRBV7 clonotypes. In one of the HIV^+ controller subjects, TRBV7 lost all ability to recognize autologous KF11 variant, K162R. These data demonstrate that dominant clonotypes do not necessarily cross-react with epitope variants, which studies from the Kalams lab previously describe at the clonal level, and suggest a potentially beneficial role for maintenance of TCR diversity even in the setting of restricted TRBV usage.

These data collectively suggest convergent evolution toward populations of epitope-specific T cells with sufficient avidity to mediate control of viremia during

chronic HIV infection, despite my observation of clonotypic diversity amongst subjects, and no observed 'public' clonotype. Based on these findings, I am able to conclude that TCR repertoire diversity specific for a dominant epitope is not a pre-requisite for control of HIV-1 viremia, and revise my model of the relationship between TCR repertoire diversity and recognition of viral variants (Figure 4-2). My revised model indicates that TCR repertoire diversity is not required for control of HIV-1, however, it can provide recognition of HIV-1 variants and perhaps improve the HIV-specific host immune response in the event of a circulating HIV-1 viral variant in vivo (Figure 4-2). Although TCR repertoire diversity is seemingly not a correlate of control of HIV-1 viremia, the structural recognition and subsequent downstream signaling that is dependent on the structure of the TCR is critical for $CD8^+$ T cell function and suppression of HIV-1. Therefore, these data highlight the importance of continuing analyses of the HIV-specific immune responses at the clonotype level, which will help define how the development and maintenance of epitope-specific TCR repertoires influences HIV disease progression. As new assays are developed for the evaluation of vaccine-induced responses, it will be important to maintain incorporation of clonotype analyses as a bridge between structure, phenotype, and the ability to control HIV replication. More extensive TCR repertoire studies that include other HLA-restricted epitopes are required to fully understand the extent of the role of TCR diversity in the control of HIV-1 viremia.

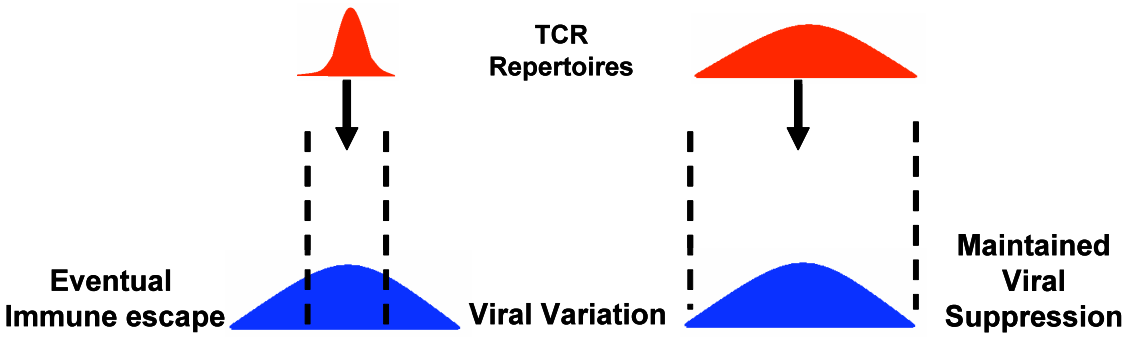


Figure 4-2. Although TCR repertoire diversity is not a pre-requisite for control of HIV-1 viremia, increased breadth of an epitope-specific TCR repertoire can provide auxiliary capability to recognize circulating viral variants.

Future studies will focus on the assessment of $CD8^+$ T cell suppression of HIV replication in vitro on the clonotype level. Although my structural and functional avidity studies did not provide evidence for the usage of TRBV7 to provide improved binding and recognition of peptide-MHC Class I complexes, we hypothesize that perhaps these data suggest an optional threshold for successful $CD8^+$ T cell responses and subsequent suppression of HIV replication. $CD8^+$ T cells will be sorted based on the expression of tetramer specificity and TRBV expression. As discussed before, the difficulty of these studies is that no commercial antibody for TRBV7 currently exists. My studies are designed to circumvent this caveat, by surface staining $CD8^+$ T cells with tetramer and all TRBV present in these tetramer-specific $CD8^+$ T cell populations, then gating TRBV7 by exclusion. These clonotype subsets of $CD8^+$ T cells will then be added to cultures of HIV super-infected $CD4^+$ target cells at appropriate E:T ratios. Cultures will be assessed for infection by viral expression of GFP in $CD4^+$ target cells, as well as the levels of p24 antigen in the culture supernatants. Future analysis will begin with the KF11-specific TCR repertoire subject group I previously described (43). Using these analyses, the Kalams lab will be able to assess differential abilities to suppress HIV replication in vitro on the clonotype level and begin to link components of an epitope-specific TCR repertoire with populations of $CD8^+$ T cells that are superior in the suppression of HIV replication.

If biased usage of TRBV genes is beneficial I anticipate that the KF11-specific TRBV7 clonotype CD8+ T cell populations will exhibit improved suppression on a

per-cell basis as compared to their KF11-specific counterparts. Further studies building upon these experiments will also include incorporating autologous virus into these infection assays. Subject autologous HIV Pol-gag sequences will be cloned into the HIV_{R5_GFP} viral construct, producing an improved representation of what each subject is being presented in vivo, in our in vitro infection model. I have observed hierarchy of avidity on the clonotype level, and I hypothesize that dominantly represented clonotypes defined by TCR sequencing will have improved recognition and suppression of HIV super-infection as compared to corresponding sub-dominant clonotypes. These experiments can provide insight into the role of dominant and sub-dominant clonotypes, and begin to answer the question of whether or not HIV vaccines need to focus on eliciting broad TCR recognizing responses. Understanding the importance of these attributes of $CD8^+$ T cell-mediated suppression of HIV-1 infection is critical in accurate and translational interpretation of HIV vaccine-elicited immune responses.

Overall my analysis provides an in depth examination of how TCR diversity can influence host control of HIV-1 infection. As my data indicates, TCR diversity may not be required for the maintained recognition of a dominant epitope during chronic HIV-1 disease, and these narrowed TCR repertoires provide delayed HIV-1 disease progression. However, at this point in time, the majority of HIV^+ subjects, even those with delayed disease progression, are susceptible to HIV immune evasion. . As my data further suggests, by maintaining some degree of TCR diversity, eventual epitope-variants that are produced can still be recognized. The decision of whether or not a HIV vaccine needs to be designed

to elicit a broad or narrow response may be dependent on the timing of administering the vaccine during the course of HIV-1 disease progression. Perhaps a vaccine that elicits a narrow TCR-founded response would be highly effective during acute infection, suppressing effectively while viral variation has not been allowed as much time to expand. Whereas, a broad TCR-founded response, elicited by a HIV vaccine, may be better utilized by the host during chronic infection as a broad response could effectively suppress HIV replication and maintain and monitor for increasingly probable HIV variation in vivo. Further studies are required in order to determine how we may interpret TCR usage in the overall prognosis of HIV disease progression. These understandings can provide improved understanding not only of vaccine design but also vaccine assessment that accurately reflects elicited immune responses in vivo.

$CD8^+$ T cell effector functions important in the control of HIV viremia

Although shown to be critical for maximum possible control of HIV-1 viremia, $CD8^+$ T cell responses eventually fail to control HIV-1 infection. The mechanisms by which the host immune system fails to control HIV-1 infection is not clearly understood, but nature provides a small group of HIV-1 infected individuals whose immune systems do exhibit active participation in the control of HIV-1 infection. By examining these subjects, great insight has been gained into what constitutes a successful immune response in the control of HIV replication (42, 89, 90, 100).

Clear understanding of not only what $CD8^+$ T cell functions are successful in suppressing HIV replication, but also the mechanism and timing by which these functions are triggered can further refine vaccine design. Further parameters are needed to make stronger connections between the immune responses elicited and overall benefits of a vaccine.

In my studies I investigated the role of $CD8^+$ T cell proliferative capacity in $CD8^+$ T cell ability to suppress HIV replication in an in vitro model of HIV super-infection. In animal studies of chronic viral infection, the expansion potential of T cells after adoptive transfer mediated control of viremia (15, 140). Observational studies in HIV infected individuals demonstrated that $CD8^+$ T cells derived from HIV controllers had increased proliferation in vitro to either anti-TCR antibody or HIV cognate peptide stimulation (89, 99). Although extensive studies indicated the correlation between $CD8^+$ T cell proliferation in vitro and control of HIV-1 viremia, the mechanism by which this effector function relates to suppression of HIV replication is not clearly defined.

Recent developments have provided the ability to examine $CD8^+$ T cell suppression of HIV replication in vitro (69, 90, 98, 144, 168). By using these newly developed assays, previous studies correlating $CD8^+$ T cell function in vitro with control of HIV-1 viremia have been extended, demonstrating a direct relationship between proliferative capacity, cytotoxic function and $CD8^+$ T suppression of HIV replication in vitro (90). Although these data provide a platform for the direct relationships between $CD8^+$ T cell function and $CD8^+$ T cell suppression, it is critical to maintain assessment of $CD8^+$ T cells that are derived

directly ex vivo. By examining $CD8^+$ T cells that are not pre-stimulated, a true reflection of the $CD8^+$ T cell state in vivo is represented in the assessment over overall ability to suppress HIV replication. Saez-Cirion et al demonstrated that $CD8^+$ T cells derived directly ex vivo from HIV^+ controllers had increased capacity to suppress viral replication in an in vitro HIV super-infection model as compared to viremic HIV^+ subjects (98). Furthermore, these studies indicated a direct relationship between $CD8^+$ T cell ability to suppress HIV replication in vitro and overall control of HIV viremia. This increased ability to suppress was connected to the expression of CD38 and HLA-DR, in a relationship that demonstrated increased proliferative capacity in HIV controllers (98). These studies are critical in the further assessment of $CD8^+$ T cell function in vivo. However, the extent by which proliferative capacity plays a role in suppression of viral replication was not defined in that work. It was unknown whether proliferation provides continual supply of an effectively suppressive $CD8^+$ T cell population, or if proliferation is directly involved in $CD8^+$ T cell-mediated suppression of HIV-1.

My studies were designed to assess this role of proliferative capacity in the control of HIV replication in vitro by evaluating the ability of $CD8^+$ T cells, directly derived ex vivo, with high proliferative capacity to suppress HIV super-infection in vitro relative to cells with lower proliferative capacity. To divide $CD8^+$ T cells based on the ability to proliferate I used the immunologic marker for senescence, CD57. Studies have shown $CD8^+CD57^+$ T cells exhibit limited proliferative capacity to cognate peptide, decreased telomere length and increased sensitivity to AICD (98, 99, 108). Despite impairment in proliferative capacity, $CD8^+CD57^+$

T cells are capable of producing IFNγ and TNFα (98, 99, 108) and have extensive effector cytotoxic capabilities (108). By dividing $CD8^+$ T cells into $CD57^+$ and $CD57^-$ subsets, I created an assay that allowed us to focus on the role of proliferation in the control of HIV replication. I hypothesized that given proliferative capacity is the strongest correlate of control of HIV-1 viremia it played a direct role in CD8+ T cell capabilities of suppression of HIV replication (Figure 4-3).

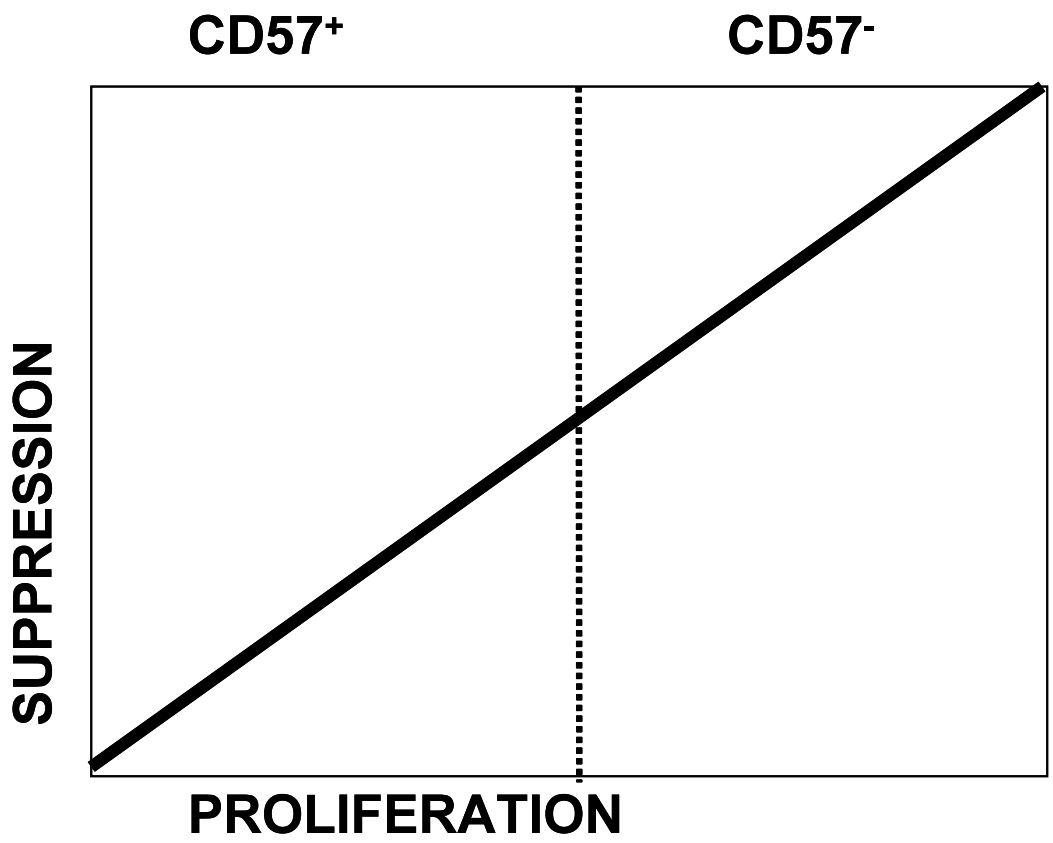


Figure 4-3. Model of proliferation playing a direct role in the suppression of HIV-1 replcation in vitro. CD57 is an immunologic marker for impaired proliferative capacity. However, $CD57^+$ cells still exhibit strong abilities of anti-viral cytokine production and direct cell lysis, two central functions of overall $CD8^+$ T cell-mediated suppression of viral infection. Utilizing CD57 as a marker to distinguish $CD8^+$ T cells that are derived directly ex vivo by proliferative capacity allows for assessing whether proliferative capacity plays a direct role in $CD8^+$ T cell suppressive capabilities ex vivo. The original hypothesis here projects proliferation to be directly involved in $CD8^+$ T cell-mediated suppression of HIV-1 replication in vitro.

I initially examined the requirement of $CD8^+$ lymphocyte presence in culture for maximum suppression of HIV replication in vitro. The removal of total $CD8^+$ lymphocytes in culture resulted in increased HIV replication in both HIV^+ and HIV^- donors, thus indicating the presence of $CD8^+$ lymphocytes to be necessary for maximum suppression of HIV replication in vitro. I hypothesized the role of $CD8^+$ T cells in the control of HIV replication to be pertinent in only HIV^+ donors, as HIV^+ donors possess HIV-specific $CD8^+$ T cells and HIV^- donors have not developed an adaptive cellular response to a pathogen they have not been exposed to. HIV^+ donors presented direct correlations between the total number of $CD8^+$ T cells present in the culture and the overall level of HIV replication in vitro. In contrast, $CD8^+$ T cells from HIV^- donors were unable to suppress HIV replication in autologous targets regardless of the E:T ratios. This examination also allowed me to gauge the sensitivity of my assay for assessing $CD8^+$ T cell-mediated suppression of HIV replication in vitro.

After determining the presence of $CD8^+$ T lymphocytes to be critical for maximum suppression of HIV replication, I next wanted to examine the ability of $CD8^+$ T cells to suppress HIV replication based on the level of proliferative capacity. Previous data supported the relationship between increased proliferative capacity and increased control of HIV-1 viremia only. Therefore I hypothesized there would be an increased ability to suppress HIV replication in vitro by the $CD8^+CD57^-$ T cell subsets relative to $CD8^+CD57^+$ T cell counterparts within individual subjects.

I observed significantly increased proliferative capacity in $CD8^+CD57^-$ T cells when compared to individual $CD8^+CD57^+$ T cell counterparts. In addition to demonstrating differences in proliferative capacity, these studies also indicated the failure to rescue $CD8^+CD57^+$ T cells from a senescent state despite incubation with soluble IL-2 and PHA-blast target cells. However, although CD57 expression was a reliable predictor of decreased proliferative capacity, I did not observe corresponding significant differences in suppression between the $CD8^+CD57^+$ and $CD8^+CD57^-$ subsets. These data contrast with recent findings reported by Migueles et al in which increased proliferation directly corresponded with increased effector function as well as suppression of HIV replication in vitro (90). I believe these discrepancies are due to my contrasting methods in $CD8^+$ T cell preparation and hypothesized that my observation of subtle differences in suppression were due to skewed frequencies of HIV-specific $CD8^+$ T cells in these two cellular subsets as well as remaining in tact effector functions in these $CD8^+$ T cells.

Epitope-specific $CD8^+$ T cells were first examined within $CD8^+$ CD57 subsets and in fact indicated increased frequency of HIV dominant epitope-specific $CD8^+$ T cells within $CD8^+CD57^-$ T cell subsets. Based on these data, $CD8^+CD57^+$ T cell populations showed increased suppression on a per epitope-specific cell basis. A broader assessment of all gag-specific $CD8^+$ T cells was required to truly assess suppression on a HIV-specific per-cell basis. ICS data revealed overall increased frequency of gag-specific $CD8^+$ T cells in $CD8^+CD57^-$ T cell populations. These data translated to increased suppression on a per gag-

specific cell basis within $CD8^+CD57^+$ T cell subsets. Previous studies had indicated increased effector functions in $CD8^+CD57^+$ T cells such as dual production of IFNγ and TNFα (86, 112). My results from gag-specific ICS stimulation indicated a trend towards increased frequencies of gag-specific $IFN\gamma^+TNF\alpha^+$ T cells in the $CD8^+CD57^+$ T cell subsets, but these frequencies did not correspond with overall control of HIV-1 viremia. My observation of increased dual cytokine producing cells in the $CD8^+CD57^+$ subset not only corresponds with previous findings, but also offers an explanation for the lack of correlation between suppression and proliferative capacity. My revised model reconciles previous findings of proliferative capacity being a strong correlate of control of HIV-1 viremia in vivo and my lack of finding evidence that tied proliferative capacity directly to the mechanism of $CD8^+$ T cell-mediated suppression of HIV-1 in vitro (Figure 4-4). Together, my data sheds light onto the role of proliferative capacity in control of HIV-1 viremia as perhaps a means to continue and maintain a population of highly effective $CD8^+$ T cells that are proficient in cytokine production and direct cell lysis, rather than directly influencing effective $CD8^+$ T cell anti-viral functions (Figure 4-4).

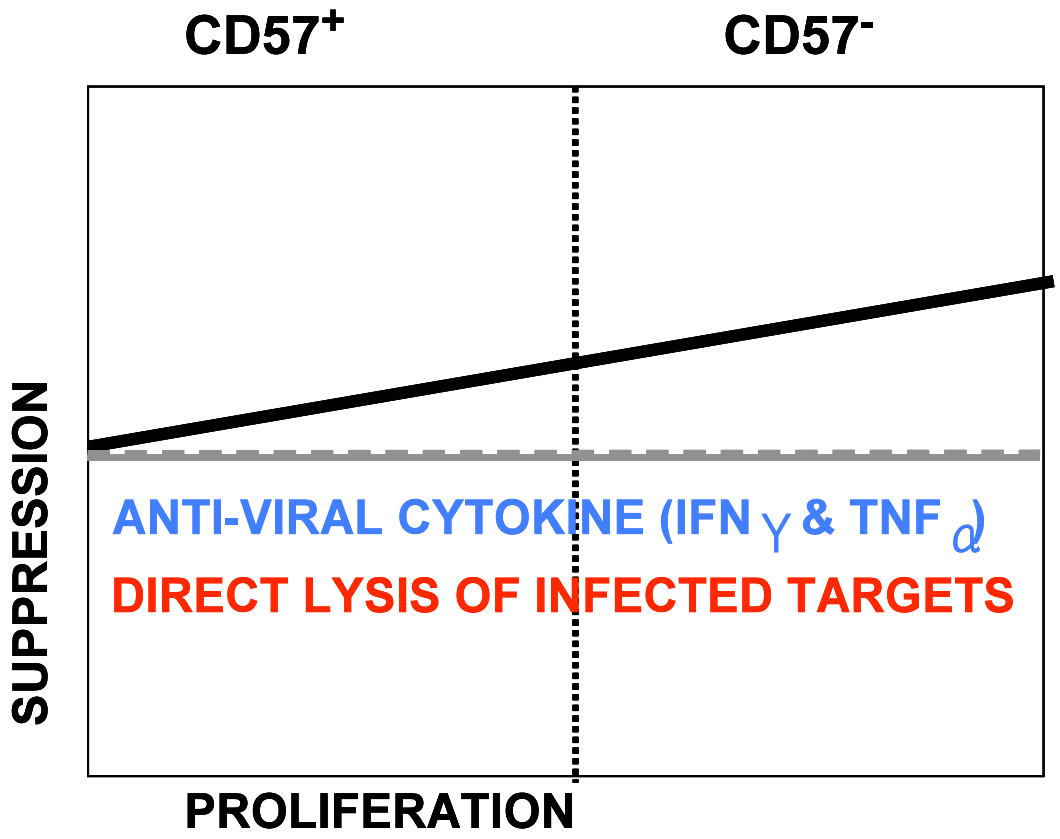


Figure 4-4. Revised model indicates that proliferative capacity does not play a direct role in CD8+ T cell-mediated suppression of HIV-1 replication. Although proliferative capacity remains a strong correlate of control of HIV-1 viremia in vivo, it does not play a direct role in $CD8^+$ T cell-mediated suppression of HIV-1 replication in vitro. Other effector functions, such as anti-viral cytokine production and direct lysis of infected targets, are capable of directly mediated $CD8^+$ T cell suppression of HIV-1 replication.

There are still questions regarding the effector function of $CD8^+CD57^+$ T cells in vivo. Understanding the answers to these questions is critical in providing accurate interpretation from the assessments of HIV vaccine-elicited immune responses, which currently include measuring CD57 expression. One aspect of $CD8^+$ T cell effector function that remains unexamined is cell migration. Several studies examining primary or $CD8^+$ T cell lines have indicated the critical importance of direct cell contact in $CD8^+$ T cell effector abilities (90, 145, 168). Le Priol et.al examined differences between $CD8^+$ $CD57^+$ and $CD8^+CD57^-$ T cells on a transcriptional and translational level (108). These data indicated a significant deficiency in CD62 expression on the surface of $CD8^+CD57^+$ T cells. Expression of CD62 is vital for lymphoid homing, a critical step in $CD8^+$ T cell ability to recognize and suppress viral infections. These studies also indicated increased expression levels of CX3CR1, a chemokine receptor, associated with non-lymphoid tissue homing.

These data suggest possible impairment of HIV suppression in vivo by $CD8^+CD57^+$ T cells due to misdirection of these cells to non-lymphoid tissues rather than the site of infection. Clinical observations corroborate these findings. There are clinical reports of increased observations of diffuse infiltrative CD8 lymphocytosis syndrome in HIV^+ subjects, which is characterized by high levels of $CD8^+CD57^+CX3CR1^+$ T cells (161). Although our my collectively suggests that proliferative capacity is not critical for direct $CD8^+$ T cell-mediated suppression of HIV replication, the necessity for proliferative capacity may still exist in vivo during HIV infection in the context of $CD8^+$ T cells that are actually

present at the site of HIV replication and immune presentation. The role of acquiring CD57 expression and the migration of CD8+ T cell and their ability to suppress is still unknown. Preliminary data from our lab suggests that over the course of the in vitro co incubation of $CD8^+CD57^-$ T cells and HIV super-infected autologous $CD4^+$ T cell targets expression of CD57 increases on these cells. Post-sort purities from original CD57 subset sorts on enriched $CD8^+$ T cells indicate greater than 98% purity of the $CD8^+CD57^-$ T cells. However, by 5 days post infection, these percentages can decrease to levels as low as 30%, as CD57 expression increases in these $CD8^+$ T cell subsets (Simons; unpublished data). Future findings in the mechanism behind acquiring CD57 and migration of T_E cells may need to be elucidated before continued investigation behind CD57 expression and anti-viral T cell functions.

Despite the caveat of neglecting analysis of cell migration in my assay, my data was still able to indicate proliferative capacity to play a minimal role in direct $CD8^+$ T cell-mediated suppression of HIV replication and shed light on the immunologic value of CD57 expression of directly derived ex vivo immune responses. Currently, there is no hypothesis-driven assessment for CD57 expression of HIV vaccine-elicited immune responses. My data indicates that perhaps despite association with immunologic senescence, increased CD57 expression may not reflect a negative feature of vaccine-elicited immune responses, allowing for clearer understanding of vaccine assessment outcomes. Future directions will focus on continuing assessment of identified $CD8^+$ T cell functions as well as features that correlate strongly with control of HIV-1

replication. Anti-viral cytokine production can be assessed simultaneous with suppression of HIV-1 replication in vitro by measuring the magnitude of cytokine production in the supernatants of the co-incubation cultures described previously. With the inclusion of negative controls that would reflect the degree of background, or non-specific, cytokine production, a wide array of anti-viral cytokines and chemokines may be assessed. This will be incorporated into the present CD57 findings to perhaps shed light on other differences in cytokine production beyond IFNγ and TNFα, but can also be incorporated into other markers of $CD8^+$ T cell loss of function, or functional exhaustion. To further understand the extent of which anti-viral cytokines play a role in $CD8^+$ T cell-mediated suppression of HIV-1 transwell experiments will also be utilized to examine whether anti-viral cytokine production physically blocks viral replication, or if anti-viral cytokine production is a reflection of an activated $CD8^+$ T cell-mediated response, such as direct cell lysis, responsible for suppression of HIV-1 replication. The immediate goals of the Kalams laboratory would be to continue from my preliminary data of improved poly-functional cytokine correspondence with increased suppression of HIV-1 replication, but future directions may move to other $CD8^+$ T cell functions such as direct cell lysis of infected targets. Currently, the Kalams laboratory is pursuing further understanding of the mechanisms behind the expression of PD-1 and associated cellular signaling in $CD8^+$ T cell-mediated suppression of HIV-1 (Conrad; unpublished), in which my assay can possibly be incorporated into.

HIV vaccines are currently administered to HIV^- study subject groups. These study groups are either high-risk groups with increased probable incidence of HIV infection, or non high-risk groups. The examination of vaccine-elicited immune responses in HIV^- subjects and possible protection offered by these vaccines in high-risk groups has proven difficult to draw distinct lines of connection between vaccines administered and subsequent elicited immune responses that can actually correlate with protection from HIV-1 infection and suppression of HIV-1 viremia. Investigating natural examples of HIV-1 control may prove to be a more straightforward approach to understanding immune responses that directly correspond with control of HIV-1 viremia. Using this resource in identifying precise immune functions that directly participate in immune control of HIV-1 replication will provide for an improved translation of vaccine-elicited immune responses to control of HIV-1 replication in vivo.
My designed in vitro assay allows for this assessment of directly derived ex vivo $CD8^+$ T cells on a small cell number basis. My assay also required little manipulation and inexpensive stimulation of target cells. Collectively, these features make my assay widely accessible to the scientific community as well as allow for assessment of $CD8^+$ T cell populations that are smaller in frequency.

In summary, my research demonstrates structural bias in the development of dominant epitope-specific TCR repertoires in chronic HIV infection. Despite no functional benefit perceived from these current data, our research suggests evidence for an optional threshold of avidity in the state of chronic infection and

antigen presentation. These results lend novel insight into the nature of the structural development of epitope-specific TCR repertoires in the context of chronic HIV infection and highlight the importance of maintaining immune analyses on the clonotype level in future HIV research endeavors. In addition, we contributed to a focused understanding of the role of proliferative capacity in the suppression of HIV replication. Importantly, this work has implications in further understanding effective assessment on a structural and functional level of elicited HIV vaccine responses and extended investigations into correlates of control of HIV-1 viremia.

LIST OF PUBLICATIONS

I. Whitmore MM, DeVeer MJ, Edling A, Oates RK, **Brenna C. Simons**, Lindner D, Williams BR. *Synergistic activation of innate immunity by double-stranded RNA and CpG DNA promotes enhanced tumor activity.* 2004. Cancer Res. 64 (16).

II. Ni J, Wen X, Yao J, Chang HC, Yin Y, Zhang M, Xie S, Chen M, **Brenna C. Simons**, Chang P, di Sant'Agnese A, Messing EM, Yeh S. *Tocopherol-Associated protein suppresses prostate cancer cell growth by inhibition of the phosphoinositide 3-Kinase pathway.* 2005. Cancer Res. 65 (21).

III. Meyer-Olson D, Brady K, Bartman MT, O'Sullivan KM, **Brenna C. Simons**, Conrad JA, Duncan CB, Lorey SL, Siddique A, Draenert R, Addo M, Altfeld M, Rosenburg E, Allen T, Walker BD, Kalams SA. *Fluctuations of functionally distinct CD8+ T cell clonotypes demonstrate flexibility of the HIV-specific TCR repertoire.* 2006. Blood 107 (6): 2373-2383.

IV. **Brenna C. Simons**, Kalams SA. *The Potential role of epitope-specific T cell repertoire diversity in the control of HIV replication.* 2007. Curr Opin HIV and AIDS. 2: 177-182.

V. **Brenna C. Simons**, VanCompernolle SE, Smith RM, Wei J, Barnett L, Lorey SL, Meyer-Olson D, Kalams SA. *Biased TRBV gene usage and a wide range of T cell receptor CDR3 diversity against a dominant HLA B57-restricted epitope demonstrates a potential role for TCR diversity in the recognition of epitope variants.* 2008. J. Immunol. 181: 5137-5146.

VI. **Brenna C. Simons**, Barnett L, Lorey SL, Wei J, Meyer-Olson D, Kalams SA. Strong suppression of HIV-1 replication mediated by non-proliferating $CD8^{+}CD57^{+}$ T cells**. Manuscript submitted.**

VII. Meyer-Olson D, **Brenna C. Simons**, Conrad JA, Smith RM, Barnett L, Lorey SL, Ramalingam R, Sadagopal SL, Schmidt RE, Kalams SA. *Lack of CD57 expression on terminally differentiated HIV-specific clonotypes provides mechanism for in vivo persistence.* In preparation.

REFERENCES

1. Douek, D. C., L. J. Picker, and R. A. Koup. 2003. T cell dynamics in HIV-1 infection. *Annu Rev Immunol* 21:265-304.
2. Simon, V., and D. D. Ho. 2003. HIV-1 dynamics in vivo: implications for therapy. *Nat Rev Microbiol* 1:181-190.
3. Sundquist, W. I., and C. P. Hill. 2007. How to assemble a capsid. *Cell* 131:17-19.
4. von Schwedler, U. K., K. M. Stray, J. E. Garrus, and W. I. Sundquist. 2003. Functional surfaces of the human immunodeficiency virus type 1 capsid protein. *J Virol* 77:5439-5450.
5. Forshey, B. M., U. von Schwedler, W. I. Sundquist, and C. Aiken. 2002. Formation of a human immunodeficiency virus type 1 core of optimal stability is crucial for viral replication. *J Virol* 76:5667-5677.
6. von Schwedler, U. K., T. L. Stemmler, V. Y. Klishko, S. Li, K. H. Albertine, D. R. Davis, and W. I. Sundquist. 1998. Proteolytic refolding of the HIV-1 capsid protein amino-terminus facilitates viral core assembly. *EMBO J* 17:1555-1568.
7. Martinez-Picado, J., J. G. Prado, E. E. Fry, K. Pfafferott, A. Leslie, S. Chetty, C. Thobakgale, I. Honeyborne, H. Crawford, P. Matthews, T. Pillay, C. Rousseau, J. I. Mullins, C. Brander, B. D. Walker, D. I. Stuart, P. Kiepiela, and P. Goulder. 2006. Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. *J Virol* 80:3617-3623.
8. Crawford, H., J. G. Prado, A. Leslie, S. Hue, I. Honeyborne, S. Reddy, M. van der Stok, Z. Mncube, C. Brander, C. Rousseau, J. I. Mullins, R. Kaslow, P. Goepfert, S. Allen, E. Hunter, J. Mulenga, P. Kiepiela, B. D. Walker, and P. J. Goulder. 2007. Compensatory mutation partially restores fitness and delays reversion of escape mutation within the immunodominant HLA-B*5703-restricted Gag epitope in chronic human immunodeficiency virus type 1 infection. *J Virol* 81:8346-8351.
9. Kelleher, A. D., C. Long, E. C. Holmes, R. L. Allen, J. Wilson, C. Conlon, C. Workman, S. Shaunak, K. Olson, P. Goulder, C. Brander, G. Ogg, J. S. Sullivan, W. Dyer, I. Jones, A. J. McMichael, S. Rowland-Jones, and R. E. Phillips. 2001. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. *J Exp Med* 193:375-386.
10. Migueles, S. A., A. C. Laborico, H. Imamichi, W. L. Shupert, C. Royce, M. McLaughlin, L. Ehler, J. Metcalf, S. Liu, C. W. Hallahan, and M. Connors. 2003. The differential ability of HLA B*5701+ long-term nonprogressors and progressors to restrict human immunodeficiency virus replication is not caused by loss of recognition of autologous viral gag sequences. *J Virol* 77:6889-6898.

11. Pantaleo, G., and A. S. Fauci. 1996. Immunopathogenesis of HIV infection. *Annu Rev Microbiol* 50:825-854.
12. Douek, D. C., J. M. Brenchley, M. R. Betts, D. R. Ambrozak, B. J. Hill, Y. Okamoto, J. P. Casazza, J. Kuruppu, K. Kunstman, S. Wolinsky, Z. Grossman, M. Dybul, A. Oxenius, D. A. Price, M. Connors, and R. A. Koup. 2002. HIV preferentially infects HIV-specific CD4+ T cells. *Nature* 417:95-98.
13. Popper, S. J., A. D. Sarr, K. U. Travers, A. Gueye-Ndiaye, S. Mboup, M. E. Essex, and P. J. Kanki. 1999. Lower human immunodeficiency virus (HIV) type 2 viral load reflects the difference in pathogenicity of HIV-1 and HIV-2. *J Infect Dis* 180:1116-1121.
14. Shin, H., and E. J. Wherry. 2007. CD8 T cell dysfunction during chronic viral infection. *Curr Opin Immunol* 19:408-415.
15. Wherry, E. J., and R. Ahmed. 2004. Memory CD8 T-cell differentiation during viral infection. *J Virol* 78:5535-5545.
16. Wooldridge, L., H. A. van den Berg, M. Glick, E. Gostick, B. Laugel, S. L. Hutchinson, A. Milicic, J. M. Brenchley, D. C. Douek, D. A. Price, and A. K. Sewell. 2005. Interaction between the CD8 coreceptor and major histocompatibility complex class I stabilizes T cell receptor-antigen complexes at the cell surface. *J Biol Chem* 280:27491-27501.
17. Tuma, R. A., and E. G. Pamer. 2002. Homeostasis of naive, effector and memory CD8 T cells. *Curr Opin Immunol* 14:348-353.
18. Trapani, J. A., and M. J. Smyth. 2002. Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol* 2:735-747.
19. Kershaw, M. H., J. A. Trapani, and M. J. Smyth. 1995. Cytotoxic lymphocytes: redirecting the cell-mediated immune response for the therapy of cancer. *Ther Immunol* 2:173-181.
20. Smyth, M. J., and J. A. Trapani. 1995. Granzymes: exogenous proteinases that induce target cell apoptosis. *Immunol Today* 16:202-206.
21. Badley, A. D., D. H. Dockrell, A. Algeciras, S. Ziesmer, A. Landay, M. M. Lederman, E. Connick, H. Kessler, D. Kuritzkes, D. H. Lynch, P. Roche, H. Yagita, and C. V. Paya. 1998. In vivo analysis of Fas/FasL interactions in HIV-infected patients. *J Clin Invest* 102:79-87.
22. Dockrell, D. H. 2003. The multiple roles of Fas ligand in the pathogenesis of infectious diseases. *Clin Microbiol Infect* 9:766-779.
23. Dockrell, D. H., A. D. Badley, A. Algeciras-Schimnich, M. Simpson, R. Schut, D. H. Lynch, and C. V. Paya. 1999. Activation-induced CD4+ T cell death in HIV-positive individuals correlates with Fas susceptibility, CD4+ T cell count, and HIV plasma viral copy number. *AIDS Res Hum Retroviruses* 15:1509-1518.
24. Balaji, K. N., N. Schaschke, W. Machleidt, M. Catalfamo, and P. A. Henkart. 2002. Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. *J Exp Med* 196:493-503.
25. Samuel, C. E. 2001. Antiviral actions of interferons. *Clin Microbiol Rev* 14:778-809, table of contents.
26. Baker, B. M., B. L. Block, A. C. Rothchild, and B. D. Walker. 2009. Elite control of HIV infection: implications for vaccine design. *Expert Opin Biol Ther* 9:55-69.

27. Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 68:4650-4655.
28. Harrer, T., E. Harrer, S. A. Kalams, T. Elbeik, S. I. Staprans, M. B. Feinberg, Y. Cao, D. D. Ho, T. Yilma, A. M. Caliendo, R. P. Johnson, S. P. Buchbinder, and B. D. Walker. 1996. Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. *AIDS Res Hum Retroviruses* 12:585-592.
29. Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone. 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* 68:6103-6110.
30. Kalams, S. A., S. P. Buchbinder, E. S. Rosenberg, J. M. Billingsley, D. S. Colbert, N. G. Jones, A. K. Shea, A. K. Trocha, and B. D. Walker. 1999. Association between virus-specific cytotoxic T-lymphocyte and helper responses in human immunodeficiency virus type 1 infection. *J Virol* 73:6715-6720.
31. Harrer, T., E. Harrer, S. A. Kalams, P. Barbosa, A. Trocha, R. P. Johnson, T. Elbeik, M. B. Feinberg, S. P. Buchbinder, and B. D. Walker. 1996. Cytotoxic T lymphocytes in asymptomatic long-term nonprogressing HIV-1 infection. Breadth and specificity of the response and relation to in vivo viral quasispecies in a person with prolonged infection and low viral load. *J Immunol* 156:2616-2623.
32. Altfeld, M., T. M. Allen, X. G. Yu, M. N. Johnston, D. Agrawal, B. T. Korber, D. C. Montefiori, D. H. O'Connor, B. T. Davis, P. K. Lee, E. L. Maier, J. Harlow, P. J. Goulder, C. Brander, E. S. Rosenberg, and B. D. Walker. 2002. HIV-1 superinfection despite broad CD8+ T-cell responses containing replication of the primary virus. *Nature* 420:434-439.
33. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 283:857-860.
34. Leslie, A. J., K. J. Pfafferott, P. Chetty, R. Draenert, M. M. Addo, M. Feeney, Y. Tang, E. C. Holmes, T. Allen, J. G. Prado, M. Altfeld, C. Brander, C. Dixon, D. Ramduth, P. Jeena, S. A. Thomas, A. St John, T. A. Roach, B. Kupfer, G. Luzzi, A. Edwards, G. Taylor, H. Lyall, G. Tudor-Williams, V. Novelli, J. Martinez-Picado, P. Kiepiela, B. D. Walker, and P. J. Goulder. 2004. HIV evolution: CTL escape mutation and reversion after transmission. *Nat Med* 10:282-289.
35. Goulder, P. J., and B. D. Walker. 1999. The great escape - AIDS viruses and immune control. *Nat Med* 5:1233-1235.
36. Goulder, P. J., C. Brander, Y. Tang, C. Tremblay, R. A. Colbert, M. M. Addo, E. S. Rosenberg, T. Nguyen, R. Allen, A. Trocha, M. Altfeld, S. He, M. Bunce, R. Funkhouser, S. I. Pelton, S. K. Burchett, K. McIntosh, B. T. Korber, and B. D. Walker. 2001. Evolution and transmission of stable CTL escape mutations in HIV infection. *Nature* 412:334-338.

37. Goulder, P. J., and D. I. Watkins. 2004. HIV and SIV CTL escape: implications for vaccine design. *Nat Rev Immunol* 4:630-640.
38. Nikolich-Zugich, J., M. K. Slifka, and I. Messaoudi. 2004. The many important facets of T-cell repertoire diversity. *Nat Rev Immunol* 4:123-132.
39. Turner, S. J., P. C. Doherty, J. McCluskey, and J. Rossjohn. 2006. Structural determinants of T-cell receptor bias in immunity. *Nat Rev Immunol* 6:883-894.
40. Van Kaer, L. 2008. Pillars article: antigen presentation: discovery of the peptide TAP. *J Immunol* 180:2723-2724.
41. Bihl, F., N. Frahm, L. Di Giammarino, J. Sidney, M. John, K. Yusim, T. Woodberry, K. Sango, H. S. Hewitt, L. Henry, C. H. Linde, J. V. Chisholm, 3rd, T. M. Zaman, E. Pae, S. Mallal, B. D. Walker, A. Sette, B. T. Korber, D. Heckerman, and C. Brander. 2006. Impact of HLA-B alleles, epitope binding affinity, functional avidity, and viral coinfection on the immunodominance of virus-specific CTL responses. *J Immunol* 176:4094-4101.
42. Kiepiela, P., A. J. Leslie, I. Honeyborne, D. Ramduth, C. Thobakgale, S. Chetty, P. Rathnavalu, C. Moore, K. J. Pfafferott, L. Hilton, P. Zimbwa, S. Moore, T. Allen, C. Brander, M. M. Addo, M. Altfeld, I. James, S. Mallal, M. Bunce, L. D. Barber, J. Szinger, C. Day, P. Klenerman, J. Mullins, B. Korber, H. M. Coovadia, B. D. Walker, and P. J. Goulder. 2004. Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. *Nature* 432:769-775.
43. Simons, B. C., S. E. Vancompernolle, R. M. Smith, J. Wei, L. Barnett, S. L. Lorey, D. Meyer-Olson, and S. A. Kalams. 2008. Despite biased TRBV gene usage against a dominant HLA B57-restricted epitope, TCR diversity can provide recognition of circulating epitope variants. *J Immunol* 181:5137-5146.
44. Flores-Villanueva, P. O., E. J. Yunis, J. C. Delgado, E. Vittinghoff, S. Buchbinder, J. Y. Leung, A. M. Uglialoro, O. P. Clavijo, E. S. Rosenberg, S. A. Kalams, J. D. Braun, S. L. Boswell, B. D. Walker, and A. E. Goldfeld. 2001. Control of HIV-1 viremia and protection from AIDS are associated with HLA-Bw4 homozygosity. *Proc Natl Acad Sci U S A* 98:5140-5145.
45. Leslie, A., D. Kavanagh, I. Honeyborne, K. Pfafferott, C. Edwards, T. Pillay, L. Hilton, C. Thobakgale, D. Ramduth, R. Draenert, S. Le Gall, G. Luzzi, A. Edwards, C. Brander, A. K. Sewell, S. Moore, J. Mullins, C. Moore, S. Mallal, N. Bhardwaj, K. Yusim, R. Phillips, P. Klenerman, B. Korber, P. Kiepiela, B. Walker, and P. Goulder. 2005. Transmission and accumulation of CTL escape variants drive negative associations between HIV polymorphisms and HLA. *J Exp Med* 201:891-902.
46. Goulder, P. J., M. Bunce, P. Krausa, K. McIntyre, S. Crowley, B. Morgan, A. Edwards, P. Giangrande, R. E. Phillips, and A. J. McMichael. 1996. Novel, cross-restricted, conserved, and immunodominant cytotoxic T lymphocyte epitopes in slow progressors in HIV type 1 infection. *AIDS Res Hum Retroviruses* 12:1691-1698.
47. Goulder, P. J., A. Edwards, R. E. Phillips, and A. J. McMichael. 1997. Identification of a novel HLA-B*2705-restricted cytotoxic T-lymphocyte epitope within a conserved region of HIV-1 Nef. *Aids* 11:536-538.

48. Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael, and S. Rowland-Jones. 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat Med* 3:212-217.
49. Leslie, A., D. A. Price, P. Mkhize, K. Bishop, A. Rathod, C. Day, H. Crawford, I. Honeyborne, T. E. Asher, G. Luzzi, A. Edwards, C. M. Rousseau, J. I. Mullins, G. Tudor-Williams, V. Novelli, C. Brander, D. C. Douek, P. Kiepiela, B. D. Walker, and P. J. Goulder. 2006. Differential selection pressure exerted on HIV by CTL targeting identical epitopes but restricted by distinct HLA alleles from the same HLA supertype. *J Immunol* 177:4699-4708.
50. Kaech, S. M., J. T. Tan, E. J. Wherry, B. T. Konieczny, C. D. Surh, and R. Ahmed. 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 4:1191-1198.
51. Zajac, A. J., J. N. Blattman, K. Murali-Krishna, D. J. Sourdive, M. Suresh, J. D. Altman, and R. Ahmed. 1998. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* 188:2205-2213.
52. Horwitz, M. S., Y. Yanagi, and M. B. Oldstone. 1994. T-cell receptors from virus-specific cytotoxic T lymphocytes recognizing a single immunodominant nine-amino-acid viral epitope show marked diversity. *J Virol* 68:352-357.
53. Yanagi, Y., A. Tishon, H. Lewicki, B. A. Cubitt, and M. B. Oldstone. 1992. Diversity of T-cell receptors in virus-specific cytotoxic T lymphocytes recognizing three distinct viral epitopes restricted by a single major histocompatibility complex molecule. *J Virol* 66:2527-2531.
54. Cornberg, M., A. T. Chen, L. A. Wilkinson, M. A. Brehm, S. K. Kim, C. Calcagno, D. Ghersi, R. Puzone, F. Celada, R. M. Welsh, and L. K. Selin. 2006. Narrowed TCR repertoire and viral escape as a consequence of heterologous immunity. *J Clin Invest* 116:1443-1456.
55. Meyer-Olson, D., N. H. Shoukry, K. W. Brady, H. Kim, D. P. Olson, K. Hartman, A. K. Shintani, C. M. Walker, and S. A. Kalams. 2004. Limited T cell receptor diversity of HCV-specific T cell responses is associated with CTL escape. *J Exp Med* 200:307-319.
56. Friedrich, T. C., A. B. McDermott, M. R. Reynolds, S. Piaskowski, S. Fuenger, I. P. De Souza, R. Rudersdorf, C. Cullen, L. J. Yant, L. Vojnov, J. Stephany, S. Martin, D. H. O'Connor, N. Wilson, and D. I. Watkins. 2004. Consequences of cytotoxic T-lymphocyte escape: common escape mutations in simian immunodeficiency virus are poorly recognized in naive hosts. *J Virol* 78:10064-10073.
57. O'Connor, D., T. Friedrich, A. Hughes, T. M. Allen, and D. Watkins. 2001. Understanding cytotoxic T-lymphocyte escape during simian immunodeficiency virus infection. *Immunol Rev* 183:115-126.
58. Barouch, D. H., J. Powers, D. M. Truitt, M. G. Kishko, J. C. Arthur, F. W. Peyerl, M. J. Kuroda, D. A. Gorgone, M. A. Lifton, C. I. Lord, V. M. Hirsch, D. C. Montefiori, A. Carville, K. G. Mansfield, K. J. Kunstman, S. M. Wolinsky, and N. L. Letvin. 2005. Dynamic immune responses maintain cytotoxic T lymphocyte

epitope mutations in transmitted simian immunodeficiency virus variants. *Nat Immunol* 6:247-252.

59. Letvin, N. L., Y. Yasutomi, L. Shen, K. A. Reimann, Z. W. Chen, J. E. Schmitz, and M. J. Kuroda. 1998. The CD8+ T lymphocyte response during primary SIVmac infection. *Adv Exp Med Biol* 452:177-179.
60. Smith, M. Z., C. J. Dale, R. De Rose, I. Stratov, C. S. Fernandez, A. G. Brooks, J. Weinfurter, K. Krebs, C. Riek, D. I. Watkins, H. O'Connor D, and S. J. Kent. 2005. Analysis of pigtail macaque major histocompatibility complex class I molecules presenting immunodominant simian immunodeficiency virus epitopes. *J Virol* 79:684-695.
61. O'Connor, D. H., B. R. Mothe, J. T. Weinfurter, S. Fuenger, W. M. Rehrauer, P. Jing, R. R. Rudersdorf, M. E. Liebl, K. Krebs, J. Vasquez, E. Dodds, J. Loffredo, S. Martin, A. B. McDermott, T. M. Allen, C. Wang, G. G. Doxiadis, D. C. Montefiori, A. Hughes, D. R. Burton, D. B. Allison, S. M. Wolinsky, R. Bontrop, L. J. Picker, and D. I. Watkins. 2003. Major histocompatibility complex class I alleles associated with slow simian immunodeficiency virus disease progression bind epitopes recognized by dominant acute-phase cytotoxic-T-lymphocyte responses. *J Virol* 77:9029-9040.
62. Vogel, T. U., M. R. Reynolds, D. H. Fuller, K. Vielhuber, T. Shipley, J. T. Fuller, K. J. Kunstman, G. Sutter, M. L. Marthas, V. Erfle, S. M. Wolinsky, C. Wang, D. B. Allison, E. W. Rud, N. Wilson, D. Montefiori, J. D. Altman, and D. I. Watkins. 2003. Multispecific vaccine-induced mucosal cytotoxic T lymphocytes reduce acute-phase viral replication but fail in long-term control of simian immunodeficiency virus SIVmac239. *J Virol* 77:13348-13360.
63. Evans, D. T., D. H. O'Connor, P. Jing, J. L. Dzuris, J. Sidney, J. da Silva, T. M. Allen, H. Horton, J. E. Venham, R. A. Rudersdorf, T. Vogel, C. D. Pauza, R. E. Bontrop, R. DeMars, A. Sette, A. L. Hughes, and D. I. Watkins. 1999. Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef. *Nat Med* 5:1270-1276.
64. Allen, T. M., D. H. O'Connor, P. Jing, J. L. Dzuris, B. R. Mothe, T. U. Vogel, E. Dunphy, M. E. Liebl, C. Emerson, N. Wilson, K. J. Kunstman, X. Wang, D. B. Allison, A. L. Hughes, R. C. Desrosiers, J. D. Altman, S. M. Wolinsky, A. Sette, and D. I. Watkins. 2000. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. *Nature* 407:386-390.
65. Vogel, T. U., T. C. Friedrich, D. H. O'Connor, W. Rehrauer, E. J. Dodds, H. Hickman, W. Hildebrand, J. Sidney, A. Sette, A. Hughes, H. Horton, K. Vielhuber, R. Rudersdorf, I. P. De Souza, M. R. Reynolds, T. M. Allen, N. Wilson, and D. I. Watkins. 2002. Escape in one of two cytotoxic T-lymphocyte epitopes bound by a high-frequency major histocompatibility complex class I molecule, Mamu-A*02: a paradigm for virus evolution and persistence? *J Virol* 76:11623-11636.
66. Friedrich, T. C., E. J. Dodds, L. J. Yant, L. Vojnov, R. Rudersdorf, C. Cullen, D. T. Evans, R. C. Desrosiers, B. R. Mothe, J. Sidney, A. Sette, K. Kunstman, S. Wolinsky, M. Piatak, J. Lifson, A. L. Hughes, N. Wilson, D. H. O'Connor, and D. I. Watkins. 2004. Reversion of CTL escape-variant immunodeficiency viruses in vivo. *Nat Med* 10:275-281.

67. Charini, W. A., M. J. Kuroda, J. E. Schmitz, K. R. Beaudry, W. Lin, M. A. Lifton, G. R. Krivulka, A. Necker, and N. L. Letvin. 2001. Clonally diverse CTL response to a dominant viral epitope recognizes potential epitope variants. *J Immunol* 167:4996-5003.
68. Chen, Z. W., Y. Li, X. Zeng, M. J. Kuroda, J. E. Schmitz, Y. Shen, X. Lai, L. Shen, and N. L. Letvin. 2001. The TCR repertoire of an immunodominant CD8+ T lymphocyte population. *J Immunol* 166:4525-4533.
69. Loffredo, J. T., B. J. Burwitz, E. G. Rakasz, S. P. Spencer, J. J. Stephany, J. P. Giraldo Vela, S. R. Martin, J. Reed, S. M. Piaskowski, J. Furlott, K. L. Weisgrau, D. S. Rodrigues, T. Soma, G. Napoe, T. C. Friedrich, N. A. Wilson, E. G. Kallas, and D. I. Watkins. 2006. SIV-specific CD8+ T cell antiviral efficacy is unrelated to epitope specificity and abrogated by viral escape. *J Virol*.
70. Allen, T. M., L. Mortara, B. R. Mothe, M. Liebl, P. Jing, B. Calore, M. Piekarczyk, R. Ruddersdorf, D. H. O'Connor, X. Wang, C. Wang, D. B. Allison, J. D. Altman, A. Sette, R. C. Desrosiers, G. Sutter, and D. I. Watkins. 2002. Tat-vaccinated macaques do not control simian immunodeficiency virus SIVmac239 replication. *J Virol* 76:4108-4112.
71. Price, D. A., S. M. West, M. R. Betts, L. E. Ruff, J. M. Brenchley, D. R. Ambrozak, Y. Edghill-Smith, M. J. Kuroda, D. Bogdan, K. Kunstman, N. L. Letvin, G. Franchini, S. M. Wolinsky, R. A. Koup, and D. C. Douek. 2004. T cell receptor recognition motifs govern immune escape patterns in acute SIV infection. *Immunity* 21:793-803.
72. Pantaleo, G., J. F. Demarest, T. Schacker, M. Vaccarezza, O. J. Cohen, M. Daucher, C. Graziosi, S. S. Schnittman, T. C. Quinn, G. M. Shaw, L. Perrin, G. Tambussi, A. Lazzarin, R. P. Sekaly, H. Soudeyns, L. Corey, and A. S. Fauci. 1997. The qualitative nature of the primary immune response to HIV infection is a prognosticator of disease progression independent of the initial level of plasma viremia. *Proc Natl Acad Sci U S A* 94:254-258.
73. Pantaleo, G., J. F. Demarest, H. Soudeyns, C. Graziosi, F. Denis, J. W. Adelsberger, P. Borrow, M. S. Saag, G. M. Shaw, R. P. Sekaly, and et al. 1994. Major expansion of CD8+ T cells with a predominant V beta usage during the primary immune response to HIV. *Nature* 370:463-467.
74. Pantaleo, G., and R. A. Koup. 2004. Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. *Nat Med* 10:806-810.
75. Gorochov, G., A. U. Neumann, A. Kereveur, C. Parizot, T. Li, C. Katlama, M. Karmochkine, G. Raguin, B. Autran, and P. Debre. 1998. Perturbation of CD4+ and CD8+ T-cell repertoires during progression to AIDS and regulation of the CD4+ repertoire during antiviral therapy. *Nat Med* 4:215-221.
76. Meyer-Olson, D., K. W. Brady, M. T. Bartman, K. M. O'Sullivan, B. C. Simons, J. A. Conrad, C. B. Duncan, S. Lorey, A. Siddique, R. Draenert, M. Addo, M. Altfeld, E. Rosenberg, T. M. Allen, B. D. Walker, and S. A. Kalams. 2006. Fluctuations of functionally distinct CD8+ T-cell clonotypes demonstrate flexibility of the HIV-specific TCR repertoire. *Blood* 107:2373-2383.
77. Douek, D. C., M. R. Betts, J. M. Brenchley, B. J. Hill, D. R. Ambrozak, K. L. Ngai, N. J. Karandikar, J. P. Casazza, and R. A. Koup. 2002. A novel approach to the

analysis of specificity, clonality, and frequency of HIV-specific T cell responses reveals a potential mechanism for control of viral escape. *J Immunol* 168:3099-3104.

78. Migueles, S. A., M. S. Sabbaghian, W. L. Shupert, M. P. Bettinotti, F. M. Marincola, L. Martino, C. W. Hallahan, S. M. Selig, D. Schwartz, J. Sullivan, and M. Connors. 2000. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci U S A* 97:2709-2714.
79. Gillespie, G. M., G. Stewart-Jones, J. Rengasamy, T. Beattie, J. J. Bwayo, F. A. Plummer, R. Kaul, A. J. McMichael, P. Easterbrook, T. Dong, E. Y. Jones, and S. L. Rowland-Jones. 2006. Strong TCR conservation and altered T cell cross-reactivity characterize a B*57-restricted immune response in HIV-1 infection. *J Immunol* 177:3893-3902.
80. Yu, X. G., M. Lichterfeld, S. Chetty, K. L. Williams, S. K. Mui, T. Miura, N. Frahm, M. E. Feeney, Y. Tang, F. Pereyra, M. X. Labute, K. Pfafferott, A. Leslie, H. Crawford, R. Allgaier, W. Hildebrand, R. Kaslow, C. Brander, T. M. Allen, E. S. Rosenberg, P. Kiepiela, M. Vajpayee, P. A. Goepfert, M. Altfeld, P. J. Goulder, and B. D. Walker. 2007. Mutually exclusive T-cell receptor induction and differential susceptibility to human immunodeficiency virus type 1 mutational escape associated with a two-amino-acid difference between HLA class I subtypes. *J Virol* 81:1619-1631.
81. Gea-Banacloche, J. C., S. A. Migueles, L. Martino, W. L. Shupert, A. C. McNeil, M. S. Sabbaghian, L. Ehler, C. Prussin, R. Stevens, L. Lambert, J. Altman, C. W. Hallahan, J. C. de Quiros, and M. Connors. 2000. Maintenance of large numbers of virus-specific CD8+ T cells in HIV-infected progressors and long-term nonprogressors. *J Immunol* 165:1082-1092.
82. Koup, R. A. 1994. Virus escape from CTL recognition. *J Exp Med* 180:779-782.
83. Betts, M. R., D. R. Ambrozak, D. C. Douek, S. Bonhoeffer, J. M. Brenchley, J. P. Casazza, R. A. Koup, and L. J. Picker. 2001. Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. *J Virol* 75:11983-11991.
84. Betts, M. R., J. P. Casazza, and R. A. Koup. 2001. Monitoring HIV-specific CD8+ T cell responses by intracellular cytokine production. *Immunol Lett* 79:117-125.
85. Douek, D. C., M. R. Betts, B. J. Hill, S. J. Little, R. Lempicki, J. A. Metcalf, J. Casazza, C. Yoder, J. W. Adelsberger, R. A. Stevens, M. W. Baseler, P. Keiser, D. D. Richman, R. T. Davey, and R. A. Koup. 2001. Evidence for increased T cell turnover and decreased thymic output in HIV infection. *J Immunol* 167:6663-6668.
86. Betts, M. R., D. A. Price, J. M. Brenchley, K. Lore, F. J. Guenaga, A. Smed-Sorensen, D. R. Ambrozak, S. A. Migueles, M. Connors, M. Roederer, D. C. Douek, and R. A. Koup. 2004. The functional profile of primary human antiviral CD8+ T cell effector activity is dictated by cognate peptide concentration. *J Immunol* 172:6407-6417.

87. Appay, V., L. Papagno, C. A. Spina, P. Hansasuta, A. King, L. Jones, G. S. Ogg, S. Little, A. J. McMichael, D. D. Richman, and S. L. Rowland-Jones. 2002. Dynamics of T cell responses in HIV infection. *J Immunol* 168:3660-3666.
88. Price, D. A., J. M. Brenchley, L. E. Ruff, M. R. Betts, B. J. Hill, M. Roederer, R. A. Koup, S. A. Migueles, E. Gostick, L. Wooldridge, A. K. Sewell, M. Connors, and D. C. Douek. 2005. Avidity for antigen shapes clonal dominance in CD8+ T cell populations specific for persistent DNA viruses. *J Exp Med* 202:1349-1361.
89. Migueles, S. A., A. C. Laborico, W. L. Shupert, M. S. Sabbaghian, R. Rabin, C. W. Hallahan, D. Van Baarle, S. Kostense, F. Miedema, M. McLaughlin, L. Ehler, J. Metcalf, S. Liu, and M. Connors. 2002. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol* 3:1061-1068.
90. Migueles, S. A., C. M. Osborne, C. Royce, A. A. Compton, R. P. Joshi, K. A. Weeks, J. E. Rood, A. M. Berkley, J. B. Sacha, N. A. Cogliano-Shutta, M. Lloyd, G. Roby, R. Kwan, M. McLaughlin, S. Stallings, C. Rehm, M. A. O'Shea, J. Mican, B. Z. Packard, A. Komoriya, S. Palmer, A. P. Wiegand, F. Maldarelli, J. M. Coffin, J. W. Mellors, C. W. Hallahan, D. A. Follman, and M. Connors. 2008. Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. *Immunity* 29:1009-1021.
91. Antia, R., C. T. Bergstrom, S. S. Pilyugin, S. M. Kaech, and R. Ahmed. 2003. Models of CD8+ responses: 1. What is the antigen-independent proliferation program. *J Theor Biol* 221:585-598.
92. Wherry, E. J., J. N. Blattman, and R. Ahmed. 2005. Low CD8 T-cell proliferative potential and high viral load limit the effectiveness of therapeutic vaccination. *J Virol* 79:8960-8968.
93. Kaech, S. M., and R. Ahmed. 2001. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat Immunol* 2:415-422.
94. Kaech, S. M., S. Hemby, E. Kersh, and R. Ahmed. 2002. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell* 111:837-851.
95. Champagne, P., G. S. Ogg, A. S. King, C. Knabenhans, K. Ellefsen, M. Nobile, V. Appay, G. P. Rizzardi, S. Fleury, M. Lipp, R. Forster, S. Rowland-Jones, R. P. Sekaly, A. J. McMichael, and G. Pantaleo. 2001. Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature* 410:106-111.
96. Refaeli, Y., and A. K. Abbas. 1998. Role of cytokines in autoimmunity. *Eur Cytokine Netw* 9:81-82.
97. Dybul, M., B. Hidalgo, T. W. Chun, M. Belson, S. A. Migueles, J. S. Justement, B. Herpin, C. Perry, C. W. Hallahan, R. T. Davey, J. A. Metcalf, M. Connors, and A. S. Fauci. 2002. Pilot study of the effects of intermittent interleukin-2 on human immunodeficiency virus (HIV)-specific immune responses in patients treated during recently acquired HIV infection. *J Infect Dis* 185:61-68.
98. Saez-Cirion, A., C. Lacabaratz, O. Lambotte, P. Versmisse, A. Urrutia, F. Boufassa, F. Barre-Sinoussi, J. F. Delfraissy, M. Sinet, G. Pancino, and A. Venet. 2007. HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. *Proc Natl Acad Sci U S A* 104:6776-6781.

99. Brenchley, J. M., N. J. Karandikar, M. R. Betts, D. R. Ambrozak, B. J. Hill, L. E. Crotty, J. P. Casazza, J. Kuruppu, S. A. Migueles, M. Connors, M. Roederer, D. C. Douek, and R. A. Koup. 2003. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. *Blood* 101:2711-2720.
100. Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. J. Wherry, H. M. Coovadia, P. J. Goulder, P. Klenerman, R. Ahmed, G. J. Freeman, and B. D. Walker. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443:350-354.
101. Wherry, E. J., J. N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed. 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77:4911-4927.
102. Sharpe, A. H., E. J. Wherry, R. Ahmed, and G. J. Freeman. 2007. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol* 8:239-245.
103. Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman, and R. Ahmed. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439:682-687.
104. Radziewicz, H., C. C. Ibegbu, M. L. Fernandez, K. A. Workowski, K. Obideen, M. Wehbi, H. L. Hanson, J. P. Steinberg, D. Masopust, E. J. Wherry, J. D. Altman, B. T. Rouse, G. J. Freeman, R. Ahmed, and A. Grakoui. 2007. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol* 81:2545-2553.
105. Petrovas, C., J. P. Casazza, J. M. Brenchley, D. A. Price, E. Gostick, W. C. Adams, M. L. Precopio, T. Schacker, M. Roederer, D. C. Douek, and R. A. Koup. 2006. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J Exp Med* 203:2281-2292.
106. Trautmann, L., L. Janbazian, N. Chomont, E. A. Said, S. Gimmig, B. Bessette, M. R. Boulassel, E. Delwart, H. Sepulveda, R. S. Balderas, J. P. Routy, E. K. Haddad, and R. P. Sekaly. 2006. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med* 12:1198-1202.
107. Urbani, S., B. Amadei, D. Tola, M. Massari, S. Schivazappa, G. Missale, and C. Ferrari. 2006. PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J Virol* 80:11398-11403.
108. Le Priol, Y., D. Puthier, C. Lecureuil, C. Combadiere, P. Debre, C. Nguyen, and B. Combadiere. 2006. High cytotoxic and specific migratory potencies of senescent CD8+ CD57+ cells in HIV-infected and uninfected individuals. *J Immunol* 177:5145-5154.
109. Walker, B. D., and P. J. Goulder. 2000. AIDS. Escape from the immune system. *Nature* 407:313-314.
110. Migueles, S. A., J. C. Tilton, and M. Connors. 2004. Advances in understanding immunologic control of HIV infection. *Curr HIV/AIDS Rep* 1:12-17.

111. Bowen, D. G., and C. M. Walker. 2005. Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature* 436:946-952.
112. Betts, M. R., B. Exley, D. A. Price, A. Bansal, Z. T. Camacho, V. Teaberry, S. M. West, D. R. Ambrozak, G. Tomaras, M. Roederer, J. M. Kilby, J. Tartaglia, R. Belshe, F. Gao, D. C. Douek, K. J. Weinhold, R. A. Koup, P. Goepfert, and G. Ferrari. 2005. Characterization of functional and phenotypic changes in anti-Gag vaccine-induced T cell responses and their role in protection after HIV-1 infection. *Proc Natl Acad Sci U S A* 102:4512-4517.
113. Migueles, S. A., and M. Connors. 2001. Frequency and function of HIV-specific CD8(+) T cells. *Immunol Lett* 79:141-150.
114. Manuel, E. R., W. A. Charini, P. Sen, F. W. Peyerl, M. J. Kuroda, J. E. Schmitz, P. Autissier, D. A. Sheeter, B. E. Torbett, and N. L. Letvin. 2006. Contribution of T-cell receptor repertoire breadth to the dominance of epitope-specific CD8+ T-lymphocyte responses. *J Virol* 80:12032-12040.
115. Altfeld, M., M. M. Addo, E. S. Rosenberg, F. M. Hecht, P. K. Lee, M. Vogel, X. G. Yu, R. Draenert, M. N. Johnston, D. Strick, T. M. Allen, M. E. Feeney, J. O. Kahn, R. P. Sekaly, J. A. Levy, J. K. Rockstroh, P. J. Goulder, and B. D. Walker. 2003. Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. *Aids* 17:2581-2591.
116. Streeck, H., M. Lichterfeld, G. Alter, A. Meier, N. Teigen, B. Yassine-Diab, H. K. Sidhu, S. Little, A. Kelleher, J. P. Routy, E. S. Rosenberg, R. P. Sekaly, B. D. Walker, and M. Altfeld. 2007. Recognition of a defined region within p24 gag by CD8+ T cells during primary human immunodeficiency virus type 1 infection in individuals expressing protective HLA class I alleles. *J Virol* 81:7725-7731.
117. Appay, V., and S. L. Rowland-Jones. 2002. The assessment of antigen-specific CD8+ T cells through the combination of MHC class I tetramer and intracellular staining. *J Immunol Methods* 268:9-19.
118. Kalams, S. A., P. J. Goulder, A. K. Shea, N. G. Jones, A. K. Trocha, G. S. Ogg, and B. D. Walker. 1999. Levels of human immunodeficiency virus type 1-specific cytotoxic T-lymphocyte effector and memory responses decline after suppression of viremia with highly active antiretroviral therapy. *J Virol* 73:6721-6728.
119. Yu, X. G., M. Lichterfeld, S. Chetty, K. L. Williams, S. K. Mui, T. Miura, N. Frahm, M. E. Feeney, Y. Tang, F. Pereyra, M. X. Labute, K. Pfafferott, A. Leslie, H. Crawford, R. Allgaier, W. Hildebrand, R. Kaslow, C. Brander, T. M. Allen, E. S. Rosenberg, P. Kiepiela, M. Vajpayee, P. A. Goepfert, M. Altfeld, P. J. Goulder, and B. D. Walker. 2006. Mutually exclusive T cell receptor induction and differential susceptibility to HIV-1 mutational escape associated with a two amino acid difference between HLA class I subtypes. *J Virol*.
120. Dong, T., G. Stewart-Jones, N. Chen, P. Easterbrook, X. Xu, L. Papagno, V. Appay, M. Weekes, C. Conlon, C. Spina, S. Little, G. Screaton, A. van der Merwe, D. D. Richman, A. J. McMichael, E. Y. Jones, and S. L. Rowland-Jones. 2004. HIV-specific cytotoxic T cells from long-term survivors select a unique T cell receptor. *J Exp Med* 200:1547-1557.

121. Lichterfeld, M., K. L. Williams, S. K. Mui, S. S. Shah, B. R. Mothe, A. Sette, A. Kim, M. N. Johnston, N. Burgett, N. Frahm, D. Cohen, C. Brander, E. S. Rosenberg, B. D. Walker, M. Altfeld, and X. G. Yu. 2006. T cell receptor cross-recognition of an HIV-1 CD8+ T cell epitope presented by closely related alleles from the HLA-A3 superfamily. *Int Immunol* 18:1179-1188.
122. Koibuchi, T., T. M. Allen, M. Lichterfeld, S. K. Mui, K. M. O'Sullivan, A. Trocha, S. A. Kalams, R. P. Johnson, and B. D. Walker. 2005. Limited sequence evolution within persistently targeted CD8 epitopes in chronic human immunodeficiency virus type 1 infection. *J Virol* 79:8171-8181.
123. Rosenberg, E. S., M. Altfeld, S. H. Poon, M. N. Phillips, B. M. Wilkes, R. L. Eldridge, G. K. Robbins, R. T. D'Aquila, P. J. Goulder, and B. D. Walker. 2000. Immune control of HIV-1 after early treatment of acute infection. *Nature* 407:523-526.
124. Rosenberg, E. S., J. M. Billingsley, A. M. Caliendo, S. L. Boswell, P. E. Sax, S. A. Kalams, and B. D. Walker. 1997. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* 278:1447-1450.
125. Addo, M. M., M. Altfeld, E. S. Rosenberg, R. L. Eldridge, M. N. Philips, K. Habeeb, A. Khatri, C. Brander, G. K. Robbins, G. P. Mazzara, P. J. Goulder, and B. D. Walker. 2001. 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* 98:1781-1786.
126. Soudeyns, H., G. Campi, G. P. Rizzardi, C. Lenge, J. F. Demarest, G. Tambussi, A. Lazzarin, D. Kaufmann, G. Casorati, L. Corey, and G. Pantaleo. 2000. Initiation of antiretroviral therapy during primary HIV-1 infection induces rapid stabilization of the T-cell receptor beta chain repertoire and reduces the level of T-cell oligoclonality. *Blood* 95:1743-1751.
127. Altfeld, M., E. S. Rosenberg, R. Shankarappa, J. S. Mukherjee, F. M. Hecht, R. L. Eldridge, M. M. Addo, S. H. Poon, M. N. Phillips, G. K. Robbins, P. E. Sax, S. Boswell, J. O. Kahn, C. Brander, P. J. Goulder, J. A. Levy, J. I. Mullins, and B. D. Walker. 2001. Cellular immune responses and viral diversity in individuals treated during acute and early HIV-1 infection. *J Exp Med* 193:169-180.
128. Lichterfeld, M., X. G. Yu, S. K. Mui, K. L. Williams, A. Trocha, M. A. Brockman, R. L. Allgaier, M. T. Waring, T. Koibuchi, M. N. Johnston, D. Cohen, T. M. Allen, E. S. Rosenberg, B. D. Walker, and M. Altfeld. 2007. Selective depletion of high-avidity human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T cells after early HIV-1 infection. *J Virol* 81:4199-4214.
129. Derby, M., M. Alexander-Miller, R. Tse, and J. Berzofsky. 2001. High-avidity CTL exploit two complementary mechanisms to provide better protection against viral infection than low-avidity CTL. *J Immunol* 166:1690-1697.
130. Yang, O. O., P. T. Sarkis, A. Trocha, S. A. Kalams, R. P. Johnson, and B. D. Walker. 2003. Impacts of avidity and specificity on the antiviral efficiency of HIV-1-specific CTL. *J Immunol* 171:3718-3724.
131. Bennett, M. S., H. L. Ng, M. Dagarag, A. Ali, and O. O. Yang. 2007. Epitope-dependent avidity thresholds for cytotoxic T-lymphocyte clearance of virus-infected cells. *J Virol* 81:4973-4980.

132. Venturi, V., K. Kedzierska, D. A. Price, P. C. Doherty, D. C. Douek, S. J. Turner, and M. P. Davenport. 2006. Sharing of T cell receptors in antigen-specific responses is driven by convergent recombination. *Proc Natl Acad Sci U S A* 103:18691-18696.
133. Lawson, T. M., S. Man, E. C. Wang, S. Williams, N. Amos, G. M. Gillespie, P. A. Moss, and L. K. Borysiewicz. 2001. Functional differences between influenza A-specific cytotoxic T lymphocyte clones expressing dominant and subdominant TCR. *Int Immunol* 13:1383-1390.
134. Lawson, T. M., S. Man, S. Williams, A. C. Boon, M. Zambon, and L. K. Borysiewicz. 2001. Influenza A antigen exposure selects dominant Vbeta17+ TCR in human CD8+ cytotoxic T cell responses. *Int Immunol* 13:1373-1381.
135. Grossman, Z., and W. E. Paul. 1992. Adaptive cellular interactions in the immune system: the tunable activation threshold and the significance of subthreshold responses. *Proc Natl Acad Sci U S A* 89:10365-10369.
136. Ibegbu, C. C., Y. X. Xu, W. Harris, D. Maggio, J. D. Miller, and A. P. Kourtis. 2005. Expression of killer cell lectin-like receptor G1 on antigen-specific human CD8+ T lymphocytes during active, latent, and resolved infection and its relation with CD57. *J Immunol* 174:6088-6094.
137. Kalams, S. A., R. P. Johnson, M. J. Dynan, K. E. Hartman, T. Harrer, E. Harrer, A. K. Trocha, W. A. Blattner, S. P. Buchbinder, and B. D. Walker. 1996. T cell receptor usage and fine specificity of human immunodeficiency virus 1-specific cytotoxic T lymphocyte clones: analysis of quasispecies recognition reveals a dominant response directed against a minor in vivo variant. *J Exp Med* 183:1669-1679.
138. Day, C. L., P. Kiepiela, A. J. Leslie, M. van der Stok, K. Nair, N. Ismail, I. Honeyborne, H. Crawford, H. M. Coovadia, P. J. Goulder, B. D. Walker, and P. Klenerman. 2007. Proliferative capacity of epitope-specific CD8 T-cell responses is inversely related to viral load in chronic human immunodeficiency virus type 1 infection. *J Virol* 81:434-438.
139. Miller, J. D., D. Masopust, E. J. Wherry, S. Kaech, G. Silvestri, and R. Ahmed. 2005. Differentiation of CD8 T cells in response to acute and chronic viral infections: implications for HIV vaccine development. *Curr Drug Targets Infect Disord* 5:121-129.
140. Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 4:225-234.
141. Ueno, T., H. Tomiyama, M. Fujiwara, S. Oka, and M. Takiguchi. 2004. Functionally impaired HIV-specific CD8 T cells show high affinity TCR-ligand interactions. *J Immunol* 173:5451-5457.
142. van Baarle, D., A. Tsegaye, F. Miedema, and A. Akbar. 2005. Significance of senescence for virus-specific memory T cell responses: rapid ageing during chronic stimulation of the immune system. *Immunol Lett* 97:19-29.
143. Horton, H., I. Frank, R. Baydo, E. Jalbert, J. Penn, S. Wilson, J. P. McNevin, M. D. McSweyn, D. Lee, Y. Huang, S. C. De Rosa, and M. J. McElrath. 2006. Preservation of T cell proliferation restricted by protective HLA alleles is critical for immune control of HIV-1 infection. *J Immunol* 177:7406-7415.

144. Sacha, J. B., C. Chung, E. G. Rakasz, S. P. Spencer, A. K. Jonas, A. T. Bean, W. Lee, B. J. Burwitz, J. J. Stephany, J. T. Loffredo, D. B. Allison, S. Adnan, A. Hoji, N. A. Wilson, T. C. Friedrich, J. D. Lifson, O. O. Yang, and D. I. Watkins. 2007. Gag-specific CD8+ T lymphocytes recognize infected cells before AIDS-virus integration and viral protein expression. *J Immunol* 178:2746-2754.
145. Yang, O. O., S. A. Kalams, A. Trocha, H. Cao, A. Luster, R. P. Johnson, and B. D. Walker. 1997. Suppression of human immunodeficiency virus type 1 replication by CD8+ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. *J Virol* 71:3120-3128.
146. Lanier, L. L., A. M. Le, J. H. Phillips, N. L. Warner, and G. F. Babcock. 1983. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. *J Immunol* 131:1789-1796.
147. Antons, A. K., R. Wang, K. Oswald-Richter, M. Tseng, C. W. Arendt, S. A. Kalams, and D. Unutmaz. 2008. Naive precursors of human regulatory T cells require FoxP3 for suppression and are susceptible to HIV infection. *J Immunol* 180:764-773.
148. Unutmaz, D., V. N. KewalRamani, S. Marmon, and D. R. Littman. 1999. Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. *J Exp Med* 189:1735-1746.
149. Sadagopal, S., S. L. Lorey, L. Barnett, R. Basham, L. Lebo, H. Erdem, K. Haman, M. Avison, K. Waddell, D. W. Haas, and S. A. Kalams. 2008. Enhancement of human immunodeficiency virus (HIV)-specific CD8+ T cells in cerebrospinal fluid compared to those in blood among antiretroviral therapy-naive HIV-positive subjects. *J Virol* 82:10418-10428.
150. Alter, G., M. P. Martin, N. Teigen, W. H. Carr, T. J. Suscovich, A. Schneidewind, H. Streeck, M. Waring, A. Meier, C. Brander, J. D. Lifson, T. M. Allen, M. Carrington, and M. Altfeld. 2007. Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. *J Exp Med* 204:3027-3036.
151. Carrington, M., M. P. Martin, and J. van Bergen. 2008. KIR-HLA intercourse in HIV disease. *Trends Microbiol* 16:620-627.
152. Martin, M. P., Y. Qi, X. Gao, E. Yamada, J. N. Martin, F. Pereyra, S. Colombo, E. E. Brown, W. L. Shupert, J. Phair, J. J. Goedert, S. Buchbinder, G. D. Kirk, A. Telenti, M. Connors, S. J. O'Brien, B. D. Walker, P. Parham, S. G. Deeks, D. W. McVicar, and M. Carrington. 2007. Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nat Genet* 39:733-740.
153. Lichterfeld, M., G. Pantaleo, and M. Altfeld. 2005. Loss of HIV-1-specific T cell proliferation in chronic HIV-1 infection: cause or consequence of viral replication? *Aids* 19:1225-1227.
154. Migueles, S. A., and M. Connors. 2002. The Role of CD4(+) and CD8(+) T Cells in Controlling HIV Infection. *Curr Infect Dis Rep* 4:461-467.
155. Kiepiela, P., K. Ngumbela, C. Thobakgale, D. Ramduth, I. Honeyborne, E. Moodley, S. Reddy, C. de Pierres, Z. Mncube, N. Mkhwanazi, K. Bishop, M. van der Stok, K. Nair, N. Khan, H. Crawford, R. Payne, A. Leslie, J. Prado, A. Prendergast, J. Frater, N. McCarthy, C. Brander, G. H. Learn, D. Nickle, C. Rousseau, H. Coovadia, J. I. Mullins, D. Heckerman, B. D. Walker, and P.

Goulder. 2007. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat Med* 13:46-53.
156. Chen, H., A. Piechocka-Trocha, T. Miura, M. A. Brockman, B. D. Julg, B. M. Baker, A. C. Rothchild, B. L. Block, A. Schneidewind, T. Koibuchi, F. Pereyra, T. M. Allen, and B. D. Walker. 2009. Differential neutralization of HIV replication in autologous CD4 T cells by HIV-specific CTL. *J Virol*.
157. Day, C. L., A. K. Shea, M. A. Altfeld, D. P. Olson, S. P. Buchbinder, F. M. Hecht, E. S. Rosenberg, B. D. Walker, and S. A. Kalams. 2001. Relative dominance of epitope-specific cytotoxic T-lymphocyte responses in human immunodeficiency virus type 1-infected persons with shared HLA alleles. *J Virol* 75:6279-6291.
158. Fauce, S. R., O. O. Yang, and R. B. Effros. 2007. Autologous CD4/CD8 co-culture assay: a physiologically-relevant composite measure of CD8+ T lymphocyte function in HIV-infected persons. *J Immunol Methods* 327:75-81.
159. Sabado, R. L., S. Kilpatrick, A. Ali, M. Dagarag, H. L. Ng, H. Cao, and O. O. Yang. 2005. Detection of HIV-1-specific CTL responses in Clade B infection with Clade C Peptides and not Clade B consensus peptides. *J Immunol Methods* 296:1-10.
160. Blackburn, S. D., H. Shin, W. N. Haining, T. Zou, C. J. Workman, A. Polley, M. R. Betts, G. J. Freeman, D. A. Vignali, and E. J. Wherry. 2009. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 10:29-37.
161. Itescu, S., L. J. Brancato, J. Buxbaum, P. K. Gregersen, C. C. Rizk, T. S. Croxson, G. E. Solomon, and R. Winchester. 1990. A diffuse infiltrative CD8 lymphocytosis syndrome in human immunodeficiency virus (HIV) infection: a host immune response associated with HLA-DR5. *Ann Intern Med* 112:3-10.
162. Petrovas, C., D. A. Price, J. Mattapallil, D. R. Ambrozak, C. Geldmacher, V. Cecchinato, M. Vaccari, E. Tryniszewska, E. Gostick, M. Roederer, D. C. Douek, S. H. Morgan, S. J. Davis, G. Franchini, and R. A. Koup. 2007. SIV-specific CD8+ T cells express high levels of PD1 and cytokines but have impaired proliferative capacity in acute and chronic SIVmac251 infection. *Blood* 110:928-936.
163. Lichterfeld, M., D. Mou, T. D. Cung, K. L. Williams, M. T. Waring, J. Huang, F. Pereyra, A. Trocha, G. J. Freeman, E. S. Rosenberg, B. D. Walker, and X. G. Yu. 2008. Telomerase activity of HIV-1-specific CD8+ T cells: constitutive up-regulation in controllers and selective increase by blockade of PD ligand 1 in progressors. *Blood* 112:3679-3687.
164. Muthumani, K., A. Y. Choo, D. J. Shedlock, D. J. Laddy, S. G. Sundaram, L. Hirao, L. Wu, K. P. Thieu, C. W. Chung, K. M. Lankaraman, P. Tebas, G. Silvestri, and D. B. Weiner. 2008. Human immunodeficiency virus type 1 Nef induces programmed death 1 expression through a p38 mitogen-activated protein kinase-dependent mechanism. *J Virol* 82:11536-11544.
165. Miura, T., M. A. Brockman, C. J. Brumme, Z. L. Brumme, J. M. Carlson, F. Pereyra, A. Trocha, M. M. Addo, B. L. Block, A. C. Rothchild, B. M. Baker, T. Flynn, A. Schneidewind, B. Li, Y. E. Wang, D. Heckerman, T. M. Allen, and B. D. Walker. 2008. Genetic characterization of human immunodeficiency virus

type 1 in elite controllers: lack of gross genetic defects or common amino acid changes. *J Virol* 82:8422-8430.
166. Miura, T., M. A. Brockman, Z. L. Brumme, C. J. Brumme, F. Pereyra, A. Trocha, B. L. Block, A. Schneidewind, T. M. Allen, D. Heckerman, and B. D. Walker. 2009. HLA-associated alterations in replication capacity of chimeric NL4-3 viruses carrying gag-protease from elite controllers of human immunodeficiency virus type 1. *J Virol* 83:140-149.
167. Picker, L. J., and D. I. Watkins. 2005. HIV pathogenesis: the first cut is the deepest. *Nat Immunol* 6:430-432.
168. Yang, O. O., S. A. Kalams, M. Rosenzweig, A. Trocha, N. Jones, M. Koziel, B. D. Walker, and R. P. Johnson. 1996. Efficient lysis of human immunodeficiency virus type 1-infected cells by cytotoxic T lymphocytes. *J Virol* 70:5799-5806.