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1 **DIFFERENTIAL ESCAPE PATTERNS WITHIN THE DOMINANT HLA-B*57:03-**
2 **RESTRICTED HIV GAG EPI TOPE REFLECT DISTINCT CLADE-SPECIFIC**
3 **FUNCTIONAL CONSTRAINTS**

4
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34 **ABSTRACT**

35 HLA-B*57:01 and HLA-B*57:03, the most prevalent HLA-B*57 subtypes in Caucasian and
36 African populations, respectively, are the HLA alleles most protective against HIV disease
37 progression. Understanding the mechanisms underlying this immune control is of critical
38 importance and yet remains unclear. Unexplained differences are observed in the impact of the
39 dominant CTL response restricted by HLA-B*57:01 and HLA-B*57:03 in chronic infection
40 towards the Gag epitope KAFSPEVIPMF ('KF11', Gag162-172). We previously showed that the
41 HLA-B*57:03-KF11 response is associated with a >1 log lower viral setpoint in C-clade infection
42 and that this response selects escape mutants within the epitope. We first examined the relationship
43 of KF11 responses in B-clade infected subjects with HLA-B*57:01 to immune control and observed
44 that a detectable KF11 response was associated with a >1 log higher viral load (p=0.02). No
45 evidence of HLA-B*57:01-KF11 associated selection pressure was identified in previous
46 comprehensive analyses of >1800 B-clade infected subjects infected. We then studied a B-clade
47 infected cohort in Barbados where HLA-B*57:03 is highly prevalent. In contrast to B-clade
48 infected subjects expressing HLA-B*57:01, we observed strong selection pressure driven by the
49 HLA-B*57:03-KF11 response for the escape mutation S173T. This mutation reduces recognition of
50 virus-infected cells by HLA-B*57:03-KF11 CTL, and is associated with a >1 log increase in viral
51 load in HLA-B*57:03-positive subjects (p=0.009). We demonstrate functional constraints imposed
52 by HIV clade relating to the residue at Gag-173 that explain the differential clade-specific escape
53 patterns in HLA-B*57:03 subjects. Further studies are needed to evaluate the role of the KF11
54 response in HLA-B*57:01-associated HIV disease protection.

55

56 **IMPORTANCE SECTION**

57 HLA-B*57 is the HLA class I molecule that affords the greatest protection against disease
58 progression in HIV infection. Understanding the key mechanism(s) underlying immune suppression
59 of HIV is of importance in guiding therapeutic and vaccine-related approaches to improve the levels
60 of HIV control occurring in nature. Numerous mechanisms have been proposed to explain the HLA
61 associations with differential HIV disease outcome but no consensus exists. These studies focus on
62 two subtypes of HLA-B*57, prevalent in Caucasian and African populations, HLA-B*57:01 and
63 HLA-B*57:03, respectively. These alleles appear equally protective against HIV disease
64 progression. The CTL epitopes presented are in many cases identical, and the dominant response in
65 chronic infection in each case is to the Gag epitope KF11. However, there the similarity ends. This
66 paper seeks to better understand the reasons for these differences and what this teaches us about
67 which immune responses are contributing to immune control of HIV infection.

68

69

70

71 **INTRODUCTION**

72 HLA polymorphism has a substantial impact on HIV disease outcome (1-5) and yet the principal
73 mechanisms underlying these effects remain unresolved (5). The most protective HLA class I
74 molecule is HLA-B*57. HLA-B*57:01 is the most prevalent subtype in Caucasian populations,
75 apparently conferring a similar level of protection against HIV disease progression as HLA-
76 B*57:03, the most prevalent subtype in African populations (6). One proposal is that the HLA-
77 B*57-mediated protection is at least in part due to the breadth of the Gag-specific CD8+ T-cell
78 response, and that HLA-associated immune control of HIV is related to the ability of the CTL
79 response to drive selection pressure on the virus, such that escape can only be achieved at
80 significant cost to viral replicative capacity (5, 7-10). However, although HLA-B*57:01 and HLA-
81 B*57:03 appear to present the identical Gag epitopes, previous studies suggest that significant
82 differences exist in the impact of these responses on immune control.

83

84 The dominant HIV-specific CD8+ T-cell response in each case is directed towards the Gag epitope
85 KAFSPEVIPMF ('KF11', Gag 162-172). Published studies in HLA-B*57:01-positive subjects have
86 been almost exclusively conducted in B clade infection and these have suggested that a response to
87 KF11 is not associated with immune control (2, 11) and that the magnitude of HLA-B*57:01-KF11
88 responses may even be higher in progressors. Evaluation of full-length viral sequences in 1888 B
89 clade infected subjects failed to identify any sequence polymorphisms within the KF11 epitope or
90 flanking it that were directly associated with HLA-B*57:01 (12) and that would have suggested
91 strong selection pressure imposed on the virus by this response. By comparison, 20 HIV amino acid
92 polymorphisms were identified elsewhere in the HIV proteome that were directly associated with
93 HLA-B*57:01. In contrast, in studies undertaken in C clade infected African subjects expressing

94 HLA-B*57:03, a KF11 response is associated with a >1 log lower viral load (13), and there is
95 strong evidence of selection pressure within this epitope, with approximately 70% of subjects
96 carrying mutants at Ala-163 and/or Ser-165, positions 2 and 4 in the epitope (P2 and P4) (9, 10, 14).

97

98 The escape mutants that are selected within KF11 in C clade-infected HLA-B*57:03-positive
99 subjects are typically A163G and S165N. The A163G mutation is selected first, reducing CTL
100 recognition, but also significantly lowering viral replicative capacity (14). The S165N mutant is
101 then selected, substantially restoring viral replicative capacity at the same time as entirely
102 abrogating recognition: an ideal result for the virus (14) that has been associated with higher viral
103 loads (15). Thus the impact of the HLA-B*57:03-KF11 response on the virus is consistent with the
104 mechanism of HLA-mediated immune control described above, with CTL activity forcing the
105 selection of viral escape mutants that reduce viral replicative capacity, whereas that of the HLA-
106 B*57:01-KF11 response is not consistent with this mechanistic model.

107

108 Initial studies proposed that the HLA-B*57:01-KF11 TCRs are highly conserved, with a dominant
109 or exclusively expressed V α 5/V β 19 TCR in 60-100% of subjects (15, 16). These HLA-B*57:01-
110 KF11 TCRs were more likely than the HLA-B*57:03-KF11 TCRs to recognise epitopes containing
111 A163G and S165N; hence these mutations would not be selected in individuals expressing HLA-
112 B*57:01 (15, 17). However subsequent studies of HLA-B*57:01-KF11 TCR usage have been
113 contradictory, with the V α 5/V β 19 TCRs identified as the dominant receptor in 0/6 and 2/10 HLA-
114 B*57:01-positive subjects, respectively (11, 18). Thus it appears unlikely that HLA B*57 subtype-
115 specific effects, namely a public TCR clonotype with high functional avidity for HLA-B*57:01-

116 KF11 (19) fully explain the observed differences in selection of KF11 escape mutants in HLA-
117 B*57:01 and HLA-B*57:03.

118

119 An alternative hypothesis is that escape mutations within KF11 are tolerated in the context of the C-
120 clade, but not in the B-clade Gag sequence, and that this may contribute to the high frequency of
121 escape mutations within the KF11 epitope in HLA-B*57:03-positive individuals infected with C-
122 clade virus. To address this possibility, we here compared a cohort of B-clade infected subjects in
123 Barbados, in which the HLA-B*57:03-subtype predominates, with a cohort of C clade infected
124 subjects in Botswana, where the HLA-B*57:03-subtype also predominates. We identified clade-
125 specific sequence differences that influence the dynamics of viral escape within the HLA-B*57:03-
126 restricted KF11 epitope. These differences were confirmed in a large multi-cohort dataset featuring
127 3298 subjects (including the Barbados and Botswana cohorts), including 1732 clade C infected
128 Africans and 1566 clade B infected North Americans.

129

130

131 **METHODS**

132 **Study cohorts and subjects**

133 HIV-1 B clade infected subjects expressing HLA-B*57:01 were studied from ART-naïve cohorts in
134 Oxford, UK (the Thames Valley Cohort, as previously described (20) and in Barcelona, Spain (21).
135 Additional study cohorts for evaluation of HLA-B*57:03 in the context of B clade and C clade
136 infection in Barbados and Botswana, respectively, were: (i) Bridgetown, Barbados (B-clade, n=246
137 Median age: 38 years IQR 31-47, female:male ratio 60:40, samples collected between 2008-2010),
138 where study subjects were attendees at the Ladymeade Reference Clinic; and (ii) Gaborone,
139 Botswana (C-clade, n=514, Median age; 27 years IQR 23-32, female:male 100:0, samples collected
140 between 2007-2008), where study subjects were antenatal women from the Mma Bana Study, as
141 previously described (8, 13, 22, 23).

142

143 Ethics approval was given by the Health Research Development Committee, Botswana Ministry of
144 Health, by the Barbados Ministry of Health, the Hospital Germans Trias i Pujol' Ethics Committee,
145 and by the Oxford Research Ethics Committee. Subjects received voluntary testing and counselling
146 and written informed consent was obtained from all individuals. Viral load in chronic infection was
147 measured using the Roche Amplicor version 1.5 assay; CD4⁺ T cell counts were measured by flow
148 cytometry. Viral load and absolute CD4 count measurements were obtained at study entry
149 (baseline) for all individuals. All study subjects were ART-naïve.

150

151 Four-digit HLA typing of the Class I locus was performed from genomic DNA as previously
152 described (24) by sequence-based typing at the ASHI* accredited HLA typing laboratory,
153 University of Oklahoma Health Sciences Centre, USA. Exons 2 and 3 of HLA Class I were

154 amplified by locus-specific PCR and then sequenced. Resolution of ambiguities was undertaken
155 according to the ASHI committee recommendations.

156

157 Additional viral sequence analyses were performed on two previously described, multi-center
158 cohorts: (1) the International HIV Adaptation Collaborative (IHAC) consisting of 1443 clade B Gag
159 sequences (12); and (2) 1470 African clade C Gag sequences from cohorts based in Durban (8),
160 Bloemfontein (25) and Kimberley (20) South Africa, Zambia and the Thames Valley area of the
161 United Kingdom (20). Where high-resolution HLA typing was unavailable, we employed a
162 published machine learning algorithm trained on a dataset of high resolution HLA class I types
163 from >13,000 individuals with known ethnicity to complete these data to high resolution. (26).

164

165 **IFN- γ ELISPOT assays**

166 IFN- γ enzyme-linked immunospot (Elispot) assays were performed as previously described (13, 27),
167 using optimally defined epitopes and 18mer overlapping peptides (OLP) with input cells/well
168 ranging from 30,000 to 100,000. The number of specific spot-forming cells (SFC) was calculated
169 by subtracting the mean number of spots in the negative control wells from the number of spots
170 counted in each well. The magnitude of epitope-specific responses was calculated as SFC per
171 million cells.

172

173 **Site-directed mutagenesis of NL43**

174 The mutation S173T (Serine to Threonine at Gag HXB2 position 173) was introduced by site
175 directed mutagenesis (Quikchange I, Stratagene,UK) into wild-type NL43 plasmid DNA, as well as
176 NL43 containing the mutations A163G and/or S165N (14). Whole plasmid DNA p83-2 (the 5'-half

177 of HIV-1_{NL43} strain) was PCR-amplified in a mutagenesis reaction with two overlapping primers,
178 containing the target mutation. Primers used for the mutagenesis reaction were: F 5'-
179 CCCAGAAGTAATACCCATGTTTACGGCATTATCAGAAGGAGC-3' and R 5'-
180 GCTCCTCTGATAATGCCGTAAACATGGGTATTACTTCTGGG-3 (the mutagenesis site is
181 underlined) .The presence of mutations were verified by DNA Gag sequencing in newly generated
182 plasmid clones. The DNA fragment ranging from SapI to Apa I restriction sites was then subcloned
183 into a new p83-2 vector to avoid potential carry over of additional mutations during the
184 mutagenesis, and the coding region sequence was verified again as previously described (28)

185

186 **Virus production and Replication kinetics**

187 Viral stocks were produced by cotransfection of the different site-directed mutant plasmids (5' half
188 of HIV-1_{NL43} strain) with p83-10_{eGFP} (3' half of HIV-1_{NL43} strain) into MT4 cells (29). Viral stocks
189 were harvested and viral RNA extracted (Qiagen, UK). The *gag* p24/p17 coding region was PCR
190 amplified and sequenced to confirm the presence of the mutations in the viral RNA and the absence
191 of any other potential polymorphisms. The 50% tissue culture-infectious dose (TCID₅₀) for each
192 viral stock was determined in MT4 using the Reed and Muench method (30). For replication
193 experiments, Jurkat, MT4 and H9 T cells were infected in triplicate with a multiplicity of infection
194 (MOI) of 0.005 in a total volume of 3ml with wild-type or mutant HIV-1 NL43 virus and incubated
195 at 37°C for 2 hours. Pellets were washed twice with PBS and cultured at 37°C and 5% of CO₂. After
196 infection around 50,000 cells were harvested daily in order to measure infectivity by percentage of
197 eGFP-positive cells by Fluorescent Activated Cell Sorting (FACS). Replication kinetics were
198 determined by calculating the mean viral slope using the LOGEST function (Microsoft Excel) and

199 converted to natural logs. Variation in replication slopes was assessed using Student's T test. All
200 statistical calculations were performed in Prism 5.0 (Graphpad).

201

202 **Amplification and sequencing of proviral DNA**

203 Gag p17/p24 sequences (Cohort; Barbados n=125, Botswana n=322) were generated from genomic
204 DNA extracted from peripheral blood mononuclear cells (PBMC) where available, amplified by
205 nested PCR using previously published primers to obtain population sequences, as previously
206 described (31). Sequencing was undertaken using the Big Dye Ready Reaction Terminator Mix
207 (V3.1) (Applied Biosystems, UK). Sequences were analysed using Sequencher v4.8 (Gene Codes
208 Corporation) and aligned by SeAl to HXB2 B-clade reference strain. Sequences were submitted to
209 Genbank and accession numbers are as follows FJ497801-FJ497875, FJ497885-FJ497899,
210 FJ497901-FJ497905, FJ497907-FJ497916, FJ497918-FJ497950.

211

212 **Identification of HLA-associated viral polymorphisms from proviral DNA**

213 HLA-associated viral polymorphisms were identified from proviral DNA using a previously
214 described method that corrects for phylogeny, HLA linkage disequilibrium and codon-covariation
215 (8, 32). A q-value statistic, representing the p-value analogue of the false discovery rate (FDR), was
216 computed for each association. The FDR is the expected proportion of false positives among the
217 associations identified at a given p-value threshold; for example, among associations $q \leq 0.2$, we
218 expect 20% to be false positives. The phylogenetically corrected methods rely on an inferred
219 phylogeny. We constructed two phylogenies for this study: (i) a phylogeny consisting of clade B
220 and C sequences from Barbados and Botswana was constructed using Phym1 v2.4.5, under the
221 general time reversible (GTR) model (33) (ii) a phylogeny consisting of N=3298 p17/p24

222 sequences from all cohorts described in this study. This phylogeny was too large for PhymI, so we
223 employed a 3 stage process to infer the phylogeny. (1) A combined alignment was created, then
224 sites with >10% missing data were removed, after which sequences with missing data in >10% of
225 remaining sites were removed (resulting in the above noted N's); (2) a phylogeny was inferred
226 separately for clade B and C alignments, using PhymI v2.4.5 under the GTR model); (3) the
227 resulting phylogenies were joined by adding single common ancestor to the two clade trees, and the
228 branch lengths were optimized using hyphy, under the GTR model (34).

229

230 **PhyloDOR ratio**

231 Identification of HLA-associated polymorphisms and assessment of differential escape between
232 viral clades and/or closely-related HLA alleles were performed as previously described (12, 32, 35).
233 Briefly, a maximum –likelihood phylogenetic tree was constructed for each gene, and a model of
234 conditional adaptation was inferred for each observed amino acid at each codon (32). In this model,
235 the amino acid is assumed to evolve independently along the phylogeny until it reaches the
236 observed hosts (tree tips). In each host, the HLA-mediated selection pressure is modeled using a
237 weighted logistic regression, in which the individual's HLA repertoire is used as predictors and the
238 bias is determined by the transmitted sequence (35). Because the transmitted sequence is not
239 observed, we average over the possible transmitted sequences, and all possible phylogenetic
240 histories, as inferred from the phylogeny. Similarly, where high resolution HLA types are not
241 available, we perform a weighted average over possible completions (12).

242

243 To test for differential escape between HLA-B*57:01 and B*57:03, or to test for clade-specific
244 effects on selection, interaction variables were added to the phylogenetically-corrected logistic

245 regression model and significance was determined via a likelihood ratio test, as previously
246 described (35).

247

248 **Effect of S173T mutation on epitope recognition by KF11-specific CD8⁺ T cells**

249 CD4⁺ T cells were enriched from PBMCs from healthy donors expressing HLA-B*57:03 using
250 negative selection (Dynabeads) and activated for 3-6 days using IL-2 (50U/ml Roche) and PHA
251 (3ug/ml). KF11-specific CD8⁺ T cells (<98% specificity) were enriched from PBMCs from HIV-
252 infected donors using tetramers as previously described (36). B*57:03-positive CD4⁺ T cells were
253 infected with NL43_{GFP} or NL43_{GFP} containing the S173T mutation as described above. To test for
254 epitope recognition, epitope-specific CD8⁺ T cells (<98% specificity) were cocultured with the
255 HIV-infected CD4⁺ T cells in the presence of CD107a antibodies (PE-Cy5), 10ug/ml Brefeldin A,
256 Golgi stop (BD), CD49d and CD28 for 6.5 hours at 37°C in a 5% CO₂ incubator. Cells were
257 stained for surface and intracellular antibodies against CD4 (APC), CD8 (Alexa Fluor 700), MIP1B
258 (FITC), p24 (PE), IFN-γ (PE-Cy7), Live/dead marker (Pacific Blue), and then immediately
259 acquired by FACS (BD LSRII).

260

261

262

263 **RESULTS**264 **B clade HLA-B*57:01-KF11 responders have higher viral setpoints than non-responders**

265 Previous studies of B clade infected subjects using peptide-MHC class I tetramers have suggested
266 that a detectable HLA-B*57:01 response is more frequently observed in progressors (including
267 those with viral loads of >90,000) than in elite controllers/long-term non-progressors (2, 11). These
268 studies however were not sufficiently powered to demonstrate a statistically significant result. We
269 therefore started by comparing responses to KF11 in B clade infected, ART-naïve individuals
270 expressing HLA-B*57:01 whose viral setpoints ranged from undetectable to 500,000 copies/ml (Fig
271 1). Here the association between KF11 responders and high viral setpoint reaches statistical
272 significance ($p=0.02$, Mann Whitney test). These findings are consistent with the earlier studies
273 cited of B clade infected subjects expressing HLA-B*57:01, and provide the opposite result to that
274 obtained in HLA-B*57:03-positive individuals infected with C clade virus (13), using the identical
275 approach of measuring IFN-g elispot responses to KF11, where a response was associated with a
276 >10-fold lower viral setpoint. Equivalent studies of KF11 responses in 17 HLA-B*57:03-positive
277 subjects infected with B clade virus similarly showed substantially lower median viral loads in
278 KF11 responders compared to non-responders (median viral load 1,629 versus 6,127 c/ml,
279 respectively), although here this difference did not reach statistical significance ($p=0.28$, data not
280 shown).

281

282 **Differential escape in the B*57:03-KF11 epitope in B-clade versus C-clade infection**

283 In order to evaluate further the potential differences between HLA-B*57:01 and HLA-B*57:03, we
284 investigated a B clade infected, ART-naïve study cohort in Barbados where HLA-B*57:03 is highly
285 prevalent. It has been noted in several other studies that HLA-B*57:03 is associated with immune

286 control of HIV in B clade and C clade infection (1-5). Consistent with these studies, HLA-B*57:03-
287 positive subjects in Barbados exhibited significantly lower median viral loads than HLA-B*57:03-
288 negative subjects (median 3,450 versus 13,350, $p=0.015$, Mann Whitney test) and significantly
289 higher CD4⁺ counts (median 565 versus 398, $p=0.003$ Mann Whitney test) (Fig. 2).

290

291 To determine the nature of any selection pressure imposed in the B clade virus through the HLA-
292 B*57:03 KF11 response, we analysed viral sequences in *gag* in the Barbados cohort in order to
293 identify associations between HLA-B*57:03 and viral polymorphisms in the region of the KF11
294 epitope. This revealed that HLA-B*57:03 expression was associated with the previously described
295 escape mutations T242N, in the epitope TW10 (TSTLQEQIGW; Gag HXB2 240-249) (7, 39), and
296 I147X, in the epitope ISW9 (ISPRTLNAW; Gag HXB2 147-155) (Table I) (40, 41). However, the
297 intra-epitope escape mutations within KF11 (KAFSPEVIPMF; Gag HXB2 162-172), namely
298 A163G and S165N, selected in approximately 70% of C-clade infected HLA-B*57:03-positive
299 subjects (3, 14), were not associated with HLA-B*57:03 in this Barbadian study cohort (Table I-II).

300

301 However, in this same Barbados cohort, we identified an HLA-B*57:03-associated viral
302 polymorphism located at Gag HXB2 position 173, which immediately flanks the C-terminus of the
303 KF11 epitope. This mutation has not been observed in association with HLA-B*57:03 in studies of
304 C-clade infected cohorts, which in any case have Threonine as the consensus residue at position 173
305 (14, 42, 43). The high frequency of selection of S173T by HLA-B*57:03-positive subjects (61%
306 versus 24% in HLA-B*57:03-positive versus HLA-B*57:03-negative subjects) together with the
307 lack of any selection of intra-epitope KF11 mutations led to the hypothesis that selection of S173T

308 in B-clade virus may mitigate against further selection of KF11 escape mutations A163G and/or
309 S165N (Table II) (see below).

310

311 We performed a further analysis using a phylogenetically-corrected method (12) to compare the
312 impact of HLA-B*57:03 on the selection of Gag escape mutants in B-clade versus C-clade HIV,
313 using data from the study cohorts in Barbados (B-clade) and in Gaborone, Botswana (C-clade). We
314 found no statistical difference between odds of HLA-B*57:03-mediated escape in the two cohorts
315 for T242N ($p=0.82$) or I147L ($p=0.29$). In contrast, we observed substantial clade differences for all
316 three KF11 escape mutations: the strength of selection for A163G and S165N was significantly
317 greater in the C clade cohort ($p=0.006$ and $p=0.08$, respectively), whereas 173T was only selected
318 in the B clade cohort ($p=0.0006$). In fact, Gag-173T, the consensus in C-clade, arises at
319 significantly *lower* frequency in HLA-B*57:03-positive subjects in Botswana ($p=0.0062$; discussed
320 further below) (Table III). These data demonstrate clade-specific differences in the impact of HLA-
321 B*57:03 on Gag escape mutant selection, with differential effects at Gag-163, 165 and 173, within
322 or immediately flanking the dominant KF11 epitope.

323

324 **Impact of S173T on recognition of virus-infected target cells and on viral setpoint**

325 The location of the HLA-B*57:03-associated mutation immediately downstream of the KF11
326 epitope suggests that the S173T mutant reduces processing of the epitope. To test whether the
327 HLA-B*57:03-associated S173T polymorphism reduces recognition of virus-infected target cells,
328 CD4⁺ T cells from HLA-B*57:03⁺ healthy subjects were infected with NL43 HIV that was either
329 wildtype, expressing Ser-173, or engineered to express the S173T viral polymorphism. Infected
330 cells were incubated with HLA-B*57:03-KF11-specific CD8⁺ T-cells (>98% specific) and the level

331 of CD8⁺ T-cell activation monitored by CD107a and MIP1 β expression. We observed that the
332 S173T mutant indeed significantly reduced recognition by the KF11-specific CD8⁺ T cells (Fig 3,
333 p=0.0038, Student's t test). In the same assay, using CD8⁺ T-cells specific for the HLA-B*57:03-
334 restricted Pol-specific epitope IATESIVIW ('IAW9'), no difference were observed in the level of
335 stimulation by the two viruses on the HLA-B*57:03-restricted IAW9-specific CD8⁺ T cells (Fig 3).
336 These data support the hypothesis that S173T specifically reduces presentation of the KF11 epitope
337 by HLA-B*57:03. Furthermore, mismatched CD4⁺ T cells induced consistently low levels of
338 stimulation confirming that activation of the KF11- and IAW9-specific CD8⁺ T cells was HLA-
339 B*57:03-dependent.

340

341 We next examined the viral setpoints and CD4 counts in HLA-B*57:03-positive subjects with and
342 without the S173T mutation. Viral loads in HLA-B*57:03-positive subjects with the B-clade
343 wildtype, Serine at Gag-173, were more than 10-fold lower than in B*57:03-positive subjects with
344 the S173T polymorphism (median viral load 520 versus 6,905 respectively; p=0.009 Mann Whitney
345 test). Furthermore, 173S was associated with a substantially higher CD4 count in HLA-B*57:03-
346 positive subjects than 173T (median CD4 count 787 versus 375 respectively; p=0.036 Mann
347 Whitney test) (Fig. 4). However no differences in median viral load or CD4 counts were observed
348 in B*57:03-negative subjects with Serine versus Threonine at Gag-173 (median viral load 14,450
349 versus 10,600 respectively; p=0.949 and median CD4 count 358 versus 374 respectively; p=0.522
350 Mann Whitney test). These data together support the conclusion that HLA-B*57:03-KF11
351 responses drive the selection of the S173T mutation in B clade infected individuals expressing
352 HLA-B*57:03; and that this is an escape mutation in that it reduces recognition of virally infected
353 targets. These findings are consistent with the hypothesis that this response contributes to HLA-

354 B*57:03-associated control of HIV, since viral loads are significantly higher in those with the
355 S173T escape mutation.

356

357 **S173T with A163G and S165N significantly reduces viral replicative capacity**

358 The observations above prompt the question: if A163G and S165N are escape mutations frequently
359 selected in HLA-B*57:03-positive subjects infected with C clade virus, why are they not selected in
360 HLA-B*57:03-positive subjects with B clade virus infection? To assess the functional significance
361 of the HLA-B*57:03-associated S173T mutation and the possible impact of this polymorphism on
362 the selection of A163G and S165N, the viral polymorphisms S173T, A163G, and S165N were
363 introduced by site-directed mutagenesis into the B-clade backbone of NL43_{GFP}. Infectious viral
364 stocks were generated by transfecting MT4 T-cells with the relevant DNA constructs. H9, MT4 or
365 Jurkat T cells were then infected and the rate of viral growth was determined by monitoring the
366 percentage of GFP-infected cells over 14 days.

367

368 Analysis of the rate of viral growth in MT4, H9 and Jurkat T-cells showed, first, that the S173T
369 polymorphism had no significant effect on viral fitness in this *in vitro* system in any of these three
370 cells lines used (Fig.5 and data not shown). We previously showed that the introduction of A163G
371 or A163G/S165N into the NL43 backbone significantly reduced viral replicative capacity, with
372 S165N acting as a partial compensatory mutant for A163G that also completely abrogated
373 recognition of KF11 (14, 15). Here we observe that the introduction of either A163G or S165N into
374 the NL43 backbone in combination with S173T also significantly reduces viral spread, but
375 substantially more so than in the absence of S173T. Furthermore, the combination of S173T and
376 both of the KF11 mutations, A163G and S165N, dramatically reduced viral spread even further,

377 indicating a significant cost to viral fitness of this combination of viral mutations in a B clade virus
378 (Fig. 5). These data together suggest that the KF11 escape mutant S173T is more commonly
379 selected in B clade infected subjects expressing HLA-B*57:03 because the cost to replicative
380 capacity is negligible, and less than that resulting from A163G or S165N. Subsequent mutations in
381 addition to S173T result in such a substantial reduction in replicative capacity, without any apparent
382 amelioration from S165N to reduce these fitness costs, that these arise very rarely (Table II).

383

384 As mentioned above, Gag-173T, the consensus in C-clade, arises at a significantly lower frequency
385 in HLA-B*57:03-positive compared to HLA-B*57:03-negative subjects in Botswana ($p=0.0062$). A
386 larger analysis of the KF11 epitope region of 1899 C-clade sequences confirmed that the presence
387 of A163G, S165N or both in combination, was significantly associated with Serine at position 173
388 and that this was the case both for HLA-B*57:03-positive and HLA-B*57:03-negative individuals
389 (Fig 6). Thus, although 173T is consensus in C-clade, it appears unfavourable in the context of the
390 KF11 intra-epitope escape mutations, supporting the findings in B-clade suggesting that this
391 combination of mutations has a detrimental impact on viral fitness.

392

393

394 **DISCUSSION**

395 HLA-B*57:01 and HLA-B*57:03 are the two most protective HLA molecules against HIV disease
396 progression in both B and C clade infection (5). These molecules differ by only two amino acids
397 (D114N and S116Y respectively), and the peptide binding motifs are almost indistinguishable (44,
398 45). In chronic infection, the dominant HIV-specific CD8⁺ T cell response in subjects expressing
399 HLA-B*57:01 or HLA-B*57:03 is to the Gag epitope KAFSPEVIPMF ('KF11', Gag HXB2 162-
400 172) (2, 10, 13). Studies of HLA-B*57:03-positive subjects infected with C clade virus indicate that
401 this KF11 response makes an important contribution to immune control (3, 15), and contributes to
402 the superiority of HLA-B*57:03 as a protective HLA class I molecule over the closely-related
403 HLA-B*57:02 and HLA-B*58:01. (10).

404

405 This study sets out to investigate the observations, first, that whilst the HLA-B*57:03-KF11
406 response is associated with significantly lower viral setpoints in C clade infection (13), studies of
407 HLA-B*57:01-KF11 responses had suggested the opposite in B clade infected individuals (2, 11);
408 and, second, whereas the HLA-B*57:03-KF11 response frequently drives escape mutations within
409 KF11 in C clade infection (A163G and S165N) (8), these are not selected in response to HLA-
410 B*57:01 responses in B clade infection (12).

411

412 We first confirmed a statistically significant association between response to the KF11 epitope in B
413 clade infected subjects expressing HLA-B*57:01 and a >1 log higher viral load. This result arose
414 from the identical assays that were used in the studies that showed a KF11 response was a
415 associated with a >1 log lower viral load in C clade infected subjects expressing HLA-B*57:03
416 (13). We next showed that HLA-B*57:03 has a similar impact in B and C clade infection in terms

417 of the escape mutations selected in the Gag epitopes ISW9 and TW10, but a differential impact in
418 the KF11 epitope. The S173T mutation selected in B clade infection reduces recognition of virus-
419 infected targets and is associated with a >1 log increase in viral setpoint. This is consistent with
420 studies in C clade infection (15) suggesting that this HLA-B*57:03 KF11 response contributes to
421 HLA-B*57:03-associated immune control of HIV infection. The clade specific differences in the
422 selection of KF11-driven escape mutants observed in Barbados (B clade infected cohort) and
423 Botswana (C clade infected cohort) were corroborated in analyses of larger datasets.

424

425 The position of S173T one residue downstream (P1') of the KF11 epitope suggests that it may be a
426 processing mutation since this residue would be involved in the cleavage site of the proteasome
427 (46). Previous studies of peptide cleavage motifs have suggested that the constitutive- and immuno-
428 proteasome have a strong preference for Alanine at P1' but prefer Serine over Threonine; thus the
429 mutation S173T could affect efficient cleavage of the C-terminal end of the KF11 epitope by the
430 proteasome (46).

431

432 We show that the HLA-B*57:03-associated S173T mutation effectively precludes further selection
433 of the KF11 intra-epitope viral mutations, A163G and S165N, since the combination of these three
434 mutations in a clade B backbone results in a virus with severely reduced replicative capacity.

435 Indeed, the close proximity of the amino acid positions 173, 163 and 165 between helix 1 and helix
436 2 of the Gag p24 structure, suggests that structural constraints prevent selection of A163G and
437 S165N if S173T has already been selected. Previous work has shown that, using a B-clade
438 backbone, and in the presence of S173, the mutation A163G reduces replicative capacity, but that
439 the further addition of S165N, as observed *in vivo*, partially restores replicative capacity (14, 47).

440 This fits with the order of selection of A163G and S165N, with S165N apparently always arising
441 subsequent to A163G (14). However in B clade infection it appears that the selection of S173T
442 prevents the selection of further mutants within the epitope because the fitness cost is too high.
443 S173T appears to be the preferred choice of viral escape from the KF11-specific response since it
444 has minimal effect on viral fitness.

445 Our inference from the data described above and from previous studies (23) indicating that the
446 HLA-B*57:03-KF11 response contributes to immune control of B and C clade HIV infection. The
447 reduced recognition of S173T-virus-infected cells by KF11-specific CTL together with the lack of
448 cost to viral replicative capacity resulting from S173T is consistent with the observation that viral
449 loads are higher and CD4 counts lower in B clade infected subjects expressing HLA-B*57:03.

450

451 In view of the substantial reduction in viral replicative capacity resulting from the
452 A163G/S165N/S173T combination in B clade infection, it is perhaps surprising to observe the
453 selection of A163G/S165N at high frequency in HLA-B*57:03-positive subjects infected with C
454 clade virus in which the vast majority of sequences carry Thr at Gag-173. It may be inferred from
455 this that the presence of consensus 173-Thr in the context of C clade Gag does not have the same
456 prohibitive effect on viral fitness, as it does not prevent the selection of A163G and S165N.
457 Nevertheless, in C clade infection, both in HLA-B*57:03-positive and HLA-B*57:03-negative
458 individuals, A163G/S165N are significantly associated with Ser at Gag-173 (Fig. 6), as opposed to
459 the consensus Thr at this position, suggesting that the combination of A163G/S165N/S173T is not
460 favoured in either B or C clade infection.

461

462 Gag-173 has been well-studied in relation to HLA-B*27, another protective HLA molecule,
463 because of the S173A mutation that accompanies the R264K escape variant within the dominant
464 HLA-B*27-restricted epitope KRWILGLNK (KK10) (48-50). It is noteworthy that in C clade
465 infection, R264K escape in HLA-B*27-positive subjects is typically accompanied by compensatory
466 mutations not at Gag-173, but at S165N (Brener *et al*, in preparation). These data underline the tight
467 constraints on amino acid substitutions in the capsid protein, the interdependence of residues at
468 certain key positions in the structure, including Gag-163, Gag-165, and Gag-173, and therefore the
469 impact that clade can have on the escape options for the virus.

470

471 These data help to explain why HLA-B*57:03 is not associated with the ‘usual’ KF11 intra-epitope
472 mutations A163G/S165N in clade B, but they do not explain why HLA-B*57:01 is not associated
473 with either the S173T flanking mutation, nor with any KF11 intra-epitope mutations. Previous
474 studies have suggested that TCR usage for the HLA-B*57:01-KF11 response allows recognition of
475 the KF11 variants (15), but these initial TCR studies indicating conservation of a ‘public’ HLA-
476 B*57:01-KF11 TCR have not been borne out by subsequent studies (11, 18). One possible
477 explanation is that the potency of the HLA-B*57:01-KF11 response is so great that a moderate
478 reduction in processed epitope would not affect killing sufficiently to be selected, however
479 preliminary data suggests that HLA-B*57:03 response is, if anything, the more potent. Further
480 studies with a large number of KF11-specific clones would be needed to establish whether clear-cut
481 differences between the responses restricted by HLA-B*57:03 and HLA-B*57:01 exist in terms of
482 potency, and the relevance of this to viral escape patterns. A recent study comparing the impact of
483 individual HLA class I molecules on immune control (viral load <2000 copies/ml) versus non-
484 control (VL>10,000 copies/ml) of B clade infection showed the identical odds ratio for protection

485 via HLA-B*57:01 in a European American cohort as via HLA-B*57:03 in an African American
486 cohort (6).

487

488 These studies therefore provide an explanation for the distinct clade-specific selection of escape
489 mutants by the HLA-B*57:03-KF11 response but do not resolve the question of why the HLA-
490 B*57:01-KF11 response does not select escape mutants. Insufficient studies have been undertaken
491 in C clade infected subjects who express HLA-B*57:01 to be certain of whether this response
492 selects no escape mutants in C clade as well as in B clade infection. The absence of the KF11
493 response in elite controllers with HLA-B*57:01 does not necessarily mean that these responses
494 have not contributed to immune control in these subjects, since it is possible that the period of
495 detectability may be transient. It is clear that many responses that are undetectable in elite
496 controllers can become detectable after peptide stimulation (51). However, if the KF11-specific
497 CTL response contributes to immune control of HIV in HLA-B*57:01-positive subjects in B clade
498 infection, it would be unique in failing to select escape mutants in the process and the mechanism
499 would be invaluable for directing successful vaccine targets.

500

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798 **FIGURE LEGENDS**

799 **Figure 1. Median Viral of KF11 responders versus nonresponders in B clade-infected**
800 **individuals expressing HLA-B*57:01**

801 PBMCs from B clade-infected, ART-naïve individuals expressing HLA-B*57:01 were analysed by
802 IFN- γ ELISPOT assay for responses to the KF11 epitope. Viral loads of responders and
803 nonresponders were compared. Mann-Whitney U tests were performed.

804

805 **Figure 2. Median Viral Load and CD4 count of B*57:03-positive versus B*57:03-negative**
806 **subjects in Barbados cohort (B-clade).**

807 B*57:03-positive subjects were compared to B*57:03-negative subjects for (A) Viral Load and (B)
808 CD4 count. Median and 5-95 percentiles are shown. Mann-Whitney U tests were performed.

809

810 **Figure 3. Effect of viral mutation S173T on epitope recognition of HIV-infected cells by KF11-**
811 **specific CD8⁺ T-cells**

812 *Ex vivo* CD4⁺ T-cells from B*57:03⁺ and B*57:03⁻ donors were infected with wildtype NL43 virus
813 or NL43 virus harbouring the S173T viral mutation. Infected CD4⁺ T-cells were then cultured with
814 KF11-specific CD8⁺ T-cells (A) or IAW9-specific CD8⁺ T-cells (B) and the level of CD8⁺ T-cell
815 activation monitored by expression of CD107 and Mip1 β . Data from both experiments were
816 standardized relative to % recognition by wildtype virus (C). Experiments were performed in
817 triplicate, mean and SD are shown. Student's t test were performed. p value summary is as follows;
818 p<0.01 *, p<0.001 **, p<0.0001 ***.

819

820 **Figure 4. Median Viral Load and CD4 count of B*57:03-positive HIV-infected subjects with**
 821 **viral polymorphisms S173 and T173.**

822 Proviral DNA sequences from B*57:03-positive subjects from the Barbados cohort (B-clade) were
 823 analysed for the presence of the viral polymorphisms S173 and T173. Viral Loads (A) and CD4
 824 counts (B) were compared. Median and 5-95 percentiles are shown. Mann-Whitney t tests were
 825 performed.

826

827 **Figure 5. Viral replication capacity of NL43_{GFP} virus with multiple B*57:03-associated viral**
 828 **mutations**

829 NL43_{GFP} virus was engineered to contain combinations of the viral mutations 173T, 163G and
 830 165N. MT4 cells were infected and monitored for GFP-positive cells over 14 days (A). The slope of
 831 the curve was calculated from the exponential growth phase using the LOGEST function and
 832 converted to natural logs (B). Experiments were performed in triplicate and mean and SD are
 833 shown. Dunnett's multiple comparison tests were performed. p value summary is as follows;
 834 $p < 0.01$ *, $p < 0.001$ **, $p < 0.0001$ ***.

835

836 **Figure 6. Frequency of KF11 mutations (A163G and S165N) and S173T in C-clade gag**
 837 **sequences (n=1899)**

838 HIV-1 p24 gag sequences (n=1899) were analysed for the presence of the KF11 mutations, A163G
 839 and S165N, in the presence of 173T and 173S. B*57:03-positive subjects (A) and B*57-negative
 840 subjects (B) were analysed. Fisher's exact tests were performed.

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845 **Table I. Summary of HLA-B*57:03-associated viral mutations in Barbados cohort (n=125).**

846 A phylogenetically-corrected method was used to determine the location of HLA-B*57:03-
847 associated viral mutations in proviral *gag* p17 and p24. Polymorphism location and HXB2 number
848 are shown. A q value cut-off of $q < 0.2$ was used.

849

850 **Table II. Summary of amino acid polymorphisms in the KF11 epitope region (HXB2 162-173)**
851 **in Barbados cohort (n=125).**

852 Proviral sequences were grouped into those from HLA-B*57:03-positive and B*57:03-negative
853 donors. A summary of all HIV-1 polymorphisms is shown for HXB2 region 162-173.

854

855 **Table III Summary of PhyloDOR of B*57:03-associated mutations located in HIV-1 p24 *gag***
856 **between Barbados [BB] and Botswana [BW] cohorts.**

857 Proviral sequences from Barbados (n=125) and Botswana (n=322) were analysed from HLA-
858 B*57:03-positive and -B*57:03-negative individuals for the presence of B*57:03-associated viral
859 mutations located in known viral epitopes in p24 *gag*. HXB2 position of mutations is shown. p
860 values were calculated from phylogenetically-corrected Odds ratios (PhyloDOR) using an online
861 tool (35).

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870 Table I
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 872

Protein	HLA	Polymorphism location	HXB2	p value	^{Q72} q value
p17	B*5703	A S V L S G G E L D R W E K I R L R P G G	15	0.00211	0.115
	B*5703	Q P S L Q T G S E E L K S L Y N T V A T L	75	0.00388	0.14
p24	B*5703	N L Q G Q M V H Q A T S P R T L N A W V K	147	0.00544	0.169
	B*5703	A F S P E V I P M F S A L S E G A T P Q D	173	0.000301	0.0218
	B*5703	R G S D I A G T T S T L Q E Q I G W M T N	242	3.02E-11	6.56E-09

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888 Table II
889

HXB2 position 162-173	890			
	B*5703 positive (n=15)		B*5703 negative (n=110)	
	n	%	n	%
K A F S P E V I P M F S	1	7	77	70
- - - - - - - - - T	7	47	25	23
- G - N - - - - - - -	0	0	1	1
- S - - - - I - - - - -	1	7	0	0
- S - - - - - - - - -	1	7	1	1
- - - K - - - - - - - -	1	7	0	0
- - - N - - - - - - - -	0	0	2	2
- - - - - I - - - - - -	0	0	1	1
- N - - - - - - - - - T	0	0	1	1
- S - - - - - - - - - T	1	7	0	0
- - - - - I - - - - - T	1	7	0	0
- - - N - - - - - - - I	1	7	0	0
- - - - - - - - - - I	1	7	0	0
- - - - - - - - - - A	0	0	1	1
- G - N - - - - - - - A	0	0	1	1

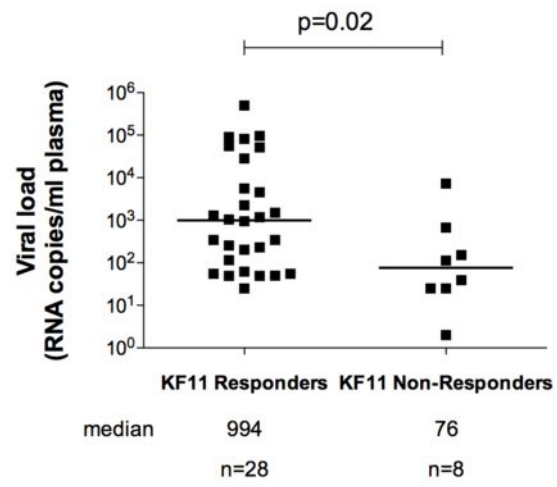
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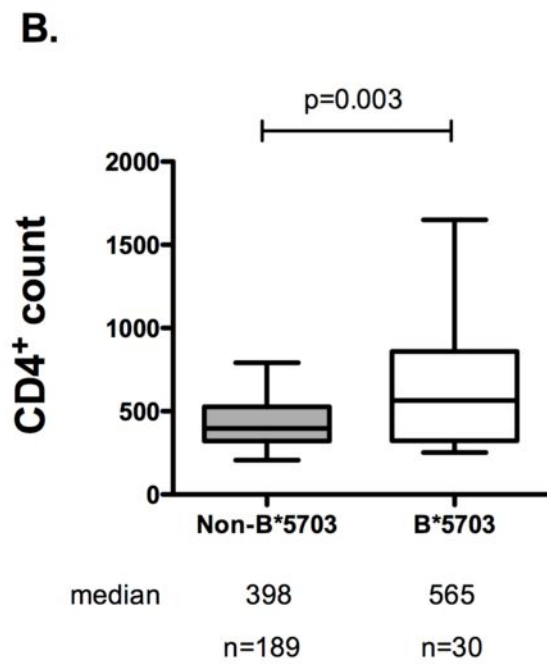
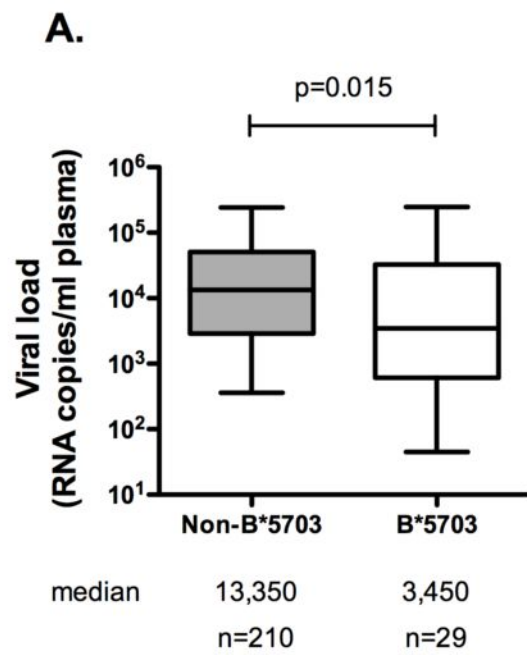
918 Table III

X (HXB2)	Country	**Log(PhyloDOR)	Odds ratio		
			p-value	Y/N	p-value
242N	Botswana	4.9	3.26E-07		
	Barbados	11.67	6.15E-14	N	0.815
147L	Botswana	10.5	4.99E-05		
	Barbados	13.26	4.00E-03	N	0.2937
163G	Botswana	13.22	0.0003		
	Barbados	9.343	0.2006	Y	0.0067
165N	Botswana	4.213	0.0025		
	Barbados	11.88	0.0887	N	0.0818
173T	Botswana	-2.798	0.0062		
	Barbados	13.52	0.0033	Y	0.0006

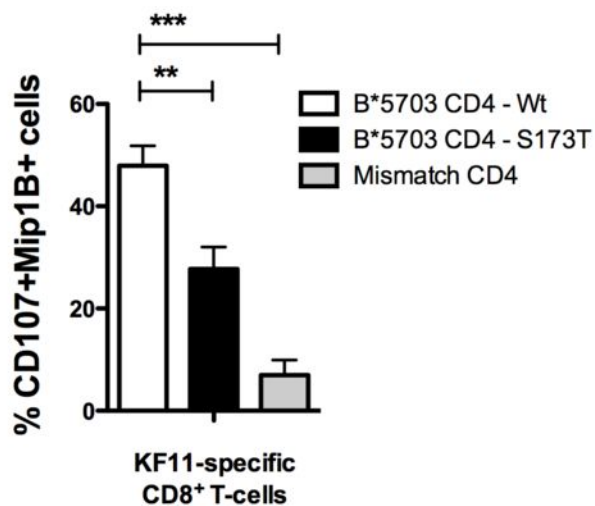
*Is B*5703's effect on X different in Botswana versus Barbados?

**Phylogenetically-corrected Odds ratio

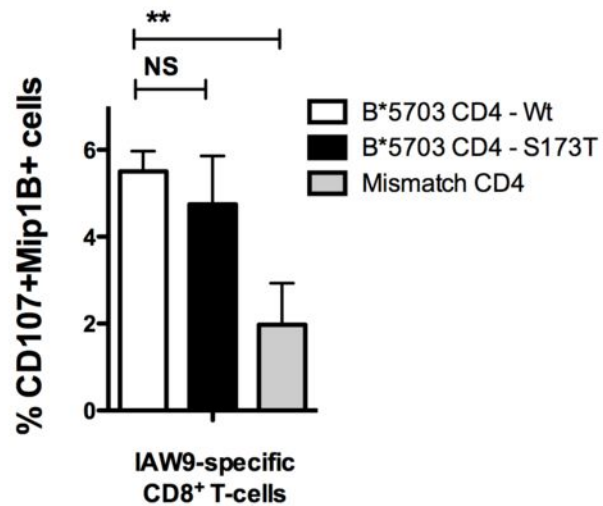




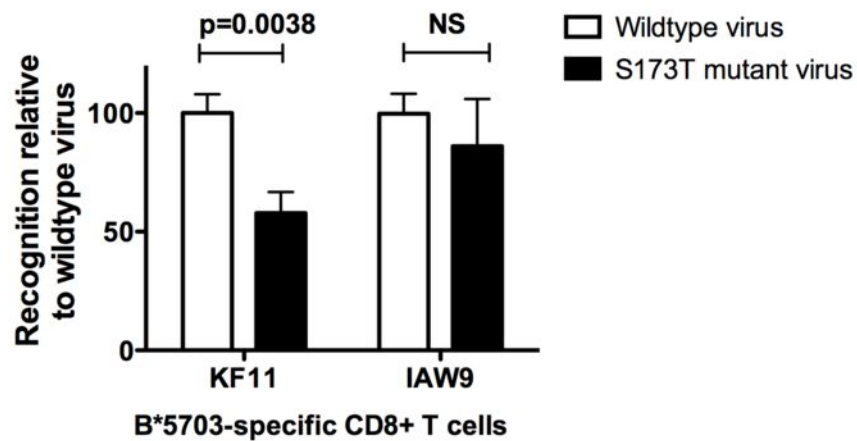
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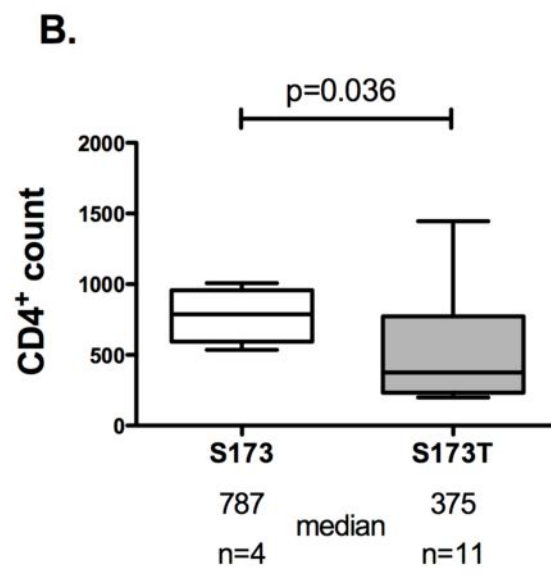
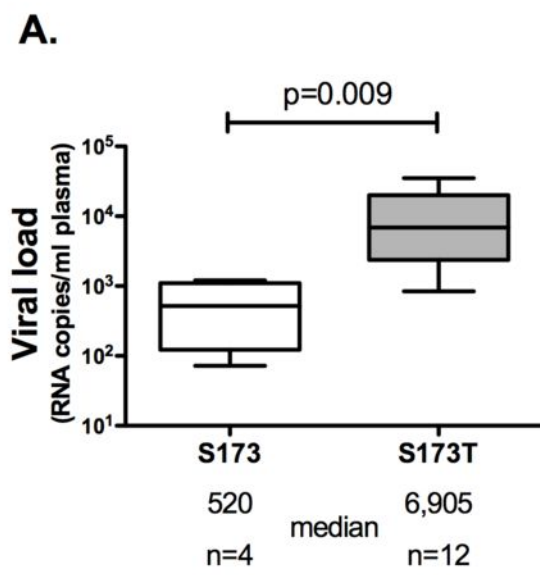


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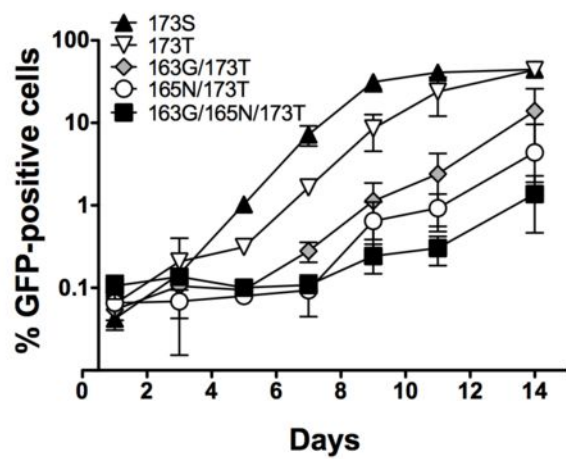


C.





A.



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