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The Dynamic Interplay between HIV-1 and T cells

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by

Christian Raul Aguilera-Sandoval

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ABSTRACT OF THE DISSERTATION

The Dynamic Interplay between HIV-1 and T cells

by

Christian Raul Aguilera-Sandoval

Doctor of Philosophy in Microbiology, Immunology and Molecular Genetics

University of California, Los Angeles, 2016

Professor Otto Orlean Yang, Committee Chair

Although it is well-documented that T cells are crucial in the pathogenesis of human immunodeficiency virus type 1 (HIV-1) yet the dynamic interplay between HIV-1 and T cells has not been fully elucidated. The effects that HIV-1 has on T cell diversity and the effects that T cell diversity has on HIV viral escape have not been well characterized; an understanding of these effects could have crucial implications for design of CTL vaccines. In particular, such information could provide insights needed to develop methods for reconstitution of sufficient diversity in the immune systems of HIV+ persons to allow CTL vaccines to be effective. Furthermore, such information could be helpful in the development of CTL vaccines against semi-conserved epitopes, so as to prevent viral escape. These are the aims this dissertation will attempt to address.

One major problem with the current approach to HIV-1 vaccine development is that the strategies currently being employed ultimately fail; this is mostly, but not entirely, due to HIV-

1's high rate of mutation. This ultimately results in the escape of the virus from vaccine-induced immunity, thereby rendering such vaccines useless. Both CD4+ and CD8+ T cells play a major role in immune responses to HIV-1. However, during the course of infection, CD4+ T cells are depleted, not only in number, but also in diversity. Limited CD4+ T cell diversity cripples the immune system, as such CD4+ T cells are not able to provide the help necessary for effective innate and adaptive immune system responses to HIV, including help to CTL, which is of particular importance for this dissertation. CTL responses constitute one of the crucial arms of the immune system that is highly responsible for responding to HIV-1 infection. However, immune defenses mediated by CTL ultimately fail in HIV infection, which is, again, also largely (but not entirely) due to high rates of HIV-1 mutation that cause constant viral escape, which, in turn, drives chronic immune activation and ultimately CTL exhaustion.

We have addressed each of these problems in this dissertation. In Chapter Two, we present results of studies in which we examined thymic output and CD4+ T cell diversity from HIV+ persons who were perinatally infected, were in treatment and had lived with the infection for over two decades. In Chapter Three, we present results of studies in which we screened for CTL responses against the gag 162-173 KAFSPEVIPMF epitope from multiple persons and identified and cloned the TCR responsible for these responses, using a novel technique TCR identification and cloning technique that we also present in this chapter. Finally, we functionally tested the cloned KF11-specific TCR to confirm that this panel was able to recognize and lyse the most common circulating variants of the KF11 epitope.

The results presented in Chapter Two of this dissertation show that HIV+ participants had reduced CD4+ T cell levels, with predominant depletion of the memory subset, but preservation of naive cells. In most of these HIV+ participants, levels of CD4+ T cells that were recent thymic

emigrants' CD4+ T cell levels were normal, and enhanced thymopoiesis was present, as indicated by higher proportions of CD4+ T cells containing TCR recombination excision circles. Memory CD4+ T cell depletion was highly associated with CD8+ T-cell activation in HIV-1-infected persons, and plasma interlekin-7 levels were correlated with levels of naive CD4+ T cells, suggesting activation-driven loss and compensatory enhancement of thymopoiesis. Deep sequencing of CD4+ T cell receptor sequences in HIV+ subjects who had high levels of compensatory enhancement of thymopoiesis revealed supranormal TCR diversity, providing additional evidence of enhanced thymic output.

In Chapter Three we introduce and describe an inexpensive new technique to quickly and efficiently identify, clone and functionally test epitope-specific TCR. Using this new technique and samples from multiple HIV+ HLA-B*5701 persons, we identified, cloned and functionally tested four KF11-specific TCR. The four identified KF11-specific TCR were able to recognize and lyse target cells that were peptide-loaded with the six most common circulating variants of KF11. These six variants make up 97% of all circulating variants, according to the Los Alamos HIV database. The functional avidity and killing efficiency of the KF11-specific TCR were also investigated. Consonant with prior supporting data on KF11-specific TCR, the functional avidity observed for these four KF11-specific TCR had a range of 89 ng/ml to 832 ng/ml. One of the KF11-specific TCR was tested for its ability to lyse HIV-infected cells. This TCR was able to lyse cells infected with three of the four variants that were previously recognized and lysed in the peptide-loaded target cells. If these TCR are validated *in vivo*, and they are to prevent viral escape, the process could be repeated with other HLA restricted epitopes in order to develop a new treatment against HIV-1.

The Dissertation of Christian Raul Aguilera-Sandoval is approved.

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2016

Dedication

A mi madre Paz Sandoval, porque siempre me has apoyado en las buenas y en las malas, dado amor incondicional, me has cuidado con todo tu ser y aconsejado para el buen camino. Sin ti no fuera lo que soy y no pudiera haber logrado lo que tengo.

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"When it is obvious that the goals cannot be reached, don't

adjust the goals,

adjust the action steps"

Confucius

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List of Acronyms

- AIDS: Autoimmune Deficiency Syndrome
- APC: Antigen Presenting Cell
- Ca: Calcium
- cART: Combination anti-retroviral therapy
- cDNA: Complementary deoxyribonucleic acid
- CDR: Complementary determining region
- CNS: Central nervous system
- cTEC: Cortical thymic epithelial cells
- CTL: Cytotoxic T lymphocyte
- DNA: Deoxyribonucleic acid
- ER: Endoplasmic reticulum
- GALT: Gut-associated lymphoid tissue
- HIV : Human Immunodeficiency Virus
- HLA: Human leukocyte antigen
- IFN: Interferon
- ITAM: Immunoreceptor tyrosine-based activation motif
- KF11: Gag 162-173 epitope KAFSPEVIPMF
- MHC: Major histocompatibility complex
- MIP: Macrophage inflammatory protein
- NK: Natural Killer cells

- PBMC: Peripheral blood mononuclear cells
- PCR: Polymerase chain reaction
- pHLA: Peptide loaded human leukocyte antigen
- Pol: Polymerase
- R10: RPMI supplemented with 10% FBS
- R10-12.5: RPMI supplemented with 10% FBS and 12.5 units of IL2/ml
- Rcf: Relative centrifugal force
- RNA: Ribonucleic Acid
- RPM: Revolutions per minute
- RTE: Recent thymic emigrants
- SFC: Spot-forming cells
- SIV: Simian immunodeficiency virus
- T/F: Transmitted / Founder
- TCR: T-Cell receptor
- TNF: Tumor necrosis factor
- TRAV: T cell receptor α chain
- TRBV: T cell receptor β chain
- TREC: T-Cell receptor excision circle

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Chapter 1. Introduction

HIV-1, Then and Now

In 1981, Dr. Michael Gottlieb, Dr. Andrew Saxon and several other physicians at UCLA first noted the immunological irregularity that later was to be recognized as a hallmark of HIV/AIDS. Five patients were suffering from opportunistic infections such as cytomegalovirus infection, pneumocystis pneumonia, mucosal candidiasis, and Kaposi's sarcoma (Gottlieb et al., 1981). These conditions, rarely observed in non-immunocompromised patients, plagued Dr. Gottlieb's patients. This led Dr. Gottlieb and his colleagues to investigate further; they found that these patients had CD4-T cell deficiency (Gottlieb et al., 1981), another immunological hallmark of HIV. These findings spurred the scientific community to research this novel immunological condition, which later, in 1983-1984, was discovered to be caused by the Human Immunodeficiency Virus I (HIV-I) (Barre-Sinoussi et al., 1983; Popovic, Sarngadharan, Read, & Gallo, 1984). This viral infection has turned into a pandemic that has resulted in millions of deaths, and it is estimated there is a minimum of 35 million people currently living with HIV worldwide. To date, no successful vaccine has been developed, despite many attempts and the dire need.

HIV-1

HIV-1 is an enveloped lentivirus that has an unspliced genome size of 9.2 kb, with a mature virion having a diameter of 100-120 nm (Kuznetsov, Victoria, Robinson, & McPherson, 2003). HIV-1 is highly genetically diverse and is divided into 3 major groups: M, N, and O (F. Gao et al., 1999; Korber et al., 2000). HIV-1 group M is the most common in worldwide infections, making up 99.6% of the reported cases. Group M has been further divided into nine clades: A-D, F-H, J, and K. An infectious mature HIV-1 virion is composed of nine viral genes,

three of which encode structural proteins and six of which encode regulatory proteins. The three genes encoding the structural proteins are *gag*, *pol*, and *env*. Each gene encodes multiple proteins. For example, *gag* encodes p17, p24, p7, and p6; *pol* encodes Protease, Reverse Transcriptase, p15, and integrase; *env* encodes gp120 and gp41.

HIV infection begins with the binding of its gp120 protein to the target cell's CD4 molecule (Maddon et al., 1986; McDougal et al., 1986) and usually either the CCR5 (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996) or CXCR4 (Y. Feng, Broder, Kennedy, & Berger, 1996) which serve as co-receptor. Consequently, the major target of HIV infection is $CD4^+$ T cells; however, dendritic cells and macrophages are also susceptible to infection (Kawamura et al., 2003; Niedecken, Lutz, Bauer, & Kreysel, 1987; Soto-Ramirez et al., 1996), although replication is less efficient than it is in $CD4^+$ T cells (Granelli-Piperno, Delgado, Finkel, Paxton, & Steinman, 1998; Kawamura et al., 2003; Steinman et al., 2003). Infection of the above target cells has been reported to occur via two different mechanisms, cell-free spread and cell-to-cell spread (C. Zhang et al., 2015). In cell-free spread, virions bud off from an infected cell and encounter and infect another target cell (C. Zhang et al., 2015). Cell-to-cell spread involves an infected cell that directly infects another target cell via a virological synapse (Arthos et al., 2008; Jolly, Kashefi, Hollinshead, & Sattentau, 2004). Alternatively, cell-to-cell spread can also occur by an infected antigen presenting cell (APC) directly transferring mature virions *in trans* to another target cell (Sattentau, 2008).

Post entry into the target cell, HIV's integrase will integrate the viral genome into the target cell's genome. HIV infection will cause increased NFkβ and NFAT expression. These proteins then bind to HIV's long terminal repeat, initiating transcription of viral RNA by the infected cell's RNA Pol II. Expression of the viral proteins in an infection of a cell results in

human leukocyte antigen (HLA) I downregulation, decreased expression of anti-viral proteins, expression and secretion of immune activating cytokines, and expression of anti-apoptotic signals.

After 3-4 weeks of a person being infected, an immune response is mounted by HIV-specific cytotoxic T lymphocytes (CTLs) is detected, reducing viremia to a relatively constant amount. This is known as the viral set point. However, the CTLs and other immune responses are never sufficient to completely eradicate the virus so far as we know. The rate of disease progression and immune dysregulation is generally correlated with the height of the set point. The amount of infectious virus circulating in an untreated adult remains relatively consistent for 2-12 years until $CD4^+$ T cells are depleted in quantity (Gottlieb et al., 1981) and/or breadth (Baum et al., 2012), creating an opportunity for opportunistic infections to begin and ultimately result in death. Since the introduction of combination anti-retroviral therapy (cART) in approximately 1995 (Palmisano & Vella, 2011), the use of cART has resulted in a decrease in viremia, increase in breadth and quantity of $CD4^+$ and $CD8^+$ T cells, semi-normalization of the immune system, decrease in immuno-activation, prevention of opportunistic infections, and delayed onset of neurodegenerative disease (Gulick et al., 1997; Hammer et al., 1997).

T Lymphocytes

All T lymphocytes are derived from pluripotent hematopoietic stem cells arising from the bone marrow that then migrate to the thymus where they mature (Donskoy & Goldschneider, 1992). T cell development in the thymus is stepwise; first, they all begin without expressing CD4 or CD8, commonly referred to as double negative thymocytes, and without having rearranged either of their T cell receptor (TCR) chain coding domains. Next, T cells rearrange and express the β-chain, which pairs with a surrogate pre-T-cell α chain, forming a

pre-T-cell receptor that is co-expressed with the CD3 complex (Spits, 2002). The successful formation of the pre-T-cell receptor and CD3 complex leads to cell proliferation, halting of rearrangements of other β-chains, and the double expression of CD4 and CD8, commonly known as double positive thymocytes. After proliferation occurs, the α-chain rearranges, replacing the pre-T-cell α-chain. After successful pairing of α and β chains, the double positive T cells undergo positive selection in the thymic cortex. Positive selection is the process in which the TCR interacts with the peptide-HLA I or II stabilized complex (pHLA I or pHLA II) of cortical thymic epithelial cells (cTECs). The T cells not able to bind pHLA with their TCRs undergo apoptosis within 3-4 days. The T cells that are able to bind with intermediate affinity are induced to differentiate into mature T cells that express either CD4 or CD8. The double positive T cells that survive positive selection then undergo negative selection (Belizario, Brandao, Rossato, & Peron, 2016). Negative selection is the deletion of double positive or $CD4^+/CD8^+$ single positive T cells whose TCR binds self-pHLA with high affinity. The vast majority of the T cells that rearrange a successful TCR are deleted either by positive or negative selection (Daley, Hu, & Goodnow, 2013; Stritesky et al., 2013; Surh & Sprent, 1994). Single positive $CD4^+$ or $CD8^+$ T cells that successfully pass positive and negative selection then migrate to the periphery as recent thymic emigrants (RTEs). Once in the periphery, pending activation through the engagement of non-self epitopes, naïve T cells need to have consistent TCR engagement and signaling with the pHLA complexes they encountered during positive selection in order to avoid apoptosis (Ebert, Jiang, Xie, Li, & Davis, 2009; Lo et al., 2009; Stefanova, Dorfman, & Germain, 2002).

Activation of T cells requires three signals. The first signal is the engagement of the T cell's TCR with its non-self cognate epitope presented by the target cell's HLA. The second

signal is the engagement of the T cell's CD28 receptor with the target cell's B7 ligand. However, signal 2 is modulated at times by the engagement of additional costimulatory interactions. Binding of CTLA-4 on the surface of T cells to the target cell's B7 ligand results in an inhibitory signal to the activated T cell. Binding of the T cell's CD40 ligand to the target cell's CD40 results in increased B7 expression by the APC and further T cell proliferation. A T cell's TCR engagement with its cognate pHLA in the absence of the costimulatory signal leads to anergy or clonal deletion through apoptosis. The third signal is received from the different cytokines produced from the presenting cell (Imboden & Stobo, 1985). The T cells that successfully develop into their effector phenotypes proliferate quickly to try to clear the pathogen and/or malignant cells. Once the cognate epitope is no longer present, most of the effector T cells die through apoptosis, while a minority differentiates into memory T cells. Memory T cells can have a lifespan of many years and, upon recognition of their cognate pHLA, they can quickly proliferate and engage in their effector function without the need of additional signals.

$CD4^+$ T cells. $CD4^+$ T cells recognize non-self antigens via the presentation of the Major Histocompatibility Complex II (MHC II) in animals or, in humans, the HLA II. Prior to exposure to non-self antigen and activation, $CD4^+$ T cells are naïve, with the phenotype $CD4^+$ $CD3^+$ $CD45RA^+$. Upon activation, $CD4^+$ T cells differentiate, proliferate, attain the phenotype $CD4^+$ CD31- $CD45RO^+$, and are subdivided based on their secreted cytokine profile. Briefly, differentiated $CD4^+$ T cells are subdivided into Th1, Th2, Th17, and Treg subtypes, which are involved in suppressing or regulating immune responses, B cell class switching, activating and supporting the growth of CTLs, and the activation of innate immune cells like dendritic cells and macrophages.

$CD8^+$ T cells. $CD8^+$ T cells recognize non-self antigens via the presentation of the Major Histocompatibility Complex I (MHC I) or, in humans, the HLA I. Pending exposure to non-self epitopes and activation, $CD8^+$ T cells are naïve. Upon activation, $CD8^+$ T cells differentiate into their effector phenotype CTLs. CTLs' effector function is to lyse virally infected and/or transformed cells that actively present non-self epitopes. CTLs lyse their target cells primarily through two different mechanisms, Ca^{2+} dependent and Ca^{2+} independent. Both mechanisms require signaling to begin with the engagement of the TCR from the CTL and the pHLA complex from the target cell. In the Ca^{2+} dependent mechanism, CTLs release lytic granules, perforin, granzyme A and B, and granulysin. Perforin will polymerize on the target cell's cell membrane to cause transmembrane pores and allow the influx of water and solutes (Podack, Young, & Cohn, 1985). Granzymes, which are serine proteases, will initiate caspase cascades that will trigger apoptosis (Greenberg & Litchfield, 1995). In the Ca^{2+} independent mechanism, the CTL's Fas ligand binds to the target cell's Fas molecule. Both mechanisms result in signaling for target cells to undergo apoptosis. Additionally, activated CTLs will produce anti-viral cytokines that will either directly or indirectly suppress viral replication (Cocchi et al., 1995; Guidotti & Chisari, 1996; Kurane, Meager, & Ennis, 1989). Some of the cytokines and chemokines produced are IFN-γ, TNF-α, and MIP-1β (Dayton, Matsumoto-Kobayashi, Perussia, & Trinchieri, 1985; Tomiyama, Matsuda, & Takiguchi, 2002). Viral infected cells exposed to TNF-α will be induced to initiate apoptosis (J. A. Levy, Mackewicz, & Barker, 1996). MIP-1β and RANTES have been shown to inhibit HIV-1 entry into $CD4^+$ T cells (Alkhatib et al., 1996; Cocchi et al., 1995) by occupying chemokine receptor 5 (CCR5), which functions as a co-receptor for most variants of HIV-1.

T Cell Receptor (TCR)

The TCR is a cell surface heterodimeric protein composed of an α and a β chain, with each chain being of about 530 amino acids (Kappler et al., 1983). Each β chain is encoded by multiple gene segments, including variable (V), diversity (D), joining (J), and constant (C) gene segments; the α chain is encoded similarly to the β chain except that it does not contain a diversity gene segment (Davis & Bjorkman, 1988). Each of these gene segments has several alleles. More specifically, there are 52 V, 2 D, 13 J, and 2 C alleles for the β chain and 70 V, 61 J, and 1 C alleles for the α chain. Each complete TCR chain is generated through somatic recombination; briefly, TCR gene segments are semi-randomly chosen, selecting a V gene segment and a D or J gene segment, depending whether it is the β or α chain, and excising out the gene segments in between so that they form T-Cell receptor excision circles (TRECs). Thereafter, the same process is repeated until the TCR has all of the VDJ-C gene segments ready for transcription and pairing of both chains. This provides a small portion of the known 10^{15}-10^{20} TCR repertoire.

However, the majority of the TCR repertoire is reached through the generation of the complementary determining region 3 (CDR3). Each chain of the TCRαβ has three CDR regions. CDR1 and CDR2 are composed of the V gene segment of each chain and have conserved interactions with the HLA complex (D. Feng, Bond, Ely, Maynard, & Garcia, 2007). Mutations at this region have been observed to decrease the efficiency of positive selection as well as reduce pHLA recognition of non-self (Scott-Browne, White, Kappler, Gapin, & Marrack, 2009). CDR3 is responsible for recognizing and binding cognate epitopes (Garcia et al., 1996). The CDR3 region is generated through a series of enzymatic reactions where a number of nucleotides are randomly added and/or deleted (Cabaniols, Fazilleau, Casrouge, Kourilsky, & Kanellopoulos, 2001; Lafaille, DeCloux, Bonneville, Takagaki, & Tonegawa, 1989). Each

CDR3 region has a limited number of epitopes it is able to recognize, each with varying degrees of affinities. The epitopes recognized are not pathogen-dependent but rather sequence-dependent and consequently can be somewhat promiscous. For example, prior studies have shown that a TCR can recognize the flu M1:58-66 epitope while also recognizing the HIV p17 gag: 77-85 epitope (Acierno et al., 2003). Additionally, a TCR can also recognize variants of an epitope. For instance, a TCR specific for the consensus sequence of the HIV epitope SLYNTVATL is also able to recognize its variants at varying degrees of avidity (Bennett, Ng, Dagarag, Ali, & Yang, 2007b). Nevertheless, TCR promiscuity is limited in order to prevent the lysis of host cells that present self-epitopes. Given the limited promiscuity of TCRs and their vast repertoire, CTLs are able to clear a myriad of pathogens. When skewing of the TCR repertoire occurs due to cell death and/or exhaustion induced by continued immune activation, a person's ability to fight off pathogens is highly impaired.

TCR Complex

The TCR complex is made up of varying proteins, the TCR, the CD3 complex, and the ζ-chain (Bragado, Lauzurica, Lopez, & Lopez de Castro, 1990). The CD3 complex is made up of three distinct chains including a CD3γ chain, a CD3δ chain, and two CD3ε chains. The TCR engages the pHLA complex, initiating a signal that is then regulated by the CD3 and the ζ-chain through a downstream sequence of phosphorylation and dephosporylation of their ITAMS, resulting in the activation of the T cell (De Palma & Gorski, 1995; Geiger, Gorski, & Eckels, 1991; Goronzy, Xie, Hu, Lundy, & Weyand, 1993; Hurley et al., 1993; Lobashevsky, Kotb, & Gaber, 1996).

Human Leukocyte Antigen (HLA)

HLAs are cell surface proteins encoded by chromosome 6. They represent the most

polymorphic locus in humans and their function is to regulate the survival and activation of T cells and natural killer (NK) cells via the presentation of self and non-self epitopes. HLA genes are highly polymorphic. According to the IMGT-HLA database (https://www.ebi.ac.uk/ipd/imgt/hla/), there are over fourteen thousand different alleles known to date. This genetic diversity within HLA genes allows the human race to combat myriad pathogens (Brahmajothi et al., 1991; Cooke & Hill, 2001; Hill et al., 1991; Thursz et al., 1995). In addition to the HLA's polymorphic nature, HLAs are also polygenic. There are two major classes, HLA Class I (HLA I) and HLA Class II (HLA II). However, not all HLA molecules are created equal. Some HLA molecules have been associated with having a predisposition for resistance or susceptibility to a pathogen (Brahmajothi et al., 1991; Cooke & Hill, 2001; Hill et al., 1991; Thursz et al., 1995). For example, HLA B*5701 is highly correlated with partial protection against HIV-1 disease progression (X. Gao et al., 2001; Hendel et al., 1999; Kaslow et al., 1996; Magierowska et al., 1999; Migueles et al., 2000), while HLA B*3501 is correlated with fast disease progression in HIV^+ persons (X. Gao et al., 2001; Itescu et al., 1992; Sahmoud et al., 1993; Scorza Smeraldi et al., 1986).

HLA I. HLA I has three major and three minor genes, HLA-A, -B, and -C and HLA-E, -F, and -G, respectively. According to the IMGT/HLA Database, there are 10,730 different HLA Class I alleles as of July 1, 2016 (see Table 1). The HLA I complex is composed of an HLA I allele whose α domains associate with β2-microglobulin (Buslepp, Wang, Biddison, Appella, & Collins, 2003). HLA I is expressed on all nucleated cells and its function is to present intracellularly processed 8-12 amino acid epitopes to CTLs. Each epitope that binds to an HLA I molecule stabilizes the pHLA complex through the engagement of generally two amino acids, one in the free amino at P2 and one in the carboxy termini of the epitope and generally two

amino acids in the epitope binding cleft of the HLA I (Madden, Garboczi, & Wiley, 1993). The stabilizing amino acids are referred to as anchor residues. HLA I expression is regulated to either clear a pathogen or provide homeostatic signals to $CD8^+$ T cells. A viral infected cell will express IFN-α, -β or -γ, which will increase the expression of HLA I molecules. Healthy cells will express normal levels of HLA I molecules to provide the homeostatic signals T cells require for survival.

Numbers of HLA Alleles											
HLA Class I Alleles	10,730										
HLA Class II Alleles	3,743										
Total HLA Alleles	14,473										
HLA Class I											
Gene	A	B	C	E	F	G					
Alleles	3,399	4,242	2,950	21	22	53					
HLA Class I-Pseudogenes											
Gene	H	J	K	L	P	V	Y				
Alleles	12	9	6	5	5	3	3				
HLA Class II											
Gene	DRA	DRB	DQA1	DQB1	DPA1	DPB1	DPB2	DMA	DMB	DOA	DOB
Alleles	7	2,018	69	911	43	644	6	7	13	12	13

Table 1. List of HLA Class I and Class II alleles.

HLA II. HLA II has eleven known genes with approximately 3,743 different alleles (see Table 1). The different HLA II genes come together to form a heterodimeric complex composed of two different chains with their respective α and β domains. HLA II complexes are capable of binding extracellularly processed epitopes of about 13-17 amino acids and are expressed by antigen presenting cells (APCs) but can also be expressed by group 3 innate lymphoid cells if induced by INF-γ. These HLA II epitope-bound complexes are presented to $CD4^+$ T cells to activate and induce proliferation and differentiation of the naïve $CD4^+$ T cells to their effector phenotypes.

Antigen Presentation via HLA I

HLA Class I molecules are used to present intracellularly processed epitopes to aid in immuno-surveillance. This process begins with cytoplasmic proteins being processed into peptides within the proteosome. The peptides are then transported into the endoplasmic reticulum (ER) by the TAP proteins, where they are further processed into 8-12 amino acid epitopes (Oancea et al., 2009; Pages et al., 1994). These processed epitopes are then loaded into HLA class I molecules through the assistance of ER chaperone proteins like Tapasis, ERp57, and calreticulin (Purcell & Elliott, 2008), thus stabilizing the HLA and forming the pHLA complex (Townsend et al., 1989). The pHLA complex then leaves the ER through the secretory pathway and is transported to the cell surface where it can be recognized by $CD8^+$ T cells, whether naïve or CTL.

$CD4^+$ T Cells Play a Major Role in HIV Pathogenesis

$CD4^+$ T cells modulate the immune system by controlling the activation and function of the innate and adaptive immune system. They are primarily located in the primary and secondary

lymphoid organs. The majority of $CD4^+$ T cells in healthy adult persons express the memory phenotype $CD4^+$, $CCR5^+$, $CD45RO^+$, tend to have a basal level of activation, and reside in the gut-associated lymphoid tissue (GALT) (Mowat & Viney, 1997), making them ideal targets for HIV. As a result, major depletion of $CD4^+$ T cells in the GALT (Brenchley et al., 2004; Mattapallil et al., 2005; Veazey et al., 1998; Veazey et al., 2000) and chronic immune activation occurs shortly post-infection (Brenchley et al., 2006). The chronic immune activation results in enhanced maturation of naïve T cells into memory T cells ($CD4^+$ $CD45RO^+$), making them also ideal targets for viral infection (Clement, Yamashita, & Martin, 1988; Schnittman et al., 1990; Sleasman, Aleixo, Morton, Skoda-Smith, & Goodenow, 1996), thus starting a vicious cycle of chronic activation and infection. Without cART intervention, $CD4^+$ T cells are eventually depleted during the course of HIV infection, all the while causing chronic immunoactivation, dysregulation, and exhaustion of innate and $CD8^+$ T cells and, ultimately, the inability to fight off opportunistic infections (Blackburn et al., 2009; Doering et al., 2012; Kao et al., 2011; Lu et al., 2014; Quigley et al., 2010). If cART is commenced, $CD4^+$ T cell counts are replenished, but the quality and breadth of the replenished $CD4^+$ T cells is highly dependent upon how quickly cART is commenced or reinstated.

CTLs Play a Major Role in HIV Pathogenesis

The effector phenotype of $CD8^+$ T cells, also known as CTLs, were first observed to be involved in suppressing HIV-1 replication in three different studies in 1994 (Borrow, Lewicki, Hahn, Shaw, & Oldstone, 1994; Koup et al., 1994; Safrit, Andrews, Zhu, Ho, & Koup, 1994). In later studies, it was shown that (i) depletion of $CD8^+$ T cells in macaques leads to a loss of viral control and disease progression, both in the acute and chronic phases of infection (Friedrich et al., 2007; Jin et al., 1999; Matano et al., 1998; Schmitz et al., 1999); (ii) HIV-1 sequences show

HLA-associated “footprints” from CTL selective pressure *in vivo* in chronically-infected individuals (Moore et al., 2002) and enrichment for HLA alleles associated with viral control in humans (Carrington & O'Brien, 2003a; Goulder et al., 1997b; Kelleher et al., 2001b; Migueles et al., 2000) and SIV-infected macaques (Loffredo et al., 2007; Yant et al., 2006); (iii) CTL responses almost exclusively drive the HIV viral evolution during acute infection (Allen et al., 2005b; Y. Liu et al., 2006; O'Connor et al., 2004a); and lastly, (iv) the HLA class I locus is the strongest genetic determinant of disease progression (International H. I. V. Controllers Study et al., 2010).

Chapter 2

Supranormal Thymic Output Up to Two Decades After HIV-1 Infection

Introduction

The hallmark of Human Immunodeficiency Virus Type 1 (HIV-1)-induced immunosuppression leading to acquired immunodeficiency syndrome is $CD4^+$ T-cell depletion, which may be caused by direct cytopathic effects of infection, immune clearance of infected cells, persistent immune activation, and likely other factors (Moir, Chun, & Fauci, 2011).In particular, immune activation is highly associated with the ongoing loss of $CD4^+$ T-cells and believed to be the cause of increased T-cell turnover during chronic infection. The precise mechanisms for this inappropriate inflammatory state are unclear, but ongoing viral replication can be a major contributor even in persons with undetectable viremia. (Chomont et al., 2009; Frenkel et al., 2003; Moir et al., 2011; Siliciano et al., 2003) .

Peripheral blood $CD4^+$ T-cell concentration is a widely used clinical predictor of the immunological status of an infected individual, with a level of less than 200/μL generally considered to reflect sharply increased risk for opportunistic infections that define AIDS.(Moir et al., 2011) However, this simple quantitative assessment does not precisely reflect immunocompetence. For example, recurrent bacterial pneumonias, malignancies, and AIDS-defining illnesses such as active cytomegalovirus infection and Pneumocystis pneumonia may occur at higher $CD4^+$ T-cell levels in children, adolescents, and adults.(Komanduri et al., 2001; Krogstad et al., 2015; Zoufaly et al., 2011) It is very likely that the clonal diversity of the $CD4^+$ T-cell population and therefore breadth of pathogen recognition is also important.(Baum et al., 2012)

Effective antiretroviral therapy (ART) suppresses HIV-1 replication, reduces immune activation, and increases peripheral blood $CD4^+$ T-cell concentrations.(Autran et al., 1997; Giorgi et al., 1998) However, the extent to which normalization of clonal T-cell diversity occurs is less well documented. In HIV-1-infected adults, the rise in $CD4^+$ T-cell levels seen after institution of ART is characterized by an initial rapid rise that is likely due to redistribution of total body memory $CD4^+$ T-cells, followed by a slower and more prolonged increase in naïve $CD4^+$ T-cells. (Autran et al., 1997; Pakker et al., 1998) By contrast, HIV-1-infected children demonstrate an early and sustained increase in naïve $CD4^+$ T-cells (Gibb et al., 2000; Jankelevich et al., 2001; Resino, Bellon, Gurbindo, Leon, & Munoz-Fernandez, 2003; Resino et al., 2006; Sleasman et al., 1999) that likely reflects greater baseline thymic function than adults, who tend to have age-related involution of thymic epithelial tissue and attrition of thymic function.(Haynes, Markert, Sempowski, Patel, & Hale, 2000)

Supporting this concept, we previously demonstrated that adolescents and young adult survivors of perinatal HIV-1 infection on ART have markers of thymopoiesis that are comparable to uninfected age-matched controls, including concentrations of peripheral blood naïve $CD4^+$ T-cells and T-cell receptor recombination excision circles (TREC) that reflect recent thymic emigrants.(Lee et al., 2006) Others have demonstrated that T-cell receptor CDR3 distribution perturbations are rapidly reduced in some children and adolescents during ART (Yin et al., 2009) suggesting that some degree of normalization of the TCR repertoire is possible. However, these measurements have not excluded qualitative abnormalities in thymopoiesis that might result from the known impact of HIV-1 on the architecture of both the thymus and

secondary lymphoid tissues.(Bohler et al., 1999; Gibb et al., 2000; Resino et al., 2003; Resino et al., 2006; Zeng, Haase, & Schacker, 2012; Zeng et al., 2011).

Thus it is unclear if $CD4^+$ T-cell clonal diversity is maintained in conjunction with recovered total $CD4^+$ T-cell numbers on ART, particularly in individuals who were infected before immunologic maturity. To address this uncertainty, we assess immune reactivity to HIV-1, thymopoiesis and $CD4^+$ T-cell diversity in a cohort of long term survivors of perinatal HIV-1 infection. These data address key questions as to whether infection early in life (during immunologic development), in conjunction with chronic infection (spanning more than 13 years), limit $CD4^+$ T-cell reconstitution.

Material and Methods

Study approval.

Healthy control and HIV-1-infected study volunteers were enrolled under protocols approved by institutional review boards of the University of California Los Angeles and Children's Hospital Los Angeles. Written informed consent was received from all participants prior to inclusion in the study**.**

Cohort and preparation of peripheral blood mononuclear cells.

All study participants were enrolled from 2003 to 2006. Individuals with known hepatitis B or C infections were excluded. Twenty control subjects and 20 HIV-1-infected subjects were described in previous reports.(Lee et al., 2006; O. O. Yang et al., 2005) Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density centrifugation gradient, washed twice with phosphate buffered saline, and viably cryopreserved. Fresh umbilical cord blood was obtained from the UCLA CFAR Virology Core. For quantitative spectratyping and pyrosequencing studies, $CD3^+CD4^+CD31^+CD45RA^+$ T-cells were purified from cryopreserved PBMC by fluorescence-activated cell sorting (FACSAria II using FACSDiva Version 6.1, Becton Dickinson). $CD4^+$ T-cells from cord blood samples were isolated by negative selection (human $CD4^+$ T-cell enrichment mixture, RosetteSep, StemCell Technologies).

Clinical laboratory tests

Complete blood counts and plasma HIV-1 RNA measurements were obtained through the Children's Hospital Los Angeles and UCLA clinical laboratories.

Volumetric tomography of thymic tissue.

Non-contrast helical computed tomography (CT) studies of the chest were performed with 3-mm collimation extending from the thoracic inlet to the lung bases, using previously described methods.(Lee et al., 2006) All female participants had negative pregnancy tests confirmed prior to imaging. Volumetric CT scans were discontinued after 49 scans (29 HIV-1-infected and 20 uninfected controls) because an interim analysis indicated futility to detect statistically significant differences with the initially planned sample size.

T-cell immunophenotyping by flow cytometry.

Whole blood T-cell staining and flow cytometry was performed as described previously, with naive $CD4^+$ T-cells defined as $CD4^+CD45RA^+CD27^+$.(Lee et al., 2006) $CD45RA^-CD4^+$ T-cells were defined as memory cells (combined central and effector subsets). Staining was also performed to quantify the $CD45RA^+CD31^+$ subset of $CD4^+$ T-cells (recent thymic emigrants) (Kimmig et al., 2002) and the $CD38^+$HLA-DR^+ subset of $CD8^+$ T-cells (activated).(Giorgi et al., 1999) Quantitation of naïve, recent thymic emigrants, and memory $CD4^+$ T-cells was not performed in one control subject and the percentage of $CD38^+$HLA-DR^+ $CD8^+$ T-cells was not determined for two other control subjects. Due to the absence of a complete blood count, the concentrations (cells/μL) of T-cell subsets of one HIV-1-infected individual are absent from panels of Figures 2 and 3.

Detection of HIV-1-specific CD8+ T-cell responses against HIV-1 by interferon γ (IFN)-γ-ELISpot analysis.

Peripheral blood HIV-1 specific $CD8^+$ T-cell responses in HIV-1 infected individuals with plasma HIV-1 levels of ≤400 RNA copies/mL at study entry were quantified by IFN-γ ELIspot analysis, as previously described.(Ibarrondo et al., 2005) In brief, purified $CD8^+$ T-cells

were screened against 53 pools of overlapping peptides spanning the total HIV-1 clade B consensus sequence proteome (NIH AIDS Reference and Reagent Repository) to determine the frequency of spot-forming cells (SFC) per added $CD8^+$ T-cells. The frequency of HIV-1-specific SFC per volume of peripheral blood was calculated by multiplying the frequency of SFC in $CD8^+$ T-cells and the number of $CD8^+$ T-cells per volume of blood.

Peripheral blood TREC analyses.

Cellular DNA was prepared from PBMCs and signal joint T-cell receptor recombination excision circles (TREC) were quantified by real time PCR as previously described (Halnon et al., 2005; Pham et al., 2003; Reiff et al., 2009), and reported as TREC/million cells. TREC were measured using isolated $CD4^+$ T-cells (Rosette-Sep beads, StemCell Technologies, Vancouver, Canada) for most participants. The number of $TREC^+$ $CD4^+$ T-cells per volume of peripheral blood was calculated by multiplying the frequency of TREC in isolated $CD4^+$T-cells and the concentration of $CD4^+$T-cells per volume of blood.

HLA and CCR5 genetic analyses.

Using PBMC DNA, HLA typing was performed by the clinical laboratory at the UCLA Immunogenetics Center, and PCR was used to determine if the □32 deletion was present at the CCR5 locus using oligonucleotide primers described by others. (Misrahi et al., 1998)

Quantitation of TCR BV family RNA transcripts.

From 3 to 15 million cryopreserved PBMC from HIV-1-infected or control subjects were stained and sorted to purify $CD31^+CD45RA^+CD4^+$ T-cells, yielding 230,000 to 700,000 cells per individual. RNA was isolated from purified lymphocytes (RNeasy MiniKit, Qiagen, Valencia CA.), and reverse-transcribed to cDNA using random primers (High Capacity Reverse Transcription Kit, Applied Biosystems, Carlsbad, CA). Quantitative spectratyping (QS) was used

to examine BV family usage as described previously. (Balamurugan, Ng, & Yang, 2010) In brief, RT-PCR was employed to determine the relative concentration of each BV gene family (IMGT nomenclature (http://www.imgt.org)), and capillary electrophoretic size resolution of each family yielded a profile of TCR sequence size distribution within each family.

Deep sequencing of TCR coding sequences.

The cDNA (6.5 to 15 μg) generated for spectratyping was PCR-amplified (Phusion High-Fidelity DNA Polymerase, New England BioLabs) for 35 cycles under the following conditions: initial denaturation 98˚C (30sec), denaturation 98˚C (10sec), annealing 62˚C (30sec), extension 72˚C (15sec) and final extension (5 min). The PCR products were then purified (PureLink PCR Purification Kit, Invitrogen) and further amplified using nested PCR (Phusion High-Fidelity DNA Polymerase) for 35 cycles under the following conditions: initial denaturation 98˚C (30sec), denaturation 98˚C (10sec), annealing 62˚C (30sec), extension 72˚C (15sec) and final extension (5 min). These PCR products were then separated in 2% agarose gels and cDNA from the appropriate bands was purified (QIA Gel Extraction Kit, Qiagen). Pyrosequencing of the nested PCR purified products using 454 FLX Titanium chemistry was performed according to the manufacturer's protocols (Roche Applied Science). The primers used for the 3 BV families were the same as those used in QS analysis (Balamurugan et al., 2010), but additionally tagged with multiplex identifier (MID) and primer key sequences (see Table 2). To check that diversity within the samples was retained during PCR amplification with the modified primers, the following control experiments were performed. First, three rounds of PCR amplification were performed on an aliquot of an umbilical cord blood DNA sample, and spectratyping was performed after each round, showing that the TCR genes of the third round of amplification remained Gaussian in size distribution (see Figure 1). Second, the PCR products from the third

round of amplification were cloned and sequenced, showing polyclonality of TCRs in all cases (see Table 3), thus demonstrating no evidence of biased amplification.

Patient Identifier	Cord Blood	BV Family	Primer
	12	29	CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACAGAGGCCA CATATGAGAGTGG
	12	03	CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACACCTAAAT CTCCAGACAAAGC
	12	19	CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACAGGAGATA TAGCTGAAGGGTA
	11	29	CGTATCGCCTCCCTCGCGCCATCAGAGACGCACTCGAGGCCA CATATGAGAGTGG
	11	03	CGTATCGCCTCCCTCGCGCCATCAGAGACGCACTCCCTAAAT CTCCAGACAAAGC
	11	19	CGTATCGCCTCCCTCGCGCCATCAGAGACGCACTCGGAGATA TAGCTGAAGGGTA
AP04		29	CGTATCGCCTCCCTCGCGCCATCAGATCAGACACGGAGGCCA CATATGAGAGTGG
AP04		03	CGTATCGCCTCCCTCGCGCCATCAGATCAGACACGCCTAAAT CTCCAGACAAAGC
AP04		19	CGTATCGCCTCCCTCGCGCCATCAGATCAGACACGGGAGATA TAGCTGAAGGGTA
AP22		29	CGTATCGCCTCCCTCGCGCCATCAGATATCGCGAGGAGGCCA CATATGAGAGTGG
AP22		03	CGTATCGCCTCCCTCGCGCCATCAGATATCGCGAGCCTAAAT CTCCAGACAAAGC
AP22		19	CGTATCGCCTCCCTCGCGCCATCAGATATCGCGAGGGAGATA TAGCTGAAGGGTA
CP04		29	CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACAGAGGCCA CATATGAGAGTGG

CP04		03	CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACACCTAAATCTCCAGACAAAGC
CP04		19	CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACAGGAGATATAGCTGAAGGGTA
CN13		29	CGTATCGCCTCCCTCGCGCCATCAGAGACGCACTCGAGGCCACATATGAGAGTGG
CN13		03	CGTATCGCCTCCCTCGCGCCATCAGAGACGCACTCCCTAAATCTCCAGACAAAGC
CN13		19	CGTATCGCCTCCCTCGCGCCATCAGAGACGCACTCGGAGATATAGCTGAAGGGTA
CN02		29	CGTATCGCCTCCCTCGCGCCATCAGATCAGACACGGAGGCCACATATGAGAGTGG
CN02		03	CGTATCGCCTCCCTCGCGCCATCAGATCAGACACGCCTAAATCTCCAGACAAAGC
CN02		19	CGTATCGCCTCCCTCGCGCCATCAGATCAGACACGGGAGATATAGCTGAAGGGTA
BN02		29	CGTATCGCCTCCCTCGCGCCATCAGATATCGCGAGGAGGCCACATATGAGAGTGG
BN02		03	CGTATCGCCTCCCTCGCGCCATCAGATATCGCGAGCCTAAATCTCCAGACAAAGC
BN02		19	CGTATCGCCTCCCTCGCGCCATCAGATATCGCGAGGGAGATATAGCTGAAGGGTA
		Reverse	CTATGCGCCTTGCCAGCCCGCTCAGCATAGTAGTGCTTCTGATGGCTCAAACAC

Forward primer (Primer A-Key):

5'-CGTATCGCCTCCCTCGCGCCATCAG-{MID}-{template-specific-sequence}-3'

Reverse primer (Primer B-Key):

5'-CTATGCGCCTTGCCAGCCCGCTCAG-{MID}-{template-specific-sequence}-3'

Table 2. List of primers used in pyrosequencing to amplify BV03, BV19 and BV29 sequences.

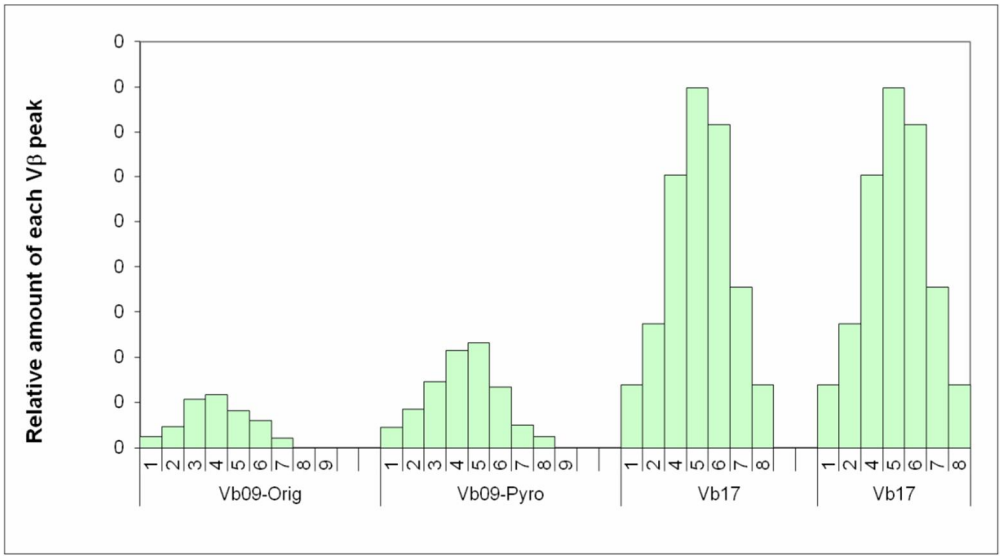


Figure 1. Nested PCR with 454-tagged BV specific primers does not result in biased PCR amplification.

A third round PCR was done on nested PCR products using the 454-pyrosequencing modified primers. Spectratyping analysis was performed on the 3rd round PCR products and a comparison was done between the spectratyping analysis of the 1st round PCR and the 3rd round PCR. The compared profiles suggest no biased amplification. This is a representative figure of the analysis done on all 3 BV families (BV03, BV19 and BV29).

Patient	Sample	BV Fam	BJ Seg	CDR3 Seq	CRD3 Length (aa)	Constant Region
CB12	1-2	19-01	2-5-01	CASSISYWETQYF	13	2
	1-3	19-01	1-1-01	CASSLIQGAYSDTEAFF	17	1
	1-4	19-01	2-1-01	CASGIAGGVYNEQFF	15	2
	1-5	19-01	2-1-01	CASSNDGRGNEQFF	14	2
AP04	2-1	19-02	1-3-01	CASSIGAGPGNTIYF	15	1
	2-4	19-02	2-1-01	CASSIRSSYNEQFF	14	2
	2-5	19-01	1-3-01	CASSIYPDRTGNTIYF	16	2
CN13	3-1	19-01	2-1-01	CASSRGLAGGTEQFF	15	2
	3-2	19-02	1-2-01	CASSIRGGPYYGYTF	15	1
	3-4	19-01	1-3-01	CASRPAVGNTIYF	13	1
	3-5	19-01	2-3-01	CASSNPLAGGADTQYF	16	2

Table 3. TCR Diversity is retained after PCR amplification with the 454-pyrosequencing modified primers.

Statistical analyses.

Clinical parameters analyzed as continuous variables were compared using two-tailed Mann Whitney U Test (except for the comparison of HIV ELISpot responses). Categorical variables were compared using Fisher's exact test. Pyrosequencing (454, Roche) of TCRs in nine samples of sorted $CD31^{+}CD45RA^{+}CD4^{+}$ T-cells (from three HIV-1-infected participants receiving ART with suppressed viremia (uVL), three uninfected controls, and three umbilical cord blood (CB) (See Table 4) yielded between 32,000 and 198,000 TCR sequences per sample. To compare the diversity of TCR sequences in these individuals, we examined TCR sequences in 3 specific BV families: BV03, BV19 and BV29 (IMGT nomenclature). These families were selected because they represented about 5% of total BV families in the $CD31^{+}CD4^{+}$ T-cells in HIV infected individuals, control study participants, and cord blood specimens that were selected. Two samples with lower yields (control BN02 and infected subject CB13 with 33,000 and 32,000 sequences respectively) were excluded from analyses that are especially sensitive to sample size.

Pyrosequencing is typically associated with significant sequencing errors (Huse, Huber, Morrison, Sogin, & Welch, 2007; Luo, Tsementzi, Kyrpides, Read, & Konstantinidis, 2012), but this will alter comparisons of diversity estimates if the error statistics do not differ across samples. Differences in the diversity of TCR coding sequences were evaluated by methods commonly employed in ecologic studies including Shannon index of diversity, sample size-corrected Shannon index, rarefaction curves, and analysis of the fraction of singleton species (that occur only once in the sample). We also used a histogram shape estimation technique using an "unseen estimator," which uses the observed distribution of species in a sample to estimate

the total number of unique species missed in sampling, as well as the full species distribution.(Valiant & Valiant, 2013)

As an additional control, sequences were also clustered with two different algorithms, as described by others.(Niu, Fu, Sun, & Li, 2010) Finally, the clusters were translated into stop codon-free amino acid sequences with verified BV and BJ flanking regions.

HIV-1 Status	Subject	Age	HLA-B	Plasma HIV-1 (RNA copies/mL)	Blood $CD4^+$ T-cells/µL
Infected[A] (uVL)	AP04	15.0	*3501, *5801	<400	811
	AP22	17.6	*4701,*5301	<400	448
	CP04	18.0	*5802, *37	<400	738
Uninfected	BN02	16.3		-	541
	CN02	19.0	-	-	923
	CN13	19.3	-	-	593
Cord Blood	CB11	NB	-	-	-
	CB12	NB	-	-	-
	CB13	NB	-	-	-

[A]All infected subjects were treated with emtricitabine, tenofovir, and ritonavir-boosted atazanavir

Table 4. TCR deep sequencing subjects.

Results

Cohort characteristics.

The study participants included 39 persons who were infected with HIV-1 as infants (22 male and 17 female) and an uninfected control group of 28 individuals (10 male and 18 female), ranging from 13.3 to 23.0 and 13.1 to 22.9 years of age respectively at the time of study (Figure 2A). Most infections (85%) were from mother to child transmission (including one by breastfeeding from a mother who acquired infection post-partum by blood transfusion), and the remainder (15%) were from blood transfusions in 1982 and 1983, including twin brothers who were described extensively in an earlier report (O. O. Yang et al., 2005). Among the infected individuals, none had the CCR5 Δ32 mutation; one and three respectively had HLA-B*27 and HLA-B*57 genotypes associated with slower disease progression (Carrington & O'Brien, 2003b), and one and none respectively had HLA-B*3502 and HLA-B*3503 associated with accelerated disease progression. All infected participants were receiving combination antiretroviral therapy (ART) at the time of study; 18 had plasma viremia <50 HIV-1 RNA copies/mL (uVL group, 11 male and 7 female) and 21 had plasma viremia ≥ 50 HIV-1 RNA copies/mL (dVL group, 11 male and 10 female). Most (77%) of the HIV-1 infected individuals had clinical or laboratory evidence of immunodeficiency (CDC class B or C) in the past (Figure 2B), although imaging revealed relatively normal thymic size overall (Figure 2C). Thus, the infected individuals represented a group of long term survivors of whom most had sustained clinically significant immunodeficiency due to HIV-1 infection at some point.

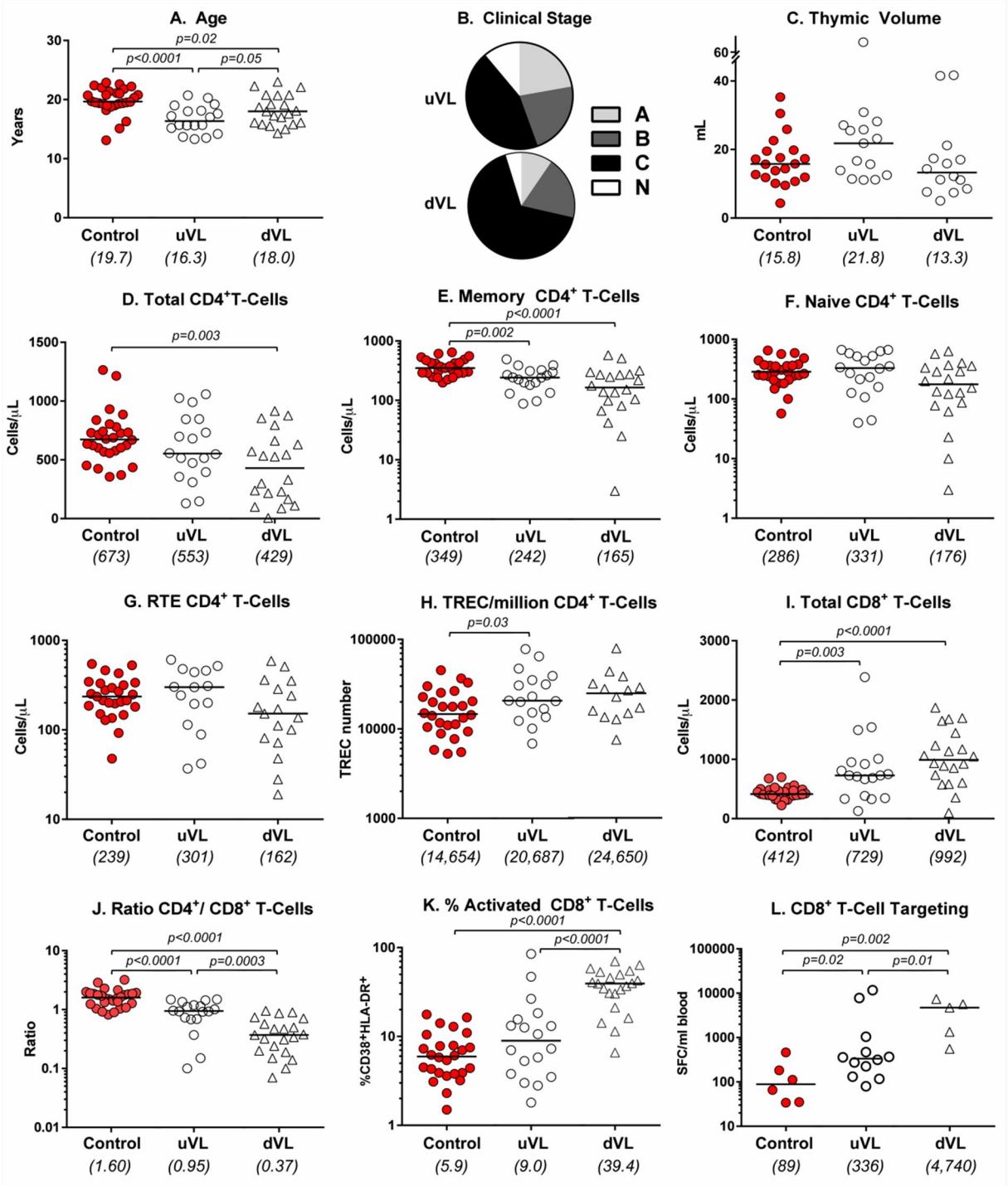

A. Age
p=0.02
p<0.0001
p=0.05
Years
Control (19.7)
uVL (16.3)
dVL (18.0)
B. Clinical Stage
uVL
dVL
A
B
C
N
C. Thymic Volume
mL
Control (15.8)
uVL (21.8)
dVL (13.3)
D. Total CD4+T-Cells
p=0.003
Cells/μL
Control (673)
uVL (553)
dVL (429)
E. Memory CD4+ T-Cells
p<0.0001
p=0.002
Cells/μL
Control (349)
uVL (242)
dVL (165)
F. Naive CD4+ T-Cells
Cells/μL
Control (286)
uVL (331)
dVL (176)
G. RTE CD4+ T-Cells
Cells/μL
Control (239)
uVL (301)
dVL (162)
H. TREC/million CD4+ T-Cells
p=0.03
TREC number
Control (14,654)
uVL (20,687)
dVL (24,650)
I. Total CD8+ T-Cells
p<0.0001
p=0.003
Cells/μL
Control (412)
uVL (729)
dVL (992)
J. Ratio CD4+/ CD8+ T-Cells
p<0.0001
p<0.0001
p=0.0003
Ratio
Control (1.60)
uVL (0.95)
dVL (0.37)
K. % Activated CD8+ T-Cells
p<0.0001
p<0.0001
%CD38+HLA-DR+
Control (5.9)
uVL (9.0)
dVL (39.4)
L. CD8+ T-Cell Targeting
p=0.002
p=0.02
p=0.01
SFC/ml blood
Control (89)
uVL (336)
dVL (4,740)

Figure 2. Clinical and immune parameters of study participants.

The HIV-1-infected participants included 39 persons, of whom 18 had plasma viremia <50 HIV-1 RNA copies/mL (uVL group, 11 male and 7 female) and 21 had plasma viremia ≥ 50 HIV-1 RNA copies/mL (dVL group, 11 male and 10 female), who were compared to a control group of healthy uninfected persons (10 male, 18 female) of similar ages (A). Most of the infected persons had had symptomatic disease in the past (B). Evaluated parameters included thymic volume (C), concentrations of blood CD4+ T-cells (D) and their characteristics (E-H), blood CD8+ T-cell concentrations (I) and the ratio of CD4+ to CD8+ T-cells (J), and CD8+ T-cell activation (K) and HIV-1 targeting (L). Filled circles represent uninfected control subjects; unfilled circles, represent uVL participants with plasma HIV-1 RNA <50 copies/ml; open triangles represent dVL individuals with plasma HIV-1 RNA 50 to<400 copies/ml. Statistically significant results ($p<0.05$), as determined by Mann-Whitney U Tests, are indicated. Bars indicate median values.

Many long term survivors of perinatal HIV-1 infection have relatively normal total and naïve CD4$^+$ T-cell concentrations on ART, despite generally depressed levels of memory CD4$^+$ T-cells.

At enrollment, peripheral blood CD4$^+$ T-cell levels were lower overall in the HIV-1-infected persons versus uninfected controls (mean 514 versus 686 cells/μL blood respectively), although the uVL group had levels similar to the controls (mean 601 versus 686 CD4$^+$ T-cells/μL respectively, Figure 2D). Examining the CD4$^+$ T-cell population phenotypically, both HIV-1-infected groups exhibited significant depletion of the memory (CD45 RA$^-$) subset (Figure 2E). By contrast, the naïve (CD45RA$^+$/CD27$^+$) subset was relatively normal to elevated in the uVL group and slightly reduced in the dVL group (neither statistically significantly different) (Figure 2F). More detailed analysis of the naïve CD4$^+$ T-cell population suggested overall normal levels of recent thymic emigrants (CD45RA$^+$/CD31$^+$)(Kimmig et al., 2002) in the μVL group and normal to reduced levels in the dVL group compared to controls (no statistically significant differences, Figure 2G). Furthermore, the frequency of the total CD4$^+$ T-cell population with T-cell receptor excision circles (TRECs) was elevated in both uVL and dVL groups compared to controls (statistically significantly only for the uVL group, Figure 2H), suggesting higher percentages of cells produced in the thymus (versus peripheral homeostatic proliferation). As a whole, these data demonstrate that these long term survivors of perinatal HIV-1 infection had depleted levels of memory CD4$^+$ T-cells, but generally exhibited quantitative restoration of naïve T-cell populations via increased thymic output on ART.

Despite suppression of viremia by treatment, perinatally-infected individuals have evidence of ongoing HIV-1-driven immune activation.

Compared to controls, both groups of HIV-1-infected subjects had significantly elevated blood $CD8^+$ T-cells levels (Figure 2I). Examining the ratio of $CD4^+$ to $CD8^+$ T-cells, it was apparent that the relative increase of $CD8^+$ T-cells and decrease of $CD4^+$ T-cells was especially marked in the dVL group (Figure 2J), suggesting an association between abnormality in the $CD8^+$ and $CD4^+$ T-cell compartments. Additionally, $CD8^+$ T-cell activation ($CD38^+$/HLA-DR^+) was increased in both infected groups versus the control group, significantly greater in the dVL versus uVL group (Figure 2K). Finally, screening of participants with <400 HIV-1 RNA copies/mL plasma (12 from the uVL group, 5 from the dVL group) for $CD8^+$ T-cell responses against the whole HIV-1 proteome (Ibarrondo et al., 2005) (Figure 2L) revealed persisting responses (predominately targeting Gag and Nef proteins similarly to infected older adults (Frahm et al., 2004))(not shown) in most persons despite undetectable or low viremia (between 50 and 400 copies/mL). Lower blood levels of memory $CD4^+$ T-cells were seen in infected individuals with higher levels of $CD8^+$ T-cell activation (Figure 3A), and there was a significant inverse correlation between the number of naïve $CD4^+$ T-cells and the plasma concentration of IL-7 in the HIV-1 infected group (Figure 3B); no such correlation was seen in the uninfected control group. These results suggest persistent generalized immune activation was present and was associated with ongoing loss of memory $CD4^+$ T-cells and secondary enhanced homeostatic proliferation of naïve $CD4^+$ T-cells in addition to the enhanced thymic output suggested by the data above.

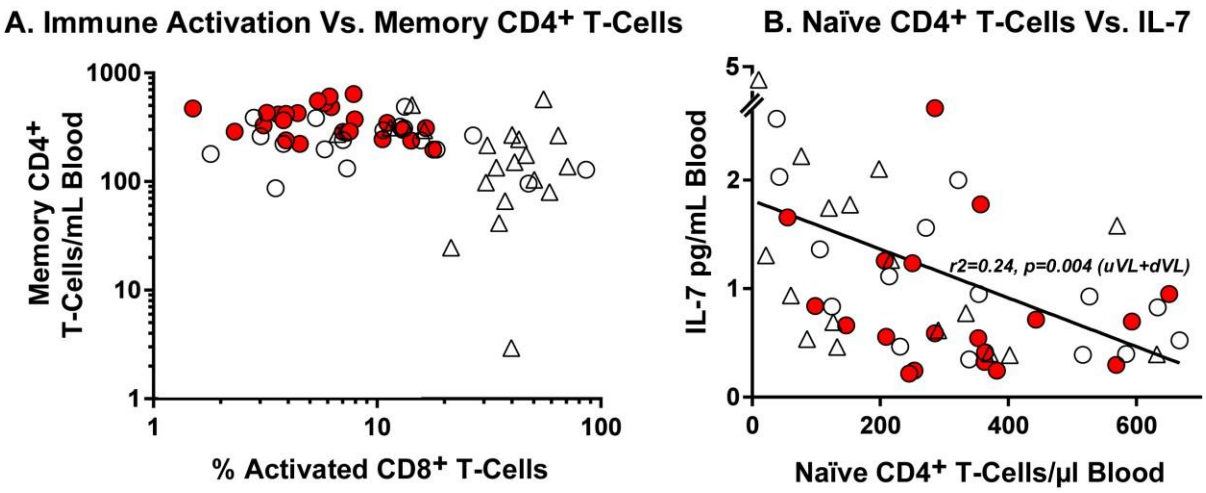


Figure 3. Relationship of immune activation to memory CD4+ T-cell loss, and resulting homeostatic proliferation of CD4+ T-cells.

A. Systemic immune activation, as reflected by CD8+ T-cell co-expression of activation markers, is plotted against blood levels of memory CD4+ T-cells (CD45RA-, including central and effector memory subsets). B. The relationship of blood levels of naïve CD4+ T-cells to plasma levels of the homeostatic cytokine IL-7 is plotted. Linear regression regression line and its associated p value is indicated for relationship between naïve T-cells and plasma IL-7 concentrations of HIV infected subjects. In both panels, filled circles represent uninfected control subjects, unfilled circles, represent uVL participants, and open triangles represent dVL individuals.

The long term survivors of perinatally HIV-1 infection exhibit increased $CD4^+$ T-cell receptor diversity and breadth.

To evaluate thymopoiesis more qualitatively, we examined the TCR repertoire of the $CD31^+$ subset of $CD4^+$ T-cells, which represent thymic emigrants and their early progeny because CD31 is lost after a few cycles of homeostatic proliferation. Quantitative spectratyping analysis (Balamurugan et al., 2008; O. O. Yang et al., 2005) revealed Gaussian distributions of TCR size populations for three control umbilical cord blood samples, as expected for unperturbed native populations (Balamurugan et al., 2010) (see Figure 4A). TCR repertoires of control subjects and uVL individuals also showed generally Gaussian distributions (data not shown), suggesting grossly diverse TCR production.

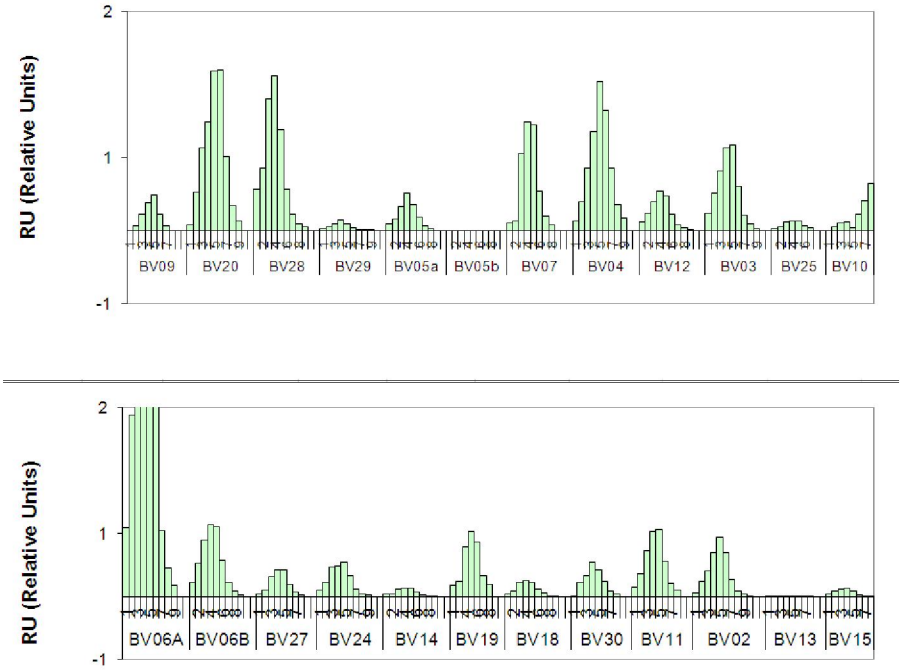
To better define the diversity of TCR production, we performed deep sequencing analysis of TCR families BV03, BV19 and BV29 (IMGT nomenclature), selected for having relatively consistent representation of ~5% of total BV families in $CD31^+CD4^+$ T-cells from representative uVL and control study participants and 3 cord blood specimens ((see Table 4, see Table 5 and See Figure 4B). The Shannon entropy index (S) for raw sequences (initially excluding BN02 (an uninfected control)) and cord blood CB13, which had insufficient sampling), ranged from 14.3 to 16.3 for CB, 11.3 to 14.1 for controls, and 13.6 to 14.1 uVL. The Shannon index of the estimated histogram from filtered sequences ranged from 16.8 to 17.2 for CB, 13.2 to 13.5 for uninfected controls, and 13.8 to 14.1 for uVL (see Table 6), indicating that TCR diversity was greatest in CB and least in controls ($p<0.01$). The two samples initially excluded (control BN02 and cord blood CB13) were also consistent with this pattern (Table 6).

As sample sizes could have biased estimates of Shannon index even after simple corrections, we also analyzed the TCR repertoire using rarefaction curves plotting the number of

unique species found in random subsamples of the total sequence population of all three BV families (see Figure 5), which revealed the same relative pattern of TCR diversity being highest in CB, intermediate in uVL subjects, and least in control subjects ($p = 0.018$). These differences also held true for BV families considered individually ($p = 0.041$, see Figure 6).

As a third approach to confirming the diversity comparisons between groups, we also performed analyses using random subsamples 24,000 sequences (corresponding to the smallest sampling size, obtained for CB13) from each individual sequence set. The number of discrete sequences, Shannon index, and fraction of singletons (number of sequences observed only once divided by the total number of observed sequences) were assessed (Table 7). Again, all parameters revealed the pattern of highest TCR diversity in CB followed by uVL, both greater than uninfected control persons ($p<0.01$). The HIV-1-infected individuals had overall more species and a higher fraction of singleton sequences. Additional analyses examining the abundance of rare and common sequences (see Figures 7 and 8) corroborated these results, supporting the overall conclusion that the recent thymic emigrant $CD4^+$ T-cells of uVL subjects had a broader TCR repertoire than uninfected subjects.

A.



B.

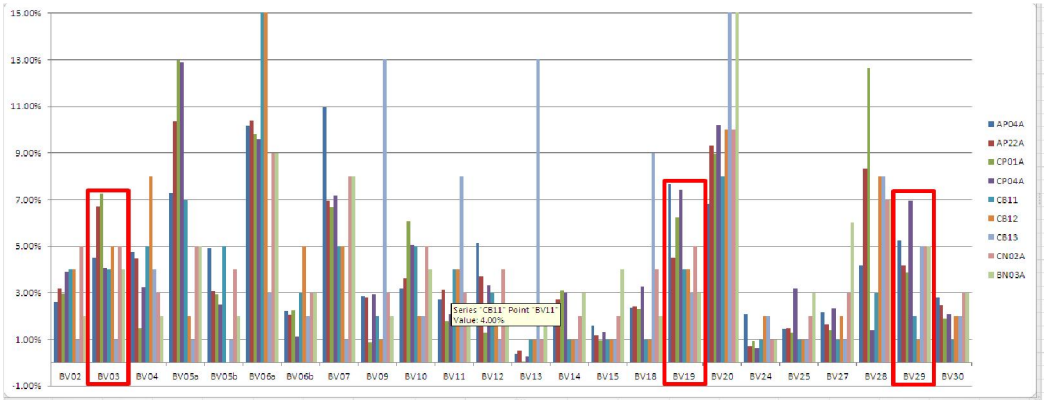


Figure 4: BV usage in cells from umbilical cord specimens and study subjects selected for TCR pyrosequencing analysis.

(A)) Representative quantitative spectratyping analysis of an umbilical cord blood sample. (B) The Spectratype of each CB, HIV+ uVL+ and seronegative control used in the pyrosequencing

was analyzed for their BV family usage. Families BV03, BV19 and BV29 had a uniform usage of approximately 5%.

	Perinatal HIV+			HIV-	Umbilical Cord Blood				
	AP04	AP22	CP04	CN02	CB11	CB12	CB13	Mean	Standard Deviation
BV03	4.5%	6.7%	4.1%	5.0%	4.0%	5.0%	1.0%	4.3%	1.7%
BV19	7.6%	4.5%	7.4%	5.0%	4.0%	4.0%	3.0%	5.1%	1.7%
BV29	5.2%	4.2%	7.0%	5.0%	2.0%	1.0%	5.0%	4.2%	2.3%

Table 5. BV usage of in CD4+ T cells from donors selected for TCR sequence analysis by pyrosequencing.

Each subject utilized about 5% of the 3 BV families chosen for 454-pyrosequencing (BV3, BV19, BV29). Each BV family was chosen based on our ability to over-sample at least 5 times during 454 sequencing, 10% usage of the BV repertoire and usage by each subject was comparable.

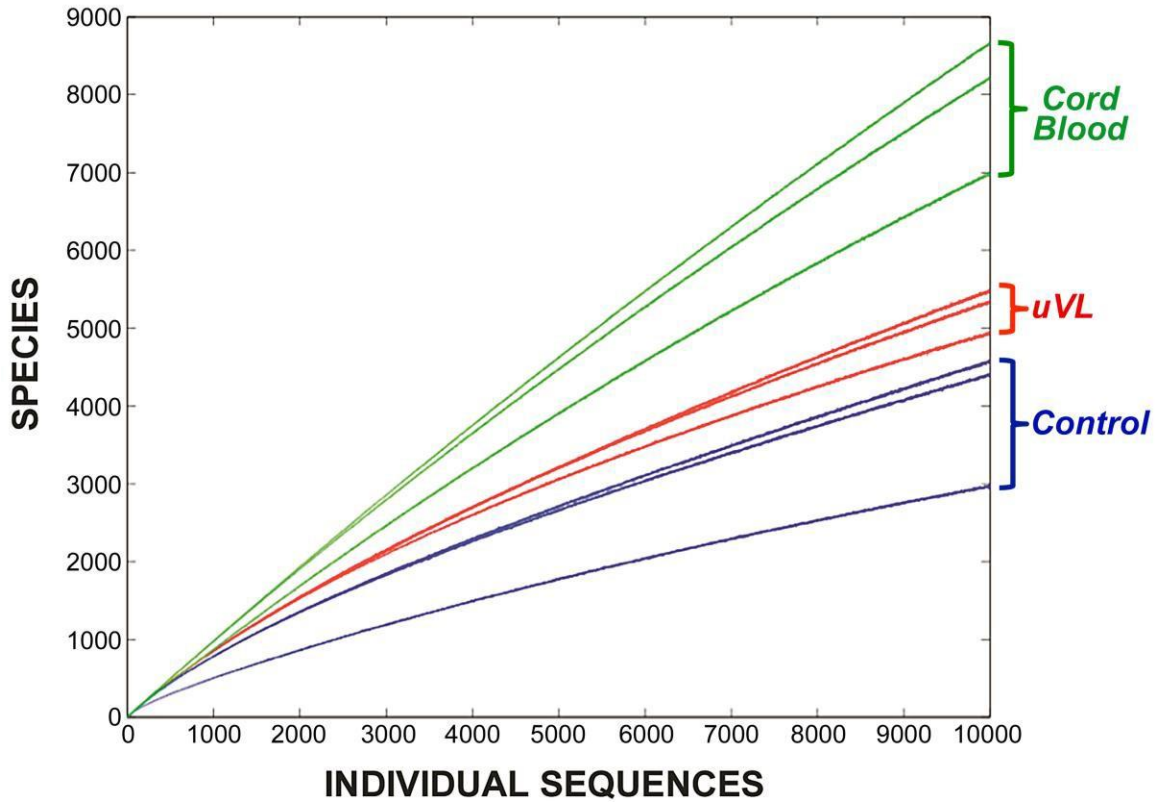


Figure 5. Rarefaction analysis of TCR species in CD31+ naïve CD4+ T-cells.

Rarefaction curves are plotted for TCR sequences isolated from each sample, indicating that diversity is greater in the uVL subjects compared to uninfected control subjects (considering the number of species at x=9809, p=0.02).

	uVL			Uninfected			Cord Blood		
	AP04	**AP22**	**CP04**	**CN13**	**CN02**	**BN02**	**CB12**	**CB11**	**CB13**
BV03	27537 (10.79)	32125 (12.62)	37942 (11.84)	112979 (11.71)	54912 (11.23)	25396 (9.63)	47801 (15.06)	82367 (15.95)	10987 (14.23)
BV19	25337 (12.27)	13015 (12.81)	23497 (11.69)	72688 (12.99)	30868 (11.02)	4734 (10.46)	10868 (14.01)	73392 (15,00)	11325 (16.86)
BV29	38031 (14.07)	3377 (14.23)	24832 (14.45)	12789 (14.08)	33727 (12.92)	2883 (11.77)	29607 (16.81)	19827 (14.66)	9627 (15.59)
Overall	90905 (14.11)	48517 (13.78)	86271 (14.14)	198456 (13.50)	119507 (13.23)	33013 (10.79)	88276 (17.15)	175586 (16.82)	31939 (16.86)

Table 6. Numbers of TCR sequences obtained by deep sequencing and Shannon Diversity Indexes (parentheses).

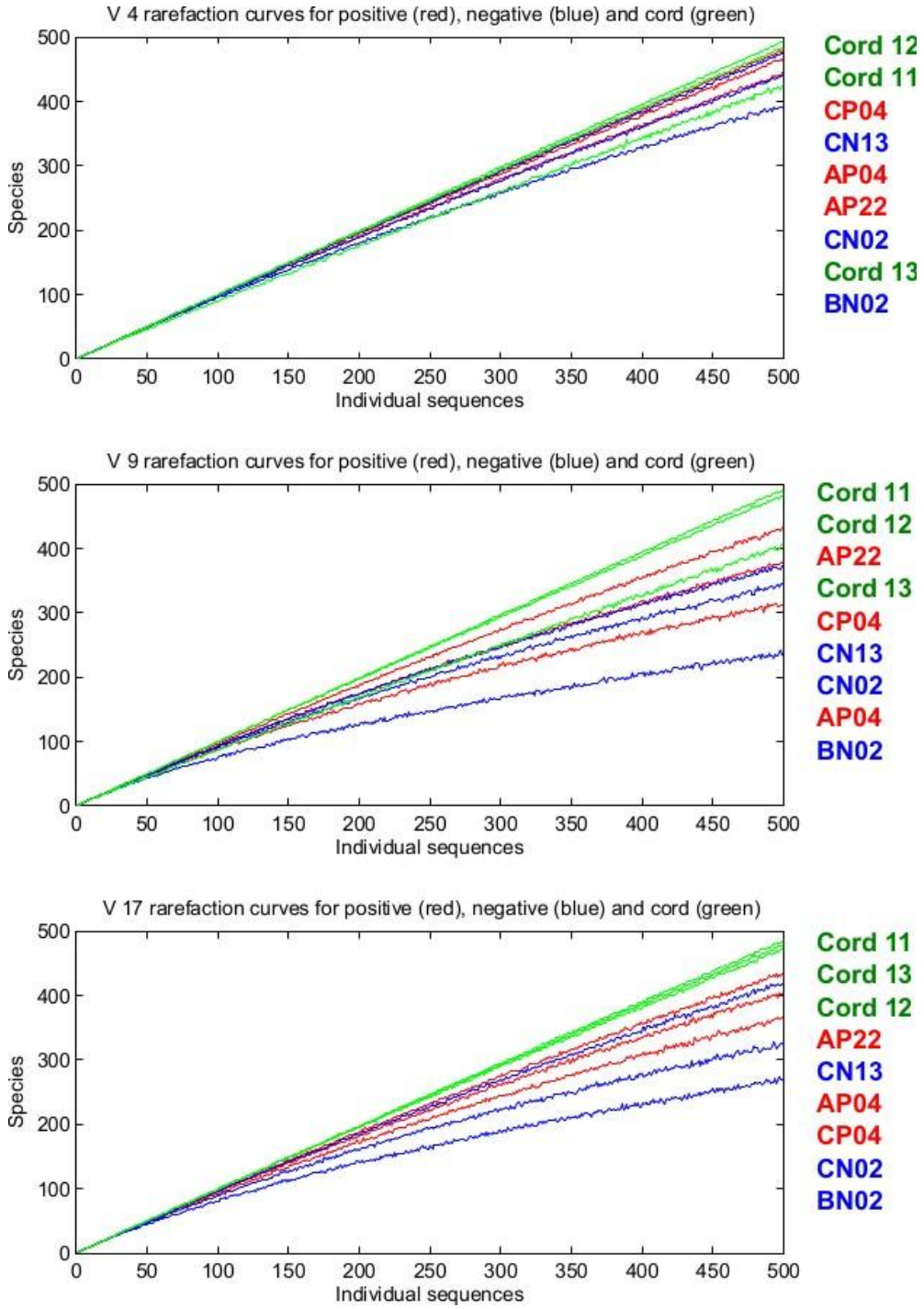


Figure 6. Rarefaction curves computed on the nine samples, divided per BV families

Average count of species - subsampled data

	Positive patients			Negative patients			Cord samples			Sample size
	AP04	AP22	CP04	CN13	CN02	BN02	Cord12	Cord11	Cord13	(#sequences)
BV 03	3047	4075	3638	3257	3176	2684	6967	8460	7354	9958
BV 19	1853	2296	1637	2026	1405	1412	3060	3153	3663	3718
BV 29	1684	1812	1867	1853	1441	1416	2047	1934	1767	2087
BV03/19 /29	10849	10775	11610	9576	9156	7129	18858	19384	20012	24369

Average Shannon index - subsampled data

	Positive patients			Negative patients			Cord samples			Sample size
	AP04	AP22	CP04	CN13	CN02	BN02	Cord12	Cord11	Cord13	(#sequences)
BV 03	9.69	11.17	10.50	10.29	9.99	8.73	12.51	12.92	11.48	9958
BV 19	10.27	10.78	9.83	10.47	9.36	8.93	11.47	11.53	11.83	3718
BV 29	10.59	10.74	10.80	10.79	10.25	10.19	10.99	10.87	10.16	2087
BV03/19 /29	12.43	12.39	12.47	11.83	11.78	9.93	13.98	14.08	13.39	24369

Average fraction of singletons - subsampled data

	Positive patients			Negative patients			Cord samples			Sample size
	AP04	AP22	CP04	CN13	CN02	BN02	Cord12	Cord11	Cord13	(#sequences)
BV 03	0.25	0.30	0.28	0.26	0.26	0.22	0.55	0.74	0.69	9958
BV 19	0.37	0.47	0.34	0.41	0.30	0.32	0.69	0.73	0.97	3718
BV 29	0.67	0.76	0.81	0.79	0.52	0.52	0.96	0.86	0.83	2087
BV03/19/29	0.34	0.34	0.38	0.32	0.30	0.23	0.66	0.66	0.78	24369

Table 7. Statistics of the TCR data after subsampling.

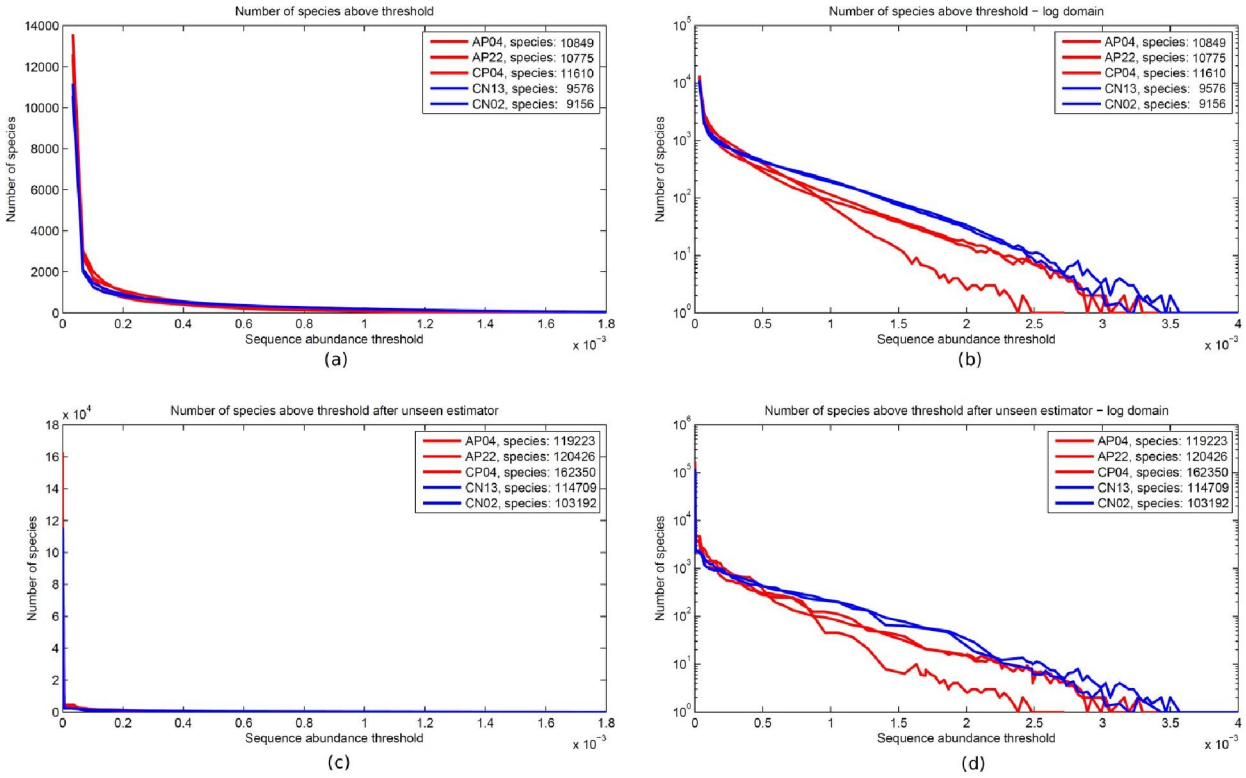


Figure 7. Abundance levels of the different species.

(a) The y axis indicates the number of species above the abundance threshold on the x axis. (b) The same representation, showing the y axis in log domain for a better insight into high frequent species. (c) and (d) depict the same graphs, after the histogram of TCR species have been reconstructed with the unseen estimator

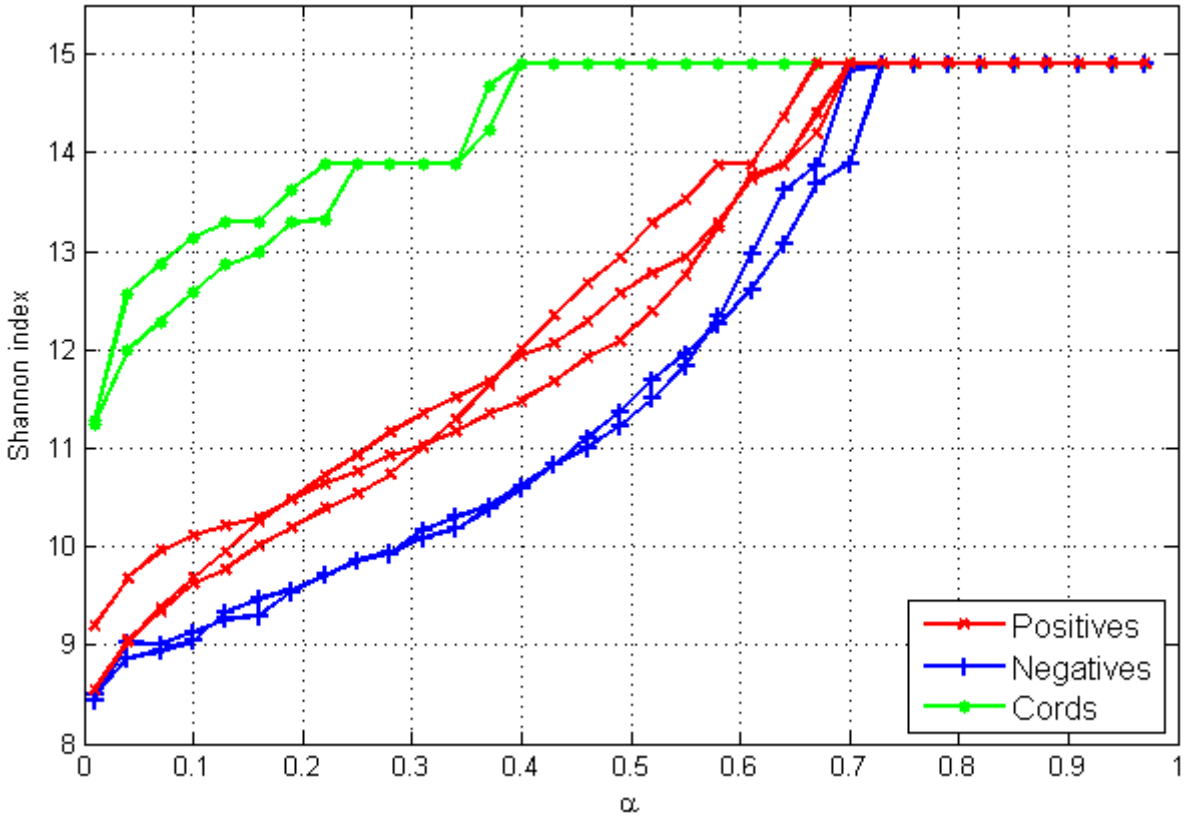


Figure 8. Nuanced patterns in the histogram of species counts.

From the subsampled species histogram (sorted by descendent frequencies) of each HIV positive or HIV negative subject, or the cord blood samples, we computed the Shannon index in contiguous bins centered in α, each region occupying 2% of total TCRs.

Discussion

Immune reconstitution following initiation of antiretroviral therapy of HIV-1 infection clearly differs between children and adults. In children, expansion of naïve T-cell populations begins soon after initiation of ART, whereas redistribution and expansion of memory T-cell populations initially predominate in adults, in whom increases in naïve $CD4^+$ T-cells are typically seen only months after therapy (Autran et al., 1997; Gibb et al., 2000; Harris et al., 2005; Jankelevich et al., 2001; Pakker et al., 1998; Resino et al., 2003; Resino et al., 2006). To investigate the nature of these differences, we performed an extensive survey of thymic function markers and found evidence of robust thymopoiesis in long-term survivors of perinatal infection (>13 years) receiving ART, compared to healthy controls of similar age. The majority in the HIV-1 infected group had evidence of abundant thymic tissue and active thymopoiesis, with naïve $CD4^+$ T-cell levels and markers (TREC) suggesting elevated production compared to uninfected persons. This was further supported by deep sequencing of TCRs in the naïve $CD4^+$ T-cell population, which demonstrated not only preserved but enhanced diversity in these long term survivors of perinatal infection versus uninfected persons. These findings are consistent with previous studies of immune reconstitution during ART (Gibb et al., 2000; Jankelevich et al., 2001; Resino et al., 2003; Resino et al., 2006; Sleasman et al., 1999), and observations that "thymic rebound" (expansion of histologically normal thymic tissue occurring after illness, stress, and cancer chemotherapy) is more common in children than adults, likely underlying age-dependent recovery of lymphocyte populations after cancer chemotherapy(Mackall et al., 1995). Of note a recent report demonstrated that restoration of naïve T-cell populations may be impaired in adult individuals with advanced HIV-1 infection, possibly due to loss of the normal stromal fibroblastic reticular cell (FRC) network in lymphoid tissue (Zeng, Southern, et al., 2012). Given

the long average duration of infection (~17 years) and histories of AIDS-defining illness in more than half of our study participants, this underscores the likely importance of age in HIV-1-induced damage to secondary lymphoid tissues and/or its reversal during ART.

As a whole, the data presented above suggest a model in which HIV-1 replication (and/or secondary immune dysregulation) drives loss of memory $CD4^+$ T-cells, leading to compensatory supranormal thymic output of naïve $CD4^+$ T-cells (see Figure 9) in these youths. Evidence of ongoing immune activation and replication is provided by the high fraction of $CD38^+$ HLA-DR^+ $CD8^+$ T-cells (Figure 2K) (Giorgi et al., 1999) and the persistence of $CD8^+$ T-cell responses to HIV (Figure 2L) which indicate ongoing HIV antigen production. We note that the persistence of broad antiviral responses to HIV during cART has previously been observed in other perinatally-infected persons and contrasts with the situation in older adults, in whom complete decay of these responses is common. (Ching et al., 2007; Frahm et al., 2004; Kalams et al., 1999; Ogg et al., 1999; Spiegel et al., 1999)

The Naïve $CD4^+$ T-cell population is composed of both $CD31^+$ and $CD31^-$ $CD45RA^+$ cells and homeostatic proliferation is thought to transform the former into the latter, resulting in a decrease in the concentration of TREC in $CD4^+$ T-cells. (Kilpatrick et al., 2008; Kimmig et al., 2002) Within the naïve $CD4^+$ T-cell population, the population size of the recent thymic emigrant ($CD31^+$ $CD45RA^+$) $CD4^+$ T-cells (Naïve RTE) is maintained in most individuals studied (Figure 2G), suggesting increased thymic output occurs to replace loss of naïve ($CD27^+CD45RA^+$) cells that differentiate into memory $CD4^+$ T-cells. The hypothesis that supranormal thymopoiesis occurs is also supported by our observation of enrichment of TREC in CD4+ T cells of uVL subjects (Figure 2H) and the enhanced diversity of TCRs in the $CD31^+$ $CD4^+$ T-cell population observed by pyrosequencing.

TCR breadth is likely an important clinical factor, as severe AIDS-defining illnesses may occur years after blood $CD4^+$ T-cell concentrations have "reconstituted" to seemingly safe levels in both children and adults receiving ART. (Komanduri et al., 2001; Krogstad et al., 2015; Zoufaly et al., 2011) Our detailed analysis of TCR repertoire substantiates and extends prior studies of HIV-1-infected adults in whom ART does not generally restore $CD4^+$ T-cell numbers to normal or fully normalize skewing of the TCR repertoire, as assessed by various tools ranging from relatively indirect to more precise measures of diversity such as CDR3 size distributions ("spectratyping" or "immunoscoping"), DNA hybridization kinetics ("Amplicot"), multiplex amplification of V-J segments, and CDR3 sequencing,(Baum & McCune, 2006; Baum et al., 2012; Connors et al., 1997; Killian et al., 2004; Pannetier et al., 1993; O. O. Yang et al., 2005) and one study in children/adolescents indicating that perturbations in TCR diversity of naïve cells begin to resolve within several months of therapy.(Yin et al., 2009) Because HIV-1 infection is typically associated with disrupted thymic architecture, involution of the thymic cortical epithelial space, and fibrosis of the peripheral lymph nodes that are required for expansion of thymic emigrants,(Haynes & Hale, 1999; Ho Tsong Fang, Colantonio, & Uittenbogaart, 2008; Schacker et al., 2002; Zeng, Southern, et al., 2012) the novel finding of substantially increased TCR breadth in the recent thymic emigrant $CD4^+$ T-cell compartment of our HIV-1-infected subjects was surprising. Supporting this observation, recent trials of administering recombinant human IL-7 to infected persons on ART have demonstrated enhanced naïve $CD4^+$ T-cell production accompanied by indirect measures of increased TCR diversity (Y. Levy et al., 2012).

Our study has several limitations, including its cross-sectional nature and the ~2.3 year average age difference between the groups of HIV-1-infected and control individuals. These concerns are mitigated by evidence that thymic architecture and function change little over this short age span (Douek et al., 1998; Harris et al., 2005; Haynes & Hale, 1999; Steinmann, Klaus, & Muller-Hermelink, 1985). Moreover, we found no evidence of a correlation between age and the number of $CD31^+$ T cells in any of the three groups studied (Controls, uVL or dVL individuals) (data not shown), consistent with earlier reports indicating that $CD31^+$ T cells decrease less than 50% between 20 and 60 years of age.(Kilpatrick et al., 2008; Kimmig et al., 2002) We also observed stability of thymopoiesis parameters over 1 to 3 years in our cohort (TREC, number and fraction of blood $CD31^+CD4^+$ T-cells, manuscript in preparation). Our assessment of TCR repertoire was limited to three BV families representing about 5% of naïve $CD4^+$ T-cells, and may not reflect the total functional repertoire, although there is no reason to suspect BV family-specific differences. Despite these limitations, the composite data support the sanguine view that thymic function and naïve T-cell homeostasis may be restored by prolonged ART in adolescent and young adult survivors of perinatal infection.

Overall, our study suggests that thymic function is resilient in most persons, even~17 years after HIV-1 infection that occurred when immunologically immature. Despite prior clinically significant immunosuppression (including AIDS defining illness and conditions indicative of moderate immune deficiency), ART appears to allow recovery of an apparently adequate TCR repertoire in many survivors of perinatal infection who have reached young adulthood, which is encouraging in light of numerous studies showing damaging effects of HIV-1 on the thymus. This appears to differ from persons infected as adults, and it is unclear whether the difference is simply due to better age-related regenerative potential and immunologic reserve,

or perhaps a difference in viral persistence or reservoirs specific to infection when immunologically immature. While the data are hopeful that long term survivors of perinatal infection are well compensated immunologically, there remain questions about whether the supraphysiologic TCR repertoire could in fact reflect an abnormality such as reduced stringency in thymic T-cell negative selection, and whether heightened thymic output will remain sustainable over longer periods of time if persistent HIV-1-driven memory $CD4^+$ T-cell loss continues. Indeed there appeared to be some persons in our cohort with low memory and naïve $CD4^+$ T-cell levels. Additional studies will be needed to determine the extent to which normalization of TCR diversity in and other T-cell parameters is indicative of true restoration of normal immune function in the setting of prolonged HIV-1 infection, and to examine the impact of detectable HIV replication and residual HIV-specific immune responses on these processes.

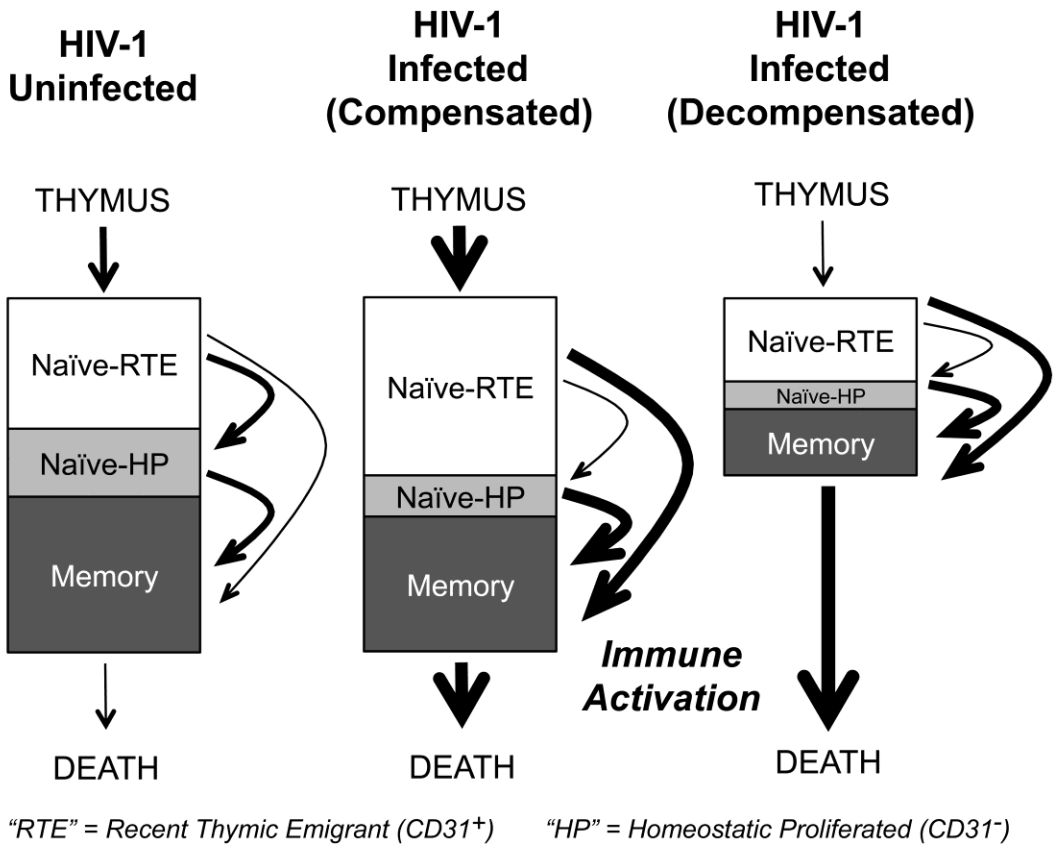


Figure 9. Schematic model of $CD4^+$ T-cell homeostasis in long term survivors of perinatal HIV-1-infection.

Box depicts the partitioning of $CD4^+$ T cells into three discrete populations following emigration from the thymus: recent thymic emigrant $CD31^+$ T cells (Naïve-RTE) that have not undergone peripheral expansion, naïve T-cells that have undergone homeostatic proliferation ($CD27^+$ $CD31^-$), and memory ($CD45RA^-$) cells. In the infected persons receiving ART, loss of memory (and possibly naïve) CD4+ T-cells is associated with enhanced thymopoiesis (thicker bold lines) and possibly less homeostatic proliferation in the naïve subset. The naïve T cell population is retained. In some individuals, this compensatory results in resulting in an increased fraction of Naïve RTE cells with relatively increased TREC content, and enrichment of TCR diversity.

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Authorship Contributions

Christian R. Aguilera-Sandoval helped design and carried out pyrosequencing studies and manuscript preparation. Paul Krogstad, Marvin Belzer, and Joseph A Church were responsible for the organization of the cohorts, subject recruitment and specimen collection, interactions with institutional review boards, and manuscript preparation. Paul Krosgtad also supervised all technical aspects of the study, apart from ELIspot and spectratyping. Otto Yang collaborated in design and implementation of the pyrosequencing studies and manuscript preparation, supervised spectratyping and ELISpot analysis. Paige Cooper and Diana Chen performed flow cytometry and spectratyping studies. M. Ines Boechat supervised and interpreted CT scan analysis, blinded to subject status. Nebojsa Jojic and Pietro Lovato helped analyze the pyrosequencing data.

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Chapter 3. Constructing HIV-Specific TCR Panels to Prevent Viral Escape

Introduction

CTLs, HIV, and Escape

Since the first recognized and treated case of HIV-1 at UCLA in 1983 (Gottlieb et al., 1981), HIV-1 has eluded our attempts at designing a successful vaccine and instead is a world epidemic that infects over 33 million individuals. One of the driving forces for HIV- 1's success is its ability to mutate and escape CTLs, as first noted in 1991 (Phillips et al., 1991). HIV-1's rate of viral replication and error during reverse transcription likely results in the generation of every combination of one or two mutations per day in an untreated person (Perelson, Essunger, & Ho, 1997), resulting in a catch-up game with our immune system, since even one amino acid mutation can abrogate CTL recognition (Bennett, Ng, Ali, & Yang, 2008a; Bennett et al., 2007b; O. O. Yang, Sarkis, Ali, et al., 2003). While HIV-1's mutation is rapid and stochastic, the mutants that survive are selected in a deterministic manner primarily in response to CTL immuno-pressure (Allen et al., 2005b; Borrow et al., 1997; Goonetilleke et al., 2009b; O'Connor et al., 2004a). Although to a lesser extent, there are other immune cells that also drive non-env HIV-1 evolution, like B-cells which apply selective pressure via immunoglobulins (Watkins et al., 1993; Wei et al., 2003), NK cells, and cytolytic $CD4^+$ T cells (Brown, 2010; Burwitz et al., 2012; Soghoian & Streeck, 2010; Tang et al., 2010).

CTL immune-pressure begins as early as three weeks post-infection (Allen et al., 2000; Fernandez et al., 2005b; McMichael, Borrow, Tomaras, Goonetilleke, & Haynes, 2010b). This is preceded by HIV-1 infecting and/or depleting about 50% (Mehandru et al., 2004; Mehandru et al., 2007) of the 10^{10} $CD4^+$ T cells that reside in the GALT (Ganusov & De Boer, 2007a) and establishing a reservoir of multiple viral strains. Nevertheless, once a CTL response is mounted,

each individual will generate a CTL response targeting 14-19 epitopes of HIV-1 (Addo et al., 2003) (Frahm et al., 2004) that will result in the lysis of 20-30% of all infected cells (Asquith, Edwards, Lipsitch, & McLean, 2006) and from the mounted response, an average of five CTL responses will yield an infected cell death rate of about 0.1 d^{-1} (Asquith et al., 2006; Ganusov et al., 2011; Goonetilleke et al., 2009a). However, which epitopes are targeted by CTLs will depend on the host's HLA haplotype. HLA-B alleles *13, -B*27, and -B*57 (Crawford et al., 2009; X. Gao et al., 2001; Itescu et al., 1992; Sahmoud et al., 1993; Scorza Smeraldi et al., 1986) have been associated with greater viral control. This control may be due to CTL responses targeting epitopes whose escape variants have a weak selective advantage (Asquith, 2008), making escape more difficult. This is because the availability of escape from CTLs hinges on two factors – the efficacy of the CTL response and the replicative cost (fitness cost) that the virus incurs from reduction in stability and/or reduction in immune evasion. Large cross-sectional studies have shown that when CTL responses target epitopes with low entropy, the viremia observed during chronic infection is lower than when CTL responses target high entropy epitopes (Kiepiela et al., 2007). Another example is demonstrated by elite controllers that are HLA-B*13^{+} (Antoni et al., 2013; Emu et al., 2008). These persons' CTLs target semi-conserved epitopes in Gag (Honeyborne et al., 2007) and Nef (Harrer et al., 2005). Through this multi-epitope targeting, these persons are able to control viremia to undetectable levels without medication because they attack HIV-1 through two mechanisms, reducing Gag fitness (i.e., viral stability by targeting the structural proteins that make up the capsid) and targeting Nef (i.e., dampening HLA-downregulation) (Shahid et al., 2015) . However, the CTL responses mounted are always escaped because no one individual is able to produce enough CTLs to target all the variants of one epitope and thereby prevent escape.

Compartmentalization of CTL Escape Variants

Escape from CTLs can occur through compartmentalization to different regions in the body and through different mechanisms, making it even more difficult for a strong and persistent CTL response to be maintained. Prior studies have shown that the viral isolates found in blood differ from those found in the gut-associated lymphoid tissue (GALT) (Chun et al., 2008) and the central nervous system (CNS) (Beck et al., 2016a) (Doherty, 1995). The HIV-1 strains in the different compartments evolve differently and give rise to different escape isolates, such that the escape variants present in the CNS will differ from those in the periphery (Beck et al., 2016a). This in its own right poses a new set of problems. Escape variants that may have been dealt with in the bloodstream could be establishing a reservoir in the CNS (Beck et al., 2016a), and later, when the CTLs specific for those escape variants have waned or died out due to a lack of epitope presence, they can arise again and result in an increase in viremia.

Mechanisms for CTL Escape

HIV-1 utilizes different mechanisms to escape CTLs, these include (i) mutations within the epitope to reduce or prevent binding to HLA molecules, (ii) mutations within the epitopes to reduce or eliminate TCR binding, (iii) mutations triggering epitope degradation, and (iv) epitope flanking mutations that prevent the production of epitopes of adequate length (Allen et al., 2004; Draenert et al., 2004; Erickson et al., 2001; Fernandez et al., 2005a; Lazaro et al., 2011; Milicic et al., 2005; Troyer et al., 2009; Wolfl et al., 2008; Yokomaku et al., 2004; S. C. Zhang et al.,

2012). This project will deal with epitope mutations that reduce or eliminate TCR binding and mutations within the epitope to reduce or prevent HLA binding.

HIV-1 escape mutations that negatively affect HLA binding. HIV-1 will reduce or abrogate HLA binding by mutating the epitope's amino acids that bind to the HLA's anchor residues, usually situated in P2 and P8 or P9 (Allen et al., 2000) (Barouch et al., 2002; Katoh et al., 2016). When HIV-1 successfully mutates these amino acids, HLA binding affinity has been observed to decrease by 50-99% (Allen et al., 2000). In one study, one amino acid mutation of the SIV tat epitope, SL8, resulted in reduced HLA affinity after 8 weeks (Allen et al., 2000); the escape variant became fixed and dominant, and the HLA affinity was observed to have been reduced between 50-80% (Allen et al., 2000). In the KAFSPEVIPMF (Gag 162-172) epitope, the mutation A163G results in 1-log less affinity to HLA-B*57 (Yu et al., 2007). Epitope mutations that negatively affect HLA binding will result in escape variants evading CTL surveillance, since the CTLs' TCR will not bind to an pHLA complex and thus unable to lyse the infected cells.

HIV escape mutations that negatively affect TCR binding. Mutations within epitopes that reduce or prevent TCR binding are another mechanism utilized by HIV-1 to escape CTL recognition (Bennett, Ng, Ali, & Yang, 2008b; Bennett, Ng, Dagarag, Ali, & Yang, 2007a; O. O. Yang, Sarkis, Trocha, et al., 2003). The epitope's stability may not necessarily be impacted and HLA binding may not change, but due to CTLs' high specificity, one mutation within the epitope may result in reduced or complete lack of recognition by the TCR (Bennett et al., 2008b; Bennett et al., 2007a; O. O. Yang, Sarkis, Trocha, et al., 2003) (Barouch et al., 2000). Not all mutations will result in this phenomenon, but in order for these mutations to arise, they must have higher replicative capacity than the consensus sequence (Loh, Petravic, Batten, Davenport, & Kent, 2008). These mutations can rise during the acute and chronic phases of infection (Allen et al.,

2005a; Bimber et al., 2009; Bimber et al., 2010; Goonetilleke et al., 2009a; O'Connor et al., 2004b). Lower TCR binding will result in lower functional avidity, as defined by Bennett et al 2011 as "the sensitizing dose of peptide for 50% maximal activity (SD_{50})" and, thus, in the persistence of those escape variants (Sunshine et al., 2015). Mutations affecting TCR binding may confer TCR escape without incurring fitness costs (Sunshine et al., 2015). These mutations will occur in epitopes with higher entropy, where fitness costs are minor or absent. The constant change within these high entropy epitopes will result in a variation of factors that impact T cell immuno-dominance. The factors that impact and determine Immuno-dominance are described as (i) the affinity of the HLA or TCR, (ii) the frequency of CTL precursors and the TCR repertoire, (iii) the kinetics of expression and level of protein present, (iv) the efficiency of epitope processing, and (v) the magnitude of the response relative to the total response within each infected person at a given time point (Bihl et al., 2006; Chen, Anton, Bennink, & Yewdell, 2000; Le Gall, Stamegna, & Walker, 2007; Osuna et al., 2014; Probst et al., 2003; Schmidt et al., 2012; Schmidt et al., 2011; Tenzer et al., 2009). The shift of immuno-dominance results from the immuno-targeting of high entropy epitopes in Nef and Env to more conserved epitopes like KF11 (Altfeld et al., 2006; Altfeld et al., 2001; Brumme et al., 2008; Pereyra et al., 2014). Because of the rapid and constant epitope mutations that result in decreased TCR binding, an infected person is unable to produce at any one given time enough TCRs to target all possible escape variants and therefore prevent viral escape and accomplish complete immune control. Fortunately, despite the constant mutations that reduce TCR binding, each TCR does not exclusively target one epitope, but is able to target other variants of that epitope (Bennett et al., 2008b).

As we have surmised, HIV-1 escapes CTLs through different mechanisms in an effort to evade the lysis of infected cells and to promote continued dissemination. However, other issues remain unaddressed – for example, when does escape happen, at what rate does this escape occur, and can the virus revert back to its consensus sequence once a CTL response to the consensus sequence dissipates.

Timing of CTL Escape Mutations

HIV-1 acute phase. The timing and rate of HIV-1 escape is influenced by the interplay among the level of viremia, the availability of target cells, immune pressure, and the fitness cost associated with the escape mutation. Early studies have shown that HIV-1 and SIV can escape CTLs during acute and chronic infection (Allen et al., 2005a; Barouch et al., 2003; Bimber et al., 2009; Bimber et al., 2010; Ganusov et al., 2011; Goonetilleke et al., 2009a; O'Connor et al., 2004b; Roberts et al., 2015). Later, other studies have shown that the majority of escape happens during acute infection, when it occurs at a higher rate; this might be due to lower initial fitness costs and a large number of target cells (Asquith et al., 2007; Davenport, Loh, Petravic, & Kent, 2008; Ganusov et al., 2011; Leslie et al., 2004), such as the 10^{10} $CD4^{+}$ T cells found in the GALT during the start of infection(Ganusov & De Boer, 2007b). Prior to a CTL response, viral diversity is primarily driven stochastically due to the large number of target cells; however, once the CTL response is initiated, viral evolution is primarily driven by CTL immuno-pressure (Allen et al., 2005a; O'Connor et al., 2004b). Escape mutations accumulate in CTL epitopes as early as 3 to 4 weeks post-infection (Allen et al., 2000; McMichael, Borrow, Tomaras, Goonetilleke, & Haynes, 2010a; Walsh et al., 2013), highly correlating with the time when a CTL response first emerges. Deep sequencing supports the notion that escape primarily occurs during acute infection, since it has revealed that the majority of early mutations leading to escape

were CTL epitopes or had an HLA footprint (Henn et al., 2012). Another reason for the high level of escape observed during acute infection is due to immunodominant CTL responses targeting high entropy epitopes in Nef, Env, and Tat (M. K. Liu et al., 2013). While there is a targeting of semi-conserved epitopes during acute infection, these responses tend to be sub-dominant. If cART is not commenced shortly after infection, early escape mutations that have very little fitness costs but (i) are resistant to CTL immunodominant responses and (ii) have a faster fixation rate will populate the latent reservoir and will re-emerge during the chronic phase, when CTL responses have weakened and CTLs are low in their lytic granules due to $CD4^+$ T cell loss (Appay et al., 2000; Bevan & Braciale, 1995; D. Zhang et al., 2003) (Asquith et al., 2006). Latent reservoir being defined as HIV strains infecting and residing in transcriptionally inactive cells. While most of the literature agrees that the majority of escape occurs during acute infection, it is noteworthy that a handful of studies have observed that, in their cohorts, the incidence of CTL escape was low during the acute phase of infection (Roberts et al., 2015). Some of these differences can be explained by the fact that they studied HIV-1 Clade C, which has been shown to have higher transmission efficiency and fitness (Rodriguez et al., 2009), or by the fact that, while analyzing their cohort, they began their analysis not post-infection but, rather, at seroconversion, which can be 1-3 weeks after infection, when a lot of the escape variants have already occurred. Lastly, the studies did not look at whether the transmitted strains had pre-existing escape variants and/or compensatory mutations.

HIV-1 chronic phase of infection. CTL escape is also observed during the chronic phase of infection, although the rate of escape decreases by 10- to 100-fold (Ganusov et al., 2011). This might be due to lower amounts of target cells, decreased immune CTL pressure due to weakening of CTL responses, and loss of $CD4^+$ T cells. Nevertheless, escape has been observed

even after 2 years or more despite effective immune control (Barouch et al., 2003) since (i) there is sufficient ongoing viral replication during undetectable levels of viremia, (ii) the number of target cells (i.e., $CD4^+$ T cells) increases with effective immune control, and (iii) the higher fitness costs that are associated with late CTL escaped are out-weighed by CTL immuno-pressure. Reactivated latently infected cells can be another source from which escape can occur, since they begin producing new virions that bear a large number of CTL resistance mutations with low fitness costs (Marsden & Zack, 2015). Lastly, CTL escape can occur in the chronic phase of HIV-1 infection since certain epitopes generate only partial immune escape (Lewis, Dagarag, Khan, Ali, & Yang, 2012)variants during acute infection or early chronic infection and only fully escape CTLs after one or more compensatory mutations have been fixed (Brockman et al., 2007; Chopera et al., 2011; Crawford et al., 2009; Crawford et al., 2007b; Jamieson et al., 2003; Kelleher et al., 2001a; D. Liu et al., 2014; Martinez-Picado et al., 2006; Seki & Matano, 2011).

Compensatory Mutations and their Effect on CTL Escape

The timing and rate of escape can also be influenced by additional factors, such as the presence of compensatory mutations, reversions, and the HLA expression of the person transmitting the virus and the recipient of the virus. Compensatory mutations are mutations that will restore replication capacity that was previously lost or mutations that allow replication by preceding an otherwise lethal mutation. Compensatory mutations can be found within the epitope, flanking the epitope, or in distal regions (Brockman et al., 2007; Chopera et al., 2011; Crawford et al., 2009; Crawford et al., 2007b; Jamieson et al., 2003; Kelleher et al., 2001a; D. Liu et al., 2014; Martinez-Picado et al., 2006; Seki & Matano, 2011) . Compensatory mutations not only can restore lost replication capacity, but may even result in replication capacity that is

even higher than the consensus sequence and still retain the ability to evade the immune system. Consensus sequence being defined as the amino acid sequence that is the most prevalent among circulating strains within the population and generally used as the baseline for replication capacity studies. The consensus sequence for the gag 162-173 epitope is KAFSPEVIPMF. The KAFSPEVIPMF (Gag 162-173) epitope has compensatory mutations that fit every one of these criteria. The A163G mutation has reduced replication capacity (Crawford et al., 2007a), but it also has 1-log less affinity to HLA-B*57 (Yu et al., 2007) and reduced TCR recognition (Yu et al., 2007). However, the S165N compensatory mutation rescues the replication capacity of the A163G mutant (Crawford et al., 2007a) while retaining the reduced HLA affinity (Yu et al., 2007) and therefore the reduced TCR recognition. This compensatory mutation lies within the epitope; it restores replication capacity while retaining the immuno-evasion benefits of the original escape. The flanking mutation S173T helps escape CTLs but, when coupled with the distal compensatory mutations S126N / L215T / H219Q / M228I, replication capacity exceeds that of the consensus sequence (Gijsbers et al., 2013). Compensatory mutations that have little or no fitness costs and help HIV-1 evade the immune system will not revert back to the consensus sequence regardless of whether they were transmitted in the transmitted/founder (T/F) virus or evolved during acute or chronic infection.

Reversion of Escape Mutations

Reversion of an escape variant to the consensus sequence has been well documented in macaques and in humans (Asquith et al., 2007; Brumme et al., 2008; Fernandez et al., 2005a; Friedrich et al., 2004; Goulder et al., 2001; Kelleher et al., 2001a; Leslie et al., 2004). The data show that, for a successful reversion, the following prerequisites must be met: (i) the wild type virus must have a higher replication capacity than its escape counterpart, (ii) the wild type virus

needs to be either the T/F or one of the strains first inoculated during infection, or be generated through stochastic mutations or as a result of immune pressure on the escape variants, (iii) there must be sufficient target cells for the wild type virus to grow in and outgrow the escape variant, and (iv) the immune pressure against the wild type must be less than the escape variant (Loh et al., 2008). As a consequence of the reversion prerequisites, reversion can occur during both acute and chronic infection. In a macaque study where they looked at the KF11 analog SIV Gag KP9 epitope (Beck et al., 2016b), macaques inoculated with the high fitness cost escape variant K165R responded in one of two ways. If the macaque's MHC could result in a CTL response against the consensus sequence, the K165R variant remained present upon the rapid development of the compensatory mutation V145A (Beck et al., 2016b). However, if the macaque could not mount a response against the consensus sequence, the K165R escape variant quickly reverted to consensus (Beck et al., 2016b). This illustrates the point that reversion occurs quickly in acute infection if the escape mutants have a high fitness cost or in the absence of CTL selective pressure against the consensus. Additionally, reversion can occur during acute infection if the newly infected person is HLA-mismatched from the HIV-1 donor, since the CTL pressure against the escape variants is absent (Kawashima et al., 2009; Leslie et al., 2004; Seki & Matano, 2011). Reversion can also occur during chronic infection (Goepfert et al., 2008; Rousseau et al., 2008). This can be a result of infection dynamics following inoculation with escape variants and the absence of wild type virus. In addition, reversion might not be observed until chronic infection when $CD4^+$ T cell levels have increased because, while the daily probability of mutation is lower, the target cell availability is higher and the replication capacity of the wild type may be more robust. Reversion might also occur during chronic infection if the CTL

response is highly impaired and cannot adequately mount a response against the wild type and if the wild type is available in newly activated cell reservoirs.

The Effects of CTL Escape on HIV-1 Disease Progression

Over the course of HIV-1 infection, escape variants accumulate in the population, despite having protective or non-protective HLAs (Crawford et al., 2009; Moore et al., 2002). In one study (Crawford et al., 2009), several HLA-B*57^{+} persons infected with HIV-1 for several years were screened for escape mutations. All subjects had mutations in three gag-p24 epitopes that have been known to cause escape, and all of the HIV-1 strains isolated had reduced viral fitness, but viral persistence was maintained and, in the event that CTL pressure became absent, disease progression would likely occur. Whether the presence and accumulation of escape variants positively or negatively impacts disease progression is still a point of contention.

One side of the argument is that CTL escape variants lead to disease progression and a poor prognosis (Allen et al., 2000; Asquith, 2008; Barouch et al., 2003; Barouch et al., 2002; Feeney et al., 2004; Geels et al., 2006; Goulder et al., 1997a; Koenig et al., 1995; Nowak et al., 1991; O'Connor et al., 2002; Oxenius et al., 2004). It is argued that, while sequential addition of escape mutations results in reduced fitness, HIV-1 can still replicate to high levels and result in disease progression (Crawford et al., 2009). Other groups have observed that the appearance of escape CTL variants has resulted in loss of virologic control and disease progression in both HIV-1 and SIV (Allen et al., 2000; Asquith, 2008; Barouch et al., 2003; Barouch et al., 2002; Feeney et al., 2004; Geels et al., 2006; Goulder et al., 1997a; Koenig et al., 1995; Nowak et al., 1991; O'Connor et al., 2002; Oxenius et al., 2004). In macaques, is has been observed that, after a mutation in a Gag epitope, CTL escape occurred and the animal's undetectable viremia underwent a burst of viral replication, clinical progression, and ultimately death from AIDS-

related complications (Allen et al., 2000). In other studies, the evolution of CTL and antibody escape variants resulted in failure of HIV-1 control and the advancement of immunodeficiency.

In the opposing viewpoint, other groups argue that the development of CTL escape variants does not contribute to disease progression and the onset of AIDS (Altfeld & Allen, 2006; Bailey, Williams, Siliciano, & Blankson, 2006; Iversen et al., 2006; Jamieson et al., 2003; Kelleher et al., 2001a; Leslie et al., 2004; Wolinsky et al., 1996). One basis for this argument is that the dramatic reduction in replicative capacity due to the fitness costs of escape results in slow disease progression and also in reduced transmission. Other groups have observed that viral escape is not always accompanied by a surge or a detectable increase in viremia. In one study (Leslie et al., 2004), HIV-1^{+} persons were followed for several years; they remained healthy despite the presence of CTL escape variants. As such, it is argued that CTL escape has no clinical disadvantage and may even be advantageous in some cases due to the lower replicative capacity. The differing viewpoints can be possibly reconciled by noting that the rate of AIDS progression due to CTL escape variants is better described as HLA-associated rate of AIDS progression, which is a fraction of the total rate of disease progression and is CTL-dependent. Thereby, while CTL escape will not result in direct progression to AIDS, it does tip the scales towards the progression of AIDS.

CTL Escape and its Effects at the Population Level

Furthermore, the evolution of CTL escape variants has changed the face of HIV-1 at the population level due to its constant adaption to HLA-driven responses (Gounder et al., 2015; Kawashima et al., 2009). The increased prevalence of CTL escape mutations coupled with compensatory mutations has further reduced the benefits of protective HLA alleles associated with slowed HIV-1 disease progression(Payne et al., 2014). The prevalence of compensatory

mutations has mitigated the fitness costs associated with the escape mutations (Brockman et al., 2010; Brockman et al., 2007; Huang et al., 2011), thus resulting in more CTL resilient HIV-1 strains. An example of this phenomenon can be seen in the Japanese population (Katoh et al., 2016), which expresses HLA-A*2402 in over 60% of its population. The HIV-1 strains observed in Japan have resulted in reduced CTL-mediated viral control and increased HIV-1 pathogenesis (in terms of lower $CD4^+$ T cell counts). Other studies have found that a large number of escape variants are being transmitted at the time of infection, suggesting a viral adaptation to host HLA molecules that is occurring at the population level.

T Cell Immunotherapy

To date, there is no HIV-1 vaccine. However, T cell immunotherapy has been considered as a possible avenue for a potential HIV-1 treatment and/or vaccine. The idea behind T cell immunotherapy is based on the promising results in the treatment of melanoma using HLA-A*02 restricted TCRs (Clay, Morse, & Lyerly, 2002; Coccoris, de Witte, & Schumacher, 2005). A problem with current T cell immunotherapies is that they are unable to cope with viral escape and thereby leave unplugged holes that the virus can use to escape CTL surveillance. Previously, an HIV-1 epitope was targeted using T cell immunotherapy; however, the treatment ultimately failed since the virus escaped, and the treatment was rendered useless (Koenig et al., 1995). Another problem with the current technology is that the methods utilized to identify and clone an epitope-specific TCR are laborious and expensive. In this work, we show a new inexpensive method to rapidly identify, clone, and functionally test a panel of TCRs that are specific to HIV-1 epitopes. This panel of TCRs targets the different variants of the HIV-1 epitope KAFSPEVIPMF (Gag 162-172) and prevents viral escape and/or greatly diminishes replicative capacity.

Materials and Methods

Cohort and Preparation of Peripheral Blood Mononuclear cells

Blood samples were collected from healthy control and non-treated chronically HIV-1–infected volunteers under University of California Los Angeles Institutional Review Board-approved protocols. PBMCs were isolated by Ficoll gradient and washed twice with Hanks' buffered saline, then viably cryopreserved.

Detection of HIV-1-Specific $CD8^+$ T cell Responses Against HIV-1 by IFN-γ ELISpot Analysis

Peripheral blood HIV-1 specific $CD8^+$ T cell responses in HIV-1 infected individuals with plasma HIV-1 levels of $\leq$400 RNA copies/mL at study entry were quantified by IFN-γ ELIspot analysis, as previously described (Ibarrondo et al., 2005). Briefly, purified $CD8^+$ T cells were screened against peptide, KAFSPEVIPMF (KF11), to determine the frequency of spot-forming cells (SFC) per added $CD8^+$ T cells. SFC were enumerated with an automated ELISPOT reader (Autoimmum Diasgnostika, Strassberg, Germany). The frequency of HIV-1-specific SFC per volume of peripheral blood was calculated by multiplying the frequency of SFC in $CD8^+$ T cells and the number of $CD8^+$ T cells per volume of blood.

HLA Genetic Analyses

Using PBMC DNA, HLA typing was performed by the clinical laboratory at the UCLA Immunogenetics Center.

Enrichment of Epitope-Specific TCRs by Cognate Peptide Stimulation of PBMC

Culture 1.5 $x10^6$ PBMC in 2 ml of R10-12.5 in a 48-well cell culture plate for each condition, i.e. cognate peptide-stimulated sample and non-peptide control. Add the cognate peptide to the experimental well to a final concentration of 0.6-1.0 μg/ml and mix well by

pipetting, and no peptide to the non-stimulated control. Incubate the plate at 37°C in a humidified cell culture incubator with 5% CO_2 for 72 hours. Without disturbing the cells, feed each well by removing 1 ml of medium and replenishing with 1 ml R10-12.5 pre-warmed to 37°C. Incubate the plate at 37°C in a humidified cell culture incubator with 5% CO_2 for 7 days, and feeding the cells with R10 every 72 hours.

Isolation of $CD8^+$ T Cells by Immunomagnetic Positive Selection

Transfer the contents of each well (after pipetting to ensure all cells are suspended) to a 2 ml microcentrifuge tube. Pellet cells by spinning at 2,400 rcf at 4°C for 3 minutes (in a FrescoTM 17 Microcentrifuge at 5000 rpm or similar). Carefully remove as much supernatant as possible without disturbing the cell pellets. Resuspend each cell pellet in 80 µl of cell separation buffer. Add 20 µl of anti-human CD8 coated Microbeads to each pellet (Miltenyi Biotech) and mix well by gently pipetting several times. Incubate for 15 minutes at 4°C. Add 1 ml of cell separation buffer to each tube and again pellet the cells at 2,400 rcf at 4°C for 3 minutes. Carefully remove as much of the supernatant as possible without disturbing the cell pellets. Resuspend each pellet with 500µl of cell separation buffer. Isolate the $CD8^+$ T cells using a MACS Cell Separator (Miltenyi) under the conditions: Program=Option, Separation Method=Possel, Wash=QuickRinse, Volume=500 µl. Place the collected $CD8^+$ T cells on ice for a maximum of one hour before RNA extraction.

RNA Isolation and Purification

Transfer each sample to a 2 ml microcentrifuge tube and pellet cells at 2,400 rcf at 4°C for 3 minutes. Carefully remove as much supernatant as possible without disturbing the cell pellets. Resuspend each pellet in 250µl of cold R10. Add 750µl of Trizol LS reagent to each pellet and lyse by pipetting several times or vortexing; if there are more than $10x10^6$ cells adjust

the amount of Trizol (additional 75µl of Trizol LS for each additional 10^6 cells). Incubate for 5 minutes at room temperature. Per 750µl of Trizol LS reagent, add 200µl of chloroform and mix vigorously using a vortexer. Incubate for 10 minutes at room temperature. Centrifuge the samples at 12,000 rcf at 4°C for 15 minutes (in a FrescoTM 17 Microcentrifuge 11,200 rpm or similar centrifuge). Carefully transfer the aqueous phase to a clean 1.5ml microcentrifuge tube, ensuring that no Trizol reagent is carried over. Add 500µl of absolute isopropanol at room temperature (optional: add 200µg of glycogen) and mix well by pipetting up and down several times to precipitate the RNA. Incubate for 10 minutes at room temperature. Centrifuge the samples again at 12,000 rcf at 4°C for 10 minutes. Discard the supernatant and add 1ml of 75% ethanol at 4°C. Mix the sample by vortexing for 3 seconds. Centrifuge the samples at 7,500 rcf at 4°C for 5 minutes (in a FrescoTM 17 Microcentrifuge at 8,800 rpm or similar centrifuge). Carefully remove the ethanol without disrupting the RNA pellet. Air-dry the RNA pellet for 10 minutes at room temperature. Dissolve the RNA pellet in 40 µl molecular grade water. Take 2 µl and measure the RNA concentration using a nanodrop spectrophotometer to ensure a yield of at least 20 ng/µl of RNA. The RNA can be immediately used for cDNA synthesis or stored at -80°C long term.

Synthesis of cDNA

For each RNA sample, prepare two microcentrifuge tubes containing 20 µl of 2x master mix using the reagents from the "High Capacity cDNA Reverse Transcription Kit" (See Table 8); mix gently by pipetting and place on ice. Add 20µl of each RNA sample (or maximum of 2 µg RNA) to each tube and pipette gently to mix. Briefly spin in a microfuge to bring all liquid to the bottom of the tubes. Place the microcentrifuge tubes in a thermal cycler and run using the conditions: Step 1=25°C for 10 minutes, Step 2=37°C for 120 minutes, Step 3=85°C for 5

minutes, Step 4=4°C hold. Once the reverse transcription reaction is completed, the cDNA can be stored at -20°C or used immediately.

COMPONENT	µl
10x Reverse Transcriptase	4
25x dNTP Mix	1.6
10x RT Random Primers	4
MultiScribe Reverse Transcriptase	2
RNase Inhibitor (10U/µl)	2
Molecular grade water	6.4
TOTAL	**20.0**

Table 8. Reverse transcription master mix.

The components for a 2x mix for reverse transcription are listed.

Purification of cDNA

Purify cDNA using the Invitrogen PureLink PCR Purification Kit as per manufacturer's instructions, except finally resuspending each sample in 40 µl molecular grade water. The purified cDNA can be stored at -20°C or used immediately for quantitative spectratyping of TCR TRAV and/or TRBV families.

Quantitation of TCR AV and TRBV Family Transcripts by Real-Time PCR

For each sample, take one of the two 40 µl cDNA reactions and add 80 µl of molecular grade water to bring the total volume to 120 µl (reserve the other for cloning, or as a backup). For the TRAV and TRBV spectratyping reactions, prepare mastermixes in 1.5ml microcentrifuge tubes with cDNA as indicated in table 9 (four mastermixes for TRAV and three mastermixes for TRBV spectratyping); keep on ice and protected from light until use. For the quantitative standards using plasmid standards, prepare mastermixes in 1.5ml microcentifuge tubes for the plasmid standard as shown in table 10; keep on ice protected from light until use. Prepare 7 serial four-fold dilutions of TRAV08 and TRBV20 plasmid standards (previously produced in a TOPO TA cloning vector, Invitrogen) in molecular grade water from 5 pg/µl (5 pg = 1.94 x 107 and 1.65 x 107 copies of the TRAV and TRBV plasmids respectively) to 1.22 fg/µl; start with 16 µl and carry forward 4 µl plus 12 µl water serially, mixing well by pipetting and changing pipet tips between each dilution. Set up a 96-well PCR plate with 19 µl of the indicated mastermixes per well as indicated in table 10 for TRAV and TRBV spectratyping, adding 1µl of each unlabeled TRAV forward primer listed in table 11A for TRAV and table 11B for TRBV to the wells indicated; mix by pipetting several times. Add 1 µl per well of the standard plasmid serial dilutions to the wells indicated in figure 10 for TRAV and figure 10 for TRBV; mix by pipetting several times. Seal the plates with a Microseal 'B' Adhesive Seal. Place the plates in an iQ5

thermal cycler and run using the real-time PCR conditions: one cycle of 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds, followed by hold at 4°C. Calculate the copy numbers of each TRAV and TRBV family based on standard curves from the diluted plasmid standards. Derive the relative concentration of each TRAV or TRBV family as the ratio of the copy number of that family versus the median copy number across all TRAV or TRBV families respectively.

COMPONENT	MASTERMIXES (µl) FOR: ALPHA	BETA
α-chain constant region reverse primer labeled with: FAM, VIC, NED, or PET* (10 mM stock solution)	9	-
α-chain constant region probe labeled with Cy5** (100 mM stock solution)	4.5	-
β-chain constant region reverse primer labeled with FAM, VIC, or NED*** (10 mM stock solution)	-	9
β-chain constant region probe labeled with Cy5**** (100 mM stock solution)	-	4.5
SsoFast Probe Supermix	90	90
cDNA	13.5	13.5
Molecular grade water	54	54
TOTAL	**171**	**171**

Table 9. Mastermixes with final working concentrations for AV and BV spectratyping.

The components for generating mastermixes for the AV and BV family real-time PCR reactions are shown. One mastermix is made for each labeled version of the constant region reverse primer (four for alpha and three for beta).

* 5’-GCAGACAGACTTGTCACTGG-3’
** 5’-(Cy5)- CTGCCGTGTACCAGCTGAGAGA-(BHQ2)-3’
*** 5’-CTTCTGATGGCTCAAACAC-3’
**** 5’-(Cy5)-TGTTCCCACCCGAGGTCGC-(BHQ2)-3’

COMPONENT	MASTERMIXES (µl) FOR: ALPHA	BETA
AV8 forward primer (10 mM stock solution)*	15	-
α chain constant region reverse primer (10 mM stock solution)**	15	-
α chain constant region probe (100mM stock solution)***	7.5	-
BV20 forward primer (10 mM stock solution)****	-	15
β chain constant region reverse primer (10 mM stock solution)*****	-	15
β chain constant region probe (100mM stock solution)******	-	7.5
SsoFast Probe Supermix	150	150
Molecular grade water	90	90
TOTAL	**277.5**	**277.5**

Table 10. Real-time PCR standard mastermixes for AV and BV families.

Mastermixes for the real-time PCR plasmid standards are indicated.

* 5'-GCATCAACGGTTTTGAGGCTG-3'
** 5'-GCAGACAGACTTGTCACTGG-3'
*** 5'-(Cy5)- CTGCCGTGTACCAGCTGAGAGA-(BHQ2)-3'
**** 5'-TCAACCATGCAAGCCTGA-3'
***** 5'-CTTCTGATGGCTCAAACAC-3'
******5'-(Cy5)-TGTTCCCACCCGAGGTCGC-(BHQ2)-3'

A

FAMILY	PRIMER SEQUENCE (5'→3')	AV GENES INCLUDED
AV01	ATGYTCTGGATGGTTTGGAGGAGA	AV1-1*01, AV1-2*01
AV02	TCAGCAGGGACGATACAACATGAC	AV2-1*01
AV03	ATCAGGTCAACGTTGCTGAAGGGA	AV3*01
AV05	GGATAAACATCTGTCTCTGCG	AV5*01
AV06	TGAAGGTCACCTTTGATACCACCC	AV6*01
AV08	GCATCAACGGTTTTGAGGCTG	AV8-1*01, AV8-2*01, AV8-3*01, AV8-4*01, AV8-6*01, AV8-6*02
AV09	CTTGGAGAAAGGCTCAGTTC	AV9-1*01, AV9-2*01
AV10	CAACTCTGGATGCAGACACAAAGC	AV10*01
AV12	GAAGATGGAAGGTTTACAGCACAG	AV12-1*01, AV12-2*01 AV12-3*01
AV13	ATTCARACAGYGCCTCARACTACTTC	AV13-1*01, AV13-2*01
AV14	AATCCGCCAACCTTGTCATCTCCG	AV14-1*01, AV14-1*02
AV16	CACTGCTGACCTTAACAAAGGCG	AV16*01
AV17	AAGCAGTTCCTTGTTGATCACGGC	AV17*01
AV18	AGTGACAGTTCCTTCCACCT	AV18*01
AV19	TCCACCAGTTCCTTCAACTTCACC	AV19*01
AV20	CCCTGAATTCCTCTTCACCCTGTA	AV20*01
AV21	TGCCTCGCTGGATAAATCATCAGG	AV21*01
AV22	TTCATCAAAACCCTTGGGGACAGC	AV22*01

AV23	TCCATGGTACCAACAATTCCCTGG	AV23*01
AV24	TGCCACTCTTAATACCAAGGAGGG	AV24*01
AV25	ACCCAGACTACAGATGTAGGAACC	AV25*01
AV26	GAAAGTCCAGTACCTTGATCCTGC	AV26-1*01, AV26-2*01
AV27	CTGTGTACTGCAACTCCTCAAGTGT	AV27*01
AV29	ACCCTGCTGAAGGTCCTACATTCC	AV29*01
AV30	GAAGCACCCGTCTTCCTGATGATA	AV30*01
AV34	AGATAACTGCCAAGTTGGATGAGAA	AV34*01
AV35	GCTGGTGAATTGACCTCAAATGG	AV35*01
AV36	GAACATCACAGCCACCCAGACCGG	AV36*01
AV38	CCCAGCAGGCAGATGATTCTCGTT	AV38-1*01, AV38-2*01
AV39	TTGATACCAAAGCCCGTCTC	AV39*01
AV40	AGCAAAAACTTCGGAGGCGG	AV40*01
AV41	ACACTGGCTGCAACAGCATC	AV41*01

B

FAMILY	PRIMER SEQUENCE (5'→3')	BV GENES INCLUDED
BV02	GCAGAAAGTCGAGTTTCTGG	BV2
BV03	CCTAAATCTCCAGACAAAGC	BV3-1
BV04	GCTTCTCACCTGAATGCCCC	BV4-1, BV4-2, BV4-3
BV05a	TCAGTGAGACACAGAGAAAC	BV5-1
BV05b	TGTGTCCTGGTACCAACAGG	BV5-3, BV5-4, BV5-5, BV5-6, BV5-7, BV5-8

BV06a	CGACAAGACCCAGGCATGGG	BV6-1, BV6-3, BV6-5, BV6-6, BV6-7, BV6-8, BV6-9
BV06b	AGACAAGATCTAGGACTGGG	BV6-4
BV07	CTCAGGTGTGATCCAATTTC	BV7-1, BV7-2, BV7-3, BV7-4, BV7-6, BV7-8, BV7-9
BV09	CGCACAACAGTTCCCTGACT	BV9
BV10	CATGGGCTGAGGCTGATC	BV10-1, BV10-2, BV10-3
BV11	TCACAGTTGCCTAAGGATCG	BV11-1, BV11-2, BV11-3
BV12	TCTGGTACAGACAGACCATG	BV12-3, BV12-4, BV12-5
BV13	GCAGGGTCCAGGTCAGGACCCCCA	BV13
BV14	AGTCTAAACAGGATGAGTCCG	BV14
BV15	ACAATGAAGCAGACACCCCT	BV15
BV18	GAGTCAGGAATGCCAAAGGA	BV18
BV19	GGAGATATAGCTGAAGGGTA	BV19
BV20	TCAACCATGCAAGCCTGA	BV20-1
BV24	GTGTCTCTCGACAGGCACAG	BV24-1
BV25	TCAACAGTCTCCAGAATAAGGACG	BV25-1
BV27	GTCTCTCGAAAAGAGAAGAG	BV27
BV28	GTCTCTAGAGAGAAGAAGGAGCGC	BV28
BV29	GAGGCCACATATGAGAGTGG	BV29-1
BV30	CAGCTCTGAGGTGCCCCAGA	BV30

Table 11. Forward primer sequences for spectratyping.

Table 11A. Forward primers for AV spectratyping. Table 11B. Forward primers for BV spectratyping.

A

STANDARD			CONTROL UNSTIMULATED					COGNATE PEPTIDE STIMULATED				
			FAM	VIC	NED	PET		FAM	VIC	NED	PET	
5.00 pg	5.00 pg	-	AV10	AV14	AV34	AV39	-	AV10	AV14	AV34	AV39	
1.25 pg	1.25 pg	-	AV08	AV22	AV02	AV38	-	AV08	AV22	AV02	AV38	
313 fg	313 fg	-	AV19	AV16	AV09	AV18	-	AV19	AV16	AV09	AV18	
78.1 fg	78.1 fg	-	AV27	AV23	AV05	AV29	-	AV27	AV23	AV05	AV29	
19.5 fg	19.5 fg	-	AV20	AV40	AV21	AV36	-	AV20	AV40	AV21	AV36	
4.88 fg	4.88 fg	-	AV35	AV24	AV13	AV17	-	AV35	AV24	AV13	AV17	
1.22 fg	1.22 fg	-	AV25	AV06	AV26	AV30	-	AV25	AV06	AV26	AV30	
0	0	-	AV12	AV14	AV34	AV39	-	AV12	AV14	AV34	AV39	

B

STANDARD			CONTROL UNSTIMULATED					COGNATE PEPTIDE STIMULATED				
			FAM	VIC	NED			FAM	VIC	NED		
5.00 pg	5.00 pg	-	BV09	BV20	BV28	-	-	BV09	BV20	BV28	-	
1.25 pg	1.25 pg	-	BV18	BV29	BV06a	-	-	BV18	BV29	BV06a	-	
313 fg	313 fg	-	BV05a	BV15	BV04	-	-	BV05a	BV15	BV04	-	
78.1 fg	78.1 fg	-	BV05b	BV27	BV02	-	-	BV05b	BV27	BV02	-	
19.5 fg	19.5 fg	-	BV03	BV25	BV24	-	-	BV03	BV25	BV24	-	
4.88 fg	4.88 fg	-	BV14	BV10	BV06b	-	-	BV14	BV10	BV06b	-	
1.22 fg	1.22 fg	-	BV19	BV30	BV11	-	-	BV19	BV30	BV11	-	
0	0	-	BV07	BV12	BV13	-	-	BV07	BV12	BV13	-	

Figure 10. Plate maps for AV and BV spectratyping reactions.

96 well plate maps are shown for real-time PCR reactions for AV (A) and BV (B) families. The dyes listed above each column of families indicate labels for those families. The blocks of 4 families (AV) or 6 families (BV) indicate groupings that are subsequently combined for analysis by capillary electrophoresis.

Size Distribution Profiling of Individual TRAV and TRBV Families to Identify Epitope-Specific TCR Peaks

Take 5µl of each real-time PCR reaction and combine into the TRAV and TRBV groupings in table 12A and table 12B**.** Mix each grouping well by pipetting and add 1.5 µl of each grouping to a 96 well PCR plate with 0.5 µl LIZ 500 size standard and 8 µl Hi-Dye Formamide. Place the 96-well PCR plate on a Mastercycler Pro (or similar thermal cycler) and denature at 94°C for 5 minutes, and immediately place the plate on ice for 5 minutes protected from light. Load 10 µl of each reaction into an ABI 3130 Sequencer (or similar unit) using the capillary electrophoresis function. Analyze the resulting histograms using GeneMapper v3.7 to determine the area under the curve of each size peak within each TRAV or TRBV family (see Figure 11). Calculate the relative concentration of each size peak within the family by multiplying the fraction of its peak area of total peaks within its family times the relative concentration of the total family. Compare the relative concentrations of each peak for the peptide-stimulated versus unstimulated control samples to identify the peaks that expanded in an epitope-specific manner (increased by at least 2 units compared to the control).

A

GROUP	CONSTANT REGION REVERSE PRIMER LABEL			
	FAM	VIC	NED	TED
1	AV10)	AV14	AV34	AV39
2	AV08	AV22	AV02	AV38
3	AV19	AV16	AV09	AV18
4	AV27	AV23	AV05	AV29
5	AV20	AV40	AV21	AV36
6	AV35	AV24	AV13	AV17
7	AV25	AV06	AV26	AV30
8	AV12	AV41	AV01	AV03

B

GROUP	CONSTANT REGION REVERSE PRIMER LABEL		
	FAM	VIC	NED
1	BV09, BV18	BV20, BV29	BV28, BV06a
2	BV05a, BV05b	BV27, BV15	BV04, BV02
3	BV03, BV14	BV25, BV10	BV24, BV06b
4	BV19, BV07	BV30, BV12	BV11, BV13

Table 12. Grouping of AV and BV PCR reactions for capillary electrophoresis spectratyping analysis.

Table 12A for alpha and table 12B for beta spectratyping PCR products, groupings of different families for capillary electrophoresis analysis are listed.

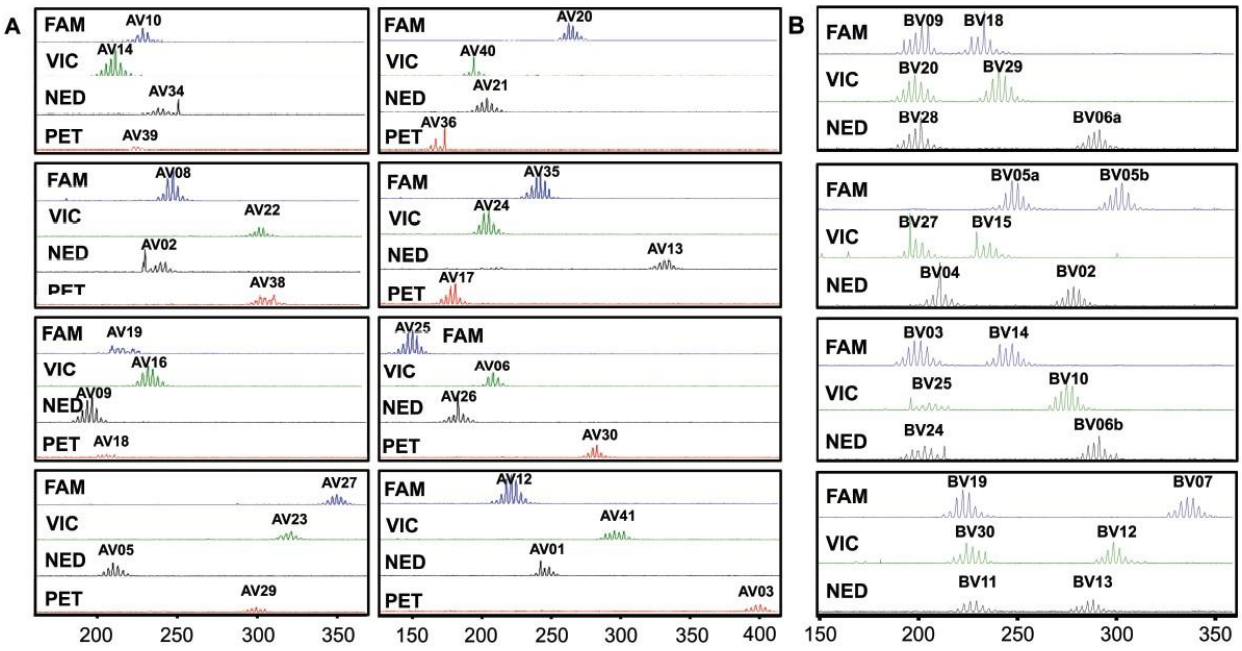


Figure 11. Maps of AV and BV family locations after capillary electrophoresis.

Examples of raw AV (A) and BV (B) spectratyping histograms generated by capillary electrophoresis are shown for a typical healthy person. Each box indicates one group of PCR reactions run together; each line represents one PCR reaction tagged by constant region reverse primers complexed to the indicated dyes.

Purification of TRAV and TRBV Family PCR Products in Preparation for Sequencing

Using the Invitrogen PureLink PCR Purification Kit, PCR purify the TRAV and TRBV families that showed epitope-specific size-peak expansions according to the manufacturer's instructions, except eluting the DNA in 50 µl molecular grade water in the last step. The DNA can be stored at -20°C or used immediately for sequencing.

Sequencing of Epitope-Specific TRAV and TRBV Chains in Families with Epitope-Specific Expansions

Prepare a sequencing reaction mix as listed in table 13 in a PCR microcentrifuge tube for each PCR product with an epitope-specific expansion; mix well by pipetting and briefly spin down. Place the tubes in a thermal cycler using the conditions: one cycle of 96°C for one minute, followed by 40 cycles of 96° for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes, followed by hold at 4°C. After PCR, the tubes can be stored at 4°C up to 2 days or used immediately for sequencing. Use the BigDye Sequencing Cleanup Kit to prepare each PCR reaction for sequencing according to the manufacturer's instructions, finally resuspending each sample in 40 µl molecular grade water. Load 10 µl of each reaction into the ABI 3130 sequence analyzer. Analyze the resulting sequences using Chromas Lite and the IMGT/V-Quest database http://www.imgt.org/IMGT_vquest/. For families that contain more than one epitope-specific expansion, it may be necessary to TOPO-TA clone the PCR product to do clonal sequencing.

COMPONENT	µl
5x BigDye Sequencing Buffer	3.75
Terminator Reaction Mix v3.1	0.5
AV or BV PCR product (100-200 ng)	(x)
Primer (10mM stock solution)*	0.5
Molecular grade water	q.s.
TOTAL	**20**

Table 13. Sequencing reaction mixes for AV or BV chains.

The components for sequencing reactions of AV or BV family PCR products are listed.

*Appropriate forward AV or BV primer (**TABLE 4**), or appropriate reverse alpha or beta constant region primer (**TABLE 3**)

Preparation of TRAV, TRBV, and Vector Link PCR Fragments for In-FusionTM

Design and obtain specific primers for TRAV and TRBV PCR amplification as shown in table 14; these primers include overlap sequences for In-FusionTM (see Figure 12). For each TRAV or TRBV family cDNA generated and the corresponding primers designed above, prepare a 50 µl PCR reaction in an 0.2 ml PCR tube using 1µl of cDNA as template, adding KOD polymerase last (table 15). For the vector link fragment between the TRAV and TRBV genes (containing the alpha constant region and the 2A ribosomal skip sequence, see Figure 12), set up the same PCR reaction conditions in table 15 in an 0.2 ml PCR tube using 5 ng of the plasmid with the template and the primers: 5'-GCCGTGTACCAGCTGCGG-3' (forward) and 5'-GGGGCCAGGGTTTTCCTCC-3' (reverse). For TRAV, TRBV, and vector link PCR amplification reactions use conditions: 95°C for 60 seconds, then 34 cycles of 95°C for 30 seconds, 58 to 63°C (1°C lower than the lowest melting temperature of the primers used) for 1 minute, 68°C for 60 seconds, followed by hold at 70°C for 7 minutes, and then hold at 4°C. Add 1µl (20 units) of DpnI enzyme to the vector link PCR product and incubate for one hour at 37°C, to digest residual plasmid template. The PCR product can be stored long term at -20°C or used immediately. Run 50 µl of each PCR reaction mixed with 6 µl loading buffer on an 2% agarose gel for 40-45min at 120V/500mA to resolve bands of about 500 base pairs for the TRAV, TRBV, and vector link PCR products. On a UV light box, excise the bands (minimizing time of exposure to UV as much as possible) and transfer each band to a 2 ml microcentrifuge tube. Use the Machery-Nagel NucleoSpin Gel and PCR Clean-up Kit to isolate DNA from the gel slices as per manufacturer's instructions except resuspending the DNA in molecular grade water rather than the supplied elution buffer. Determine DNA concentration using 2 µl in a nanodrop

spectrophotometer; the 260:280 OD ratio should be 1.8 to 1.95. The DNA can be used for immediately for In-FusionTM, or stored long term at -20°C.

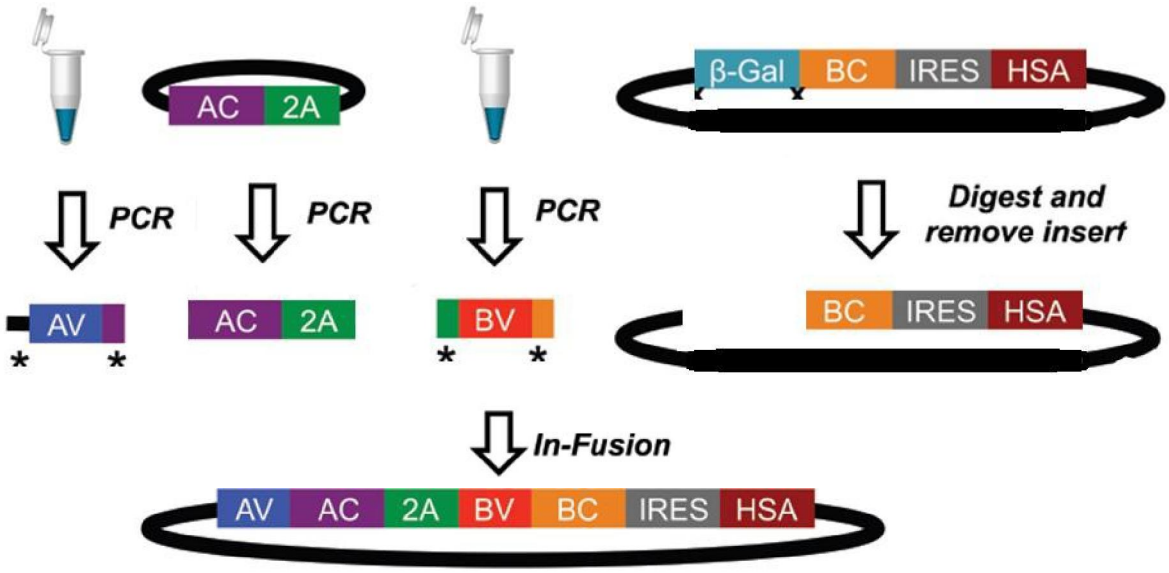


Figure 12. TCR lentiviral expression vector and In-Fusion strategy.

The cDNA from CD8+ T cells that have been highly enriched for epitope-specificity is PCR amplified for the AV and BV chains of interest using primers with overhangs : AV PCR to produce a fragment overlapping the 5' end of the lentiviral vector and the alpha constant (AC) chain, and BV PCR by to produce a fragment overlapping the 2A ribosomal skip sequence of the AC-2A vector linker fragment and the beta constant (BC) chain. The AC-2A vector linker fragment is produced by PCR of a plasmid with that insert. The outer vector fragment is produced by digestion of vector plasmid to remove a beta -galactosidase place-holder insert. The final vector (bottom) produced by In-Fusion™ contains genes for complete alpha and beta chains flanking the 2A ribosomal skip sequence, followed by an IRES that drives expression of heat stable antigen (HSA) reporter, all within a lentiviral vector in a plasmid.

AV= Alpha chain variable region

AC= Alpha chain constant region

2A = Ribosomal skip sequence ("self-cleaving

peptide") BV= Beta chain variable region

BC= Beta chain constant region

IRES= Internal ribosomal entry site

HSA= Heat stable antigen reporter (murine CD24)

J}-Gal = Beta galactosidase

* = Complementary overhang

A

	PRIMER SEQUENCE (5'→3')	
	ALPHA	**BETA**
FORWARD (VARIABLE)	**CGCCCGGGGGGGAT CCGCCACCX**	**GAAAACCCTGGCCCCX**
REVERSE (CONSTANT)	**CAGCTGGTACACGGC** AGGGT	**TCGAACACGGCCACC**TCAGGTGGGAACAC GTTTTTCAGGTC

B

	FAMILY	FORWARD PRIMER SEQUENCE (5'→3')	Estimated T_m
AV Families	AV01	CGCCCGGGGGGGATCCGCCACCATGTGGGGAGYTTTCC TTCTYTATGTTTC	63.9°C
	AV02	CGCCCGGGGGGGATCCGCCACCATGGCTTTGCAGAGCA CTCTGGG	64.2°C
	AV03	CGCCCGGGGGGGATCCGCCACCATGGCCTCTGCACCCA TCTCGA	64.0°C
	AV04	CGCCCGGGGGGGATCCGCCACCATGAGGCAAGTGGCGA GAGTGATC	64.4°C
	AV05	CGCCCGGGGGGGATCCGCCACCATGAAGACATTTGCTG GATTTTCGTTCCTGTT	64.4°C

	AV06	CGCCCGGGGGGGATCCGCCACCATGGAGTCATTCCTGGGAGGTGTTTTG	65.0°C
	AV07	CGCCCGGGGGGGATCCGCCACCATGGAGAAGATGCGGAGACCTGTC	64.4°C
	AV08-1	CGCCCGGGGGGGATCCGCCACCATGCTCCTGTTGCTCATACCAGTGC	64.6°C
	AV08-1/-4/-6	CGCCCGGGGGGGATCCGCCACCATGCTCCTGCTGCTCGTCCC	63.4°C
	AV08-3	CGCCCGGGGGGGATCCGCCACCATGCTCCTGGAGCTTATCCCACTG	64.4°C
	AV09-1	CGCCCGGGGGGGATCCGCCACCATGAATTCTTCTCCAGGACCAGCGATTG	65.1°C
	AV09-2	CGCCCGGGGGGGATCCGCCACCATGAACTATTCTCCAGGCTTAGTATCTCTG	64.0°C
	AV10	CGCCCGGGGGGGATCCGCCACCATGAAAAAGCATCTGACGACCTTCTTGGT	63.9°C
	AV12	CGCCCGGGGGGGATCCGCCACCATGATGAWATCCTTGAGAGTTTTACTRGTSATC	63.9°C
	AV13-1	CGCCCGGGGGGGATCCGCCACCATGACATCCATTCGAGCTGTATTTATATTCCTG	64.5°C
	AV13-2	CGCCCGGGGGGGATCCGCCACCATGGCAGGCATTCGAGCTTTATTTATGTAC	64.0°C

	AV14	CGCCCGGGGGGGATCCGCCACCATGTCACTTTCTAGCCTGCTGAAGGT	63.2°C
	AV15	CGCCCGGGGGGGATCCGCCACCATGTATACGTATGTAACAAACCTGCGCGT	63.9°C
	AV16	CGCCCGGGGGGGATCCGCCACCATGAAGCCCACCCTCATCTCAGTG	64.4°C
	AV17	CGCCCGGGGGGGATCCGCCACCATGGAAACTCTCCTGGGAGTGTCTTTG	65.0°C
	AV18	CGCCCGGGGGGGATCCGCCACCATGCTGTCTGCTTCCTGCTCAGG	64.2°C
	AV19	CGCCCGGGGGGGATCCGCCACCATGCTGACTGCCAGCCTGTTGAG	64.2°C
	AV21	CGCCCGGGGGGGATCCGCCACCATGGAGACCCTCTTGGGCCTG	63.7°C
	AV22	CGCCCGGGGGGGATCCGCCACCATGAAGAGGATATTGGGAGCTCTGC	63.0°C
	AV23	CGCCCGGGGGGGATCCGCCACCATGGACAAGATCTTAGGAGCATCATTTTTAGTTC	64.7°C
	AV24	CGCCCGGGGGGGATCCGCCACCATGGAGAAGAATCCTTTGGCAGCCC	64.6°C
	AV25	CGCCCGGGGGGGATCCGCCACCATGCTACTCATCACATCAATGTTGGTCTTATG	64.4°C

	AV26-1	CGCCCGGGGGGGATCCGCCACCATGAGGCTGGTGGCAAGAGTAACTG	64.6°C
	AV26-2	CGCCCGGGGGGGATCCGCCACCATGAAGTTGGTGACAAGCATTACTGTACTC	64.0°C
	AV29	CGCCCGGGGGGGATCCGCCACCATGGCCATGCTCCTGGGGG	63.1°C
	AV30	CGCCCGGGGGGGATCCGCCACCATGGAGACTCTCCTGRAAGTGCYTTC	64.8°C
	AV34	CGCCCGGGGGGGATCCGCCACCATGGAGACTGTTCTGCAAGTACTCCTA	63.4°C
	AV35	CGCCCGGGGGGGATCCGCCACCATGCTCCTTGAACATTTATTAATAATCTTGTGGATG	63.8°C
	AV38-1	CGCCCGGGGGGGATCCGCCACCATGACACGAGTTAGCTTGCTGTGGG	64.6°C
	AV38-2	CGCCCGGGGGGGATCCGCCACCATGGCATGCCCTGGCTTCCTGT	64.0°C
BV Families	BV02	GAAAACCCTGGCCCCATGGATACCTGGCTCGTATGCTGG	64.4°C
	BV03-1	GAAAACCCTGGCCCCATGGGCTTCAGGCTCCTCTGCT	64.0°C
	BV03	GAAAACCCTGGCCCCATGGGCTKCAGGCTCCTCTGCT	64.9°C
	BV04	GAAAACCCTGGCCCCATGGGCTGCAGGCTGCTCTG	63.4°C
	BV05-	GAAAACCCTGGCCCCATGGGCCCYGGGCTCCTCT	64.2°C

	3/-4/-5/-7		
	BV05-6/-8	GAAAACCCTGGCCCCATGGGMCCCRGGCTCCTCTKC	66.7°C
	BV06-1/-9	GAAAACCCTGGCCCCATGAGCATCGGGCTCCTGTGC	63.7°C
	BV06-2/-3/-7/-8	GAAAACCCTGGCCCCATGAGCCTCGGGCTCCTGTG	63.4°C
	BV06-4	GAAAACCCTGGCCCCATGAGAATCAGGCTCCTGTGCTGTG	64.6°C
	BV06-5	GAAAACCCTGGCCCCATGAGCATCGGCCTCCTGTGC	63.7°C
	BV06-6	GAAAACCCTGGCCCCATGAGCATCAGCCTCCTGTGCTG	64.2°C
	BV07-2/-3/-4/-5/-8/-9	GAAAACCCTGGCCCCATGGGSACCAGSCTCCTCTKC	64.7°C
	BV07-6/-7	GAAAACCCTGGCCCCATGGGYACCAGTCTCCTATGCTG	63.3°C
	BV09	GAAAACCCTGGCCCCATGGGCTTCAGGCTCCTCTGC	63.7°C

	BV10	GAAAACCCTGGCCCCATGGGCACVAGGYTSTTCTTCTATG	63.3°C
	BV11	GAAAACCCTGGCCCCATGRGYACCAGGCTYCTCTGC	62.8°C
	BV14	GAAAACCCTGGCCCCATGGTTTCCAGGCTTCTCAGTTTAGTGT	63.7°C
	BV15	GAAAACCCTGGCCCCATGGGTCCTGGGCTTCTCCAC	63.7°C
	BV19	GAAAACCCTGGCCCCATGAGCAACCAGGTGCTCTGCTGT	64.4°C
	BV20	GAAAACCCTGGCCCCATGCTGCTGCTTCTGCTGCTTCTG	64.4°C
	BV25	GAAAACCCTGGCCCCATGACTATCAGGCTCCTCTGCTACAT	63.2°C
	BV27	GAAAACCCTGGCCCCATGGGCCCCCAGCTCCTTG	63.1°C
	BV28	GAAAACCCTGGCCCCATGGGAATCAGGCTCCTCTGTCG	64.2°C
	BV30	GAAAACCCTGGCCCCATGCTCTGCTCTCTCCTTGCCC	64.0°C

Table 14. Primer design for AV/BV PCR amplification for In-Fusion™.

A. The reverse primers, and base sequences for designing forward AV or BV primers are given. Underlined sequences indicate regions for overlap with adjacent vector fragments for In-Fusion™. "X" indicates variable gene-specific leader sequences (http://www.imgt.org/genedb/tableC.action), which should be added to yield a primer melting temperature of approximately 64˚C. B. Examples of working forward primers designed in our laboratory are given.

COMPONENT	µl
10x Buffer for KOD Hot Start DNA Polymerase	5
MgSO4 (25 nM stock solution)	3
dNTPs (2 mM each stock solution)	5
Template DNA*	x
Forward primer (10 mM stock solution)	1.5
Reverse primer (10 mM stock solution)	1.5
Molecular grade water	q.s.
KOD Hot Start DNA Polymerase (1 U/µl)	1
TOTAL	**50**

Table 15. PCR reaction mixes for preparing TCR and intervening vector link fragments for In-Fusion™.

* cDNA for AV and BV constructs, or plasmid containing the vector link between AV and BV chains (including the alpha constant region and 2A ribosomal skip sequence) for the vector link sequence between AV and BV genes.

Preparation of Outer Vector for In-FusionTM

In a 1.5 ml microcentrifuge tube, digest 10 µg of the lentiviral outer vector (see Figure 12) with the restriction enzymes BamHI-HF and Bsu36I (NEB) using 30 units of each enzyme, and 8µl CutSmart Buffer (NEB) in a total volume of 80 µl for 3 hours at 37°C to remove the LacZ insert gene to be replaced by the TRAV and TRBV genes. Run the digested DNA on an agarose gel and isolate the 8433 bp band DNA except using a 0.5% agarose gel, after which the DNA can be used immediately or stored at -20°C.

In-FusionTM Reaction to Produce Lentiviral Vector Constructs

For each TCR expression vector to be generated, combine 10.9 ng of the TRAV PCR product, 10.6 ng of the TRBV PCR product, 11.1 ng of the vector link PCR product, and 100 ng of the outer vector (final molecular ratio of 2:2:2:1 respectively) in a microcentrifuge tube. Add 2 µl of the enzyme from the In-FusionTM HD Cloning Kit and bring the volume to 10µl total with molecular grade water; pipet gently to mix. Briefly spin down in a microfuge to bring all liquid to the bottom of the tube. In a thermal cycler, incubate at 42°C for 30 minutes, followed by 4°C for 30 minutes. Use 2 µl of the above In-FusionTM reaction to transform One Shot® Stbl3™ Chemically Competent *E. coli* as per the manufacturer's protocol (Life Technologies)- Note these cells do not have β-galactosidase screening capability. Streak an LB-agar plate containing 50 µg/ml ampicillin, then incubate inverted overnight at 37°C, after which the plate can be stored at 4°C. Inoculate 5 or more colonies separately into 14 ml polystyrene round-bottom tubes with 3 ml of LB-broth containing ampicillin (50 µg/ml) and incubate overnight at 37°C in an orbital shaker incubator at 200 rpm. Isolate plasmid DNA using the Biopioneer Plasmid Preparation Kit as per manufacturer's instructions; the purified plasmid DNA can be used immediately or stored at -20°C. Digest 5 µl of plasmid DNA with 5 units each of the

restriction enzymes BamHI-HF and XbaI and 2 µl of the CutSmart Buffer in a microcentrifuge tube in a total volume of 20 µl for 1 hour at 37°C. Electrophorese the digested DNA to confirm a vector band of 8433 bp and insert fragment(s) totaling approximately 2000 bp. Sequence confirm plasmids with the appropriate sized inserts using the PCR primers in table 16 and sequencing conditions**.**

SEQUENCE (5'→3')	LOCATION
TTAAGACCCATCAGATGTTTC	Upstream of AV
GCCGTGTACCAGCTGCGG	Beginning of AC
TGGAGGAAAACCCTGGCC	Upstream of BC
AGAGGTGCACAGCGGAGTCAG	Beginning of BC

Table 16. TCR vector sequencing primers.

Primers (forward) for confirming TCR sequences in the final assembled expression vector are listed.

Generating Pseudotyped TCR Lentiviral Vectors

For each construct being tested, seed 1.5-2 x106 293 T cells in 10 ml D10 medium in a 25 cm3 flask, and culture for approximately 24 hours in a humidified 5% CO2 tissue culture incubator until 60 to 75% confluent. Immediately before lipofection, replace the medium with 10 ml fresh D10 pre-warmed to 37°C. In a microcentrifuge tube, premix 1.67 μg of the plasmid containing the TCR expression construct generated above, 1.67 μg of the packaging construct plasmid (pCMVΔR8.2DVPR), and 0.83 μg of Vesicular Stomatitis Virus envelope expression plasmid to achieve a molecular ratio of 2:2:1 in a total volume of 200 μl of pre-warmed DMEM (without serum); pipet to mix. Add 6.6 μl of the lipofection reagent BioT (Bioland Scientific LLC) and pipet gently to mix. Add 300 μl of pre-warmed DMEM to bring the total volume to 500 μl and pipet gently to mix; leave at room temperature for 10 to 15 minutes. Add the DNA/lipofectamine mixture to the cells in the flask drop-wise, distributing it over the cells by tilting the flask after each drop; return the flask to the cell incubator. After 16 to 24 hours, replace the medium with 10 ml pre-warmed D10 and return to the incubator. After another 24 hours, harvest and save the medium at 4°C; replace with 10 ml pre-warmed D10 and return to the incubator. After another 24 hours remove the medium and pool with the harvested medium from the prior day. Prepare a 0.45 μm low protein-binding syringe filter (Corning) by running through 5 ml of DMEM without serum. Using a 12 ml syringe, filter the collected medium. Concentrate the virus by ultracentrifuging at 175,000 rcf (SW32 TI rotor at 28,000 rpm in an Optima L-90K ultracentrifuge or equivalent) for 2 hours at 4°C. Carefully aspirate and discard the supernatant, taking care not to disturb the pellet. Resuspend the pellet in 200 μl RPMI without serum and store in two aliquots of 100 μl each at -80°C until use.

TCR Testing Using a Jurkat Reporter Cell Line

Pellet two aliquots of 500,000 Jurkat reporter cells expressing CD8 and containing an NFAT-dependent GFP expression construct in a 15 ml conical centrifuge tube by centrifuging at 484 rcf at room temperature (in a Sorvall Legend RT at 1500 rpm or similar). Aspirate the medium and resuspend each pellet in 50 µl of R20 medium. Thaw a 100 µl aliquot of lentiviral vector and add to one aliquot; pipet gently to mix. Add 100 µl D10 to the other aliquot; pipet gently to mix. Incubate for 4 hours at 37°C in a rotating mixer at 24 rpm. Add 850 µl of R10 pre-warmed to 37°C to each aliquot, pipet gently to mix, and transfer to a 24-well plate to culture for 48 to 72 hours in the tissue culture incubator before testing.

Testing the TCR-Transduced Jurkat Reporter Cells for Epitope Reactivity

Pellet two aliquots of 100,000 target cells with the appropriate HLA type for the epitope/TCR in 1.5 ml microcentrifuge tubes at 2,400 rcf at 4°C for 3 minutes (in a FrescoTM 17 Microcentrifuge at 5,000 rpm or similar centrifuge); these can be HLA matched immortalized B or T lymphocytes, or other cells transfected/transduced with the gene for the HLA. Remove the supernatants and vortex to resuspend the cell pellets, adding 200 µl of R20 and the cognate peptide at a final concentration of 1 µg/ml to one aliquot and no peptide (or an irrelevant negative control peptide) to the other aliquot. Culture with the caps loosened in the tissue culture incubator for 30 to 60 minutes while continuing to prepare the Jurkat reporter cells. Pellet 200,000 of the non-transduced Jurkat reporter cells and 150,000 of the previously TCR-transduced Jurkat reporter cells in 1.5 ml microcentrifuge tubes at 2,400 rcf at 4°C for 3 minutes. Aspirate and discard supernatants; vortex to break up the cell pellets. Bring both tubes of the Jurkat effector cells to a concentration of 0.5×10^6 cells/ml in R20 pre-warmed to 37°C by adding 400 µl to the non-transduced cells and 300 µl to the transduced cells; mix by pipetting.

Bring both tubes of the target cells to 0.5 x 10^6 cells/ml by adding 150 µl of R20 pre-warmed to 37°C each; mix by pipetting. In a 96-well round bottom tissue culture plate, set up the conditions listed in table 17 by adding 100 µl of cells or R20 to each well, bringing all wells to a final volume of 200 µl. Incubate the plate in the tissue culture incubator for 24 hours.

JURKAT REPORTER CELLS	STIMULUS	STAINING	PURPOSE
Non-Transduced	None	Isotype (PE) Isotype (APC) Isotype (PerCP-Cy5.5)	Negative stain control
	None	α-CD8 (PE) Isotype (APC) Isotype (PerCP-Cy5.5)	PE stain control
	None	Isotype (PE) α-CD8 (APC) Isotype (PerCP-Cy5.5)	APC stain control
	None	Isotype (PE) Isotype (APC) α-CD8 (PerCP-Cy5.5)	PerCP-Cy5.5 stain control
TCR-Transduced	PMA/Ionomycin	Isotype (PE) Isotype (APC) Isotype (PerCP-Cy5.5)	GFP control
	Target cells without peptide	α-HSA (PE) α-CD3 (APC) α-CD8 (PerCP-Cy5.5)	Unstimulated control
	Target cells with cognate peptide	α-HSA (PE) α-CD3 (APC) α-CD8 (PerCP-Cy5.5)	Epitope recognition test

Table 17. Experimental test conditions for TCR-transduced Jurkat reporter cells.

Test conditions for evaluating TCR responsiveness after transduction of Jurkat reporter cells are listed.

Isotype (PE) = Rat IgG2b Isotype Control-PE Clone KLH-G2B-1-2 (Southern Biotec, https://www.southernbiotech.com/ProductDetails.aspx?catno=0118-09&ttl=Rat+IgG2b-PE, S0118-09)
Isotype (APC) = Anti-Mouse IgG2a Isotype Control-APC Clone m2a-15F8 (Southern Biotec, http://www.ebioscience.com/mouse-igg2a-antibody-m2a-15f8-apc.htm, 17-4210-80)
Isotype (PerCP-Cy5.5) = PerCP/Cy5.5 Mouse IgG1, κ Isotype Ctrl Antibody (BioLegend, http://www.biolegend.com/percp-cy55-mouse-igg1-kappa-isotype-control-4205.html, 400149)
α-CD8 (PE) = Anti-Human CD8-PE Clone SK1 (http://www.bdbiosciences.com/us/applications/clinical/blood-cell-disorders/ivd-reagents/cd8-leutrade-2a-pe-sk1/p/340046, 340046)
α-CD8 (APC)= Anti-Human CD8-APC Clone SK1 (http://www.bdbiosciences.com/us/applications/clinical/blood-cell-disorders/asr-reagents/cd8-apc-sk1/p/340659, 340659)
α-CD8 (PerCP-Cy5.5)= Anti-human CD8a clone RPA-T8 (eBioscience, http://www.ebioscience.com, 45-0088-41)
α-HSA (PE)= Anti-mouse CD24 clone M1/69 (eBioscience, http://www.ebioscience.com, 12-0242-81)
α-CD3 (APC)= Anti-Human CD3 clone OKT3 (eBioscience, http://www.ebioscience.com, 50-0037-41)

Preparation of Jurkat Reporter Cells for Flow Cytometric Assessment

To wash the cells, spin the 96-well plate at 484 RCF for 10 minutes at 4°C (in a Sorvall Legend RT centrifuge at 1,500 rpm or similar). Remove the supernatants, taking care not to disrupt the cell pellets. Add 200 µl of PBS with 1% FBS to each well and resuspend the cells by pipetting. Repeat the spin and wash. Spin down the cells and remove the supernatants, taking care not to disrupt the cell pellets. Protected from direct light, add 95 µl of PBS with 1% FBS to each well, resuspending the cells by pipetting, and add 0.8 µl of the antibodies**.** Incubate the plate at 4°C for 25 minutes in the dark. Spin and wash the plate twice with PBS with 1% FBS, and resuspend each pellet in 200 µl PBS with 1% paraformaldehyde, after which the plate can be stored for up to 24 hours at 4°C before analysis.

Flow Cytometric Assessment of Jurkat Reporter Cells Transduced with TCR

Calibrate the flow cytometer for negative gating and compensation using isotype and single color control samples 136. Assess the TCR-transduced Jurkat reporter cells (CD8-expressing gated cells) for transduction efficiency (HSA expression), TCR expression (CD3 expression), and epitope recognition (GFP expression), using the gating strategy shown in figure 13**.**

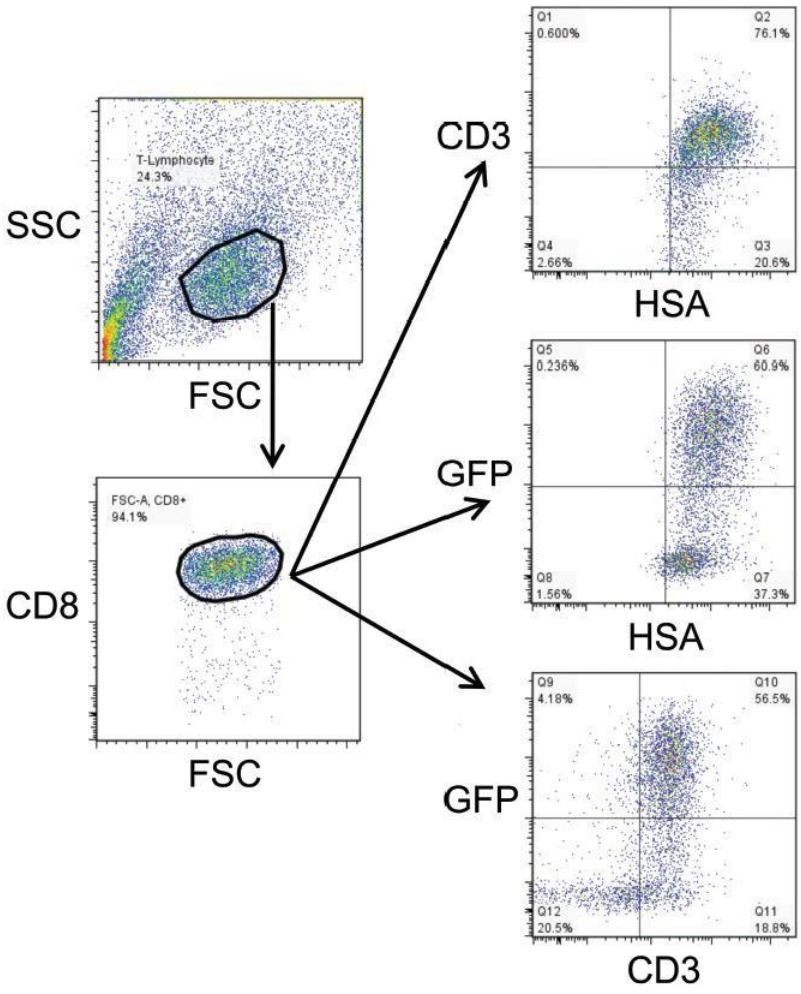


Figure 13. Gating stratey to assess epitope-specific TCR triggering in Jurkat reporter cells. Cells in the live gate (by forward scatter and side scatter) that are CD8+ (left panels) are examined for expression of: the lentiviral vector heat stable antigen (HSA) reporter reflecting successful transduction, CD3 reflecting expression of a T cell receptor, and green fluorescent protein (GFP) reflecting T cell receptor signaling (right panels). In this example, transduced cells (HSA+) express intact TCRs (CD3+), and TCR-transduced cells respond to the epitope (GFP+).

HIV-1 Permissive Target Cells

The cell lines Jurkat (expressing HLA-B*3501 or HLA-B*5701), H9 (expressing HLA-B*3501 or HLA-B*5701) and T1 (expressing HLA-B*3501 or HLA-B*5701) were maintained in RPMI 1640 medium supplemented with L-glutamine, penicillin-streptomycin, and 10% heat-inactivated fetal calf serum, as described elsewhere (O. O. Yang et al., 1996).

Generation of Pseudotyped Non-T-Cell Receptor Lentivirus Stocks

Lentivirus was produced by transfection of 293T cells with the above lentiviral vector constructs in conjunction with the lentiviral packaging vector pCMV[DELTA]R8.2DVPR (Paranjape, Gadkari, Lubaki, Quinn, & Bollinger, 1998) and the vesicular stomatitis virus envelope protein G expression vector pHCMVG (Rutebemberwa et al., 2004) using Fugene HD transfection reagent (Roche, Nutley, New Jersey, USA). Supernatants from 2 and 3 days after transfection were combined, passed through a 0.22 micron filter, and concentrated by centrifugation at 28 000g for 120 min at 4°C. Virus concentration was assessed by Flow Cytometry by staining against the reporter CD24.

HIV-1 Mutagenesis and Stocks

Virus stocks were generated from plasmid DNA as described elsewhere (reference). The NL4-3 sequence served as the backbone for the HIV-1 mutants produced for this study, with a single amino acid mutation in Nef to ablate HLA class I down-regulatory function and avoid this confounding effect on CTL activity. Consensus sequences were obtained from the Los Alamos National Laboratory HIV Sequence Database (http://www.hiv.lanl.gov/content/hiv-db). Production of the clade B consensus KF11 sequence KAFSPEVIPMF using overlapping polymerase chain reaction mutagenesis was as reported elsewhere (reference). The different KF11 variants were produced using the QuikChange mutagenesis kit (Stratagene) by changing

the clade B consensus sequence from KAFSPEVIPMF to the different KF11 variant sequences as listed in table 18.

Variant	% of Circulating KF11 variants
KAFSPEVIPMF	**91.8**
------I----	**2.16**
---N-------	**1.27**
-N---------	**0.75**
-S---------	**0.52**
-G-N-------	**0.15**
------T----	**0.15**
Total % of Circulating KF11 Variants	**96.8**

Table 18. List of the most common circulating KF11 epitope variants within clade B.

The above variants are the most common circulating variants for HIV-1 epitope KF11 (gag 162-172). Together, these variants comprise ≈97% of all circulating HIV-1 clade B strains.

Functional avidity measurements and chromium release assays

CTL functional avidity was measured via standard chromium release assays with five-fold peptide dilutions ranging from 5.0 ug/ml to 64 pg/ml using 10^4 target cells (Cr^{51}-labeled T1 cells) in 96-well U-bottom plates with 10^5 effector cells (TCR-transduced subcloned $CD8^+$ T cells). Chromium release in the supernatant was assessed after 3.5 h using a microscintillation counter (MicroBeta 1450; Wallac-Perkin Elmer, Waltham, Massachusetts, USA), and specific lysis was calculated as follows: (observed chromium release – spontaneous chromium release) ÷ (maximal chromium release – spontaneous chromium release). SD_{50} values (the concentrations of peptide needed to achieve half maximal specific lysis) were calculated via nonlinear regression using Graphpad Prism 5 (Graphpad Software, La Jolla, California, USA).

Results

Cells Lines Engineered to Express HLA-B*5701

Immortalized B-cells from an HLA-B*5701^{+} subject were used to PCR amplify HLA-B*5701. The PCR product amplified had 15nt overhangs that were complementary to our lentiviral vector. In-Fusion HD cloning was then used to clone the HLA-B*5701 gene into the lentiviral vector. The newly made HLA-B*5701 lentiviral vector was then used to make pseudotyped lentivirus and transduce the CD4^{+} T1 cell line (Salter, Howell, & Cresswell, 1985) that normally expresses HLA-A*02 and HLA-B*40. The T1-transduced cells' HLA-B*5701 expression was then verified via flow cytometry. Upon confirmation of HLA-B*5701 expression, the T1-HLA-B*5701 cells were subcloned to attain a uniform clonal population of T1 cells that highly expressed HLA-B*5701. After 4 weeks of culturing the subclones cells in R10, the expression of HLA-B*5701 was again confirmed by flow cytometry (see Figure 14).

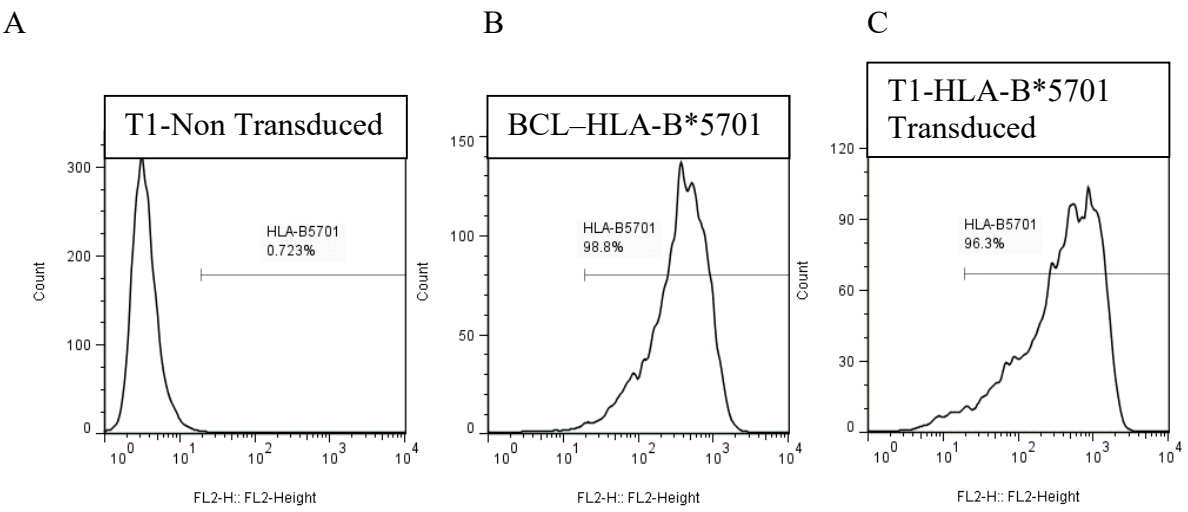


Figure 14. Stable high expression of HLA-B*5701 in transduced and subcloned T1 cells. Samples were stained with anti-HLA-B*5701 to confirm the level of expression HLA-B*5701 in the transduced T1 cells. Panel A : Non-transduced T1 cells. Panel B : Immortalized B-cells isolated from an HLA-B*5701 positive subject and used as a positive control. Panel C : Transduced and subcloned T1-HLA-B*5701 cell line. The immortalized B-cells and the transduced subclone T1-HLA-B*5701 cell line have comparable HLA-B*5701 expression, with mean fluorescence intensity of 448 and 573, respectively.

KF11-Specific TCR α β Chains were Identified

Seven subjects with HIV-1 chronic untreated infection were assessed by INF-γ EliSpot Assays for their response against KAFSPEVIPMF (see Figure 15). The PBMC of subjects with a positive INF-γ response were co-cultured for seven days with and without 1ug/ml of KAFSPEVIPMF peptide for Quantitative Spectratyping (QS) analysis (Balamurugan et al., 2010). Eight TRAV and TRBV chains were identified and sequenced (See Table 19). TRAV 05-01 and TRBV 19-01 have been previously described as public TCR sequences specific for the KAFSPEVIPMF. These same sequences were also identified in this study as specific for KAFSPEVIPMF. At times, more than one TRAV or TRBV were identified for one subject. A limitation of QS is that TRAV and TRBV chains are analyzed separately. Consequently, if more than one expansion takes place it is uncertain which combination of TRAV and TRBV is correct.

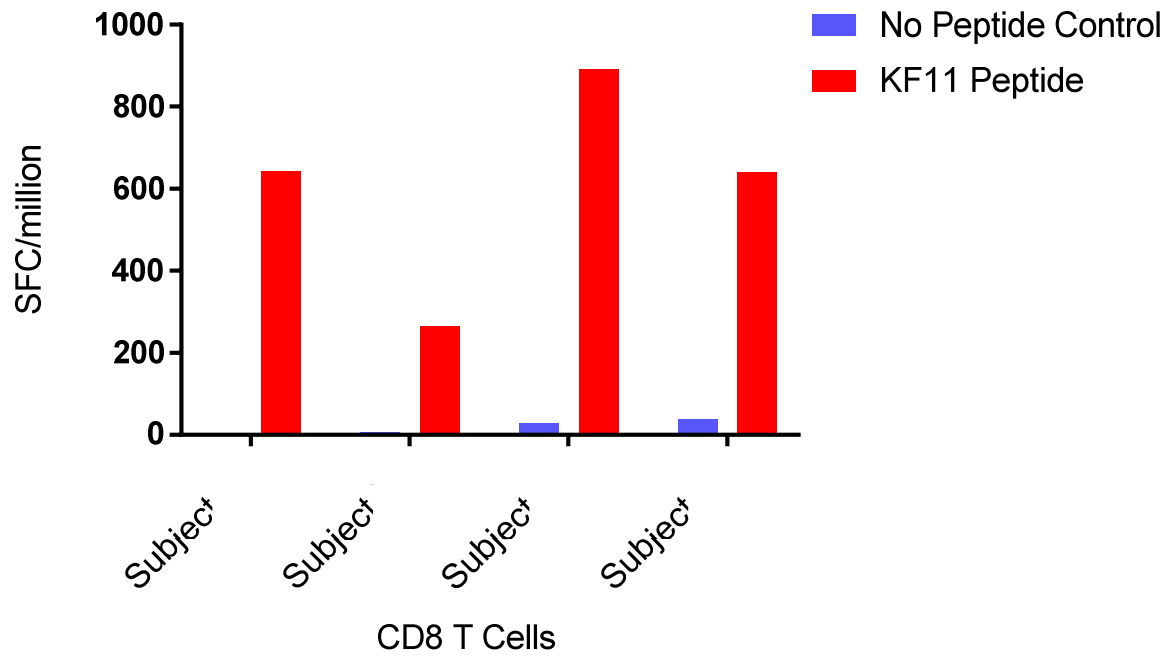


Figure 15. Screening for KF11-specific CTL responses. KF11-specific CTL responses were measured by INF-γ EliSpot assays using polyclonally expanded CD8+ T cells from each of the four subjects. Responses are reported as the number of spot-forming cells (SFC) per million CD8+ T cells.

A

AV	AJ	A-CDR3
05-01	13-01	CAVLGGYQKVTF
05-01	13-01	CAVSGGYQKVT
05-01	13-02	CAASGGYQKVTF
05-01	22-01	CAESSGSARQLTF
05-01	31-01	CAEGARLMF
12-03	15-01	CAMSAGQAGTALIF
14/D4-01	26-01	CAMRDNYGQNFVF
14/D4-02	49-01	CAMRESVATGNQFYF

B

BV	BJ	BD	B-CDR3
05-08	02-06	01-01	CASSVGFGANVLTF
06-05	02-05	02-02	CASSYSQQGGETQYF
07-08	02-05	01-01	CASSPREGAGETQYF
07-09	02-05	02-02	CASSPTLAGIQETQYF
14-01	02-05	02-01	CASSPRDSKETQYF
15-02	01-02	01-01	CATSGTEYGYTF
19-01	01-02	01-01	CASSGSYGYTF
19-01	01-02	01-01	CASTGTYGYTF

Table 19. List of TRAV and TRBV chains identified by spectratyping against HIV-1 epitope, KAFSPEVIPMF.

Table 19A is the list of TRAV chains identified. Table 19B is the list of TRBV chains identified.

CONSTRUCTION OF LENTIVIRAL TCR EXPRESSION VECTORS

cDNA from KAFSPEVIPMF stimulated PBMC was used to amplify the identified TRAV and TRBV. As described in detail in the Materials and Methods section, the PCR products were used clone the TRAV and TRBV into a pCCL lentiviral transfer vector under control of the Ubiquitin-C promoter. Nine different constructs were made (See Table 20).

AV	AJ	A-CDR3	BV	BJ	BD	B-CDR3
05-01	13-01	CAVLGGYQKVTF	19-01	01-02	01-01	CASTGTYGYTF
05-01	13-01	CAVSGGYQKVT	19-01	01-02	01-01	CASSGSYGYTF
05-01	13-02	CAASGGYQKVTF	07-08	02-05	01-01	CASSPREGAGETQYF
05-01	13-02	CAASGGYQKVTF	07-09	02-05	02-02	CASSPTLAGIQETQYF
05-01	22-01	CAESSGSARQLTF	15-02	01-02	01-01	CATSGTEYGYTF
05-01	31-01	CAEGARLMF	06-05	02-05	02-02	CASSYSQQGGETQYF
12-03	15-01	CAMSAGQAGTALIF	19-01	01-02	01-01	CASTGTYGYTF
14/D4-01	26-01	CAMRDNYGQNFVF	14-01	02-05	02-01	CASSPRDSKETQYF
14/D4-02	49-01	CAMRESVATGNQFYF	05-08	02-06	01-01	CASSVGFGANVLTF

Table 20. List of KF11-specific TCR constructs cloned into the pCCL vector.

TRC Constructs Can Recognize KF11 Peptide Loaded Target Cells

As described in detail in the Materials and Methods section, the pCCL-KF11-specific TCR pseudotyped lentiviruses were screened in modified Jurkat J.RT3-T3.5 reporter cells (Birkholz et al., 2009; Schaft, Lankiewicz, Gratama, Bolhuis, & Debets, 2003). These reporter cells lack an endogenous TRBV chain and therefore do not express CD3 unless an exogenous TCR is introduced (Ohashi et al., 1985; Schneider, Schwenk, & Bornkamm, 1977). Additionally, this Jurkat cell line has been modified to express CD8 and contain an NFAT-dependent GFP reporter gene, yielding green fluorescence if a functional TCR is engaged (Macian, 2005). T1-HLA B*5701 cells were peptide pulsed for 45 minutes with either KAFSPEVIPMF or one of its variants. The KF11-peptide loaded T1-HLA B*5701 cells were then co-cultured overnight with modified Jurkat cells transduced with KF11-specific TCR. The modified Jurkat cells were then screened for GFP expression. All nine constructs expressed the cloned TCR as referenced by the positive CD3 expression. However, only four of the nine constructs, S14-KF11-B5701-TRAV5TRBV15, S52-KF11-B5701-TRAV5TRBV19, S68-KF11-B5701-TRAV5TRBV19 and S94-KF11-B5701-TRAV14TRBV5, recognize the KAFSPEVIPMF variants as evidenced by GFP expression (See Figure 16).

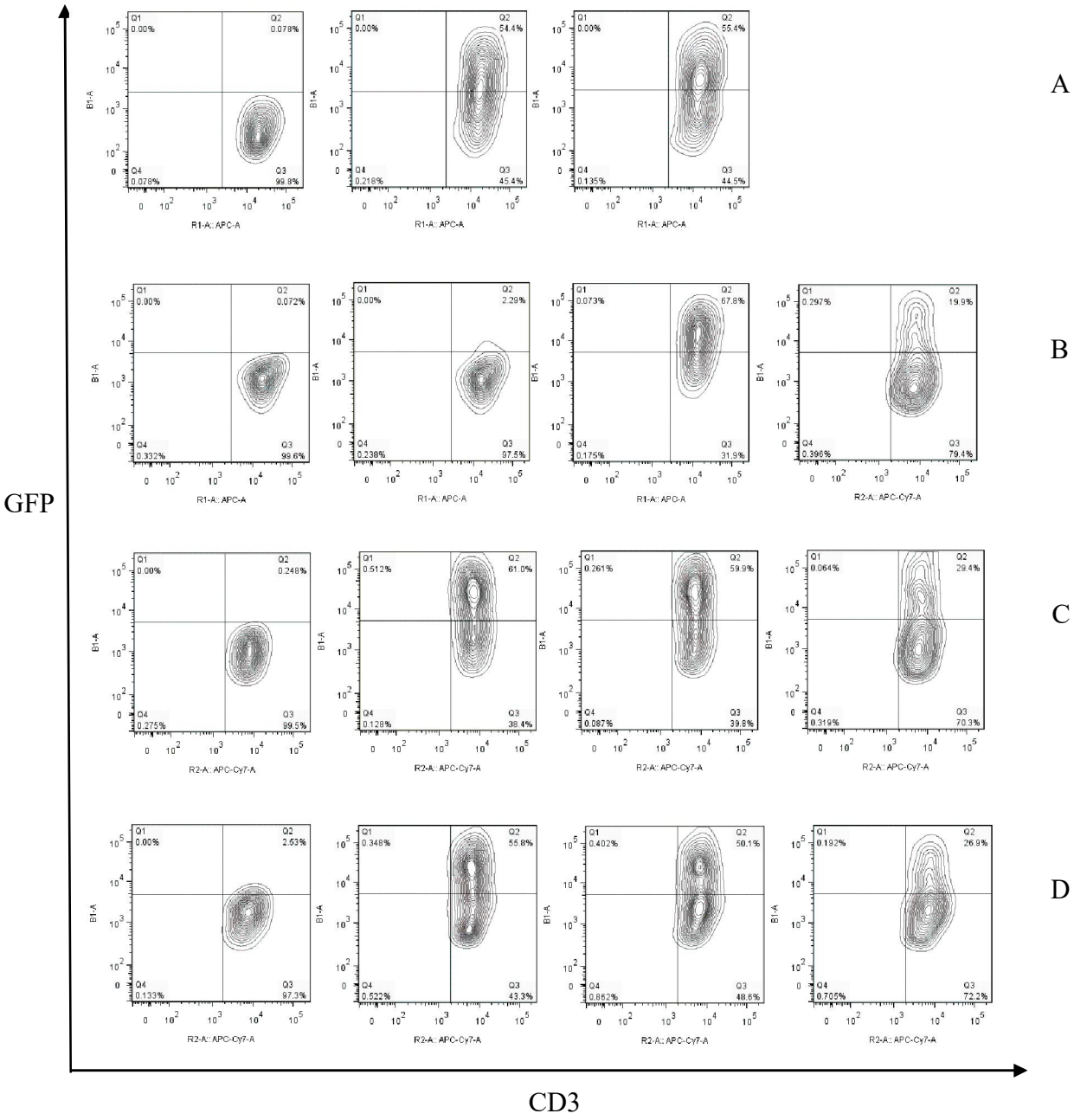


Figure 16. KF11-Specific TCR are able to recognize KF11 variants and induce GFP expression in the Jurkat-GFP system.

Modified jurkat-CD8+ cells were transduced with KF11-specific TCR to assess whether or not the TCR could recognize KF11 variants and induce GFP expression as a result of a successful TCR/pHLA engagement. The different TCR construct recognize different KF11 variants and to

a varying degree as measured by GFP expression. Row A: TCR construct S14-KF11-B5701-TRAV5TRBV15 is able to recognize the KF11 V168I and V168T variants. Row D: TCR construct S52-KF11-B5701-TRAV5TRBV19 is able to recognize the KF11 V168T and S165N variants but not the KF11 V168I variant. Row C: TCR construct S68-KF11-B5701-TRAV5TRBV19 is able to recognize the KF11 V168I and V168T variants and to a lesser extent, the KF11 S165N variant. Row B: TCR construct S94-KF11-B5701-TRAV14TRBV5 is able to recognize the KF11 V168I and V168T variants and to a lesser extent, the KF11 S165N variant.

Primary Bulk CD8$^+$ T Cells Transduced with KF11-Specific TCR recognize and Lyse KAFSPEVIPMF Peptide Loaded Target Cells

Healthy primary CD8$^+$ T cells were transduced with pCCL-KF11-specific TCR pseudotyped lentiviruses and 48 hours later screened for CD24 expression. Samples with >90% CD24 expression and an MFI of 2 logs higher than the untransduced control were preliminarily screened for their ability to lyse target cells loaded with KAFSPEVIPMF peptide. Constructs S14-KF11-B5701-TRAV5TRBV15, S52-KF11-B5701-TRAV5TRBV19, S68-KF11-B5701-TRAV5TRBV19 and S94-KF11-B5701-TRAV14TRBV5 recognized and lysed the T1-HLA B*5701 cells loaded with KAFSPEVIPMF peptide.

Primary CD8$^+$ T Cells Demonstrate Stable Clonal Expression of Exogenous TCR

The bulk CD8$^+$ T cells that were transduced with one of the four KF11 TCR constructs were sorted for CD24 expression. The sorted transduced CD8$^+$ T cells were then subcloned with one, three and five cells per cell in six 96-well round bottom plates. Each construct was co-cultured with feeder cells, stimulated with 12F6 antibody and maintained with R10-12.5 for approximately six to seven weeks. Each sample was then screened for CD24 expression using flow cytometry. Samples with a clonal population expressing CD24 with about two MFI logs higher than non-transduced controls were then re-stimulated and maintained in R10-12.5. After two additional weeks, the samples were re-screened for CD24 expression to ensure high stable expression of CD24.

The Combined KF11-Specific TCR Panel can Lyse T1 Cells that are Peptide Loaded with KF11 WT Peptide or All of Its Most Common Circulating Variants

Primary CD8$^+$ T cell subclones transduced with KF11-specific TCR were screened for their ability to lyse target cells loaded with KAFSPEVIPMF peptide or its variants using the ^{51}Cr

killing assay with an effector to target ratio of 10:1. Each of the four different constructs are able to lyse the consensus sequence of the KF11 epitope, however, each construct has its own unique pattern of variant coverage. The S14-KF11-B5701-TRAV5TRBV15 construct recognized and lysed target cells loaded with KAFSPEVIPMF and the A163S, S165N and A163G S165N KF11 variants. The S52-KF11-B5701-TRAV5TRBV19 construct recognized and lysed target cells loaded with KAFSPEVIPMF and the A163S, A163G S165N, V168I variants. The S68-KF11-B5701-TRAV5TRBV19 construct recognized and lysed target cells loaded with KAFSPEVIPMF and the A163S, S165N, A163G S165N, V168I, V168T variants. The S94-KF11-B5701-TRAV14TRBV5 construct recognized and lysed target cells loaded with KAFSPEVIPMF and the S165N, V168I, V168T (See Figure 17). When combined, these four KF11-specific TCR are able to recognize and lyse cells that are peptide loaded with all the most common circulating strains of the KF11 epitope.

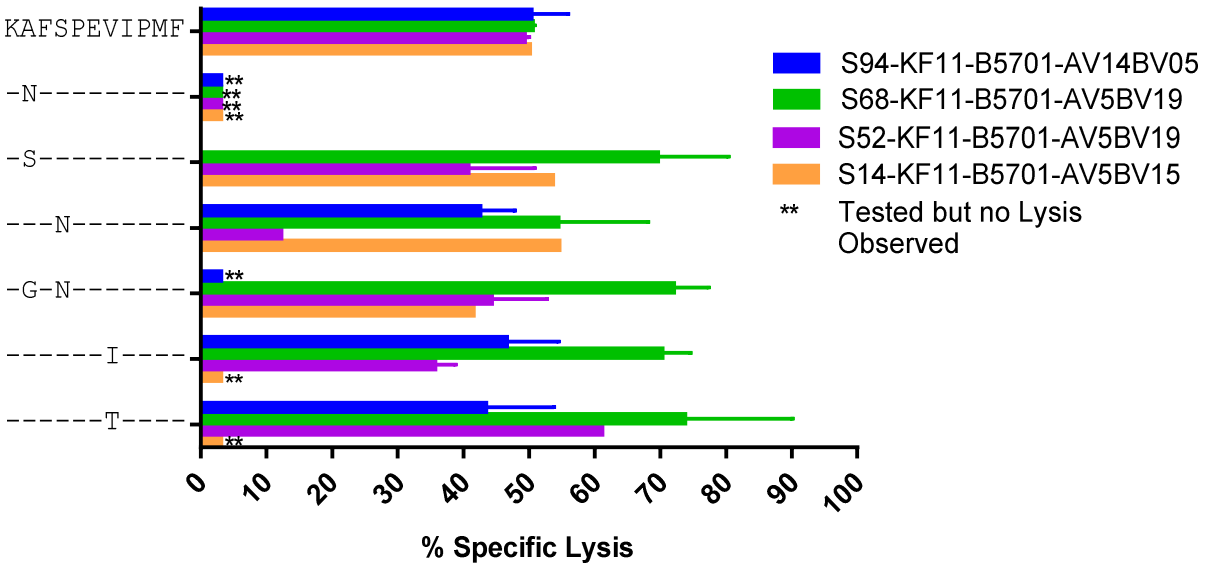


Figure 17. Viral variant coverage of KF11-specific TCR.

Each KF-11 specific TCR construct was tested for its ability to lyse the different KF11 variants using peptide loaded T1-HLA-B57*01 target cells with an E:T ratio of 10:1. Each KF11-specific TCR construct has a different recognition pattern for KF11 variants.

TCR Avidity Differs Between Each KF11-Specific TCR Construct and KF11 Epitope Sequence

To assess whether the KF11-specific TCR subclones varied in their sensitivity to the KF11 epitope and its variants, the primary CD8+ T cell subclones transduced with the different KF11-specific TCR were tested in chromium release assays against T1-B*5701-expressing target cells that were loaded with varying concentrations of KF11 wild type peptide and KF11 variant peptides (see Table 18). The functional avidities, defined as the sensitizing dose of peptide for 50% maximal activity (SD_{50}), were calculated for each KF11 TCR versus each KF11 variant (See Figure 18). The SD_{50} values for S52-KF11-B5701-TRAV5TRBV19, S68-KF11-B5701-TRAV5TRBV19 and S94-KF11-B5701-TRAV14TRBV5 varied over a range of 32ng/ml to 2,167ng/ml, 28ng/ml to 2,118ng/ml and 609ng/ml to 894ng/ml, respectively, across the different combinations of KF11 epitope variants.

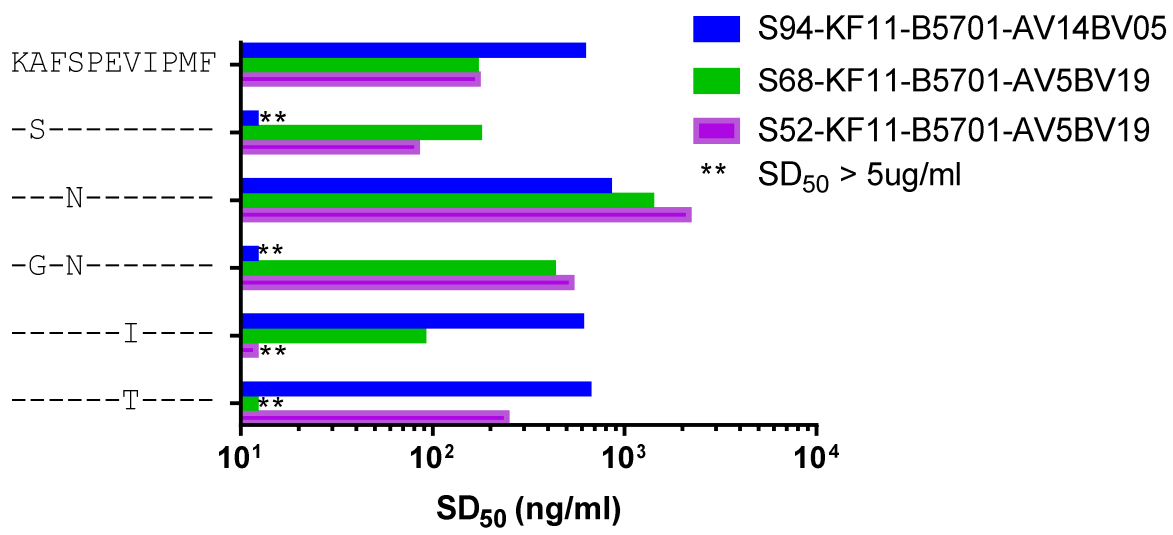


Figure 18. Functional Avidity of KF11-specific TCR constructs against KF11 variants.

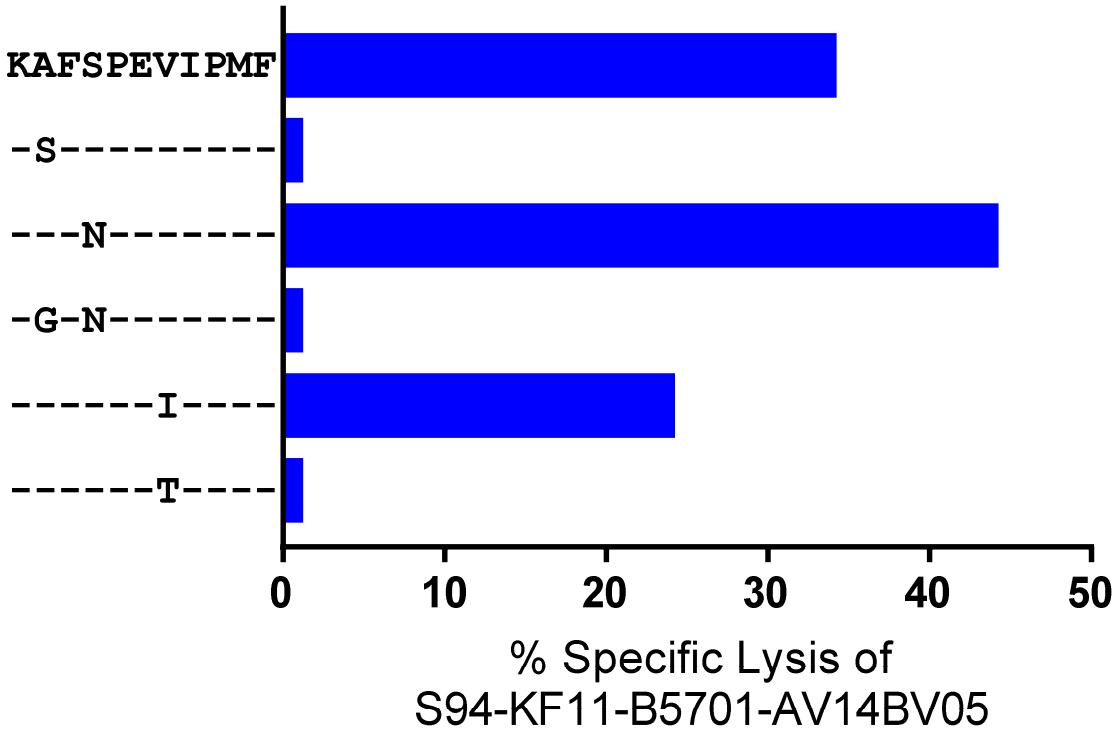
Each TCR has a different SD50 for the targeted epitope. The 2N variant was not tested since it was not lysed by any of the KF11 TCR.

Primary CD8$^+$ T Cells Transduced with KF11-specific TCR Can Lyse Cells Infected with HIV- containing KF11 WT and its variants

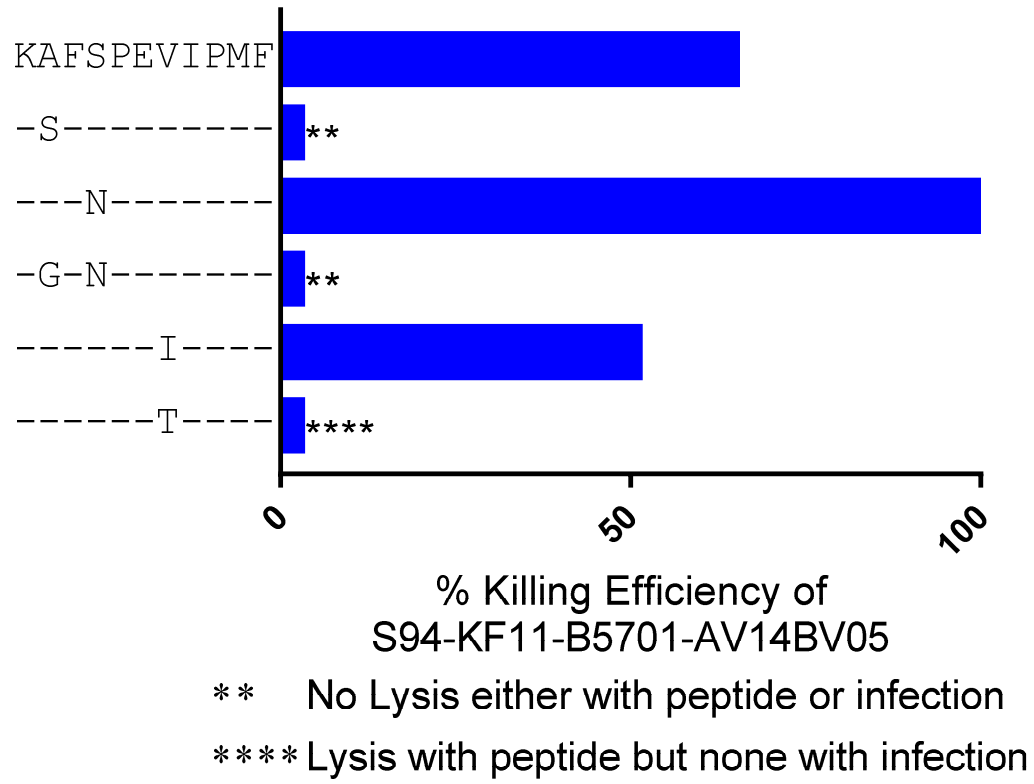
The primary CD8+ T cell subclone transduced with the KF11 specific TCR, S94-KF11-B5701-TRAV14TRBV5, was re-transduced with the same KF11 TCR construct to have physiological levels of TCR expression. The re-transduced subclone expressing the KF11 TCR S94-KF11-B5701-TRAV14TRBV5 was tested in chromium release assays against HIV infected T1-B*5701 target cells. The target cells were infected with HIV vector, NL4-3 M20A VPR-CD24. The HIV vector contained either the KF11 consensus epitope or each of its variants. The re-transduced subclone was able to lyse the HIV vector containing the KF11 consensus sequence, S165N variant, and the V168I variant (See Figure 19 panel A). Except for the KF11 V168T variant, the KF11 specific TCR, S94-KF11-B5701-TRAV14TRBV5, is able to lyse the same KF11 epitope pattern in both, peptide loaded and in HIV infected cells (See Figure 19 panel A). The observed lysis against the infected cells was then used to determine the percent killing efficiency of the KF11 specific TCR, S94-KF11-B5701-TRAV14TRBV5 against the KF11 variants. % Killing efficiency was calculated by [(Observed killing from infected cells) ÷ (Theoretical Max Killing as determined by % lysis of peptide loaded cells)] X [% of infected cells] . The highest observed killing efficiency was between the KF11 variants was S165N > consensus > V168I and with no observed lysis of infected cells for variant V168T (See Figure 19 panel B). In a prior study (Bennett, 2007), it was observed that lysis of infected cells was restricted to a strict threshold of 1log difference of SD_{50}. In this study, no lysis of infected cells was observed when a variant differed by more than 1log from the highest avid epitope variant. All variants targeted by KF11 specific TCR, S94-KF11-B5701-TRAV14TRBV5, were within

1log and as such killing of infected cells was observed except for the V168T variant (See Figure 19 Panel C).

A


KAFSPEVIPMF
-S---------
---N-------
-G-N-------
------I----
------T----
0
10
20
30
40
50
% Specific Lysis of
S94-KF11-B5701-AV14BV05

B



** No Lysis either with peptide or infection

**** Lysis with peptide but none with infection

C

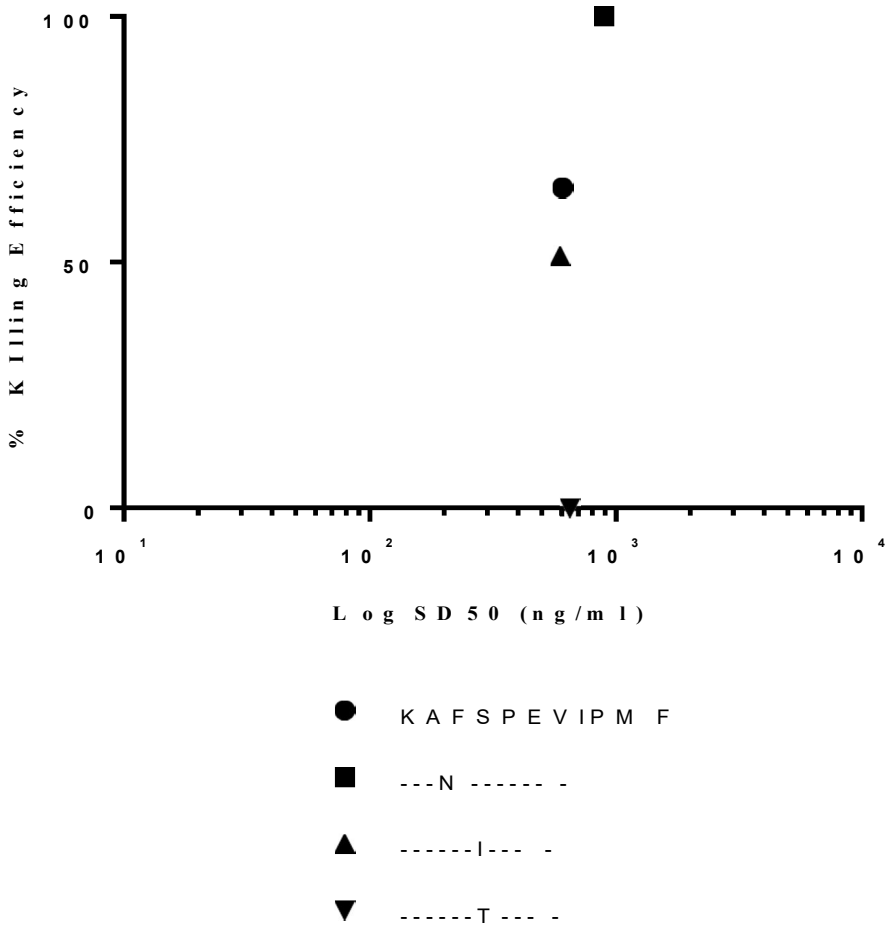


Figure 19. Recognition and killing pattern of target cells infected with HIV-containing KF11 variants.

(A)T1-B*5701 target cells were infected with NL4-3 M20A VPR-CD24 that encoded for the different KF11 variants. The target cells were infected for 3 days and then used in a 51Cr killing assay using the KF11-specfic TCR construct S94-KF11-B5701-AV14BV05. TCR construct S94-KF11-B5701-AV14BV05 lysis pattern of infected cells was the same as with peptide loaded cells except for the V168T variant. (B) KF11-specfic TCR construct S94-KF11-B5701-

AV14BV05 has the highest % killing efficiency for variant S165N at 100% followed by consensus at 65%, then V168I with 51%. No killing efficiency reported for variant V168T since there was no observed lysis of cells infected with this variant. (C) % Killing efficiency was plotted against SD50 values for KF11-specfic TCR construct S94-KF11-B5701-AV14BV05. Despite the difference of an SD50 of about 300ng/ml between the different KF11 variants, lysis was still observed.

Discussion

Viral escape by HIV-1 is one of the leading causes of why we lack an effective HIV-1 vaccine. HIV-1 is able to quickly mutate to render current treatments useless and to avoid immuno-surveillance. Despite recent successes with T cell immunotherapy against melanoma, one of the challenges in its transfer to HIV-1 treatment is viral escape and cost. In a prior attempt to utilize T-cell immunotherapy against HIV-1, an individual was passively transferred an HIV-specific CTL. However, the epitope quickly mutated and escaped the CTL, rendering the CTL useless. Additionally, the current technology to identify, isolate and clone CTL is very laborious and expensive.

The earliest methodology to generate epitope-specific TCR genes was cloning epitope-specific T-cells followed by isolation of TCR sequences, but this laborious and reagent-intensive method is not amenable to high throughput. The current state-of-the art is cloning TCR genes from molecular libraries generated from populations of epitope-specific cells identified by binding of 4 MHC-epitope multimers sorted by flow cytometry (Douek et al., 2002; Szymczak et al., 2004). From the total sorted cell population a cDNA library of AV and BV genes is prepared, from which many molecular clones are screened. Within the mixture from a polyclonal response, the proper pairing of these genes is unknown, requiring individualized functional testing different pairings of intermediate vectors in primary CD8+ T cells, after which a final cloning step is required to combine the AV and BV chain genes into a single vector (Kobayashi et al., 2013; S. Yang et al., 2008). Although a recently described single cell PCR protocol addresses some of these limitations (Kobayashi et al., 2013), that technique is not widely used and requires highly specialized equipment and expertise associated with single cell sorting.

There are several limitations to this strategy. MHC-epitope multimers may be unavailable, especially for rare MHC types/subtypes, and every newly identified epitope requires production of a new multimer. Cell sorting requires significant infrastructure, expertise, and expense, and is inadequate for epitope-specific cells that are below the limit of reliable detection by flow cytometry. High frequency clones in the bulk cDNA library may obscure rare subpopulations of TCRs, given limitations in the numbers of AV or BV genes from the library that can be feasibly cloned and screened. These caveats are addressed by our approach.

Our protocol is founded upon spectratyping, a tool originally developed by Pannetier *et al* (Pannetier et al., 1993) to analyze the diversity of TCRs based on the size distributions within variable gene families. By enriching the frequency of epitope-specific TCRs through cognate peptide stimulation of PBMC, spectatyping has been developed further as a tool by Killian *et al* for indicating epitope-specific clonal expansions (Killian et al., 2005). Most recently we modified this assay to be quantitative to reveal epitope-specific TCR sequences without need for cloning the bulk PCR products of AV and BV families. Taking advantage of this capability for rapid definition of epitope-specific TCR gene sequences, we have devised a simple method to combine these sequences into a modified version of a lentiviral construct developed by Szymczak *et al (Szymczak et al., 2004)* for dual expression of AV and BV genes, further coupled with a rapid screening strategy using Jurkat cells that express green fluorescent protein (GFP) in a TCR signaling-dependent manner (Birkholz et al., 2009; Schaft et al., 2003).

Our strategy circumvents the requirement for cell sorting and MHC-epitope multimer complexes, which is a major limitation to current methodology. Because expansions of epitope-specific TCRs are identified within individual variable gene families after enrichment of epitope-specific cells, there is greater sensitivity to find low frequency epitope-specific responses; a high

frequency expansion in one family does not obscure a low frequency expansion in another.

Like the current state-of-the-art approach, ours does not directly distinguish proper pairing of the identified AV and BV chains. However, the quantitative measurements of clonal expansions can suggest likely pairings (e.g. a high frequency AV expansion pairs with a high frequency BV expansion because they come from the same cell), and the use of a one-step molecular combination method (Zhu, Cai, Hall, & Freeman, 2007) greatly accelerates the process of producing complete TCR lentiviral vectors for functional screening, circumventing the need for generating and screening intermediate single AV and BV chain vectors. Finally, functional screening is typically performed using transduced primary CD8+ T cells, requiring high titer vectors and assay(s) such as cytokine release or cytolysis in response to the epitope. Alternatively, we harness a Jurkat cell line that conveniently tests TCR functionality by NFAT-dependent GFP expression (Birkholz et al., 2009; Schaft et al., 2003). These cells are easily maintained, highly transducible with lentiviral vectors even at the low titers achieved with small-scale virus stock production, and efficiently express exogenous TCRs given their endogenous TCR beta chain deletion. Thus, overall our protocol creates a more inexpensive, rapid and efficient method for producing final TCR lentiviral vectors starting from PBMCs.

Using our new technique, we identified and cloned TCRs specific for the gag epitope, KAFSPEVIPMF or KF11. This epitope is HLA-B*5701 restricted and its viral variants either have a high fitness cost or are lethal without compensatory mutations. Additionally, in contrast to many other HIV-1 epitopes, the top six circulating variants make up about 97% of all variants in the population according to the Los Alamos HIV database. HLA-B*5701 has been strongly correlated with HIV-1 elite non-progressors. As such, the KF11 epitope has been widely studied. These studies have revealed that viral variants have reduced replicative capacity or a

high fitness cost and how they require compensatory mutations to lessen or negate those fitness costs. Additionally, these studies have also revealed that there are public TCR against the KF11 epitope. Public TCR are TCR that are commonly found in persons sharing the same HLA and targeting the same epitope. Some of the known public TCR chains against KF11 include TRAV 5 and TRBV 19. Of the four TCR we identified and confirmed their functionality against KF11, two of them have the TRAV 5 and TRBV 19 chains but differed in their CDR3 sequence. Another TCR had the public TCR chain TRAV 5 but utilized the private TCR chain TRBV 15. Lastly, the final TCR identified utilized both private TCR chains TRAV14 and TRBV 15. From this study, using peptide loaded target cells, the two TCR that have the two public TCR chains, TRAV 5 TRBV 19, were able to lyse all six tested variants. Whereas the other two TCR constructs either had one public TCR chain, TRAV 5, or lacked any public TCR chains but instead had, TRAV 14 TRBV5. These two TCR were able to lyse four of the six most common KF11 variants tested.

Next we wanted to examine the avidity of the KF11-specific TCR. For this, we measured the amount of peptide the TCR requires for 50% of its maximal lysis, or its SD_{50}. To date, the field of TCR immunology follows the school of thought that the more avid the TCR, the better the TCR. Some studies suggest that highly avid TCR control HIV more effectively (Berger et al., 2011; Bihl et al., 2006; Mothe et al., 2012) and are thus better candidates for TCR immunotherapy. However, this may not be necessarily correct. In a prior study, three TCR specific for gag epitope SL9 were examined and tested for their SD_{50} (Bennett et al., 2007a). The SD_{50} of the SL9 TCR ranged from one to ten nanograms/ml (Bennett et al., 2007a). In a separate study, the avidity for HIV gag epitope, KRWIILGLNK (KK10) was measured using its SD_{50}. The SD_{50} of the KK10 TCR ranged from thirteen to 201 nanograms/ml (Berger et al.,

2011). Both of these epitopes are targeted during the acute phase of infection. Whereas the KF11 epitope is generally targeted during the chronic phase of infection. This may be due to the lower avidity observed with KF11. In a prior study, the SD_{50} of the KF11 TCR ranged from 152 to 443ng/ml (Berger et al., 2011). These data support the results we obtained in the KF11-specific TCR we tested, S52-KF11-B5701-TRAV5TRBV19, S68-KF11-B5701-TRAV5TRBV19 and S94-KF11-B5701-TRAV14TRBV5 varied over a range of 82ng/ml to 531ng/ml, 89ng/ml to 426ng/ml and 596ng/ml to 832ng/ml, respectively, across the different combinations of KF11 epitope variants. The KF11 epitope has been highly correlated with HIV control; any variant from the consensus sequence has either high fitness costs or is lethal without a compensatory mutation, suggesting that the KF11 epitope is crucial for the survival of HIV. When taken together with the available published SD_{50} data and our results, the notion that SD_{50} is the crucial factor to determine the efficacy of a TCR, disease progression and/or importance of an epitope seems erroneous. Although more TCR's SD_{50} need to be tested, I believe that early targeted epitopes will have higher avidity while epitopes targeted during chronic infection will have a lower avidity. This suggests that avidity alone may not necessarily be the determinant of the efficacy of a TCR, disease progression and/or importance of an epitope. Instead, SD_{50} should only be used as a comparison between TCR targeting the same epitope and not TCR that target different epitopes.

Conclusions made on SD_{50} values should also be made judiciously since SD_{50} is only half the story for the efficacy of a TCR. As previously described in Benett 2011, SD_{50} measurements do not take into account "protein expression, proteasome processing, epitope transport, and HLA binding". To take into account all of these factors, we tested the killing efficiency of the S94-KF11-B5701-TRAV14TRBV5 construct against infected cells with each of the targete KF11

variants. Except for the V168T variant, all of the variants that were targeted and lysed in the peptide loaded system, were also targeted and lysed in infected cells. Despite having an SD_{50} of 609ng/ml for the KF11 consensus sequence, and the S165N variant an SD_{50} of 832ng/ml, the S165N variant had a higher killing efficiency of 100% versus the consensus' of 65%. This would suggest that it is likely that this TCR was originally raised against the S165N variant but the TCR is also able to recognize and lyse the consensus and V168I variant.

It was previously reported that TCR have a narrow threshold of 1log SD_{50} variation which can translate to lysis of infected cells (Bennett et al., 2007a). Any epitope targeted by a TCR outside the 1log SD_{50} range would not be recognized and lysed in infected cells. However, the observation made was using TCR whose SD50 ranged from 1 to 10ng/ml or 10 to 100ng/ml. The difference between the highest and lowest SD50 for which killing of infected cells was observed was always <100 ng/ml. However, we found that even with an SD_{50} difference of about 449ng/ml, the S94-KF11-B5701-TRAV14TRBV5 construct was still able to lyse infected cells. While the prior conclusion about the narrow threshold of 1log SD_{50} variation for lysis of infected cells is supported by these findings, we can expand the prior conclusion to stating that highly avid TCR with an SD_{50} range of 1 to 100ng/ml are more sensitive to a deviation of peptide concentration than TCR that are less avid with an SD_{50} range of 100 to 1000ng/ml. This however would need to be tested with more TCR due to our small sample size of 4 TCR.

The goal of this project was to identify and clone a panel of TCR by combining epitope-specific TCR from multiple persons that would either prevent viral escape or limit the routes of escape to variants with high fitness cost. Towards this, we have successfully identified and clone four different KF11-specific TCR isolated from four different persons. Albeit in a peptide loaded system, these TCR can target and lyse all six of the most common variants of the KF11-

epitope that make up 97% of all circulating HIV-1 strains. These TCR may also recognize other variants but this has not yet been done. We have also demonstrated that at least one of our KF11-specific TCR can also target and lyse cells infected with the different targeted KF11 variants.

Funding

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Authorship Contributions

Dr. Otto Yang conceived the project idea to prevent viral escape through the construction of an antigen specific TCR panel. Dr. Otto Yang and Christian Aguilera-Sandoval conceived the TCR cloning protocol. Dr. Otto Yang oversaw the development of this project. Dr. Otto Yang, Christian Aguilera-Sandoval and Dr. Christian Hoffman developed the TCR cloning protocol, together developed the tables and figures and text for the TCR cloning portion of the project. Christian Aguilera-Sandoval and Dr. Diana Chen developed the modified Jurkat-$CD8^{+}$-GFP system. Christian Aguilera-Sandoval identified, cloned and functionally tested the KF11-specific TCR panel and developed the tables and figures for the functional testing of the KF-11-specific TCR panel. Hwee Ng did the majority of the ELISpot assays.

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I would like to thank Dr. Arumugam Balamurugan for expert advice on quantitative spectratyping. I would like to thank Hwee Ng for expert advice on cell culture. I would like to thank Ayub Ali for expert advice for molecular biology. I would also like to thank Hwee Ng, Brian Diep, and Eduardo Martin for technical assistance. I would like to thank Dr. Irvin Chen for expert advice on lentiviral vector construction.

Ch. 4 Discussion

Abstract

Chapter Four is subdivided into two major sections. The first section summarizes the major findings of, and discussions in, this dissertation. The second section will describe potential future work, and includes two sub-sections. The first sub-section discusses two issues: (1) the potential future characterization of the TCR repertoire in different segments of the HIV+ population, including fast progressors, elite non-progressors, and HIV+ persons who are treatment-naïve compared to those in modern cART treatment; and (2) the possibility in the future of complete characterization of TCRs that target early and late HIV epitopes. This latter characterization can include a comparison of the SD_{50} values of these epitopes to confirm whether high avidity TCR have a lower threshold between SD_{50} and killing efficiency compared to low avidity TCR. Lastly, in the final sub-section, we will discuss the potential future testing of the KF11-specific TCR panel constructed in this study, including the testing of its efficacy *in vivo*.

Summary of results

The overall aim of this dissertation was to gain a better understanding of the effects of HIV on T lymphocytes. Specific questions addressed include: (1) how HIV skews TCR repertoire and its effect on thymic output, and (2) how, despite having a negatively affected TCR repertoire, a panel of epitope-specific TCR can be identified and constructed to prevent viral escape.

We wanted to see if HIV infection skews TCR repertoire and thymic output, since it has been well-documented that thymic infrastructure is often damaged in HIV+ persons. Since the thymus is responsible for the development and production of T lymphocytes, it is safe to assume that T cell development and output is affected. To examine this, we performed an extensive analysis of parameters of thymic function in perinatally HIV-1-infected persons, ranging from 13 to 23 years of age. To our surprise, we found that the HIV+ persons had reduced CD4 T-cell levels, with predominant depletion of the memory subset but preservation of naive cells. RTE CD4 T-cell levels were normal in most infected individuals, and enhanced thymopoiesis was indicated by higher proportions of CD4 T cells containing TCR recombination excision circles. Memory CD4 T-cell depletion was highly associated with CD8 T-cell activation in HIV-1-infected persons and plasma interlekin-7 levels were correlated with levels of naive CD4 T cells, suggesting activation-driven loss and compensatory enhancement of thymopoiesis was occurring. Deep sequencing of CD4 T-cell receptor sequences in infected persons revealed supranormal diversity, thus providing additional evidence of enhanced thymic output. These results provide hope that, despite the damage caused by HIV-1 to thymic infrastructure, thymic output and TCR repertoire can be reconstituted with treatment. Nevertheless, this conclusion is

limited to children and young adults who have a higher likelihood of an “immune-comeback”, as opposed to older adults whose thymic output may be decreased, and whose TCR repertoire may have been skewed by viral infections throughout their lives. This last issue can be easily studied by thoroughly characterizing the TCR repertoire and thymic output of adults in cART versus treatment-naïve and healthy individuals. A further conclusion is that the development of an HIV vaccine has the potential to not only aid in getting rid of HIV but also can provide hope that the immune system of an HIV+ person can be reconstituted so they can live healthy.

To date there is no HIV vaccine available. However, T cell immunotherapy has shown promise in battling melanoma. In 1995, T cell immunotherapy was tried as a potential new treatment against HIV-1. Unfortunately it failed. However, it did not fail because of a lack of T cell responses to HIV-1 in the immunotherapy, but rather because HIV-1 was able to mutate and escape the TCR used. Therefore, the obvious goal for successful TCR immunotherapy would be the development of a T cell immunotherapy strategy that HIV could not mutate around . No one person can develop enough TCR at any given one time to cover all possible variants of an HIV epitope. However, if epitope-specific TCR from multiple HIV+ persons were pooled together, a panel of TCR could potentially be identified and constructed to target all possible variants of a specific HIV epitope, thereby preventing escape.

A problem with identifying and constructing a panel of TCR is that the current technology required is laborious and expensive. However, as previously demonstrated in Chapter Three, we have developed a new inexpensive strategy to quickly and efficiently identify and clone epitope-specific TCR. One of the advantages of this strategy is not it is not limited to HIV- specific TCR, but can be utilized in any disease that elicits a CTL response. Briefly, the TCR are: (1) identified via quantitative spectratyping, (2) the identified TCRαβ are

then cloned into a lentiviral vector through one single cloning InFusion reaction, and (3) the cloned TCR's functionality against the desired epitope is tested using our modified Jurkat-CD8+-GFP flow cytometry system.

Using our new TCR identification and cloning technique, we screened PBMC from HIV+ HLA-B*5701+ persons for CD8 responses directed against the gag 162-173 KF11 epitope. Four KF11- specific TCR were identified and cloned into a lentiviral vector. These four TCR constructs were named S14-KF11-B5701-TRAV5TRBV15, S52-KF11-B5701-TRAV5TRBV19, S68- KF11-B5701-TRAV5TRBV19 and S94-KF11-B5701-TRAV14TRBV5. Together, these four

KF11-specific TCR constructs were able to recognize and lyse cells that were peptide-loaded either with the KF11 consensus sequence or with any of the six most common circulating variants. According to the Los Alamos HIV Database, these six variants compose about 97% of all circulating strains of HIV-1 of Clade B. The SD_{50} values for these KF11-specific TCR constructs were measured and the difference between the highest and lowest SD_{50} for each construct against each of the six variants was always less than ten-fold. The highest SD50 (82ng/ml) was for construct S52-KF11-B5701-TRAV5TRBV19, which targets the KF11 A163S variant, while the lowest SD_{50} (832ng/ml) was for construct S94-KF11-B5701-TRAV14TRBV5, which targets the KF11 S165N variant. These data match previous results that found that KF11-specific TCR have SD_{50} values in the hundreds of ng/ml. These data also support a model in which low avidity TCR are used after high avidity TCR have been used. For example, TW10 is an epitope targeted during acute phase of infection and has an SD_{50} ranging from 1-100ng/ml, while KF11 is targeted during the chronic phase of infection and has an SD_{50} ranging from 100-900ng/ml. We next tested construct S94-KF11-B5701-TRAV14TRBV5

against HIV-infected cells to confirm that our TCR constructs were able to lyse HIV-infected cells and not only peptide-loaded cells. Construct S94-KF11-B5701-TRAV14TRBV5 was able to lyse cells infected with three of the four KF11 variants that were lysed in the peptide loaded system. The highest killing efficiency (100%) for construct S94-KF11-B5701-TRAV14TRBV5 was observed against the KF11 S165N variant, suggesting that this TCR was originally raised against this variant. However, this has not been confirmed since no sequencing of the virus infecting this person has been done.

In conclusion, we have demonstrated that we have compiled an antigen- specific panel against the major variants of a particular epitope that could potentially prevent viral escape. When taken together, these results suggest that if a TCR panel to prevent escape could be developed as a new therapy against HIV, it might be possible to restore the CD4 count and repertoire of HIV+ persons to either normal levels, or potentially even supranormal levels of T cell diversity.

Potential Future Work:

As is typical of science (and one reason why most of us do research), these findings have raised more questions than answers; the questions raised can and should be addressed with future work by others. It was surprising to have found that perinatally infected persons who have been chronically infected for more than a decade, and who have been on treatment, have supranormal thymic output up to 2 decades after HIV-1 infection. The current scientific literature appears to suggest that thymic output and TCR diversity have been addressed in adults infected with HIV; however, it is our belief that these studies have been superficial, since the majority have looked at TREC levels as a measurement of diversity. However, TREC level are a rough tool that actually measures output, but not necessarily diversity. To address the question of diversity, the TRAV, TRAJ, TRBV, TRBJ and the actual sequences of the CDR3 need to be defined. While in many adults undetectable levels of viremia occur as a result of taking cART, we have shown that immuno-activation persists and a perturbation of the TCR repertoire can be suspected. This issue should be addressed – this can be done by determining which treatments promote immune reconstitution by checking whether the TCR repertoire in such cART-treated subjects matches those of healthy, HIV(-), persons of a similar age. Additionally, it would be interesting to also characterize the TCR repertoire of HIV+ persons who are known elite non-controllers and those who are fast-progressors.

To date, we have tested TCR construct S94-KF11-B5701-TRAV14TRBV5's killing efficiency of infected cells and confirmed it has the same lysis pattern for infected cells and peptide-loaded target cells. However, we must test the killing efficiency of the remaining three

KF11-specific TCR to determine if they are also able to lyse HIV-infected cells with the same lysis pattern as observed with the peptide-loaded target cells. The SD_{50} of these three KF11-specific TCR should also be plotted against their killing efficiency to test whether they have a one log threshold of SD_{50} values that is related to their ability to lyse HIV-infected cells. Lastly and more importantly, the TCR panel constructed in this dissertation should be: (1) pooled together and co-cultured with cells infected with different HIV-KF11 variants to test if such a panel is able to prevent viral escape; (2) tested *in vivo* to confirm that each TCR is able to induce sufficient immuno-pressure to cause escape; and (3) tested *in vivo* to confirm that it is able to prevent viral escape in a physiological setting.

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