1   Macaque-human differences in SARS-CoV-2 Spike antibody response elicited by vaccination or

2   infection

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4   Short title: Macaque vs. human antibodies to SARS-CoV-2 Spike

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Abstract

Macaques are a commonly used model for studying immunity to human viruses, including for studies of SARS-CoV-2 infection and vaccination. However, it is unknown whether macaque antibody responses recapitulate, and thus appropriately model, the response in humans. To answer this question, we employed a phage-based deep mutational scanning approach (Phage-DMS) to compare which linear epitopes are targeted on the SARS-CoV-2 Spike protein in humans and macaques following either vaccination or infection. We also used Phage-DMS to determine antibody escape pathways within each epitope, enabling a granular comparison of antibody binding specificities at the locus level. Overall, we identified some common epitope targets in both macaques and humans, including in the fusion peptide (FP) and stem helix-heptad repeat 2 (SH-H) regions. Differences between groups included a response to epitopes in the N-terminal domain (NTD) and C-terminal domain (CTD) in vaccinated humans but not vaccinated macaques, as well as recognition of a CTD epitope and epitopes flanking the FP in convalescent macaques but not convalescent humans. There was also considerable variability in the escape pathways among individuals within each group. Sera from convalescent macaques showed the least variability in escape overall and converged on a common response with vaccinated humans in the SH-H epitope region, suggesting highly similar antibodies were elicited. Collectively, these findings suggest that the antibody response to SARS-CoV-2 in macaques shares many features with humans, but with substantial differences in the recognition of certain epitopes and considerable individual variability in antibody escape profiles, suggesting a diverse repertoire of antibodies that can respond to major epitopes in both humans and macaques.

Author summary

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50   Non-human primates, including macaques, are considered the best animal model for studying

51   infectious diseases that infect humans. Vaccine candidates for SARS-CoV-2 are first tested in

52   macaques to assess immune responses prior to advancing to human trials, and macaques are

53   also used to model the human immune response to SARS-CoV-2 infection. However, there may

54   be differences in how macaque and human antibodies recognize the SARS-CoV-2 entry protein,

55   Spike. Here we characterized the locations on Spike that are recognized by antibodies from

56   vaccinated or infected macaques and humans. We also made mutations to the viral sequence

57   and assessed how these affected antibody binding, enabling a comparison of antibody binding

58   requirements between macaques and humans at a very precise level. We found that macaques

59   and humans share some responses, but also recognize distinct regions of Spike. We also found

60   that in general, antibodies from different individuals had unique responses to viral mutations,

61   regardless of species. These results will yield a better understanding of how macaque data can

62   be used to inform human immunity to SARS-CoV-2.

Introduction

64   The COVID-19 pandemic has created a pressing need to understand immunity to SARS-CoV-2,

65   both in the setting of vaccination and infection. This has prompted numerous studies in non-

66   human primates (NHPs), which are considered the most relevant animal model for studying

67   many infectious diseases of humans. Various NHP models have been employed to study the

68   immunogenicity and protective efficacy of SARS-CoV-2 vaccine candidates, with most studies

69   using macaque species including rhesus macaques (*Macaca mulatta*) [1-23], cynomolgus

70   macaques (*Macaca fascicularis*) [8, 24-32], and pigtail macaques (*Macaca nemestrina*) [22, 33-

71   35]. Some of these models have also been used to study infection and re-infection [35-39]. In

72   the NHP model, studies typically measure virus neutralizing antibody responses to vaccination

73   or infection. However, no study has investigated the fine binding specificities of both

74   neutralizing and non-neutralizing SARS-CoV-2 antibodies in macaques and how they compare to

75   the human responses they are meant to model.

76   Coronaviruses such as SARS-CoV-2 enter host cells using their Spike glycoprotein, which is

77   composed of trimeric S1 and S2 subunits. Receptor-binding S1 homotrimers protrude out from

78   the surface of the virion like a crown, giving this family of viruses its name, while the fusion-

79   mediating S2 trimers anchor the protein to the viral membrane. On S1, the receptor-binding

80   domain (RBD) of SARS-CoV-2 Spike protein binds to angiotensin-converting enzyme 2 (ACE2) on

81   host cells [40, 41]. For subsequent membrane fusion to occur, the Spike protein must be

82   cleaved by host cell proteases at the S1/S2 boundary and at an S2' site located just upstream of

83   the fusion peptide (FP) of S2 [42], leading to substantial conformational changes that likely

84   unmask new epitopes of S2 to immune cells [43].

85   Antibodies to SARS-CoV-2 Spike protein are especially interesting as a potential correlate of

86   protection, as they have the capacity to block infection and kill infected cells [44-47]. There has

87   understandably been great interest in studying neutralizing antibodies against the RBD, given

88   that such antibodies can directly block interaction with host cells. While RBD-directed

89   antibodies indeed contribute disproportionately to neutralization [48], the majority of the anti-

90   Spike plasma IgG response in convalescent individuals is directed to epitopes outside of the

91   RBD [49, 50]. RBD-directed antibodies are also less likely to maintain activity against future viral

92   strains, given the increasing number of variants of concern that harbor mutations in the RBD

93   and have reduced sensitivity to neutralization by immune plasma [51]. Additionally, growing

94   evidence from studies in humans and animal models indicates that non-neutralizing antibodies

95   play a role in protection [52-57].

96   Previous studies have used Phage-DMS [58], a tool that combines phage display of linear

97   epitopes with deep mutational scanning, to interrogate the fine binding specificities and escape

98   profiles of binding antibodies against all domains of Spike in infected and vaccinated humans

99   [59, 60]. These studies have shown that infection-induced human polyclonal antibodies

100   consistently bind linear epitopes in the FP and stem helix-heptad repeat 2 (SH-H) epitope

101   regions, with patient-to-patient variability in escape profiles [59]. Comparatively, mRNA

102   vaccination induces a broader antibody response across Spike protein with more consistent

103   escape profiles [60].

104   In this study, we built on this foundation by using Phage-DMS to study the binding and escape

105   profiles of antibodies in vaccinated and convalescent macaques in comparison to humans. Our

106   data reveal broad overlap in some major epitopes targeted by both macaques and humans,

107   though neither vaccinated nor convalescent macaques perfectly model the human response.

108   We also find considerable variability in individuals' antibody escape pathways in most epitope

109   regions in both macaques and humans. The broadest responses were seen in vaccinated

110   humans and re-infected rhesus macaques, groups that also share more concordant escape

111   profiles. These results have implications for the interpretation of COVID-19 macaque research

112   studies.

Results

114   Four groups were included in this study: vaccinated pigtail macaques, vaccinated humans,

115   convalescent (re-infected) rhesus macaques, and convalescent humans (Table 1). The

116   vaccinated macaques received a replicating mRNA (repRNA) vaccine encoding the full-length

117   wildtype (not pre-fusion stabilized) SARS-CoV-2 A.1 lineage Spike protein formulated with a

118   cationic nanocarrier [35, 61]. The vaccine was delivered as a prime-only 25ug (n=3) or 250ug

119   (n=6) dose or prime-boost 50ug dose (n=2), with plasma collected 42 days after the first dose

120   (n=9) or 14 days after the second dose (n=2). The vaccinated humans received two doses of the

121   100ug Moderna mRNA-1273 vaccine encoding the pre-fusion stabilized full-length SARS-CoV-2

122   A.1 lineage Spike protein and formulated with a lipid nanoparticle. Serum was collected from

123   human vaccinees 36 days after the first dose (7 days after the second dose). The convalescent

124   macaques were infected twice with SARS-CoV-2, with infections spaced six weeks apart and

125   serum collected 56 days after the first infection (14 days after the second infection). The

126   convalescent humans were naturally infected once with SARS-CoV-2 and exhibited mild disease,

127   with a median of 67 days between symptom onset and sample collection. Details of individual

128   participants are available in Table S1.

129 **Table 1.** Details of samples used in the current study.

Group	Number of samples	Age range (years)	Treatment	Time of sample collection
Vaccinated pigtail macaques	11	3 ½ - 6	repRNA vaccine encoding full-length SARS-CoV-2 Spike[a]	42 days post 1st dose
Vaccinated humans	15	18 - 55	100ug mRNA vaccine encoding full-length pre-fusion stabilized SARS-CoV-2 Spike (Moderna)	36 days post 1st dose
Convalescent rhesus macaques	12	2 ½ - 5	Infected twice with SARS-CoV-2 six weeks apart[a]	56 days post 1st infection
Convalescent humans	12	28 - 52	Naturally infected once with SARS-CoV-2 (mild disease)	Median 67 (IQR 62, 70) days post symptom onset

130 [a]Within each group of macaques, subgroups received slightly different treatments (described in Table S1).

Enrichment of wildtype peptides

132 To compare which regions of Spike protein are recognized by human and macaque antibodies,

133 we examined the enrichment of wildtype peptides by antibodies from each individual (Fig 1A).

134 Broadly speaking, binding was observed in the NTD, CTD, FP, and stem helix-HR2 epitope

135 regions as reported previously in human studies [59, 60]. Epitope regions (shown as different

136 colors on Fig 1) were defined as previously [60]: NTD, amino acid 285-305; FP, 805-835; stem

137 helix-HR2 (SH-H), 1135-1170. For the CTD, the bounds of epitope regions were expanded and

138 altered from previous studies based on macaque antibodies recognizing a wider area than

139 previously seen in humans: CTD-N', 526-593; CTD-C', 594-685 (S1A Fig). Several additional

140 epitopes that flank previously-defined regions were also identified in this analysis: pre-FP, 777-

141 804; post-FP, 836-855 (S1B Fig); and HR2, 1171-1204 (S1C Fig). Specific epitope regions can be

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142   visualized on the structure of a Spike protein monomer in Fig 1B. In addition to these defined

143   regions, we noted that one convalescent rhesus macaque appeared to weakly recognize an

144   epitope at the beginning of the S2 subunit (amino acid 686-710, Fig 1A).

145   In general, we did not detect responses in the RBD because many epitopes in this region are

146   known to be conformational, and Phage-DMS only has the power to detect epitopes that

147   include linear sequences. Epitopes in the RBD have been extensively detailed elsewhere [62,

148   63]. However, we did detect strong binding to an RBD epitope in some vaccinated pigtail

149   macaques (Fig 1A). This same region was enriched in samples from before vaccination in four of

150   the five pigtail macaques with baseline samples available (S2 Fig). Pre-infection serum from the

151   twelve rhesus macaques did not show any consistent responses (S2 Fig). Because the RBD

152   response in pigtail macaques was present prior to vaccination with SARS-CoV-2 Spike, we did

153   not investigate it further as a response to vaccination.

154   To quantify differences in the epitopes targeted by different groups, the enrichment of wildtype

155   peptides was summed across each epitope region for every individual. Because the main

156   research question is whether responses in macaques model those in humans, two comparisons

157   were performed: vaccinated macaques vs. vaccinated humans and convalescent macaques vs.

158   convalescent humans (Fig 2).

159   In concordance with a qualitative assessment of the enrichment heatmap in Fig 1A, vaccinated

160   humans preferentially recognized the following epitope regions compared to vaccinated

161   macaques: NTD (Mann-Whitney $p \leq 0.01$), CTD-C' ($p \leq 0.0001$), and FP ($p \leq 0.05$) (Fig 2A).

162   Meanwhile, convalescent macaques recognized the following epitope regions more than

163   convalescent humans: CTD-N' ($p \leq 0.01$), pre-FP ($p \leq 0.001$), and post-FP ($p \leq 0.01$) (Fig 2B). All

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164 groups consistently recognized the SH-H epitope region (Fig 2). While vaccination appeared to

165 induce a stronger response against HR2 than infection (Fig 1A), there were no significant

166 differences in response driven by species (Fig 2). Within each group of macaques (vaccinated

167 and convalescent), subgroups received slightly different treatments (Table S1), so similar

168 analyses were performed comparing these subgroups; no comparisons were significant at a

169 threshold of p=0.05 (Kruskal-Wallis test, S3 Fig).

170 Taken together, these findings indicate: 1) vaccinated humans were the only group to

171 consistently recognize peptides from both the NTD and CTD-C' epitope regions, which are in

172 close physical proximity to one another (Fig 1B); 2) convalescent humans had a limited

173 response to the CTD-N'; 3) compared to other groups, convalescent macaques had a notably

174 more robust response to regions upstream and downstream of the main FP epitope region; 4)

175 vaccinated macaques did not recognize the FP as strongly as other groups; and 5) vaccination

176 seemed to induce a stronger response against HR2 than infection in both macaques and

177 humans.

178 ## Defining and comparing escape pathways

179 To assess differences in the binding characteristics of human and macaque antibodies on a

180 more granular level, we next examined the mutations in Spike that reduced antibody binding in

181 each epitope region of interest. Because the antibody escape pathways for vaccinated humans

182 have been described previously [60], we did not examine the NTD and CTD-C', which are

183 exclusively recognized by this group. Instead, we focused on comparing escape profiles

184 between groups in the following epitope regions: CTD-N', FP, and SH-H. We first represent the

185 data as scaled differential selection values in logo plot form, as commonly shown in previous

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186   studies. Importantly, scaled differential selection is highly correlated with peptide binding as

187   measured by competition ELISA [58]. To summarize the data represented by the logo plots by

188   group, summed differential selection values across each epitope region were also calculated.

189   This metric represents the overall magnitude of escape at each locus regardless of the specific

190   amino acid substitution, with negative values indicating a decrease in binding compared to the

191   wildtype amino acid, and positive values indicating enhanced binding (see "Materials and

192   Methods"). Finally, escape similarity scores were calculated between pairs of individuals to

193   quantify similarity in escape profiles (see "Materials and Methods" and S4 Fig).

194   **CTD-N'**

195   Vaccinated macaques, vaccinated humans, and convalescent macaques recognized peptides in

196   the CTD-N' (AA 526-593), whereas convalescent humans generally did not (Fig 2B). Within this

197   epitope region, the individual escape profiles showed notable variability both within and

198   between groups (S5 Fig). For example, across all groups, some individuals showed relatively

199   high sensitivity to mutations between sites 558-567, while others had a response focused more

200   downstream around AA 577-586. There was also substantial variability in which loci in the CTD-

201   N' had the highest relative magnitude of escape, and sometimes even in the directionality of

202   scaled differential selection at a given locus. For example, some individuals had antibodies that

203   bound mutated peptides better than wildtype at AA 555 (e.g., convalescent macaque 353)

204   while others exhibited reduced binding to mutated peptides (e.g., convalescent macaque 358).

205   The same was true for site 560 (e.g., vaccinated humans M24 and M26 exhibited improved and

206   disrupted binding to mutated peptides, respectively).

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207   To summarize the trends observed in the individual findings, we calculated summed differential

208   selection values for each individual at each site and generated boxplots by group (Fig 3A). In

209   addition to the aforementioned regions of escape common to all groups, convalescent

210   macaques also showed considerable escape between AA 529-535, with vaccinated macaques

211   also showing a less consistent response in this area (Figs. 3A and S5). The complexity and

212   variability of the escape pathways also prompted us to quantify the similarity in escape

213   between and within groups. Escape similarity scores largely corresponded to areas of high

214   magnitude of escape. Sites with low-magnitude summed differential selection values indicate

215   loci where mutations have no notable impact, and therefore those escape profiles reflect

216   fluctuations in peptide enrichments due to noise, which drives a lower escape similarity score

217   at those sites (Fig 3A, lower panel). At some sites (e.g., 560, as described above), low scores

218   were also the result of some samples showing negative differential selection and others

219   showing positive differential selection, a comparison that was assigned the highest cost in our

220   escape similarity score algorithm.

221   To test the similarity of escape profiles across the CTD-N' epitope region, escape similarity

222   scores were aggregated across the region and computed both within and between groups.

223   These are shown as boxplots, with each point representing a pairwise comparison between

224   individual samples (Fig 3B). For example, every vaccinated macaque was compared to every

225   other vaccinated macaque (a within-group comparison) and to every vaccinated human (a

226   between-group comparison). We included a comparison of convalescent macaques and

227   vaccinated humans, given visual similarities between their patterns of escape (Fig 3A).

228   Convalescent macaques showed the highest within-group similarity in escape profiles, meaning

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229   their escape profiles were more consistent than those of the vaccinated macaques or

230   vaccinated humans (Fig 3B). Between-group escape similarity scores were on par with the

231   within-group scores for the vaccinated macaques and humans, indicating that although there

232   was substantial variability in individual profiles, this was not driven by sample groups.

233   **FP**

234   Escape profiles were examined in the FP epitope region (AA 805-835) for the three groups that

235   showed significant wildtype enrichment in this area: vaccinated humans, convalescent

236   macaques, and convalescent humans. As in the CTD-N', overall there was variability in

237   individual escape profiles, though the convalescent macaques showed a more consistent

238   pattern of escape than other groups (S6 Fig). Within the FP, most sites of escape fell between

239   AA 811-825 for all groups (Fig 4A). The convalescent macaques again exhibited the highest

240   escape similarity scores (Fig 4B). The median within-group escape similarity scores in the FP

241   were on par with those in the CTD-N' (Fig 3B), indicating approximately equal variability in

242   antibody escape in these epitope regions. The between-group escape similarity scores were

243   generally similar to each other and to the human within-group scores (Fig 4B).

244   **SH-H**

245   All four groups consistently recognized peptides spanning the SH-H epitope region (AA 1135-

246   1170). Major sites of escape were located between AA 1145-1158 for all groups (Fig 5A). The

247   individual logo plots in the SH-H suggested a consistent response among vaccinated humans

248   and convalescent macaques, with more variability in the remaining groups (S7 Fig). This finding

249   is supported by the within-group escape similarity scores for those groups trending higher

250   across the epitope region (Figs. 5A lower panel and 5B). The median epitope region-wide

251   escape similarity scores for vaccinated humans and convalescent macaques were also higher in

252   the SH-H than in the CTD-N' or FP, confirming a more concordant response. The median

253   between-group escape similarity score for vaccinated humans and convalescent macaques was

254   on par with their median within-group scores, indicating that the escape profile of a vaccinated

255   human looks as similar to that of a convalescent macaque as it does to another vaccinated

256   human (Fig 5B). The similarity between these two groups was higher than the similarity

257   between convalescent macaques and humans, as well as between vaccinated macaques and

258   humans (Fig 5B). Despite this overall trend, two vaccinated humans had more unique escape

259   profiles (S7 Fig, M26 and M19) and are responsible for a cluster of lower-similarity outlier

260   points (Fig 5B, "Vaccinated Humans" and "Conv. Mac. vs. Vacc. Hum.").

261   The pairwise comparison between participant 352 (a convalescent macaque) and M21 (a

262   vaccinated human) generated an escape similarity score closest to the median for all

263   comparisons between these groups. Logo plots for these individuals are shown in Fig 5C as a

264   representative example of the striking between-group similarity. The most consistent sites of

265   escape for both groups were AAs 1148, 1152, 1155, and 1156 (Figs. 5A and S7). While some

266   differences exist, there was not nearly as much variability as in the CTD-N' (S5 Fig) and FP (S6

267   Fig).

Other epitope regions

269   In addition to the epitope regions described above, the convalescent macaques strongly

270   recognized the pre-FP and post-FP, which were not targeted by human antibody responses (S8

271   Fig). Escape profiles in the pre-FP appeared highly consistent among individual macaques, with

272   major sites of escape at AAs 795, 798, 800, and 802. Profiles were more variable in the post-FP,

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273   likely due in part to low enrichment of wildtype peptides in this epitope region for some

274   individuals (S8 Fig).

Comparison of vaccinated humans and convalescent macaques

276   It was notable that the vaccinated humans and convalescent macaques showed the most

277   similarity in escape profiles across all epitope regions, most strikingly in the SH-H. Thus, we also

278   asked whether they showed similarity in the epitopes they targeted by comparing the

279   enrichment of wildtype peptides in these groups in each epitope region (S9 Fig). Vaccinated

280   humans recognized the following epitope regions more strongly than convalescent macaques:

281   NTD (Mann-Whitney $p \leq 0.0001$), CTD-C' ($p \leq 0.0001$), and HR2 ($p \leq 0.001$). Convalescent

282   macaques preferentially recognized the pre-FP ($p \leq 0.0001$) and post-FP ($p \leq 0.001$) epitope

283   regions. This suggests some diversity in the epitopes targeted, but similarity of antibody escape

284   patterns within epitopes targeted by both groups.

Discussion

286   In this study, we aimed to assess whether the antibody binding specificities to SARS-CoV-2

287   Spike in macaques are a useful model for the human response. Our results indicate important

288   similarities between macaques and humans; for example, both have antibodies that recognize

289   major epitopes in the CTD, FP, and SH-H. However, many differences are also apparent, with

290   some groups showing responses to unique epitopes, such as two physically proximal epitopes in

291   the NTD and CTD that are recognized by antibodies from vaccinated humans but not macaques.

292   Additionally, epitope regions flanking the FP were recognized by antibodies from convalescent

293   macaques, while antibodies from convalescent humans did not recognize the flanking regions

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294   but showed a strong response within the FP itself. We found considerable diversity in the

295   pathways of escape between individuals, and this was not specific to either macaques or

296   humans, suggesting a diverse repertoire of antibodies that can respond to the major epitopes in

297   both groups. Overall, these results suggest that macaques and humans share recognition of

298   certain major epitopes. The differences that exist could be due to species (macaque vs. human),

299   but could also be influenced by differences in the specific type and number of exposures to

300   antigen in each group.

301   Other studies have characterized human monoclonal antibodies against some of the epitopes

302   we report here, many of them with neutralizing or other activities. As previously reported by

303   our group [60], we found that antibodies from vaccinated humans bound peptides spanning a

304   30 amino acid segment at the C-terminus of the NTD. Interestingly, most if not all neutralizing

305   human mAbs targeting the SARS-CoV-2 NTD to date have been shown to target a single

306   supersite on the "tip" of Spike, distinct from the epitope we detected at the C-terminus [49, 64-

307   70]. An NTD mAb with Fc effector function [71], as well as several NTD mAbs that enhance

308   infection in vitro [65, 72], also bind sites upstream of the C-terminal epitope. Therefore, future

309   studies are warranted to investigate the function of antibodies binding the new NTD epitope

310   detected by Phage-DMS. In the CTD, we detected broad antibody binding, with vaccinated

311   macaques, vaccinated humans, and convalescent macaques enriching peptides in the CTD-N'

312   epitope region, and vaccinated humans also recognizing peptides spanning the remainder of

313   this domain (CTD-C'). Polyclonal antibodies targeting sites within the CTD-N' and CTD-C' have

314   been isolated from human sera and shown to have neutralizing activity [73]. Interestingly, the

315   neutralizing epitope on the CTD-C' (AA 625-636) [73] is physically adjacent to the NTD epitope

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316   we describe (AA 285-305), raising the possibility that a conformational epitope extending to the

317   NTD is recognized by neutralizing antibodies from vaccinated humans. Depleting human serum

318   of FP-binding antibodies reduced its neutralization capacity [74]; these antibodies are of high

319   interest, both due to their potential to block membrane fusion, and given the high sequence

320   conservation among the FPs of diverse coronaviruses [75, 76]. We found that convalescent

321   rhesus macaque sera strongly recognized the pre- and post-FP epitope regions, but to our

322   knowledge, functional antibodies against these regions have not been previously described.

323   Finally, the SH-H epitope region we describe is in the stem helix, a region known to be highly

324   conserved across coronaviruses. Broadly neutralizing [77-79] stem helix antibodies have been

325   isolated and suggest an avenue for rational design of a pan-coronavirus vaccine. Interestingly, a

326   mAb raised against the MERS-CoV stem region protected mice against SARS-CoV-2 challenge,

327   despite having no neutralizing activity against SARS-CoV-2 in vitro [80]. The detection of broad

328   antibody binding across Spike supports the continued investigation of non-RBD epitopes, which

329   remain understudied. Some of the epitopes we describe may also be the target of non-

330   neutralizing Fc-effector antibodies [81], and/or antibodies that enhance infection via Fc-

331   independent [72] or Fc-dependent [82] mechanisms. This latter concept may be important in

332   the pathogenesis of COVID-19, though this remains speculative.

333   Previous work elucidated that pathways of antibody escape to SARS-CoV-2 Spike protein can be

334   quite variable in convalescent humans, with vaccination inducing a more consistent response

335   [60]. In the current study, we found considerable variability in escape profiles in the FP and

336   CTD-N' in both macaques and humans, though the convalescent rhesus macaques had more

337   concordant escape profiles than other groups. Variability in escape patterns suggests that a

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338   diversity of antibodies are targeting these epitopes. Intra-species germline diversity in

339   immunoglobulin genes may help explain why individuals with similar exposures often mount

340   distinct responses [83, 84]. On the other hand, escape profiles were more consistent in the SH-

341   H, where the responses of convalescent macaques and vaccinated humans appeared to

342   converge. This conservation of response suggests that highly similar antibodies are dominating

343   the antibody repertoire against this epitope. Convergent antibody responses to SARS-CoV-2

344   have been reported within human populations [85-87], and our findings here suggest that

345   antibodies from different species may also be able to converge on the same "public" antibody

346   repertoires in a functional sense, despite genetic differences. While a shared escape profile

347   among individuals could suggest that viral escape mutations are more likely to emerge on a

348   population level, another factor to consider is the effect of the mutations on viral fitness. Key

349   domains of the S2 subunit (such as the SH-H epitope) have essential functions and high

350   sequence conservation, suggesting a low tolerance for mutation and thus for escape. Indeed,

351   previous work determined that sites of escape identified by Phage-DMS are not typically

352   mutated at a high frequency in circulating strains of SARS-CoV-2 [59].

353   While our focus was on understanding how macaques and humans respond to a similar

354   exposure (i.e., vaccination or infection), we also noted similarities in response between re-

355   infected macaques and vaccinated humans. These groups both exhibited the broadest

356   recognition across Spike, although the epitope regions they targeted were somewhat different.

357   As described above, these groups also had highly similar antibody escape profiles in the SH-H.

358   The vaccinated humans and re-infected macaques both received two exposures to high doses

359   of antigen. It is plausible that re-exposure directed initially diverse antibodies to converge on a

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360   more focused response in both scenarios. While it is known that vaccination and infection

361   induce distinct humoral responses against Spike [60, 88, 89], our data suggest that a second

362   exposure may generate antibodies that better match the vaccine-induced response.

363   This study had several limitations. Because the Phage-DMS library displays peptides 31AA in

364   length, discontinuous or conformational epitopes are not readily detected using this method.

365   Additionally, epitopes that may normally be glycosylated are exposed for antibody binding in

366   Phage-DMS. There also are known germline-encoded differences in the properties of

367   immunoglobulin subclasses and Fc receptors between macaques and humans, leading to

368   differences in antibody function that cannot be assayed using Phage-DMS [90]. Additionally,

369   our sample set includes variables that limit our ability to draw conclusions about species-

370   specific (macaque vs. human) differences in antibody response. The vaccinated macaques and

371   humans both received RNA vaccines encoding full-length Spike protein, but there were

372   differences in vaccine technology, including: 1) the use of mRNA in the human vaccine vs.

373   repRNA in the macaque vaccine, 2) the stabilization of Spike in its pre-fusion state in the human

374   vaccine, 3) the dosage and number of doses delivered, and 4) the formulation used to deliver

375   the RNA. Despite these differences, we found commonalities in some of the epitopes targeted

376   by antibodies from both groups. Additionally, the convalescent rhesus macaques were

377   experimentally infected twice with high titers of virus, compared to the convalescent humans

378   who were naturally infected once. This important discrepancy could be the reason why the

379   response in re-infected macaques aligned more closely with vaccinated humans than

380   convalescent humans. Studies of re-infected humans would help address this possibility.

19

381 Our findings suggest that while vaccinated and convalescent macaques and humans share

382 recognition of some major epitopes, each group has a unique antibody binding profile.

383 Antibody escape profiles suggest a diversity of individual responses to most epitopes.

384 Important avenues for future study include comparing macaque and human responses to the

385 RBD and evaluating species differences in antibody function. Continued investigation of

386 immunogenic epitopes in conserved regions of Spike is also warranted to inform the

387 development of immunity that is more robust in the face of viral escape.

388 # Materials and Methods

389 ## Samples

390 ### Vaccinated pigtail macaques

391 Plasma was collected from 11 pigtail macaques immunized with a replicating RNA (repRNA)

392 vaccine expressing full-length SARS-CoV-2 Spike protein. A subset of these animals was

393 previously described [35]. All animals were housed at the Washington National Primate

394 Research Center (WaNPRC), an accredited facility of the American Association for the

395 Accreditation of Laboratory Animal Care International (AAALAC). All procedures were

396 approved by the University of Washington's Institutional Animal Care and Use Committee

397 (IACUC) (IACUC #4266-14). Individual macaques received the vaccine by intramuscular

398 injection in either a Lipid InOrganic Nanoparticle (LION) [35] or a Nanostructured Lipid Carrier

399 (NLC) [61] formulation, delivered in a single priming dose of 25ug (n=3) or 250ug (n=6) or in a

400 prime-boost regimen with 50ug doses spaced 4 weeks apart (n=2). All samples were collected 6

401   weeks post-prime immunization. A subset of these animals also previously received an

402   experimental hepatitis B vaccine as part of another study (n=5).

Convalescent rhesus macaques

404   Serum was collected from 12 rhesus macaques housed at the Rocky Mountain Laboratories

405   (National Institutes of Health [NIH]), 14 days after the second of two SARS-CoV-2 infections

406   spaced 42 days apart. Prior to infection, macaques were variably depleted of CD4+ T cells, CD8+

407   T cells, CD4+ and CD8+ T cells, or neither, as part of another study. Details of macaque

408   treatment and regulatory approvals are as published previously [39].

Vaccinated humans

410   We obtained serum from 15 individuals who received two 100ug doses of the Moderna mRNA-

411   1273 vaccine as part of a phase I clinical trial (NCT04283461) [91]. Phage-DMS results from

412   these samples were reported previously [60]. Because samples were de-identified, this study

413   was approved by the Fred Hutchinson Cancer Research Center Institutional Review Board as

414   nonhuman subjects research. Only samples from individuals aged 18-55 years were included in

415   the current study to better match the young age range of the macaques.

Convalescent humans

417   Plasma was collected from 12 individuals post-mild COVID-19 illness as part of the Hospitalized

418   or Ambulatory Adults with Respiratory Viral Infections (HAARVI) study in Seattle, WA. Phage-

419   DMS results from these samples were reported previously [59, 60]. This research was approved

420   by the University of Washington Institutional Review Board (IRB number STUDY00000959).

421   Again, the sample set was restricted to only include individuals aged 18-55 years to better

422   match other sample groups.

21

423   All plasma and sera were heat inactivated at 56°C for 1 hour prior to use. Full details of all

424   samples are available in Tables 1 and S1.

Phage-DMS, Illumina library preparation and deep sequencing

426   The experimental protocol was performed exactly as described previously [59]. Briefly, an

427   oligonucleotide pool was synthesized that contains sequences coding for peptides of 31 amino

428   acids that tile along the length of the Wuhan-Hu-1 Spike protein sequence [92] in 1 amino acid

429   increments. For each peptide with the wildtype sequence, 19 variations were included that

430   have a single mutation at the middle amino acid, resulting in a total library size of 24,820

431   unique sequences. The oligonucleotide pool was cloned into T7 phage, followed by

432   amplification of the phage library; this step was performed twice independently to generate

433   biological duplicate phage libraries. The phage library was incubated with a serum or plasma

434   sample, then bound antibody-phage complexes were immunoprecipitated using Protein A and

435   Protein G Dynabeads (Invitrogen). Bound phage were lysed, and DNA was amplified by PCR and

436   cleaned prior to sequencing on an Illumina MiSeq or HiSeq 2500 with single end reads.

437   Demultiplexing and read alignment were also performed as described previously [60].

Replicate curation

439   Biological replicates were analyzed in parallel to assess reproducibility of results. For simplicity,

440   results from only one biological replicate are shown and described, with the same figures

441   generated with the second biological replicate available to view online at

442   https://github.com/matsengrp/phage-dms-nhp-analysis. Within each biological replicate, "in-

443 line" technical replicates were run for some samples. In these cases, the technical replicate with

444 the highest mapped read count was selected for analysis.

Wildtype enrichment and defining epitope regions

446 The enrichment of wildtype peptides was calculated as described previously to quantify the

447 proportion of each peptide in an antibody-selected sample relative to the proportion of that

448 peptide in the input phage library [58]. On enrichment plots, the locus of each peptide is

449 defined by its middle amino acid. Enrichment values of wildtype peptides were summed across

450 epitope regions of interest for statistical comparisons between groups ("Summed WT

451 enrichment" on figures). Mann-Whitney U tests were performed with multiple comparisons

452 adjustment using the Bonferroni-Dunn method.

Escape profile comparison

454 The effect of a mutation on antibody-peptide binding was quantified as "differential selection,"

455 which is the log fold change in the enrichment of a mutation-containing peptide compared to

456 the wildtype peptide. This number is multiplied by the average of the wildtype peptide

457 enrichments at that site and its two adjacent sites to get a "scaled differential selection" value,

458 as described previously [60]. The enrichment values of the adjacent wildtype peptides are

459 included in this calculation to make the analysis less susceptible to noise. Negative differential

460 selection values represent reduced binding compared to wildtype, while positive differential

461 selection values indicate that the mutation enhanced binding. "Summed differential selection"

462 is the sum of the 19 scaled differential selection values for all mutations at a site, and gives a

463 sense of the overall magnitude of escape at that site.

464   The comparison of two escape profiles is quantified by an escape similarity score computed in

465   the framework of an optimal transport problem [93]; this algorithm was described in detail at

466   https://matsengrp.github.io/phippery/esc-prof.html. An overview of the method is shown in S4

467   Fig. Escape profiles are commonly portrayed as logo plots using scaled differential selection

468   values (S4A Fig). At each site, escape data in logo plot form can instead be represented as

469   binned distributions, with each mutation making some contribution to the total amount of

470   escape at that site based on its scaled differential selection value (S4B Fig). For each site, an

471   optimal transport problem computes the most efficient way to transform one individual's

472   escape distribution into that of a different individual (S4C Fig). The cost to "exchange" amino

473   acid contributions between profiles is based on the similarity between the amino acids being

474   exchanged, as defined by the BLOSUM62 matrix [94]. More "movement" between dissimilar

475   amino acids drives up the total cost of the transport; therefore, a higher cost indicates less

476   similar profiles. Escape similarity scores are the inverse of the total cost of transforming one

477   profile into another. Scores were calculated between pairwise combinations of individuals to

478   compare escape profile variability within and between sample groups.

479   **Protein structure**

480   The structure of a SARS-CoV-2 Spike glycoprotein monomer in the closed state (PDB 6XR8) was

481   examined to visualize epitope regions [95]. Coloring was added using UCSF ChimeraX-1.2.5,

482   developed by the Resource for Biocomputing, Visualization, and Informatics at the University of

483   California, San Francisco, with support from National Institutes of Health R01-GM129325 and

484   the Office of Cyber Infrastructure and Computational Biology, National Institute of Allergy and

485   Infectious Diseases [96].

24

Code, software, and data availability

486 All analyses were performed in RStudio version 1.3.1093, Python version 3.6.12, GraphPad

487 Prism version 9.0.1, and the phip-flow and phippery software suite

488 (https://matsengrp.github.io/phippery/). The phip-flow tools perform read alignment using

489 Bowtie2 [97] in a Nextflow [98] pipeline script. The escape profile comparisons are done with

490 phippery in Python 3.6.12 and depend on the NumPy [99], pandas [100, 101], xarray [102], POT

491 [103], and biopython [104] packages. All code and instructions for running this analysis are

492 available at https://github.com/matsengrp/phage-dms-nhp-analysis.

Acknowledgements

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Figure legends

Fig 1: Enrichment of wildtype peptides. (A) The x axis indicates each peptide's location along

SARS-CoV-2 Spike protein, and each entry on the y axis is an individual sample. All enrichment

values over 20 are plotted as 20 to better show the lower range of the data. Above the

heatmap, domains of Spike are shown with grey boxes, with the S1/S2 and S2' cleavage sites

indicated with arrows. The epitope regions defined in the current study are shown as colored

boxes (from left to right: NTD in red, CTD-N' in green, CTD-C' in cyan, pre-FP in pink, FP in black,

post-FP in orange, SH-H in purple, and HR2 in blue). (B) Defined epitope regions shown on a

structure of one monomer of SARS-CoV-2 Spike in the pre-fusion conformation (PDB 6XR8 [ref

95]). The amino acid loci spanned by each epitope are listed. The HR2 epitope (AA 1171-1204)

could not be resolved on the structure and is not shown.

Fig 2: Differences in enrichment of wildtype peptides by group. Wildtype enrichment values

were summed for all peptides within each epitope region. Box plots summarize the data by

group. (A) compares vaccinated pigtail macaques to vaccinated humans, while (B) compares

convalescent rhesus macaques to convalescent humans. Multiple Mann-Whitney U tests were

performed, with p values corrected for the number of comparisons in each plot (8) using the

Bonferroni-Dunn method. ****, $p \leq 0.0001$; ***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$.

Fig 3: Comparison of escape profiles in the CTD-N'. (A) The top three panels show boxplots

depicting the summed differential selection values of all samples in a group at each locus.

Negative values represent sites where the binding interaction between antibody and peptide

was weakened when peptides were mutated, whereas positive values represent enhanced

binding. The bottom panel shows the mean escape similarity score for all pairwise comparisons

807   between samples in each group, calculated at every locus. See S4 Fig for a description of the

808   escape similarity score algorithm. (B) Within- and between-group region-wide escape similarity

809   scores, summarized as boxplots. Each point represents a pairwise comparison between two

810   samples. The contribution of a site's score to the total escape similarity score is weighted based

811   on its relative contribution to the summed differential selection values across the region. P

812   values are not computed due to lack of independence between data points.

813   **Fig 4: Comparison of escape profiles in the fusion peptide (FP).** (A) and (B) Data are shown as

814   described in Fig 3.

815   **Fig 5: Comparison of escape profiles in the stem helix-HR2 region (SH-H).** (A) and (B) Data are

816   shown as described in Fig 3. (C) Logo plots for participant 352 (a convalescent macaque) and

817   M21 (a vaccinated human) showing the effect of specific mutations on antibody binding at each

818   site. The comparison between these samples had an escape similarity score closest to the

819   median value for all pairwise convalescent macaque vs. vaccinated human comparisons and

820   thus can be considered representative of the similarity between these groups. The 352 – M21

821   comparison is shown in red on (B).

Supporting information

823   **S1 Fig: Enrichment of wildtype peptides varies by group in newly defined epitope regions.** The

824   locus numbers are shown on the x axis, and each individual is represented in a different color.

825   (A) Wildtype enrichment by group from AA 526-685, spanning the CTD-N' and CTD-C' epitopes.

826   (B) Wildtype enrichment by group from AA 777-855, spanning the pre-FP, FP, and post-FP

827   epitopes. (C) Wildtype enrichment by group from AA 1135-1204, spanning the SH-H and HR2

828   epitopes.

829   **S2 Fig: Enrichment of wildtype peptides in baseline macaque samples compared to post-**

830   **vaccination or post-infection samples.** The x axis indicates each peptide's location along SARS-

831   CoV-2 Spike protein, and each entry on the y axis is an individual sample. Sample groups are

832   indicated on the left. The same macaques that contributed baseline samples also contributed

833   post-vaccination or post-infection samples. All enrichment values over 20 are plotted as 20 to

834   better show the lower range of the data. Above the heatmap, domains of Spike are shown with

835   grey boxes, with the S1/S2 and S2' cleavage sites indicated with arrows. The epitope regions

836   defined in the current study are shown as colored boxes (from left to right: NTD in red, CTD-N'

837   in green, CTD-C' in cyan, pre-FP in pink, FP in black, post-FP in orange, SH-H in purple, and HR2

838   in blue).

839   **S3 Fig: Differences in enrichment of wildtype peptides by macaque subgroups.** Wildtype

840   enrichment values were summed for all peptides within each region of Spike that showed

841   enrichment. Each point represents an individual macaque. No significant differences were

842   found by Kruskal-Wallis test at a threshold of p=0.05. LION: Lipid InOrganic Nanoparticle; NLC:

843   Nanostructured Lipid Carrier.

844   **S4 Fig: Use of optimal transport to quantify similarity between amino acid escape profiles.** (A)

845   Profile 1 and 2 show example logo plots for two samples across the same region. Negative

846   scaled differential selection values represent mutations that reduce antibody binding. Amino

847   acids of the same color indicate similar chemistry (e.g., green = polar). (B) At each location (in

848   this example, the boxed site in panel A), the profiles are represented as binned distributions

849   where each bin corresponds to the contribution to escape for an amino acid substitution. (C)

850   The optimal transport solution to transform one profile to the other is computed, where the

42

851   cost to "exchange" an amino acid contribution in Profile 1 to an amino acid contribution in

852   Profile 2 is derived from the BLOSUM62 matrix. For the purposes of the schematic, the number

853   of dollar signs associated with each line denotes the relative cost of each move (i.e., more

854   dollar signs = more costly = moving between amino acids that are less similar). (D) To quantify

855   similarity between profiles, an escape similarity score is calculated as the inverse of the total

856   cost to perform the transformation. For more details, see

857   https://matsengrp.github.io/phippery/esc-prof.html. Created with BioRender.com.

858   **S5 Fig. Logo plots for all vaccinated macaques, vaccinated humans, and convalescent**

859   **macaques in the CTD-N' epitope region.**

860   **S6 Fig. Logo plots for all vaccinated humans, convalescent macaques, and convalescent**

861   **humans in the FP epitope region.**

862   **S7 Fig. Logo plots for all vaccinated macaques, vaccinated humans, convalescent macaques,**

863   **and convalescent humans in the SH-H epitope region.**

864   **S8 Fig. Logo plots for all convalescent macaques in the pre-FP and post-FP epitope regions.**

865   **S9 Fig: Differences in enrichment of wildtype peptides in vaccinated humans and**

866   **convalescent macaques.** As in Fig 2, wildtype enrichment values were summed for all peptides

867   within each epitope region of Spike. Multiple Mann-Whitney U tests were performed, with p

868   values corrected for the number of comparisons (8) using the Bonferroni-Dunn method. ****, p

869   ≤ 0.0001; ***, p ≤ 0.001; **, p ≤ 0.01; *, p ≤ 0.05.

Figure 1

Figure 2
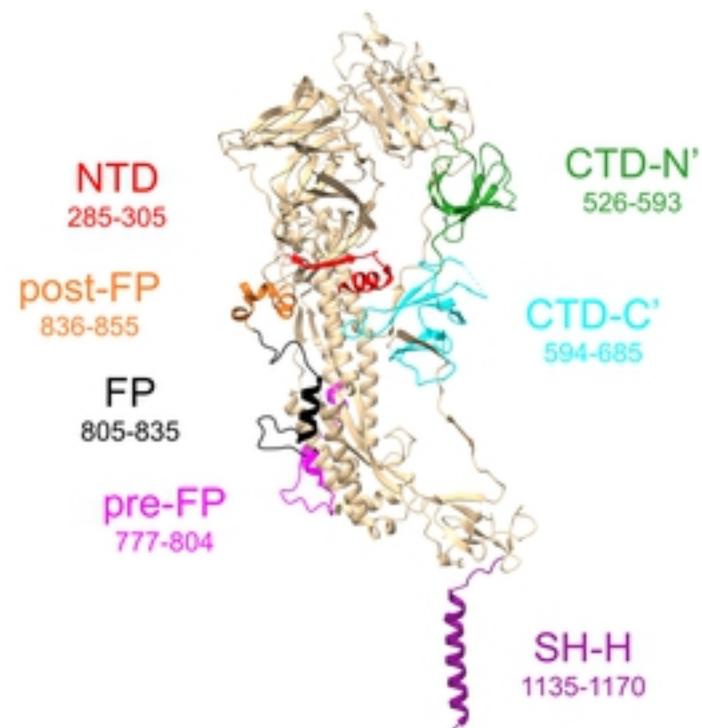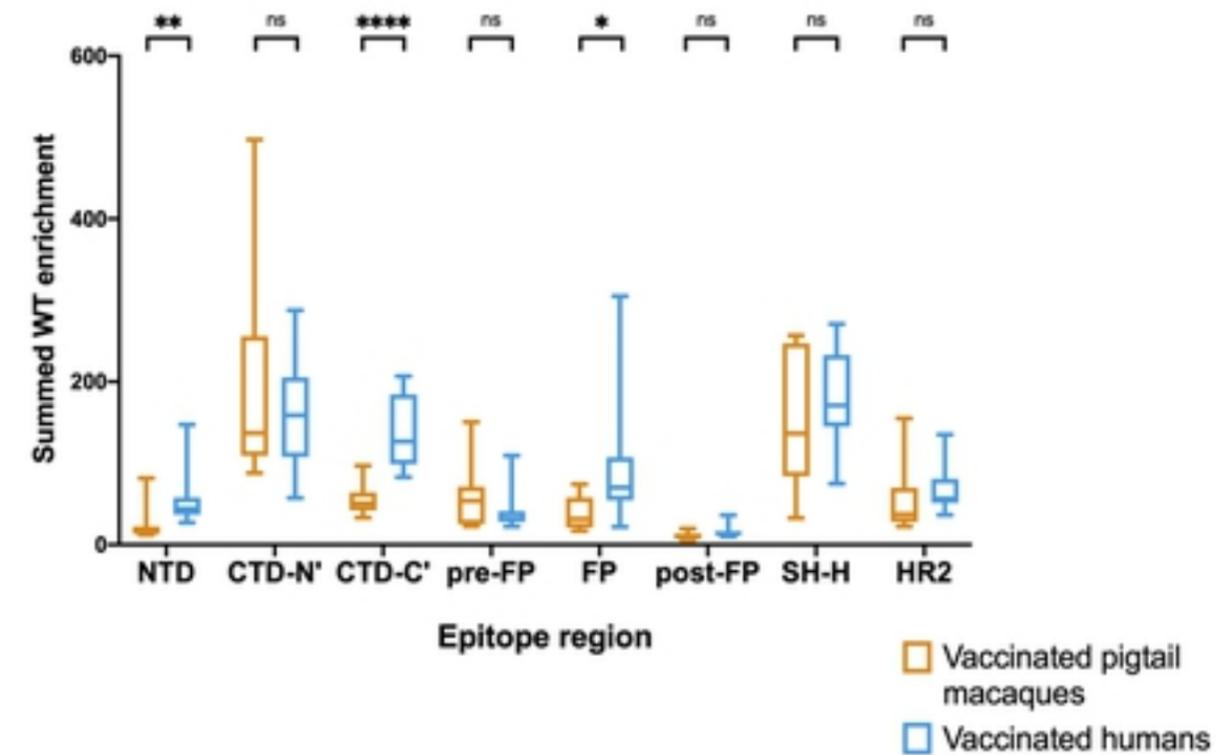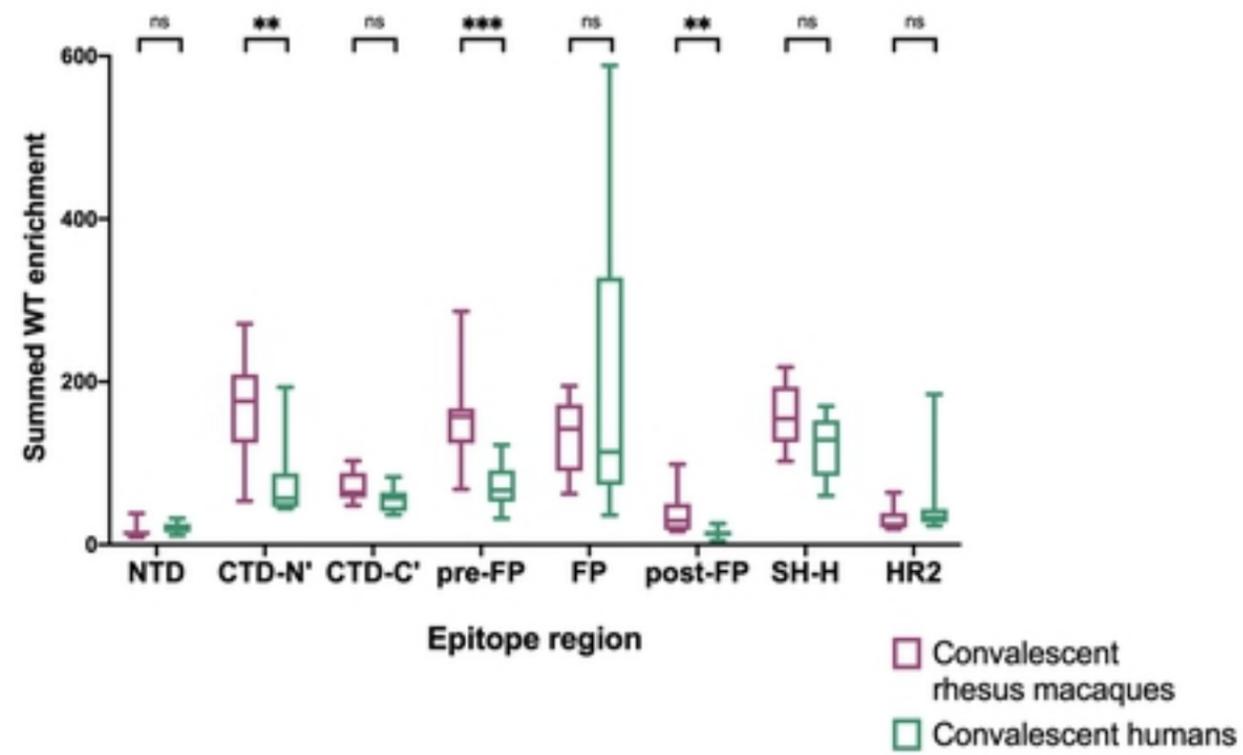
Figure 3

Figure 4

Figure 5