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1 **Title Page:**

2 Comparative Pathogenesis of Asian and African-Lineage Zika Virus in Indian Rhesus 3 Macaque's and Development of a Non-Human Primate Model Suitable for the Evaluation 4 of New Drugs and Vaccines

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20 **Article Summary:** Zika virus strains of African and Asian lineage show differential infectivity
21 in Indian rhesus macaques yet the Asian lineage model has demonstrated utility for vaccine
22 efficacy studies.

23 **Key Words:** Zika virus; ZIKV; Rhesus macaques; Non-human primates; NHP; infection; natural
24 history; Asian-lineage; African-lineage

25

26 **Abstract:**

27 The establishment of a well characterized non-human primate model of Zika virus (ZIKV)
28 infection is critical for the development of medical interventions. In this study, challenging
29 Indian rhesus macaques (IRMs) with ZIKV strains of the Asian lineage resulted in dose
30 dependent peak viral loads between days 2 and 5 post infection; and a robust immune response
31 which protected the animals from homologous and heterologous re-challenge. In contrast,
32 viremia in IRMs challenged with an African lineage strain was below the assays lower limit of
33 quantitation and the immune response was insufficient to protect from re-challenge. These
34 results corroborate previous observations but are contrary to reports using other African strains
35 obviating the need for additional studies to elucidate the variables contributing to the disparities.
36 Nonetheless, the utility of an Asian lineage ZIKV IRM model for countermeasures development
37 was verified by vaccinating animals with a formalin inactivated reference vaccine and
38 demonstrating sterilizing immunity against a subsequent subcutaneous challenge.

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39 **Introduction:**

40 Since its introduction into the Americas, Zika virus (ZIKV) has been the subject of a
41 widespread outbreak linked to a number of different fetal abnormalities including congenital
42 microcephaly [1-6] and neurological disorders such as Guillain-Barre syndrome (GBS) [7].
43 Previously, human infections with ZIKV were infrequent or underreported and associated with
44 only mild symptoms including headache, myalgia, rash and a self-limiting fever; thus no
45 vaccines or therapeutics have been developed against ZIKV despite the fact that the virus has
46 been known since 1947 [8]. Given the magnitude of the recent outbreak and association with
47 significant clinical manifestations there is a heightened need for medical interventions to prevent
48 or treat ZIKV infections in humans. Concomitant with the need for new vaccines and
49 therapeutics is the need for appropriate animal models which can be used to support pre-clinical
50 efficacy studies.

51 ZIKV is a mosquito-borne virus belonging to the *flavivi*rus genus of the *Flaviviridae*
52 family and was first isolated in the Zika forest region of Uganda from the blood of a sentinel
53 rhesus macaque [9]. Since that time, the virus has spread from Africa into Asia with little
54 incidence until 2007 when it was associated with an outbreak characterized by rash,
55 conjunctivitis, and arthralgia on the Island of Yap in the Federal States of Micronesia [10]. This
56 outbreak was followed by an epidemic of ZIKV infection in French Polynesia in 2013 and 2014
57 that was correlated temporally with a 20 fold higher incidence rate of GBS [11, 12]. A
58 retrospective case-control study confirmed the association of ZIKV with increased incidence of
59 GBS [13] and a second study identified an increased incidence of congenital cerebral
60 malformations in fetuses and newborns associated with ZIKV infection during this time [14].
61 Subsequent ZIKV outbreaks were reported from numerous islands in the Pacific prior to its

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62 emergence in Brazil in March of 2015 [15, 16]. As of March 2017, the World Health
63 Organization Situation Report identified 84 countries, territories and subnational areas with
64 evidence of vector-borne ZIKV transmission; 31 countries or territories who have reported
65 microcephaly and other central nervous system malformations possibly associated with ZIKV
66 infection; and 33 countries or territories who have reported and increased incidence of GBS
67 potentially associated with ZIKV infection [17].

68 Prior to 2015, animal models of ZIKV infection were limited. However, with the
69 increased magnitude of the recent ZIKV outbreaks and severity of clinical disease in both adults
70 and in the developing fetus there has been a heightened interest in developing animal models to
71 better understand the pathogenesis of different geographic and temporal ZIKV isolates [18].
72 Recent research efforts with mice have focused predominantly on the use of
73 immunocompromised strains which lack receptors for type I interferon (IFN α/β), type II
74 interferon (IFN γ), or which lack other components of the innate antiviral response [19-23].
75 Infection of immunocompromised mice with ZIKV is frequently lethal depending on the specific
76 immune deficiency however most of these studies used different ZIKV strains, doses, and routes
77 of administration making it difficult to draw conclusions. While a lethal challenge model
78 provides a definite endpoint for efficacy studies a central tenant to animal model development
79 under the animal rule (21 CFR Parts 314.600-314.650 and 21 CFR Parts 601.90-601.95) is that
80 disease progression in the model recapitulate that observed in humans. The
81 immunocompromised nature of these models also limit their utility for vaccine efficacy studies.
82 Although no overt disease is observed in wild-type immunocompetent mice challenged with
83 ZIKV, viral RNA in addition to infectious viruses can be detected in serum and tissues
84 depending on the ZIKV isolate and the route of inoculation; and these models have been used for

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85 vaccine efficacy studies [19, 23-25]. However, restrictions on the volume and frequency of
86 samplings that can be taken from mice due to their small size also limit the utility of
87 immunocompetent mice for efficacy studies.

88 Non-human primates (NHP's) have also been studied extensively following the
89 emergence of ZIKV in the America's and rhesus macaques are an obvious first choice given the
90 fact that the virus was originally isolated from this species in 1947 [9]. Consequently, several
91 different groups have evaluated ZIKV pathogenesis in rhesus macaques and the resultant model
92 has been used for vaccine efficacy studies [26-31]. Cynomolgus macaques and pigtail macaques
93 have also been evaluated [28, 32, 33] and all species have been found to be similarly susceptible
94 and affectively recapitulate the human condition; however, each of these studies utilized
95 different ZIKV isolates of either African or Asian lineages, different routes of inoculation, and
96 different challenge doses again making it difficult to make comparisons. The objective of this
97 study is to provide a definitive evaluation of ZIKV natural history utilizing highly characterized
98 virus stocks of African and Asian lineages to establish an Indian rhesus macaque (IRM) model
99 for product evaluation under the animal rule. In the process, significant differences in the
100 infectivity of IRMs to ZIKV strains of Asian and African lineages were noted and are contrary to
101 previous reports with other strains of the African lineage [34]. The implications of these
102 differences are discussed below.

103

104 **Materials and Methods:**

105 **Care and use of animals:** This study was designed to use the fewest number of animals
106 possible, consistent with the objective of the study, the scientific needs, contemporary scientific

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107 standards, and in consideration of applicable regulatory requirements. Study design was
108 reviewed by the Institutional Animal Care and Use Committee (IACUC) at Southern Research.
109 Animals were socially housed during the quarantine and pre-study phases, then single housed
110 following challenge phases of the study. Animals were housed in stainless steel cages that meet
111 requirements as set forth in the Animal Welfare Act (Public Law 99-198) and the *Guide for the*
112 *Care and Use of Laboratory Animals* (8th Edition, Institute of Animal Resources, Commission on
113 Life Sciences, National Research Council; National Academy Press; Washington D.C.; 2011).
114 Animals were housed in an environmentally monitored and ventilated room. Fluorescent
115 lighting provided illumination approximately 12 hours per day to simulate the natural diurnal
116 lighting and minimize housing associated stress.

117 **Viruses, Cell Culture:** Vero cells were grown in Dulbecco's Minimal Essential
118 Medium (DMEM, Lonza, Walkersville, MD), supplemented with 10% Fetal Bovine Serum
119 (FBS), NEAA and L-Glutamine according to standard culture conditions. ZIKV strain
120 PRVABC59 was isolated in 2015 from human serum collected in Puerto Rico and obtained from
121 the Center for Disease Control and Prevention (Division of Vector-borne Infectious Diseases,
122 CDC, Fort Collins, CO). The PLCal_ZV strain (NR-50234) was isolated from a human who had
123 traveled to Thailand in 2013 and was obtained through the Biodefense and Emerging Infections
124 Research Resources Repository (BEI Resources, Manassas, VA), National Institute of Allergy
125 and Infectious Diseases (NIAD), of the National Institute of Health (NIH). ZIKV, IbH_30656,
126 NR-50066 was obtained through BEI Resources, NIAID, NIH, as part of the WRCEVA program
127 and was isolated from human blood collected in Nigeria in 1968. Master and working stocks of
128 each virus strain were amplified in Vero cells (with minimal passages after receiving them in
129 house from the primary source) and quantified using standard plaque assay on Vero cells to

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130 determine the plaque forming unit (PFU) titer. All stocks were determined to be free of
131 mycoplasma using MycoAlert Mycoplasma Detection Kit (Lonza, LT07-118). Endotoxin levels
132 for stocks were determined by QCL-1000 kit (Lonza, 50-647U) and found to be <0.1 EU/mL.
133 Sterility was also verified by both blood agar and potato dextrose slant cultures for at least 14
134 days.

135 **Natural History Study:** A total of 36- IRM's seronegative by ELISA for Dengue, West
136 Nile and ZIKV were subdivided into nine groups, each group containing 2 males and 2 females
137 assigned randomly based on body weight. Animals weighed between 3.0 to 7.5 kg and 2-5 years
138 age at the study start.

139 For the initial challenge, animals were inoculated subcutaneously (SC) with a ZIKV
140 isolate from either the Asian-lineage (PRVABC59 or PLCal_ZV) or the African-lineage
141 (IbH_30656) at the indicated doses (Table 1). Biological fluid samples (blood, urine, and saliva)
142 were collected between Days 0-30 post initial challenge (Figure 1). Following a (2) week resting
143 period the 12 animals originally challenged with each isolate in Phase 1 were distributed into two
144 groups composed of one male and one female from each low, medium and high dosing group
145 and re-challenged at a dose of $1x10^6$ PFU/animal using either the identical strain they were
146 previously exposed to or a different strain as indicated in Table 2. Serum collection during the
147 re-challenge phase paralleled the periodicity of the collection time points of the initial challenge
148 phase, between days 45-75 as per (Figure 1).

149 **Primers and Probes:** PCR primers and probes were designed to the viral Envelope
150 region of each ZIKV genome, proximal to those described in [35] utilizing modified sequences
151 optimizing primer/probe binding for strains PRVABC59 (Genbank Accession KU501215.1),
152 PLCal_ZV (Genbank Accession KX694532.1) and IbH_30656 (Genbank Accession

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153 HQ234500.1). All genomic ZIKV strain analysis used for primer and probe design for
154 PRVABC59 and IbH_30656 were as previously described [36]. For the PLCal_ZV strain, we
155 used the previously described primers Zika Dual-For and Zika Dual-Rev [36] and a sequence
156 optimized probe (5'6FAM-TGC-CCA-ACA/ZEN/CAA-GGC-GAA-GCC-TAC-CT-3'IABkFQ).
157 The probe contains a 5'-6FAM reporter, an internal ZEN quencher and a 3' IBFQ Iowa Black
158 quencher. All primers and probes were synthesized by Integrated DNA Technologies (IDT,
159 Coralville IA). Primer and probe combinations were fully characterized for compatible melting
160 temperatures (Tm), self-dimer and hairpin potential as previously described [32]. Primers and
161 protocols used for the generation of the RNA template used for the standard curve for absolute
162 quantitation were as described in [36].
163 **Biological Sample Collection:** Biological fluid samples (serum, urine and saliva) were
164 collected from anesthetized IRM at multiple times after the initial challenge on Day's 0-30 and
165 re-challenge on Day's 45-79 (Figure 1). Briefly, for the initial challenge (Day 0) blood was
166 collected daily (Day 0-10), then thereafter on Day 15, 20, 25 and 30. After re-challenge on day
167 45, blood was collected daily from Day 45-55 and thereafter on Day 60, 65, 70 and 75. Collected
168 blood samples were immediately processed to serum using serum separator tubes (SST) with
169 brief centrifugation and stored at or below -70°C. Urine was collected by either cystocentesis
170 (needle) or catheter. Saliva (or drool) was collected directly, if saliva could not be collected oral
171 cavity swabs were taken and immersed in 1mL DPBS without Calcium and Magnesium (Cat. #
172 17-512F, Lonza Walkersville, MD). Both urine and saliva collections occurred on the same
173 schedule, post initial challenge Day 5, 10, 15, 20, 25, and 30 and post re-challenge Day 50, 55,
174 60, 65, 70 and 75. Upon collection and the preparation of small aliquots, urine and saliva were
175 frozen at or below -70°C.

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176 **Viral Load by qRT-PCR:** Viral RNA was extracted from collected biological fluids to
177 quantify viral load. Briefly, using the QIAmp Viral RNA Mini kit, (Qiagen, 52906) total RNA
178 was extracted and purified from a biological sample volume of 140µl (as per manufacturer) and
179 eluted into 60µL of nuclease-free water (Ambion, AM9939). Five µl of purified RNA from each
180 test article was used in a 20µl qRT-PCR reaction consisting of Fast Virus 4x Master Mix
181 (Applied Biosystems, 4444436) containing 500nM forward and reverse primers with 200nM
182 probe. Cycling parameters for the QuantStudio Flex 6 instrument include: an initial reverse
183 transcription (RT) step for 5 minutes at 53°C, followed by 1 min. at 95°C and 45 cycles of 2 step
184 cycling at 95°C for 5s and 60°C for 50s. A standard curve for absolute quantitation using
185 positive control RNA template was established over the dynamic range of 6-logs (1E6 – 1E1)
186 with each dilution in triplicate of test samples. As reported in (Koide et al., 2016) the Ct values
187 obtained for each of the 6-log dilutions for each strain specific primer/probe combination
188 perform consistently (within 1 Ct) of each other. NCBI Sequence alignment was used for the
189 development of primers and probes that would work specifically with IBH30656 and other
190 strains used in this study. Additionally, the lower limit of quantitative detection (LLOQ) is
191 approximately 10 copies/20uL PCR reaction. Depending on the volume/weight of the extracted
192 test sample and the elution volumes, the resulting dilution factor is typically between 80 and 100
193 fold thus the LLOQ for this assay is recorded as 500 copies/ml
194 **Viral Load by Plaque Assay:** The 3 serum samples from each primary and secondary
195 challenge containing the highest viral load as determined by qRT-PCR were further assessed by
196 plaque assay on Vero cells. Briefly, Vero cells were cultured in 6-well plates to approximately
197 90-100% confluent monolayers and cells were exposed to 200 µl of 4-fold serially diluted
198 samples. Three dilutions of each sample were tested in duplicate. Plates were incubated at 37ºC

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199 and 5% CO_2 for 1 hour before the addition of an overlay media consisting of EMEM and 0.5%
200 agarose. Plates were incubated for 3 to 5 days until discernable plaques were formed then fixed
201 and stained with crystal violet.

202 **Indirect ELISA:** Purified African lineage Zika virus lysate (The Native Antigen
203 Company, Oxfordshire, U.K.) was diluted to 0.5µg/mL in carbonate-bicarbonate buffer (Thermo
204 Fisher Scientific). Lysate was generated by culturing virus in Vero cells, clarified, concentrated
205 via sucrose gradient ultracentrifugation, then lysed in Triton X-100, and heat-inactivated. Use of
206 a whole virus lysate provides magnitudes of additional linear epitopes, when compared to use of
207 a single viral protein (Env or NS1 only). This allows for increased epitope presentation,
208 presumably including targets present on both African and Asian lineages. Specificity to ZIKV
209 was confirmed by comparing OD values of vehicle-only treated sera to naïve sera, which
210 produced indistinguishable results (data not shown).

211 High binding MAXISORP™ 96-well plates (Thermo Fisher Scientific) were coated with
212 100µL of diluted antigen and refrigerated at 2-8°C overnight. Plates were washed five times with
213 0.05% Tween 20 in PBS, then blocked for 30 minutes at 37°C using 5% dry milk (Quality
214 Biological, Maryland, USA). Plates were washed five times then incubated for 1 hour at 37°C
215 with serially diluted sera samples. Signal was detected using goat anti-human HRP conjugated
216 IgG (SeraCare, Milford, MA) diluted 1:2000 in 5% milk. Following wash, 100µL of ABTS 1-
217 component peroxidase substrate (SeraCare, Milford, MA) was added to each well in low light.
218 Plates were covered and incubated at room temperature for fifteen to twenty minutes, then 100µL
219 of 1% sodium dodecyl sulfate (Fisher Scientific) was added to stop the reaction. Absorbance
220 optical density (OD) was read at 405nm on a SpectraMax i3 using Softmax Pro software
221 (Sunnyvale, CA). Averaged OD values were plotted against the log of the reciprocal of their

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222 dilution (ex. $Log_{10}100$, $Log_{10}12800$). A four-parameter non-linear regression method (GraphPad
223 Prism 5 software, La Jolla, CA) was used for data analysis. Using the formula from curve fitting,
224 data points between each dilution step were extrapolated, expanding the data from eight to one
225 thousand OD values. The log of the reciprocal dilution that corresponded to the extrapolated OD
226 value at a cut-point was identified. The antilog of each identified value at the cut-point was
227 calculated. This value is the reciprocal of the lowest serum dilution capable of producing a
228 positive result for anti-Zika IgG antibodies.

229 An endpoint cut-off was defined as follows: more than twenty IRM serum samples were
230 tested using commercially available kits for Zika Virus (XpressBio, Frederick, MD) and Dengue
231 Virus (Calbiotech, El Cajon, CA and Abcam, Cambridge, MA). Sera testing negative for both
232 viruses on all kits were run on the above ELISA, with the exception that samples were diluted
233 1:100 only. Naive sera averaged an OD value of 0.064. The cut point OD value (0.148) was
234 calculated by adding two times the standard deviation of the OD values to the average OD value.

235 **Focus Reduction Neutralization Test (FRNT):** The FRNT assay for ZIKV was
236 performed on serum samples collected at the time points indicated. Briefly, Vero cells seeded at
237 a concentration of approximately $1x10^5$ to $2x10^5$ cells/ml in a 96-well plate were incubated
238 approximately 24 hours. On the day of assay, input virus and serially diluted serum samples were
239 mixed and incubated for 1 hour at 37°C ± 1°C in the dilution plate. The supernatant was
240 decanted from cell-seeded 96-well plates then 100 µl of virus/serum mixture was transferred
241 from the dilution plate and added to the cells. After adsorption for 1 hour, overlay media was
242 added to the plate and incubated at 5% CO_2 overnight. Next day, the plates were stained using
243 broad spectrum pan-flavivirus antibody (MAB10216, Millipore, Burlington, MA) and Goat anti-
244 mouse IgG (H+L) HRP- conjugated secondary antibody (5220-0341, SeraCare Life Sciences,

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245 Milford, MA). TrueBlue Peroxidase Substrate [5510-0030, SeraCare Life Sciences, Milford,
246 MA] was added to the plates and spots were analyzed by BioSpot scanner. Each dilution of sera
247 was tested in triplicate. Neutralizing antibody titers were reported as the inverse of the serum dilution
248 estimated to reduce the number of input virus by 50% ($FRNT_{50}$). The percent neutralization at each
249 dilution was calculated by the ratio of average foci counts of the replicates to the average foci of the input
250 virus wells. $FRNT_{50}$ titers were estimated by point-to-point linear regression between the two dilutions
251 that span 50% neutralization

252 **Efficacy and Immunogenicity Testing of Inactivated Vaccine:** Formalin Inactivated
253 PRVABC59 Vaccine (lot number 2016) [37], with a concentration of 5 µg/0.5 mL from Walter
254 Reed Army Institute of Research (WRAIR, Springfield, MD) was used for the study. Prior to
255 Study Day 0, four (4) IRMs were randomized into two groups (2 animals/group) according to
256 gender/weight using Provantis Software. On Days 0 and 28, all animals were anesthetized and
257 inoculated intramuscularly (IM) with inactivated-PRVABC59 vaccine (Group 1) or SC with
258 Minimal Essential Medium supplemented with 1% FBS as a vehicle control (Group 2). On Day
259 56, all macaques were anesthetized and challenged SC with 0.5 mL of ZIKV strain PRVABC59
260 with a target challenge dose of 1E5 PFU per animal. Blood samples were collected at Day 0
261 (prior to immunization), Day 14, Day 28 (prior to boost) and Day 56 (prior to challenge0 to
262 determine anti-ZIKV neutralizing antibody (Nab) titer's by FRNT. Viral load was assessed by
263 qRT-PCR on serum samples collected daily between days 56 and 66; and again on Day 85.
264

265 **Results:**

266 **Clinical Observations:** All of the animals were monitored twice daily during the
267 challenge and re-challenge phases. With the exception of some mild erythema, described below,
268 none of the animals showed overt signs of clinical manifestations associated with ZIKV infection

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269 throughout the study. On Day 1, ZIKV challenge associated mild erythema at the injection site
270 was observed in 5 animals however there was no correlation with challenge dose or strain and
271 lesions resolved by Day 2 (data not shown). Mild erythema at the inguinal region was also
272 observed in one animal challenged with PLCal_ZV at 1E6 PFU on Day 6 and another
273 challenged with the PRVABC59 strain at 1E5PFU on Day 21. ZIKV re-challenge associated
274 mild erythema at the injection site was observed in a total of 13 animals distributed throughout
275 the study groups on Day 46 and one animal on Day 47, but resolved afterwards (data not shown).

276 **Virus Detection Following Primary Infection:** Following challenge, the dose
277 preparations were back titered via plaque assay and targeted doses were confirmed for all but the
278 high, medium and low dose groups for IbH_30656 and the low dose group for PLCal_ZV which
279 were slightly more than 0.5 logs below the targeted dose (data not shown). Serum, saliva and
280 urine samples collected at the time points indicated in Figure 1 were tested by qRT-PCR to
281 estimate the relative level of virus replication in each animal. Analysis of the serum viral RNA
282 load (Figure 2) showed that animals challenged with PRVABC59 developed peak concentrations
283 at Day 2 in the mid and high dose groups as compared to Day 3 in the low dose group (Figure
284 2a). Regardless of the dose concentration viral RNA fell below the LLOQ after Day 4 in serum
285 from animals challenged with PRVABC59. Animals challenged with PLCal_ZV demonstrated
286 similar serum viral RNA kinetics to PRVABC59 at the mid and high dose levels, but peak RNA
287 concentration was delayed to Day 4 and persisted until after Day 6 in the low dose group (Figure
288 2b). Animals challenged with the IbH_30656 (African) strain demonstrated only low levels of
289 viral RNA in serum samples collected throughout the study that were generally at or below the
290 LLOQ (Figure 2c). Only the highest dose group produced and average viral RNA concentration
291 above the LLOQ on Day 2 that was transient.

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292 Serum samples with high viral RNA concentrations in the qRT-PCR assay (above the
293 LLOQ) were selected for testing by standard plaque assay to quantify infectious virus particles
294 present in serum and the comparative results are presented in Supplementary Table-1 and 2.
295 Based on these criterion the viral plaque assay was only performed on samples from IRMs
296 challenged with PRVABC59 and PLCal_ZV. Infectious virus particles were detected in most
297 samples confirming viremia; however viral PFU titers were generally 2 to 3 logs lower as
298 compared to viral genome levels. In some cases, no plaque titers were detected despite RNA
299 copy numbers as high as 1.12E5/ml thus there was no consistent correlation between RNA copy
300 number and plaque titer; and qRT-PCR analysis was confirmed to be a more reliable method for
301 the detection of virus in serum.

302 Detectable levels of Viral RNA were also observed in urine and saliva samples collected
303 throughout the study from animals challenged with the PRVABC59 and PLCal_ZV strains.
304 However, RNA detection from individual animals was sporadic and irrespective of dose
305 concentration as demonstrated in Figure 3 for animals challenged with PRVABC59. In this case
306 low levels of viral RNA (5.3E0 to 6.9E1 copies/ml on average) were detected in urine samples
307 up to Day 30 (Figure 3A) yet only one animal challenged with the lowest dose had RNA levels
308 above the LLOQ on Day 10 post infection. Viral RNA ranging from 3.9E0 to 9.7E2 copies/ml on
309 average were also observed in saliva samples up to day 25 but again only one animal from the
310 medium dose group had detectable RNA above the LLOQ on Day 5 post-infection (Figure 3B).
311 Results were similar with samples collected from animals challenged with PLCal_ZV; however
312 viral RNA was only detected in 4 urine samples collected from animals challenged with
313 IbH_30656 collected over the course of this study while none of the saliva samples had
314 detectable RNA levels (data not shown). The most notable observation from the analysis of urine

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315 and saliva samples from animals challenged with Asian lineage viruses is that viral RNA is most
316 reliably detected in saliva samples collected 5 days' post infection as demonstrated for
317 PRVABC59 in Figure 3.

318 **Immune Response Following Primary Infection:** Anti-ZIKV antibodies induced
319 following challenge with varying doses of Zika viruses are illustrated in Figure 4 and Table 3.
320 IgG antibodies were measured using an in-house quantitative ZIKV ELISA against a purified
321 African lineage ZIKV lysate, and enumerated using four-point non-linear regression analysis.
322 Following challenge, a dose dependent anti-ZIKV immune response was detected by Day 10 in
323 all groups though higher levels of IgG were observed in IRMs challenged with Asian lineage
324 strains (PRVABC59 and PLCal_ZV) as compared to the African lineage strain (IbH_30656).
325 Antibody titers were weak for IbH_30656 infected animals presumably due to very low infection
326 of the animals. The highest average IgG titers on Day 10 were observed in the high dose groups
327 challenged with PLCal_ZV (3494), followed by PRVABC59 (1890) and IbH_30656 (717). By
328 day 15, antibody titers in the high dose groups increased to 10039 (PLCal_ZV), 8560
329 (PRVABC59), and 3331 (IbH_30656); however, by day 30, average IgG antibody titers from
330 IRMs challenged with PLCal_ZV and IbH_30656 began to decrease to 7685 and 1790,
331 respectively, while PRVABC59 continued to increase to 10486. IRMs challenged with
332 PLCal_ZV and PRVABC59 at the median dose level had averaged titers of 732 and 1026
333 respectively on Day 10 as compared to an average titer of 226 in those challenged with
334 IbH_30656. Titers peaked at this dose level in all groups on Day 15 with titers reaching 7715
335 (PLCal_ZV), 7122 (PRVABC59) and 1040 (IbH_30656). By Day 30, PLCal_ZV titers in the
336 median dose group remained sustained at 7151, while PRVABC59 titers decreased slightly to
337 5127 and IbH_30656 dropped to 521. In the lowest dose groups only one of four animals

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338 challenged with IbH_30656 and three of four animals challenged with PLCal-ZV had an IgG
339 Antibody titer above the LLOQ; whereas all animals challenged with PRVABC59 at this dose on
340 this Day had a detectable titer. By Day 15, the average IgG titers increased to 3558 and 8380 in
341 IRMs challenged with PRVABC59 and PLCal_ZV respectively and titers continued to increase
342 on Day 30 reaching 5573 (PRVABC59) and 9341 (PLCal_ZV). IgG antibody titers in IRMs
343 challenged with the low dose of IbH_30656 also increased to 562 on Day 15 but fell on Day 30
344 to 414.

345 **Virus Detection Following Secondary Challenge:** After primary challenge and the
346 conclusion of the natural history study, animals were regrouped as described in the materials and
347 methods section; and challenged on Day 45 either with the same isolate they were previously
348 exposed to or cross-challenged with an isolate of different geographic origin (Table 2). The
349 targeted dose of 2.0E6 PFU/ml was confirmed by plaque assay (data not shown); and serum was
350 collected to assess viral load and immunological responses as before (Figure 1). In animals
351 previously challenged with PRVABC59, viral RNA that was below the LLOQ was detected in
352 only 4 samples collected on Days 45, 46 or 49 following re-challenge with PRVABC59; and two
353 samples collected on Day 47 from animals challenged with PCal_ZV (Figure 5). Following
354 homologous challenge, no viral RNA was detected in serum samples from IRM's previously
355 challenged with PLCal_ZV; however, RNA that was below the LLOQ was detected in at least
356 one animal on Days 45 through 49 when challenged with the heterologous PRVABC59. Re-
357 challenging IRMs with IbH_30656 resulted in detectable levels of RNA in multiple samples on
358 Days 45 to 47 that were similar to the levels seen following primary challenge but again below
359 the LLOQ. In contrast, challenging IRM's with PRVABC59 following a primary challenge with
360 IbH_30656 resulted in detectable levels of viral RNA that were attenuated by only two logs as

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361 compared to naïve animals challenged with PRVABC59 (Figure 4 vs. Figure 1a). In this case,
362 peak concentrations of viral RNA were achieved between Days 2 and 3 post challenge and
363 persisted out to Day 5 before falling below the LLOQ (Figure 4).
364 Immune Response Following Secondary Challenge: Figures 6 and Table 4 illustrate the
365 anti-ZIKV immune response at 5 and 30 Days post-secondary challenge (Study Days 50 and 75,
366 respectively) with homologous and heterologous ZIKV challenge. Average IgG antibody titers
367 ranged between 10141 and 12099 on Day 50 when IRMs received homologous or heterologous
368 secondary challenges with high doses of PRVABC59 or PLCal_ZV. IgG concentrations
369 remained high at Day 75 for IRM's that received a secondary challenge with PLCal_ZV (Figure
370 6A and B), while titers of animals re-challenged with PRVABC59 decreased. Re-challenging
371 IRM's with IbH_30656 did not increase the average IgG immune response on Days 50 or 75;
372 however, challenging IRMs with PRVABC59 following an initial exposure to IbH_30656
373 resulted in increased IgG concentrations on Day 75 of 10456 on average as shown in Figure 6C.
374 **Immunogenicity and Efficacy of Inactivated ZIKV Vaccine:** To demonstrate the
375 utility of the resultant model, groups of 2 IRMs were immunized twice (Day 0 and 28) with
376 inactivated PRVABC59 vaccine (Group 1) or vehicle alone (Group 2) followed by SC challenge
377 with PRVABC59 virus on Day 56. Immunogenicity of the inactivated vaccine was evaluated by
378 measuring Nab's as shown in Figure 7A. FRNTs on sera collected on Day 14 demonstrated
379 seroconversion in both animals in the vaccinated group with an average $FRNT_{50}$ titer of 294. By
380 Day 28, prior to boost, $FRNT_{50}$ titers had decreased to 127 on average; however, following the
381 boost and prior to challenge on Day 56, $FRNT_{50}$ titers averaged 3270 in the two vaccinated
382 animals. In contrast ZIKV neutralizing antibodies were not observed in the vehicle control
383 animals.

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384 Efficacy of the vaccine was evaluated by assessing serum viral loads in the vaccinated
385 and control animals via qRT-PCR as shown in Figure 7B. Viremia was below the LLOQ in
386 vaccinated animals throughout the study; whereas control animals showed significant viremia
387 starting on Day 57 and peaking on Day 58. Individual peak viral titers in the unvaccinated
388 animals ranged from 6.2E4 to 2.8E5 GE/mL and viral titers fell below the LLOQ by Day 62, 6
389 days post challenge, similar to what was observed in preliminary studies.

390

391 **Discussion:**

392 In 2016, more than 40,000 symptomatic Zika disease cases, not including congenital
393 disease cases, were reported in the United States and its territories alone
394 (https://www.cdc.gov/zika/reporting). In 2017, the number has decreased to less than 1000;
395 however, the global incidence remained high with as many as 84 countries, territories or
396 subnational areas reporting evidence of vector-borne ZIKV transmission [17]. In the same report,
397 at least 13 countries have also reported evidence of person to person transmission underscoring
398 the need to better understand the factors contributing to the epidemiology and pathogenesis of
399 ZIKV; and to develop new vaccines and therapies to prevent or treat disease in humans.
400 Paramount to addressing these needs are animal models which recapitulate the human condition
401 and NHP's have arguably proven to be the most appropriate model to fulfill these requirements.
402 The rhesus macaque in particular has been the most extensively utilized to study the
403 pathogenesis of multiple different ZIKV isolates from the Asian-lineage following SC exposure;
404 however, only one isolate of the African-lineage, MR766, has been evaluated via this route to
405 this point [34]. MR766 represent the prototype ZIKV strain originally isolated from rhesus
406 macaques in Uganda in 1947 and was passaged at least 149 times in suckling mouse brains [9].

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407 IRM's challenged with ZIKV strain MR766 via the SC route resulted in plasma viral loads that
408 were similar to that seen in IRM's challenged with a French Polynesian strain (H/FP/2013)
409 isolated in 2013 [34]. Primary infection of IRM's with the MR766 strain also resulted in a robust
410 immune response which protected the animals from subsequent challenge with H/FP/2013. In a
411 separate study, cynomolgus macaques were demonstrated to be refractory to infection with the
412 IbH_30656 strain of ZIKV which was isolated in Nigeria in 1968 [32]. The IbH_30656 strain
413 falls under the West African subclade of the African-lineage ZIKV isolates as compared to
414 MR766 which falls under the East African subclade [38, 39] thus it is unclear if the disparity in
415 these results are related to the phylogenetic differences, the extensive passage history of MR766
416 in mouse brains, or the genetic background of the NHP's. In yet another study, both rhesus
417 macaques and cynomolgus macaques developed detectable viremia following intravaginal
418 (IVAG) and intrarectal (IR) challenge with a ZIKV strain isolated from mosquitoes collected in
419 Senegal in 1984 [29]. This strain (ArD 41525) is also of the West African subclade and closely
420 related to IbH_30656 [38, 39] further complicating the interpretation of these results. The
421 IbH_30656 strain was chosen for this comparative study because it is a historical African isolate
422 but unlike MR766 was not passaged in animals with the intent of increasing virulence. The
423 objective of this study was not to provide an exhaustive comparison of Asian and African lineage
424 viruses but to verify previous data with the IbH_30656 strain in cynomolgus macaques; and
425 challenge the dogma from a single study with MR766 that African lineage isolates are equally
426 pathogenic to Asian lineage isolates in rhesus and that an immune response generated to the
427 African-lineage isolates are protective against Asian-lineage viruses. The ultimate goal of these
428 studies was to perform a definitive analysis of ZIKV isolates from Asian and African-lineage

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429 using qualified virus stocks and establish a NHP model of ZIKV suitable for vaccine and
430 antiviral evaluation.

431 To this end, master and working stocks of ZIKV isolates PRVABC59, PLCal_ZV, and
432 IbH_30656 were prepared in a qualified bank of Vero cells; and confirmed for purity, quantity,
433 mycoplasma contamination, and endotoxin levels. All stocks met the acceptance criteria prior to
434 use in the study. Comparison of the genome copies (as measured by RT-qPCR) and PFU data
435 suggested that generally the genome copies were about 2-3 log higher than the PFU levels. IRMs
436 determined to be naïve to flavivirus exposure were challenged with ZIKV via the SC route and at
437 doses between 1E4 and 1E6 PFU as has been done previously [27] to most closely mimic the
438 natural route of vector-borne transmission and at theoretical concentrations reported for West
439 Nile Virus in mosquito saliva [40]. More recent studies suggest that the maximum concentration
440 of ZIKV in mosquito saliva is closer to 1E3 PFU and that replication kinetics are delayed when
441 ZIKV is delivered via mosquito bite as compared to the SC route at a dose of 1E4 PFU [41];
442 however, there was significant variability in mosquito delivery such as the number of mosquitoes
443 feeding on any given animal; and other aspects of ZIKV infection including tissue distribution
444 were not significantly altered justifying the dose and route used in these studies. Following
445 challenge, a transient rash was observed in some IRMs similar to previous report [34, 42].
446 Previous reports have also reported mild weight loss after infection of IRMs with the French
447 Polynesian strain H/FP/2013 [27] and elevations in body temperatures [26, 28]. In contrast, no
448 significant changes in body weight or temperatures were observed in these experiments.

449 Similar to previously published IRM studies with Asian lineage ZIKV strains and MR766
450 [27, 28, 34, 43, 44], serum RNA levels in PRVABC59 and PLCal_ZV infected NHPs peaked at
451 Day 2 to 3 depending on the dose and persisted until Day 6. No obvious differences in serum

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452 RNA levels were observed between sexes (data not shown). Virus shedding in urine and saliva
453 that persisted to Day 30 and Day 25, respectively, was also observed similar to previous reports
454 [26, 27, 42]; however at significantly reduced concentrations (1E2-1E3 genome copies/mL here
455 versus 1E4-1E6 genome copies/mL in other reports). Differences in virus shedding in urine and
456 saliva might be due to the ZIKV strain used, the method by which the sample was collected, or
457 differences in the sensitivities and detection limits of the qRT-PCR assays; and highlights the
458 need to standardize these methods between labs so that accurate comparisons can be made.
459 Regardless, the sporadic nature of virus shedding in urine and saliva reported here and elsewhere
460 limits the utility of these specimens for assessing clinical progression of ZIKV and efficacy of
461 medical interventions as compared to viremia. The immune response to ZIKV PRVABC59 and
462 PLCal_ZV as measured by IgG response was also similar to previous reports [42, 43]. All
463 animals challenged with these strains had detectable IgG titers by Day 10 that peaked at Day 15
464 or Day 30 depending on the challenge dose. Higher IgG titers were generally observed in IRM's
465 challenged with PLCal_ZV as compared with PRVABC59; however, the immune response
466 following exposure to either PLCal_ZV or PRVABC59 was sufficient to protect IRM's from
467 homologous and heterologous re-challenge as demonstrated by serum viral RNA titers that were
468 below the LLOQ.

469 In contrast to IRM's challenged with PRVABC59 and PLCal_ZV, viremia following
470 primary challenge with ZIKV strain IbH_30656 was below the LLOQ consistent with what was
471 observed previously in cynomolgus macaques [32]. Consistent with the low level infectivity, the
472 immune response to challenge with the IbH_30656 isolate was also low and although detectable
473 serum IgG responses were observed in all animals by Day 15 it was insufficient to protect IRMs
474 from heterologous challenge with the PRVABC59 strain. While these results appear contrary to

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475 what was reported previously in IRMs challenged with the MR766 strain it is most likely that
476 this failure to protect is due to the low level immune response as opposed to the failure of the
477 antibodies to cross protect [34].

478 These results serve to rule out the genetic background of the NHP model as a factor
479 contributing to the differential infectivity of African-lineage ZIKV strains; however, it is yet to
480 be determined what viral genetic factors contribute to the disparity. Both the IbH_30656 and
481 MR766 isolates have been subject to multiple laboratory passages since their initial isolation
482 more than 50 years ago. Sequence comparisons performed in 2012 demonstrated that MR766
483 and IbH_30656 differ by 7% at the nucleotide level and 2.2% at the amino acid level; whereas
484 the ArD 41519 strain which was isolated more recently and also productively infected IRMs via
485 the IVAG and IR routes differs from MR766 by 1.7% at the amino acid level despite a 7%
486 nucleotide divergence [39]. These results serve to narrow the amino acids changes to be assessed
487 in future studies aimed at discerning the viral genetic factors contribute to differential
488 pathologies of ZIKV strains from the African-lineage and should aid in determining the changes
489 that have contributed to recently emerging pathologies associated with Asian-lineage strains.

490 Until then, the results presented here and elsewhere support the use of IRMs and the
491 ZIKV PRVABC59 strain as a model of Asian-lineage ZIKV infection for the evaluation of new
492 vaccines and therapeutics. The PRVABC59 strain represents a human clinical isolate of low
493 passage which is a prerequisite for animal model development under the animal rule.
494 Additionally, infection of IRMs with as little as 1E4 PFU of virus results in detectable viremia
495 that can be assessed by qRT-PCR. Vaccinating IRMs with an inactivated PRVABC59 vaccine
496 resulted in Nab's of similar titers and kinetics to those reported previously [31]; and vaccinated
497 animals were protected from challenge with 1E5 PFU of live ZIKV PRVABC59 as determined

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498 by the absence of viremia as compared to sham vaccinated animals. This model utilizing
499 qualified master and working virus banks will serve as a valuable resource suitable for
500 submission of preclinical efficacy data on new ZIKV vaccines and therapeutics to the FDA.

501

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507

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598

599

600

601

602 **Tables:**

603 **Table 1.** Animal groupings of (36) Indian Rhesus Macaques subdivided into 9 groups for initial
604 challenge with the ZKV isolate and the dose indicated.

605

Group Number	Animal Number	Isolate	Target Dose (PFU/animal)	Delivered Dose (PFU/animal)
1	4 (2M/2F)	PRVABC59	$1x10^4$	$5.5x10^3$
2	4 (2M/2F)	PRVABC59	$1x10^5$	$9.1x10^4$
3	4 (2M/2F)	PRVABC59	$1x10^6$	$7.6x10^5$
4	4 (2M/2F)	PLCal_ZV	$1x10^4$	$3.0x10^3$
5	4 (2M/2F)	PLCal_ZV	$1x10^5$	$9.0x10^4$
6	4 (2M/2F)	PLCal_ZV	$1x10^6$	$6.5x10^5$
7	4 (2M/2F)	IBH 30656	$1x10^4$	$2.8x10^3$
8	4 (2M/2F)	IBH 30656	$1x10^5$	$3.4x10^4$
9	4 (2M/2F)	IBH 30656	$1x10^6$	$3.9x10^5$

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Table 2. Animal groupings of (36) Indian Rhesus Macaques for the re-challenge phase resulting in some animals being re-challenged with the identical geographical isolate or cross challenged with a ZKV isolate from a different geographical location at dose indicated.

Group Number	Animal Number	Initial Challenge	Secondary Challenge	Target Dose (PFU/animal)
1	6 (3M/3F)	PRVABC59	PRVABC59	$1x10^6$
2	6 (3M/3F)	PRVABC59	PLCal_ZV	$1x10^6$
3	6 (3M/3F)	PLCal_ZV	PLCal_ZV	$1x10^6$
4	6 (3M/3F)	PLCal_ZV	PRVABC59	$1x10^6$
5	6 (3M/3F)	IBH 30656	IBH 30656	$1x10^6$
6	6 (3M/3F)	IBH 30656	PRVABC59	$1x10^6$

Figures:

Figure 1. Key Study Challenge and Sample Collection Time Points. Challenge Phase I animals were subdivided into cohorts and inoculated on Day 0 with a primary challenge ZIKV isolate. After the primary challenge and a resting period, animals were regrouped and challenged on Day 45 with either the same isolate or cross-challenged with an isolate of different geographic origin. Blood, saliva and urine samples were collected as indicated for viral load analysis by RT-qPCR or plaque assay.

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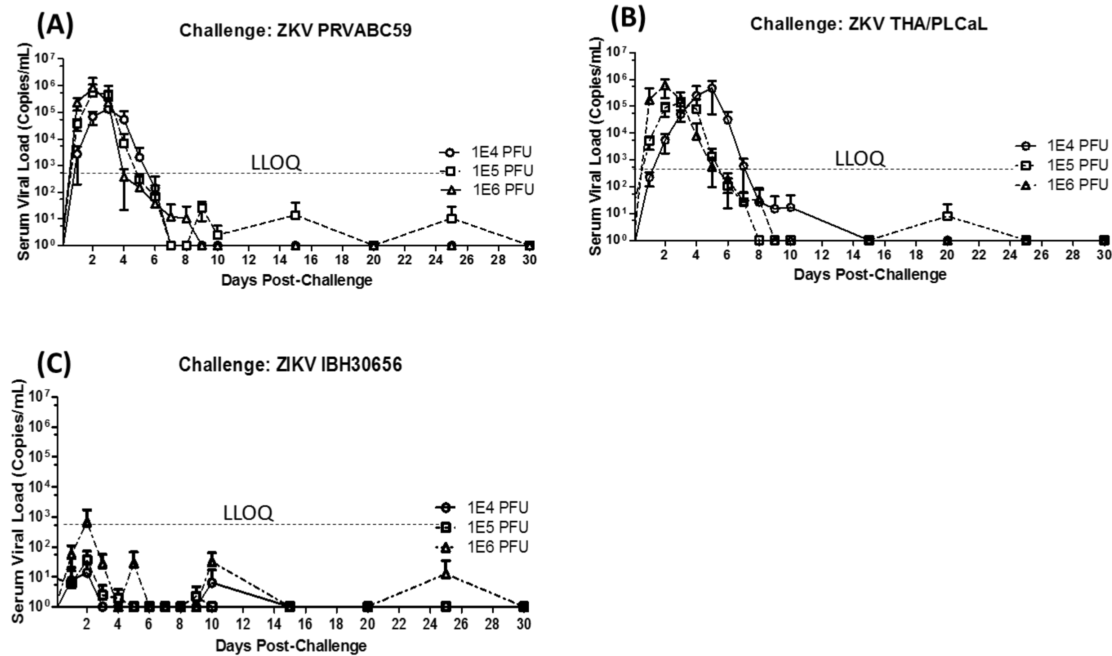

621

622 **Figure 2.** Serum viral loads in IRMs infected with different ZIKV isolates: Serum Viral load in
623 the IRM's (N=4 per dose group) infected with different doses of ZIKV isolates as indicated in
624 the figures. A) PRVABC59 B) PLCal_ZV C) IBH_30656. Viral load is reported as copies/mL
625 from serum purified from blood collected at multiple time points post challenge between Days
626 (0-30). Lower Limit of Quantitation (LLOQ) is shown by the dotted line.

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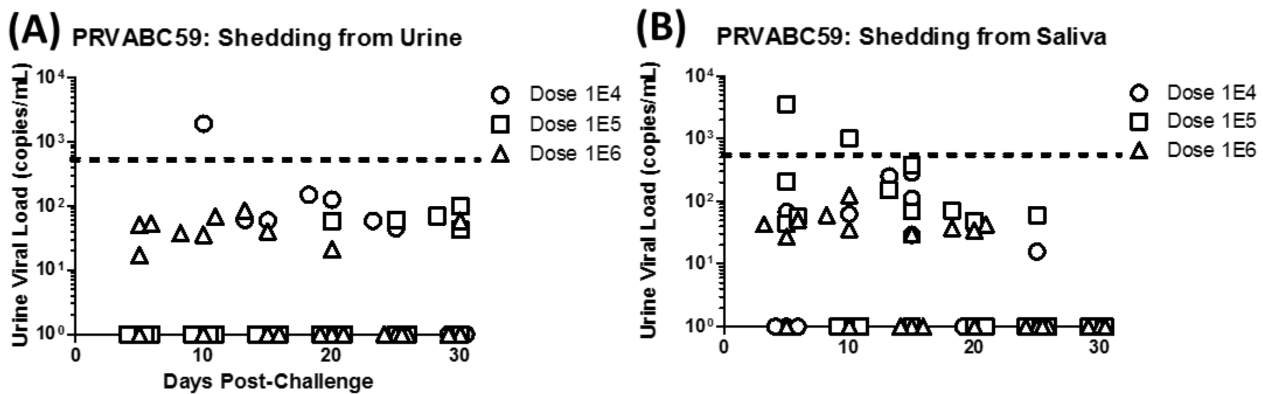
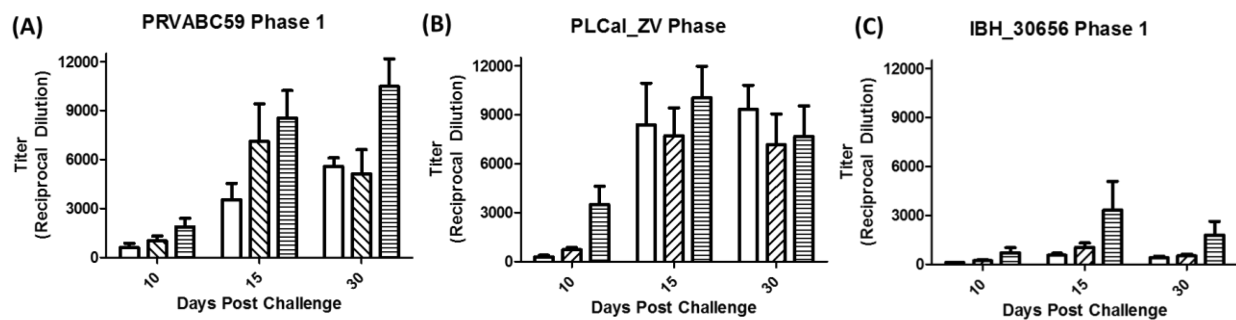


629

630 **Figure 3.** Viral RNA shedding in PRVABC59 infected IRM: Urine and saliva was collected at
631 multiple time points post challenge between Days (0-30) from IRM infected with ZIKV
632 PRVABC59 and subjected to qRT-PCR assay. ZIKV RNA concentrations (copies/ml) are presented
633 in the urine (A) and saliva (B). Circles represent data from single animals in the 1E4 dose group,
634 squares represent the 1E5 dose group, and triangles represent the 1E6 dose group. Urine could not
635 be collected from one IRM on Day 5 (dose 1E4) and day 25 (dose 1E5). Lower Limit of
636 Quantitation (LLOQ) is shown by the dotted line.



637

638 **Figure 4.** Anti-Zika IgG production after initial challenge with Zika Virus. Animals were
639 challenged with 1E4 PFU, 1E5 PFU, or 1E6 PFU with one of three ZIKV isolates and anti-ZIKV
640 IgG was detected in serum by ELISA. No fill 1E4, Hatched lines 1E5, Horizontal lines 1E6.

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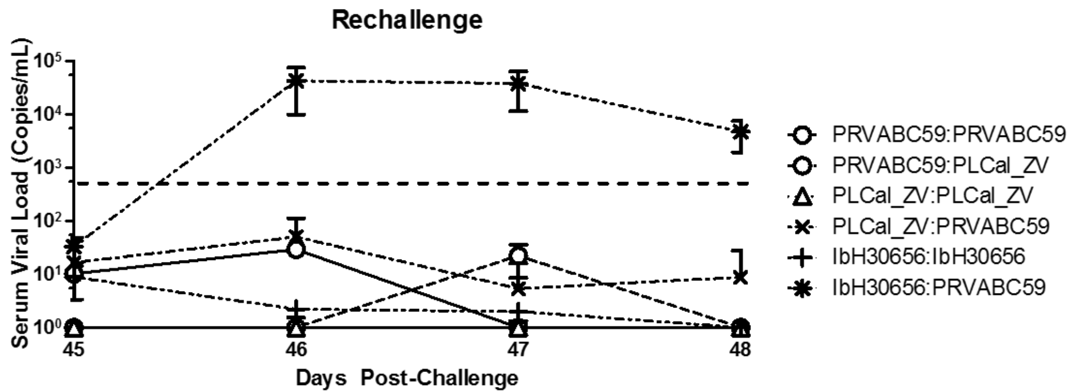


642

643 **Figure 5**. **Serum viral load post rechallenge in IRMs:** Serum viral load was determined by qRT-

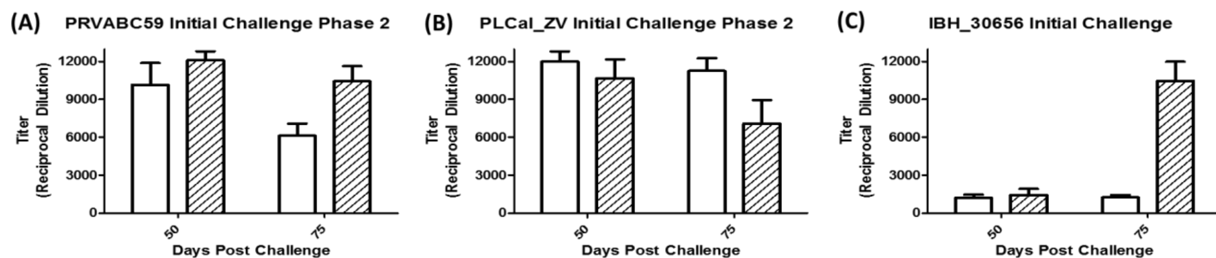
644 PCR in IRMs (N=6 per group) previously infected and re-challenged with either PRVABC59

645 (PRV), PLCal_ZV(PLCal), or IBH_30656 (IBH) at a dose of 1E6 PFU/IRM. Viral RNA is

646 reported as copies/mL of serum purified from blood collected at multiple time points post re-

647 challenge between Days (45-50). Note, for the IBH:IBH group day (50) viral load was determined

648 from N=3. Lower Limit of Quantitation (LLOQ) is shown by the dotted line.



649

650 **Figure 6. Anti-Zika IgG production after secondary challenge with Zika virus.** Antibody

651 titers on Study Days 50 and 75 during Phase II. Animals from each group in Phase I were

652 reassigned based on previous challenge strain and titer, then re-challenged with 1E6 PFU of

653 PRVABC59, PLCal_ZV, or IBH_30656. A) Primary challenge with PRVABC59 followed by

654 secondary challenge with PRVABC59 (No fill) or PLCal_ZV (Hatched lines); B) Primary

655 challenge with PLCal_ZV followed by secondary challenge with PLCal_ZV (No fill) or

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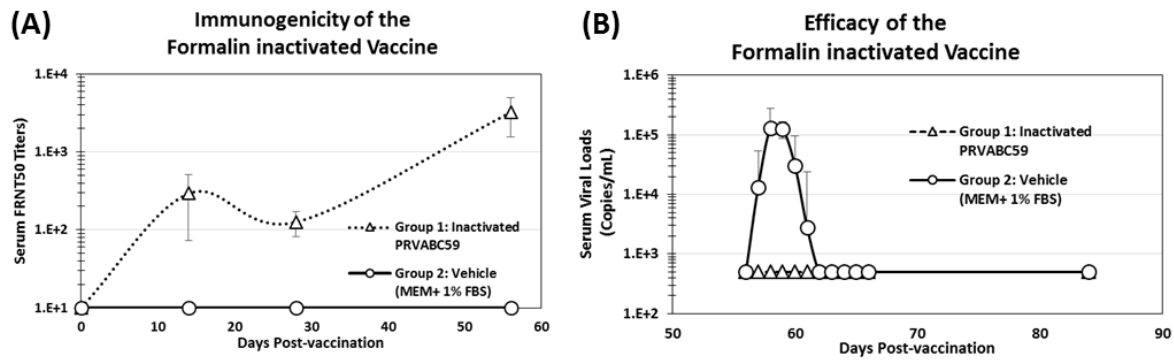
656 PRVABC59 (Hatched lines); and C) Primary challenge with IbH_30656 followed by secondary
657 challenge with IBH_30656 (No fill) or PRVABC59 (Hatched lines).

658



659

660 **Figure 7 Immunogenicity (A) and Efficacy (B) of Formalin inactivated Zika vaccine in the**
661 **IRM model:** IRMs were vaccinated with formalin inactivated ZIKV vaccine or the sham control
662 at day 0, followed by challenge with 1E5 PFU of PRVABC59 per IRM on day 54. Serum viral
663 loads after the challenge and FRNT50 titers after vaccination were measured by using qRT-PCR
664 assay and ZIKV neutralization assay as described in the method section. Triangles- Vaccinated
665 animals, Circles- Vehicle control.

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668 **Supplementary Data:**

669

670 **Table 1.** PRVABC59 viral load in serum detected by quantitative RT-PCR (Genome Copies/mL)

671 and Viral Plaque Assay (PFU/mL).

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				Day Post Infection							
				1		2		3		4	
Group	Animal	Dose	Sex	RT-PCR	Plaque	RT-PCR	Plaque	RT-PCR	Plaque	RT-PCR	Plaque
1	5366	$1.0x10^4$	M	-	-	1.63E4	6.25E1	1.80E4	2.50E1	9.70E3	5.75E2
	5362	$1.0x10^4$	M	6.29E3	2.50E1	8.05E4	3.38E2	6.10E4	2.50E1	-	-
	5388	$1.0x10^4$	F	-	-	9.60E4	2.50E2	1.12E5	6.25E1	8.82E4	0
	5376	$1.0x10^4$	F	-	-	8.48E4	3.75E2	3.44E5	5.75E2	1.12E5	0
2	5360	$1.0x10^5$	M	4.04E4	6.25E1	1.39E6	3.18E3	1.20E6	1.33E3	-	-
	5368	$1.0x10^5$	M	2.64E4	1.25E2	2.39E5	9.25E2	1.39E5	2.88E2	-	-
	5389	$1.0x10^5$	F	2.17E4	1.50E2	3.80E5	1.29E3	4.72E5	8.88E2	-	-
	5378	$1.0x10^5$	F	6.22E4	6.25E1	1.26E5	4.00E2	5.66E4	3.75E1	-	-
3	5374	$1.0x10^6$	M	2.21E5	1.50E2	6.07E5	1.13E3	-	-	-	-
	5363	$1.0x10^6$	M	9.02E4	5.00E1	2.34E5	4.38E2	1.19E5	4.00E2	-	-
	5375	$1.0x10^6$	F	2.41E5	6.25E1	1.47E5	2.13E2	2.54E4	1.25E1	-	-
	5381	$1.0x10^6$	F	3.63E5	3.75E1	2.43E6	5.75E2	7.80E5	5.38E2	-	-

679 **Table 2.** PLCal_ZV viral load in serum detected by quantitative RT-PCR (Genome Copies/mL) and Viral

680 Plaque Assay (PFU/mL).

				Day Post Infection											
				1		2		3		4		5		6	
Group	Animal	Dose	Sex	RT-PCR	Plaque	RT-PCR	Plaque	RT-PCR	Plaque	RT-PCR	Plaque	RT-PCR	Plaque	RT-PCR	Plaque
4	5370	$1.0x10^4$	M	-	-	-	-	7.53E4	1.00E2	2.00E5	2.50E1	2.83E4	0	-	-
	5359	$1.0x10^4$	M	-	-	-	-	-	-	2.42E4	0	2.21E5	3.00E2	2.74E4	2.50E1
	5392	$1.0x10^4$	F	-	-	-	-	3.88E4	0	7.05E5	8.38E2	7.02E5	1.25E2	-	-
	5383	$1.0x10^4$	F	-	-	-	-	-	-	8.26E4	7.50E1	9.46E5	4.50E2	7.55E4	0
5	5369	$1.0x10^5$	M	3.11E3	0	5.41E4	6.00E2	7.08E4	2.00E2	-	-	-	-	-	-
	5358	$1.0x10^5$	M	-	-	5.39E4	1.00E2	2.22E5	2.50E1	2.29E5	2.50E1	-	-	-	-
	5380	$1.0x10^5$	F	-	-	9.03E4	3.00E2	9.11E4	5.00E1	3.21E4	0	-	-	-	-
	5384	$1.0x10^5$	F	-	-	1.56E5	1.88E2	1.67E5	6.25E1	5.90E4	0	-	-	-	-
6	5367	$1.0x10^6$	M	3.86E4	1.75E2	5.90E5	1.55E3	3.57E5	1.00E2	-	-	-	-	-	-
	5372	$1.0x10^6$	M	4.28E4	5.00E1	2.37E5	7.00E2	1.37E4	1.25E1	-	-	-	-	-	-
	5379	$1.0x10^6$	F	-	-	4.26E5	1.00E2	9.63E4	1.25E1	2.92E4	0	-	-	-	-
	5377	$1.0x10^6$	F	6.14E5	1.58E3	1.17E6	1.15E3	2.52E5	2.50E2	-	-	-	-	-	-

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686 **Table 3.** Zika IgG ELISA data for Phase I of Natural History study

Phase I						Average		
Group	Challenge Dose (PFU/Animal)	Animal ID	Day			Day		
			10	15	30	10	15	30
1	1E4 PRVABC59	**5366**	218.6	1755.4	4126.5	606.4	3558.1	5573.2
		5362	1431.5	6357.5	5769.1			
		5388	399.1	2866.8	6739.0			
		5376	376.5	3252.6	5658.1			
2	1E5 PRVABC59	**5360**	938.2	2866.8	1925.1	1025.9	7121.5	5127.4
		5368	1789.8	8888.3	9018.7			
		5389	395.3	>12800	5337.8			
		5378	980.1	3930.8	4227.9			
3	1E6 PRVABC59	**5374**	2237.8	9421.6	>12800	1890.3	8559.7	10486.3
		5363	3174.6	>12800	>12800			
		5375	1383.7	5011.2	10741.7			
		5381	765.1	7006.0	5603.4			
4	1E4 PLCal_ZV	**5370**	542.0	>12800	>12800	294.9	8379.9	9341.2
		5359	<100	4008.0	8104.8			
		5392	345.0	>12800	6026.8			
		5383	192.6	3911.8	10433.2			
5	1E5 PLCal_ZV	**5369**	1101.3	>12800	>12800	732.3	7714.7	7151.4
		5358	633.1	6513.8	5769.1			
		5380	714.8	5576.2	5286.2			
		5384	480.0	5968.6	4750.5			
6	1E6 PLCal_ZV	**5367**	6837.9	>12800	>12800	3494.1	10039.1	7685.4
		5372	2360.6	9987.0	3950.0			
		5379	1925.1	4569.5	7572.1			
		5377	2852.9	>12800	6419.6			
7	1E4 IBH_30656	**5361**	123.8	732.4	371.1	106.0	562.3	414.2
		5365	<100	787.7	623.9			
		5385	<100	242.0	444.1			
		5390	<100	487.0	217.5			
8	1E5 IBH_30656	**5371**	173.1	1746.9	843.1	226.2	1039.7	520.8
		5357	395.3	1019.0	395.3			
		5387	227.2	980.1	511.3			
		5386	109.1	412.9	333.5			
9	1E6 IBH_30656	**5364**	765.1	3447.8	1664.1	717.2	3330.9	1790.4
		5373	1562.3	8263.8	4207.4			
		5382	313.1	768.8	885.1			
		5391	228.3	843.1	405.0			

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688 **Table 4. Zika IgG ELISA data for Phase II of Natural History study**

Phase II					Average	
Group	Re-Challenge Dose (PFU/Animal)	Animal ID	Day 50	Day 75	Day 50	Day 75
1	1E6 PRVABC59	**5366**	>12800	8104.8	10141.3	6110.5
		5360	3009.4	1878.9		
		5374	>12800	7833.9		
		5388	12731.8	7248.3		
		5389	6706.4	4891.0		
		5375	>12800	6706.4		
2	1E6 PLCal_ZV	**5362**	>12800	12426.4	12098.5	10423.0
		5368	8591.2	7390.4		
		5363	>12800	>12800		
		5376	>12800	>12800		
		5378	>12800	11005.7		
		5381	>12800	6115.3		
3	1E6 PLCal_ZV	**5370**	>12800	>12800	11985.1	11247.5
		5369	>12800	>12800		
		5367	>12800	>12800		
		5392	>12800	>12800		
		5380	>12800	9106.7		
		5379	7910.4	7178.2		
4	1E6 PRVABC59	**5359**	>12800	>12800	10672.3	7093.0
		5358	8845.2	4867.3		
		5372	3988.5	3009.4		
		5383	>12800	3284.3		
		5384	>12800	5797.2		
		5377	>12800	>12800		
5	1E6 IBH_30656	**5361**	331.9	516.3	1203.5	1245.6
		5371	1184.5	1156.1		
		5364	2205.5	1860.7		
		5385	924.6	1128.4		
		5387	1330.9	1219.5		
		5382	1243.4	1592.9		
6	1E6 PRVABC59	**5365**	1249.5	>12800	1431.7	10455.7
		5357	1184.5	>12800		
		5373	3672.5	>12800		
		5390	302.6	4459.9		
		5386	1370.3	>12800		
		5391	811.0	7074.4		

Initial Challenge
1e4 PRVABC59
1e5 PRVABC59
1e6 PRVABC59
1e4 PLCal_ZV
1e5 PLCal_ZV
1e6 PLCal_ZV
1e4 IBH_30656
1e5 IBH_30656
1e6 IBH_30656

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