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Functional Properties and Epitope Characteristics of T-Cells Recognizing Natural HIV-1 Variants

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Abstract

To understand how broad recognition of HIV-1 variants may be achieved we examined T-cell reactivity in newly-infected persons as well as vaccine recipients to a broad spectrum of potential T-cell epitope (PTE) variants containing conservative, semi-conservative and non-conservative amino acid substitutions. Among early-infected persons T-cells recognized epitope variants with one substitution at a significantly higher frequency versus those with two (P=0.0098) and three (P=0.0125) substitutions. Furthermore T cells recognized variants containing conservative substitutions at a higher frequency versus those containing semi-conservative (P=0.0029) and non-conservative (P<0.0001) substitutions. Similar effects were observed on recognition of variants by vaccine induced T-cells. Moreover even when variants were recognized, the IFN-γ and granzyme B responses as well as T-cell proliferation were of lower magnitude. Finally, we show that epitope distribution is strongly influenced by both processing preferences and amino acid entropy. We conclude that induction of broad immunity is likely to require immunogen sequences that encompass multiple variants. However, cost-effective design of peptide and sequence based vaccine immunogens that provide maximal coverage of circulating sequences may be achieved through emphasis on virus domains likely to be T-cell targets.

1. INTRODUCTION

The enormous genetic diversity in HIV-1 is a major challenge in the design of vaccines that would protect against the wide range of HIV-1 circulating strains. Group-M HIV-1 dominates

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the worldwide pandemic and consists of several distinct subtypes [1]. Proteins within subtypes vary by approximately 5–25% and between subtypes by 10–40%, the lower bound representing conserved proteins and the upper bound representing variable proteins. A globally efficacious vaccine will need to elicit broadly reactive immune responses [2,3]. Such a vaccine is likely to require immunogen sequences that induce T-cells recognizing epitopes that are similar to or cross-react with those in circulating viruses. Although, cross-clade T-cell responses have been reported [4–15] our understanding of the numbers and types of amino-acid differences that are tolerated and how these impact T-cell functional properties is limited. Such an analysis requires a definition of the immune responses at the epitope level comparing T-cell reactivity to variants containing conservative versus semi- conservative and non-conservative amino acid substitutions.

In this study we examined the ability of T-cells to recognize a broad spectrum of potential T cell epitope (PTE) variants in newly-infected persons within the context of autologous virus sequences and in uninfected persons who were recipients of HIV-1 vaccines. We chose subjects with early HIV-1 infection since the immune responses detected in these subjects are more likely to be relevant for immune control, compared to later in infection when the viruses may have escaped key CTL responses. Additionally, we chose to examine a group of vaccine recipients since it is not known if the spectrum of epitope variants recognized in the context of vaccination and infection are similar. We assessed responses to a standardized peptide panel that represented PTE contained in diverse circulating HIV-1 strains. Compared to the consensus (CON) peptides, which have at each site the most common amino acid residue across a sequence alignment, the peptide reagents based on the PTE approach offer improvements in the assessment of T-cell responses [16,17]. PTE sequences are naturally occurring in HIV-1 strains and encompass sequences among virus variants circulating at moderate to high frequencies [18].

Most T-cell epitopes identified by central-sequence peptides in infected persons cluster in domains with low sequence variability, with few epitopes being detected in regions of moderate and high sequence variability [19]. This could partially be attributed to sequence variability between infecting and testing antigen sequences and consequently poor sensitivity for detecting epitopes in more variable domains with such peptide sets. Indeed T-cell responses detected by stimulation with a PTE peptide set are significantly greater in breadth and magnitude compared to those detected with a CON peptide set [20]. Therefore in this study we examined epitope distribution characteristics in infected persons using the PTE peptide set with its expanded epitope coverage.

Our findings indicate that while Nef-specific T-cells capable of recognizing multiple variants are commonly induced during early infection, semi- and non-conservative substitutions and those affecting more than one residue are infrequently tolerated. Further investigations of immune responses in recipients of HIV-1 vaccines consisting of Ad5 encoding Gag/Pol (clade B) and Env (clades A, B and C) corroborated these findings. We provide further evidence that there are considerable differences in the quality of T-cell responses recognizing multiple epitope variants. The data have wider implications in relation to vaccine immunogenicity, suggesting that while vaccine-induced T-cells may be able to recognize epitopes both in and outside the subtype represented in the vaccine, the quality of the response to epitopes outside the subtype may not be adequate to provide protection against highly diverse circulating strains and rapidly changing virus populations. Thus, induction of broad immunity may require vaccines that encompass multiple sequence variants. Furthermore, we show that epitope distribution is strongly influenced by both processing preferences and amino acid entropy. This information provides critical insight into the cost-effective design of peptide and sequence based vaccine immunogens as well as peptide test reagents that provide maximal coverage of

circulating sequences through emphasis on domains that are likely to be T-cell targets [21–24].

2. METHODS

Study population

Subjects with primary HIV-1 infection were recruited and enrolled at the University of Washington (UW) Primary Infection Clinic (PIC). The duration of infection was typically defined as the time from the onset of clinical signs and/or symptoms suggestive of acute retroviral syndrome [25–27]. All participants were men who have sex with men, and presumed to be infected with clade B viruses [28]. Some patients at various time points following diagnosis elected to receive combination antiretroviral therapy (ART) with nucleoside reverse transcriptase inhibitors, and either a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor. Participants in the HVTN054 vaccine trial were recruited through the HIV Vaccine Trials Network (HVTN) and received one dose of Ad5 encoding Gag/Pol (clade B) and Env (clades A, B and C) [Producer: Vaccine Research Center (VRC), National Institutes of Health (NIH)]. Immunogenicity assessment was performed 28 days after vaccination. Uninfected volunteers at low risk for HIV-1 infection were recruited through the Fred Hutchinson Cancer Research Center HIV Vaccine Trials Unit to serve as donor controls. The Human Subjects Review Board at the institutions approved the studies, and all volunteers provided written consent prior to participation.

Plasma HIV-1 RNA levels, T-cell subset analyses, and HLA typing

Plasma HIV-1 RNA was determined by quantitative branched-chain DNA (bDNA, Chiron, Emeryville, CA, USA) and ultrasensitive reverse transcriptase-polymerase chain reaction (RT-PCR, Roche Molecular Systems, Branchburg, NJ, USA) assays. RNA levels were expressed as copies/ml, and the lower levels of sensitivity were 500 copies/ml (bDNA assay) and 50 copies/ml (RT-PCR assay) [29]. Absolute blood CD4+ T-cell counts were measured by consensus flow cytometry. HLA typing was performed at the Puget Sound Blood Center by sequence-specific primer PCR as previously described [30].

Analysis of PTEs from circulating strains and PTE coverage

The design of PTE peptides has been previously described [18]. In brief, given that MHC class I molecules typically present antigenic peptides that are 8–10 aa in length [31–33], we considered 9 aa as the minimal epitope length, and defined PTE as all possible unique 9-mers contained in the virus sequences. Within this mixture of unique PTEs, each was then classified by its frequency of occurrence relative to the other PTEs. To design longer peptides such as 15-mers, each unique 15 amino acid sequence embedded in the group of virus sequences is extracted. These naturally-occurring 15-mers are then ordered by their PTE coverage level, which is the summation of the PTE frequencies associated with each of the individual embedded 9-mers. Thus, the higher the PTE coverage, the greater the likelihood that the peptide represents the virus sequences. A peptide reagent set is then built in a step-wise fashion until the desired cumulative PTE coverage is reached.

The nef PTE peptide set used to assess immune responses in newly-infected subjects covered all PTE contained among 5% or more of the subtype B virus sequences [20]. The Env, Gag, and Pol PTE peptide sets used to assess immune responses in the vaccine recipients covered all PTE contained in 15% or more of the global viral sequences.

IFN-γ and granzyme B ELISpot assay

HIV-1 Nef PTE peptides (n=90) were synthesized at Mimotopes (Raleigh, NC, U.S.A.); and the Gag, Env, Pol, and truncated peptides to fine map epitopes were synthesized at BioSyn (Lewisville, TX, USA). All peptides were used at a final concentration of 2 μg/ml unless stated otherwise, and the final concentration of DMSO never exceeded 1% in the assays.

IFN-γ ELISpot assays were performed to detect HIV-1-specific IFN-γ secreting cells as previously described [34], with modifications as follows. Granzyme B ELISpot assays were performed using monoclonal antibodies from Mabtech (Sweden) and following the manufacturer's directions. Phytohemagglutinin (Murex Biotech, Dartford, United Kingdom) stimulation at 2 μg/ml was used as a positive control and no peptide stimulation was used as a negative control. Spot-forming cells (SFC) were counted using the Immunospot (Cellular Technology Ltd., Cleveland, Ohio, USA) optical reader. The number of antigen-specific IFN-γ-and granzyme B-secreting cells was calculated by subtracting the SFC in the negative control wells from those in the antigen-stimulated wells. Peptides were first tested in pools and all responses were confirmed at the single peptide level [35,36]. Responses > 3-fold background and > 50 SFC/10^6 cells above background were scored positive.

To determine the functional avidity of the T-cell response, the standard IFN-γ ELISpot assay was performed with the optimal epitopic peptides at serial concentrations between 10,000 and 0.1 ng/ml. SFC frequencies were plotted against the $\log_{10}$ peptide concentration with ORIGIN 6.0 professional software (Microcal Software, Inc. Northampton, MA) and the effective peptide concentration that elicited 50% of the maximum T-cell response, defined as the EC_{50}, was determined with the Sigmoidal Fit tool.

Measurement of T-cell proliferation by CFSE labeling [37,38]

Briefly, thawed, washed, and overnight-rested PBMC were incubated with 1.3μg/mL (1.25μM) Carboxyl Fluorescein Succinimidyl Ester (CFSE) (Molecular Probes, Eugene, OR) for 8 minutes; quenched with cold FBS; and washed with PBS. The cells were then resuspended in complete media (10^6 cells/ml), plated in 24-well plates at 1 ml per stimulus and cultured for 5 days. Positive and negative controls included cells incubated with anti-CD3 mAb (30 ng/mL) and anti-CD28 mAb (1 μg/mL), and with medium alone. The decrease in CFSE signal is used to monitor proliferation of each cell subset.

Sequencing of autologous viruses

DNA was purified from 2 × 10^6 cryopreserved PBMC using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA), according to the manufacture's recommendations. HIV-1 Nef sequences were amplified by standard nested PCR as previously described [39]. Primers DS7 (F) 8169–8198 and TMMNEF6 (R) 9607–9631 were used in the first round PCR resulting in a 1.5 Kb product. Primers DS9 (F) 8678–8697 and DS8 (R) 9527–9550 were used in the second round PCR resulting in an 872 bp product. The PCR products were cloned into the TA vector using the TOPO TA Cloning Kit according to the manufacturer's protocol (Invitrogen, San Diego, CA) and selected for sequencing as described previously [40] to avoid template resampling [41]. Plasmids from individual clones were isolated and sequenced using the M13 355–370 and T7 328–347 forward and M13 205–221 and T3 243–262 reverse primers with ABI Prism Big Dye Terminator Cycle sequence reagents (Applied Biosystems, Foster City, CA). Computational analysis of the sequences was performed using ExPASy Translate Tool (http://us.expasy.org/tools/dna.html), ClustalW (http://www.ebi.ac.uk/clustalw/) and BioEdit.

Scoring of changes in amino acid sequences

Amino acid substitutions between sequences were scored for similarity using the BLOSUM matrix [42]. The matrix is derived from blocks of aligned sequences from homologous proteins with a certain level of identity. Conservative changes have positive scores and non-conservative changes have negative scores with the scale extending from −4 to +3. Conservative amino acid substitutions are likely to conserve the physical and chemical properties necessary to maintain the structure and function of the protein, whereas non-conservative substitutions are likely to disrupt essential structural and functional features of the protein.

Calculation of amino acid entropy and proteasomal cleavage scores

Amino acid variability was calculated as the entropy. The average entropy score for each 9-mer or a defined optimal epitope was calculated from Shannon entropy for each residue of B clade protein alignments, using only complete Nef protein sequences and only a single protein from multi-sequence sets derived from a single subject. Proteasomal cleavage prediction scores were determined based on a cleavage prediction algorithm (NetChop 3.0, [43] that has been shown to be highly predictive of C-termini in epitopes.

Statistical analysis

Effects of numbers and relative conservation of amino acid substitutions on recognition of T-cell epitope variants in subjects with primary HIV-1 infection and in HIV-1 vaccine recipients, as well as entropy and C-terminus cleavage scores for targeted optimal epitopes and the group of all Nef subtype B 9-mers, were assessed using the Wilcoxon Signed Rank Test. Entropy vs. C-terminus cleavage scores for the group of all Nef subtype B 9-mers was examined using the Spearman correlation coefficient.

3. RESULTS

Recognition of epitope variants and autologous virus sequence analyses

T-cell recognition of a total of 150 Nef variants, encompassing 35 epitopes (mean 4.3 variants/epitope), was assessed in 17 newly HIV-1-infected subjects with demonstrated Nef responses. At the time of analysis of the immune response, the subjects were infected for a median duration of 45 (range 7 – 134) days post onset of symptoms of primary HIV-1 infection (DPS), and 2 subjects had received ART for > 1 week (duration 42 and 57 days). For each of the 35 epitopic domains, the optimal epitope was either confirmed using peptide truncations or inferred based on the subject's HLA type and published MHC class I restricted CD8+ T-cell epitopes.

Autologous HIV-1 nef sequences were amplified by standard nested PCR in eight subjects and an average of 20 (range 10 – 33) Nef sequences were analyzed. Amino acid substitutions were scored for similarity using the BLOSUM matrix. Scores of +2 and +3 were imputed conservative, 0 and +1 semi-conservative and −1 and below non-conservative. Notably, of 19 epitopes sequenced in these subjects, the autologous consensus sequence either matched the subtype B consensus (n=14 epitopes) or differed by a single conservative amino acid (aa) substitution (n=4 epitopes) except for one epitope that contained two semi-conservative substitutions. With the referent sequence being either the autologous (when available, N=19), or the subtype B consensus (N=16), 73 of the 115 peptide variants examined across the 35 epitopes contained one aa substitution, 35 contained two, and seven contained three substitutions. T-cells recognized 52.1% (38 of 73) of the variant epitopes containing one substitution with a < 0.5 log difference in response magnitude in contrast to 25.7% (9 of 35, P=0.0098) that contained two substitutions and none (0 of 7, P=0.0125) that contained three substitutions (Figure 1A). Variants containing conservative substitutions were recognized at a

significantly higher frequency [61.1% (22 of 36)] vs. those containing semi-conservative [37.9% (11 of 39), P=0.0029] and non-conservative substitutions [7.5% (3 of 40), P<0.0001] (Figure 1B). In no instance did we detect a response to a variant epitope in the absence of a response to the autologous consensus.

Next in HVTN 054 vaccine enrollees we characterized T-cell recognition of epitope variants 28 days after one dose of the Ad5 encoding Gag/Pol (clade B) and Env (clades A, B, C) using global PTE peptides encompassing Gag (n=330), Env (n=492) and Pol (n=509). Responses were analyzed in 7 subjects in whom T-cells targeted epitopes (n=16) with amino acid variability so that recognition or non-recognition of peptide variants could be assessed. With the referent sequence being the vaccine sequence, 45 of the 73 peptide variants examined across the 16 epitopes contained one aa substitution, 21 contained two, and seven contained three substitutions. T-cells recognized 57.8 % (26 of 45) of the variant epitopes containing one substitution with a < 0.5 log difference in response magnitude in contrast to 19.0% (4 of 21, P=0.003) that contained two substitutions and none (0 of 7, P=0.0098) that contained three substitutions (Figure 1C). Variants containing conservative substitutions were recognized at a significantly higher frequency [68.2% (15 of 22)] vs. those containing semi-conservative [34.6% (9 of 26), P=0.0032] and non-conservative substitutions [24% (6 of 25), P=0.0024] (Figure 1D).

In summary, HIV-1-specific T-cells capable of recognizing multiple variants are frequently induced during early infection as well as following vaccination. However, semi- and non-conservative substitutions and those affecting more than one residue are infrequently tolerated in the context of both infection and vaccination.

IFN-γ and granzyme B responses to epitope variants

We further characterized T-cell recognition of several optimal epitope variants in the Nef protein measuring the IFN-γ and granzyme B responses to serial dilutions of peptide variants in a sub-group of HIV-1-infected subjects where PBMC were available for these analyses. Due to lack of sufficient PBMC these studies could not be done in vaccine recipients.

T-cells from subject 1188 recognized all five variants of the WF9/B1503 epitope tested producing IFN-γ [20], although only one of these variants was detected among the autologous sequences at 93 DPS. However, the magnitude and avidity of the responses to the variants showed significant differences and we found that the granzyme B response pattern mimicked the IFN-γ pattern. Amino acid substitutions in presumed T-cell receptor (TCR) contact sites e.g. WKFDSSLAF (R→S, position 6, BLOSUM score −1) and WKFDSHLAF (R→H, position 6, BLOSUM score 0) were associated with a 5–10-fold reduction in magnitude compared to responses to the WKFDSRLAF epitope (Figure 2A). Similarly in subject 1692 (at 50 DPS), the highest IFN-γ and granzyme B responses were elicited by the same two RW8 epitope variants (RYPLTFGW, RFPLTFGW). Semi-conservative substitutions at presumed TCR contact sites (RYPLTLGW, RYPLCFGW and RFPLCFGW) had large effects on IFN-γ and granzyme B responses (Figure 2B).

In summary, T-cells from newly-infected persons recognize multiple epitopic variants, although the magnitude and avidity of the responses can be highly variable. Multiple factors influence recognition, including the number, the type (i.e., conservative vs. non-conservative aa substitutions), and the position of the substitutions within the epitope. Even when epitope variants are recognized, the magnitude and/or avidity of IFN-γ and Granzyme B responses can be significantly lower.

Functional heterogeneity among T-cell responses to epitope variants

To further define the quality of the T-cell response to different variants, we examined T-cell proliferation by CFSE staining. In subject 1238 we characterized the AL9/Cw08-restricted response examining IFN-γ and granzyme B production as well as proliferation to six variants (AAVDLSHFL, AAV; GAVDLSHFL, GAV; AAIDLSHFL, AAI; GALDLSHFL, GAL; AALDLSHFL, AAL; and GAFDLSHFL, GAF). We observed high magnitude (542 – 726 SFC/10^6 PBMC, 1 μg/ml) and avidity (0.008 – 0.020) IFN-γ responses to the AAV, GAV and AAI variants and relatively lower magnitude (136 – 179 SFC/10^6 PBMC at 1 μg/ml) and avidity (0.202 – 0.909 μg/ml) responses to the GAL and AAL variants with no response to the GAF variant. To determine if there were differences in the granzyme B production and proliferative responses to the epitope variants we assessed granzyme B production and the $CFSE_{lo}$ frequencies of cells. We detected the strongest granzyme B and T-cell proliferative responses to the same three variants, AAV, GAV and AAI, as the IFN-γ responses (Figure 3A).

In subject 1212 we characterized the AL9/B62 response examining IFN-γ production and T-cell proliferation to six variants (Figure 3B). The IFN-γ responses to all six variants were strongly avid (EC_{50} 0.0004 – 0.07 μg/ml) and of high magnitude (120 – 270 SFC/10^6 PBMC, 1 μg/ml) and we observed robust T-cell proliferation to all AL9 epitope variants with $CFSE_{lo}$ frequencies in the range of 12 – 30%. Overall, strongly avid, high magnitude IFN-γ responses were associated with granzyme B production and high frequencies of proliferating antigen-specific T-cells.

Distribution of reactive PTE peptides vs. frequency distribution in virus isolates

We previously showed that the reactive Nef domains detected with both the CON and PTE peptide sets were similar, with the greatest frequency of responses spanning amino acids 65–103 and 113–147, and minor clusters between amino acids 1–27 and 177–206 [20]. Although majority of T-cell responses were detected following stimulation with the most frequent PTE among subtype B virus isolates, there were several high frequency PTE (peptides # 5, 11, 12, and 13) that were not targeted at all by any of the 18 Nef responders [20]. (PTE peptides with lower numbers are those present at the highest frequency among the virus sequences in the database).

We next examined the extent to which processing likelihoods and amino acid sequence entropy influenced epitope distribution, comparing the average entropy and C-terminus cleavage scores for the defined optimal epitopes with that of the entire group of 9-mers (overlapping by one aa, N=206) spanning the entire Nef sequence. Sequence variability was addressed by averaging the Shannon entropy for each amino acid residue in the 9-mer using the subtype B Nef alignments in the Los Alamos database. We considered the epitope data set generated with the use of PTE peptides to be optimal to address these issues since the peptide set has been designed to encompass virus heterogeneity and thus to improve the detection of epitopes in domains with moderate heterogeneity.

The median entropy score for 50 optimally defined 8 to 10-mer epitopes targeted in 17 Nef responders, which included a total of 23 unique epitopes, was 0.236 (range 0.126 – 0.574) compared to 0.363 (range 0 – 1.237) for the entire group of 9-mers spanning the Nef protein sequence ($P<0.0001$) (Figure 4A). The median C-terminus cleavage scores for the targeted epitopes was 0.960 (0.046 – 0.980) compared to 0.202 (0.022 – 0.980) for the entire group of 9-mers ($P<0.0001$) (Figure 4B). Notably, 45 of the 50 (90%) targeted epitopes had entropy scores <0.3 and 43 of the 50 (86%) epitopes had cleavage scores >0.5, with scores for 38 of the 50 (76%) being > 0.9. No significant correlation was observed between the average entropy and the C-terminus cleavage scores for the 9-mers ($P=0.453$, $r=-0.053$). Epitope distribution relative to entropy (top panel) and cleavage scores (bottom panel) is shown (Figure 4C). Our

findings provide evidence for a strong role for both processing preferences and amino acid entropy in determining epitope distribution.

4. DISCUSSION

Here in a comprehensive analysis we defined the extent of amino acid diversity in epitope variants that is tolerated by HIV-1-specific T-cells in the context of infection and vaccination. We hypothesized a greater breadth of variant epitope recognition by T-cells among infected persons compared to vaccine recipients because of the generation of sequence variants through virus replication during infection. However, T-cell recognition of variant epitopes was similar in the two settings as also were the effects of numbers and conservation amino acid substitutions on the recognition of variant epitopes. It is plausible that the distinct antigen conditions during the priming of T-cell responses in infection versus vaccination and differences in antigen processing and T-helper function have counter effects on variant epitope recognition. Alternately, T-cell recognition of variant epitopes may be independent of these factors.

While Gag, Pol, and Env responses were analyzed in the vaccinees, Nef responses were examined in early infection. Gag, Pol, and Env responses could not be analyzed in early infection due to PBMC limitations. Instead we selected Nef as a prototype because, although a small protein, Nef contains many highly immunogenic regions, is frequently targeted in early infection, and has a broad range of sequence diversity with domains that are highly conserved and others that are highly variable [20,44]. Recognizing the caveat we have refrained from making direct comparisons in the response patterns between infected subjects and vaccinees. We have kept our primary focus on the analysis of the effects of numbers and conservation of substitutions in amino acid residues on variant epitope recognition. We speculate that our findings will be similar if we tested responses to Gag, Pol and Env epitope variants in early infection. We expect these data to be useful in developing algorithms to predict cross- and intra-clade recognition of variant epitopes by T-cells induced following vaccination and infection, accounting for the numbers and relative conservation of amino acid substitutions between the variants and the infecting/consensus/vaccine sequence.

Altfeld et al previously demonstrated that T-cell responses to 29% (12 of 42) of targeted peptides in Gag, Tat, and Vpr were only detected with peptides representing the autologous virus strain compared to the HIV-1 clade B consensus sequence [45]. Specifically, the use of autologous peptides allowed the detection of significantly stronger HIV-1-specific T-cell responses in the more variable regulatory and accessory HIV-1 proteins Tat and Vpr ($P = 0.007$). Overall our data are in agreement with these findings. While, we demonstrate that many epitope variants can be recognized even when not detected among the autologous virus populations, in further detailed analyses we show that cross-reactive responses are predominantly directed to variants containing single conservative or semi-conservative amino acid substitutions. It is difficult to say with certainty if T-cell responses to variant epitopes were primed without exposure to the variant sequences since the possibility that the variant sequences were present among the autologous virus population at a prior point in time cannot be excluded. Conversely, in no instance did we detect a response to a variant epitope without a response to the autologous consensus sequence epitope.

Furthermore even when T-cells showed an IFN-γ response upon reactivity with epitope variants, the magnitude and/or avidity of IFN-γ and Granzyme B responses and T-cell proliferation was significantly reduced. We postulate that amino acid differences in the peptide variants affect the binding of the peptide variants with the HLA molecules and that of the peptide-MHC (pMHC) complexes with the TCR which then contributes to the difference in responses. For example, epitope variants may deliver negative signals to T-cells specific for

the agonist peptide-MHC complexes [46] and the relative avidity of the TCR-pMHC interaction may influence the cytokine response profiles [47,48].

Our findings lead us to hypothesize that vaccine-induced T-cells will proliferate and produce high magnitude IFN-γ and granzyme B responses when challenged with either homologous virus sequences or those that differ from the vaccine sequence by single conservative substitutions at the targeted specificities. By contrast, if the challenge sequences contain multiple or semi- and non-conservative substitutions in the targeted epitopes, then more likely than not, the T-cells will exhibit poor proliferation with low or no granzyme B production and low magnitude IFN-γ responses. We further hypothesize that broad T-cell recognition encompassing proliferation and high magnitude cytokine production directed at several epitope specificities may require vaccines that encompass multiple sequence variants.

Since PTE peptides, as well as similar [49] and combined central sequence [50] peptide sets made by others, are designed to maximally cover known circulating viral sequences, they are especially suited for analysis of the breadth of potentially protective immune responses in recipients of HIV-1 preventive vaccines and in infected individuals. While in this study we employed the PTE peptides to evaluate vaccine immunogenicity, this and similar approaches [21,23] lay the framework for the design of a vaccine immunogen with expanded T-cell epitope coverage for protection against highly diverse and rapidly changing virus populations.

Several prior studies have addressed factors that contribute to immunogenicity of highly targeted domains. These include processing preferences for proteasomal cleavage [22,51–53]; the frequency of C-terminus amino acids that inhibit HLA binding [53,54]; and constraints on the amino acids that can be accommodated in the peptide-binding groove of the HLA molecules [31,55,56]. Comparing published experimentally defined HIV-1 CTL epitope distributions to global protein sequence alignments, Yusim et al showed that epitopes are concentrated in relatively conserved regions and that highly variable regions that lack epitopes have a paucity of predicted proteasome processing sites and an enrichment for amino acids that do not serve as C-terminal anchor residues [19,57]. In a subsequent study of CTL responses using a clade B consensus sequence peptide set spanning all HIV proteins Frahm et al showed that the frequency of recognition for each peptide was highly correlated with the relative conservation of the peptide sequence, the presence of predicted proteasome cleavage sites within the C-terminal half, and a reduced frequency of amino acids that impair binding to the restricting class I molecules [19,57].

The unique aspect of our study was the use of PTE peptides to address the issue. Since, sequence variability between infecting and testing antigens, especially when the latter are based on central-sequences, could limit the ability to detect epitopes in relatively variable domains [45], we examined epitope distribution characteristics using the PTE peptide set with its expanded epitope coverage. T-cell responses detected by stimulation with these peptides are significantly greater in breadth and magnitude compared to those detected with CON peptides [20]. Our findings with the use of a peptide set with significantly expanded epitope coverage indicate that conservation of the peptide sequence remains a critical factor in driving epitope distribution in addition to the C-terminus cleavage scores. We show that the entropy scores for the targeted epitopes are significantly lower ($P<0.0001$) and the C-terminus cleavage scores significantly higher ($P<0.0001$) compared to the group of 9-mers spanning the protein sequence. We postulate that cost-effective design of peptide and sequence based vaccine immunogens as well as peptide test reagents should emphasize domains that are likely to be T-cell targets [21–24].

In summary, we have defined the extent of amino acid diversity in epitope variants that is tolerated by HIV-1-specific T-cells in the context of infection and vaccination. In more detailed

analyses, although, in a small number of subjects we show significant differences in the magnitude of the IFN-γ response to the variants as well as in other functional properties such as proliferation and granzyme B production, particularly in the presence of multiple or semi- and non-conservative amino acid substitutions. Therefore, when assessing response to variant epitopes in studies of cross-clade T-cell responses, a qualitative assessment for the presence or absence of an IFN-γ response is not sufficient. Our data support the strategic design of vaccines that generate T-cell populations with optimal effector activities through inclusion of multiple sequence variants. Finally, we show that epitope distribution is strongly influenced by both processing preferences and amino acid entropy. Thus, cost-effective design of peptide and sequence based vaccine immunogens that provide maximal coverage of circulating sequences may be achieved through emphasis on virus domains with high likelihood of being T-cell targets.

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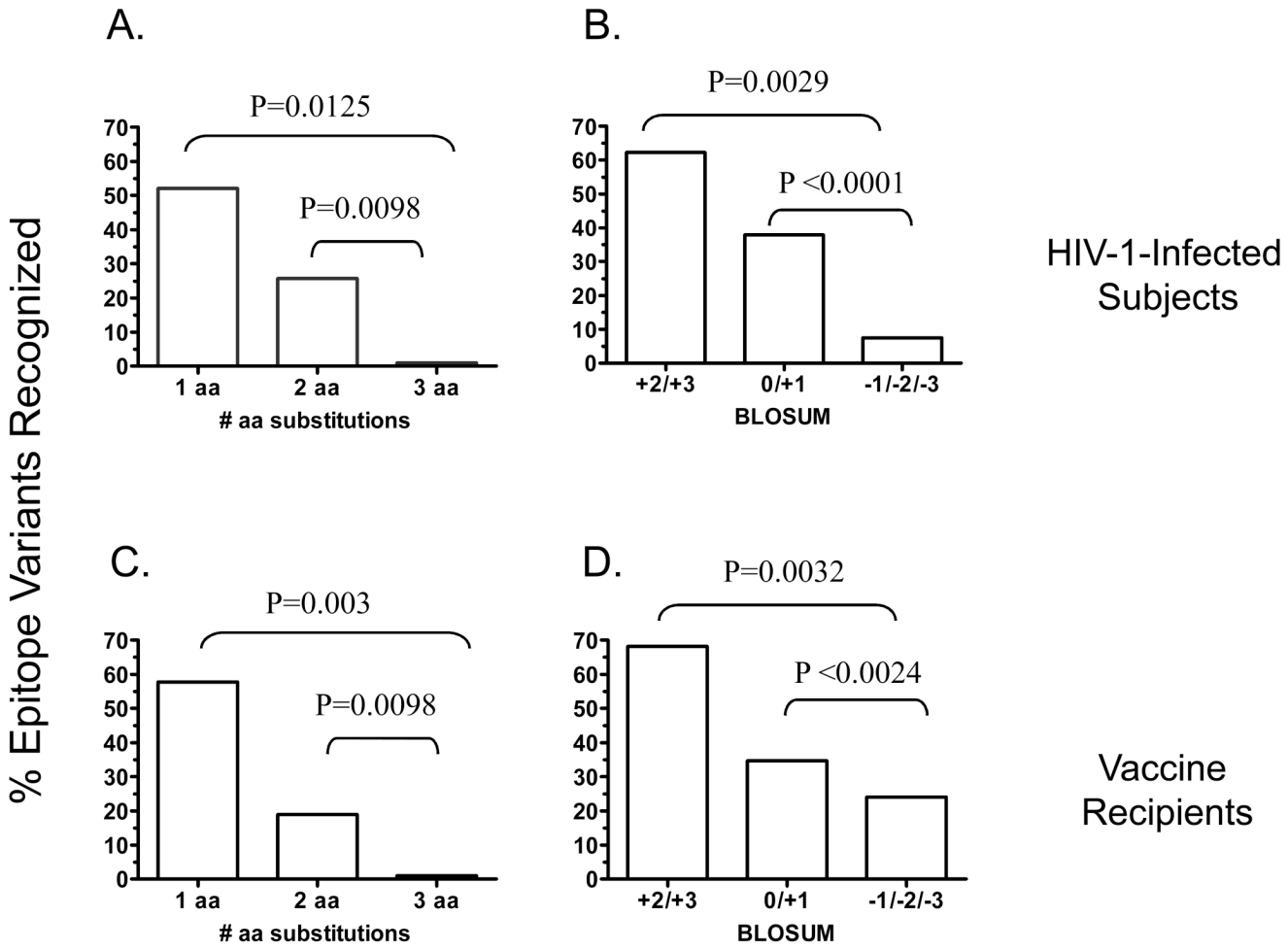
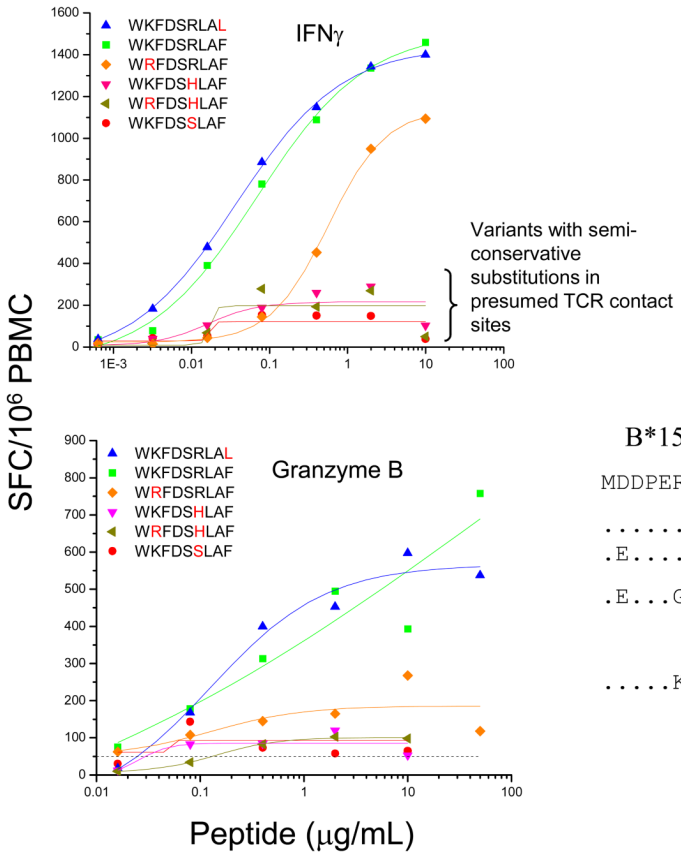


Figure 1. Effects of numbers and conservation of amino acid substitutions on recognition of T-cell epitope variants in subjects with primary HIV-1 infection (A, B) and in HIV-1 vaccine recipients (C, D)

PBMC were stimulated with HIV-1 PTE peptide sets in a matrix format and tested for IFN-γ production using an ELISpot assay. Responses were examined to the Nef protein in subjects with primary infection and to Gag, Env and Pol in the vaccinees. Responses > 3-fold background and > 50 SFC/10^6 cells above background were scored positive. All responses were confirmed at the single peptide level. Shown is the frequency of recognition of epitope variants containing one, two and three amino acid substitutions; and conservative, semi-conservative and non-conservative substitutions.



B*1503 motif x-[QK]-x-x-x-x-x-[YF]

MDDPEREVLEWKFDSRLAFHHMARELHPE

..... (5/15)
.E.....M..... (5/15)
.E...G...I.....L..... (2/15)
.....K...V.....K..L..V..... (3/15)

Figure 2A

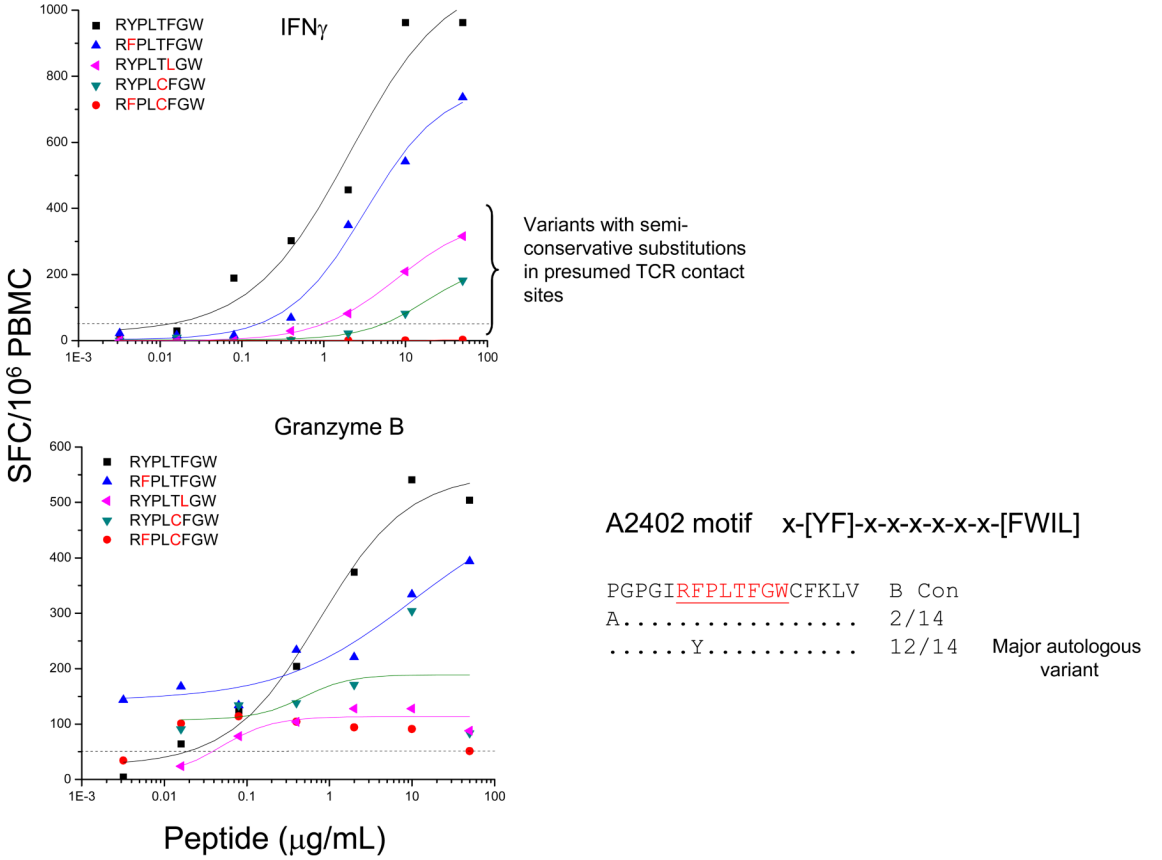
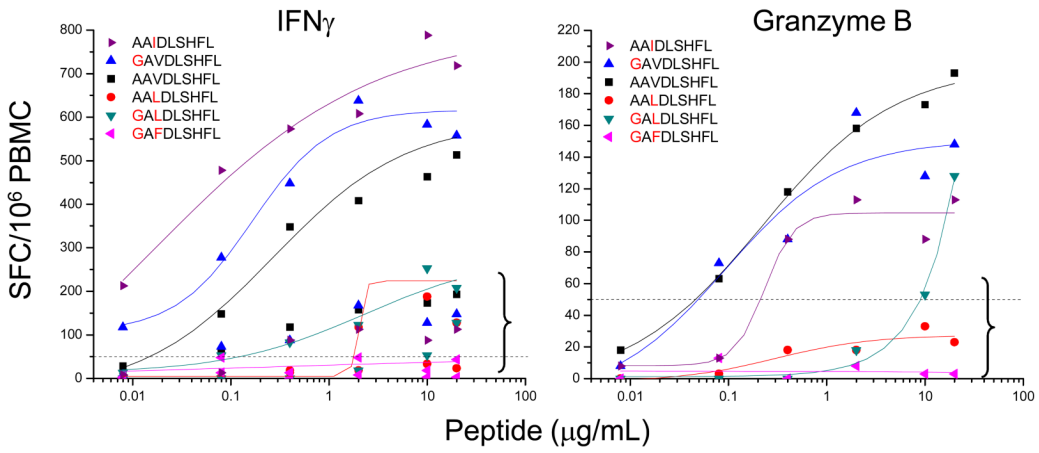


Figure 2B

Figure 2. IFN-γ and Granzyme B responses to optimal epitope variants in the Nef protein

Standard IFN-γ and granzyme B ELISpot assays were performed by stimulating with the optimal epitopic peptides and variants at serial concentrations between 10,000 and 0.1 ng/ml. Shown here are PBMC responses from subjects 1188 (WF9 epitope variants) (A) and 1692 (RW8 epitope variants) (B). The SFC frequencies are plotted against the $\log_{10}$ peptide concentration. CON B sequence for each epitope is indicated on the top. Identity of autologous sequence to amino acid in the CON sequence is indicated by a dot.



Variants with semi-conservative substitutions in presumed TCR contact sites

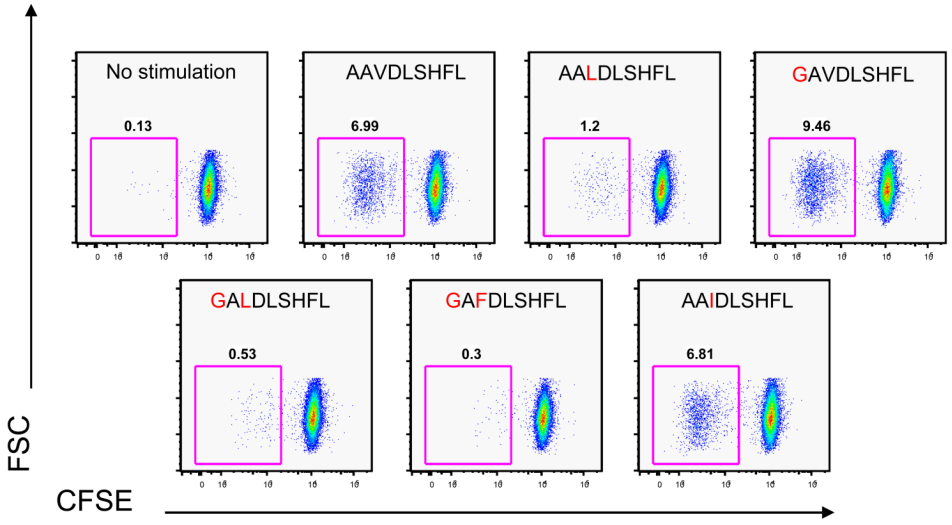


Figure 3A

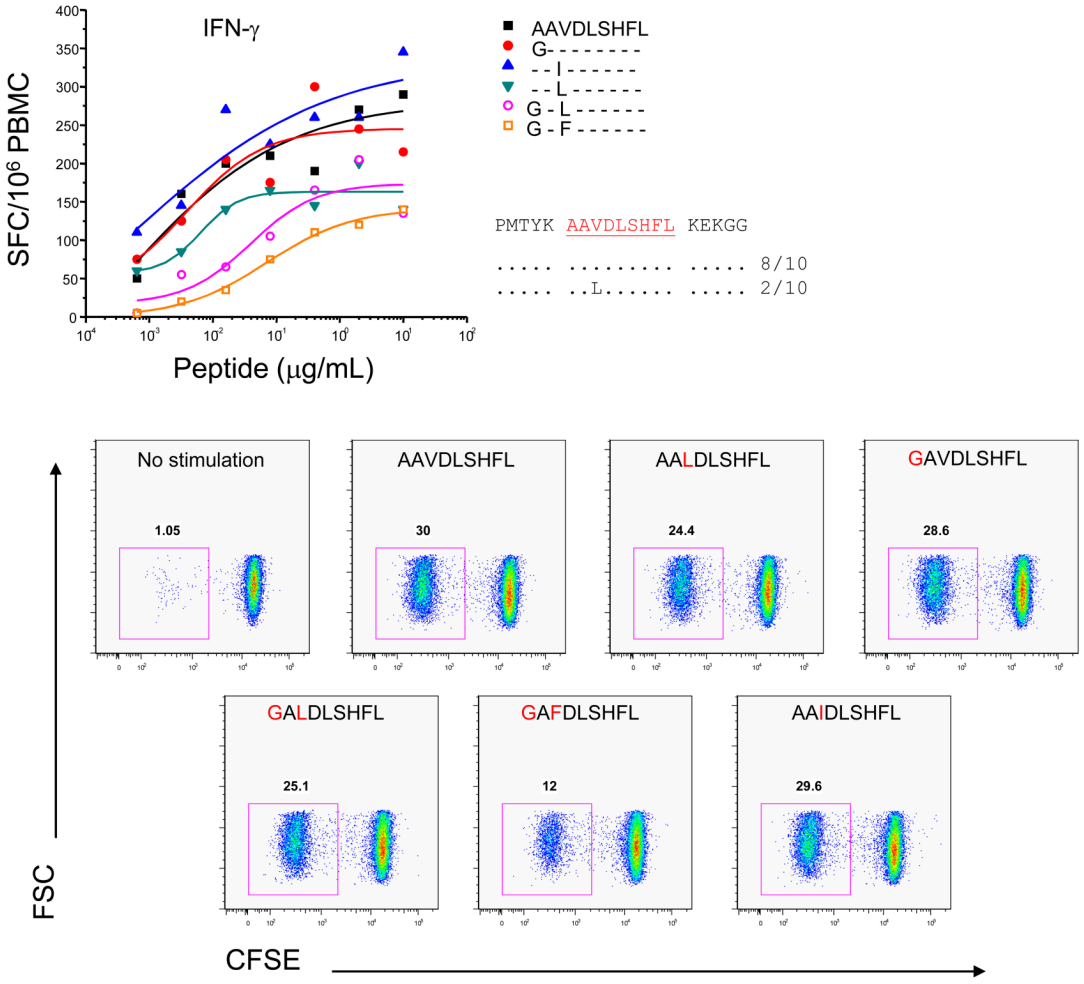


Figure 3B

Figure 3. T-cell proliferation in response to stimulation with Nef epitope variants relative to IFN-γ and granzyme B responses

Standard IFN-γ and granzyme B ELISpot assays were performed by stimulating with the optimal epitopic peptides and variants at serial concentrations between 10,000 and 0.1 ng/ml. Shown here are PBMC responses from subjects 1238 (AL9 epitope variants) (A) and 1212 (AL9 epitope variants) (B). The SFC frequencies are plotted against the $\log_{10}$ peptide concentration. Autologous sequence where available is shown and identity of autologous sequence to amino acid in the CON sequence is indicated by a dot. The decrease in CFSE signal was used to monitor proliferation. Positive and negative controls included cells incubated with anti-CD3 mAb (30 ng/mL) and anti-CD28 mAb (1 μg/mL), and with medium alone.

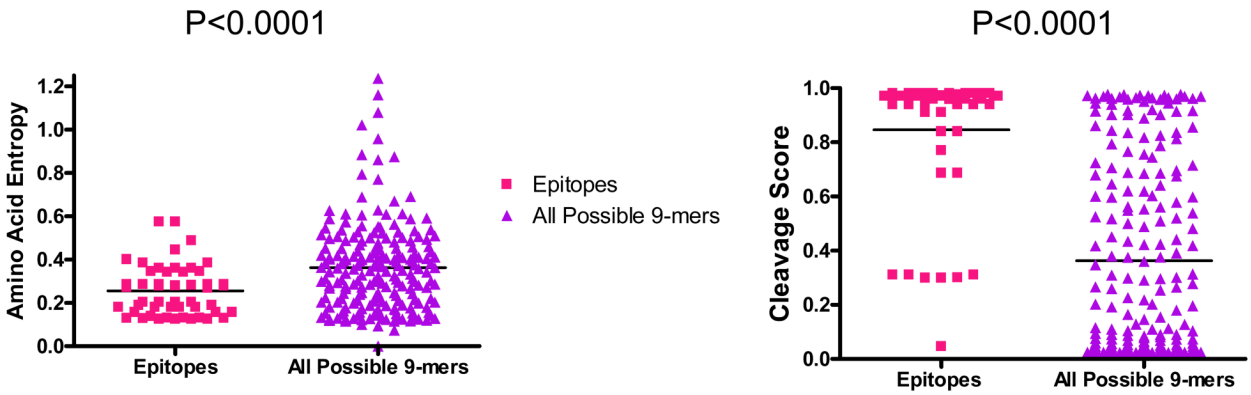


Figure 4 A, B

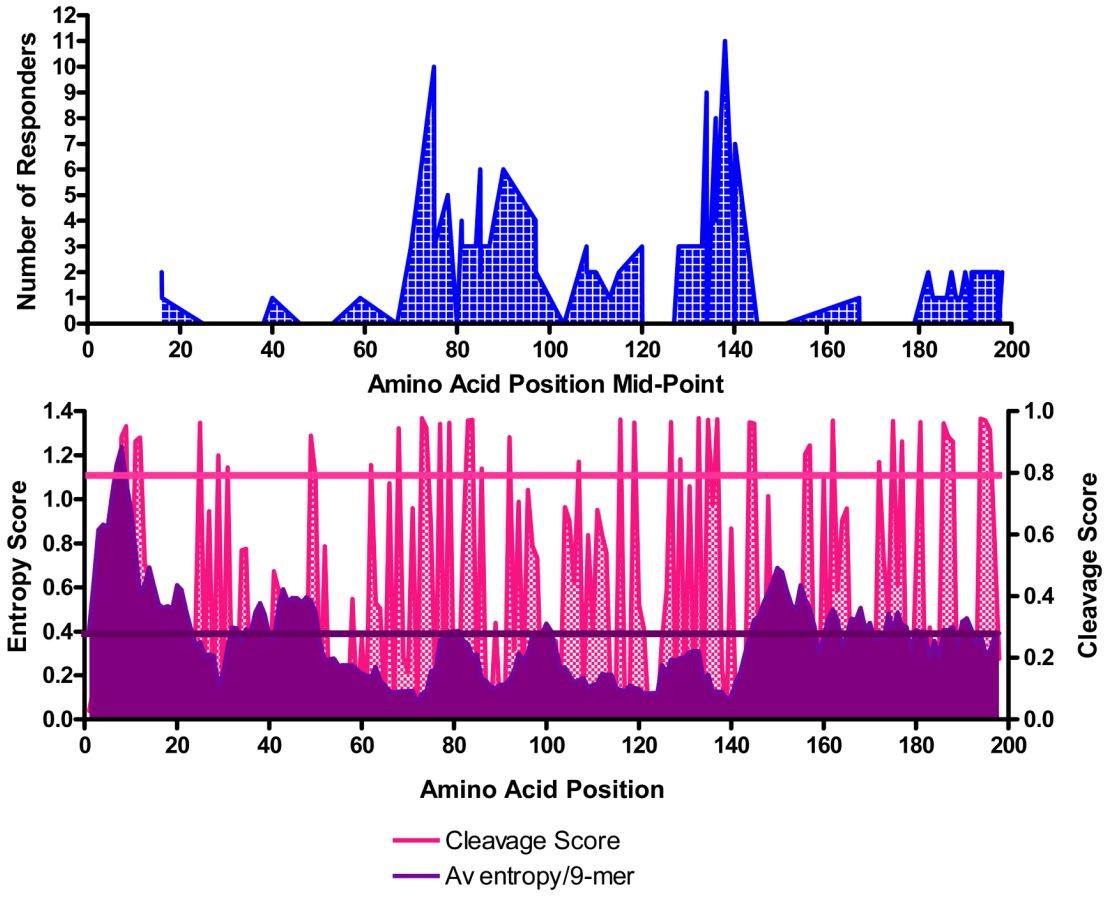


Figure 4C

Figure 4. Epitope distribution in the Nef protein relative to entropy and cleavage scores

Amino acid entropy (A) and proteasomal cleavage scores (B) were compared between 50 optimal epitopes versus all possible 9-mers spanning the nef sequence. Epitope distribution relative to entropy and cleavage scores is shown from the N- to C-terminus (C). Entropy and C-terminus cleavage scores for targeted optimal epitopes and the group of all Nef subtype B 9-mers were compared by the use of Wilcoxon Signed Rank Test.

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Table 1

Demographic, clinical, and virologic characteristics of the primary infection study population.

			Enrollment Time Point			Sampling Time Point				HLA Class I		
Subject ID[A]	Age	Ethnicity[B]	Days Post Infection	HIV-1 RNA copies/ml	CD4+ T-cells/mm^3	Days Post Infection	Treatment duration (days)	HIV-1 RNA copies/mL	CD4+ T-cells/mm^3	A	B	Cw
1188	23	H	79	73,074	409	93	5	<500	375	23	35, 15	02, 07
1212	37	C	11	5,060,000	757	16	No	102,045	925	02, 23	44, 62	03, 04
1216	33	C	129	26,000	300	134	2	38,400	626	03,24	07,39	07
1238	42	C	45	99,600	367	45	No	99,600	367	01, 03	07, 14	07, 08
1242	30	C	42	96,900	874	105	57	343	787	03, 33	14, 35	04, 08
1362	24	C	8	8,240,000	878	8–680	No	117,077	871	02, 25	18, 51	01, 12
1484	27	C	12	4,340,700	300	19	No	4,340,700	830	02,03	07,35	04,07
1490	41	C	55	286,272	568	62	No	286,272	568	02, 11	15, 35	03, 04
1576	32	C	22	3,717,000	332	63	42	4,129	552	11, 33	49, 51	07, 15
1596	36	C	19	260,605	700	20	No	260,605	265	03,26	38,35	04,12
1599	30	C	46	404,463	265	46	No	404,463	265	01, 02	08, 18	7
1686	38	H	55	155,545	186	70	No	219,312	241	23, 25	35, 40	03, 04
1689	28	H	11	2,240,958	1206	11	No	94,708	745	02, 03	35, 40	03, 04
1690	42	C	21	1,031,477	396	21	No	400,163	528	01	08	07
1692	42	B	50	2,461	612	50	No	2,461	612	23, 30	08, 42	07, 17
1693	41	H	7	377,063	559	7	No	1,912,195	472	01, 03	08, 35	04, 07
1698	45	H	11	734,413	458	11	No	262,437	531	NT	NT	NT

[A] All subjects were male.

[B] Ethnicity: C, Caucasian; H, Hispanic; B, Black