

1 Article

2 A synthetic peptide CTL vaccine targeting nucleocapsid confers 3 protection from SARS-CoV-2 challenge in rhesus macaques

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30 Abstract

31
32 **Background:** Persistent transmission of severe acute respiratory syndrome
33 coronavirus 2 (SARS-CoV-2) has given rise to a COVID-19 pandemic. Several
34 vaccines, evoking protective spike antibody responses, conceived in 2020, are
35 being deployed in mass public health vaccination programs. Recent data sug-
36 gests, however, that as sequence variation in the spike genome accumulates,
37 some vaccines may lose efficacy.

38 **Methods:** Using a macaque model of SARS-CoV-2 infection, we tested the effi-
39 cacy of a peptide-based vaccine targeting MHC Class I epitopes on the SARS-
40 CoV-2 nucleocapsid protein. We administered biodegradable microspheres
41 with synthetic peptides and adjuvants to rhesus macaques. Unvaccinated con-
42 trol and vaccinated macaques were challenged with 1×10^8 TCID₅₀ units of

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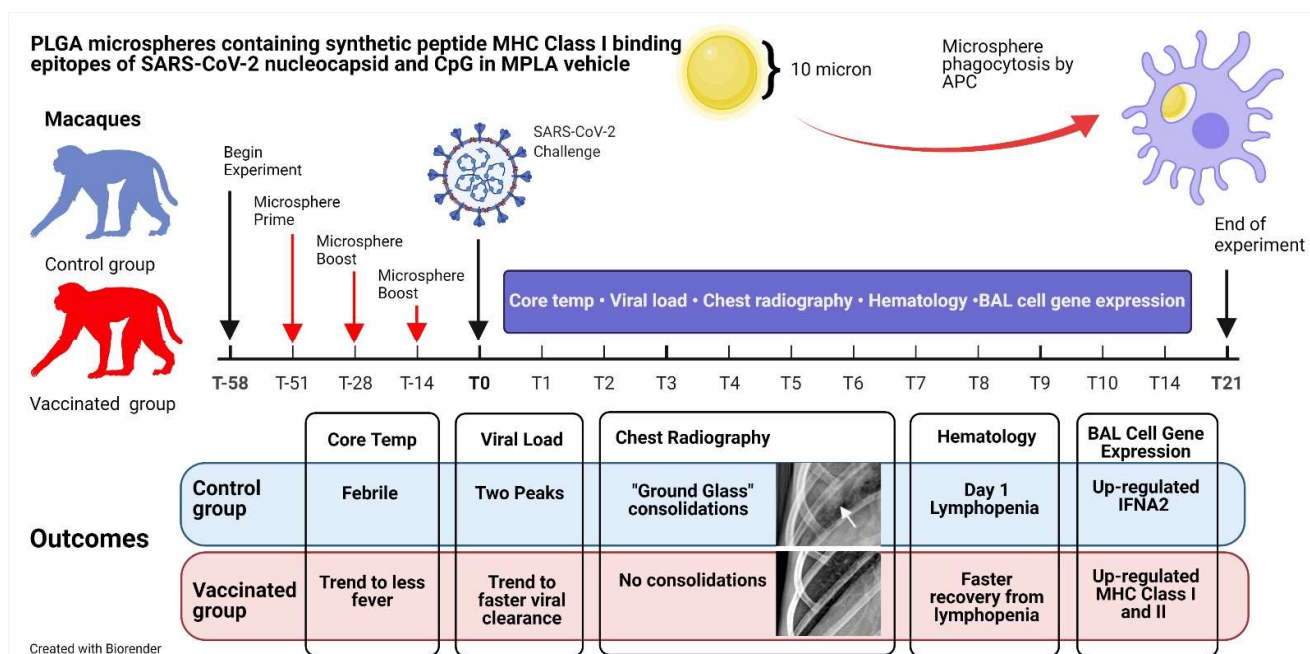
SARS-CoV-2, followed by assessment of clinical symptoms, viral load, chest radiographs, sampling of peripheral blood and bronchoalveolar lavage (BAL) fluid for downstream analysis.

Results: Vaccinated animals were free of pneumonia-like infiltrates characteristic of SARS-CoV-2 infection and presented with lower viral loads relative to controls. Gene expression in cells collected from BAL samples of vaccinated macaques revealed a unique signature associated with enhanced development of adaptive immune responses relative to control macaques.

Conclusions: We demonstrate that a room temperature stable peptide vaccine based on known immunogenic HLA Class I bound CTL epitopes from the nucleocapsid protein can provide protection against SARS-CoV-2 infection in non-human primates.

Keywords: SARS-CoV-2; animal model; macaque; vaccine; MHC Class I peptide; T-cell

Graphical Abstract



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68 **1. Introduction**

69

70 As of April 2021, over 1.6% of the world population have had confirmed COVID-19 disease and the
71 new case rate is about one-half million per day. Less than 2 % of the world's population are vac-
72 cinated against SARS-CoV-2[1]. Confounding efforts to reach herd immunity to COVID-19 disease
73 include, but are not limited to the following: 1) the spread of SARS-CoV-2 mutations affecting the
74 efficacy of current iterations of vaccines and therapeutic biologics[2], 2) the speed of SARS-CoV-2
75 vaccine deployment, development, and manufacture, and 3) in the context of global public health,
76 issues related to vaccine hesitancy, cold supply chain requirements, the total manufacturing cost
77 per dose, and ease of administration.

78

79 We have previously described a novel vaccine platform [3,4], which may address many of the above
80 concerns and now report on its efficacy in a rhesus macaque model of SARS-CoV-2 infection. Ma-
81 caque models of COVID-19 disease have been previously reported and have served as critical tools
82 for understanding disease pathology and for the development and testing of vaccines and thera-
83apeutics [5-21]. While the clinical course of SARS-CoV-2 infection in macaques is milder relative to
84 that observed in humans [6,8,20,21], the macaque model remains the gold standard for preclinical
85 evaluation of COVID-19 vaccines [10,19,22-27]. Our overall approach focuses on promoting protective
86 T-cell immunity using synthetic peptides delivered in biodegradable microspheres together with
87 Toll-like receptor (TLR) 4 and 9 adjuvants and differs from current COVID-19 vaccines against spike
88 proteins. The synthetic peptide sequences applied are based on known immunogenic HLA Class I
89 bound epitopes that have previously been characterized in either SARS-CoV-1 [28] or SARS-CoV-2
90 [29-31] infections. We provide evidence that application of this vaccine platform in SARS-CoV-2 chal-
91 lenged macaques provides protection from pneumonia-like pathology observed in virally chal-
92 lenged but unvaccinated control non-human primate (NHP) subjects, reduces viral loads as com-
93 pared to unvaccinated controls, and induces changes in the gene expression patterns in recovered
94 BAL cells consistent with enhanced antigen presentation capacity and markers of T-cells.

95

96 **2. Materials and Methods**

97

98 **A. Macaque MHC Class I Typing**

99

100 The MHC Class I genes of a cohort of 15 rhesus macaques (Envigo, Alice, TX, USA) were molecu-
101 larly-typed by the University of Wisconsin-Madison National Primate Research Center. Mamu
102 (*Macaca mulatta*) MHC class I alleles were typed amplifying the genomic DNA of each subject using
103 a panel of specific primers for exon 2 of all known MHC-A and MHC-B alleles encoded by each
104 subject's target DNA. Resulting amplicons were sequenced by the Illumina MiSeq method [32]. The
105 primer panel contained specific primer pairs able to amplify all possible MHC-A and MHC-B alleles
106 encoded by each macaque. Sequencing data analysis provided a high-resolution haplotype for the
107 MHC-A and MHC-B alleles carried by each subject. Following analysis, we selected four of the 15
108 macaques for vaccination based on peptide MHC binding predictions as described below.

109

110 **B. Vaccine Design/Peptide Selection and Manufacture**

111

112 *In silico design and selection of SARS-CoV-2 CTL epitopes.*

113 The overall strategy and rationale used in the selection of synthetic peptides used to stimulate po-
114 tential CTL immune responses in SARS-CoV-2 infected humans has been previously described [3,4].
115 We selected SARS-CoV-2 nucleoprotein as the target of CTL attack based on the following rationale:
116 1) survivors of SARS-CoV-1 have shown a memory T-cell response to nucleoprotein at least 2 years
117 after infection [28], 2) there is >90% amino acid sequence homology between SARS-CoV-1 and SARS-
118 CoV-2 nucleoprotein (the homology for the selected CTL epitopes used in this report is 100%) [33],
119 and 3) in general, there is a lower frequency of mutations resulting in amino acid substitutions (rel-
120 ative to Spike protein) that might affect the immunogenicity of the selected CTL epitopes repre-
121 sented by synthetic peptides within the vaccine formulation [34]. The lower mutation frequency may
122 reflect the hypothesis that amino acid substitutions in nucleoprotein may impact viral fitness [35].
123 We reviewed previous literature and MHC peptide-binding databases [36] and selected five amino
124 sequences representing SARS-CoV-2 nucleoprotein with predicted strong *in vitro* affinity for HLA
125 Class I molecules[37], and/or documented or predicted immunogenic potential[4,28-31,33,38,39]. To-
126 gether this set of peptides yielded potential broad coverage of HLA haplotypes (>90% worldwide)
127 (**Supplemental data, Table S1A**). The predicted binding of this set of peptides was examined within
128 the MHC genotypes of the cohort of 15 rhesus macaques [40] available to us and the best correspond-
129 ence between selected peptides and rhesus MHC Class I genotype was selected (**Supplemental**
130 **data, Table S1B**). Because the predicted peptide macaque MHC binding coverage for the peptide
131 LLLDRLNQL was incomplete in the available genotypes, we added an additional peptide (AS-
132 AFGMSR) with predicted strong Mamu MHC Class I binding to the formulation for a total of six
133 peptides.

134

135 *Microsphere preparation and adjuvant formulation*

136 The peptide epitopes used in this study were delivered *in vivo* by intratracheal instillation of a
137 formulation of Poly-L-lactide-co-glycolide (PLGA) microspheres containing the corresponding syn-
138 thetic nine-mer peptides and TLR-9 agonist CpG oligonucleotide adjuvant in a vehicle containing
139 TLR-4 agonist monophosphoryl lipid A (MPLA). The rationale for the choice of the delivery plat-
140 form and the basic manufacturing scheme used in production has been previously reported [3,4,41].
141 Briefly, room temperature solutions of a synthetic peptide, CpG oligonucleotide, and mannose were
142 mixed with a solution of PLGA in acetone/water followed by sonication. The formulation was then
143 processed through a precision spray-drying device (Buchi Corporation, New Castle, DE, USA) and
144 passed through a drying chamber (air at room temperature) to allow evaporation of the acetone.
145 The dry microsphere stream was analyzed in real-time through a laser particle size analyzer (Spray-
146 Tech, Malvern Instruments, Malvern, PA) before collection (Buchi cyclone drier) as a dry powder
147 for reconstitution at the time of delivery using a 2% DMSO aqueous solution containing MPLA (20
148 µg/ml). Each microsphere contained peptide loaded at approximately 0.1% by weight and CpG
149 0.01% by weight. Monitoring of the microsphere diameters allowed the production of microspheres
150 with a mean diameter of 10 ± 2 microns. This diameter was selected for formulation to ensure de-
151 livery via phagocytosis of no more than 1-4 microspheres per antigen-presenting cell (APC) which
152 have an average diameter of 13 microns [3]. cGMP manufacturing protocols were employed using
153 GMP grade synthetic peptides (Peptides International, Louisville, KY, USA), CpG oligonucleotides

154 (Trilink Biosciences, San Diego, CA, USA), and MPLA (Avanti Polar Lipids, Alabaster, AL, USA).
155 The CpG oligonucleotide and MPLA used in this study were manufactured using the same chemical
156 compositions as equivalent materials used in FDA-approved vaccines. Assessment of thermal sta-
157 bility of the synthetic peptides within the microspheres has been previously reported [3]. Peptide
158 content and structure in microspheres were determined by HPLC after two months of room tem-
159 perature storage. We found that over 99% of the peptide was maintained structurally intact (data
160 not shown).

161

162 **C. Animal Studies**

163

164 *Ethics statement*

165 The animal research protocols used in this study were performed in strict accordance with the rec-
166 ommendations in the Guide for Care and Use of Laboratory Animals, Eighth Edition (National
167 Academy Press, Washington, D.C., 2011). The University of Texas Medical Branch (UTMB) facility
168 where these studies were conducted is accredited by the Association for Assessment and Accredi-
169 tation of Laboratory Animal Care. The protocols were approved by the UTMB Institutional Animal
170 Care and Use Committee (Protocol Numbers 2004051 [natural history/control study] and 2003033
171 [vaccination study]) and complied with the Animal Welfare Act, the U.S. Public Health Service Pol-
172 icy, and other Federal statutes and regulations related to animals and experiments involving ani-
173 mals. All hands-on manipulations, including immunizations and biosampling, were performed
174 while animals were sedated via ketamine (5 mg/kg)/dexmedetomidine (0.025 mg/kg) intramuscular
175 injection. All efforts were made to minimize suffering.

176

177 *Macaques*

178 Adult Indian origin rhesus macaques (*Macaca mulatta*, n = 7 [5 male, 2 female], 46-48 months old) or
179 Vietnamese origin cynomolgus macaques (*Macaca fascicularis*, n = 1 female, 84 months old), individ-
180 ually identified via unique tattoo, were obtained from Envigo/Covance (Alice, Texas, USA). All an-
181 imals were considered healthy by a veterinarian before being placed on study. Macaques were
182 individually housed in stainless steel nonhuman primate caging equipped with squeeze backs for
183 the duration of the studies. For continuous core body temperature measurements, a DST micro-T
184 implantable temperature logger (Star-Oddi, Gardabaer, Iceland) was surgically implanted into the
185 peritoneal cavity of each animal prior to study initiation; data recording was set to 10- or 15-min
186 intervals for control and vaccinated macaques, respectively. Certified primate Diet 5048 was pro-
187 vided to the macaques daily. Drinking water (RO) was provided ad libitum through an automatic
188 watering system. To promote and enhance the psychological well-being of the animals, food enrich-
189 ment consisting of fresh fruits and vegetables was provided daily. Environmental enrichment in-
190 cluding various manipulatives (Kong toys, mirrors, and puzzles) was also provided.

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192 **D. Immunization, Virus Challenge, Post-Challenge Monitoring and Biosampling**

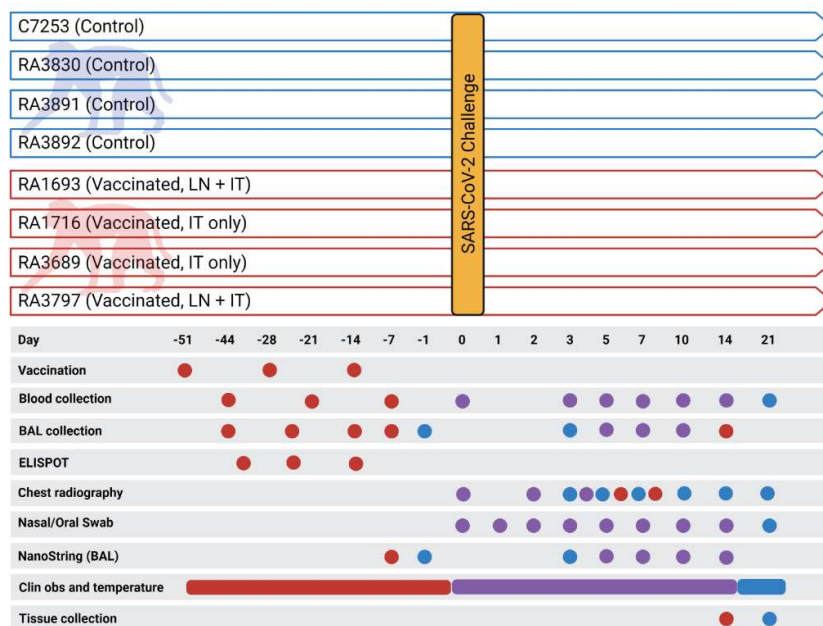
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194 *Immunization and ELISPOT analysis*

195 Immunizations were performed on the selected MHC-typed rhesus macaques (n = 4) via ultra-
196 sound-guided inguinal lymph node injection (LN) and/or intratracheal instillation (IT). Twenty mg

197 of vaccine microsphere preparation in 1ml was used for each LN injection (two injections / dose /
198 animal) and 100mg of vaccine microspheres in a 5 ml volume was used for each IT administration.
199 Specifically, on Day -51 (51 days prior to virus challenge), two of the macaques (**Figure 1**) were
200 administered 2 mL of vaccine via LN injection (1 mL per node). Subsequent administration of the
201 vaccine occurred via the IT route (5 mL) only as described previously[42]. Remaining vaccine
202 doses, administered on Days -28 and -14, were delivered via IT only (5 mL per dose) to the rhesus
203 vaccination group. On Days -44, -21, and -7 (7 days post-vaccination), femoral vein peripheral
204 blood (8 mL) was collected from each animal into a BD Vacutainer® CPT™ Cell Preparation Tube
205 with Sodium Heparin (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and processed
206 to peripheral blood mononuclear cells (PBMCs) per manufacturer instructions. Collected PBMCs
207 were assessed for immunoreactivity via ELISPOT. In brief, ELISPOT assay plates (MabTech Inc.,
208 Cincinnati, OH, USA) specific for the detection of primate IFN γ were used according to manufac-
209 turer instructions. BAL cell concentrations were adjusted to 1×10^5 cells per mL in a complete growth
210 medium. Diluted BAL cells were dispensed (100 μ L/well) into a 96-well plate after which 100 μ L of
211 complete growth medium (CGM, negative control), Concanavalin A in CGM at 10 μ g per well (pos-
212 itive control), and various concentrations of specific (i.e., immunizing) and non-specific peptides
213 (**Supplemental data, Table S1B**) were added. Peptides used for immunization were added to wells
214 at a concentration of 50 μ M. All samples were assayed in duplicate. Plates were incubated at
215 37°C/5% CO $_2$ for 20-22 hours after which plates were thoroughly washed. Conjugated detection
216 antibody was then added and incubated followed by additional washing. Wells were developed
217

218 Figure 1



220
221

222 **Figure 1 Legend.** Schematic of the experimental protocol. Unvaccinated (control) macaques are
223 represented by blue coloring. Vaccinated macaques are represented by red coloring. Overlapping
224 tasks are represented by purple coloring. Graphic created with BioRender.com.

225 using TMB as a substrate. Counts were performed at Cellular Technology Corporation (Shaker
226 Heights, OH, USA) using an Immunospot Analyzer and all well images were quality-controlled on
227 site. All spot-forming cell counts reported are the result of averaging counts from the duplicate
228 50 μ M immunization-specific peptide wells.

229

230 *Virus challenge*

231 On Day 0, macaques were administered 1-5 $\times 10^8$ TCID₅₀ SARS-CoV-2 (USA_WA1/2020) via com-
232 bined mucosal atomization (1 mL as delivered using a MAD Nasal™ Intranasal Mucosal Atomiza-
233 tion Device per manufacturer instructions) and intratracheal instillation (4 mL). Intratracheal instil-
234 lations were performed as described above for delivery of the vaccine. The virus suspension was
235 prepared on the day of challenge from frozen seed stock (kindly provided by Dr. Chien-Te [Kent]
236 Tseng at UTMB) initially generated (one passage) in Vero C1008 (E6) cells (BEI Resources, NR-596,
237 Lot 3956593) from original material provided by the Centers for Disease Control and Prevention in
238 January 2020. Next-generation sequencing confirmed a 100% consensus sequence-level match to the
239 original patient specimen (GenBank accession MN985325.1).

240

241 *Post-challenge monitoring and chest radiography*

242 Animals were monitored and scored twice daily for clinical signs of disease including alterations in
243 activity/appearance (i.e., hunched posture), food consumption/waste output, and were scored
244 based on general appearance, activity, food consumption, and outward changes in breathing pat-
245 terns. Prospectively defined criteria that required immediate euthanasia included severe dyspnea
246 and/or agonal breathing and prostate posture/reluctance to move when stimulated. No animals met
247 endpoint criteria during the study. Ventrolateral chest radiography was performed on the days in-
248 dicated (**Figure 1**) using a portable GE AMX-4+ computed radiography system per manufacturer
249 instruction. DICOM data files were independently evaluated by two independent investigators
250 blinded to group assignment with large animal imaging experience via a four-pattern approach
251 (analyses of consolidation, interstitial areas, nodules or masses, and atelectasis).

252

253 *Biosampling*

254 Blood, nasal cavity samples, and BAL fluid were collected at the indicated times (**Figure 1**). Femoral
255 vein peripheral blood was collected via Vacutainer® into standard collection tubes containing eth-
256 ylenediaminetetraacetic acid (EDTA). Hematology was performed on EDTA blood using the Abaxis
257 VETSCAN® HM5 Hematology Analyzer (Abaxis, Inc., Union City, CA, USA). Nasal cavity samples,
258 collected using sterile cotton-tipped medical swabs, were placed into 0.5 mL sterile phosphate-buff-
259 ered saline (PBS) for viral load analysis. For BAL fluid collection, animals were sedated as previ-
260 ously described and placed in ventral recumbency. The trachea was visualized and cannulated by
261 an appropriately sized rubber feeding tube. Following the placement of the feeding tube, 20mL of
262 sterile PBS was introduced into the lung and recovered manually through the feeding tube via sy-
263 ringe. This was repeated for a total of 40mL per animal. The total collected volume from each animal
264 (10-30mL) was pooled and centrifuged under ambient conditions (10 min at 500 x g) after which the
265 supernatant was removed. The resulting cell pellet was resuspended in 2 mL of sterile PBS. From
266 this, 1 mL was used for ELISPOT analysis as described for PBMCs. The remaining volume was used
267 for viral load analysis and gene expression profiling.

268 **E. Viral Load Analysis**

269

270 *Infectious viral load (TCID₅₀)*

271 Nasal swab and BAL cells suspension samples were serially diluted and incubated with 2×10^4 Vero
272 C1008 (E6) cells (BEI Resources, NR-596, Lot 3956593) in 100 μ l of culture medium (MEM/2% FBS)
273 in 96-well flat-bottom plates (n = 5 replicate wells per dilution). Each plate contained negative and
274 positive control wells inoculated with culture medium and diluted virus stock, respectively. Cul-
275 tures were incubated at 37°C/5% CO₂ for 96h after which cytopathic effect was measured via mi-
276 croscopic observation. The TCID₅₀/mL value for each sample was calculated as previously de-
277 scribed[43]. Macaque C75243 (cynomolgus) was not included in this study.

278

279 *qRT-PCR*

280 Nasal swab and BAL cell suspension samples (50 μ L) were added to TRIzol® LS Reagent (250 μ L)
281 and allowed to incubate under ambient conditions for 10 min. Samples were processed to RNA
282 using Zymo Direct-zol™ RNA Mini Prep kits per manufacturer instructions. RNA samples were
283 analyzed via qRT-PCR targeting the SARS-CoV-2 E gene. Probe (Integrated DNA Technologies,
284 Coralville, IA, USA) was labeled at the 5'-end with fluorophore 9-carboxyfluorescein (6-FAM) and
285 included an internal quencher (ZEN) and a 3'-end quencher (IowaBlackFQ, IABkFQ). Master Mix
286 was prepared by combining forward primer (250 nM, 5'-ACAGGTACGTTAATAGTTAATAGCGT-
287 3'), reverse primer (250 nM, 5'-ATATTGCAGCAGTACGCACACA-3'), and probe (375 nM, 5'-
288 6FAM-ACACTAGCC/ZEN/ATCCTTACTGCGCTTCG-IABkFQ-3') with 12.5 μ L of 2X QuantiFast
289 Probe Mix (QIAGEN), 0.25 μ L of 2X QuantiFast RT Mix (QIAGEN), and PCR-grade water (fill to 20
290 μ L). To the Master Mix, a test sample (5 μ L) was added resulting in a final volume of 25 μ L per reac-
291 tion. Real-time analysis was performed using the Bio-Rad CFX96™ Real-Time PCR Detection Sys-
292 tem. Thermocycling conditions were as follows: Step 1, 1 cycle, 50°C for 10 minutes; Step 2, 1 cycle,
293 95°C for 10 minutes; Steps 3-5, 45 cycles, 95°C for 10 seconds, 60°C for 30 seconds, single read. Neg-
294 ative controls included reaction mixtures without RNA. For quantification purposes, viral RNA ex-
295 tracted from the virus seed stock with a known TCID₅₀/mL titer was used. All qRT-PCR results are
296 expressed as TCID₅₀/mL equivalents. Macaque C75243 (cynomolgus) was not included in this study.

297

298 **F. Gene Expression Profiling**

299

300 BAL samples were processed to RNA as described above for qRT-PCR analysis. RNA quantity and
301 quality were assessed using a NanoDrop™ Lite Spectrophotometer (ThermoFisher Scientific, Wal-
302 tham, MA, USA). Samples, normalized to 20ng/ μ L, were analyzed by NanoString Technologies (Se-
303 attle, WA, USA) using the nCounter® SPRINT™ Profiler gene expression profiling using the Non-
304 Human Primate Immunology V2 Panel containing 754 genes that encompass 17 immune-related
305 signaling pathways with isoform coverage for both *Macaca mulatta* and *Macaca fascicularis*. Probe
306 sets that did not cover both *Macaca* species were eliminated resulting in a probe set of 730 genes.
307 Raw gene expression data sets received from NanoString Technologies were processed to remove
308 background signals and normalized using the nSolver™ V.3.0 digital analyzer software. Back-
309 ground signal correction was accomplished by subtracting the NanoString negative control genes.

310 Gene expression normalization was performed using the 16 internal reference genes included in the
311 panel.

312

313 **G. Study Termination**

314

315 At scheduled study termination time points (14 and 21 days post-challenge for vaccinated and con-
316 trol macaques, respectively), animals were humanely euthanized via intravenous administration of
317 a pentobarbital-based euthanasia solution under deep anesthesia followed by bilateral thoracotomy.

318

319 **H. Statistical Analysis**

320

321 Descriptive statistics were performed using Microsoft Excel. Hypothesis testing was performed by
322 considering the null hypothesis of the absence of an association between the compared variables.
323 The statistical strength of associations of continuous data was tested using Students *t*-testing. Qlu-
324 core Omics Explorer 3.5 (Qlucore), Metascape (Metascape.org) was used to identify the discriminat-
325 ing variables within the NanoString gene expression data sets from BAL sample analysis that were
326 most significantly different between vaccinated and control subjects. The identification of signifi-
327 cantly differential variables between the two groups was performed by fitting a linear model for
328 each variable. The set of genes (87 variable genes out of a total of 730 genes) was identified using a
329 p-value of 0.05, at least a three-fold change, and a q-value cutoff of 0.1. P-values were adjusted for
330 multiple testing using the Benjamini-Hochberg method [44]. Gene expression data were scaled to a
331 mean = 0 and a variance = 1 before clustering. Hierarchical clustering of gene expression in BAL was
332 performed using a supervised weighted average linkage two comparisons approach. The metric
333 used in scaling dendrogram arms was Pearson's correlation coefficient.

334

335 **3. Results**

336

337 **A. Primary Clinical Outcomes**

338

339 *Clinical signs, body temperature alterations and hematology.*

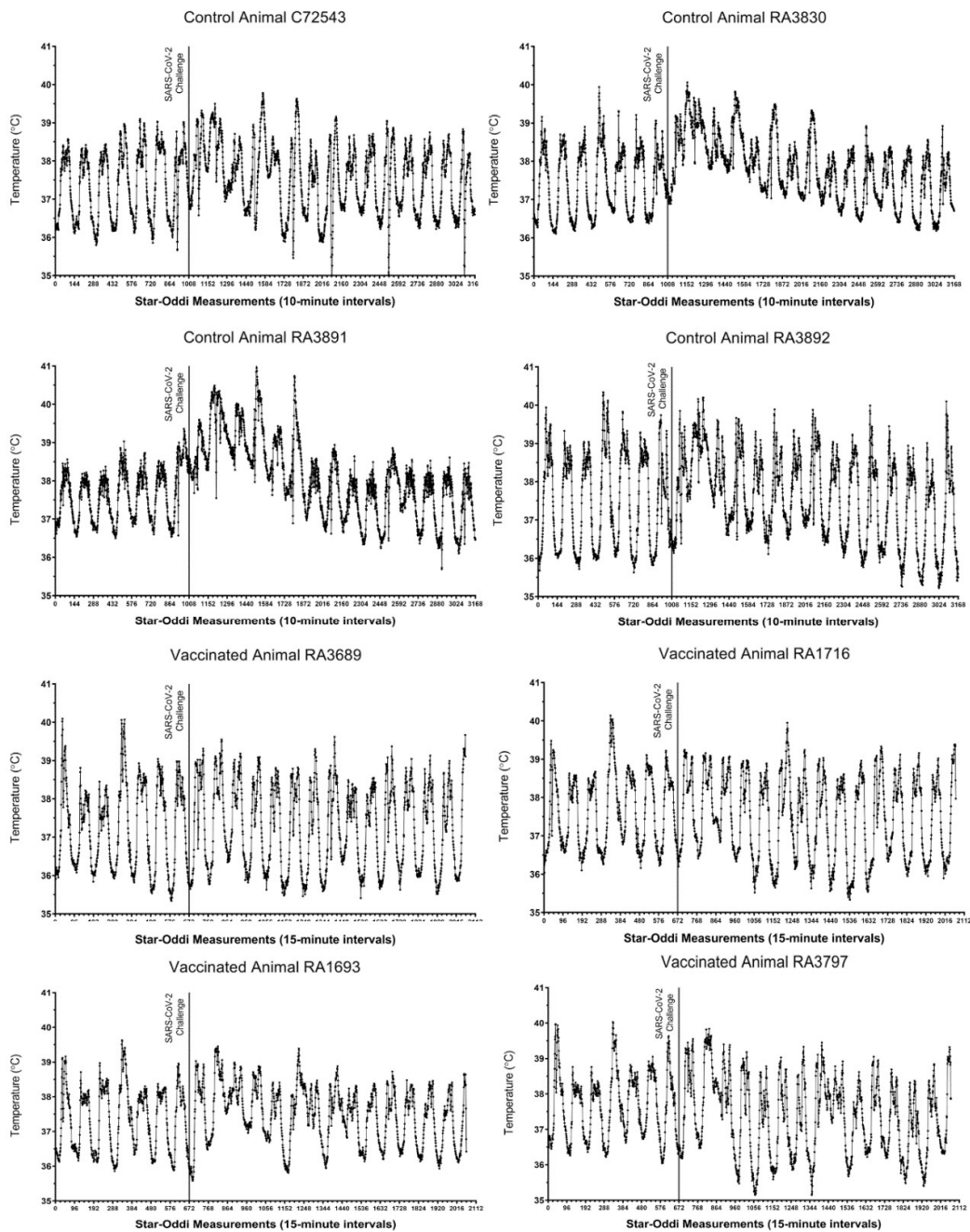
340 Following SARS-CoV-2 challenge, outward clinical signs measured in control macaques included
341 acute mild lethargy and respiratory distress. All vaccinated animals were normal throughout the
342 post-challenge study period. Core body temperatures, as measured via implanted Star-Oddi DST
343 temperature loggers, demonstrated a disruption in the diurnal cycle and mild fever lasting 2-5 days
344 post-challenge in all four-control animals (**Figure 2, top panels**). Conversely, only two of the four
345 vaccinated macaques (RA1693 and RA3797) presented with similar findings, although diurnal cycle
346 disruption was of shorter duration (1-2 days) and the febrile response was milder (**Figure 2, bottom**
347 **panels**). No alterations were measured in the remaining two vaccinated animals. Prior to virus chal-
348 lenge, vaccinated macaques presented with occasional disruptions in the diurnal temperature asso-
349 ciated with the vaccination procedure (**Supplemental Data, Figure S1**).

350

351 Automated hematology analyses were performed on peripheral blood samples (**Supplemental**
352 **Data, Figure S2**). Overall, the number of white blood cells was significantly increased in control

353 **Figure 2**

354



355

356

357 **Figure 2 Legend. Core body temperature alterations in control and vaccinated macaques follow-**
358 **ing SARS-CoV-2 challenge.** For each animal, seven days of pre-challenge baseline temperature
359 measurements are shown. Each tick on the x-axis represents 6 hours or 36 individual logger mea-
360 surements.

361 subjects on post-challenge days 3 and 5. We observed general lymphopenia in all macaques on day
362 1 post-challenge. By day 3 post-challenge, lymphocyte counts significantly increased in vaccinated
363 subjects relative to unvaccinated control subjects. Peripheral blood monocyte counts generally
364 peaked on days 1 through 5 in all animals but remained significantly elevated in vaccinated ma-
365 caques at the end of the study. Neutrophil counts generally rose by day 1 post-challenge in all sub-
366 jects. There was a transient significant elevation in neutrophils on day 7 post-challenge in vaccinated
367 animals.

368

369 *Viral load*

370 Following SARS-CoV-2 challenge, nasal swab and BAL fluid samples were collected throughout the
371 post-challenge period for analysis of infectious viral load and viral RNA via TCID₅₀ and qRT-PCR
372 assays, respectively. Infectious virus was measured from nasal swabs of control and vaccinated ma-
373 caques beginning one-day post-challenge (**Figure 3** [top panel]). By Day 7, 3 of the 4 unvaccinated
374 animals continued to demonstrate infectious viral shedding, albeit at low levels. In contrast, infec-
375 tious virus could be measured in only one of the vaccinated macaques at the same time point. By 10
376 days post-challenge, infectious viral loads were undetectable in nasal swab samples from all ani-
377 mals. Viral RNA in nasal swabs generally reflected infectious viral loads. By Day 7, three of the four
378 vaccinated animals demonstrated a 100-fold decrease in nasal swab viral RNA relative to the un-
379 vaccinated controls (**Figure 3** [bottom panel]). By 14 days post-challenge, SARS-CoV-2 RNA levels
380 were undetectable in nasal swab samples from all subjects. Infectious viral load data were used to
381 calculate an average viral clearance rate post-challenge for each rhesus macaque. The average viral
382 clearance rate from Days 2 through 10 was 4-5 fold higher in two of the four vaccinated macaques
383 (RA1693 and RA3797) relative to the unvaccinated controls (**Supplemental data, Figure S3**).

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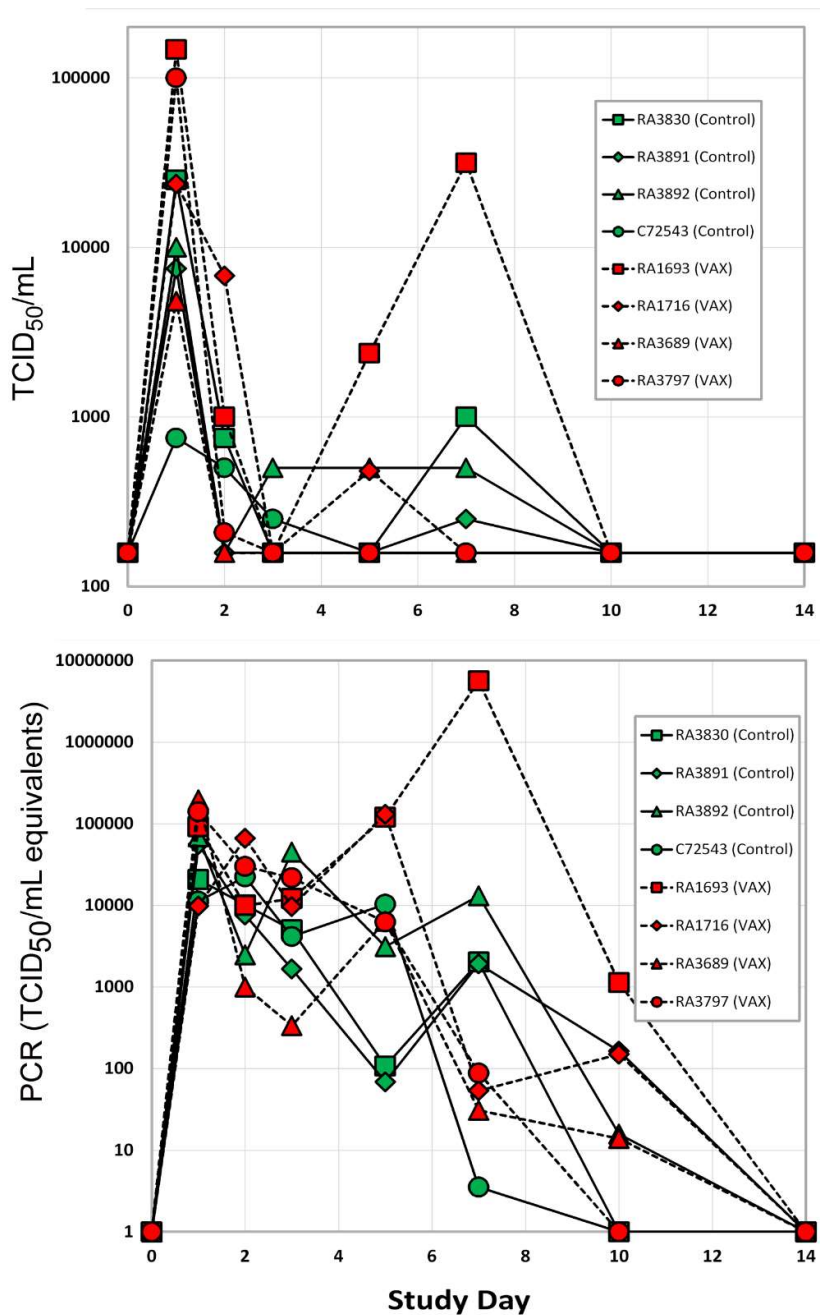
385 *Macaque chest radiography*

386 SARS-CoV-2 challenge in unvaccinated controls resulted in mild-to-moderate lung abnormalities,
387 similar to those previously reported for macaques [6,15,16,20,45-48]. These were predominantly lim-
388 ited to the caudal lung relative to baseline images, peaked 3-5 days post-challenge, and were quali-
389 tatively characteristic of subclinical or mild-to-moderate human COVID-19 (e.g., ground-glass opac-
390 ities with or without reticulation, paving, silhouetting, and/or linear opacities). The mild to moder-
391 ate interstitial pneumonitis seen on the ventrolateral chest radiographs of unvaccinated subjects are
392 consistent with focal infiltrates representing a complex of interstitial macrophages, neutrophils, and
393 plasmacytoid dendritic cells [49]. Abnormalities in control animals resolved by Days 10-21. In con-
394 trast, vaccinated macaques lacked the appearance of ground-glass opacities in all regions of the lung
395 throughout the study period (**Figure 4** and **Supplemental data, Figures S4-S9**). We did observe,
396 however, modest bilateral increases in reticulation in vaccinated macaques on Days 3-5, but these
397 abnormalities also resolved by Day 10-21. Bronchoalveolar lavage has been reported to affect com-
398 puterized tomography X-ray results in healthy rhesus macaques [50]. In our study, however, the
399 pattern of reported changes in vaccinated (i.e., healthy) animals on which BAL was performed was
400 more similar to increases in reticulation versus the patchy consolidations observed in the unvac-
401 cinated controls.

402

403 **Figure 3.**

404



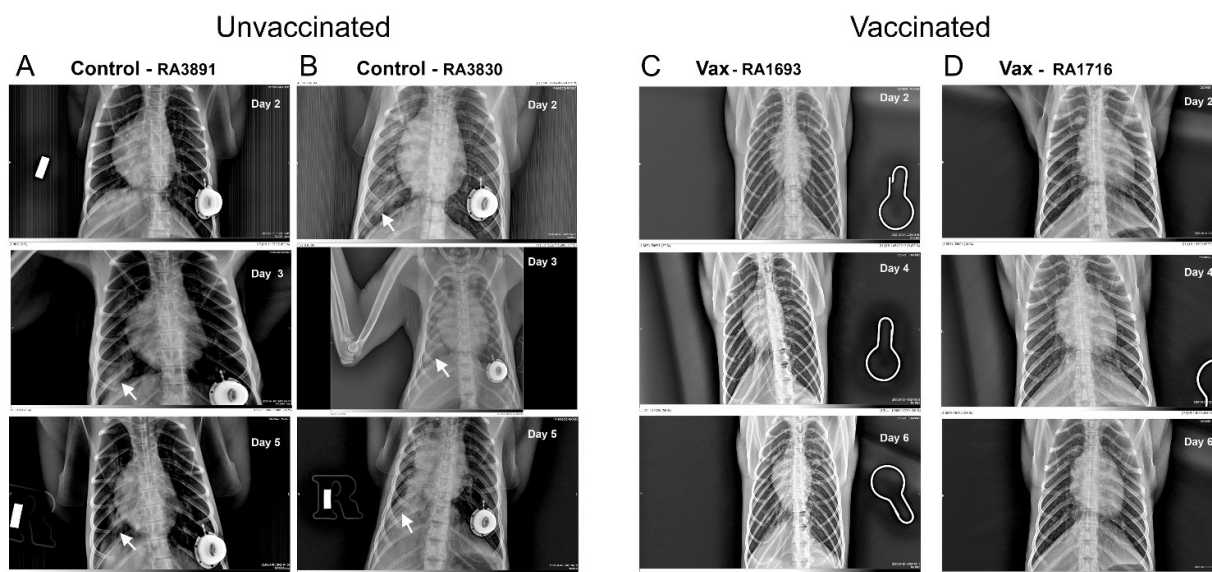
405

406

407 **Figure 3 legend.** Viral load in nasal swab samples as measured via TCID₅₀ assay (top) and qRT-
408 PCR (bottom). The LLOD of the plaque assay was 150 units. Red symbols are vaccinated rhesus
409 macaques, control unvaccinated rhesus subjects are shown in green symbols.

410 **Figure 4**

411



412

413

414 **Figure 4 Legend. Representative chest radiographs of control and vaccinated macaques follow-**
415 **ing SARS-CoV-2 challenge.** As shown, control macaques (left columns A and B) demonstrated a
416 progression of pulmonary infiltrates during the acute period (Days 2-5) of disease post-challenge.
417 In contrast, vaccinated macaques (right columns C and D) lacked similar abnormalities. White ar-
418 rows indicate areas of mild to moderate pulmonary infiltrates seen as ground glass consolidations.

419 **B. Secondary Outcomes**

420

421 *Analysis of gene expression patterns in BAL cells*

422 We identified a set of 87 genes in BAL samples collected 5-7 days post-challenge from control ani-
423 mals with statistically significant differential expression (as measured from changes in accumula-
424 tion of their specific transcripts) versus BAL samples collected from vaccinated animals during the
425 same time points (**Figures 5 and 6**). We selected to focus on the Day 5 and Day 7 samples to capture
426 a possible peak of adaptive immune responses to SARS-CoV-2 challenge as suggested by previous
427 reports [51,52]. Several of the identified differentially regulated genes were of particular interest in
428 the context of adaptive viral T cell immunity (**Tables 1 and 2**). Several differentially regulated im-
429 mune response genes laying outside the main window of interest (i.e., Day 5 alone, Day 7 alone, or
430 Day 10 alone) were also identified (**Tables 1 and 2 and Supplemental data, Figure S10**). For exam-
431 ple, on Day 5 in unvaccinated macaques, we found up-regulation of IFIT3 and IL-1RAP. The ex-
432 pression levels of these transcripts have been previously reported to correlate with viral loads in a
433 SARS-CoV-2 rhesus macaque model of COVID-19 disease [5].

434

435 In BAL samples collected on Days 5 and 7, we observed statistically significant up-regulation of
436 MHC Class I genes, MHC Class II and associated accessory genes (CD74 invariant chain, HLA-DM),
437 and T cell markers (CD8 and IL2) in the vaccinated group relative to the unvaccinated control ma-
438 caques. We also observed statistically significant down-regulation of Interferon alpha 2 (IFNA2),
439 the negative regulator of T cell expansion, PD-L1, the decoy receptor for IL1 α and IL1 β inhibiting
440 signaling, and FoxJ1, a regulator of Th1 cell activation [53] in the vaccinated group relative to the
441 unvaccinated control macaques.. This pattern suggests enhanced antigen presentation and CD 4/8+
442 T cell response capacity in BAL cells from vaccinated macaques relative to the controls. In control
443 animals, we observed up-regulation of several genes (CCR1, CSF3R, IFNA2, IL-1RN, IL-1RAP, IL-
444 1R2, and SOXS3) previously reported to be activated during SARS-CoV-2 infection in rhesus ma-
445 caques [5]. These genes appeared to be down-regulated, relative to controls, in vaccinated macaques.

446

447 The vaccine formulation containing synthetic peptide cytotoxic T cell epitopes, CpG, and MPLA
448 adjuvants was delivered primarily via intratracheal instillation. While we did not observe any ad-
449 verse clinical signs of respiratory distress in vaccinated subjects, we examined BAL cell gene ex-
450 pression for lymphokines and cytokines associated with the observed mixed Th1/Th2 patterns ob-
451 served in asthma [54] (**Supplemental data, Figures S11 and S12**). In BAL samples collected on Days
452 5 and 7, we did not observe significant differences in expression of IL4, IL5, IL6, IL9, IL10, IL13,
453 CXCL10, CCL5, CCL7, CCL22, CX3CL1, or CXCL1. We did observe a general trend of higher ex-
454 pression for many of the cytokines in BAL cell samples collected from vaccinated macaques pre-
455 challenge compared to unvaccinated pre-challenge controls. This effect was generally transient, di-
456 minishing by Day 5 post-challenge. This pattern of cytokine and lymphokine expression did not
457 suggest that BAL cells assumed a phenotype associated with a Th2 response.

458

459 **Figure 5**

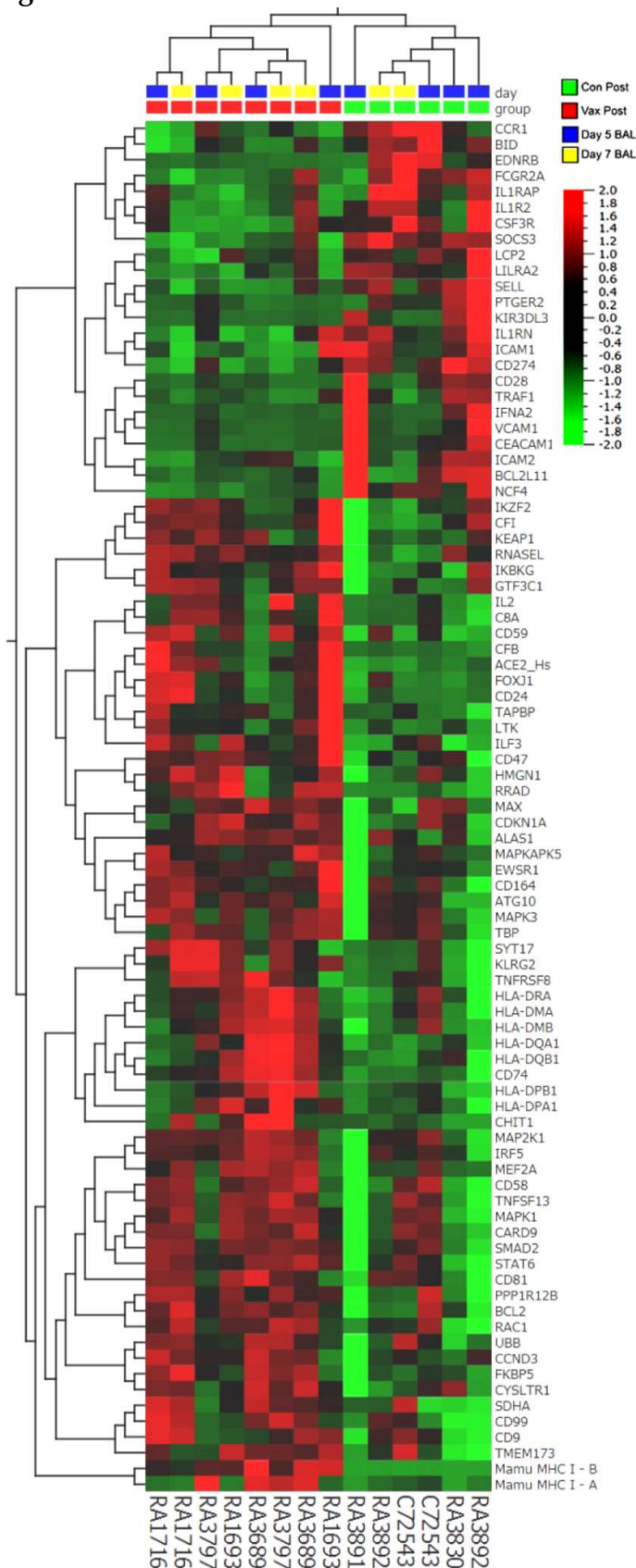
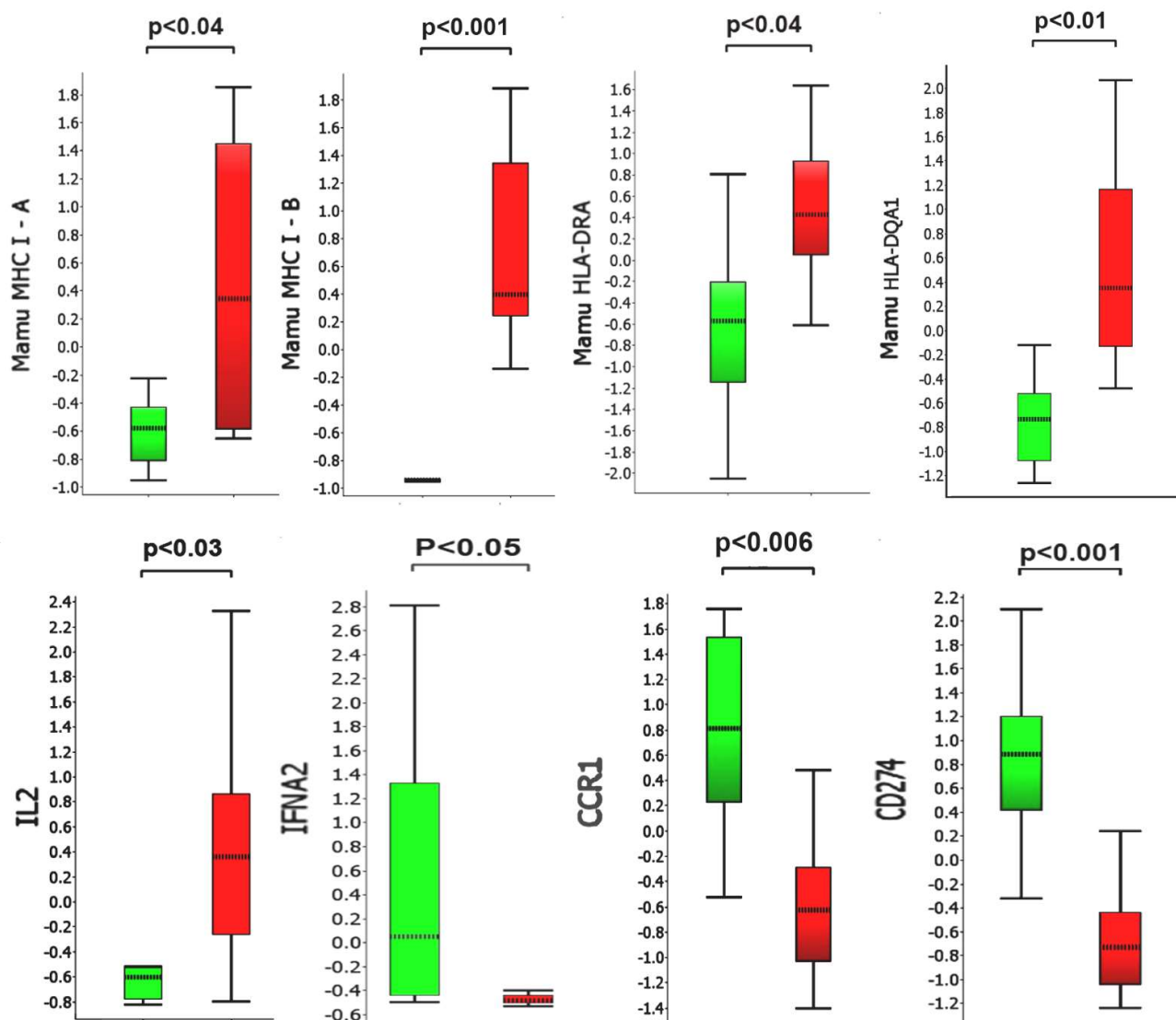


Figure 5 legend. Hierarchical clustering of gene expression in BAL samples collected from control and vaccinated macaques 5- and 7- days post SARS-CoV-2 challenge. Heatmap shows significantly ($p < 0.05$) up-regulated (red) genes (63 genes > 3 -fold) and down-regulated (green) genes (24 genes $< 1/3$ fold) from a total of 730 genes analyzed using the NanoString Non-Human Primate Immunology V2 Panel and identifies a set of genes possibly associated with protection from SARS CoV-2 challenge.

497 **Figure 6**

498



499

500

501 **Figure 6 Legend. Comparison of selected transcripts up- or down-regulated in collected BAL**
502 **samples 5-7 days post-challenge. Y-axis values represent fold differences in average scaled**
503 **counts. Green bars and red bars represent control and vaccinated macaques, respectively. P-values**
504 **below 0.05 were considered significant.**

505 **Table 1** - Differentially regulated genes in BAL cells obtained from SARS-CoV-2 challenged vac-
 506 cinated versus control macaques
 507

| Up-regulated Transcripts on Days 5 and 7 Post-Challenge ¹ | | | | | |
|--|---|--|---|--|---------------------------|
| Gene ID | Function | Distribution | Gene ID | Function | Distribution |
| Mamu MHC1 A | Antigen presentation to CD8+ T cells ¹ | Low cell type specificity ¹ | CD8 | Co-receptor for TCR binding to MHC C1 | T cells |
| Mamu MHC1 B | Antigen presentation to CD8+ T cells | Low cell type specificity | IL2 | Differentiation/maturation of T-cells | CD4+ and CD8+ T cells |
| TAPBP | MHC Class I Antigen presentation | Low cell type specificity | CD81 | Costimulatory signal with CD3 | Low cell type specificity |
| HLA-DRA ² | Antigen presentation to CD4+ T cells | Professional APC | CD9 | Cell adhesion, recognized by CD81 | Low cell type specificity |
| HLA-DQA1 ² | Antigen presentation to CD4+ T cells | Professional APC | CD59 | Inhibitor of the complement membrane attack complex | Low cell type specificity |
| HLA-DQB1 ² | Antigen presentation to CD4+ T cells | Professional APC | CD24 | Cell adhesion molecule | Eosinophils and B cells |
| CD74 | MHC Class II Antigen presentation | Professional APC | CD47 | High affinity receptor for thrombospondin-1 | Low cell type specificity |
| HLA-DMA | MHC Class II Antigen presentation | Professional APC | CD58 | Ligand of the T-lymphocyte CD2 glycoprotein | Low cell type specificity |
| HLA-DMB | MHC Class II Antigen presentation | Professional APC | CD164 | Facilitates adhesion of CD34+ cells | Low cell type specificity |
| Up-regulated Transcripts on Day 5 or 7 Post-Challenge | | | Up-regulated Transcripts on Day 10 Post-Challenge | | |
| IL17 B | Proinflammatory cytokine | Low cell type specificity | IL6R | Low affinity receptor for Interleukin 6. | Neutrophil |
| CX3CL1 | Chemotactic for T cells and monocytes | Low cell type specificity | ABL1 | Tyrosine-protein kinase, role cell growth and survival | Low cell type specificity |
| CD99 | Facilitates T cell adhesion | Low cell type specificity | TYK2 | Tyrosine-protein kinase Initiation of type I IFN signaling | Low cell type specificity |

¹Gene annotations supplied by the human protein atlas [55,56]

²Macaque equivalent of human HLA Class II

509 **Table 2** - Differentially regulated genes in BAL cells obtained from SARS-CoV-2 challenged vac-
 510 cinated versus control macaques
 511

| Down-regulated transcripts on Days 5 and 7 Post-Challenge ¹ | | | | | |
|--|--|---|---|---|-------------------------|
| Gene ID | Function | Distribution | Gene ID | Function | Distribution |
| IFNA2 | Inhibition of viral replication | Macrophages, eosinophils | CD28 | Provides co-stimulatory signals required for T cell activation. Receptor for CD80 | T cell |
| CCR1 | C-C chemokine receptor, recruitment of immune effector cells | Macrophages | IL1RAP | Coreceptor with IL1R1 in the IL-1 signaling system | Neutrophil |
| CD274 | Ligand of PD-1 (PDL1), inhibits expansion of antigen-specific CD8 ⁺ T cells and CD4 ⁺ helper cells | Monocytes, granulocytes | IL1R2 | Decoy receptor for IL1 α , IL1 β (IL1B) inhibiting signaling | Macrophage, neutrophils |
| Down-regulated Transcripts on Day 5 or 7 Post-Challenge | | | Down-regulated Transcripts on Day 10 Post-Challenge | | |
| CD80 | Receptor for CD28 and CTLA-4 on T cells | B cells and monocytes, APCs | HLA-DRA ² | Antigen presentation to CD4 ⁺ T cells | Professional APC |
| IFNGR2 | β chain of the gamma interferon receptor | B cells, APCs and neutrophils | HLA-DMA | Antigen presentation to CD4 ⁺ T cells | Professional APC |
| IL8 | C-X-C chemokine for recruitment of neutrophils | Macrophages, epithelial and endothelial cells | LY96 | Confers responsiveness to LPS | Macrophages |
| IL21 | Regulates proliferation of mature B and T cells in response to activating stimuli | Activated CD4 ⁺ T cells, NKT cells | CTSC | Cathepsin protease | Macrophages |
| DPP4 | Protease upregulated in SARS-CoV-2 [57] Possible viral entry receptor [58] | T cell CD2 | TyroBP | Mediates NK cell activation | Macrophages, monocytes |

¹Gene annotations supplied by the human protein atlas [55,56]

²Macaque equivalent of human HLA Class II

513 A major difference between pre-challenge BAL samples was that vaccinated macaques had received
514 prior intratracheal instillation of vaccine formulation containing CpG and MPLA adjuvants. To as-
515 sess the possible effects and efficacy of our vaccination procedure and formulation, we compared
516 the expression of 60 genes previously reported to be up-regulated, not by antigenic stimulation, but
517 by adjuvants alone [59-63]. We found 30 out of 60 genes examined to be significantly ($p < 0.05$) and
518 differentially (> 2 fold) regulated in BAL cells obtained from vaccinated but unchallenged macaques
519 (Day -7) relative to samples from unchallenged control animals (Day-1) (**Supplemental data, Figure**
520 **S13**). This pattern suggests that BAL-associated cells from vaccinated animals were stimulated by
521 the adjuvants in the vaccine formulation. As a further measure of vaccination efficiency, we meas-
522 ured BAL immunoreactivity to the peptides via ELISPOT prior to SARS-CoV-2 challenge (**Supple-**
523 **mental data, Figure S14**). We observed modest immunoreactivity to one of the six CTL peptides
524 (LL9) in 3 out of the 4 vaccinated pre-challenge macaques. We did not detect immunoreactivity to
525 the peptide antigens using samples of peripheral blood mononuclear cells (data not shown).

526 527 **4. Discussion**

528
529 In our study, SARS-CoV-2 infection in unvaccinated control macaques progressed similar to previ-
530 ous reports using this model. The post-viral challenge period was clinically characterized by 1) two
531 waves of infectious viral particle recovery in the nasal tissues, 2) lymphopenia on day 1 post-chal-
532 lenge in all animals, and 3) progressive development of pneumonia-like infiltrations visible on chest
533 x-rays as “ground-glass like consolidations”, but few changes associate with human SARS CoV-2
534 infection such as loss of appetite, respiratory distress, vomiting and/or diarrhea. We observed that
535 the kinetics of change in viral loads observed in control rhesus macaques were similar to those pre-
536 viously reported [11]. Additionally, the lung tissue abnormalities revealed by chest radiography
537 were similar in the kinetics of progression and depth to previous reports [6,15,16,20,45-48]. Together,
538 these observations suggest that SARS CoV-2 infection in the Rhesus model is clinically mild, a con-
539 clusion confirming some [6,8,11], but not all [64] previous reports. SARS-CoV-2 infection in micro-
540 sphere/adjuvant vaccinated macaques progressed in a pattern different from the unvaccinated con-
541 trols. Specifically, disease was characterized by a trend toward diminished recovery of infectious
542 viral particles from nasal tissues; enhance recovery of peripheral blood lymphocytes counts, and a
543 significant absence of pneumonia-like infiltrates in the lung. Together, these observations suggest
544 that vaccination conferred some degree of protection against SARS CoV-2 induced disease.

545
546 Supporting this clinical conclusion were our studies of the gene expression profiles in serially har-
547 vested BAL cells from SARS CoV-2 challenged macaques and immunoreactivity of the BAL cells. In
548 samples collected from vaccinated (but pre-viral challenge) macaque BAL cells, we found distinct
549 changes in gene expression associated with the use of TLR 4 and 9 agonists, including up-regulation
550 of CSF1 [61], IRF7 [62], and IL10[60] suggesting effective delivery of the adjuvant portion of the vac-
551 cine formulation. When we examined HLA Class I restricted immunoreactivity of the pre-challenge
552 but vaccinated macaque BAL cells towards the vaccinating peptides, we found modest to low reac-
553 tivity towards one of the peptides (LLDRLNQL) in three of four NHP subjects vaccinated.

554

555 Following vaccination and viral challenge, we found evidence of up-regulation of both Mamu MHC
556 Class I and Class II genes in macaque BAL cells relative to unvaccinated, viral challenged macaques.
557 Upregulation of HLA Class I and Class II molecules in peripheral blood mononuclear cells has been
558 reported following the measles /mumps/rubella (MMR) vaccine in MMR naïve individuals [65]. In-
559 creased signatures of M1-type macrophage APC transcripts in the BAL of SARS-CoV-2 infected
560 rhesus macaques has been previously reported [5]; however our finding of upregulated expression
561 of MHC Class II genes, a hallmark of professional APCs, appears to be unique to the BAL of our
562 vaccinated macaques. Likewise, we found increased up-regulation of the IL2 genes in vaccinated
563 relative to unvaccinated macaques. A similar finding has been reported in humans where higher IL
564 2 levels distinguish mild/asymptomatic forms of COVID-19 disease from the moderate/severe forms
565 [66]. Following viral challenge in our study (specifically, Day 5 to 7), we found down-regulation of
566 IFN α 2 genes in BAL cells recovered from vaccinated subjects relative to their unvaccinated controls.
567 The observation perhaps reflects the decreased viral loads found in vaccinated NHP subjects [5].

568
569 While antibodies are a critical component of the protective humoral immune response to pathogens,
570 antibodies that promote disease have been described and categorized as ADE [67] or VAERD, such
571 as that described in MERS-CoV or RSV patients [68-70]. Since our vaccine only delivered low molec-
572 ular weight nonameric synthetic peptides, unconjugated to any carrier, we did not expect to gener-
573 ate a significant anti-peptide humoral response. Rather, during the development and testing of the
574 microsphere synthetic peptide COVID 19 vaccine, we were mindful of evoking Th2 biased immune
575 responses, particularly those that occur in the absence of Th1 responses or appropriate T regulatory
576 cell responses [71]. The gene expression patterns of BAL cells obtained from the vaccinated ma-
577 caques in our study suggested that we did not provoke an unbalanced Th2 response by vaccination.

578
579 We note several weaknesses in our approach to demonstrate the efficacy of this experimental vac-
580 cine: 1) SARS-CoV-2 infection in Rhesus macaques resulted in only mild disease, which appears to
581 resolve by day 10-14 post-infection. Having noted this in previous reports on the SARS-CoV-2 /rhe-
582 sus model, we tried to induce more severe forms of infection using higher doses of infectious parti-
583 cles than previous reports. Based on clinical signs, we found little effect of the increased dose of the
584 virus on Rhesus SARS-CoV-2 disease severity. 2) Because of the limited amount of cells recovered
585 from the BAL, we were where unable to perform confirmatory quantitative RT-PCR analysis of the
586 unique gene expression patterns found in vaccinated versus control BAL. Nevertheless, we found
587 that many of our observations have been previously reported from studies in similar models, 3) Not
588 all the macaque chest radiographs were performed on the same day in control versus vaccinated
589 subjects, a reflection of the logistic difficulties in working under BSL $\frac{3}{4}$ conditions. We are confident,
590 however, we have captured the chest radiographic abnormalities induced by SAR-CoV-2 infection
591 of macaques (i.e. conspicuous consolidations and infiltrations prevalent in the caudal lobe of the
592 right lung), and adequately shown their absence in vaccinated viral challenged macaques, 4) we
593 only included one cynomolgus macaque in our control group. This was primarily due to macaque
594 availability at the time the study was conducted. However, this appears to have been both a strength
595 [64] and weakness of the experimental design, as we found similar lung pathology in the cynomol-
596 gus macaque as well as similar patterns of BAL gene expression as the controls Rhesus subjects [72],

597 and 5) we did not study the effects of adjuvant alone on SARS-Cov-2 infection in the Rhesus ma-
598 caques. Previous experience with this microsphere CTL vaccine platform in a murine Ebola virus
599 model has shown that adjuvant alone was not sufficient to confer protection against lethal virus
600 challenge. Protection was conferred only when the corresponding synthetic CTL peptide epitopes
601 were delivered in the microsphere [4].

602
603 We believe this report is the first demonstration of efficacy in a preclinical NHP model of SARS
604 CoV-2 infection of a synthetic peptide-based vaccine based on known and persistently immuno-
605 genic HLA Class I bound CTL peptide epitopes of SARS nucleoprotein [28]. The SARS-CoV-2 nucle-
606 oprotein genomic sequences have shown significantly reduced mutations rates compared to spike
607 protein. As such, it may represent an additional target for vaccination, perhaps in the context of a
608 booster vaccine used following SARS-CoV-2 spike protein vaccines based on recombinant protein,
609 mRNA, or adenoviral vectors [73-75]. The ready ability to change the sequence of the synthetic pep-
610 tide HLA Class I restricted CTL epitopes used in the system is an attractive feature, given the ob-
611 served rates of mutation in SARS-CoV-2 as it spreads in the human population in the future. A
612 second potentially attractive feature of this vaccine approach is that it can be delivered by aerosoli-
613 zation to the respiratory mucosa, a route previously demonstrated to generate efficiently lung-
614 dwelling tissue-resident memory T cells [76,77].

615 **5. Conclusions**

616 We demonstrate that Rhesus macaques receiving the microsphere vaccine formulation prior to viral
617 challenge are protected from pneumonia-like lung abnormalities that characterize SARS CoV-2 in-
618 fection in unvaccinated control macaques. Analysis of gene expression of cells obtained from bron-
619 chiolar lavage shows unique signatures consistent with the hypothesis that vaccination with this
620 platform induces a protective T cell response in viral challenged macaques.

622 **6. Conflict of Interest / Competing Interests Disclosures and Contributions**

623 R.R., T.Bl., S.B., S.C., R.C., T.H., L.W., P.L., and C.H. are employees of Flow Pharma, Inc. compen-
624 sated in cash and stock, and are named inventors on various issued and pending patents assigned
625 to Flow Pharma. Some of these patents pending are directly related to the study presented here.
626 P.H. is a member of Flow Pharma's Scientific Advisory Board. T.Be. is a Flow Pharma stockholder.
627 The remaining authors declare that the research was conducted in the absence of any commercial
628 or financial relationships that could be construed as a potential conflict of interest. All authors made
629 substantial contributions to: (1) the conception and design of the study (R.R., S.B., S.C., T.Br., J.C.,
630 P.H., C.H., T.Be.), or acquisition of data (T.Br. C.M. J.C., C.H.), or analysis and interpretation of data
631 (R.R., P.H., T.Br., J.C.), (2) drafting the article or revising it critically for important intellectual con-
632 tent (P.H., T.Br. S.B., T.H., T.Bl., P.L., R.R.) (3) and all authors have approved the final version of the
633 submitted manuscript.

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637
638
639

640

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643 mercial, or not-for-profit sectors.

644

645 **9. Data Availability Statement:**

646 The original DICOM files (> 20 MB/file) are available upon request.

647

648 **10. Appendix: none**

649

650 **11. References**

651

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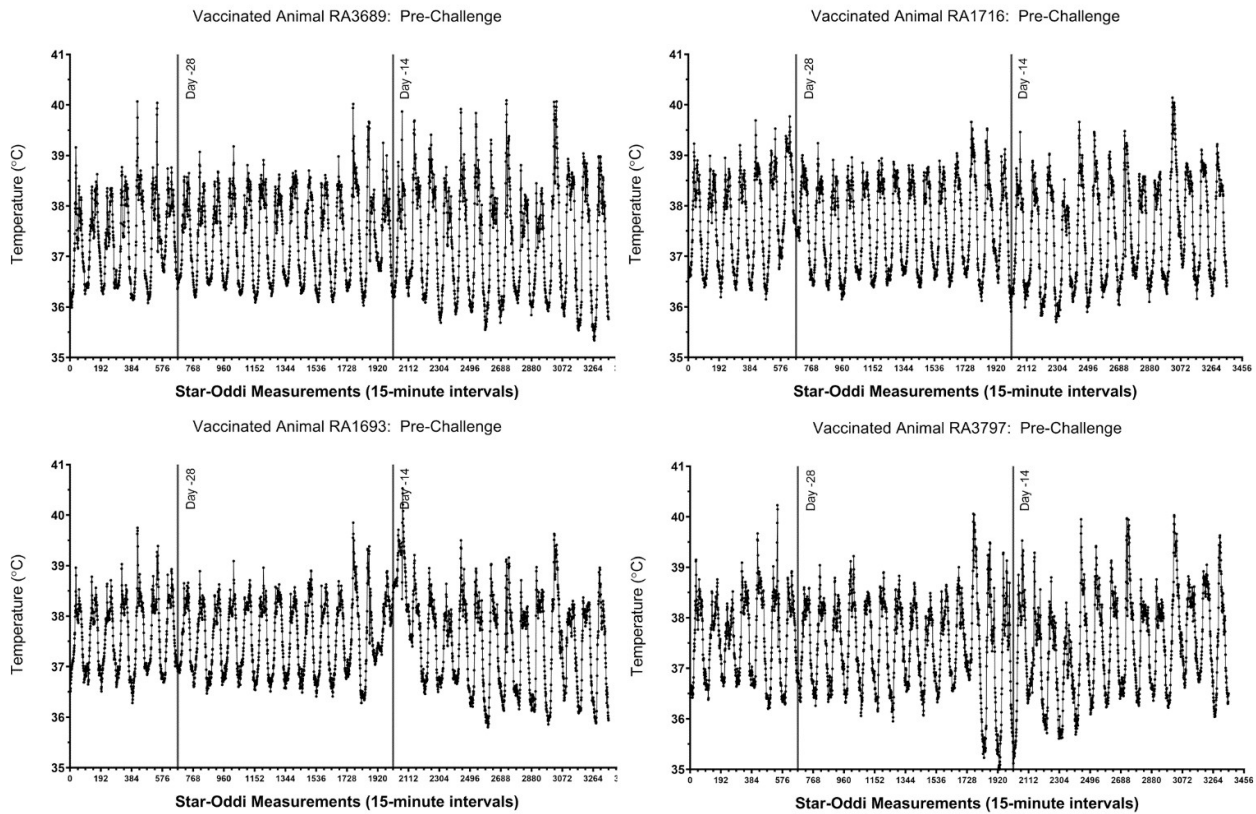
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922 **Supplemental Materials**

923

924 **Figure S1**

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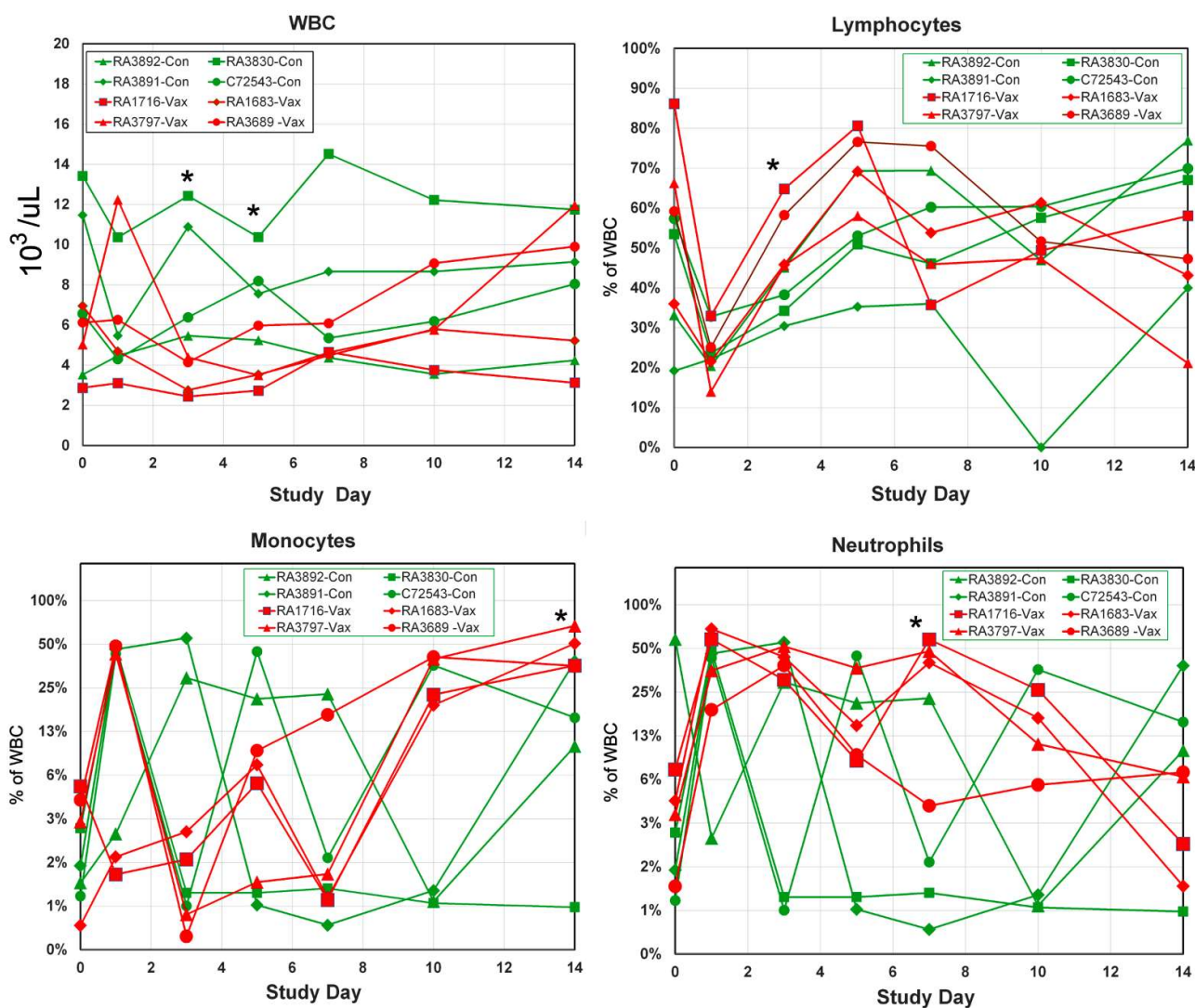
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928 **Supplemental Data, Figure S1 legend.** Core body temperature alterations in vaccinated macaques
929 prior to SARS-CoV-2 challenge. For each animal, 35 days of pre-challenge temperature measure-
930 ments are shown. Each tick on the x-axis represents 12 hours or 48 individual logger measure-
931 ments.

932 **Figure S2**

933



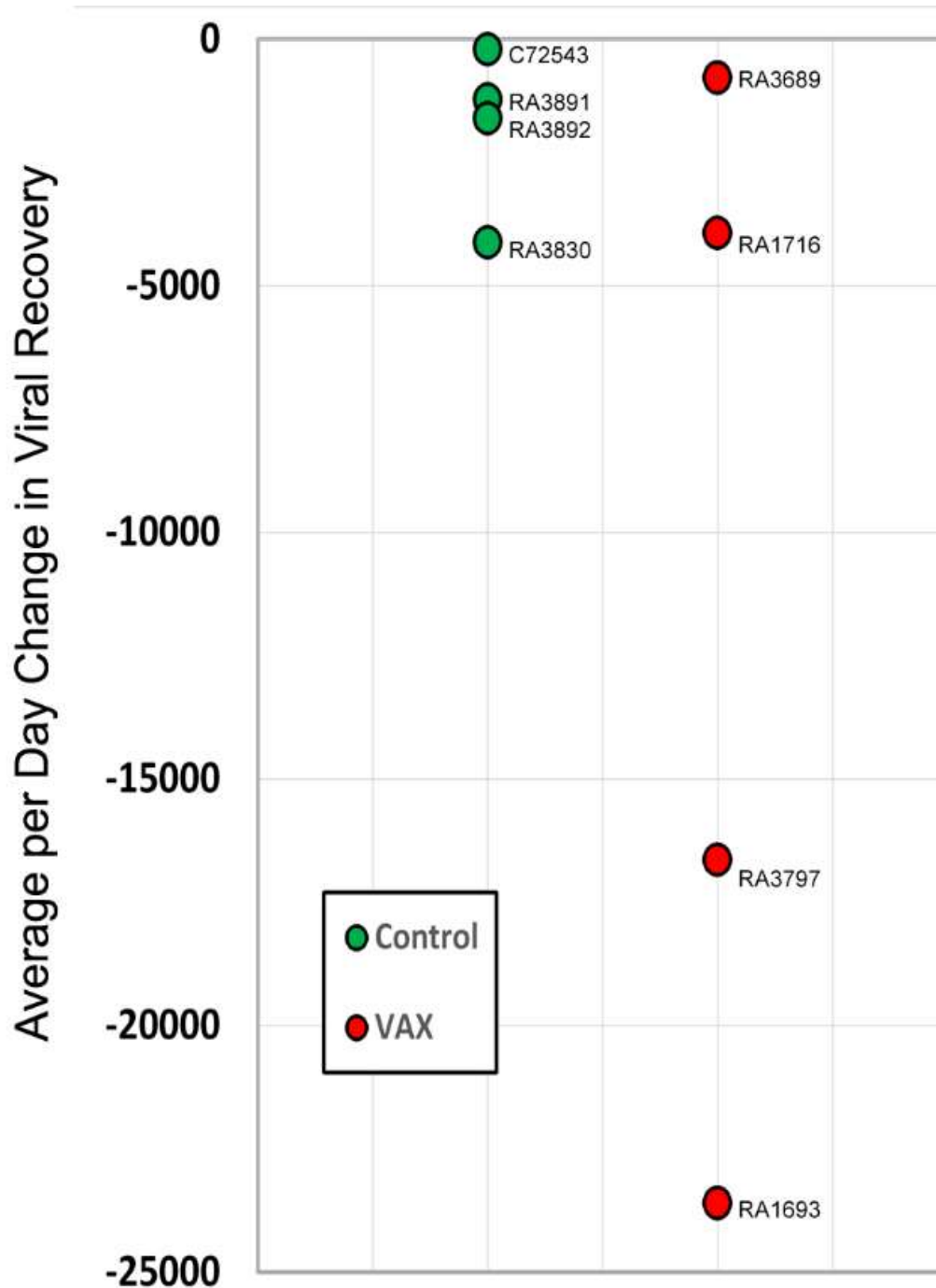
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936 **Supplemental Data, Figure S2 Legend.** Hematological analysis in control and vaccinated macaques
937 challenged with SARS-CoV-2. The counts of white blood cells (WBC) (upper left panel), the percent
938 of lymphocytes in WBC (upper right panel), the percent of monocytes in the WBC (lower left panel),
939 and the percent of neutrophils in the WBC (lower right panel) were analyzed. An asterisk indicates
940 a statistically significant difference (p < 0.05) between control and vaccinated macaques by Students
941 t-test.

942 **Figure S3**

943



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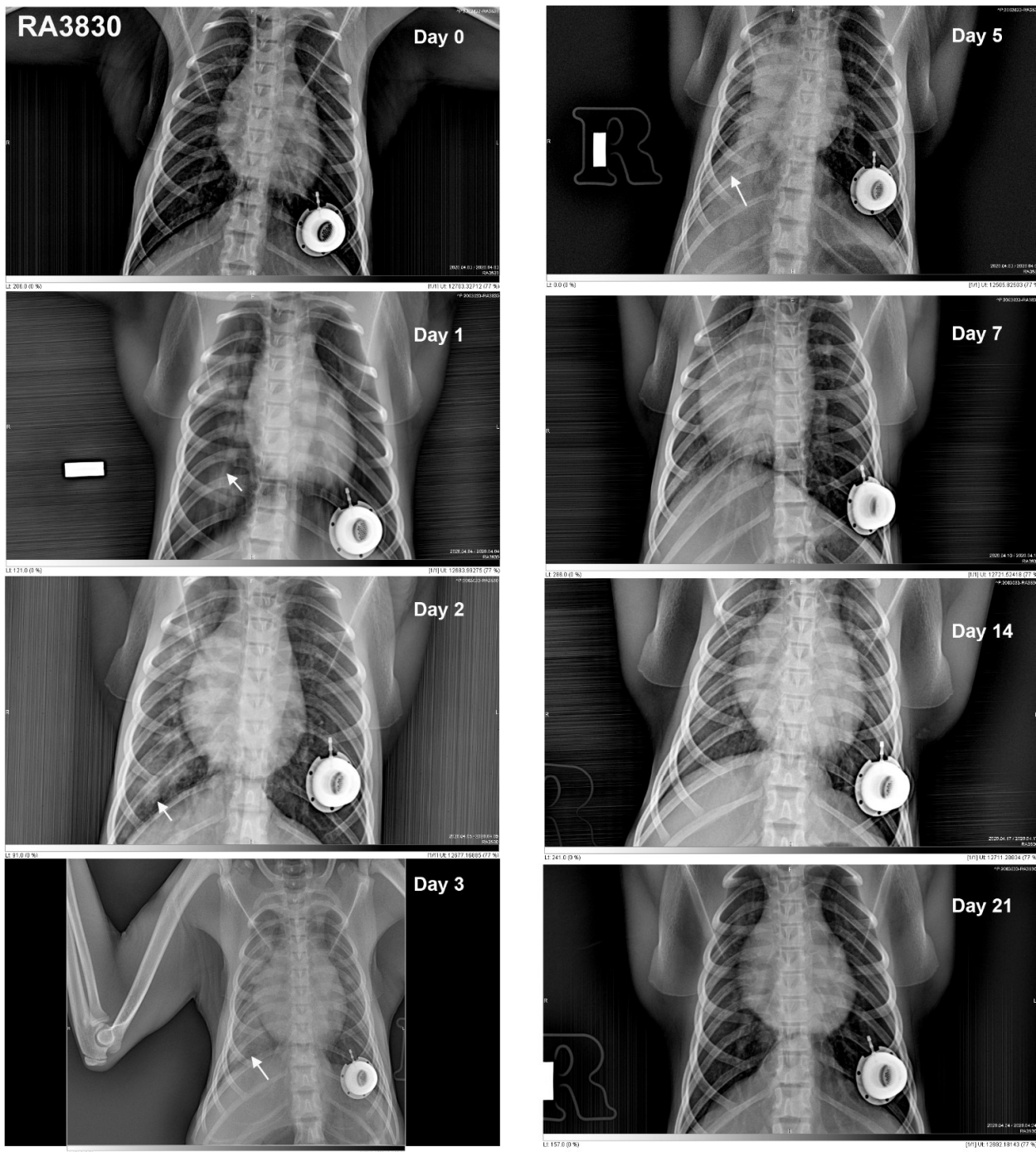
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946 **Supplemental Data, Figure S3 Legend. Viral clearance rates in control and vaccinated SARS CoV-**
947 **2 challenged macaques.** From the total viral loads measurements in nasal swab samples from SARS-
948 CoV-2 challenged macaques, the daily viral clearance rates (i.e. $TCID_{50}/mL_{day\ n-1}$ minus $TCID_{50}/mL_{day\ n}$)
949 were calculated and averaged over a nine-day period. Red symbols are vaccinated macaques
950 subjects, control unvaccinated macaque subjects are shown in green symbols.

951

952 **Figure S4**

953



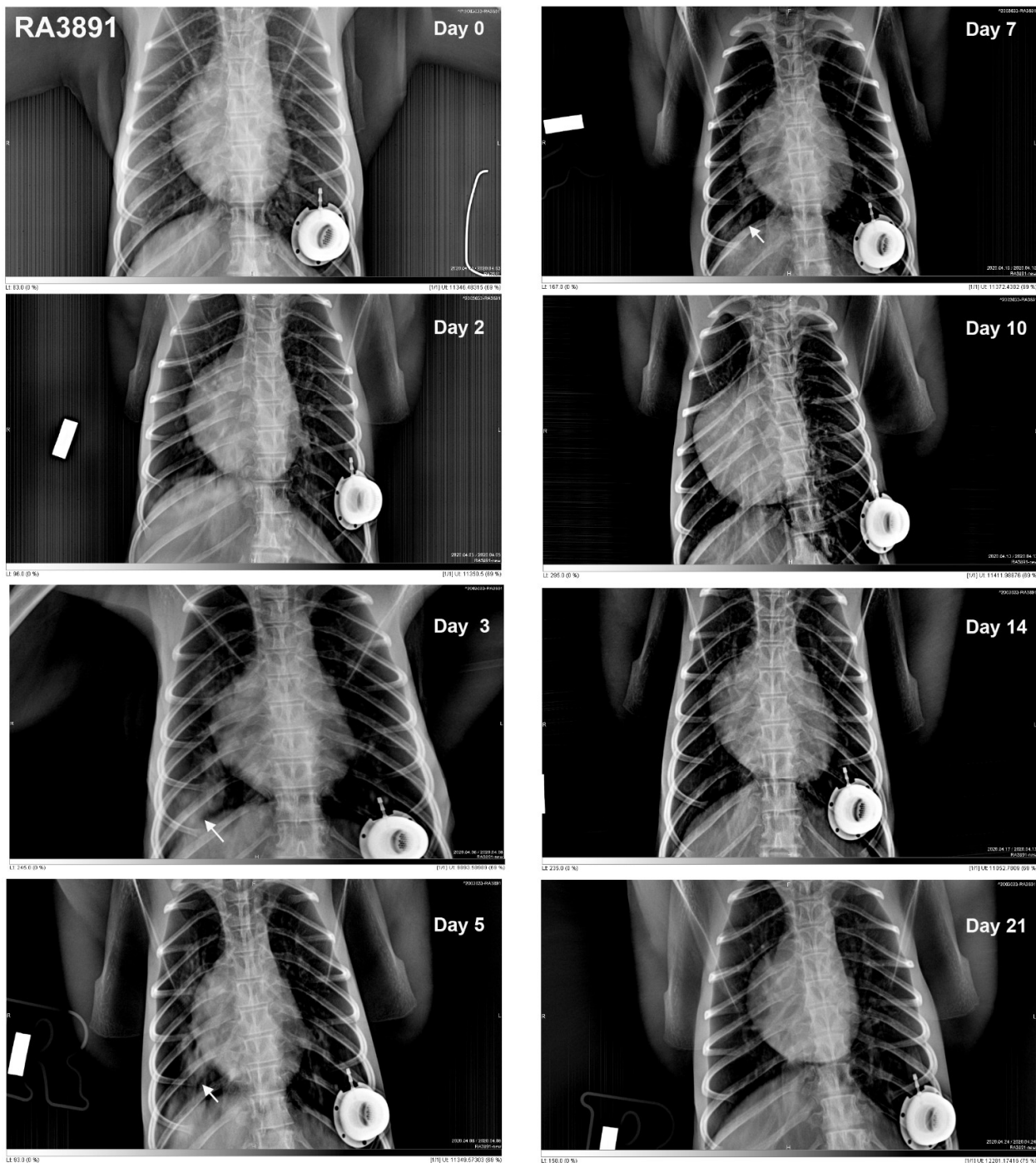
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956 **Supplemental Data, Figure S4 legend.** Chest radiographs of control rhesus macaque RA3830 fol-
957 lowing SARS-CoV-2 challenge. As shown, this animal demonstrated a progression of pulmonary
958 infiltrates during the acute period (Days 2-5) of disease post-challenge which resolved by study
959 termination (Day 21). White arrows indicate areas of mild to moderate pulmonary infiltrates seen
960 as ground glass consolidations.

961 **Figure S5**

962



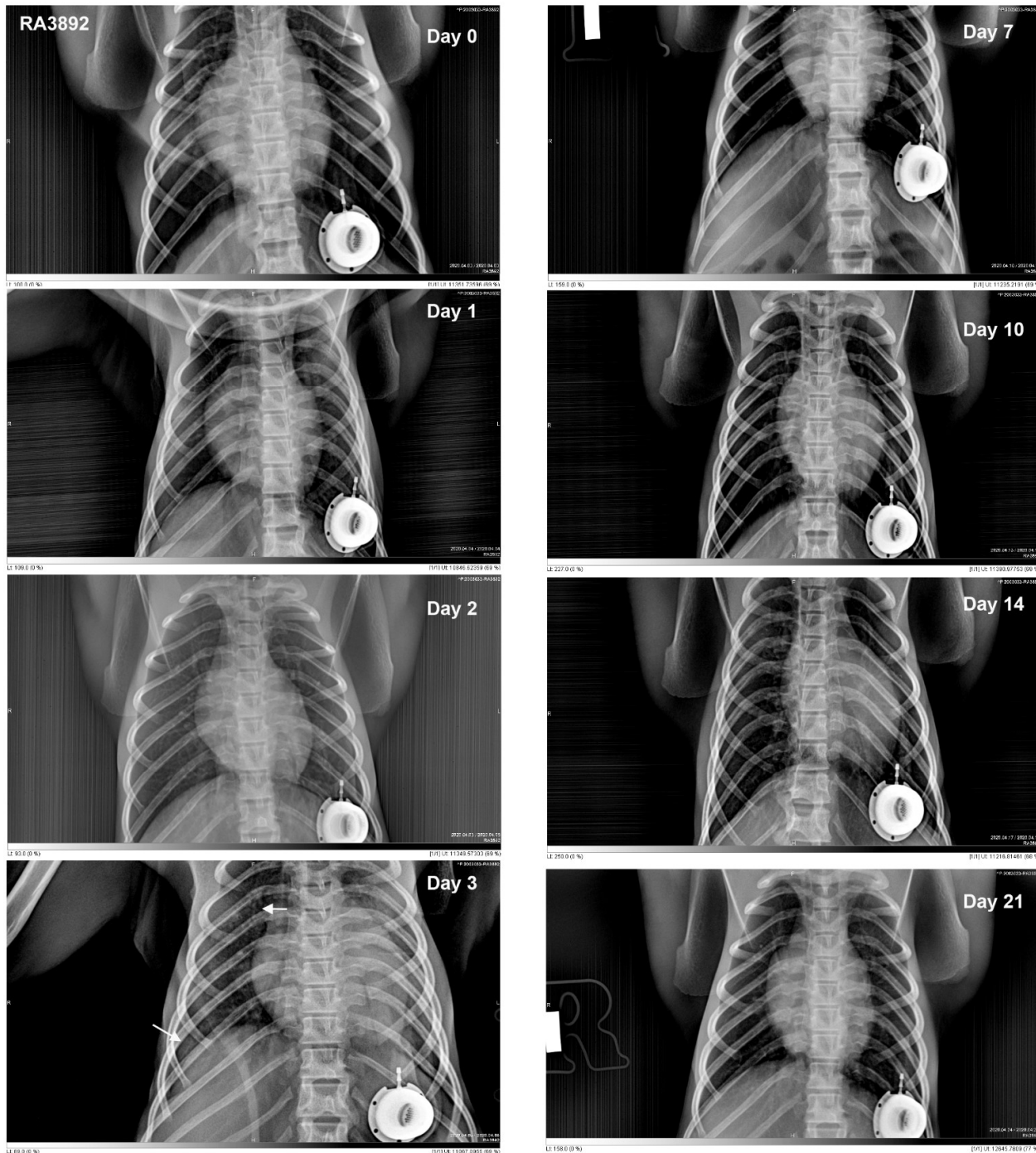
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965 **Supplemental Data, Figure S4 Legend.** Chest radiographs of control rhesus macaque RA3891 fol-
966 lowing SARS-CoV-2 challenge. As shown, this animal demonstrated a progression of pulmonary
967 infiltrates during the acute period (Days 3-7) of disease post-challenge which resolved by study
968 termination (Day 21). White arrows indicate areas of mild to moderate pulmonary infiltrates seen
969 as ground glass consolidations.

970 **Figure S6**

971



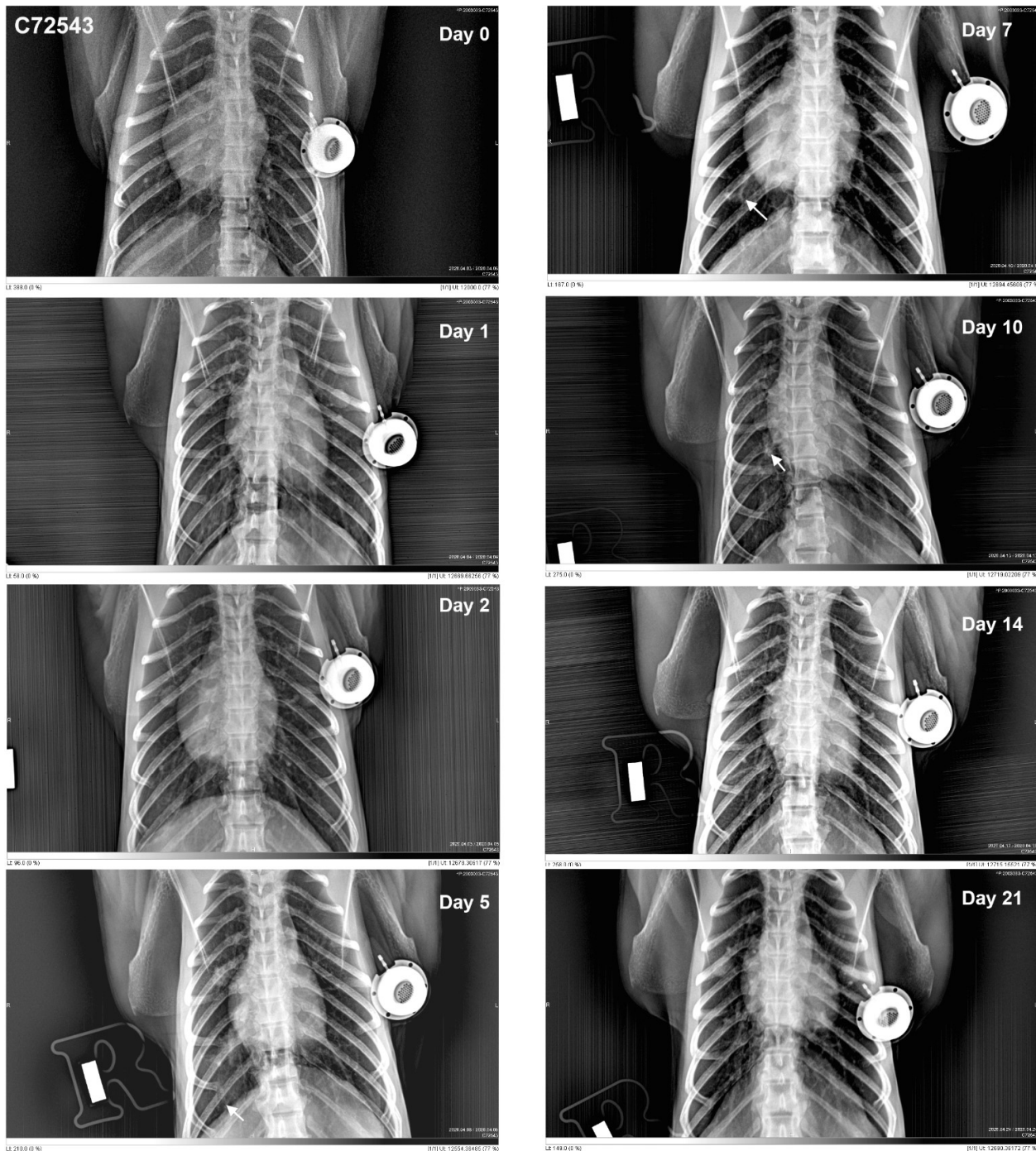
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974 **Supplemental Data, Figure S6 Legend.** Chest radiographs of control rhesus macaque RA3892 fol-
975 lowing SARS-CoV-2 challenge. As shown, this animal demonstrated a progression of pulmonary
976 infiltrates during the acute period (Day 3) of disease post-challenge which resolved by study termi-
977 nation (Day 21). White arrows indicate areas of mild to moderate pulmonary infiltrates seen as
978 ground glass consolidations.

979 **Figure S7**

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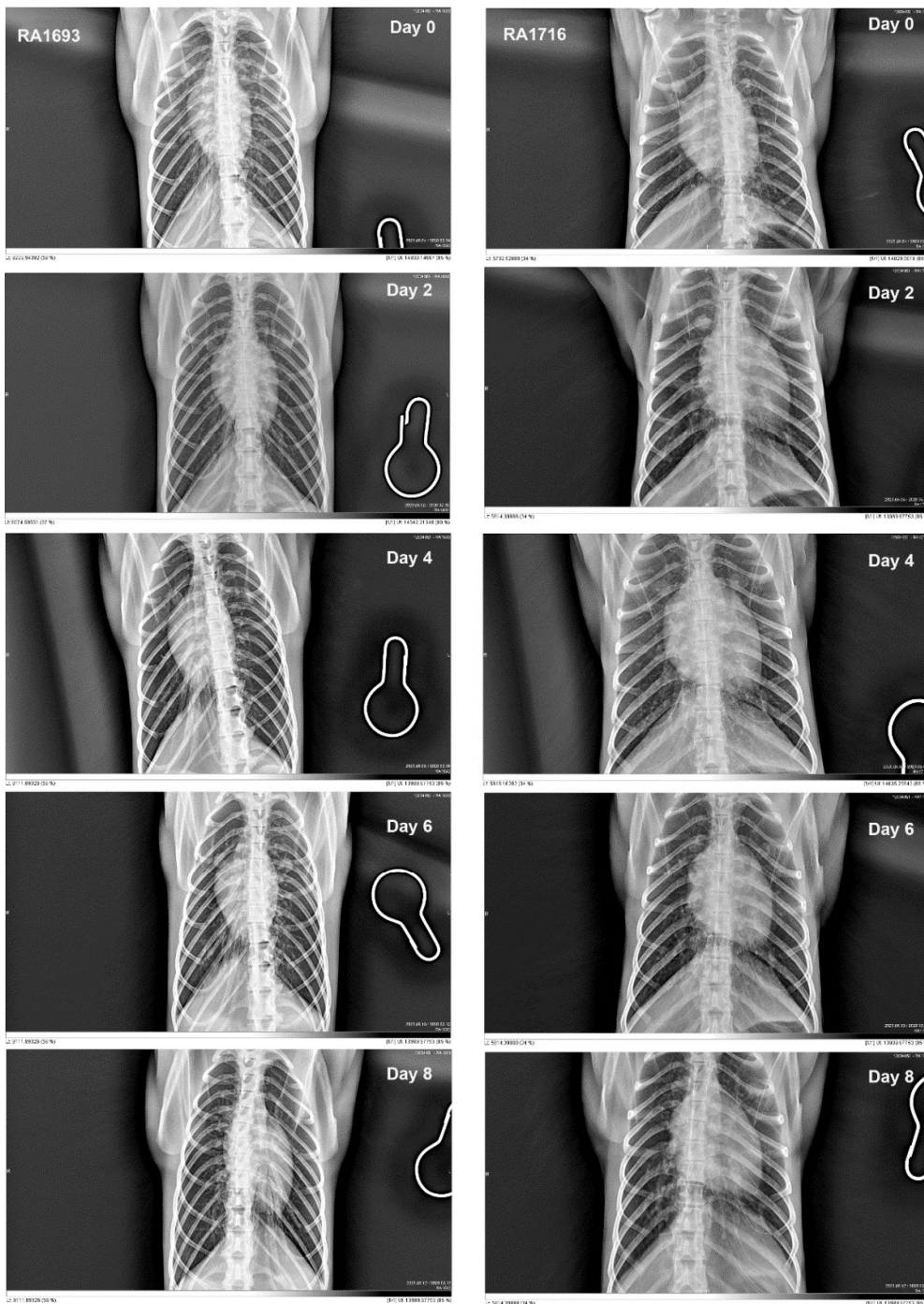
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983 **Supplemental Data, Figure S7 Legend.** Chest radiographs of control cynomolgus macaque C72543
984 following SARS-CoV-2 challenge. As shown, this animal demonstrated a progression of pulmonary
985 infiltrates 5-10 days post-challenge which resolved by study termination (Day 21). White arrows
986 indicate areas of mild to moderate pulmonary infiltrates seen as ground glass consolidations.

987 **Figure S8**

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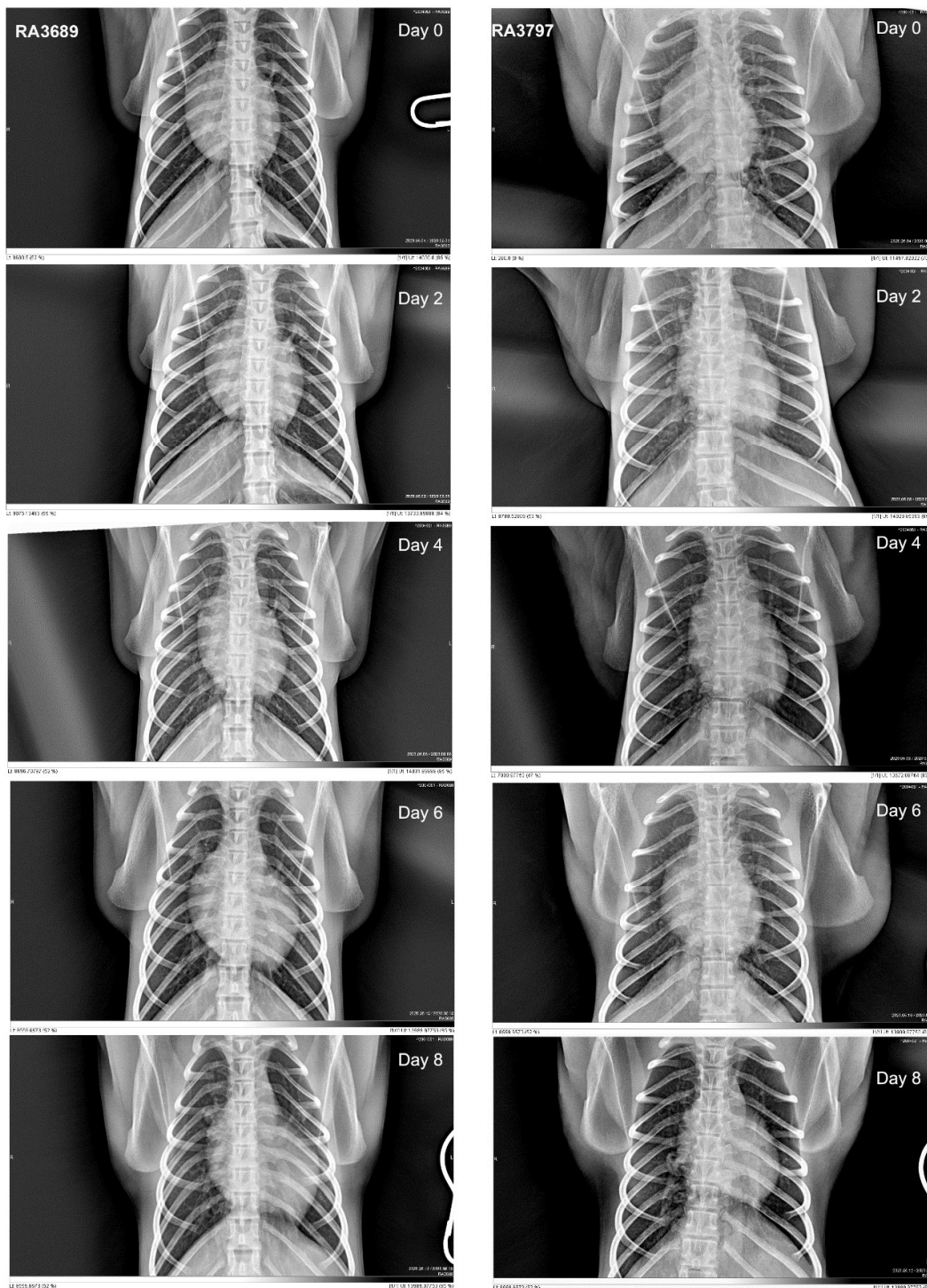
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991 **Supplemental Data, Figure S8 Legend.** Chest radiographs of vaccinated rhesus macaques RA1693
992 and RA1716 following SARS-CoV-2 challenge. With the exception of increased reticulation relative
993 to baseline, few abnormalities were observed in collected radiograph images. Note the absence of
994 infiltrates or consolidation typically seen in the unvaccinated control population.

995 **Figure S9**

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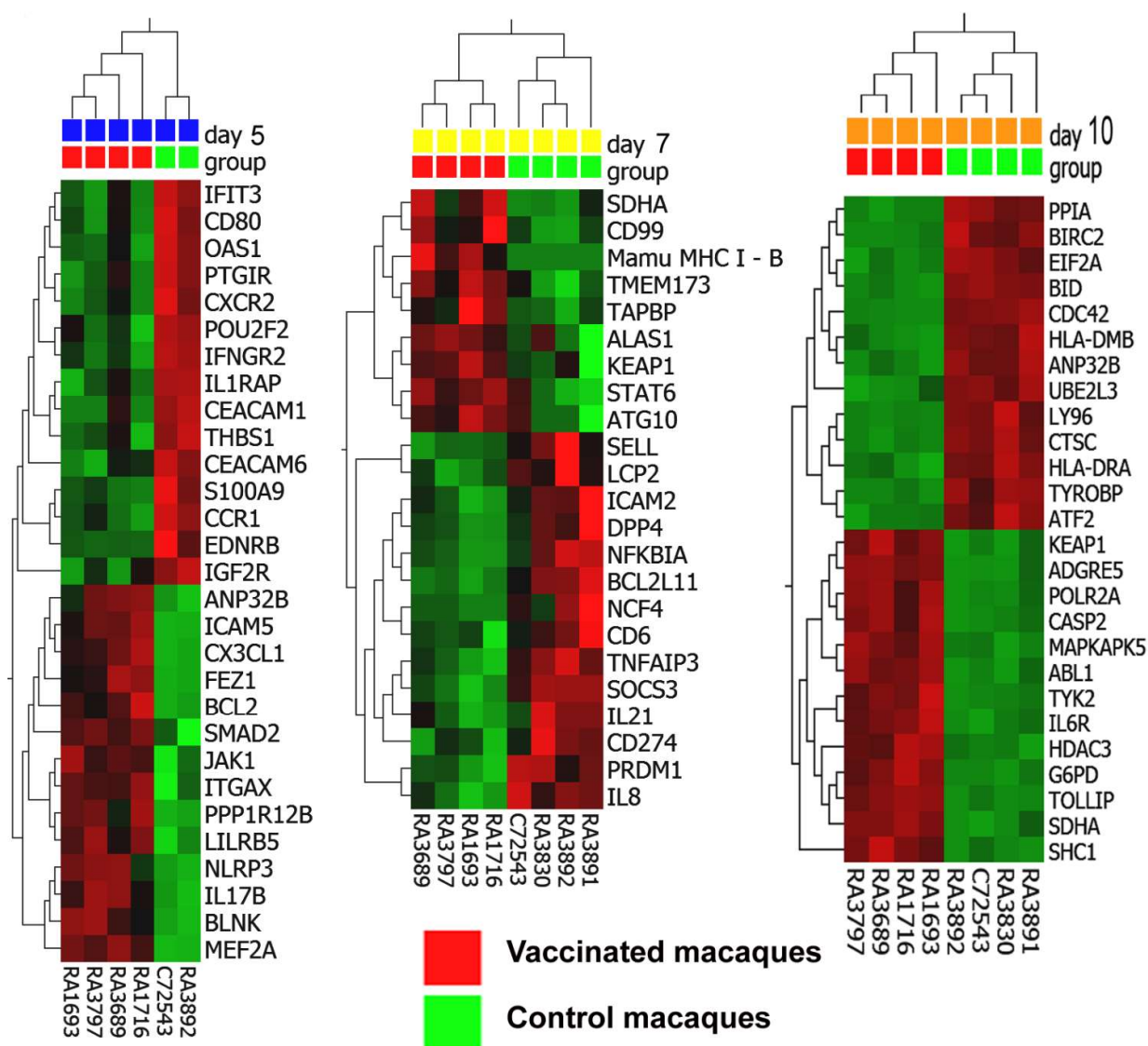
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Supplemental Data, Figure S9 Legend. Chest radiographs of vaccinated rhesus macaques RA3689 and RA3797 following SARS-CoV-2 challenge. Radiographs are unremarkable, other than showing increased reticulation relative to baseline, appearing on days 2 through 4, clearing on later imaging. In particular, note lack of focal infiltrates or consolidations.

1002 **Figure S10**

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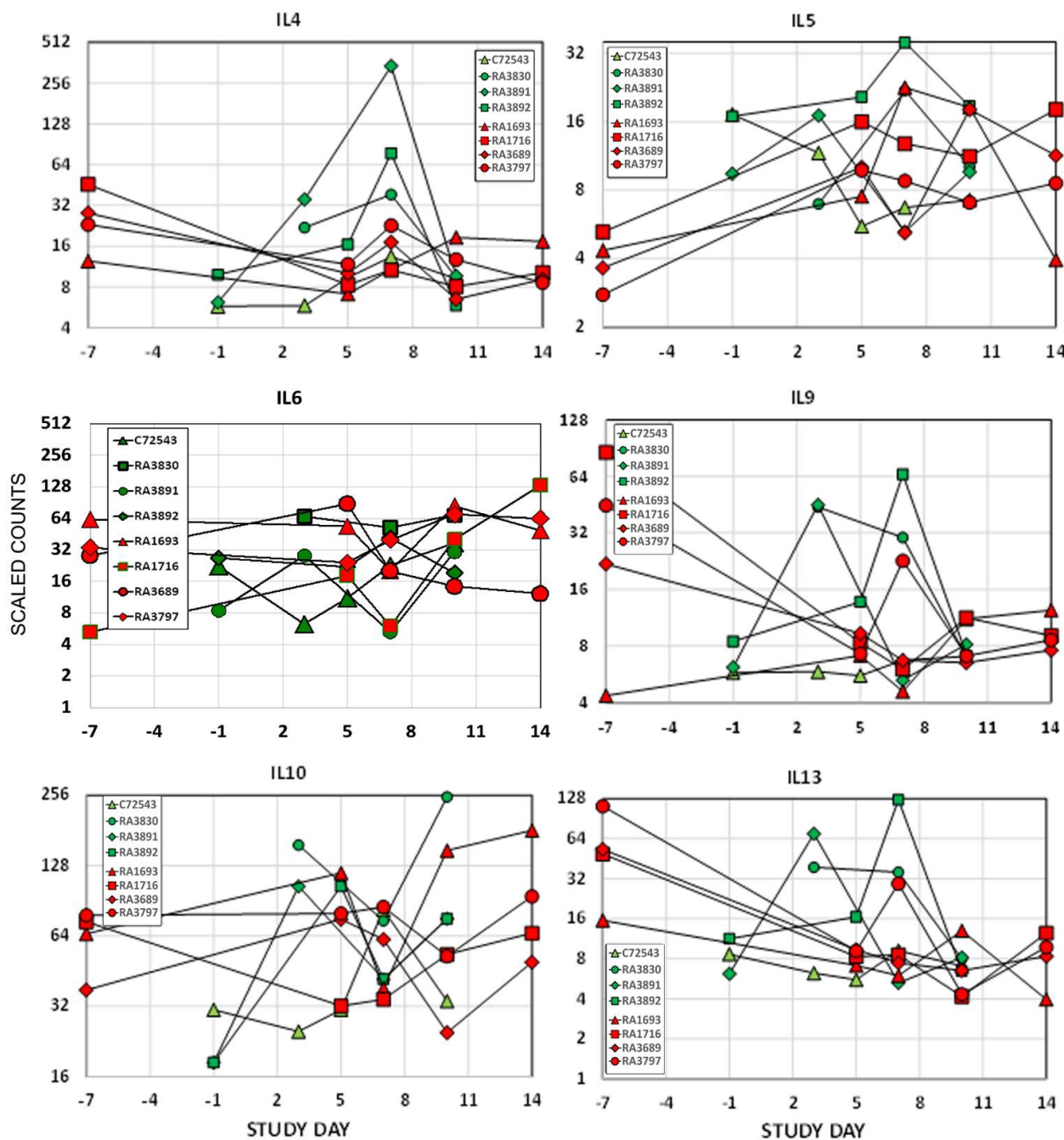


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1006 **Supplemental Data, Figure S10.** Hierarchical clustering of gene expression in BAL samples col-
 1007 lected from control and vaccinated macaques on Day 5 (left panel), Day 7 (middle panel), and Day
 1008 10 (right panel). 5- and 7- days post SARS-CoV-2 challenge. Heatmap shows significantly (p<0.05)
 1009 up-regulated (red) transcripts and down-regulated (green) transcripts from a total of 730 genes an-
 1010 alyzed using the NanoString Non-Human Primate Immunology V2 Panel.

1011 **Figure S11**



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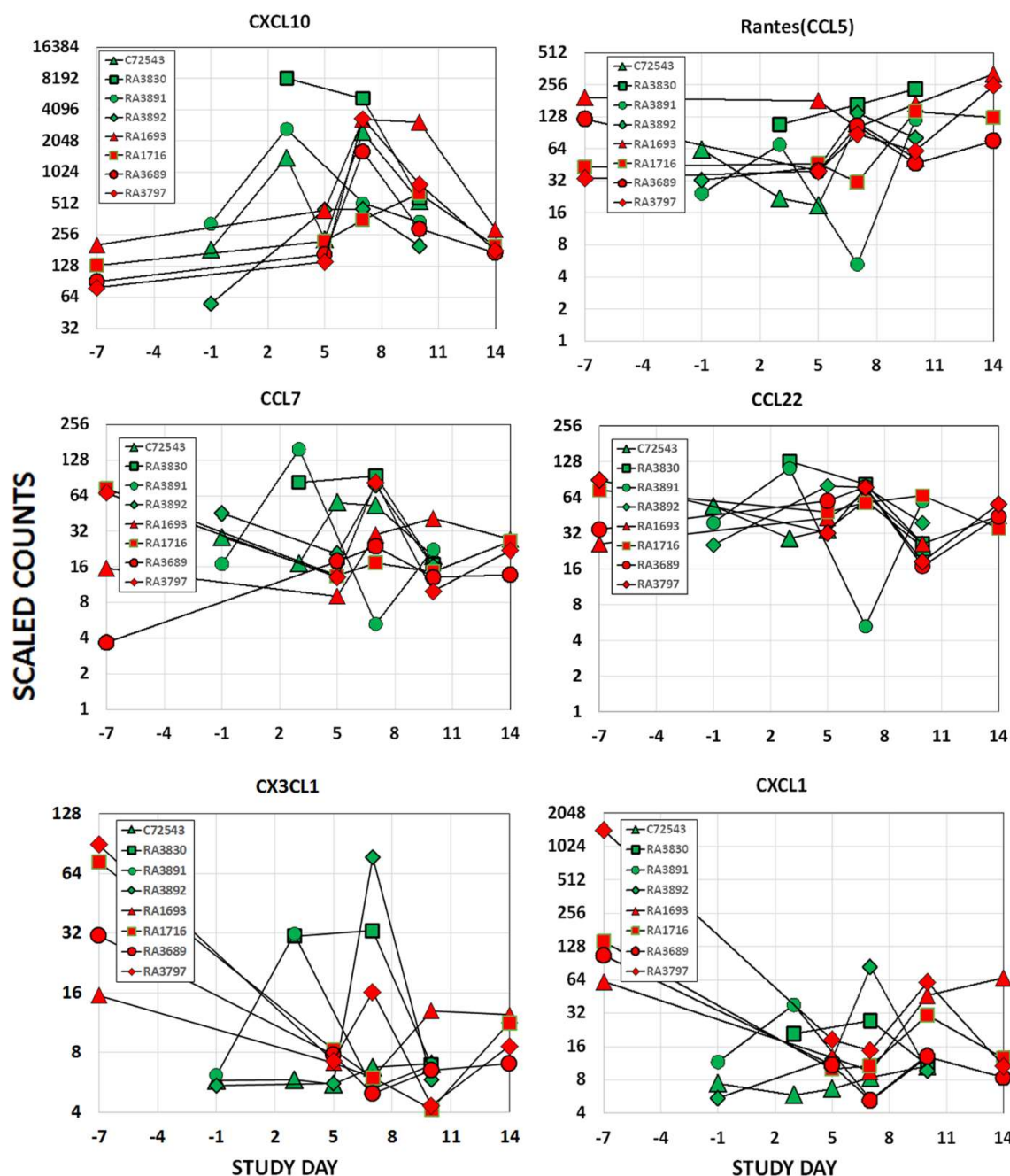
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Supplemental Data, Figure S11 Legend. Intratracheal vaccination with the adjuvanted microsphere peptide vaccine did not promote the expression of Th₂ type interleukin cytokine transcripts in collected BAL samples relative to levels measured in control macaques. BAL cell gene expression (shown as scaled counts on the y-axis) of cytokines associated with Th₂ T cell responses were plotted by study day (x-axis) for each animal prior to and following the SARS-CoV-2 challenge. No statistically significant differences between control and vaccinated macaques were found.

1020 **Figure S12**

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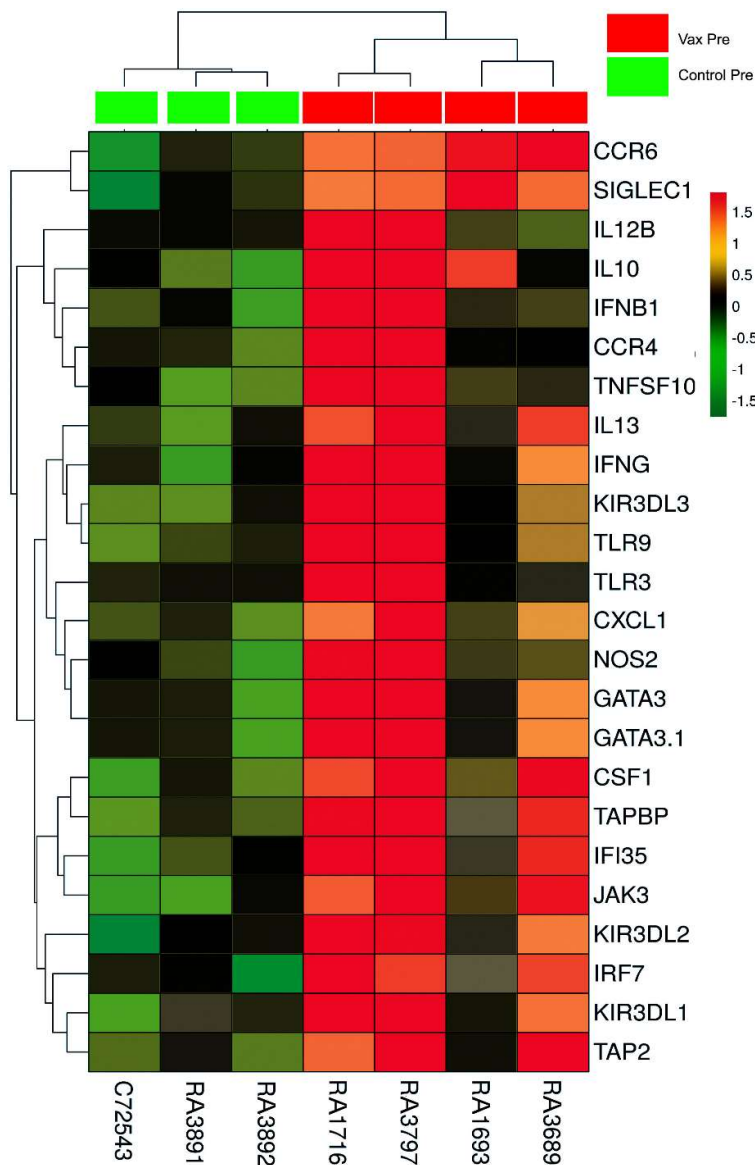
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Supplemental Data, Figure S12. Intratracheal vaccination with the adjuvanted microsphere peptide vaccine did not promote the expression of Th₂ type interleukin cytokine transcripts in collected BAL samples relative to levels measured in control macaques. BAL cell gene expression (shown as scaled counts on the y-axis) of cytokines associated with Th₂ T cell responses were plotted by study day (x-axis) for each animal prior to and following the SARS-CoV-2 challenge. No statistically significant differences between control and vaccinated macaques were found.

1029 **Figure S13.**

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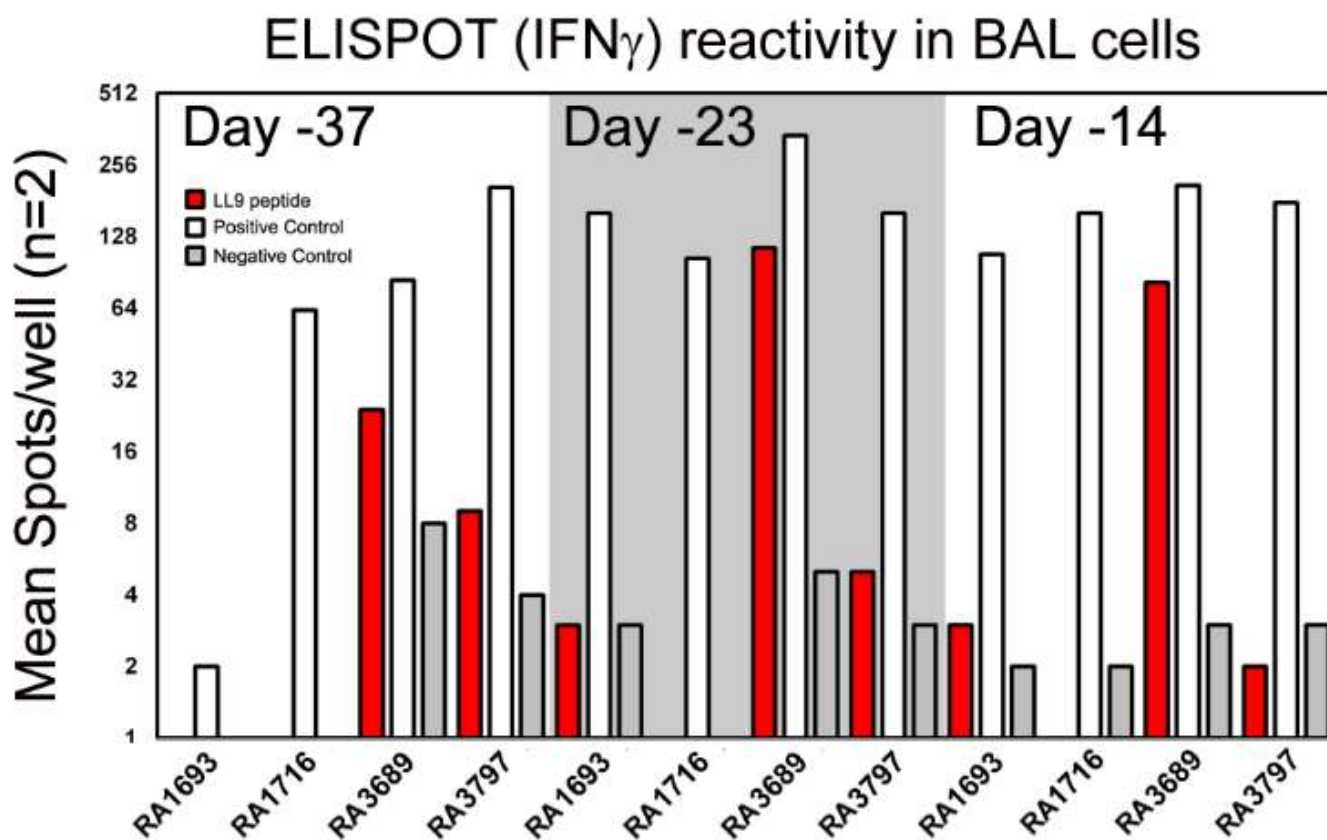
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1033 **Supplemental Data, Figure S13 Legend.** Hierarchical clustering of adjuvant-related transcript ex-
1034 pression in BAL samples collected from control and vaccinated macaques prior to virus challenge
1035 (indicated as Control Pre and Vax Pre). Gene expression analysis identified differentially regulated
1036 genes in BAL samples obtained on Day -1 from control animals and Day -7 from vaccinated ma-
1037 caques. Heatmap shows significant ($p < 0.05$) differential expression of a series of genes that had
1038 previously been identified (see main text) as being regulated by adjuvants alone. Up-regulated (red);
1039 down-regulated (green). Macaque RA 3830 was not sampled on day - 1.

1040 **Figure S14**

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1044 **Supplemental Data, Figure S14 Legend.** Immunoreactivity of BAL-associated cells from vac-
1045 cinated macaques to immunizing peptides prior to SARS-CoV-2 challenge. Dates shown are assay
1046 date rather than sampling date. Concanavlin A was used as a positive control.

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1048 **Supplemental Materials**

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1050 **Supplemental data –Table S1A and S1B**

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| Supplemental Table S1A | | | | | | | | | | | |
|-------------------------------|--|---------------------------|--------------------------|--------------------------|--------------------------|---------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--|
| CTL epitope HLA coverage | HLA allele (genotypic frequency % world) | | | | | | | | | | Aggregate HLA Class I Coverage % |
| | HLA A*01:01 (10.09) | HLA A*02:01 (24.39) | HLA A*03:01 (9.77) | HLA A*11:01 (8.99) | HLA A*23:01 (3.06) | HLA A*24:02 (12.59) | HLA A*29:02 (2.18) | HLA A*30:02 (1.36) | HLA A*31:01 (3.02) | HLA A*68:01 (3.29) | |
| Epitope | | | | | | | | | | | |
| LSPRWYFYF | + | - | - | + | + | + | + | + | + | - | 60.51 |
| LLLDRLNQL | - | + | - | - | - | - | - | - | - | - | 39.08 |
| KTFPTEPK | - | - | + | + | - | - | - | - | + | + | 40.03 |
| GMSRIGMEV | - | + | - | - | - | - | - | - | - | - | 39.08 |
| ASAFFGMSR | - | - | + | + | - | - | - | - | + | + | 40.03 |
| QQQGQTVTK | - | - | - | + | - | - | - | - | + | - | |
| Epitope Count | 1 | 2 | 2 | 4 | 1 | 1 | 1 | 1 | 4 | 2 | 91.51 ² |

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Notes: 1. + Indicates positive in-vitro assays for MHC binding and/or T-cell recognition [36]. 2. Calculated as previously described[78]

| Supplemental Table S1B | | | | | | | |
|---|---------------------|----------------------|----------------------|----------------------|---------------------|----------------------|---------------------|
| Rhesus Mamu MHC Class I coverage – predicted binding | | | | | | | |
| NHP Subject | R1693 ¹ | R1716 | | R3689 | | R3797 | |
| Epitope | | | | | | | |
| LSPRWYFYF | | <i>A7*01:03</i> | | | | <i>A1*002:02</i> | <i>B*068:01(WB)</i> |
| LLLDRLNQL | <i>A1*026:01</i> | <i>B:086:01 (WB)</i> | | <i>A1*026:01</i> | <i>B*056:01(WB)</i> | <i>B*056:01(WB)</i> | <i>B*068:01(WB)</i> |
| | | | | <i>A1*004:01(WB)</i> | | <i>A1*004:01(WB)</i> | |
| KTFPTEPK | <i>B*013:01(SB)</i> | <i>B*083:01</i> | <i>B:086:01 (WB)</i> | <i>B*066:01</i> | | <i>B*066:01</i> | |
| GMSRIGMEV | <i>A1*026:01</i> | | | <i>A1*026:01</i> | | | |
| ASAFFGMSR | <i>B*013:01(WB)</i> | <i>B*083:01</i> | | <i>B*066:01</i> | | <i>B*066:01</i> | |
| QQQGQTVTK | <i>B*013:01(SB)</i> | | | | | | |

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Notes: 1. A typical rhesus MHC haplotype may contain two or three expressed Mamu-A genes, and up to nineteen distinct Mamu-B-like loci[79], 2. Mamu MHC in *italics* are predicted to bind based on HLA homology and in-vitro analysis [40]. 3. Mamu MHC in regular font are predicted to bind based on NetMHCpan 4.1, with a weak binder (WB) at top 2% percentile rank and strong binder (SB) at top 0.5% percentile rank.