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Protective efficacy of rhesus adenovirus COVID-19 vaccines against mouse-adapted SARS-CoV-2

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24 **Abstract**

25 The global COVID-19 pandemic has sparked intense interest in the rapid development of
26 vaccines as well as animal models to evaluate vaccine candidates and to define immune correlates
27 of protection. We recently reported a mouse-adapted SARS-CoV-2 virus strain (MA10) with the
28 potential to infect wild-type laboratory mice, driving high levels of viral replication in respiratory
29 tract tissues as well as severe clinical and respiratory symptoms, aspects of COVID-19 disease in
30 humans that are important to capture in model systems. We evaluated the immunogenicity and
31 protective efficacy of novel rhesus adenovirus serotype 52 (RhAd52) vaccines against MA10
32 challenge in mice. Baseline seroprevalence is lower for rhesus adenovirus vectors than for human
33 or chimpanzee adenovirus vectors, making these vectors attractive candidates for vaccine
34 development. We observed that RhAd52 vaccines elicited robust binding and neutralizing
35 antibody titers, which inversely correlated with viral replication after challenge. These data support
36 the development of RhAd52 vaccines and the use of the MA10 challenge virus to screen novel
37 vaccine candidates and to study the immunologic mechanisms that underscore protection from
38 SARS-CoV-2 challenge in wild-type mice.

39

40 **Importance**

41 We have developed a series of SARS-CoV-2 vaccines using rhesus adenovirus serotype 52
42 (RhAd52) vectors, which exhibits a lower seroprevalence than human and chimpanzee vectors,
43 supporting their development as novel vaccine vectors or as an alternative Ad vector for boosting.
44 We sought to test these vaccines using a recently reported mouse-adapted SARS-CoV-2 (MA10)
45 virus to i) evaluate the protective efficacy of RhAd52 vaccines and ii) further characterize this
46 mouse-adapted challenge model and probe immune correlates of protection. We demonstrate

47 RhAd52 vaccines elicit robust SARS-CoV-2-specific antibody responses and protect against
48 clinical disease and viral replication in the lungs. Further, binding and neutralizing antibody titers
49 correlated with protective efficacy. These data validate the MA10 mouse model as a useful tool to
50 screen and study novel vaccine candidates, as well as the development of RhAd52 vaccines for
51 COVID-19.

52

53 **Introduction**

54 A critical component of the evaluation of vaccine candidates for COVID-19 has been the
55 development of pre-clinical challenge models. Transgenic mice [1-5], hamsters [6-9], and non-
56 human primates [10-12] have been shown to support viral replication and, to varying degrees,
57 clinical disease following infection with SARS-CoV-2 [13]. We recently described a mouse-
58 adapted virus (MA10) to enable challenge of standard, wild-type laboratory mice and recapitulate
59 several key features of human disease, such as viral replication in respiratory tract tissues and
60 severe infection-associated weight loss [14]. This model has been explored to evaluate small
61 molecule antivirals and candidate monoclonal antibodies for prophylactic or therapeutic
62 applications, as well as prototype vaccine candidates [14-17]. For example, initial studies using
63 viral replicon particles expressing SARS-CoV-2 Spike protein demonstrate the capacity of
64 vaccines to restrain MA10 infection and disease [14].

65 This model has not yet been utilized to study the characteristics of vaccine-elicited immune
66 responses that protect against clinical disease and viral replication. Thus, we sought to test a series
67 of candidate rhesus adenovirus serotype 52 (RhAd52) [18] vector-based vaccines expressing
68 engineered versions of SARS-CoV-2 Spike. RhAd52 vectors have lower seroprevalence in human
69 populations than Ad26 vectors, which recently received FDA Emergency Use Authorization as a

70 COVID-19 vaccine [19, 20]. We aimed to probe correlates of protection, including whether similar
71 immune parameters such as neutralizing antibody titers emerge in the mouse model as predictors
72 of challenge outcome, as has been observed in hamsters and nonhuman primates [9, 10, 21]. These
73 data will inform applications of the MA10 virus to study key questions about clinical disease,
74 infection, or both.

75

76 **Results**

77 **Immunogenicity of RhAd52 vectors**

78 We designed a series of replication incompetent viral vector vaccines using rhesus
79 adenovirus serotype 52 (RhAd52) vectors [18, 22] that encode variations of the SARS-CoV-2
80 Spike (S) protein (Fig 1A). Similar to our previous reports with human adenovirus serotype 26
81 (Ad26) vectors [9, 23, 24], inserts included: i) unmodified S, ii) truncations of the cytoplasmic tail
82 (S.dCT) or the transmembrane region (S.dTM), or iii) select fragments, including the S1 domain
83 and the receptor binding domain (RBD). In some cases, immunogens were modified with mutation
84 of the furin cleavage site and the addition of proline mutations to stabilize protein prefusion
85 conformation (PP) [25-27]. To explore the potential of candidate RhAd52 vaccines to elicit
86 humoral immune responses, groups of wild-type BALB/c mice were immunized with 10^9 viral
87 particles (VPs) of these vaccines via the intramuscular route (Fig 1B). Peripheral blood was
88 collected on a biweekly basis to monitor antibody responses in serum.

89 At week 2 following the initial immunization, 100% of mice immunized with RhAd52
90 vaccines, irrespective of the immunogen insert, exhibited SARS-CoV-2 S-specific binding
91 antibodies by enzyme-linked immunosorbent assay (ELISA) (Fig 1C). These responses generally
92 increased over the time frame of 2-8 weeks post-prime. At week 8, RBD-specific binding

93 responses were also observed in 100% of mice immunized with RhAd52 candidates (Fig 1D).
94 Furthermore, antibody function was assessed using *in vitro* assays to quantify the potential to
95 neutralize either a pseudotyped virus [10, 28, 29] (Fig 1E) or live SARS-CoV-2 virus [10, 28, 30,
96 31] (Fig 1F). Similar to the binding results, neutralizing titers were elicited by several of the
97 RhAd52 candidate vaccines, with the lowest responses observed following immunization with the
98 S1 domain insert, in which a subset of mice exhibited no detectable neutralizing responses at week
99 8. Mice received a second identical dose of the respective RhAd52 vectors at week 8. Two weeks
100 following the boost immunization, median S-specific ELISA titers were found to increase by
101 approximately 10-fold for all the vaccine candidates (Fig 1G). These data demonstrate the
102 immunogenicity of a homologous boost with a second immunization of a RhAd52 vector.

103 We next designed a series of immunization regimens that we hypothesized would i) allow
104 direct comparison of protective efficacy of single-shot versus two-dose prime-boost schedules, and
105 ii) generate a range of binding and neutralizing antibody responses that could enable analyses of
106 correlates of protection following challenge [21, 24, 28]. Briefly, groups of mice were immunized
107 with a prime and a matched boost with the seven candidate RhAd52 vaccines, as in Fig 1B. At the
108 time of boost (i.e., week 8), additional groups of mice were immunized with a single dose of select
109 vaccines, RhAd52.S, RhAd52.S.dCT, and RhAd52.S.PP. At week 12, serum was collected to
110 assess antibody responses prior to viral challenge. Expansion of S-specific (Fig 2A) and RBD-
111 specific (Fig 2B) binding antibody titers was again observed in all vaccinated mice. Groups of
112 mice that received the two-dose regimens exhibited approximately one-log higher median titers
113 compared with groups administered a single immunization. Furthermore, consistent with our
114 previous data using a DNA vaccination platform in non-human primates [28], the S1 insert drove
115 the lowest binding responses.

116 In evaluating neutralizing antibody activity elicited by these vaccine regimens, similar
117 patterns were observed with pseudovirus (Fig 2C) and live virus (Fig 2D) assays. Among the
118 groups administered prime-boost dosing schedules, high neutralizing antibody titers were
119 observed across all vaccines with the exception of the S1 immunogen, for which 50%
120 neutralization titer (NT50) values were below the assay limit of detection for several mice. Groups
121 that received only one dose of select inserts exhibited lower neutralizing titers compared with
122 boosted mice, with detectable responses in a subset of mice that, on average, were approximately
123 one log lower than the boosted groups that received full length or truncated (e.g., S.dCT) vaccines.
124 As consistent trends were observed across binding and neutralizing titers in the relative magnitude
125 of responses elicited by various candidate vaccines, correlation analyses were performed to assess
126 the relationship between these immunologic readouts (Fig 2E). Highly significant ($P < 0.0001$,
127 Spearman correlation) strong positive correlations were observed between binding ELISA titers to
128 S and RBD proteins and capacity to neutralize either pseudovirus or live SARS-CoV-2 virus.

129

130 **Protective efficacy of RhAd52 vector vaccines against MA10 challenge**

131 At week 12, all groups of mice were challenged to evaluate whether vaccine-elicited
132 responses protected from clinical disease and viral replication in this mouse-adapted model [14].
133 Vaccinated mice were challenged on day 0 with 10^4 PFU SARS-CoV-2 MA10 via the intranasal
134 route (Fig 3A). Half of the mice were followed through day 4 post-challenge and body weight was
135 monitored daily for signs of clinical disease. At the terminal time point, lung tissue was collected,
136 and outgrowth assays were performed to quantify replication-competent virus (i.e., plaque forming
137 units (PFU) in this key respiratory tract tissue. In parallel, half of the mice were sacrificed at day
138 2 post-challenge to measure virus in the lungs. We hypothesized this approach would allow

139 evaluation of the potential to restrain clinical symptoms of disease as well as enable a virologic
140 endpoint at the time of typical peak viral load (i.e., day 2 post-challenge).

141 As expected, the sham control group exhibited significant weight loss following MA10
142 challenge, with a median loss of 15.2% of body weight at day 4 post-challenge (Fig 3B-3C). All
143 vaccine regimens provided robust protection from clinical signs of infection in terms of body
144 weights ($P < 0.0001$, one-way ANOVA with Dunnett's multiple comparisons test), with body
145 weight generally remaining stable irrespective of the RhAd52 insert or whether a single or two-
146 dose vaccine regimen was employed. Analyses of lungs revealed differences in the level of
147 replication-competent virus detected in respiratory tract tissues among mice largely protected from
148 weight loss (Fig 4A). In sham control mice at day 2 post-challenge, high levels of virus were
149 recovered from lung, with a median titer of 3.1×10^7 PFU/lung (Fig 4B). In contrast, two-dose
150 regimens with full-length (i.e., S, S.PP) or truncated (i.e., S.dCT, S.dTM, S.dTM.PP) S
151 immunogens provided a dramatic reduction in viral titer, with a greater than a 6 log drop in median
152 titer. In nearly all mice in these groups, no replication competent virus was recovered from the
153 lungs (i.e., PFU < 100/lung). Immunization with two doses of the S fragment immunogens –
154 RhAd52.S1 and RhAd52.RBD – restrained the level of virus in the lung, with a median titer of 3.9
155 $\times 10^5$ and 5.0×10^3 PFU/lung, respectively. Similarly, in the single-shot groups, RhAd52.S and
156 RhAd52.S.dCT provided significant but incomplete protection, reducing the viral burden in the
157 lung to 3.3×10^2 and 1.0×10^4 PFU/lung, respectively. Finally, a single shot of RhAd52.S.PP
158 dramatically reduced viral load, with no detectable viral outgrowth from lung tissues in 100% of
159 mice. Of note, the S.PP insert previously proved optimal in nonhuman primates [24] and was
160 advanced into clinical trials [19, 20].

161 Similar analyses at day 4 revealed the level of virus in lung tissue was several logs lower

162 (median 4.4×10^4 PFU/lung) than at day 2 post-challenge (Fig 4C), consistent with our previous
163 finding that tissue viral loads peak at day 1-2 post-challenge and gradually resolve over
164 approximately 7 days [14]. Across vaccine regimens, virus levels were largely below the limit of
165 detection of the outgrowth assay, with low levels observed in a subset of mice in the RhAd52.S
166 and RhAd52.S1 two-dose groups. Together, these data suggest that all vaccines led to a reduction
167 in respiratory tract tissue viral loads at the typical peak of infection as well as significantly
168 decreased the persistence of virus in the lungs.

169

170 **Exploration of immune correlates of protection**

171 We next evaluated possible correlations between vaccine-elicited immune responses prior
172 to challenge and peak viral levels following challenge. A highly significant ($P < 0.0001$) correlation
173 was observed between pre-challenge S ELISA titers and peak (i.e., day 2) lung PFU (Fig 4D).
174 Furthermore, the resulting Spearman correlation coefficient ($R = -0.7499$) suggests a strong inverse
175 relationship between pre-challenge binding antibody levels and virologic outcome post-challenge.
176 Similar highly significant ($P < 0.0001$) inverse correlations were observed between three additional
177 pre-challenge immunologic metrics and viral replication in the lung: i) RBD ELISA titers ($R = -$
178 0.7234), ii) pseudovirus neutralization titers ($R = -0.7446$, and iii) live virus neutralization titers
179 ($R = -0.7744$). Together, these data suggest that multiple vaccine-elicited humoral immune
180 responses are inversely correlated with viral replication in respiratory tract tissue following MA10
181 SARS-CoV-2 challenge.

182

183 **Discussion**

184 Our data indicate that RhAd52 vectors expressing SARS-CoV-2 S antigens elicit robust

185 and protective humoral immune responses in mice. Based on baseline seroprevalence as well as
186 the expanded global use of Ad5, Ad26, and ChAdOx1 vaccines [19, 20, 32-35], developing
187 additional adenoviral vectors for COVID-19 vaccines is critical. This approach could be important
188 for developing future boosting vectors or to tune the innate immune signatures induced [36].
189 Similar to our recent reports in hamsters [9], non-human primates [24, 28], and humans [19], a
190 robust correlation was observed between binding and neutralizing antibody responses.
191 Furthermore, although single-shot vaccines were highly protective, we observed increased
192 immune responses using a homologous prime-boost strategy. In particular, the expansion of
193 neutralizing antibody responses, as measured by both pseudovirus and live virus assays, in mice
194 is encouraging, as this metric has emerged as a potential correlate of protection in hamster and
195 non-human primate challenge models [9, 10, 21, 24, 28].

196 Importantly, the MA10 virus has previously been shown to drive significant clinical disease
197 (i.e., weight loss) as well as replication localized in respiratory tract tissues, characteristics of
198 interest for modeling severe COVID-19 disease. In contrast, nonhuman primate models for
199 COVID-19 generally do not develop severe clinical disease [10-13]. The MA10 mouse model has
200 proven useful for screening candidate therapeutics, but it remains relatively unexplored for testing
201 vaccines [14-17]. The results from our challenge studies using RhAd52 vaccines suggest that
202 candidate vaccines significantly protected against clinical disease and virus replication in lung
203 tissue. However, only select immunization regimens drove full suppression of replicating virus in
204 the lungs, as measured by viral outgrowth assays. Moreover, our data show that the recently-
205 reported mouse-adapted virus MA10 exhibits robust humoral immune correlates of vaccine
206 protection [9, 10, 21], which will prove useful in future studies of vaccines and other interventions
207 using this model.

208 Future studies could further explore mechanistic correlates of protection, such as defining
209 how vaccine candidates tune systemic pro-inflammatory cytokine secretion typically triggered by
210 viral infection, as well as exploring the role of T cell responses in the context of MA10 challenge.
211 No signs of disease enhancement (e.g., enhanced weight loss) were observed with sub-protective
212 immune responses, an important finding due to concerns of antibody-dependent enhancement.
213 Together, these data support the MA10 mouse-adapted virus as a tool to screen vaccine candidates.
214 This approach could help to test novel immunogens, delivery systems, or dosing regimens,
215 harnessing a relatively high throughput, tractable small animal model, wild-type mice. These
216 studies could be employed to identify promising approaches to advance to large animal pre-clinical
217 and, subsequently, early clinical trials. Moreover, similar mouse challenge models could be
218 developed for the newly described SARS-CoV-2 variants of concern.

219

220 **Materials and Methods**

221 **RhAd52 vectors.**

222 RhAd52 vectors were constructed with seven variants of the SARS-CoV-2 Spike (S)
223 protein sequence (Wuhan/WIV04/2019; GenBank MN996528.1). Sequences were codon
224 optimized and synthesized. Replication-incompetent, E1/E3-deleted RhAd52-vectors were
225 produced in HEK 293B-55K.TetR cells as previously described [23], with the E1 region replaced
226 by a transgene cassette encoding for the S sequence of interest. Vectors were sequenced and tested
227 for expression before use.

228

229 **Animals and study design.**

230 Female BALB/c mice (The Jackson Laboratory) were randomly allocated to groups. Mice

231 received RhAd52 vectors expressing different versions of the SARS-CoV-2 S protein or sham
232 controls ($N=10$ per group). Animals received a single immunization of 10^9 viral particles (VPs)
233 of RhAd52 vectors by the intramuscular route without adjuvant. In some cases, eight weeks later,
234 mice received a homologous boost immunization. At indicated timepoints, peripheral blood was
235 collected via the submandibular route to isolate serum for immunologic assays. For viral challenge,
236 mice were administered 1×10^4 PFU MA10 SARS-CoV-2 in a volume of $50\mu\text{L}$ via the intranasal
237 route [14]. Following challenge, body weights were assessed daily. Subsets of animals were
238 euthanized on days 2 and 4 post-challenge for viral outgrowth assays. All animal studies were
239 conducted in compliance with all relevant local, state and federal regulations and were approved
240 by the Beth Israel Deaconess Medical Center and University of North Carolina at Chapel Hill
241 Institutional Animal Care and Use Committees.

242

243 **ELISA.**

244 S and RBD-specific binding antibodies were assessed by ELISA essentially as described
245 [10, 28]. Briefly, plates were coated with $1 \mu\text{g ml}^{-1}$ of SARS-CoV-2 S protein (Sino Biological)
246 or SARS-CoV-2 RBD protein (Aaron Schmidt, Massachusetts Consortium on Pathogen
247 Readiness), diluted in $1\times$ PBS, and incubated at 4°C overnight. After incubation, plates were
248 washed once with a wash buffer (0.05% TWEEN-20 in $1\times$ PBS) and blocked with $350 \mu\text{l}$ of casein
249 per well. The block solution was discarded after 2-3 hours of incubation at room temperature and
250 plates were blotted dry. Three-fold serial dilutions of mouse serum in casein block were added to
251 wells and plates were incubated for 1 hour at room temperature. Plates were then washed three
252 times and rabbit anti-mouse IgG HRP (Jackson ImmunoResearch), diluted 1:1000 in casein block,
253 was added to wells and incubated at room temperature in the dark. After 1 hour, plates were washed

254 three times, and 100 μ l of SeraCare KPL TMB SureBlue Start solution was added to each well.
255 Development was halted with the addition of 100 μ l of SeraCare KPL TMB Stop solution per well.
256 The absorbance at 450 nm was recorded using a VersaMax microplate reader. ELISA endpoint
257 titers were defined as the highest reciprocal serum dilution that yielded an absorbance > 0.2. The
258 raw OD values were transferred into GraphPad Prism for analysis. A standard curve was
259 interpolated using a sigmoidal four-parameter logistic (4PL) fit. To quantify the endpoint titer, the
260 interpolation function was used to calculate the dilution at which the OD value would be equal to
261 a value of 0.2.

262

263 **Pseudovirus neutralization assay.**

264 A SARS-CoV-2 pseudovirus expressing a luciferase reporter gene was generated in an
265 approach similar to as described previously [10, 28, 29]. Briefly, the packaging construct psPAX2
266 (AIDS Resource and Reagent Program), luciferase reporter plasmid pLenti-CMV Puro-Luc
267 (Addgene) and S protein expressing pcDNA3.1-SARS CoV-2 S.dCT were co-transfected into
268 HEK293T cells using lipofectamine 2000 (Thermo Fisher Scientific). After 48 hours, supernatant
269 was collected and pseudotype viruses were purified by filtration with a 0.45- μ m filter. To
270 determine the neutralization activity of the antisera from vaccinated animals, HEK293T-hACE2
271 target cells were seeded in 96-well tissue culture plates at a density of 1.75×10^4 cells per well and
272 incubated overnight. Three-fold serial dilutions of heat-inactivated serum were prepared and
273 mixed with 50 μ l of pseudovirus. The mixture was incubated at 37 °C for 1 hour before adding to
274 HEK293T-hACE2 cells. 48 hours after infection, cells were lysed in Steady-Glo Luciferase
275 (Promega) according to the manufacturer's instructions. Neutralization titers were defined as the
276 sample dilution at which a 50% reduction in relative light units was observed relative to the average

277 of the virus control wells.

278

279 **Live virus neutralization assay.**

280 Live virus neutralization of sera was determined using a nanoLuciferase-expressing SARS-
281 CoV-2 virus (SARS-CoV-2-nLuc), bearing wild-type spike protein, as described [37, 38], with
282 slight modification. Briefly, Vero E6 cells were seeded at 2×10^4 cells per well in a 96-well plate
283 24 hours before the assay. 90 PFU of SARS-CoV-2-nLuc virus were mixed with serial diluted sera
284 at 1:1 ratio and incubated at 37 °C for 1hour. An 8-point, 3-fold dilution curve was generated for
285 each sample with starting concentration of 1:20. Virus and serum mix was added to cells and
286 incubated at 37 °C + 5% CO₂ for 48 hours. Luciferase activity was measured by Nano-Glo
287 Luciferase Assay System (Promega) following manufacturer protocol using SpectraMax M3
288 luminometer (Molecular Device). Fifty percent neutralization titer (NT50) was calculated in
289 GraphPad Prism by fitting the data points to a sigmoidal dose-response (variable slope) curve.

290

291 **PFU assay.**

292 Lung viral titers were determined by plaque assay. Briefly, right caudal lung lobes were
293 homogenized in 1mL PBS using glass beads and serial dilutions of the clarified lung homogenates
294 were added to a monolayer of Vero E6 cells and overlaid with a solution of 0.8% agarose and
295 media. After three days, plaques were visualized via staining with Neutral Red dye and counted.

296

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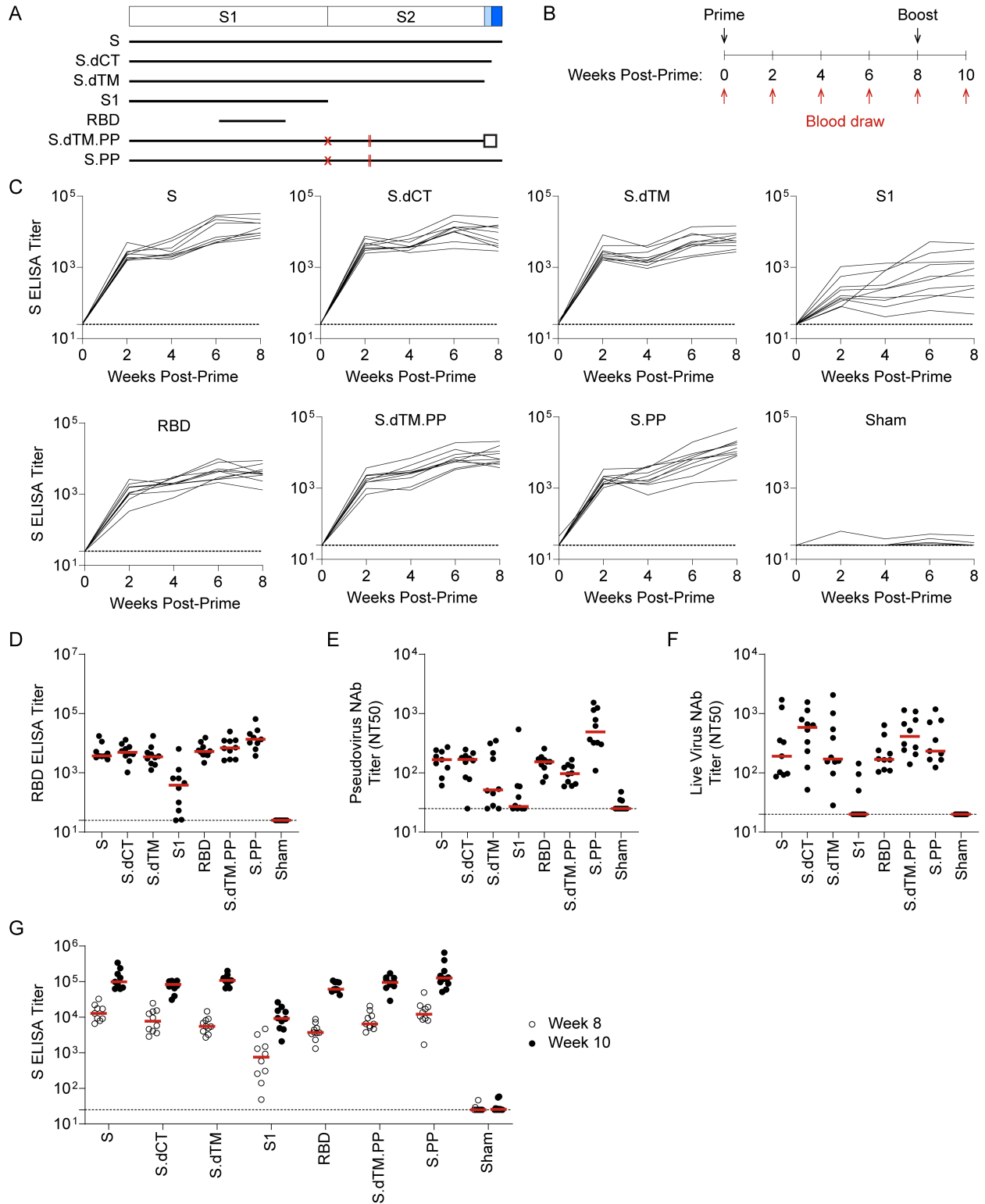
313

314 **Author Contributions**

315 L.H.T., L.E.G., D.R.M., R.S.B., and D.H.B. designed and planned experiments. Z.F.L., J.L., and
316 D.H.B. designed the RhAd52 vector vaccines. Z.F.L. and F.N. synthesized and characterized
317 RhAd52 vectors. L.H.T. and S.H.M. performed the immunogenicity phase of the mouse studies.
318 L.H.T., H.W., and K.A.M. performed ELISA assays. J.Y. and A.C. performed the pseudovirus
319 neutralization assays. D.R.M. performed the live virus neutralization assays. D.R.M., L.E.G., and
320 A.S. performed the MA10 challenge phase of the mouse studies. D.R.M., L.E.G., A.S., K.H.D.,
321 and S.R.L. performed the viral outgrowth plaque assays. L.H.T., L.E.G., and D.R.M. analyzed data
322 and performed statistical analyses. L.H.T. and D.H.B. wrote the manuscript with input from and

323 review by all authors.

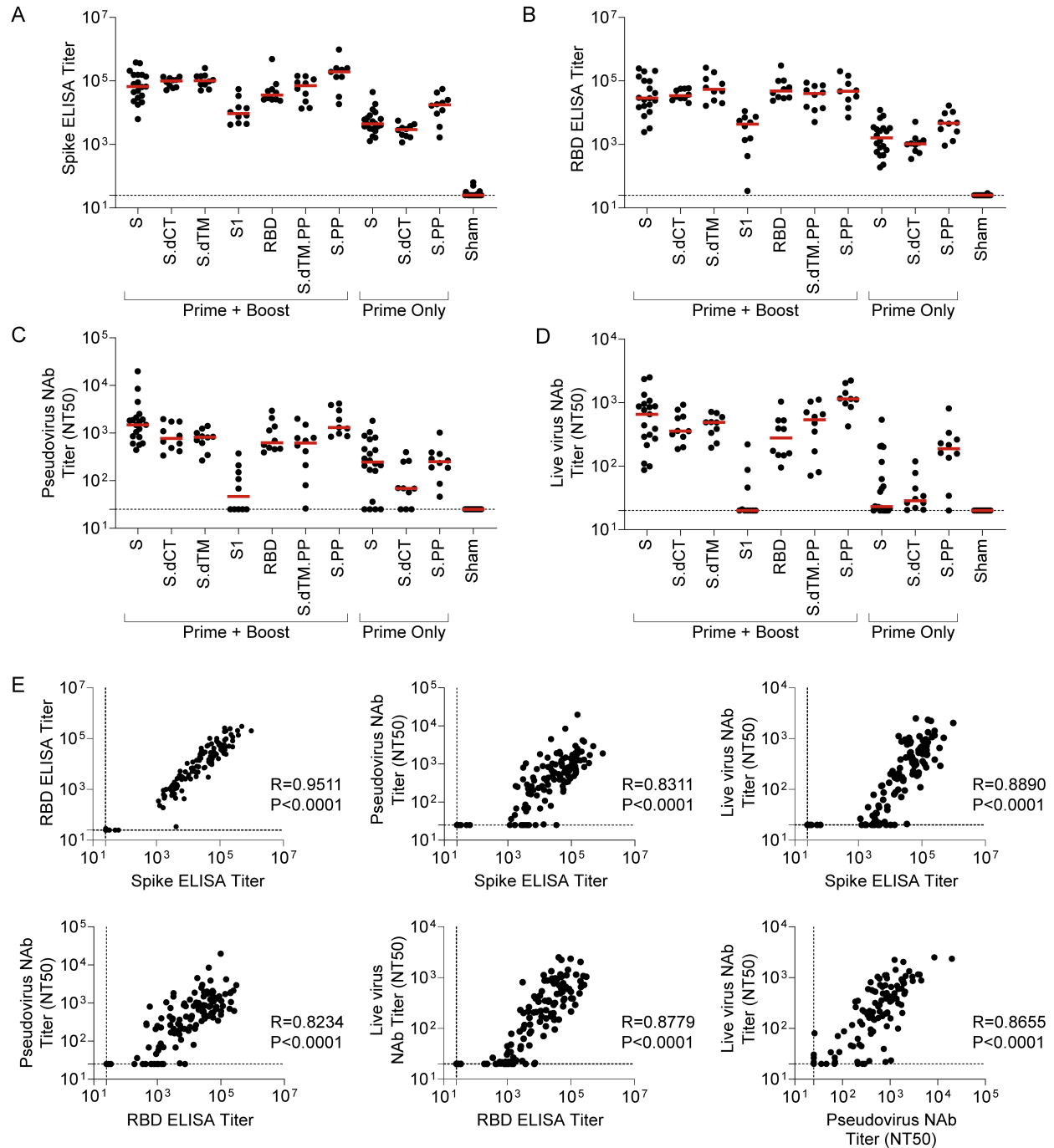
324 **Figures and Figure Captions**



325 **Fig 1. RhAd52 vaccines elicit robust S-specific binding antibody responses.** A) A series of
326 replication incompetent RhAd52 vectors, encoding for variations on the SARS-CoV-2 Spike (S)

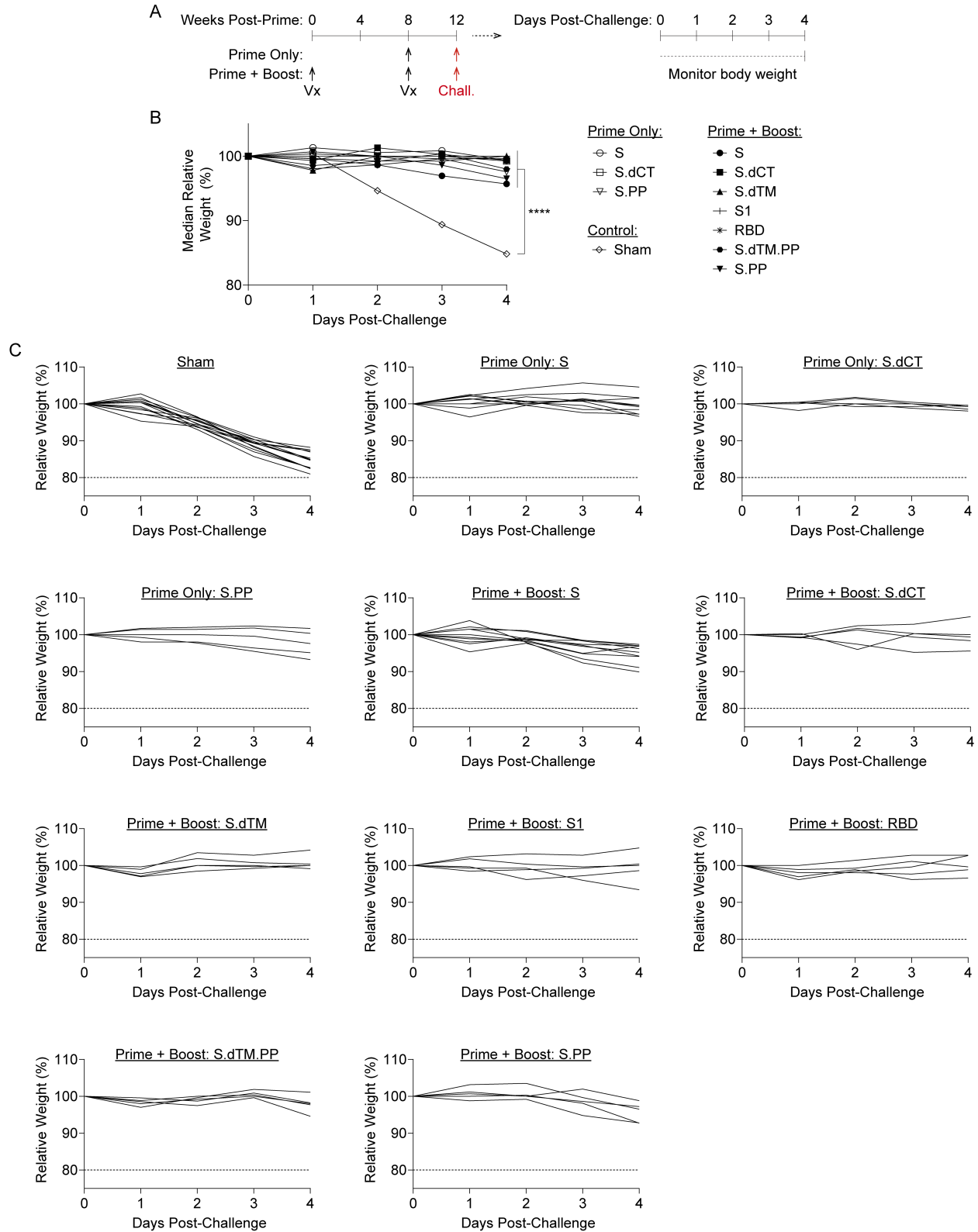
327 protein, was designed. Inserts included i) S, ii) deletion of the cytoplasmic tail (S.dCT), iii) deletion
328 of the transmembrane domain and cytoplasmic tail (S.dTM), iv) the S1 domain with a foldon
329 trimerization tag, v) the receptor binding domain (RBD) a foldon trimerization tag, vi) deletion of
330 the transmembrane domain and cytoplasmic tail, with mutation of the furin cleavage site (red X),
331 addition of stabilizing proline mutations (red lines), and a foldon trimerization tag (S.dTM.PP),
332 and vii) S with mutation of the furin cleavage site (red X) and addition of stabilizing proline
333 mutations (red lines) (S.PP). B) To explore the immunogenicity of these vaccine candidates, wild-
334 type BALB/c mice were immunized at week 0 with 10^9 viral particles (VPs) of candidate RhAd52
335 vaccines or sham. Peripheral blood was collected at baseline and every two weeks following
336 vaccination to monitor antibody responses in serum. Eight weeks post-prime, mice were
337 administered a homologous boost to explore the potential to boost responses. C) For each RhAd52
338 insert, as well as sham controls, S-specific binding antibody responses were quantified through
339 enzyme-linked immunosorbent assay (ELISA) in serum every two weeks post-prime. D) The
340 distribution of RBD-specific ELISA titers at week 8 across candidate RhAd52 vectors. Red lines
341 indicate the median titer of each group. Neutralizing activity of vaccine-elicited antibody responses
342 were assessed through E) pseudovirus or F) live SARS-CoV-2 virus *in vitro* neutralization assays.
343 The 50% neutralization titer (NT50) is displayed, with the median of each vaccine regimen
344 indicated with a red line. G) The distribution of S-specific ELISA titers at week 8 (open circles)
345 and week 10 (two weeks post-boost, closed circles) was measured to characterize the
346 immunogenicity of a homologous boost with candidate RhAd52 vaccines. Red lines indicate the
347 median titer of each group. For panels C-G, N=9-10 mice/group and endpoint binding titers are
348 reported. Representative data from one of two similar experiments are shown.

349
350



351
 352 **Fig 2. Serum binding and neutralizing antibody responses are tightly linked following**
 353 **RhAd52 vaccination.** Groups of mice were administered a prime (week 0) and a boost (week 8)
 354 of 10^9 VP the indicated RhAd52 vaccines. At the time of boost (i.e., week 8), additional groups of
 355 mice were administered a single dose (i.e., Prime Only) of 10^9 VP of select indicated RhAd52
 356 vaccines. At week 12, serum was analyzed. A) S-specific and B) RBD-specific ELISA titers are
 357 shown, with median titer for each regimen indicated with a red line. Neutralizing activity of
 358 vaccine-elicited antibody responses were assessed through C) pseudovirus or D) live SARS-CoV-
 359 2 virus neutralization assays. The 50% neutralization titer (NT50) is displayed, with the median of

360 each vaccine regimen indicated with a red line. E) Spearman correlation analyses of binding and
361 neutralizing antibody responses are displayed. For panels A-E, data are pooled from two similar
362 experiments. For panels A-D, N=9-10 mice/group for all regimens, with the exception of
363 RhAd52.S Prime + Boost (N=19), RhAd52.S Prime Only (N=20), and Sham (N=23). In Panel E,
364 data from all vaccine regimens are pooled to explore the relationship between binding and
365 neutralizing antibody function independent of the RhAd52 insert.

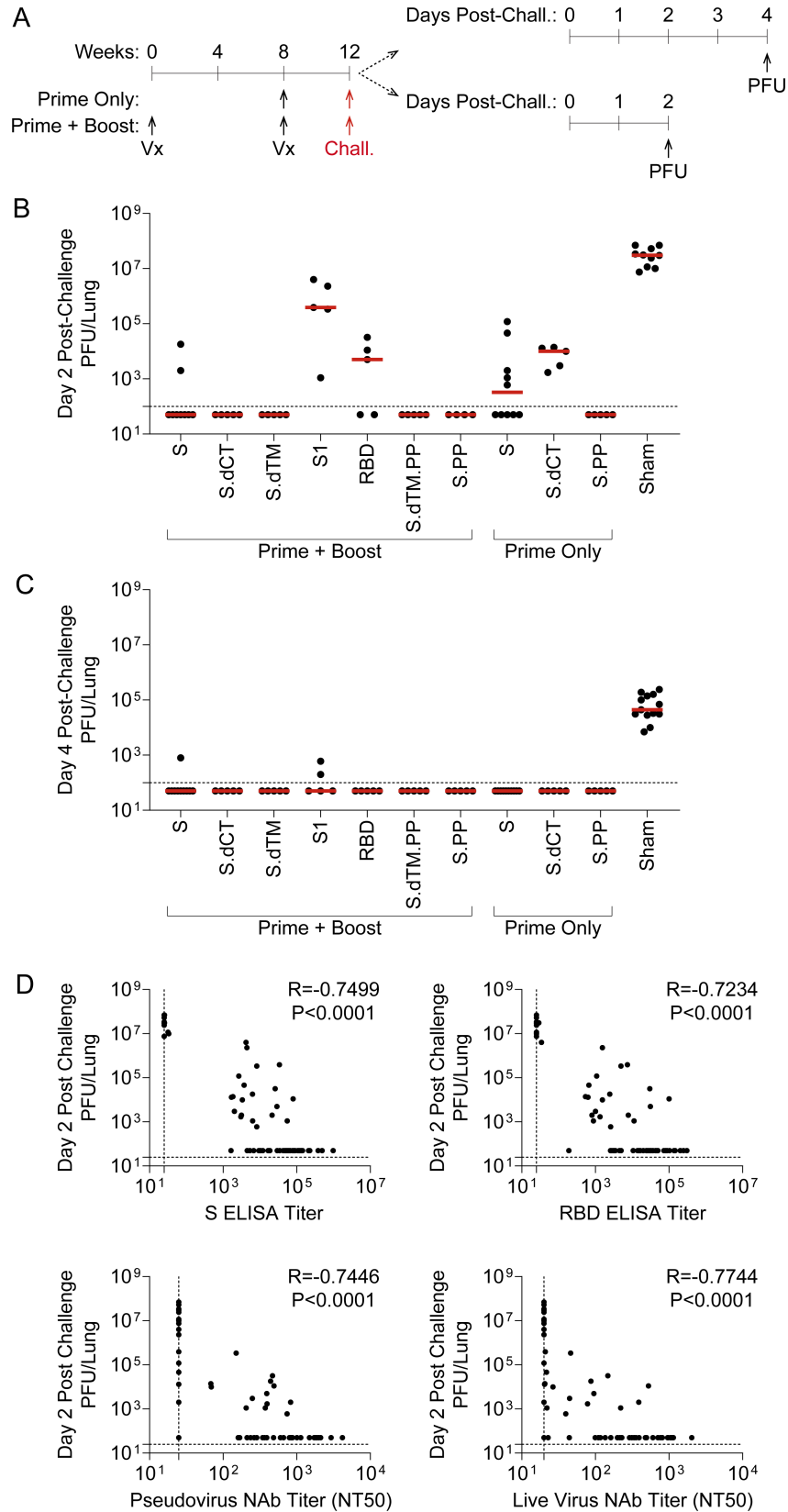


366 **Fig 3. RhAd52 vaccines protect from clinical disease following mouse-adapted SARS-CoV-**
 367 **2 challenge.** A) Groups of mice were immunized with either a prime and boost or a prime only

368 of 10^9 viral particles (VPs) of RhAd52 candidate vaccines following the indicated timeline. At
369 week 12, mice were challenged with 10^4 PFU of MA10 SARS-CoV-2 via the intranasal route.
370 After challenge, a subset of mice was followed through day 4 post-challenge to monitor for signs
371 of clinical disease. B) Relative body weight following MA10 SARS-CoV-2 challenge in mice
372 immunized with the indicated vaccine regimens. Median value of each group is displayed.
373 $P < 0.0001$ indicates results of a one-way ANOVA analysis followed by Dunnett's multiple
374 comparisons, comparing vaccinated groups to the sham control group. C) Traces of relative body
375 weight in individual mice, immunized with the indicated RhAd52 vaccine regimen, following
376 challenge. For panels B-C, data are pooled from two similar experiments. $N = 5$ mice/group for all
377 regimens, with the exception of RhAd52.S Prime + Boost ($N = 10$), RhAd52.S Prime Only
378 ($N = 10$), and Sham ($N = 13$).

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380



381 **Fig 4. RhAd52 vaccine-elicited antibody responses link to restraint of viral replication in the**
 382 **lung following mouse-adapted SARS-CoV-2 challenge.** A) Groups of mice were immunized

383 with either a prime and a boost or a prime only of 10^9 viral particles (VPs) of RhAd52 candidate
384 vaccines. At week 12, mice were challenged with 10^4 PFU of MA10 SARS-CoV-2 via the
385 intranasal route. A subset of mice was monitored through day 4 post-challenge; at the terminal
386 timepoint, lungs were harvested to measure virus via outgrowth assays to quantify plaque forming
387 units (PFU) per tissue. The second subset of mice were followed through day 2 post-challenge for
388 similar PFU assays at the time of peak viral replication. B-C) Quantification of PFU per lung at
389 B) day two and C) day 4 post challenge. Median of each group indicated by the red line. N=4-5
390 mice/group for all regimens, with the exception of RhAd52.S Prime + Boost (N=9), RhAd52.S
391 Prime Only (N=10), and Sham (N=10). D) Spearman correlation analyses of pre-challenge serum
392 binding or neutralizing antibody responses with day two post-challenge viral titers in lung are
393 displayed. For panels B-C, data are pooled from two similar experiments. In Panel E, data from all
394 vaccine regimens are pooled to explore the relationship between pre-challenge binding and
395 neutralizing antibody responses and virologic endpoint independent of the RhAd52 insert.

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