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2 **Positive selection in CD8+ T-cell epitopes of influenza nucleoprotein revealed by a**
3 **comparative analysis of human and swine viral lineages**

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5 **Running title: Positive selection on influenza CD8+ T-cell epitopes**

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25 **ABSTRACT**

26 Numerous experimental studies have demonstrated that CD8⁺ T-cells contribute to
27 immunity against influenza by limiting viral replication. It is therefore surprising that
28 rigorous statistical tests have failed to find evidence of positive selection in the epitopes
29 targeted by CD8⁺ T-cells. Here we use a novel computational approach to test for
30 selection in CD8⁺ T-cell epitopes. We define all epitopes in the nucleoprotein (NP) and
31 matrix protein (M1) with experimentally identified human CD8⁺ T-cell responses, and
32 then compare the evolution of these epitopes in parallel lineages of human and swine
33 influenza that have been diverging since roughly 1918. We find a significant enrichment
34 of substitutions that alter human CD8⁺ T-cell epitopes in the NP of human versus swine
35 influenza, consistent with the idea that these epitopes are under positive selection.
36 Furthermore, we show that epitope-altering substitutions to human influenza NP are
37 enriched on the trunk versus the branches of the phylogenetic tree, indicating that viruses
38 that acquire these mutations have a selective advantage. However, even in human
39 influenza NP, sites in T-cell epitopes evolve more slowly than non-epitope sites,
40 presumably because these epitopes are under higher inherent functional constraint.
41 Overall, our work demonstrates that there is clear selection from CD8⁺ T-cells in human
42 influenza NP, and illustrates how comparative analyses of viral lineages from different
43 hosts can identify positive selection that is otherwise obscured by strong functional
44 constraint.

45

46

47 **IMPORTANCE**

48 There is a strong interest in correlates of anti-influenza immunity that are protective
49 against diverse viral strains. CD8+ T-cells provide such broad immunity, since they target
50 conserved viral proteins. An important question is whether T-cell immunity is sufficiently
51 strong to drive influenza evolution. Although many studies have shown that T-cells limit
52 viral replication in animal models and are associated with decreased symptoms in
53 humans, no studies have proven with statistical significance that influenza evolves under
54 positive selection to escape T-cells. Here we use comparisons of human and swine
55 influenza to rigorously demonstrate that human influenza evolves under pressure to fix
56 mutations in nucleoprotein that promote escape from T-cells. We further show that
57 viruses with these mutations have a selective advantage since they are preferentially
58 located on the “trunk” of the phylogenetic tree. Overall, our results show that CD8+ T-
59 cells targeting nucleoprotein play an important role in shaping influenza evolution.

60 INTRODUCTION

61 Both arms of the adaptive immune system help control influenza replication:
62 antibodies neutralize virus [1] and direct the clearance of infected cells [2], while CD8+ T-
63 cells kill infected cells that display viral peptides on their MHC class I molecules [3], [4].
64 While antibodies against the viral surface protein hemagglutinin (HA) provide the most
65 potent protection when they are well matched to the viral strain [5]–[7], T-cells offer
66 broader protection against diverse strains since they tend to recognize epitopes in more
67 conserved internal viral proteins such as nucleoprotein (NP) and matrix protein (M1) [3],
68 [4], [8], [9].

69 Studies in both mice [10]–[14] and humans [9], [15], [16] have shown that pre-
70 existing influenza-specific CD8+ T-cells reduce the severity of disease and enhance viral
71 clearance. For instance, pre-existing virus-specific CD8+ T-cells correlated with
72 decreased symptoms in humans infected during the 2009 H1N1 pandemic [15]. Similarly,
73 T-cells specific for NP were associated with decreased incidence of symptomatic
74 infection over a multi-year study of a large human cohort [9], and CD8 T-cell responses
75 correlate with recovery from severe H7N9 infection [16]. Therefore, experimental and
76 epidemiological work demonstrates that CD8+ T-cells contribute to immunity against
77 influenza.

78 Because humans are repeatedly infected with influenza over their lifetimes, one
79 might expect viruses to be under evolutionary pressure to accumulate substitutions in
80 epitopes targeted by immune memory. Indeed, there are numerous examples of the
81 fixation of antibody-escape mutations in HA [17], [18], consistent with the notion that this
82 protein evolves under strong selection from antibodies. Several studies have also
83 described influenza mutations that escape recognition by CD8+ T-cells [19]. In a mouse

84 study, viral mutations arose that conferred T-cell escape in RAG-1 deficient mice
85 expressing an influenza NP specific TCR [20]. Rimmelzwaan and coworkers identified
86 the fixation of mutations in the NP of human H3N2 that mediated escape from CD8+ T-
87 cells by altering the epitope recognized by the T-cell receptor [21]–[23] or abrogating
88 binding of the epitope to MHC class I [24]. Valkenburg *et al* described the emergence of
89 CD8+ T-cell escape mutations in a persistently influenza-infected infant [25]. These
90 elegant studies demonstrate that influenza accumulates substitutions that escape CD8+
91 T-cells as well as antibody-mediated immunity.

92 But these studies do not prove that positive selection for CD8+ T-cell escape is an
93 important driving force in influenza's evolution, since many sites in the viral genome will
94 fix substitutions given enough time [26]–[28]. To rigorously establish the presence of
95 positive selection, the field of molecular evolution has developed statistical tests to
96 discern whether a subset of sites is evolving faster than expected. Most of these tests
97 compute nonsynonymous and synonymous distances (referred to as dN and dS,
98 respectively), and then test for sites with statistical evidence that the accumulation of
99 nonsynonymous substitutions exceeds that of synonymous substitutions ($dN/dS > 1$) [29],
100 [30]. These tests consistently find overwhelming evidence for positive selection in the
101 antigenic sites of influenza hemagglutinin [31]–[33], but little evidence for positive
102 selection in CD8+ T-cell epitopes [33]. One study did report that CD8+ T-cell epitopes in
103 NP have a greater dN/dS than other sites [34]; however this study only made a pairwise
104 comparison of two sequences and included no tests for statistical significance. Below, we
105 have used several state-of-the-art tests to verify that CD8+ T-cell epitopes have neither
106 an elevated frequency of sites with $dN/dS > 1$ nor an elevated rate of nonsynonymous

107 substitutions. Therefore, by standard criteria, CD8+ T-cell epitopes are not under positive
108 selection.

109 The results of these statistical tests for positive selection seem at odds with the
110 extensive body of experimental work described above. We hypothesized that the
111 discrepancy arises because known CD8+ T-cell epitopes are under strong functional
112 constraint [34]–[37]. If epitopes are highly constrained, then even strong positive
113 selection might fail to elevate the rate of nonsynonymous substitutions in epitopes above
114 that at less constrained non-epitope sites. To address this possibility, we developed new
115 statistical tests that take advantage of the fact that some lineages of human influenza are
116 paralleled by lineages of swine influenza that are not under selection from human CD8+
117 T-cells. Using these tests, we show that CD8+ T-cell epitopes in NP evolve significantly
118 faster in human influenza than in swine influenza. Furthermore, we show that
119 substitutions in these epitopes are enriched on the trunk of the phylogenetic tree,
120 indicating that viruses that acquire them have a selective advantage that promotes their
121 evolutionary spread. Overall, our work provides clear statistical evidence that
122 complements prior experimental studies showing that CD8+ T-cell epitopes are under
123 selection in human influenza [22], and suggests that the failure of conventional tests to
124 identify this selection is due to high levels of functional constraint in epitopes.

125 **METHODS AND MATERIALS**

126 **Inference of phylogenetic trees and mutation counts.**

127 M1 and NP protein-coding sequences were downloaded from the Influenza Virus
128 Resource (<http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html>) [38].

129 For human influenza, we assembled sequence sets by taking sequences for H1N1
130 (1918 to 1957), H2N2 (1957 to 1968), and H3N2 (1968 to 2013) – if there were less than
131 three sequences per year then we retained them all; when there were more than three for
132 a year then we randomly selected three to retain. For swine influenza, we similarly
133 assembled sequence sets containing up to 3 sequences per subtype per year for H1N1
134 (1918 to 2013), H1N2 (1999 to 2013), and H3N2 (1998 to 2013). For swine influenza, the
135 first available sequence is from 1933. We excluded sequences previously classified as
136 misannotated [39] or that were strong outliers based on a molecular clock analysis using
137 RAxML [40] and Path-O-Gen (<http://tree.bio.ed.ac.uk/software/pathogen/>). The sequence
138 sets are in Supplemental Files 1-4.

139 There are gaps in sequence availability in earlier years (most prominently, there
140 are no sequences from between 1918 and the early 1930s). Therefore, we have reduced
141 power to identify substitutions in these early years. However, since our comparisons are
142 between human and swine influenza, and since both lineages have similarly sparse
143 sequences in these early years, these gaps seem unlikely to systematically bias our
144 study, although they may reduce its power.

145 We translated the sequences and inferred separate human and swine influenza
146 phylogenies for each protein using Bayesian Evolutionary Analysis by Sampling Trees
147 (BEAST) [41] with a strict molecular clock, a Jones-Taylor-Thornton (JTT) [42] model of
148 substitution, and a constant population size demographic model. **Fig. 1** shows the

149 maximum clade credibility trees rendered with FigTree
150 (<http://tree.bio.ed.ac.uk/software/figtree/>). The trunk of each tree (dark lines in **Fig. 1**) was
151 defined by tracing from the most recent sequence back to the oldest sequence. We used
152 a stochastic mapping technique [43]–[45] implemented via the “MarkovJumps” feature in
153 BEAST to estimate the posterior mean number of substitutions at each site for each
154 phylogenetic tree and along the trunk of each tree. The times to most recent common
155 ancestor referred to in the **Fig. 1** legend were estimated by a BEAST analysis of the joint
156 swine and human influenza lineages. The dates of fixation of the CD8+ T-cell escape
157 substitutions characterized by Rimmelzwaan and colleagues [21]–[24] refer to estimates
158 obtained from Figure 2 - Supplement 2 of [37]. Supplemental File 9 lists all substitutions
159 that are present along the trunk of at least 90% of the trees sampled from the posterior
160 for each viral protein and lineage, along with the posterior-mean estimate of the date at
161 which the substitution fixed on the trunk.

162

163 **Identification of CD8+ T-cell epitope sites.**

164 We downloaded all epitopes with an experimentally identified CD8+ T-cell response
165 (source organism *Influenza A virus* and host *Homo sapiens*) from the Immune Epitope
166 Database (<http://www.iedb.org>) [46]. We identified unique epitopes as described in the
167 RESULTS section using a previously described software package
168 (<https://github.com/jbloom/epitopefinder/>) [47], and then determined the number of unique
169 epitopes (E_r) to which each site r contributes (see **Fig. 2**). The epitopes and the counts of
170 epitopes per site are in Supplemental Files 5 to 8.

171

172 **Conventional dN/dS tests for positive selection.**

173 We used DataMonkey (<http://www.datamonkey.org/>) [48] to perform two types of dN/dS
174 analyses, the hierarchical Bayes method FUBAR (Fast Unconstrained Bayesian
175 AppRoximation) [30] and the maximum-likelihood method FEL (Fixed Effects Likelihood)
176 [29]. The maximum clade credibility tree from BEAST was used as the input phylogeny
177 for FUBAR and FEL, and a REV (general reversible model) codon substitution model was
178 specified for FEL. For both methods, we calculated the percentage of sites for which the
179 estimated dN/dS ratio was greater than one, and the percentage of sites for which there
180 was strong statistical support for this ratio being greater than one (posterior probability >
181 0.95 for FUBAR; P-value < 0.05 for FEL).

182

183 **Statistics on substitutions at each site in human and swine influenza lineages.**

184 The posterior mean estimate of the number of nonsynonymous substitutions S_r at each
185 site r was extracted from the BEAST trees. These estimates were used to compute the
186 average substitution rates across all epitope sites (sites that fell in at least one epitope)
187 and across all non-epitope sites, both for the entire tree and for the trunk alone. We also
188 defined a statistic, F , which represents the average number of epitopes changed per
189 substitution. This statistic is defined as

$$190 \quad F = \frac{\sum_r E_r \times S_r}{\sum_r S_r}$$

191 where E_r is the number of unique epitopes to which site r contributes.

192 We performed statistical tests of whether we could reject the null hypothesis that
193 there was no difference between the F statistics for human versus swine and for the trunk
194 versus the tree. To do this, we calculated the ratio of these statistics for human versus

195 swine or trunk versus tree, and then created a null distribution by repeatedly recalculating
196 the statistics after randomizing the epitope counts E_r among sites. The P-values
197 represent the fraction of time the randomized statistic is greater than the actual statistic in
198 10^4 randomizations.

199

200 **Availability of data and computer code.**

201 Data and computer code are available at

202 <https://github.com/hmmachko/TcellEpitopeComparisons>

203 RESULTS

204 Parallel human and swine influenza lineages can reveal selection by CD8+ T-cells.

205 Our goal is to determine whether epitopes targeted by human CD8+ T-cells are
206 under selection in influenza viruses that circulate in human hosts. The two most highly
207 expressed influenza proteins are NP and M1 [49], and epitopes in these proteins are
208 major targets of CD8+ T-cells [3], [4], [8], [9]. The NP and M1 in contemporary human
209 H3N2 influenza have circulated in humans since at least 1918 [50], [51]. For both genes,
210 this unbroken lineage consists of H1N1 viruses from 1918 to 1957, H2N2 viruses from
211 1957 to 1968, and H3N2 viruses from 1968 to the present. The red lines in **Fig. 1** show
212 phylogenetic trees of NP and M1 from this human influenza lineage.

213 This human influenza lineage is closely paralleled by a swine influenza lineage
214 descended from the common ancestor of the virus that caused concurrent pandemics in
215 humans and swine in 1918 [50], [52]. NP and M1 of this lineage have circulated
216 exclusively in swine since 1918 [50], [52]. The blue lines in **Fig. 1** show phylogenetic
217 trees of NP and M1 from this swine influenza lineage. The phylogenetic trees show that
218 both human and swine influenza undergo substantial genetic evolution in NP and M1;
219 however, this fact alone does not reveal what forces drive this evolution. Influenza
220 genetic evolution can be driven by positive selection, but it can also be driven by
221 stochastic forces such as genetic hitchhiking or drift [26]–[28].

222 The parallel lineages of human and swine influenza enable us to perform an
223 internally controlled analysis of whether CD8+ T-cells represent an important selective
224 force in driving influenza evolution, since human CD8+ T-cells target epitopes in human
225 but not swine influenza. There are two reasons that we can be confident that swine
226 influenza is not under selection from human CD8+ T-cells. First, the MHC class I

227 molecules that restrict CD8+ T-cell epitopes are highly variable among species; therefore
228 epitopes displayed to human CD8+ T-cells will differ from those displayed to swine CD8+
229 T-cells [53], [54] (note that our approach does not require the human and swine epitopes
230 to be completely non-overlapping; it simply assumes that the MHC alleles are sufficiently
231 diverged that not all epitopes targeted by humans are also targeted by swine). Second,
232 swine influenza is under weaker selection from immune memory than human influenza
233 because pigs are infected only once or a few times during their short lives [55]–[59].
234 Therefore, swine influenza is probably under less pressure from CD8+ T-cells in general,
235 and whatever pressure does exist will generally focus on different epitopes than those
236 targeted by human T-cells.

237

238 **Experimentally identified human CD8+ T-cell epitopes.**

239 We aimed to identify CD8+ T-cell epitopes targeted by individuals in the human
240 population. There are two plausible ways to do this: computationally predict peptides that
241 bind to MHC class I, or collate epitopes that have been experimentally identified as
242 eliciting responses from CD8+ T-cells isolated from humans. We chose to use
243 experimentally identified epitopes since computational predictions are imperfect [60], and
244 only a fraction of peptides that bind MHC class I are targets of cytolytic CD8+ T-cells [61],
245 [62]. We extracted all influenza epitopes from the Immune Epitope Database [46]
246 between 8 and 12 amino acids in length with an experimentally identified human CD8+ T-
247 cell response. We retained all epitopes that aligned to at least one strain from our human
248 and swine influenza lineages with no more than one amino-acid mismatch. We classified
249 epitopes as redundant if they shared 8 or more amino acids and were in the same MHC
250 class I group [63] (or supertype [64] if the group was not specified). We identified 133

251 unique epitopes in the seven proteins that did not reassort in the human influenza
252 pandemics of 1957 or 1968 (NS1, NS2, PB2, PA, M1, M2, and NP). Of the 133 epitopes,
253 62 were in NP (47%) and 29 were in M1 (22%), consistent with reports that these two
254 proteins are major targets of CD8⁺ T-cells [9]. **Fig. 2** shows the number of epitopes to
255 which each site in NP and M1 contributes; individual sites are involved in anywhere
256 between zero and nine epitopes.

257 These experimentally identified epitopes probably do not represent an exhaustive
258 list of all sites targeted by human T-cells. In particular, some epitopes in historical strains
259 may be overlooked since most studies use recent viral strains. However, since our
260 analyses are internally controlled (we compare either human to swine influenza, or the
261 trunk of the tree to side branches), missing some epitopes should not systematically bias
262 our results.

263 Our approach identifies sites that contribute to epitopes in any of the influenza
264 strains under consideration. Mutations to an epitope can mediate escape by abrogating
265 peptide binding to MHC class I or by changing the sequence of the bound peptide such
266 that it is no longer recognized by memory T-cells. Both types of escape have been
267 experimentally demonstrated in the NP of human H3N2. An example of escape by
268 abrogation of MHC-binding is the R384G mutation that fixed in 1993 [24]. Three
269 examples of escaping T-cell recognition but not MHC-binding are the D421E/I425V
270 mutations that fixed in 1979 [22], [23], the K103R mutation that fixed in 1980 [21], and the
271 S259L mutation that fixed in 1990 [21]. Additionally, mutations outside an epitope can
272 affect its processing [65], although we are unaware of documented examples of extra-
273 epitopic escape mutations that have fixed in human influenza. Our analysis cannot
274 distinguish among these types of escape, since most experiments identify epitopes

275 without characterizing how prior or subsequent mutations affect their processing, MHC
276 binding, and recognition by T-cells.

277 We therefore classify sites according to the number of epitopes to which they
278 contribute in *any* of our viral strains without attempting to determine whether the epitopes
279 are present across all the viral strains. This approach is usually valid when mutations
280 alter T-cell recognition without affecting processing or MHC binding, since epitopes that
281 escape existing T-cells via such mutations will often soon be targeted by new T-cells [21].
282 But our approach is imperfect for mutations that abrogate binding to MHC and so
283 eliminate the epitope from all subsequent strains. However, since the NP and M1
284 homologs are closely related (the maximal protein-sequence divergence among the
285 sequences in **Fig. 1** is just 12%), even if a site fixes just a single mutation that eliminates
286 an epitope, this would represent a substantial elevation in its rate of evolution over the
287 timeframe of interest. Therefore, classifying sites by the number of epitopes as in **Fig. 2**
288 should identify positions in NP and M1 that will exhibit an increased rate of substitution if
289 there is T-cell selection.

290

291 **Conventional dN/dS tests fail to find positive selection in CD8+ T-cell epitopes.**

292 One might hypothesize that immune selection from CD8+ T-cells would lead to a greater
293 proportion of epitope than non-epitope sites with dN/dS > 1. We therefore used two state-
294 of-the-art methods (a hierarchical Bayes approach [29] and a maximum-likelihood
295 approach [30]) to identify sites in human and swine influenza with dN/dS > 1, and
296 partitioned the sites based on whether or not they were involved in at least one
297 experimentally identified epitope. As shown in **Fig. 3A,B**, the proportion of sites with
298 dN/dS > 1 was actually lower for epitope than non-epitope sites in human influenza.

299 Additionally, we calculated the proportion of sites with strong statistical evidence of dN/dS
300 >1 (**Fig. 3C,D**). In no instance is there a greater proportion of epitope versus non-epitope
301 sites with dN/dS >1. Our findings are consistent with previous work that has failed to find
302 evidence for positively selected sites in NP or M1 [33]. So overall, state-of-the-art dN/dS
303 tests fail to identify enhanced positive selection in T-cell epitopes in NP.

304
305 **Epitope sites do not evolve faster than non-epitope sites, although the rate is**
306 **higher on the “trunk” of the human influenza NP lineage.**

307 We next estimated the number of nonsynonymous substitutions at each site in NP and
308 M1 for both the full swine and influenza trees in **Fig. 1**, and for the “trunks” of these trees
309 (dark lines in **Fig. 1**). The rationale for examining the trunk separately is that we expect
310 beneficial substitutions to be enriched on the trunk since they will confer a selective
311 advantage that favors the propagation of sequences that contain them. Consistent with
312 this idea, studies of human H3N2 influenza HA have found that presumably beneficial
313 substitutions that alter antigenicity are enriched on the trunk versus the entire tree [66],
314 [67].

315 **Fig. 4** shows the ratio of the substitution rate at epitope versus non-epitope sites.
316 In none of the cases (NP or M1, swine or human influenza, trunk or tree) is the ratio
317 substantially greater than one when taken over the whole tree – so in no case are the
318 epitope sites evolving faster than the non-epitope ones. This result helps explain why
319 the dN/dS tests fail to find evidence for positive selection in the epitopes – for NP and M1
320 (unlike for HA), epitope sites simply don't evolve faster than their non-epitope
321 counterparts.

322 However, further examination of **Fig. 4** reveals an interesting trend. Although there
323 are no instances where epitope sites are evolving substantially faster than non-epitope
324 ones, the ratio of epitope to non-epitope substitution rate is greater for the NP of human
325 influenza than for the NP of swine influenza. This trend is particularly pronounced along
326 the trunk of the tree, which is exactly where we would expect to see the largest increase
327 in rate if epitope substitutions confer a selective advantage. To test if this trend was
328 indicative of statistically significant CD8+ T-cell selection in NP, we undertook the more
329 nuanced analysis in the next section.

330

331 **Substitutions alter more NP epitopes in human than swine influenza, especially on**
332 **the trunk.**

333 The foregoing analyses simply subdivided sites as epitope or non-epitope based on
334 whether they fall in at least one experimentally identified epitope. But as **Fig. 2** shows,
335 some sites are involved in far more unique epitopes than others. To account for this, we
336 defined a new statistic (which we denote as F) that gives the average number of unique
337 epitopes that are altered per substitution. **Fig. 5A** shows this F statistic for the trunk and
338 entire tree for NP and M1 for both human and swine influenza. There appears to be little
339 difference in this F statistic for M1 between the trunk and the entire tree, and between
340 human and swine influenza. But for NP, F is greater for human influenza than swine
341 influenza, with the increase much larger for the trunk than for the entire tree. This result
342 indicates that the average substitution alters more NP epitopes in human influenza than
343 swine influenza, and that this trend is especially pronounced on the trunk of the tree –
344 exactly as we would expect under selection for epitope-altering substitutions.

345 To test if the trend is statistically significant, we computed the ratio of F for human
346 versus swine influenza, and then calculated a null distribution by repeatedly randomizing
347 the epitopes among sites. For M1, the actual ratio falls near the center of the null
348 distribution (**Fig. 5B**), confirming that there is no enhancement of the rate of fixation of
349 epitope-altering substitutions in the M1 of human influenza. But for NP, the actual ratio
350 falls near the top the null distribution for both the trunk and the tree (**Fig. 5B**). In
351 particular, for the trunk there is strong statistical support ($P = 0.0002$) for rejecting the null
352 hypothesis that epitope-altering substitutions are equally likely to fix in human and swine
353 influenza. This result indicates that there is selection for substitutions that alter CD8+ T-
354 cell epitopes in the NP of human influenza.

355 To corroborate this statistical finding of an enhanced rate of epitope-altering
356 substitutions along the trunk of human versus swine influenza, we undertook a further
357 sub-analysis of the small subset of epitopes for which specific T-cell escape mutations
358 have been identified. The vast majority of epitopes that comprise the analysis in **Fig. 5**
359 were identified by relatively high-throughput studies that characterize peptides eliciting T-
360 cell responses without identifying escape mutations. However, a series of meticulous
361 studies by Rimmelzwaan and coworkers [21], [22], [24] have identified the sites of
362 specific escape mutations for a small number of epitopes. **Table 1** shows that
363 substitutions are fixed at all of these sites along the trunk of human influenza, but that
364 swine influenza has not fixed substitutions at any of these sites. This finding lends further
365 support to the idea that human T-cells exert positive selection on human but not swine
366 influenza.

367 We next tested whether selection for epitope-altering substitutions in human
368 influenza NP was stronger on the trunk than the rest of the tree, as would be expected if

369 such substitutions confer a selective advantage. We computed the ratio of the F statistic
370 for the trunk versus the entire tree, and generated null distributions for this statistic by
371 randomizing the epitopes among sites (**Fig. 6**). For M1, the actual ratios are near the
372 center of the null distributions. But for NP, the actual ratio is near the top of the null
373 distribution ($P = 0.003$) for human influenza, and near the bottom for swine influenza ($P =$
374 0.01). This result demonstrates that epitope-altering substitutions to NP are significantly
375 enriched on the trunk of the human influenza lineage, suggesting viruses that fix these
376 mutations are more fit than other strains. The depletion of epitope-altering substitutions
377 on the swine lineage can also be given a clear explanation: If the known CD8+ T-cell
378 epitopes in NP are in functionally constrained regions of the protein (as has been
379 suggested by a variety of experimental studies [34]–[37]), then substitutions in these
380 epitopes will often be deleterious in swine influenza lineages that experience no human
381 CD8+ T-cell selection, and so will be relatively depleted on the trunk. So overall, the
382 results in this section provide strong statistical evidence of positive selection in the CD8+
383 T-cell epitopes of human influenza NP.

384

385 **DISCUSSION**

386 We describe the first rigorous statistical evidence that CD8+ T-cell epitopes are under
387 positive selection in human influenza. Our work adds to a growing body of evidence
388 suggesting an important role for T-cell immunity in shaping influenza evolution. Prior
389 studies have shown that T-cells help protect against human influenza [9], [15] and have
390 detailed specific instances of T-cell escape [21]–[25]. Our work shows that T-cell
391 selection increases the rate at which mutations fix in epitopes of NP, and indicates that
392 viruses with these substitutions have a selective advantage that makes them more likely
393 to fall along the trunk of the phylogenetic tree.

394 Our results also explain why conventional dN/dS tests fail to detect positive
395 selection in CD8+ T-cell epitopes. Known human CD8+ T-cell epitopes tend to be under
396 high functional constraint [34]–[37]. It is unclear whether this is because T-cells inherently
397 target conserved epitopes, because repeated infections preferentially boost T-cells that
398 target conserved epitopes, or because there is a bias towards experimentally identifying
399 conserved epitopes. But in any case, the fact that known epitopes are under high
400 constraint means that even fairly strong positive selection may not enhance the
401 nonsynonymous substitution rate to a level detectable by conventional dN/dS tests. This
402 contrasts with antibody epitopes in HA, where the ability of dN/dS tests to detect
403 antibody-mediated positive selection is probably augmented by the fact that antigenic
404 sites are disproportionately tolerant of point mutations [68].

405 The novel approach that we developed ameliorates this problem by comparing the
406 evolution of epitopes between human and swine influenza, or between the entire
407 phylogenetic tree and only its trunk. These comparisons should better control for site-to-
408 site variation in functional constraint, since the comparisons are always between

409 homologous sites that should be subject to similar functional constraints. Admittedly,
410 there may also be other differences in functional constraints between human and swine
411 influenza beyond T-cells, but unless these differential constraints are systematically
412 biased towards occurring at T-cell epitopes they should not alter the fundamental validity
413 of our approach. By making comparisons in this way, we demonstrated clear positive
414 selection in CD8+ T-cell epitopes in NP, both in human versus swine influenza and in the
415 trunk versus the entire phylogenetic tree.

416 One interesting aspect of our study is that we found positive selection in NP but
417 not M1. This finding is consistent with a recent large-scale study that found that NP was
418 the only protein for which the presence of pre-existing memory T-cells correlated with
419 decreased symptomatic infections [9]. However, our study does not preclude an
420 important role for T-cells against M1, which contains an immunodominant HLA-A2
421 epitope spanning residues 58-66 [69], [70]. One study has argued that T-cells targeting
422 this epitope are ineffectual [70], although this interpretation is disputed [71], [72]. But
423 experiments have also shown that this epitope is under high constraint [34]. If an epitope
424 is completely intolerant of mutations, it will of course be unable to accumulate
425 substitutions regardless of the strength of selection. It remains unclear if our failure to
426 detect positive selection in M1 reflects a lack of effective immunity targeting this protein
427 or strong constraints that simply prevent the fixation of escape mutations.

428 It is well established that antibodies are strong drivers of repeated selective
429 sweeps in the evolution of human influenza [66], [73]. The fact that we can detect positive
430 selection by CD8+ T-cells even in the presence of these antibody-driven selective
431 sweeps demonstrates the importance of T-cell immunity in driving viral evolution. The

432 existence of such selection is consistent with modeling studies showing that T-cell
433 immunity that reduces the infectious period can strongly favor viral escape [74].

434 There is considerable interest in developing vaccines that elicit stronger T-cell
435 immunity to better protect against diverse influenza strains [3]. Our demonstration of the
436 evolutionary importance of T-cell selection suggests that this interest is well founded. In
437 addition, our results suggest that attempts to forecast the seasonal evolution of influenza
438 [75], [76] could benefit from examining changes in T-cell as well as antibody epitopes.

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690

691 **Figure 1. Phylogenetic trees for human and swine influenza M1 and NP.**

692 Maximum clade credibility trees for NP and M1. The swine influenza lineage is in blue,
693 and the human influenza lineage is in red. The dark blue and red lines represent the
694 trunk. The dotted black lines indicate that human and swine influenza lineages share a
695 recent common ancestor (estimated times to most recent common ancestor are 13 and 6
696 years for M1 and NP, respectively).

697

698 **Figure 2. The distribution of CD8 T-cell epitopes in M1 and NP.**

699 The number of unique experimentally identified CD8+ T-cell epitopes to which each site
700 contributes for M1 and NP.

701

702 **Figure 3. Conventional dN/dS tests do not detect positive selection in T-cell**
703 **epitopes.**

704 State-of-the-art methods for detecting positive selection fail to find any enrichment in sites
705 with $dN/dS > 1$ at epitopes. Percentage of sites estimated to have $dN/dS > 1$ using (A)
706 the hierarchical Bayesian approach implemented in FUBAR and (B) the maximum-
707 likelihood approach implemented in FEL. (C) The percentage of sites for which FUBAR
708 reports a posterior probability > 0.95 that $dN/dS > 1$. (D) The percentage of sites for
709 which FEL reports $dN/dS > 1$ with $P < 0.05$.

710

711 **Figure 4. Epitope sites do not evolve faster than non-epitope sites.**

712 Shown are the ratios of the average nonsynonymous substitution rate of epitope sites
713 versus nonepitope sites. When the substitution rate is computed across the entire tree,
714 the epitope sites always have a lower substitution rate than the non-epitope sites.

715 However, along the trunk of the tree for NP from human influenza, the substitution rate in
716 epitopes is slightly greater than the substitution rate at non-epitopes.

717

718 **Figure 5. The average substitution changes more NP epitopes in human influenza**
719 **than swine influenza.**

720 The F statistic is the average number of epitopes altered per substitution. (A) The
721 average substitution alters a similar number of epitopes in M1 in both human and swine
722 influenza, and for both the entire tree and along the trunk alone. However, for NP the
723 average substitution alters substantially more epitopes in human than swine influenza,
724 particularly along the trunk of the tree. (B) The increased rate of epitope-altering
725 substitutions to NP along the trunk of human versus swine influenza is statistically
726 significant. The violin plots show the null distribution of the ratio of F for human to swine
727 influenza, with the median shown by the red lines and the actual value shown by the red
728 circles. The P-values (fraction of the null distribution \geq the actual value) are: M1 trunk
729 0.49, M1 tree 0.65, NP trunk 0.0002, NP tree 0.098.

730

731 **Figure 6. Epitope-altering substitutions to human influenza NP are enriched on the**
732 **trunk of the tree.**

733 The ratio of the average number of epitopes altered per substitution (F) for the trunk
734 versus the entire tree. The violin plots show the actual values versus the null distributions
735 as in **Fig. 5B**. There is no difference between the trunk and the entire tree for M1 (P-
736 values are: M1 human influenza: 0.59 and M1 swine influenza: 0.57). But for NP, epitope-
737 altering substitutions are significantly enriched on the trunk of the human influenza tree

738 relative to the rest of the tree ($P = 0.003$), and significantly depleted on the trunk of the
739 swine influenza lineage ($P = 0.01$ for depletion) relative to the rest of the tree.

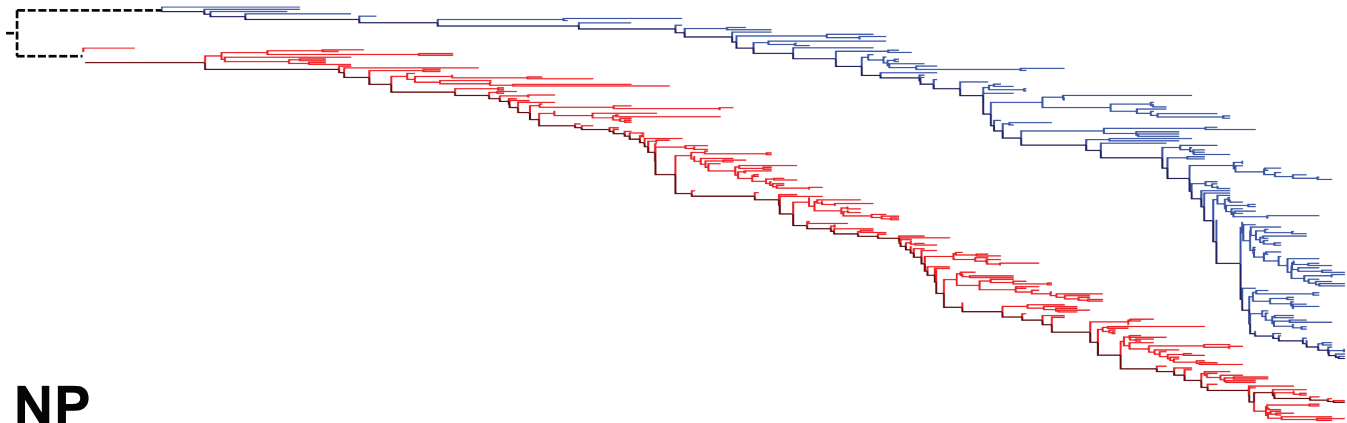
740

741 **Table 1. Number of trunk substitutions for the small subset of NP epitopes where**
742 **specific escape substitutions have been experimentally validated.**

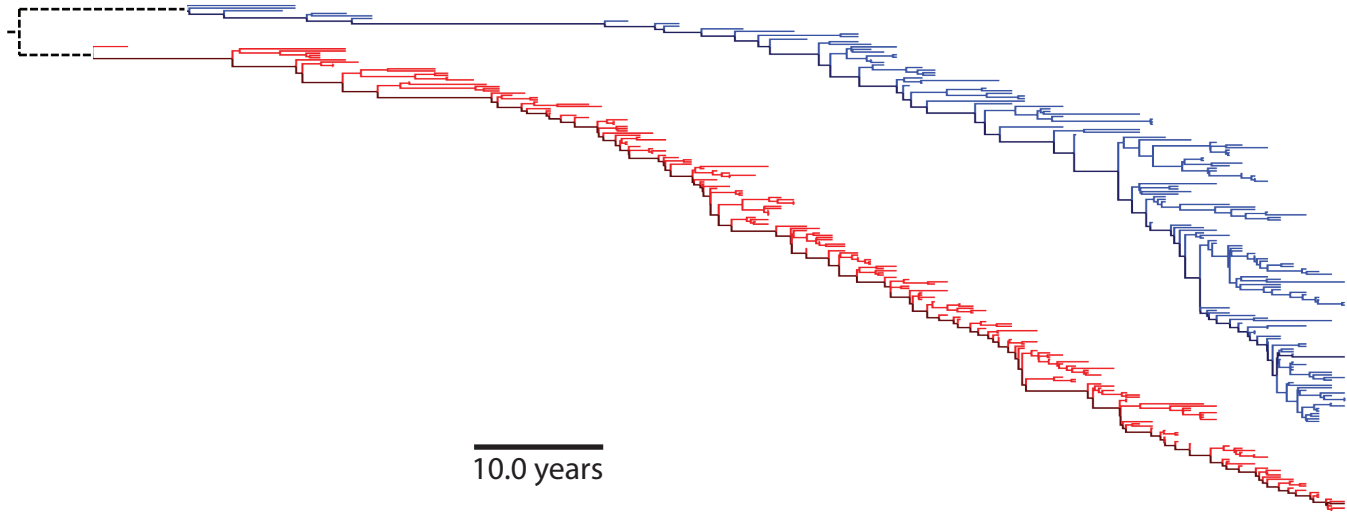
site in NP	Experimental evidence	Number of trunk substitutions for human influenza	Number of trunk substitution for swine influenza
103	K103R escapes recognition by a HLA-B*1503-restricted T-cell [21]	2 (K103R in 1981, R103K in 1997)	none
259	S259L escapes recognition by a HLA-B*4002-restricted T-cell [21]	2 (L259S in 1972, S259L in 1990)	none
384	R384G abrogates MHC binding in HLA-B*2705 [24]	1 (R384G in 1990)	none
421	D421E escapes recognition by a HLA-B*3501-restricted T-cell [22]	1 (D421E in 1977)	none
425	I425V escapes recognition by a HLA-B*3501-restricted T-cell [22]	2 (I425V in 1975, V425I in 1999)	none

743

M1

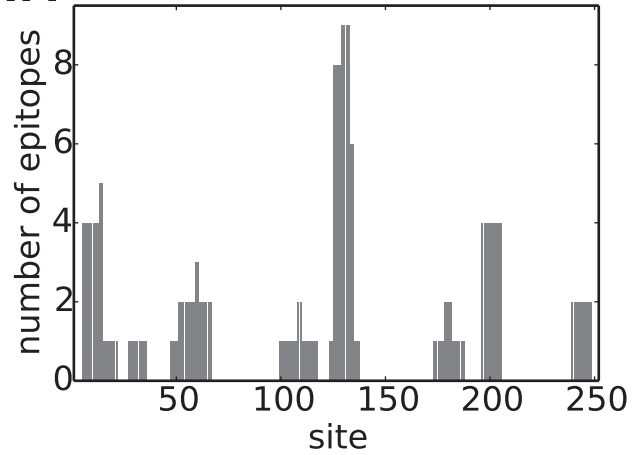


NP

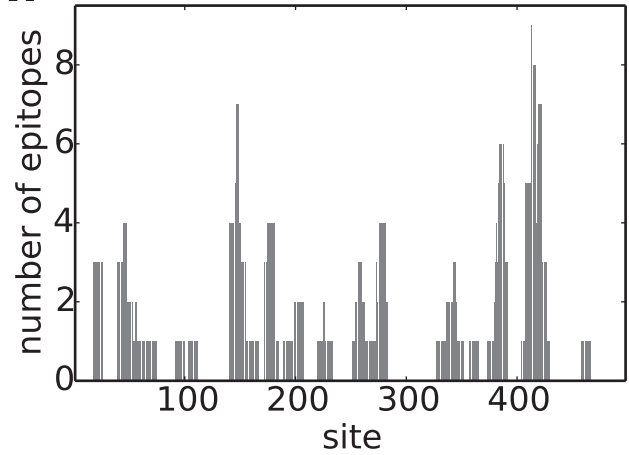


10.0 years

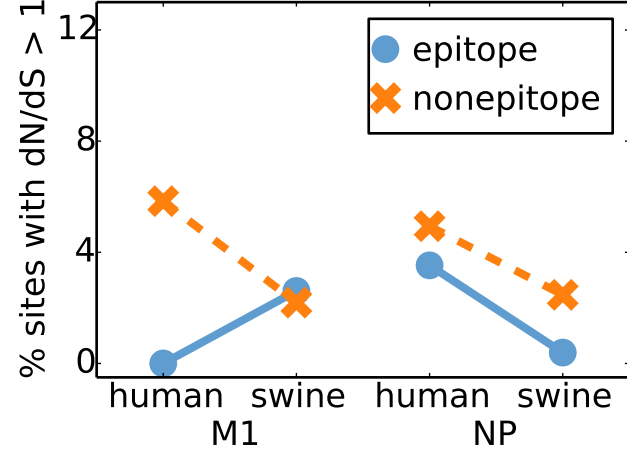
M1



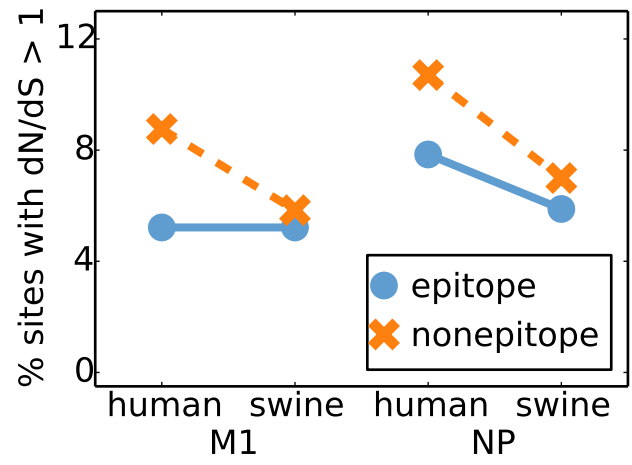
NP



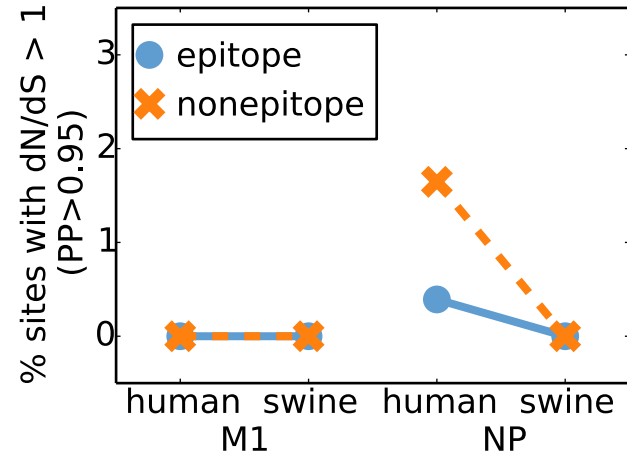
A



B



C



D

