

# 1 **African-lineage Zika virus replication dynamics and maternal-fetal** 2 **interface infection in pregnant rhesus macaques**

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29 Running head (54 characters): African-lineage Zika virus in pregnant rhesus macaques  
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38 **ABSTRACT**

39 Following the Zika virus (ZIKV) outbreak in the Americas, ZIKV was causally associated with  
40 microcephaly and a range of neurological and developmental symptoms, termed congenital  
41 Zika syndrome (CZS). The isolates responsible for this outbreak belonged to the Asian lineage  
42 of ZIKV. However, in-vitro and in-vivo studies assessing the pathogenesis of African-lineage  
43 ZIKV demonstrated that African-lineage isolates often replicated to high titer and caused more  
44 severe pathology than Asian-lineage isolates. To date, the pathogenesis of African-lineage  
45 ZIKV in a translational model, particularly during pregnancy, has not been rigorously  
46 characterized. Here we infected four pregnant rhesus macaques with a low-passage strain of  
47 African-lineage ZIKV and compared its pathogenesis to a cohort of four pregnant rhesus  
48 macaques infected with an Asian-lineage isolate and a cohort of mock-infected controls. Viral  
49 replication kinetics were not significantly different between the two experimental groups and  
50 both groups developed robust neutralizing antibody titers above levels considered to be  
51 protective. There was no evidence of significant fetal head growth restriction or gross fetal  
52 harm at delivery in either group. However, a significantly higher burden of ZIKV vRNA was  
53 found in maternal-fetal interface tissues in the macaques exposed to an African-lineage isolate.  
54 Our findings suggest that ZIKV isolates of any genetic lineage pose a threat to women and their  
55 infants.

56

57 **IMPORTANCE**

58 ZIKV was first identified over 70 years ago in Africa, but most of our knowledge of ZIKV is  
59 based on studies of the distinct Asian genetic lineage, which caused the outbreak in the  
60 Americas in 2015-16. In its most recent update, the WHO stated that improved understanding  
61 of African-lineage pathogenesis during pregnancy must be a priority. Recent detection of  
62 African-lineage isolates in Brazil underscores the need to understand the impact of these  
63 viruses. Here we provide the first comprehensive assessment of African-lineage ZIKV infection  
64 during pregnancy in a translational non-human primate model. We show African-lineage  
65 isolates replicate with similar kinetics to Asian-lineage isolates and are capable of infecting the  
66 placenta. However, there was no evidence of more severe outcomes with African-lineage  
67 isolates. Our results highlight both the threat that African-lineage ZIKV poses to women and  
68 their infants and the need for future epidemiological and translational in-vivo studies with  
69 African-lineage ZIKV.

70

71 **INTRODUCTION**

72 Zika virus (ZIKV) gained global notoriety in 2015 when it caused a large epidemic of febrile  
73 illness in the Americas and, for the first time, was causally associated with birth defects in  
74 infants born to mothers who became infected while pregnant (1). Why was ZIKV, which was  
75 first isolated in Uganda in 1947, not causally linked to birth defects prior to this outbreak in the  
76 Americas? Several hypotheses have emerged to explain why congenital ZIKV infection seems  
77 like a new complication, including that women in Africa are exposed to and therefore immune  
78 to the virus before childbearing age; that ZIKV circulating in the Americas acquired mutations

79 that increased its ability to cause congenital ZIKV syndrome (CZS); or that ZIKV disease is  
80 enhanced by prior flavivirus immunity.

81  
82 ZIKV circulates as two genetic lineages: African and Asian. The vast majority of animal model  
83 and epidemiological studies of ZIKV to date have focused on Asian-lineage viruses because  
84 they were responsible for the outbreak in the Americas. Therefore, relatively little is known  
85 about the pathogenic potential of African-lineage viruses, particularly with regard to fetal  
86 outcomes. In cell culture experiments, African-lineage ZIKV isolates have been shown to  
87 replicate to higher titers and induce increased cell lysis as compared to Asian-lineage ZIKV (2–  
88 6). Particularly notable was the demonstration that African-lineage ZIKV isolates cause more  
89 rapid and more severe cytopathic effects (CPE) than Asian-lineage isolates in human  
90 embryonic stem-cell derived trophoblasts, which are cells critical for the development of the  
91 placenta (3, 4). Furthermore, in both pregnant and non-pregnant immunocompromised mouse  
92 models, African-lineage isolates have consistently shown both increased mortality and  
93 increased fetal harm as compared to Asian-lineage strains (5–8). Several experiments have  
94 been conducted with African-lineage ZIKV isolates in non-pregnant macaques. Two of the  
95 isolates used in these studies have extensive passage histories in mice and therefore cannot  
96 be considered natural ZIKV isolates (9). Still, in one study the virus replicated in the rhesus  
97 macaque host, but not as robustly as Asian-lineage isolates. A second study showed no  
98 replication of the isolate in Mauritian cynomolgus macaques, while a third study showed  
99 replication comparable to Asian-lineage viruses and subsequent protection against  
100 heterologous challenge (10–12). A low-passage isolate has been used in several non-human  
101 primate models and showed modest replication when inoculated intrarectally and  
102 intravaginally, and robust replication when inoculated subcutaneously (13, 14).

103  
104 In the July 2019 epidemiological update on ZIKV, the WHO identified the assessment of fetal  
105 outcomes following infection with African-lineage viruses as a priority (15). This is underscored  
106 by recent findings of African-lineage isolates in South America, including evidence of fetal harm  
107 in a non-human primate naturally exposed to an African strain of ZIKV most closely related to  
108 the prototype strain, MR766 (16, 17). While the ability of African-lineage ZIKV to infect the  
109 maternal-fetal interface and cause fetal harm has been rigorously studied in cell culture and  
110 immunocompromised mice, it remains unclear how translational these findings are to  
111 humans.

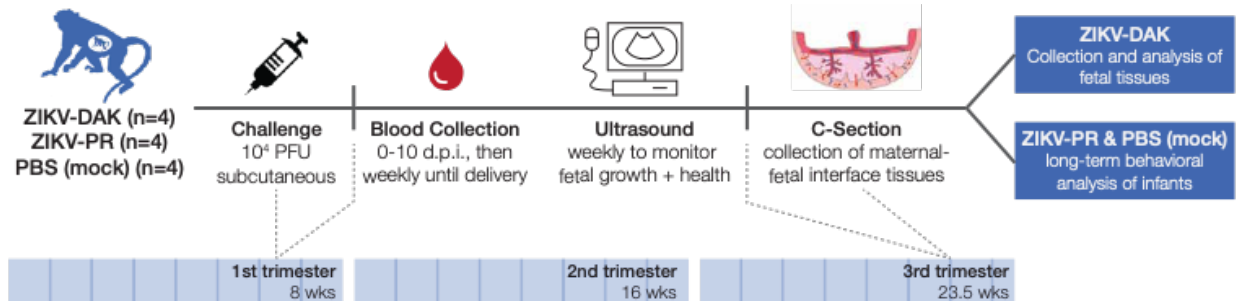
112  
113 To address this gap, we aimed to assess the pathogenic potential of a low-passage African-  
114 lineage ZIKV isolate during pregnancy in our well-established non-human primate model of  
115 ZIKV (18, 19). Recently, we demonstrated that the low-passage and highly pathogenic African-  
116 lineage ZIKV strain ZIKV/Aedes africanus/SEN/DAK-AR-41524/1984 (ZIKV-DAK; BEI  
117 Resources, Manassas, VA) replicated to higher titer in maternal serum and caused significantly  
118 greater fetal harm as compared to Asian-lineage ZIKV in *Irfnar1*<sup>-/-</sup> C57BL/6 mice (8). Notably,  
119 placental pathology in mice infected with ZIKV-DAK was more severe than in mice infected  
120 with an Asian-lineage virus. Since contemporary ZIKV isolates from Africa are not readily

121 available, this strain is one of the most recent, low-passage isolates available for pathogenesis  
122 studies.

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124 We infected four pregnant macaques with ZIKV/Aedes-africanus/SEN/DakAr41524/1984 (ZIKV-  
125 DAK) during the late first trimester, monitored fetal health and growth throughout pregnancy  
126 and assessed fetal outcomes (presence of vRNA, gross abnormalities) at delivery at gestational  
127 day 155, approximately 1.5 weeks prior to full term. We compare data from a cohort of four  
128 pregnant macaques infected with ZIKV-DAK to a cohort of four pregnant macaques infected  
129 with Zika-virus/H.sapiens-tc/PUR/2015/PRVABC59\_v3c2 (ZIKV-PR), a low-passage Asian-  
130 lineage isolate. This virus, isolated from a human in Puerto Rico in 2015, has been well  
131 characterized in rhesus macaques (10, 18–22). Although we did not find evidence of more  
132 severe fetal outcomes following infection with an African-lineage virus as compared to Asian-  
133 lineage virus, data from this study supports the hypothesis that ZIKV of both African- and  
134 Asian-lineage pose a threat to women and their infants.

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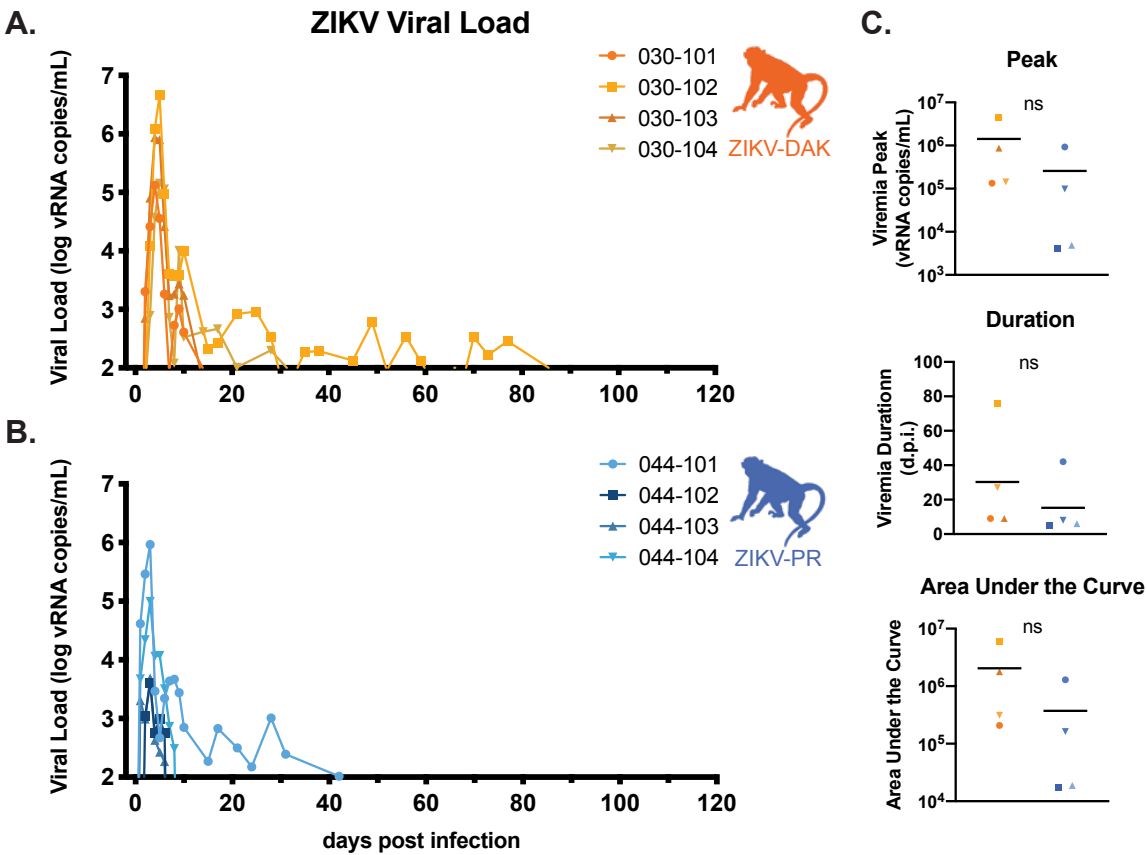
137 **Figure 1. Study overview.** Groups of four pregnant macaques were challenged between gestation days  
138 (gd) 45 and 50 (late first trimester) with either ZIKV-DAK, ZIKV-PR, or PBS (mock). Following viral  
139 challenge, blood was collected daily from 0-10 d.p.i., then twice weekly until viremia resolved, and then  
140 once weekly until delivery. Ultrasounds were performed once weekly to measure fetal health and growth.  
141 Between gd 155-160 (1.5 weeks prior to full term), infants were delivered via cesarean section, and  
142 maternal-fetal interface tissues, including the placenta, fetal membranes, umbilical cord, and placental  
143 bed were collected. Infants born to dams inoculated with ZIKV-DAK were humanely euthanized, and a  
144 comprehensive set of tissues were collected. Infants born to dams challenged with ZIKV-PR or PBS  
145 (mock) were paired with their mothers and followed for long-term behavioral analysis.

## 146 RESULTS

### 147 ZIKV-DAK replicates to high titer in macaques and with similar replication to ZIKV-PR

148 Four pregnant rhesus macaques (*Macaca mulatta*) were subcutaneously inoculated with  $10^4$   
149 PFU of ZIKV-DAK between gestation day 45 and 50, late in the first trimester (Figure 1). The  
150 first trimester is associated with the greatest risk of CZS in pregnant women and is both a time  
151 of active neurological development and a time before which many women know that they are  
152 pregnant (23, 24). Following inoculation, plasma was collected daily for 10 days post infection  
153 (dpi), then twice weekly until viremia resolved, and then once weekly for the remainder of  
154 gestation. Negative viremia was defined as two consecutive timepoints with viral loads below  
155 the limit of quantification of our ZIKV QRT-PCR assay (100 copies/mL plasma). Virus replicated  
156 to high levels ( $10^5$ - $10^6$  vRNA copies/mL) in all four macaques, with viremia persisting through  
157 day 10 for all four macaques; two macaques had prolonged viremia out to 28-77 dpi (Figure

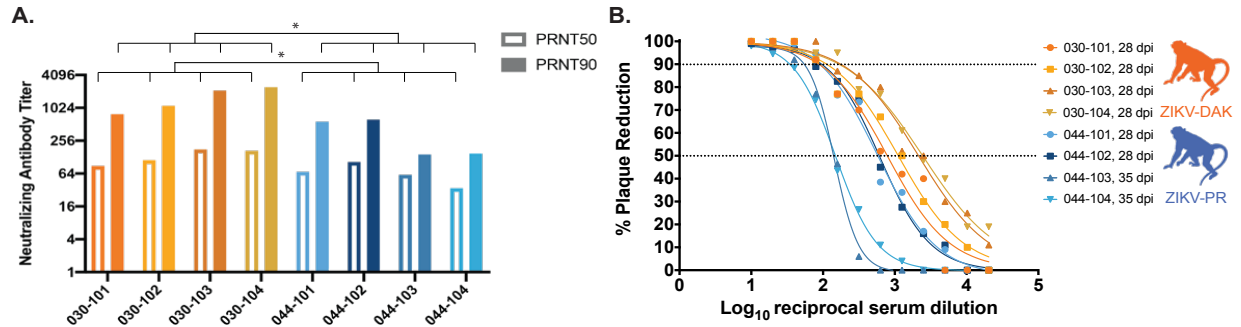
158 2A). When compared to a cohort of four macaques infected with ZIKV-PR using the same  
159 inoculation and sampling regimen, there were no statistically significant differences in viremia  
160 peak, duration, or area under the curve, suggesting that this African-lineage isolate replicates  
161 in pregnant macaques with similar kinetics to Asian-lineage isolates (Figure 2B).  
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164 **Figure 2. ZIKV-DAK and ZIKV-PR Viral replication kinetics. A-B.** Viral load was determined using  
165 ZIKV-specific QRT-PCR of RNA isolated from plasma. Only values above the assay's limit of  
166 quantification (100 copies/ml) are shown. **C.** There were no statistically significant differences in the  
167 peak, duration, or area under the curve of viremia between the two groups (two-sample t tests).

### 168 ZIKV-DAK induces a robust nAb response

169 By 28 dpi, all macaques infected with either ZIKV-DAK or ZIKV-PR, regardless of viremia  
170 duration, had developed a robust neutralizing antibody titer (Figure 3A). The PRNT50 and 90  
171 titers developed in response to ZIKV-DAK infection are significantly higher than those  
172 developed in response to ZIKV-PR infection (Figure 3B). The PRNT90 titers in macaques  
173 exposed to ZIKV-DAK are greater than the titers of macaques in a different study infected with  
174 the mouse-adapted isolate ZIKV-MR766, which were shown to be protective against  
175 heterologous challenge (10). Therefore, we expect that the immune response produced in  
176 these macaques infected with ZIKV-DAK during pregnancy would be protective against  
177 secondary ZIKV challenge.



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**Figure 3. Neutralizing antibody titers.** Plaque reduction neutralization tests (PRNT) were performed on serum samples collected between 28–35 days post infection to determine the titer of ZIKV-specific neutralizing antibodies. Neutralization curves were constructed using GraphPad Prism. PRNT90 and PRNT50 values were estimated using non-linear regression analysis and are shown on the bar graph in (A) and indicated with dotted lines in (B). PRNT50 and 90 titers were compared using an unpaired, parametric t-test. ZIKV-DAK-infected macaques had significantly higher PRNT50 ( $p=0.0371$ ) and PRNT90 ( $p=0.0243$ ) titers than ZIKV-PR-infected macaques.

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### No reduction in fetal growth during gestation

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Sonographic imaging was conducted weekly beginning one week prior to inoculation to evaluate fetal health (heart rate), overall fetal growth (abdominal circumference, femur length) and head growth (head circumference, biparietal diameter). No gross fetal or placental anomalies were observed. Varying amounts of placental calcification were noted on ultrasound in all four macaques exposed to ZIKV-DAK; however, calcifications were also observed in macaques infected with ZIKV-PR and in mock-inoculated macaques, suggesting that these qualitative observations are normal for gestational age or unrelated to viral infection.

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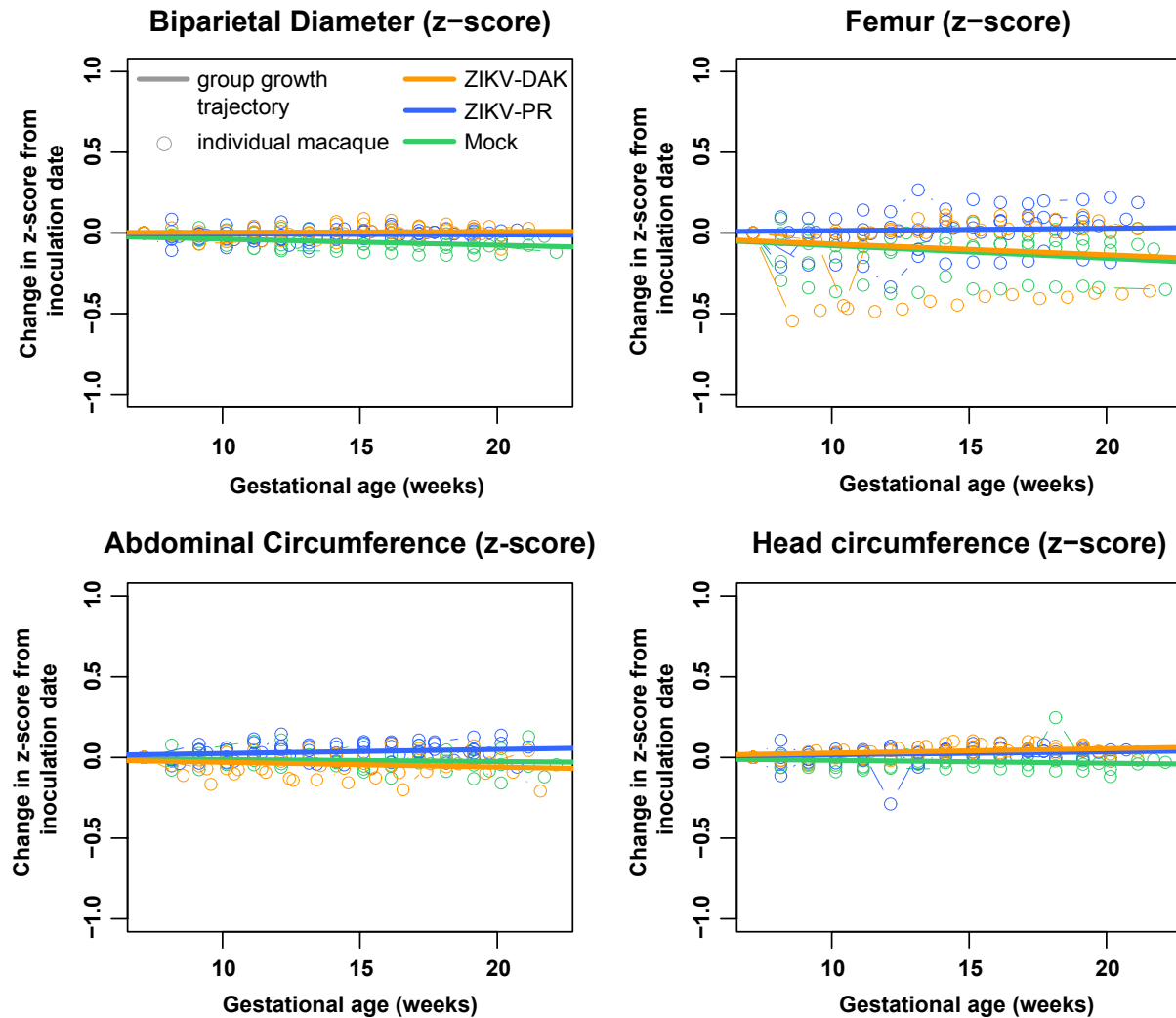
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Femur length, abdominal circumference, head circumference, and biparietal diameter measurements were compared to normative data developed from  $n=85$  cynomolgus and rhesus macaques at the California National Primate Research Center (25, 26). We calculated the number of standard deviations that each fetal measurement differed from the normative data (z-score) at that gestational age. A linear mixed effects model with animal-specific random effects was used to evaluate the change in the outcome measures between gestation days 50–160. Growth was quantified by calculating the slope parameters for each experimental group. We then compared fetal growth in each group both to the normative data and to each of the other groups (Figure 4). When compared to the normative data, mock-infected animals had significantly reduced biparietal diameter growth ( $p=0.0207$ ), while ZIKV-PR and ZIKV-DAK had a very modest, but statistically significant increase in head circumference growth ( $p=0.0230$ ;  $p=0.0179$ ). All other values were not significantly different from the normative data. When each of the experimental groups were compared to the mock-infected group, there was no significant reduction or increase in any of the growth measurements in the experimental group, suggesting that infection with either lineage of ZIKV did not restrict or enhance fetal growth.



211

212 **Figure 4. Intrauterine fetal growth.** Sonographic imaging was performed weekly to measure fetal  
213 health and growth. Normative measurement data from the California National Primate Research Center  
214 was used to calculate z-scores for each weekly measurement for each macaque. The change in the z-  
215 score from the baseline measurement is plotted for each macaque with an open circle. Growth  
216 trajectories were quantified by calculating the regression slope parameters from baseline for each  
217 experimental group (solid line). When compared to the normative data, mock-infected animals had  
218 significantly reduced biparietal diameter growth ( $p=0.0207$ ); ZIKV-PR and ZIKV-DAK had a very modest,  
219 but statistically significant increase in head circumference growth ( $p=0.0230$ ;  $p=0.0179$ ).

#### 220 **No evidence of vertical transmission at delivery**

221 At approximately gestation day 155 (full term is  $165\pm 10$  days in rhesus macaques), fetuses of  
222 ZIKV-DAK-infected dams were delivered via cesarean section and humanely euthanized. No  
223 gross abnormalities were noted in any of the infants at delivery. A comprehensive set of  
224 maternal biopsies, maternal-fetal interface tissues, and fetal tissues were collected for vRNA  
225 measurements and histopathological analysis. In the fetus, emphasis was placed on collecting  
226 tissues that may be involved in transmission of the virus and tissues that are likely to be sites  
227 of ZIKV replication, including the central nervous system. Infants of ZIKV-PR-infected dams  
228 were delivered via cesarean section at approximately gestation day 160 and are being

229 assessed for long-term neurodevelopmental sequelae. As a result, no fetal tissues were  
230 collected for comparison. No ZIKV RNA was detected in any of the fetal tissues collected at  
231 the time of delivery from the four ZIKV-DAK pregnancies (Supplemental table 2). Because  
232 pregnancies were allowed to go to term, we cannot exclude the possibility that ZIKV-DAK was  
233 vertically transmitted earlier in gestation but cleared from the fetus before delivery.  
234 Histopathological examination of fetal tissues revealed evidence of minimal to mild neutrophilic  
235 lymphadenitis in 3 of 4 ZIKV-exposed animals. Because we also observed neutrophilic  
236 lymphadenitis in four mock-infected animals that underwent the same experimental regimen,  
237 this inflammation may be a feature of normal development or from experimental procedures  
238 rather than viral infection.

239

#### 240 **ZIKV is present in a variety of maternal-fetal interface tissues at delivery**

241 Macaques typically have a bidiscoid placenta. To understand ZIKV distribution in the placenta,  
242 each placental disc was dissected into its individual cotyledons (perfusion domains), and a  
243 sample from the decidua, chorionic villi, and chorionic plate were taken from each cotyledon  
244 for both viral loads and histology (27). To understand ZIKV distribution in the maternal-fetal  
245 interface, additional samples were taken from the fetal membranes, uterine placental bed, and  
246 umbilical cord for both viral loads and histology. To assess the presence of virus in the dam, a  
247 biopsy of the mesenteric lymph node, liver, and spleen was taken from dams exposed to ZIKV-  
248 DAK for viral loads and histology. Only mesenteric lymph node biopsies were collected from  
249 dams exposed to ZIKV-PR. In the ZIKV-DAK dams, 3 of 12 biopsies, representing 2 different  
250 tissue types from 2 different macaques, were positive (Supplementary Table 2). In the ZIKV-PR  
251 dams, 1 of 4 mesenteric lymph node biopsies were positive.

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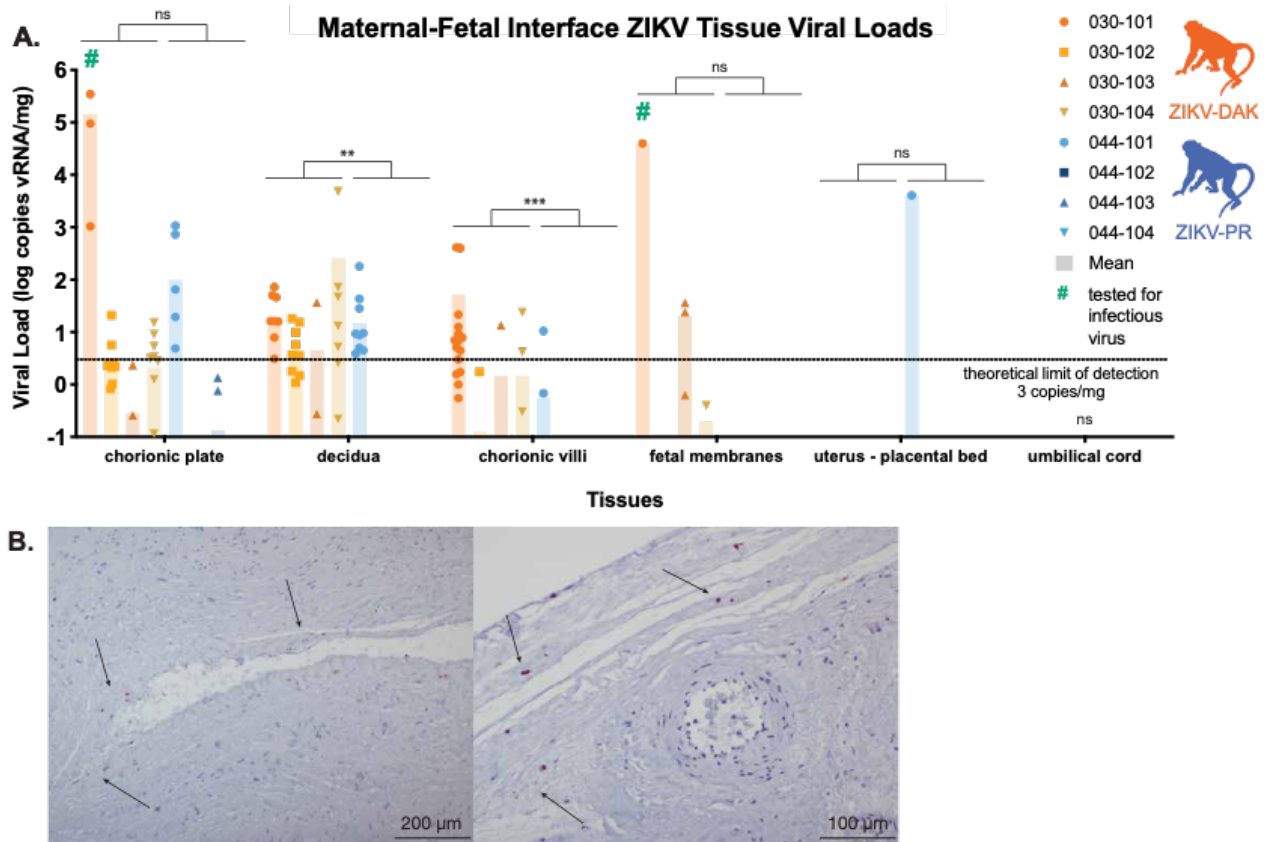
253 ZIKV RNA was present in the placenta and maternal-fetal interface in all four ZIKV-DAK  
254 infected animals to varying degrees regardless of the duration of viremia (Figure 5A). The  
255 highest burden was found in the decidua (basalis), chorionic plate, and chorionic villi and at  
256 lower levels in the fetal membranes. No vRNA was identified in the placental bed of the uterus  
257 or umbilical cord tissues (Figure 5A) or in the amniotic fluid or umbilical cord blood  
258 (Supplemental Table 2). In contrast, there were fewer positive tissues in the maternal-fetal  
259 interface tissues of macaques infected with ZIKV-PR. All but one of the tissues positive for  
260 ZIKV-PR RNA were from a single macaque, 044-101. No vRNA was detected in the umbilical  
261 cord (Figure 5A), amniotic fluid, or umbilical cord plasma (Supplemental Table 2) in ZIKV-PR  
262 infected animals. When compared to the cohort of macaques infected with ZIKV-PR, there is a  
263 significantly greater burden of ZIKV vRNA present in the ZIKV-DAK cohort in the decidua,  
264 chorionic plate, and chorionic villi (Figure 5A). There were no significant differences in the vRNA  
265 burden in the fetal membranes, uterine placental bed, or umbilical cord.

266

267 To assess whether there was replicating virus present in the placenta at delivery (105-113 days  
268 post infection), three high viral load ( $>10^3$  copies/mg) chorionic plate samples and one fetal  
269 membrane sample from 030-101 were tested for the presence of infectious virus using plaque  
270 assay. Three of the four tissues tested (two chorionic plates and one fetal membrane tissue)  
271 were positive for infectious virus via plaque assay (Supplemental Table 3). To further



272 understand the distribution of vRNA within the placenta, tissue sections of the placental  
273 cotyledons from 030-101 were evaluated using in-situ hybridization (ISH). ISH probes for the  
274 ZIKV genome were used to identify ZIKV RNA in the tissue sections. 11 of the 17 cotyledons  
275 tested were positive for ZIKV RNA, primarily in the chorionic plate, which is consistent with  
276 both QRT-PCR and plaque assay results (Figure 5B).  
277



278 **Figure 5. vRNA at the maternal-fetal interface.** For each macaque, tissue biopsies were collected  
279 from the chorionic plate, chorionic villi, and decidua from each placental cotyledon; one to three  
280 biopsies were collected from the fetal membranes; and one biopsy was collected from the uterine  
281 placental bed and umbilical cord. Viral load was determined by ZIKV-specific QRT-PCR from RNA  
282 isolated from tissue samples. **A.** Viral load of maternal-fetal interface tissues. A non-parametric Mann-  
283 Whitney test was used to assess statistical significance between each experimental group in samples  
284 containing more than the theoretical limit of detection of 3 copies vRNA/mg tissue (\*\*p<0.01, \*\*\*p<0.001).  
285 **B.** Representative images of in situ hybridization performed on fixed tissue sections from each of the  
286 placental cotyledons from 030-101. Positive staining for ZIKV RNA (red, arrows) was identified in 11 of  
287 the 17 cotyledons tested, primarily in the chorionic plate.  
288

### 289 Comprehensive histological examination of placental tissues

290 To better understand the impact of in-utero ZIKV-DAK infection, maternal-fetal interface  
291 tissues were evaluated microscopically. Gross histopathological evaluation of the maternal-  
292 fetal interface tissues of ZIKV-DAK-exposed animals revealed a primary finding of transmural  
293 infarction of the central section of the placenta (Supplementary Figure 1). Transmural placental  
294 infarctions are areas of ischemic necrotic placental villi extending from the trophoblastic shell

295 of the basal plate to the chorionic plate and are considered to be a result of a lack of  
 296 oxygenated maternal blood flow. These infarctions were present in all four macaques infected  
 297 with ZIKV-DAK. In contrast, this was observed in 2 of 4 macaques infected with ZIKV-PR and 1  
 298 of 4 mock-infected macaques.

299  
 300 Further histological analysis examined a cross-section of each of the individual placental  
 301 cotyledons for the presence of chronic histiocytic intervillitis (CHIV), infarctions, villous  
 302 stromal calcifications, and vasculopathy (Table 1). We also compared placental weights. There  
 303 were no statistically significant differences in weight or pathological findings between the  
 304 experimental and control groups for any of the features. The presence of infarctions, not often  
 305 observed in mock-infected controls, could indicate that the pathology observed is a result of  
 306 normal placental maturation and aging or a result of weekly anesthesia from experimental  
 307 procedures. Regardless, these evaluations underscore the need to include mock-infected  
 308 controls when evaluating tissues for viral pathogenesis. In order to quantitatively assess the  
 309 pathologies present in the maternal-fetal interface, a central cross-section of each placental  
 310 disc was scored for 22 functional features (Supplementary Table 4; Supplementary Figure 3).  
 311 There were no statistically significant differences between either of the experimental groups  
 312 and the mock-infected controls for any of the scored features; however, there was a trend  
 313 toward increased chronic and acute villitis in the ZIKV-DAK exposed animals.  
 314

**Table 1. Placental cotyledon pathology**

Group	Dam	% CHIV+ cotyledons	Infarcted cotyledons/total cotyledons (%)	Villous stromal calcifications (present/absent)	Vasculopathy (present/absent)	Placental weight (g)
Mock	044-105	0.0	5.88	Present	Absent	111.08
	044-106	0.0	12.5	Present	Absent	106.5
	044-107	0.0	0.0	Present	Present	144.48
	044-108	0.0	45.5	Present	Absent	122.92
ZIKV-DAK	030-101	0.0	43.8	Absent	Absent	131.54
	030-102	0.0	0.0	Absent	Absent	111.4
	030-103	0.0	66.7	Absent	Present	135.32
	030-104	0.0	57.9	Present	Present	124.71
ZIKV-PR	044-101	0.0	25.0	Present	Absent	172.59
	044-102	0.0	33.3	Present	Absent	123.87
	044-103	0.0	0.0	Absent	Absent	134.49
	044-104	0.0	18.2	Absent	Absent	120.48

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## 318 **DISCUSSION**

319 Here we provide the first comprehensive analysis of a low-passage, African-lineage ZIKV  
320 isolate in pregnant non-human primates. The data presented here demonstrate that this  
321 African-lineage ZIKV isolate is capable of robust replication in rhesus macaques. Infection  
322 induces a strong neutralizing antibody response, at or above titers that have been shown to be  
323 protective against subsequent challenge two years following primary challenge (28). Regular  
324 monitoring of fetal growth via ultrasound did not reveal any significant intrauterine growth  
325 restriction as compared to mock-infected animals. ZIKV infection of the placenta has been  
326 shown to be focal (21); therefore, in addition to assessment of well-established sequelae of  
327 viral infection at the maternal-fetal interface, we completed an extensive virological and  
328 histological evaluation of the placenta at delivery. Viral load testing of tissues from the  
329 extensive dissection of the placental discs into individual cotyledons and specific segments  
330 thereof revealed a higher burden of ZIKV in the chorionic plate in animals exposed to ZIKV-  
331 DAK, while plaque assay and ISH testing of high viral load samples confirmed the presence of  
332 infectious virus in the chorionic plate. ZIKV vRNA was also regularly found in the decidua and  
333 chorionic villi, and to a lesser extent in the fetal membranes. Despite a high burden of ZIKV in  
334 the chorionic plate – the fetal side of the placenta – there was no evidence of vertical  
335 transmission at delivery, even though infectious ZIKV was detected in the chorionic plate of  
336 one animal. This suggests that the vRNA burden in the maternal-fetal interface is not a robust  
337 predictor of clinical outcome for the fetus, but it does not preclude the possibility that infants  
338 may develop clinical sequelae later in life due to viral exposure, or placental insufficiency  
339 during gestation. Many normal-appearing infants exposed to ZIKV in utero develop  
340 neurodevelopmental delays in the years after birth (29–32).

341  
342 This cohort of macaques infected with an African-lineage virus was compared to a cohort of  
343 macaques infected with an Asian-lineage virus and a mock-infected control group. Based on  
344 previous studies in cell culture and mice, we expected to see a more severe phenotype in the  
345 macaques that were infected with the African-lineage virus (2, 3, 3, 4, 4, 5, 5–8). We expected  
346 this more severe phenotype to manifest as enhanced viral replication (as determined by higher  
347 peak or longer duration of viremia), gross fetal abnormalities at delivery, or fetal demise.  
348 However, the only feature that was significantly different between the experimental groups was  
349 an increase in the burden of vRNA in the chorionic plate, chorionic villi and decidua.

350  
351 To date, few studies of Asian-lineage viruses in non-human primates have shown clear  
352 evidence of fetal harm, despite a clear association between Asian-lineage ZIKV and CZS. A  
353 minority of human pregnancies known to be affected by ZIKV result in CZS (5-14%) or fetal  
354 loss (4-7%) (33); therefore, it is perhaps unsurprising that there is limited evidence of fetal harm  
355 in small non-human primate studies. In this study, ZIKV-DAK infection of pregnant macaques  
356 resembled infection of ZIKV-PR across several parameters, including infection of MFI tissues.  
357 Therefore, although we did not observe direct fetal harm, our findings suggest that African-  
358 lineage viruses have similar capacity to cause fetal harm as Asian-lineage viruses. This data  
359 suggests that African-lineage ZIKV poses a threat to women and their infants, which should be  
360 taken into account when providing public health guidance. While African-lineage ZIKV had

361 been thought to be geographically confined to Africa, recent studies have identified African-  
362 lineage isolates in South America (16, 17). This highlights the need for continuing study of ZIKV  
363 of both genetic lineages.

364

365 A significant limitation of this study is the small sample size ( $n=4$ ) in each of the experimental  
366 groups. Particularly when studying a pathogen whose most severe effects are only found in a  
367 minority of cases (33), modeling rare events in a small study is difficult and we cannot capture  
368 the full range of disease experienced by women infected with ZIKV during pregnancy. We also  
369 tested a single inoculation dose, virus strain, and inoculation time point; different experimental  
370 conditions may reveal different outcomes, which could include more stark differences between  
371 the lineages. Furthermore, this study focused on characterizing the pathogenesis of African-  
372 lineage ZIKV as compared to Asian-lineage but did not seek to understand the mechanisms of  
373 the vertical transmission of ZIKV or the potential mechanisms underlying differences between  
374 the lineages. Future studies should investigate these mechanisms and conduct more thorough  
375 epidemiological studies of African-lineage ZIKV, which may shed light on the reasons why ZIKV  
376 had not been associated with fetal harm prior to the outbreak in the Americas.

377

## 378 **METHODS**

### 379 **Experimental design**

380 This study was designed to assess the pathogenic potential of a low-passage African-lineage  
381 ZIKV isolate during pregnancy in a non-human primate model. Four pregnant Indian origin  
382 rhesus macaques (*Macaca mulatta*) were inoculated subcutaneously with  $1 \times 10^4$  PFU of ZIKV-  
383 DAK between 44-50 days of gestation (term is  $165 \pm 10$  days). Macaques were monitored  
384 throughout the remainder of gestation. At approximately gestation day 155, infants were  
385 delivered via c-section and humanely euthanized. A comprehensive set of maternal biopsies,  
386 maternal-fetal interface and fetal tissues were collected for analysis. For the Asian-lineage  
387 group, four pregnant Indian origin rhesus macaques (*Macaca mulatta*) were inoculated  
388 subcutaneously with  $1 \times 10^4$  PFU of ZIKV-PR between 44-50 days of gestation (term is  $165 \pm 10$   
389 days). Macaques were monitored throughout the remainder of gestation. At approximately  
390 gestation day 160, infants were delivered via cesarean section and monitored for long-term  
391 development. A comprehensive set of maternal biopsies and maternal-fetal interface were  
392 collected for analysis. A cohort of four pregnant PBS-inoculated animals served as a control  
393 group and underwent the same experimental regimen, including the sedation for all blood  
394 draws and ultrasounds, as the ZIKV-PR cohort. Data used in this manuscript are publicly  
395 available at [openresearch.labkey.com](http://openresearch.labkey.com) under study ZIKV-030 (ZIKV-DAK) and ZIKV-044 (ZIKV-  
396 PR and mock).

397

### 398 **Ethical approval**

399 This study was approved by the University of Wisconsin College of Letters and Sciences and  
400 Vice Chancellor for Research and Graduate Education Centers Institutional Animal Care and  
401 Use Committee (Protocol numbers: G005401 and G006139).

402

### 403 **Care and use of macaques**

404 All macaque monkeys used in this study were cared for by the staff at the WNPRC in  
405 accordance with the regulations and guidelines outlined in the Animal Welfare Act and the  
406 Guide for the Care and Use of Laboratory Animals and the recommendations of the Weatherall  
407 report (<https://royalsociety.org/topics-policy/publications/2006/weatherall-report/>). All  
408 macaques used in the study were free of *Macacine herpesvirus 1*, simian retrovirus type D  
409 (SRV), simian T-lymphotropic virus type 1 (STLV), and simian immunodeficiency virus. For all  
410 procedures (including physical examinations, virus inoculations, ultrasound examinations, and  
411 blood collection), animals were anaesthetized with an intramuscular dose of ketamine (10  
412 mg/kg). Blood samples were obtained using a vacutainer system or needle and syringe from  
413 the femoral or saphenous vein.

414

### 415 **Cells and viruses**

416 ZIKV/*Aedes-africanus*/SEN/DakAr41524/1984 (ZIKV-DAK) was originally isolated from *Aedes*  
417 *africanus* mosquitoes with a round of amplification on *Aedes pseudocutellaris* cells, followed by  
418 amplification on C6/36 cells and two rounds of amplification on Vero cells. ZIKV-DAK was  
419 obtained from BEI resources (Manassas, VA). Zika-virus/*H.sapiens*-  
420 tc/PUR/2015/PRVABC59\_v3c2 (ZIKV-PR) was originally isolated from a human in Puerto Rico  
421 in 2015, with three rounds of amplification on Vero cells, was obtained from Brandy Russell  
422 (CDC, Fort Collins, CO, USA). African Green Monkey kidney cells (Vero; ATCC #CCL-81) were  
423 maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal  
424 bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 100  
425 U/ml penicillin, 100 µg/ml of streptomycin, and incubated at 37°C in 5% CO<sub>2</sub>. *Aedes albopictus*  
426 mosquito cells (C6/36; ATCC #CRL-1660) were maintained in DMEM supplemented with 10%  
427 fetal bovine serum (FBS; Hyclone, Logan, UT), 2mM L-glutamine, 1.5 g/L sodium bicarbonate,  
428 100 U/ml penicillin, 100 µg/ml of streptomycin, and incubated at 28°C in 5% CO<sub>2</sub>. The cell lines  
429 were obtained from the American Type Culture Collection, were not further authenticated, and  
430 were not specifically tested for mycoplasma. Virus stocks were prepared by inoculation onto a  
431 confluent monolayer of C6/36 cells; a single, clarified stock was harvested for each virus, with  
432 a titer of 7.3 x 10<sup>8</sup> PFU/ml for ZIKV-DAK and 1.58 x 10<sup>7</sup> PFU/ml for ZIKV-PR. Deep sequencing  
433 with limited PCR cycles confirmed that the ZIVK-DAK virus stock was identical to the reported  
434 sequence in GenBank (KY348860) at the consensus level. Five nucleotide variants were  
435 detected at 5.1-13.1% frequency (Supplementary Table 1). PCR-free deep sequencing did not  
436 detect any evidence of Dezydougou virus, an insect-specific *Negevirus* that is present in some  
437 ZIKV-DAK stocks. Amplicon deep sequencing of ZIKV-PR virus stock using the methods  
438 described in Quick, et al. (34) revealed two consensus-level nucleotide substitutions in the  
439 stock as compared to the reported sequence in GenBank (KU501215), as well as seven other  
440 minor nucleotide variants detected at 5.3-30.6% frequency (Supplementary Table 1).

441

### 442 **Plaque Assay**

443 All titrations for virus quantification from virus stocks and screens for infectious ZIKV from  
444 macaque tissue were completed by plaque assay on Vero cell cultures as previously described

445 (35). Briefly, duplicate wells were infected with 0.1 ml aliquots from serial 10-fold dilutions in  
446 growth media and virus was adsorbed for one hour. Following incubation, the inoculum was  
447 removed, and monolayers were overlaid with 3ml containing a 1:1 mixture of 1.2% oxoid agar  
448 and 2X DMEM (Gibco, Carlsbad, CA) with 10% (vol/vol) FBS and 2% (vol/vol)  
449 penicillin/streptomycin (100 U/ml penicillin, 100 µg/ml of streptomycin). Cells were incubated at  
450 37°C in 5% CO<sub>2</sub> for four days for plaque development. Cell monolayers were then stained with  
451 3 ml of overlay containing a 1:1 mixture of 1.2% oxoid agar and 2X DMEM with 2% (vol/vol)  
452 FBS, 2% (vol/vol) penicillin/streptomycin, and 0.33% neutral red (Gibco). Cells were incubated  
453 overnight at 37 °C and plaques were counted.

454

#### 455 **Inoculations**

456 Inocula were prepared from a viral stock propagated on a confluent monolayer of C6/36 cells.  
457 The stocks were thawed, diluted in PBS to 10<sup>4</sup> PFU/ml and loaded into a 1 mL syringe that was  
458 kept on ice until challenge. Animals were anesthetized as described above and 1 ml of inocula  
459 was delivered subcutaneously over the cranial dorsum. Animals were monitored closely  
460 following inoculation for any signs of an adverse reaction.

461

#### 462 **Ultrasound measurements**

463 Ultrasound measurements were taken according to the procedures described previously (19).  
464 Briefly, dams were sedated with ketamine hydrochloride (10mg/kg) for weekly sonographic  
465 assessment to monitor the health of the fetus (heart rate) and to take fetal growth  
466 measurements, including the fetal femur length (FL), biparietal diameter (BPD), head  
467 circumference (HC), and abdominal circumference (AC). Weekly fetal measurements were  
468 plotted against mean measurement values and standard deviations for fetal macaques  
469 developed at the California National Primate Research Center (25, 26). Additional Doppler  
470 ultrasounds were taken as requested by veterinary staff.

471

472 Gestational age standardized growth parameters for fetal HC, BPD, AC, and FL were evaluated  
473 by calculating gestational age specific z-values from normative fetal growth parameters. Linear  
474 mixed effects modeling with animal-specific random effects was used to analyze the fetal  
475 growth trajectories with advancing gestational age. In order to account for differences in fetal  
476 growth parameters at the date of inoculation, changes in fetal growth parameters from date of  
477 inoculation (~day 50) were analyzed. That is, changes in fetal growth parameters from date of  
478 inoculation were regressed on gestational age (in weeks). An autoregressive correlation  
479 structure was used to account for correlations between repeated measurements of growth  
480 parameters over time. The growth trajectories were quantified by calculating the regression  
481 slope parameters which were reported along with the corresponding 95% confidence intervals  
482 (CI). Fetal growth was evaluated both within and between groups. All reported P-values are  
483 two-sided and P<0.05 was used to define statistical significance. Statistical analyses were  
484 conducted using SAS software (SAS Institute, Cary NC), version 9.4.

485

#### 486 **Viral RNA isolation from blood**

487 Viral RNA was isolated from macaque blood samples as previously described (18, 35). Briefly,  
488 plasma was isolated from EDTA-anticoagulated whole blood on the day of collection either  
489 using Ficoll density centrifugation for 30 minutes at 1860 x g if the blood was being processed  
490 for PBMC, or it was centrifuged in the blood tube at 1400 x g for 15 minutes. The plasma layer  
491 was removed and transferred to a sterile 15 ml conical and spun at 670 x g for an additional 8  
492 minutes to remove any remaining cells. Viral RNA was extracted from a 300  $\mu$ L plasma aliquot  
493 using the Viral Total Nucleic Acid Kit (Promega, Madison, WI) on a Maxwell 16 MDx or Maxwell  
494 RSC 48 instrument (Promega, Madison, WI).

495

#### 496 **Viral RNA isolation from tissues**

497 Tissue samples, cut to 0.5 cm thickness on at least one side, were stored in RNAlater at 4°C for  
498 2-7 days. RNA was recovered from tissue samples using a modification of the method  
499 described by Hansen et al., 2013 (36). Briefly, up to 200 mg of tissue was disrupted in TRIzol  
500 (Lifetechnologies) with 2 x 5 mm stainless steel beads using the TissueLyser (Qiagen) for 3  
501 minutes at 25 r/s twice. Following homogenization, samples in TRIzol were separated using  
502 Bromo-chloro-propane (Sigma). The aqueous phase was collected and glycogen was added  
503 as a carrier. The samples were washed in isopropanol and ethanol precipitated. RNA was fully  
504 re-suspended in 5 mM tris pH 8.0.

505

#### 506 **Quantitative reverse transcription PCR (QRT-PCR)**

507 vRNA isolated from both fluid and tissue samples was quantified by QRT-PCR as previously  
508 described(8). The RT-PCR was performed using the SuperScript III Platinum One-Step  
509 Quantitative RT-PCR system (Invitrogen, Carlsbad, CA) on a LightCycler 96 or LightCycler 480  
510 instrument (Roche Diagnostics, Indianapolis, IN). Viral RNA concentration was determined by  
511 interpolation onto an internal standard curve composed of seven 10-fold serial dilutions of a  
512 synthetic ZIKV RNA fragment based on a ZIKV strain derived from French Polynesia that  
513 shares >99% similarity at the nucleotide level to the Puerto Rican strain used in the infections  
514 described in this manuscript.

515

#### 516 **Statistical analysis of viral loads**

517 Plasma viral load curves were generated using GraphPad Prism software. The area under the  
518 curve of 0-10 d.p.i. was calculated and a two-sample t-test was performed to assess  
519 differences in the peak, duration, and area under the curve of viremia between macaques  
520 infected with ZIKV-DAK and ZIKV-PR. To compare differences in the viral burden in the  
521 maternal-fetal interface, a non-parametric Mann-Whitney test was used to assess differences  
522 in each of the maternal-fetal interface tissues. GraphPad Prism 8 software was used for these  
523 analyses.

524

#### 525 **Plaque reduction neutralization test (PRNT)**

526 Macaque serum was isolated from whole blood on the same day it was collected using a  
527 serum separator tube (SST). The SST tube was centrifuged for at least 20 minutes at 1400 x g,  
528 the serum layer was removed and placed in a 15 ml conical and centrifuged for 8 minutes at

529 670 x g to remove any additional cells. Serum was screened for ZIKV neutralizing antibody  
530 utilizing a plaque reduction neutralization test (PRNT) on Vero cells as described in (37) against  
531 ZIKV-PR and ZIKV-DAK. Neutralization curves were generated using GraphPad Prism 8  
532 software. The resulting data were analyzed by non-linear regression to estimate the dilution of  
533 serum required to inhibit 50% and 90% of infection.

534

### 535 **Cesarean section and tissue collection**

536 Between 155-160 days gestation, infants were delivered via cesarean section and tissues were  
537 collected. The fetus, placenta, fetal membranes, umbilical cord, and amniotic fluid were  
538 collected at surgical uterotomy and maternal tissues were biopsied during laparotomy. These  
539 were survival surgeries for the dams. For fetuses born to dams infected with ZIKV-DAK, the  
540 fetus was euthanized with an overdose of sodium pentobarbital (50 mg/kg) and the entire  
541 conceptus (fetus, placenta, fetal membranes, umbilical cord, and amniotic fluid) was collected  
542 and submitted for tissue collection and necropsy. For fetuses born to dams infected with ZIKV-  
543 PR, the infant was removed from the amniotic sac, the umbilical cord clamped, and neonatal  
544 resuscitation performed as needed. The placenta, amniotic fluid, and fetal membranes were  
545 then collected. Infants were placed with their mothers following the dam's recovery from  
546 surgery.

547

548 Tissues were dissected as previously described(19) using sterile instruments that were  
549 changed between each organ and tissue type to minimize possible cross contamination. Each  
550 organ/tissue was evaluated grossly in situ, removed with sterile instruments, placed in a sterile  
551 culture dish, and sectioned for histology, viral burden assay, and/or banked for future assays.  
552 Sampling priority for small or limited fetal tissue volumes (e.g., thyroid gland, eyes) was vRNA  
553 followed by histopathology, so not all tissues were available for both analyses. A  
554 comprehensive listing of all specific tissues collected and analyzed is presented in Figure 5A  
555 (maternal-fetal interface tissues) and Supplementary Table 2 (maternal biopsies and fetal  
556 tissues). Biopsies of the placental bed (uterine placental attachment site containing deep  
557 decidua basalis and myometrium), maternal liver, spleen, and a mesenteric lymph node were  
558 collected aseptically during surgery into sterile petri dishes, weighed, and further processed for  
559 viral burden and when sufficient sample size was obtained, histology.

560

561 In order to more accurately capture the distribution of ZIKV in the placenta, each placental disc  
562 was separated, fetal membranes sharply dissected from the margin, weighed, measured, and  
563 placed in a sterile dish on ice. A 1-cm-wide cross section was taken from the center of each  
564 disc, including the umbilical cord insertion on the primary disc, and placed in 4%  
565 paraformaldehyde. Individual cotyledons, or perfusion domains, were dissected using a scalpel  
566 and placed into individual petri dishes. From each cotyledon, a thin center cut was taken using  
567 a razor blade and placed into a cassette in 4% paraformaldehyde. Once the center cut was  
568 collected, the decidua and the chorionic plate were removed from the remaining placenta.  
569 From each cotyledon, pieces of decidua, chorionic plate, and chorionic villi were collected into  
570 two different tubes with different media for vRNA isolation and for other virological assays.

571



## 572 **Histology**

573 Following collection, tissues were handled as described previously (35). All tissues (except  
574 neural tissues) were fixed in 4% paraformaldehyde for 24 hours and transferred into 70%  
575 ethanol until processed and embedded in paraffin. Neural tissues were fixed in 10% neutral  
576 buffered formalin for 14 days until processed and embedded in paraffin. Paraffin sections (5  
577  $\mu\text{m}$  for all tissues other than the brain (sectioned at  $8\mu\text{m}$ )) were stained with hematoxylin and  
578 eosin (H&E). Pathologists were blinded to vRNA findings when tissue sections were evaluated  
579 microscopically. Photomicrographs were obtained using a bright light microscope Olympus  
580 BX43 and Olympus BX46 (Olympus Inc., Center Valley, PA) with attached Olympus DP72  
581 digital camera (Olympus Inc.) and Spot Flex 152 64 Mp camera (Spot Imaging) and captured  
582 using commercially available image-analysis software (cellSens DimensionR, Olympus Inc. and  
583 spot software 5.2).

584

## 585 **Placental Histology Scoring**

586 Pathological evaluation of the cross-sections of each of the placental cotyledons were  
587 performed blinded to experimental condition. Each of the cross sections were evaluated for the  
588 presence of chronic histiocytic intervillitis (CHIV), infarctions, villous stromal calcifications,  
589 and vasculopathy. A three-way ANOVA was performed to assess statistical significance among  
590 groups for each parameter, including placental weight.

591

592 Two of three boarded pathologists, blinded to vRNA findings, independently reviewed the  
593 central cross section of each placental disc and quantitatively scored the placentas on 22  
594 independent criteria. Six of the criteria are general criteria assessing placental function, 2  
595 assess villitis, three criteria assessing the presence of fetal malperfusion, and 11 criteria  
596 assessing the presence of maternal malperfusion. The scoring system was developed by Dr.  
597 Michael Fritsch, Dr. Heather Simmons, and Dr. Andres Mejia. A summary table of the criteria  
598 scored and the scale used for each criterion can be found in Supplementary Table 3. Once  
599 initial scores were assigned, pathologists met to discuss and resolve any significant  
600 discrepancies in scoring. Scores were assigned to each placental disc for most parameters,  
601 unless the evaluation score corresponded to the function of the entire placenta.

602

603 For criteria that are measured on a quantitative scale, median scores and interquartile range  
604 were calculated for each experimental group. For criteria that were measured on a binary  
605 “present/not present” scale, the cumulative incidence in each experimental group was  
606 calculated as a frequency and a percentage. For quantitative criteria, a non-parametric  
607 Wilcoxon rank test was used to calculate statistical significance between each of the groups  
608 and between the mock-infected group and the two ZIKV-infected groups. For binary features,  
609 Fisher’s exact test was used to calculate statistical significance between each of the groups  
610 and between the mock-infected group and the two ZIKV-infected groups. To determine  
611 whether chronic villitis correlated with the criteria assessing fetal malperfusion and whether  
612 chronic deciduitis correlated with the criteria assessing maternal malperfusion, scores were  
613 adjusted to be on the same scale (i.e., converting measures on a 0-1 scale to a 0-2 scale) so

614 that each parameter carried equal weight in the combined score. A non-parametric  
615 Spearman's correlation was used to determine the correlation.

616

### 617 **In situ hybridization**

618 In situ hybridization was conducted on cross sections of placental cotyledons as previously  
619 described(20). Briefly, tissues were fixed in 4% PFA, alcohol processed, and paraffin  
620 embedded. Commercial ISH probes against the ZIKV genome (Advanced Cell Diagnostics, Cat  
621 No. 468361, Newark, California, USA) were used. ISH was performed using RNAscope® Red  
622 2.5 Kit (Advanced Cell Diagnostics, Cat No. 322350) according to the manufacturer's  
623 instructions.

624

### 625 **Data availability**

626 All of the data used for figure generation and statistical analysis in this manuscript can be  
627 found at <https://github.com/cmc0043/african-lineage-zikv-in-pregnant-macaques>. In the  
628 future, primary data that support the findings of this study will also be available at the Zika  
629 Open Research Portal (<https://openresearch.labkey.com/project/ZEST/begin.view>). Data for the  
630 ZIKV-DAK infected cohort can be found under study ZIKV-030; data for ZIKV-PR and mock-  
631 infected cohorts can be found under ZIKV-044. Raw FASTQ reads (BioProject: PRJNA673500)  
632 and a FASTA consensus sequence (BioProject: PRJNA476611) of the challenge stock of  
633 ZIKV/*Aedes africanus*/SEN/DAK-AR-41524/1984 are available at the Sequence Read Archive.  
634 Raw FASTQ reads of the challenge stock of ZIKV PRVABC59 are available at the Sequence  
635 Read Archive, BioProject accession number PRJNA392686. The authors declare that all other  
636 data supporting the findings of this study are available within the article and its supplementary  
637 information files.

638

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647

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