CLONOTYPIC DOMINANCE WITHIN THE HIV-EPITOPE-SPECIFIC T CELL RECEPTOR REPERTOIRE CORRELATES WITH PHENOTYPIC AND FUNCTIONAL IMPAIRMENT

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CHAPTER I

BACKGROUND AND RESEARCH OBJECTIVES

Introduction

In theory, pathogen-specific T cell responses are capable of recognizing virtually every potential antigen (1); thus, appropriately tuned T cell responses can be an incredibly powerful component in the defense against infection. T cell responses are governed by myriad cellular signals which inform them to 'go' or 'stop' or 'speed up' or 'slow down.' In chronic human immunodeficiency virus (HIV) infection, T cell responses are often unable to function normally upon stimulation. Understanding the cellular signaling networks that influence immune responses in chronic infections like HIV is crucial to our broader understanding how T cell immunity is initially generated and subsequently maintained over time.

The context of HIV disease and research

HIV remains a pressing public health concern in communities large and small. Advances in antiretroviral therapy (ART) (2-6), findings from vaccine trials (7, 8), detailed genetic studies (9), mathematical models of epidemiology (10, 11), as well as improvements in animal model systems (12, 13) have led us closer to an understanding of how HIV causes disease in humans. Ultimately, these

advances serve to highlight not only deficiencies in immune protection against HIV but also in our understanding of the processes which govern immunity.

In the majority of natural HIV infections, the immune response is characterized by systemic immune activation and progressive immune dysfunction leading to the eventual collapse of the immune response and acquired immunodeficiency syndrome (AIDS) (14). There do exist a small number of infected individuals who suppress viral replication to very low levels for long periods of time in the absence of drug therapies (15-17), and so we presume that natural immune control of HIV replication is possible. Despite mammoth research efforts over nearly 30 years, concrete correlates of immune protection in HIV, such as minimum antibody titers or consistent T cell responses remain elusive. In HIV disease, the study of natural and effective immune responses and results from vaccine trials may be our best hope to establish useful correlates of protection.

We can note that one recent human vaccine trial had high expectations for success and generated immune responses, but had no impact on infection rates (7) and another human trial with lower expectations for success actually conferred a modest degree of protection from HIV infection (8). The immunological and molecular details of these trials are being investigated intensely (18, 19). Even without achieving fully protective immunity, the data from these vaccine trials and others will provide useful insights into HIV immunopathogenesis.

Human HIV infection has likely been under greater scrutiny than any other disease system in history, and this intensive focus has advanced our understanding of immunology, virology, and host-pathogen interactions as well as diverse fields in the social, economic, and interventional sciences. The impact of this scrutiny on academic research is undeniable, far-reaching, and mostly comprised of independent searchers and researchers seeking to fit small pieces of knowledge into a rapidly advancing picture of human health and disease (20). My work is a piece of this puzzle directed toward understanding the molecular characteristics of the HIV-specific cytotoxic T lymphocyte (CTL) response in natural HIV infection as well as the molecular mechanisms that contribute to T cell dysfunction over time. These data also may provide useful insight into the immunopathogenesis of HIV infection.

The host – pathogen interface

The composition of immune systems in diverse mammals is stunningly similar (21), speaking to the long evolutionary history behind its current iteration in humans. The immune systems of mice are, in large part, representative of those found in primates, and immunity in non-human primates is an even better representative of human immunology. Thus, much of the foundational knowledge of immune function is derived from experiments conducted in small animal models which are more amenable to manipulation and experimentation than humans (22). The immune system, at its most basic level, is rooted in the idea that keeping self and non-self separate confers advantage to the host organism.

The segregation of self is of greatest import when considering the non-self actors represented by microbial pathogens seeking to infect (or invade) and parasitize a host.

Compared to the human host, HIV has an incredibly short generational time and a capacity for very rapid evolution (23). The host immune response, on the other hand, has a great diversity of cells and corresponding functions to draw upon. An organism's immune system is comprised of a variety of leukocytes (white blood cells) resident in tissues, circulating in the periphery, and stashed away in replenishing organs. The constant tension between microbes seeking to reproduce within a host and the host immune cells seeking to maintain the host's integrity by denying pathogens a suitable environment for reproduction lends itself to military analogies. We often portray microbes as attackers and the immune system as the body's defender. In anthropomorphized evolutionary terms, the pathogen and host are simply pawns as each organism attempts to maximize its reproductive potential which sometimes comes at the expense of the other (24). With HIV cast as the attacker in this case, and the human immune system acting as defender with many small but critical units, the stage is set to discuss this host-pathogen pair in more detail.

Innate and adaptive immune responses

Innate immune responses are comprised of cells which have the capacity to recognize self from non-self at a relatively low level of resolution by identifying pathogens on the basis of common molecular characteristics. In a normal immune response, the sum of these initial immune responses either eliminates a pathogen and clears infection or slows its growth while also working to prime the development of an adaptive immune response. In this way, non-specific innate immune responses act as a bridge from initial pathogen recognition to the initiation of a pathogen-specific adaptive immune response.

HIV has evolved several mechanisms to subvert innate responses, which indicates how important these initial steps are in the development of immune responses to the virus (25). Innate immune responses on their own are not likely to protect individuals from acquisition of HIV in many, if any, cases (26), and some features of the innate response may even contribute to increased viral replication (27, 28). Infection of host cells is but one of the tactics HIV employs (as do all viruses) to avoid recognition by parts of the innate immune response and impair subsequent host immune responses.

In contrast to innate immune responses which are non-specific but recognize invading pathogens rapidly, adaptive T cell responses are somewhat slower in their initiation, but they identify pathogens at a much higher level of resolution. T cells (leukocytes expressing an immunoglobulin T cell receptor) are activated in

response to specific physical and chemical signals from innate cells which have come in contact with a pathogen. T cells recognize pathogen-derived peptide antigens (epitopes), mount a defense which is diverse in function, eradicate the pathogen, and eventually, and ideally, contract to form memory cells which can reactivate more rapidly and defend more vigorously upon secondary infection or re-exposure to the pathogen (29). The ideal process of antigen recognition, expansion, clearance, and contraction is marked by dysfunction on a number of levels in natural HIV infection (30-33), and some of the factors which contribute to impaired T cell immunity form the basis for this dissertation research.

Antigen presentation and epitope variability

A critical component of the adaptive immune response is successful antigen presentation on the surface of cells which have been exposed to or infected by a pathogen. Antigen presenting cells (APC) can be professional leukocytes dedicated to the task of scouring the body for antigens and activating lymphocytes (specialized leukocytes) with specific signals or they might be non-leukocyte, pathogen-infected cells. Often, professional APCs comprise part of the innate immune response itself. In the case of viral infections like HIV, antigen presentation takes place on the surface of HIV-infected cells, which also often happen to be a specific sub-set of T cells. HIV-infected cells can harbor actively replicating virus, and if replication is allowed to proceed, infected cells can produce millions of infectious viral particles per day (23). Subsequent recognition of HIV-infected cells by HIV-specific T cells can lead to killing of the infected cell

before the viral life-cycle is complete and results in suppression of viral replication.

As a part of cellular protein synthesis, especially synthesis of viral proteins in infected cells, small peptide fragments are produced and are presented on the cell's surface in complex with surface molecules known as major histocompatibility complex (MHC) proteins. MHC-I molecules are immunoglobulin cell-surface proteins specifically recognized by T cells expressing the CD8 co-receptor. A binding cleft in the membrane distal domain of the MHC-I protein can bind small peptide epitopes from protein synthesis. Each different MHC-I molecule is derived from a distinct MHC-I gene and is capable of binding a set of protein peptide epitopes. Figure 1-1 provides a schematic diagram of the MHC-I molecule.

Epitopes bound in MHC-I molecules are usually between 8-14 amino acids in length and restricted in structure and sequence such that they can make stable molecular interactions with amino acids lining the MHC-I binding cleft. The complex comprised of a peptide epitope bound within the cleft of the MHC-I molecule is known as a peptide-MHC-I complex (pMHC).

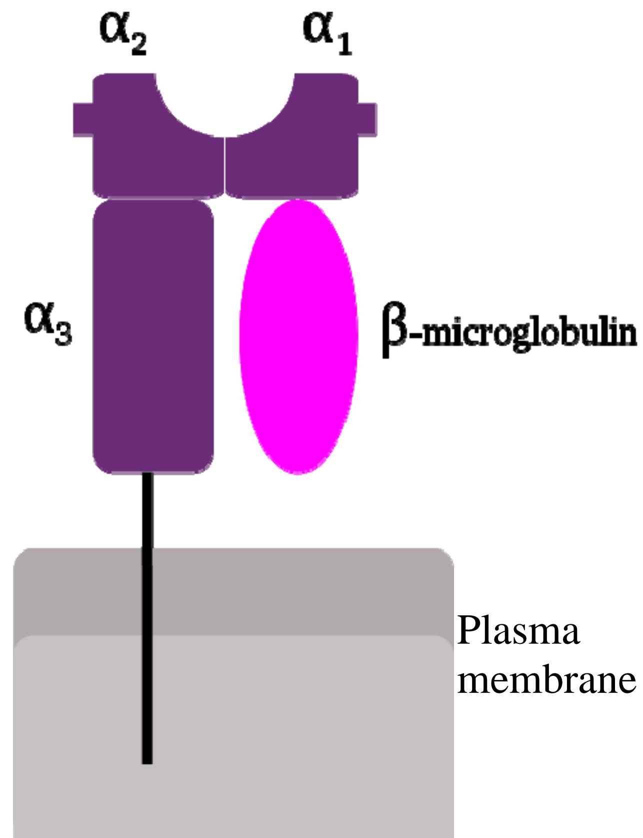


Figure 1-1 – Class I Major Histocompatibility Complex. 3 alpha-subunits are shown with the B-microglobulin protein. At the membrane distal region the epitope binding cleft is formed by the alpha-1 and alpha-2 subunits. Graphic adapted from atropos235, Wikimedia commons.

Human populations retain an exceptional diversity of and in MHC genes, especially in the MHC-I family of genes which number in the thousands of alleles and likely represent a necessity for the population to preserve the capacity to present many different peptide antigens. Every individual expresses several different MHC-I genes, and it is this complement of surface proteins which allows for a distinct set of pathogen peptides to be displayed. Genetic studies indicate that the expression of MHC alleles and their role in moderating T cell responses are critically linked to disease outcome [9, 34] which serves to reinforce the importance of T cell responses in the defense against HIV infection.

Suppression of viral replication by T cells represents a powerful selective force in the evolution of viral genetic sequences which can be observed in both the global HIV population as well as within intra-individual HIV populations. The HIV-reverse transcriptase protein is responsible for copying HIV RNA into viral DNA, and it makes many errors as it transcribes the genetic information (34, 35). Many of these errors result in the production of viruses which cannot replicate, however, random errors sometimes result in mutations that improve viral fitness and provide advantage under selection pressure. Recent research indicates that up to two thirds of viral mutations may occur as a result of T cell selection pressure (36, 37). Considering the potential variability of T cell epitope sequences in HIV, T cells with a capacity to cross-react with multiple viral epitopes may be valuable as the immune response seeks to recognize and suppress viral variants.

Development of T cells

T lymphocyte progenitors are found in the bone marrow and migrate to the thymus for maturation through a course of proliferation, differentiation, and positive and negative selection events. The T cell receptor (TCR) complex on the cell surface is the defining structure for T lymphocytes, and in ~95% of circulating T cells, it is comprised of a set of dimerized alpha (α) chain and beta (β) chain immunoglobulin superfamily proteins. TCR α and β chain genes are rearranged during the maturation process to produce variable and unique protein surface dimers capable of recognizing pathogen epitopes.

The initial expression of a TCRβ chain and the successful pairing of that molecule with a TCRα chain forms a functional TCR that locks the T cell into expression of a single TCRαβ dimer. Future activation and clonal expansion of this T cell will result in a genetically and structurally identical population of sister T cells. T cells expressing unique, clonal TCR are selected in the thymus through a series of deterministic events which ensure that the TCR can recognize and be activated by non-self antigens presented in the context of an individual's expressed MHC molecules, but that these cells are not overly reactive in response to self-antigens. This process is repeated millions of times and produces a diverse population of T cells with the ability to recognize many different pathogen epitopes.

Through selection and recombination from various germline sequences during T cell maturation, and with the addition of random nucleotides between the recombined and rearranged TCR gene segments, each chain of the αβTCR has a similar structural backbone but also encodes a unique sequence of amino acids in its three hypervariable, complementarity determining region (CDR) loops. The first and second CDR loops are responsible for most of the TCR's affinity for major histocompatibility complex (MHC) molecules while the third CDR loop (CDR3) is primarily responsible for discrimination of different peptides bound by the MHC. The unique amino acid sequences within the CDR3 can be used to determine T cell clonality. The research reported in this dissertation focuses on the properties of expansions of epitope-specific clonotypes identified in this manner.

Appropriate recognition of the host’s MHC molecules is a critical component of the T cell selection process and is concurrent with selection for T cells that recognize pMHC-I in a relatively narrow range of affinities (29, 38). The TCR complex found on the surface of functional T cells is comprised of several invariant CD3 and co-receptor molecules, that aid in the physical connection between the TCR and pMHC (TCR:pMHC). These molecules also coordinate and carry signals from the exterior of the cell across the membrane and into the cell where the signals are distributed into a variety of signaling pathways with overlapping and unique functions. Figure 1-2 provides a schematic diagram of the TCR molecule.

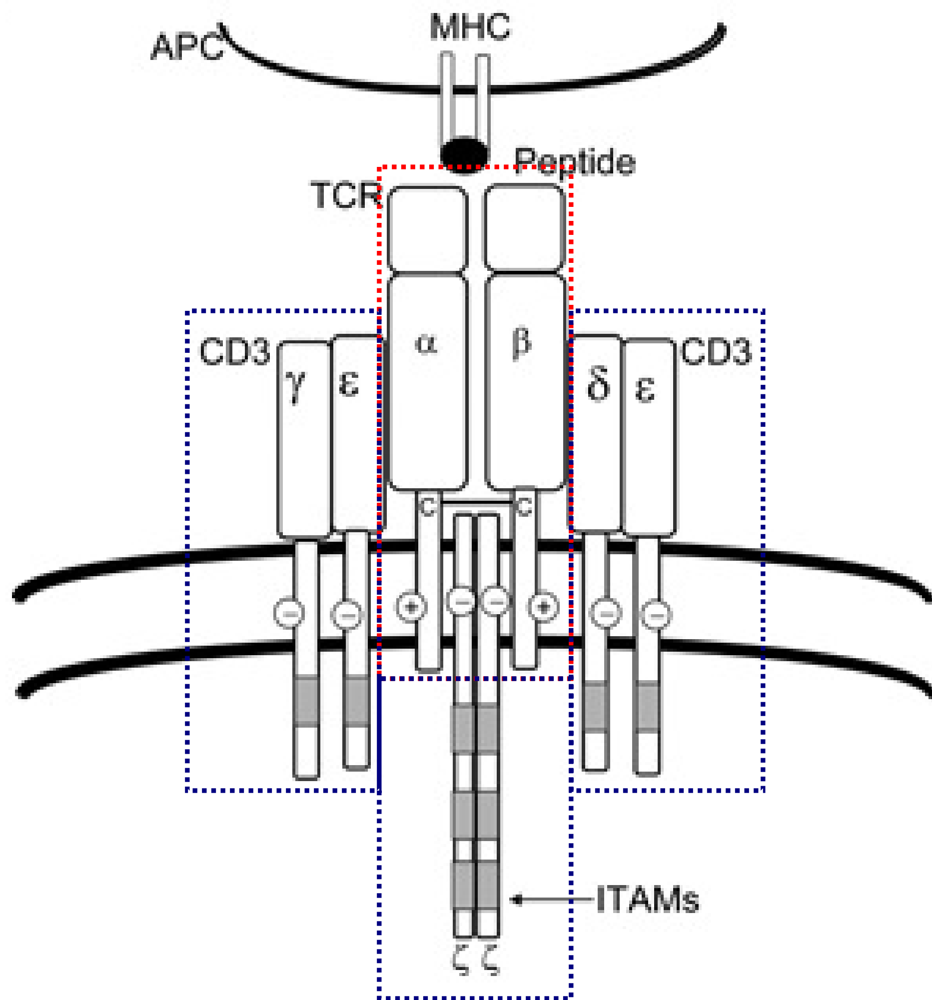


Figure 1-2 – The TCR antigen receptor/CD3 signaling complex in proximity to pMHC. Schematic diagram of the TCRαβ dimer (red box) with invariant CD3 signaling complex (blue boxes). Figure adapted from Paul WE, *Fundamental Immunology*, 2008.

T cell antigen recognition and activation

T cell activation is an extraordinarily complex series of physical connections between T cell and APC. Bi-directional signal transduction events originate at the cell surface and cascade through each cell with broad effect, eventually initiating distinct transcriptional programs leading to protein production and phenotypic changes. The CD8 co-receptor on T cells serves to enhance and stabilize the physical TCR:pMHC connection and likely also serves to recruit intracellular signaling molecules proximal to the TCR complex on the inner surface of the membrane (29).

Initial T cell activation has been simplified into a 'two signal' hypothesis (39, 40) whereby an initial signal derived from the binding of a T cell's antigen receptor to its cognate pMHC complex confers specificity to the ensuing response. A second, TCR independent, signal is transduced through one or more immunoregulatory receptor molecules which license T cell activation and enhance function, clonal expansion, and differentiation (41). Importantly and recently, investigators discovered that many T cells also express inhibitory immunoregulatory receptors which serve to attenuate or limit cellular activation (42-46). Figure 1-3 provides a schematic diagram of the two signal paradigm for T cell activation.

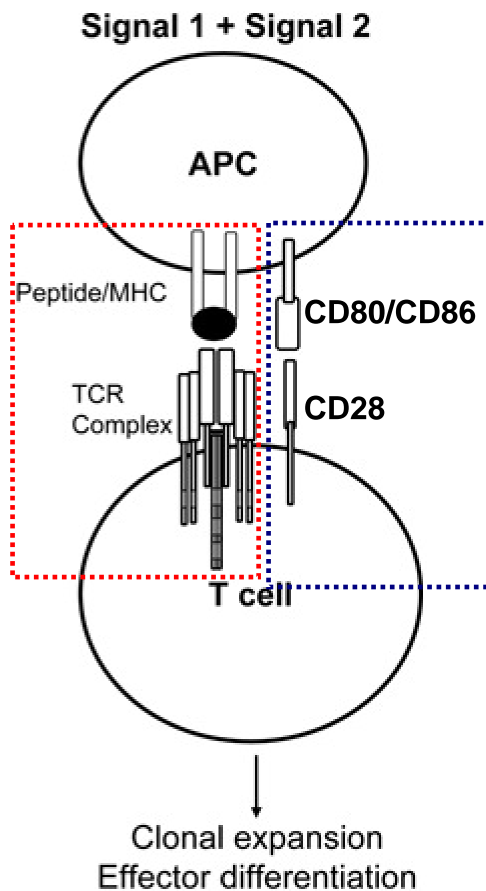


Figure 1-3 – Productive activation of T cells requires an antigen independent co-stimulatory signal. Schematic diagram depicting TCR:pMHC contacts, signal 1 (red box), and the prototypical CD28/CD80(86) co-stimulatory signal 2 (blue box). Figure adapted from Paul WE, *Fundamental Immunology*, 2008.

The TCR-CDR3 loops (especially that of the TCRβ chain) confer much of the specificity a particular TCR, and thus a particular T cell, has for its optimally preferred (cognate) peptide antigen – Signal 1 (38). In reality, a single TCR can recognize many peptide epitopes that are structurally similar but may differ in sequence known as the T cell's capacity for cross-reactivity. The strength with which the TCR binds to pMHC influences T cell function and phenotype and, to a large degree, ability to suppress viral replication (47).

A prototypical stimulatory second signal occurs between the CD28 receptor molecule expressed on activated T cells and CD80 (B7.1) and CD86 (B7.2) ligand molecules expressed on the APC surface (41, 48). Ligation of the CD28 molecule triggers a signaling cascade that has far-reaching effects including intracellular calcium release. Calcium release enhances cell trafficking, proliferation, and differentiation programs, phosphorylation of intracellular signaling molecules which eventually initiate T cell specific gene production in support of continued function, and even cytoskeletal rearrangements which promote and enhance cell contact and formation of a long-lived immune synapse. Activation of T cells also often triggers the release of granules containing pre-loaded with cytokines and cytotoxic molecules necessary for effector function and for the killing of virally-infected cells (29). In fact, strong expression, production, and release of cytolytic granules has been linked to control of viral replication (49), and poor expression has been linked to T cell dysfunction in chronic HIV infection (33).

More recent advances in the understanding of immunoregulatory molecules and signaling networks downstream of the initial TCR signal suggest that the ‘two signal’ model is likely oversimplified, although it remains a useful point of reference (50). An early finding, which helped to launch the field of inhibitory receptor signaling, was that activated T cells also express the cytotoxic T lymphocyte antigen-4 (CTLA-4) surface receptor. CTLA-4 is structurally related to CD28, expressed shortly after cellular activation, and like CD28, also binds CD80 and CD86 but transduces an inhibitory signal which reduces T cell activation and proliferation (44-46). The notion that second signals could inhibit antigen-specific T cell activity suggested a molecular mechanism which could mediate immune tolerance, autoimmunity, and immunopathology. Since the discovery of CTLA-4 as an inhibitory immunoregulatory receptor, several other molecules have been identified and characterized which have similar T cell regulatory properties, among them, Programmed Death-1 (PD-1) (51, 52).

T cell responses in HIV infection

HIV infection results in high viral loads and widespread immune activation, each of which likely contributes to a progressively dysfunctional immune response. Numerous studies in human and non-human primate models confirm that CD4+ T cells in the mucosa are almost completely depleted in early infection and are not repleted as fully as those in the periphery (53-55). The incurred loss of gut immunity allows for the translocation of microbes and microbial products such as lipo-polysaccharide (LPS) across the intestine which contributes to further

increases in immune activation. Increased activation, in turn, exacerbates HIV infection as greater numbers of activated T cells serve as targets for viral infection and replication (56). Peak viremia of millions to tens of millions of copies of HIV RNA per mililitre of peripheral blood occurs within about 14 days of initial infection (57). Studies of the relatively benign natural infection of sooty mangabys with SIV_{sm} indicate that the enhanced immune activation seen in humans may play an important role in the progressive acquisition of immunodeficiency not seen in natural hosts (58, 59). One of the few direct immune correlates of disease progression in humans is the positive association between the activation marker CD38 and viral load and its negative correlation with CD4 T cell counts (60, 61).

T cell responses appear in vivo coincident with initial reductions in HIV viremia and several studies indicate that they contribute directly to suppression of viral replication (62-65). The extent of their contribution to initial control remains incompletely defined in large part due to the recent discovery that the CD4+ T cell compartment is decimated very early in infection and thus the cells HIV replicates within are gone (53-56, 66). Depletion of CD8+ T cells in SIV infection of macaques results in increasing viral loads, and decreases in viral loads are associated with the repletion of those cells (63, 64). Specific viral immune escape mutations are temporally associated with loss of control of viremia as the recognition of viral epitopes by TCR is abrogated (36, 62, 67).

Several additional lines of evidence suggest that epitope-specific T cell responses drive HIV genetic evolution to escape T cell recognition (68, 69) and that individuals expressing rare MHC alleles have delayed disease progression that is related to expression of specific MHC alleles (9, 70, 71). Much of this evidence supports the idea that epitope-specific CD8+ T cell responses are a major component to the initial control of HIV viremia in acute infection as well as the primary contributor to suppression of ongoing viral replication during chronic HIV infection.

T cell dysfunction and impaired memory development in the setting of poor or incomplete CD4+ T cell help have been described for some time (72-74), although the exact mechanisms by which CD4+ T cells ‘help’ CTL are not precisely known (75). T cell dysfunction in the setting of chronic viral infection and ongoing antigen exposure and not always as a result of the loss or absence of CD4+ T cells has been noted in numerous infection systems including mouse LCMV (76-78), SIV infection in macaques (59), as well as in human HIV and HCV infections (33, 72, 79, 80) and represents a pressing issue in natural infection.

Dysfunctional or exhausted T cells are partially or wholly impaired for cytokine production, granule release, proliferation, and survival (80-83). It remains unclear whether T cell dysfunction in HIV is directly attributable to the dramatic loss of CD4+ T cells and the help they provide or to the persistence of viral antigen

exposure, but both features of HIV immunopathogenesis likely play major roles. Progressive T cell dysfunction occurs over time in many chronic infections and results in a hierarchical and predictable loss of effector T cell functions (77) beginning with high level functions like proliferation and production of IL-2 and ending with full functional exhaustion and eventual deletion. The inhibitory receptor molecule PD-1 has recently been identified as playing a significant and partially reversible role in regulating T cell function in chronic infections like HIV (78, 84-86).

Structure, function, and signaling in the PD-1 pathway

A role for the 288-amino acid programmed death-1 (PD-1 also CD279) in regulating apoptosis was determined through a genetic screen of B cell hybridomas undergoing cell death (51). Subsequent investigations of genetically engineered mice without the gene encoding PD-1 (*Pdcd1*) (knockout mouse = *Pdcd1*$^{-/-}$) suffer from various T cell mediated autoimmune disorders and *Pdcd1*$^{-/-}$ on autoimmune-prone backgrounds have accelerated immunopathological phenotypes (87, 88). Continued study of these phenotypes revealed PD-1's important role in mediating T cell function, especially in autoimmunity and T cell tolerance (89). Further characterization of polymorphisms in the human *Pdcd1* gene brought to light associations with rheumatoid arthritis and systemic lupus erythematosus (90, 91). The identification of the primary ligand molecules for PD-1 (PD-L1 and PD-L2) and subsequent characterization of their tissue distribution throughout the periphery on cells within epithelial and endothelial tissues as well

as wide expression within the immune compartment suggests that the PD-1 pathway is involved in both peripheral and central tolerance (92).

The extracellular domain of PD-1 shares close structural similarity with other immunoregulatory CD28-superfamily members CD28, CTLA-4, and BTLA (B and T lymphocyte attenuator) and has no intrinsic enzymatic activity. Rather, it seems to serve as an adapter molecule receiving its extracellular signal in ligand binding and then functioning to recruit other signaling molecules to the intracellular side of the immune synapse (93).

Natively monomeric, PD-1 is a 288 amino acid transmembrane immunoglobulin (Ig) surface protein. The extracellular, N-terminal Ig-variable-like domain binds with its two ligands, PD-L1 (B7H1) and PD-L2 (B7H2). PD-L1 is widely expressed and is thought to be the ligand most responsible for PD-1's influence attenuating autoimmunity. PD-L1 knockout mice have a severe and early autoimmune phenotype. PD-L2 expression is limited to APC and activated lymphocytes and is responsible for attenuating active, antigen-specific immune responses (92). Interestingly, the crystal structure of PD-1 bound to PD-L1 has extremely high homology to the antigen binding domain of antibodies (94) perhaps suggesting that the receptor:ligand complex could have an independent affinity for a third molecule further complicating this signaling pathway.

PD-1 is thought to discharge its inhibitory role by recruiting phosphatase enzymes to the inner cell membrane during an active immune response. Putative phosphatases would subsequently interact with signaling proteins downstream of the activated TCR and dephosphorylate activated proteins, thereby dampening the early immune response (95). Recruitment activity has been localized to the ~70 amino acid cytoplasmic tail, which contains two canonical signaling motifs, a membrane proximal immunoreceptor tyrosine-based inhibitory motif (ITIM) and membrane distal immunoreceptor tyrosine-based switch motif (ITSM) in an identical arrangement to the CD33-related sialic acid binding Ig-like lectin (Siglec) proteins. The inhibitory motifs may recruit protein tyrosine phosphatases Src homology region 2 domain-containing phosphatase-1 (SHP-1, PTPN6) and SHP-2 (PTPN11) in addition to other unknown inhibitory factors (96). The SHP-1 and SHP-2 phosphatases, although likely active in PD-1 signal inhibition, have not been directly demonstrated in concert with PD-1 signaling, which suggests that PD-1 may recruit these as well as yet unidentified inhibitory molecules to the membrane (93).

Upon the engagement of PD-1 with its ligands, various phosphatases are recruited to the cytoplasmic face of the membrane. While the specific phosphatase molecules remain unknown, inhibitory signaling pathways downstream of PD-1 ligation are better defined. PD-1 engagement blocks phosphoinositol-3-kinase, and through inhibition of PI3k, PD-1 decreases resistance to apoptosis and reduces the cell’s capacity to utilize glucose, two

global pathways which negatively influence T cell activation. PI3k induces expression of Bclxl, which itself is an inhibitor of apoptosis, and experimental evidence suggests that increased PD-1 expression is associated with increased cell death. Furthermore, PI3k also induces expression of Akt, which upregulates proteins necessary for glucose metabolism and provides experimental evidence linking expression of PD-1 to reduced proliferative capacity (95). Recent gene expression analysis in epitope-specific T cells also suggests that PD-1 ligation also serves to upregulate basic leucine transcription factor ATF-like (BATF) which inhibits AP-1 and downstream events including differentiation and proliferation (97). Thus, the PD-1 signaling pathway negatively influences T cell activation through diverse mechanisms that inhibit activation networks and upregulate transcription of inhibitory elements. Figure 1-4 depicts a schematic of PD-1 signaling inhibition.

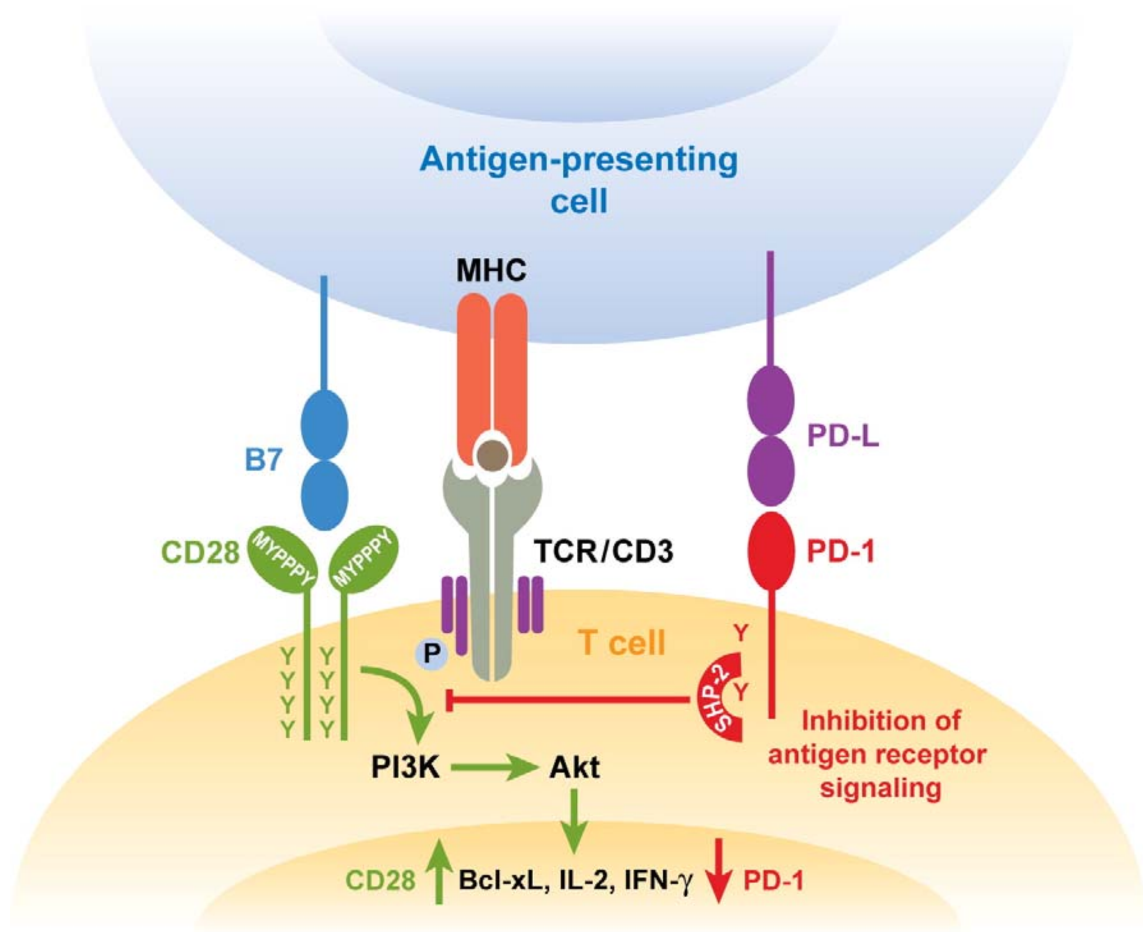


Figure 1-4 – PD-1-mediated signaling inhibition. PD-1 Ligation recruits intracellular phosphatases and dampens TCR signaling by inhibiting PI3K activity which influences glucose metabolism and cell survival. Adapted from Keir et al. 2008, Annual Review of Immunology.

PD-1 expression in disease

The PD-1 pathway has been implicated as a factor exacerbating disease in several chronic infections including *Helicobacter pylori*, schistosomiasis, and leishmania (89, 98). Recent studies have shed more light on the manner in which PD-1 influences antigen-specific immune responses in chronic viral infections. Lymphocytic choriomeningitis virus (LCMV) infection in mice has been an important model system in the study of immune dysfunction in chronic infection for many years (99). The infection characteristics of two related LCMV strains are altered such that the Armstrong strain causes acute infection, which is rapidly cleared, and the clone 13 strain causes chronic infection that persists without clearance. As in other chronic infections with persistent antigen exposure, immune responses exhibit features consistent with immune exhaustion and dysfunction (76, 100). The availability of genetically similar viruses with distinct patterns of immunopathogenesis makes possible experiments comparing the same epitope-specific T cell responses in the context of acute or chronic infection…and so these experiments came to pass.

Recently, a seminal study utilized the acute/chronic LCMV infection system to investigate the influence of PD-1 signaling in T cell dysfunction (78). Microarray analysis indicated dramatic upregulation of PD-1 in chronic LCMV infection. Subsequent analysis of *ex vivo* expression levels of PD-1 on the surface of T cells from acute and chronic infections confirmed that T cells in chronic infection expressed high levels which were sustained over time whereas PD-1 expression

on T cells in acute infection was downregulated within a few days after clearance of virus. Further experiments demonstrated that interruption of the PD-1 signaling pathway using antibodies to block PD-L1 was sufficient to induce modest reductions in viral load and restore some degree of T cell function *in vitro*, *in vivo*, and in the absence of CD4+ T cell help. A final interesting note from the study indicated that *Pdcd1*$^{-/-}$ mice could clear infection by the Armstrong strain with normal kinetics and immune responses but that *Pdcd1*$^{-/-}$ mice infected with the chronic clone 13 strain died of overwhelming immunopathology in less than 7 days. Together, these results suggested for the first time that immunomodulation of the PD-1 pathway could lead to improved outcomes in chronic viral infection and that the inhibitory signals from the PD-1 pathway likely also play a role in limiting immunopathological damage from an overactive immune response in situations of persistent antigen exposure.

Following initial characterizations in LCMV, several groups showed that PD-1 expression was upregulated on T cells in various chronic viral infections in humans, including Hepatitis B (101)and C (102) (HBV and HCV), cytomegalovirus (CMV) (103), HIV (84-86), and simian immunodeficiency virus (SIV) infection (104). These groups noted that in ART-naïve individuals, PD-1 expression correlates positively with viral load and negatively with CD4 counts, both of which are standards for measuring disease progression. Further experiments indicated positive correlations between PD-1 expression and apoptosis and inverse correlations between PD-1 expression and proliferation

potential. Importantly, and as shown previously in the mouse model, interrupting the PD-1 signaling pathway alleviated T cell dysfunction, reduced apoptosis, and increased proliferation and cytokine production in epitope-specific T cells from humans and non-human primates.

Recent evidence implicates the exposure of epitope-specific T cells to their cognate antigen to increased PD-1 expression (105, 106) and suggests that the PD-1 signaling pathway may be an intrinsic and protective mechanism that works to reduce immunopathology in infections with ongoing immune activation and exposure. The expression patterns of PD-1 on clonotypes within epitope-specific responses were unknown and unexplored prior to this dissertation work.

The immunomodulatory role of Interleukin-7 and its surface receptor

Direct cell contact between receptor and ligand molecules on the surface of APCs and T cells is but one method of cell signaling which can influence T cell responses, development, and maintenance. Cytokines are immunomodulatory signaling proteins that play a central role in lymphocyte development, proliferation, survival, and function (107). These (mostly) unbound signaling molecules are produced and released by numerous cell types (many of them leukocytes), and they can have local paracrine or autocrine (108) effects as well as systemic pyrogenic effects (109). Mutations in cytokine genes or the genes for their receptors have wide-ranging deleterious effects on leukocyte and lymphocyte development and function. Many cytokines share common structural

motifs and thus receptor molecules for structurally related cytokines are similar in their own structures (110). The γc family cytokines derive their name from the fact that the cell surface receptors for each molecule share a common element in their γc receptor subunit. This family of cytokines includes interleukin-7 (IL-7), which is known to transmit essential and potent signals promoting naïve T cell development and effector T cell survival and development of memory (111).

While evidence is clear that IL-7 signals influence immature lymphocyte development, we also know that IL-7 has an additional role in regulating T cell homeostasis in naïve and effector populations, even in the absence of antigen exposure (82, 110). Stromal and epithelial cells in the bone marrow and thymus and fibroblasts in the secondary lymphatics constitutively produce and secrete IL-7. Investigations of IL-7 signaling and its influence on cell survival have been approached by assessing the distribution of the IL-7-specific receptor (CD127) on different cell populations (112).

The IL-7 receptor is comprised of the common y-chain (CD132) and the IL-7 receptor alpha subunit (CD127) (110). The shared y-chain receptor subunit invokes the recruitment of the Jak-Stat and PI3k pathways for cellular activation. The unique CD127 subunit, on the other hand, seems most responsible for the upregulation of anti-apoptosis molecules such as B cell lymphoma-2 (Bcl-2) and for the downregulation of pro-apoptotic factors like Bcl-2 associated X protein (BAX). Reduced cell surface expression of CD127 and susceptibility to apoptosis

has been noted in sub-populations of recently activated and short-lived effector T cells.

Blattman et. al. studied epitope-specific responses in LCMV in the context of viral mutation, epitope escape, and subsequent reduction of exposure to cognate epitopes. They describe an inverse relationship between the expression of CD127 and PD-1 on epitope-specific T cells after the appearance of epitope escape variants and concurrent reduction in antigen exposure (106). My work demonstrates a similar pattern of CD127 and PD-1 expression on dominant and sub-dominant epitope-specific clonotypes in chronic HIV infection.

Polyclonal epitope-specific T cell responses

Clonal selection theory is a fundamental concept in immunobiology (113) and informs the existence of epitope-specific CTL populations that express genetically and structurally unique antigen receptors (T cell clonotypes) but which recognize the same pMHC molecule. T cells with broad reactivity for many peptide antigens are generated in the thymus, and it is thought that the human body has as many as a hundred or so T cells that recognize every peptide antigen potentially bound by that individual's MHC molecules (1).

Upon viral infection, circulating T cell clonotypes with a range of antigen affinities may each recognize, receive co-stimulation, and clonally expand in response to the same HIV peptide epitope (114). Concurrent expansion of these clonotypes

yields an eventual polyclonal population, which can be defined by its recognition of a single viral peptide epitope and comprises an epitope-specific population. Epitope-specific populations include at least one clonotype and often contain many clonotypes. Distinct T cell populations within epitope-specific responses can be identified by their usage of a finite number of TCRβ elements and corresponding genetic analysis of the TCRβ can establish clonality by confirming the expression of a unique CDR3 (115, 116). Statistical models can quantify the diversity of the epitope-specific TCR repertoire in terms of clonotype number and frequency (117) as well as in terms of the variety in amino acid sequences within TCRβ-CDR3 (118, 119). Research from different groups and studying different viral infections supports the notion that clonotypic and repertoire diversity plays a role in improved recognition of viral epitope variants (120, 121).

T cell clonotypes – fundamental units of the T cell response

Cytotoxic T lymphocytes are defined by their antigen receptors and their interaction with pMHC molecules. As such, T cell clonotypes form the fundamental units of T cell immunity (47). Daughter/sister cells within a clonotypic population have structurally identical T cell receptors. In this way, a polyclonal epitope-specific T cell response contains clonotypic populations that differ in their avidity for antigen and thus their capacity for functional responses and in their cell surface phenotypes. A significant body of literature describes HIV-epitope-specific T cell populations (84, 112, 122-126), and in this work, epitope-specific responses are often treated as homogenous populations of T

cells. However, a relatively small amount of research has been conducted to establish the important relationships between clonotypic recognition of epitope antigens, their function and phenotype, maintenance of the repertoire, or the manner in which individual clonotypic constituents might influence the epitope-specific response (127-133). The data presented in this dissertation defines some of the relationships which mediate the phenotype and function of epitope-specific clonotypes.

Identifying epitope-specific T cell Clonotypes

In the relatively recent past, multiple discoveries in monoclonal antibodies have been combined and applied using various technologies like flow cytometry to revolutionize the way immunobiology research is conducted (20, 134). Lymphocyte populations express patterns of surface proteins that are consistent and exclusive to different, distinct cell-type lineages these surface protein expression patterns define. The distribution of many of these populations is perturbed in HIV+ individuals compared to HIV- individuals (30, 135, 136). Monoclonal antibodies have been developed which bind specifically to various cell surface proteins. The conjugation of these monoclonal antibodies to fluorescent molecules allows immunologists to label cells with multiple 'colors' that can be used to define their lineage and cell surface phenotype. Multi-parameter flow cytometry is used to analyze the presence or expression of many different fluorescent labels on single cells. In this way, peripheral blood cells from individuals can be labeled with a variety of fluorescently-tagged antibodies and

populations and sub-populations of their cells can be identified and compared to one another.

Epitope-specific T cells in peripheral circulation can be positively identified through the use of MHC-I tetramer reagents (137). ‘Tetramers’ are comprised of 4 MHC-I molecules, each of which have been loaded with a peptide and bound to one another and to a fluorescently-labeled molecule. By incubating T cells specific for the pMHC complex with cognate tetramers, epitope-specific cells can be fluorescently labeled and analyzed using flow cytometry. Epitope-specific populations can then be isolated using flow assisted cell sorting (FACS) or their sub-populations can be analyzed via multi-parameter flow cytometry (138).

Individual T cell clonotypes within an epitope-specific response can be determined using several methods. Sorting epitope-specific populations and sequencing and analyzing the TCRβ chain CDR3 region is one method (127). A second method uses monoclonal antibodies to evaluate TRBV usage, but this cannot conclusively determine clonality within a TRBV population. Using flow cytometry and antibodies to identify TRBV usage as presented in the following research, clonotypes identified through genetic analyses can be confirmed and their surface phenotypes can be measured.

The ideal vaccine

Successful vaccines stimulate acute immune responses that then contract into memory responses and subsequently protect the host from infection or pathogenesis. The discovery of adjuvants allowed enhancement of the immune response to many vaccine antigens and resulted in increased vaccine effectiveness (139). For many years, adjuvants have improved immune responses to vaccination with broad action and without a tremendous degree of specificity. Now, with a deeper understanding of the signaling networks which govern immune responses, the notion that vaccines and adjuvants could be designed to stimulate immune responses in a disease- or pathology-specific manner has finally come within reach.

An optimal vaccine for HIV would generate an immune response that completely blocks infection by providing sterilizing cellular and humoral immunity. While these features might be ideal, vaccines that manage to provide sub-optimal responses and protection from pathology could also be beneficial in the absence of sterilizing immunity. Controlling infection by reducing symptoms to sub-clinical levels or decreasing viral replication and reducing subsequent transmission would both be favorable outcomes (140). Future vaccines may incorporate features to inhibit the PD-1 signaling network on epitope-specific T cells or enhance memory development or homeostatic proliferation stimulating the IL-7 receptor molecule in order to directly influence specific cell types.

Thus far, HIV vaccines in humans have not elicited strong, durable immune responses that provide lasing immunity (7, 8, 18, 19). With few exceptions, experimental vaccines have also not shown efficacy in reducing viral loads to a great enough extent to influence disease outcomes. Research in SIV vaccination systems shows promise for approaches using electroporation of plasmid DNA encoding viral proteins (141). Recent investigations using T cell stimulatory cytokines to alleviate immune inhibition in concert with DNA or protein vaccination also provide encouraging results (142).

Experimental evidence in mouse model systems of chronic LCMV infection suggest that interruption of the PD-1 signaling pathway can alleviate immune impairment and improve epitope-specific proliferation and cytokine production (143, 144). Subsequent vaccination protocols using PD-1 in the SIV infection macaque model provide similar results more relevant to HIV infection in humans (145). Strategies that incorporate PD-1 blockade into human papilloma virus (HPV) vaccination also show promise for enhancing T cell responses against HPV-mediated tumor cells (146). These successful experimental vaccination protocols, including concurrent stimulation of immune responses alongside interruption of immune inhibitory signaling, demonstrate that immune responses can be tuned to be more specific, robust, and durable than with stimulatory vaccination alone. My research will help inform the immunological effects these strategies may impart on the epitope-specific TCR repertoire.

Research Objectives

My research defines the relationships between clonotypic dominance in the epitope-specific TCR repertoire and surface and functional phenotypes on epitope-specific clonotypes. These data provide insight into the immunological consequences of constant antigen exposure in chronic HIV infection and the influence of antiretroviral therapy and subsequent antigen reduction on clonotypic repertoire diversity and phenotype.

In Chapter II, my work defines the relationship between high expression of the inhibitory marker PD-1 and low expression of the memory marker CD127 on dominant clonotypes within the epitope-specific TCR repertoire during chronic HIV infection. Furthermore, this work indicates that PD-1 expression on individual clonotypes may be influenced by avidity for cognate antigen, but that TCR avidity is not a singular mediator of clonotypic dominance within the epitope-specific TCR repertoire. Finally, this work shows that sub-dominant clonotypes retain higher levels of function compared to dominant clonotypes and suggests that they may play a role in the suppression of viral epitope variants.

My work described in Chapter III represents data derived from longitudinal studies of TCR repertoire diversity and clonotypic phenotype within epitope-specific T cell responses before and after the initiation of ART. These data clearly indicate that immune responses undergo several levels of remodeling after initiation of ART; the frequency of epitope-specific responses is reduced, memory

distribution is altered on T cell populations, and activation levels are reduced although the clonotypic hierarchy remains intact in most epitope-specific responses studied. Additional analysis of TCR repertoire diversity indicates a distinct pattern of TCR repertoire narrowing after initiation of ART. Despite unambiguous immunological changes after initiation of ART, pre-ART PD-1 expression patterns on dominant and sub-dominant clonotypes remain durable even after the initiation of ART. Taken together, these data suggest that overall maintenance of repertoire diversity may depend on high levels of generalized antigen exposure, but that epitope-specific T cells may still remain activated even during viral suppression as a result of low level antigen sensing.

The prospect of enhancing the quantity and quality of immune responses to HIV through manipulation of immunomodulatory signaling pathways is a tantalizing goal. My findings better clarify the immunomodulatory networks which influence the generation, function, and survival of epitope-specific clonotypic T cells in chronic infection. As a result, this work provides insight into the potential effects of immunomodulatory therapies on the epitope-specific T cell receptor repertoire.

CHAPTER II

DOMINANT CLONOTYPES WITHIN HIV-SPECIFIC T CELL RESPONSES ARE PD-1^{HI} AND CD127^{LOW} AND DISPLAY REDUCED VARIANT CROSS-REACTIVITY

Abstract

HIV-epitope-specific T cell responses are often comprised of clonotypic expansions with distinct functional properties. In HIV+ individuals, we measured PD-1 and IL-7Rα expression, MHC-I tetramer binding, cytokine production, and proliferation profiles of dominant and sub-dominant T cell receptor clonotypes to evaluate the relationship between the composition of the HIV-specific T cell repertoire and clonotypic phenotype and function. Dominant clonotypes are characterized by higher PD-1 expression and lower C127 expression compared to sub-dominant clonotypes and TCR avidity positively correlates with PD-1 expression. At low peptide concentrations, dominant clonotypes fail to survive in culture. In response to stimulation with peptides representing variant epitopes, sub-dominant clonotypes produce higher relative levels of cytokines and display greater capacity for cross-recognition compared to dominant clonotypes. These data indicate that dominant clonotypes within HIV-specific T cell responses display a phenotype consistent with ongoing exposure to cognate viral epitopes and suggest that cross-reactive, sub-dominant clonotypes may retain greater

capacity to suppress replication of viral variants as well as to survive in the absence of strong antigenic signaling.

Introduction

Evidence indicates that CD8+ T cell responses are a critical component of the natural immune responses to HIV (63, 64, 68). Epitope-specific CD8+ T cell responses appear to be impaired as a result of unique conditions present in HIV infection, namely constant antigen exposure (147) and overwhelming immune activation leading to exhaustion and eventual deletion of HIV-specific T cell responses (14). Our understanding of the mechanisms that underlie impaired T cell responses and their contributions to viral control remains incomplete.

Reversible T cell exhaustion has been associated with the expression of high levels of Programmed Death-1 receptor (PD-1), especially on epitope-specific CD8+ T cells (78). PD-1 is a surface-expressed transmembrane signaling protein with extracellular homology to CD28 superfamily molecules and is upregulated on activated lymphocytes (89). The role of PD-1 in the development of functional T cell memory and resolution of acute infections is increasingly well defined using model systems such as lymphocytic choriomeningitis virus (LCMV) and in human infections such as human hepatitis B (78, 101). In the setting of chronic viral infection, however, the immunomodulatory role of PD-1 signaling becomes more

complex as the necessity to limit immunopathology can also dampen effective T cell responses that might contribute to viral clearance (31, 143).

In HIV infection, PD-1 expression on T cell populations correlates positively with viral load (84) and likely contributes to increased sensitivity to apoptosis (85, 148). PD-1 signaling blockade has been shown to restore some T cell function in LCMV infection as well as in vitro with T cells from HIV+ individuals (78, 86). A reduction in the expression of cytokine receptor molecules such as IL-7Rα (CD127) on epitope-specific T cells may also play an important role in the natural control of HIV (106, 149). Reduced T cell capacity to respond to homeostatic cytokines such as IL-7 represents a point of dysregulation in the maintenance of functional, long-lived antigen-specific memory (112, 150).

Both quantitative and qualitative features of T cell responses are likely important for control of chronic viremia. The frequency of T cells that produce cytokine or proliferate in response to activation by cognate antigen is an important measure of the magnitude of the immune response (49, 122, 151), but qualitative aspects of CD8+ T cell responses such as the composition of the HIV-specific T cell receptor repertoire have been shown to be important in chronic viral infections such as hepatitis C virus infection (120) and HIV-1 infection (127). Activation or antigen exposure profiles of T cell subsets (80, 106), differentiation (67), or clonotypic antigen sensitivity (47) continue to provide important insight into potential mechanisms governing the generation and maintenance of optimal T

cell responses to chronic viral infections. Our previous work suggests that individual T cell clonotypes within HIV-epitope-specific responses are capable of responding independently to changes in viral load (127) and recognizing circulating viral variants (129).

The relationship between the composition of the clonotypic T cell receptor repertoire and clonotypic phenotype or function has not been clearly defined in model systems or natural infections. We found that dominant clonotypes express relatively higher levels of PD-1 and relatively lower levels of CD127 in comparison to corresponding sub-dominant clonotypes. PD-1 expression correlated strongly with the ability of clonotypes to bind MHC-I tetramers, and while dominant and sub-dominant clonotypes were able to respond to stimulation with HIV peptide epitopes matching circulating sequence, sub-dominant clonotypes were more cross-reactive in response to common variant peptide epitopes. Additionally, dominant clonotypes displayed an impaired ability to survive in culture at low levels of antigen stimulation. These data provide insight into the relationships between the structural composition of HIV-specific CD8+ T cell responses, the relative antigen exposure of clonotypes within the epitope-specific TCR repertoire, and the functional capacity of these clonotypes in ongoing HIV infection.

Materials and Methods

Individual Cohort and HLA-typing. This cohort was organized within the Vanderbilt-Meharry CFAR and was comprised of anti-retroviral therapy naïve patients recruited through the Comprehensive Care Center (Nashville, TN). All individuals were typed for HLA Class I by DCI Tissue Typing Laboratory (Nashville, TN). This study was approved by the Institutional Review Board at Vanderbilt University, and all participating individuals provided written informed consent.

Flow cytometric evaluation of lymphocyte surface molecules. Lymphocyte subsets were evaluated using fresh and cryopreserved peripheral blood mononuclear cells and a combination of monoclonal antibodies. CD3-AlexaFluor-700 (BD), CD4-PE-Texas Red (Caltag), CD8-Pacific Orange (Caltag), CD14-PerCP (BD), CD19-PerCP (BD), CD56-PE-Cy5 (BD), Viaprobe (BD), CD127-biotin (eBioScience), Streptavidin-APC-Cy7 (BD), PD-1-pure (Mouse IgG1, clone EH12:2H7, BioLegend), goat-anti-mouse IgG-Pacific Blue (Molecular Probes), anti-TRBV-PE/FITC (Beckman-Coulter) and MHC-I tetramers-PE/APC. MHC-I tetramers: HLA-B*08-EI8 (EIYKRWII), HLA-B*08-FL8 (FLKEKGGL), HLA-B*15-GY9 (GLNKIVRMY), HLA-B*15-TY11 (TQGYFPDWQNY), HLA-B*27-KK10 (KRWIILGLNK) – synthesized by the NIH Tetramer Core Facility, Atlanta, GA. HLA-B*57-KF11 (KAFSPEVIPMF), HLA-B*57-IW9 (ISPRTLNAW), and HLA-B*57-QW9 (QASQEVKNW) – synthesized by Beckman-Coulter.

Cells were labeled with MHC-I tetramers at 21ºC for 10 minutes. Anti-PD-1 antibody was added to the suspension and incubated for a further 20 minutes. Cells were washed and labeled in separate steps with intervening washes with pacific blue conjugated goat anti-mouse antibody, normal goat Ig blocking antibody, anti-CD127-biotin, streptavidin APC-Cy7, and the remaining directly conjugated surface antibodies listed above. Gating strategy shown in Figure 2-1.

Identification of dominant and sub-dominant clonotypes and TRBV populations. The phenotype of T cell clonotypes was determined by a combination of labeling with tetramer, anti-TRBV antibodies, and antibodies to cell surface markers. Single TCR clonotypes identified by sequencing, and which comprised more than 50% of the epitope-specific population were considered dominant. In TCR repertoires where no clonotype comprised more than 50% of the total, the largest population was considered dominant, and the remaining populations were considered sub-dominant. Monoclonal antibodies are not available to label TRBV7, so in the five cases where the dominant TRBV7 clonotype was not directly labeled, TCR beta chain sequence data informed the identification of sub-dominant populations that were directly labeled. In these cases the unlabeled fraction of tetramer+ cells represented the dominant clonotypes. We determined TRBV repertoires for 11 epitopes in this study by using TRBV antibody panels (IOTest Beta Mark, TCR V-beta repertoire kit, Beckman Coulter). Dominant TRBV populations were definitively labeled within these responses, and sub-dominant populations were defined as tetramer+/TRBV-.

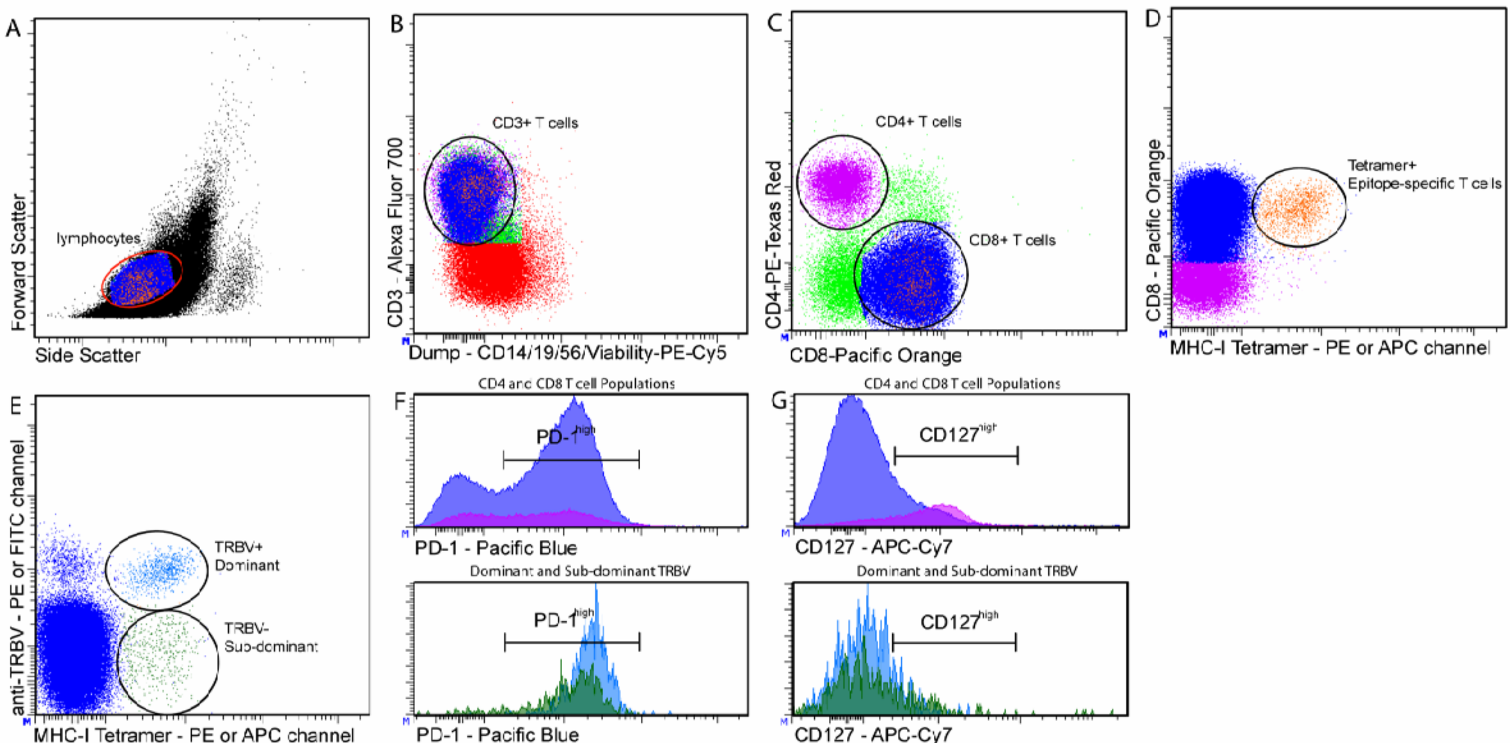


Figure 2-1 - Flow Cytometry Gating Strategy. Lymphocytes were discriminated on the basis of cell size and granularity using forward- and side-scatter, A. CD3+ lymphocytes were gated from non-viable cells, and CD14+, CD19+, and CD56+ cells, B. CD4+ and CD8+ T cell populations were selected, C. Tetramer+ and TRBV+/- sub-populations were selected from the parent CD8+ population, D and E. Surface expression of PD-1 and CD127 was measured on parent CD4+ and CD8+ T cell populations as well as tetramer+ and constituent dominant and sub-dominant TRBV populations, F and G.

cDNA synthesis and TCR sequencing. Epitope-specific T cells were labeled with appropriate MHC-I tetramers and sorted by FACS to >95% purity on a FACSAria cell sorter (BD). RNA was extracted from sorted cells and anchored RT-PCR was performed with total RNA as previously described (128). PCR product was cloned into *E.coli* and sequenced on an ABI 3130xl automated sequencer (PE Applied Biosystems, Norwalk, CT). After editing and alignment using Sequencher (Gene Codes Corp., Ann Arbor, MI), TRBV/TRBJ usage was determined using the human TCR gene database (http://imgt.cines.fr/). T cell receptor variable region classification system of the ImMunoGeneTics database (IMGT) is used throughout this manuscript.

Sequencing of autologous virus. Population viral sequence was obtained using viral RNA isolated from plasma (Qiagen) and reverse transcribed in one step (Qiagen) using HIV-Gag and HIV-Nef specific primers. DNA was amplified by PCR with the following primers: 5gag5–28 5'-GCG AGA GCG TCA GTA TTA AGC G-3', 3gag1668–1693 5'-TCT GAG GGA AGC TAA AGG ATA CAG TT-3', 3gag1398-1420 5'-AAA ATT AGC CTG TCT CTC CCC AT-3', 5nef1-19 5'-ATG GGT GGC AAG TGG TCA A-3', 3nef691-708 5'-TGC TAG GCG GCT GTC AAA-3'. Resulting PCR fragments were gel purified (Qiagen) and sequenced bi-directionally on an ABI 3130xl automated sequencer using the same primers. Sequencher (Gene Codes) was used to edit and align sequences and identification was made using the Los Alamos HIV Sequence Database (http://www.hiv.lanl.gov/).

Intracellular Cytokine Staining. Intracellular cytokine staining assays were performed using 10ug/ml of indicated peptide, anti-CD28 and anti-CD49d MAbs (1 ug/mL each; BD) and GolgiPlug at 1ug/mL (BD). Cells were stimulated for 6 hours and labeled with surface and intracellular antibodies. Surface staining panel: CD3 (AlexaFluor-700, BD), CD4 (PE-Texas Red, Caltag), CD8 (Pacific Blue, BD), CD14/CD19/CD56 (PerCP, BD), Fixable Live-Dead Aqua (Invitrogen). Intracellular cytokine production: IFN-γ (PE-Cy7, BD) and TNF-α (APC, BD). Positive (Staphylococcus enterotoxin B) and negative (unstimulated/media) controls were included for each individual. Reported cytokine production was subtracted from negative control values. Epitope variant panels: B*08-FL8 [Consensus-FLKEKGGL, Variant 1-FL**r**EKGGL, Variant 2-FLK**d**KGGL], B*08-EI8 [Consensus-EIYKRWII, Variant 1-**d**IYKRWII, Variant 2-EIYKRWI**v**], B*27-KK10 [Consensus-KRWIILGLNK, Variant 1-KRWII**m**GLNK, Variant 2-KRWI**v**LGLNK], B*57-QW9 [Consensus-QASQEVKNW, Variant 1-QA**t**Q**d**VKNW, Variant 2-QA**t**QEVKNW] (peptide synthesis – Genemed, CA).

Tetramer binding analysis. PBMC were washed in FACS buffer, resuspended, aliquoted, and labeled for 30 minutes at RT with tetramer (APC-conjugated) at the following dilutions from manufactured stock – 1:25, 1:50, 1:100, 1:200, 1:400 final concentrations (~16uM to 4uM). With 5 minutes remaining for tetramer incubation, Live/Dead Fixable Aqua Dead Cell stain (Invitrogen) was added to each aliquot of PBMC. At 30 minutes, labeled cells were immediately washed with PBS and resuspended. Cells were fixed with 2% paraformaldehyde and washed in PBS. Fixed PBMC were first labeled with anti-TRBV-FITC conjugated

antibodies and subsequently with antibodies to surface markers CD3, CD4, CD8, and CD14/19/56 (fluorescent antibodies and manufacturers as detailed above) for 30 minutes at room temperature. Surface antibodies were fixed to cells a final time and analyzed immediately.

In vitro culture and proliferation. PBMC were labeled with CFSE and cultured for 4 days in the presence or absence of peptide epitopes at the indicated concentrations. Cell culture media was supplemented with 1U/ml IL-2. Epitope-specific and clonotypic proliferation was assessed by co-staining live cells with tetramer and anti-TRBV antibodies and measuring CFSE dilution.

Statistical analysis. Comparisons between whole CD4+, CD8+, and epitope-specific T cell populations were performed using Mann-Whitney tests. All paired comparisons were made using Wilcoxon matched pairs test. Fisher's exact test for proportions was used to determine significance between PD-1 and CD127 expression on dominant and sub-dominant populations. Spearman rank correlation was used to test for the relationship between PD-1 expression and avidity for tetramer. All statistics were calculated using GraphPad Prism, v5.01.

Flow cytometry. All samples were sorted and data acquired on a FACSAria (BD) cell sorter. Data was analyzed using FACSDiva (BD) software. Plots shown using $\log_{10}$ fluorescence; histograms are $\log_{10}$ fluorescence vs. count.

Results

Epitope-specific T cell populations express high levels of PD-1

We evaluated the degree of PD-1 expression on total CD4+, CD8+, and HIV-specific CD8+ T cell populations in 22 chronic HIV+ patients off anti-retroviral therapy (Figure 2-2). These individuals had varying levels of disease progression (Table 2-1, median VL=2474 copies/ml, range=<50-382,000; median CD4=688, range=132-1374). PD-1 expression (Mean fluorescence intensity, MFI) was measured on CD4+, CD8+, and 35 CD8+, HIV-epitope-specific T cell populations identified by MHC class I tetramers (Table 2-1, mean 1.6 epitopes/individual, range 1-5 epitopes/individual). As has been observed by other groups (84, 85), we found PD-1 expression to be higher on HIV-specific CD8+ T cell populations when compared to total CD4+ ($p=0.007$, mean 2.4 fold higher) and CD8+ ($p=0.0003$, mean 1.9 fold higher) T cell populations (Figure 2-2A and 2-2B).

PD-1 expression on CD4+, CD8+, and HIV-specific CD8+ T cell populations was often bi-modal, and we were able to measure the percentage of PD-1^{high} cells within a given T cell population. Tetramer+, HIV-specific populations have a larger fraction of PD-1^{high} cells than CD4+ or parent CD8+ T cell populations ($p=0.0001$ and $p<0.0001$, Figure 2-2A and 2-2C). Despite overall high levels of PD-1 expression on epitope-specific T cells, we observed PD-1 expression as low as 40% on some epitope-specific populations, which may represent a subset of epitope-specific cells capable of greater function than PD-1^{high} populations.

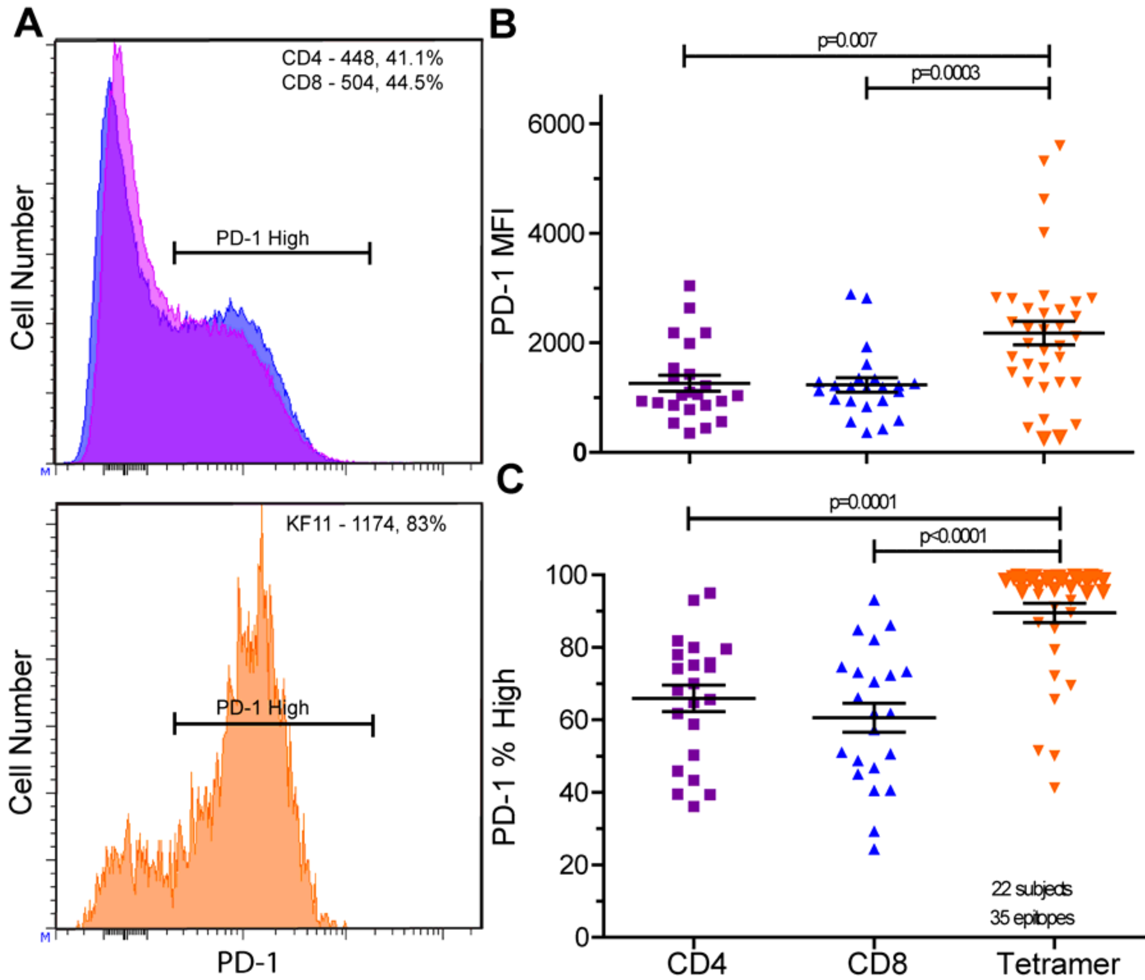


Figure 2-2 – PD-1 is highly expressed in a bi-modal pattern on epitope-specific T cells in HIV+ individuals. Histograms showing PD-1 expression on T cell populations in a single individual, PD-1 MFI and percentage PD-1^{high} values are provided in the corner of each histogram, CD8+ (top panel, purple), CD4+ (top panel, blue), and HIV-specific, tetramer+ (bottom panel, orange), A. PD-1 MFI is higher on tetramer+ cells compared to CD4+ T cells (p=.007) and CD8+ T cells (p=.0003), B. Percentage of tetramer+ PD-1^{high} cells is higher than the percentage CD4+ PD-1^{high} T cells (p=.0001) and CD8+ PD-1^{high} T cells (p<.0001), C. N=35 epitope-specific responses in 22 HIV+ individuals.

Table 2-1 – PD-1 Study Cohort Demographic Data

Patient ID	# epitopes studied	CD4 Count	CD8 Count	Plasma Viral Load (HIV copies/ml)	Sex	Race[#]	Age (years)	Duration of Infection (years)	HLA-A1, -A2, -B1, -B2
10001[**]	2	378	1539	32030	M	W	32	2	2, 2, 14, 15
10002[†**]	3	664	1199	94	M	W	43	21	3, 31, 27, 57
10004[†**]	2	203	1091	50	M	W	59	21	3, 30, 7, 57
10015[**]	1	717	1462	98778	M	W	42	3	1, 3, 8, 52
10022[†**]	2	724	1254	292	M	W	36	16	1, 31, 8, 27
10027[†**]	5	775	1300	1893	M	W	66	14	1, 2, 8, 57
10035	1	429	663	2679	M	AA	52	21	3, 33, 27, 44
10038	1	510	646	1888	M	W	48	9	2, 2, 27, 44
10040	2	1161	972	50	F	W	49	14	1, 31, 44, 57
10060	1	688	1042	722	M	AA	30	4	1, 33, 42, 57
10069	1	1032	2005	2474	M	AA	45	4	1, 30, 53, 57
10070[**]	1	782	391	7115	F	AA	28	10	23, 74, 57, 58
10071[†]	2	743	414	50	F	AA	45	14	1, 66, 8, 57
10076	1	700	1500	21339	M	AA	54	6	2, 30, 35, 57
10086[**]	1	132	876	76427	M	W	39	17	1, 29, 8, 44
10094	1	546	928	14621	M	W	44	8	1, 3, 7, 8
10105[†**]	2	289	513	35050	F	AA	54	4	1, 23, 8, 44
10138[**]	2	231	NA	46800	M	W	46	6	24, 29, 15, 44
10141[**]	1	542	NA	382000	M	W	46	6	1, 2, 15, 37
20002	1	612	578	1556	M	W	56	2	1, 2, 7, 27
20004	1	720	1094	3714	M	W	42	1	3, 32, 18, 27
20018[**]	1	1374	1035	1886	M	AA	24	2	2, 26, 40, 57

[#] W - white, AA - African American

[†] subjects followed longitudinally

** subjects in CD127 sub-cohort

NA - not available

Dominant TRBV populations within HIV-specific T cell responses are predominantly clonotypic and express higher levels of PD-1 and lower levels of CD127 compared to sub-dominant TRBV populations

We next evaluated TRBV usage and clonotypic composition within HIV-specific PD-1^{high} and PD1^{low} populations. To identify T cell receptor usage within HIV-specific CD8+ T cell populations, we sequenced FACS-isolated HIV-specific CD8+ T cell in combination with direct staining of PBMCs with HIV-epitope-specific MHC-I tetramers and an anti-TRBV monoclonal antibody panel as previously described (67, 129). Twenty-one of 35 HIV-specific CD8+ T cell responses were sequenced to determine TRBV, CDR3, and corresponding TRBJ regions (Table 2-2) with subsequent repertoire confirmation using monoclonal anti-TRBV antibodies. Within each epitope-specific TCR repertoire, we identified a single, dominant CDR3 clonotype, although sometimes this dominant clonotype was found with other clonotypes within a single TRBV family (Table 2-2). For example, subject 10002 recognizes the HLA B*5701-restricted epitope IW9. Although we identified 8 clonotypes responding to this epitope, one TRBV27-TRJ27 clonotype comprises 64% of the sequences. Two other clonotypes also use TRBV27, but combined, they only contribute to 6% of the total sequences. In this case, staining with anti-TRBV27 antibody was used to identify the dominant T cell clonotype for phenotypic analysis. We noted highly significant concordance between our two methods for repertoire determination (Figure 2-3A, $r=0.86$, $p<0.0001$).

Table 2-2 - TRBV CDR3 Sequences of Epitope-specific Populations

PID	Epitope	TRBV	CDR3	TRBJ	Sequence % freq	TRBV labeling	TRBV%	#
10001	TY11	TRBV10-3	CAISERAIRGTSGLTDTQYF	TRBJ2-3	56	Vb12	64	50/67
		TRBV10-3	CAISECAIRGTSGLTDTQYF	TRBJ2-3	2			1/67
		TRBV10-3	CAISERAIRGTSGLTDTQYL	TRBJ2-3	2			1/67
		TRBV4-2	CASSQAAGGRAFF	TRBJ1-1	25	Vb7.1	36	11/67
		TRBV4-2	CASSQAEGGQNHF	TRBJ2-7	9			1/67
		TRBV4-2	CASSLAPGGIAFF	TRBJ1-1	5			3/67
		TRBV4-2	CASSQAAGGRAIF	TRBJ1-1	2			1/67
10001	GY9	TRBV11-2	CASSLDSGFLEQYF	TRBJ2-7	44	Vb21.3	44	18/36
		TRBV10-3	CAISESGGRVDEQYF	TRBJ2-7	36	Vb12	21	13/36
		TRBV12-3	CASSPPSSYNEQFF	TRBJ2-7	3	Vb8	11	1/36
		TRBV4-3	CASSLQGAPEQFF	TRBJ2-1	8	Vb7.2		3/36
		TRBV11-3	CTSRLDPGFLEQYF	TRBJ2-7	3	Vb21		1/36
10002	KK10	TRBV7-2	CASSLYGEYEQYF	TRBJ2-7	79	Ab unavailable		30/38
		TRBV13	not identified by seq			Vb23	4	
		TRBV6-6	CASSQGTTDTQYF	TRBJ2-3	21			8/38
10002	IW9	TRBV27	CASRPGQGGYEQY	TRBJ2-7	64	Vb14	62	29/45
		TRBV27	CASSSSTGQQPQH	TRBJ1-5	4			2/45
		TRBV27	CASRTQRWETQY	TRBJ2-5	2			1/45
		TRBV7-9	CASSLAQGWKTQY	TRBJ2-5	20	Ab unavailable		9/45
		TRBV7-9	CASSIQGLRATNEKLF	TRBJ1-4	2			1/45
		TRBV7-8	CASRSPLGYEQY	TRBJ2-7	2			1/45
		TRBV12-4	CASSSGTSGSAGYNEQF	TRBJ2-1	2			1/45
		TRBV5-1	CASSTNNEQF	TRBJ2-1	2			1/45
10002	QW9	TRBV27	CASRTQRWETQY	TRBJ2-5	100	Vb14	93	13/13
		TRBV4	not identified by seq			Vb7.2	7	
10004	QW9	TRBV3-1	CASSQGPGERAGFNYEQY	TRBJ2-7	56	Vb9	55	4/16
		TRBV28	CASSLGYGYT	TRBJ1-2	19	Vb3	29	3/16
		TRBV27	CASSKGRYNEQF	TRBJ2-1	25	Vb14	12	9/16
10004	KF11	TRBV7-9	CASPHPDRPNYGYT	TRBJ1-2	78	Ab unavailable		39/50
		TRBV7-9	CASGGEFYGYT	TRBJ1-2	16			8/50
		TRBV19	CASSLTYGYT	TRBJ1-2	2	Vb17	26	1/50
		TRBV19	CASSSRTGGYGYT	TRBJ1-2	2			1/50
		TRBV24	CATSDRMDNEQF	TRBJ2-1	2			1//50
10022	KK10	TRBV12-4	CASSIAGGGEDTQY	TRBJ2-3	31	Vb8	45	26/84
		TRBV6-5	CASRKGQGDWEAF	TRBJ1-1	5	Vb13.1	35	4/84
		TRBV20-1	CSARGWVSNNQETQY	TRBJ2-5	57	Vb2	20	48/84
		TRBV20-1	CSARDPLPEASGGAGTDTQY	TRBJ2-3	2	Vb2		1/84
		TRBV19	CASTPPGF	TRBJ1-2	1	Vb17		2/84

Table 2-2 (continued) - TRBV CDR3 Sequences of Epitope-specific Populations

		TRBV27	CASSQWTGELF	TRBJ2-2	4	Vb14		1/84
10022	FL8	TRBV6-2	CASSFIPGQGTHYSNQPQH	TRBJ1-5	78	Vb13.2	75	7/9
		TRBV10-3	CAIRPFLGQDDNYGYT	TRBJ1-2	11	Vb12	11	1/9
		TRBV28	CASSLRGTGELF	TRBJ2-2	11	Vb3	14	1/9
10027	KF11	TRBV10-3	CAIGGHDYGYT	TRBJ1-2	45	Vb12	54	40/88
		TRBV6-5	CASSSLVNTGELF	TRBJ2-2	23	Vb13.1	10	20/88
		TRBV6-5	CALTGGDYGYT	TRBJ1-2	7			6/88
		TRBV20-1	CSARGWVSNNRETQY	TRBJ2-5	14	Vb2	10	12/88
		TRBV20-1	CAASTSAVLGKKGSQETQY	TRBJ2-5	2			2/88
		TRBV20-1	CSAREKGSQETQY	TRBJ2-5	8			7/88
		TRBV28-1	CASSGPGGEQY	TRBJ2-7	1			1/88
10027	FL8	TRBV2	CASSELGARVYEQYF	TRBJ2-7	67	Vb22	70	37/42
		TRBV10-1	CASSESSREVSYNSPLHF	TRBJ1-6	6	Vb12	11	3/42
		TRBV4-2	CASKEELSNTGELFF	TRBJ2-2	23			1/42
		TRBV29-1	CSVGDQGGSEQYF	TRBJ2-7	4			1/42
10027	EI8	TRBV13-6	CASTGGRGSPLHF	TRBJ1-6	100	Vb13.1	95	45/45
		TRBV9	Not identified by sequence			Vb1	2	
		TRBV20	Not identified by sequence			Vb2	1	
		TRBV28	Not identified by sequence			Vb3	1	
10060	IW9	TRBV7-8	CASSQDRIHTEAF	TRBJ1-1	94	Ab unavailable		52/54
		TRBV13-2	CASSLGLDETQYF	TRBJ2-5	6		5	2/54
10070	KF11	TRBV7-9	CASSLGGGYT	TRBJ1-2	50	Ab unavailable		22/44
		TRBV7-8	CASEDFKNIQY	TRBJ2-4	16			7/44
		TRBV7-9	CASSPGQTNYGYT	TRBJ1-2	14			6/44
		TRBV7-9	CATPGEVLSPNYGYT	TRBJ1-2	2			1/44
		TRBV7-9	CASSLGGGQNGYT	TRBJ1-2	2			1/44
		TRBV7-6	CASSSMGGGTDTQY	TRBJ2-2	2			1/44
		TRBV7-9	CASSLAGGYT	TRBJ2-2	2			1/44
		TRBV11-2	CASSDGTGVGLGYT	TRBJ1-2	5	Vb21.3	12	2/44
		TRBV11-2	CASSDGQGRLGYT	TRBJ1-2	2			1/44
		TRBV14	CASSPRDSQETQY	TRBJ2-5	2	Vb16	8	1/44
10071	KF11	TRBV5-1	CASYNFGQYGYT	TRBJ1-2	75	Vb5	67	20/25
		TRBV7-6	CASSPMDLLDEQY	TRBJ2-7	25	Ab unavailable		5/25
10071	FL8	TRBV2	CASSELGATIYEQY	TRBJ2-7	40	Vb22	46	8/20
		TRBV4-1	CASSQEMNRVVGNEQF	TRBJ2-1	25	Vb7.1	26	7/20
		TRBV6-9	CASTRPGQGTYNEFQ	TRBJ2-1	35	Ab unavailable		5/20
10076	KF11	TRBV2	CASRGGSGELF	TRBJ2-2	63	Vb22	87	34/54
		TRBJ7-9	CASSGFRDRVNEQY	TRBJ2-7	33	Ab unavailable		18/54
10086	EI8	TRBV9	CASSVVGDSRETQYF	TRBJ2-5	52	Vb1	80	24/46
		TRBV9	CGSSVVGDSRETQYF	TRBJ2-5	2			1/46
		TRBV9	CASSTLRDSREKLFF	TRBJ1-4	4			2/46
		TRBV9	CASSTLGDSREKLFF	TRBJ1-4	7			3/46
		TRBV9	CASSADGSFYEQYF	TRBJ2-7	2			1/46
		TRBV27	CASSLVGQGARQPQHF	TRBJ1-5	2	Vb14	9	1/46

Table 2-2 (continued) - TRBV CDR3 Sequences of Epitope-specific Populations

		TRBV27	CASSLGSAKNIQYF	TRBJ2-4	2			1/46
		TRBV2	CASSEPPGVRGEAFF	TRBJ1-1	9			4/46
		TRBV4-1	CAGSQEFLNRRYF	TRBJ2-5	7			3/46
		TRBV5-1	CASSLSGSGWQETQYF	TRBJ2-5	9			4/46
		TRBV7-2	CASSLLPDPRSSGGYTF	TRBJ1-2	2			1/46
		TRBV7-8	CASSLLDGTRDQQYF	TRBJ2-7	2			1/46
10138	GY9	TRBV5-1	CASSEAGGTEAFF	TRBJ1-1	46	Vb5.1	47	21/46
		TRBV9	CASSVEGTILTDTQYF	TRBJ2-3	13	Vb1	47	6/46
		TRBV9	CASSVEGTIHTDTQYF	TRBJ2-3	9			4/46
		TRBV13	CASSLQQTLGAFF	TRBJ1-1	9	Vb23.1	3	4/46
		TRBV13	CASSPQQTLGAFF	TRBJ1-1	9			4/46
		TRBV20-1	CSALVEGDEQFF	TRBJ2-1	4			2/46
		TRBV20	CSAIVGSAYEQYF	TRBJ2-7	2			1/46
		TRBV29	CSVDGPTGGYTF	TRBJ1-2	4			2/46
		TRBV29	CASSQGLAGDEQYF	TRBJ1-2	2			1/46
		TRBV29	CSASLGGRISGANVLTF	TRBJ1-2	2			1/46
		TRBV14	CASSQDLRGARGYTF	TRBJ1-2	2			1/46
		TRBV14	CASSQGTGSTDTQYF	TRBJ2-3	2			1/46
		TRBV4-2	CASSQDSSGRVTGELFF	TRBJ2-2	2			1/46
10141	GY9	TRBV27	CASSDNGGDRSPGELFF	TRBJ2-2	54	Vb14.1	67	21/39
		TRBV27	CASSPSFPPDTQYF	TRBJ2-3	10			4/39
		TRBV27	CASSPGGGELFF	TRBJ2-2	3			1/39
		TRBV6-6	CASSSPGGVTEAFF	TRBJ1-1	10	Vb13.6	10	4/39
		TRBV6-6	CASSYSVVEAAAEAFF	TRBJ1-1	2			1/39
		TRBV20-1	CSARDRADRVLIPDTQYF	TRBJ2-3	8	Vb2.1	6	3/39
		TRBV20-1	CSASPVGGAYEQYF	TRBJ2-7	5			2/39
		TRBV6-5	CASRLGRLAYEQYF	TRBJ2-7	3			1/39
		TRBV6-5	CASSTLTGEDSGPQHF	TRBJ1-5	3			1/39
		TRBV3-1	CASSQGLAGDEQFF	TRBJ2-1	3			1/39
20018	KF11	TRBV7-9	CASELSGNTIY	TRBJ1-3	70	Ab unavailable		35/51
		TRBV7-9	CASSYLNTIY	TRBJ1-3	2			1/51
		TRBV7-9	CASEGGNTIY	TRBJ1-3	2			1/51
		TRBV7-9	CATEASGNTIY	TRBJ1-3	12			6/51
		TRBV7-9	CASEITRDRRNTIY	TRBJ1-3	2			1/51
		TRBV7-6	CASSSWTGQDEQF	TRBJ2-1	2			1/51
		TRBV7-9	CASSGFTGFANEAF	TRBJ2-6	2			1/51
		TRBV28-1	CATSDLMDNEQF	TRBJ2-1	4	Vb3	7	2/51
		TRBV24-1	CATSDLMDNEQF	TRBJ2-1	2			1/51
		TRBV5-6	CASILTSGRNEQF	TRBJ2-1	2	Vb5.2	3	1/51

[a] Dark shading identifies dominant clonotype

[b] Light shading identifies sub-dominant population(s) used for comparison (unshaded clonotypes were unlabeled)

[c] TRBV populations directly labeled with antibodies are bordered

[d] IMGT nomenclature for TRBV designation is used throughout the table and accompanying text

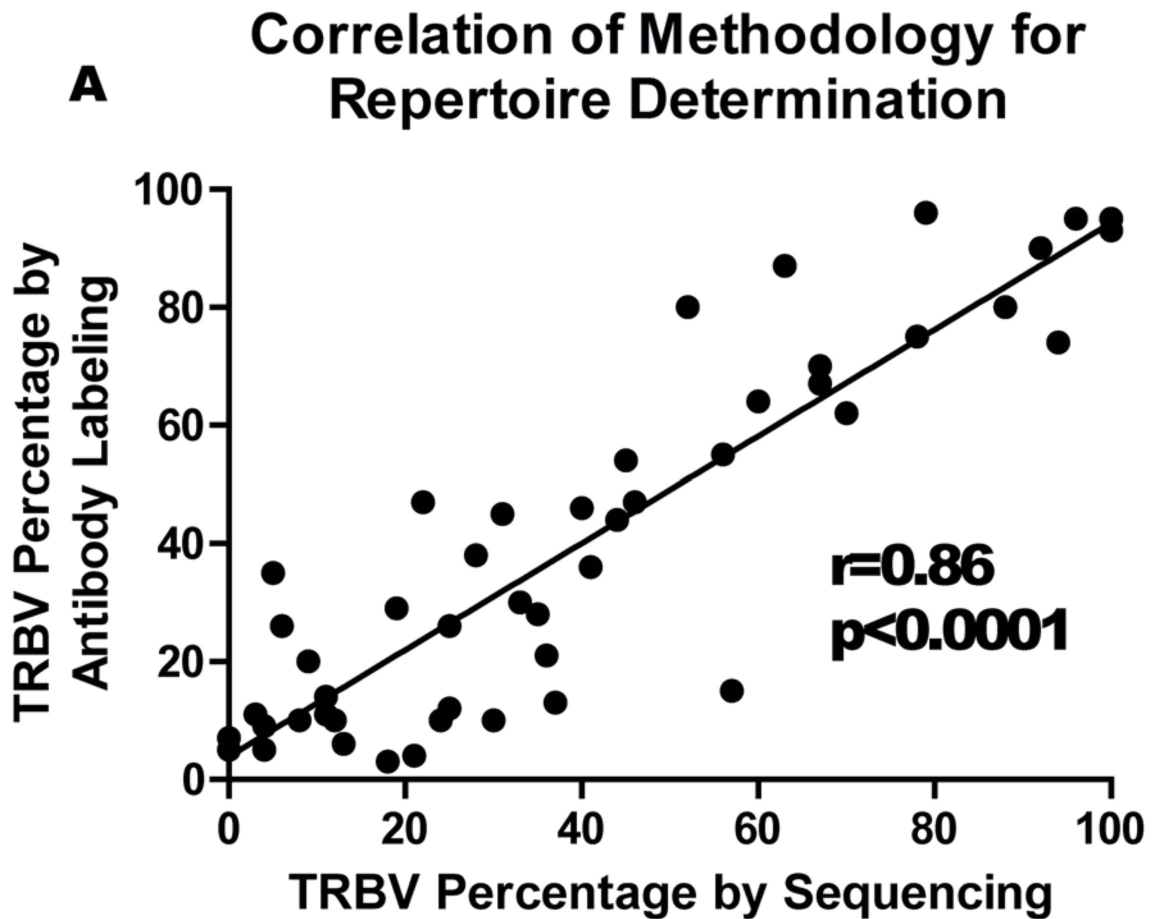


Figure 2-3 – Correlation between methods of repertoire determination. Epitope-specific TRBV repertoires were determined using two methodologies as described in Materials and Methods (sort/sequence and antibody labeling). Comparison of TRBV repertoire composition using these methodologies correlated strongly (r=0.86) and with high significance (p<0.0001, Spearman correlation), A.

We show representative plots of PD-1 expression on corresponding dominant and sub-dominant TRBV populations in a single HIV-epitope-specific T cell response (Figure 2-4A). Within HIV-epitope-specific responses, PD-1 expression is higher on dominant TRBV populations compared to sub-dominant TRBV populations when measured by MFI (p=0.001, Figure 2-4B) or frequency of PD-1^{high} cells (p=0.0001, Figure 2-4C). We evaluated multiple HIV-epitope-specific populations in 9 of 22 individuals studied (range 2-5 epitopes/individual, Table 2-1 and Table 2-3). We did not find a correlation between the degree of dominance within the repertoire and the degree of PD-1 expression on dominant and sub-dominant clonotypes within epitopes, suggesting that the magnitude of expansion within a parent population is not the sole determinant of PD-1 expression.

If we limit our phenotypic analysis to those epitopes for which we have sequence confirmation that the dominant TRBV population is monoclonal, the relationships we highlight between clonotypic dominance and PD-1 and CD127 expression remain statistically significant (PD-1 MFI, p=0.0398; CD127 MFI, p=0.0342). Additionally, there are several ways to define clonotypic dominance within epitope-specific TCR repertoires in the absence of a single, highly dominant clonotype.

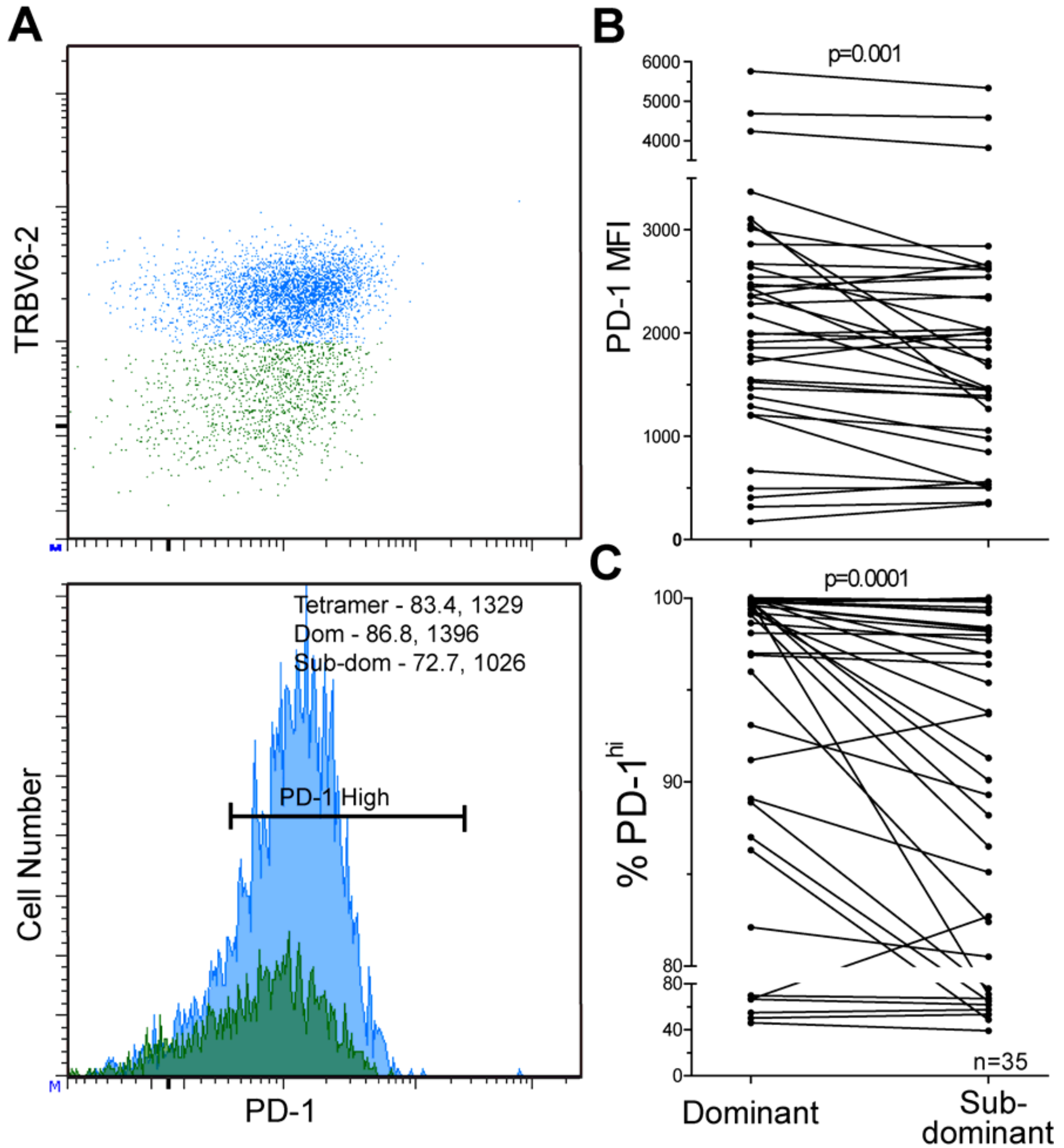


Figure 2-4 – PD-1 expression is higher on dominant TRBV compared to sub-dominant TRBV populations within epitope-specific responses. Dot plot and histogram showing PD-1 expression on dominant (blue) and sub-dominant (green) TRBV populations in a single epitope-specific response. PD-1 MFI and percentage PD-1^{high} values are provided in the upper corner histogram for the dominant and sub-dominant TRBV populations, A. PD-1 expression is higher on dominant TRBV compared to subdominant TRBV as measured by MFI, B (p=0.001) and percentage PD-1^{high} , C (p=0.0001). N= 35 epitope-specific populations in 22 HIV+ individuals.

Table 2-3 - TRBV Repertoire Data

PID	Epitope	% of CD8	TRBV Repertoire[a]	Method[b,c]
10001	TY11	2.2	10-3 (64%), 4-2 (36%)	sort-sequence
	GY9	1.5	11-2 (44%), 10-3 (21%), 12 (11%)	sort-sequence
10002	KK10	5.6	13-1 (4%), 7-2 (96%)	sort-sequence
	IW9	2.9	27 (62%), 7-9, 12-4, 5-1 (38%)	sort-sequence
	QW9	1.6	27 (92%), 4 (7%)	sort-sequence
10004	KF11	1.4	19 (26%), 7-9, 24 (74%)	sort-sequence
	QW9	1.1	3-1 (55%), 28 (29%), 27 (12%)	sort-sequence
10015	EI8	1.2	9 (71%), 2 (10%), 4 (5%)	TRBV
10022	KK10	19	12 (45%), 6-5 (40%), 2 (15%)	sort-sequence
	FL8	1.3	6-2 (75%), 10-3 (11%), 28 (14%)	sort-sequence
10027	KF11	0.5	10-3 (54%), 6-5 (10%, mult cdr3), 20-1 (10% multi cdr3)	sort-sequence
	FL8	0.8	22 (70%), 12 (11%)	sort-sequence
	EI8	0.4	6 (95%), 9 (2%)	sort-sequence
	QW9	0.8	28 (85%)	TRBV
	IW9	0.7	27 (86%), 4 (5% multi-Vb)	TRBV
10035	KK10	5.5	6 (62%), 19 (16%), 20 (15%), 12 (4%), 27 (3%)	TRBV
10038	KK10	1.3	6-2 (32%), 3 (24%), 4-2 (11%), 19 (20%)	TRBV
10040	QW9	2	6 (95%)	TRBV
	KF11	2	9 (94%)	TRBV
10060	IW9	1.5	13-1 (4%), 7-8 (96%)	sort-sequence
10069	QW9	2.6	6 (90%)	TRBV
10070	KF11	10	11-2 (12%), 27 (8%), 7 (80%)	sort-sequence
10071	KF11	1.8	5 (67%), 7 (33%)	sort-sequence
	FL8	3.7	2 (46%), 4-1 (26%)	sort-sequence
10076	KF11	7.4	2 (87%), 7 (13%)	sort-sequence
10086	EI8	1.8	9 (80%), 27 (9%)	sort-sequence
10094	FL8	3.7	27 (77%), 28 (7%)	TRBV
10105	FL8	1.8	6-2 (70%)	sort-sequence
	EI8	1.8	8-1 (45%)	sort-sequence
10138	GY9	2.1	5-1 (47%), 9 (47%), 13 (3%)	sort-sequence
	TY11	2.2	29 (52%), 9 (13%), 5-1 (12%), 13-2 (10%), 12 (8%)	sort-sequence
10141	GY9	0.8	27 (67%), 6-6 (10%), 20 (6%)	sort-sequence
20002	KK10	5.3	5-2 (45%)	TRBV
20004	KK10	2.4	27 (68%), 4-3 (12%)	TRBV
20018	KF11	11.2	28-1 (7%), 5-6 (3%), 7 (90%)	sort-sequence

[a] **TRBV Repertoire** - as determined by antibody labeling
[b] **sort-sequence** - tetramer+, epitope-specific cells were sorted and subjected to TCR sequence analysis before co-staining with anti-TRBV antibodies

[c] **TRBV** - tetramer+, epitope-specific cells were co-stained with anti-TRBV antibodies

However, even using a more stringent criterion that dominant clonotypes must comprise more than 70% of the TRBV repertoire (19 epitope-specific responses in 14 individuals fit this criteria), comparison between dominant and sub-dominant clonotypes yields significant relationships for MFI and % PD-1^{high} (p=0.03, MFI and p=0.001, % PD-1^{high}, figure 2-5A and 2-5B). These data support our observations that dominant clonotypes express higher levels of PD-1 despite relative differences in dominance within the clonotypic repertoire.

We also evaluated CD127 expression on dominant and sub-dominant TRBV populations in a sub-cohort of 12 individuals, which included analysis of 19 epitope-specific responses (noted in Table 2-1). In contrast to higher PD-1 expression observed on dominant TRBV populations, CD127 expression was lower on dominant TRBV populations as measured by MFI (p=0.007, Figure 2-6B) and frequency of $CD127^{hi}$ (p=0.05, Figure 2-6C) compared to corresponding sub-dominant TRBV populations. The PD-1 expression pattern described above on dominant and sub-dominant TRBV populations remains intact in this smaller cohort (p=0.006, PD-1 MFI).

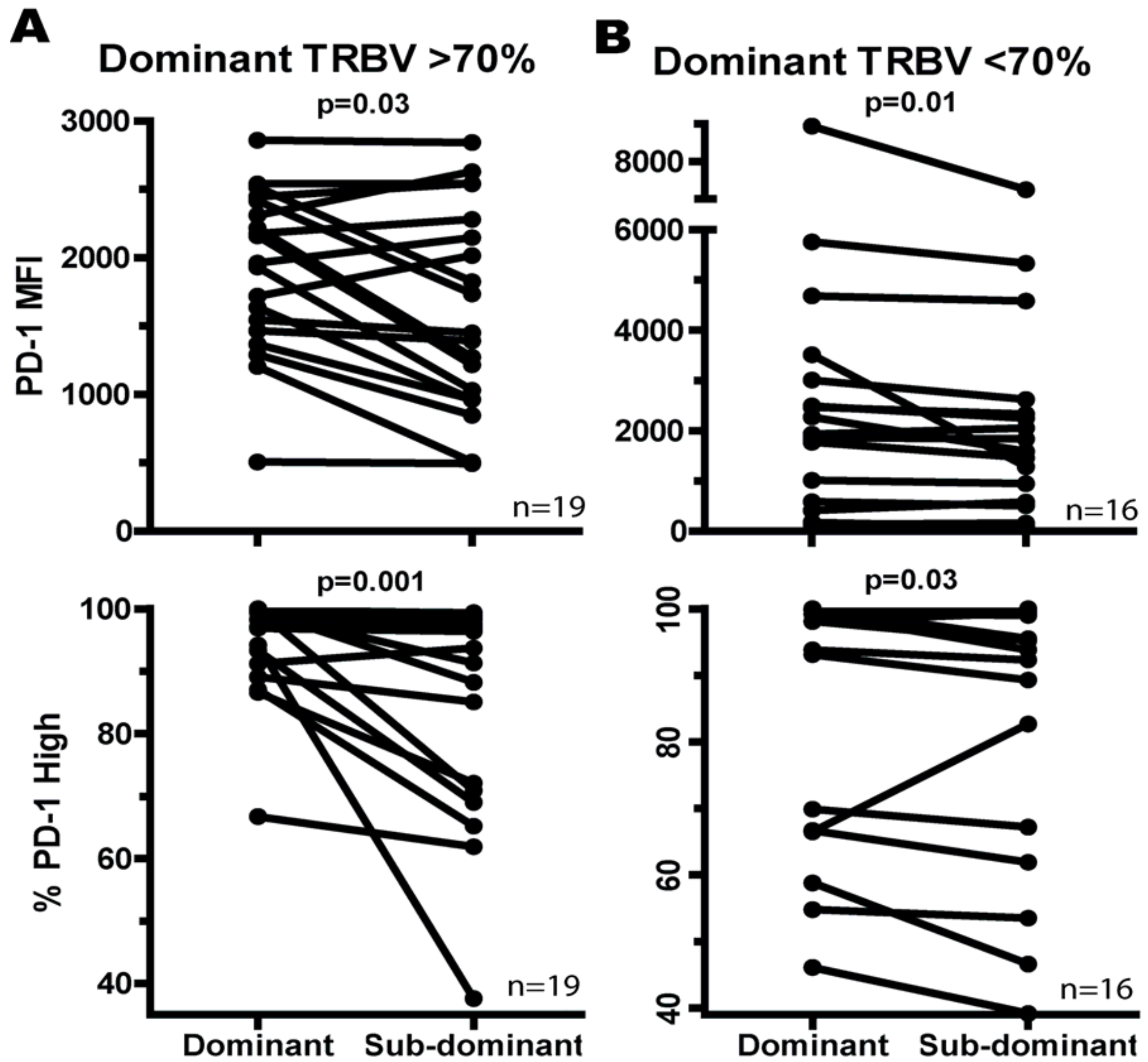


Figure 2-5 – Alternative analysis of PD-1 expression on dominant clonotypes. PD-1 expression was measured by MFI (upper charts) and percentage PD-1high (lower charts) was compared between dominant and sub-dominant TRBV populations when dominant populations comprised more than 70%, B (n=19, p=0.03, p=0.001), or less than 70%, C (n=16, p=0.01 and p=0.03) of the overall epitope-specific repertoire (Wilcoxon matched pairs test was used for these comparisons).

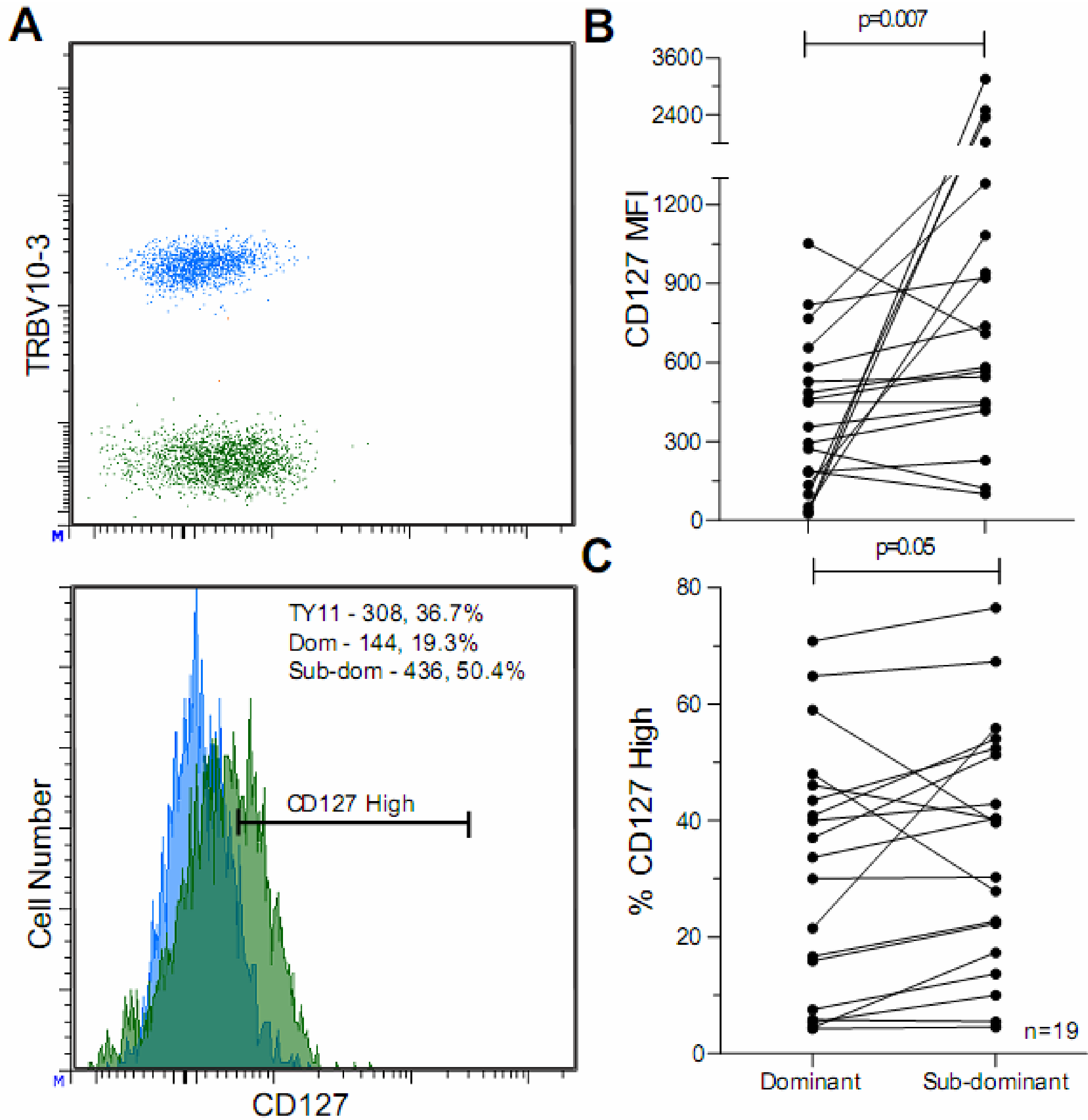


Figure 2-6 – CD127 expression is lower on dominant TRBV compared to sub-dominant TRBV populations within epitope-specific responses. Dot plot and histogram showing CD127 expression on dominant (blue) and sub-dominant (green) TRBV populations for a single epitope-specific response. MFI and percentage CD127^{high} values are provided in the corner of the histogram for the dominant and sub-dominant TRBV populations, A. CD127 expression is lower on dominant TRBV compared to sub-dominant TRBV as measured by MFI, B (p=0.007) and percentage CD127^{high}, C (p=0.05). Measurements from 19 epitope-specific populations in 12 HIV+ individuals.

Within this sub-cohort of epitopes labeled with PD-1 and CD127, 15 of 19 dominant TRBV populations displayed a PD-1^{high} phenotype and 15 of 19 displayed a $CD127^{low}$ phenotype compared to their corresponding sub-dominant population.

However, there was not complete concordance between these populations. The majority of dominant clonotypes (11 of 19) displayed the combination of higher PD-1 expression and lower CD127 expression. In contrast, there were no instances (0 of 19) in which the sub-dominant clonotype had both higher PD-1 expression and lower CD127 expression ($p<.0001$). In summary, our data suggest that clonotypic dominance within the epitope-specific TCR repertoire is associated with a PD-1^{high}/$CD127^{low}$ phenotype.

PD-1^{high}/$CD127^{low}$ phenotype on dominant clonotypes in HIV-specific responses is stable over time

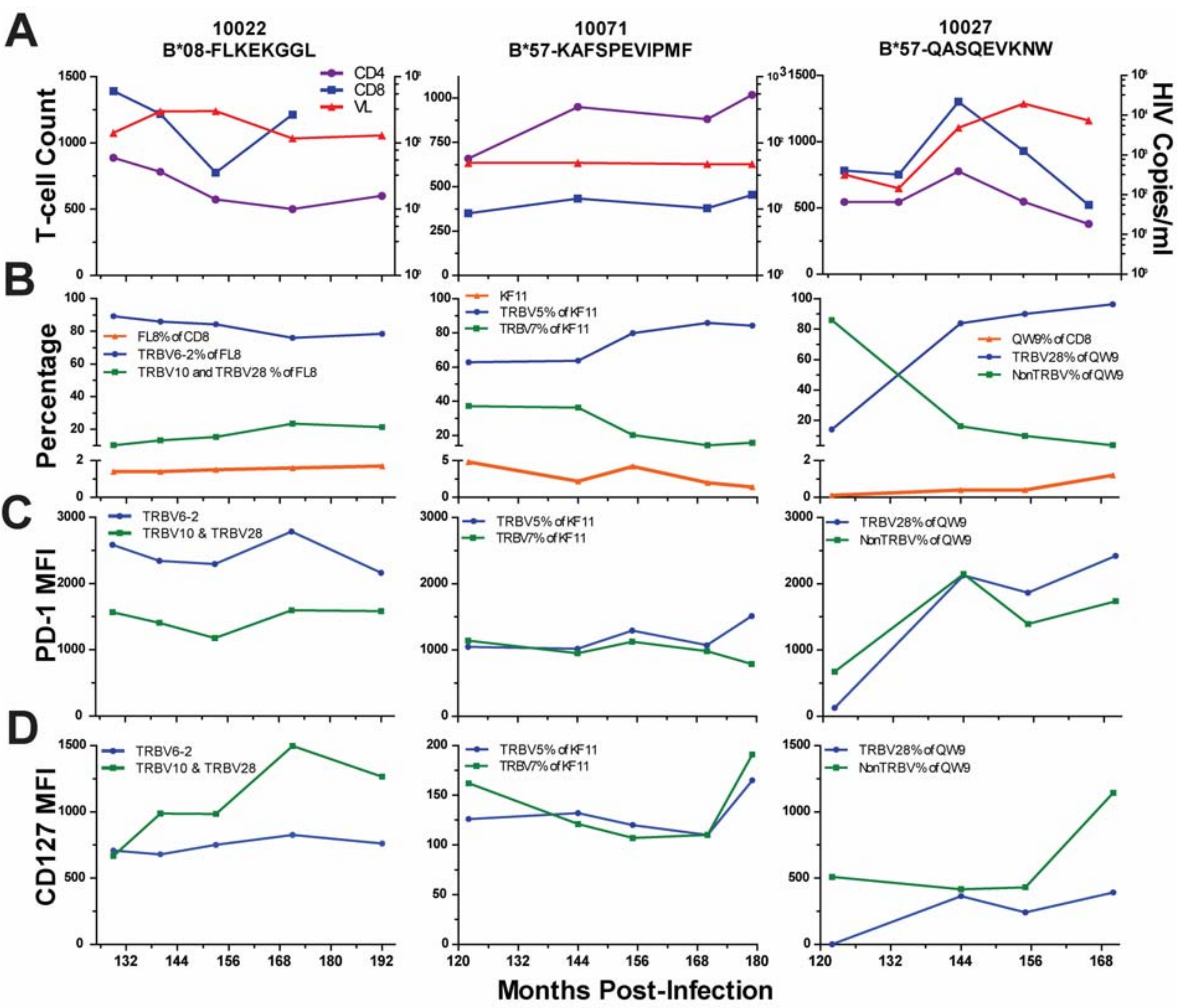
To characterize the stability of PD-1 and CD127 expression on dominant and subdominant TRBV population, we performed a longitudinal analysis of HIV-specific responses from 3 individuals. Figure 2-7 details longitudinal viral load and CD4+ and CD8+ T cell number (Figure 2-7A), epitope-specific CD8+ T cell frequency and corresponding TRBV repertoire composition (Figure 2-7B), and PD-1 and CD127 expression (Figure 2-7C and 2-7D) on TRBV populations for the dominant clonotype within the HLA-B*08-FL8 response in 10022, the dominant TRBV population within the HLA-B*57-QW9 response in 10027 (this epitope-specific T cell population was not sequenced), and the dominant

clonotype within the HLA-B*57-KF11 response in 10071 for the most recent 6 years of their infections (duration of infection 16, 16, and 15 years, respectively). 10022 and 10071 are long-term controllers with stable viral loads and CD4+ T cell counts and 10027 is a chronically infected individual with progressive disease (increasing viral load and decreasing CD4+ T cell counts).

Although expression levels of PD-1 and CD127 on the TRBV clonotypes within these HIV-specific responses are dynamic, the association of higher PD-1 expression and lower CD127 expression with TRBV dominance remains consistent over the 6 years of our analysis. The B*08-FL8-specific TRBV repertoire in 10022 is relatively stable over time. The dominant TRBV2 population in this individual maintains higher PD-1 expression over time whereas the sub-dominant TRBV populations have higher and increasing CD127 levels over the same period. The B*57-KF11-specific TRBV repertoire in 10071 is characterized by an increasingly dominant TRBV5 population and a corresponding increase in PD-1 expression compared to the subdominant TRBV7 population.

Figure 2-7 – Longitudinal analysis of epitope-specific TCR repertoire dynamics and clonotypic PD-1 and CD127 expression. Absolute CD4+ (circles) and CD8+ (squares) T cell counts (left-hand axis) and viral load (dashed line, triangles, RNA copies/ml, right-hand axis), A. Epitope-specific responses as a percentage of total CD8+ parent population (triangles, below y-axis split) and TRBV % of epitope-specific response (dominant clonotype, solid line, circles and subdominant clonotype, dashed line, squares, above y-axis split), B. PD-1 expression, C, and CD127 expression, D, on dominant (solid line, circles) and sub-dominant (dashed line, squares) TRBV populations within epitope-specific responses.

Figure 2-7 – Longitudinal analysis of epitope-specific TCR repertoire dynamics and clonotypic PD-1 and CD127 expression.



In 10027, the B*57-QW9-specific TCR repertoire fluctuates early in our observations and as the TRBV28 population becomes dominant, its PD-1 expression levels increase. Over this time period, 10027 experienced declining T cell counts and increasing viral load with an overall increase in PD-1 expression on CD8+ T cells. The dominant circulating viral sequence in 10022 and 10027 was determined for the B*27-FL8 and B*57-QW9 epitopes at a midpoint in this analysis and corresponded to the peptide sequence within the tetramers in each case. 10071 maintained viral loads of <50 copies/ml during this study, and we were unable to generate viral sequences from this individual.

We also evaluated mean PD-1 and CD127 expression levels at early and late timepoints on 10 additional epitope-specific responses and determined a similar and statistically significant expression pattern on dominant and sub-dominant TRBV populations (Figure 2-8). These longitudinal data suggest that dominance within the epitope-specific TRBV repertoire is associated with a more pronounced PD-1^{hi}/CD127^{lo} phenotype over time and may be related to the course of disease.

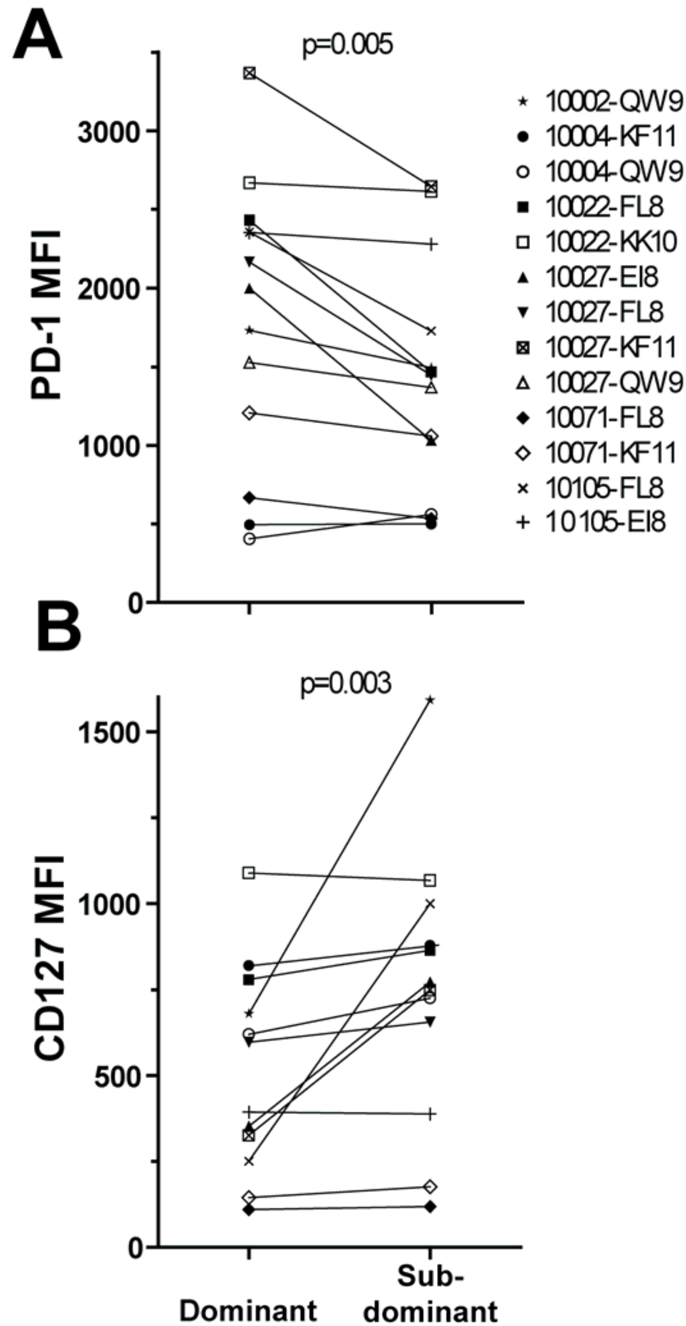


Figure 2-8 – PD-1^{high}/CD127^{low} phenotype on dominant TRBV populations is a stable relationship over time. PD-1 and CD127 expression levels (MFI) were measured at multiple time points on dominant and sub-dominant TRBV populations and mean values were calculated for these populations over all time points. Dominant populations had higher average expression of PD-1 MFI (p=.005), A, and lower average expression of CD127 MFI (p=0.003), B. Measurements from 13 (12 for the CD127 comparison) epitope-specific populations in 6 HIV+ individuals.

Tetramer binding characteristics of TRBV populations correlate with PD-1 expression but are not directly related to dominance within the epitope-specific TCR repertoire

We next investigated whether differences in tetramer binding characteristics were related to dominance within the TRBV repertoire. Our group and others have previously described differential tetramer binding on epitope-specific T cell clonotypes (127, 133) and we observed a similar phenomenon in this study (Figure 2-9A). Several groups have previously used differential levels of tetramer binding to define T cell receptor avidity (133, 152), and so we measured tetramer binding (MFI) on TRBV populations over a 16-fold range of tetramer concentration and determined tetramer-binding curves for dominant and sub-dominant clonotypes of 9 epitope-specific responses in 4 individuals (Figure 2-9B and Table 2-4). Non-linear regression analysis indicated that TRBV populations with lower half-maximal values have higher maximal binding values in 8 of the 9 epitopes tested. Thus, we used tetramer MFI on labeled TRBV populations as a surrogate measure of TCR avidity for tetramer complexes.

We compared tetramer binding levels on corresponding dominant and sub-dominant TRBV populations. While there was a trend suggesting that dominant TRBV populations have higher avidity for tetramer than corresponding sub-dominant populations, this pairing was not statistically significant (Figure 2-9C, p=.09). We found a positive and significant correlation between clonotypic avidity for tetramer and clonotypic PD-1 expression (Figure 2-9D, r=0.34, p=0.004). These data indicate that while clonotypic avidity for tetramer does not strictly

govern dominance within the repertoire, it may influence the degree of PD-1 expression.

Figure 2-9 – Tetramer binding correlates to PD-1 expression on epitope-specific T cell clonotypes. We used MHC-I tetramers to label epitope-specific T cell populations at a range of tetramer concentrations. Dot plots from highest and lowest tetramer concentrations show variable tetramer binding on clonotypes, A. Epitope-specific clonotypes were labeled at increasing tetramer concentrations from ~0-16uM. Representative graphs of tetramer binding curves are shown for 10004-QW9, 10022-KK10, 10027-FL8, and 10071-FL8 (patient-epitope) for whole epitope-specific populations (triangles), and TRBV populations (dominant-circles, sub-dominant-squares, sub-sub-dominant-diamonds), B. Comparison of tetramer binding levels (tetramer MFI) on dominant and sub-dominant TRBV populations for 35 epitopes and 22 individuals ($p=0.09$), C. Spearman correlation of tetramer binding and PD-1 expression on dominant (open circles) and sub-dominant (closed circles) clonotypes, ($r=0.34$, $p=0.004$), D.

Figure 2-9 – Tetramer binding correlates to PD-1 expression on epitope-specific T cell clonotypes.

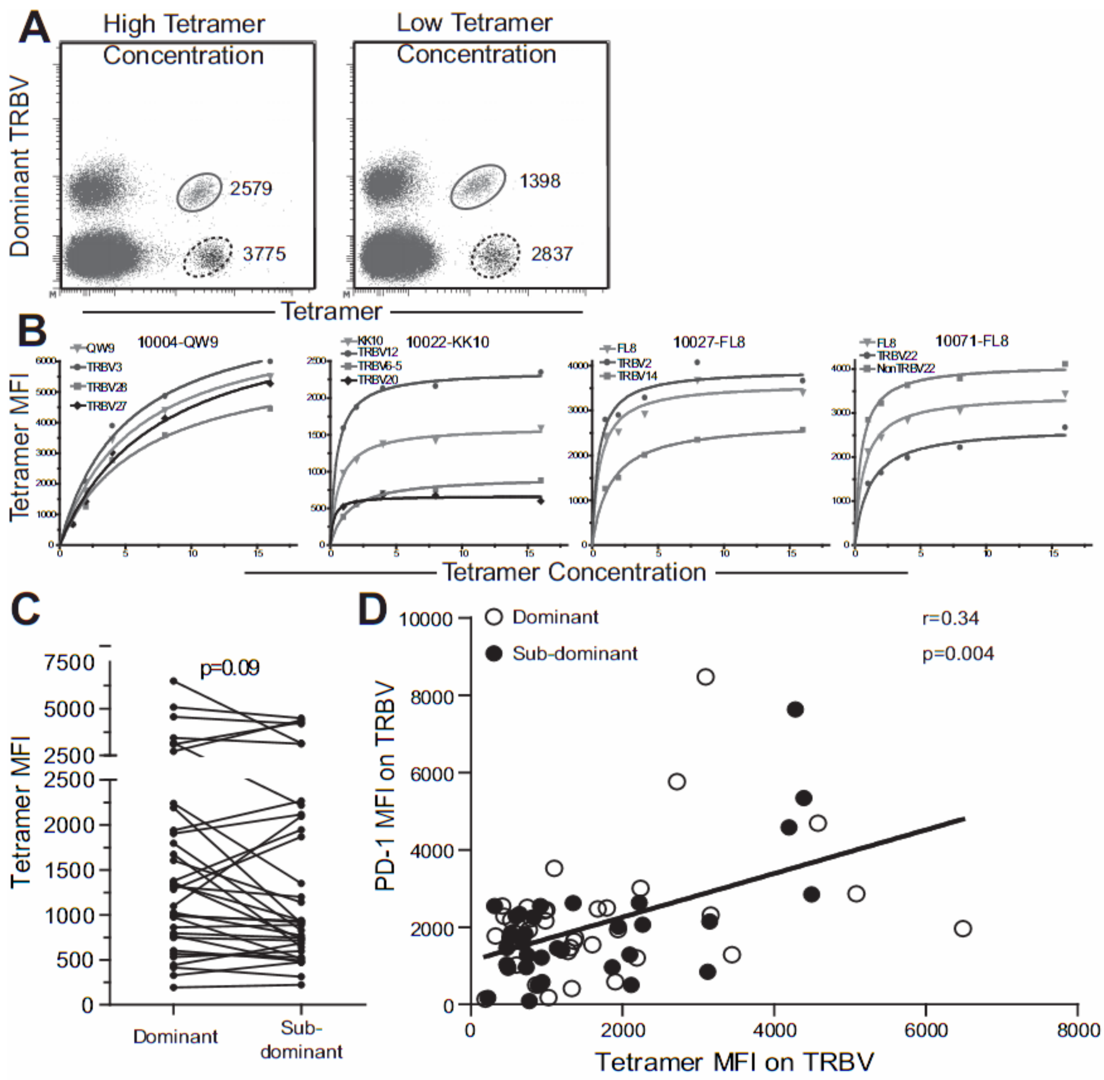


Table 2-4 - Tetramer Binding Characteristics

		TRBV			
		Tetramer concentration at 1/2 B_{max}(uM)		B_{max} (MFI)	
PID	Epitope	Dom	Sub-dom	Dom	Sub-dom
10004	KF11	6.64	3.40	9899	6596
	QW9	1.26	1.58	7985	6329
10022	KK10	0.13	0.35	2370	930
	FL8	0.36	0.23	3009	2340
10027	FL8	5.30	11.00	6542	5116
	QW9	0.12	0.36	3910	2760
	KF11	3.28	3.34	7811	3701
10071	FL8	0.62	2.38	915	2323
	KF11	0.12	0.28	4107	2678

Sub-dominant TRBV populations display greater cytokine production capacity and cross-recognition in responses to epitope variant peptides

We assessed the capacity of dominant and sub-dominant TRBV populations to produce cytokines after stimulation with consensus and variant peptides. Two common viral sequence variants for each of 4 HIV-epitopes were tested in 7 individuals. We performed viral sequencing in these individuals and found that circulating viral sequence matched the consensus epitopes used in the tetramer reagents in each individual except for 10094, who harbored a circulating sequence variant at the FL8 epitope which matched the FLK**d**KGGL variant we used in our functional assay (Table 2-5).

Taking our analysis of the B*27-KK10 response in 10022 as an example, the dominant TRBV12 clonotype comprises 45% of the B*27-KK10 response which is 19% of total CD8+ T cells. The maximal possible cytokine production by the TRBV12 clonotype is therefore 8.6% of total CD8+ T cells. Likewise, maximal cytokine production for the sub-dominant clonotypes (TRBV6-5 and TRBV20-1, together 55% of the KK10-tetramer+ population) is 10.4% of total CD8+ T cells. We determined the relative cytokine capacity (RCC) of dominant and sub-dominant TRBV populations by dividing cytokine production of the TRBV population by the frequency of that TRBV population within the tetramer population. By virtue of being a ratio, the RCC value for each TRBV population illustrates the extent to which it reaches its own maximal cytokine production potential without regard to its absolute percentage within the TCR repertoire.

Table 2-5 – Circulating Viral Sequence

	Subject ID	Epitope	epitope sequence
WT epitope	10022	B*27-KK10	KRWIILGLNK
circulating epitope			----------
		B*08-FL8	FLKEKGGL

	10027	B*57-KF11	KAFSPEVIPMF

		B*57-QW9	QASQEVKNW

	10071	B*57-KF11	KAFSPEVIPMF
			not determined
	10086	B*08-EI8	EIYKRWII

	10094	B*08-FL8	FLKEKGGL
			---**D**----
	20002	B*27-KK10	KRWIILGLNK

	20004	B*27-KK10	KRWIILGLNK

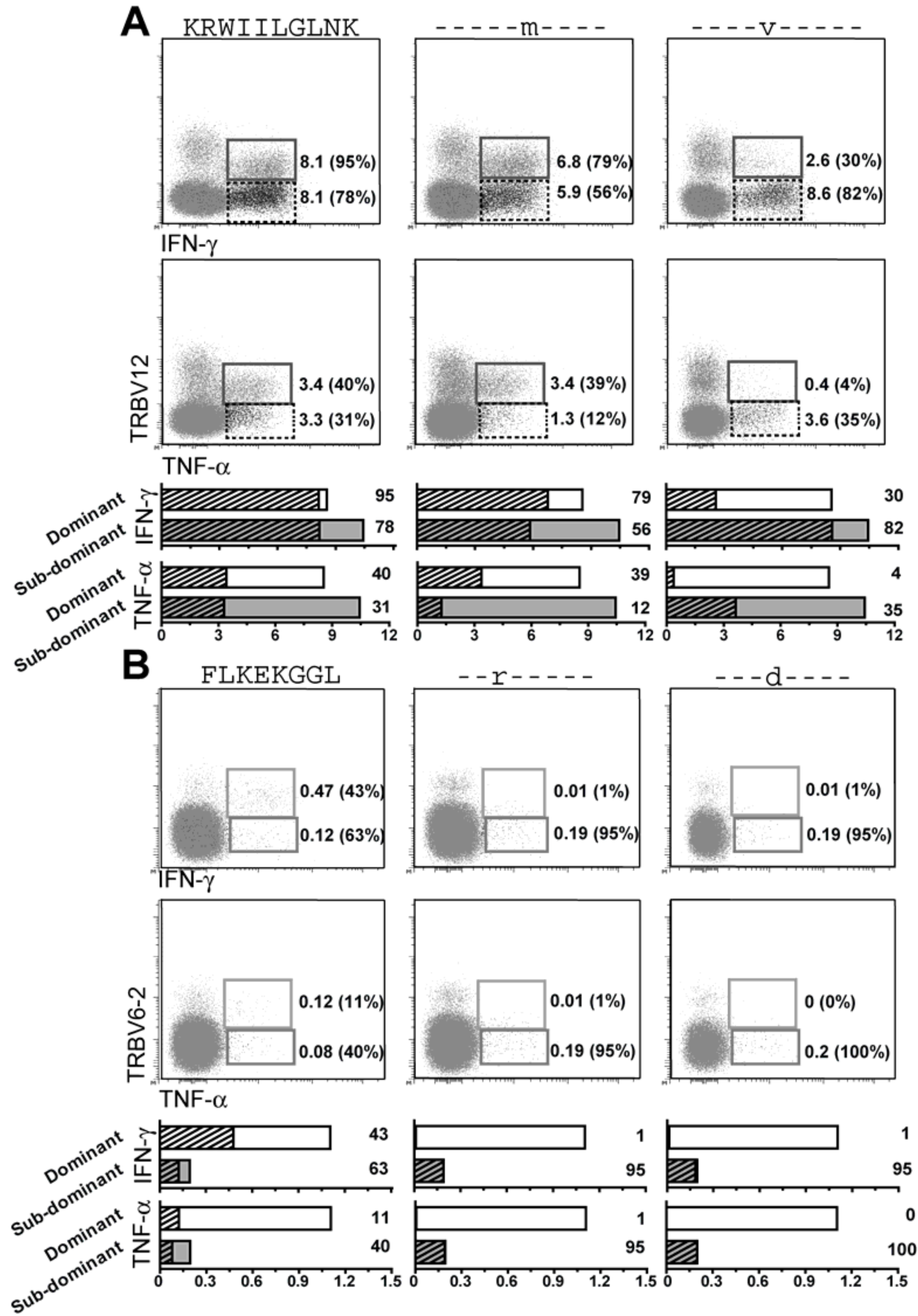
Representative plots are shown in Figure 2-10A detailing cytokine production (IFN-γ – upper plots; TNF-α – lower plots) by the dominant TRBV12 clonotype and the sub-dominant clonotypes in response to stimulation with consensus and variant peptides for the HLA-B*27-KK10 epitope. In response to stimulation with consensus peptide, the TRBV12 clonotype reached absolute cytokine production levels of 8.1% (IFN-γ) and 3.4% (TNF-α) of total CD8+ T cells and the sub-dominant clonotypes reached cytokine production levels of 8.1% (IFN-γ) and 3.3% (TNF-α) of total CD8+ T cells. TRBV12 RCC values are 95% (IFN-γ) and 40% (TNF-α). The sub-dominant clonotypes together comprise a larger part of the TCR repertoire than the dominant TRBV12 clonotype, and so despite similar levels of absolute cytokine production, their corresponding RCC values are lower at 78% (IFN-γ) and 31% (TNF-α). The strong cytokine response and high RCC values for the dominant TRBV12 clonotype suggest that these cells recognize consensus peptide more effectively than the sub-dominant clonotypes. Stimulation with the KRWI**Im**GLNK variant peptide yielded similar results to those from consensus stimulation. In response to stimulation with the KRW**Iv**LGLNK peptide, the dominant TRBV12 clonotype reached lower levels of absolute cytokine production and had lower RCC ratios for both IFN-γ and TNF-α compared to the sub-dominant clonotypes. The sub-dominant clonotypes preferentially recognized the KRW**Iv**LGLNK peptide, produced their highest levels of absolute cytokine at 8.6% (IFN-γ) and 3.6% (TNF-α) of total CD8+ T cells, and reached their highest RCC ratios of 82% (IFN-γ) and 35% (TNF-α).

The B*08-FL8 response in this individual is represented in similar fashion in Figure 2-6B and yields similar results.

Comparison of RCC values for the clonotypic cytokine responses in a further 8 epitopes from 6 additional individuals (total, 10 epitopes in 7 individuals; Figure 2-6C-D) reveals that both dominant and sub-dominant TRBV populations are capable of cytokine production to consensus peptides (Figure 2-6C, $p>0.05$ for IFN-γ and TNF-α production). In response to stimulation with common variant peptides, sub-dominant TRBV populations have higher RCC ratios for IFN-γ production (Figure 2-6D, $p=0.04$) with a trend toward higher sub-dominant RCC ratios for TNF-α production as well (Figure 2-6D, $p=0.08$). These results indicate that while dominant and sub-dominant clonotypes are capable of producing cytokines in response to stimulation with consensus and variant peptide epitopes, sub-dominant clonotypes seem to retain greater capacity for cross-recognition and secretion of multiple cytokines in response to the common viral epitope variants we tested.

Figure 2-10 – Sub-dominant TRBV populations have high cytokine production potential in response to stimulation with variant peptides. IFN-γ and TNF-α production was assessed by ICS on dominant and sub-dominant TRBV populations. Dot plots showing dominant (solid box) and sub-dominant (dashed box) clonotypic cytokine production (IFN-γ, upper plots and TNF-α, lower plots) in response to stimulation with consensus and variant peptides for the B*27-KK10, A, and B*08-FL8, B, responses in 10022. Within each plot, absolute cytokine production for clonotypic populations as a percentage of total CD8+ T cells is shown to the right of each indicated population as well as relative cytokine capacity (RCC, in parentheses). Graphs representing cytokine production for each response are located below corresponding plots. Bars represent maximal cytokine production for dominant (grey) and sub-dominant (unfilled) clonotypes, absolute cytokine production (% of total CD8+ T cells) is represented by the hatched area within each bar, and RCC for each clonotype and condition is noted to the right of each bar, A and B.

Figure 2-10 – Sub-dominant TRBV populations have high cytokine production potential in response to stimulation with variant peptides.



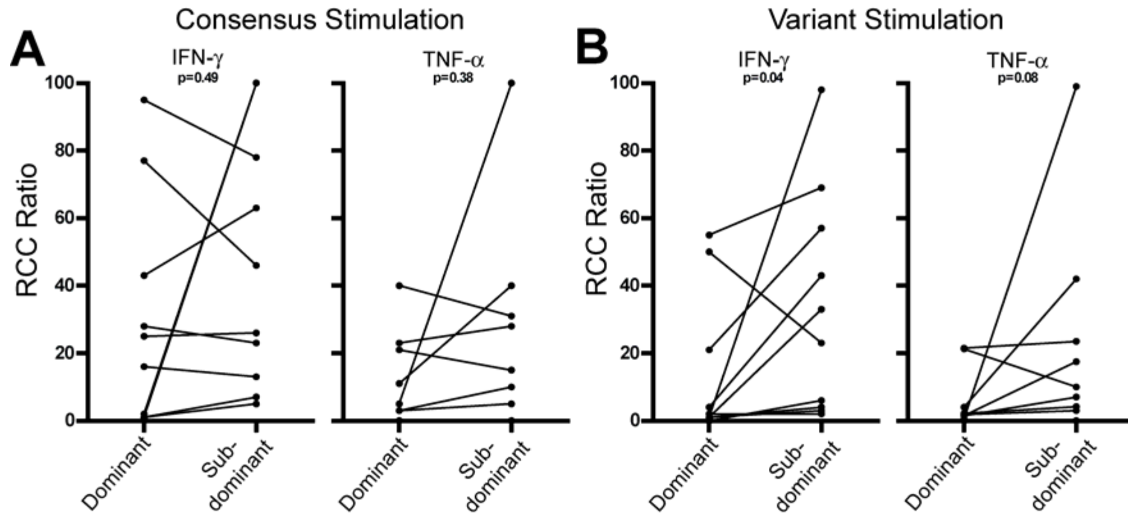


Figure 2-11 – Sub-dominant TRBV populations have high cytokine production potential in response to stimulation with variant peptides. IFN-γ and TNF-α production was assessed by ICS on dominant and sub-dominant TRBV populations. Comparison of clonotypic RCC ratios for IFN-γ and TNF-α production in response to stimulations using peptide matching consensus, A (p=0.42 IFN-γ and p=0.38 TNF-α), and variant (p=0.04 IFN-γ and p=0.08 TNF-α), B. Measurements from 10 epitopes in 7 HIV+ individuals.

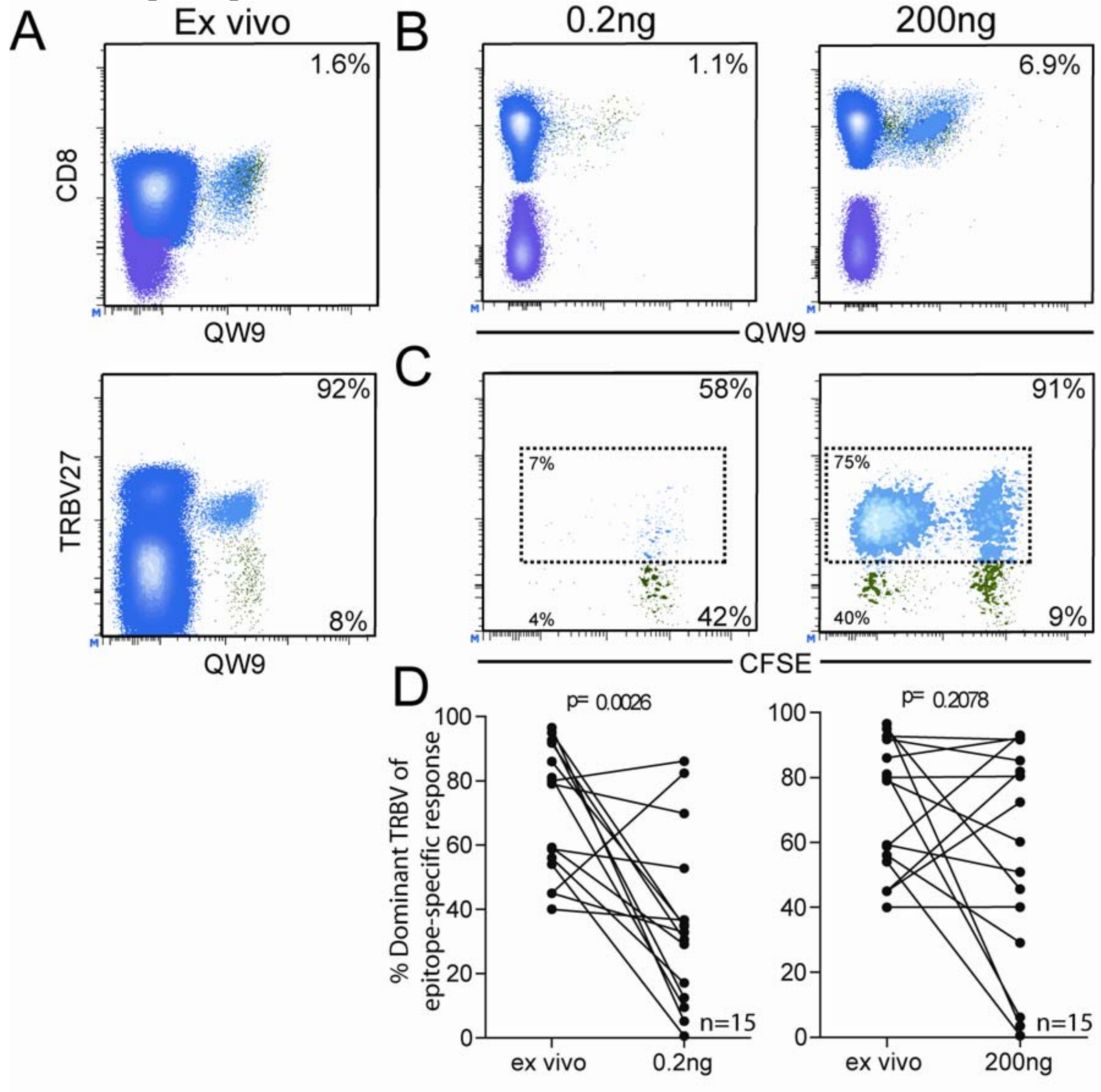
Dominant TRBV populations display a survival defect in culture

Proliferation upon antigen exposure is an important measure of T cell function and has been associated with improved control of viral replication (49). We labeled T cells with CFSE and cultured them with varying concentrations of peptide for 4 days to assess changes to the epitope-specific TRBV repertoire and capacity for proliferation of dominant and sub-dominant TRBV populations. The ex vivo epitope-specific response and its clonotypic repertoire is shown for the B*57-restricted-QW9 response in 10002 (Figure 2-12A). Representative plots are shown to illustrate epitope-specific populations (Figure 2-12B) and CFSE dilution (Figure 2-12C) for the dominant and sub-dominant clonotypes after 4 days of culture with low (0.2 ng/ml) and high (200 ng/ml) concentrations of optimal peptide antigen. At the 200 ng/ml peptide concentration, the dominant TRBV27 clonotype made up 91% of the total repertoire at the end of the 4 day stimulation period, reflecting the ex-vivo repertoire. However, at the 200 pg/ml concentration, the TRBV27 clonotype comprised 58% of the repertoire. Therefore while both dominant and sub-dominant TRBV clonotypes proliferate well in response to stimulation with higher concentrations of consensus peptide, the dominant clonotype does not survive as well at lower peptide concentrations and therefore does not maintain the same degree of dominance in vitro. Moreover, as measured by the percentage of $CFSE^{low}$ cells, the dominant TRBV27 clonotype proliferates better than the sub-dominant clonotypes in response to stimulation with consensus peptides, reflecting in vitro what happens naturally in vivo.

Aggregate data from 15 epitopes in 7 subjects indicate that dominant TRBV populations fail to maintain their dominance at low concentrations of peptide ($p=0.0026$, Figure 2-12D). Conversely, dominant TRBV populations more effectively maintain their level of dominance at higher concentrations of peptide stimulation ($p=0.2078$, Figure 2-12D). In this series of experiments, the addition of antibody to block PD-1/PD-L1 interaction did not significantly alter the relative proliferative capacity of dominant and subdominant TRBV populations over the short duration of this assay (data not shown). These results suggest that while clonotypic constituents may not expand well at low concentrations of stimulation, sub-dominant clonotypic populations are better able to survive culture conditions with low levels of antigen.

Figure 2-12 – Dominant epitope-specific TRBV populations display a survival defect at low peptide concentrations which is alleviated by increasing antigen stimulation. PBMC were cultured in the presence of peptide antigen at the indicated concentrations and TRBV repertoire composition was assessed by flow cytometry on day 4. Relevant percentages of parent are shown to the right of each population. Representative contour plots showing ex vivo T cell populations: B*57-QW9+ T cells (upper plot) as a percentage of CD8+ T cells; B*57-QW9 epitope-specific population and its constituent dominant (blue) and sub-dominant (green) TRBV populations (lower plot) as a percentage of the epitope-specific population, A. MHC-I tetramer labeling after 4 day culture in the presence of different concentrations of peptide antigen, B. TRBV repertoire composition was determined by antibody labeling for the dominant TRBV as a part of the B*57-QW9+ population. Dominant (blue) and sub-dominant (green) TRBV populations are indicated on each plot and their percentage composition of the B*57-QW9 response is shown at the right of each box. CFSE^{low} percentages are shown for each population in the upper left corner of each box, C. Aggregate data was compiled, and statistical comparisons were made between epitope-specific TRBV repertoire composition ex vivo and after 4 days of proliferation in culture. Dominant populations fail to maintain dominance at low peptide concentrations ($p=0.0026$), but repertoire composition is not significantly altered at higher concentrations ($p=0.2078$), D. Wilcoxon signed rank test. Measurements from 15 epitope-specific populations in 7 HIV+ subjects.

Figure 2-12 – Dominant epitope-specific TRBV populations display a survival defect at low peptide concentrations which is alleviated by increasing antigen stimulation.



Discussion

Several groups have observed enhanced global expression of PD-1 on T cells in HIV+ individuals, with the highest level of PD-1 expression on HIV epitope-specific cells (84-86). A detailed analysis by Day et al. found that different epitope-specific responses, even within the same individual, had differing degrees of PD-1 expression (84). This has led to speculation that the degree of PD-1 expression could be linked to the efficacy of viral control for individual epitopes (84, 153).

In this study, we evaluated constituent clonotypes within epitope-specific responses and determined that clonal dominance within epitope-specific responses is associated with a PD-1^{high}/CD127^{low} phenotype, that PD-1 expression correlates with clonotypic TCR avidity for tetramer, and that dominant clonotypes display defects in their ability to respond to variant peptide epitopes and survive in the absence of strong antigen signals. We found that the most dominant clonotype within an epitope-specific response tended to have the highest level of PD-1 expression (p=0.001) and the lowest level of CD127 expression (p=0.007). We did not see a relationship between the overall magnitude of a response (or the degree of clonotypic expansion within a response) and PD-1 expression, suggesting that PD-1 expression may not be directed related to the level of T cell expansion or exhaustion, but could mark T cells which have recently been exposed to their cognate antigen (154).

In LCMV infection downregulation of PD-1 and upregulation of CD127 occurs after viral epitope escape (106), suggesting that ongoing antigen exposure is a key factor in pushing T cells toward a PD-1^{high}/CD127^{low} phenotype. Lichterfeld et al described progressive reductions in CD127 expression on high avidity HIV-epitope-specific clonotypes which were eventually deleted (155), and more recent work by Steeck et. al found PD-1 expression on HIV-1 epitope-specific T cells decreased after in-vivo selection for escape mutations (147). While this recent work highlights the relationship between, PD-1, and CD127 expression on epitope specific responses (106, 112), the data we present here is the first to our knowledge which describes differential expression of these markers on individual T cell clonotypes and links dominance to specific differences in clonotypic function.

We have shown that epitope-specific T cell populations are often comprised of a single dominant and various sub-dominant clonotypic populations that can respond variably to changes in viremia (127) and that these clonotypes have differing abilities to recognize epitope variants (129). Our more recent work demonstrates a relationship between TCR use and memory phenotype (128). Thus, our new finding that dominant and sub-dominant T cell clonotypes have phenotypic and functional characteristics linked to antigen sensing is yet another indicator that the fine-specificity of individual T cell clones plays a role in the evolution of epitope-specific immune responses.

The majority of individuals we sequenced had dominant circulating sequences matching HIV Clade-B consensus, with the exception of 10094 (Table and data not shown), and this subject still preferentially recognized the consensus peptide over the circulating variant. Despite their PD-1^{high}/CD127^{low} phenotype, we present evidence that dominant T cell clonotypes able to recognize circulating viral sequences have the capacity to produce multiple cytokines after stimulation with consensus and variant peptide epitopes and that subdominant clonotypes have increased ability to recognize common HIV-1 epitope variants. Improved recognition of viral variants by sub-dominant clonotypes might also be influenced by the diversity of TCR clonotypes within these sub-dominant populations. Each of the epitope-specific responses we assessed is comprised of a single dominant clonotype and at least one and in some cases more than one sub-dominant clonotypes. Effective recognition of variant epitopes may also be a reflection of increased diversity within sub-dominant TRBV populations.

Immune selection pressure mediated by CD8+ T cells can lead to viral mutation and epitope escape from immune recognition (156-158), therefore the frequency of circulating epitope variants and the degree to which individual clonotypes are able to recognize these variants may also play a role in the development and maintenance of the epitope-specific TCR repertoire. A recent study from van Bockel et. al. offers insight into the relationship between clonal evolution within the TCR repertoire in HIV+ individuals and viral epitope variation (131). Their work highlights TCR repertoire remodeling within HLA-B*27-restricted responses

to a viral epitope known to consistently undergo immune-mediated mutational escape (67). The authors in this study found that in the presence of epitopes that varied from consensus, dominant T cell clonotypes were maintained over time and expressed higher levels of CD127 compared to subdominant clonotypes. In contrast to van Bockel et. al. we found dominant clonotypes to have lower levels of CD127 compared to subdominant clonotypes. Our study was different in that we evaluated 35 different epitope responses (representing 8 discrete HIV epitopes) in 22 individuals, and in the majority of cases the circulating viral sequence corresponded to the tetramer peptide sequence. These findings are broadly complementary to our own; both data sets indicate that dominant clonotypes are surprisingly persistent in vivo over time and support the notion that broad epitope-specific TCR repertoires may contain clonotypes capable of recognizing and suppressing viral sequence variants.

While we cannot rule out the possibility that some HIV+ individuals in our cohort harbored viral variants not covered by the consensus or variant epitope sequences we selected, the recently reported associations between PD-1 expression and epitope escape (106) highlight the importance of this line of inquiry for future longitudinal in vivo and in vitro studies. The relationship between epitope exposure, recognition, escape, and corresponding epitope-specific T cell phenotype and functional capacity seems to be tightly related, although the effects of persistent exposure to antigen and viral escape on repertoire composition or clonotypic impairment have yet to be determined. In

this cross-sectional study, we were unable to assess whether higher avidity clones had been deleted earlier in infection or whether circulating virus had already escaped immune control for all the epitopes studied.

Despite the higher expression of PD-1 on dominant clonotypes, and the relative failure of these dominant clonotypes to survive at low peptide concentrations in vitro, blockade of the PD-1 signaling pathway did not result in significant enhancement of clonotypic proliferation or survival. Studies evaluating the effect of PD-1 blockade on proliferative capacity have typically found modest increases in proliferation (78, 84, 86). The lack of enhanced proliferation we saw may be due to the short duration of our assays, and to the inclusion of relatively healthy subjects with low viral loads. Future studies with combinations of PD-1/PD-L blockade and cytokine combinations may help us determine to what extent dominant clonotypes can be "rescued" in vitro.

Previous reports in mouse influenza models (159) and human EBV/CMV infection (133) indicate that T cell avidity for antigen is positively correlated with dominance in the epitope-specific TRBV repertoire. Our data support the notion that clonotypic TCR avidity is associated with higher expression of PD-1, but suggest that the association between overall TCR avidity and clonal dominance may be weaker in the setting of chronic HIV infection. Prior studies evaluated either acutely resolved or chronic viral infections with limited antigen variability and low levels of ongoing antigen exposure during chronic infection, and those conditions could account for the discrepancies between our study and this

previously published work. It remains to be determined if the associations between epitope-specific clonotypic dominance, phenotype, and function which we report in HIV infection also apply to other infections in humans and model systems.

T cell phenotype and function is determined not only by the fundamental interaction between TCR:pMHC but represents a sum of inhibitory and stimulatory signals emanating from surface receptor molecules such as PD-1 and CD127. Recent work from Almeida et al. (47) suggests that a composite measure for T cell function such as 'antigen sensitivity' might encompass not only avidity for antigen but a wide range of influential factors such as antigen receptor density, coreceptor-mediated signals, as well as activation status and expression of inhibitory signaling molecules. We suggest that the composition, phenotype, and functional profile of the clonotypic repertoire may be necessarily dynamic in order to respond to a highly variable pathogen such as HIV.

The following model accommodates our observations and experimental results: dominant clonotypes preferentially expand to circulating viral epitopes in vivo. Dominant clonotypes express a surface phenotype consistent with ongoing antigen exposure and activation. Continued exposure to cognate antigen may erode the capacity of dominant clonotypic responses as a result of accumulated PD-1 signal inhibition and a reduction in homeostatic turnover from reduced CD127 expression. Sub-dominant clonotypes expand sub-optimally to circulating

viral epitopes in vivo and express a phenotype consistent with reduced exposure to antigen. Sub-dominant populations may recognize non-circulating or low-level variants more effectively than dominant populations and are exposed to relatively lower levels of their preferred cognate antigen resulting in lower overall antigen exposure and concomitant activation. This sparing effect results in the maintenance of a population of cells better able to survive in the absence of strong antigenic signaling. These data also suggest that higher avidity clonotypes develop a relatively PD-1^{high} phenotype compared to lower avidity clonotypes and is consistent with the observation that higher avidity responses are deleted early in infection (155). It remains to be determined whether TCR repertoire composition or clonotypic phenotype in HIV is significantly different in individuals with confirmed viral escape or in the absence of antigen, although data from LCMV infection and HIV infection suggests that this might be the case (106, 131, 147).

A diverse epitope-specific TCR repertoire comprised of clonotypes capable of recognizing and suppressing both circulating and variant epitopes would be a beneficial outcome from either prophylactic vaccine strategies or for strategies seeking to broaden existing immune responses in established HIV infections. Furthermore, manipulation of immunomodulatory surface proteins such as PD-1 or CD127 as a part of vaccination protocols could influence qualitative and quantitative aspects of the epitope-specific immune response including antigen sensitivity or clonotypic repertoire (143). Effective immunological strategies to

control chronic infections like HIV may require not only the generation or stimulation of antigen-specific cells but also a coordinated manipulation of inhibitory pathways.

CHAPTER III

ANTI-RETROVIRAL THERAPY REDUCES THE MAGNITUDE AND T CELL RECEPTOR REPERTOIRE DIVERSITY OF HIV-SPECIFIC T CELL RESPONSES WITHOUT CHANGING T CELL CLONOTYPE DOMINANCE OR PHENOTYPE

Abstract

After initiation of highly active anti-retroviral therapy (ART), HIV viral loads decrease as do the magnitudes of HIV-epitope-specific immune responses. In chronic HIV infection, the virus-specific T cell receptor repertoire allows the host to respond to viral epitope diversity; however, the effects of antigen reduction on the T cell receptor (TCR) repertoire of epitope-specific CD8+ T cell populations are not well established. We determined the TCR repertoires of 14 HIV-specific CD8+ T cell responses from 8 HIV+ individuals before and after initiation of ART. We used multiparameter flow cytometry to measure the distribution of memory T cell subsets, and the surface expression of PD-1, CD127, and CD38 on overall T cell populations and T cell clonotypes within epitope-specific responses from these individuals. Post-ART, we noted decreases in the frequencies of circulating epitope specific T-cells ($p=0.02$), decreases in the number of T-cell clonotypes found within epitope-specific T cell receptor repertoires ($p=0.024$), and an overall reduction in the amino acid diversity within these responses ($p<0.0001$). Despite

this narrowing of the T cell response to HIV, the overall hierarchy of dominant T cell receptor clonotypes remained stable compared to pre-ART. CD8+ T cells underwent redistributions in memory phenotypes and a reduction in CD38 and PD-1 expression post-ART. Despite extensive remodeling at a structural and phenotypic level, PD-1 was expressed at higher levels on dominant clonotypes within epitope-specific responses before and after initiation of ART. These data suggest that antigen burden drives TCR diversity, and that dominant clonotypes are sensitive to very low levels of antigen.

Introduction

Successful anti-retroviral therapy (ART) reduces viral loads and decreases the level of T cell activation but the effect of HIV antigen reduction on the TCR repertoire of epitope-specific T cell responses remains poorly defined (160). The level of generalized T cell activation as measured by expression of CD38 is a strong, independent predictor of disease progression (61, 161). More recent work has shown a positive correlation between increased expression of PD-1 on T cells and the level of viremia (85, 112). In addition to being an independent risk factor for disease progression in the absence of ART, sustained high levels of immune activation in the presence of ART are associated with poorer levels of CD4+ T cell recovery (162). Other phenotypic markers such as CD45R0, CCR7, CD27, and CD28 define T cell memory subsets, which are altered as a result of HIV infection (32, 124, 136). After the initiation of ART, the distribution of memory

T cell subsets improves, indicating broad remodeling of T cell populations with successful treatment and antigen reduction (163). Few studies have evaluated the effect of ART on HIV-specific T cell populations in detail and even these have not analyzed the epitope-specific TCR repertoire in detail (160, 164-166).

Virus-specific CD8+ T cell responses are a critical component of the natural immune response to HIV (63-65). However, quantitative features of T cell responses such as the frequency or magnitude of HIV-specific T cell responses do not correlate well with control of viral replication or disease progression (122, 123). On the other hand, qualitative features of T cell responses such as epitope-specific proliferation (49) and breadth of T cell effector function have been shown to correlate well with control of viremia (125) and may represent important determinants of disease outcome. Indeed, qualitatively superior, polyfunctional HIV-specific T cells have been shown to emerge after suppression of viremia with ART (167); however, after initiation of ART, the magnitude of HIV-epitope-specific immune responses also contract (168). Clonotypic and amino acid diversity within the epitope-specific TCR repertoire is a qualitative feature of T cell responses that may be associated with control of viremia (120, 121), but there is little data to inform our understanding of how the TCR repertoire may be affected by ART.

We hypothesized that the structural composition and clonotypic phenotype of epitope-specific responses may also be altered in individuals undergoing ART. In

this study, we compared amino acid and clonotypic diversity within the HIV-epitope-specific TCR repertoire before and after initiation of ART. Furthermore, we analyzed memory phenotypes and expression of CD38 and PD-1 on dominant and sub-dominant clonotypes from these epitope-specific responses. After initiation of ART, we noted changes in memory subset distributions, and decreased activation of CD4+ and CD8+ T cell populations. The magnitude of HIV epitope-specific T cell responses decreased after ART, and this was accompanied by a reduction in TCR repertoire diversity as measured by the number of discrete clonotypes found within epitope-specific responses, as well as by measurement of TRBV-CDR3 amino acid diversity. However, dominant T cell clonotypes typically remained dominant even post ART, and these T cell populations retained a PD-1^{high} phenotype compared to sub-dominant clonotypes. These findings provide insights into the forces which likely drive the evolution of the TCR repertoire, namely, that high levels of persistent antigen exposure may drive TCR repertoire diversity, and that even in the setting of sustained reduction in circulating antigen, dominant T cell clonotypes maintain a phenotype consistent with exhaustion.

Materials and Methods

Individual Cohort and HLA-typing. This cohort was organized within the Vanderbilt-Meharry CFAR and was comprised of patients recruited through the Comprehensive Care Center (Nashville, TN) who underwent initiation of ART during the course of follow up. All individuals were typed for HLA Class I by DCI Tissue Typing Laboratory (Nashville, TN). This study was approved by the Institutional Review Board at Vanderbilt University, and all participating individuals provided written informed consent.

T-cell sorting and TCR sequencing. Epitope-specific T cells were labeled with appropriate MHC-I tetramers and sorted by FACS to >95% purity on a FACSAria cell sorter (BD). TCR Repertiore Sequencing was carried out as described (169). Briefly, total RNA was isolated from sorted class I tetramer-specific cells with RNA-STAT-60 (Teltest inc., TX, USA). Anchored RT-PCR was performed using a modified version of the SMART (switching mechanism at 5' end of RNA transcript) procedure (Clontech, Mountain View, CA) and a TCRB constant region 3' primer (5'-ATT CCT TTC TCT TGA CCA TG-3'). cDNA amplification was performed using the TCR constant region based primer (5'-TTC ACC CAC CAG CTC AGC TC-3') and 10X Universal Primer A Mix (Clontech, Mountainview, CA) . PCR products of 600–700 base pairs were gel purified (Qiagen, Valencia, CA), ligated into the TOPO TA vector (Invitrogen, Carlsbad, CA), and used to transform chemically competent Top 10 *E. coli* cells (Invitrogen, Carlsbad, CA). Bacterial colonies were selected, and screened for the presence of insert using

PCR with M13 primers. Selected colonies were sequenced with the Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems, Foster City CA) and capillary electrophoresis on a PRISM automated sequencer (Applied Biosystems, Foster City CA). In frame TCR Sequences were edited and aligned using Sequencher (Gene Codes Corp., Ann Arbor, MI) and compared to the human TRBV genes database (http://imgt.cines.fr) (170). The TRBV classification system of the international ImMunoGeneTics database (171) is used unless noted.

Measurement of T cell receptor diversity: The diversity of the TCR CDR3 region was performed was determined using the Shannon entropy (*H*) (118, 172) calculation for protein sites as described previously (119) by the formula: $H = -\Sigma p_i \log_2 p_i$ where p_i is the fraction of residues at a site that is amino acid type *i*. For amino acids in the CDR3, *H* can range from 0 (site contains only one amino acid in all sequences) to 4.32 (all amino acids are represented equally at this site). Positions that contained >50% gaps were excluded from analysis. Statistical values are expressed as mean or median plus or minus SEM. The diversity of TCR clonotypes was calculated using the Simpson's Diversity Index (173) using the formula $Ds=1- \Sigma ni(ni-1)/N(N-1)$, where ni is the TCR clone size of the clonotype and N is the total number of TCR sequences sampled. Comparisons between groups were performed using Mann-Whitney test or two-tailed Student t test, depending on data normality. Correlations were calculated using Spearman Rank test. GraphPad Prism v5.03 software was used for statistical analysis.

Flow cytometric evaluation of lymphocyte surface molecules. Lymphocyte subsets were evaluated using with anti-CD3-AlexaFluor-700 (BD), CD4-PE-Texas Red (Caltag), CD8-Pacific Orange (Caltag), CD14-PerCP (BD), CD19-PerCP (BD), CD38-PE-Cy7 (E-biosciences), CD45RO-PE-TexasRed (E-biosciences), CD56-PE-Cy5 (BD), CCR7-pure (Mouse IgG2, clone 150503, R&D Systems), Viaprobe (BD), anti-PD-1-pure (Mouse IgG1, clone EH12:2H7, BioLegend), goat-anti-mouse IgG-Pacific Blue (Molecular Probes), anti-TRBV-PE/FITC (Beckman-Coulter). Epitope-specific T cell populations were stained with MHC-I tetramers HLA-B*08-EI8 (EIYKRWII), HLA-B*08-FL8 (FLKEKGGL), HLA-B*15-GY9 (GLNKIVRMY), and HLA-B*15-TY11 (TQGYFPDWQNY) (NIH Tetramer Core Facility, Atlanta, GA) and HLA-B*57-KF11 (KAFSPEVIPMF), HLA-B*57-IW9 (ISPRTLNAW), and HLA-B*57-QW9 (QASQEVKNW) (Beckman-Coulter).

Cells were labeled with MHC-I tetramers at 21ºC for 10 minutes. Anti-PD-1 or anti-CCR7 antibody was added to the suspension and incubated for a further 20 minutes. Cells were washed and labeled with pacific blue-conjugated goat anti-mouse antibody, washed and blocked with mouse normal IgG antibodies, and labeled with the remaining directly conjugated surface antibodies listed above. Cells were then washed a final time before acquisition on a BDFACSAria instrument. Data was analyzed using BDDiva software v6.1.

Results

Epitope-specific immune responses contract after ART

ART dramatically reduces viral loads in HIV+ individuals and many individuals experience increases in CD4 T cell count or reductions in the phenotypic activation status of T cells. We determined the composition of the epitope-specific T cell receptor (TCR) clonotypic repertoire as well as clonotypic cell surface phenotype of 14 epitope-specific CD8+ T cell responses from 8 HIV+ individuals before and after the initiation of highly active anti-retroviral therapy (ART) (Table 3-1). Six of eight individuals had increases in CD4+ T cell counts, and the cohort had a mean reduction in viral load of 2.3 log ($p=.01$; paired T test). One of the two individuals who failed to reconstitute CD4+ T cells is 10004. This individual had abnormally low CD4 T cell counts from his inclusion in our cohort in 2003, and it is not surprising to note that the CD4 compartment is not fully reconstituted in this individual. The mean number of days before suppression of viremia to below 50 copies/ml was 170. All individuals were eventually suppressed to below 50 copies/ml, and 5 of 8 individuals were fully suppressed to undetectable levels at the time of repertoire and phenotypic analysis (Table 3-1).

Table 3-1 – Pre-post ART Study Cohort

Patient ID	Epitopes	Pretherapy		Post therapy CD4	Days to reach <50 copies/ml after HAART	TCR sequence analysis and phenotyping (days)
		CD4	VL (copies RNA/ml)			
10001	B*1501 - GY9, TY11	308	5.0	598	288	288
10004	B*5701 - KF11, QW9	203	1.69	167	11	1109
10027	B*5701 - KF11, QW9 B*08 - FL8, EI8,	378	3.87	637	35	428
10086	B*08 - EI8	132	4.88	500	219	1125
10105	B*08 - FL8, EI8	289	4.54	430	384	406
10138	B*1501 - GY9	291	3.20	305	190	137
10141	B*1501 - GY9	464	5.46	572	170	7
20023	B*1501 - GY9,	538	3.54	413	60	46

Using MHC-I tetramer reagents, we identified and tracked 14 distinct HIV-epitope-specific CD8+ T cell populations before and after initiation of ART. Overall, epitope-specific T cell populations measured post-ART contracted as a percentage of the parent CD8+ T cell population compared to pre-ART measurements (73% median decrease, Figure 3-1A, p=0.02). However, this effect was not universal. For example, The B*57-KF11 response in 10004 increased as a percentage of the parent CD8+ T cell population after initiation of ART, while the B*57-QW9 response in the same individual did not increase to a similar degree indicating that individual epitope-specific responses can respond independently to reductions in viral load.

T cell clonal dominance is maintained after ART

Epitope-specific TCR repertoires were determined by labeling epitope-specific populations with MHC-I tetramers, isolating these cells using FACS, and directly sequencing the T cell receptor-B variable region. Clonotypic dominance within the repertoire was determined by sequencing and confirmed by co-labeling tetramer+ populations with anti-TRBV antibodies. TCR repertoire data generated independently using these two methods corresponds well and with a high degree of significance (Figure 3-2, Pearson r=0.85, p<0.0001).

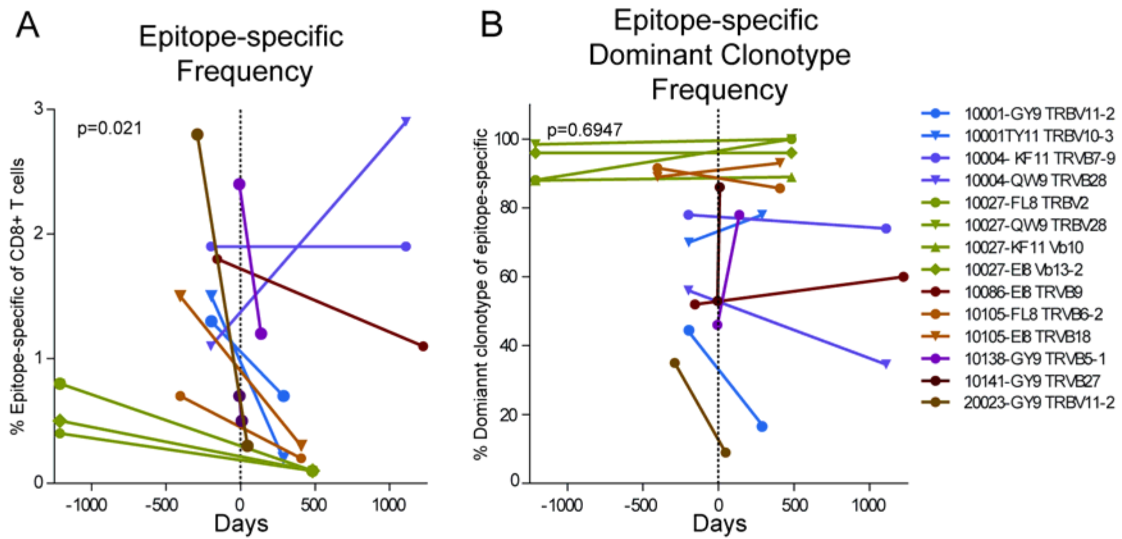


Figure 3-1 – Epitope-specific responses contract but clonotypic dominance remains intact. Epitope-specific responses contract after initiation of ART (p=0.021, n=14),A. Dominant clonotypes maintain relative dominance after initiation of ART (p=0.6947, n=12), B. Patient identifier, epitope-specific response, and dominant TRBV population are indicated in the figure legend.

Several clonotypes varied in their relative frequency within the epitope-specific repertoire after initiation of ART, but dominant clonotypes remained dominant after initiation of ART in the majority of cases. No statistically significant patterns emerged from our analysis of changes within the TCR repertoire after initiation of ART (Figure 3-1B). Dominant clonotypes in the 10001 B*15-TY11 and 20023-B*15-GY9 responses contracted significantly after initiation of ART to the point that they made up only minor clonotypic populations in the TCR repertoire. No relationship was apparent between contractions in the TCR repertoire and corresponding phenotypic measures in our study. We next evaluated the TCR repertoire of these epitope-specific immune responses in more detail.

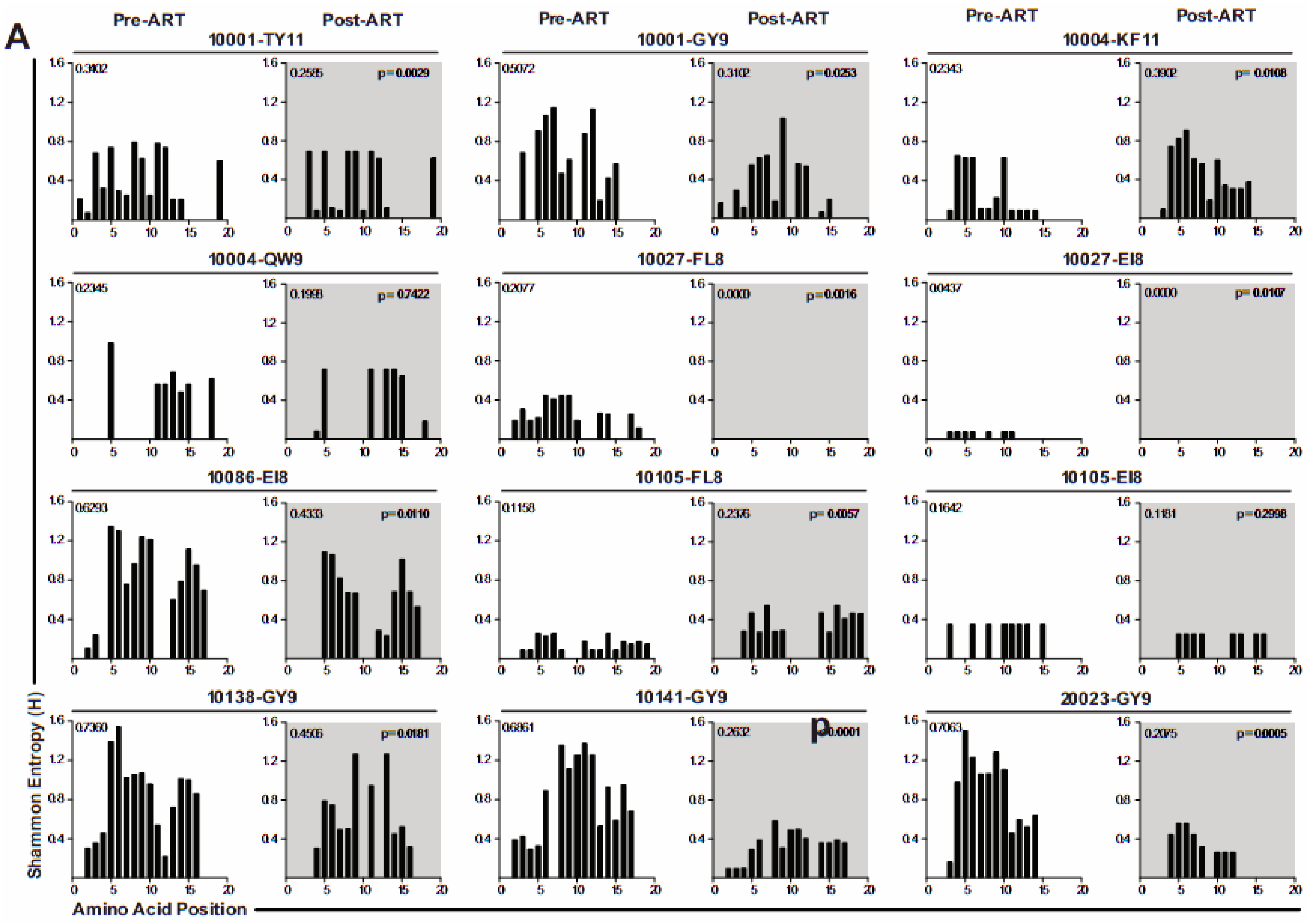
The epitope-specific T cell receptor repertoire narrows after ART

We were interested in determining the effect of ART on the clonotypic composition and structural diversity of the T cell receptor repertoire within epitope-specific responses. Diversity within the TCR repertoire can be measured using several methods. Shannon entropy (H) provides a useful metric to assess the degree of overall amino acid diversity within the TRBV-CDR3 (118, 119) (120, 129). Diversity analysis using Simpson's diversity index can account for both abundance of a clonotype as well as its proportion within the repertoire (117). We used both methods to evaluate T cell repertoire diversity before and after ART.

We calculated entropy (H) values for each amino acid position within the TRBV-CDR3 regions of 12 epitope-specific responses in 8 individuals before and after initiation of ART (Figure 3-2A). Taking individual amino acid positions in each of the epitope-specific populations into account, post-ART entropy was reduced compared to pre-ART ($p<0.0001$). The total number of clonotypes measured post-ART was reduced from a mean of 6.5 clonotypes/response to 3.9 clonotypes/ response (Figure 3-3A, $p=0.02$). The mean entropy values for each CDR3 region (i.e. the average of the individual CDR3 amino acid entropy values) for epitope-specific populations post-ART decreased compared to pre-ART values (Figure 3-3B, $p=0.0269$). Additionally, by measuring the number of clonotypes and their relative abundance in the epitope-specific TCR repertoire, we also show that diversity is reduced after initiation of ART (Simpson's diversity index, Figure 3-3C, $p=0.025$). It is important to note that despite the smaller magnitudes of epitope-specific responses after initiation of ART, there was no correlation between the number of cells sorted and the number of clonotypes determined (Spearman correlation, $r=0.33$, $p=0.15$). This is consistent with our prior data (129). Taken together, these data provide compelling evidence that the epitope-specific TCR repertoire narrows after initiation of ART in terms of overall amino acid diversity as well as in the number of clonotypes within each response.

Figure 3-2. TCR repertoire diversity is reduced after initiation of ART. Shannon entropy (H) diversity calculation of TRBV CDR3 amino acids of 12 immune responses from 8 HIV-infected individuals. Pre-ART and Post-ART (shaded) entropy for each amino acid position within the TRBV-CDR3 is represented by the bars in each graph. Mean entropy values for each epitope-specific response are shown in the upper left corners of each graph. Comparisons of mean entropy values pre-ART and post-ART are shown in bold in the upper right corner of each graph, A.

Figure 3-2. TCR repertoire diversity is reduced after initiation of ART.



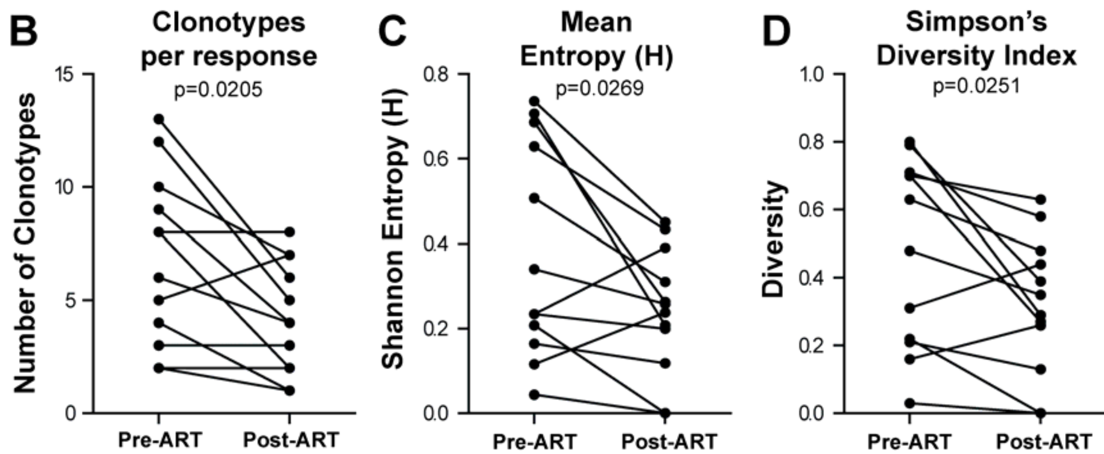


Figure 3-3 – TCR repertoire diversity is reduced after initiation of HAART. Total number of clonotypes within epitope-specific decreased after initiation of HAART (p=0.0205, 12 epitopes), A. Mean entropy decreased in 10/12 evaluated responses, (p=0.0537, 11 epitopes), B. Reductions in diversity by Simpson's diversity index (p=0.0251), C.

T cell populations undergo memory redistribution and reductions in activation status after initiation of ART

T cell phenotype is governed by environmental signals and is likely influenced by the level of antigen burden. Compared to uninfected controls, T cell populations in HIV infection are associated with skewed T cell memory differentiation (32) and increased expression levels of CD38 and PD-1 (174). We used expression of CD45RO and CCR7 to measure memory distribution on T cell populations before and after initiation of ART. Our longitudinal analysis shows that CCR7+/CD45RO- (T_{naive}) and CCR7+/CD45RO+ (T_{cm}) populations are reconstituted after initiation of ART. Mean T_{naive} populations increase by 2.5 fold and T_{cm} populations increase by 1.8 fold (Figure 3-4A, B; T_{naive}, p=0.0078 and T_{cm}, p=0.0494). However, epitope-specific populations did not undergo significant changes in memory phenotype after initiation of ART.

In a similar fashion, we compared post-ART expression of CD38 and PD-1 to pre-ART expression (Figure 3-5A). Our analysis determined a 3.1 fold reduction in CD38 mean fluorescence intensity (MFI) and a 2.2 fold reduction in PD-1 MFI on CD8+ T cell populations (Figure 3-5B, CD38, p=0.0547; PD-1, p=0.0078). We did not observe statistically significant changes in the expression of these markers on epitope-specific populations. Overall, these results suggest that parent T cell populations in individuals receiving ART undergo phenotypic changes in memory distribution and activation status but that these changes are not directly mirrored in epitope-specific populations.

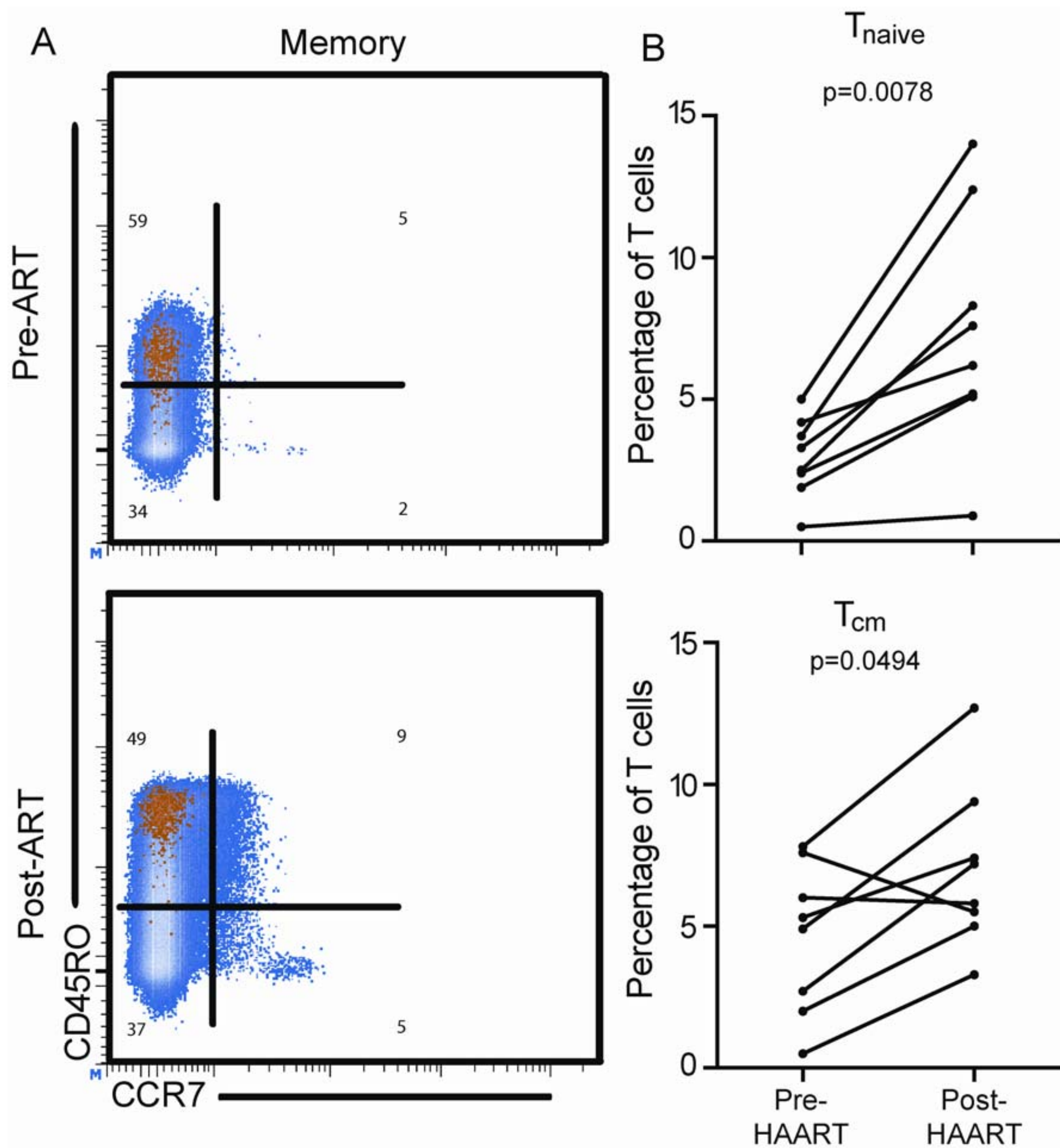


Figure 3-4 – CD8+ T cell memory populations are reconstituted after initiation of ART. CD8+ and epitope-specific T cells were co-labeled with CCR7 and CD45RO to determine memory distribution, A. CD8+ T cell Tnaive and Tcm populations increased after initiation of ART (T_{naive}, p=0.0078 and T_{cm}, p=0.0494).

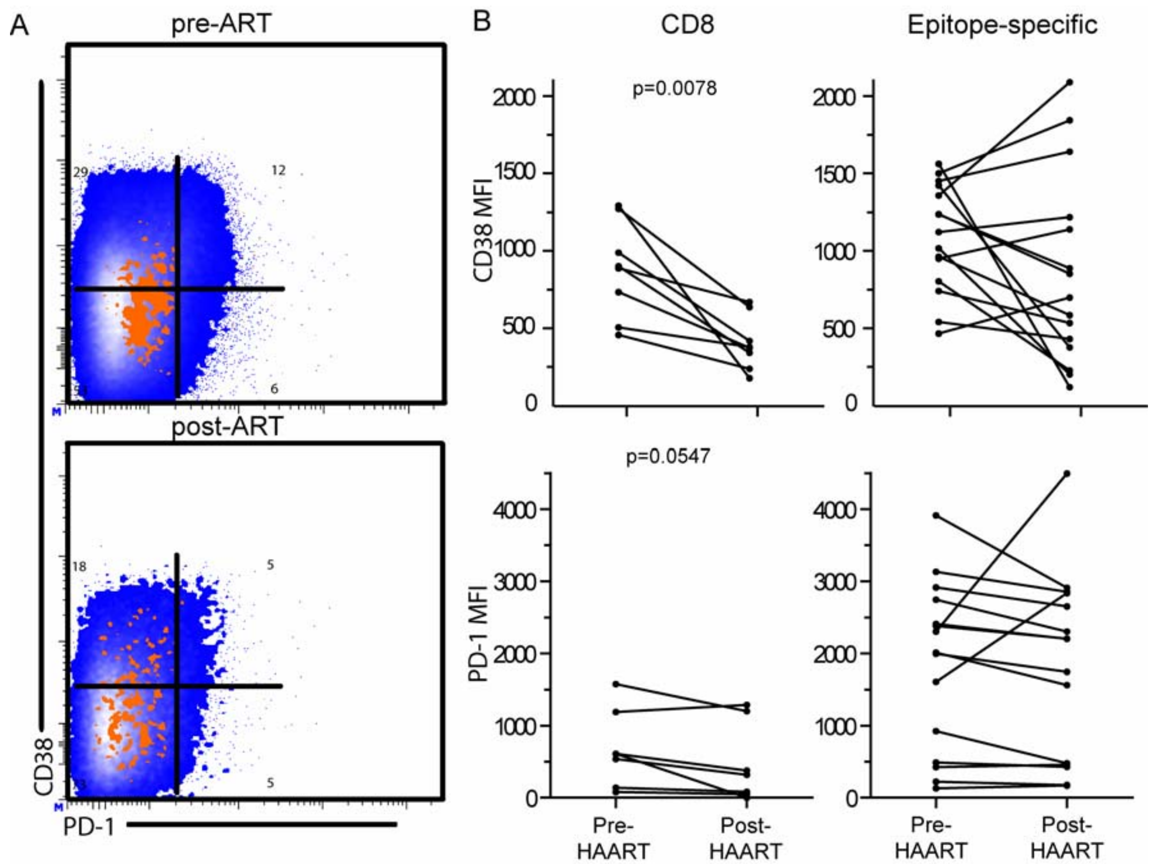


Figure 3-5 – CD8+ T cell activation is reduced after ART while epitope-specific changes are variable. CD8+ and epitope-specific T cells were co-labeled with CD38 and PD-1 at pre-ART and post-ART timepoints, A. CD38 and PD-1 expression on CD8+ T cells is reduced after initiation of ART (p=0.0078), B.

PD-1 expression patterns remain stable after initiation of ART

Expression of PD-1 is closely related to antigen exposure and T cell exhaustion in several infection systems (93, 175, 176), and we have previously shown that PD-1 is more highly expressed on dominant clonotypes within epitope-specific T cell populations (Conrad et. al. 2011). Having demonstrated clonotypic remodeling after initiation of ART, we were interested in determining whether initiation of ART would influence the relationship between clonotypic dominance and PD-1 expression.

Epitope-specific T cell clonotypes were identified using tetramer reagents and anti-TRBV antibodies and PD-1 expression was determined by co-labeling these populations with anti-PD-1 antibodies (Figure 3-6A). As previously reported, we observed a distinct pattern of PD-1 expression on dominant and sub-dominant clonotypes within HIV-epitope-specific T cell populations whereby dominant clonotypes within epitope-specific populations expressed higher levels of PD-1 than did corresponding sub-dominant clonotypes. This pattern of higher PD-1 expression on dominant clonotypes remained statistically significant post-ART (Figure 3-6B). We noted no significant relationships between clonotype dominance and expression of CD38, CD127, or CD57 (data not shown). These results indicate that while overall immune activation may decrease on the parent CD8+T cell population after initiation of ART, the environmental signals which influence PD-1 expression on epitope-specific clonotypes remain intact.

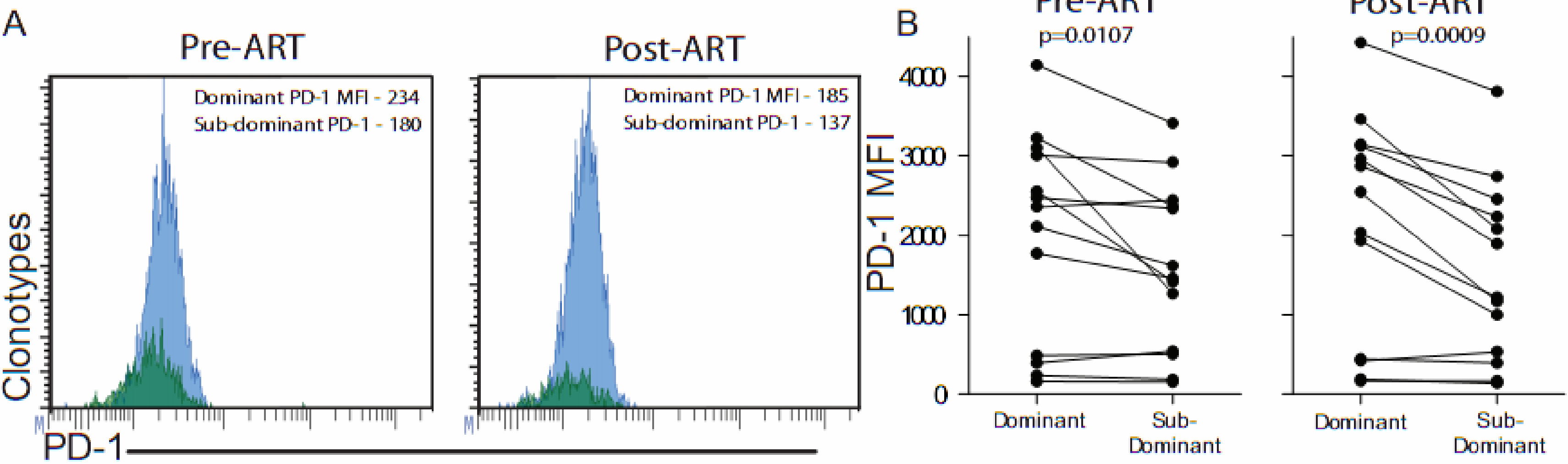


Figure 3-6 – Dominant clonotypes express higher levels of PD-1 than sub-dominant clonotypes before and after ART. Epitope-specific T cell populations were co-labeled with tetramers and anti-TRBV and anti-PD-1 antibodies at pre-ART and post-ART timepoints, A. PD-1 expression (MFI) on epitope-specific dominant and sub-dominant TRBV populations was compared at pre-ART and post-ART timepoints (p=0.0107, preART; p=0.0009, post-ART), B.

Discussion

Clonotypes within the epitope-specific T cell receptor repertoire recognize viral epitopes with an exquisite specificity (115), and antigen exposure almost certainly drives their phenotypic qualities (106) (Conrad, Ramalingam et al. 2011). Our data here demonstrate that after initiation of ART, epitope-specific T cell responses contract but that clonotypic dominance within the TCR repertoire is most often maintained, that the TCR repertoire narrows by number of clonotypes and by amino acid diversity within the repertoire, and finally that despite the clear effects within the immune system, dominant clonotypes remain PD-1^{high} compared to sub-dominant clonotypes. While other studies have noted phenotypic changes on T cell populations (61, 163) and contraction of epitope-specific responses (164, 177), this is the first report to our knowledge which assess diversity with in the TCR repertoire and clonotypic phenotype before and after initiation of ART.

Our studies here analyzed the epitope-specific TCR repertoire in 14 epitopes in 8 HIV+ individuals before and after initiation of ART using a variety of methodologies. The first method involved simply counting the number of clonotypes identified by sequencing. Method two used Simpson's diversity index (117) to assess the number of clonotypes in a response and, importantly, also accounts for the frequency of each clonotype as a part of the whole population. The third method used calculations to determine entropy (H) by assessing the

variability of amino acids substitutions within the TCRβ-CDR3 expressed in a given epitope-specific TCR repertoire. Each method indicated with statistical significance that the clonotypic TCR repertoire narrows after initiation of ART.

We have previously shown that reduced diversity within the epitope-specific T cell receptor (TCR) clonotypic repertoire is associated with epitope escape in chronic HCV infection and data from SIV-infected macaques show a similar effect whereby narrow TCR repertoires drive immune escape Our group has shown the dynamic nature of the HIV epitope-specific T cell receptor repertoire over the course of chronic infection (127). Individual T cell clonotypes differ in their fine specificity for wild-type and variant viral epitopes (115), and this extends to populations of T cell clonotypes to recognize circulating in-vivo epitope variants (127, 129). We recently demonstrated that epitope specific clonotypes have a skewed maturation phenotype (128), and our most recent extension of this work shows that dominant clonotypes express a PD-1^{high}/CD127^{low} surface phenotype and recognize circulating viral variants, whereas sub-dominant clonotypes are better able to recognize and suppress epitope variants [Conrad et al, 2011]. Taken together with the data presented here, we suggest that individual T cell clonotypes may preferentially contribute to recognition of discrete virus populations, and that a diverse T cell receptor repertoire may provide more effective control of viral replication.

We noted that epitope-specific T cell populations decreased in relative frequency after suppression of viral replication under ART. Presumably, and as suggested previously (164, 177), this contraction is a result of reductions in antigen exposure and corresponding decreases in antigen-mediated T cell expansion. Within these lower frequency epitope-specific responses, however, dominance within the clonotypic hierarchy was largely maintained after ART suggesting that the features of the immune response which are responsible for maintaining the clonotypic hierarchy are independent of absolute antigen levels. Various lines of research support the theory that TCR avidity for antigen is the driving factor in establishing and maintaining dominance within the TCR repertoire (133, 159) although clonotypic dominance may also result in deletion from the repertoire(155).

If avidity is a primary biophysical determinant of dominance within the repertoire, it would fit the criteria outlined above of acting as a relative factor independent of absolute or systemic levels of antigen. An alternative theory to explain the unperturbed maintenance of the clonotypic hierarchy might be that with the dramatic reduction in viral antigen exposure, epitope-specific clonotypes become ambivalent to change and are maintained in the status quo. If this were the case, we might expect to observe deletion of the more highly dysfunctional dominant clonotypes, and indeed, in two epitope-specific TCR repertoires, we do observe a dramatic shift in dominance where the frequency of the pre-ART dominant clonotype contracts to much lower levels post-ART and previously sub-dominant

clonotypes increase in frequency. If, in these cases, the dominant clonotype had been generated in response to a viral variant epitope which had not been fixed in the viral population and after suppression of viremia another epitope sequence became predominant, even at low levels, we might expect a distinct lonotype to establish dominance in the TCR repertoire. Future studies should be directed at evaluating epitope sequence variation in the context of ART-mediated viral suppression as well as a further characterization of the mechanisms which influence maintenance of epitope-specific clonotypic hierarchies in the absence of antigenic activation or stimulation.

We assessed cell surface phenotype on various cell populations. Activation and memory profiles on CD8+ T cells clearly change after initiation of ART. CD38 and PD-1 expression are reduced on CD8+ T cells but no pattern of overall reduction was apparent on epitope-specific populations in our study. In line with other studies indicating activation levels are strong prognostic indicators of disease progress and viral load (61, 163), reductions in activation are a positive indicator of clinical improvement in these individuals. We noted consistent redistributions within CD8+ T memory subsets which indicated Tnaive and Tcm repopulations after initiation of ART. Again, these results are in line with previously reported data (136, 178) which indicate that ART initiates a rebound of immune responses which more closely resemble those found in uninfected individuals. The changes we note on CD8+ T cell population support our hypothesis that these individuals underwent significant immune remodeling after initiation of ART.

While the changes we observed in activation and memory phenotype on CD8+ T cells after initiation of ART have been reported previously in other cohorts, the measurement of phenotypic changes after initiation of ART on epitope-specific clonotypes has not been reported. Recent findings from our group established a relationship between dominance in the epitope-specific TCR repertoire and a PD-1^{high}/CD127^{low} phenotype (Conrad, Ramalingam et al. 2011). By assessing epitope-specific clonotypic T cell populations before and after initiation of ART, we noted that dominant clonotypes had a PD-1^{high} phenotype compared to sub-dominant clonotypes which had been established during chronic infection and which endured after initiation of ART. We did not note statistically significant relationships between clonotypic dominance and expression of CD38, CD127, CD57 or memory distribution before or after initiation of ART. The absence of these relationships conforms to results from a previous study in chronic HIV infection in the absence of ART which also found no relationships between dominance and CD57 expression or memory distribution (128).

Our findings in regard to the durable nature of the PD-1high phenotype on dominant clonotypes have implications in a few different scenarios. As mentioned above, if dominance is established as a result of TCR avidity and if PD-1 expression is predicated on antigen sensing as has been suggested by other groups in both the LCMV and SIV models as well as in naturally controlled HIV infection (105, 106), then, as in the maintenance of the TCR repertoire hierarchy

mentioned above, we might expect PD-1 expression patterns to be maintained by relative antigen exposure rather than as a result of absolute levels of circulating antigen. Furthermore, considering our observations that PD-1 and CD38 expression patterns were not universally reduced on epitope-specific populations or on their clonotypic constituents, we hypothesize that these cells continue to receive some level of antigen stimulation even after the dramatic antigen reductions seen after initiation of ART. Indeed, the increased expression of PD-1 and CD38 we noted on a few epitope-specific populations may suggest that these responses actually saw relative increases in antigen exposure after initiation of ART. A more detailed dissection of the mechanisms at work at the epitope and clonotypic level is necessary to more fully understand how epitope-specific responses evolve over time in the context of ART-mediated suppression of viremia.

With their expression of identical TCR, HIV-epitope-specific clonotypes represent the fundamental unit of the T cell response to HIV. A more complete understanding of how epitope-specific clonotypes are maintained over time in the presence and absence of their cognate antigen is of great interest as we seek to elicit effective, durable T cell responses via vaccination. Overall, the results we report here indicate that despite the dramatic decreases in viral load, increases in CD4 count, and decreases in systemic activation, the mechanisms which govern clonotypic expansion and phenotype within epitope-specific T cell responses remain intact at some level. While TCR avidity may be a key component, it is

likely not a singular determinant of clonotypic repertoire diversity or phenotype. Moreover, the mechanisms at work in these responses and the manner in which they interact remains obscure, and a more complete understanding of their nature requires further investigation.

CHAPTER IV

DISCUSSION AND FUTURE OBJECTIVES

Clonotypes are the fundamental units of T cell immunity

A fine line separates immune responses which recognize, suppress, and clear pathogens from immune responses which are either too weak or dysfunctional to clear pathogens or too strong and cause immunopathology. The host immune response must have the capacity to recognize pathogen-derived antigens with exquisite specificity and mount a defense just strong enough to clear infection without causing collateral damage. Numerous lines of empirically- and experimentally-derived knowledge from cellular and genetic studies inform us that T cell responses are critical components of the immune response to suppress viral replication and control HIV disease (30, 63, 64, 68-71, 179). Despite this knowledge, no clear correlates for immune protection have been defined in HIV infection (140, 180). Recent findings, including my own work on epitope-specific clonotypic dominance and surface phenotype, yield insight into the features which regulate immune responses and mediate dysfunction in natural immune responses to chronic viral infections (14, 30, 33, 128, 165).

Much of the work from our research group is founded on the notion that clonotypic diversity within the epitope-specific TCR repertoire contributes to improved recognition of highly variable epitopes within pathogen infections, and

several lines of inquiry support this (1, 120, 121, 127, 129, 181). Despite an unambiguous understanding that epitope-specific responses are usually comprised of multiple independent clonotypes which recognize antigens to differing degrees and with different functional outcomes (115, 127-130, 133), a relatively meager effort has been directed toward defining the phenotypic and functional nature of clonotypes within epitope-specific responses. The clonotypes which constitute heterogeneous epitope-specific T cell populations are the fundamental units of a T cell response to antigen (47). As such, studies of epitope-specific clonotypes can provide insight into the mechanisms which allow for their recognition of antigen, drive their subsequent expansion, and mediate their capacity to produce effector functions and proliferate in response to activation.

Clonotypic dominance and dysfunction

In characterizing *ex vivo* epitope-specific TCR repertoires in chronically infected, ART-naïve individuals, my work defined a marked PD-1^{high}/CD127^{low} phenotype on dominant clonotypes within CD8+ HIV-epitope-specific T cell responses (Conrad et. al, 2011). Functional experiments indicated that dominant clonotypes are primed to respond to circulating viral epitopes, that they are likely impaired for in vitro survival in the absence of antigen signaling, and that sub-dominant clonotypes may preferentially recognize and suppress variant epitopes. These ideas are represented graphically in Figure 4-1.

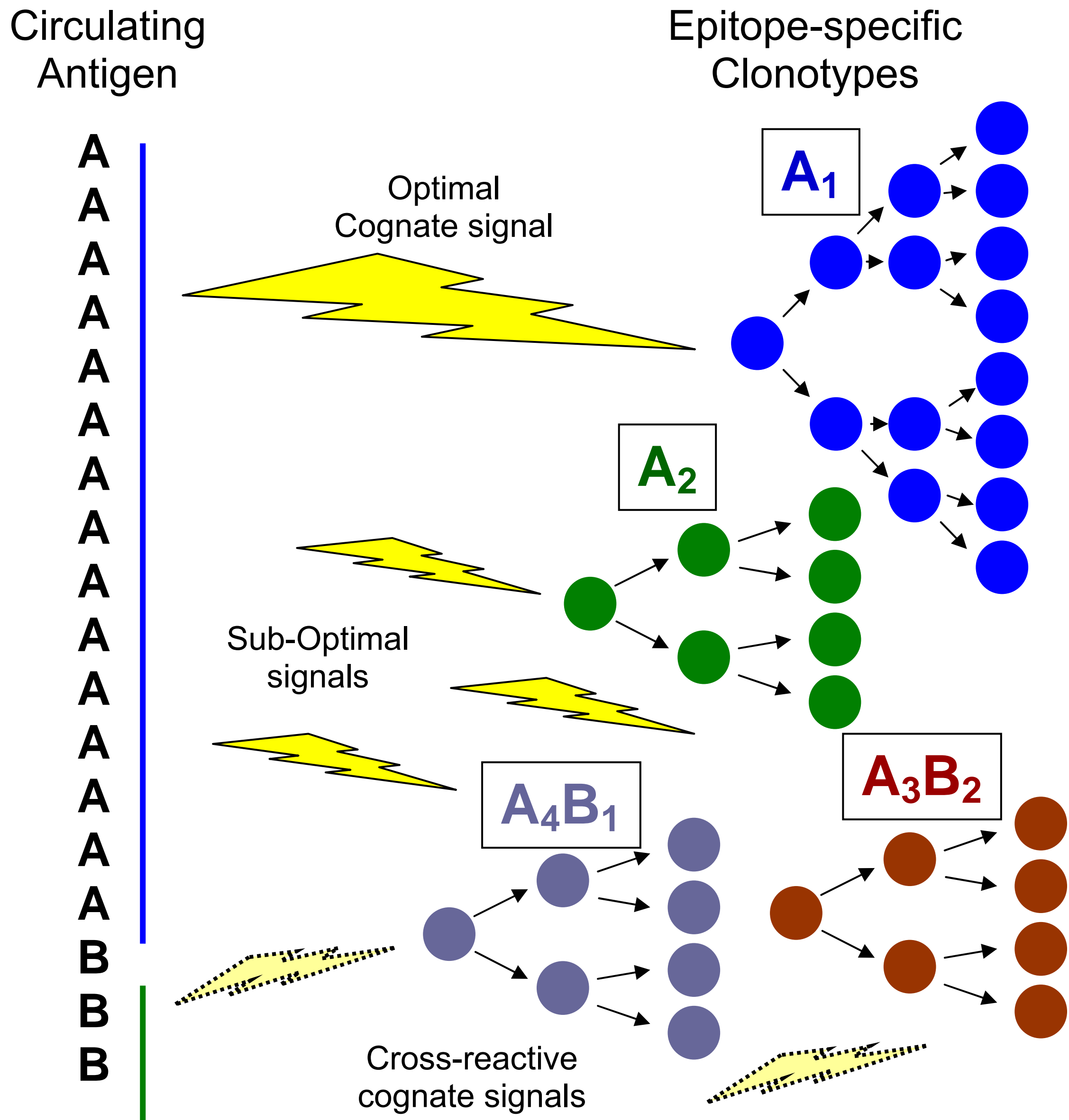


Figure 4-1 – Maintenance of the clonotypic hierarchy. Dominance within the clonotypic repertoire is established in response to circulating viral epitopes (clonotype A_1). Sub-dominant clonotypes respond sub-optimally to circulating sequence (clonotype A_2) and cross-react with low-level viral variant epitopes (clonotypes A_3B_2 and A_4B_2).

Furthermore, my findings indicate that clonotypic PD-1 expression patterns in naturally-controlled, chronic HIV infection are durable in the face of immune recovery, TCR repertoire narrowing, and suppression of viral replication subsequent to the initiation of ART. These research findings illuminate a distinct phenotypic and functional heterogeneity which segregates to independent clonotypes within epitope-specific responses, and it suggests that sensing cognate antigen drives clonotypic phenotype and expansion within the repertoire to a large degree.

My work established that dominance in the HIV-epitope-specific clonotypic hierarchy is associated with a PD-1^{high}/CD127^{low} surface phenotype, one consistent with T cell impairment and immune exhaustion (80). Expression of PD-1, a co-inhibitory surface receptor, on whole T cell populations in HIV+ individuals has been associated with increased viral loads and decreased CD4 T cell count (84, 86) and expression on epitope-specific T cell populations has been linked directly to T cell exhaustion and ongoing antigen exposure in chronic infections in mouse models (106) and natural human infections (147). Moreover, various studies of PD-1 expression determined that interruption of PD-1 signaling improved cytokine production and proliferation in epitope-specific T cell populations (78, 84, 86) and reduced sensitivity of PD-1^{high} populations to apoptosis (85). Reduced expression of CD127, a component of the IL-7 receptor complex, has been implicated as a marker of impaired memory differentiation in HIV infection (112).

My studies involved HLA-B-restricted HIV-epitopes from chronically infected individuals for reasons primarily related to reagent and sample availability. HLA-B-restricted HIV-epitopes are more commonly recognized and exert more selective pressure on viral replication (68), but a few HLA-A alleles restrict highly immunodominant HIV epitopes which are widely recognized in human populations (71). Additionally, limited viral sequence data was generated in order to confirm that circulating viral sequences matched the consensus sequences used experimentally in our tetramer reagents. Finally, selection of viral epitope variants in my studies was based on the most common epitope variants (Los Alamos HIV Database) rather than informed by deep sequencing of autologous viral sequence to identify circulating and low level variants.

In order to extend my initial findings, future research directions must be oriented around the strengths and expertise of our research group in characterizing epitope-specific TCR repertoires which arise in response to viral infection. Two broad lines of inquiry will enable us to better define mechanisms that underlie recognition of viral epitopes, development and maintenance of the TCR repertoire over time, and suppression of viral replication: 1 – Comprehensive investigation of TCR repertoires specific for consensus and variant epitopes as well as for the corresponding in vivo viral epitope sequences in HIV+ individuals over time, preferably beginning at time points prior to establishment of viral set point and chronic infection; 2 – Parallel determination of surface phenotype,

functional capacity, and signaling properties of epitope-specific clonotypes. This future work will require the investigation of additional HLA-A-restricted epitopes from HIV and other common viral infections, a more comprehensive strategy for establishing the presence or absence of viral sequence variants in circulation within an individual, as well as subsequent experiments informed by autologous sequence using viral variants to assess clonotypic function and signaling characteristics.

Viral epitopes and epitope-specific TCR repertoires in HIV+ individuals

Our research group has at its disposal many of the key elements necessary to expand our understanding of TCR repertoire composition and maintenance in diverse, natural human viral infections namely a cohort of chronically infected HIV+ individuals, facilities to perform ex vivo sorting of live T cell populations, and expertise in culture of primary T cells. Several very accessible lines of inquiry are possible based on the recent availability of tetramer reagents which facilitate identification of clonotypes within HLA-A-restricted epitope-specific responses to HIV, CMV, and EBV.

Epitope-specific responses to two other chronic viral infections, cytomegalovirus (CMV) and Epstein Barr virus (EBV), are commonly detectable at relatively high frequencies within individuals in our HIV+ cohort. Research from other groups suggests that clonotypic TCR hierarchies in epitope-specific responses to CMV and EBV are stable over time but vary in differentiation status (182) and that T

cell responses to these herpesviruses may have some degree of cross-reactivity (183). The evolutionary history of HIV infection in humans is significantly shorter than that of CMV or EBV (31, 184), and comparison of T cell responses specific for these viruses in the context of HIV co-infection may yield useful insight into the mechanisms which allow for effective suppression of EBV and CMV replication while replication of HIV remains partially or completely uncontrolled. While some data has been reported using epitope-specific responses to CMV and EBV as convenient internal controls for investigations of HIV-epitope-specific responses (84-86), little is known about the phenotypic or functional nature of constituent clonotypes and their response to changes in corresponding viral antigen exposure. Moreover, a better understanding of the T cell responses to these viral infections in the context of HIV-mediated immunosuppression may provide insight into the dynamics of T cell responses and herpesvirus reactivation in the related field of transplant immunology (185, 186).

Research groups have studied the effects of epitope variation on T cell activation and function (187) and have studied the dynamics of epitope-specific repertoires upon epitope-specific escape in a limited manner (131), but little research has put all the pieces of this puzzle together in a comprehensive manner. Experiments which carefully measure the changing composition of epitope-specific TCR repertoires as they react to a variable antigenic epitope should be undertaken. This will require the occurrence of a few relatively special events - namely epitope-specific responses and clonotypic repertoires amenable to

longitudinal interrogation and viral epitopes which mutate to escape recognition of the TCR (as opposed to mutations which abrogate binding to the HLA molecule). Fortunately, recent developments in massively parallel sequencing technology (188, 189) show some promise in the ability to determine detailed analyses of both TCR repertoires and viral quasispecies. If these technologies are able to be scaled down to the point where small populations of sorted T cells or viral sequences from individuals with low levels of viremia could be sequenced, they may become a necessary component of future studies.

In natural HIV infection, epitope-specific T cells are likely exposed to and activated by their specific cognate antigens as well as by related, but non-cognate viral variants or escape mutants. The complex interplay of constant exposure and activation of epitope-specific clonotypes by related, but distinct antigens has not been well treated in the literature, and this avenue of investigation should be pursued. Indeed, activation as measured by CD38 expression on T cells is a strong, independent predictor of disease progression (61) and is thought to be one of the underlying causes of immunopathogenesis in HIV disease (56, 58). Understanding how epitope variation affects the activation status of epitope-specific T cell populations is necessary in the assessment of T cell responses in the context of ongoing viral replication and immune activation found in chronic HIV infection.

Within our current and growing cohort of HIV+ individuals, a natural initial extension of my work would be further characterization of the phenotype and function of clonotypic constituents within epitope-specific T cell responses to HIV, CMV, and EBV in the same HIV+ individuals. These studies should include longitudinal investigations of TCR repertoires directed against epitopes restricted by HLA-A and HLA-B alleles and should be done in coordination with careful viral sequencing to determine not only corresponding circulating epitope sequences but also low frequency viral epitope escape variants. Several groups have demonstrated that epitope escape can influence surface expression of PD-1 and CD127 (106, 147, 187), but none at the clonotypic level. Van Bockel et. al. have recently published investigations indicating that dominant clonotypes within a single HIV-epitope-specific response persist over time and cross-react with epitope variants suggesting that exposure to circulating and low level variants likely impacts the composition of the clonotypic repertoire (131), findings my work largely agrees with. This group used novel tetramer reagents constructed using a common epitope variant peptide. Their findings indicated significant overlap in the clonotypes found within TCR repertoires which recognize both consensus and variant epitopes. Future experiments should include the use of both consensus and variant tetramer reagents to determine TCR repertoires which correspond to consensus and autologous variant epitopes. I hypothesize that in vivo dominant epitope-specific clonotypes will be directed against the predominant circulating viral variant, perhaps a consensus epitope, and that clonotypes identified as sub-dominant with consensus tetramer may actually be

dominant clonotypes when assessed using tetramer constructed with low-level autologous variant epitopes.

Clonotypic dominance in the TCR repertoire

The mechanisms which govern clonotypic dominance within the TCR repertoire have been scrutinized by a number of different research groups, and TCR avidity for cognate antigen was defined as a prominent determinant in the selection of dominant clones within an epitope-specific response (133, 190-192). However, TCR avidity for antigen seems also to be a double edged sword. For the individual, maintenance of high avidity clonotypes is associated with lower viral set points in chronic infection, but the high avidity clonotypes themselves are the ones more likely to be deleted from a given response (155). Studies preceding establishment of HIV viral set point or simply closer to acute infection may yield interesting insight into the clonotypic dynamics within epitope-specific responses as well as show a more significant relationship between dominance and avidity.

Clonotypes in my studies were defined by expression of a unique TRB-CDR3 or distinct TRBV usage, and statistical analysis of clonotypic PD-1 and CD127 expression patterns shows a relationship between surface phenotype and dominance within the TCR repertoire. These results imply that antigen signaling through unique clonotypic TCR likely influences cell surface phenotype. There was no statistically significant relationship to indicate that TCR avidity drove clonotypic dominance in our cohort, although there was a modest trend in that

direction. There was, however, a positive correlation between TCR avidity and PD-1 expression on clonotypes regardless of hierarchical status in the repertoire indicating that stronger recognition of antigen may be a determinant of PD-1 mediated dysfunction on clonotypes. A similar analysis of dominance and memory phenotypes showed no significant relationship (128). These findings may be reconciled by considering that our cross-sectional study used tetramers constructed only with consensus peptide epitopes and might have assessed epitope-specific TCR repertoires in individuals for which HIV had already undergone escape mutation. In 'escaped' epitopes, the *in vivo* TCR repertoire would be primarily directed against a circulating variant epitope rather than the consensus peptide epitope in the tetramer. Almeida et. al. described T cell antigen sensitivity as a primary determinant of T cell function and suppressive capacity (47). These investigations, which utilized unimpaired clonal T cell populations that recognized the same HLA-B*27-KK10 epitope but with different functional sensitivities, highlight the necessity to investigate T cell responses at their most fundamental level, that of the epitope-specific clonotype.

Future efforts to define the relationship between clonotypic antigen sensing may include experiments to define the functional qualities (sensitivity, proliferative, and suppressive capacities) of sister clonotypes within the same epitope-specific response. In order to have enough cells for experimentation, it may even be necessary to sort these clonotypic populations and grow out clones as previously described (47, 115, 130). We have some expertise in labeling small epitope-

specific clonotypes and isolating these cells using FACS. While this is not a trivial task, sorting clonotypes of a given epitope-specific population and culturing these cells to suppress viral replication is feasible and such experiments might even be enhanced with the addition of reagents to inhibit or activate cell surface receptors like PD-1 or CD127 and thereby alter clonotype function. It would be of great interest if the functions of epitope-specific clonotypes could be tuned using exogenous signals such as cytokines or inhibitory signal blockade and might lend support to the notion that the epitope-specific repertoire itself could be manipulated in the context of artificial antigen stimulation or vaccination.

Relieving clonotypic dysfunction through signaling inhibition

Beginning with initial descriptions of CTLA-4 as a negative co-stimulatory molecule (44-46), our understanding of co-stimulatory networks on lymphocytes has developed rapidly in recent years. Other co-stimulatory molecules such as PD-1, BTLA (B and T lymphocyte attenuator), and ICOS (inducible co-stimulator) have been described since that time (42, 48, 98) and act in concert to regulate T cell responses through unique and overlapping functions, especially in the context of continuous antigen exposure and cellular activation as seen in chronic HIV infection. Since the seminal reports of reversible PD-1 mediated T cell impairment in mouse and human infections (78, 84, 86, 193), several other groups have investigated myriad molecules and signaling interactions which regulate T cell responses (95, 97, 154, 194) in chronic HIV infection.

It is clear that in the context of chronic HIV infection, ongoing viral replication, antigen exposure, and persistent immune activation, natural immune mechanisms are rendered dysfunctional and inadequate to the task of suppressing viremia (14, 31). It seems that some large part of the immune dysfunction may be caused by inhibitory mechanisms such as PD-1 (154, 195) which likely evolved to reduce autoimmunity and control runaway cellular activation after acute infection (78, 93, 98). The tantalizing feature of this sort of immune dysfunction, however, is that signaling pathways originating from ligation of surface receptors may be interrupted outside the cell before they are received and thus the dysfunction might be alleviated. With a growing understanding of the intracellular events differentiating signaling pathways like CTLA-4 and PD-1 (194) and downstream transcriptional events (97), it may be possible to inhibit the inhibitory pathways at an intracellular level. Signaling interventions show promise in mouse and non-human primate vaccination systems where immune stimulation by vaccination in concert with interruption of the inhibitory PD-1 signaling pathway gives rise to strong cellular immune responses which show improved efficacy in controlling viral replication (144, 145). Our findings indicate that cell surface receptor phenotypes are related to dominance within the TCR repertoire and TCR avidity which suggests that vaccination protocols which include immunomodulation might influence the clonotypic composition of the repertoire.

In conclusion, my work demonstrates that clonotypic phenotype and function are closely associated to expansion within the TCR repertoire and antigen sensing. These novel findings can inform further, more detailed investigations of epitope-specific responses in chronic controlled viruses as well as investigations to provide insight into the cellular mechanisms involved in maintaining epitope-specific clonotypic repertoires to variable and static viral epitopes. Future studies will seek to better understand the development, maintenance, and functional capacity of the TCR repertoire in the face of chronic viral infection. The immunopathogenesis of HIV poses difficult questions for the field, but as we gain insight into how immune responses develop and are maintained, answers reveal themselves. Ultimately, the insights we provide can inform our approaches for immunomodulatory therapies for HIV and other infections.

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