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Mobilizing Subdominant HIV-Specific CTLs As A Novel Vaccine Strategy

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Dedication

This dissertation, along with all of the hard work that was put into completing this dissertation is dedicated to the two loves of my life, Dylan Thomas and Dominic Jude. The two of you are the light of my life and the reason I wake up every morning. All I do is for you and you give me more motivation then you could ever know. I cannot imagine my life without you, and I thank God every day for giving me the wonderful opportunity to be your mother. Most importantly, you have taught me to be a better person and to never give up on your dreams.

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WE DID IT!!!!!

Love, Mom

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by

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Abstract

To overcome the extreme antigenic diversity of HIV, there is increasing interest in developing vaccines that target CD8 T cell responses to conserved regions of the viral proteome. Understanding the immunogenic potential of these domains is therefore critical for the success of this vaccine strategy. CD8 T cell responses to conserved domains restricted by the "neutral" and highly prevalent HLA-A2 allele have not been extensively characterized. Here we describe a novel essentially invariant 10-mer epitope in HIV Gag ($p24_{14-23}$, RTLNAWVKVV (RV10)) in that it is expressed by the majority HIV isolates from all clades. Here we examined the character of the CD8 T cell response to this subdominant epitope in healthy volunteers and in HIV-infected individuals.

RV10 was identified with *in vitro* immunized CD8 T cells from eight seronegative HLA-A*0201 (A*02) carriers. In brief, CD8 T cells purified by immunobeads were primed with autologous dendritic cells (DCs) transduced by a lentiviral vector to express Gag. Antigen-specific T cells were further expanded by three weekly re-stimulations with autologous monocytes pulsed with 123 15-mer overlapping peptides (OLPs) spanning across the entire Gag protein. Reactivities to individual OLPs were identified by IFNγ-ELISPOT assays using matrix peptide pools followed by interrogation at the single peptide level. Of particular interest, OLPs 7908 and/or 7909 encoding RV10 were recognized by CD8 T cells immunized from all donors. RV10 was fine mapped from five cultures using a cassette of truncated peptides within OLP 7908. Interestingly, the 10-mer peptide encodes two well-known HLA-A2-restricted 9-mer epitopes, Gag ($p24_{15-23}$, TLNAWVKVV (TV9)) and Gag ($p24_{14-22}$, RLNAWVKVV (RV9)). This 10-mer Gag sequence appears to enjoy a paradoxically high T cell repertoire reserve

despite its subdominance as judged by the rarity of reported responses in HLA-A*02+ HIV-infected individuals.

To analyze the character of the CD8 T cell responses to RV10, TV9 and RV9, parallel cultures were generated from four healthy HLA-A*02 carriers. All cultures were stable with distinct populations of tetramer-binding cells. RV10- and TV9-specific T cells were consistently cross-reactive as measured by functional assays such as cytotoxicity and polychromatic flow cytometry. In contrast, they were less reactive to RV9. On the other hand, RV9-specific T cell cultures recognized the cognate peptide efficiently but were minimally cross-reactive to the other two peptides. Interesting to note, the three epitopes sharing the same 8-mer antigenic core display two distinct patterns of reactivities. To show that the three peptides are naturally processed and presented by MHCI molecules of infected cells, CD8 T cells were primed with autologous transduced DCs and re-stimulated with autologous transduced B cells expressing Gag. Tetramer-binding RV10-, TV9- and RV9-specific CD8 T cells were found in cultures from four of four donors, thereby indicating that these reactivities were immunized by epitopic determinants naturally processed and presented by both APCs.

TCRB gene usage by TV9- and RV10-specific CD8 T cells sorted to >98% purity were compared by an unbiased template-switch anchored rt-PCR . While a single TCR can recognize both peptides, no clonal overlap was found between three sets of parallel cultures of TV9 and RV10 cells. These observations suggest that the repertoires for the two peptides are very diverse and furthermore, cross-recognition among the clonotypes is common.

Alanine scanning mutagenesis revealed that substitution of asparagine (N) (position 4 of RV10 and position 3 of TV9) was associated with the most precipitous decline in recognition by RV10- and TV9-T cells, respectively. Moreover, recognition by RV10-specific T cells appeared

to be more dependent on tryphtophan in position 6 than their TV9 counterparts. Of note, alanine substitutions at all positions of both peptides did not significantly reduce binding to HLA-A*02. In some instances, binding was somewhat increased. In summary, the arginine residues in these peptides appear to serve as a key TCR contact site, thereby accounting for reciprocal cross-recognition. RV10-specific T cells appeared to recognize the phenylalanine residue as well. This provides compelling albeit indirect evidence that RV10 is a distinct epitope.

Here we have analyzed in detail the character of CD8 T cell responses to a set of three nested HLA-A*0201 epitopes that share a core 8-mer antigenic determinant in common. This finding has implications in T cell-based HIV vaccine design for the one of the most common HLA allele worldwide. Specifically, our data suggest a new mechanism of immunological escape ("CTL escape") by this highly variant virus. The addition of an arginine to the TV9 sequence, a seemingly useful HLA-A*02 epitope, results in immune interference due to the simultaneous presentation of cross-reactive epitopes by the same MHCI molecule, thereby preventing a full-scale activation at priming and over the long-term maintenance of Gag-specific CD8 T cell expansion. Understanding this immunological mechanism may be of great potential importance given the multiplicity of overlapping CD8 epitopes and their tendency to cluster in various HIV protein regions.

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Chapter 1: Background and Significance

1.1 HIV and AIDS Pandemic

HIV is the human immunodeficiency virus that can lead to AIDS (acquired immune deficiency syndrome). At the end of 2010, an estimated 34 million people were living with HIV globally, which included 2.7 million new infections[1].

In particular, the HIV epidemic is a serious public health issue in the Latino community. They are disproportionately affected by HIV and in 2009, Latinos accounted for 20% of new HIV infections in the United States while representing approximately 16% of the total US population [1].

Latino men who have sex with men (MSM) are particularly affected by HIV, as their infection rate is nearly three times as high as that of whites[1].

Latina women are also affected by this epidemic and accounted for 21% of new infections in 2009 and was more than four times that of white women [1].

Statistics show that 1 in 36 Latino men will be diagnosed with HIV, as will 1 in 106 Latina women, within their lifetime. In 2007, HIV was the fourth leading cause of death among Latinos aged 35–44 and the sixth leading cause of death among Latinos aged 25–34 in the US[1].

Many factors contribute to the HIV epidemic in Latino communities, which include as poverty, migration patterns, lower educational attainment, inadequate health insurance, limited access to health care or language barriers. These factors may limit Latinos' awareness about HIV infection risks and opportunities for counseling, testing, and treatment. It is imperative for the global community, and in particular the Latino communities, to continue to work towards an HIV Vaccine to help slow down and eventually end this epidemic.

1.2 CD8+ T cells and viral control.

There is a large body of evidence to support that $CD8^+$ T cells are critical for the control of HIV infections. The most direct came from studies in the nonhuman primate simian immunosuppressive virus (SIV)-macaque model. Monkeys depleted of $CD8^+$ lymphocytes and then infected with SIV were unable to control the virus [2]. In chronically infected monkeys, transient $CD8^+$ cell depletion by administration of anti-CD8 monoclonal antibody was followed by increased viral loads, which returned to pretreatment levels with the recovery of $CD8^+$ T cells [3].

1.3 Association between possession of particular HLA class I alleles and successful containment of HIV.

Certain HIV-specific CD8+ T cell responses contribute significantly to viral control and are related to better prognosis in infected people. Unfortunately these responses occur only in rare individuals carrying a few HLA-B alleles. In fact, the majority of HLA alleles do not correlate with control of HIV infection [4]. The most effective CTL targets are those that force the virus to mutate and lose replicative capability [5]. These epitopes reside in highly conserved regions of the HIV proteome; particularly Gag [6-8]. Since each HLA class I molecule presents a limited and unique set of peptides, the genotype of any individual is one factor that dictates the repertoire of CTL responses that he or she is capable of mounting.

The value of using conserved regions of the HIV proteome for the purposes of vaccination has been recognized for some time [9-11]. However, while these domains are populated with $CD8^+$ T cell epitopes [12], most have not been correlated with protection in carriers of the majority of HLA class I alleles. We propose that the use of conserved regions as

vaccines for non-protective class I alleles will depend on whether it is feasible to improve the intrinsic immunogenicity of natural sequences.

1.4 HLA-A2 and HIV infection.

HLA-A2, the most prevalent allele worldwide[13], must be considered in terms of coverage. CTL responses restricted by HLA-A*0201 do not appear to suppress virus effectively *in vivo*, consistent with the notion that "inactive" forms of CTL epitopes may have been fixed in the circulating pool of viral strains.

1.5 SL9 and TV9 are attractive vaccine targets.

Gag is preferentially targeted in infection [14, 15]. Single-point mutations in p17 [16] or p24 [17] can have profound effect on viral assembly and infectivity. Therefore, a vaccine targeting the highly conserved Gag protein with crucial viral functions may exact a high viral fitness cost [18, 19]. **SL9** has attributes that are potentially important for a vaccine target. These include a high level of expression of SL9-HLA class I complexes on infected [20, 21] or transduced cells [22], which may be related to its unique processing requirement [23]. SL9 is relatively conserved across clades[24]. SL9-CTLs have also been detected in GALT [25, 26], suggesting an important role in mucosal immunity as well.

TV9 resides in the first of seven α-helices of the N-terminal of p24. Linker-insertion mutations between residues 26-27 (A and W) destroy HIV's ability to replicate [27]. TV9 is highly conserved across clades, with one common variant (155 of 371 reported [24]) where V in position 9 is conservatively replaced by I. This epitope overlaps by five residues with the protective HLA-B*57-restricted ISPRTLNAW (IW9) determinant recognized early by slow progressors [28]. Despite the small number of reports of TV9 reactivity in patients, there are

indications that CTL responses to this epitope can control virus [29]. Above all, an elevated and sustained TV9-specific response was noted in one person who remained uninfected despite parenteral exposure to a highly replicating HIV strain [30]. The functional sensitivity of the *in vivo* TV9 reactivity, a factor considered important for the inhibition of HIV *in vivo* [31], was also relatively high [29]. The potential of TV9 as a vaccine target deserves investigation since there may be few alternatives for the most prevalent HLA class I allele in the world.

1.6 De novo/in vitro priming, mapping and characterization of CD8+ reactivities to HIV conserved sequences from healthy seronegative donors.

Almost all HIV epitopes have been defined by analyzing $CD8^+$ T cell responses to infection *in vivo*. Although informative, they offer insights into a CTL repertoire that has made significant accommodations to the ravages of a remarkably persistent viral infection. In terms of prophylactic vaccines, analysis of the pre-infection virus-specific precursor T cell pools in healthy donors will help complete the picture. While *in vitro* and *in vivo* primed-and-expanded HIV-specific CTLs are generated by greatly different selection processes, specificity of these responses is pre-determined by the available T cell repertoire since the TCR does not undergo somatic hypermutation [32]. *In vitro* priming-and-culture offers the only practical option to study subdominant responses, particularly those that are not only small in magnitude but are infrequently recognized in infections. Our culture conditions consistently capture many peptide-specific TCR clonotypes from individuals, allowing a more representative evaluation of the specificity of the T cell repertoire.

Ex vivo-primed CTLs to the dominant HLA-A2-SL9 (Gag $p17_{77-85}$) were found to be aberrantly sensitive to cytokine-mediated activation-induced cell death (AICD) and uniquely independent of IL-2 supplementation for proliferation, producing copious amounts of autocrines [22, 33]. Sensitivity to AICD may account for the paucity of SL9 reactivity during the proinflammatory acute infection and the ability to support its own proliferation may explain the paradoxical dominance during the CD4-diminished chronic infection [34-36]. Moreover, SL9-$CD8^+$ T cells from patients proliferated better without added IL-2 *in vitro*, suggesting that cells primed *in vitro* share some characteristics with those mobilized in natural infection [33].

Although TV9 responses have only been reported in a few patients [29], there are indications to suggest a role in viral control [30, 37, 38]. Since TV9 is infrequently recognized during infection, *in vitro* priming is the only practical option to study this response. Using established protocols, we found that TV9 subdominance is not due to poor immunogenicity, a lack of cognate TCR repertoire or potential avidity properties.

1.7 Subdominant epitopes can control virus replication.

Immunodominance refers to the hierarchy of epitopes based on their ability to elicit prevailing T cell responses [39, 40]. Prior immunizations against a subdominant antigen can reduce the response against a dominant antigen upon immunization against both antigens, suggesting that hierarchy can be altered in some cases by increasing the precursor frequency to the subdominant antigen [41-43].

That subdominant reactivities can contribute significantly to durable lentiviral control is supported by a study of elite controller macaques (ECs) infected by the pathogenic SIVmac239 [44]. Transient depletion of $CD8^+$ T cells with mAb resulted in large increases in viremia.

Control was re-instated with the return of $CD8^+$ (and $CD4^+$) responses. Of interest, the repertoire of $CD8^+$ reactivities differed drastically from that in acute infection; previously subdominant SIV-specific populations expanded the most. This study suggests that durable control can be attained with a small number of effective recall responses including those that are subdominant during acute infection.

There is evidence that subdominant $CD8^+$ T cell responses restricted by common HLA alleles targeting highly conserved regions throughout HIV can play a role in the control of infection [45]. An explanation for enhanced antiviral pressure by responses to highly conserved Gag epitopes over that targeting other HIV proteins may be greater loss of fitness cost with escape mutations [5]. It was also proposed that functional quality and maturation phenotype of these HIV-specific $CD8^+$ T cells may be important [7, 46-50].

1.8 Project Hypothesis. Understanding the immunological basis for the subdominant status of conserved region of p24 is necessary for the rationale design of a more effective Gag vaccine for individuals carrying HLA-A2, the most common HLA class I allele worldwide.

1.9 Specific Aims

Specific Aim 1 will determine whether T cells, induced to the immunodominant SL9 epitope, dominate those primed to subdominant epitopes such as TV9 *in vitro* using autologous DCs transduced to express Gag.

Specific Aim 2 will study the immunological basis for the subdominance of TV9.

Chapter 2: Analysis of $CD8^+$ T cells primed ex vivo by transduced DCs

2.1 Introduction

Immunodominance in T cell responses to viruses is not completely understood. Two major factors involved in immunodominance are the amount of peptide-MHC ClassI molecules on the surface of antigen presenting cells and the number of naïve T cells available to recognize such complexes [51]. A consequence of immunodominance is that potentially effective subdominant responses could be masked. This is particularly important in HIV where there is rapid escape from dominant T cell responses in infected individuals. We explored the hierarchy of CD8 T cell responses to Gag using an *in vitro* system to try to identify potentially efficacious subdominant epitopes.

2.2 Materials and Methods

Healthy seronegative donors and HLA typing. This study was approved by the Human Investigation Committee at the University of Texas at El Paso. All subjects provided written informed consent.

DNA was isolated from ten milliliters of heparinized blood using QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA) for high resolution HLA typing by the NIH-HLA Laboratory (Bethesda, MD).

Generation of dendritic cells (DCs) from adherence-purified monocytes. PBMC from fresh heparinized blood were isolated by density gradient centrifugation using Lymphocyte Separation Medium (Mediatech, Herndon, VA). Monocytes were isolated by adherence to plastic for 2 hr in serum-free HEPES-buffered RPMI 1640 (Mediatech). Adherent cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 100 U/ml of penicillin and 100 µg/ml streptomycin, 1 mM sodium pyruvate, 1x MEM nonessential amino acids (Life Technologies, Rockville, MD), and 10% autologous serum ("complete medium") supplemented with 1000 U/ml GM-CSF (Leukine Sargramostim, Bayer HealthCare Pharmaceuticals, Wayne, NJ) and 500 U/ml IL-4 (Peprotech, Rocky Hill, NJ). Half of the medium was replaced on day 3 with an equal volume of fresh medium containing cytokines. DCs used after 7 days in culture were considered as immature DCs (34).

Generation of activated B cells. CD40L activated-B cells were and fed with cytokine-containing medium with Cyclosporin A (CsA, Sigma) generated as described previously [52]. In brief, PBMC were cultured on irradiated NIH3T3 cells transfected with CD154 (tCD40L) [53] in the presence of IL-4 (2 ng/ml; Peprotech) and 0.63 µg/ml CsA in Iscove's modified Dulbecco's

medium (IMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) for 7 days. The expanding cells were transferred onto freshly prepared irradiated t-CD40L cells every 3 to 4 days.

Lentivirus preparation and titration. The Gag gene of the NL4-3 laboratory HIV strain (plasmid p83-2) was cloned into the pLenti6/V5 Directional TOPO® expression vector (Invitrogen) and provided by Dr. Otto O. Yang. To produce a lentivirus stock, highly purified plasmid DNA (SNAP MidiPrep Kit) was added to the ViraPower Packaging Mix, an optimized mixture of three plasmids (pLP1[Gag/Pol], pLP2[rev and LTR polyadenylation signal] and pLP/VSVG). The mixture was co-transfected into 293FT cells by Lipofectamine (Invitrogen). Virus was concentrated from supernatants by centrifugation at 21,000 rpm for 2 hr at 4°C.

Viral titer (transducing unit, TU) was determined according to manufacturer's instructions. Briefly, one ml of ten-fold serial dilutions of the virus stock was added to subconfluent monolayers of HT-1080 cells in 6-well plates in MEM containing 10% FBS and 5µg/ml Blasticidin (Invitrogen). After 14 days, cell colonies were stained with crystal violet and counted.

Transduction of DCs and B cells. Four-day-old DCs and seven-day-old B cells were transduced with the lentiviral vector at a multiplicity of infection (MOI) of 2. The percent of transduced DCs or B cells was determined 3 days later by intracellular p24 staining (FITC-conjugated mAb KC57; Beckman Coulter, Miami, FL).

Staining of peptide-specific CTL with tetrameric HLA-A*0201-peptide complexes (tetramers). HLA-A*0201-TV9,-RV10 and -RV9 monomers were prepared by the National Institutes of Health/National Institute of Allergy and Infectious Diseases Tetramer Facility (Atlanta, GA). Tetramers were made by conjugating to fluorochrome-labeled streptavidin (BD

Biosciences) by the Tetramer Facility tetramerization protocol. Cultured T cells were washed, re-suspended in cold staining buffer containing PBS, 0.2% BSA, and 0.02% sodium azide, and then incubated with 1 μg/ml of tetramer and 100 ng/ml of Qdot-labeled anti-CD8 mAb for 30 min at 4°C. The cells were washed twice with staining buffer and analyzed promptly by flow cytometry. Controls used to gate for specific tetramer binding included the staining of the cells of interest by an irrelevant tetramer and any nonspecific binding of the tetramer under study to cultured T cells with other specificities.

2.3 Results

Studies of T cell competition for SL9 and TV9

In vitro priming protocol: To study the hierarchy of naturally processed and presented epitopes, autologous monocyte-derived DCs transduced with a lentiviral vector expressing full length Gag cloned from the NL4-3.1 virus, developed by Dr. O.O. Yang, were used to prime naïve $CD8^+$ T cells. DCs were generated from adherent monocytes by culturing with GM-CSF and IL-4 and used on d7 as APCs. DCs were transduced on d4 by the lentiviral vector Gag-1 at a multiplicity of infection (m.o.i.) of 2 and used 3 days later when the expression of Gag was highest as shown by intracellular staining for p24 (Figure 1a).

The purity of $CD8^+$ T cells in each preparation was verified by flow cytometry, staining with CD8-PerCp, CD3-FITC and CD4-PE mAbs (BD Biosciences) and confirmed to be ≥98% $CD3^+CD8^+$ T cells within the small lymphocyte population gated by forward and side scatter ($CD4^+$ cells were ≤ 2%) (data not shown). Starting with highly homogenous $CD8^+$ cells appeared to be critical for consistent generation of antigen-specific T cells, possibly due to interference by T_{reg} cells within the contaminating $CD4^+$ population.

Transduced DCs (t-DCs) were co-cultured with naïve circulating $CD8^+$ T cells purified from peripheral blood of healthy donors at the DC:T cell ratio of 1:5 (d0). The cultures were re-stimulated on d7 and d14 with autologous B-cells transduced (Figure 1B) with with Gag-1. IL-7 was added on the day of priming or re-stimulation. IL-2 was added 4 days after priming and 1 and 4 days after re-stimulation. Induction of epitope-specific T cells was determined by staining with tetramers specific for SL9, TV9, RV10, and RV9 (Figure 1C). All three subdominant epitopes are located in p24, while SL9 resides upstream in p17 of Gag.

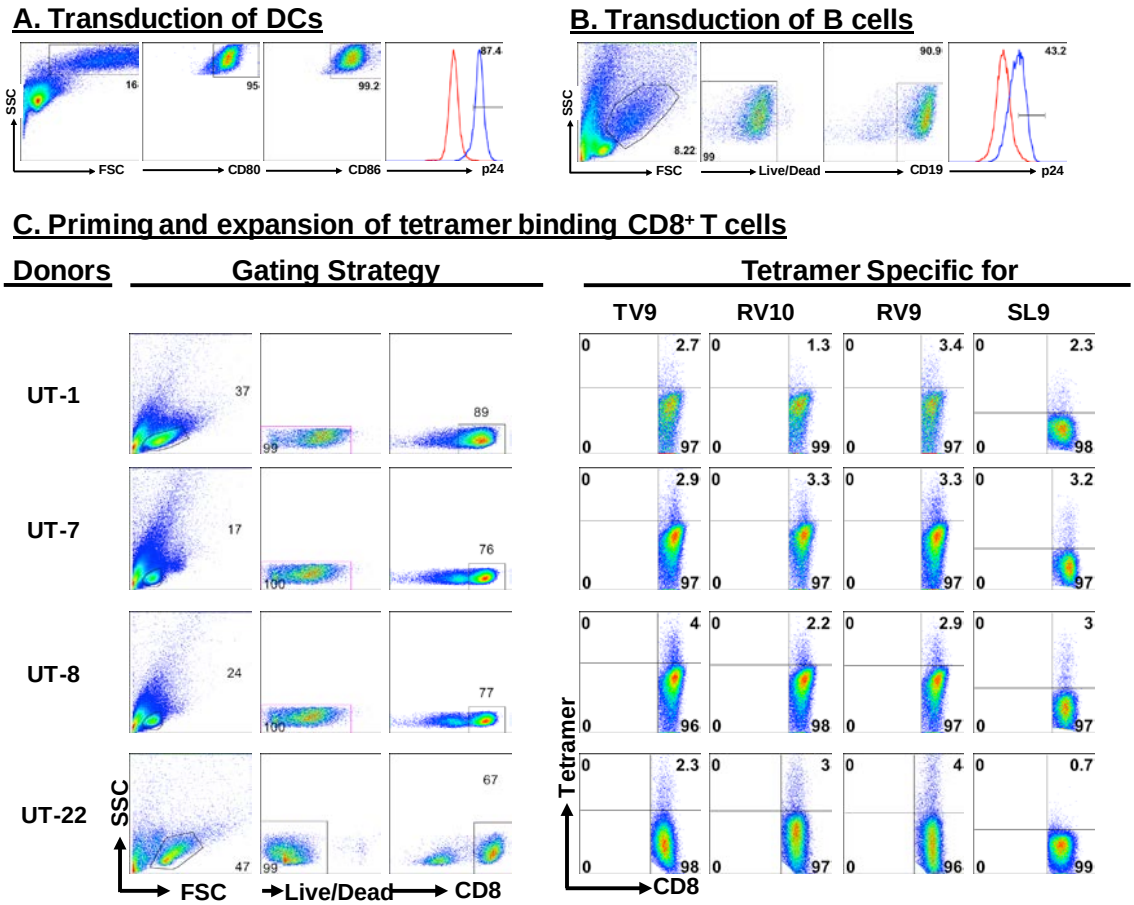

Figure 1. Gag epitope-specific CD8+ T cells induced by APCs transduced to express Gag. Autologous monocyte-derived DCs (A) or cultured B cells (B) transduced with a lentiviral vector encoding HIV Gag were stained 3 days later for intracellular p24 expression. DCs were identified by SSC and FSC as well as expression of CD80 and 86. B cells were identified by expression of CD19. Tranduced DCs were used to prime purified circulating CD8+ T cells from healthy donors at the DC:T cells ratio of 1:5 (d0). T cell cultures were subsequently restimulated on d7 and d14 with transduced B cells. Epitope-specific T cells were identified by staining with tetramers specific for TV9, RV10, RV9 and SL9 (C). These cells were monitored over 4 weeks, with tetramer staining at each week. Data shown is from d28.

2.4 Discussion

` Tetramer staining indicates that in 3 of 4 cultures, T cells induced to the immunodominant SL9 epitope; do not dominate those primed to subdominant epitopes such as TV9. This data suggests that the presence of the chronically dominant SL9 eptiope does not seem to affect the emergence of other subdominant epitopes.

This pattern of emergence of epitopes may be different than what we typically see in acute HIV infection because SL9 is seen during the chronic phase. In acute infection there are responses to Gag, Nef and Envelope proteins although SL9 is not one of them. Although this is not the case in acute infection, in general the chronic dominant epitope did not affect the emergence of the subdominant epitopes.

With this observation we decided to further investigate other possible mechanisms of what could be contributing to the subdominance of other emerging subdominant epitopes.

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Chapter 3: Analysis of the pre-infection virus-specific precursor T cell pools in healthy donors

3.1 Introduction

While empirical trial-and-error approaches have led to many successful vaccines in the past, development of an HIV vaccine requires a greater understanding of why the natural antiviral immune responses fail to resolve infections. [54-59].

Most HIV-1 epitopes, including those found for HLA-A2 carriers, have been discovered by mapping responses in HIV-1 infected persons, and yet most people do not elaborate effective CTL responses [4, 9]. Therefore, the responses that have been mapped are insufficient and do not control viral replication for most individuals. Studying the pre-infection CD8+ T cell repertoire to determine if there are useful CTL epitopes presented in the context of HLA-A2 is critical.

We analyzed the pre-infection virus-specific precursor T cell pools in healthy donors to empirically map HIV-1 Gag as a relevant approach to uncover new subdominant A*0201 epitopes.

3.2 Materials and Methods

Healthy seronegative donors and HLA typing, generation of dendritic cells (DCs) from adherence-purified monocytes, lentivirus preparation and titration, and transduction of DCs is same as described in Section 2.2 Material and Methods.

Peptides. TV9 (TLNAWVKVV), RV10 (RTLNAWVKVV), RV9 (RTLNAWVKV) and alanine substituted peptides were purchased from Genemed Synthesis (San Antonio, TX). Gag-specific 15-mer overlapping peptides (OLPs) were obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health.

MHC-peptide binding assays. Quantitative assays to measure the binding of peptides to HLA A*0201, A*0202, A*0203, A*0206, and A*6802 molecules are based on the inhibition of binding of a radiolabeled standard peptide and performed by Drs. Alessandro Sette and John Sidney. MHC molecules were purified by affinity chromatography from EBV-transformed cell lines, and assays performed as described previously [60-62]. Under the conditions used, where [radiolabeled probe] < [MHC] and IC50 ≥ [MHC], the measured IC50 values are reasonable approximations of the true Kd values.

Generation of antigen-specific $CD8^{+}$ T cells by *in vitro* immunization with 123 Gag overlapping peptide-pulsed APCs. DCs were transduced with a lentiviral vector encoding Gage and were harvested 3 days later with Cell Dissociation Solution Non-enzymatic (Sigma-Aldrich Corp., St. Louis, MO), re-suspended at 0.5×10^6 cells/ml in RPMI and then irradiated at 3000 cGy. DCs were plated at 0.1×10^6 cells per well of a 48-well cluster plate after addition of 3 μg/ml of β2-microglobulin (MP Biomedicals Solon, OH) and 1% human albumin (Sigma) for

3 hr at 37°C. To each well, circulating $CD8^+$ T cells purified by immunobeads (Dynal, Oslo, Norway) were added at the T cell:DC ratio of 5:1 in complete medium supplemented with 10 ng/ml IL-7 (Peprotech). IL-2 (20 I.U./ml, Proleukin, Prometheus Inc., San Diego, CA) was added one and four days after stimulation. Half a million of T cells were re-stimulated weekly with fresh autologous adherent monocytes derived from 1.4 x 10^6 PBMC per well plated in RPMI containing β2-microglobulin and human albumin with 2 μg/ml of the 123 Gag OLPs for 3 hrs.

IFN-γ ELISPOT analysis. Cultured $CD8^+$ T cells were plated at 100,000 cells per well in 96-well polyvinylidene plates pre-coated with anti-IFN-γ mAb (human ELISPOT kit, BD Biosciences) and incubated overnight in the presence of peptides (at 2 μg/ml each) at 37°C in a CO_2 incubator. Wells containing $CD8^+$ T cells and media alone were included as negative controls. ELISPOT plates were developed using 3-Amino-9-Ethylcarbazole (AEC) as chromogen according to the manufacturer's instructions. Spots were counted using an ImmunSpot Series 1 Analyzer (Cellular Technology Ltd., Cleveland, OH), and the numbers of specific spot forming cells (sfc) were calculated by subtracting those detected in the negative control wells.

3.3 Results

Mapping novel epitopes from A*0201 healthy donors.

We used t-DCs to prime naïve $CD8^+$ T cells to identify epitopes that are naturally processed and presented and are recognized by human T cell repertoires. Naïve $CD8^+$ T cells from HLA-A2+ healthy donors were primed with autologous t-DCs expressing Gag. Gag-specific T cells were re-stimulated discontinuously every 7 days by autologous monocytes pulsed with a pool of 15-mer peptides overlapping by 11 amino acid residues (OLPs) spanning the entire Gag protein. Reactivities to individual OLPs were assessed by IFN-γ ELISPOT assays with C1R-A2wt target cells using the strategy of screening OLP matrix pools followed by interrogation at the individual OLP level in responsive pools. All positive responses (>no Ag control+3 SD) were confirmed by a second determination.

Table 1 summarizes reactivities directed at Gag-OLPs in eight $A2^+CD8^+$ T cell cultures. Responses to one or both OLPs 7908 and 7909 were detected in eight of eight cultures and indeed, were predominant in five. Interestingly, the TV9 sequence is embedded in both OLPs. Grey highlighted regions within the table are known HLA-A*0201 restricted epitopes, confirming we are able to detect a range of specificities.

Table 1: Mapping from healthy donors. HLA-A*0201-restricted reactivities to Gag 15-mer OLPs in CD8+ T cell cultures from healthy donors primed by DC transduced to express Gag and re-stimulated by monocytes pulsed with 123 OLP spanning Gag. The number of SFCs was determined 21 to 42 days post priming. Cultures were monitored over a 42 day period.

Donor	OLP	Location on Gag	OLP Sequence	SFCs/10^5 $CD8^+$ T cells
WS-12	7879	p17 (29-43)	Y K L K H I V W A S R E L E R	25
	7880	p17 (33-47)	H I V W A S R E L E R F A V N	27
	7908	p24 (13-27)	Q A I S P R **T L N A W V K V V**	3,760
	7909	p24 (17-31)	P R **T L N A W V K V V** E E K A	2,760
	7910	p24 (21-35)	N A W V K V V E E K A K S P E	42
	7911	p24 (25-39)	K V V E E K A K S P E V I P M	148
	7916	p24 (45-59)	E G A T P Q D L N T M L N T V	30
	7917	p24 (49-63)	P Q D L N T M L N T V G G H Q	499
	7938	p24 (133-147)	W **I I L G L N K I V** R M Y S P	219
	7939	p24 (137-151)	G L N K I V R M Y S P T S I L	456
	7943	p24 (153-167)	I R Q G P K **E P F R D Y V D R**	20
	7954	p24 (197-211)	D C K T I L K A L G P A A T L	369
	7955	p24 (201-215)	I L K A L G P A A T L E E M M	175
	7985	p2p7p1p6 (90-104)	P E P T A P P E E S F R F G E	565
	Gag pool			**2,060**
WS-9	7882	p17 (41-55)	L E R F A V N P G L L E T S E	172
	7908	p24 (13-27)	Q A I S P R **T L N A W V K V V**	78
	7909	p24 (17-31)	P R **T L N A W V K V V** E E K A	26
	7937	p24 (129-143)	I Y K R W **I I L G L N K I V** R	15
	7954	p24 (197-211)	D C K T I L K A L G P A A T L	19
	7955	p24 (201-215)	I L K A L G P A A T L E E M M	20
	7978	p2p7p1p6 (62-76)	D C T E R Q A N F L G K I W P	3,712
	7979	p2p7p1p6 (66-80)	R Q A N **F L G K I W P S H K G**	7,648
	7980	p2p7p1p6 (70-84)	**F L G K I W P S H K G** R P G N	7,616
	Gag pool			**6,848**
WS-11	7904	p17p24 (129-11)	S Q N Y P I V Q N L Q G Q M V	25
	7905	p24 (1-15)	P I V Q N L Q G Q M V H Q A I	36
	7908	p24 (13-27)	Q A I S P R **T L N A W V K V V**	650
	7909	p24 (17-31)	P R **T L N A W V K V V** E E K A	935
	7963	p2p7p1p6 (2-16)	E A M S Q V T N S A T I M M Q	37
	Gag pool			**1,350**
WS-10	7908	p24 (13-27)	Q A I S P R **T L N A W V K V V**	151
	7935	p24 (121-135)	N P P I P V G **E I Y K R W I I**	140
	7944	p24 (157-171)	P K **E P F R D Y V D R F** Y K T	65
	7963	p2p7p1p6 (2-16)	E A M S Q V T N S A T I M M Q	67
	Gag pool			**160**
WS-4	7908	p24 (13-27)	Q A I S P R **T L N A W V K V V**	4,820
	7909	p24 (17-31)	P R **T L N A W V K V V** E E K A	5,100
	7917	p24 (49-63)	P Q D L N T M L N T V G G H Q	245
	7985	p2p7p1p6 (90-104)	P E P T A P P E E S F R F G E	321
	Gag pool			**4,840**
UT-7	7874	p17 (10-24)	S G G E L D R W E K I R L R P	50
	7908	p24 (13-27)	Q A I S P R **T L N A W V K V V**	350
	Gag pool			**340**
UT-9	7908	p24 (13-27)	Q A I S P R **T L N A W V K V V**	99
	7909	p24 (17-31)	P R **T L N A W V K V V** E E K A	43
	7944	p24 (157-171)	P K **E P F R D Y V D R F** Y K T	183
	7957	p24 (209-223)	A T L E E M M T A C Q G V G G	11
	7960	p24 (221-235)	V G G P G H K A R V L A E A M	43
	7993	p2p7p1p6 (122-136)	P L A S L R S L F G N D P S S	327
	7994	p2p7p1p6 (126-137)	L R S L F G N D P S S Q	190
	Gag pool			**627**
UT-10	7878	p17 (25-39)	G K K K Y K L K H I V W A S R	840
	7908	p24 (13-27)	Q A I S P R **T L N A W V K V V**	4,300
	7909	p24 (17-31)	P R **T L N A W V K V V** E E K A	2,780
	7936*	p24 (13-27)	P V G E I Y K R W I I L G L N	1,234
	Gag pool			**2,920**

Fine Mapping of 7908.

Fine epitope mapping of OLP 7908 was performed using five T cell cultures using a panel of peptides truncated by one residue from either the N- and C-termini (P1 to P12) (**Table 2**). Consistent reactivities to the P5 10-mer, RV10 peptide, were detected in all T cell cultures. On the other hand, four cultures showed strong responses to P6, which is TV9. Of interest, significant responses were noted for P7 in three cultures, despite deletion of the V-residue at the C-terminal. These data suggest that the presence of three nested epitopes, (TV9, TLNAWVKVV; RV10, RTLNAWVKVV; and RV9, RTLNAWVKV) within a 10-mer sequence (RTLNAWVKVV). This was confirmed by showing that the cultures responded to these peptides.

These results showed that the subdominant stature of TV9 was not due to inefficient pMHCI presentation. Intriguing, they also strongly suggest high pre-infection precursor pools in many individuals.

Table 2: Fine mapping using a panel of 12 peptides progressively truncated by one residue from the NH2- or the COOH-terminal reveals three minimal epitopic peptides in OLP-7908. Data shown for the truncated peptides are the mean of duplicate assays. Elispot assays were performed with frozen samples of WS-10, -11 and -12 and UT-7, -9 and -10 CD8+ T cells in culture between 21 to 42 days post priming. Reactivity to the minimal peptides was assessed in separate experiments.

Peptides	Sequences	SFCs per 10^5 $CD8^+$ T cells				
		WS-10	WS-12	UT-7	UT-9	UT-10
OLP-7908	QAISPRTLNAWVKVV	154±7.5	1,676±234	227±96.5	683±31.8	840±40
P1	AISPRTLNAWVKVV	171±45.5	1,906±84	395±25	593±95	753±10
P2	ISPRTLNAWVKVV	144±0.5	1,890±70	315±55	505±149	677±1.75
P3	SPRTLNAWVKVV	181±62.5	1,708±82	302±51.5	535±191	729±17.25
P4	PRTLNAWVKVV	142±19.5	1,631±29	302±71.5	522±96	818±8.75
P5	RTLNAWVKVV	147±20.5	1,650±260	305±45	576±190	764±96
P6	TLNAWVKVV	0	1,356±16	328±12	362±2	590±63
P7	QAISPRTLNAWVKV	0	1,575±35	135±55	414±50	651±69.5
P8	QAISPRTLNAWVK	0	15±15	0	53±25	0
P9	QAISPRTLNAWV	2±1	146±4	0	0	0
P10	QAISPRTLNAW	0	0	0	0	0
P11	QAISPRTLNA	0	0	23±13	0	4±2
P12	QAISPRTLN	0	343±17	0	0	16±7.75
TV9	**TLNAWVKV**V	0	1,166±60	225.5±104.5	371±8	382
RV10	R**TLNAWVKV**V	60	1,623±83	266±67	403±7	462
RV9	R**TLNAWVK**V	0	335±19	45±35	272±128	102
RK8	RTLNAWVK	0	0	0	0	0
TV8	TLNAWVKV	0	678±36	16	0	497±133.25

Binding Affinities of TV9, RV10 and RV9 peptides.

Following the identification of these three overlapping epitopes we wanted to assess their binding affinities to the MHC ClassI molecule. Quantitative assays to measure the binding of peptides to HLA A*0201, A*0202, A*0203, A*0206, and A*6802 molecules are based on the inhibition of binding of a radiolabeled standard peptide and performed by Drs. Alessandro Sette and John Sidney. As listed in **Table 3**, TV9 has the strongest affinity to HLA-A*0201. RV10 has a lower affinity than TV9, with RV9 having the lowest affinity out of the three. SL9 was used in comparison because it is a well know A2 binder. This experiment will have to be repeated as it was only performed once.

Table 3. Bindinig Affinities. Binding affinities of TV9, RV10 and RV9 to HLA-A2 supertype molecules. The dash indicates that IC50 >10,000 nM.

Peptide	Sequence	IC_{50} (nM)				
		A*0201	A*0202	A*0203	A*0206	A*6802
TV9	T L N A W V K V V	468	179	108	1,965	23,937
RV10	R T L N A W V K V V	3,124				
RV9	R T L N A W V K V	5,841				
SL9	S L Y N T V A T L	367	79	19	--	--

3.4 Discussion

Mapping from healthy donors revealed a high pre-cursor frequency targeted towards a particular OLP, 7908 which contains the TV9 subdominant epitope. After fine-mapping to confirm that the response was to TV9, another interesting finding emerged with the identification of three overlapping epitopes. Of interest one of those epitopes was a novel 10-mer A*02-CD8 epitope that was mapped to the conserved CTL-sensitive p24 domain. The10-mer encodes 3 nested A*02-epitopes that share a core 8-mer antigenic determinant. All 3 epitopes bind to HLA-A*0201, and have paradoxically high CD8 precursor pools in naïve individuals**.** These data initiate some insight into why this area in p24 contains subdominant epitopes.

The there is a range of binding of the epitopes to the MHC Class I molecule based on the binding assay done by Sette's group. Please note that this experiment was only performed one time with all three epitopes, therefore statistical analysis could not be done. I do have further data to show that TV9 and RV10 have similar binding based on mean fluorescence ratio in a T2 stabilization assay described in Chapter 5.

With the new finding of overlapping epitopes we needed to understand this region better and had to come up with a strategy to study it. The responses in patients samples to TV9 is quite low and would be difficult to try to characterize and since we have evidence that healthy donors have a high pre-cursor frequency to these responses we decided to generate parallel cultures. Therefore, to further investigate this region and understand the functionality of these specificities we generated parallel cultures to all three epitopes.

3.5 Acknowledcements

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Chapter 4: Generation of parallel cultures to TV9, RV10 and RV9

4.1 Introduction

Characterization of subdominant epitopes such as TV9, RV10 and RV9 is particularly difficult due to the small number of reactive T cells and/or responders. For this reason, as well as previous data indicating high precursor frequencies for such epitopes, we continued to use healthy A*02 donors to generate parallel cultures to TV9, RV10 and RV9.

4.2 Materials and Methods

Generation of antigen-specific CD8$^{\pm}$ T cells by *in vitro* immunization with peptide-pulsed APCs. DCs were harvested with Cell Dissociation Solution Non-enzymatic (Sigma-Aldrich Corp., St. Louis, MO), re-suspended at 0.5 x 10^6 cells/ml in RPMI and then irradiated at 3000 cGy. DCs were plated at 0.1 x 10^6 cells per well of a 48-well cluster plate after addition of 10 µg/ml of peptide, 3 µg/ml of β2-microglobulin (MP Biomedicals Solon, OH) and 1% human albumin (Sigma) for 3 hr at 37°C. To each well, circulating CD8$^+$ T cells purified by immunobeads (Dynal, Oslo, Norway) were added at the T cell:DC ratio of 5:1 in complete medium supplemented with 10 ng/ml IL-7 (Peprotech). IL-2 (20 I.U./ml, Proleukin, Prometheus Inc., San Diego, CA) was added one and four days after stimulation. Half a million of T cells were re-stimulated weekly with fresh autologous adherent monocytes derived from 1.4 x 10^6 PBMC per well plated in RPMI containing β2-microglobulin and human albumin with 10 µg/ml of the cognate peptide for 3 hr.

Staining of peptide-specific CTL with tetrameric HLA-A*0201-peptide complexes (tetramers). HLA-A*0201-TV9,-RV10 and -RV9 monomers were prepared by the National Institutes of Health/National Institute of Allergy and Infectious Diseases Tetramer Facility (Atlanta, GA). Tetramers were made by conjugating to fluorochrome-labeled streptavidin (BD Biosciences) by the Tetramer Facility tetramerization protocol. Cultured T cells were washed, re-suspended in cold staining buffer containing PBS, 0.2% BSA, and 0.02% sodium azide, and then incubated with 1 µg/ml of tetramer and 100 ng/ml of Qdot-labeled anti-CD8 mAb for 30 min at 4°C. The cells were washed twice with staining buffer and analyzed promptly by flow cytometry. Controls used to gate for specific tetramer binding included the staining of the cells

of interest by an irrelevant tetramer and any nonspecific binding of the tetramer under study to cultured T cells with other specificities.

4.3 Results

Tetramer Staining of Parallel Cultures.

Tables 1 and 2 suggest that most $A2^+$ carriers possess the repertoire for all three peptides. This was further confirmed by the generation of parallel T cell cultures to each of the three peptides from four of four donors (**Figure 3**). T cell cultures were primed with peptide-pulsed autologous DCs and re-stimulated every 7 days thereafter with peptide-pulsed monocytes. Induction of peptide-specific T cells was determined at various time points by staining with tetramers for these peptides. $0.2x10^6$ T cells were stained with 10μg/ml tetrameric pMHCI at 4°C for ~30 minutes. Samples were run on LSR-II (BD Biosciences) and data was collected and analyzed in FACS Diva software **(Figure 2).** Numbers in the upper right quadrant represent the percentage of $tetramer^+CD8^+$ stained cells. An irrelevant tetramer was used to gate for specific binding by tetramers. As shown in **Figure 2**, there are clear tetramer populations for all three specificities for all donors' studies thus far. The frequencies vary from donor to donor, but there are consistently high $tetramer^+$ populations, ranging from 17% to 96% for the TV9- and RV10-specific cultures among all donors. Alternatively, there are consistently low tetramer+ populations ranging from 0.82% to 25% for the RV9-specific cultures. This data suggest that RV9 T-cells may have the lowest avidity of all three specificities based on low tetramer staining intensity. Avidity has been described as the strength of the interaction between a T cell and its target antigen. Studies have shown that avidity may be related to the response of the activated T cell, which implicates that RV9-specific T cells may require more antigen in order to be fully activated [63].

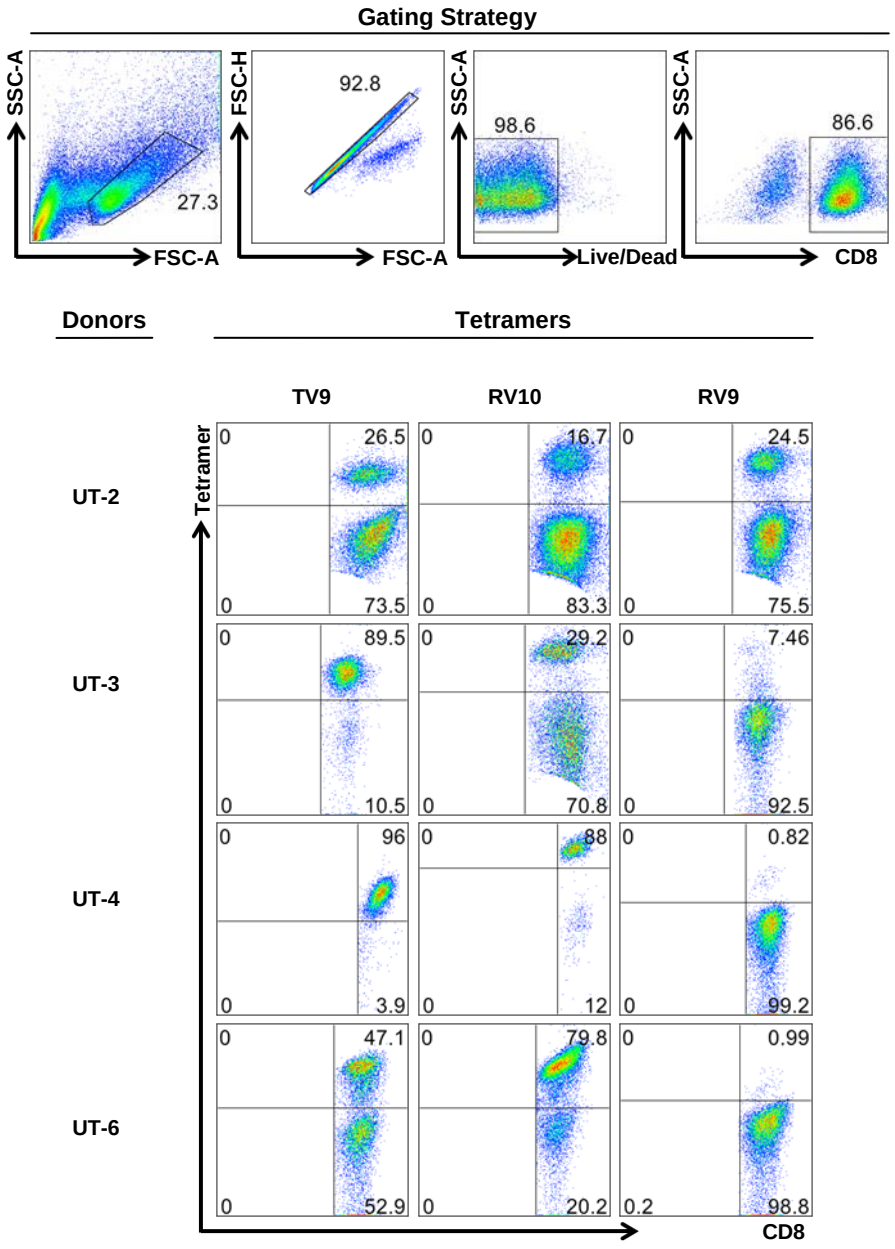

Figure 2. Tetramer of parallel cultures. Percent tetramer-binding cells in parallel cultures from four donors of TV9-, RV10- and RV9-CTLs at day 42.CTLs were primed with peptide-pulsed DCs and re-stimulated with peptide-pulsed monocytes. T cell cultures were stained every 7 days to determine specificity. This was repeated a total of 6 times.

4.4 Discussion

TV9-, RV10- and RV9-specific cultures were readily generated from four donors. This confirms our previous data that there is a high CD8 precursor pool in naïve individuals for each epitope. With these available specific T cell lines, we were able to further investigate the functionality to each specificity.

As stated earlier, the generation of the parallel cultures was imperative to study the functionality of the subdominant responses that we most likely would not be able to do with patient samples. Generation of these robust cultures not only gave us enough cells to carry out the characterization assays but allowed us to work with quality T cells.

It is well known within the HIV community that it is very difficult to generate quality specific $CD8^+$ T cells in large quantities for characterization purposes. Generation of the parallel cultures from healthy donors was our solution to overcome these problems, which we were successfully able to do.

4.5 Acknowledgements

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Chapter 5: Functional characterization of parallel cultures

5.1 Introduction

We hypothesized, that the overlapping of epitopes, within this region of p24, were contributing to the overall subdominance to the specificities. We examined the functional characteristics of the reactivities to determine if we could possibly identify, if any, the best epitope within the three. We sought to see if CD8 dependency played a role in the cytotoxicity of the responses, the polyfunctionality (degranulation, cytokine and chemokine production) of each response, their ability to suppress virus *in vitro*, TCR usage and responses in A2 patients.

5.2 Materials and Methods

Target cell lines. Cytotoxicity or intracellular cytokine secretion was assessed against peptide-pulsed T2 cells or C1R cells expressing either full-length wild-type HLA-A*0201 (C1R-$A2^{wt}$), a point-mutated variant (C1R-$A2^{CD8null}$) that does not bind the CD8 coreceptor or a point-mutated variant (C1R-$A2^{CD8enhanced}$) that enhances binding to the CD8 coreceptor [64].

Cytotoxicity assay. C1R-$A2^{wt}$, C1R-$A2^{CD8null}$, C1R-$A2^{CD8enhanced}$ target cells were labeled with sodium chromate (^{51}Cr, PerkinElmer, Waltham, MA) and pulsed with an appropriate peptide for 1 h at 37°C. After washing, the cells were admixed with T cells at different E:T ratios in 96-well round-bottom plates. After an incubation period of 4 h, supernatants were harvested and mixed with scintillation fluid (Optiphase SuperMix; PerkinElmer-Wallac, Gaithersburg, MD) for counting in a MicroBeta counter (PerkinElmer-Wallac). Cells not pulsed with peptide were used as negative controls. Specific percent lysis was calculated using the following formula: ((cpm experimental – cpm spontaneous)/(cpm total – cpm spontaneous)) x 100.

Cytotoxicity/Functional Sensitivity Assay. T2 cells were labeled with sodium chromate (51Cr, PerkinElmer, Waltham, MA) and pulsed with an appropriate peptide at different concentrations for 1 h at 37°C. After washing, the T2 cells were admixed with T cells at a constant E:T ratios in 96-well round-bottom plates. After an incubation period of 4 h, supernatants were harvested and mixed with scintillation fluid (Optiphase SuperMix; PerkinElmer-Wallac, Gaithersburg, MD) for counting in a MicroBeta counter (PerkinElmer-Wallac). T2 cells not pulsed with peptide were used as negative controls. Specific percent lysis was calculated using the following formula: ((cpm experimental – cpm spontaneous)/(cpm total – cpm spontaneous)) x 100.

Flow cytometric analysis. Directly-conjugated mAbs to CD8 (Qdot-605-3B5), CD107a (FITC-H4A3), CD107b (FITC-H4B4), IFN-γ (PE-Cy7-4S.B3), IL-2 (PerCP-Cy5-MQ1-17H12), TNF-α (Alexa Fluor 700-MAb11) and Granzyme B (Alexa 647-GB11) were purchased from BD Pharmingen (San Diego, CA). Perforin (PE-B-D48) was purchased from Cell Sciences (Canton, MA). Vβ family-specific mAbs were obtained from Beckman Coulter (Miami, FL). Intracellular cytokine production and degranulation were determined after a 4-hr stimulation with peptide-pulsed (10 μg/mL) T2 cells at a T cell:target cell ratio of 1:1 as described previously [65]. Briefly, CD107a/b-FITC and Golgi Stop and Golgi Plug (BD Biosciences) were added to the cells at the beginning of the incubation period. After 4 hr at 37°C, the cells were washed in staining buffer and then stained with the LIVE/DEAD Fixable Near IR Dead Cell Stain Kit (Invitrogen) and CD8-Qdot for 30 min at 4°C. Cells were permeabilized by the BD Cytofix/Cytoperm reagent (BD Biosciences) for 20 min at room temperature in the dark. Cells were washed twice with the perm/wash buffer, re-suspended in staining buffer, and left overnight at 4°C.

Cells were stained in a single step with the intracellular antibodies for 30 min at 4°C, washed once in perm/wash buffer, and re-suspended in fixed buffer. Cells were analyzed using a LSR II flow cytometer (BD Biosciences). Gating was performed on small lymphocytes, singlets, and viable $CD8^+$ T cells. More than 10,000 CD8 events were collected for each sample. Boolean gating analysis was carried out once positive gates were established for each functional parameter. This analysis resulted in 64 possible combinations of the 6 measured functions. Data analysis was performed using FlowJo 7.7.8 software (TreeStar), Pestle for background subtraction, and SPICE for frequency analysis and generation of pie charts. Pestle and SPICE were kindly provided by Dr.M. Roederer Vaccine Research Center, NIH, Bethesda, MD.

***In vitro* viral suppression assay.** The ability of $CD8^+$ T cells to inhibit HIV replication was determined *in vitro* [66]. PBMC from HLA-A*0201^+, HIV-uninfected donors were acutely-infected by 4 hr exposure to HIV NL4-3 at 80 $TCID_{50}$/ml. Infected PBMCs were co-cultured with T cells at a 4:1 ratio in complete medium containing IL-2 (25 U/ml). The concentrations of p24 in the supernatants were determined by ELISA on day 3 and day 6.

Identification of TCRβ-chain sequences by quantitative clonotypic PCR. Tetramer-binding cells were sorted viably to >99% purity for RNA-based analysis of TCRβ gene expression using a modified anchored RT-PCR as described previously [67]. The IMGT nomenclature is used to designate TCR gene usage [68].

T2 stabilization Assay. T2 cells were split the day before the experiment so they were growing in log phase. T2 cells were washed 1x with warm complete media and resuspended at $4x10^6$/ml in CM + 1% FBS + 4ug/mL β2m. Aliquots of 50ul of cells were placed into each well of a 96-well U-bottom plate. Peptide dilutions were added into each well and plates were incubated overnight at 37°C in a 5% CO2 incubator. 3 wells of T2 cells with no peptide were included for staining controls. The following day primary antibody, bb7.2 was added to each well and incubated for 30 minutes at 4°C. Immediately following, secondary antibody, gαm IgG FITC was added to each well and incubated for 30 minutes at 4°C. Cells were washed twice and run on LSRII. Data analysis was performed using FlowJo 7.7.8 software. Fluorescence ratio was calculated by (mean fluorescence of T2 cells loaded with peptide)/(mean fluorescence of T2 cells without peptide).

Cytotoxicity/Alanine Scanning Assay. T2 cells were labeled with sodium chromate (51Cr, PerkinElmer, Waltham, MA) and pulsed with an appropriate alanine substituted peptides for 1 h at 37°C. After washing, the T2 cells were admixed with T cells at a constant E:T ratio in 96-well

round-bottom plates. After an incubation period of 4 h, supernatants were harvested and mixed with scintillation fluid (Optiphase SuperMix; PerkinElmer-Wallac, Gaithersburg, MD) for counting in a MicroBeta counter (PerkinElmer-Wallac). T2 cells not pulsed with peptide were used as negative controls. Specific percent lysis was calculated using the following formula: ((cpm experimental – cpm spontaneous)/(cpm total – cpm spontaneous)) x 100.

5.3 Results

CD8 dependency of cytotoxic activity mediated by TV9-, RV10- and RV9-CTLs.

Homogeneous TV9-, RV10- and RV9-cultures were assessed for their dependence on CD8 compensation using peptide pulsed C1R target cells expressing wild type HLA-A*0201 (C1Rwt), pointed mutated HLA-A*0201 that enhances binding of CD8 (C1RCD8Enhanced) and point mutated HLA-A*0201 that cannot bind CD8 (C1RCD8Null) in chromium release cytotoxicity assays. Lysis of TV9-, RV10- and RV9-pulsed C1RCD8Null cells by TV9-, RV10- and RV9-CTLs, respectively, was substantially reduced compared to lysis of peptide pulsed C1Rwt and C1RCD8Enhanced cells. These results indicate the nullification of CD8 binding impaired and hindered functional activation of specific T cells in the majority of the cultures (Figure 4A-L).

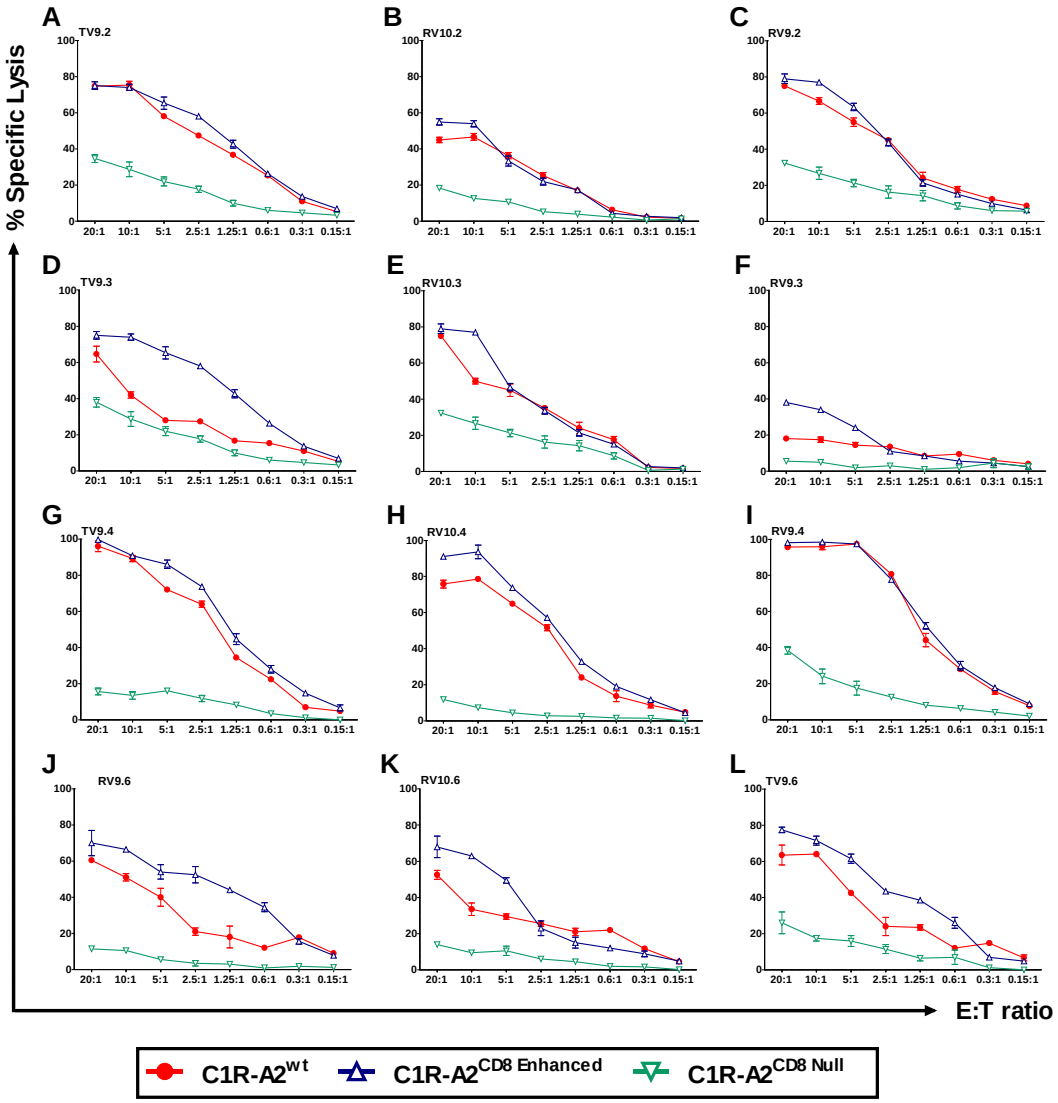

Figure 3. CD8 dependency of cytotoxic activity mediated by TV9-, RV10- and RV9-CTLs. Parallel cultures to TV9, RV10 and RV9 were assayed for cytotoxicity in a chromium release assay using C1R-A2CD8Enhanced, C1R-A2wt and C1R-A2CD8null targets loaded with 10µg/ml at a range of E:T ratios. Lysis of C1R cells alone was subtracted to give percent specific lysis. Data shown as mean ±SEM of triplicate assays and are representative of two independent experiments.

Functional sensitivity and cross reactivity of TV9-, RV10- and RV9-specific T cells.

Functional sensitivity of these cultures were also assessed using peptide pulsed T2 target cells expressing wild type HLA-A*0201. TV9- and RV10- specific T cell lines had a higher avidity to their cognate peptides, as compared to RV9-specific T cells, with EC50's ranging from 10^{-10} to 10^{-7}. RV9-specific T cells had EC50's ranging from 10^{-8} to 10^{-6}. This is consistent with low avidity tetramer binding of the RV9 cultures. Of note, TV9 and RV10 cell lines are consistently cross reactive with each other. Whereas, RV9 cell lines are slightly crossreactive with TV9 and RV10 epitopes and typically at very high concentrations of peptide. This was quite interesting, considering the nine-mer and ten-mer were crossreactive with each other but the two overlapping nine-mers were not. Further substantiation of the crossreactivity between TV9 and RV10 was confirmed with intracellular cytokine staining.

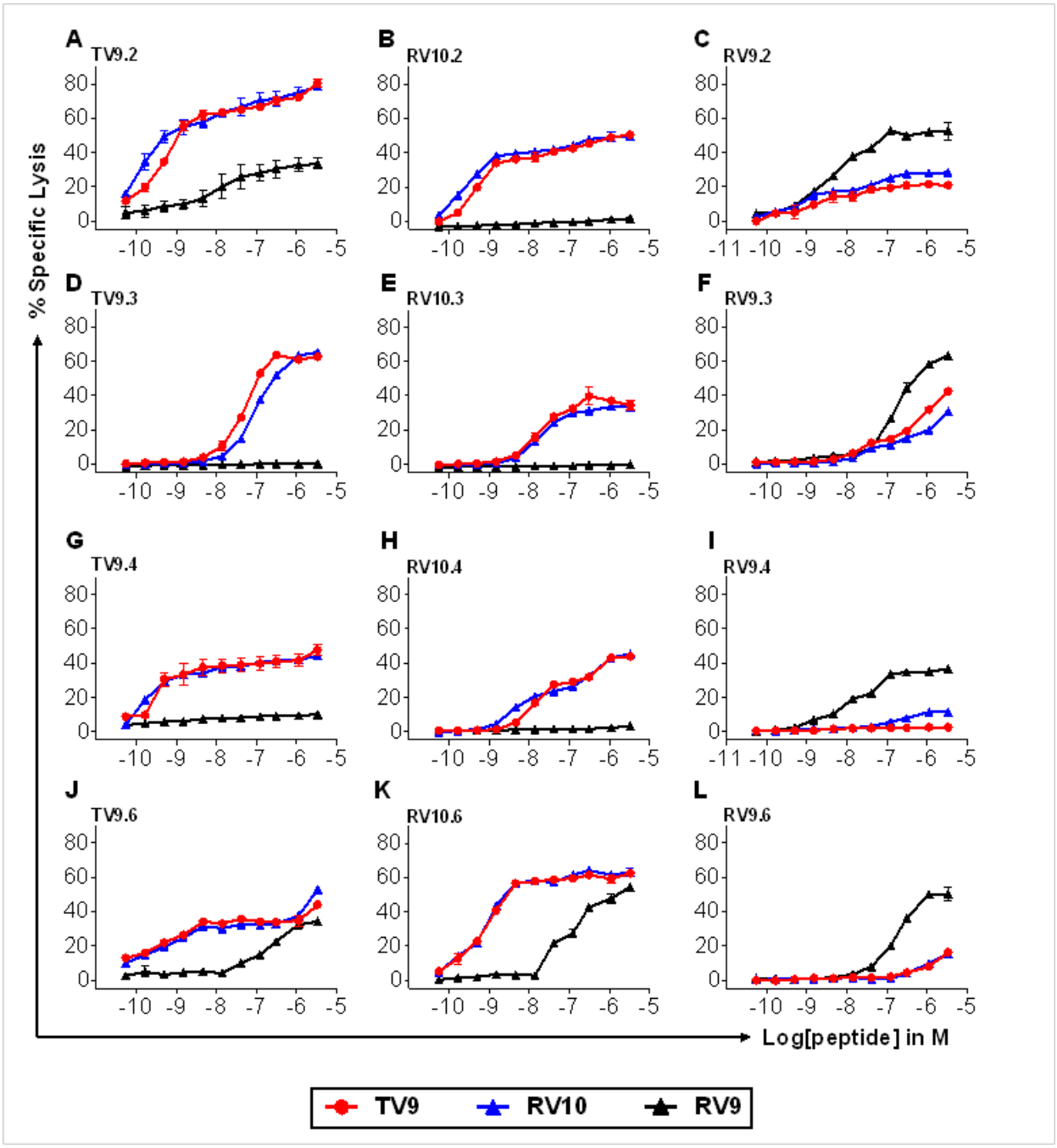

Figure 4. Functional sensitivity and cross reactivity of TV9-, RV10- and RV9-specific T cells. Parallel cultures to TV9, RV10 and RV9 were assayed for functional sensitivity and cross reactivity in a chromium release assay using T2 targets loaded with a range of peptide concentrations. Lysis of C1R cells alone was subtracted to give percent specific lysis. Data shown as mean ±SEM of triplicate assays and are representative of two independent experiments.

Analysis of crossreactive and polyfunctional subsets in TV9-, RV10- and RV9-specific CD8+ T cell cultures.

An important role for the fraction of the anti-HIV $CD8^+$, polyfunctional T cell fraction during chronic infection has previously been suggested [69]. We characterized the functional profile of CD8 responses directed against TV9, RV10 and RV9. This profile included the upregulation of CD107a/b as well as the induction of IFN-γ, IL-2, TNF-α, Perforin and Granzyme B. These responses revealed consistent cross-recognition between TV9 and RV10 (confirming data for cytotoxicity shown in **Figure 4**). Of note, the hierarchy of polyfunctionality of specific T cell responses directed towards their cognate peptide is as follows: TV9>RV10>RV9. The TV9.3 culture contains a small subset of specific T cells with all 6 functions, four large fractions of 5, 4 and 3 functions, and a small fraction of single parameter cells. The specific cells in the RV10.3 culture range from 5 functions to 1 function, with the largest subset having dual functionality. RV9.3 specific cells have a large subset of double function cells, with smaller proportions of 5, 4, 3 and single function cells. Although a weak stimulation, when TV9 and RV10 specific T cells are stimulated with RV9 peptide, there is a large subset of cells mediating only a single function. Alternatively, it is remarkable to find that when RV9 specific cells are stimulated with TV9 and RV10 peptide, there is a larger fraction of 5-, 4-, and 3-functional subsets as compared to stimulation with its cognate peptide.

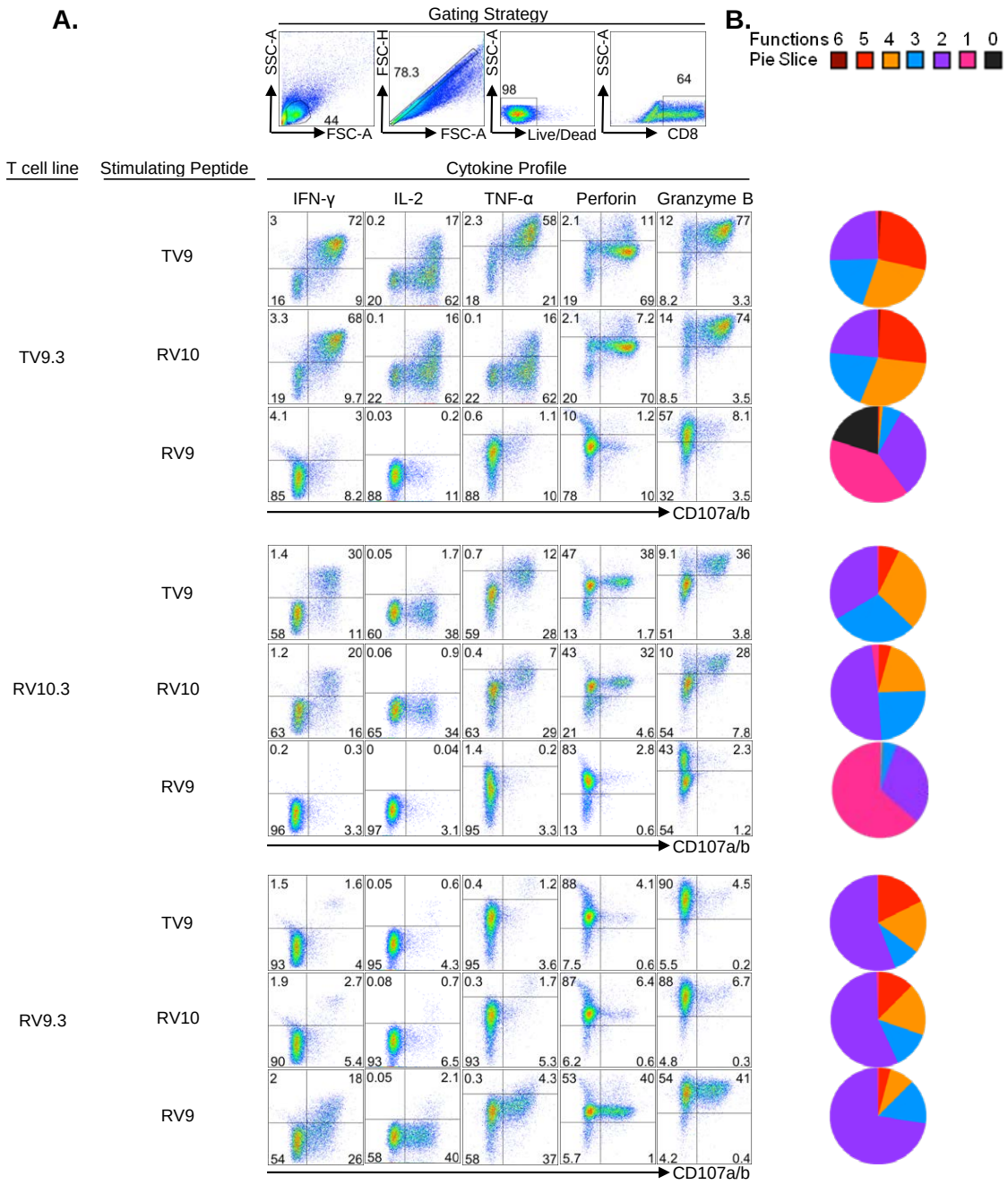

Figure 5. Analysis of crossreactive and polyfunctional subsets in TV9-, RV10- and RV9-specific CD8+ T cell cultures. Cross-reactive and polyfunctional epitope-specific $CD8^+$ T-cell responses were measured by flow cytometry following a 4 hour stimulation with TV9-, RV10- and RV9-peptides. **A**) Psuedocolor plots show the percentage of $CD8^+$ T-cells expressing CD107a/b, IFN-γ, IL-2, TNF-α, Perforin and Granzyme B. **B)** The pie charts represent the relative contribution to the total response of cells exhibiting each functional parameter or combination of functional parameters. The color code for grouped functions (pie slice) is reported above the pie charts. This data represents a single experiment.

Distinct TCR clonotype usage in parallel cultures.

TCR diversity in a given epitope-specific response is important for effective immune control [70]. Due to cross reactivity of TV9 and RV10 it was important to examine and determine if there was any overlap in clonotype usage. Tetramer-binding cells from parallel cultures were sorted viably to >99% purity for RNA-based analysis of TCRβ gene expression. Due to low avidity tetramer for RV9 we were only able to get TCR data from parallel culture UT.2 for this particular specificity. As seen in **Table 4** there is no clonal overlap between any of the specificities or between any of the parallel cultures. This implies that there must be another mechanism of cross-recognition since they are not utilizing public TCRs.

Table 4. TCRB usage. CDR3 amino acid sequence and percent frequency in TV9-, RV-10 and RV9-CTL cultures. The IMGT nomenclature for TCRVB is entirely consistent with Arden nomenclature used for staining by Vβ-specific mAbs. This data represents a single experiment.

CTL	IMGT TRB	Vb (Arden)	CDR3	TRBJ	Frequency (%)
TV9-2	4-2	7.3	C A S S Q D P S G D T D T Q Y	2-3	33.33
	6-2/6-3	13.2a/13.2b	C A S S P D W P N E Q F	2-1	16.05
	9	1.1	C A S S V E G L A E A Y N E Q F	2-1	9.88
	9	1.1	C A S S V D G W L D E Q F	2-1	9.88
	4-3	7.2	C A S S Q D V A G G Y N E Q F	2-1	8.64
	12-3/12-4	8.1/8.2	C A S S H N E K L F	1-4	7.41
	6-6	13.6	C A S S Y S L G T D E Q Y	2-7	4.94
	20-1	2.1	C S A S D W G G N E Q Y	2-7	2.47
	5-6	5.2	C A S S L V G G S F G E L F	2-2	2.47
	6-2/6-3	13.2a/13.2b	C A S S P D W S N E Q F	2-1	1.23
	5-6	5.2	C A S S A A P G Q I S D G Y T	1-2	1.23
	15	24.1	C A T S S I G P L D G Y T	2-3	1.23
	19	17.1	C A S S I G P L D G Y T	1-2	1.23
RV10-2	29-1	4.1	C S V G D G Y E Q Y	2-7	45.83
	29-1	4.1	C S V R H P H L S D E Q F	2-1	27.78
	20-1	2.1	C S A S W A G Q L N Y G Y T	1-2	15.28
	25-1	11.1	C A S S E S A W Y E Q H	2-7	5.56
	3-1	9.1	C A S T D T G E L F	2-2	2.78
	7-9	6.4	C A S S S L L D E Q Y	2-7	1.39
	20-1	2.1	C S A S W L A G G F G E T Q Y	2-5	1.39
RV9-2	30	20.1	C A W S V V Y E Q Y	2-7	55.29
	30	20.1	C A W S D S G G S D E Q Y	2-3	35.29
	28	3.1	C A S G N G A S Y G Y T	1-2	4.71
	30	20.1	C A W S V L G V T S T D T Q Y	2-3	1.18
	30	20.1	C A R S V V Y E Q Y	2-7	1.18
	5-6	5.2	C A G T D Y N E Q F	2-1	1.18
	30	20.1	C A W S V G T E A F	1-1	1.18
TV9-3	5-1	5.1	C A S S G T T G V D E Q Y	2-7	61.45
	29-1	4.1	C S E V G L P D S P N Y G Y T	1-2	15.66
	2	22.1	C A S S A D S F P K P P Q Y	2-3	8.43
	27	14.1	C A S S L L E G V P N T G E L F	2-2	4.82
	12-3/12-4	8.1/8.2	C A S S L L G T N G Y T	1-2	2.41
	2	22.1	C A S R E D A G A Y E Q Y	2-7	2.41
	29-1	4.1	C S V G G L P D P N Y G Y T	1-2	1.20
	29-1	4.1	C S V E G L P D P N Y G Y T	1-2	1.20
	5-1	5.1	C A S S G T T G V D K Q Y	2-7	1.20
	5-1	5.1	C A S S G T A G V D K Q Y	2-7	1.20
RV10-3	19	17.1	C A S S I T S G N Y N E Q F	2-1	38.27
	19	17.1	C A S S T G L E Q F	2-1	24.69
	6-1	13.3	C A S S E Q G E A F	1-1	24.69
	6-1	13.3	C A S S E G T R E Y	2-7	7.41
	19	17.1	C T S S I S G N Y N E Q F	2-1	1.23
	6-6	13.6	C A S T G L A T E Q Y	2-7	1.23
	19	17.1	C A S C T G L E Q F	2-1	1.23
	12-3/12-4	8.1/8.2	C A S S Y N E Q F	2-1	1.23
TV9-6	2	22.1	C A S R Q G G Y D E Q Y	2-7	46.91
	28	3.1	C A S S L A L G G Y Q E T Q Y	2-5	11.11
	20-1	2.1	C S V W T G G P Y E Q Y	2-7	8.64
	12-3/12-4	8.1/8.2	C A S S S L G L Q F	2-1	8.64
	24-1	15.1	C A T S D N L S P R S S Y N E Q F	2-1	3.70
	27	14.1	C A S S W V A G E G T G E L F	2-2	3.70
	12-3/12-4	8.1/8.2	C A S S P G E T Q Y	2-5	3.70
	12-3/12-4	8.1/8.2	C A S V D I H E A F	1-1	3.70
	7-9	6.4	C A S S P K A G Q G A Y D E Q Y	2-7	2.47
	19	17.1	C A S S I W A A P N E Q F	2-1	2.47
	2	22.1	C A S S E L A G V G D T Q Y	2-3	1.23
	20-1	2.1	C A S S E L A G V G D T Q Y	2-7	1.23
	2	22.1	C A S R Q G G Y G E Q Y	2-7	1.23
	12-3/12-4	8.1/8.2	C A S T H P G E A F	1-1	1.23
RV10-6	28	3.1	C A G T G Q G A D E Q Y	2-2	72.50
	11-2	21.3	C A S S D F P H P D E Q F	2-7	11.25
	24-1	15.1	C A T S D F G T S G E F	2-1	6.25
	28	3.1	C A S S S Y T G E L F	2-1	2.50
	6-4	13.5	C A S S D M G L G Y T	1-2	7.50

TV9-, RV10- and RV9-CTLs can suppress HIV replication *in vitro*.

The ability of TV9-, RV10- and RV9-CTLs to suppress replication of NL4-3.1 *in vitro* was assessed (**Figure 6**). Suppression of HIV-1 replication in acutely infected HLA-A*0201^{+} T1 cells by TV9-, RV10- and RV9-CTLs was determined 3 and 6 days post-infection. p24 in the supernatants was measured by p24 ELISA. The Δnef NL4-3.1 virus was designed to enhance expression of surface peptide-MHCI complexes in case there was no or low suppression with NL4-3.1 virus. Similar antiviral efficacy between three sets of RV10-CTL cultures was observed at two time points and overall had highest suppression among all three specificites. UT.2 had overall higher suppression in TV9 and RV10 cultures but not in RV9. Culture UT.3 had overall lower suppression for all specificities compared to the other T cell cultures studied. UT.6 had high suppression among all three specificities. No significant difference in suppression was observed between NL4-3.1 virus and Δnef NL4-3.1 virus.

The ability of TV9-, RV10- and RV9-specific T cells to suppress virus *in vitro* indicates that these responses can be effective T cell responses and quality vaccine candidates.

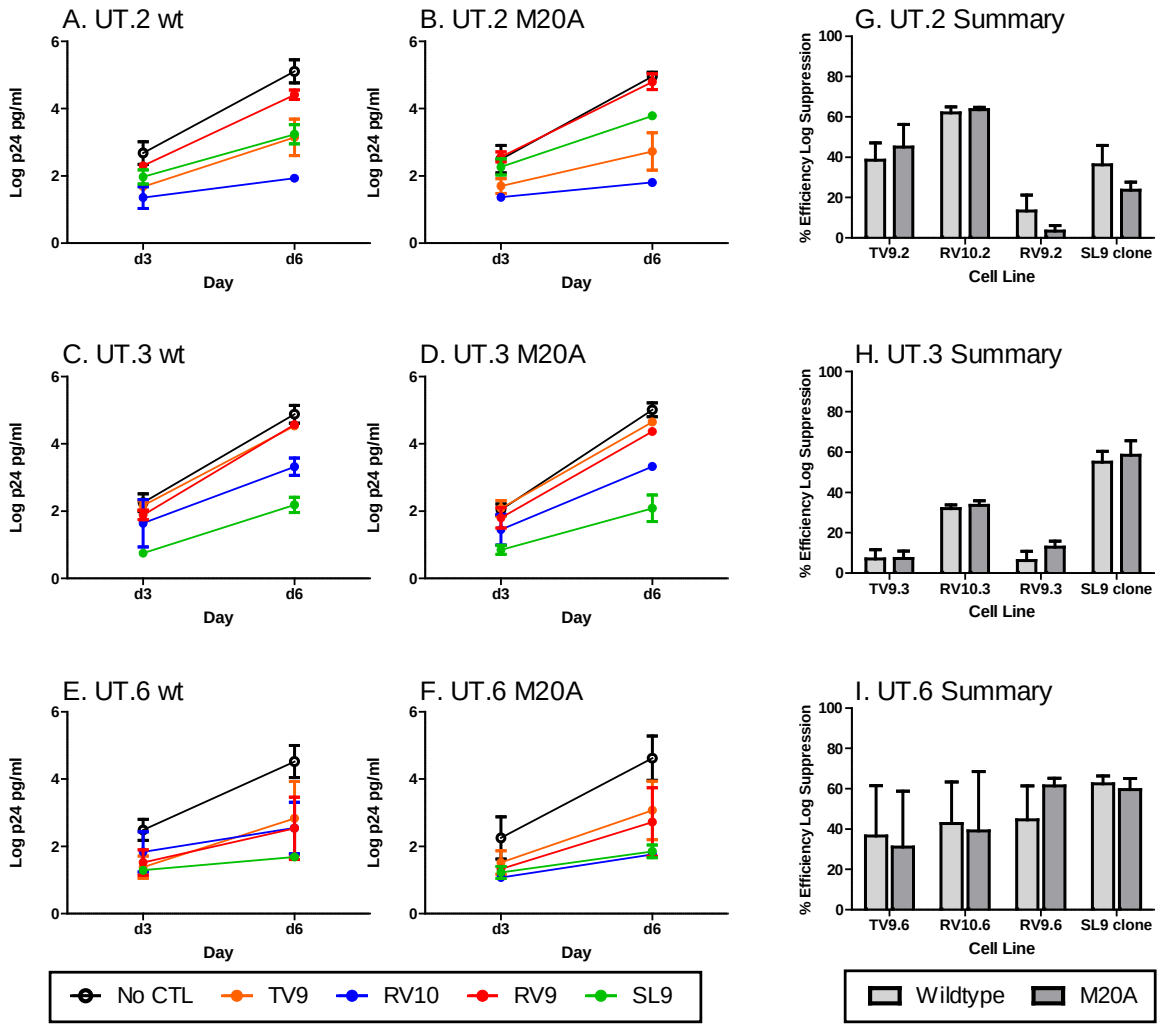

Figure 6. TV9-, RV10- and RV9-specific CTL suppression of HIV-1. T1 cells were acutely infected with the indicated viruses and cultured in the absence or presence of TV9-, RV10-, RV9- and SL9-specific CTLs. (A-F)Raw p24 values overtime are shown. (G-I) The mean efficiency of virus suppression (log10 units of p24 reduction) is plotted for each virus. This data represents a single experiment.

T2 stabilization and alanine scanning of TV9 and RV10 substituted peptides.

Due to the crossreactivity between TV9 and RV10 and distinct clonotype usage, we wanted to investigate the potential TCR contact sites of these epitopes. A panel of alanine substituted peptides to TV9 and RV10 were synthesized and tested for stabilization of the MHC Class I molecule as well as TCR recognition by specific T cell lines. **Table 5** list the sequences of substituted peptides used in the T2 Stabilization Assay and subsequent chromium release assay. Peptides substituted with alanine in all positions of the TV9 and RV10 sequences have similar or increased binding, to HLA-A*0201, as compared to the native sequence **(Figure 7A)**. This data shows evidence that RV10 and TV9 possible have similar binding to the MHC Class I molecule. The binding assay previously shown will need to be repeated to confirm that the binding affinities of TV9 and RV10.

TV9 and RV10 specific CTLs can recognize alanine substituted peptides but RV10 CTLs tend to be more sensitive to substitutions than TV9 **(Figure 7B)**. Asparagine (N), postion A3 in the TV9 substituted peptides, is important for recognition by TV9-specific CTLs. The loss of recognition is not due to lack of binding to the MHC ClassI molecule, implicating it as a potential TCR contact site. RV10-specific CTLs are sensitive to substitutions at Asparagine (N) and Tryptophan (W), positions A4 and A6 in the RV10 substituted peptides, respectively. These are also an indication of possible TCR contact sites for RV10 CTLs. Since both TV9 and RV10 CTLs are sensitive to substitutions at N, this may possibly be, a shared TCR contact site used for cross recognition of both epitopes.

Table 5. Alanine scanning library of TV9 and RV10. Seventeen peptide sequences were synthesized with the substitution of a native amino acid for an alanine, shown in bold, for each position of TV9 (A) and RV10 (B). The native sequence corresponds to position 4 in TV9 and position 5 in RV10.

A. TV9 alanine substituted peptides

	Position								
Peptide Name	P1	P2	P3	P4	P5	P6	P7	P8	P9
TV9-A1	**A**	L	N	A	W	V	K	V	V
TV9-A2	T	**A**	N	A	W	V	K	V	V
TV9-A3	T	L	**A**	A	W	V	K	V	V
TV9-A4(native)	**T**	**L**	**N**	**A**	**W**	**V**	**K**	**V**	**V**
TV9-A5	T	L	N	A	**A**	V	K	V	V
TV9-A6	T	L	N	A	W	**A**	K	V	V
TV9-A7	T	L	N	A	W	V	**A**	V	V
TV9-A8	T	L	N	A	W	V	K	**A**	V
TV9-A9	T	L	N	A	W	V	K	V	**A**

B. RV10 alanine substituted peptides

	Position									
Peptide Name	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
RV10-A1	**A**	T	L	N	A	W	V	K	V	V
RV10-A2	R	**A**	L	N	A	W	V	K	V	V
RV10-A3	R	T	**A**	N	A	W	V	K	V	V
RV10-A4	R	T	L	**A**	A	W	V	K	V	V
RV10-A5(native)	**R**	**T**	**L**	**N**	**A**	**W**	**V**	**K**	**V**	**V**
RV10-A6	R	T	L	N	A	**A**	V	K	V	V
RV10-A7	R	T	L	N	A	W	**A**	K	V	V
RV10-A8	R	T	L	N	A	W	V	**A**	V	V
RV10-A9	R	T	L	N	A	W	V	K	**A**	V
RV10-A10	R	T	L	N	A	W	V	K	V	**A**

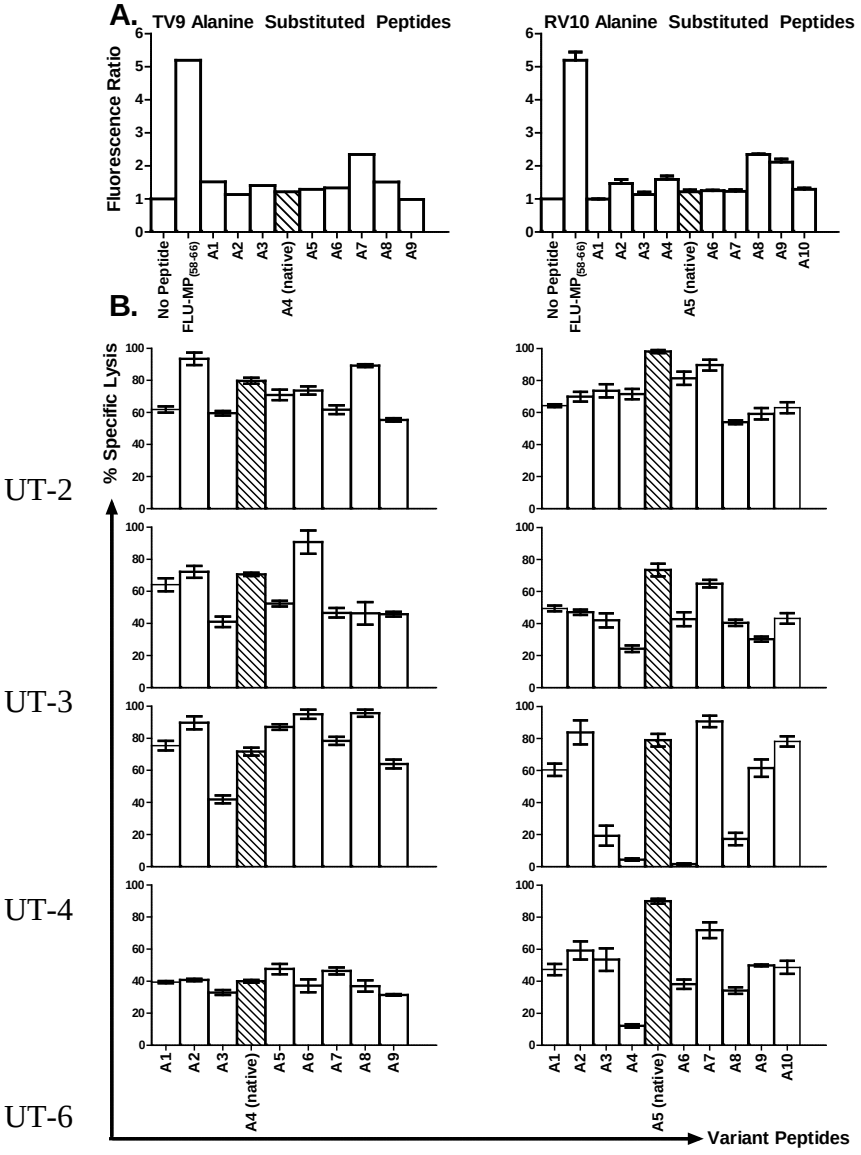

Figure 7. A. Relative Binding of TV9 and RV10 variant peptides to HLA-A*0201 as determined by the T2 stabilization assay. Fluorescence ratio was calculated by (mean fluorescence of T2 cells loaded with peptide)/(mean fluorescence of T2 cells without peptide). Assays were performed in triplicate, and the values represent the mean ± SD. B. Analysis of the recognition of alanine-scan substitutions of TV9 and RV10 variant peptides to by chromium release assay. Assays were performed in triplicate, and the values represent the mean ± SD.

Gag Specific Subdominant T cell Responses in HIV infected patients.

Many cell surface markers, activation profiles, and functional parameters of *ex vivo* HIV-specific $CD8^+$ T-cells have been shown to correlate with control of viremia [71]. We wanted to determine if there were subdominant $CD8^+$ T cells responses in HLA-A*0201 HIV-infected patients as determined by degranulation and upregulation of cytokines.

Briefly, frozen PBMC was defrosted and $CD8^+$ T cells were isolated via positive bead selection. Remaining PBMC was irradiated, plated and pulsed with 10µg/ml of SL9, TV9, RV10 and RV9 peptides, 1% human albumin and 2µg/ml β2M. Isolated $CD8^+$ T cells were added to wells and stimulated for 7 days. Day 7 post stimulation, and ICS assay was performed to determine if there was any recall responses to these epitopes. **Table 6** summarizes the total number of patients studied, patient's clinical status and responses to epitopes tested. A total of 10 A2 patients were tested with clinical status' ranging from elite controllers to progressors. Previous reports have indicated TV9 as an infrequently targeted epitope in natural HIV infection [51]. Our data is consistent with previous findings as only one patient out of ten responded to TV9. Of importance, that patient was an elite controller, A2-EC.1, classified by viral load <50 copies/mL and not on highly active anti-retroviral therapy **(Figure 8)**. The polyfunctionality of the TV9 responses ranges from five functions to one function.

Another important finding in this set of data are the responses in a good controller A2-GC.1, classified by viral load in the range of 50-200 copies/mL and not on highly active anti-retroviral therapy, to RV10 and RV9 **(Figure 8)**. The polyfunctionality of the RV10 and RV9 responses range from four functions to one function. This is similar to pattern to the polyfunctionality identified in the parallel cultures, in which TV9>RV10/RV9. Although, responses to the subdominant epitopes are lower compared to the chronic dominant SL9 epitope,

this data provides further suggestions that immunodominant epitopes do not mask the emergence of subdominant epitopes.

Table 6. Summary table of HLA-A*0201 HIV infected patient samples tested for subdominant responses to TV9, RV10 and RV9.

# Patients Tested	Response to			
	SL9	TV9	RV10	RV9
EC-4 (VL<50 copies/mL)	3/4	1/4	1/4	1/4
GC-2 (VL 50-200 copies/mL)	1/2	0/2	1/2	1/2
C-2 (VL 200-2000 copies/mL)	1/2	0/2	0/2	0/2
P-2 (VL <2000 copies/mL)	2/2	0/2	0/2	1/2
Total Patients= 10	7/10	1/10	2/10	3/10

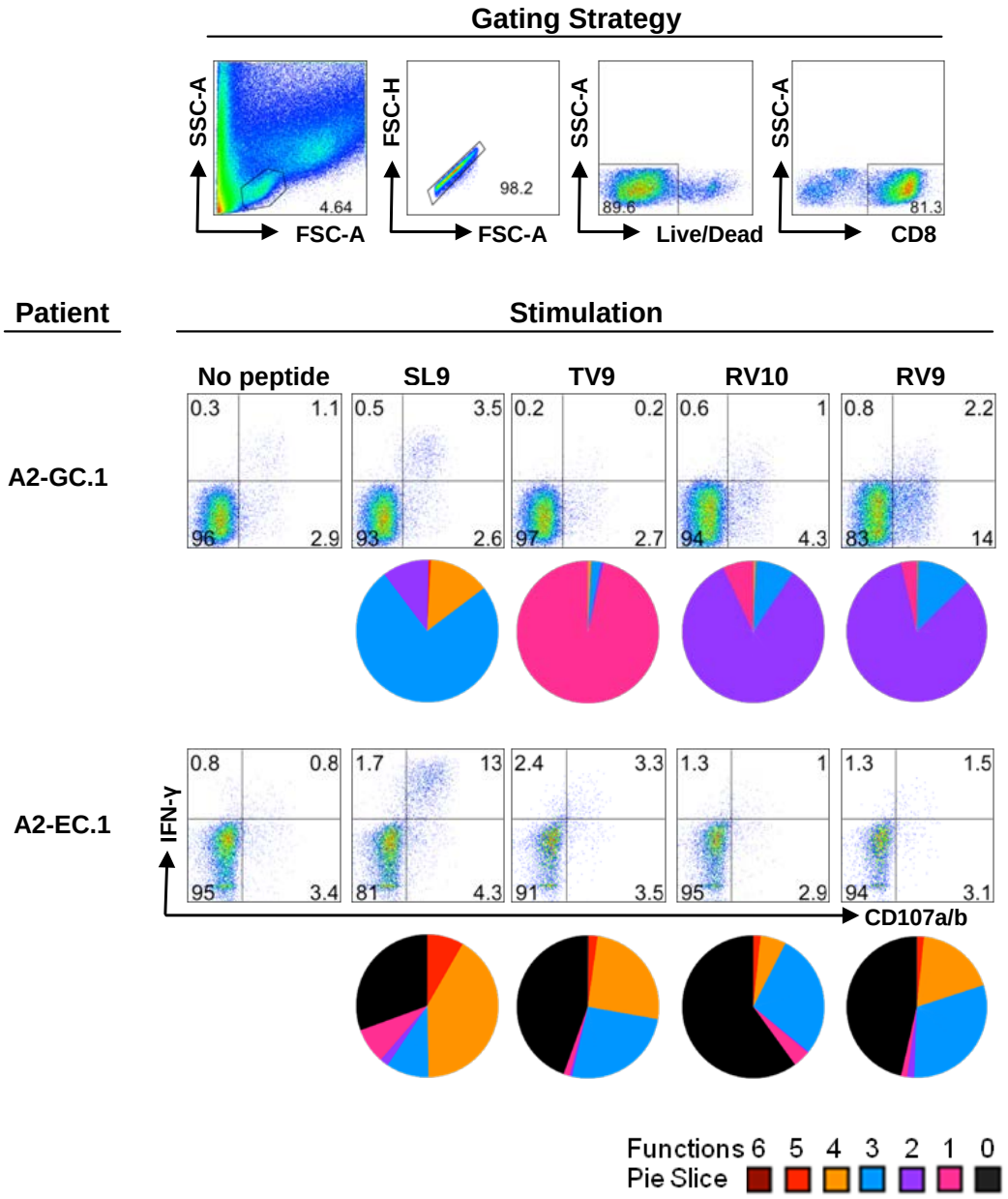

Figure 8. Functionality of Gag Specific Subdominant Responses in a Good Controller (GC) and an Elite Controller (EC). Representative flow cytometric plots showing CD107a/b versus IFN-γ from a GC (top row) and an EC (bottom row). Percentages represent the proportion of INF- γ and CD107a/b expressing cells. Pie charts represent the average CD8+ T-cell functional profile is shown for GC and EC. Responses are grouped according to the number of positive functions. This data represents a single experiment.

5.4 Discussion

Our data indicates that CD8 binding is necessary for functional activation of specific T cells in the majority of the TV9-, RV10-, and RV9-specific T cell cultures. TV9- and RV10-specific T cell lines had a higher avidity to their cognate peptides, as compared to RV9-specific T cells. All specific T cell lines were polyfunctional, although TV9 had the maximum amount of functional subsets among the cultures. TV9 and RV10 peptide-specific $CD8^+$ T cell cultures were consistently crossreactive in all assays tested. Despite consistent crossreactivity, there was distinct TCR clonotype usage in three of four parallel cultures.

There was similar antiviral efficacy between three sets of RV10-CTL cultures, which had the highest percent suppression of the three specificities.

TV9 and RV10 alanine substituted peptides showed no loss in binding to the MHC Class I molecule but did have a loss of T cell recognition when asparagine (N) was substituted. This is an indication that N is a possible shared TCR contact site that plays a role in cross recognition.

TV9-, RV10- and RV9-responses were identified in two of ten patients, both of whom are able to control virus and are classified as controllers based on their viral load and lack of HAART. Although responses were not as strong as, the known chronic dominant epitope SL9, they were present and polyfunctional. TV9 had the leading amount of polyfunctional subsets, which was a similar pattern found in the parallel cultures from healthy donors.

5.5 Acknowledgements

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Chapter 6: Final Conclusions and Future Directions

6.1 Overview and Final Conclusions

Subdominance of these epitopes may result from immune interference caused by simultaneous presentation of partially crossreactive epitopes, which leads to suboptimal TCR activation.

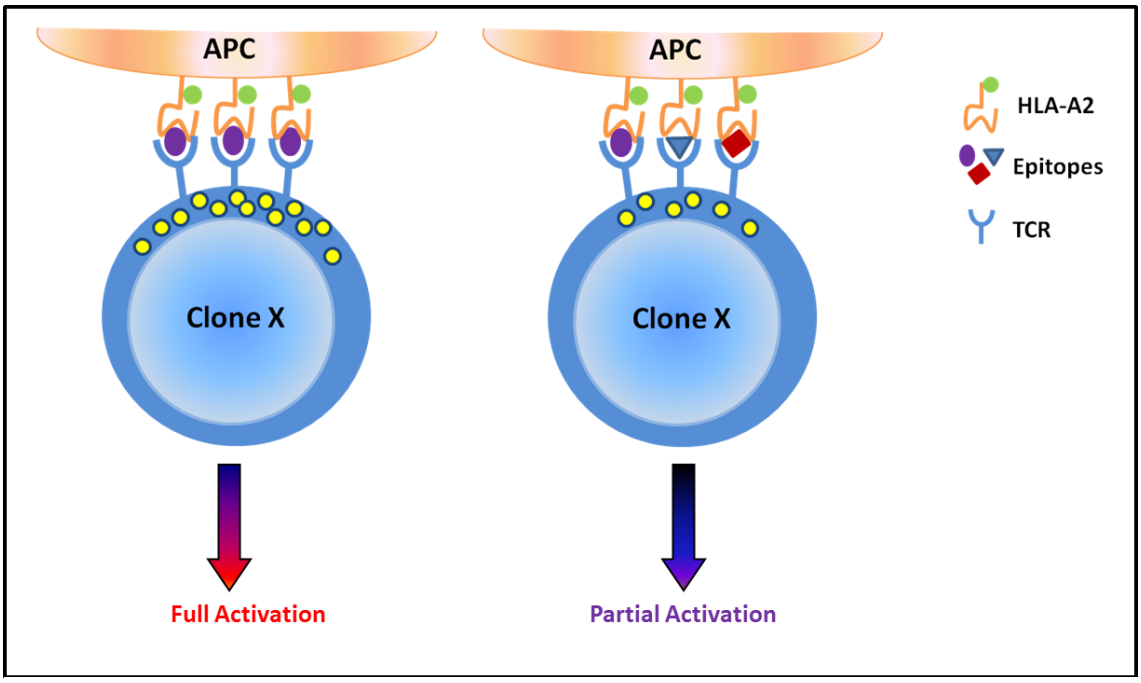

Figure 9. Mechanism of subdominance. Schematic of a potential mechanism of simultaneous presentation of partially crossreactive epitopes that could lead to the subdominance of TV9, RV10 and RV9 epitopes.

Understanding the immunological mechanism that contributes to the CD8 epitope hierarchy of HIV conserved sequences provides the insights necessary to develop novel vaccination strategies to increase the magnitude and diversity of epitope-specific cell populations and to target responses to conserved epitopes.

6.2 Future Directions

Future work will look for immune interference in other HIV conserved domains, since overlapping epitopes is not uncommon across the viral proteome. We will also look at focusing the response to TV9 by eliminating RV10 and RV9 epitopes by replacement or removal of amino acid residues. Flanking regions will also be utilized to increase the presentation of the TV9 epitope alone.

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Curriculum Vitae

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Pathobiology- Biological Sciences

Margaret C. Costanzo earned her Bachelor of Science degree in Biology from the University of Texas at El Paso (UTEP) in 2006. She decided to become a biomedical researcher after an inspiring research internship at the University of Texas at San Antonio in her sophomore year. During her undergraduate years, she was selected for the National Institutes of Health (NIH) Research Initiative for Scientific Enhancement (RISE) program for seven semesters based upon her outstanding academic record.

Ms. Costanzo began her doctoral training in Pathobiology in June, 2006 at UTEP. During this period, her achievement was recognized by her winning several highly competitive, university-wide awards including a tuition scholarship from the National Science Foundation (NSF) program "Alliance for Graduate Education and the Professoriate (AGEP)", a RISE doctoral fellowship and an individual award from the NIH program "Research Supplement to Promote Diversity in Health-Related Research." Ms. Costanzo has presented her research at national and international meetings, in particular the prestigious Keystone Symposium on HIV Vaccines in 2009, 2010 and 2012 for which she was awarded an internationally competitive travel scholarship each time. Ms. Costanzo has worked under the auspices of Dr. June Kan-Mitchell, Professor of Biological Sciences, on the development of new HIV vaccines. The title of her dissertation is "Mobilizing Subdominant HIV-specific CTLs as a Novel Vaccine Strategy". To further her training in HIV immunology, Ms. Costanzo will be a Postdoctoral Fellow in her mentor's laboratory.

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