

1 **Antisense-Derived HIV-1 Cryptic Epitopes Are Not Major Drivers of Viral Evolution**

2 **during the Acute Phase of Infection**

3

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16

17 **Running Title:** Cryptic epitopes and viral evolution

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24 **ABSTRACT**

25 While prior studies have demonstrated that CD8 T cell responses to cryptic epitopes (CE) are
26 readily detectable during HIV-1 infection, their ability to drive escape mutations following acute
27 infection is unknown. We predicted 66 CE in a Zambian acute infection cohort based on escape
28 mutations occurring within or near the putatively predicted HLA-I restricted epitope. The CE
29 were evaluated for CD8 T cell responses in patients with chronic and acute HIV infection. Of
30 the 66 predicted CE, 10 were recognized in 8/32 and 4/11 patients with chronic, and acute
31 infection respectively. The immunogenic CE were all derived from a single antisense reading
32 frame within *pol*. However, when these CE were tested using longitudinal study samples, CE
33 specific T cell responses were detected but did not consistently select for viral escapes. Thus,
34 while we demonstrated that CE are immunogenic in acute infection, the immune responses to CE
35 are not major drivers of viral escape in the initial stages of HIV infection. This latter finding may
36 be due to either the subdominant nature of CE-specific responses, the low antigen sensitivity,
37 and magnitude of CE responses during acute infections.

38

39 **IMPORTANCE**

40 Although prior studies demonstrated that cryptic epitopes of HIV-1 induce CD8 T cell responses,
41 evidence supporting that targeting these epitopes to drive HIV escape mutations have been
42 substantially limited and none have addressed this question following acute infection. In this
43 comprehensive study, we utilized longitudinal viral sequencing data obtained from three separate
44 acute infection cohorts to predict potential cryptic epitopes based on HLA-I associated viral
45 escape. Our data shows that cryptic epitopes are immunogenic during acute infection and many
46 of these responses are elicited towards translation products of HIV-1 antisense reading frames.

47 However, despite cryptic epitope targeting, our study did not find any evidence of early CD8
48 mediated immune escape. Nevertheless, improving cryptic epitope specific CD8 T cell responses
49 may still be beneficial in both preventative and therapeutic HIV-1 vaccines.

50

51 INTRODUCTION

52 CD8 T cells play a vital role in controlling intracellular pathogens by recognizing epitopes
53 presented on infected cells. In human immunodeficiency virus type 1 (HIV-1) infections, CD8 T
54 cells have been shown to be essential in control of viremia and disease progression, and part of
55 this evidence comes from the fact that they drive HIV escape mutations that may impact overall
56 viral fitness (1). However, due to the mutable nature of HIV, achieving a broader range of
57 epitope recognition by targeting beyond the scope of the nine conventional proteins of the virus
58 may be necessary for augmenting the efficacy for both preventative and therapeutic vaccines (2,
59 3). Several studies have described a lesser known form of epitopes within several viruses
60 including HIV and SIV, termed cryptic epitopes (CE)(4-24). These epitopes are predominantly
61 derived from the translation of two sense and three antisense alternative open reading frames.
62 Translation products of any of these five alternate reading frames (ARF) were found to be
63 recognized by CD8 T cells during the acute and chronic stages of infection (6, 21, 25-29). While
64 CE usually induce T cell responses of lower magnitude when compared to responses induced by
65 their main reading frame counterparts, past studies have demonstrated that immune responses to
66 these ARF epitopes are observed in many infected individuals or are immunoprevalent during
67 the course of the disease. (25, 27). Furthermore, HIV was demonstrated to escape CE-specific
68 CD8 T cell responses in chronic infection, demonstrating that they exert significant selective
69 pressure on viral replication *in vivo*. However, the contribution made by such responses in
70 driving viral evolution during acute and early HIV-1 infection has not been adequately
71 addressed.

72 Here, we utilized a variety of methods to predict CE that potentially induced CD8 T cell
73 responses during the acute phase of infection. By validating the epitope predictions from one of

74 the acute cohorts through immunogenicity screening assays, we identified ten previously
75 undescribed CE, to be immunogenic in both acute and chronic infection. However, no reliable
76 CE responses can be detected when using similar methods of epitope prediction in two additional
77 acute cohorts. From this study, we conclude that CE are indeed present during early stages of
78 infection and can be targeted by T cells. Nevertheless, due to the overall subdominant T cell
79 response seen to these epitopes, they appear to contribute minimally in driving viral adaptation.

80

81 **RESULTS**

82 **Cryptic epitope predictions and T cell responses**

83 To analyze the role of CE targeting during acute infections, we adopted an approach similar to
84 previous studies, utilizing viral sequence data obtained from a serodiscordant cohort (30, 31). We
85 first identified sites of HLA-I associated polymorphisms within or near previously predicted CE
86 that may have evolved to escape CD8 T cell responses during the first two years of infection in
87 78 acutely infected Zambian patients. Due to the limited Zambian sample availability, and to
88 facilitate analysis in subtype B HIV infected patients, we narrowed the list of all predicted
89 epitopes down to 66 putative CE for testing (Table 1). The CE were first tested as seven distinct
90 peptide pools (6-10 peptides/pool) for recognition by PBMCs from chronically infected HIV-1
91 subtype B patients in an IFN- γ ELISpot assay. Thirty-two patient samples were used. 78%
92 (25/32) patients were not on active ART, with the median viral load of the cohort being 3,380
93 RNA copies/ml and median absolute CD4 count of 646 (Table 2). We observed T cell responses
94 to the CE peptide pools in 25% of the subjects tested (8/32, Table 2, shaded in gray and Figure
95 1A). In contrast, CE pools did not elicit T cell responses in any of the 16 HIV seronegative
96 donors ($p < 0.0001$) evaluated. Next, we deconvoluted the positive pool responses to the single

97 peptide level. Single peptide responses were successfully deconvoluted from six out of the eight
98 CE pool responders, which allowed us to identify ten novel immunogenic CE (Table 3) with no
99 significant homology to HIV-1 proteins encoded by the main reading frame (Table 4).

100 Interestingly, all immunogenic CE were derived from the antisense frame 3 (RF3) of *pol*. The
101 magnitude of the responses to these CE in the six patients tested varied, ranging from 63 to 487
102 SFU/10⁶ PBMC (Figure 1B, median magnitude of 133 SFU/10⁶ PBMC).

103 **Polyfunctionality of T cells responding to CE**

104 Since T cell responses to cryptic epitopes WL9 and FY11 were frequently observed in the
105 ELISpot assays, ICS assays were employed to determine CD4/CD8 restriction of CE-reactive T
106 cell responses. The CE specific CD8 T cells produced IFN- γ and upregulated perforin expression
107 in an *ex vivo* intracellular cytokine staining assay, confirming that CE could elicit antiviral
108 responses (Figure 2A). To demonstrate the ability of these cells to kill virally infected targets, we
109 generated CD8 T cell lines using two rounds of peptide pulsed monocytes as antigen presenting
110 cells and tested their effector functions in an ICS assay as described in methods. CD8 T cells
111 specific for WL9 and FY11 were able to expand after the two-week culture and were highly
112 specific for CE based on effector molecule expression after re-stimulation (Figure 2B).
113 Furthermore, these CE specific cell lines were capable of killing HIV(NL4.3) infected HLA-I
114 matched target cells *in vitro*, demonstrating their effectiveness at eliminating HIV infected target
115 cells (Figure 2C, p-value <0.001).

116 **CD8 T cell responses to *pol* CE in acute infection**

117 We next analyzed samples from the Zambian cohort to determine whether T cell responses to the
118 10 *pol* CE epitopes could drive viral escape during acute and early infection. With limited
119 quantities of PBMC samples remaining from the cohort, we were only able to identify two

120 Zambian patients with PBMCs available who showed a sequence-based mutation in or around
121 any of the CE. These patients each had a synonymous mutation in *pol*, which generated a fixed
122 amino acid change near the CE-KL10 epitope. For ZA1, the mutation did not occur until 24
123 months post estimated date of infection, and for ZA2 the mutation occurred very early,
124 detectable by month 3 after transmission of the virus (Figure 3A). No T cell responses were
125 detected by IFN- γ ELISPOT assay in ZA1 at either 590 or 841dpi. The magnitude of the IFN- γ
126 response for ZA2 at 280 days post infection was below our criteria for a positive response, but
127 the IFN- γ level was subsequently increased to 150 SFU/10⁶ PBMC at 389 days post infection
128 (Figure 3B). To examine whether CE specific T cell responses were indeed stronger in earlier
129 stages of infection, we examined 11 subtype B individuals with PBMC samples collected within
130 the first three months of infection (Table 2). When tested, 4/11 patients showed T-cell responses
131 to at least one of the novel CE identified, with the highest magnitude of response being detected
132 at 443 SFU/10⁶ PBMC (Figure 3C). CE Antigen sensitivity testing was further performed using
133 acute patient sample (Figure 4A and B). Although at higher EC50 levels, the CE tested show
134 comparable avidity to some main reading frame epitopes tested in the past (32, 33). To
135 strengthen our finding, we examined whether the emergence of synonymous mutations in early
136 HIV infections resulted from CD8 T cell targeting of cryptic epitopes in two additional acute
137 cohorts (Rwandan/Ugandan and CHAVI001, Supplemental Table 1). T cell responses to putative
138 autologous virus CE were tested using IFN- γ ELISpot assays as described previously (*ex-vivo*
139 and cultured). No CE-specific T cell responses were detected during acute/early infection in the
140 16 individuals studied (data not shown), suggesting that although CE can be targeted by CD8 T
141 cells, it may not be common for these responses to drive viral escapes during early stages
142 infection.

144 **DISCUSSION**

145 Beginning with the discovery of antisense reading frame protein specific antibodies in the serum
146 of HIV-1 infected individuals nearly two decades ago (22), alternative reading frame
147 transcription and translation have remained an exciting and active facet of viral immunology
148 research (8, 9, 19, 23, 34). Several groups have since then reported immunogenicity of peptides
149 derived from the ARF of HIV-1 that can trigger CD8 T cell activation and effector functions.
150 Although some studies have attempted to unravel the biological role of these alternate reading
151 frame derived proteins and their derivative epitopes, the exact role of cryptic epitopes and their
152 relationship with host immunity remains an enigma. The potential ability for HIV to produce up
153 to six different amino acid combinations per single genomic coding region remains a tantalizing
154 idea for broadening T cell responses in vaccine designs. Although many have described the
155 ability of CE to trigger CD8 T cell responses in both acute and chronic stages of HIV-1 and SIV
156 infection (20, 25, 27, 35, 36), our findings here do not indicate that CE-specific CD8 T cells
157 select for HIV escape mutations. This conclusion is based on longitudinal data performed on 16
158 patients following acute infection whereby we did not demonstrate evolutionary pressure from
159 cryptic epitope-specific CD8 T cells. This finding is in sharp contrast to what was observed for
160 CD8 T cells targeting epitopes in the main reading frame in the CHAVI001 cohort (42). 11
161 participants of this cohort demonstrated an average of 4 CD8 T cell responses (range: 2-11) to
162 the main reading frame epitopes that were temporally associated with escape. In a similar
163 fashion, the two Rwandan individuals had 12 and 13 total T cell responses to the main reading
164 frame (48). When all immune data were combined, a median of 3 main reading frame CD8 T cell
165 responses were detected which were associated with viral escape in these cohorts. These
166 numbers are significantly different than what was seen for cryptic epitopes tested, where we

167 showed only one possible detectable T cell responses in the 176 cryptic epitopic regions tested
168 (Fisher's exact test: $p < 0.0001$).

169 The Zambian sequence data allowed us to identify ten novel cryptic epitopes, each being derived
170 from the antisense reverse frame 3 in the *pol*-coding region of HIV. Perhaps the most intriguing
171 and perplexing observation in our study is the fact that half of the novel immunogenic CE do not
172 appear to be a translation product of an ORF initiated from the typical AUG^{met} start codon. In
173 addition, *in silico* analysis of HIV-1 consensus sequence shows an enrichment of CUG^{leu}
174 initiated ORF within reverse frame 3 of the virus (data not shown). With *pol* being the largest
175 gene of the virus, this observation could help explain why all our immunogenic CE were derived
176 from the reverse frame 3 of *pol*. Nevertheless, this is a similar phenomenon to what was
177 previously observed by Berger et al. (27), where an immunogenic CE of HIV from the
178 alternative forward frame was also found to be initiated by a leucine (e.g., CUG^{leu}) start codon.

179 As in this prior study, we too show killing of virus-infected cells by a CD8 T cell line specific to
180 a cryptic epitope translated from a leucine initiated ORF (Figure 2C). Furthermore, the studies of
181 Shastri et al. in recent years have provided evidence in both cell lines and animal models, of how
182 CUG^{leu} start codons are frequently used by cells to generate epitopes that can trigger T cell
183 activation (37-40). Moreover, using *in silico* analysis, we have found an enrichment of CUG
184 ORF within the antisense RF3 of HIV-1 consensus sequence (data not shown), providing further
185 anecdotal explanation to our data. In conjunction with our observation of multiple CE being
186 translated from a CUG initiated ORF, we hypothesize that the production of HIV CE relies
187 heavily on the utilization of alternative start codons. It has been shown that the translation
188 efficiency of CUG initiated ORFs appears to depend heavily on other molecular mechanisms and
189 environmental factors can impact the protein translation process. In particular, inflammation and

190 cytokine milieu seem to have a dramatic effect on the translation efficiency of CUG^{leu} initiated
191 ORF (41). When pro-inflammatory cytokines such as type I and type II interferons and tumor
192 necrosis factor α were added exogenously to cell lines, CUG^{leu} ORF-encoded peptides were, in
193 turn, more efficiently produced. We speculate that CE production from CUG^{leu} initiated ORFs
194 may therefore be modulated by the levels of inflammatory cytokines produced during different
195 stages of infection.

196 We have previously shown that the magnitude of CD8 T cell responses to CE are often lower
197 during acute than in chronic HIV-1 infection (25). Unfortunately, with the limited availability of
198 acute Zambian samples, we were unable to directly test the magnitude of T cell responses to the
199 predicted CE immediately after infection. Nevertheless, in the longitudinal Zambian samples
200 obtained, we could capture archival T cell responses to CE-KL10 definitively in one subject
201 (ZA2) but not in the other (ZA1). The lone T cell responses to CE-KL10 was detected at time-
202 points that were well into the chronic stage of infection. The kinetics of selection of T cell escape
203 mutations during acute and early HIV-1 infection is strongly dependent on both the
204 immunodominance of the epitope-specific T cell response, and the fitness costs associated with
205 acquisition of escape-conferring mutations in and around the epitope sequence (42-46). Although
206 acquisition of escape mutations in epitopes derived from alternative open reading frames may
207 potentially incur lower average costs to viral fitness than for epitopes derived from the main
208 reading frames, the fact that responses to CE are typically of very low magnitude during acute
209 infection, likely explains why virus escape is slower and not detected until later in infection.

210 Another potential explanation for the lack of escape can be the result of lower antigen avidity
211 noticed for some of the CE in acute samples (Figure 4). In line with all observations, an SIV-
212 macaque study published by Harris et al. provided evidence for strong CD8 T cell responses to

213 CE mainly when the infecting SIV strain has had an accumulation of mutations in epitopes
214 derived from the main reading frame (47). This would indicate that HIV does evolve to escape
215 CE-T cell responses but does so more during the chronic stages of infection, where CE are
216 generated more frequently due to the accumulation of both inflammatory stimuli and viral escape
217 has occurred from responses to immunodominant epitopes in the main reading frame (41, 47).
218 Together, our data support the notion that cryptic epitopes are translated by HIV during acute
219 stages of infection, but due to their subdominant nature during natural infections, selection
220 pressure on these epitopes is likely minimal. However, with vaccination strategies that
221 specifically target cryptic epitopes, frequencies of these responses would likely increase,
222 augmenting the immune pressure targeted at these ARF regions of the virus. Future studies are
223 needed to determine if vaccine boosted HIV-CE immune responses can enhance the overall
224 quality of response directed against the virus.

225 MATERIALS AND METHODS

226 **Patient Cohorts:** Autologous HIV-1 sequence and HLA-I allelic data from three separate acute
227 infection cohorts were used in this study for cryptic epitope prediction. These included data from
228 a small cohort of two Rwandans and two Ugandans, Zambian (N=78) patients enrolled in the
229 International AIDS Vaccine Initiative (IAVI) protocol C study, and patients enrolled in the
230 CHAVI001 study (N=12). Putative CE predicted based on information from the Zambian cohort
231 (see below) were tested in 32 patients with chronic infection and 11 with acute infection enrolled
232 at the 1917 HIV-1 clinic at the University of Alabama at Birmingham (UAB). Healthy donor
233 PBMC from the Alabama Vaccine Research Clinic were used as HIV-1 seronegative controls.
234 IRB approval was obtained, and all participants consented to this study. Demographic details of
235 the 1917 clinic cohort can be found in Table 2.

236 **CE prediction and peptide synthesis:** For the patients in the Rwandan/Ugandan and
237 CHAVI001 cohorts, HIV-1 single genomic amplification (SGA) and sequencing were performed
238 at serial post-infection timepoints as previously described (42, 48) (CHAVI001 cohort sequences
239 can be accessed via Los Alamos HIV sequence database,
240 <http://www.hiv.lanl.gov/components/sequence/HIV/search/search.html>, PubMed ID 23221345),
241 and viral sequences were evaluated for the emergence of synonymous mutations (with respect to
242 the main reading frame). Peptides were designed that overlapped the region containing each
243 synonymous mutation, and putative optimal epitopes in this region were also predicted using
244 either Microsoft EpiPred (<http://boson.research.microsoft.com/bio/epipred.aspx>), or LANL ELF
245 software (http://www.hiv.lanl.gov/content/sequence/ELF/epitope_analyzer.html). Following
246 acute HIV infection of the patients in the Zambian cohort, bulk plasma viral sequences were
247 previously analyzed at the time of infection and every 3-6 months after that for two years (30,
248 31). We used prior predicted CE using HLA-I associated polymorphism in the ARF of HIV in
249 subtype C chronically infected patients (25). We then applied these CE predictions to the
250 longitudinal sequence data obtained from 78 patients in a linked transmission cohort in Zambia
251 to determine if synonymous mutations were fixed in or within 3 amino acids of these epitopes
252 during the first two years of follow up. A fixed mutation was defined as being present for at least
253 the penultimate and the final sequencing time points. A list of likely previously predicted
254 epitopes (25) containing the fixed mutation were generated based on HLA-I of the recipient and
255 the sequence flanking the fixed mutation. Additional criteria were adopted in selecting CE
256 including predicted CE that had a posterior probability greater than 50%, greater than 80%
257 peptide homology to subtype B consensus sequence at the amino acid level, and the mutation
258 observed in least three or more individuals. Based on these criteria, 66 epitopes were selected for

259 testing in the Zambian cohort. All peptides were synthesized in a 96 well peptide array format
260 (New England Peptides (Gardner, MA) or as previously described (42)). The peptides were
261 reconstituted in DMSO and stored at -80°C. The peptides were tested in pools (6-10
262 peptides/pool), and responses were further mapped to the single peptide level.

263 **IFN- γ ELISpot assay:** ELISpot assays were performed as previously described by our group
264 and others (25, 26, 28, 42, 44, 48-50). In brief, nitrocellulose 96-well plates were first coated
265 with anti-IFN- γ monoclonal antibody. PBMCs, rested for 2-6 hrs after thawing, were then
266 incubated in the plates (100,000~200,000 cells/well) in at least duplicates with the appropriate
267 antigen for 20-24 hrs at 37°C in 5% CO₂. The final concentrations of CE used were 2 μ M
268 (peptide pools) and 10 μ M (single peptide). PBMCs were washed off (PBS and Tween-20) after
269 overnight culture, and biotinylated anti-IFN- γ antibody (Mabtech) was added to the plates for 2hr
270 at room temperature. Another round of washing was performed, followed by the addition of
271 streptavidin-conjugated alkaline phosphatase (Mabtech) for 45 minutes. Finally, NBT/BCIP
272 (Southern Biotech) was added to develop spots as readout. ELISpot reader (C.T.L.) counted
273 individual spot forming units (SFU), and the counts were then normalized to SFU/10⁶ PBMCs.
274 Criteria for positive ELISpot responses were as described previously (25, 26, 42, 48, 50).

275 **Intracellular cytokine staining (ICS) assay:** Cryopreserved PBMCs were stained in an ICS
276 assay following stimulation with appropriate CE peptides at 10 μ M final concentration. In brief,
277 co-stimulatory monoclonal antibodies anti-CD28 and anti-CD49d, each at 2 μ g/ml was added to
278 each tube containing 10⁶ PBMCs. Cells were then stimulated with the appropriate peptide,
279 monensin, and brefeldin A for 12 hrs at 37°C. The cells were then washed and stained with
280 Live/Dead cell dye (Invitrogen) for 30 min, washed again, and stained for surface markers for 30
281 min. Surface labeling of cells was done using anti-CD3 (Pacific Blue), anti-CD8 (V500), anti-

282 CD4 (Alexa Fluor 780) conjugated antibodies. Anti-CD14 and anti-CD19 labeled with PerCp-
283 Cy5.5 were used as dump channels. Cells were washed with PBS before treating with
284 Cytotfix/Cytoperm reagent (BD) for 20 min at RT. Following permeabilization, cells were
285 labeled with intracellular antibodies for 30 min at room temperature Intracellular staining was
286 performed using anti-IFN γ (Alexa Fluor 700), anti-IL-2 (APC), anti-TNF α (PE-Cy7), and anti-
287 Perforin (PE). Stained cells were fixed with 2% paraformaldehyde and acquired on a BD LSRII
288 flow cytometer. Data collected were analyzed using FlowJo software version 9.9.7 (Tree Star,
289 Inc.).

290 **CD8 T cell line generation:** Epitope-specific CD8⁺ T cell lines were generated from patient
291 PBMC initially identified with a positive response to CE based on the IFN- γ ELISpot assay. As
292 previously described by our group, cell lines were *in vitro* expanded over the course of 14 days
293 using two rounds of irradiated monocytes pulsed with peptide as antigen presenting cells (26,
294 44). In brief, at the end of 2 weeks, CD8 T cell lines were re-stimulated with cognate antigen in
295 the presence of co-stimulatory antibodies and intracellular transport inhibitors (monensin,
296 brefeldin A) for 12 hrs and analyzed via flow cytometry for effector function, as described
297 above.

298 ***In-vitro* viral killing assay (iVKA):** To obtain target cells needed for viral infection, PBMC
299 from HIV seronegative donors were first depleted of CD8⁺ T cells using magnetic bead
300 separation (CD8 Dynabeads, Invitrogen). CD8⁻ PBMC were then activated with PHA (5ug/ml)
301 and IL-2 (50U/ml) for two days before infection with HIV NL4.3 virus at MOI of 0.5. Two days
302 post infection, 1 x 10⁵ cells of the CE-FY11 specific CD8⁺ T cell line (HLA-A*0201/A*0201,
303 B*1503/B*3501, C*0210/0401) were then added to infected HLA-matched (Match target 1:
304 HLA-A*0201/A*0201, B*5501/B*5801, C*0303/C*0701, Match target 2: HLA-

305 A*0201/A*0201, B*4402/B4402, C*0501/0501), and completely HLA-mismatched targets
306 (HLA-A*0301/A*3101, B*0702/B*4001, C*0304/C*0702) at 0:1, 1:1, at 3:1 effector/target
307 (E/T) ratios and co-cultured in duplicate for 48 hrs at 37°C in 5% CO₂. Cells were then stained
308 with Live/dead stain and surface markers using anti-CD3 (Pacific Blue), anti-CD8 (V500), anti-
309 CD4 (Alexa Fluor 780), anti-CD14 (Percpcy5.5) conjugated antibodies. Following PBS wash,
310 cells were first permeabilized with Perm A reagent (Invitrogen), then stained intracellularly with
311 Gag p24-PE antibody (Beckman Coulter) diluted in Perm B buffer (Invitrogen) according to the
312 manufacturer's instructions. Gates for p24 expression were set based on uninfected targets.
313 Percentage of killing for each E/T ratio was determined by the following formula:
314 $\% \text{ killing} = 1 - \left(\frac{\% \text{ P24+effector}}{\% \text{ P24 without effector}} \right) \times 100$. For purpose of calculating statistical significance,
315 values obtained for the two HLA matched targets were combined (Figure 2C).
316 **Antigen sensitivity:** Serial 10-fold dilutions of peptides were used in IFN- γ ELISPOT assays to
317 stimulate functional responses. CE antigen sensitivity was measured as the peptide concentration
318 eliciting 50% of the maximal IFN- γ response, or EC50, which was calculated and graphed using
319 GraphPad Prism software (version 7.0). Additional evaluations were based on response
320 magnitude (SFU/10⁶ PBMCs).
321 **Statistics:** Fisher's tests, paired test, and nonparametric Mann-Whitney U test were used to
322 determine statistical significance in this study. An area-under-the-curve based paired *t*-test was
323 used to determine significance between p24 expression of match and mismatch targets in iVKA.
324 GraphPad Prism (ver. 7.0) was used to perform these tests, and significance is determined as *p*
325 value <0.05.

326

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341 PAG, PB, MKPL, and NG analyzed the data. BJP, AB, and PAG wrote the manuscript.
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343

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515 **FIGURE LEGENDS**

516 **Figure 1. T cell responses to predicted cryptic epitope (CE) in chronically HIV infected**
517 **individuals. (A)** PBMC obtained from thirty-two chronically HIV infected individuals (HIV+)
518 were evaluated for responses to 10 predicted CE using an IFN- γ ELISpot assay. Samples
519 obtained from HIV seronegative (HIV-) donors served as a control group. PBMC were
520 stimulated overnight with CE peptide pools (6-10 peptides/pool) and the number of cytokine
521 producing cells was enumerated and is shown as spot forming units (SFU) per million PBMC.
522 Each dot represents the average magnitude of response for one individual to a single peptide
523 pool. A positive response is defined as ≥ 55 SFU/ 10^6 PBMC (dotted line), and three times greater
524 magnitude than unstimulated control (media wells). Mann-Whitney U test was used to determine
525 significance between the two groups. **(B)** Deconvoluted responses to single peptides in six
526 chronic patients are shown. Controls and criteria for positive responses were identical to those of
527 (A).

528 **Figure 2. *Ex-vivo* and *in-vitro* functional responsiveness of T cells responding to cryptic**
529 **epitopes. (A)** Representative flow cytometry data shown for an individual who tested positive
530 for CE-WL9 in an IFN- γ ELISpot assay. PBMCs were incubated with peptide for 12 hrs before
531 antibody staining for flow cytometry-based analysis. Cell population shown in panel were pre-
532 gated on live CD3+ lymphocyte population, and excluded cells expressing CD14, CD19, and
533 CD4. **(B)** WL9 and FY11 specific cells were expanded in vitro by culturing for two weeks with
534 peptide pulsed monocyte stimulation (see methods). Cell populations were pre-gated in the same
535 format as (A), with the panel depicting secretion of IFN γ , TNF α , and perforin in the top, middle,
536 and bottom respectively. **(C)** A CD8+ T cell line specific for CE-FY11 was generated using
537 peptide pulsed monocyte stimulation and co-cultured with HLA A*02 matched and mismatched,

538 NL4.3 infected CD4⁺ T-cell targets (see methods). Percentage of killing was determined by the
539 following equation: $100 - (\% \text{ p24}^+ \text{ staining without any effector} / \% \text{ p24}^+ \text{ at the different E/T}$
540 ratios).

541 **Figure 3. Analysis of cryptic epitope targeting in acutely HIV-1 infected individuals. (A)**
542 Amino acid (AA) sequence of CE-KL10 is shown for two Zambian subtype C infected patients
543 (ZA1 and ZA2) who were predicted to respond to this epitope. Longitudinal changes in AA at
544 the polymorphic site within each CE are shown for each of these individuals. **(B)** IFN- γ ELISpot
545 assays were performed on PBMC cryopreserved at the indicated number of days after the
546 estimated day of infection (dpi) from ZA1 and ZA2, using 10 μ M of CE-KL10 as the antigen.
547 Error bars represent mean magnitude of responses in triplicate wells. A positive response was
548 defined as ≥ 55 SFU/10⁶ PBMC (dotted line), and three times greater magnitude than
549 unstimulated control (media alone). **(C)** Eleven subtype B acutely infected individuals were
550 tested for T cell responses to 10 CE, and 4/11 had one or more positive T cell responses. The
551 magnitude of the response to each of the 10 peptides in these four individuals are shown in
552 SFC/10⁶ PBMC. All samples used here were cryopreserved within three months of the estimated
553 date of infection.

554 **Figure 4. Antigen sensitivity of immunogenic cryptic epitopes. (A)** Serial 10-fold dilution of
555 cryptic peptides were used stimulate USAHI-3 PBMC in an ELISpot assay. Mean with standard
556 deviation of IFN- γ (x-axis) at different concentrations of each peptide (y-axis) is displayed.
557 Dotted line represents limit of positive response. **(B)** Data in panel (A) normalized to show
558 percent maximal IFN- γ response induced at each peptide concentration. Dotted line shows the
559 half-maximal effective concentration (EC50).

Table 1. Cryptic epitopes predicted from HLA-I associated polymorphisms

ARF ^a	Gene	Predicted HLA-I	Peptide Sequence	PP ^b	Esc/Rev ^c	Conserv ^d
FF2	<i>gag</i>	A74, A3301	PTRGGQGISFR	0.69	3/3	0.81
FF3	<i>gag</i>	A0205, B0801	EMWKRTPNERL	0.64	3/8	0.87
FF3	<i>gag</i>	B39, B4201	FPQITLWQRPL	0.79	6/6	0.97
FF3	<i>gag</i>	A74	NLAFPQGEAR	0.51	3/3	0.88
RF1	<i>gag</i>	A340201	EPVYIVSK	0.68	1/4	0.94
RF1	<i>gag</i>	A3001	FPRGPAIFGY	0.87	2/4	0.85
RF1	<i>gag</i>	A0201	GLMYHLPL	0.94	0/7	0.97
RF1	<i>gag</i>	C0802	HPCYLFLKV	0.86	1/4	0.80
RF1	<i>gag</i>	B5101	EPVYIVSKGF	0.56	0/4	0.93
RF2	<i>gag</i>	B57	HAFKVLGDMAW	0.66	2/2	0.84
RF2	<i>gag</i>	B58	HAFKVLGDMDW	0.78	4/2	0.78
RF2	<i>gag</i>	C0804	HAVIISSNVA	0.77	3/1	0.90
RF2	<i>gag</i>	B4201	LPQLKHLTIL	0.56	3/4	0.82
RF2	<i>gag</i>	B1402	WRKFPGPLPL	0.66	5/2	0.88
RF2	<i>gag</i>	C0401, C17	YCFPPGLNL	0.63	4/6	0.78
RF3	<i>gag</i>	A0202	FLLCHFPLV	0.83	1/7	0.97
RF3	<i>gag</i>	B5703	LLLLPMHSKF	0.80	0/3	0.87
RF3	<i>gag</i>	A2402	SFPLALTF	0.66	1/6	0.82
RF3	<i>gag</i>	A4301	WLLDVPPLY	0.96	0/3	0.84
RF2	<i>nef</i>	A2402	GYLTPGPGVF	0.59	2/2	0.89
FF2	<i>pol</i>	B5702	IAGLSMIYRSW	0.70	2/1	0.81
FF2	<i>pol</i>	B5301	IPLPNYGTSW	0.91	0/5	0.88
FF2	<i>pol</i>	B5703	ITAIGEQQW	0.88	0/6	0.84
FF2	<i>pol</i>	A33	KIWPSHKGR	0.73	7/2	0.95
FF2	<i>pol</i>	A0301, A340201, A66	QIRRLNYK	0.68	4/3	0.84
FF2	<i>pol</i>	A2402	QYWMWGMHI	0.99	0/4	0.85
FF2	<i>pol</i>	A0202, A2402, A6801, B1402, B3001	WMWGMHIFQFL	0.82	2/17	0.79
FF2	<i>pol</i>	A3201	YIMTHQKT _w	0.61	1/12	0.76
FF3	<i>pol</i>	A33	GLPVMGTSTQR	0.66	0/3	0.82
RF1	<i>pol</i>	B1801	CPFVRMEF	0.76	1/5	0.85
RF1	<i>pol</i>	B15	FITASYYFCY	0.62	2/5	0.88
RF1	<i>pol</i>	A0201	FLFPCVWVWYSL	0.80	0/4	0.81
RF1	<i>pol</i>	B15	FLHCLCLFY	0.53	5/2	0.94
RF1	<i>pol</i>	A0301, A68	FLMFFVWCGK	0.74	1/5	0.92
RF1	<i>pol</i>	B4201	FPSLRKHIL	0.80	1/3	0.78
RF1	<i>pol</i>	B0801, C18	FYKYLIL	0.63	1/2	0.85
RF1	<i>pol</i>	A2902	GLDFCHATLEY	0.56	1/7	0.85
RF1	<i>pol</i>	C0501	HIQYCHFF	0.83	0/6	0.83
RF1	<i>pol</i>	A2902	LYFCSMLLY	0.86	1/3	0.86
RF1	<i>pol</i>	B1801	NPWCFIVYTRY	0.65	0/7	0.84
RF1	<i>pol</i>	A4301	QIIHILIDDY	0.95	1/2	0.76
RF1	<i>pol</i>	A4301	QLLYFCSMLPY	0.70	0/3	0.79
RF1	<i>pol</i>	A29	WTTVYLSMY	0.85	1/2	0.87
RF1	<i>pol</i>	C18	YCHLYCCLKVL	0.87	2/3	0.80
RF1	<i>pol</i>	B18	YPQVDPFLFY	0.93	0/10	0.76

RF1	<i>pol</i>	A24	YYYAFHGYF	0.94	1/2	0.84
RF2	<i>pol</i>	A2902	AMGSFSSWY	0.73	0/6	0.76
RF2	<i>pol</i>	B5301	APAMGSFSSW	0.73	1/5	0.78
RF2	<i>pol</i>	B3501	KPSSKGTEKY	0.58	5/1	0.79
RF2	<i>pol</i>	A30, A3002	KSDPTYKSSY	0.73	2/6	0.90
RF2	<i>pol</i>	A0201	SIACNSVFFV	0.54	1/3	0.89
RF2	<i>pol</i>	B3501	YPAPFVLVSL	0.52	5/1	0.89
RF3	<i>pol</i>	C18	DYSLLVHVWL	0.68	2/3	0.87
RF3	<i>pol</i>	A0201	ELPKSLEFFYV	0.70	0/4	0.81
RF3	<i>pol</i>	A26	FLLILYHLLLY	0.54	0/4	0.94
RF3	<i>pol</i>	B1801, B4201	FPWLFFAL	0.66	1/5	0.79
RF3	<i>pol</i>	A0101, A29, A2902, A4301	GTIILLGEGY	0.63	6/14	0.81
RF3	<i>pol</i>	C0501	HNILHVLVL	0.82	1/3	0.84
RF3	<i>pol</i>	A02, A0201, B15:16	KLLLMLLFFL	0.81	5/8	0.86
RF3	<i>pol</i>	B35	LPRSLEFFY	0.59	0/4	0.86
RF3	<i>pol</i>	B42	RPFPLLVL	0.71	0/4	0.77
RF3	<i>pol</i>	A0202	SLIFSFLTLL	0.65	0/6	0.76
RF3	<i>pol</i>	A3201, A6801, A6802, B58	STMSPMFLFGW	0.80	5/15	0.76
RF3	<i>pol</i>	A02	TMFPMFLFGWV	0.81	1/4	0.79
RF3	<i>pol</i>	A0202	WLFFALPLL	0.74	0/5	0.92
RF3	<i>pol</i>	B5702	YQTPTQESRW	0.52	0/5	0.95

^aFF= Forward reading Frame and RF= Reverse reading Frame

^bPP=posterior probability

^cesc/rev = escapes or reversions

^dconservation= score based on average percentage of occurrence for listed amino acids within HIV subtype C infected cohort

Table 2. Demographics and clinical parameters of chronic and acute clade B HIV infected individuals

^a ID	Gender	Age	^b CD4	^c VL	^d Risk Factor	^e ART
CHI-1	M	54	705	47	IVDU	No
CHI-2	F	31	606	4684	Heterosexual	No
CHI-3	M	51	468	6900	Heterosexual	No
CHI-4	F	45	829	132	heterosexual	No
CHI-5	F	38	772	12700	heterosexual	No
CHI-6	F	46	650	33543	Heterosexual	No
CHI-7	F	63	827	47	heterosexual	No
CHI-8	F	42	717	47	heterosexual	No
CHI-9	M	62	593	5857	Heterosexual	No
CHI-10	M	52	474	36538	MSM	No
CHI-11	M	44	848	561	MSM	No
CHI-12	M	26	454	435	IVDU	No
CHI-13	M	52	1155	47	MSM	No
CHI-14	M	35	522	3860	MSM	No
CHI-15	F	47	667	132	Heterosexual	No
CHI-16	M	34	681	2359	MSM	No
CHI-17	M	43	686	277	Heterosexual	No
CHI-18	M	52	711	112	MSM	No
CHI-19	F	27	641	1260	Heterosexual	No
CHI-20	M	42	632	305	Heterosexual	No
CHI-21	F	22	656	47	Heterosexual	No
CHI-22	F	37	731	324	Heterosexual	No
CHI-23	F	51	550	14100	Heterosexual	No
CHI-24	M	52	567	8424	Heterosexual	No
CHI-25	M	31	289	5258	MSM	No
CHI-26	F	46	357	119	Heterosexual	Yes
CHI-27	F	46	1562	47	Heterosexual	Yes
CHI-28	F	52	445	49	Heterosexual	Yes
CHI-29	F	53	360	187	Heterosexual	Yes
CHI-30	M	42	892	229	MSM	Yes
CHI-31	F	24	619	13371	IVDU	Yes
CHI-32	M	43	240	5934	IVDU	Yes
USAHI-1	M	34	503	2090000	IVDU	No
USAHI-2	F	31	619	492		No
USAHI-3	M	19				Yes
USAHI-4	M	28		<20		Yes
USAHI-5	M	33				Yes
USAHI-6	M	28	868	1083	MSM	Yes
USAHI-7	M	33	534	461		Yes
USAHI-8	M	30	995	64	Heterosexual	Yes
USAHI-9	M	29	1429	683		Yes
USAHI-10	M	49	1129	152	MSM	Yes
USAHI-11	M	50	541	1580	MSM	Yes

Patients with grayed-out boxes tested positive for at least one CE used in the study

^aCHI=chronic infection and USAHI=acute infection

^bAbsolute CD4 count per mm³ blood

^cCopies of HIV RNA detected per mm³ of blood

^dRoute of virus acquisition. MSM-men who have sex with men and IVDU- intravenous drug user

^eActive antiretroviral therapy status

Table 3. Novel immunogenic cryptic epitopes (CE) identified during acute and chronic HIV-1 infection

Name	AA Sequence	HLA-I ^a	Frequency ^b	
			Acute	Chronic
FY11	FLILYHLLLY	A*26	18%	6%
FL8	FPWLFFAL	B*1801, B*4201	0%	3%
GY10	GTIILLGEGY	A*0101, A*29, A*4301	18%	3%
HL9	HNILHVLVL	Cw*0501	0%	3%
KL10	KLLLMLLFFL	A*02	18%	6%
RL10	RPFPFLIVL	B*42	18%	3%
SW11	STMSPMFLFGW	A*3201, A*6801, A*6802, B*58	9%	3%
TV11	TMFPMFLFGWV	A*02	9%	9%
WL9	WLFFALPLL	A*0202	0%	13%
YW10	YQTPTQESRW	B*5702	9%	3%

All ten epitopes described here are translated from the antisense, reverse frame 3 of the *pol* coding region.

^aEpiPred predicted HLA-I restriction.

^bFrequency of US acute (n=11), and chronic (n=32) patient cohort responding to each CE.

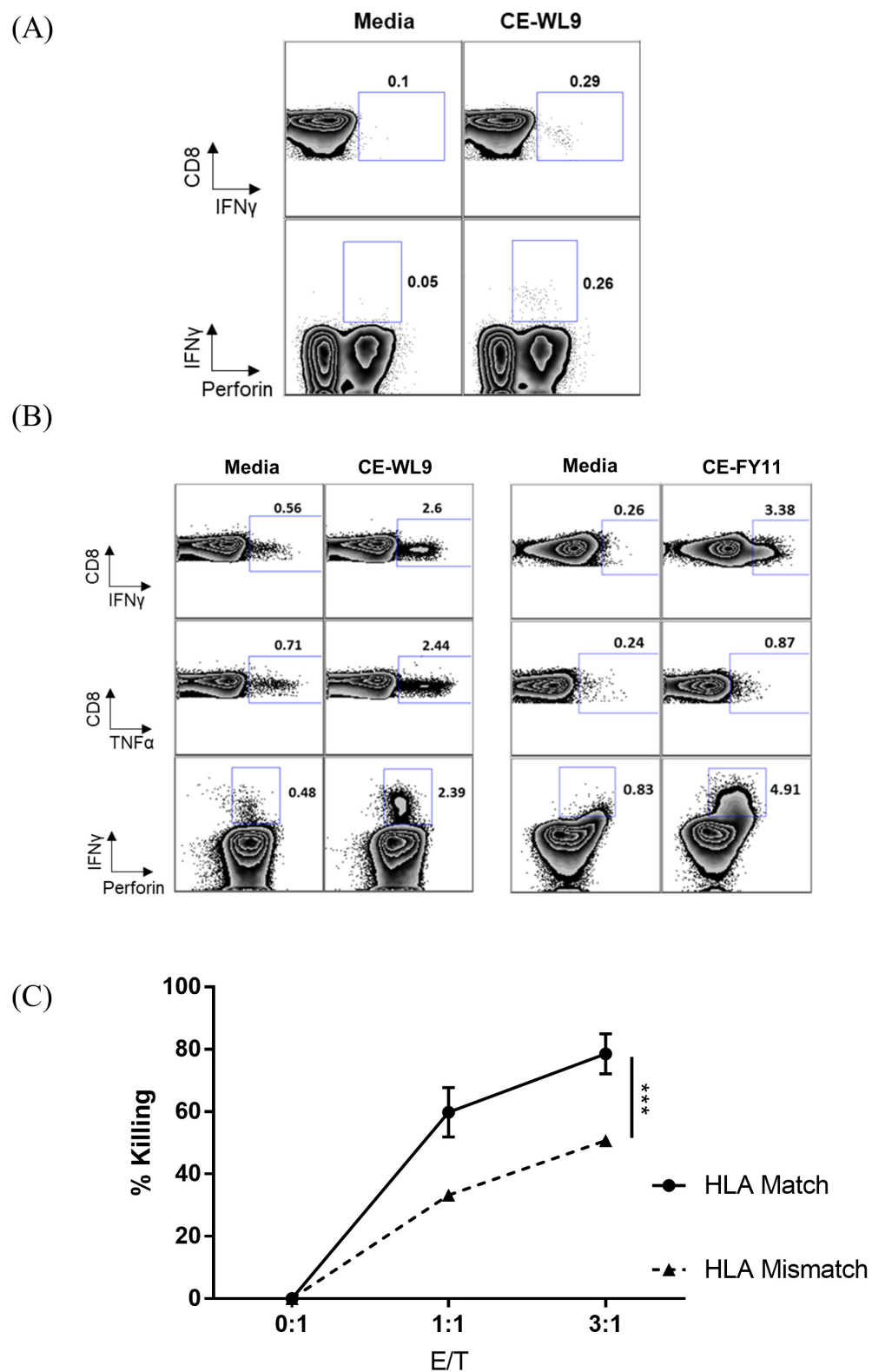
Table 4. Amino acid sequence homology of cryptic epitopes compared to HXB2 derived sequences

CE sequence	HXB2 sequence ^a	HXB2 protein region ^b	% homology ^c
FLLILYHLLLY	LDLWIYHTQG	Nef	36
FPWLFFAL	FLWMGYEL	Pol	38
GTIILLGEGY	VTVLDVGDAY	Pol	30
HNILHVLVL	HNWATHACV	Env	36
KLLLMLLFFL	RILQQLLIH	Vpr	40
RPFPFLIVL	QPIPIVAIVA	Vpu	40
STMSPMFLFGW	QAISPRTLNA	Gag	36
TMFPMFLFGWV	VRYPPL-TFGW	Nef	36
WLFFALPLL	WEFVNTPL	Pol	44
YQTPTQESRW	FKLPIQKETW	Pol/RT	30

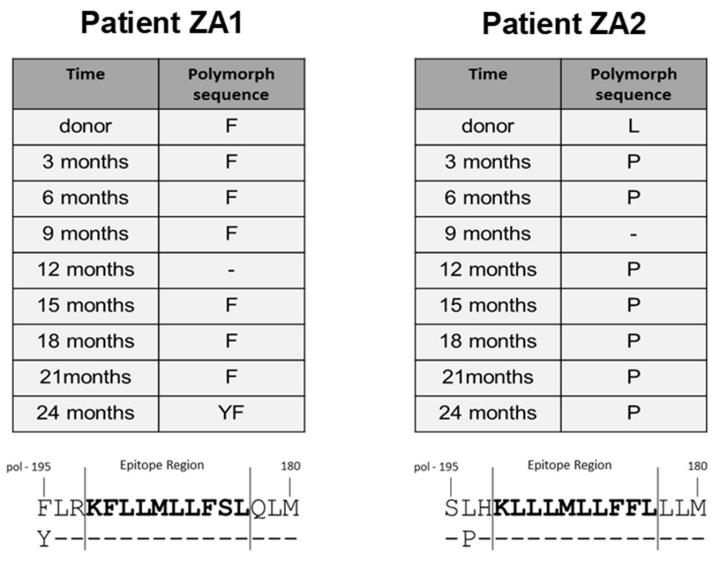
^aClosest matching HXB2 AA sequence based on LANL HIV sequence locator

^bHIV-1 protein where the HXB2 sequence is located

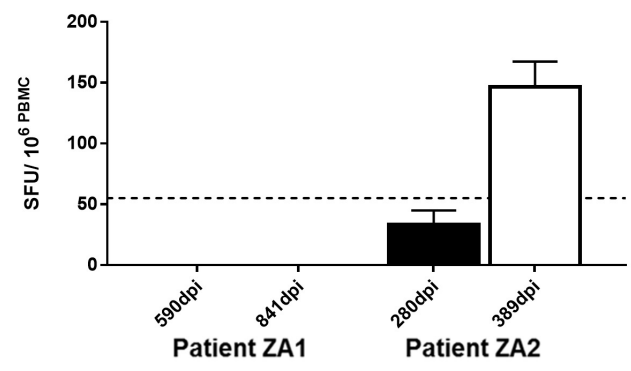
^c% identity between CE and HXB2 sequences



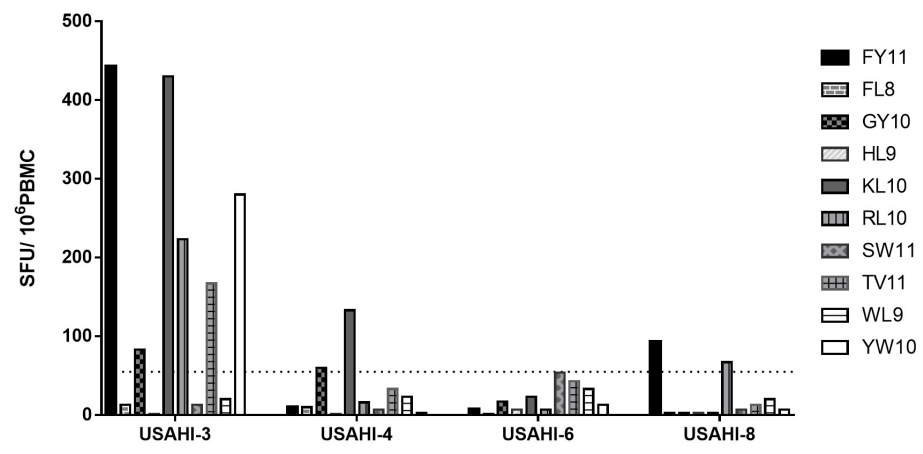
(A)



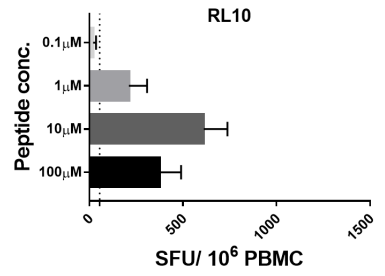
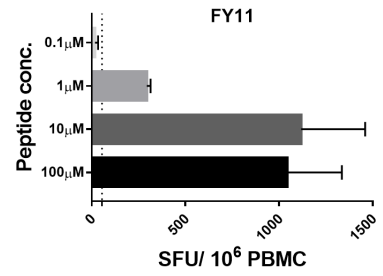
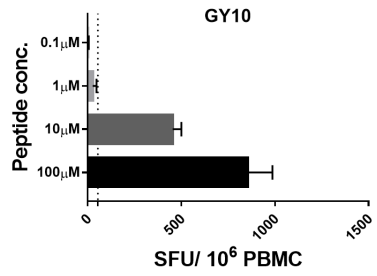
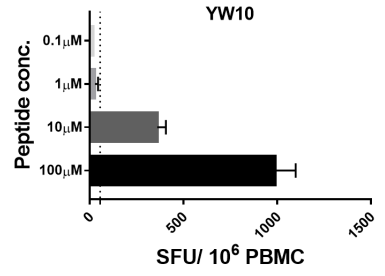
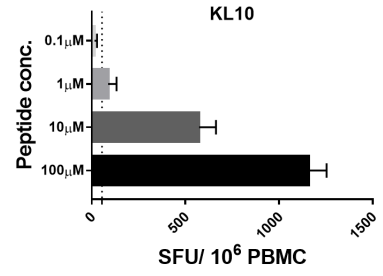
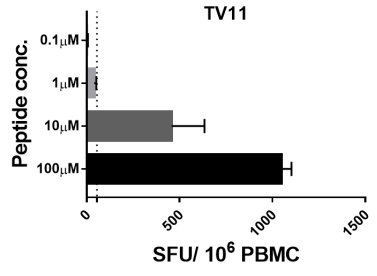
(B)



(C)



(A)



(B)

