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

Sarah van Tol[1], Paige Fletcher[1], Friederike Feldmann[2], Reshma K. Mukesh[1], Julia R. Port[1], Shane Gallogly[1], Jonathan E. Schulz[1], Joseph F. Rhoderick[1], Rebecca Makinson[3], Aaron Carmody[4], Lara Myers[4], Jamie Lovaglio[2], Brian J. Smith[2], Atsushi Okumura[1], Carl Shaia[2], Greg Saturday[2], Andrea Marzi[1], Teresa Lambe[3], Vincent J. Munster[1], Neeltje van Doremalen[1*]

1. Laboratory of Virology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, USA
2. Rocky Mountain Veterinary Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, USA
3. Oxford Vaccine Group, Department of Paediatrics, University of Oxford, Oxford, UK
4. Research Technologies Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, USA

*Corresponding author: Neeltje van Doremalen, 903 south 4th street, Hamilton, MT, USA. neeltje.vandoremalen@nih.gov

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22 Abstract

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

37

38 Key words: Sudan virus, filovirus, vaccine, animal model, NHP, adenovirus-vectored vaccine,
39 Ebola virus

40 Background

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

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

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

84 Results

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

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

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

153 expression of IL-4, IL-6, IL-8, IL-15, and IP-10 in the prime boost group compared to the other
154 two groups on D3 or day of necropsy (Supplementary Figure 1).
155 In liver tissue, multiple cytokines and chemokines were significantly elevated in the prime boost
156 group compared to the prime and control groups (Supplementary Figure 2, IFN-α2, IFN-γ, IP-10,
157 MIP-1α, MIP-1β). No differences in cytokines and chemokines levels were observed between
158 groups in lung tissues.
159 At time of necropsy, sixteen different samples (liver, spleen, adrenal gland, six lung lobes,
160 inguinal, axillary, mesenteric and mediastinal lymph nodes, aqueous humor, and urine) were
161 collected and investigated for the presence of SUDV viral RNA. Viral RNA was detected in all
162 tissues with no differences between groups detected (Figure 4). The highest viral load was
163 detected in liver, spleen, adrenal gland, and inguinal lymph node. Viral RNA was lowest in
164 aqueous humor.
165 Histologically, all animals demonstrated lesions typical of SUDV infection including multifocal
166 to coalescing hepatocellular degeneration and necrosis with acute inflammation. Splenic lesions
167 consist of abundant micro-fibrin thrombi and splenic white pulp necrosis and loss with abundant
168 fibrin effacing the red pulp. Pulmonary lesions in the control group were minimal and included
169 rare microthrombi in two of six animals and minimal neutrophilic infiltrates in one animal. Prime
170 and prime boost vaccinated animals showed minimal to mild neutrophilic interstitial infiltrates
171 and mild subacute perivascular inflammation in all animals across all pulmonary lobes (Figure
172 5).
173
174 Discussion

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

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

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

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

266 vaccinated cynomolgus macaques from SVD and elicited GP binding antibodies, but the cellular
267 immune response was not evaluated (*39*). A quadrivalent VesiculoVax platform combines four
268 distinct, attenuated rVSVs encoding the GPs of EBOV, SUDV, MARV, and Lassa virus (*22*) or
269 Bundibugyo (*40*) into a single shot. When delivered using a prime-boost regimen, the
270 quadrivalent VesiculoVax protects against SUDV, EBOV, and MARV, elicits production of
271 binding and neutralizing antibodies, and activates antigen-specific T cells in cynomolgus
272 macaques. Of note, IFN-γ producing antigen-specific T cells were only detected in a subset of
273 animals (*22*). When administered a week prior to challenge, all vaccinated animals infected with
274 SUDV, BDBV, or MARV were protected while 80% of macaques challenged with EBOV
275 survived (*40*). Thus, combining different antigens in one vaccine has protected cynomolgus
276 macaques against SUDV in previous studies. A notable difference here is that both antigens were
277 expressed in the same vector; whereas, in the studies described above vaccines encoding a single
278 GP were mixed. When antigens are expressed in the same vector, they will be produced by the
279 same cells, whereas if they are not expressed in the same vector, they may be produced by
280 different cells, potentially resulting in a stronger immune response.
281 The lack of protection despite vaccine-elicited SUDV GP-specific responses in this model and
282 protection in small animal models suggests that we need a better understanding of the correlates
283 of protection for SUDV vaccines. Overall, this study highlights the importance of evaluating
284 Ebolavirus disease vaccines in multiple models and demonstrates the importance of
285 understanding protective efficacy for any infectious agent in both animal models and human
286 hosts.
287
288 <u>Materials and Methods</u>

289 *Ethics statement*

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

303

304 *Generation of ChAdOx1-biEBOV*

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

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

335 distribution weight (RDW), platelets, mean platelet volume (MPV), white blood cells (WBC),
336 neutrophil count (abs and %), lymphocyte count (abs and %), monocyte count (abs and %),
337 eosinophil count (abs and %), and basophil count (abs and %). Serum chemistries were
338 completed on a VetScan VS2 Chemistry Analyzer (Abaxis, Union City, CA) and the following
339 parameters were evaluated: glucose, blood urea nitrogen (BUN), creatinine, calcium, albumin,
340 total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline
341 phosphatase (ALP), total bilirubin, globulin, sodium, potassium, chloride, and total carbon
342 dioxide. Upon reaching endpoint criteria, animals were euthanized, and necropsy was performed.
343 The following tissues were collected: muscle at injection site, liver, spleen, adrenal gland, 6 lung
344 lobes, inguinal, axillary, mesenteric and mediastinal lymph nodes, aqueous humor, and urine.
345 Urine was not collected from three of the macaques, one in each study group.
346
347 *Cells and Virus*
348 SUDV Gulu (NC_006432.1) was obtained from the US Army Medical Research Institute of
349 Infectious Diseases and propagated once on Vero E6 cells. Deep sequencing revealed no
350 contaminants; however, compared to the reference sequence, four base pair changes (two in NP,
351 one in VP40, and one non-coding in GP (*17*)) were noted. VeroE6 cells were maintained in
352 DMEM supplemented with 10% fetal bovine serum, 1 mM l-glutamine, penicillin (50 U/ml), and
353 streptomycin (50 µg/ml; DMEM10). Mycoplasma testing is performed at regular intervals, and
354 no mycoplasma was detected.
355
356 *RNA extraction and quantitative reverse-transcription polymerase chain reaction*

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

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

391

392 *sGP ELISA*

393 The SUDV sGP-specific sandwich ELISA was performed as previously described (*43*) with
394 minor differences. SUDV sGP was captured using the 1 µg/mL polyclonal rabbit anti-SUDV
395 sGP antibody (cat. # 0302-030, IBT Bioservices). Dilutions of recombinant SUDV sGP (cat. #
396 0570-001, IBT Bioservices) at known concentrations served as standards.

397

398 *Pseudovirus neutralization*

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

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

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

448 histological sections from each of the six lung lobes resulting in eighteen evaluated sections for
449 each macaque. Histologic lesion severity was scored according to a standardized scoring system
450 evaluating the presence of pathology: 0, no lesions; 1, minimal (1-10% of lobe affected); 2, mild
451 (11-25%); 3, moderate (26-50%); 4, marked (51-75%); 5, severe (76-100%). Presence of viral
452 antigen was scored per lung lobe according to a standardized scoring system: 0, none; 1,
453 rare/few; 2, scattered; 3, moderate; 4, numerous; 5, diffuse. A representative lesion from each
454 group was selected for figures.
455
456 *Statistical analysis*
457 Kruskall-Wallis analysis was conducted to compare differences between groups, followed by
458 Mann-Whitney test if differences were found to be significant using GraphPad Prism version
459 8.3.0. Statistical tests used are identified in the figure legends.

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462 AM, NvD acquired, analyzed, and interpreted the data; SvT and NvD wrote the manuscript. All
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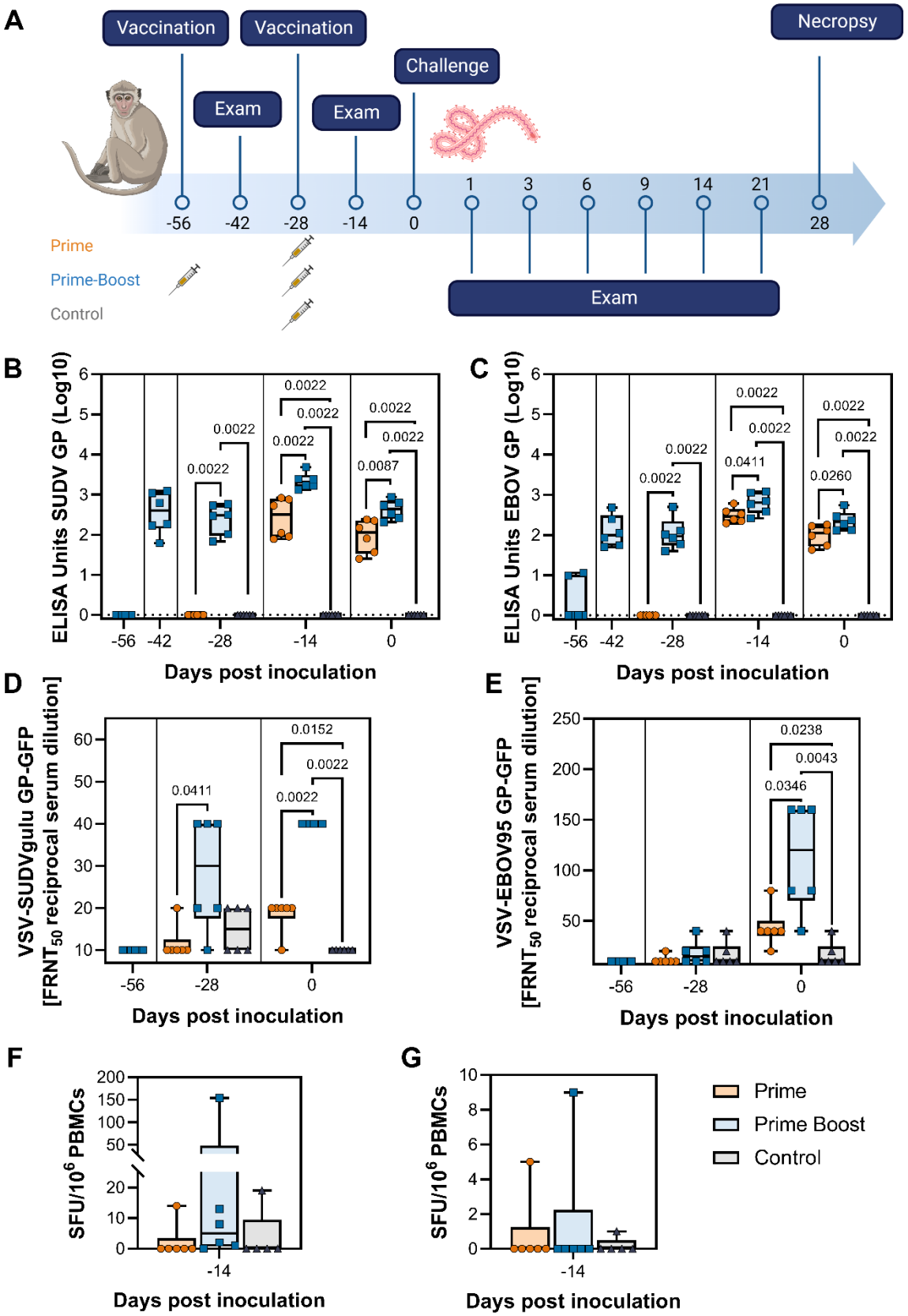
A
Vaccination
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Necropsy
-56
-42
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0
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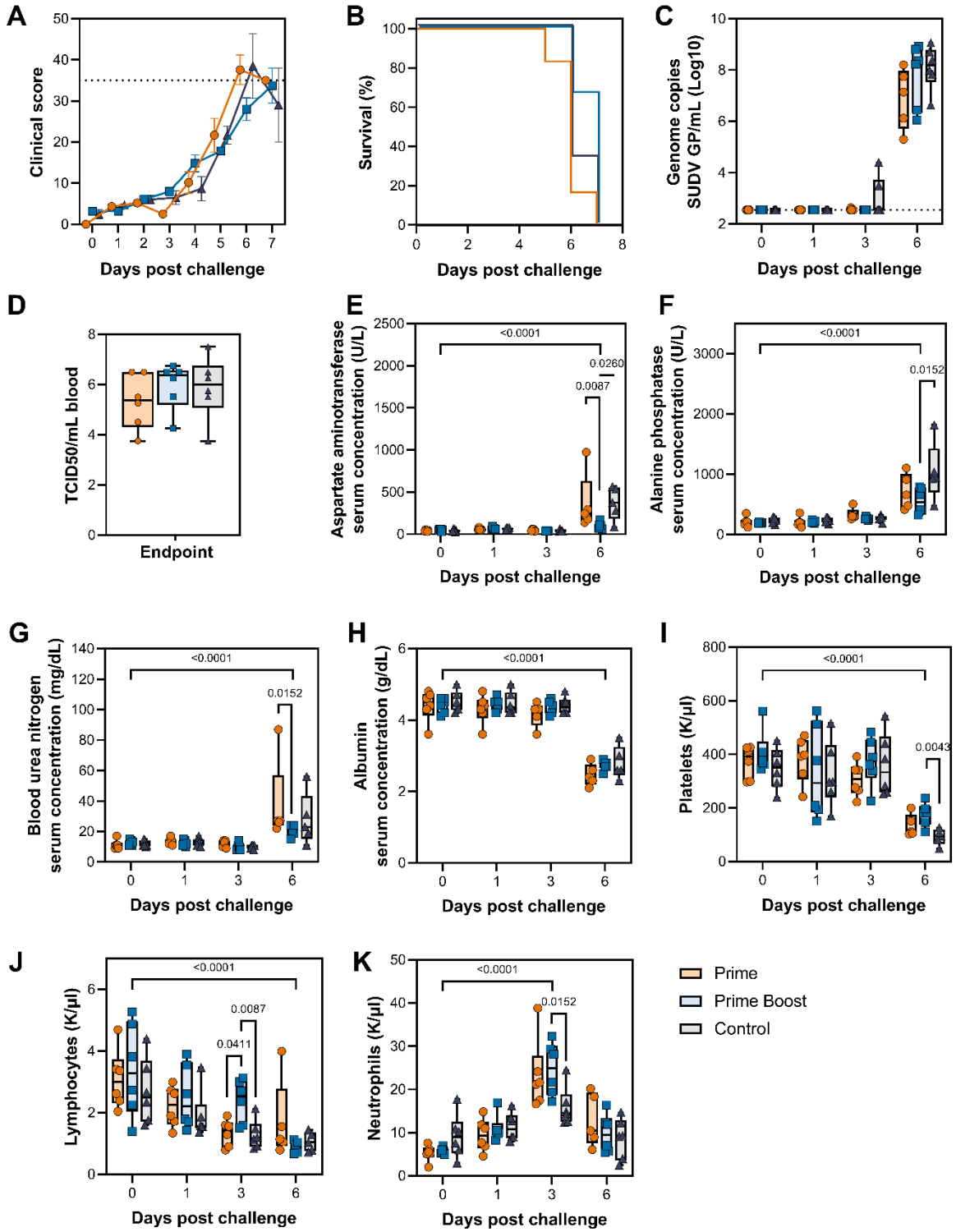
3
6
9
14
21
28
Prime
Prime-Boost
Control
Exam
B
ELISA Units SUDV GP (Log10)
Days post inoculation
C
ELISA Units EBOV GP (Log10)
Days post inoculation
D
VSV-SUDVgulu GP-GFP [FRNT50 reciprocal serum dilution]
Days post inoculation
E
VSV-EBOV95 GP-GFP [FRNT50 reciprocal serum dilution]
Days post inoculation
F
SFU/10^6 PBMCs
Days post inoculation
G
SFU/10^6 PBMCs
Days post inoculation
Prime
Prime Boost
Control

692

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

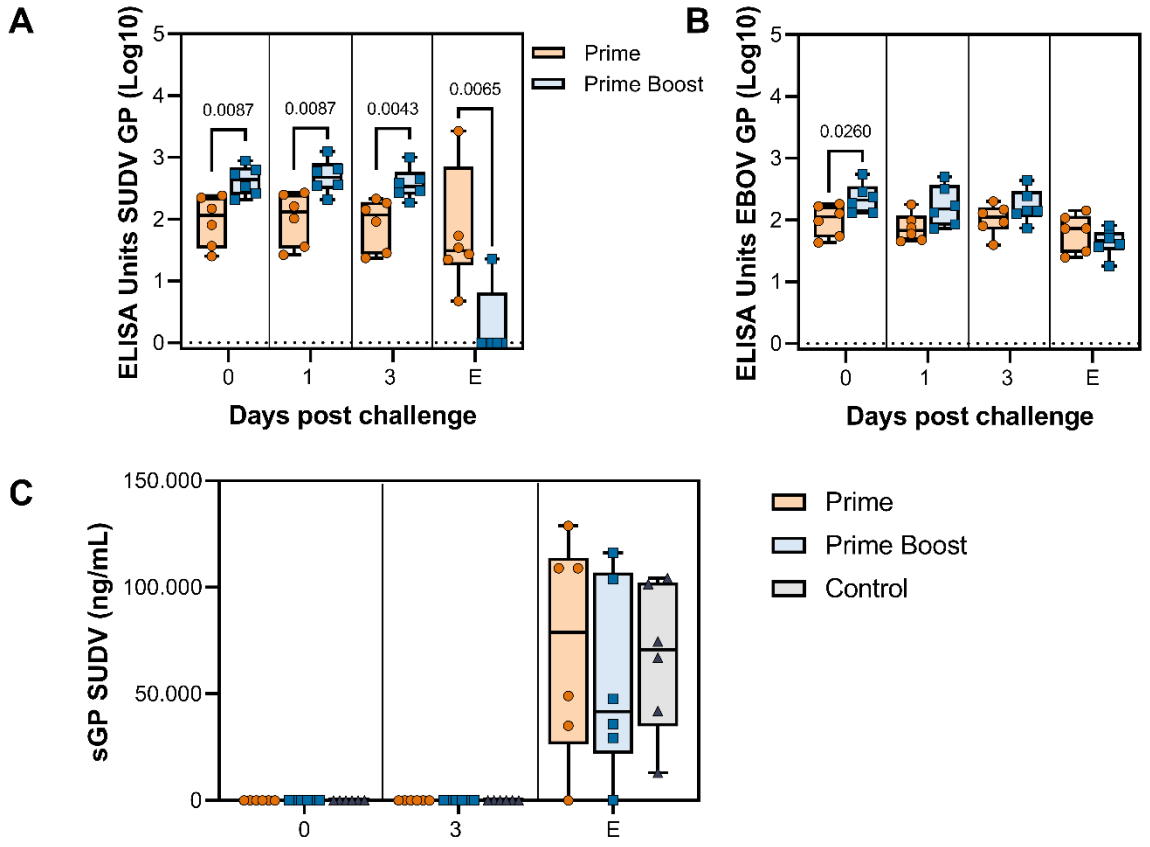


705

706 **Figure 2. Survival and clinical signs upon challenge of macaques with SUDV Gulu.** Clinical

707 score (A) survival (B), and viremia (C) are shown. The following parameters were measured in

708 blood of NHPs: Aspartate aminotransferase (AST, E), alkaline phosphatase (ALP, F), blood urea
709 nitrogen (BUN, G), albumin (H), platelets (I), lymphocytes (J), and neutrophils (K). Significance
710 was determined using Kruskall Wallis test followed by Mann Whitney test. Dotted line shows
711 (A) clinical score at which euthanasia is required, (C) or lower limit of detection.
712



713

714 **Figure 3. Binding antibody levels and sGP in serum obtained from macaques post**

715 **challenge.** Binding antibody levels against SUDV GP (A) and EBOV GP (B) are shown for the

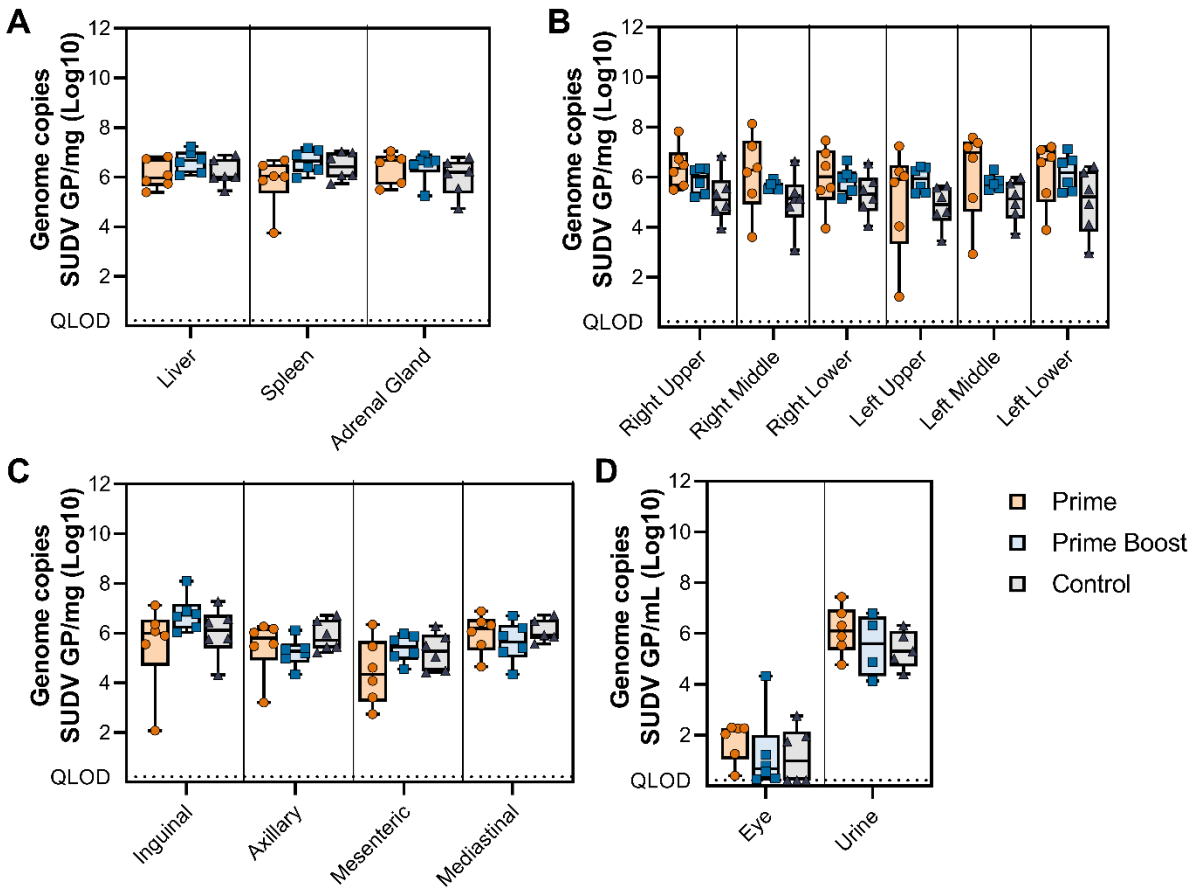
716 prime and prime boost group post challenge. No binding antibody was detected in control

717 animals. The dotted line shows the limit of detection. C. Soluble GP (sGP) levels were

718 determined in sera using capture ELISA. Significance was determined using Kruskall Wallis test

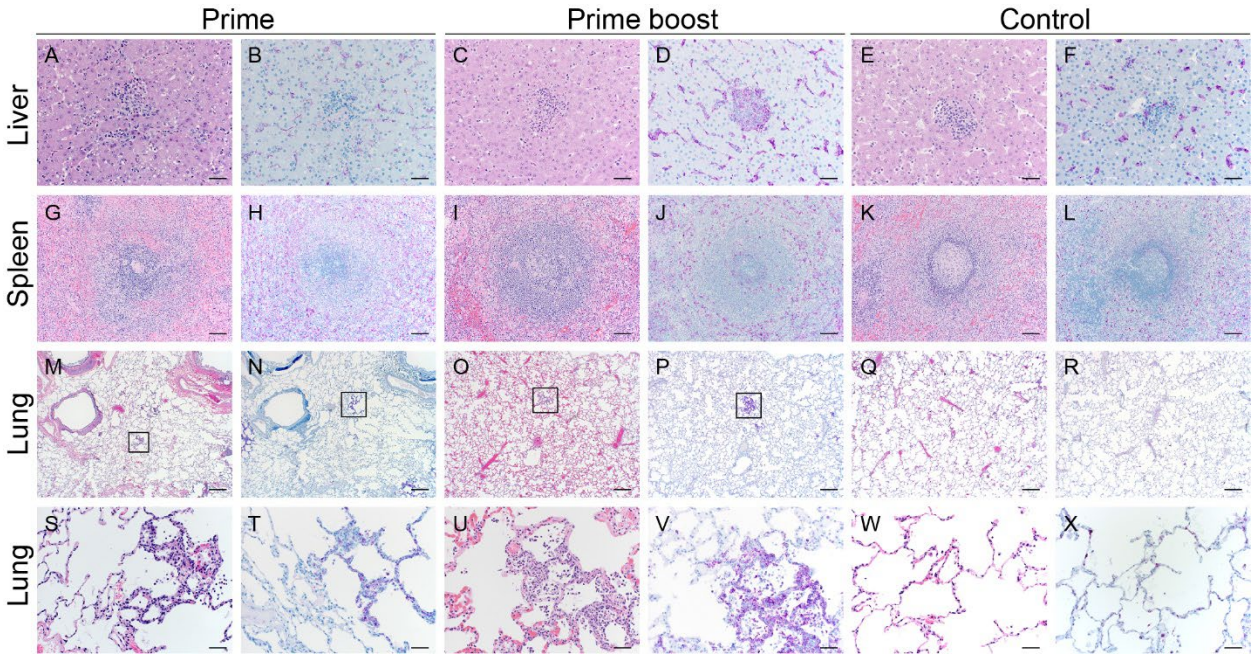
719 followed by Mann Whitney tests. E = Endpoint. GP = glycoprotein. SUDV=Sudan virus.

720 EBOV=Ebola virus.



721

722 **Figure 4. Presence of SUDV viral RNA in tissues obtained from NHPs at necropsy.** Viral
723 RNA load was determined in liver, spleen, adrenal gland (A), lung (B), multiple lymph nodes
724 (C), and aqueous humor collected from the eye as well as urine (D). Log10 transformed values
725 are shown, which were compared to a standard curve of known copies of viral SUDV RNA.
726 Significance was determined using Kruskall Wallis test followed by Mann Whitney tests. QLOD
727 = Qualitative limit of detection.

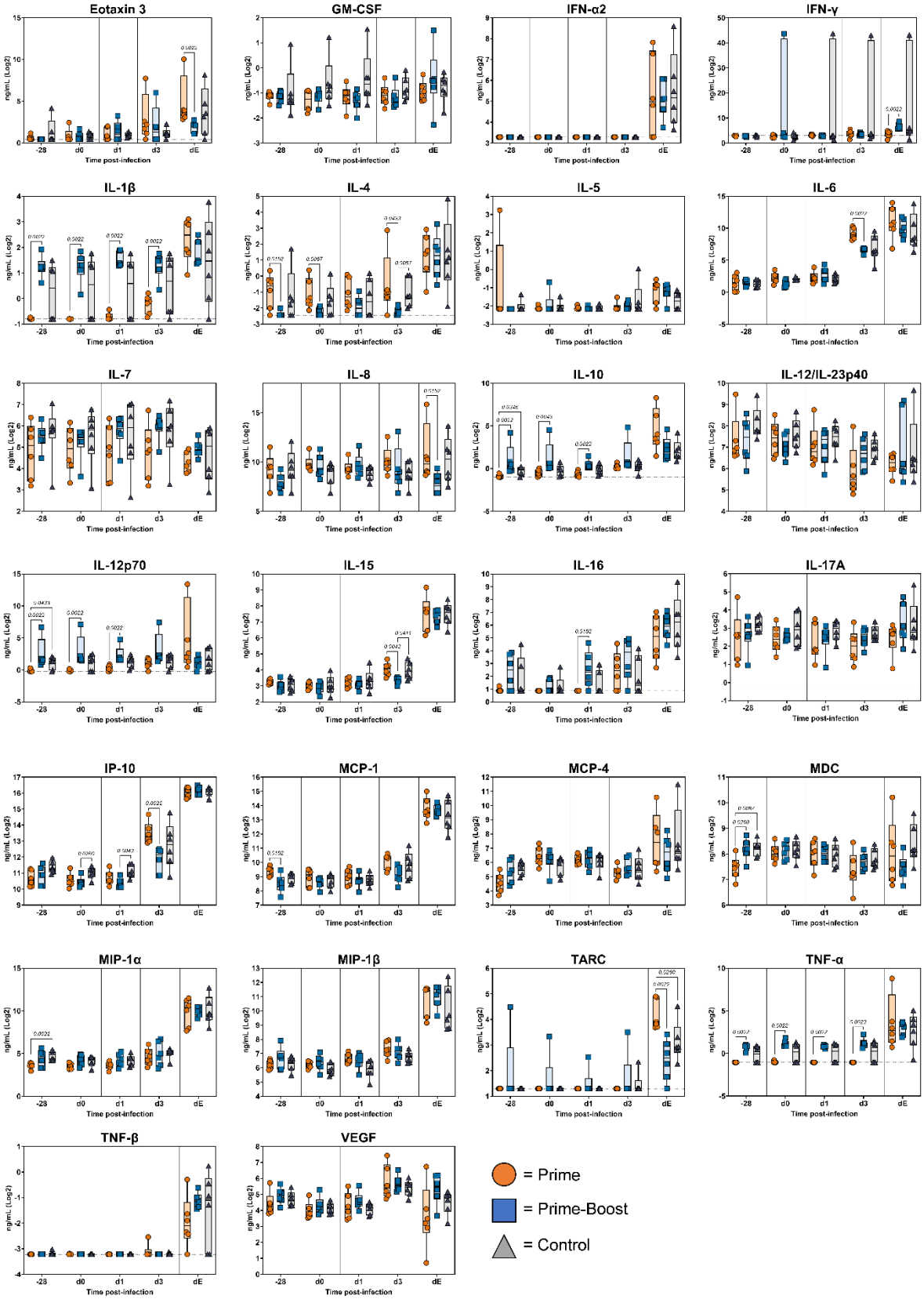


728

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

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

743 200X. Bar 200X=50 µm, 100X=100 µm and 20X=500 µm.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GM-CSF
IFN-α2
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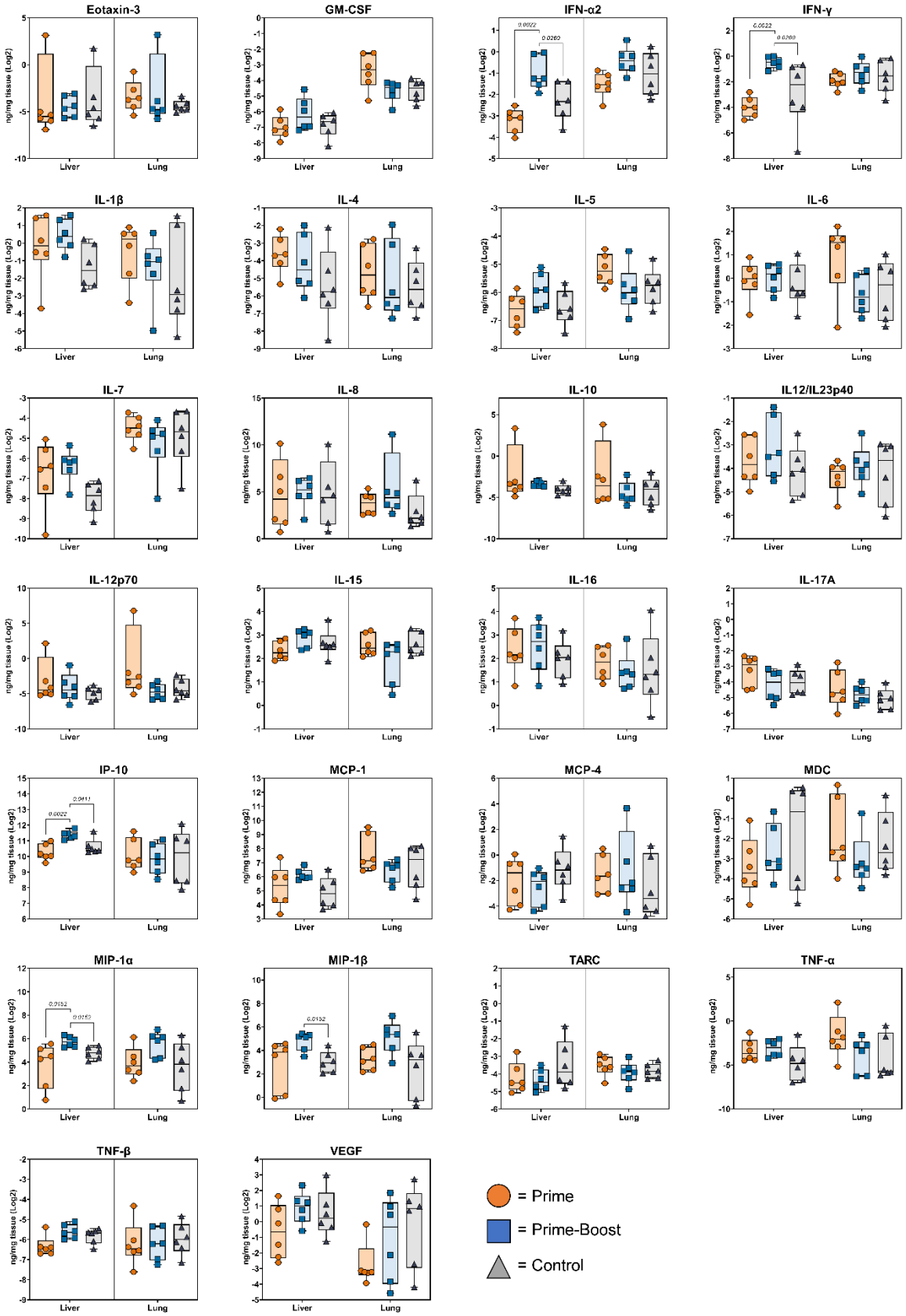
IL-5
IL-6
IL-7
IL-8
IL-10
IL-12/IL-23p40
IL-12p70
IL-15
IL-16
IL-17A
IP-10
MCP-1
MCP-4
MDC
MIP-1α
MIP-1β
TARC
TNF-α
TNF-β
VEGF
ng/mL (Log2)
Time post-infection
-28
d0
d1
d3
dE
= Prime
= Prime-Boost
= Control

744

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

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

747 concentrations were measured using the Meso Quickplex.


Eotaxin-3
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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

748

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

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

751 concentrations were measured using the Meso Quickplex.