Characterization of the Mamu-A*01-Restricted CD8-Positive T Lymphocyte Immunodominance Hierarchy in Simian Immunodeficiency Virus-Infected Rhesus Monkeys

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CHARACTERIZATION OF THE MAMU-A*01-RESTRICTED CD8-POSITIVE T LYMPHOCYTE IMMUNODOMINANCE HIERARCHY IN SIMIAN IMMUNODEFICIENCY VIRUS-INFECTED RHESUS MONKEYS

ABSTRACT

CD8^{+} cytotoxic T lymphocytes (CTLs) play a critical role in controlling human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication. The CTL responses that are thought to be the most protective against HIV and SIV are those that are of high frequency, recognize multiple epitopes, and perform multiple antiviral functions. Therefore, current vaccines aim to elicit CTLs possessing these characteristics. However, the phenomenon of immunodominance likely limits the potential of vaccines from generating such CTL responses by restricting the breadth of epitopes recognized by CTLs and the frequency and functionality of these CTL responses. In this dissertation, we explored the relationship between SIV epitope dominance and the functionality of the epitope-specific CTL populations. We also examined factors that contribute to the development of SIV epitope immunodominance hierarchies.

We initially investigated the relationship between SIV epitope dominance and the antiviral functionality of the epitope-specific CTL populations in rhesus monkeys. We performed a gene expression analysis in dominant and subdominant epitope-specific CTLs during the acute phase of SIV infection and observed differential expression of a number of genes during this time. Subsequent *in vitro* functional studies of these epitope-specific CTL populations during the chronic phase of infection confirmed the presence of differences in maturation phenotype

and functional capacity of dominant and subdominant epitope-specific CTLs. These studies demonstrate a relationship between epitope dominance and antiviral functionality of epitope-specific CTLs and suggest that dominant and subdominant epitope-specific CTLs may differ in their protective role against HIV acquisition and replication. This has important implications for vaccine design.

In subsequent studies, we investigated the contribution of the binding of the peptide:MHC (pMHC) complex to the T cell receptor (TCR) in the development of immunodominance hierarchies. Using surface plasmon resonance, we measured the kinetics and the affinity of the interactions between dominant and subdominant epitope pMHC complexes with their respective TCRs. We found that epitope dominance was associated with higher affinities of pMHC:TCR binding. These findings indicate a molecular interaction that may be manipulated in vaccine-induced CTL responses to enhance their frequency and functional capacity.

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ATTRIBUTIONS

I performed the experiments presented in this dissertation, with the exceptions described below.

Chapter 2

Ana Maria Gonzales and Amy Shi Hung conducted the experiments for gene expression analyses including the sorting of cells, RNA extraction, RNA amplification, and RNA hybridization. The Illumina BeachChip was processed by Kevin Shianna and David Goldstein at the Duke Center for Human Genome Variation. Ana and Amy also collected the plasma for viral load quantification. Rachel Lovingood at the Duke Human Vaccine Institute generated the plasma viral load data. Hsun-Hsien Chang at Children's Hospital, Boston analyzed the gene expression data.

Chapter 3

The SPR data shown in this chapter were a result of a collaboration with Dr. S. Munir Alam's laboratory at the Duke Human Vaccine Institute. Evan Cale sorted the cells that were used to generate the data presented in Figure 10.

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INTRODUCTION

HIV AND AIDS

Acquired immunodeficiency syndrome (AIDS) is caused by infection with the human immunodeficiency virus (HIV) [1-5]. Currently, 33 million individuals are infected with this virus and 1.8 million people die from AIDS each year [6]. To combat spread of infection, intense efforts are being made to develop a vaccine that will confer protection from HIV infection. However, successful development of a HIV vaccine will require an improved understanding of the immune response to the virus.

HIV belongs to the lentivirus genus of the retrovirus family. It contains nine reading frames that encode 15 proteins. Three of these reading frames encode polyproteins that are further cleaved into the final protein products: gag encodes the structural proteins matrix, capsid, nucleocapsid, and p6; Env encodes the structural proteins gp120 and gp41; and Pol encodes the enzymes reverse transcriptase, protease, and integrase. The other proteins encoded by the virus are accessory proteins that are required during various stages of the virus lifecycle and include vif, vpr, nef, tat, rev, and vpu [7].

Infection of a cell with HIV results in the permanent integration of the viral DNA into the host's DNA, resulting in a lifelong persistent infection in the host [8, 9]. Entry of HIV into a host cell requires the interaction of the envelope protein gp120 with the host cell-surface proteins CD4 [10, 11] and either CCR5 or CXCR4 [12-14]. CD4 is expressed on cells of the immune system including CD4$^+$ T cells, macrophages, and dendritic cells (DCs) [15-17]. HIV preferentially infects activated memory CD4$^+$ T cells as these cells also express CCR5 and can support viral replication [18-22]. Following infection, the virus spreads with exponential replication from the site of infection, to local lymphoid tissue, to other mucosal sites, and eventually throughout the entire

body [22-25]. Within a week after infection, due to the preferential infection of activated

CCR5^{+}CD4^{+} T cells and the abundance of these cells at mucosal sites, up to 20% of gut-associated CD4^{+} T cells are infected and up to 80% are destroyed [26-28]. Virus replication in the blood peaks around day 21 and then declines [29-31], reaching a steady state two to six months after infection [32, 33]. This decline of virus replication is associated with a rebound in CD4^{+} T cell numbers that remain steady for many months. But the levels of CD4^{+} T cells eventually begin to decline slowly over the following years and this is associated with a decay and dysfunction of many other aspects of the immune system that eventually lead to AIDS [34, 35].

Use of nonhuman primates as an AIDS model. As currently there is no appropriate small animal model of HIV infection, nonhuman primates (NHPs) have become a useful and necessary model for studies of HIV. African NHPs harbor viruses that are closely related to HIV, called simian immunodeficiency viruses (SIVs) [36]. Although SIVs usually do not cause disease in their natural hosts, infection of non-natural NHP hosts such as Asian primates, can result in disease. This was initially observed when captive rhesus monkeys (*Macaca mulatta*) developed AIDS-like symptoms due an infection of SIV from sooty mangabeys (*Cercocebus torquatus*, SIVsm), a species endemically infected with SIV with minimal pathology [37-40].

The infection of rhesus monkeys with strains of SIVsm has become the most frequently used NHP model of HIV. A major advantage of using NHP AIDS models is the ability to exert experimental control over a number of variables that often confound studies in humans such as

viral sequence variability, route of infection, and dose of infection. In addition, there are many similarities between SIV and HIV both genetically and in the diseases that they cause in NHPs and humans, respectively. Humans and NHPs have similar genetic composition of their immune system including human leukocyte antigens (HLA) class I, HLA class II, and T cell receptors (TCRs) [41-44]. SIV and HIV have closely related nucleotide sequences [45, 46]. Additionally, SIV and HIV have similar cell tropisms, both using the CD4 molecule as their receptor and CCR5 as their coreceptor [47-51], resulting in infection and destruction of the CD4$^+$ T cell population. Chronic infection with SIV or HIV results in similar AIDS-like syndrome namely weight loss, immune activation, wasting, and increased susceptibility to opportunistic infections [26, 48, 52].

However, some differences between HIV infection in humans and SIV infection in rhesus monkeys should be considered when extrapolating findings in monkeys to humans. Although, both infections result in an early peak in viral load followed by a decrease and eventual set point, the viral kinetics are somewhat different. In rhesus monkeys, viral load peaks about one week before it does in humans. The viral setpoint also occurs earlier in rhesus monkeys and usually at higher levels. Additionally, the time to progression to AIDS occurs earlier in rhesus monkeys, usually in 0.5 to 3 years versus 8 to 10 years in humans [53]. Finally, rhesus monkeys do not have an HLA-C ortholog and they have greater polymorphisms within their MHCs [54, 55].

Correlates of protection*.* From studies in both HIV-infected humans and SIV-infected monkeys, we have developed an understanding of the factors that are involved in, or serve as surrogate measurements of, protection from infection and disease progression. As HIV and SIV

preferentially infect CD4$^+$ T cells, and CD4$^+$ T cell are essential for multiple aspects of the immune system, there is a strong negative correlation between the number of CD4$^+$ T cells in peripheral blood and disease progression. Indeed, CD4$^+$ T cell count is used clinically as the basis for deciding when to initiate anti-retroviral therapy [56] and a rise in CD4$^+$ T cell count is used as a measurement of effectiveness of anti-retroviral therapy in humans [57, 58].

Most viruses are sensitive to neutralizing antibodies. Therefore, it is not surprising that neutralizing antibodies have also been associated with SIV and HIV control. Depletion of B cells in SIV-infected rhesus monkeys inhibited production of neutralizing antibodies and was associated with an increase in viral load [59]. Passive transfer of neutralizing antibodies to rhesus monkeys prior to challenge prevents infection [60-63]. Additionally, as HIV has been shown to rapidly mutate in the envelope region to become neutralization-resistant, it is clear that neutralizing antibodies are applying immune pressure [64, 65]. Finally, a recent vaccine trail using a recombinant canarypox vector prime and gp120 protein boost (the Thai ALVAC/AIDSVAX trial (RV144)), showed a marginal effect at reducing HIV acquisition. This protective effect was associated with antibodies rather than CD8$^+$ T cell-mediated responses [66].

Many lines of evidence suggest that CD8$^+$ T cells also play a substantial role in control of viral replication. First, there is a temporal association between the decrease in viral load during acute infection and the rise in virus-specific CD8$^+$ T cells [67, 68]. This suggests that CD8$^+$ T cells may be playing a role in the decrease in viral load. Indeed, antibody-mediated CD8$^+$ cell depletion in rhesus monkeys either before or during SIV infection was associated with a substantial rise in viral load during acute infection and more rapid disease progression [69, 70].

Additionally, the frequency of virus-specific CD8^{+} T cells during chronic infection is negatively correlated with viral load [71]. Second, there is a strong correlation between certain major histocompatibility complex (MHC) class I alleles, in both humans and rhesus monkeys, and superior viral control. This suggests that MHC class I presentation of viral epitopes to CD8^{+} T cells likely plays a role in viral control and that certain alleles are more protective than others [72]. Finally, mutations in the viral sequences that result in loss of recognition by the CD8^{+} T cells that leads to disease progression preferentially occur within MHC class I-restricted viral epitopes [73-87]. This suggests that the epitope-specific CD8^{+} T cells are applying immune pressure.

Although most agree that a vaccine capable of generating high titers of broadly neutralizing antibodies would be ideal, creating such a vaccine has been challenging [88-92]. Considering the clear role that CD8^{+} T cells play in controlling HIV and SIV, much effort has been directed at developing a vaccine that would elicit virus-specific CD8^{+} T cells that could control infection and possibly induce sterilizing immunity. Rhesus monkeys receiving vaccines designed to induce virus-specific CD8^{+} T cell responses were often able to control virus more efficiently than unvaccinated controls [93-98]. Based on their success in rhesus monkeys, some have been taken to human clinical trials, but with limited success. The STEP trial used recombinant adenovirus type 5 vectors expressing the HIV proteins gag, pol, and nef. Although this vaccine increased the frequencies of virus-specific CD8^{+} T cell responses, as determined by IFNγ ELISPOT, ultimately it was neither protective against HIV acquisition nor did it decrease viral loads following acquisition [99]. Additionally, although the vaccine used in the RV144 trial showed a marginal effect at reducing HIV acquisition, the CD8^{+} T cell-mediated responses in the

protected individuals, as measured by IFNγ and IL-2 production, were weak [66]. Assuming that CD8^{+} T cells are truly important for HIV protection and control, there are at least three explanations for these disappointing results. First, although CD8^{+} T cells were elicited, perhaps they were not high enough frequency. Second, CD8^{+} T cells elicited may not have possessed the effector functions that were necessary for protection. Third, the vaccine-elicited CD8^{+} T cells did possess the appropriate protective functions, but these functions were not measured and, therefore, were not correlated to protection. These two vaccine trials illustrate the lack of understanding of the role of CD8^{+} T cells in HIV infection. The optimal characteristics of CD8^{+} T cells that convey protection, and how to generate these responses by vaccination, remain unclear and require further investigation.

CD8^{+} T CELLS

T cells are a subset of lymphocytes that can be divided broadly into CD4^{+} T cells and CD8^{+} T cells. Via their TCR, CD4^{+} T cells recognize epitope peptides that are presented by MHC class II molecules on professional antigen-presenting cells (APCs), usually derived from extracellular pathogens [100]. CD4^{+} T cells generally are responsible for producing soluble molecules that direct the immune response to that particular pathogen: for example, promoting antibody production and facilitating the development of protective CD8^{+} T cell memory [101, 102]. On the other hand, the TCR expressed by CD8^{+} T cells recognizes epitopes that are presented by MHC class I molecules, usually derived from intracellular pathogens, such as viruses [16]. As all nucleated cells express MHC class I molecules, presentation of MHC class I-restricted epitopes to CD8^{+} T cells does not require professional APCs [16]. Although CD8^{+} T

cells also can produce soluble molecules, their most influential role during infection is the direct killing of infected cells [103-113].

Effector functions. CD8^{+} T cells can kill target cells by two different mechanisms, both of which result in apoptosis-induced death of the target cell: 1) the induction of signaling through the Fas death receptor (CD95) and 2) the release of perforin and granzymes [114]. The perforin/granzyme-dependent cytotoxic mechanism is more important for the control and clearance of pathogens [115-118], while the Fas-mediated mechanism is more important for the downregulation of the adaptive immune response following clearance of a pathogen and maintenance of peripheral tolerance by removal of autoreactive cells [119].

Effector CD8^{+} T cells express perforin and granzymes and store them in organelles specialized for secretion called granules [120]. The particular pattern of expression of the different granzymes (-A, -B, -H, -K, and -M), together with perforin, depends upon the maturation stage of the CD8^{+} T cell [121-127]. Upon recognition of a target cell, a CD8^{+} T cell releases the contents of these granules directly toward the target cell, resulting in the initiation of apoptosis and death of the target cell [128-131]. Perforin is responsible for damaging the membrane of the target cell and allowing entrance of the granzymes into the cytoplasm [132-136]. Once in the cytoplasm, the granzymes induce apoptosis via multiple mechanisms including a caspase-dependent pathway, a mitochondrial-dependent pathway, and induction of reactivate oxygen radicals [137]. For the killing of infected cells by CD8^{+} T cells, perforin is

absolutely necessary [113, 116, 138-140], while certain granzymes, notably granzyme A and granzyme B, are more important than others [141-148].

One method frequently used to identify cellular responses that may be protective against HIV is the comparison of responses between HIV-infected individuals who control viral replication well, termed long-term non-progressors (LTNP), and individuals who do not control viral replication well, termed progressors. The rationale is that differences identified between these two groups of individuals may reveal factors that that contribute to viral control. Another useful method has been comparing the cellular responses against HIV to those responses against other chronic viruses that are usually better-controlled such as Epstein-Barr Virus (EBV) and cytomegalovirus (CMV). From such studies, we have identified multiple cellular functions that may contribute to control of HIV replication.

In HIV, there is substantial evidence that killing of infected cells by CD8^{+} T cells is important for control of viral replication. Initial studies of cytotoxicity in the context of HIV demonstrated that HIV-specific CD8^{+} T cells were deficient in their cytotoxic capacity [149] and that this was associated with a deficiency in perforin content [150], suggesting lack of cytotoxicity as an explanation for poor viral control. It was later shown that although perforin expression was indeed deficient in many HIV-specific CD8^{+} T cells analyzed *ex vivo* from infected individuals, re-stimulation of cells from HIV-infected individuals resulted in proliferation that was associated with an upregulated production of perforin and that this function was preferentially maintained in LTNP [151]. Later studies discovered that perforin can be upregulated without the requirement for proliferation, providing CD8^{+} T cells with a mechanism to rapidly replenish their cytotoxic capacity without the lag of proliferation [152]. Analysis of

this function of rapid perforin upregulation found that HIV-specific CD8^{+} T cells from LTNPs also exhibited an enhanced capacity to upregulate perforin directly after stimulation compared to CD8^{+} T cells from progressors [153]. Finally, CD8^{+} T cells from LTNP have also been shown to possess *in vitro* suppression of viral replication [154] and killing of target cells [155] that is superior to CD8^{+} T cells from progressors.

Soluble molecules produced by CD8^{+} T cells include: IL-2, IL-4, IL-5, IL-8, IL-10, IL-17, MIP-1β, MIP-1α, RANTES, IFNγ, GM-CSF, and TNFα [156-159]. The most extensively characterized effector molecules produced by CD8^{+} T cells are IL-2, IFNγ, and TNFα. IL-2 is essential for the expansion of CD8^{+} T cells during primary and secondary responses as well as the development of effector functions [160-163]. Therefore, although not technically an antiviral effector molecule, the production of IL-2 by CD8^{+} T cells is frequently used as a measurement of the quality of the CD8^{+} T cell response. IFNγ acts on multiple cell types and has multiple functions during infections, notably, the upregulation of MHC class I and MHC class II epitope processing and presentation machinery and induction of expression of antiviral proteins [164, 165]. TNFα contributes to pathogen clearance possibly by inhibiting viral replication or stimulating the generation of reactive oxygen radicals [166-174].

The role of many of these soluble molecules have been studied in HIV and SIV infection and some have been found to be associated with protection and control of viral replication. IL-2 has been identified as a soluble molecule produced by CD8^{+} T cells that is important for viral control [175-177]. IL-2 production is linked to CD8^{+} T cell proliferation and is, therefore, thought to be important for this process [175, 177], which is preserved in LTNP [151]. IFNγ is another

soluble molecule that has been associated with control of these viruses. The decline of CD4^{+} T cells associated with the progression to AIDS is associated with a decline in IFNγ production by CD8^{+} T cells [178, 179], a function that appears also to be preserved in LTNP [176]. TNFα, although frequently measured, has rarely been identified as being independently associated with protection. Although, a study comparing progressive versus non-progressive disease in SIV-infected rhesus monkeys did find as association of TNFα production by CD8^{+} T cells with control [180]. Finally, the β-chemokines MIP-1β, MIP-1α, and RANTES share a common receptor, CCR5, the same receptor used by many HIV and SIV isolates [12, 181, 182]. The binding of these chemokines to CCR5 can inhibit entry of HIV and SIV into target cells [183] and the production of these chemokines by CD8^{+} T cells during infection has been associated with viral control [184-186].

As an alternative to seeking associations of individual CD8^{+} T cell functions with viral control, many studies have begun to perform analyses that consider multiple CD8^{+} T cell functions. These studies evaluate the ability of a single cell to simultaneously perform multiple functions including IL-2, IFNγ, TNFα, and MIP-1β production in addition to cytotoxicity. In these analyses, cytotoxicity is often measured by cell surface expression of CD107 following antigenic stimulation, a surrogate measurement for degranulation of cytotoxic granules [187]. Such analyses of a cell's ability to perform multiple functions are considered a more global assessment of their "quality". Those cells that can simultaneously perform multiple antiviral functions are termed "polyfunctional" or "multifunctional". It has been shown that the quality of CD8^{+} T cells from progressors is different than those from LTNPs; the CD8^{+} T cells from LTNPs are often more polyfunctional compared to those from progressors. This has been

demonstrated in CD8^{+} T cells from both peripheral blood [188, 189] and from mucosal tissues [190, 191]. Although it is possible that the enhanced protective capacity that polyfunctional cells provide is simply due its more efficient antiviral functionality (a single cell can provide multiple functions), that is probably not the entire reason. Polyfunctional cells also produce more of each effector molecule per cell as compared to monofunctional cells, when measured by the fluorescence intensity of staining of these effector molecules by flow cytometry [192] .

Activation of CD8^{+} T cells. Although capable of recognizing a pathogen-derived epitope, naïve CD8^{+} T cells (those lacking previous exposure to antigen) are unable to confer protection against pathogens due to their lack of effector function and low frequency [193]. Recognition of cognate epitope peptide by the TCR of naïve CD8^{+} T cell provides signals that activate these cells to undergo massive expansion, and to differentiate and acquire effector functions [194-200]. Activation of naïve CD8^{+} T cells occurs in lymphoid tissues [201, 202] and requires three distinct signals. The primary signal, termed signal 1, is the TCR's recognition of a foreign epitope bound by an MHC class I molecule on an APC or infected cell [203, 204]. The second signal, signal 2, results from the interaction of a CD8^{+} T cell costimulatory receptor, usually CD28, with its ligands, such as CD80 and CD86, on the APC [205-207]. The third signal, signal 3, is an inflammatory signal, determined by the binding of soluble inflammatory molecules to their receptors on the naïve CD8^{+} T cell [208-210]. Signal 1 without signal 2 and/or 3 will result in incomplete activation and may render the CD8^{+} T cell refractory to further stimulation [211-214]. In addition, the quality and strength of these different signals shape the frequency, maturation, and function of the resulting CD8^{+} T cells.

Maturation of antigen-experienced CD8^{+} T cell subsets and identification of these subsets.

Naïve epitope-specific CD8^{+} T cells circulate through secondary lymphoid organs, surviving by stimulation through MHC class I and by the binding of the homeostatic cytokine IL-7 to the IL-7 receptor (CD127)[215-219]. The expression of the chemokine receptor CCR7 and the adhesion molecule CD62L allow them to enter secondary lymphoid organs where they can contact cognate foreign epitope presented by MHC class I on APCs [220, 221]. Recognition of cognate epitope by the TCR provides the CD8^{+} T cell with signals that allow it to undergo massive expansion and to mature and acquire effector functions [200]. The expression on the naive CD8^{+} T cells of the costimulatory molecules CD28 and CD27, as well as the phosphatase CD45RA, enhance the stimulatory signal initiated through the TCR [222-226].

The expansion of naïve CD8^{+} T cells is accompanied by a maturation program through which they acquire effector functions that allow them to combat the pathogen[227, 228]. This expansion can often be detected by changes in the expression of various cell cycle-associated molecules such as Ki-67, which is upregulated, and Bcl-2, which is downregulated during this expansion period [228-231]. Activated cells also downregulate the cell surface expression of CD62L and CCR7, allowing them to circulate in peripheral tissues rather than lymphoid tissues. They also downregulate the expression of CD127 and CD45RA and upregulate the expression of a different form of the CD45 phosphatase, CD45R0 [228, 232]. They also express a number of additional surface molecules that are often used to identify activated CD8^{+} T cells including PD-1, HLA-DR, CD38, and CD69 [228, 233, 234].

Following clearance of the pathogen, the majority of the effector cells die by apoptosis [200, 235]. However, a small percentage of these cells survive and give rise to a pool of long-lived memory cells [200, 228]. These memory cells persist in the host, often for the remainder of its lifetime, and provide enhanced protection if re-exposed to the same pathogen [236-241]. The transition from effector to memory cell requires a number of transcriptional changes that occur nonsimultaneously and gradually over time [228, 242, 243]. Although there appears to be a continuum over which these changes occur, and therefore enormous heterogeneity of phenotype and function within the memory pool [222], memory cells have been broadly categorized as either 1) central memory or 2) effector memory [244, 245]. These memory subsets have been distinguished by a number of unique functional characteristics as well as expression of particular cell surface molecules.

Based on studies that measured the telomere lengths of different human CD8^{+} T cell subsets, which is a measurement of replicative history and proliferative potential [246-250], the central memory subset is considered the least-mature memory subset [251-253]. Cells found in this subset express many of the molecules found on naïve CD8^{+} T cells, such as CCR7, CD62L, CD28, CD27, and CD127 [217, 225, 245, 251, 254-259]. They similarly lack some of the effector functions such as the production of the cytolytic effector molecules perforin and granzyme [121, 127, 225, 251]. However, they have a number of functions that naïve cells do not have, notably, the capacity to expand more rapidly and produce IL-2 upon re-exposure to cognate epitope-MHC I [243, 260-262]. They also have some production of soluble effector molecules such as IFNγ and TNFα [263, 264]. Unlike naïve cells that express CD45RA, central memory cells continue to express CD45RO [121].

Some studies also have described a memory stage that appears to be an intermediate

between central and effector memory, which was designated transitional memory. This stage is

usually described as having many of the phenotypic markers of central memory cells, except

the lack of expression of CCR7 [225]. In this transitional memory stage, cells start to upregulate

the expression of the granzymes A and K; however they usually do not yet express perforin, and

therefore do not have immediate cytotoxic capacity [125-127].

Cells found in the effector memory stage do not express CCR7 or CD62L but have

upregulated expression of the cytokine receptor CXCR3, allowing them to enter peripheral

tissues, rather than secondary lymphoid tissues [265]. Some show loss of expression of CD127

[266-268]. They do not express the costimulatory receptor CD28 and some also show loss of the

costimulatory receptor CD27 [225]. Loss of these costimulatory molecules contributes to the

reduced expansion capacity that these cells exhibit following antigen re-exposure [269, 270]. In

contrast to cells in the central memory subset, cells in the effector memory subset display an

increased capacity to produce many effector molecules, including IFNγ and TNFα [263]. They

maintain expression of granzyme A, lose some expression of granzyme K, and now show

expression of both granzyme B and low amounts of perforin [121, 125-127]. Therefore, they

possess some immediate cytotoxicity [225, 271, 272].

Maturation of memory cells into fully mature effector cells is often defined by the

expression of a large amount of perforin and granzymes A and B, and therefore increased

cytotoxic capacity [121, 125-127]. Interestingly, effector cells have usually lost expression of

granzyme K [125, 126]. They retain some level of IFNγ and TNFα production, but have lost the

ability to produce IL-2 [273]. Many have further lost the expression of CD27, although some retain it [251, 263]. They have also lost the expression of CD127 [175, 266-268]. This subset often has increased expression of the surface molecule CD57 and the inhibitory receptor KLRG-1, both of which have been associated with the decreased proliferative capacity that is highly associated with this subset, often referred to as immunological senescence [121, 274-280]. Many effector CD8^{+} T cells have also re-gained the expression of the phosphatase CD45RA and this is often considered a marker of terminal differentiation [252, 281].

Although the particular pathway though which memory cells are generated from the effector cells that survive following primary infection remains disputed, it is clear that over time, following resolution of a pathogen, central and effector memory populations are formed, both of which possess the potential to give rise to functional effector cells if re-exposed to antigen [282]. Although some studies have concluded that central and effector memory cells represent distinct differentiation fates [283], others have found that given enough time in the absence of antigen following clearance of the pathogen, effector memory cells will give rise to the less mature central memory cells [243, 284-287]. However, this is not the case in the setting of unresolvable, chronic infections, such as EBV, CMV, HCV, and HIV. Under these conditions, CD8^{+} T cells are chronically exposed to antigen and the development of memory does not occur as it does in the setting of acute infection [260, 288, 289]. Phenotypically, epitope-specific CD8^{+} T cells in chronic infections can be found in the stereotypical central, transitional, and effector memory subsets; however, instead of the eventual conversion into the less mature subsets, these cells seem to be fixed at these different stages of memory development. Additionally, the distribution of cells among the different memory subsets differs among different pathogens

[254, 260, 284, 287, 288]. For example, HCV-, EBV-, HIV-, and CMV-specific CD8^{+} T cells are predominately central memory, central/transitional memory, effector memory, and terminally differentiated effector memory, respectively [260, 288, 290]. Although it is not completely clear why the distribution of memory subsets differs for cells specific for different persistent pathogens, it is thought that the amount and quality of antigenic and inflammatory stimulation likely plays a role [232, 287, 291, 292].

As mentioned above, the majority of HIV- and SIV-specific CD8^{+} T cells have been characterized as early effector memory (usually defined as CCR7^{-}CD28^{-}CD27^{+}CD45RA^{-}) [126, 254, 260, 293]. More recently, HIV- and SIV-specific CD8^{+} T cells have been shown also to have reduced expression of CD127 [294]. The role of CD127 on the CD8^{+} T cells during HIV infection remains unclear. Although its expression pattern may simply be a reflection of maturation stage, it has also been suggested that the reduced expression of C1D27 may contribute to immune dysfunction [295, 296].

The lack of HIV- and SIV-specific CD8^{+} T cells displaying more mature memory phenotypes has led some to speculate that this may represent a defect in these cells and that this defect may contribute to the inability of these cells to control viral replication. In support of the concept that more mature effector memory cells are more protective, it has been found that LTNPS contain a greater frequency of HIV-specific CD8^{+} T cells with a more mature phenotype [154, 297-300]. In addition, a recent study has tested CMV as a SIV vaccine vector, which had been demonstrated to induce CD8^{+} T cells displaying more mature phenotypes than previously-used vaccine vectors [301]. This study found that those monkeys that received an

SIV vaccination delivered by the CMV vector were provided greater protection than those monkeys receiving the vaccine by an adenovirus vector [301, 302].

The chronic antigenic stimulation that CD8^{+} T cells experience during unresolvable infections is often associated with the development of functional impairment of these cells, termed exhaustion. Exhaustion was first described in the setting of chronic lymphocytic choriomeningitis virus (LCMV) infection in mice where epitope-specific CD8^{+} T cells were found to have a reduced cytotoxic and IFNγ-producing capacity [303, 304]. Since then, the phenotypic and functional defects of exhausted cells have been more extensively defined and exhausted CD8^{+} T cells have been found in the setting on multiple chronic infections including HIV, SIV, HBV, and HCV [305-314]. The functional defects are gradually acquired; there is a progressive loss of proliferative potential accompanied by a loss of functions starting with the production of IL-2, followed by cytotoxicity, TNFα, and finally IFNγ [315, 316].

As the phenotypic and functional characteristics of exhausted cells have become further elucidated, it has been suggested that exhaustion may be a distinct pathway of differentiation [316]. This is supported by the unique transcriptional profile that is associated with these cells [317, 318]. However, others argue that exhaustion is associated with differentiation in the setting of chronic exposure to antigen [319-322]. Regardless of their origin, exhausted cells have been found to display a unique combination of cell surface molecules, many of which are also used to define different effector and memory subsets, thus complicating their identification. Similar to terminally differentiated cells, exhausted cells have downregulated the expression of CD62L, CD127, CCR7, and CD28 [323-326]. They also express low levels of the

anti-apoptotic molecule bcl-2 [326]. They upregulate the expression of a number of inhibitory molecules including KLRG1, PD-1, 2B4, CD160, CTLA-4, LAG-3, and TIM-3 [318, 327, 328]. They also show increased expression of CD69, CD95, and CD44 [318, 323, 326].

Exhausted epitope-specific CD8^{+} T cells in the setting of HIV and SIV infection have now been described in multiple studies and their presence is thought to play a role in the inability of CD8^{+} T cells to ultimately control HIV and SIV replication. Exhausted HIV and SIV epitope-specific CD8^{+} T cells express multiple inhibitory molecules including PD-1, TIM-3, CD160, 2B4 [311, 312, 329-331]. These molecules are more highly expressed on CD8^{+} T cells from individuals with progressive disease compared to LTNP [332]. The increased expression of these molecules on HIV- and SIV-specific CD8^{+} T cells is associated with functional defects including decreased proliferation and cytokine production following re-stimulation [307, 311, 312, 329, 332] that can be reversed following blockade of PD-1 either *in vitro* [307, 311, 312, 332, 333] or *in vivo* [314].

Transcriptional regulation of CD8^{+} T cell maturation. The transition of naïve cells into effector and memory cells, as well as the development of exhaustion, is accompanied by global changes in their transcriptional program. In order to understand the process of CD8^{+} T cell maturation, and also to better identify cells at specific stages of maturation, much work has been done to identify the factors that control these transcriptional changes. A number of major transcriptional regulators have been identified and they include Eomesodermin (Eomes), T-bet, Blimp-1, and Bcl-6.

T-bet and Eomes are major regulators of CD8^{+} T cell maturation. Both T-bet and Eomes contribute to the production of the effector molecules IFNγ, perforin, and granzyme B [334-336]. Combined deficiencies in both T-bet and Eomes leads to a lack of memory CD8^{+} T cells, suggesting that they have overlapping roles in memory formation and/or maintenance [337, 338]. However, T-bet expression is preferentially upregulated in shorter-lived effector cells, suggesting that high expression levels favor effector formation rather than memory formation. However, low levels of T-bet expression have been shown to permit memory formation [334, 339]. On the other hand, Eomes expression is preferentially increased in memory cells, suggesting that it favors the generation and maintenance of memory [339, 340]. Consistent with their opposing roles in CD8^{+} T cell maturation, IL-12 inversely regulates the expression of these transcription factors; IL-12 induces the expression of T-bet while inhibiting the expression of Eomes [337, 340].

Bcl-6 is a transcriptional repressor that promotes the generation of memory CD8^{+} T cells, particularly central memory [341, 342]. Consistent with this, Bcl-6 has been found to promote proliferation, which is a function associated with central memory cells, and inhibit expression of granzyme B, which is associated with effector cells [341, 343]. A homologue of Bcl-6, Bcl-6b, also plays a role in the proliferative capacity of memory cells [344].

Bcl-6 antagonizes, and is antagonized by, another transcription factor, Blimp-1 [345]. Consistent with this, Blimp-1 has roles that are opposite to that of Bcl-6; Blimp-1 promotes apoptosis and the production of granzyme and perforin while it inhibits proliferation and the production of IL-2 [345-348]. It is more highly expressed in effector CD8^{+} T cells, relative to

memory cells, suggesting that is favors terminal differentiation [345-347, 349]. Consistent with this, Blimp-1 expression is associated with increased T-bet expression and decreased Eomes [345]. Interestingly, very high levels of Blimp-1 are found in exhausted cells and are associated with increased levels of multiple inhibitory receptors [350]. On the other hand, T-bet does not appear to favor exhaustion; rather, it promotes sustained T cell responses during chronic infections and represses the expression of the inhibitory receptor PD-1 [351].

The aforementioned transcription regulators have major roles in CD8^{+} T cell maturation; however, there are other transcriptional regulators that have also been described to have roles in maturation, although their roles are not as well-defined. Id2 is a transcription factor that promotes survival of CD8^{+} T cells during the expansion phase and generation of memory, particularly effector memory [352]. XBP-1 is a transcription factor downstream of Blimp-1. It has also been found to be preferentially expressed in terminally differentiated CD8^{+} T cells and is required for the formation of this subset [353]. Bmi1 is a transcriptional repressor that enhances the proliferative and cytotoxic responses of effectors during expansion. It is also preferentially expressed in long-lived memory precursors, suggesting that it may be involved in subsequent memory formation [354]. Gfi-1 represses the expression of CD127 in effector cells while GABPα promotes its expression [355, 356]. RBP-J and CREB1 are transcription factors that contribute to the expression of granzyme B via Notch2 [357]. Tcf-1 and Lef-1 are transcription factors that favor formation of a subset of memory CD8^{+} T cells [358]. STAT4 plays a role in IFNγ production [359]. Runx3 induces Eomes expression and contributes to granzyme B, perforin, and IFNγ production. Notch1 also regulates Eomes expression as well as granzyme B and perforin expression [360]. STAT5 controls the expression of a number of genes, notably

granzyme B [361]. MBD2 is a transcriptional repressor that plays a role in inhibiting granzyme production and enhancing proliferative capacity in the memory population [362].

With regard to the transcriptional regulation of the state of exhaustion, transcription factors NFAT2 and BATF appear to play a role. Exhausted cells show increased levels of NFAT2 [318]. In these cells, the translocation of NFAT2 to the nucleus is impaired and is associated with their reduced cytokine production and cytotoxicity [155, 363]. Additionally, PD-1 ligation on exhausted cells results in increased expression of the transcription factor BATF, which contributes to the defect in proliferation and cytokine production in these cells [317].

peptide:MHC:TCR interactions in determining CD8+ T cell responses . Many studies have found that the "dose" of antigen that a CD8+ T cell is exposed to affects the subsequent frequency and/or functionality of the cell. "Dose" generally refers to how much antigen the cell is exposed to; dose is determined by both the amount of antigen available at a given time and by the duration that the antigen is available.

Antigen duration is generally determined by the duration of infection with a pathogen. This is, of course, affected by the effectiveness of the immune response in pathogen clearance. Studies of antigen duration have found that prolonged antigen duration during primary infection usually results in higher frequencies of epitope-specific CD8+ T cells during the primary phase [364-367] and/or memory phase [368]. These increased frequencies have been shown to be at least in part determined by prolonged antigen exposure promoting survival [365, 366]. Antigen duration has, in some cases, also been shown to affect the composition of memory

subsets [368], possibly by determining the rate of central and effector memory formation following pathogen clearance [286].

The amount of antigen available at a given time is determined in part by the amount of protein produced by the pathogen that is available for epitope processing and also by the efficiency of multiple steps of epitope processing. This ultimately determines the density of peptide:MHC (pMHC) complexes that are displayed on the cell surface and therefore available for CD8^{+} T cell recognition. pMHC density has been shown to influence frequency of epitope-specific CD8^{+} T cells in the primary response with greater doses resulting in higher frequencies [369].

With respect to the role of the epitope processing on pMHC density, the factors that affect this include the efficiency of release of the epitope from the protein [370-372], the efficiency of the entry of the epitope into the endoplasmic reticulum (ER), and the loading of the epitope onto an MHC class I molecule [373-377]. The affinity of the epitope peptide for the MHC class I molecule also plays a major role determining pMHC density. Low peptide:MHC affinities reduce formation of peptide:MHC complexes in the ER, reducing the amount of pMHC complexes transported to the cell surface, thus reducing density [378, 379]. Once on the cell surface, the peptide:MHC affinity and complex stability will also contribute to pMHC density by affecting how long a pMHC complex will be displayed on the cell [380-383]. This is turn determines if the threshold is reached for CD8^{+} T cell activation [378, 384-388]. When the pMHC cell surface density is very low, TCR binding may produce a signal in the CD8^{+} T cells; however, this signal may be suboptimal and may induce anergy [389]. Alternatively, some low

densities may be sufficient to elicit functional CD8^{+} T cell responses, although, these responses have lower frequencies during the effector or memory phases compared to those elicited at relatively higher densities of pMHC [369, 390, 391]. Most studies of pMHC density have generally found only an effect on proliferation, although a few studies have demonstrated that increased density also enhanced *in vivo* function [384].

Multiple aspects of the interaction of a TCR with a pMHC complex also influence the frequency and function of the responding CD8^{+} T cells. Much work has been done to identify epitope peptides that bind the same TCR but that their binding results in different quantities or qualities of CD8^{+} T cell functions. These epitope peptides, often referred to as altered peptide ligands (APLs), and the TCRs that recognize them, have provided useful systems to analyze the role of pMHC:TCR interactions on T cell activation and function [392-396]. Studies of the role of APL binding to their TCRs were initially limited to TCRs expressed on cell lines. These studies established that the potency of a pMHC for T cell activation was associated with alterations in TCR signal transduction [396, 397] that were associated with differences in the subsequent proliferation, cytotoxicity, and cytokine-producing abilities of the T cell [388, 393, 398-409]. Although some studies have suggested that the potency of APLs is determined by differences in thermodynamics, in structure, or in conformation [410-415], in most cases, the functional differences elicited by these APLs have, been attributed to differences in the strength of the binding between a pMHC complex and a TCR [409, 416-418].

The particular parameter of the pMHC:TCR binding that is most important for determining strength and quality of CD8^{+} T cell stimulation remains a matter of debate. Some

studies suggested that the affinity was the most important parameter in predicting CD8^{+} T cell

activation [388, 399, 400]. Subsequent studies found that although affinity was associated with

ligand potency, it was the dissociation component that was more important, with slower

dissociation rates being more stimulatory because it allowed the TCR to interact with the pMHC

long enough to transmit the intracellular signals required for activation [403-409]. The

subsequent finding that extremely slow dissociation rates were inhibitory [401] led to the

model in which it was proposed that there was an optimal dissociation rate for activation;

dissociation rates that were too fast did not allow complete TCR signaling, but those that were

too slow did not allow the pMHC to engage multiple TCRs [419]. More recently, the apparent

discrepancy between the role of affinity versus dissociation rate has been somewhat resolved.

Studies in both CD4^{+} T cells and CD8^{+} T cells demonstrated that both affinity and dissociation

rate determine ligand potency, but each under different conditions. For pMHC with faster

association rates from the TCR, affinity is the better predictor of T cell activation, while those

with slower association rates, the dissociation rate is a better correlate [420-422].

CD8^{+} T cell immunodominance hierarchies. Among the hundreds to thousands of MHC class I-

restricted epitopes that are encoded in viral proteins, only a very small fraction of them end up

eliciting CD8^{+} T cell responses [423-426]. This phenomenon has been termed

immunodominance [427]. Among those epitopes that elicit responses, not all of them have the

same capacity to elicit a high frequency of CD8^{+} T cells. Instead, there is usually a few epitopes

that elicit a relatively high frequency of epitope-specific CD8^{+} T cell responses, while the

remaining epitopes elicit CD8^{+} T cells at substantially lower frequencies [426, 428, 429]. The

epitopes can be ranked based on the frequencies of the CD8^{+} T cells that they elicit and this

rank has been termed the immunodominance hierarchy [427]. The epitopes that elicit very

high-frequencies of CD8^{+} T cells are termed dominant epitopes; while those that elicit relatively

lower-frequencies of cells are termed subdominant epitopes [427].

Immunodominance hierarchies are best studied in a genetically homogenous

population, such as inbred mice. Infection of a group of mice with the same genetic

background, particularly the same MHC haplotype, results in all the mice generating epitope-

specific CD8^{+} T cell responses to the same few epitopes and the relative frequencies of those

cells within each mouse being identical [427]. That is, the immunodominance hierarchy is

identical in each mouse. This reproducibility of the immunodominance hierarchy has been

interpreted as being an intrinsic property of an epitope [430, 431]; although, numerous host

factors, pathogen-associated factors, and endogenously-administered reagents can modify it

[432-434]. Countless studies have tried to identify the factors that influence immunodominance

hierarchies and numerous factors have been found. Generally, these factors influence either

the density of pMHC complexes on the surface of an APC or the ability of a CD8^{+} T cell to

respond to these pMHC complexes.

Almost all of the immunodominance of a peptide, i.e. whether it elicits a CD8^{+} T cell

response or not, is determined by one or more steps in the peptide processing and

presentation pathway [427, 435, 436]. This includes the kinetics of expression of the protein

from which the peptide is derived [437], the intracellular abundance of this protein [427], the

efficiency of peptide release from the protein by the proteasome [371, 377, 438-440], entrance into the ER via TAP [377, 441], and interaction with ER proteins such as ERAAP and tapasin [377, 442]. But the most frequently-correlated factor is the affinity of the peptide for the MHC class I molecule [378, 443-448]. As discussed previously, each of these factors ultimately determine if the epitope will be presented on the surface of the APC and, if so, if it will be presented at a sufficient density and for a sufficient period of time to be immunogenic.

The factors that determine the immunodominance hierarchy, i.e. the relative frequencies of the different epitope-specific CD8^{+} T cells, once the epitopes are on the cell surface at sufficient densities are sometimes different than those that simply determine if a peptide will be recognized or not. All of the aforementioned steps in antigen processing and presentation play a role in establishing immunodominance hierarchies since they regulate the density and duration of antigen presentation that, as discussed earlier, impacts that signal strength provided to a CD8^{+} T cell and therefore expansion capacity. Additionally, there are some situations where the presence of the dominant epitope actively inhibits the CD8^{+} T cell response to the subdominant epitope, a phenomenon termed immunodomination [435, 449, 450]. This is sometimes simply a result of the dominant epitope more efficiently competing for peptide processing machinery and therefore being more highly represented on the APC surface, thus being more immunogenic [451]. In other cases of immunodomination, the presence of the dominant epitope-specific CD8^{+} T cells suppress the expansion of the subdominant epitope-specific CD8^{+} T cells [452-455]. Other factors that have been associated with the frequency of epitope-specific CD8^{+} T cells in an immunodominance hierarchy include the number of circulating naive epitope-specific CD8^{+} T cells, thymic selection, direct- versus cross-

presentation, and the TCR α and β chain usage [434, 456-461]. Many of these latter-mentioned

factors affect immunodominance hierarchies by determining the number of naïve epitope-

specific CD8^{+} T cells available to respond rather than ability of an epitope-specific CD8^{+} T cell to

expand following stimulation.

One major understudied aspect of immunodominance is the potential functional

differences that may be associated with dominant and subdominant epitope-specific CD8^{+} T

cells. Indeed, if the dominance of an epitope is determined, for example, by its greater

representation on the surface of an APC or the preferential selection of TCRαβ pairs that are

more sensitive to being triggered, then one might expect that the stronger stimulation that the

CD8^{+} T cell receives by this epitope may also impart that population with functional differences.

This issue has been addressed to some extent, but mostly in mouse models, and mostly in

models of acute infection. A study of LCMV epitope-specific CD8^{+} T cells by Rodriguez et al

found that subdominant epitope-specific CD8^{+} T cells developed less cytotoxic capacity,

although greater cytokine-producing capacity, than dominant epitope-specific CD8^{+} T cells

during acute infection [462]. However, the mechanism determining these differences was not

determined. A caveat to this study was that these functional studies were measured after DNA

immunization followed by LCMV infection; thus they were looking at secondary responses

rather than primary responses. Another study in the murine LCMV model found a positive

correlation between the frequency of LCMV epitope-specific CD8^{+} T cells and the rapidity of

IFNγ secretion during primary infection, suggesting that more rapid secretion of IFNγ may

confer an expansion advantage to dominant epitope-specific CD8^{+} T cells [463]. Another study

by Baron and colleagues investigated the functional differences between a dominant and

cryptic epitope (an epitope that does not generate a response unless the dominant epitope is absent). In this case, responses directed towards the cryptic and dominant epitope could be elicited simultaneously if they were presented on different APCs. In so doing, they found that the cryptic epitope-specific CD8^{+} T cells displayed an altered maturation development as measured by both gene and protein expression. Specifically, the cryptic epitope-specific CD8^{+} T cells showed relatively lower levels of expression of CD127 and granzyme A and increased levels of KLRG1 and CD62L during the primary response [464].

Although there have been studies of CD8^{+} T cell immunodominance hierarchies in HIV and SIV, they remain scarce, leaving the determinants of immunodominance hierarchies mostly undefined. Those studies that do exist often focus on associations between the patterns of immunodominance hierarchies and viral control [465-469]. A few studies have addressed the factors that influence immunodominance hierarchies. These studies have found that many of the antigen processing steps that influence immunodominance in other infections also play a role in establishing HIV epitope immunodominance hierarchies including proteasomal release, affinity for TAP, modifications by ERAAP, and affinity for MHC [377, 440]. Other factors have been found that are specific to HIV. For example, a study by Liu *et al* found that epitopes that were more frequently found in viral sequences that were conserved within a population were more likely to be subdominant [470]. Comparisons of functional differences between HIV dominant and subdominant epitope-specific CD8^{+} T cells are rare. There has been one study of the functionality HLA-B27-restricted HIV-specific CD8^{+} T cell immunodominance hierarchy. In this study, they found that higher-frequency epitope-specific CD8^{+} T cells were associated with

superior *in vitro* viral suppression; although, subsequent functional studies could not identify a particular function that may have determined this suppression capacity [471].

CURRENT INVESTIGATION

Elicitation of high frequencies of protective CD8+ T cells is an objective of current HIV vaccine development. However, neither the nature of the CD8+ T cell required for protection nor the nature of the stimulation required to elicit such protective CD8+ T cells is known. Current evidence suggests that the particular phenotype, and therefore function, of epitope-specific CD8+ T cells likely plays a role in protective capacity. Rational vaccine design will require further knowledge of how to manipulate CD8+ T cells to achieve the desired phenotype and function.

Manipulation of immunodominance hierarchies will likely be required for optimal generation of protective CD8+ T cells by vaccines. However, the factors that determine the establishment of CD8+ T cell immunodominance hierarchies in HIV-infected individuals remain unknown. Additionally, the relationship between epitope dominance and functionality of the epitope-specific CD8+ T cell is not clear. Differences in antiviral functionality between dominant and subdominant epitope-specific CD8+ T cells may indicate that certain CD8+ T cell specificities should be targeted by vaccines. The studies presented in this dissertation aimed to shed light on these issues. In Chapter 2 of this dissertation, we explored the relationship between the functionality and the frequency of epitope-specific CD8+ T cells found the Mamu-A*01-restricted immunodominance hierarchy in SIV-infected rhesus monkeys. In chapter 3, we

explored the relationship between the pMHC:TCR binding interactions and the frequency of

epitope-specific CD8$^+$ T cells found in SIV-infected rhesus monkeys.

PHENOTYPE AND FUNCTION OF DOMINANT AND SUBDOMINANT EPITOPE-SPECIFIC CD8$^+$ T CELLS IN SIV-INFECTED RHESUS MONKEYS

INTRODUCTION

CD8^{+} T cells play an important role in controlling HIV and SIV replication in infected individuals and SIV-infected monkeys [67-87, 472]. Additionally, studies of individuals who have been repeatedly exposed to HIV but have remained uninfected suggest that the CD8^{+} T cell response in some individuals may act to prevent HIV infection [473-478]. Based on these observations, the vaccine field remains interested in methods to enhance immunogen-elicited HIV-specific CD8^{+} T cell responses that can provide protection from acquisition of infection or can enhance control of viral replication. Consensus in the field is that the most protective HIV-specific CD8^{+} T cells in infected individuals are those that exhibit the capacity to simultaneously perform multiple antiviral functions and maintain proliferative capacity in the setting of chronic antigen exposure [188, 189, 479, 480]. Therefore, current vaccine strategies aim at generating such polyfunctional HIV epitope-specific CD8^{+} T cells.

Very little is known about the nature of the antigen stimulation that is required to generate epitope-specific CD8^{+} T cells with polyfunctional capacities. It is possible that MHC class I-restricted dominant and subdominant epitopes may elicit qualitatively or quantitatively different functional responses by their respective epitope-specific CD8^{+} T cells, and this may influence the preferences for which epitopes to include in vaccine constructs. However, the relationship between the immunodominance of an epitope and the functionality of the epitope-specific CD8^{+} T cells remains to be characterized fully. A few studies have explored this relationship between epitope dominance and epitope-specific CD8^{+} T cell function, and some have found evidence that functional differences do indeed exist between dominant and subdominant epitope-specific CD8^{+} T cells [188, 462-464]. However, many of these studies have

been conducted in mice using models of acute infection, and these findings may not be applicable to cellular responses in the setting of chronic viral infection in humans.

Identifying HIV-infected individuals during the acute phase of infection is extremely difficult due to the variability and non-specificity of symptoms and the limitations of current diagnostic screening [481]. Therefore, studies of the human immune response against HIV during the acute phase of infection are currently limited. As a result, the evolution of the anti-HIV immune response that occurs during the first few weeks following infection is poorly understood. Additionally, many confounding variables including date of infection, route of infection, heterogeneity of infecting virus, and heterogeneity of the host's genetic composition further limit the ability to conduct HIV infection studies in an experimentally controlled setting [33, 481]. Therefore, the SIV-infected rhesus monkey model has proven invaluable for the study of antiviral immune responses due to the ability to control many of these potentially confounding factors and access to samples during the known acute phase of infection.

Many studies in SIV-infected rhesus monkeys have focused on the relative contribution to viral control of different epitope-specific CD8^{+} T cell responses and the MHC class I alleles by which they are restricted [482-485]. One of these MHC class I alleles is Mamu-A*01. The CD8^{+} T cell epitopes restricted by this allele have been extensively characterized and the relative frequencies of the epitope-specific CD8^{+} T cells in infection studies have been found to be highly reproducible [450, 482, 486-490]. Moreover, the epitope immunodominance hierarchy in Mamu-A*01-positive rhesus monkeys is particularly stable due to the rare occurrence of epitope sequence evolution that occurs only during late infection [85, 466, 491]. Therefore, the

SIV-infected *Mamu-A*01*[+] rhesus monkey model is a useful model in which to study immunodominance hierarchies.

The following study was conducted to determine if qualitative differences exist between dominant and subdominant epitope-specific CD8^{+} T cells in this well-characterized, highly reproducible, and stable model. We conducted a longitudinal gene expression analysis during acute SIV infection to screen for functional differences between dominant and subdominant Mamu-A*01-restricted epitope-specific CD8^{+} T cells. Using this approach, we were able to evaluate the gene expression patterns in these SIV epitope-specific CD8^{+} T cells as early as seven days following SIV inoculation. We observed multiple differences in gene expression during the acute phase of infection between the dominant and subdominant epitope-specific CD8^{+} T cells that suggested phenotypic and functional differences between these epitope-specific cells. Using *in vitro* functional assays, we were able to confirm these differences and that they were maintained through the chronic phase of infection. The present data demonstrate that there is indeed a relationship between CD8^{+} T cell epitope dominance and the functionality of the responding epitope-specific CD8^{+} T cells. These findings add to our understanding of the basic immunology of immunodominance hierarchies and also may inform vaccine design.

The dominant p11C- and the subdominant p54AS-specific CD8$^+$ T cells of SIVmac251-infected rhesus monkeys express different gene transcripts. We used the SIV-infected rhesus monkey as a model to explore whether functional differences exist between dominant and subdominant epitope-specific CD8$^+$ T cells during acute viral infection. We infected six *Mamu-A*01$^+$* rhesus monkeys with SIVmac251. The Mamu-A*01-restricted dominant SIV Gag p11C- and subdominant SIV Env p54AS-specific CD8$^+$ T cells were characterized and sorted to ≥ 95% purity weekly during the first ten weeks post-inoculation. Consistent with previous reports of the kinetics of expansion of these two epitope-specific CD8$^+$ T cells [450], the differences in frequency between these two epitope-specific CD8$^+$ T cells were apparent as early as 14 days following infection and were maintained throughout the duration of infection (Fig. 1B). Interestingly, on day 7, while none of the monkeys contained detectable p11C tetramer$^+$ CD8$^+$ T cells, all of the monkeys contained detectable p54AS tetramer$^+$ CD8$^+$ T cells. The frequencies of the p54AS$^+$ CD8$^+$ T cells on day 7 were generally very low (<0.05%); however, two monkeys, 112-06 and 133-06, showed substantial responses of 2.3% and 11.9% p54AS tetramer$^+$ CD8$^+$ T cells, respectively on day 7 (Fig 1A). Finally, the plasma viral loads peaked around day 14 at approximately 7 logs of viral RNA/mL and reached setpoint around day 28 at approximately 5.5 logs of viral RNA/mL (Fig. 1C).

To identify differences in gene expression patterns between the dominant p11C- and subdominant p54AS-specific CD8$^+$ T cells, we measured whole genome RNA expression in these epitope-specific CD8$^+$ T cells at each of the weekly timepoints post infection. We also isolated

Figure 1- Frequencies of p11C- and p54AS-specific CD8$^+$ T cells and plasma viral loads during primary infections of *Mamu-A*01*$^+$ rhesus monkeys
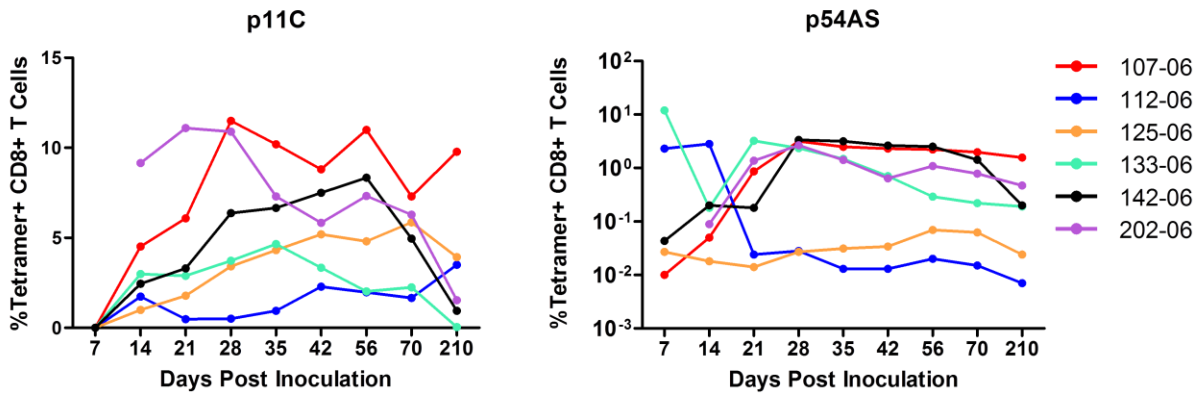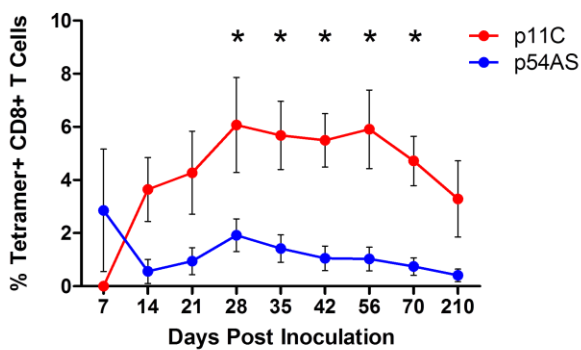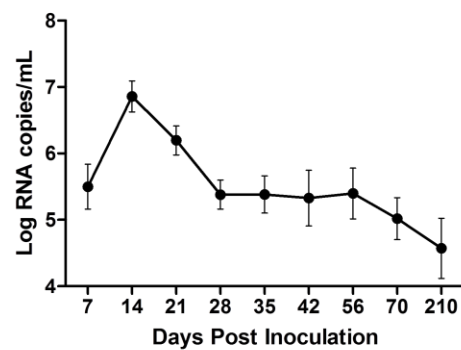
A) Frequencies of the p11C- and p54AS-specific CD8$^+$ T cells for each monkey. Left, p11C tetramer$^+$ CD8$^+$ T cells shown on a linear scale. Right, p54AS tetramer$^+$ CD8$^+$ T cells shown on a Log$_{10}$ scale. B) Mean frequencies of the p11C- and p54AS-specific CD8$^+$ T cells. Error bars indicate ± SEM. C) Plasma SIV RNA levels in the peripheral blood. Error bars indicate mean ± SEM.* indicates p ≤ 0.05 using Wilcoxon signed rank test.

total naïve CD8$^+$ T cells (CD95$^-$CD28$^+$) before inoculation to establish the baseline expression of each transcript. Gene transcripts were detected using an Illumina HT-12 human BeachChip. Previous work in our laboratory had compared detection of rhesus monkey gene transcripts between Illumina's human BeachChips and Affymetrix's rhesus monkey GeneChips and found that they detected similar genes. In addition, the reproducibility of detection of transcripts was substantially higher using the Illumina human BeadChip than the Affymetrix rhesus GeneChip. Finally, the Illumina human BeadChip required less starting RNA, needing only 50 ng according to manufacturer specifications while the affymetrix GeneChip required microgram levels of starting RNA. This was important since we would be collecting small numbers of cells and therefore isolating small amounts of RNA. Therefore, for the reasons of superior reproducibility, less required starting RNA, and adequate homology, the Illumina Human BeachChip was chosen as the gene expression platform for these studies [492].

To characterize functional differences that may exist between these two epitope-specific CD8$^+$ T cells based on their RNA expression profiles, we generated lists of genes that are known to be associated with specific CD8$^+$ T cell functions (Table 1). These genes were grouped into the following categories: maturation, cytotoxicity, cell cycle and apoptosis, and cytokines and chemokines. For each of these genes, we identified those whose expression was significantly different between the two epitope-specific CD8$^+$ T cells on at least one timepoint and whose median fold difference of expression (p11C/p54AS) was greater than 1.5 or less than -1.5. The expression patterns over time of the genes that met these two criteria are shown in Figures 2, 3, and 4 for all of the timepoints that were evaluated. For each timecourse graph (Fig. 2A, 3A, and 4A), we also included the expression of the gene in the naïve CD8$^+$ T cells

Table 1- Genes analyzed for differential expression

MATURATION	CYTOKINES AND CHEMOKINES	CELL CYCLE AND APOPTOSIS				
B3GAT1		AIFM1	CASP8	DLG1	MAP2K1	RHEBL1
BCL6		AIFM2	CASP9	DLG3	MAP2K2	RHOA
BMI1	CCL18	AKT1	CCNA1	DLG4	MAP3K5	RING1
CCR7	CCL20	ANAPC1	CCNA2	E2F1	MAP3K7	RPS6KB1
CD27	CCL3	ANAPC10	CCNB1	E2F2	MAPK1	RPS6KB2
CD28	CCL4	ANAPC11	CCNB2	E2F3	MAPK14	RRAS
CXCR3	CCL5	ANAPC13	CCNC	E2F4	MAPK7	RRAS2
EOMES	CCL7	ANAPC16	CCND1	E2F5	MAX	SCRIB
GFI1	CSF2	ANAPC2	CCND2	E2F6	MCL1	SFN
ID2	CX3CL1	ANAPC4	CCND3	E2F7	MDM2	SKP2
IL2RA	CXCL10	ANAPC5	CCNDBP1	ECT2	MDM4	SKP2
IL7R	CXCL11	ANAPC7	CCNE1	EGR1	MEF2A	SMAD1
ITGAL	CXCL5	APAF1	CCNE2	EIF4EBP1	MEF2B	SMAD2
KLF2	CXCL9	APC	CCNF	ENDOG	MEF2C	SMAD3
KLRG1	FASLG	ARAF	CCNG2	EP300	MEF2D	SMAD4
MBD2	IFNA1	ARHGAP19	CCNH	FADD	MGA	SMAD5
PRDM1	IFNB1	ATM	CDC16	FAS	MKI67	SMAD6
SELL	IFNG	ATR	CDC20	FASLG	MNAT1	SMAD7
SPN	IFNW1	AURKB	CDC23	FBX05	MXD1	SMAD9
TBX21	IL10	BAD	CDC25A	FLT3	MXD3	SMC2
XBP1	IL12A	BAK1	CDC25B	FOSL1	MYB	SMC4
	IL13	BARD1	CDC25C	FOSL2	MYC	SNRPE
	IL16	BAX	CDC27	FOXO1	NAIP	SOD2
	IL17A	BBC3	CDC2L2	FOXO1	NCAPD2	SP1
	IL17D	BCL2	CDC42	FOXO3	NCAPG	STK11
	IL17F	BCL2A1	CDC42EP4	FOXO3	NCAPH	SUV39H1
	IL18	BCL2L1	CDCA8	FOXO4	NFKB1	TACC3
	IL1A	BCL2L10	CDK1	FOXO4	NFKB2	TCL1A
CYTOTOXICITY	IL2	BCL2L11	CDK10	FZR1	NOL3	TERT
AP3B1	IL22	BCL2L14	CDK11A	GABPA	NRAS	TFDP1
C1ORF9	IL26	BCL2L2	CDK11B	GADD45A	NUSAP1	TFDP2
CSPG5	IL29	BCL3	CDK13	HDAC1	PCNA	THAP5
CTSC	IL3	BCL6	CDK16	HDAC2	PEA-15	TNFR1
EBAG9	IL4	BID	CDK2	HDAC3	PKMYT1	TNFRSF10A
GNLY	IL5	BIK	CDK3	HRAS	PLK1	TNFRSF10B
GZMA	IL6	BIN1	CDK4	HRK	PMAIP1	TNFRSF21
GZMB	IL8	BIRC2	CDK5	HTRA2	PPARA	TNFRSF25
GZMH	IRF4	BIRC3	CDK6	HUS1	PPKACA	TNFSF10
GZMK	LTA	BIRC5	CDK7	ID1	PPKACB	TP53
IQGAP1	LTB	BIRC6	CDKN1A	ID2	PRKAR1A	TP53AIP1
JAKMIP1	MIF	BIRC7	CDKN1B	ID3	PRKAR1B	TP73
LAMP2	OSM	BIRC8	CDKN1C	ID3	PRKAR2A	TRADD
LYST	SPP1	BLK	CDKN2A	ID4	PRKAR2B	TRAF2
M6PR	TGFB1	BMF	CDKN2B	IGF1	PRKDC	TRIM25
PRF1	TNF	BNIP3	CDKN2C	IGFBP3	PRPS1	TSC1
RAB27A	TNFSF11	BNIP3L	CDKN2D	IRF1	RAC1	TSC2
SMPD1	TNFSF14	BNIP3L	CEBPA	IRF2	RAD1	TSC22D1
SNAP23	XCL1	BOK	CEBPB	IRF3	RAF1	TTK
SNAP25	XCL2	BRAF	CENPF	IRF4	RALA	UBE2C
SRGN		BRCA1	CFLAR	IRF8	RALB	UHRF1
STOML2		BRCA2	CFLAR	IRF9	RALGDS	WEE1
STX11		BTRC	CHEK1	JUN	RB1	XIAP
STXBP2		CABIN1	CHEK2	JUNB	RBL1	YWHAB
SYTL1		CASP1	CKS1B	KAT2B	RBL2	YWHAE
SYTL2		CASP10	CKS2	KIT	REL	YWHAG
TFF1		CASP12	CPEB1	KLF10	RELA	YWHAH
TRIP10		CASP2	CUL1	KLF4	RELB	YWHAQ
UNC13D		CASP3	DAP	KLF5	RGL1	YWHAZ
VAMP7		CASP6	DAXX	KLF6	RGL2	YY1
VAMP8		CASP7	DIABLO	KRAS	RHEB	ZMIZ1

that was measured on day 0. Unfortunately, there were four timepoints for which we were unable to obtain expression data for both epitope-specific CD8^{+} T cells in all six animals, limiting the study's power to detect statistically significant difference in gene expression. We generated expression data from both epitope-specific CD8^{+} T cells from all six animals on days 14, 21, 56 and 70. On day 7, data were obtained only for one animal; on days 35 and 42, for four animals; and on day 28, for five animals.

Of the genes involved in CD8^{+} T cell maturation, we identified CCR7, SELL (CD62L), and IL7R (CD127) as genes that were differentially expressed between these cells based on the abovementioned criteria (Fig. 2). CCR7 was more highly expressed on the p54AS-specific CD8^{+} T cells with a significant fold difference of -2.4 on day 14. SELL was more highly expressed on the p54AS-specific CD8^{+} T cells with significant fold differences of -1.8 on day 14, -2.0 day 21, and -1.7 on day 56. IL7R was more highly expressed on the p54AS-specific CD8^{+} T cells with significant fold differences of -1.8 on day 14, -1.5 on day 56, and -1.9- on day 70.

Each of these genes are known to be expressed very highly on naïve CD8^{+} T cells and their expression decreases upon activation to effector CD8^{+} T cells. These genes are sometimes re-expressed during the memory phase [217, 251, 255-259]. Consistent with the expected expression profiles of these genes, all were expressed at relatively high levels in the naïve CD8^{+} T cels, and their expression decreased within one week following infection, a period when the epitope-specific CD8^{+} T cells are known have already entered the CD8^{+} T cell effector and memory phases [288, 293]. The trend of expression of these genes associated with maturation was similar for both the p11C- and the p54AS-specific CD8^{+} T cells; however, all of these genes

Figure 2- Differences in expression of maturation-association genes between dominant p11C-and subdominant p54AS-specific CD8$^+$ T cells
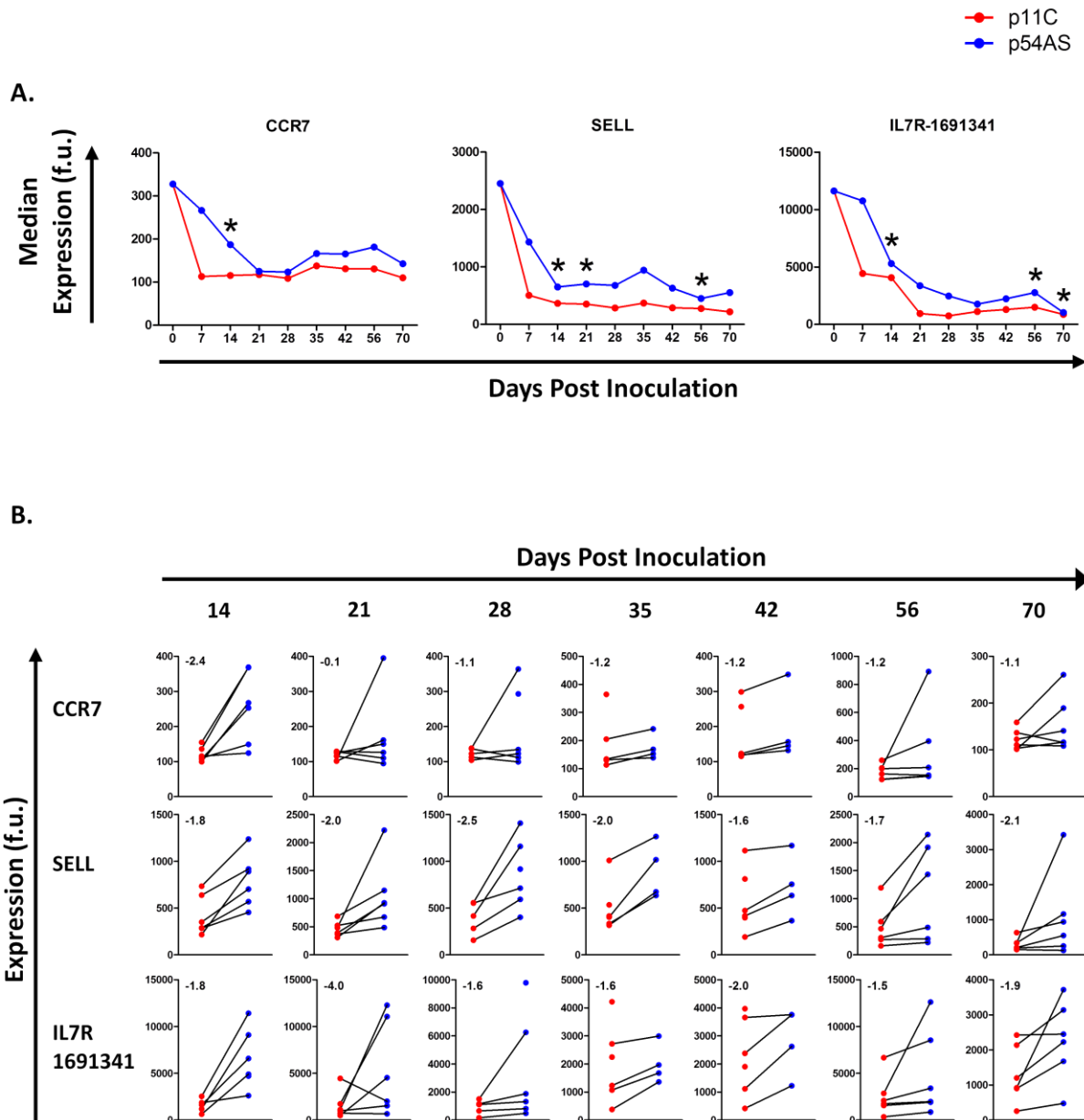
Figure 2 (continued). The RNA expression of genes involved in maturation in the dominant p11C- and subdominant p54AS-specific CD8^{+} T cells was examined using the Illumina HT-12 Human BeadChip. Fold differences in gene expression were calculated by dividing the expression in the p11C-specific CD8^{+} T cells by the expression in the p54AS-specific CD8^{+} T cells (p11C/p54AS). When p54AS expression values were larger than p11C values, the negative reciprocal was calculated. Fold changes of ≥+1.5 or ≤ -1.5 with a $p \leq 0.05$, using a Wilcoxon signed rank test were considered significant. The normalized raw expression values, in fluorescence units (f.u.), of genes that were significant on at least one time point are shown (CCR7, SELL, and IL7R). For those genes that had more than one probe on the BeadChip, the probe IDs are included in the gene name. A) The median raw expression value for each epitope-specific CD8^{+} T cell for each timepoint. The expression values of these genes from total naïve CD8^{+} T cells measured on day 0 are also shown. Red, p11C. Blue, p54AS. *indicates the days on which the fold change met the significance criteria. B) Each individual expression value for each epitope-specific CD8^{+} T cell for each timepoint, including the values for which its matching pair is missing. Data from only one monkey were obtained on day 7, therefore this timepoint was omitted. Fold change values are indicated in upper left corner.

were found to be expressed at relatively higher levels in the subdominant p54AS-specific CD8$^+$ T cells compared to the dominant p11C-specific CD8$^+$ T cells. Although not significant, this trend in differential expression remained consistent for both SELL and IL7R for all timepoints examined, showing high fold differences (Fig. 2B) and p values ranging from 0.1 to 0.06. The difference in CCR7 expression was lost after day 14. The finding that CCR7, SELL, and IL7R were all being expressed at relatively higher levels in the subdominant p54AS-specific CD8$^+$ T cells than in the p11C-specific CD8$^+$ T cells suggested that the p54AS-specific were at a less mature stage than the p11C-specific CD8$^+$ T cells.
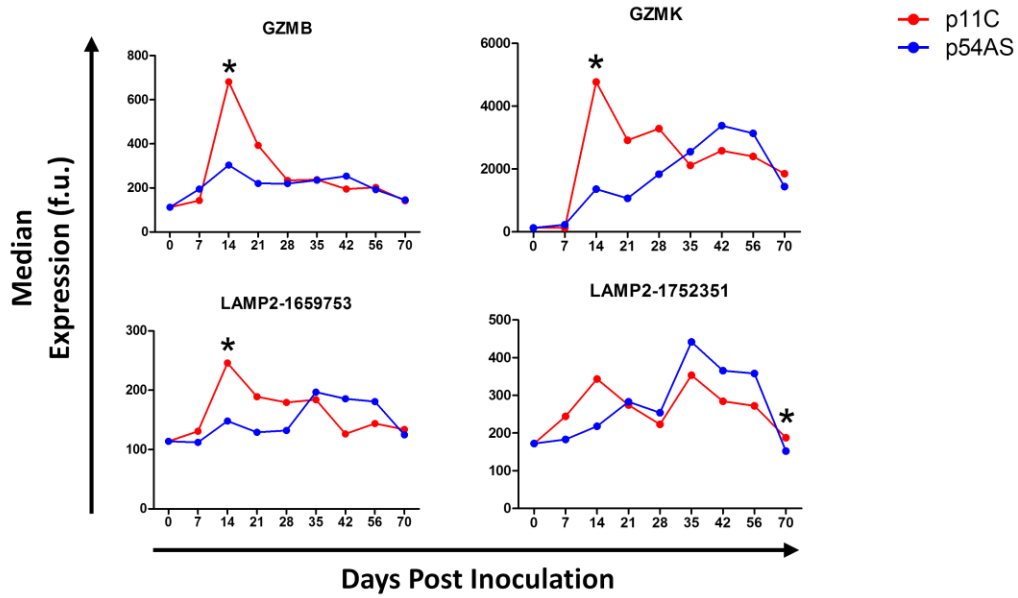
Of the genes involved in cytotoxicity, three met the pre-determined criteria for being differentially expressed following SIV infection: GZMB (granzyme B), GZMK (granzyme K), and LAMP2 (CD107b) (Fig. 3). Granzyme B and granzyme K are well-defined cytotoxic effector molecules of CD8$^+$ T cells [493]. The expression of these each of these cytolytic molecules, and their gene transcripts, are known to be absent in naïve CD8$^+$ T cells and to increase as these cells transition to central memory, effector memory, and finally to effector subsets [251]. Consistent with the expected expression profiles of these genes, they were expressed at very low levels in the naïve CD8$^+$ T cells, and expression increased following infection. LAMP2 encodes a protein that is associated with lytic granules, although its function is not completely clear [494]. We observed that the expression of LAMP2 increased following infection in SIV epitope-specific CD8$^+$ T cells, remained high during acute infection, and returned close to baseline at onset of chronic infection. All of the cytotoxicity-associated genes that were found to be differentially expressed were expressed more highly in the dominant p11C-specific CD8$^+$ T cells compared to the subdominant p54AS-specific CD8$^+$ T cells. GZMB was more highly

expressed in the p11C-specific CD8^{+} T cells with a significant fold difference of 1.5 on day 14. GZMK was more highly expressed in the p11C-specific CD8^{+} T cells with a significant fold difference of 1.9 on day 14. LAMP2 (1659753) was more highly expressed in the p11C-specific CD8^{+} T cells with a significant fold difference of 1.5 on day 14. LAMP2 (1752351) was more highly expressed in the p11C-specific CD8^{+} T cells with a significant fold difference of 1.8 on day 70. These differences were particularly pronounced during peak viral load on day 14 (Fig 1C). The trend in differential expression was still apparent on day 21, but it was lost on the subsequent timepoints (Fig 3A and 3B). The kinetics of expression for all of these genes also appeared to differ between the p11C- and p54AS-specific CD8^{+} T cells; expression in the p11C-specific CD8^{+} T cells peaked on day 14, while expression in the p54AS-specific CD8^{+} T cells peaked between day 35 and 56 following infection. The two LAMP2 probes detected similar trends of expression; although, probe 1659753 detected significant differential expression on day 14, while probe 1752351 detected significant differential expression on day 70. Finally, it is notable that both GZMA (granzyme A) and PRF1 (perforin), which are also key molecules involved in CD8^{+} T cell cytotoxicity, followed a similar trend of differential expression as GZMB and GZMK, both being more highly expressed in the p11C-specific CD8^{+} T cells (Fig. 3C). Expression of GZMA peaked on day 14 with a 1.4 fold difference in expression (p = 0.09). Expression of PRF1 peaked on day 21 with a 1.4 fold difference in expression (p = 0.03).

The higher expression of GZMB, GZMK, and LAMP2 in the dominant p11C-specific CD8^{+} T cells suggested that these cells may have greater cytotoxic capacity than the p54AS-specific CD8^{+} T cells. It has been demonstrated that as CD8^{+} T cells mature, their expression of cytolytic effector molecules increases [121, 127, 251, 263]. Therefore, the increased expression of these

Figure 3- Differences in expression of cytotoxicity-associated genes between dominant p11C- and subdominant p54AS-specific CD8⁺ T cells
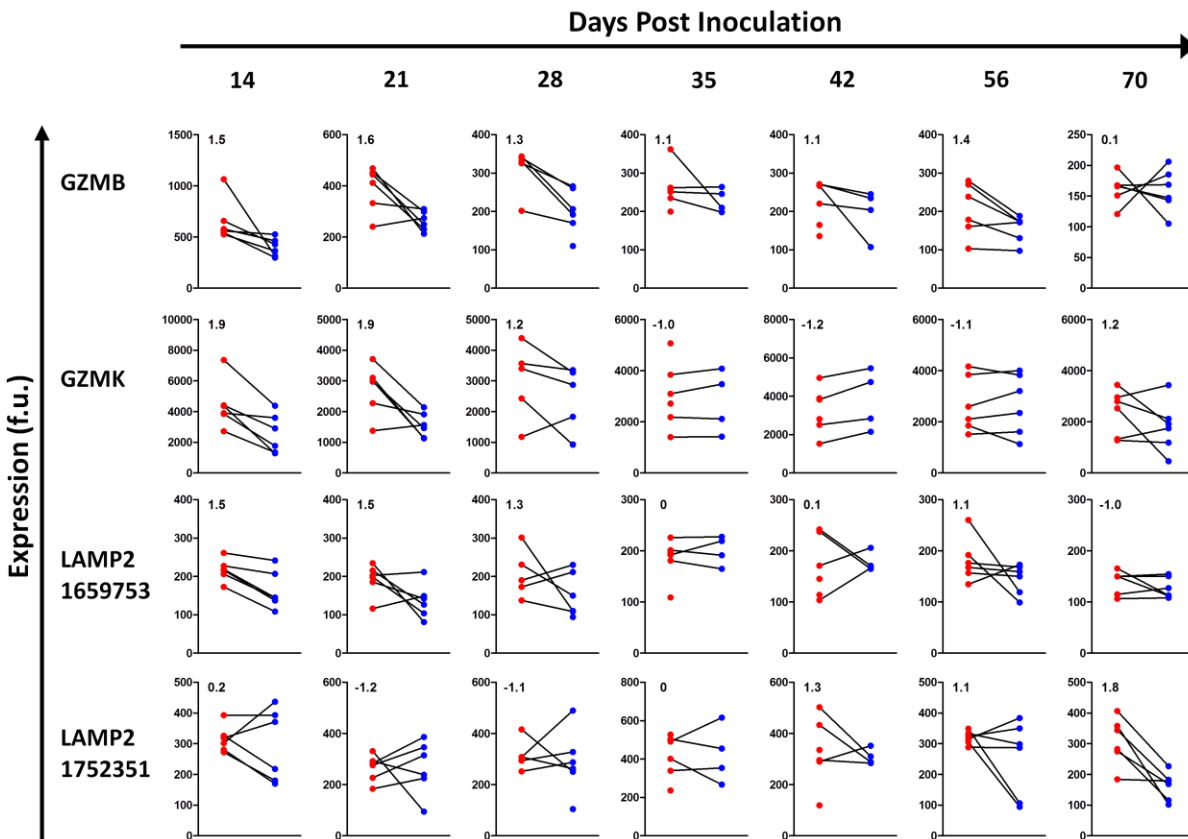
Figure 3 (continued)

C.
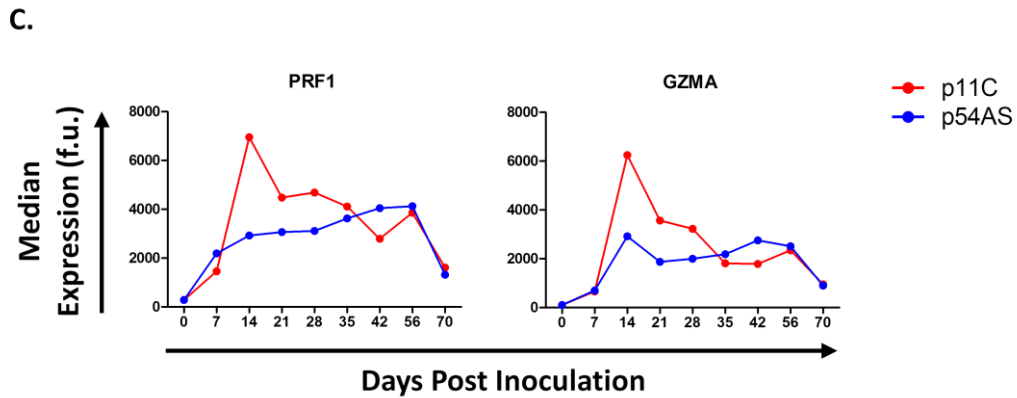
The RNA expression of genes involved in cytotoxicity found to be significantly differentially expressed between the dominant p11C- and subdominant p54AS-specific CD8^{+} T cells (GZMB, GZMK, LAMP2-1659753, and LAMP2-1752351). Data were analyzed as described in Fig. 2. A) The median raw expression value for each epitope-specific CD8^{+} T cell population for each timepoint. B) Each individual expression value for each epitope-specific CD8^{+} T cell population for each timepoint, including the values for which its matching pair is missing. Fold change values are indicated in upper left corner. C) The median raw expression values for each epitope-specific CD8^{+} T cell population for the notable genes GZMA and PRF1. Red, p11C. Blue, p54AS.

cytolytic molecules in the p11C-specific compared to the p54AS-specific CD8^{+} T cells suggested

that the p11C-specific CD8^{+} T cells were more mature than the p54AS-specific CD8^{+} T cells,

consistent with the decreased expression of CCR7, SELL, and IL7R in the p11C-specific CD8^{+} T

cells described above.

Of the 300 cell cycle- and apoptosis-associated genes, nine were differentially expressed

after SIV infection between the p11C- and p54AS-specific CD8^{+} T cells: ATM, AURKB (aurora B

kinase), BIRC3, CASP2 (caspase 2), CCND2 (cyclin D2), CDC42, NUSAP1, UBE2C, and UHRF1 (Fig.

4). UBE2C encodes a ubiquitin-conjugating enzyme (E2) that provides the ubiquitination activity

of the anaphase-promoting complex or cyclosome (APC/C), a complex that coordinates cell

cycle progression [495-497]. AURKB encodes the kinase aurora B, a kinase required for multiple

steps of mitosis, which is highly expressed in proliferating cells [498, 499]. Interestingly, the

ubiquitination and degradation of aurora B that is required for cell cycle progression is

regulated by the APC/C complex that includes UBE2C [500, 501]. Upon infection, the expression

of both UBE2C and AURKB increased in SIV epitope-specific CD8^{+} T cells, relative to naïve CD8^{+} T

cells and decreased toward the end of acute infection. Both were found to be more highly

expressed in the p11C-specific CD8^{+} T cells on day 56 with fold difference values of 1.6 and 1.5

of UBE2C and AURKB, respectively.

Figure 4- Differences in expression of cell cycle- and apoptosis-associated genes between

dominant p11C- and subdominant p54AS-specific CD8^{+} T cells
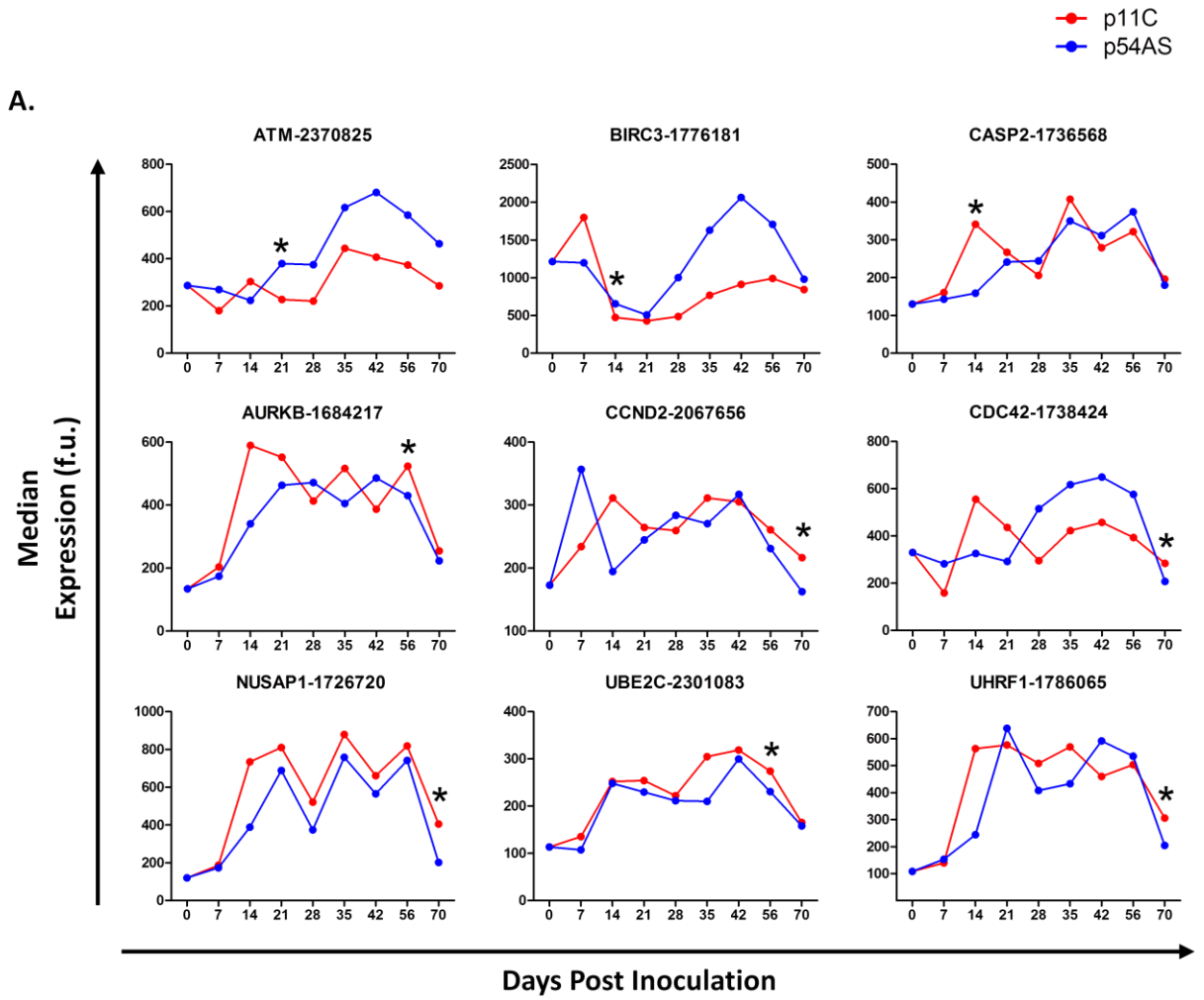
Figure 4 (continued)

B.

Figure 4 (continued) B. Paired expression (f.u.) plots for genes ATM 2370825, BIRC3 1776181, CASP2 1736568, AURKB 1684217, CCND2 2067656, CDC42 1738424, NUSAP1 1726720, UBE2C 2301083, and UHRF1 1786065 across Days Post Inoculation (14, 21, 28, 35, 42, 56, 70). p11C (red) and p54AS (blue).
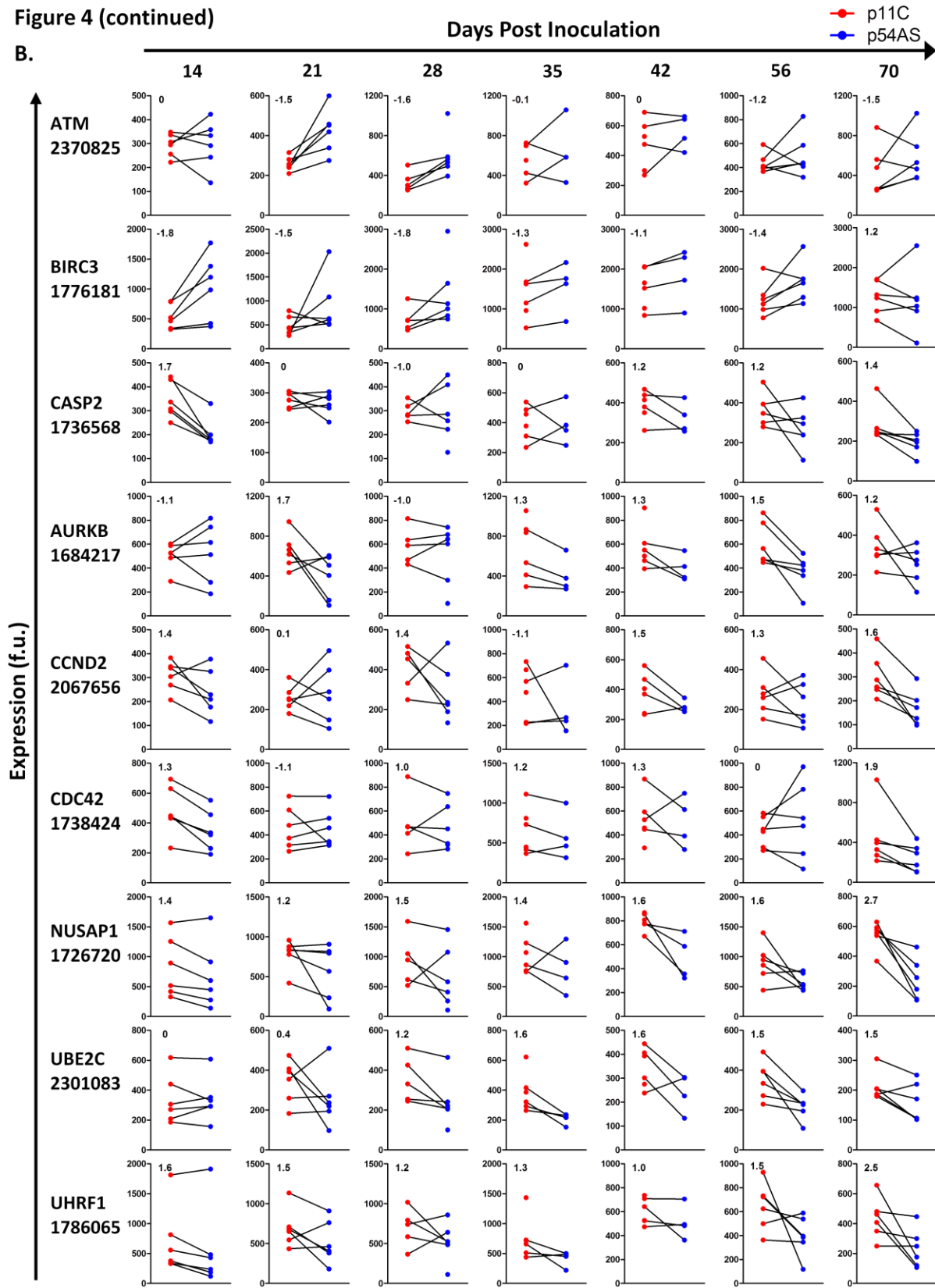
Figure 4 (continued). The RNA expression of genes involved in cell cycle and apoptosis in the dominant p11C- and subdominant p54AS-specific CD8$^+$ T cells was examined using the Illumina HT-12 human beadchip. Data were analyzed as described in Fig. 2. A) The median raw expression value for each epitope-specific CD8$^+$ T cell population for each timepoint. B) Each individual expression value for each epitope-specific CD8$^+$ T cell population for each timepoint, including the values for which its matching pair is missing. Fold change values are indicated in upper left corner. Red, p11C. Blue, p54AS.

ATM is a serine/threonine protein kinase that inhibits cell cycle progression via induction of p53 expression upon detection of DNA damage [502]. Data in the present study showed that expression of ATM increased following infection in both SIV epitope-specific CD8^{+} T cells, and its expression was found to be significantly increased in the p54AS-specific CD8^{+} T cells, with a -1.5-fold difference on day 21.

BIRC3 encodes cIAP2, a protein with multiple anti-apoptotic functions, including inhibition of caspases-7 and -9, activation of NFKB, and ubiquitination Smac/DIABLO and caspases-3 and -7 [503]. The present data showed that BIRC3 expression in the SIV epitope-specific CD8^{+} T cells decreased upon initial infection and then gradually increased after day 21. BIRC3 was differentially expressed on day 14, showing higher expression levels in the p54AS-specific CD8^{+} T cells with a -1.8-fold difference.

The gene CASP2 encodes caspase-2, a protease that induces apoptosis following cellular stress or death receptor signaling, and it also inhibits the cell cycle at the G2/M phase in response to DNA damage [504, 505]. CASP2 expression was found to be relatively low in naïve CD8^{+} T cells and increased in the SIV epitope-specific CD8^{+} T cells following infection. It was found to be expressed at 1.7-fold higher levels in the p11C-specific CD8^{+} T cells on day 14.

CCND2 encodes cyclin D2, a cyclin that pairs with CDK4 and CDK6 in promoting cell cycle progression through G1 [506]. The expression of CCND2 showed a modest increase in the SIV epitope-specific CD8^{+} T cells through day 42 and then started to decrease after day 42. By day 70, expression of CCND2 in the p54AS-specific CD8^{+} T cells had reached baseline levels while expression remained relatively higher in the p11C-specific CD8^{+} T cells by 1.6-fold.

The CDC42 gene encodes a GTPase that plays a role in cytoskeletal reorganization by promoting vesicular transport [507]. Increased expression of CDC42 has been shown to facilitate cytoskeletal changes that favor cell division [508, 509]. Compared to naïve CD8^{+} T cells, expression of CDC42 showed a modest increase in SIV epitope-specific CD8^{+} T cells following infection. We observed a 1.9-fold higher expression of this gene in the p11C-specific CD8^{+} T cells on day 70 following infection compared to the p54AS-specific CD8^{+} T cells. Interestingly, CDC42 is also required for the cytoskeletal changes necessary for the polarization of cytotoxic granules towards their targets [510]. Consistent with the increased expression of cytotoxicity-related genes in the p11C-specific CD8^{+} T cells on day 14, CDC42 also showed a modest increase in the p11C-specific CD8^{+} T cells of 1.3-fold on day 14.

NUSAP1 encodes a pro-proliferative protein that is selectively expressed by proliferating cells. It is a microtubule-binding protein that is involved in spindle assembly during mitosis [511-514]. Expression of NUSAP1 has been shown to increase in epitope-specific CD8^{+} T cells following infection compared to naïve CD8^{+} T cells [227]. The present data showed that NUSAP1 expression was very low in naïve CD8^{+} T cells and increased in SIV epitope-specific CD8^{+} T cells during acute infection. Although both p11C- and p54AS-specific CD8^{+} T cells manifested similar trends of NUSAP1 expression, there was a difference observed on day 70. While expression in p54AS-specific CD8^{+} T cells declined to baseline levels, it remained significantly higher in the p11C-specific CD8^{+} T cells by 2.7-fold.

UHRF1 is an ubiquitin ligase that has been shown to play a role in maintaining genomic stability and promoting cell cycle progression. In its absence, cells undergo DNA damage-

induced apoptosis [515, 516]. Expression of UHRF1 has been shown to increase in epitope-specific CD8^{+} T cells following infection compared to naïve CD8^{+} T cells [227]. The present data showed that the expression of UHRF1 was very low in naive CD8^{+} T cells, increased in the SIV-epitope-specific CD8^{+} T cells during acute infection, and declined at the onset of chronic infection. Expression of UHRF1 in p11C- and p54AS-specific CD8^{+} T cells followed a similar trend; however, it was 2.5-fold more highly expressed in p11C-specific CD8^{+} T cells on day 70.

The dominant epitope-specific CD8^{+} T cell population was enriched with cells that exhibit a more mature phenotype. The present gene expression data suggested that functional differences existed between the dominant p11C-specific and the subdominant p54AS-specific CD8^{+} T cells. There were clear differences between these cells in differentiation- and cytotoxicity-associated gene expression that suggested that the dominant p11C-specific CD8^{+} T cells were more mature and contained greater cytotoxic potential than the subdominant p54AS-specific CD8^{+} T cells. We also observed differences in the expression of multiple proliferation- and apoptosis-associated genes between these cell populations; however, due to the complexity of the regulation of these processes, it was unclear what the implications of the differential expressions of these genes might be on proliferation and apoptosis in these cells. Finally, although we did not observe measureable differences of cytokine or chemokine gene expression patterns between these cells when measured _ex vivo_, we expected that differences would be manifested upon _in vitro_ stimulation of these cells because of differences in their maturation status. Therefore, we decided to conduct a series of functional assays to confirm

and extend the observations made in the RNA expression data set. In addition to p11C- and p54AS-specific CD8^{+} T cells, we chose to include in these functional studies an additional Mamu-A*01-restricted CD8^{+} T cell specificity of even lower frequency, the CD8^{+} T cells that recognize the SIV Pol p68A epitope [450]. Moreover, we included in these analyses cells from SIVsmE660-infected monkeys in addition to cells from the SIVmac251-infected monkeys used in the gene expression studies. The immunodominance hierarchies of the p11C-, p54AS/E660-, and p68A-specific CD8^{+} T cells were similar in both infections (Fig. 5). All of the following data were obtained using PBMCs isolated from these chronically-infected monkeys.

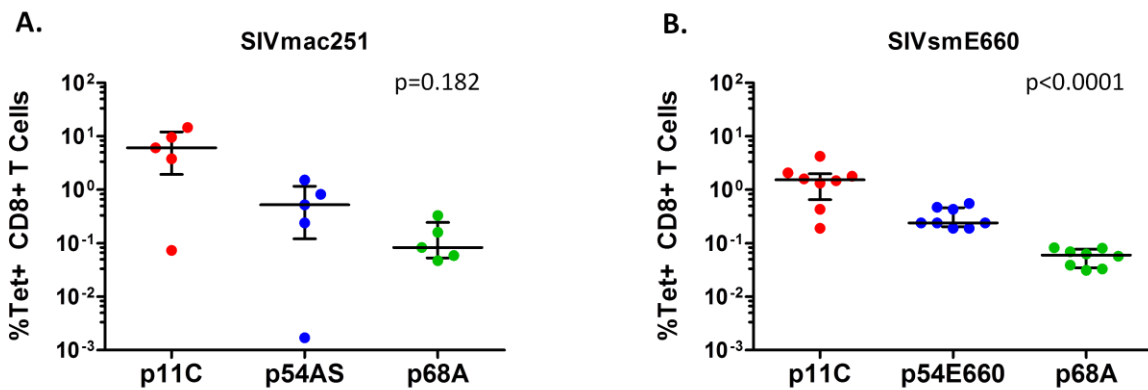**Figure 5- Mamu-A*01-restricted CD8^{+} T cell immunodominance hierarchies in SIVmac251- and SIVsmE660-infected rhesus monkeys**

Frequencies of the p11C-, p54AS/E660-, and p68A-specific CD8^{+} T cells in peripheral blood of chronically infected (A)SIVmac251- and (B)SIVsmE660- infected rhesus monkeys. Error bars indicate the median ± interquartile range. p values were determined using a Friedman test.

We first employed cell surface phenotyping to confirm the differences in CD8$^+$ T cell maturation between the p11C- and p54AS-specific CD8$^+$ T cells, which was suggested by the differential expression of the CCR7, SELL, and IL7R transcripts. We isolated PBMCs from both chronically infected SIVmac251- and SIVsmE660-infected *Mamu-A*01*$^+$ rhesus monkeys. Cells were stained with tetramers that recognize the p11C-, p54AS/E660-, and p68A-specific CD8$^+$ T cells, as well as antibodies against CCR7, CD28, CD27, and CD45RA, and analyzed by flow cytometry. Within each tetramer-positive population, we analyzed the differential expression of these molecules and categorized cells as central memory (CCR7$^+$CD28$^+$CD27$^+$CD45RA$^-$), transitional memory (CCR7$^-$CD28$^+$CD27$^+$CD45RA$^-$), or effector/effector memory (CCR7$^-$CD28$^-$CD27$^{+/-}$CD45RA$^{+/-}$) (Fig. 6).

The phenotypic composition of HIV and SIV epitope-specific CD8$^+$ T cells has previously been studied and it has been demonstrated that the vast majority are early effector memory cells (CCR7$^-$CD28$^-$CD27$^+$CD45RA$^-$) [126, 254, 260, 293]. Consistent with these observations, we found within each tetramer-positive CD8$^+$ T cell population a large proportion of cells that were CCR7$^-$CD28$^-$CD27$^+$CD45RA$^-$ (Fig. 6). However, there were some subtle differences between the SIVsmE660- and SIVmac251-infected animals, including a slight skewing of the cells in the SIVsmE660-infected monkeys to the less mature central and transitional memory phenotypes. Moreover, fewer cells from SIVsmE660-infected monkeys displayed an early effector memory phenotype compared to the cells from the SIVmac251-infected animals.

When comparing the p11C- and p54AS-specific CD8$^+$ T cells, we found that the p11C-specific cells displayed a more mature cell surface phenotype than the p54AS-specific cells. This

finding was consistent with the gene expression data which showed that that the CCR7, CD62L, and SELL genes were expressed at lower levels in the p11C-specific CD8$^+$ T cells, suggesting they were more mature than the p54AS-specific CD8$^+$ T cells. In the SIVmac251-infected monkeys, this was most dramatically seen with the higher representation of cells with the CCR7$^-$ CD28$^-$CD27$^+$CD45RA$^+$ effector memory phenotype in the p11C-specific CD8$^+$ T cell population. There were also decreases in the less mature central and transitional memory subsets in the p11C-specific population compared to the p54AS-specific population of CD8$^+$ T cells.

A comparison of p11C- and p54E660-specific CD8$^+$ T cells from SIVsmE660-infected monkeys revealed a slightly different pattern. Like the cells from the SIVmac251-infected monkeys, p11C-specific CD8$^+$ T cells from SIVsmE660-infected monkeys also showed an increased representation of more mature cells compared to the p54E660-specific cells. However, unlike in SIVmac251-infected animals, there was also a dramatic difference in the representation of the CCR7$^-$CD28$^-$CD27$^+$CD45RA$^-$ effector memory subset, with the p11C-specific CD8$^+$ T cells containing a greater frequency of cells with this phenotype than the p54E660-specific CD8$^+$ T cells. There were also clear increases of other effector/effector memory subsets within the p11C-specific population relative to the p54AS-specific population. Similar to cells from SIVmac251-infected monkeys, there was also a decrease in the central and transitional memory subsets in the p11C-specific population compared to the p54E660-specific CD8$^+$ T cell populations.

Figure 6- The dominant p11C-specific CD8^{+} T cell population contained a greater proportion of more mature cells than the subdominant epitope-specific CD8^{+} T populations
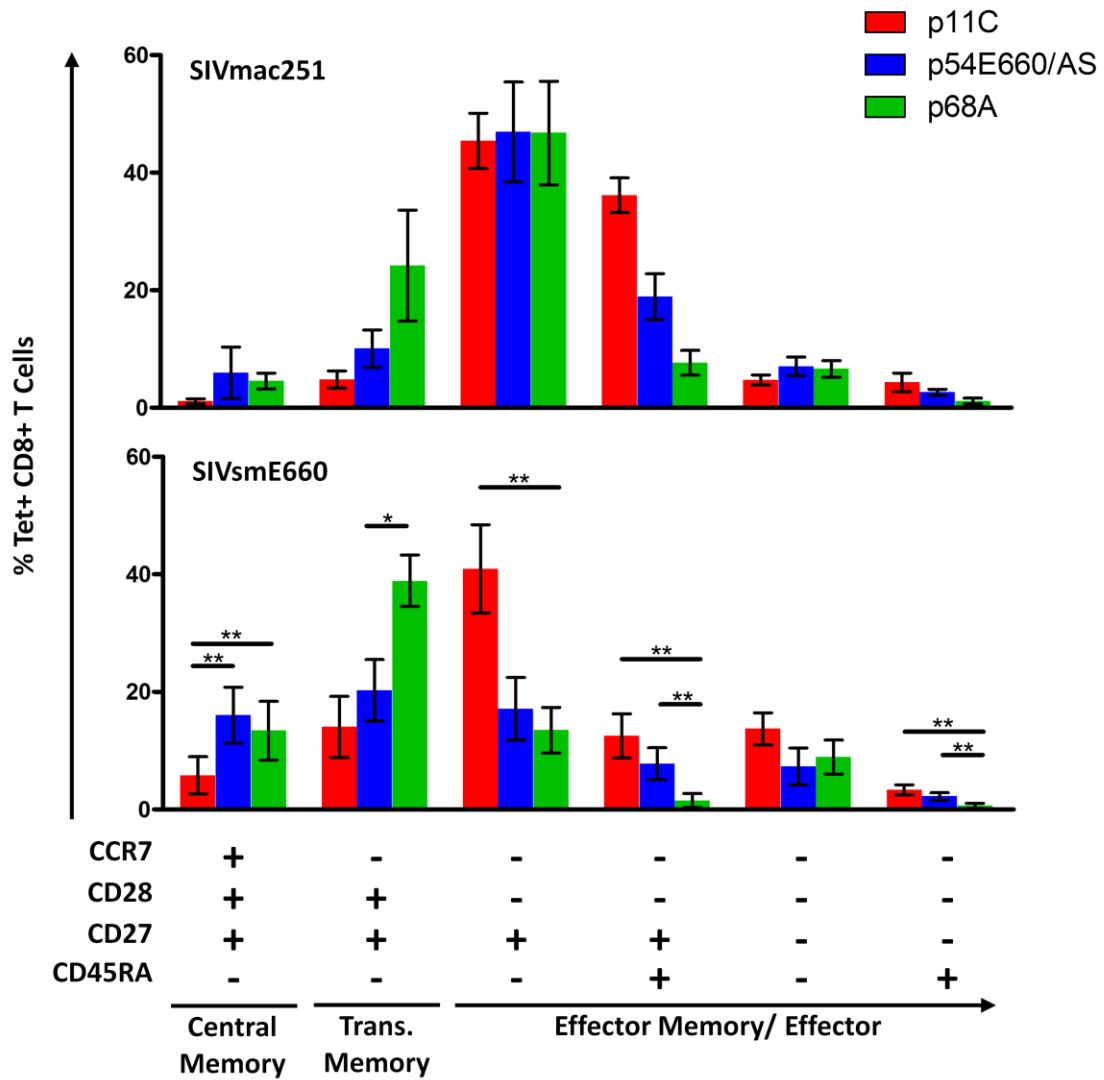
Figure 6 (continued). PBMCs were stained with p11C, p54AS/E660, and p68A peptide/Mamu-A*01 tetramers and antibodies and analyzed by flow cytometry. Flow data were gated for single, CD20$^-$CD3$^+$CD4$^-$CD8$^+$ lymphocytes. The phenotypic profile of each tet$^+$ population was determined by its differential expression of CCR7, CD28, CD27, and CD45RA, as indicated by the + and - signs below the bar graphs. Bars represent the mean ± SEM of the percent of the tet$^+$ cells that comprise each phenotypic category. Top, SIVmac251-infected *Mamu-A*01*$^+$ rhesus monkeys (n=8). Bottom, SIVsmE660-infected *Mamu-A*01*$^+$ rhesus monkeys (n=3-4). p values were calculated using a Wilcoxon signed rank test, and significant p values were determined using a Bonferroni correction for a 3-way comparison. * p ≤ 0.017, ** p ≤ 0.003.

An analysis of the phenotype of the subdominant p68A-specific CD8^{+} T cells revealed an association between the frequency of the epitope-specific CD8^{+} T cells and their phenotype. We found that the p68A-specific cells showed even less maturation than the p54AS-specific of cells. This trend was true in both SIVmac251- and SIVsmE660-infected monkeys.

In summary, we found that the p11C-specific CD8^{+} T cell population contained a greater frequency of more mature cells than the p54AS-specific population, consistent with the gene expression differences observed in the maturation-associated genes. We also found that there appeared to be a relationship between the frequency of epitope-specific CD8^{+} T cells and maturation phenotype, as the even lower-frequency p68A-specific CD8^{+} T cells showed a phenotype that was less mature than the p54AS/E660-specific CD8^{+} T cells. We found these trends to be true in monkeys infected with two different SIV isolates.

The dominant p11C-specific CD8^{+} T cell population contained a greater frequency of cytolytic molecules and more cytolytic molecules per cell than subdominant epitope-specific CD8^{+} T cell populations. The relationship between the expression of cytolytic molecules and the maturation of CD8^{+} T cells has been well-defined. As CD8^{+} T cells become more mature, the expression of cytolytic molecules, such as granzymes and perforin, increases [127, 175, 253, 254, 263, 517, 518]. Consistent with this finding, the gene expression data in the present study suggested that not only did the p11C-specific CD8^{+} T cells have a gene expression profile that was characteristic of more mature cells, but that it also exhibited increased expression of genes involved in cytotoxicity when compared to the p54AS-specific CD8^{+} T cells (Fig. 3). Using cell

surface staining and flow cytometric analysis, we found that the differences in maturation that were suggested by the gene expression studies were also seen at the level of protein production (Fig. 6). To confirm if differences in cytolytic molecule gene expression were similarly reflected in differences in protein production, we stained PBMCs from these chronically infected monkeys with tetramers and antibodies specific for perforin and granzyme B. The p11C-specific CD8+ T cell population had a greater frequency of cells that contained perforin and granzyme B than the p54AS/E660-specific CD8+ T cell population, which in turn had a greater frequency of perforin- and granzyme B-containing cells than the p68A-specific CD8+ T cell population (Fig. 7). We also found that the p11C- specific CD8+ T cells contained a greater amount of perforin and granzyme B on a per-cell basis than the p54AS/E660-specific cells, which in turn contained a greater amount of these proteins than the p68A-specific cells (Fig. 7C). This is consistent with previous findings that demonstrated that perforin- and granzyme-containing CD8+ T cells that display more mature phenotypes have more perforin and granzyme per cell [263].

In summary, we found that there was an association with the frequency of epitope-specific CD8+ T cells and their content of cytolytic molecules. Not only did CD8+ T cell populations that recognize more dominant epitopes contain a greater frequency of perforin and granzyme B that those CD8+ T cell populations recognizing subdominant epitopes, but those cells also contained more perforin and granzyme on a per-cell basis.

Figure 7- The dominant p11C-specific CD8$^+$ T cell population contained a greater frequency of cytolytic molecules and more cytolytic molecules per cell than subdominant epitope-specific CD8$^+$ T cell populations
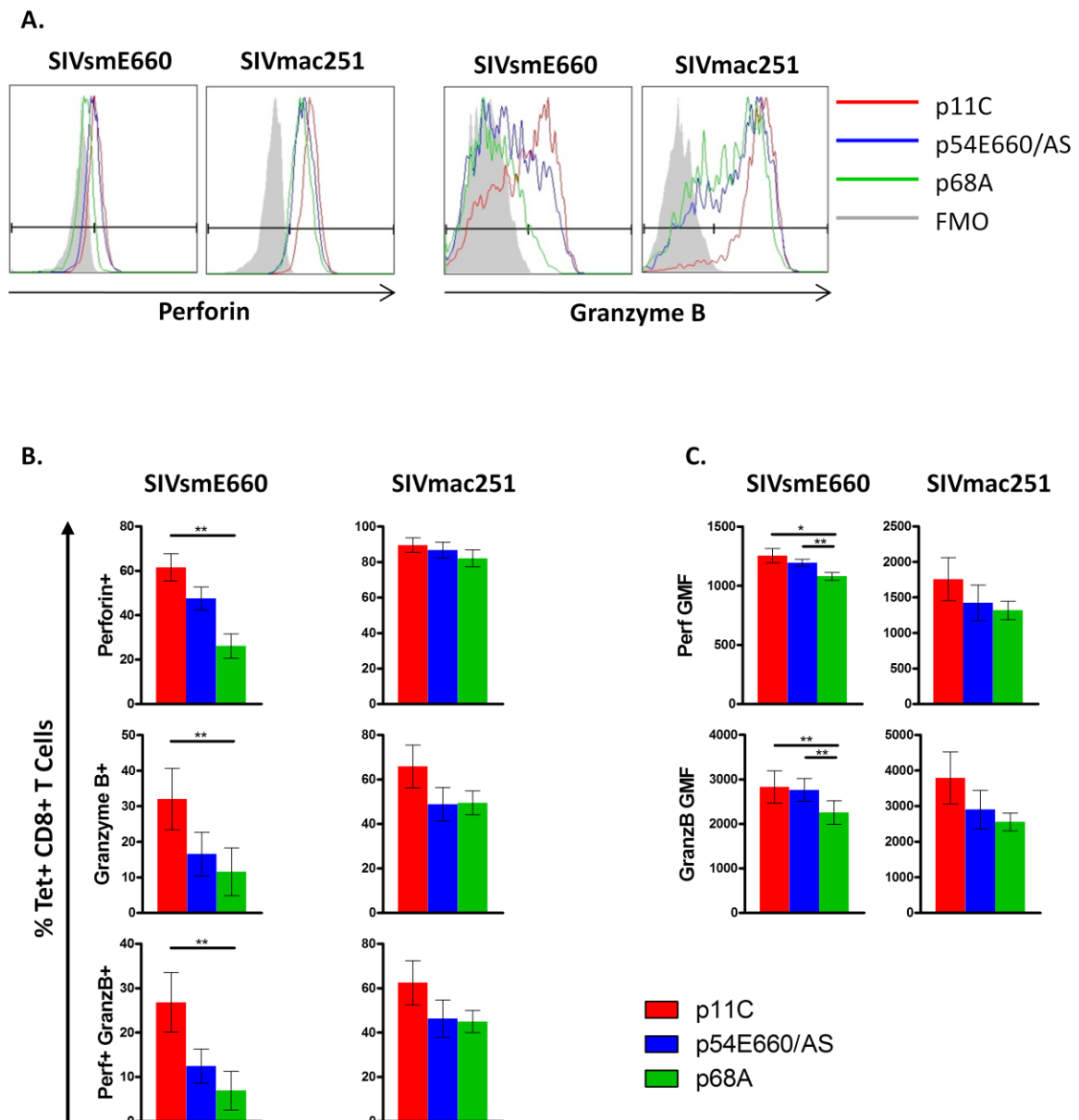
Figure 7 (continued). PBMCs were stained with p11C, p54AS/E660, and p68A peptide/Mamu-A*01 tetramers and antibodies specific for surface and intracellular molecules, and analyzed by flow cytometry. Expression of perforin and granzyme B was analyzed on cells gated for single, $CD3^+CD4^-CD8^+$ lymphocytes. A) Histograms show representative perforin (left) and granzyme B (right) staining in tet^+ populations from one SIVsmE660-infected (left) and one SIVmac251-infected (right) monkey. Filled gray histograms show the fluorescence of the cells in the FITC (perforin) and Alexa700 (granzyme B) channels for the FMO samples, which were used to set the positive gates for perforin and granzyme B staining. B) The percent of perforin and/or granzyme B staining in each epitope-specific $CD8^+$ T cell population. Bars represent mean ± SEM of the percent of the epitope-specific $CD8^+$ T cells that contained perforin and/or granzyme B. C) The geometric mean fluorescence (GMF) of perforin and granzyme B staining within each $perforin^+$ and granzyme B^+ epitope-specific $CD8^+$ T cell population. Bars represent mean ± SEM of the GMF. p values were calculated using a Wilcoxon signed rank test, and significant p values were determined using a Bonferroni correction for a 3-way comparison. * $p \leq 0.017$, ** $p \leq 0.003$.

The dominant p11C-specific CD8^+ T cells exhibited decreased antigen-specific expansion compared to subdominant epitope-specific CD8^+ T cells. The gene expression data in the present study showed differences in the expression of some genes involved in cell cycle and apoptosis that suggested that there may be differences in proliferative capacity between the dominant p11C- and subdominant p54AS-specific CD8^+ T cells. However, it was unclear what impact the differential expression of these genes had on the relative expansions of these cells upon antigen stimulation during chronic infection. Since we had shown that the p11C-specific population contained a greater percentage of more mature and more cytotoxic cells than the subdominant epitope-specific populations, we predicted that these dominant epitope-specific cells would have a relatively lower expansion capacity than the subdominant epitope-specific CD8^+ T cells, as has been described for more mature effector memory and effector CD8^+ T cells [245, 260]. To explore this possibility, we stimulated PBMCs from SIV-infected *Mamu-A*01*^+ rhesus monkeys with optimal epitope peptides and followed the expansion of the epitope-specific CD8^+ T cells over a 14 day-period. We also stained PBMCs with tetramers on day 0 in order to calculate the fold change of expansion at each timepoint relative to day 0. We did this for p11C-, p54AS/E660-, and p68A-specific CD8^+ T cells from both SIVmac251- and SIVsmE660-infected monkeys.

We found that the dominant p11C-specific CD8^+ T cells had a reduced capacity to expand when compared to both of the subdominant epitope-specific CD8^+ T cells and this trend was consistent between cells from both SIVsmE660- and SIVmac251-infected monkeys (Fig. 8). The relative expansion capacities of the subdominant p54AS/E660- and p68A-specific populations were different between cells from the two different infections; the p54E660-

Figure 8- The dominant p11C-specific CD8$^+$ T cells exhibited decreased antigen-specific expansion compared to subdominant epitope-specific CD8$^+$ T cells
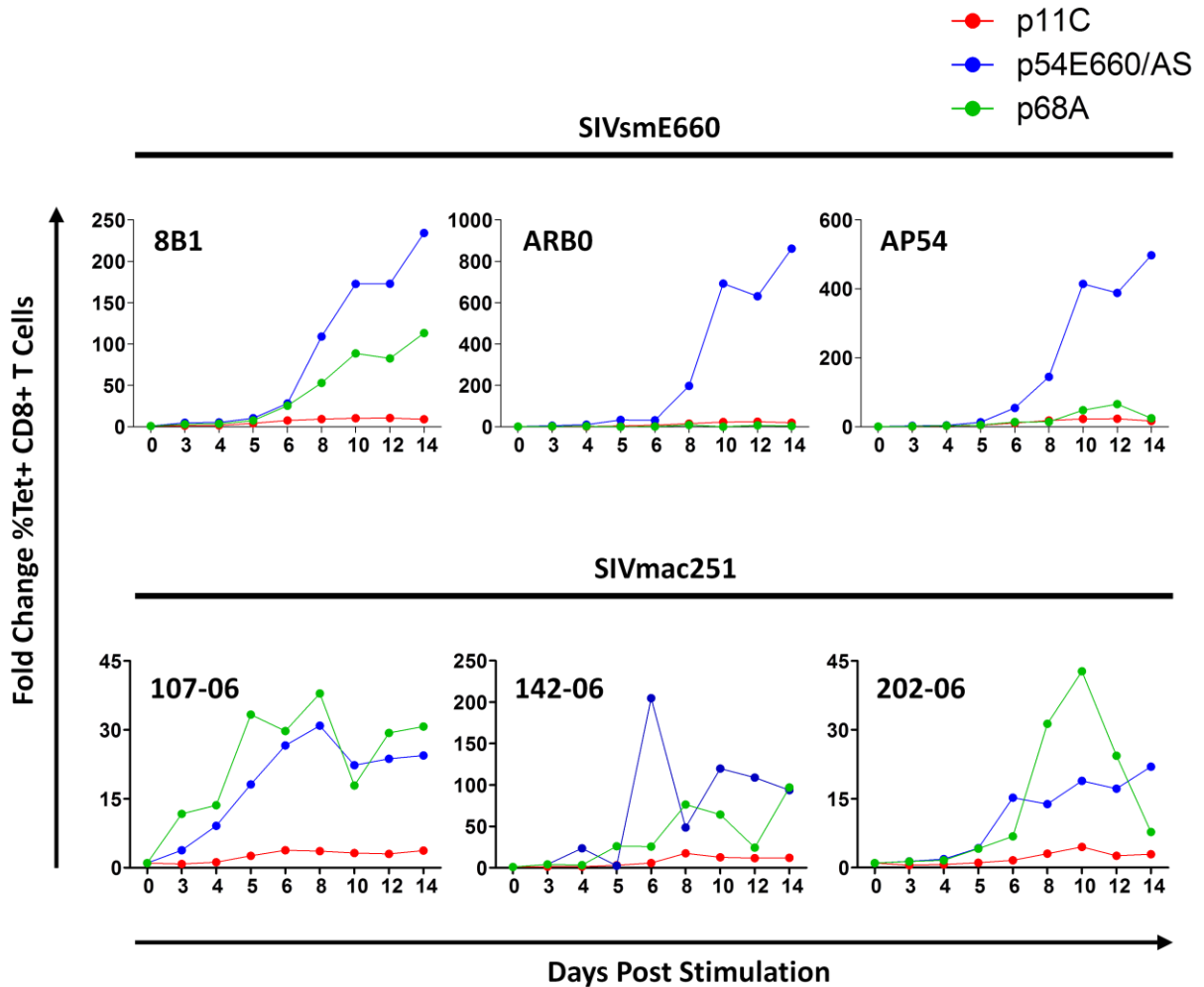
PBMCs were stimulated *in vitro* with peptide, harvested on days 3, 4, 5, 6, 8, 10, 12, and 14 following stimulation, and analyzed following tetramer and antibody staining by flow cytometry. Flow data were gated for single, live, CD3$^+$CD4$^-$CD8$^+$ tet$^+$ lymphocytes. The fold change of the percent of each tet$^+$ population, relative to day zero, was calculated. Data from three SIVsmE660- (top panel) and three SIVmac251-infected (bottom panel) *Mamu-A*01$^+$* rhesus monkeys are shown.

specific cells from the SIVsmE660-infected monkeys usually showed enhanced expansion potential compared to the p68A-specific cells from the same monkeys while the p54AS- and p68A-specific cells from the SIVmac251-infected monkeys were comparable in their ability to expand.

The dominant p11C-specific CD8$^+$ T cell population contained a lower frequency of cytokine- and chemokine-producing cells than the subdominant epitope-specific CD8$^+$ T cell populations.
Although the gene expression data did not demonstrate that any cytokines or chemokines were differentially expressed by the p11C- and p54AS-specific CD8$^+$ T cells , we considered that differences in their expression might be observed following antigen stimulation. Phenotypic and functional data suggested that the p11C-specific CD8$^+$ T cell population, with its greater frequency of more mature cells, might exhibit a lower production of cytokines and chemokines than the p54AS/E660-specific CD8$^+$ T cell population, and in turn the p54AS/E660-specific CD8$^+$ T cell population might show lower production than the p68A-specific CD8$^+$ T cell population. To investigate this, we isolated PBMCs from both SIVmac251- and SIVsmE660-infected _Mamu-A*01$^+$_ rhesus monkeys and stimulated them with optimal epitope peptides and measured IL-2, TNFα, IFNγ, and MIP1-β production in a standard intracellular cytokine staining (ICS) assay. We found that each of these cytokines and chemokines were produced at reduced frequencies by the p11C-specific CD8$^+$ T cell population (Fig 9B). The p54AS/E660- and p68A-specific populations contained comparable frequencies of cytokine- and chemokine-producing cells.

Figure 9- The dominant p11C-specific CD8^{+} T cell population contained a lower frequency of cytokine- and chemokine-producing cells than the subdominant epitope-specific CD8^{+} T cell populations
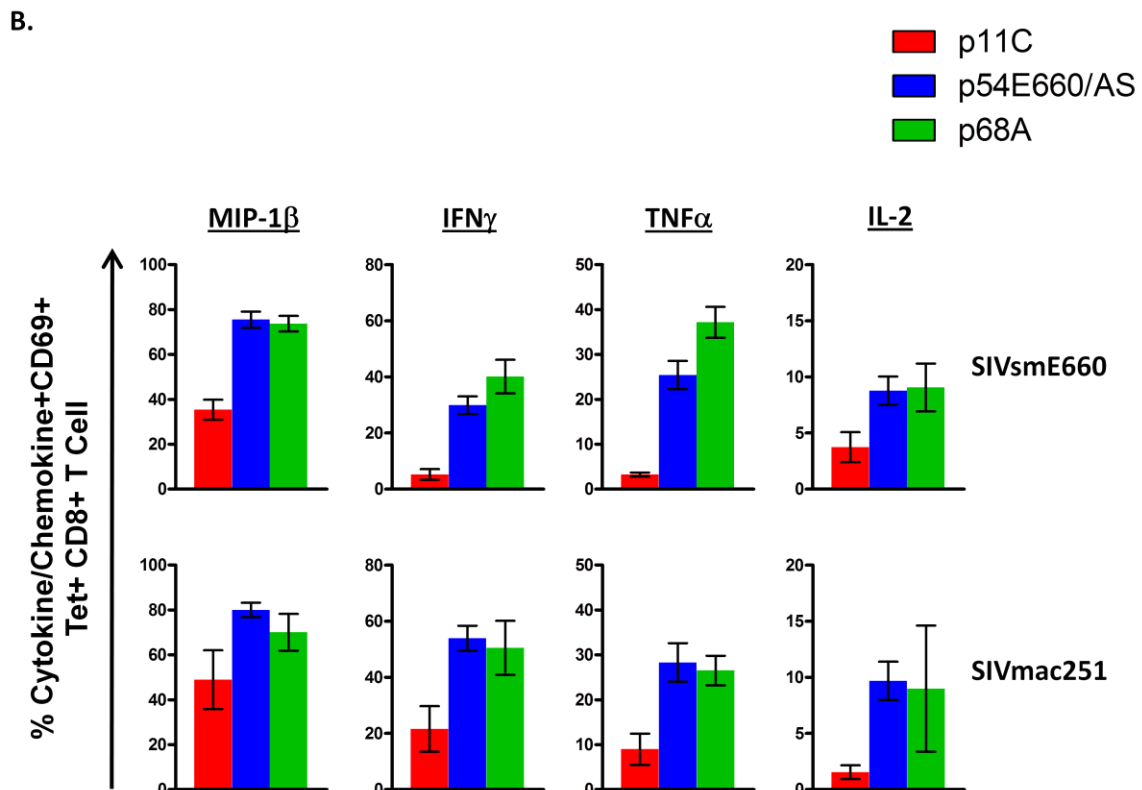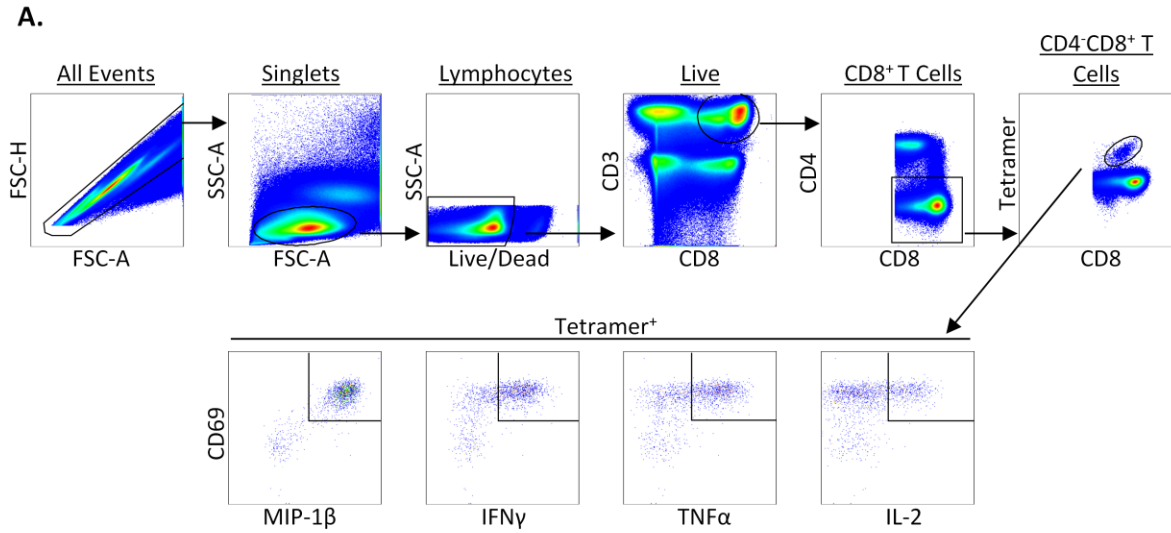
Figure 9 (continued)
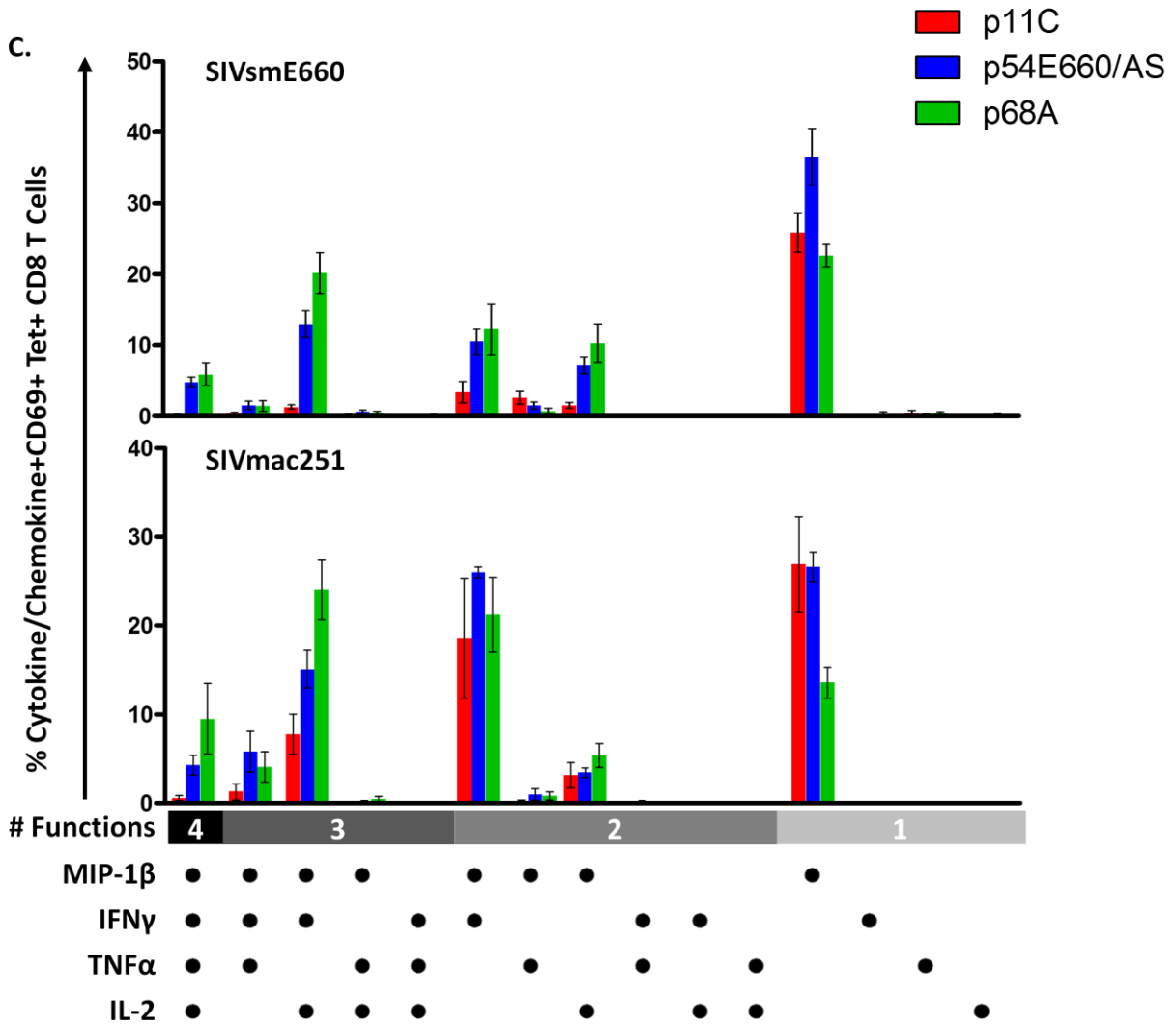
Figure 9 (continued). PBMCs were stimulated *in vitro* with p11C, p54AS/E660, or p68A epitope peptides and with anti-CD28 and anti-CD49d antibodies and incubated for six hours, with the addition of monensin and brefeldin A after the first hour. Cells were then stained with surface and intracellular molecule-specific antibodies and analyzed by flow cytometry. A) An example of the gating strategy used for analysis of the flow cytometry data. Flow data were gated for single, live, $CD3^+CD4^-CD8^+CD69^+$ lymphocytes. Unstimulated and PMA/Ionomycin-stimulated samples were used to determine the positive gates for CD69, IFNγ, TNFα, IL-2, and MIP-1β. B) Individual analyses of cytokine and chemokine production. Bars represent the mean ± SEM of the percent of each epitope-specific $CD8^+$ T cell population that produced the indicated cytokine (IFNγ, TNFα, and IL-2) or chemokine (MIP-1β). Top, SIVsmE660-infected animals (n=6). Bottom, SIVmac251-infected animals (n=3-5). C) Polyfunctional analysis of cytokine and chemokine production. The polyfunctionality of each epitope-specific $CD8^+$ T cell population was determined using Boolean gating of each individually-gated cytokine and chemokine population. Bars represent the mean ± SEM of the percent of epitope-specific $CD8^+$ T cells producing the combination of molecules indicated by the dots below.

We also determined the impact of this decreased production of soluble molecules by the dominant p11C-specific cell population on its polyfunctional properties compared to that of the subdominant epitope-specific cells. Using a Boolean analysis of the expression of each of these soluble molecules, we determined the extent of polyfunctionality, defined as responses producing more than one cytokine or chemokine, of each epitope-specific cell population. We found that the p11C-specific population contained a much lower frequency of polyfunctional cells than the subdominant epitope-specific populations (Fig 9C). Cells in the four molecule-producing category were essentially absent in the p11C-specific populations from both SIVmac251- and SIVsmE660-infected monkeys. There were a small number of p11C-specific cells in the MIP1-β^+ IFNγ^- TNFα^+ IL-2$^+$ triple-positive category and a larger number in the MIP1-β^+ IFNγ^+ TNFα^- IL-2$^-$ double-positive category. Most of the p11C-specific cells produced MIP1-β-only or did not produce any of the measured molecules (quadruple-negative cells not plotted on graph). This finding is consistent with previous reports that more mature cytotoxic CD8$^+$ T cells are more frequently MIP1-β-only producers compared to less mature populations [153, 299].

The dominant p11C-specific CD8$^+$ T cells did not exhibit greater exhaustion. The present data suggested that there was a relationship between the frequency of epitope-specific CD8$^+$ T cells and maturation, as measured by differences in phenotype and function. However, exhaustion due to chronic antigenic stimulation also causes changes in the phenotypic and functional profiles of cells that sometimes resemble increased maturation [320, 323]. Exhaustion is a

progressive development of functional defects that initially manifests as a defect in IL-2

production, and sometimes reduced cytotoxicity, followed by loss of TNFα and finally IFNγ

production. These changes are also associated with a progressive loss of proliferative potential

[316]. These functional defects are often associated with changes in expression of cell surface

molecules such as an increase in expression of inhibitory molecules PD-1 and CTLA-4, as well as

changes in expression of molecules involved in gene expression such as the transcription factor

BLIMP-1 [350]. To examine whether the differences that we observed were a result of different

extents of exhaustion among the epitope-specific CD8$^+$ T cells , we sorted the p11C- and p54AS-

specific CD8$^+$ T cells from the SIVmac251-infected monkeys at a chronic timepoint, day 210. We

obtained gene expression data from these cells and looked for differential expression of a

number of genes that are well-known to be modulated in exhausted cells.

Comparison of the pattern of expression of these genes between the p11C- and p54AS-

specific cells did not suggest that there were different degrees of exhaustion (Table 2). In fact,

most of the genes were expressed at the same level by the p11C- and p54AS-specific cells, as

indicated by a fold change near one. We also observed differences in expression of genes that

are also involved in maturation (PRF1, IL7R, SELL, and CCR7); although, these differences were

not significant. There was one gene, LAG3, whose expression was significantly higher in the

p11C-specific cells (p=0.05). However, differential expression of this gene alone, without

coincident differential expression of other genes, is not suggestive of different extents of

exhaustion [328].

Table 2- Expression of exhaustion-associated genes in dominant p11C- and subdominant

p54AS-specific CD8$^+$ T cells

Gene	Alias	Median Fold Change (p11C/p54AS)
faslg	FasL	-1.2
cd244	2B4	1.0
cblb	Cbl-b	1.3
cd69 (1651316)	CD69	1.1
havcr2	TIM-3	1.1
lag3	LAG-3	1.7*
pdcd1	PD-1	-1.1
ctla4 (1763487)	CTLA-4	-1.1
cd44 (1778625)	CD44	-1.0
prdm1 (1655077)	BLIMP-1	1.1
cd27	CD27	1.0
cd160	CD160	-1.2
Spn (1658017)	CD43	-1.1
il2ra	IL-2R	-1.1
prf1	Perforin	1.5
il7r (1691341)	IL-7Ra	-2.3
sell	CD62L	-3.8
ccr7	CCR7	-2.0

Data are from either week 30 or 31 post inoculation with SIVmac251. For genes where there was more than one probe on the beadchip, the ID number of the Illumia probe that was used is provided in parentheses next to the gene. * p≤0.05 using a Wilcoxon signed rank test. n=5.

DISCUSSION

In the present study, we have identified an association between the frequencies of

Mamu-A*01-restricted SIV epitope-specific CD8^{+} T cells in rhesus monkeys and the phenotype

and function of these cells. Using a longitudinal whole genome expression analysis, we showed

that the phenotypic and functional differences among these SIV epitope-specific CD8^{+} T cells

are evident as soon as these cells are detectable in the blood, one to two weeks following SIV

infection. Subsequent flow cytometric analysis confirms the presence of these differences and

that they are maintained during chronic SIV infection. To our knowledge, this is the first time

that a longitudinal whole genome expression profiling approach has been undertaken to

characterize multiple HIV or SIV epitope-specific CD8^{+} T cells within an individual during acute

infection.

The gene expression analysis in the present study revealed that a number of genes that

encode molecules involved in CD8^{+} T cell maturation were differentially expressed between the

dominant p11C- and subdominant p54AS-specific CD8^{+} T cells. These findings were similar to

those in a study by Baron and colleagues of a dominant and a cryptic H2-D[b]–restricted epitope-

specific CD8^{+} T cell population in mice [464]. In that study, gene expression analysis of these

two epitope-specific CD8^{+} T cells was performed on day 14 following immunization with

antigen-loaded DCs. Of over 39,000 transcripts analyzed, they found only 15 that were

differentially expressed, based on their pre-determined criteria, between the dominant and

cryptic epitope-specific CD8^{+} T cells. Several of these 15 transcripts were also found in our study

to be differentially expressed between dominant and subdominant SIV epitope-specific CD8^{+} T

cells. Similar to our study, they found IL7R and GZMA to be more highly expressed in the

dominant epitope-specific CD8^{+} T cells, while SELL was more highly expressed in the cryptic epitope-specific CD8^{+} T cells. A reexamination of our data indicated that some of the other genes that were found to be significantly differentially expressed in the Baron *et al* study also had high fold change values or low p values in our study: EOMES (on day 21, fold change -1.6 and p=0.06), KLRG1 (on day 14, fold change -1.2 and p=0.03), S100A4 (on day 21, fold change 1.5 and p=0.06, and on day 56, fold change 1.5 and p=0.06), VAMP5 (on day 56, fold change 1.4 and p=0.03 and on day 70, fold change 1.5 and p=0.06), and CSF1R (on day 210, fold change - 1.5 and p=0.06). The patterns of differential expression of these genes in the present study were also the same as in the Baron *et al* study, that is, genes more highly expressed by the dominant epitope-specific CD8^{+} T cells were also found to be more highly expressed by the dominant p11C-specific cells in our study. Baron *et al* subsequently used flow cytometry to measure the expression of the proteins encoded by a few of these differentially expressed genes on the epitope-specific CD8^{+} T cells over time following immunization. They found that the difference in the expression of these molecules were present at all time points examined during the first few weeks following immunization. However, analysis of the expression of these molecules at a late time point (day 100) found that these populations now displayed similar expression. The discrepancy between the maintenance of phenotypic differences observed in the present study and the lack of maintenance of these differences in the study by Baron *et al* is likely due to the fact that the epitope-specific CD8^{+} T cell responses in the Baron *et al* study were induced by immunization, whereas ours were induced by viral infection. Therefore, the maintenance of the phenotypic differences that was observed in the present study was likely due to the chronic antigenic stimulation that these cells were experiencing, whereas in the

mouse study, the dominant and cryptic epitope-specific CD8^{+} T cells were able to develop

similar memory responses as antigen was presumably cleared. However, the similarity in the

differential gene expression patterns between the dominant and subdominant/cryptic epitope-

specific CD8^{+} T cells during the primary immune response between our study and the Baron *et*

al study suggests that it may be a common feature of epitope-specific CD8^{+} T cell

immunodominance hierarchies.

Our analysis of cell cycle- and apoptosis-related genes did not show any major

differences in gene expression between the dominant p11C- and subdominant p54AS-specific

CD8^{+} T cells that would suggest differences in proliferation or death. However, cell cycle and cell

death are very intricately-regulated processes and are not simply determined by the presence

or absence of gene expression. They often depend on many other factors including cellular

localization, presence of post-transcriptional modifications, stage in cell cycle, and the

coordinated expression of additional genes [519]. Therefore, drawing conclusions about the

rate of cell division or cell death based on the expression of one or a few individual genes may

be misleading. However, from the expression patterns of the genes that we found to be

differentially expressed between the p11C- and p54AS-specific CD8^{+} T cells during acute

infection, several trends were evident. First, there was differential expression of apoptosis-

related genes early during infection. The pro-apoptotic gene CASP2 was more highly expressed

in the p11C-specific CD8^{+} T cells on day 14 while the anti-apoptotic gene BIRC3 was more highly

expressed in the p54AS-specific CD8^{+} T cells on day 14. This is consistent with the p11C-specific

CD8^{+} T cells being more enriched in terminally differentiated effector cells, which are known to

be more susceptible to apoptosis [199]. Second, we observed a number of pro-proliferation

genes being more highly expressed in the p11C-specific CD8^{+} T cells at the end of acute

infection (AURKB and UBE2C on day 56 and, CCND2, CDC42, NUSAP1, and UHRF1 on day 70).

The reason for this is unclear. It may be the p11C-specific CD8^{+} T cells were experiencing

greater levels of stimulation causing these cells to divide more frequently than the p54AS-

specific CD8^{+} T cells.

Our analysis of cytokine and chemokine gene expression failed to reveal differences

between the dominant and subdominant epitope-specific CD8^{+} T cells. This finding was neither

consistent with the differences that we found in the expression of other genes involved in CD8^{+}

T cell maturation, nor was it consistent with the findings in our ICS experiments, which clearly

demonstrated that these two populations have different capacities to produce multiple

cytokines. Upon further examination of the gene expression data, we found that the expression

of these cytokine and chemokine genes never changed over the course of infection when

compared to the naïve baseline measurements. This lack of the detection of cytokine and

chemokine gene expression above baseline values was most likely due to the absence of

antigenic stimulation of the cells prior to sorting. Since expression of cytokine genes often

requires re-stimulation immediately prior to assaying [520], the design of our gene expression

experiment likely prevented us from detecting any differences in the expression of these

cytokine and chemokine genes.

To confirm our finding that the dominant p11C-specific CD8^{+} T cells were more mature

than the subdominant epitope-specific CD8^{+} T cells, we performed an *ex vivo* measurement of

cellular perforin and granzyme B content without stimulation, as the cellular content of these

proteins has been demonstrated to be linked to CD8^{+} T cell maturation [121, 127, 251, 263].

Consistent with our expectations, we detected more perforin and granzyme B content in dominant compared to subdominant epitope-specific CD8^{+} T cells. However, we did not perform a functional cytotoxicity assay or measure cell surface expression of CD107a following stimulation, and therefore cannot confirm that one CD8^{+} T cell specificity is more or less cytotoxic than the other. However, a recent study by Vojnov and colleagues performed an *ex vivo* viral suppression assay with sorted SIV epitope-specific CD8^{+} T cells and found that p11C (GagCM9)-specific CD8^{+} T cells exhibited substantially more viral inhibition than the p54AS (EnvTL9)-specific CD8^{+} T cells [521]. Additionally, a study by Wolint and colleagues demonstrated that central memory, effector memory, and effector LCMV epitope-specific CD8^{+} T cells all degranulated to similar levels, as measured by CD107a cell surface expression, despite the fact that they differed in their cytotoxic capacities [518]. Instead, the differences in cytotoxic potency of the cells were determined by amount of granzyme B stored in these cells prior to antigenic stimulation. Therefore, based on cytotoxicity assays performed by other groups and our measurements of cytolytic molecule content, it is likely that the dominant p11C-specific CD8^{+} T cells were more cytotoxic than the subdominant p54AS/E660- and p68A-specific CD8^{+} T cells.

Consistent with our findings that the subdominant SIV epitope-specific CD8^{+} T cells exhibited a less mature phenotype, we found that they possessed a greater expansion capacity compared to the dominant epitope-specific CD8^{+} T cells. Interestingly, a study by Friedrich *et al* also suggested that subdominant epitope-specific CD8^{+} T cells had greater proliferative capacities than dominant epitope-specific CD8^{+} T cells [522]. In that study, CD8^{+} lymphocytes in

rhesus monkeys chronically infected with SIV were antibody-depleted and the natural re-population of the SIV-specific CD8^{+} T cell pool was subsequently observed. Using tetramers to compare the percent of epitope-specific CD8^{+} T cells post-antibody depletion compared to pre-antibody depletion, they found that those SIV epitope-specific CD8^{+} T cells that were considered subdominant prior to CD8^{+} cell depletion showed relatively higher frequency responses following re-population, and some even became the dominant population. This suggests that the subdominant epitope-specific CD8^{+} T cells expanded more extensively than the dominant epitope-specific CD8^{+} T cells during CD8^{+} T cell re-population. This is consistent with the findings in the present study and suggests that the higher proliferative capacity of subdominant CD8^{+} T cells compared to dominant CD8^{+} T cells during chronic infection may be a common feature of the SIV-specific CD8^{+} T cell response.

Although we found similar trends in the phenotypes of epitope-specific CD8^{+} T cells in monkeys infected with the SIVmac251 and SIVsmE660 viral isolates, we did observe some minor differences. When comparing the maturation states of epitope-specific CD8^{+} T cells, we found that the populations from monkeys infected with SIVmac251 were slightly more mature than their counterparts from monkeys infected with SIVsmE660. Consistent with these small differences in maturation, we found that those epitope-specific CD8^{+} T cells taken from SIVmac251-infected monkeys had a significantly greater content of cytolytic molecules compared to their counterparts in SIVsmE660-infected monkeys. It is unclear in the NHP field whether one of these viral isolates is more pathogenic, more inflammatory, or generates a greater antigenic load than the other. However, the observation that SIVsmE660 is more sensitive to neutralization [523, 524] and causes a disease that progresses more slowly than

SIVmac251 [52, 525-527] suggests that SIVmac251 may be a more pathogenic virus. Therefore, it is tempting to speculate that SIVmac251 infection results in higher antigenic load than SIVsmE660 and that this provides greater CD8$^+$ T cell stimulation, which in turn drives the SIV-specific CD8$^+$ T cell cells to be slightly more mature than those equivalent cells generated by SIVsmE660 infection.

HIV and SIV epitope-specific CD8$^+$ T cells displaying a fully differentiated effector phenotype are found only at low frequencies in the majority of HIV-infected humans and SIV-infected monkeys [260]. It is thought that this inability of epitope-specific CD8$^+$ T cells to mature fully contributes to the failure of these cells to ultimately control viral replication [150, 254, 260, 528]. In support of this, it has been found that individuals who spontaneously control viral replication, termed long-term non-progressors, contain a greater frequency of HIV-specific CD8$^+$ T cells with a more mature phenotype [154, 297-300]. In addition, a recent study testing cytomegalovirus (CMV) as a SIV vaccine vector (due to reports that CMV induces more mature CD8$^+$ T cell phenotypes than previously used vaccine vectors [301]), found that this vaccine provided greater protection that was manifested by enhanced control of viral replication following SIV challenge [301, 302]. Interestingly, previous studies have demonstrated that the p11C-specific CD8$^+$ T cells are predominantly responsible for control of SIV in *Mamu-A*01$^+$* rhesus monkeys as the selection of viral escape mutations within the p11C epitope results in loss of epitope recognition by the p11C-specific CD8$^+$ T cells leading to rapid disease progression [85, 486]. It is possible that the strong protection afforded by the p11C-specific CD8$^+$ T cell population is, at least in part, due to its increased number of more mature cells that likely exhibit increased cytotoxicity towards SIV-infected cells [150, 153, 154, 529]. Thus, the present

data support the concept that more mature epitope-specific CD8^{+} T cells provide enhanced

protection against HIV and SIV and that vaccines should aim to generate such cells. Because our

data suggest that dominant epitopes may generate more frequently epitope-specific CD8^{+} T

cells with more mature phenotypes, it may be beneficial to include such epitopes in vaccine

constructs.

MATERIALS AND METHODS

Animals. *Mamu-A*01+ Mamu-B*17- Mamu-B*08-* Indian-origin rhesus monkeys (*Macaca mulatta*) were selected for these studies after PCR-based MHC typing as previously described [530]. All monkeys were housed in accordance with the guidelines outlined in the *NIH Guide for the Care and Use of Laboratory Animals* and with the approval of the Institutional Animal Care and Use Committee of Harvard Medical School and the National Institutes of Health. All monkeys were infected intrarectally with either SIVmac251 or SIVsmE660. SIVmac251 infection was administered by a single high dose challenge. SIVsmE660 was administered by a repeated low-dose challenge.

Antibodies and flow cytometry reagents. Conjugated antibodies and staining reagents included anti-MIP-1β-phycoerythrin (PE) (clone D21-1351), anti-CD3-Pacific Blue (PB) (clone SP34.2), anti-CD3-PE-Cy7 (clone SP34.2), anti-CD3-PerCP-Cy5.5 (clone SP34.2), anti-CD3-APC-Cy7 (clone SP34.2), anti-CD3-Horizon V450 (clone SP34.2), anti-CD4-PerCP-Cy5.5 (clone L200), anti-CD4-AmCyan (clone L200), anti-CD4- fluorescein isothiocyanate (FITC) (clone 19thy5D7), anti-CD8α-allophycocyanin (APC) (clone SK1), anti-CD8α-APC-Cy7 (clone SK1), anti-CD8α-AlexaFluor700 (clone RPA-T8), anti-CD8α-FITC (clone SK1), anti-CD8α-APC-H7 (clone SK1), anti-CD69-electron-coupled dye (ECD) (clone TP1.55.3, Beckman Coulter), anti-CD20-Horizon V450 (clone L27), anti-CD45RA-ECD (clone 2H4, Beckman Coulter), anti-CCR7-FITC (clone 150503, R&D Systems), anti-CCR7-PerCP-Cy5.5 (clone 150503), anti-CD27-APC-e780 (clone 0323, eBioscience), anti-CD28-PE-Cy7 (clone 28.2, eBioscience), anti-granzyme B-AlexaFluor700 (clone GB11), anti-perforin-FITC (clone Pf344, MabTech), anti-IFNγ-PE-Cy7 (clone B27), anti-TNFα- AlexaFluor700 (clone

Mab11), anti-IL-2-APC (clone MA1-17H12), anti-CD95-PE (clone dx2), anti-CD95-APC (clone

dx2), and Aqua LIVE/DEAD Fixable Dead Cell Stain (Invitrogen). All reagents are from BD

Biosciences unless indicated otherwise.

Monomers and tetramers. The following peptides were synthesized by New England Peptide,

LLC and were purified at >95% by HPLC: p11C (CTPYDINQM), p54AS (TVPWPNASL), p54E660

(TVPWPNETL), and p68A (STPPLVRLV). The p11C, p54AS, p54E660, and p68A peptide-Mamu-

A*01 monomeric and tetrameric complexes were prepared as previously described [531, 532].

Tetrameric complexes for flow cytometry were prepared using either streptavidin-PE

(Prozyme), -APC (Prozyme), -AlexaFluor488 (Invitrogen), or -Qdot655 (Invitrogen).

Blood processing. Peripheral blood mononuclear cells (PBMCs) were isolated from

ethylenediaminetetraacetic acid (EDTA)-preserved blood using Ficoll-paque (GE Healthcare)

density gradient centrifugation. PBMCs were collected from the buffy layer and washed twice

with a wash buffer of PBS (Gibco) supplemented with 2% fetal bovine serum (FBS, HyClone)

(wash buffer). When necessary, red blood cells were removed by lysis with ACK buffer (150mM

ammonium chloride, 10mM potassium bicarbonate, 30mM EDTA in water) for 3 minutes,

followed by one wash.

Sorting of epitope-specific CD8^{+} T cells for gene expression analysis. Peripheral blood was

collected weekly for 10 weeks post-inoculation with SIVmac251. In addition, blood was

collected a week prior to infection and on the day of infection (day 0) for sorting of total

CD8$^+$CD4$^-$CD3$^+$CD28$^+$CD95$^-$ naïve CD8$^+$ T cells. For each timepoint, 10 mL of blood was collected in EDTA, and PBMCs were isolated as described above. Cells were stained with p11C and p54AS tetramers for 15 min at 4°C in the dark. Cells were washed once and then stained with a mixture of CD3 and CD8 antibodies for 10 min at 4°C in the dark. Cells were washed, resuspended in cold PBS at 10^7 cells/mL, and sorted. Gates were set to include single CD3$^+$CD8$^+$ lymphocytes. The p11C$^+$ and p54AS$^+$ cells within these gates were sorted into RNAprotect (Qiagen) at 4°C. Sorting was performed using a FACS-Vantage flow cytometer/cell sorter (BD).

RNA extraction. RNA was isolated from sorted tetramer-positive cells using a Trizol (Invitrogen) extraction protocol. Briefly, the cell pellet was resuspended in 1 mL of Trizol and incubated for 5 min at room temperature (RT). A volume of 200 μL of chloroform was added and shaken vigorously by hand for 15 sec and then incubated at RT for 2 to 3 min. Samples were centrifuged at 13,000 rpm for 15 min at 4°C. The colorless upper aqueous phase was collected and transferred to a new tube containing 2 μL of linear acrylamide. An equal volume of isopropyl alcohol was then added and mixed. The mixture was incubated at RT for 10 min and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected, and the RNA was washed with 1 mL of 70% ethanol and centrifuged at 10,500 rpm for 5 min at 4°C. The supernatant was completely removed, and the RNA pellet was allowed to air-dry. The RNA was then resuspended in RNase-free water and stored at -80°C. RNA integrity was tested using an Agilent Bioanalyzer.

RNA amplification. RNA was amplified from Trizol-extracted total RNA using the TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0 (Epicentre Biotechnologies) according to the manufacturer's instructions. The amplification was comprised of two rounds, including two steps of cDNA synthesis and *in vitro* transcription (IVT). The first strand cDNA synthesis was primed with a T7-Oligo(dT) primer and was catalyzed by either Superscript II or III Reverse Transcriptase (Invitrogen). Then, double-stranded cDNA was synthesized from the first-strand template by a DNA polymerase, and the IVT was catalyzed by a T7 polymerase. The first IVT round ran for 4 hr and the second IVT round for 9 hr, with biotin-UTP added in the second IVT. For the first RNA purification, Zymo RNA Clean and Concentrator columns (Zymo Research) were used, and for the second RNA purification, RNeasy MinElute Cleanup Kit (Qiagen) was used. Amplified biotinylated antisense-RNA (aRNA) was resuspended in RNase-free water and stored at -80°C. A Nanodrop ND-1000 (ThermoScientific) was used to determine the aRNA concentration, and an Agilent Bioanalyzer was used to determine the aRNA integrity.

Plasma RNA viral loads. Plasma was collected from the top layer following ficoll density gradient centrifugation and frozen at -80°C until use. Viral RNA levels from these samples were measured using an ultra-sensitive branched DNA amplification assay (Bayer Diagnostics, Berkeley, CA).

Microarray processing. Amplified aRNA was hybridized to Illumina Human HT-12 Expression BeadChips according to the manufacturer's instructions and was stained with Streptavidin Cy3 for detection (Illumina, San Diego, CA, USA). The Human HT-12 BeadChip assays 48,000

transcripts. The BeadChips were built with sequences derived from the National Center for Biotechnology Information Reference Sequence (NCBI RefSeq) database (Build 36.2, Release 22). Arrays were scanned according to the manufacturer's instructions. Processing of the raw array data was performed using Illumina BeadStudio software.

Statistical analysis of gene expression. We used Matlab (Mathworks, Natick, MA, USA) to perform statistical analysis. The analysis began with quantile-normalizing of the whole expression data to remove system noise. Fold-differences of individual transcripts were computed as the ratios of the median expression levels of p11C- to those of p54AS-specific CD8$^+$ T cells. When p54AS expression values were larger than p11C values, the negative reciprocal was calculated. A Wilcoxon signed-rank test was used to evaluate the significance of the differences in gene expression between p11C- and p54AS-specific CD8$^+$ T cells.

Quantification of epitope-specific CD8$^+$ T cells. Freshly isolated PBMCs from chronically infected monkeys were resuspended in wash buffer, stained with tetramer for 15 min at RT in the dark, and then stained with a cocktail of CD3-, CD4-, and CD8-specific antibodies for 15 min at RT in the dark. Cells were washed once, resuspended in a solution of 2% paraformaldehyde (Polysciences) in PBS, and analyzed by flow cytometry.

Cell surface maturation phenotyping. Cells were stained as described for the quantification of epitope-specific CD8$^+$ T cells above. The staining cocktail contained CD3-, CD8-, CD4-, CD20-, CD27-, CD28-, CCR7-, and CD45RA-specific fluorochrome-labeled antibodies. Fluorescence

minus one (FMO) samples were also prepared to set the analysis gates for positive CD27 and

CD45RA staining. In these FMO samples, either the CD27- or the CD45RA-specific antibody was

omitted from the cocktail.

Ex vivo intracellular staining of perforin and granzyme B. Cells were stained as described for

the quantification of epitope-specific CD8^{+} T cell populations above. The staining cocktail

contained CD3-, CD4-, and CD8-specific antibodies. Cells were then washed twice, resuspended

in Cytofix/Cytoperm (BD Biosciences), and incubated for 20 min at 4°C. Cells were washed twice

with a 1X solution of Perm/Wash buffer (BD), resuspended in a solution of 1X Perm/Wash

buffer containing perforin- and granzyme B-specific antobodies and incubated for 30 minutes at

RT in the dark. FMO samples were also prepared to set the analysis gates for positive perforin

and granzyme B staining. In these FMO samples, either the perforin- or the granzyme B-specific

antibody was omitted from the cocktail. Cells were washed twice with 1X Perm/Wash buffer,

resuspended in a solution of 1% paraformaldehyde in PBS, and then analyzed by flow

cytometry.

Cytokine and chemokine production analysis. Freshly isolated PBMCs were resuspended in

R10, a medium containing RPMI 1640 (Cellgro) supplemented with 10% heat-inactivated(HI)

FBS and the antibiotics penicillin and streptomycin (Pen/Strep, Gibco), and rested for 1 hr at

37°C. Cells were washed with R10 and resuspended in a solution of 1 μg/mL each of anti-CD28

(BD) and anti-CD49d (BD) antibodies, 1 nM peptide, and tetramer in R10. Alternatively, control

cells were either left unstimulated or stimulated with a final concentration of 10 ng/mL phorbol

12-myristate 13-acetate (Sigma) and 1 μg/mL ionomycin (Sigma). Cells were incubated at 37°C for 6 hours, with the addition GolgiStop (BD Biosciences) and Golgiplug (BD) at a final dilution of 1:1,500 and 1:1,000, respectively, after the first hour. Following the 6 hour stimulation, cells were stored overnight at 4°C. Cells were then washed once with wash buffer, resuspended in PBS and aqua LIVE/DEAD (Invitrogen) at a final dilution of 1:500, and incubated for 20 min at RT in the dark. Cells were washed once with PBS and then stained following the *ex vivo* intracellular staining protocol above. The cell surface staining included tetramer and antibodies specific to CD4 and CD8, and the intracellular antibodies cocktail contained specific to CD3, CD69, IL-2, IFNγ, TNFα, and MIP-1β.

Expansion of epitope-specific CD8^+ T cells. Following isolation of fresh PBMCs, cells were resuspended in R10. The lymphocyte concentration was measured using the Guava EasyCyte automatic cytometer (Millipore) and then adjusted to approximately $3x10^6$ lymphocytes/mL . A small sample of cells was also stained with tetramer and antibodies for CD3, CD4 and CD8, and analyzed by flow cytometry to measure the frequency each CD8^+ T cell specificity before peptide-stimulated expansion. Cells were plated in either 96-, 48-, or 24-well flat bottomed culture plates and stimulated with a final concentration of 1 nM epitope peptide. A final concentration of 20 U/mL IL-2 (Hoffmann-LaRoche) was added on day 3, and media supplemented with IL-2 was changed thereafter as needed. Samples were harvested on days 3, 4, 5, 6, 8, 10, 12, and 14 post-stimulation, resuspended in PBS, and stained with aqua LIVE/DEAD. Cells were washed and stained with tetramer and CD3-, CD4-, and CD8-specific

fluorochrome-labeled antibodies. Cells were washed once, resuspended in a solution of 2%

paraformaldehyde, and analyzed by flow cytometry.

Flow cytometry data collection and analysis. All flow cytometry data were collected on an LSRII

(BD). In order to collect and analyze comparable numbers of each CD8$^+$ T cell specificity,

different numbers of PBMCs were used for each p11C, p54, and p68A sample. Input numbers of

PBMCs were dependent on the expected relative frequency of each epitope-specific

population. For example, if the expected magnitudes of the p11C-, p54-, and p68A-specific CD8$^+$

T cell populations were 10%, 1%, and 0.1%, respectively, then the ratio of cells used for each

sample was 1:10:100. Staining volumes, washing volumes, and amounts of staining reagents

used were adjusted for each sample to ensure the same cell-to-reagent concentration ratios

were used for all samples. Flow cytometric data was analyzed using FlowJo (Tree star).

Statistical analysis of flow cytometric data was conducted in GraphPad Prism 5.

ACKNOWLEDGEMENTS

Role of pMHC:TCR Binding Interactions in Determining CD8$^+$ T Cell Immunodominance Hierarchies in SIV-Infected Rhesus Monkeys

INTRODUCTION

It is believed that an HIV vaccine strategy aimed at eliciting highly-functional epitope-specific CD8^{+} T cells will need to elicit CD8^{+} T cells that are of high frequency [71, 96, 98, 533, 534] and that broadly recognize multiple HIV-derived epitopes [297, 535]. However, the phenomenon of immunodominance may limit the potential of current CD8^{+} T cell-based vaccine strategies by restricting the number of vaccine-encoded epitopes that are able to elicit CD8^{+} T cell responses and by limiting the frequencies of these vaccine-elicited CD8^{+} T cells. Therefore, an effective vaccine must be able to overcome these limitations imposed by immunodominance in order to generate high-frequency CD8^{+} T cells of broad specificity. To do this, a better understanding of the factors underlying the establishment of immunodominance hierarchies is required.

The strength and duration of the interactions between an epitope peptide and its restricting MHC molecule, as well as the interaction between a peptide:MHC class I complex (pMHC) and its cognate TCR influence the potency of the T cell response. Parameters including pMHC cell surface density [380, 386, 387, 536], pMHC:TCR affinity [388, 398-400], and pMHC:TCR dissociation [393, 401-409] have all been shown to determine the potential and extent to which a particular T cell becomes activated. Such factors also determine the extent to which the activated T cell, in response to antigen stimulation, exhibits cytotoxic capabilities [353, 386-388, 398, 399, 402, 403, 536], produces cytokines [380, 398, 400, 406, 408, 409, 536], or proliferates [393, 404, 407, 536].

The clear role that the peptide:MHC:TCR interactions have on determining T cell activation and proliferation suggests that differences in these interactions may play a role in determining the immunodominance hierarchy of multiple epitopes restricted by the MHC class I allele. Indeed, the peptide:MHC interaction, and specifically its dissociation rate, is frequently correlated with epitope dominance [378, 443-448]. Interestingly, few studies have assessed the impact of the pMHC:TCR interaction on immunodominance hierarchies. One study in the murine herpes simplex virus model used pMHC tetramers to measure dissociation rates of tetramers constructed with one dominant and one subdominant epitope peptide from their respective TCRs. Although there was a weak trend that the tetramer constructed with the subdominant epitope displayed a faster dissociation rate from the TCR compared to the tetramer with the dominant epitope, the authors concluded that these dissociation rates were similar [537]. The use of tetramers, which decreases the apparent dissociation rate though avidity effects, likely limited their ability to resolve dissociation rate differences between the two epitopes. Another study in the murine influenza virus model also used tetramers to measure the dissociation rate of a dominant and two subdominant epitopes from their respective TCRs. This study found an association between epitope dominance and dissociation rate [538]. However, the TCR had a faster dissociation rate from the dominant epitope tetramer than from the subdominant epitopes. This was opposite of the hypothesized relationship between epitope dominance and pMHC:TCR dissociation based on previous studies showing an association between antigen-induced cell proliferation and the pMHC:TCR dissociation rate [393, 404, 407, 536]. Both of these studies were conducted in murine systems and used tetramer dissociation as a surrogate measurement of the strength of pMHC:TCR interactions.

The role of pMHC:TCR interactions in determining immunodominance hierarchies in humans remains largely unexplored.

To date, the tetramer-binding assay has been the most frequently used technique to measure pMHC:TCR interactions because it provides the convenience of being used on intact cells [406, 539, 540]. This is useful because it allows for the measurement of the pMHC:TCR interaction to be performed on a polyclonal population of cells. It also allows the TCR to be maintained in its physiological context (embedded in the outer membrane of the CD8^{+} T cell and accompanied by its associated surface molecules that contribute to the overall interaction). However, its utility in accurately predicting physiologic pMHC:TCR binding affinities is limited [541, 542]. In addition, the multiple valency of the tetramer and their propensity to aggregate can complicate the accurate measurement of these interactions [543]. In contrast, surface plasmon resonance (SPR) has been the gold standard for the measurement of overall affinity and also association and dissociation rates of almost any molecular interaction [544-548]. However, until recently, measurements of pMHC:TCR interactions using SPR has been limited to the study of a few individual recombinant TCRs [409, 549, 550]. Although this is useful to compare functional outcomes of individual T cell clones, it is not conducive to studying entire epitope-specific CD8^{+} T cell populations generated *in vivo*, which are almost always polyclonal.

Our laboratory has recently developed an SPR-based technique that permits measurement of the TCR interaction of polyclonal epitope-specific CD8^{+} T cell populations for monomeric pMHC complexes [551]. In the present study, we have employed this technique to evaluate the role of pMHC:TCR binding affinity in determining the well-defined Mamu-A*01-

restricted epitope immunodominance hierarchy in SIV-infected rhesus monkeys. We found that

the frequencies of the different epitope-specific CD8$^+$ T cells within this hierarchy were

associated with the various parameters involved in the pMHC:TCR interaction. Specifically, we

found that greater epitope dominance was associated with slower pMHC:TCR dissociation rates

and higher affinities. These findings indicate a role of the pMHC:TCR interaction in determining

immunodominance hierarchies and suggest that manipulation of this interaction may be a

means by which the frequencies of epitope-specific CD8$^+$ T cells can be enhanced for

immunotherapy and vaccine design.

RESULTS

We evaluated the role of pMHC:TCR binding in determining immunodominance hierarchies using a cohort of seven SIVsmE660-infected *Mamu-A*01*^{+} rhesus monkeys. The frequencies of the dominant p11C- and subdominant p54E660- and p68A-specific CD8^{+} T cells in these monkeys had been previously measured and are shown in Figure 5B in Chapter 2. We chose to measure the pMHC:TCR interactions using a novel SPR-based technique in which the TCRs being evaluated were derived from polyclonal epitope-specific CD8^{+} T cell populations sorted directly *ex vivo* from infected monkeys. This technique involved the use of a mild detergent to generate detergent-resistant micro-domain (DRM) preparations that were enriched in TCRs. This technique also permitted immobilization of the TCR on the Biacore™ L1 chip in a more physiological setting surrounded by associated membrane proteins, such as CD8, which were free to move laterally within the membrane lipid environment [551]. The technique was initially validated using large numbers of T cell hybridomas. This guaranteed that large numbers of TCRs would be bound to the Biacore™ L1 chip and that the strength of the signal from the binding of the soluble monomeric pMHCs to these TCRs would be sufficient for analysis. However, *ex vivo* epitope-specific CD8^{+} T cells, such as those that were the focus of our studies, are often present at very low frequencies in the peripheral blood. Therefore, the DRMs that would be prepared from peripheral blood samples would contain lower numbers of TCRs compared to DRMs prepared from T cell hybridomas. It was previously unknown whether these small numbers of TCRs would be sufficient to detect a specific binding signal by SPR. Therefore, we first determined if we could detect a signal from small numbers of epitope-specific CD8^{+} T cells.

We used pMHC tetramers to sort by flow cytometry 1×10^3, 3×10^3, 5×10^3, and 10×10^3 epitope-specific CD8$^+$ T cells from peripheral blood of SIV-infected monkeys. Each of these sorted cell preparations were added to a sample of 1×10^6 total CD8$^+$ T cells. DRMs were purified from these samples and captured on a Biacore™ L1 chip, and the binding of soluble pMHC monomers in the fluid phase to the TCRs in these DRM preparations was measured. Binding of a control pMHC monomer was also evaluated to establish the level of non-specific binding. This control pMHC monomer was constructed with the same MHC as the experimental monomer, but bound to a peptide which the CD8$^+$ T cells in these samples did not recognize. We detected specific signals of similar magnitude from the samples containing 10×10^3, 5×10^3, and 3×10^3 epitope-specific CD8$^+$ T cells (Fig. 10). A weaker signal was detected in the sample containing 1×10^3 epitope-specific CD8$^+$ T cells. Therefore, we determined that we would need a minimum of 3×10^3 epitope-specific CD8$^+$ T cells to detect a specific signal, although detection of a specific signal would likely also be dependent on the affinity of the TCR in question.

Epitope-specific CD8$^+$ T cells for the following experiments were isolated by flow cytometry. Cells were stained for sorting with anti-CD3 and -CD8 antibodies and a tetramer constructed with the Mamu-A*01-resticted SIV epitope TL8. Any cells that stained positively with this tetramer were excluded from the sorted CD3$^+$CD8$^+$ cells. A monomer constructed with the TL8 epitope peptide was used as a negative binding control in the SPR assays such that any

Figure 10- Determination of minimum input of epitope-specific CD8$^+$ T cells required for SPR analysis
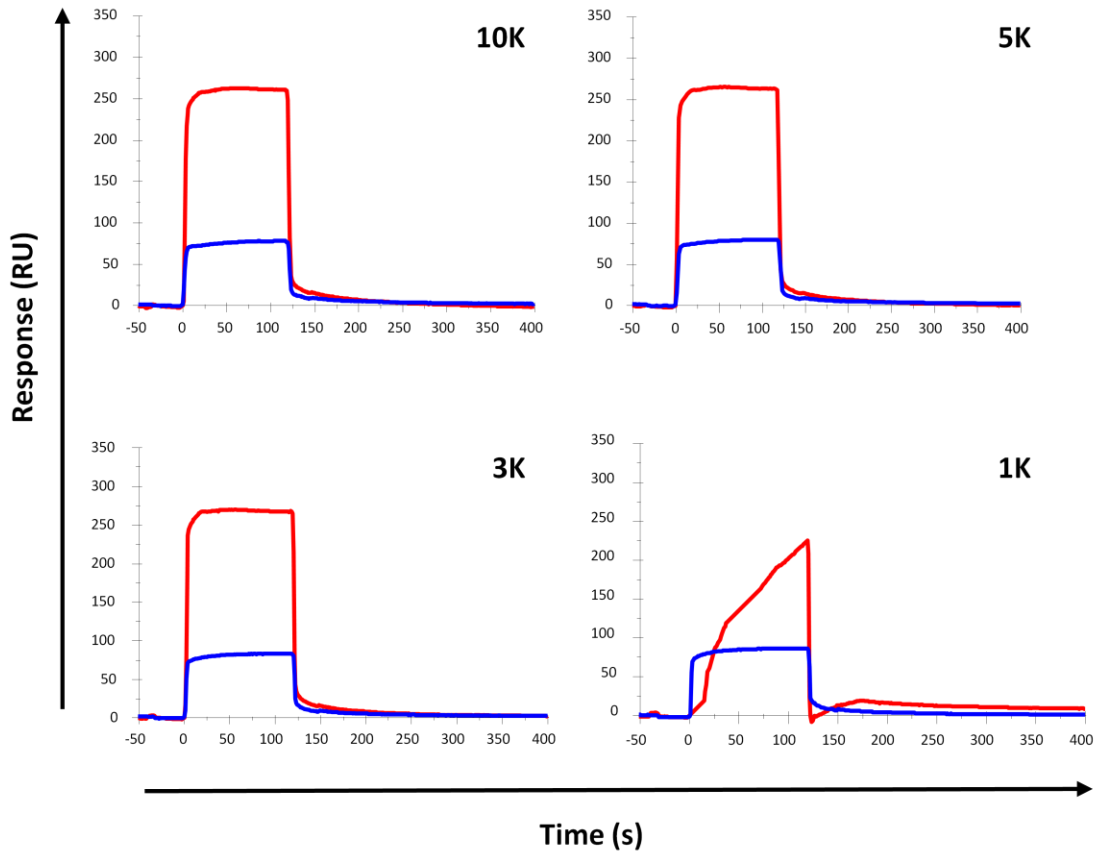

1x10^3 (1K), 3x10^3 (3K), 5x10^3 (5K), and 10x10^3 (10K) epitope-specific CD8$^+$ T cells were sorted, and each cell population was added to 1x10^6 total CD8$^+$ T cells. DRMs were purified and captured on a Biacore™ L1 chip. Wildtype (red) and control (blue) pMHC monomers were flowed over these DRMs at 100 µg/mL and specific binding, measured in resonance units (RU), was detected.

signal detected from the binding of the TL8 monomer was considered non-specific and was subtracted from the signals of the other monomers.

For initial measurements of the binding of the p11C-, p54E660- and p68A-specific TCRs to their respective pMHC complexes, we sorted total TL8-negative CD8^{+} T cells from seven *Mamu-A*01^{+}* SIVsmE660-infected monkeys. A small amount of cells was also stained with p11C, p54E660, and p68A tetramers to measure the frequency of each CD8^{+} T cell specificity in the sample. From these frequencies, we estimated the total number of each CD8^{+} T cell specificity in our sorted sample. The estimations for each CD8^{+} T cell specificity were greater than 3,000 for all samples. Initial binding assays with DRMs purified from these samples demonstrated that specific binding could be detected from the monomers constructed with the dominant p11C and subdominant p54E660 epitope peptides. The binding signal from the p54E660 monomer was weaker than p11C monomer binding at the same concentration. However, we were unable to detect specific binding of the monomers constructed with the more subdominant p68A epitope peptide at any of the concentrations of monomers that were evaluated (Fig. 11). We then performed titrations of the p11C and p54E660 pMHC monomers on the DRM preparations from each monkey, and we were able to detect specific binding within the range of concentrations evaluated (25 to 200 µg/mL for p11C monomers and 50 to 200 µg/mL for p54E660 monomers) (Fig. 12). From these titrations, we used a curve fitting analysis to calculate the association rate (k_{on}) and the dissociation rate (k_{off}) of both the p11C and p54E660 pMHC monomer binding to DRMs from all seven monkeys (Table 3). The p11C monomers bound to the DRM samples with a median k_{on} of 7.38×10^3/Ms (range $3.56\text{-}28.60 \times 10^3$/Ms), which was faster than the p54E660 monomers that had a median k_{on} of 0.96×10^3/Ms

Figure 11- Detection of p11C, p54E660, and p68A monomer binding
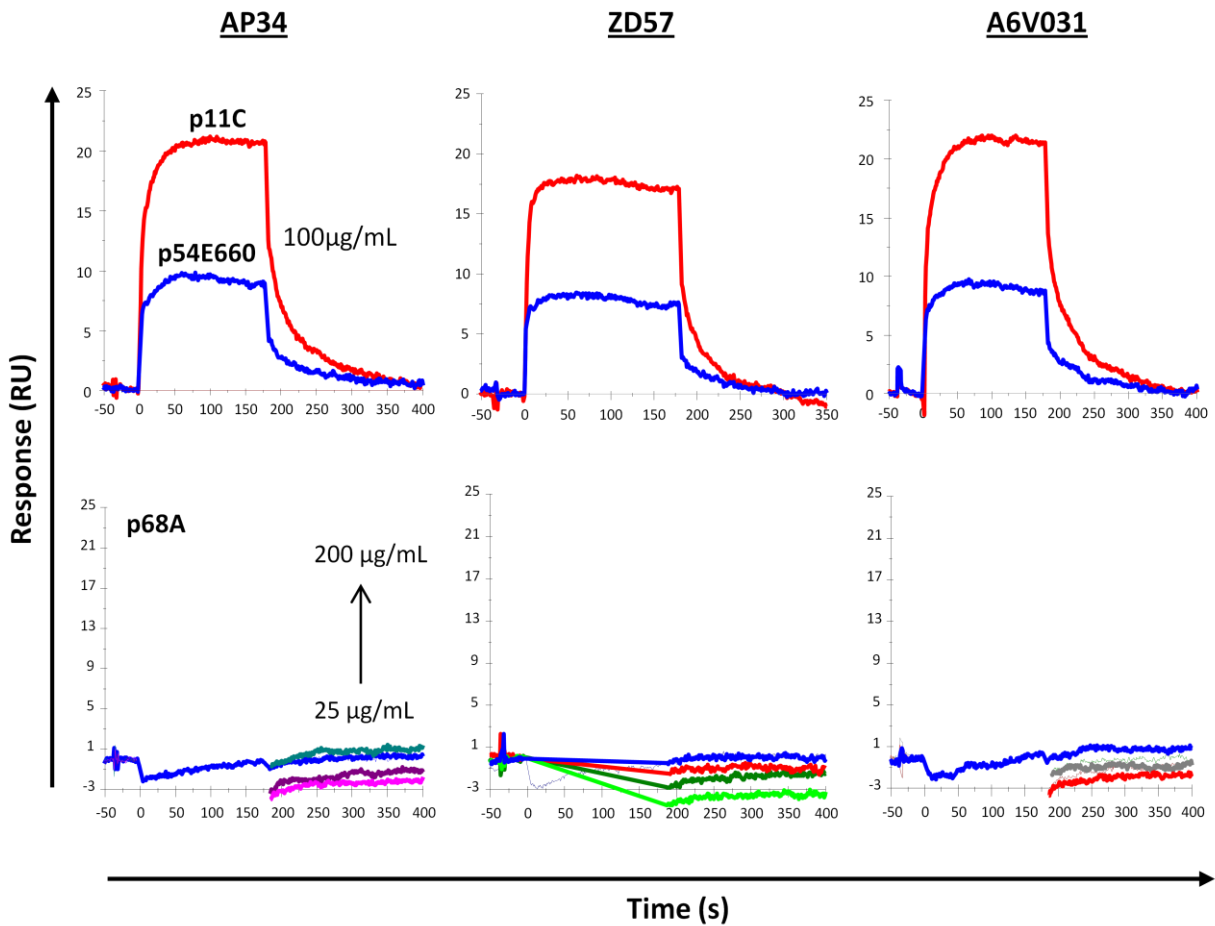
Total CD8$^+$ T cells were sorted from SIVsmE660-infected rhesus monkeys. DRMs isolated from these cells were evaluated for specific binding with pMHC monomers constructed with p11C, p54E660, and p68A epitope peptides. Representative data are shown for binding of monomers to DRMs prepared from three monkeys (AP34, ZD57, and A6V031). Top, overlaid readings of the binding of p11C (red) and p54E660 (blue) pMHC monomers at 100 µg/mL. Bottom, overlaid readings of the binding of p68A pMHC monomers at the following concentrations: 25, 50, 100, 150, and 200 µg/mL. Binding of the control monomer TL8 at the same concentration has been subtracted from all readings.

Figure 12
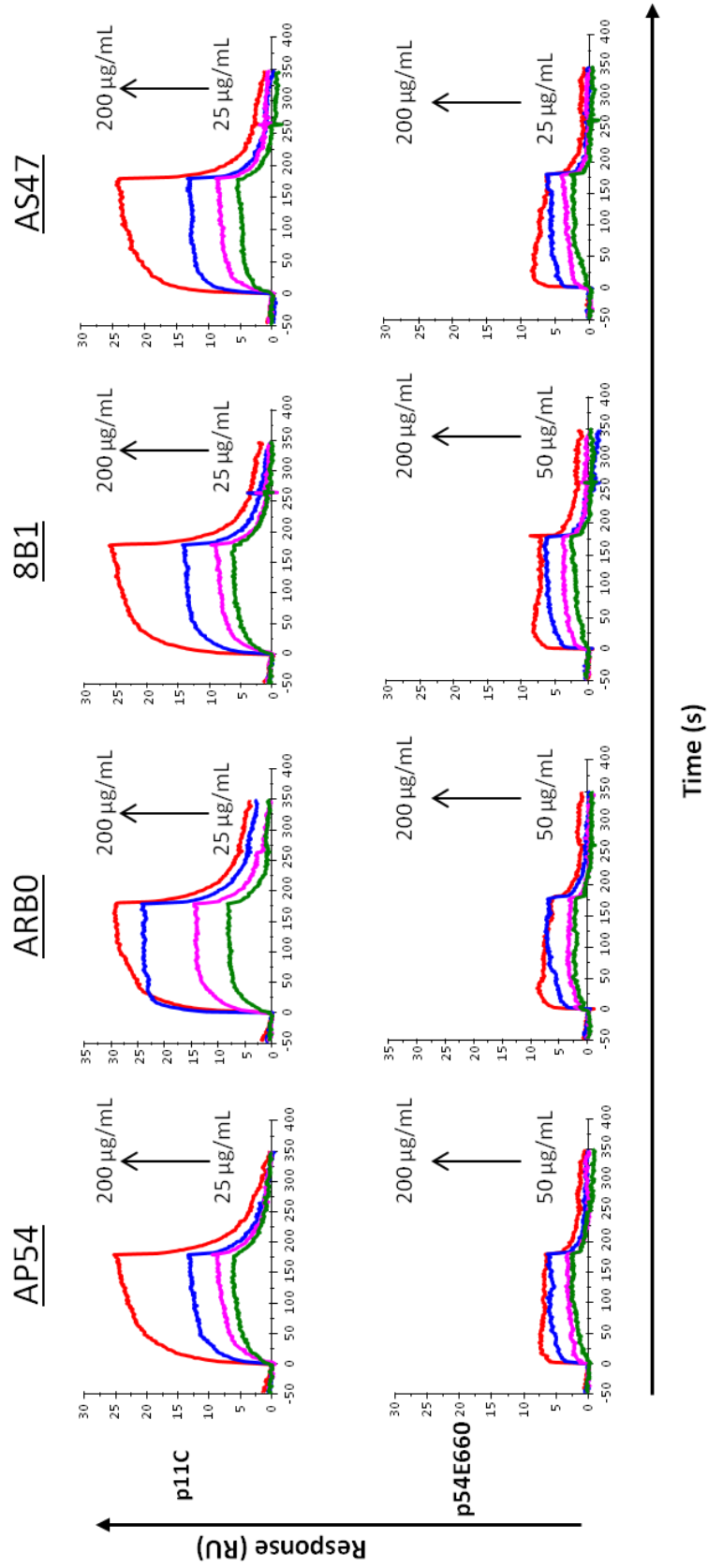
Figure 12 (Continued)
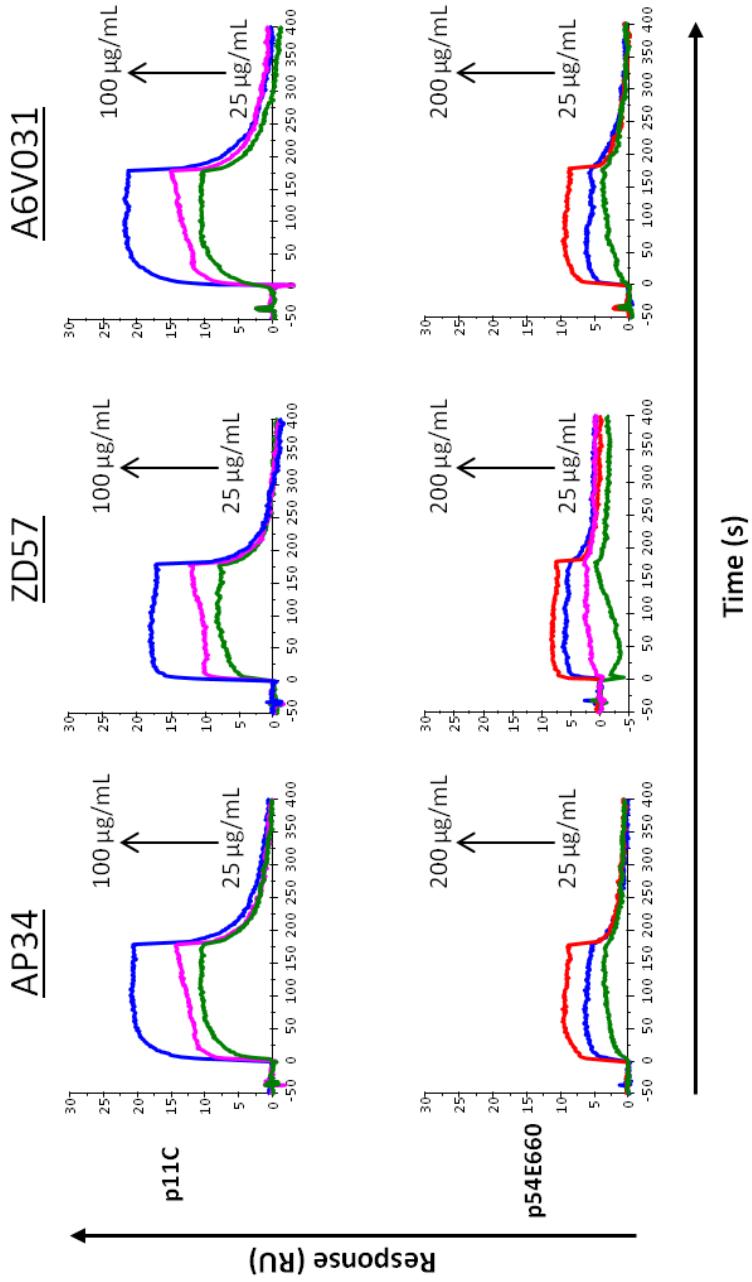
Figure 12 (continued)- p11C and p54E660 monomer titrations for calculation of kinetics and affinity

Binding of p11C and p54E660 pMHC monomers to DRMs purified from CD8^{+} T cells sorted from SIVsmE660-infected rhesus monkeys. Top panels, p11C monomer binding. Bottom panels, p54E660 monomer binding. Binding of the control monomer TL8 at the same concentration has been subtracted from all readings. p11C monomers were run at 25, 50, 100, and 200 µg/mL. The ARB0 plot shows a 150 µg/mL run in place of the 100 µg/mL. The AP34, ZD57, and A6V031 plots do not show the 200 µg/mL run. p54E660 monomers were run at 25, 50, 150, and 200 µg/mL for AP54, ARB0, 8B1, and AS47 and at 25, 100, and 200 µg/mL for AP34, ZD57, and A6V031. The ZD57 plot includes an additional 50 µg/mL run.

(range 0.66-1.87x10^3/Ms). The p11C monomers also dissociated more slowly from the DRMs with a median k_{off} of 0.02/s (range 0.02-0.03/s) compared to the p54E660 monomers that dissociated with a median k_{off} of 0.03/s (range 0.02-0.06/s). The equilibrium dissociation constant (K_d) values were derived from these k_{on} and k_{off} values. Binding of the p11C monomers had a median K_d of 2.01μM (range 1.03-6.77μM), which was lower than the binding of the p54E660 monomers which had a median K_d of 32.00 μM (range 22.00-43.00μM). Thus, monomers constructed with the dominant p11C peptide epitope showed faster association rates and slower dissociation rates that resulted in higher affinities (lower K_d) compared to monomers constructed with the subdominant p54E660 epitope peptide.

We speculated that the inability to detect the binding of the p68A monomers to the DRM samples may be due to the interaction between the p68A pMHC complex with cognate TCRs being substantially weaker than the binding of the p11C and p54E660 pMHCs to their cognate TCRs. The difficulty in detecting this weak binding was also likely exacerbated by the fact that the p68A-specific CD8^{+} T cells were the lowest frequency epitope-specific CD8^{+} T cells, and therefore the p68A-specific TCRs were the least represented in the DRMs. Therefore, we repeated the sorting of CD8^{+} T cells from multiple bleeds from four of the SIVsmE660-infected monkeys and the cells from these sorts were pooled for DRM purification. These samples contained two- to six-fold more p68A-specific CD8^{+} T cells than samples from the previous experiments.

We were able to detect a specific binding signal for p68A pMHC monomer binding to the DRM samples containing greater numbers of p68A-specific CD8^{+} T cells and with the pMHC

Figure 13- Detection of p68A monomer binding
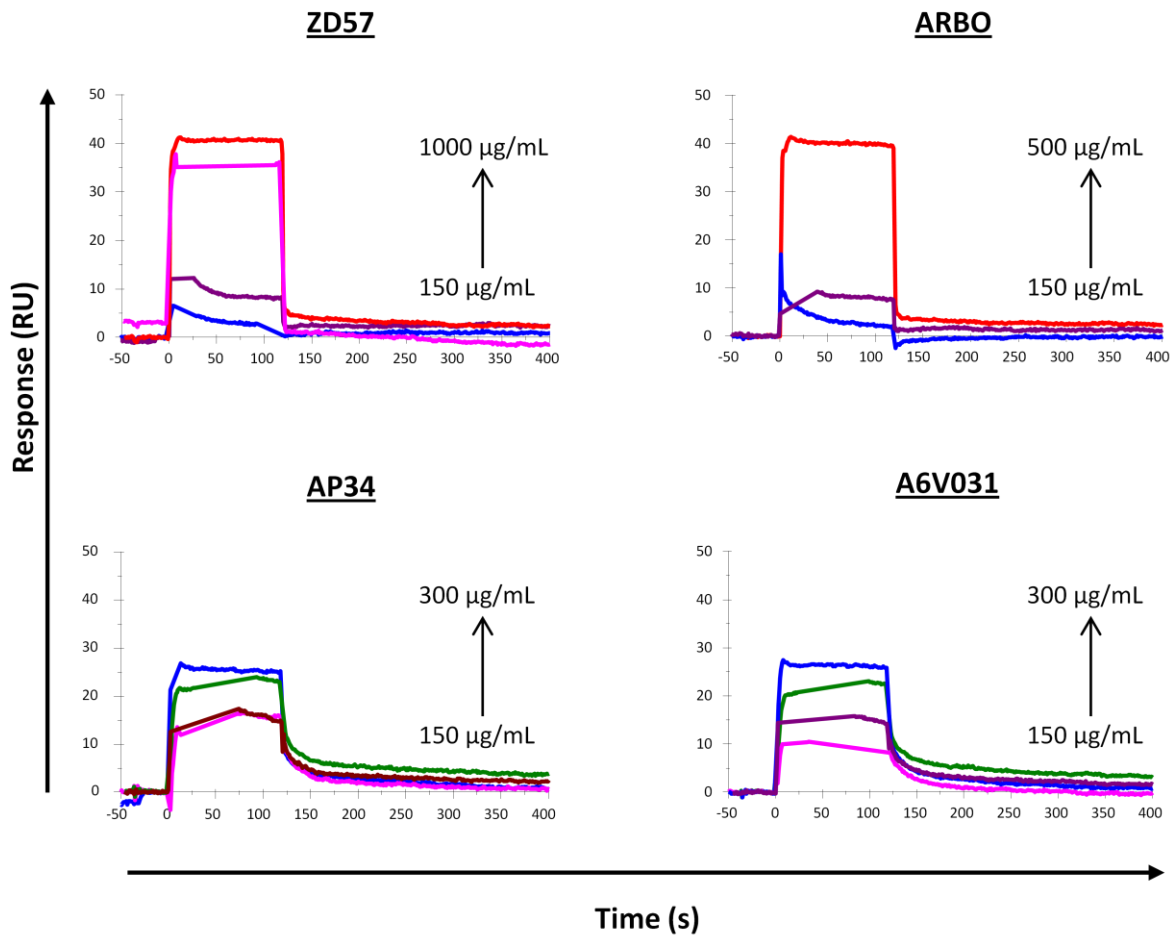
p68A-specific CD8^{+} T cells were collected from multiple tetramer sorts and combined for DRM isolation. Titrations of p68A pMHC monomers were performed at 150, 200, 500, and 1000 µg/mL for ZD57, 150, 200, and 500 µg/mL for ARB0, 150, 200, 250, and 300 µg/mL for AP34, and 150, 200, 250, and 300 µg/mL for A6V031. Binding of the control monomer TL8 at the same concentration has been subtracted from all readings.

injected at higher concentrations (>100 µg/mL) (Fig 13). However, the binding of these

monomers exhibited extremely fast association and dissociation rates that could not be

measured using curve fitting analysis, although we were able to estimate the binding values.

Previous measurements of p11C and p54E660 monomer binding to DRMs indicated that p11C

had the fastest association rate. The association rate of the p68A monomer to its respective

TCRs was likely to be at least at fast as that of the quantifiable p11C monomer, although more

likely to be even faster. Therefore, we estimated that the k_{on} for p68A would be greater than

10×10^3/Ms. Knowing that the Biacore™ instrument's lower limit of detection of dissociation is

about 1/s, we estimated that the dissociation of the p68A monomer from its respective TCR

was at least as fast as 1/s. Using these estimated k_{on} and k_{off}, the estimated affinity of the p68A

monomer for its respective TCRs would approach at least 100 µM.

The estimated values for p68A monomer binding to DRMs are included in Table 3 for

comparison with p11C and p54E660 monomers. Although the p11C monomers bound their

cognate TCRs faster than p54E660 monomers, p68A monomers bound with the fastest

association rate. In addition, the dissociation rates of the p11C monomers were slower than

p54E660 monomers; p68A monomers had the fastest dissociation rate. Therefore, the

calculated K_d values were lowest for p11C monomers, intermediate for p54E660 monomers,

and highest for p68A monomers. In summary, epitope dominance was associated with higher

affinities that were driven by the faster association rate of the dominant p11C epitope and the

exceptionally fast dissociation rate of the more subdominant p68A epitope.

Table 3- pMHC:TCR binding values

	k_{on} (x10^{3}M^{-1}s^{-1})	k_{off}(s^{-1})	K$_d$(μM)
p11C	7.38 (3.56-28.60)	0.02 (0.02-0.03)	2.01 (1.03-6.77)
p54E660	0.96 (0.66-1.87)	0.03 (0.02-0.06)	32.00 (22.00-43.00)
p68A	≥ 10[a]	≥ 1[b]	≥ 100[c]

k_{on}, association rate. k_{off}, dissociation rate. K$_d$, equilibrium dissociation constant. Values indicate the median among readings from seven monkeys. Parentheses indicate range. [a] k_{on} of the p68A monomers was estimated as being at least as fast as the fastest measured monomer, p11C. [b] k_{off} of p68A monomers was estimated as being at least as fast as the limit of detection of the Biacore™ instrument, 1/s. [c] K$_d$ of p68A monomer binding calculated using estimated values of the k_{on} and k_{off} using the equation k_{off}/k_{on}.

DISCUSSION

In the present study, we have addressed the previously unexamined role of the strength of pMHC:TCR binding in determining the differences in frequencies of epitope-specific CD8^{+} T cells that underlie the establishment of immunodominance hierarchies. We found that the SIV epitope-specific CD8^{+} T cells in rhesus monkeys that were of higher frequency expressed TCRs that had higher affinities for their pMHC complexes. These differences in affinity were driven by differences in both association and dissociation rates.

These findings were made possible using a novel SPR-based technique that employs the Biacore™ L1 chip to immobilize cell membrane preparations, referred to as DRMs, onto the solid sensor surface [552]. This technique has many advantages over SPR-based approaches previously used to study pMHC interactions with TCRs. The technique we employed in the present study did not limit us to assessing the pMHC affinity of only one or a few cloned TCRs. Rather, we were able to measure the average pMHC:TCR affinity of a polyclonal population of epitope-specific CD8^{+} T cells sampled *ex vivo*. The lipophilic anchor of the L1 chip directly embeds itself into lipid bilayers, obviating the need for artificial adsorption of membrane preparations onto the sensor chip. Other cell surface proteins involved in TCR engagement with pMHC and TCR signaling, such as CD8, CD3, CD2, LFA-1, and CD28, likely also are enriched in the DRM fraction and anchored to the chip with the TCR. Finally, the TCRs were able to move laterally in the immobilized lipid membrane, allowing the assessment of their interactions in a physiological context. This SPR technique, therefore, enables the measurement of the affinity of pMHC for TCRs expressed in as close to their native environment as possible.

The mechanism by which the pMHC:TCR binding affinity or its kinetics may influence the frequencies of dominant and subdominant SIV epitope-specific CD8^{+} T cells is unclear. The most intuitive explanation is that TCR affinity and/or kinetics influence the rate of cellular expansion upon antigen recognition. Indeed, this explanation is supported by evidence from multiple *in vitro* and *in vivo* studies. A study by Dzutsev and colleagues sorted from mice CD8^{+} T cell clones that recognized the H-2D[d]-restricted HIV Env p18 epitope either with either high or low affinity. Naïve mice were administered either the high or the low affinity p18-specific CD8^{+} T cells and then immunized with a vaccinia virus (VV) encoding the p18 epitope to stimulate proliferation of the p18-specific CD8^{+} T cells. Cell division was measured by the amount of BrdU incorporated into the proliferating cells following administration of the VV. They found that the high affinity population incorporated more BrdU than the low affinity population, demonstrating that the high affinity clones divided more rapidly than the low affinity clones. This study demonstrated that TCR affinity affects the rate of proliferation [553].

Another study by Schmid and colleagues generated TCRs that recognized the same pMHC, but with varying affinities. These different-affinity TCRs were expressed in a T cell line and loaded with CFSE, and the T cells' abilities to proliferate following stimulation with APCs expressing their cognate epitopes were assessed by CFSE dilution *in vitro*. T cells expressing the higher affinity TCRs proliferated more extensively than those expressing the lower affinity TCRs, as demonstrated by greater CFSE dilution of the cells expressing the higher-affinity TCRs [554]. Thus, this study is another demonstration of the role of TCR affinity in determining epitope-elicited proliferation rate of CD8^{+} T cells.

Finally, a study by Hommel and colleagues performed a highly quantitative *in vitro* CFSE-based study to measure the differences in proliferation and cell death of OVA-specific OT-1 TCR transgenic CD8^{+} T cells when stimulated by altered peptide ligands (APLs) of different affinities for the OT-1 TCR. They found that stimulation with high-affinity APLs was associated with an increased rate of proliferation that resulted from both higher numbers of cells entering the cell cycle following stimulation and lower rates of cell death during the proliferation stage [555].

An alternative explanation for the differences in frequency of dominant and subdominant epitope-specific CD8^{+} T cells was provided by a study by Zehn and colleagues that evaluated the *in vivo* expansion of OT-1 CD8^{+} T cells in response to a low and a high affinity APLs. CFSE-labeled OT-1 CD8^{+} T cells were grafted into mice and these mice were infected with a strain of *Listeria monocytogenes* that expressed either the low or the high affinity APL. By measuring CFSE dilution of the stimulated OT-1 CD8^{+} T cells *in vivo,* this study found that OT-1 cells proliferated at the same rate regardless of whether they were stimulated with the high or the low affinity APL. However, the OT-1 cells stimulated by the low affinity ligand stopped proliferating before the OT-1 cells stimulated by the high affinity ligand [556]. Therefore, the differences in frequency of the OT-1 cells that were observed following high versus low affinity ligand stimulation were a result of differences in the duration of the expansion period, and not the rate of expansion.

The remarkably rapid dissociation rate, and consequently the lower affinity, of the p68A pMHC interaction with its TCR observed in our study raises the question of how such a weak interaction can elicit an epitope-specific CD8^{+} T cell response. Until recently, it was generally

accepted that more potent TCR ligands were those exhibiting slower rates of dissociation from the TCR [403, 405-409]. Slow dissociation rates were thought to better enable more complete signal transduction through the TCR and thus allow complete T cell activation. However, some studies have described interactions where T cells were potently activated by TCR ligands even though they had relatively fast TCR dissociation rates [398, 557-560]. Recent studies have attempted to reconcile this apparent discrepancy and found that the potency of ligands with very fast dissociation rates can be compensated by very fast association rates [420, 422]. In these studies, it was found that potent $CD8^+$ T cell stimulation with ligands displaying fast dissociation rates was dependent on multiple re-binding events of the same pMHC complex with the same TCR. The faster association rates of these ligands thus allowed these re-binding events to occur after rapid dissociation, before the pMHC or TCR diffused away laterally in the membrane. Therefore, the considerably fast dissociation rate of the p68A pMHC complex measured in the present study may be compensated by the considerably fast association rate to allow the p68A epitope to elicit a $CD8^+$ T cell response.

The present study found that the interaction of the dominant p11C pMHC with its TCR was characterized by K_d values of approximately 2μM and k_{off} values of approximately 0.02/s. Other studies in our laboratory have recently used the same SPR technique to measure the pMHC:TCR interaction of the dominant epitope peptide p199RY restricted by the rhesus MHC class I molecule Mamu-A*02. In these studies, the interaction of the dominant p199RY pMHC with its TCR was characterized by similar K_d values ranging 1.1-1.5 μM and similar k_{off} values ranging from 0.01-0.03/s [551]. In that study, however, the binding of subdominant Mamu-A*02-restricted epitope peptide pMHCs were not evaluated. Yet, the similarities in the affinities

and the dissociation rates between the Mamu-A*01- and Mamu-A*02-restricted dominant epitopes further substantiate the concept that pMHC:TCR binding may influence the dominance of an epitope peptide. We speculate that measurement of a Mamu-A*02-restricted subdominant pMHC:TCR binding interaction would show that it has affinities and kinetics similar to that of the subdominant p54E660 pMHC:TCR binding measured in the present study.

In summary, the data in the present study demonstrated a relationship between the affinity of a TCR for its pMHC and the frequency of those epitope-specific CD8^{+} T cells in SIV-infected rhesus monkeys. The precise mechanism by which TCR affinity determines the frequency of epitope-specific CD8^{+} T cells is unclear, although it likely relates to the ability of the cells to expand. A high frequency of epitope-specific CD8^{+} T cells are thought to be required for optimal protection from pathogens such as HIV. Therefore, the pMHC:TCR interaction may be able to be manipulated by vaccines or immunotherapies to enhance the frequencies of epitope-specific CD8^{+} T cells, potentially enhancing protection.

MATERIALS AND METHODS

Monomers and tetramers. The following peptides were synthesized by New England Peptide,

LLC and were purified at >95% by HPLC: p11C (CTPYDINQM), p54AS (TVPWPNASL), p54E660

(TVPWPNETL), p68A (STPPLVRLV), and TL8 (TTPESANL). The p11C, p54AS, p54E660, p68A, and

TL8 peptide-Mamu-A*01 monomeric and tetrameric complexes were prepared as previously

described [531, 532]. Monomers used in surface plasmon resonance studies were further

quantified using an *RC DC* protein kit (Bio-Rad). Tetrameric complexes for flow cytometry were

prepared using either streptavidin-PE (Prozyme), -APC (Prozyme), -AlexaFluor488 (Invitrogen).

Preparation of detergent-resistant micro-domains (DRMs). Freshly isolated PBMCs were

enriched for CD8$^+$ T lymphocytes using Miltenyi's magnetic-activated cell sorting (MACS) kit for

isolation of untouched NHP CD8$^+$ T cells according to the manufacturer's instructions. Isolated

CD8$^+$ T cells were then stained at 4°C with tetramers and with CD3- and CD8-specific

fluorochrome-labeled antibodies. Cells were resuspended in PBS at a concentration of 10^7/mL

for sorting. Sorting was performed on an Aria flow cytometer/cell sorter (BD). Cells were sorted

at 4°C into 2% FBS in PBS. Gates were set for single CD3$^+$CD8$^+$ lymphocytes. For initial TCR

binding analyses, cells were only stained with the TL8 tetramers and those cells that were

positive for TL8 were excluded from the sorted CD3$^+$CD8$^+$ population. For repeated TCR binding

analysis for enrichment of p68A-specific TCRs, cells were stained with TL8, p54E660, and p68A

tetramers and multiple populations were simultaneously sorted: p54E660$^-$ p68A$^-$ TL8$^-$, p54E660$^+$

p68A$^-$ TL8$^-$, and p54E660$^-$ p68A$^+$ TL8$^-$. These sorted populations were subsequently combined

during purification of the DRMs. For samples with low total cell number, there was concern

over not having enough cell mass and of losing the DRM pellet during the ultracentrifugation steps. To these samples extra DRMs from cells not specific for any of the epitopes being evaluated were added to provide extra cellular mass to the pellet. These extra DRMs were obtained from sorted single $CD3^+CD8^+$ lymphocytes that were negative for all evaluated epitope specificities ($p11C^-p54E660^-p68A^-TL8^-$). All sorted cell populations were resuspended in cold TNE buffer (25 mM Tris-HCL pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.1% sodium azide, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 μg/mL aprotinin) and stored at -80°C until analysis. Frozen samples were lysed by subjection to 3 freeze-thaw cycles. Brij 58 was added to a final concentration of 1% and incubated on ice for 1 hr. Cell lysates were spun at 100,000 g for 10 min at 4°C. Supernatants were transferred to new tubes and spun at 202,000 g for 2 hr. Pellets were resuspended in cold PBS and sonicated with three pulses of 55W using an interval pulse off time of 15 sec (Misonix Sonicator 3000). The cell membrane preparations were extruded 11 times though a 100 nM polycarbonate filter and immediately used for SPR binding assays.

Surface plasmon resonance (SPR) measurements. SPR binding analyses using T cell DRMs were carried out as previously described for protein-lipid interactions [410, 546, 547, 552, 561-564] and for reconstituted G protein-coupled receptors in liposomes [564]. DRMs were captured on a Biacore™ L1 sensor chip, which utilizes an alkyl linker for anchoring lipids. Before capturing DRMs, the surface on the Biacore™ L1 chip was cleaned with a 60 s injection of 40 mM OGP at 100 μL/minute, and the chip and fluidics were washed with excess buffer to remove any traces of detergent as described previously [561]. TCR-containing DRMs were captured by slowly injecting the DRM preparation at 5 μL/min over the Biacore™ L1 sensor chip. Monomeric pMHC

complexes constructed with p11C, p54E660, p68A and TL8 epitope peptides were injected at 50

μL/minute for 3 min at concentrations ranging from 25 μg/mL to 200 μg/mL for initial

measurements and up to 1,000 μg/mL of p68A on repeated samples. The specific binding signal

was obtained by subtracting the non-specific signal from TL8 pMHC monomer binding to the

TL8-depleted DRM preparation when injected at the same concentration. The global curve

fitting to the Langmuir equation was used to derive kinetic rate constants (k_{on} and k_{off}) for

calculation of equilibrium dissociation constant K_d as described earlier [410, 416]. All SPR

measurements were carried out on a Biacore™3000 instrument, and data analyses were

performed using BIAevaluation 4.1 software (GE Healthcare).

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GENERAL DISCUSSION

The studies presented here were performed to gain insight into the relationship between epitope dominance and the functionality of epitope-specific CD8$^+$ T cells and to identify factors that contribute to this relationship. Until now, there have been no studies relevant to HIV that determined if dominant and subdominant epitope-specific CD8$^+$ T cells differ in their antiviral capacities. In the present study, we have investigated this in SIV-infected rhesus monkeys and found that dominant SIV epitope-specific CD8$^+$ T cell populations were enriched in more mature cells compared to subdominant SIV epitope-specific CD8$^+$ T cell populations. The increased maturation of the dominant p11C-specific CD8$^+$ T cells was accompanied not only by decreased cytokine production and proliferative capacity but also by increased cytotoxic capacity. Thus, we have demonstrated that dominant and subdominant SIV epitope-specific CD8$^+$ T cells, indeed, differ in antiviral functional capacity.

Although an understanding of the relationship between epitope dominance and functionality may inform vaccine development, it would be advantageous also to identify the factors that contribute to these differences so that a vaccine can be developed that can more efficiently manipulate the epitope-specific CD8$^+$ T cells. Therefore, we sought to identify factors that influenced the establishment of the differences in frequency of epitope-specific CD8$^+$ T cells within an immunodominance hierarchy. Many studies had demonstrated that pMHC:TCR binding determines epitope-specific CD8$^+$ T cell proliferation and expansion and therefore frequency [553-556]. For that reason, we chose to examine if pMHC:TCR binding similarly determined immunodominance hierarchies. In so doing, we found an association between epitope dominance and pMHC:TCR binding, with the dominant p11C epitope exhibiting higher affinities than subdominant epitopes.

As previously discussed, it is likely that the differences in the strength of the p11C, p54E660, and p68A pMHC binding to their TCRs that was measured in this study contributed to the differences in frequency of their epitope-specific CD8$^+$ T cells. However, we found that these differences in frequency also were associated with differences in maturation and function of these cells. It is tempting to speculate that these differences in maturation and function resulted from the differences in pMHC:TCR binding that we measured here. Indeed, many studies that have evaluated the role of pMHC:TCR binding found that differences in proliferation were often accompanied by differences in function including cytotoxicity and the production of cytokines [380, 393, 398, 400, 404, 406-409, 536]. The majority of these studies were performed using cell lines where evaluations of maturation are not appropriate. However, there are a few more recent studies of the role of pMHC:TCR binding on T cell stimulation that have used cells from mice either directly *ex vivo* or even *in vivo*. These studies suggested that pMHC:TCR binding may indeed affect CD8$^+$ T cell maturation and are discussed below.

A study by Denton and colleagues used APLs that bind the mouse OT-1 transgenic TCR with different affinities. They found that *in vitro* stimulation of naïve OT-1 CD8$^+$ T cells with the APLs of different TCR affinities resulted in variable extents of CD62L downregulation- the stronger the affinity of the APL for the TCR, the more dramatic the CD62L downregulation [417]. This suggested that downregulation of CD62L immediately following stimulation was a surrogate measurement for the strength with which an epitope peptide binds its TCR. This is consistent with the findings in the present study, where we found that the dominant p11C-specific CD8$^+$ T cells had lower expression of the gene encoding CD62L (SELL) during acute infection compared to the subdominant p54AS-specific CD8$^+$ T cells and that this was associated

with their TCR affinity (the p11C monomers showed higher affinity for their TCRs than the p54AS monomers did for their TCRs).

Another study by Smith-Garvin and colleagues introduced mutations into the SLP-76 protein, an essential adaptor protein for TCR signal transduction and T cell activation [565]. These mutations were shown to dampen the signaling transmitted by TCR binding, as indicated by reduced calcium mobilization and reduced phosphorylation of signal transduction proteins following TCR crosslinking. They found this dampened TCR signaling was associated with altered *in vivo* CD8^{+} T cell maturation that was indicated by an accelerated transition to the less mature central memory phenotype. Specifically, following infection with LCMV, epitope-specific CD8^{+} T cells with the mutant SLP-76 displayed a more rapid surface protein re-expression of CD62L and IL-7Rα following initial infection and increased percentages of CD27^{+} and CXCR3^{+} epitope-specific CD8^{+} T cells throughout the immune response [566]. These experiments demonstrated that differences in the strength of TCR signal transduction can influence subsequent CD8^{+} T cell maturation. Interestingly, the differences in maturation phenotype that they observed between the stronger-signaling wildtype CD8^{+} T cells and the weaker-signaling mutant CD8^{+} T cells were similar to the differences we observed between the dominant and subdominant SIV epitope-specific CD8^{+} T cells in our study. The affinity of the p11C-specific TCRs was greater than that of the p54E660 TCRs which was greater than that of the p68A TCRs. These TCR affinities were in turn associated with their phenotype; the p11C-specific CD8^{+} T cells showed lower expression of CD62L, IL-7Rα, and CD27 in both acute and chronic SIV infection than p54E660-specific CD8^{+} T cells, which showed lower expression of these molecules than p68A-specific CD8^{+} T cells.

Finally, the role of TCR affinity in determining maturation has been more extensively characterized for CD4$^+$ T cells and findings from this field may be informative for this discussion. Similar to CD8$^+$ T cells, stimulation of CD4$^+$ T cells with APLs of weaker TCR affinities results in altered TCR signal transduction and altered effector function such as reduced IL-2 production and proliferation. Most strikingly though, is the affect of APL stimulation on CD4$^+$ T cell lineage decision. Whereas a TCR signal initiated by binding of a strong-affinity ligand promotes T$_H$1 differentiation, binding of a weaker ligand diverts CD4$^+$ T cells down the T$_H$2 pathway [567-569]. Additionally, weak TCR stimulation in the absence of an inflammatory stimuli favors generation of Foxp3$^+$ regulatory T cells [570-573]. Therefore, differences in TCR signaling that occur between strong and weak affinity interactions with the TCR can influence T cell maturation, but these differences may be more subtle for CD8$^+$ T cells than for CD4$^+$ T cells. Additional studies need to be conducted to develop a more thorough understanding of the role of TCR affinity on CD8$^+$ T cell maturation.

The poor *in vitro* expansion capacity that we observed of the p11C-specific CD8$^+$ T cells compared to the subdominant epitope-specific CD8$^+$ T cells during chronic infection at first appeared inconsistent with the substantially higher frequency of the p11C-specific CD8$^+$ T cells. However, these findings were not necessarily in disagreement and can be explained by the differences in antigen-elicited responses between naïve and memory CD8$^+$ T cells. The initial stimulation that the p11C-, p54-, and p68A-specific naïve CD8$^+$ T cells received elicited different levels of expansion, resulting in the differences in frequency that define the immunodominance hierarchy. As discussed previously, these differences in frequency may be due to the differences in pMHC:TCR binding that the present study shows. We also show that

following initial stimulation by SIV infection, the different SIV epitope-specific CD8^{+} T cells

develop different extents of maturation. As maturation determines proliferative capacity (less

mature cells exhibit greater capacities to expand than more mature cells) [245, 261], the

enrichment of the p11C-specific CD8^{+} T cell population with more mature cells likely resulted in

its decreased capacity to expand upon *in vitro* re-exposure to peptide epitope. Therefore, the

establishment of the SIV epitope immunodominance hierarchy was determined by the

differential abilities of the naïve p11C-, p54-, and p68A-specific CD8^{+} T cells to expand in the

primary response; the differences in expansion capacity observed during chronic infection were

determined by their differences in maturation.

   The finding that the protective dominant p11C-specific CD8^{+} T cells may be elicited by

stronger TCR interactions suggests that epitope-specific CD8^{+} T cells of lower frequency or

functionality may be enhanced by manipulation of the TCR interaction. Indeed, various

approaches to manipulate the peptide:MHC:TCR interaction for enhancement of CD8^{+} T cell

responses have been described and some have even been evaluated in clinical trials. First,

administration of tetramers to mice primed epitope-specific CD8^{+} T cell responses and

facilitated enhanced rejection of tumors expressing the epitope that the tetramer was

constructed with [574]. This suggests that tetramers constructed with APLs that confer

enhanced TCR binding may be used to enhance CD8^{+} T cell responses *in vivo*. Second, TCRs have

been engineered to have higher affinities for pMHC complexes and recognition of pMHCs by T

cells expressing these higher-affinity TCRs resulted in enhanced *in vitro* cytokine and chemokine

production as well as enhanced suppression of infected target cells. Such higher-affinity TCRs

have been engineered not only for recognition of the mouse QL9:H-2L[d] pMHC [575] but also for

the human HIV SL9:HLA-A*02 pMHC [576]. As TCR-transduced T cells have been used successfully for cancer therapies in humans [577-579], T cells expressing enhanced-affinity TCR for pathogen-derived epitope peptides is therefore a possibility. Finally, APLs of melanoma epitopes that enhance CD8^{+} T cell functionality have been identified. Vaccination of cancer patients with these APLs has shown modest results in eliciting epitope-specific CD8^{+} T cells that has resulted in cancer regression [580, 581]. Although the APLs in these cases were selected based on their enhancement of peptide:MHC binding [582], it is possible that APLs engineered to have enhanced pMHC:TCR binding may be used in a similar manner [383, 583-586].

Further studies are required to substantiate the findings of the present study. Analyses of the relationships between frequency of epitope-specific CD8^{+} T cells and the functionality of these cells should be examined, not only in immunodominance hierarchies restricted by other rhesus monkey MHC class I alleles, but also in mice and human CD8^{+} T cell immunodominance hierarchies. Of interest would be the finding that this relationship exists for HIV-specific CD8^{+} T cell immunodominance hierarchies. This would confirm that similar mechanisms exist for determining frequency and functionality of both HIV- and SIV-specific CD8^{+} T cells and therefore would validate the use of the SIV monkey model to test manipulations of immunodominance that are relevant to HIV.

The murine LCMV-infection model would be an ideal system to further dissect the relationship between TCR affinity, and the frequency and functionality of the responding CD8^{+} T cells. The LCMV epitope immunodominance hierarchy in LCMV-infected mice is well-characterized and APLs of multiple dominant and subdominant epitopes could be generated.

Epitope-specific CD8$^+$ T cells could be elicited with wildtype epitopes or their APLs. We would expect that the APLs would alter the frequency of responding epitope-specific CD8$^+$ T cells according to their TCR affinity. Any changes in phenotype of the CD8$^+$ T cells elicited by the APLs, compared to that of the cells elicited by the wildtype epitope, can then be identified and further investigated.

In summary, the findings in these studies enhance our understanding of CD8$^+$ T cell immunodominance hierarchies, particularly in the context of HIV and SIV infection, and have important implications for vaccine design. We revealed that dominant and subdominant SIV epitope-specific CD8$^+$ T cells differ in their antiviral capacities; dominant epitope specific CD8$^+$ T cells exhibited enhanced cytotoxicity but reduced proliferation and production of cytokines and chemokines compared to subdominant epitope-specific CD8$^+$ T cells. These differences were a result of different extents of CD8$^+$ T cell maturation. Gene expression analyses showed that the divergence in maturation and function occurs immediately following SIV infection.

The differences in frequency of dominant and subdominant SIV epitope-specific CD8$^+$ T cells in this study were associated with differences in the strength with which their TCRs bind their cognate pMHC complexes. Specifically, epitope dominance was associated with relatively higher affinities. While the relatively higher affinity of the p11C epitope was driven by its faster TCR association rate compared to the subdominant p54E660 and p68A epitopes, the relatively lower affinity of the p68A epitope was driven by its faster dissociation rate from its TCR compared the p54E660 and p11C epitopes. In light of the findings of the differences in antiviral

function also observed in this study, these differences in TCR affinity suggest that they may play

a role in the differences in CD8$^+$ T cell maturation and function.

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