A bivalent Adenovirus-Vectored Vaccine induces a measurable humoral response, but does not protect cynomolgus macaques against a lethal challenge with Sudan virus

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23 Abstract

24 The most recent Sudan virus (SUDV) outbreak in Uganda was first detected in September 2022
25 and resulted in 164 laboratory-confirmed cases and 77 deaths. Currently, there are no approved
26 vaccines or therapeutics against SUDV. In the current study, we investigated the protective
27 efficacy of ChAdOx1-biEBOV in cynomolgus macaques using a prime or a prime-boost
28 regimen. ChAdOx1-biEBOV is a replication-deficient simian adenovirus vector encoding SUDV
29 and Ebola virus (EBOV) glycoproteins (GPs) at the E1 and E4 loci, respectively. Intramuscular
30 vaccination induced SUDV and EBOV GP-specific IgG responses and neutralizing antibodies.
31 Upon challenge with SUDV, vaccinated animals, regardless of vaccination scheme, showed
32 signs of disease like those observed in control animals, and no difference in survival outcomes
33 were measured among all three groups. Viral load in blood samples and in tissue samples
34 obtained after necropsy were not significantly different between groups. Overall, this study
35 highlights the importance of evaluating vaccines in multiple animal models, including nonhuman
36 primates, and demonstrates the importance of understanding protective efficacy in both animal
37 models and human hosts.

38 Background

39 Of the six viruses described in the genus *Orthoebolavirus (1)*, four are known to cause
40 Ebolavirus disease in humans, namely Ebola virus (EBOV), Sudan virus (SUDV), Bundibugyo
41 virus (BDBV), and Taï Forest virus (TAFV). Although EBOV is responsible for most outbreaks
42 (*2*), SUDV has caused eight outbreaks in Sudan and Uganda since first discovered in 1976 (*3–9*).
43 SUDV disease (SVD) has been documented in nearly 1,000 human cases and has a combined
44 case fatality rate (CFR) of 50% (*3–9*). The most recent SUDV outbreak was first detected in
45 Uganda's Mubende district in September 2022 (*9*, *10*). During the months-long outbreak, there
46 were 164 laboratory-confirmed cases and 77 deaths (CFR 47%). The severity of SVD and the
47 increasing potential for wider spread of SUDV outbreaks warrant the development of medical
48 countermeasures.

49 Current licensed vaccines and therapeutics against EBOV exist, but the cross-protection against
50 SVD in humans has not been demonstrated. The United States Food and Drug Administration
51 (FDA)-approved EBOV vaccine, Ervebo®, utilizes a recombinant vesicular stomatitis virus
52 (rVSV) backbone expressing EBOV Kikwit's glycoprotein (GP) (*11*). In the pre-clinical stage,
53 Ervebo® protects against lethal EBOV challenge but not SVD in cynomolgus macaques (*12*). A
54 second EBOV vaccine approved by the European Medical Agency utilizes a prime-boost
55 approach of replication-incompetent adenovirus (Ad26) encoding the EBOV Mayinga GP
56 (Ad26.ZEBOV, Zabdeno) followed by non-replicating modified vaccinia Ankara–vectored
57 vaccine encoding the EBOV Mayinga, SUDV Gulu, and MARV Musoke GPs and the
58 nucleoprotein of the Tai Forest virus (MVA-BN-Filo, Mvabea). The animal rule regulatory
59 pathway, which allows for efficacy data generated in well-characterized animal models of human
60 disease to demonstrate effectiveness in place of phase II or III efficacy trails in humans,

61 facilitated the licensure of the Zabdeno/Mvabea prime-boost vaccination regimen (*13*). This two-
62 shot vaccination is efficacious against SUDV in non-human primates (NHPs) (*14*), but the
63 effectiveness against SVD in humans is unknown.
64 Furthermore, the two FDA-approved immunotherapeutics, InmazebTM and EbangaTM, target
65 the GP of multiple EBOV strains but do not cross-protect against SUDV (*15*). This lack of
66 protection is expected, given that EBOV and SUDV GPs share less than 56% amino acid
67 identity. A bivalent vaccine that targets both EBOV and SUDV could provide protection against
68 both viruses which have historically caused most outbreaks. Several vaccines that target SUDV
69 (*16–20*) or multiple filoviruses (*12*, *21*, *22*) are being developed and tested in clinical trials,
70 including ChAdOx1-biEBOV vaccine.
71 The ChAdOx1 platform is a replication-deficient simian adenovirus vector, that has been used to
72 generate vaccines which are efficacious against multiple pathogens (*23–26*), including severe
73 acute respiratory syndrome coronavirus-2 (SARS-CoV-2) . The ChAdOx1 nCoV-19 vaccine
74 against SARS-CoV-2 has been approved in more than 170 countries. ChAdOx1-biEBOV uses
75 the same backbone as ChAdOx1 nCoV-19 but encodes SUDV GP and EBOV GP at the E1 and
76 E4 loci, respectively. ChAdOx1-biEBOV protects type I interferon receptor (Ifnar1[-]) knockout
77 mice against challenge with SUDV Boniface. Here, we evaluated the ChAdOx1-biEBOV
78 efficacy in cynomolgus macaques using either a prime only or prime boost vaccination regimen
79 with SUDV challenge occurring 28 days after the last vaccination. Despite eliciting binding and
80 neutralizing antibodies against SUDV GP, neither vaccine regimen protected against a lethal
81 challenge with SUDV.

82 Results

83 Eighteen female cynomolgus macaques were divided into three groups of six. One group
84 received ChAdOx1-biEBOV 28 days prior to challenge (D-28; prime), one group received
85 ChAdOx1-biEBOV at D-56 and D-28 (prime boost), and one group received ChAdOx1 GFP at
86 D-28 (control). Vaccines were administered via the intramuscular (IM) route with 2.5 x 10^{10}
87 virus particles/animal (Figure 1A). Exams were conducted at the time of vaccination and 14 days
88 thereafter. Serum was collected on days of vaccination, exam, and challenge, and the levels of
89 binding antibodies against SUDV and EBOV GP were measured. Fourteen days post-prime
90 vaccination, antibody levels against SUDV GP varied between 62-1084 arbitrary ELISA units
91 (geometric mean of 294). Fourteen days post-boost vaccination, antibody levels against SUDV
92 GP had increased to 1346-4824 arbitrary ELISA units (geometric mean of 2179). At time of
93 challenge, antibody levels against SUDV GP varied between 30-241 arbitrary ELISA units
94 (geometric mean of 92) for the prime group and 206-882 arbitrary ELISA units (geometric mean
95 of 419) for the prime boost group, 4.6-fold increase over the prime group (Figure 1B). Binding
96 antibodies against EBOV GP followed a similar trend: Antibodies could be detected 14 days post
97 prime vaccination, with arbitrary ELISA units between 50 and 604 (geometric mean of 192) and
98 were increased upon boost vaccination (between 262-1200 arbitrary ELISA units, geometric
99 mean of 618). On the day of challenge, antibodies in the prime boost group were significantly
100 higher (2.3 fold) compared with the prime group (Figure 1C). We investigated the ability of
101 serum antibodies to neutralize SUDV or EBOV using a pseudo-neutralization assay based on
102 rVSV, as previously reported (*17*). Virus-neutralizing (VN) antibodies against SUDV could be
103 detected 28 days after a single vaccination, the VN titers varied between 1:10-1:40. At the time
104 of challenge, all prime-boost sera had a VN titer of 1:40 against SUDV (Figure 1D). Twenty-

105 eight days after a single vaccination, VN titers against EBOV were between 1:10-1:80, whereas
106 all prime-boost sera at time of challenge had VN titers varying between 1:40-1:160 (Figure 1E).
107 The cellular response from peripheral blood mononuclear cells (PBMCs) after vaccination with
108 ChAdOx1-biEBOV was investigated using ELIspot at D-14. Surprisingly, although a robust
109 humoral response was detected, minimal to no interferon-producing T cells were detected upon
110 stimulation with peptides specific for either SUDV GP (Figure 1F) or EBOV GP (Figure 1G) at
111 14 days post final vaccination.
112 Each macaque was challenged with 10^3 focus-forming units (FFU) SUDV Gulu, administered in
113 1 mL via the IM route (*17*). Clinical scores for all three groups increased starting on D4, with no
114 significant differences observed between groups (Figure 2A). All macaques developed clinical
115 signs of SVD, including petechial rash in four of six macaques for each group. All were
116 euthanized between D5-7 when they reached end point criteria, and no significant differences in
117 survival were observed between groups (Figure 2B). Viremia was first detected on D3 in two out
118 of six controls and was present in all eighteen macaques on D6, with no significant differences
119 between groups (Figure 2C). Infectious virus was detected in the blood of all 18 macaques at
120 necropsy, with no differences in titers between groups (Figure 2D). Serum chemistry values were
121 significantly different on D6 compared to D0 for the following analytes (Figure 2): increased
122 concentrations of aspartate aminotransferase (AST), increased alkaline phosphatase (ALP),
123 increased blood urea nitrogen (BUN), and decreased albumin. Hematologic values were
124 significantly different on D6 compared to D0 for the following analytes (Figure 2): decreased
125 platelet count, decreased lymphocyte count, and on D3 compared to D0 for increased neutrophil
126 count. Levels of AST and ALP were significantly decreased in the prime boost group compared
127 to the control or prime groups on D6 (Figure 2E-F). Increased blood urea nitrogen (BUN) was

128 observed in all macaques by D6, albeit significantly less in the prime boost group (Figure 2G).
129 Platelet levels in the control group were significantly lower than in the prime boost group on D6
130 (Figure 2I). Lymphocytes were significantly higher in the prime boost group compared to the
131 control and prime groups on D3 (Figure 2J). Neutrophils were significantly higher in the prime
132 boost group compared to the control group on D3 (Figure 2K).
133 Although high binding antibody levels against SUDV GP and EBOV GP were detected at
134 challenge, by necropsy the levels of binding antibody had dropped in both vaccine groups, albeit
135 to a lower extent in the prime boost group (Figure 3A-B). We hypothesize that this is due to the
136 presence of sGP and virus with GP on its surface in serum, which would affect our ability to
137 detects SUDV-specific antibodies. Using a SUDV sGP-specific ELISA, sGP was detected in
138 most serum samples obtained on the day of necropsy but not at earlier time points (Figure 3C).
139 Interestingly, we were unable to detect sGP in one sample each for the prime and prime boost
140 group. In these serum samples, SUDV GP-specific binding antibody levels were the highest
141 within the respective vaccine group.
142 A panel of twenty-six different cytokines and chemokines was measured in sera (D-28, 0, 1, 3,
143 and endpoint) as well as in liver and lung tissue homogenates collected at necropsy. In serum
144 (Supplementary Figure 1), an upregulation of multiple cytokines and chemokines could be
145 measured at time of necropsy compared to earlier timepoints (interferon alpha 2 (IFN-α2),
146 interleukin-1 beta (IL-1β), IL-4, IL-6, IL-10, IL-15, IL-16, interferon gamma inducible protein-
147 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1
148 alpha (MIP-1α), MIP-1β, thymus and activation-regulated chemokine (TARC), tumor necrosis
149 factor alpha (TNF-α), and TNF-β). Multiple significant differences were observed between
150 groups at different timepoints, including an increased expression of IFN-γ, and decreased

151 expression of IL-4, IL-6, IL-8, IL-15, and IP-10 in the prime boost group compared to the other
152 two groups on D3 or day of necropsy (Supplementary Figure 1).
153 In liver tissue, multiple cytokines and chemokines were significantly elevated in the prime boost
154 group compared to the prime and control groups (Supplementary Figure 2, IFN-α2, IFN-γ, IP-10,
155 MIP-1α, MIP-1β). No differences in cytokines and chemokines levels were observed between
156 groups in lung tissues.
157 At time of necropsy, sixteen different samples (liver, spleen, adrenal gland, six lung lobes,
158 inguinal, axillary, mesenteric and mediastinal lymph nodes, aqueous humor, and urine) were
159 collected and investigated for the presence of SUDV viral RNA. Viral RNA was detected in all
160 tissues with no differences between groups detected (Figure 4). The highest viral load was
161 detected in liver, spleen, adrenal gland, and inguinal lymph node. Viral RNA was lowest in
162 aqueous humor.
163 Histologically, all animals demonstrated lesions typical of SUDV infection including multifocal
164 to coalescing hepatocellular degeneration and necrosis with acute inflammation. Splenic lesions
165 consist of abundant micro-fibrin thrombi and splenic white pulp necrosis and loss with abundant
166 fibrin effacing the red pulp. Pulmonary lesions in the control group were minimal and included
167 rare microthrombi in two of six animals and minimal neutrophilic infiltrates in one animal. Prime
168 and prime boost vaccinated animals showed minimal to mild neutrophilic interstitial infiltrates
169 and mild subacute perivascular inflammation in all animals across all pulmonary lobes (Figure
170 5).
171
172 Discussion

173 We have investigated the efficacy of ChAdOx1-biEBOV against a uniformly lethal challenge
174 with SUDV in cynomolgus macaques. Despite the presence of antigen-specific binding and
175 neutralizing antibodies, we did not observe differences in the clinical signs of disease in
176 vaccinated macaques compared to controls. All macaques reached endpoint criteria and had to be
177 euthanized.
178 Correlates of protection have not yet been determined for SUDV but have been investigated for
179 EBOV. Recombinant adenovirus serotype 5 (rAd5) encoding EBOV GP is protective against
180 lethal EBOV challenge in cynomolgus macaques. Despite the vaccine eliciting robust EBOV-
181 specific antibodies, passive transfer of purified IgG only protected one out of four macaques. In
182 contrast, CD8+ T cell depletion in vaccinated macaques four days prior to challenge resulted in
183 loss of protection, highlighting the need for T cell mediated responses (*27*). These results parallel
184 with our findings that ChAdOx1-biEBOV immunization resulted in the production of a robust
185 humoral response but did not elicit strong T cell-mediated immunity. Another study utilized
186 immunogenicity and protective efficacy data of the heterologous two-dose vaccine regimen of
187 Ad26.ZEBOV followed by MVA-BN-Filo in a lethal EBOV cynomolgus macaque model and
188 identified binding antibodies against GP as the strongest correlate to survival post-EBOV
189 challenge (*28*). In a previous study using VSV-EBOV vaccination, cynomolgus macaques were
190 T or B cell-depleted before and after vaccination. When they were CD8+ T cell-depleted, they
191 survived a subsequent EBOV challenge four weeks later. CD4+ T cell-dependent survival was
192 time-related: If CD4+ T cells were depleted before vaccination, it reduced EBOV GP-specific
193 antibody development, and macaques did not survive EBOV challenge; whereas, if CD4+ T cells
194 were depleted after vaccination, and macaques were able to develop a normal EBOV-specific
195 antibody response, they survived an EBOV challenge (*29*). Thus, correlates of protection appear

196 to be vaccine platform- and animal model-dependent but suggest an important role for both
197 humoral and cellular immunity dependent on platform.
198 It should be noted that the virus neutralizing antibody response was higher against EBOV than it
199 was against SUDV using a pseudotype neutralization assay (Figure 1D/E). It is unknown what
200 level of neutralizing antibody is needed to protect against overt disease. A previous VSV-SUDV
201 vaccination study in cynomolgus macaques demonstrated complete protection against a lethal
202 SUDV challenge despite low levels of serum neutralizing antibody titers (*17*). It is therefore
203 likely that a multi-component immune response with high levels of cellular and humoral
204 immunity will be best placed to confer protection in the macaque challenge model.
205 Without knowledge of correlates of protection against SUDV, which are likely animal model and
206 vaccine platform dependent, NHP studies are helpful to inform selection of vaccine candidates
207 for clinical development. If needed, NHP studies can be performed relatively fast and challenge
208 outcome can be known within five weeks of study start if utilizing a prime only regimen.
209 Preclinical vaccine studies in NHPs do not necessarily correlate with results obtained in small
210 animal models. Although ChAdOx1-biEBOV was protective against lethal SUDV challenge in
211 Ifnar1[-] mice, this was not the case in cynomolgus macaques. Guinea pigs vaccinated with
212 VSV-EBOV, which provides full protection against guinea pig-adapted (GPA-) EBOV
213 challenge, are partially protected against GPA-SUDV (*30*). However, this finding is not
214 replicated in NHPs (unpublished work). Likewise, we previously showed low immunogenicity of
215 Curevac's CVnCoV SARS-CoV-2 mRNA vaccine candidate in NHPs (*31*), despite the induction
216 of a humoral response in mice and hamsters and a subsequent reduction in replication of SARS-
217 CoV-2 in hamsters (*32*). Ensuing Phase III clinical trials failed to reach the threshold of 50%
218 vaccine efficacy set by the WHO (*33*). Although small animal models are an essential initial

219 model for vaccine evaluation, when possible, efficacy in NHPs should be evaluated ideally
220 before large-scale clinical trial studies.
221 Unfortunately, it is not clear from our results why vaccination with ChAdOx1-biEBOV did not
222 provide protection against challenge. Considering that the amino acid identity between SUDV
223 Gulu GP (AAP88031.1), the challenge strain, and Boniface GP (Q66814.1), included in
224 ChAdOx1-biEBOV is 94.67%, we cannot exclude that protective epitopes differ between these
225 strains; however, vaccination with a rVSV expressing SUDV Boniface has conferred protection
226 against SUDV Gulu (*22*). Since the Uganda 2022 outbreak strain's (OQ672950.1) nucleotide
227 identity is 99.3% with the Gulu strain (KU182912.1) and 94.82% with the Boniface strain
228 (FJ968794.1), SUDV Gulu was selected as the challenge strain. Binding and neutralizing
229 antibodies were elicited against SUDV GP; however, the role of antibodies in protecting
230 cynomolgus macaques against filoviruses when vaccination is done with an adenovirus differs
231 depending on the study design (*27*, *28*). Both binding antibody levels and VN titers were
232 significantly higher in the prime-boost macaques than the prime only, but we did not observe
233 substantial changes in measurements of disease. We measured a minimal T cell response,
234 through ELIspot, after vaccination, even though human volunteers develop a robust cellular
235 response following vaccination with ChAdOx1-biEBOV (unpublished data). We have seen a
236 weak cellular immune response following vaccination of NHPs with the ChAdOx1 vector
237 previously (*23*), in contrast with what has been shown in human volunteers (*34*). This suggests
238 that either the response to ChAdOx1 in NHPs differs from that observed in humans, or that
239 PBMCs are not the correct sample to investigate cellular responses in NHPs vaccinated with
240 ChAdOx1.

241 As for EBOV, translation of the SUDV GP gene results in three different products: soluble GP
242 (sGP, 70-80%), GP (20-30%), and small sGP (low frequency) (*35*). Furthermore, the presence of
243 sGP reduces neutralizing antibody titer *in vitro* (*36*). Thus, we hypothesized that the presence of
244 SUDV sGP and GP in NHP sera after challenge would be able to bind SUDV GP antibodies and
245 thereby reduce the detection of binding antibodies via ELISA. In line with this hypothesis, the
246 two samples in the prime and prime boost group in which we could not detect SUDV sGP had
247 the highest binding SUDV GP antibody levels at the day of necropsy.
248 Finally, the EBOV GP-specific response may play a role in the lack of protection against SUDV.
249 *In vitro,* antibodies against EBOV GP have been associated with enhanced cellular entry (*37*).
250 However, it is unclear whether this plays a role *in vivo,* and in this study, no disease
251 enhancement was observed in vaccinees compared to controls. Indeed, when significant
252 differences were observed, they pointed towards a slight improvement of vaccinees over
253 controls, such as in the platelet count. Furthermore, the efficacy of vaccines that combine
254 filovirus antigens in NHPs has yielded variable results depending on the challenge filovirus.
255 Callendret *et al*. mixed Ad26 or Ad35 adenoviral vector-based filovirus GP vaccines expressing
256 Marburg virus (MARV) Angola GP, EBOV Mayinga GP, or SUDV Gulu GP. Cynomolgus
257 macaques were primed with a mixture of vaccines expressing the different GPs in Ad26 vectors,
258 primed with a mixture of vaccines expressing the different GPs in Ad35 vectors four weeks later,
259 and finally challenged four weeks later. Upon lethal challenge with SUDV Gulu, three out of
260 four animals survived (*38*). In a follow-up study with an increased vaccination dose of 4×10^{10}
261 virus particles/vector (1.2×10^{11} total virus particles/animal), 100% of animals survived a SUDV
262 Gulu challenge, which was significantly correlated to binding antibodies to GP (*14*). A bivalent
263 recombinant protein vaccine that included EBOV Kikwit and SUDV GPs protected all

264 vaccinated cynomolgus macaques from SVD and elicited GP binding antibodies, but the cellular
265 immune response was not evaluated (*39*). A quadrivalent VesiculoVax platform combines four
266 distinct, attenuated rVSVs encoding the GPs of EBOV, SUDV, MARV, and Lassa virus (*22*) or
267 Bundibugyo (*40*) into a single shot. When delivered using a prime-boost regimen, the
268 quadrivalent VesiculoVax protects against SUDV, EBOV, and MARV, elicits production of
269 binding and neutralizing antibodies, and activates antigen-specific T cells in cynomolgus
270 macaques. Of note, IFN-γ producing antigen-specific T cells were only detected in a subset of
271 animals (*22*). When administered a week prior to challenge, all vaccinated animals infected with
272 SUDV, BDBV, or MARV were protected while 80% of macaques challenged with EBOV
273 survived (*40*). Thus, combining different antigens in one vaccine has protected cynomolgus
274 macaques against SUDV in previous studies. A notable difference here is that both antigens were
275 expressed in the same vector; whereas, in the studies described above vaccines encoding a single
276 GP were mixed. When antigens are expressed in the same vector, they will be produced by the
277 same cells, whereas if they are not expressed in the same vector, they may be produced by
278 different cells, potentially resulting in a stronger immune response.
279 The lack of protection despite vaccine-elicited SUDV GP-specific responses in this model and
280 protection in small animal models suggests that we need a better understanding of the correlates
281 of protection for SUDV vaccines. Overall, this study highlights the importance of evaluating
282 Ebolavirus disease vaccines in multiple models and demonstrates the importance of
283 understanding protective efficacy for any infectious agent in both animal models and human
284 hosts.
285
286 <u>Materials and Methods</u>

287 *Ethics statement*

288 The Institutional Animal Care and Use Committee of Rocky Mountain Laboratories, National
289 Institutes of Health (NIH) approved all animal experiments. Experiments were carried out in an
290 AAALAC International–accredited facility, following the guidelines and basic principles in the
291 Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act, U.S. Department of
292 Agriculture, and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory
293 Animals. Cynomolgus macaques were singly housed in adjacent primate cages, which allowed
294 for social interactions. The animal room was climate-controlled with a fixed light-dark cycle (12-
295 hour light/12-hour dark). Commercial monkey chow was provided twice daily. The diet was
296 supplemented with treats, vegetables, or fruit daily. Water was available *ad libitum*.
297 Environmental enrichment consisted of a variety of human interaction, manipulanda, commercial
298 toys, videos, and music. Animals were monitored at least twice daily. The Institutional Biosafety
299 Committee approved work with SUDV under biosafety level 4 conditions and subsequent sample
300 inactivation for removal of specimens from high containment (*41*, *42*).

301

302 *Generation of ChAdOx1-biEBOV*

303 Generation of ChAdOx1-biEBOV was done as described previously (Flaxman GP paper if
304 published). Briefly, EBOV GP (Makona-Kissidougou-C15 GenBank:KJ660346.1) and SUDV
305 GP (Boniface, UniProtKB Q66814.1) were codon-optimized for humans. Antigen cassettes were
306 cloned into an ENTRY plasmid and recombined *in vitro* with ChAdOx1-biDEST containing a
307 Gateway destination cassette in both the E1 and the E4 loci, respectively. E1 and E4 expression
308 cassettes both contained the TetR-repressible CMV promoter; E1 cassettes contained the BGH
309 polyA sequence, while E4 expression cassettes contained the SV40 polyA sequence. SUDV GP

310 was encoded in the E1 cassette, whereas EBOV GP was encoded in the E4 cassette. Viral vectors
311 were produced at the Viral Vector Core Facility at the Jenner Institute. All adenovirus vectors
312 were produced in the T-REx-293 cell line (Thermo Fisher Scientific), which allows for
313 transcriptional repression of the vaccine antigens during vector production. Vectors underwent
314 quality control (including titration, identity PCR, and sterility testing) before being used in *in*
315 *vitro* and *in vivo* studies.
316
317 *Study design*
318 Eighteen cynomolgus macaques of Indonesian origin, all female, were sorted by weight and then
319 randomly divided into three groups of six animals each (prime group 8-11 years old, 3.2-6.0kg;
320 prime boost group 8-13 years old, 3.2-5.2kg; control group 7-11 years old, 3.4-4.7kg). Animals
321 were vaccinated with 2.5 x 10^{10} virus particles per animal of ChAdOx1-biEBOV or ChAdOx1
322 GFP intramuscularly (250 µl in each caudal thigh), as done previously (*23*). Vaccine was diluted
323 in sterile phosphate-buffered saline (PBS). All animals received a vaccination at D-28, and the
324 prime boost group received an additional vaccination at D-56. On D0, animals received 1 x 10^4
325 $TCID_{50}$ of SUDV Gulu (500 µl in each caudal thigh). Clinical scoring was done daily post
326 challenge and based on the evaluation of the following criteria: general appearance and activity,
327 appearance of skin and coat, discharge, respiration, feces and urine output, and appetite. Clinical
328 exams were conducted on D−56, −42, −28, −14, 0, 1, 3, and 6. All animals reached endpoint
329 criteria between D5-7. Hematology analysis was completed on a ProCyte DX (IDEXX
330 Laboratories, Westbrook, ME, USA) and the following parameters were evaluated: red blood
331 cells (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean
332 corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell

333 distribution weight (RDW), platelets, mean platelet volume (MPV), white blood cells (WBC),
334 neutrophil count (abs and %), lymphocyte count (abs and %), monocyte count (abs and %),
335 eosinophil count (abs and %), and basophil count (abs and %). Serum chemistries were
336 completed on a VetScan VS2 Chemistry Analyzer (Abaxis, Union City, CA) and the following
337 parameters were evaluated: glucose, blood urea nitrogen (BUN), creatinine, calcium, albumin,
338 total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline
339 phosphatase (ALP), total bilirubin, globulin, sodium, potassium, chloride, and total carbon
340 dioxide. Upon reaching endpoint criteria, animals were euthanized, and necropsy was performed.
341 The following tissues were collected: muscle at injection site, liver, spleen, adrenal gland, 6 lung
342 lobes, inguinal, axillary, mesenteric and mediastinal lymph nodes, aqueous humor, and urine.
343 Urine was not collected from three of the macaques, one in each study group.
344
345 *Cells and Virus*
346 SUDV Gulu (NC_006432.1) was obtained from the US Army Medical Research Institute of
347 Infectious Diseases and propagated once on Vero E6 cells. Deep sequencing revealed no
348 contaminants; however, compared to the reference sequence, four base pair changes (two in NP,
349 one in VP40, and one non-coding in GP (*17*)) were noted. VeroE6 cells were maintained in
350 DMEM supplemented with 10% fetal bovine serum, 1 mM l-glutamine, penicillin (50 U/ml), and
351 streptomycin (50 µg/ml; DMEM10). Mycoplasma testing is performed at regular intervals, and
352 no mycoplasma was detected.
353
354 *RNA extraction and quantitative reverse-transcription polymerase chain reaction*

355 RNA was extracted from whole blood using a QiaAmp Viral RNA kit (Qiagen) according to the
356 manufacturer's instructions. Tissue was homogenized and extracted using the RNeasy kit
357 (Qiagen) according to the manufacturer's instructions. A SUDV GP viral RNA–specific assay
358 (F: CAAAGGGAAGAATCTCCGACC, R: CAGGGGAATTCTTTGGAACC, P: FAM-
359 GGCCACCAGGAAGTATTCGGACC-BHQ; designed using the Sudan ebolavirus isolate Ebola
360 virus/H.sapiens-tc/UGA/2000/Gulu-808892 (KR063670.1) sequence) was used for the detection
361 of viral RNA. RNA (5 µl) was tested with QuantStudio (Thermo Fisher Scientific) according to
362 instructions of the manufacturer. To create standards, SUDV Gulu stocks were extracted using
363 QIAamp viral RNA kits per manufacturer's instructions. RNA was serially diluted and
364 quantified using the One-Step RT-ddPCR system (Bio-Rad). Briefly, 5 µL of RNA was
365 combined with recommended volumes and concentrations of supermix, reverse transcriptase,
366 DTT, primers, probe, and molecular grade water. This mix was then combined with Automated
367 Droplet Generation Oil for Probes using the Automated Droplet Generator. Once the droplets
368 were generated, PCR was performed using recommended cycling conditions. Reverse
369 transcription was performed at 50°C and anneal/extension was performed at 60°C. After thermal
370 cycling, positive droplets were quantified with the QX200 Droplet Reader. Finally, a range of
371 copies/rxn that gave cycle threshold values between 20-30 were selected as controls and used to
372 quantify RNA in this study.
373
374 *SUDV GP and EBOV GP ELISA*
375 The presence of SUDV GP- and EBOV GP-specific binding antibodies in macaque sera was
376 determined using ELISA, carried out in Nunc MaxiSorp flat bottom 96-well plates (Thermo Fisher
377 Scientific) coated with 50 ng/well of trimeric ectodomain SUDV GP or EBOV GP

378 (NativeAntigen) in DPBS followed by overnight incubation at 4°C. The wells were blocked with
379 100 µl of casein in PBS (ThermoFisher) for 1 h at room temperature and then incubated with
380 diluted sera in triplicate for 1 h at 37°C. Serially diluted (1:100 to 1:51200) sera obtained from a
381 SUDV-challenged macaques at 40 days post challenge was utilized as a standard curve and diluted
382 1:1600 as an internal control. IgG antibodies were detected by using affinity-purified polyclonal
383 antibody peroxidase labelled goat-anti-monkey IgG (Seracare) at a dilution of 1:2500 in casein in
384 PBS for 1 h at 37°C. The absorbance was measured at 405 nm after the addition of TMB two
385 component peroxidase substrate (Seracare, 5120-0047) for 5-10 min followed by the addition of
386 stop solution (Seracare, 5150-0021). Wells were washed 6x with PBST 0.1% Tween in between
387 each step. Arbitrary ELISA units were calculated based on the standard curve. All samples diluted
388 at 200x with an OD value of less than 0.250 were given a value of 1.

389

390 *sGP ELISA*

391 The SUDV sGP-specific sandwich ELISA was performed as previously described (*43*) with
392 minor differences. SUDV sGP was captured using the 1 µg/mL polyclonal rabbit anti-SUDV
393 sGP antibody (cat. # 0302-030, IBT Bioservices). Dilutions of recombinant SUDV sGP (cat. #
394 0570-001, IBT Bioservices) at known concentrations served as standards.

395

396 *Pseudovirus neutralization*

397 Neutralization assays with VSV-EBOV-GFP and VSV-SUDV-GFP were performed as
398 previously described (*17*). The VSV-SUDV-GFP assay was optimized to include incubation of
399 serum dilution mix on the Vero E6 cells for 16 hours at 37ºC. Samples were analyzed on the

400 FACSymphony™ A5 Cell Analyzer (BD Biosciences, San Jose, CA, USA) and the GFP-
401 positive cell count was analyzed using FlowJo v10 Software (BD Life Sciences).
402
403 *ELIspot assay*
404 Peripheral blood mononuclear cells (PBMCs) were isolated from ethylene diamine
405 tetraaceticacid (EDTA) whole blood on certain exam days (D-56, -42, -28, -14, and 0) using
406 Leucosep tubes (Greiner Bio-one International) and Histopaque-1077 density-gradient cell-
407 separation medium (Sigma-Aldrich) according to the manufacturers' instructions and frozen in
408 liquid nitrogen. After thawing of cells, cells were plated at a concentration of approximately
409 100,000 cells per well and were stimulated for 6 hours with contiguous peptide pools that
410 spanned the length of the EBOV or SUDV GP at a concentration of 2 µg/mL per peptide. As a
411 positive control, cells were stimulated with eBioscience™ Cell Stimulation Cocktail
412 (ThermoFisher). As a negative control, cells were incubated in media with equivalent amounts of
413 DMSO. The ImmunoSpot Human IFNγ Single-Colour Enzymatic ELISpot Assay Kit was
414 performed according to the manufacturer's protocol (Cellular Technology). Analysis was
415 performed using the CTL ImmunoSpot Analyzer and ImmunoSpot Software (Cellular
416 Technology).
417
418 *Measurement of cytokines and chemokines*
419 Sera was collected on all exam days. Tissue (Lower left lung lobe, and liver) was weighted,
420 homogenized in 750 µl of DMEM, spun down for 10 min at 8000 rpm, after which supernatant
421 was collected. Sera and tissue supernatant were irradiated (4 MRad) and analyzed on the
422 MesoQuick Plex (MSD, K15203D) at a 4x and 10x dilution, respectively. The U-PLEX

423 Biomarker Group 1 (NHP) Assay kit (MSD, K15068L) was used to test the presence of twelve
424 cytokines (GM-CSF, IFNα2a, IFN-γ, IL-1β, IL-4, IL-6, IL-10, IL-12 p70, IL-15, IP-10, MCP-1,
425 and TNF-α), the V-PLEX Cytokine Panel 1 Human kit (MSD, K15050D) was used to test the
426 presence of seven additional cytokines (IL-5, IL-7, IL-12/IL-23p40, IL-16, IL-17A, TNF-β, and
427 VEGF-A), and the V-PLEX Chemokine Panel 1 Human kit (MSD, K15047D) was used to test
428 the presence of seven additional chemokines (Eotaxin-3, IL-8 (HA), MCP-1, MDC, MIP-1α,
429 MIP-1β, and TARC). Data were extracted from the plates using the MSD Workbench 4.0
430 software and qualitative lower limit of detection (QLOD) were calculated for each parameter. All
431 values below the QLOD were changed to the QLOD. Cytokine and chemokine concentrations
432 were calculated per mg of tissue and mL of sera.
433
434 *Histology and immunohistochemistry*
435 Necropsies and tissue sampling were performed according to IBC-approved protocols. Tissues
436 were perfused with 10% neutral-buffered formalin and fixed for a minimum of 7 days. Tissues
437 were placed in cassettes and processed with a Sakura VIP-6 Tissue Tek, on a 12-hour automated
438 schedule, using a graded series of ethanol, xylene, and PureAffin. Embedded tissues are
439 sectioned at 5 µm and dried overnight at 42°C prior to staining. Specific anti-Ebolavirus
440 immunoreactivity was detected using an in-house rabbit polyclonal anti-Ebola Zaire VP40
441 antibody at a 1:2000 dilution. Vector Laboratories ImmPress VR horse anti-rabbit IgG polymer
442 (# MP-6401) was used as a secondary antibody. The tissues were stained using the Discovery
443 Ultra automated stainer (Ventana Medical Systems) with a Roche Tissue Diagnostics Discovery
444 purple kit (#760-229). The tissue slides were examined by a board-certified veterinary anatomic
445 pathologist blinded to study group allocations. Pulmonary pathology was evaluated in three

446 histological sections from each of the six lung lobes resulting in eighteen evaluated sections for
447 each macaque. Histologic lesion severity was scored according to a standardized scoring system
448 evaluating the presence of pathology: 0, no lesions; 1, minimal (1-10% of lobe affected); 2, mild
449 (11-25%); 3, moderate (26-50%); 4, marked (51-75%); 5, severe (76-100%). Presence of viral
450 antigen was scored per lung lobe according to a standardized scoring system: 0, none; 1,
451 rare/few; 2, scattered; 3, moderate; 4, numerous; 5, diffuse. A representative lesion from each
452 group was selected for figures.
453
454 *Statistical analysis*
455 Kruskall-Wallis analysis was conducted to compare differences between groups, followed by
456 Mann-Whitney test if differences were found to be significant using GraphPad Prism version
457 8.3.0. Statistical tests used are identified in the figure legends.

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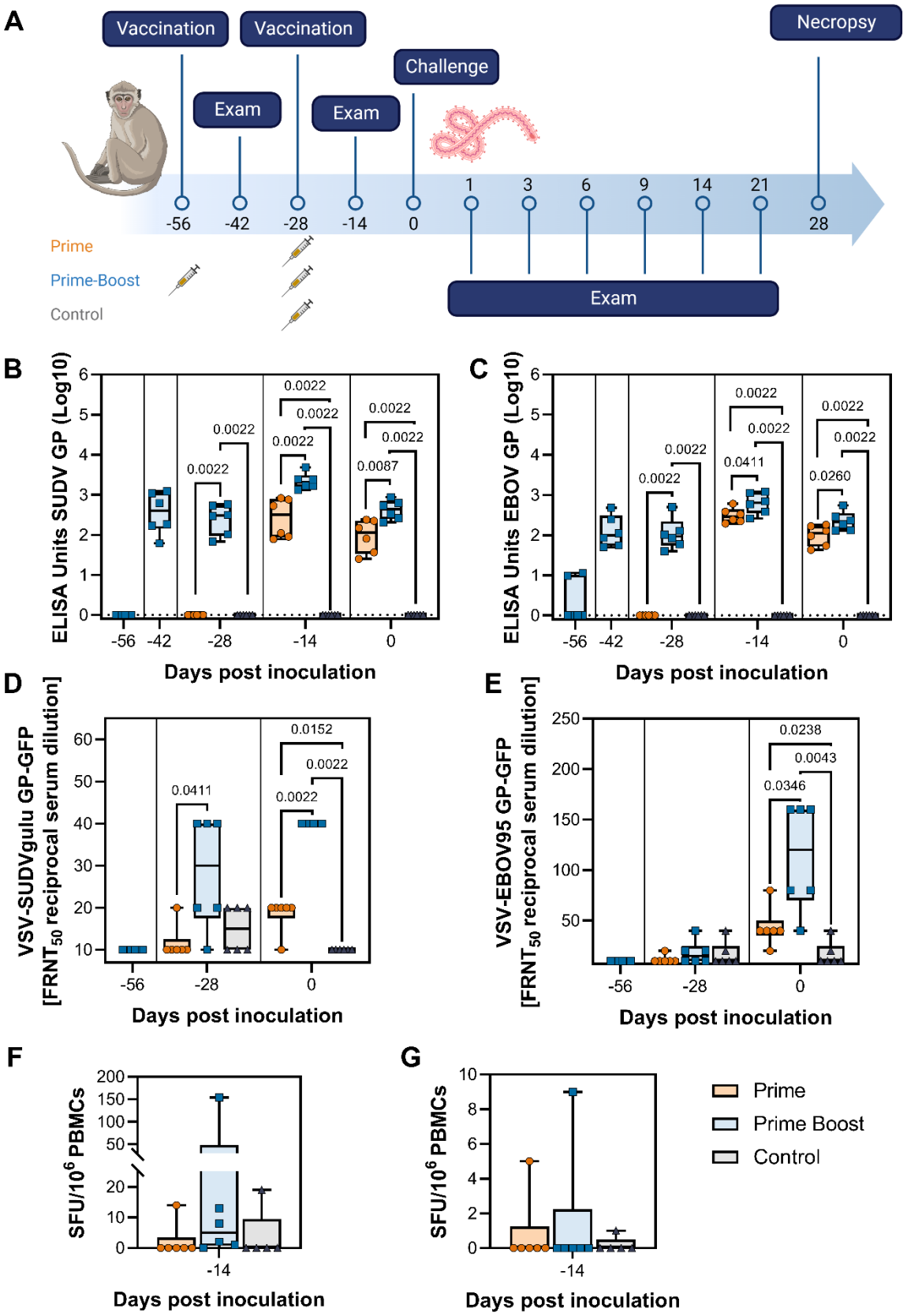

A
Vaccination
Vaccination
Exam
Exam
Challenge
Necropsy
-56
-42
-28
-14
0
1
3
6
9
14
21
28
Prime
Prime-Boost
Control
Exam
B
ELISA Units SUDV GP (Log10)
Days post inoculation
C
ELISA Units EBOV GP (Log10)
Days post inoculation
D
VSV-SUDVgulu GP-GFP [FRNT50 reciprocal serum dilution]
Days post inoculation
E
VSV-EBOV95 GP-GFP [FRNT50 reciprocal serum dilution]
Days post inoculation
F
SFU/10^6 PBMCs
Days post inoculation
G
SFU/10^6 PBMCs
Days post inoculation
Prime
Prime Boost
Control

686

687 **Figure 1. Immunological responses in cynomolgus macaques upon vaccination with**
688 **ChAdOx1-biEBOV.** A. Overview of experimental design. B/C. Binding antibodies against
689 trimeric SUDV GP (B) or EBOV GP (C) at different time points post vaccination and challenge.
690 Log10 transformed values are shown, which were compared to a standard curve of positive sera.
691 D/E. Virus neutralizing antibodies against pseudovirus VSV-SUDV (D) or VSV-EBOV (E) at
692 different time points post challenge. $FRNT_{50}$ of green fluorescence protein-positive cell count is
693 shown. F/G. SUDV GP (F) or EBOV GP (G) specific T cell responses in PBMCs isolated from
694 vaccinated or controls animals at D-14 normalized to D−56 or D-28 days post-challenge
695 response. Significance was determined using Kruskall Wallis test followed by Mann Whitney
696 test. The dotted line shows the limit of detection. GP = glycoprotein. FRNT50=50% fluorescence
697 reduction. SUDV=Sudan virus. EBOV=Ebola virus. VSV=vesicular stomatitis virus, SFU=spot-
698 forming units.

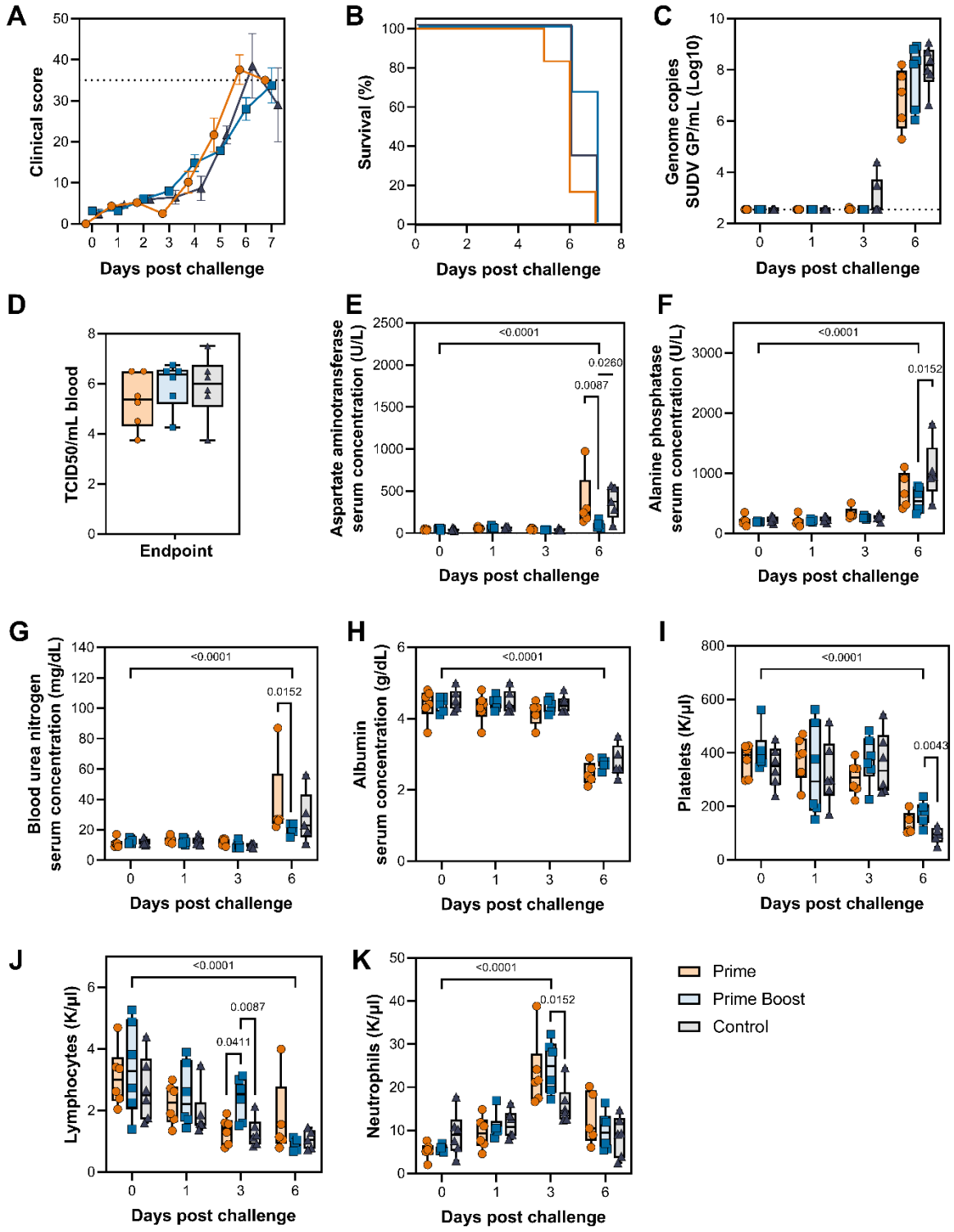


Figure 2. Survival and clinical signs upon challenge of macaques with SUDV Gulu. Clinical score (A) survival (B), and viremia (C) are shown. The following parameters were measured in

702 blood of NHPs: Aspartate aminotransferase (AST, E), alkaline phosphatase (ALP, F), blood urea
703 nitrogen (BUN, G), albumin (H), platelets (I), lymphocytes (J), and neutrophils (K). Significance
704 was determined using Kruskall Wallis test followed by Mann Whitney test. Dotted line shows
705 (A) clinical score at which euthanasia is required, (C) or lower limit of detection.
706

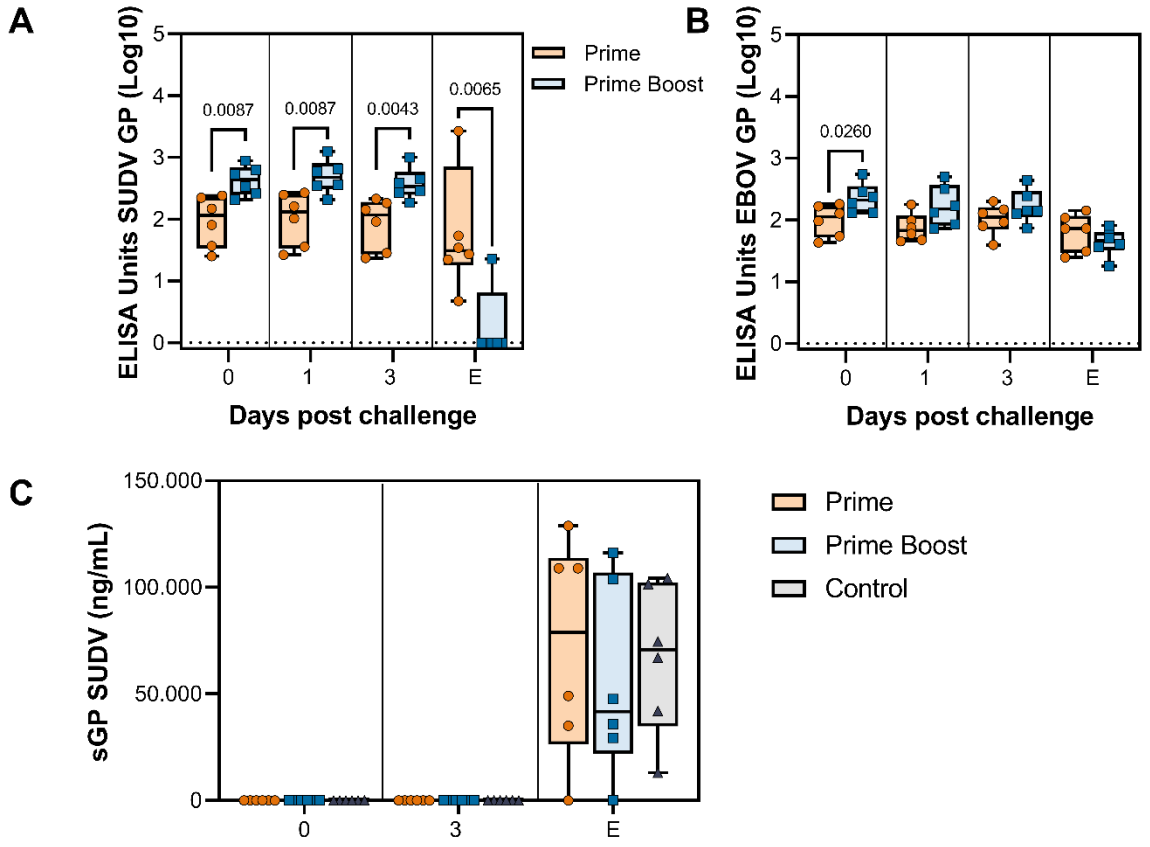
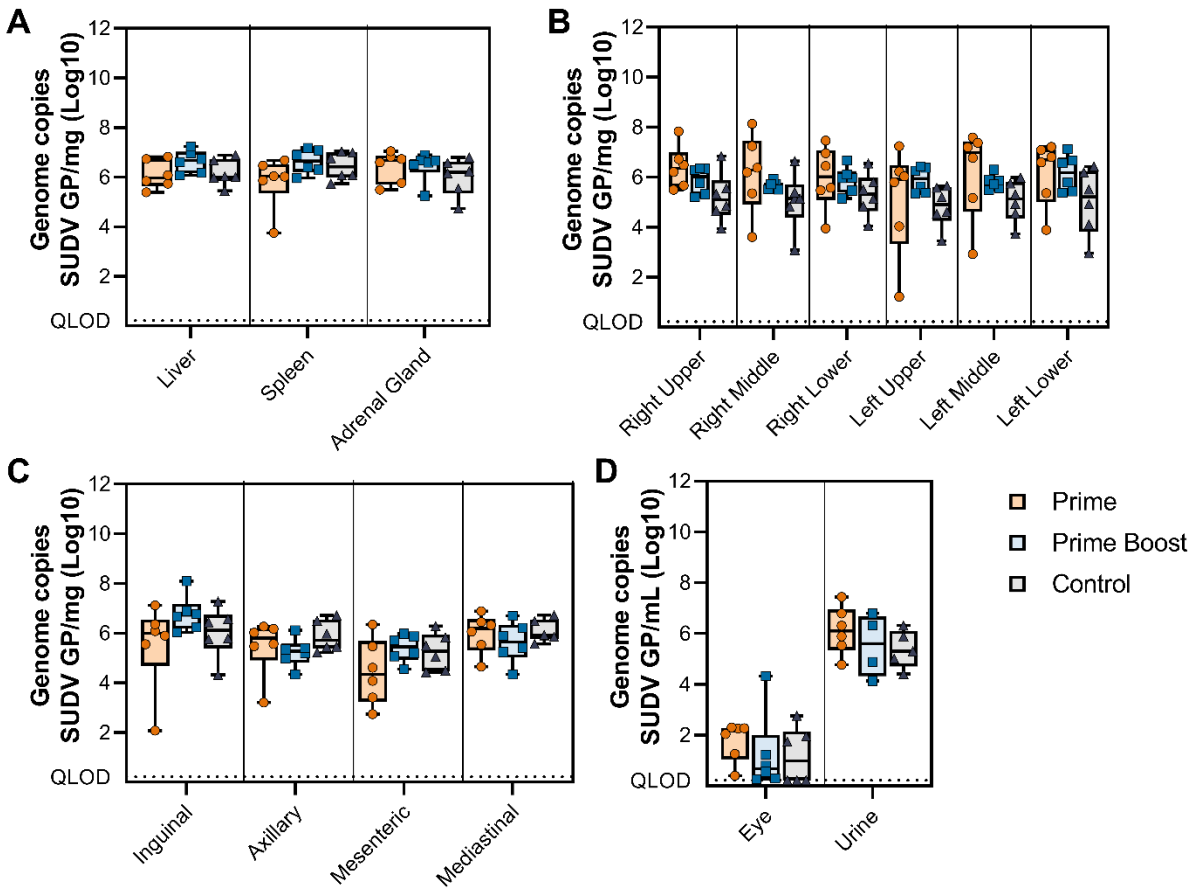


Figure 3. Binding antibody levels and sGP in serum obtained from macaques post challenge. Binding antibody levels against SUDV GP (A) and EBOV GP (B) are shown for the prime and prime boost group post challenge. No binding antibody was detected in control animals. The dotted line shows the limit of detection. C. Soluble GP (sGP) levels were determined in sera using capture ELISA. Significance was determined using Kruskall Wallis test followed by Mann Whitney tests. E = Endpoint. GP = glycoprotein. SUDV=Sudan virus. EBOV=Ebola virus.



715

716 **Figure 4. Presence of SUDV viral RNA in tissues obtained from NHPs at necropsy.** Viral

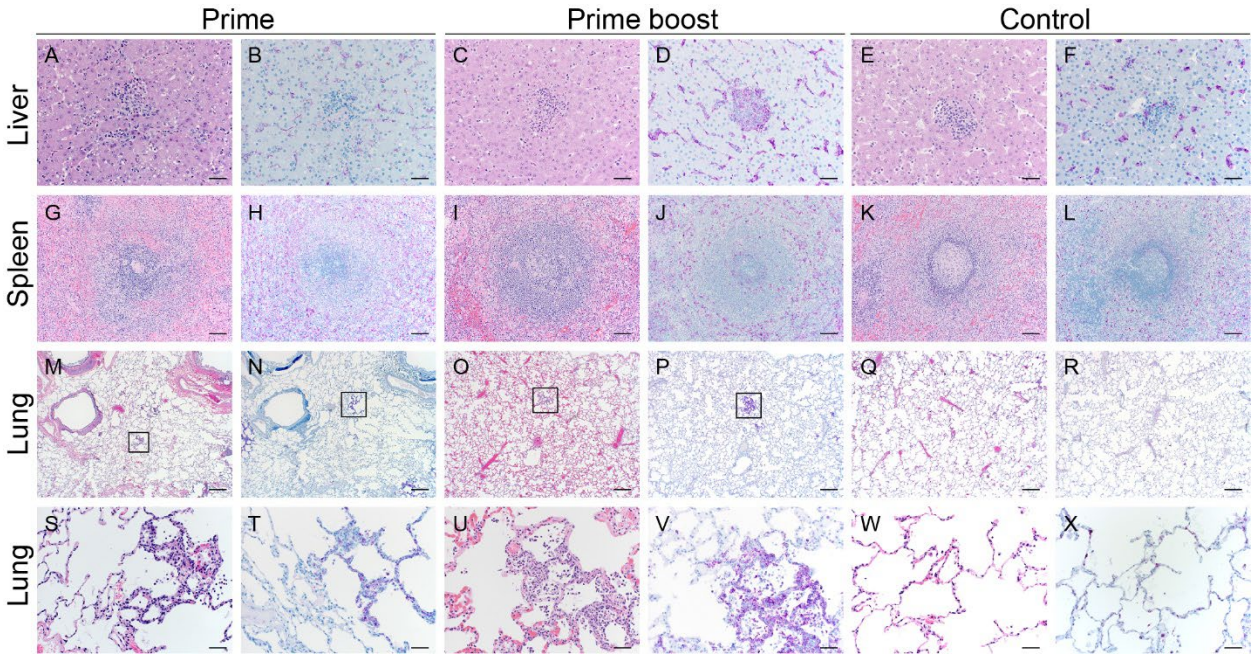
717 RNA load was determined in liver, spleen, adrenal gland (A), lung (B), multiple lymph nodes

718 (C), and aqueous humor collected from the eye as well as urine (D). Log10 transformed values

719 are shown, which were compared to a standard curve of known copies of viral SUDV RNA.

720 Significance was determined using Kruskall Wallis test followed by Mann Whitney tests. QLOD

721 = Qualitative limit of detection.

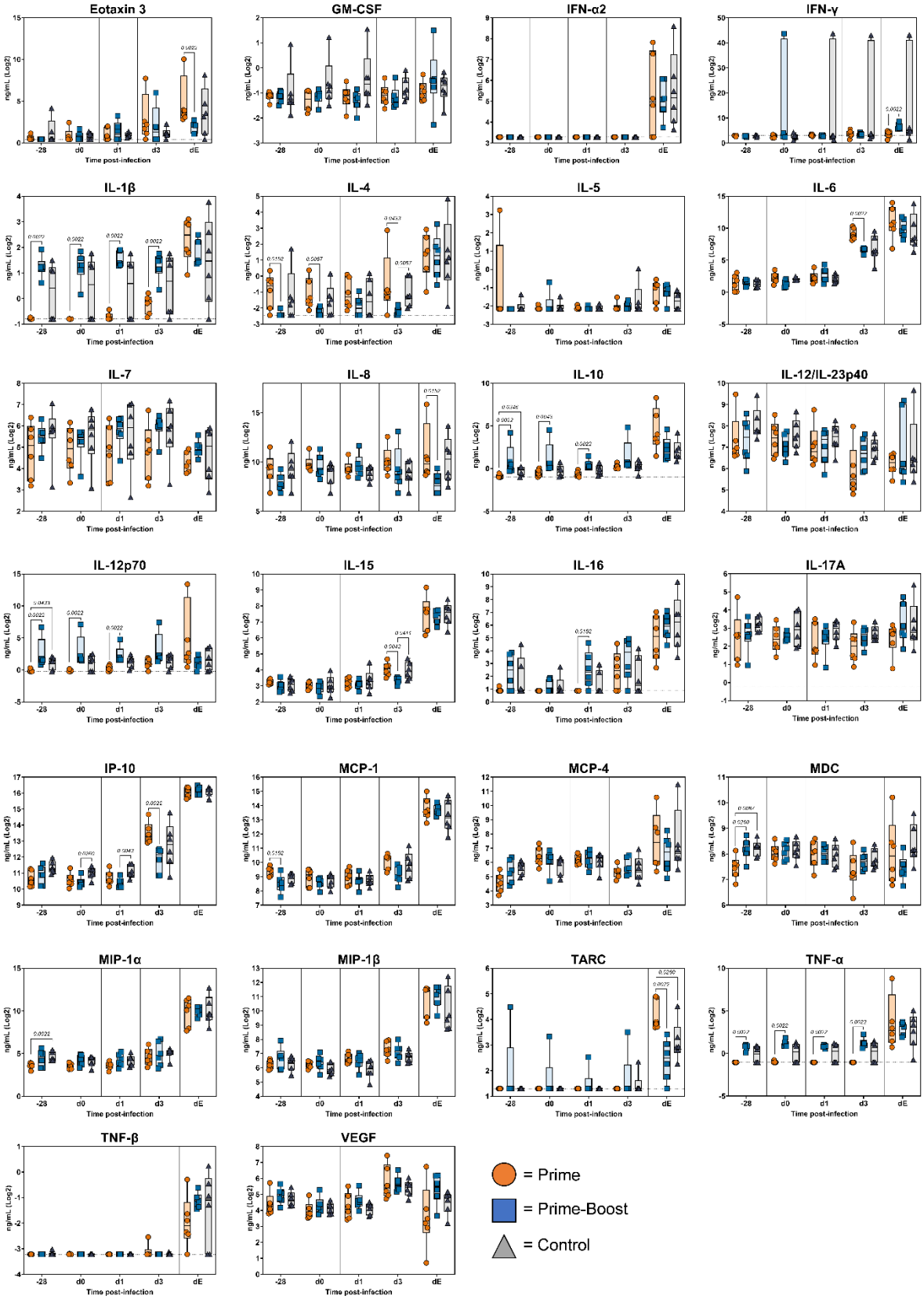


722

723 **Figure 5. Histopathological effects of ChAdOx1-biEBOV vaccine in the cynomolgus**
724 **macaque model of SUDV infection.** Tissue sections were stained with hematoxylin and eosin
725 (H&E- A,C,E,G,I,K,M,O,Q,S,U,W) or polyclonal rabbit serum against Ebola virus viral protein
726 40 for detection of viral antigen (purple) by immunohistochemistry (IHC-
727 B,D,F,H,J,L,N,P,R,T,V,X). Liver pathology was similar in all groups consisting of mild-
728 moderate acute inflammation with multifocal hepatocellular necrosis and abundant Kupffer cell
729 and hepatocyte IHC immunoreactivity. Splenic pathology was similar in all groups consisting of
730 minimal-moderate acute inflammation with moderate numbers of red pulp fibrin thrombi; IHC
731 immunoreactivity was prevalent in numerous macrophages. Pulmonary pathology in the prime
732 and prime boost vaccinated animals had minimal-mild neutrophilic interstitial infiltrates shown
733 in the black box (M-P) at 20X magnification and enlarged in S-V at 200X magnification. Control
734 animals did not show evidence of pathology in 5/6 animals with one showing minimal interstitial
735 infiltrates. IHC immunoreactivity in all groups was scatted-numerous in macrophages and

736 endothelial cells. The following magnification was used; liver 200X, spleen 100X, lung 20X and

737 200X. Bar 200X=50 µm, 100X=100 µm and 20X=500 µm.

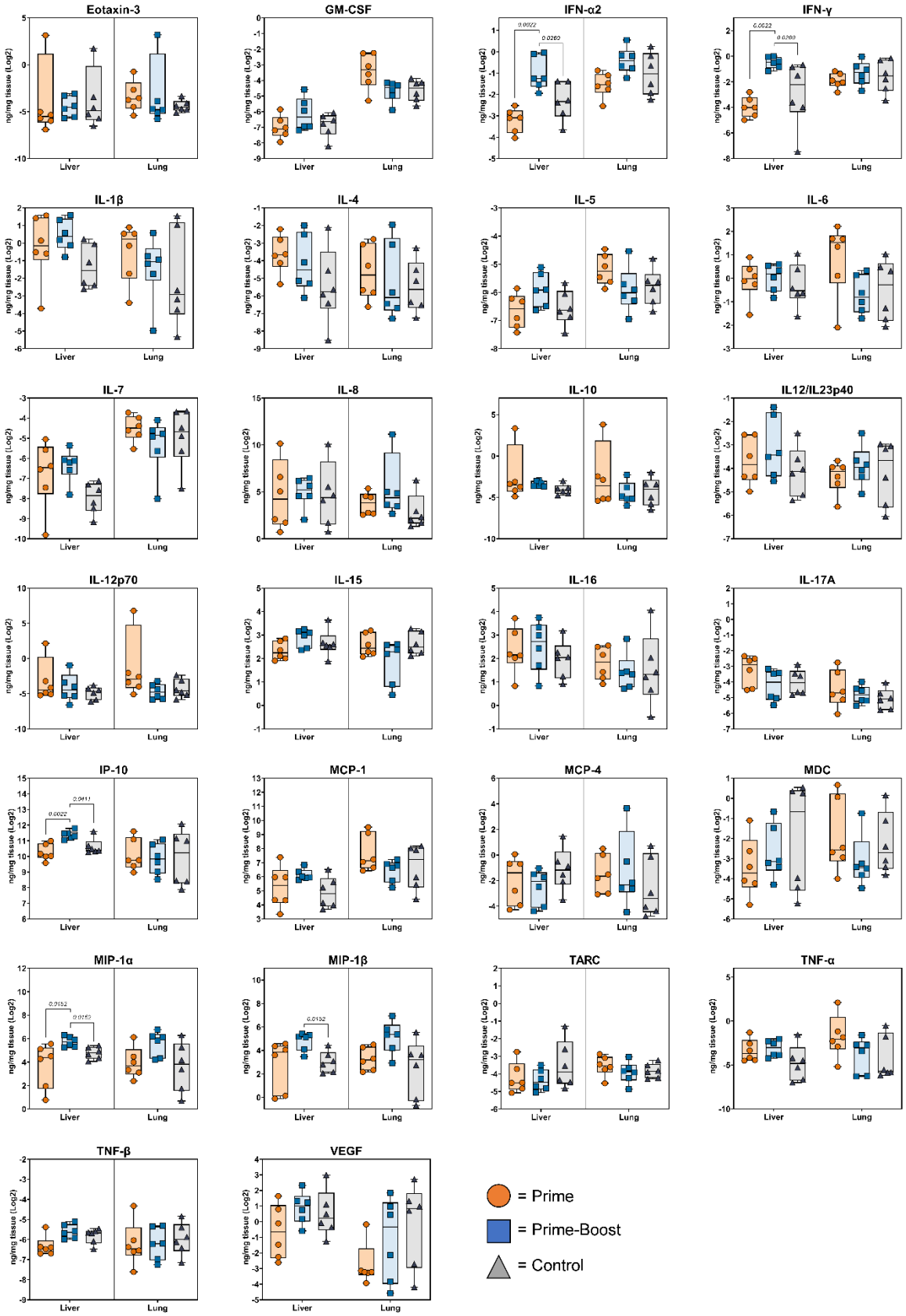

Eotaxin 3
GM-CSF
IFN-α2
IFN-γ
IL-1β
IL-4
IL-5
IL-6
IL-7
IL-8
IL-10
IL-12/IL-23p40
IL-12p70
IL-15
IL-16
IL-17A
IP-10
MCP-1
MCP-4
MDC
MIP-1α
MIP-1β
TARC
TNF-α
TNF-β
VEGF
ng/mL (Log2)
Time post-infection
-28
d0
d1
d3
dE
= Prime
= Prime-Boost
= Control

738

739 **Supplementary Figure 1. Cytokines and chemokines in serum from cynomolgus macaques**

740 **challenged with SUDV.** Serum was collected at exam and cytokine and chemokine

741 concentrations were measured using the Meso Quickplex.


Eotaxin-3
GM-CSF
IFN-α2
IFN-γ
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IL-4
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IL-7
IL-8
IL-10
IL12/IL23p40
IL-12p70
IL-15
IL-16
IL-17A
IP-10
MCP-1
MCP-4
MDC
MIP-1α
MIP-1β
TARC
TNF-α
TNF-β
VEGF
ng/mg tissue (Log2)
Liver
Lung
= Prime
= Prime-Boost
= Control

742

743 **Supplementary Figure 2. Cytokines and chemokines in tissues from cynomolgus macaques**

744 **challenged with SUDV.** Tissues were collected at necropsy and cytokine and chemokine

745 concentrations were measured using the Meso Quickplex.