1   **ZIKV disrupts placental ultrastructure and drug transporter expression in mice**

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27   **protein (Bcrp), Abca1, Abcg1, ultrastructure, cytokine, chemokine**

28

29 Abstract

30 Congenital Zika virus (ZIKV) infection can induce fetal brain abnormalities. Here, we
 31 investigated whether maternal ZIKV infection affects placental physiology and
 32 metabolic transport potential and impacts the fetal outcome, regardless of viral presence
 33 in the fetus at term. Low (10^3 PFU-ZIKVPE243; low ZIKV) and high (5×10^7 PFU-
 34 ZIKVPE243; high ZIKV) virus titers were injected into immunocompetent (ICompetent
 35 C57BL/6) and immunocompromised (ICompromised A129) mice at gestational day
 36 (GD) 12.5 for tissue collection at GD18.5 (term). High ZIKV elicited fetal death rates of
 37 66% and 100%, whereas low ZIKV induced fetal death rates of 0% and 60% in
 38 C57BL/6 and A129 dams, respectively. All surviving fetuses exhibited intrauterine
 39 growth restriction (IUGR) and decreased placental efficiency. High-ZIKV infection in
 40 C57BL/6 and A129 mice resulted in virus detection in maternal spleens and placenta,
 41 but only A129 fetuses presented virus RNA in the brain. Nevertheless, pregnancies in
 42 both strains produced fetuses with decreased head sizes ($p < 0.05$). Low-ZIKV-A129
 43 dams had higher IL-6 and CXCL1 levels ($p < 0.05$), and their placentas showed increased
 44 CCL-2 and CXCL-1 contents ($p < 0.05$). In contrast, low-ZIKV-C57BL/6 dams had an
 45 elevated CCL2 serum level and increased type I and II IFN expression in the placenta.
 46 Notably, less abundant microvilli and mitochondrial degeneration were evidenced in the
 47 placental labyrinth zone (Lz) of ICompromised and high-ZIKV-ICompetent mice but
 48 not in low-ZIKV-C57BL/6 mice. In addition, decreased placental expression of the drug
 49 transporters P-glycoprotein (P-gp) and breast cancer resistance protein (Bcrp) and the
 50 lipid transporter Abca1 was detected in all ZIKV-infected groups, but Bcrp and Abca1
 51 were only reduced in ICompromised and high-ZIKV ICompetent mice. Our data
 52 indicate that gestational ZIKV infection triggers specific proinflammatory responses
 53 and affects placental turnover and transporter expression in a manner dependent on

54 virus concentration and maternal immune status. Placental damage may impair proper
55 fetal-maternal exchange function and fetal growth/survival, likely contributing to
56 congenital Zika syndrome.

57 **1. Introduction**

58 Congenital Zika virus (ZIKV) infection can be associated with adverse
59 pregnancy outcomes. Neonates born from ZIKV-positive pregnancies may develop
60 severe neurological abnormalities, placental pathologies and intrauterine growth
61 restriction (IUGR), among other complications (1). ZIKV vertical transmission has
62 become a major public health issue worldwide, especially in Brazil, where more than
63 200,000 ZIKV-positive cases have been confirmed and over 2,000 congenital
64 microcephaly births have been reported (2–6). These numbers represent a 20-fold rise in
65 the incidence of congenital microcephaly in Brazil during the years of the ZIKV
66 pandemic, with similar increases reported elsewhere in Latin America (2,3,7).
67 Importantly, while the ZIKV pandemic is currently thought to be controlled, evidence
68 points to a possible silent ZIKV spread across the Americas (8,9), highlighting the need
69 for improved knowledge of the possible routes of vertical ZIKV transmission and its
70 association with disruptive inflammatory and developmental phenotypes and the need
71 for new avenues of prevention and treatment.

72 Previous studies have investigated the possible pathways involved in vertical
73 ZIKV transmission. Miranda and colleagues (10) showed that in humans, ZIKV
74 infection changed the pattern of tight junction proteins, such as claudin-4, in
75 syncytiotrophoblasts. Jurado et al. (2016) suggested that the migratory activities of
76 Hofbauer cells (feto-placental macrophages) could help disseminate ZIKV to the fetal
77 brain (11). Other recent studies have shown that placental villous fibroblasts,
78 cytotrophoblasts, endothelial cells and Hofbauer cells are permissive to ZIKV, and

79 placentae from ZIKV-infected women had chorionic villi with a high mean diameter
80 (11–14). Furthermore, in 2019, Rathore et al. demonstrated that pregnant mice carrying
81 high levels of antibodies against dengue virus (DENV) exhibited increased ZIKV
82 vertical transmission associated with severe microcephaly-like syndrome,
83 demonstrating another possible mechanism of antibody-dependent vertical ZIKV
84 transmission (15). However, at present, further studies are required to identify the
85 precise mechanism of maternal-fetal ZIKV transmission.

86 Many mouse models have been developed to identify how ZIKV overcomes
87 placental defenses. Initially, limited information was obtained due to the apparent
88 inability of the virus to infect wild-type (WT) mice (16). ZIKV NS5 targets the
89 interferon signaling pathway in humans but not in mice (17). Thus, WT mice show no
90 clear evidence of clinical disease (17,18) and are of limited use in modeling the disease.
91 However, mice lacking an interferon signaling response show evidence of disease and
92 have been widely used to investigate ZIKV infection during pregnancy (8,17,19).

93 The interferon system, especially type III interferon, is a key mechanism of host
94 defense and a viral target for immune evasion (20). Type III interferons have a role in
95 protection against ZIKV infection in human syncytiotrophoblasts from term placenta
96 (21). Luo et al. have shown that inhibition of Toll-like receptors 3 and 8 inhibits the
97 cytokine output of ZIKV-infected trophoblasts (22). In addition, viral replication
98 coincides with the induction of proinflammatory cytokines, such as interleukin [IL]-6.
99 This cytokine has a crucial role in inflammation and affects the homeostatic processes
100 related to tissue injury and activation of stress-related responses (23,24). ZIKV infection
101 can trigger an inflammatory response with IL-6 release (11,25).

102 Maternal infection has profound effects on placental permeability to drugs and
103 environmental toxins. Changes in the expression and function of specific ABC

104 transporters in the placenta and yolk sac following infective and inflammatory stimuli
 105 have been demonstrated (26–30). ABC transporters are efflux transporters that control
 106 the biodistribution of several endogenous and exogenous substrates, including
 107 xenobiotics (antiretrovirals and synthetic glucocorticoids), steroid hormones (estrogens
 108 and androgens), nutrients (folate and cholesterol) and immunological factors
 109 (chemokines and cytokines) within the maternal-fetal interface (31). The best described
 110 ABC transporters in the placenta are P-glycoprotein (P-gp; also known as multidrug
 111 resistance protein 1, MDR1), breast cancer resistance protein (Bcrp) and the lipid Abca1
 112 and Abcg1 transporters. P-gp and Bcrp transporters are responsible for preventing fetal
 113 accumulation of xenobiotics and environmental toxins that may be present in the
 114 maternal circulation, whereas Abca1 and Abcg1 control the placental exchange of
 115 cytotoxic oxysterol and lipid permeability throughout pregnancy; therefore, they play an
 116 important role in fetal protection and placental lipid homeostasis (26).

117 Despite the limited number of studies showing ZIKV infection in
 118 immunocompetent mice, intrauterine inoculation with a high virus titer was previously
 119 demonstrated to result in decreased fetal viability, with worse outcomes following
 120 infection in early gestation (32). In another report, intravenous infection on a very early
 121 embryonic day resulted in fetal demise even though the virus was not found in the fetal
 122 compartment in most of the treated animals (33). In the present study, we hypothesize
 123 that maternal exposure to ZIKV affects placental function, including placental
 124 ultrastructure and ABC transporter (P-gp, Bcrp, Abca1 and Abcg1) protein expression,
 125 even in the absence of vertical transmission and that these effects are dependent on viral
 126 infective titers and maternal immune status.

127 **2. Materials and methods**

128 ***2.1 Virus preparation and storage***

129 The Brazilian ZIKV_{PE243} (GenBank ref. number KX197192) strain was isolated
130 from a febrile case during the ZIKV outbreak in the state of Pernambuco, Brazil and
131 was kindly provided by Dr Ernesto T. Marques Jr. (Centro de Pesquisa Aggeu
132 Magalhães, FIOCRUZ, PE). Viruses were propagated in C6/36 cells, and viral titers
133 were determined by plaque assays in Vero cells, as previously described (34).
134 Supernatants of noninfected C6/36 cells cultured under the same conditions were used
135 as mock controls.

136 ***2.2 Animal experimentation and study design***

137 Two mouse strains were used in the study: immunocompetent (ICompetent)
138 C57BL/6 and immunocompromised (ICompromised) (type 1 *Ifnr*-deficient) A129
139 strains. Since we were unable to consistently produce viable pregnancies by mating
140 A129 males and females in our experimental settings, we mated A129 females (n=15)
141 with C57BL/6 males (n=4) to produce ICompromised C57BL6/A129 pregnancies,
142 whereas ICompetent C57BL/6 pregnancies were obtained by mating male (n=6) and
143 female (n=35) C57BL/6 mice (8-10 weeks old). Animals were kept in a controlled
144 temperature room (23°C) with a light/dark cycle of 12 hours and *ad libitum* access to
145 water and food. After detection of the proestrous/estrous phase via vaginal cytology,
146 copulation was confirmed by visualization of the vaginal plug and considered
147 gestational day 0.5 (GD0.5). Maternal weight was monitored for confirmation of
148 pregnancy; thus, females were weighed on GD0.5 and GD12.5, and females with a
149 weight gain greater than 3 g were considered pregnant and entered randomly in the
150 experimental groups. Experimental protocols were approved by the Animal Care
151 Committee of the Health Sciences Center, Federal University of Rio de Janeiro (CEUA-
152 036/16 and 104/16) and registered with the Brazilian National Council for Animal
153 Experimentation Control.

154 On GD12.5, pregnant mice (ICompetent and ICompromised pregnancies) were
155 injected with a single intravenous (i.v.) titer of ZIKV or mock control. ICompetent and
156 ICompromised pregnant mice were randomly subdivided into three experimental
157 groups: the mock (control) group, which received an injection of supernatant from
158 noninfected C6/36 cells (ICompetent mock and ICompromised mock); the high-ZIKV-
159 titer group, inoculated with 5×10^7 plaque-forming units (PFU) of ZIKV_{PE243}
160 (ICompetent high and ICompromised high); and the ZIKV low-titer group, injected
161 with 10^3 PFU of ZIKV_{PE243} (ICompetent low and ICompromised low).

162 On the morning of GD18.5, all animals were euthanized with a sodium
163 phenobarbital overdose of 300 mg/kg. Maternal blood was collected via cardiac
164 puncture, centrifuged (10 min, 4000 g) and stored at -20°C. The maternal brain and
165 spleen and all placentae and all fetuses were dissected, collected and weighed, followed
166 by fetal head isolation and measurement. The three placentae closest to the mean weight
167 in a litter were selected for further analysis and cut in half using umbilical cord insertion
168 as a reference (35–37). One-half of the placental disk was frozen in liquid nitrogen for
169 qPCR, and the other half was fixed overnight in buffered paraformaldehyde (4%,
170 Sigma-Aldrich, Brazil) for ultrastructural and protein expression/localization analysis.
171 Matched fetal heads, maternal brains and spleens were frozen in liquid nitrogen for
172 qPCR. Of important note, all fetuses obtained from ICompromised pregnancies were
173 heterozygous.

174 ***2.3 ZIKV RNA quantification via RT-qPCR***

175 ZIKV load was evaluated in maternal blood, brains and spleens and in the
176 placentae and fetal heads. Brains, spleens, placentae and fetal heads were macerated in
177 RPMI medium (Gibco™ RPMI 1640 Medium) normalized by the ratio of 0.2 mg of
178 tissue to every 1 µl of medium and plotted per gram of tissue. The macerated volume

179 was centrifuged at 4500 g for 5 min to remove tissue residues, and then, 500 µl of the
180 centrifuged volume was used for RNA extraction using 1 mL of TRIzol reagent (Life
181 Technologies, Thermo Fischer, USA). Treatment with DNase I (Ambion, Thermo
182 Fischer, USA) was performed to prevent contamination by genomic DNA. cDNA was
183 synthesized using a cDNA High Capacity Kit (Applied Biosystems, Thermo Fischer,
184 USA) according to the manufacturer's instructions by subjecting the samples to the
185 following cycle: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. qPCR was
186 performed using a StepOnePlus Real-Time qPCR system, TaqMan Master Mix
187 Reagents (Applied Biosystems, Thermo Fischer, USA) and primers and probes specific
188 for the protein E sequence (38). Samples were then subjected to the following cycle:
189 50°C for 2 min, followed by 40 cycles of 95°C for 10 min, 95°C for 15 sec, and 60°C
190 for 1 min.

191 **2.3.1 RT-qPCR**

192 The placenta was macerated in 1.5 mL of TRIzol reagent (Life Technologies,
193 Thermo Fischer, USA). RNA extraction was performed following the manufacturer's
194 protocol. cDNA was prepared using Power SYBR Green PCR Master Mix (Life
195 Technologies, Thermo Fisher, USA). The reaction was carried out for selected genes
196 using intron-spanning primers (Table 1) and the StepOnePlus Real-Time PCR system
197 (Life Technologies, Thermo Fischer, USA). Samples were subjected to the following
198 cycle: 95°C for 10 min, followed by 40 amplification cycles consisting of DNA
199 denaturation for 30 sec at 95°C and annealing of primers for 30 sec at 60°C. The
200 threshold cycle (Ct) was determined for each gene of interest and for the reference
201 genes glycerol 3-phosphate dehydrogenase (*Gapdh*) and RNA Polymerase II Subunit A
202 (*Polr2a*). The relative expression of each gene was calculated using $2^{-\Delta\Delta CT}$ (39) and

graphically expressed as the fold-increase. The efficiency was calculated using the standard curve method. The melting curves were analyzed for each sample.

Table 1: Primer sequences for the real-time PCR assay.

Gene	Primer sequences	GenBank accession no.
<i>ZIKV</i>	5'CCGCTGCCCAACACAAG3' 5'CCACTAACGTTCTTTTGCAGACAT3'	
<i>Il6</i>	5'TCATATCTTCAACCAAGAGGTA3' 5'CAGTGAGGAATGTCCACAAACTG3'	NM_031168.2
<i>Il1b</i>	5'GTAATGAAAGACGGCACACC3' 5'ATTAGAAACAGTCCAGCCCA3'	XM_006498795.4
<i>Il10</i>	5'TAAGGGTTACTTGGGTTGCCAAG3' 5'CAAATGCTCCTTGATTCTGGGC3'	NM_010548.2
<i>Ifng</i>	5'AGCAACAGCAAGGCGAAA3' 5'CTGGACCTGTGGGTTGTTGA3'	NM_008337.4
<i>Ifn1</i>	5'CTGGAGCAGCTGAATGGAAAG3' 5'CTTGAAGTCCGCCCTGTAGGT3'	NM_010510.1
<i>Gapdh</i>	5'CTTTGTCAAGCTCATTTCTGG3' 5'TCTTGCTCAGTGTCTTGC3'	XM_017321385.2
<i>Tnf</i>	5'CCTCACACTCAGATCATCTTCTCA3' 5'TGGTTCTCTTTGAGATCCATGC3'	NM_013693.3
<i>Polr2a</i>	5'TCTGCCAAGAATGTGACGCT3' 5'CCAAGCGGCAAAGAATGTCC3'	NM_001291068.1

2.4 Detection of cytokines and chemokines in maternal serum and the placenta

Initially, placental tissue was homogenized in extraction buffer (50 mM Tris, 150 mM NaCl, 1X Triton, 0.1% SDS, 5 mM EDTA, 5 mM NaF, 50 mM sodium pyrophosphate, 1 mM sodium orthovanadate, pH 7.4) containing complete protease inhibitor cocktail (Roche Applied Science, Germany) with TissueLyser LT (Qiagen, Germany). The protein concentration of each sample was analyzed using a Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Analysis of the cytokines IL-6 and IL-1β and the chemokines monocyte chemoattractant protein-1 (MCP-1/CCL2) and chemokine (C-X-C motif) ligand 1 (CXCL1) in maternal serum and placenta was performed with MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel – Immunology Multiplex Assays (MCYTOMAG-70K, Merck Millipore, Germany) following the manufacturer's recommendations. The plate with samples and magnetic beads was analyzed on a

219 MAGPIX® System (Merck Millipore, Germany). The analyses were performed using
220 Luminex xPonent® for MAGPIX® v software. 4.2 (Luminex Corp., USA). For
221 each reaction well, the MAGPIX Luminex® platform reports the median fluorescence
222 intensity (MFI) for each of the analytes in the sample. The levels of each analyte were
223 then calculated against the standard curve. The ratio between the value obtained and the
224 protein quantification for each sample was determined and plotted.

225

226 ***2.5 Virus titration using plaque assays***

227 Blood from mock- and ZIKV_{PE243}-infected mice was collected from the base of
228 the tail at 4 hours, 48 hours and 144 hours following the appropriate treatments and
229 subsequently centrifuged at 400 g for 30 min for plasma separation. Samples obtained at
230 different periods post infection were titrated using a plaque assay. Vero cells (obtained
231 from ATCC® CCL81™) (African green monkey kidney epithelial cell line) were plated
232 in 24-well plates at 4×10^4 cells per well in Dulbecco's modified Eagle's medium
233 (DMEM) (GIBCO, Thermo Fisher, USA) supplemented with 5% fetal bovine serum
234 (FBS) (GIBCO, Thermo Fisher, USA) and 1% gentamicin (10 µg/ml) (GIBCO, Thermo
235 Fisher, USA) and cultured overnight for complete adhesion at 37°C with 5% CO₂.
236 Then, the medium was removed, and the cells were washed with 1x PBS and incubated
237 with serial (base 10) dilutions of virus in FBS-free medium. After 90 min of incubation
238 under gentle shaking, the medium was removed, and the cells were washed with 1x PBS
239 and cultured with 1.5% carboxymethylcellulose (CMC) supplemented with 1% FBS
240 (GIBCO, Thermo Fisher, USA). After 5 days, the cells were fixed overnight with 4%
241 formaldehyde and stained with 1% crystal violet in 20% methanol (ISOFAR, Brazil) for
242 1 hour. Plaques were counted, and the virus yield was calculated and expressed as
243 plaque-forming units per milliliter (PFU/ml).

244 **2.6 Histological, immunohistochemistry and TUNEL analyses of the placenta**

245 Placental fragments were fixed overnight and subjected to dehydration
246 (increasing ethanol series; ISO FAR, Brazil), diaphanization with xylol (ISO FAR,
247 Brazil) and paraffin (Histopar, Easypath, Brazil). Sections (5 µm) were prepared using a
248 Rotatory Microtome CUT 5062 (Slee Medical GmbH, Germany) and subjected to
249 immunohistochemistry and TUNEL analyses.

250 For immunohistochemistry, blocking of endogenous peroxidase was performed
251 with 3% hydrogen peroxide diluted in PBS, followed by microwave antigenic recovery
252 in Tris-EDTA (pH=9) and sodium citrate (pH=6) buffers (15 min for Tris-EDTA buffer
253 and 8 min for citrate buffer). Sections were washed in PBS + 0.2% Tween and exposed
254 to 3% PBS/BSA for 1 hour. Sections were then incubated overnight at 4°C with the
255 following primary antibodies: anti-Ki-67 (1:100 – [M3064]; Spring Bioscience, USA),
256 anti-P-gp (1:500 – Mdr1[sc-55510]; Santa Cruz Biotechnology, USA), anti-Bcrp (1:100
257 – Bcrp [MAB4146]; Merck Millipore, USA), anti-Abcg1 (1:100 – [PA5-13462];
258 Thermo Fisher Scientific, USA) or anti-Abca1 (1:100 – [ab18180]; Abcam Plc, UK).
259 The next day, sections were incubated with the biotin-conjugated secondary antibody
260 SPD-060 (Spring Bioscience, USA) for 1 hour at room temperature. Three washes were
261 performed with PBS + 0.2% Tween followed by incubation with streptavidin (SPD-060
262 - Spring Bioscience, USA) for 30 min. Sections were stained with 3,3-diamino-
263 benzidine (DAB) (SPD-060 - Spring Bioscience, USA), counterstained with
264 hematoxylin (Proquímios, Brazil), dehydrated, diaphanized and mounted with a
265 coverslip and Entellan (Merck, Germany).

266 For analysis of apoptotic nuclei, terminal deoxynucleotidyl transferase dUTP
267 nick-end labeling (TUNEL) staining was performed using an ApopTag® In Situ
268 Peroxidase Detection Kit (S7100, Merck Millipore, USA) according to the

269 manufacturer's recommendations and as previously described (36). All negative controls
270 were prepared with omission of the primary antibody.

271 Image acquisition was performed using a high-resolution Olympus DP72
272 (Olympus Corporation, Japan) camera coupled to an Olympus BX53 light microscope
273 (Olympus Corporation, Japan). For nuclear quantification of Ki-67 and TUNEL
274 immunolabeling, Stepanizer software (40) was used. For this analysis, we evaluated 15
275 images from different random fields of the Lz (labyrinth zone) and Jz (junctional zone)
276 for each animal, in a total of five animals from each ICompetent group and three
277 animals from each ICompromised group. A total of 360 digital images (40X) randomly
278 captured per placental region (Lz and Jz) were evaluated in each experimental group.
279 The total number of immunolabeled Ki-67 or TUNEL nuclei in each digital image was
280 normalized by the total image area to obtain an index of the estimated number of
281 proliferative and apoptotic nuclei in the entire histological section analyzed. Analysis
282 was undertaken by two investigators blinded to the treatment.

283 Quantification of P-gp, Bcrp, Abca1 and Abcg1 staining was performed using
284 the Image-Pro Plus, version 5.0 software (Media Cybernetics, USA) mask tool. The
285 percentage of viable tissue area was considered upon exclusion of negative spaces. A
286 total of 360 digital images (40X) randomly captured per placental region (Lz and Jz)
287 were evaluated in each experimental group. Analysis was undertaken by two
288 investigators blinded to the treatment.

289 ***2.7 Transmission electron microscopy (TEM)***

290 Sections of the placental Lz and Jz were fixed in paraformaldehyde 4% (Sigma-Aldrich,
291 Brazil) for 48 hours, postfixed with osmium tetroxide (Electron Microscopy Sciences,
292 USA) and potassium ferrocyanide (Electron Microscopy Sciences, USA) for 60 min and
293 dehydrated with an increasing series of acetone (30%, 50%, 70%, 90% and two of

100%) (ISO FAR, Brazil). Sections were subsequently embedded with EPOXI resin (Electron Microscopy Sciences, USA) and acetone (1:2, 1:1 and 2:1, respectively). After polymerization, ultrafine sections (70 nm) were prepared (Leica Microsystems, USA) and collected into 300 mesh copper grids (Electron Microscopy Sciences, USA). Tissue was contrasted with uranyl acetate and lead citrate and visualized using a JEOL JEM-1011 transmission electron microscope (JEOL, Ltd., Akishima, Tokyo, Japan). Digital micrographs were captured using an ORIUS CCD digital camera (Gatan, Inc., Pleasanton, California, EUA) at 6000× magnification. An overall qualitative analysis of the Lz and Jz in different groups was performed by investigating the ultrastructural characteristics of the mitochondria and the ER cisterns. The qualitative evaluation consisted of analyzing disruption of the mitochondrial membranes, mitochondrial morphology, preservation of mitochondrial cristae and matrix intensity (41). Ultrastructural analysis of nuclear morphology and the presence of microvilli in trophoblast sinusoidal giant cells was also undertaken. Analysis of ER cisterns was performed by evaluating the dilation of their lumen (42).

2.8 Statistical analysis

GraphPad Prism 8 software (GraphPad Software, Inc., USA) was used for statistical analysis. A D'Agostino & Pearson normality test was used to evaluate normal distribution, and outliers were identified using a Grubbs test. The data are expressed as the mean $\pm$ SEM or individual values. One-way ANOVA followed by Tukey's posttest was used for comparisons between different inbred groups, whereas Student's t-test or a nonparametric Mann-Whitney test was performed to compare the outbred groups. Differences were considered significant when $p < 0.05$. Pregnancy parameters were evaluated using the mean value of all fetuses and placentae in a litter per dam and not the individual conceptus, i.e., the mean value. In Figures 1 and 2, "n" represents the

number of dams. For MET and immunostaining data, placentae closest to the mean weight of all placentae were selected from each litter; “n” represents the number of litters (35–37).

3. Results

3.1 Weight gain during pregnancy is dependent on maternal immune status in ZIKV-infected mice.

To determine the effect of ZIKV infection on fetal and placental phenotypes at term (GD18.5), we infected ICompetent C57BL/6 and immunocompromised (ICompromised) A129 mice with ZIKV at GD12.5 (Figure 1A). Given the very distinct susceptibility of C57BL/6 and A129 mice to ZIKV, systemic infection models were established by injecting high (5×10^7 PFU) and low (10^3 PFU) virus inoculum titers. As shown in Figure 1B, ICompetent C57BL/6 mice in all groups and ICompromised A129 dams inoculated with mock and low ZIKV titers exhibited higher maternal weight at GD18.5 than at GD12.5 and GD0.5 ($p < 0.05$). On the other hand, ICompromised A129 mice presented significant weight loss at GD18.5, despite showing an increase at GD12.5 in relation to GD0.5 (Figure 1B).

3.2 Immunocompetent and immunodeficient mice have distinct term placental and fetal phenotypes in response to high and low ZIKV titer challenges in mid-pregnancy.

The fetuses from C57BL/6 dams and sires were called ICompetent. The fetuses from the mating of A129 dams and C57BL/6 sires were called ICompromised. High-ZIKV ICompetent mice exhibited 34% fetal loss, whereas high-ZIKV-A129 mice had 100% fetal loss (Figure 1C). In the low-ZIKV groups, C57BL/6 mice had no (0%) fetal death, while A129 mice exhibited a 42% fetal death rate (Figure 1C). Fetal and fetal head sizes were decreased in A129 mice compared to those in C57BL/6 dams infected

with the high ZIKV titer ($p=0.05$; Figure 1 D-G). However, no changes in fetal weight or fetal head size were observed when the mice were infected with the low ZIKV titer (Figure 1 D-G).

Figure 1: ZIKV infection induced fetal changes during pregnancy. **A**) Experimental design: matings of C57BL/6 dams x C57BL/6 sires (mock $n=11$ dams; high ZIKV $n=15$ dams; low ZIKV $n=9$ dams) and A129 dams x C57BL/6 sires ($n=3$ dams/group). The mock control consisted of noninfected C6/36 cell supernatants; high ZIKV titers consisted of 5×10^7 plaque-forming units (PFU) of ZIKV_{PE243}, and low ZIKV titers consisted of 10^3 PFU of ZIKV_{PE243}. Fetuses from C57BL/6 dams and sires were termed ICompetent, whereas fetuses from A129 dams and C57BL/6 sires were termed ICompromised. **B**) Maternal weight gain throughout pregnancy, $*=p < 0.05$, one-way ANOVA. **C**) Uterine horn and survival rates following ZIKV exposure (arrows show resorption sites). **D**) Fetal/reabsorption images (#=example of fetal reabsorption). **E**) Fetal weight, **F**) fetal head weight and **G**) fetal head weight/fetal weight ratio. One-way ANOVA followed by Tukey's posttest was used to assess changes among ICompetent groups, whereas an unpaired Student's t-test or nonparametric Mann-Whitney test was used to assess differences between ICompromised groups. Values are the mean of individual plotted values.

3.3 ZIKV is detected in the fetal brain of ICompromised, but not ICompetent mice.

ZIKV RNA was detected in the spleens of pregnant ICompetent C57BL/6 mice inoculated with the highest ZIKV titer, confirming acute systemic infection. Viral RNA

365 was also detected in the majority of the placentae of those mice (Figure 2A-B) but not
 366 in the maternal and fetal C57BL/6 brains (Figure 2A-B), suggesting that the virus was
 367 not transmitted to the fetuses. Although low ZIKV inoculation resulted in virus
 368 detection in the spleens of pregnant C57BL/6 mice, infection was not evidenced in the
 369 placentae or fetal brains. In contrast, ZIKV RNA was detected in all analyzed organs
 370 from ICompromised A129 dams, including the maternal brain and spleen and the
 371 placenta and fetal brain (Figure 2A-B). Viremia in maternal plasma was evaluated at 4,
 372 48 and 144 hours after infection. Within 4 hours, the presence of the virus was verified
 373 in the serum (high ICompetent=637.5 PFU/mL, low ICompetent=740 PFU/mL and low
 374 ICompromised=325 PFU/mL), indicating that the virus was correctly inoculated.
 375 Afterwards, ZIKV RNA was detected in ICompromised dams at 48 and 144 hours post
 376 inoculation but not in ICompetent dams (Figure 2C).

377

Figure 2: Viral load detection in maternal and fetal tissues after ZIKV infection. Pregnant ICompetent and ICompromised mice were inoculated i.v. with low and high doses of ZIKV. A-B) ZIKV RNA was measured via RT-qPCR in tissue samples obtained from maternal spleen and brain (A) and from placenta and fetal brain (B). C) The presence of ZIKV infectious particles in the plasma of infected dams was evaluated with a plaque assay 4, 48, and 144 hours post infection in ICompetent (mock n=11 dams; high ZIKV n=15 dams; low ZIKV n=9 dams) and ICompromised (mock n=3 dams; high ZIKV n=3 dams; low ZIKV n=3 dams) mice.

3.4 ZIKV infection induces distinct systemic and placental inflammatory responses in ICompetent and ICompromised mice.

The maternal serum and placental protein levels of specific cytokines and chemokines related to fetal death and preterm delivery (43–45) were evaluated to probe whether midgestation ZIKV infection would induce a maternal inflammatory response at term in our two distinct models. Since A129 infected with 10^7 ZIKV-PFU showed 100% fetal loss, we proceeded using 10^7 PFU inoculation in C57BL/6 mice and 10^3 PFU inoculation in both C57BL/6 and A129 mice.

CCL2 was elevated in the serum of low-ZIKV ICompetent mice, but no other alteration was systemically detected in any ICompetent mice at this time point (Figure 3A-B). On the other hand, ICompromised dams showed significantly increased CXCL1 and IL-6 levels in the serum and a strong trend for an enhancement in CCL2 (Figure 3C). Analysis of cytokine and chemokine expression in the placenta demonstrated that the CXCL1 and CCL2 chemokines were also upregulated in ICompromised but not ICompetent mice (Figure 3D-F). Surprisingly, IL-6 protein expression was augmented in some of the low-ZIKV ICompetent mice (58%; $p < 0.05$) but not in high-ZIKV mice or ICompromised mice (Figure 3D-F).

We also assessed the placental mRNA expression of a range of cytokines related to placental infective responses: *Ifng*, *Il6*, *Ifn1*, *Tnf*, *Il1b* and *Il10*. All the infected mice showed a significant increase in *Tnf* mRNA expression, with higher levels detected in

406 ICompetent mice (Figure 3G-I). The low-ZIKV ICompetent mice presented modest but
407 significant *Ifn1* expression, which was not detected in the high-ZIKV group (Figure
408 3G). Additionally, both low-ZIKV-infected groups (ICompetent and ICompromised)
409 presented increased *Ifng* mRNA expression (Figure 3G and 3I, $p < 0.05$). Interestingly,
410 placental *Il6* mRNA levels were only elevated in ICompromised pregnancies compared
411 to mock pregnancies ($p=0.05$) (Figure 3I). *Il1b* and *Il10* remained unchanged in all
412 groups analyzed (Figure 3 G-I).

413

414

415 **Figure 3: ZIKV during pregnancy promotes an inflammatory response in the**
416 **maternal serum and in the placenta.** A) Levels of IL-1 β , IL-6, CCL-2 and CXCL-1
417 in the maternal serum (A-C) and placenta (D-F) at GD18.5 in matings of C57BL/6 dams x
418 C57BL/6 sires (ICompetent fetuses - mock n=11 dams; high ZIKV n=15 dams; low
419 ZIKV n=9 dams) and A129 dams x C57BL/6 sires (ICompromised fetuses n=3
420 dams/group). Placental mRNA expression (G-I) of *Ifng*, *Ifn1*, *Il1b*, *Il6*, *Il10* and *Tnf*.
421 Broken lines show the expression levels in both lineages in the mock group. One-way
422 ANOVA followed by Tukey's posttest was used to assess changes among ICompetent
423 groups, whereas an unpaired Student's t-test or nonparametric Mann-Whitney test was

424 used to assess differences between ICompromised groups. The values are expressed as
425 the mean $\pm$ SEM.

426

427 ***3.5 ZIKV affects placental proliferation, apoptosis and ultrastructure in a viral load-***
428 ***and maternal immune status-dependent manner.***

429 We did not observe changes in placental weight or in the fetal:placental weight
430 ratio in any of the groups investigated (data not shown). However, since we detected the
431 presence of ZIKV RNA in the placentas, we investigated the cellular proliferation (Ki-
432 67⁺ cells) and apoptotic ratio in the Lz and Jz of the mouse placenta. Increased Ki-67
433 staining was observed in the Lz of high- and low-ZIKV-treated ICompetent animals
434 compared to mock animals (Figure 4A-D, $p=0.005$ and $p=0.015$), whereas the apoptotic
435 ratio in the Lz was increased in high-ZIKV dams and decreased in low-ZIKV dams
436 ($p=0.01$, Figure 4E-H). Although no differences were observed in Ki67 staining (Figure
437 5A-D), a similar apoptotic pattern was detected in the Jz of ICompetent pregnancies
438 ($p=0.008$ and $p=0.004$, respectively; Figure 5E-H). In contrast, in ICompromised dams,
439 Lz Ki-67 staining was decreased ($p=0.001$; Figure 4I-L), while the apoptotic reaction
440 was increased in ZIKV-infected animals ($p=0.05$; Figure 4M-P). Jz from ICompromised
441 offspring exhibited increased Ki-67 labeling and no differences in the apoptotic reaction
442 ($p=0.001$; Figure 5 I-L and Figure 5M-P).

443

444 **Figure 4: Labyrinthine remodeling in ICompetent and ICompromised placentae is**
445 **affected by gestational ZIKV infection.** A total of 180 digital images (40X) randomly
446 captured from the whole labyrinth zone (Lz) of each placenta per dam were evaluated.
447 Immunolabeled nuclei from each digital image were quantified and normalized by the
448 total digital image area to obtain an index of the estimated number of proliferative and
449 apoptotic nuclei in the entire histological section. (**A and I**) Quantification and (**B-D**
450 **and J-K**) representative photomicrographs of Ki-67⁺ stained nuclei in the Lz of
451 ICompetent (n=6 placentae from 6 independent dams/group) and ICompromised (n=3
452 placentae from 3 independent dams/group) placentae, respectively. (**E and M**)
453 Quantification and (**F-H and N-O**) representative photomicrographs of apoptotic nuclei
454 (TUNEL) in the Lz of ICompetent (n=5 placentae from 5 independent dams/group) and
455 ICompromised (n=3 placentae from 3 independent dams/group) placentae, respectively.
456 (**L and P**) Negative controls. One-way ANOVA followed by Tukey's posttest was used
457 to assess changes among ICompetent groups, whereas an unpaired Student's t-test or
458 nonparametric Mann-Whitney test was used to assess differences between
459 ICompromised groups. The values are expressed as the mean $\pm$ SEM. Images were
460 captured at 40X. Scale bar=50 μ m.

461

Figure 5: Junctional zone remodeling in ICompetent and ICompromised placentae is affected by gestational ZIKV infection. A total of 180 digital images (40X) randomly captured from the whole junctional zone (Jz) of each placenta per dam were evaluated. Immunolabeled nuclei from each digital image were quantified and normalized by the total digital image area to obtain an index of the estimated number of proliferative and apoptotic nuclei in the entire histological section. (A and I) Quantification and (B-D and J-K) representative photomicrographs of Ki-67⁺ stained nuclei in the Jz of ICompetent (n=6 placentae from 6 independent dams/group) and ICompromised (n=3 placentae from 3 independent dams/group) placentae, respectively. (E and M) Quantification and (F-H and N-O) representative photomicrographs of apoptotic nuclei (TUNEL) in the Jz of ICompetent (n=5 placentae from 5 independent dams/group) and ICompromised (n=3 placentae from 3 independent dams/group) placentae, respectively. (L and P) Negative controls. One-way ANOVA followed by Tukey's posttest was used to assess changes among ICompetent groups, whereas an unpaired Student's t-test or nonparametric Mann-Whitney test was used to assess differences between ICompromised groups. The values are expressed as the mean $\pm$ SEM. Images were captured at 40X. Scale bar=50 μ m.

3.6 Placental ultrastructure is differently impacted by high- and low-titer ZIKV infection in ICompetent and ICompromised strains.

Lz ultrastructural analyses of ICompetent and ICompromised-mock animals detected sinusoidal trophoblastic giant cells exhibiting regular microvilli, euchromatic

nuclei, preserved mitochondrial ultrastructure and regular narrow ER cisternae (Figure 6A and 6B). In sharp contrast, high-ZIKV ICompetent (Figure 6C) infected placentae showed fewer villi in the sinusoidal giant trophoblastic cells, degenerated mitochondria, granular ER with dilated cisterns and euchromatic nuclei. The sinusoidal giant trophoblastic cells in the low-ZIKV ICompetent mice (Figure 6D) also had fewer villi and degenerated mitochondria than those in the mock placentae, but no effect on the ER or euchromatic nuclei observed. Low-ZIKV ICompromised infected placentae (Figure 6E) showed fewer villi in the sinusoidal giant trophoblastic cells, degenerated mitochondria, granular ER with dilated cisterns and euchromatic nuclei.

493

Figure 6: Associated ultrastructural changes in the placental Lz after ZIKV infection. Transmission electron photomicrographs of ICompetent mock (A), ICompromised mock (B), ICompetent high (C), ICompetent low (D) and ICompromised low (E) groups (n=5/group). We observed dilatation in the ER cisterns of ICompetent high placentas. Additionally, there was a reduction in the microvilli in both the ICompetent high and ICompetent low placentas. In the ICompromised low group, we found fragmented ER and microvillus reduction. All infected groups showed degenerate mitochondria. GER=granular endoplasmic reticulum; Δ=dilated granular endoplasmic reticulum; *=fragmented granular endoplasmic reticulum;

503 Mt=mitochondria; MtD=degenerate mitochondria; Mv=microvilli; EN=euchromatic
504 nuclei; SS=sinusoidal space; TGC=trophoblastic giant cell. Scale bar=2 μ m.

505 The Jz of mock ICompetent and ICompromised placentae (Figure 7A and 7B)
506 exhibited euchromatic nuclei, with evident heterochromatin, preserved mitochondria
507 and narrow cisternae in a granular ER. High-ZIKV ICompetent Jz had degenerated
508 mitochondria, granular ER with dilated cisterns and euchromatic nuclei (Figure 7C).
509 Low-ZIKV ICompetent (Figure 7D) placentae exhibited euchromatic nuclei with
510 evident heterochromatin, preserved mitochondria and narrow cisternae in a granular ER,
511 whereas low-ZIKV ICompromised placentae had degenerated mitochondria, granular
512 ER with dilated cisterns and euchromatic nuclei (Figure 7E).

513

514 **Figure 7: Associated ultrastructural changes in the placental Jz after ZIKV**
515 **infection.** Transmission electron photomicrographs of ICompetent mock (A),
516 ICompromised mock (B), ICompetent high (C), ICompetent low (D) and
517 ICompromised low (E) groups (n=5/group). We found deteriorating mitochondria and
518 dilated reticulum endoplasmic cisterns in both the high ICompetent and low
519 ICompromised groups. GER=granular endoplasmic reticulum; Δ=dilated granular
520 endoplasmic reticulum; Mt=mitochondria; MtD=degenerate mitochondria;

521 Mv=microvilli; EN=euchromatic nuclei; SS=sinusoidal space; TGC=trophoblastic giant
522 cell; HA=heterochromatin area. Scale bar=2 μ m.

523

524 **3.7 ZIKV differentially affects placental expression of drug and lipid ABC** 525 **transporter systems.**

526 Evaluation of key ABC transporters in the Lz of mock and ZIKV-infected
527 ICompetent and ICompromised placentae revealed that immunolabeling of the drug P-
528 gp and Bcrp efflux transporter systems was primarily present at the cellular membranes
529 of the sinusoidal trophoblastic giant cells, with diffuse cytoplasmic Bcrp staining.
530 Labeling of the Abca1 and Abcg1 lipid efflux transporters was moderately and
531 heterogeneously distributed within the Lz. Less Lz-P-gp was observed in ICompetent
532 mice infected with both high- and low-ZIKV infective regimens than in mock-treated
533 animals (p=0.001 and p=0.002, respectively; Figure 8A-E), whereas reduced Bcrp and
534 Abca1 staining was observed in high-ZIKV-infected mice (p=0.003 and p=0.004,
535 Figure 8F-J and Figure 8K-O, respectively). No changes in Abcg1 were observed in any
536 of the ICompetent experimental groups (Figure 8P-T). P-gp, Bcrp and Abca1
537 transporter immunostaining was downregulated in ICompromised low ZIKV-treated
538 animals (p=0.001, p=0.05 and p=0.05, Figure 9A-D, Figure 9E-H and Figure 9I-L,
539 respectively). No changes in Abcg1 were observed in any of the ICompromised
540 experimental groups (Figure 9M-P).

541

542 **Figure 8: ZIKV infection decreases P-gp, Bcrp and Abca1 expression in the**
543 **placental Lz of infected mice in the ICompetent groups.** A total of 180 digital
544 images (40X) randomly captured from the whole labyrinth zone (Lz) of each placenta
545 per dam were evaluated. Immunolabeling in each digital image was quantified by
546 calculating the percentage area of the total stained labyrinthine tissue after exclusion of
547 the total negative space. (A) Quantification and (B-D) representative photomicrographs
548 of P-gp staining in the Lz of ICompetent (n=6 placentae from 6 independent
549 dams/group) placenta. (F) Quantification and (G-I) representative photomicrographs of
550 Bcrp staining in the Lz of ICompetent (n=6 placentae from 6 independent dams/group)
551 placenta. (K) Quantification and (L-N) representative photomicrographs of Abca1
552 staining in the Lz of ICompetent (n=6 placentae from 6 independent dams/group)
553 placenta. (P) Quantification and (Q-S) representative photomicrographs of Abcg1
554 staining in the Lz of ICompetent (n=6 placentae from 6 independent dams/group)
555 placenta. (E, J, O, T) Negative controls. One-way ANOVA followed by Tukey's post-
556 test. The values are expressed as the mean $\pm$ SEM. Images were captured at 40X. Scale
557 bar=50 μ m.
558

559

560 **Figure 9: ZIKV infection decreases P-gp, Bcrp and Abca1 protein expression in**
561 **the placental Lz of infected mice in the ICompromised groups.** A total of 180 digital
562 images (40X) randomly captured from the whole labyrinth zone (Lz) of each placenta
563 per dam were evaluated. Immunolabeling in each digital image was quantified by
564 calculating the percentage area of the total stained labyrinth zone tissue after exclusion
565 of the total negative space. (A) Quantification and (B-C) representative
566 photomicrographs of P-gp staining in the Lz of ICompromised (n=3 placentae from 3
567 independent dams/group) placenta. (E) Quantification and (F-G) representative
568 photomicrographs of Bcrp staining in the Lz of ICompromised (n=3 placentae from 3
569 independent dams/group) placenta. (I) Quantification and (J-K) representative
570 photomicrographs of Abca1 staining in the Lz of ICompromised (n=3 placentae from 3
571 independent dams/group) placenta (M) quantification and (N-O) representative
572 photomicrographs of Abcg1 staining in the Lz of ICompromised (n=3 placentae from 3
573 independent dams/group) placenta. (D, H, L, P) Negative controls. Unpaired Student's
574 t-test or nonparametric Mann-Whitney test was used to assess differences between
575 ICompromised groups. The values are expressed as the mean $\pm$ SEM. Images were
576 captured at 40X. Scale bar=50 μ m.

577

578 Next, the impact of ZIKV on ABC transporters in the Jz layer (structural and
579 endocrine layers of the mouse placenta) was assessed. P-gp and Bcrp were
580 predominantly localized at the cellular membranes of spongiotrophoblast cells, whereas
581 Abca1 and Abcg1 exhibited membrane and cytoplasmic staining. P-gp staining was

decreased in Jz cells from the low-ZIKV ICompromised placentae ($p=0.006$), with no other alterations observed (Figure 10A-T and Figure 11A-P).

584

585

Figure 10: ZIKV infection did not impact P-gp, Bcrp, Abca1 or Abcg1 protein expression in the placental Jz of infected mice in the ICompetent groups. A total of 180 digital images (40X) randomly captured from the whole junctional zone (Jz) of each placenta per dam were evaluated. Immunolabeling in each digital image was quantified by calculating the percentage area of the total stained junctional zone tissue after exclusion of the total negative space. (A) Quantification and (B-D) representative photomicrographs of P-gp staining in the Jz of ICompetent ($n=6$ placentae from 6 independent dams/group) placenta. (F) Quantification and (G-I) representative photomicrographs of Bcrp staining in the Jz of ICompetent ($n=6$ placentae from 6 independent dams/group) placenta. (K) Quantification and (L-N) representative photomicrographs of Abca1 staining in the Jz of ICompetent ($n=6$ placentae from 6 independent dams/group) placenta. (P) Quantification and (Q-S) representative photomicrographs of Abcg1 staining in the Jz of ICompetent ($n=6$ placentae from 6 independent dams/group) placenta. (E, J, O, T) Negative controls. One-way ANOVA followed by Tukey's post-test. The values are expressed as the mean $\pm$ SEM. Images were captured at 40X. Scale bar=50 μ m.

602

603

Figure 11: ZIKV infection decreases Bcrp protein expression in the placental Jz of infected mice in the ICompromised group. A total of 180 digital images (40X) randomly captured from the whole junctional zone (Jz) of each placenta per dam were evaluated. Immunolabeling in each digital image was quantified by calculating the percentage area of the total stained junctional zone tissue after exclusion of the total negative space. (A) Quantification and (B-C) representative photomicrographs of P-gp staining in the Jz of ICompromised (n=3 placentae from 3 independent dams/group) placenta. (E) Quantification and (F-G) representative photomicrographs of Bcrp staining in the Jz of ICompromised (n=3 placentae from 3 independent dams/group) placenta. (I) Quantification and (J-K) representative photomicrographs of Abca1 staining in the Jz of ICompromised (n=3 placentae from 3 independent dams/group) placenta. (M) Quantification and (N-O) representative photomicrographs of Abcg1 staining in the Jz of ICompromised (n=3 placentae from 3 independent dams/group) placenta. (D, H, L, P) Negative controls. Unpaired Student's t-test or nonparametric Mann-Whitney test was used to assess differences between ICompromised groups. The values are expressed as the mean $\pm$ SEM. Images were captured at 40X. Scale bar=50 μ m.

4. Discussion

In this study, we investigated several fetal and placental features at term (GD18.5) in ICompetent (C57BL/6) and ICompromised (A129) mice exposed to ZIKV

626 at mid-pregnancy (GD12.5). Fetal survival rates, systemic and placental inflammatory
627 responses, placental ultrastructure and cell turnover, as well as the expression of key
628 drug (P-gp and Bcrp) and lipid (Abca1) efflux transporter systems in the placenta, were
629 consistently impacted by ZIKV in both strains. The magnitude of the effects was clearly
630 related to the infective titer (high and low) of ZIKV and maternal immune status
631 (ICompetent-C57BL/6 x and ICompromised-A129), and fetal alterations were not
632 exclusively dependent on virus detection in the fetuses.

633 Infection of ICompetent mice with ZIKV did not result in viremia in the initial
634 postinoculation phase, although viral RNA was detected in the maternal spleen in both
635 the high- and low-ZIKV groups, confirming systemic infection. This is consistent with a
636 previous report (46). Our data demonstrate that pregnant ICompetent C57BL/6 mice
637 were more susceptible to high ZIKV titers than to low ZIKV infective. Since viral RNA
638 was only detected in the placentae of high ZIKV-infected mice, fetal survival rates and
639 weights were impacted to a greater extent in those mice. Strikingly, even though the
640 virus was not present in the fetal brain (at least at term), fetal and fetal head weights
641 were lower in mice subjected to the high-ZIKV titer regimen, suggesting that high
642 infective viral load in mid-pregnancy, even in ICompetent individuals, can induce
643 IUGR and lower fetal head weight despite a lack of transmission to the fetal brain (47).
644 On the other hand, ICompromised placentae and fetal brains had detectable viral
645 transcripts, with no changes in weight, which is consistent with previous data (19). In
646 fact, in our models, the presence (ICompromised) or absence (ICompetent) of the virus
647 in the fetal brain did not correspond to fetal head size (decreased in only high
648 ICompetent). The data from ICompetent and ICompromised placentae demonstrate how
649 important the maternal immunological status is to control viremia, fetal survival and
650 accessibility of the virus to the fetal brain. The reason for the reduction in fetal brain

size in C57BL/6 mice in the absence of fetal brain infection requires further investigation. It is possible that fetal brains in the ICompetent mice may have been exposed to ZIKV earlier in pregnancy, when viremia was present in the maternal blood, and this may have severely compromised brain development. Of note, one limitation of our study is that we measured fetal head weight instead of cortical thickness. Future studies should investigate whether high- and low-ZIKV exposure alters cortical thickness in ICompetent and ICompromised offspring.

A distinct inflammatory profile was also detected in the three analyzed groups. At the protein level, low-ZIKV-ICompromised dams exhibited increased maternal IL-6 and CXCL-1 and placental CCL-2 and CXCL-1, whereas low-ZIKV-ICompetent dams had increased maternal CCL2 and placental IL-6 levels. CCL-2 and CXCL-1 are related to fetal death and preterm delivery (43–45) and could be associated with pronounced fetal injury detected upon ICompromised pregnancy. In addition, IL-6 was previously demonstrated (48) to be related to fetal response syndrome, characterized by activation of the fetal immune system. This syndrome is known to increase fetal morbidity and affect several organs, such as the adrenal gland, brain and heart (32,48–51). At the mRNA level, *Il6* expression was only detected in ICompromised placentae at term and may indicate a sustained harmful response in these mice until term. The IFN signaling pathway may be triggered by ZIKV (17) and is one of the key mechanisms of host defense and a viral target for immune evasion (20), but we only detected a slight increase in *Ifn1* in low-ZIKV ICompetent mice at term. However, we cannot rule out the possibility that these cytokines might have been produced earlier. Our findings showed that *Ifng* expression was significantly enhanced in both ICompetent and ICompromised low-ZIKV-derived placentae but not in high-ZIKV-infected mice. Although we could not assess cytokine expression in high-ZIKV ICompromised

676 placentas, one may extrapolate that low-ZIKV infection could result in stimulation of
677 *Ifng* producing cells, which has been previously shown to be protective for ZIKV-
678 infected mice (52).

679 Both ICompromised and ICompetent mice showed increased expression of
680 placental *Tnf* mRNA, which has been demonstrated to be directly related to placental
681 damage, abortion and premature birth (53–56). In addition, an increased *Tnf* response is
682 related to impaired placental hormone production and trophoblastic invasion and
683 increased apoptosis in pregnancy (57,58). Although we did not assess TNF- α protein
684 levels in the placenta and maternal blood, this response could be implicated in the
685 overall damage detected.

686 Although differences in placental weight were not observed, ZIKV infection
687 mid-pregnancy had a profound effect on placental cellular turnover, dependent on titer,
688 strain and/or placental compartment. The Lz is responsible for fetal and maternal
689 nutrient, gas and waste exchange, while the Jz provides structural support, nutrient
690 storage and hormone synthesis (35). ZIKV induced a consistent increase in Lz
691 proliferation in all groups. However, the Lz apoptotic rate was increased only in the
692 high-ZIKV-ICompetent and ICompromised groups and decreased in low-ZIKV-
693 ICompetent mice. The mechanisms underlying these differences are unknown but may
694 be related to the distinct maternal and placental proinflammatory responses and/or to the
695 direct effect of the virus on the placenta (59). Increased Lz apoptosis in the high-
696 ICompetent-ZIKV group may be one of the mechanisms driving the lower fetal and
697 fetal head weight detected in this group. In this context, changes in placental turnover
698 can determine placental maturation and function and lead to fetal distress and
699 developmental abnormalities (60). An increase in the Lz apoptotic ratio may signify
700 damage to this placental layer, which is consistent with the fact that diverse pathological

701 lesions associated with congenital disorders were described in placentae from women
702 infected by ZIKV at different stages of pregnancy (61). Conversely, no proliferative
703 changes were observed in the Jz in high-ZIKV and low-ZIKV ICompetent mice, while
704 increased and decreased apoptotic rates were detected. It follows that the lack of Jz-Ki-
705 67 induction may suggest that this layer is less capable of restoring proliferation in
706 response to high-ZIKV challenge, and this may be related to decreased fetal growth.

707 Our placental ultrastructural analysis detected consistent differences across
708 ZIKV-exposed groups. The Lz and Jz layers from both strains exhibited signs of ER
709 stress, i.e., dilated ER cisterns or fragmented ER granular structures. These alterations
710 may result from the accumulation of folded or poorly folded viral proteins in the ER
711 lumen (42,62,63). The *Flaviridae* family uses the ER to replicate (64), and according to
712 Offerdahl et al. (2017)(63), there is evidence of ZIKV interacting with this organelle,
713 promoting an increased release of Ca^{+2} from the ER to the cell cytoplasm, thereby
714 causing an increase in the production of reactive oxygen species (ROS) (65,66).

715 The mitochondrial ultrastructure in the Lz and Jz layers was severely impacted
716 by ZIKV exposure. We found evidence of mitochondrial degeneration, i.e.,
717 mitochondrial membrane rupture, absence of mitochondrial ridges and a less electron-
718 dense mitochondrial matrix, in all the treated groups. Placental mitochondrial
719 dysfunction is associated with IUGR (67,68) and may be related, at least in part, to the
720 lower fetal weight observed in high-ZIKV-ICompetent fetuses along with the altered
721 placental apoptotic and proliferative patterns. Furthermore, mitochondrial dysfunction
722 together with ER stress is likely to modify the placental ROS balance and generate local
723 oxidative stress (69), which is associated with impaired fetal development (70). Of
724 importance, associations between mitochondrial disruption, ER stress and placental cell
725 senescence have been reported. Senescence is characterized as an irreversible

726 interruption of the cell cycle and acquisition of a senescence-associated secretory
727 phenotype (SASP) that promotes the release of cytokines, such as IL-1, IL-6, IL-8 and
728 proinflammatory proteases (70). Therefore, the increased expression of IL-6 detected in
729 the placentas of ICompromised mice suggests a SASP profile, which may be related to
730 changes in the ER and mitochondrial ultrastructure, accompanied by important changes
731 in apoptosis and cell proliferation. The interactions between mitochondria and the ER
732 are critical for homeostasis and cell signaling (71). In conjunction with the ER,
733 mitochondria can regulate cell death mediators in response to hypoxia and inflammation
734 (72). The increase in apoptosis observed in the high-titer ICompetent groups and the
735 low-titer ICompromised group may be related to the mitochondrial damage and ER
736 stress observed. In fact, we observed an important decrease in microvillus abundance in
737 sinusoidal giant trophoblast cells. Previously, we observed a decrease in microvillus
738 density in the Lz of pregnancies exposed to malaria in pregnancy (MiP) (36). Together,
739 our data show that different gestational infective stimuli (MiP and ZIKV) are capable of
740 damaging placental microvillus abundance and impairing proper fetal-maternal
741 exchange function and fetal growth/survival.

742   Next, to investigate whether maternal ZIKV exposure may influence fetal
743 protection, we evaluated the placental localization and expression (semiquantitative) of
744 the ABC efflux transporter systems P-gp, Bcrp, Abca1 and Abcg1, which are highly
745 enriched in labyrinthine microvilli and in human syncytiotrophoblasts. These efflux
746 transporters exchange drugs, environmental toxins, cytotoxic oxysterols and lipids
747 within the maternal-fetal interface (26). We found a consistent decrease in labyrinthine
748 P-gp expression in all ZIKV-exposed groups, demonstrating that ZIKV infection during
749 pregnancy has the potential to increase fetal exposure to P-gp substrates, such as
750 synthetic glucocorticoids, antibiotics, antiretrovirals, antifungals, stomach-protective

751 drugs, and nonsteroidal anti-inflammatory drugs (26). Furthermore, Jz-P-gp was
752 decreased in ICompromised placentae. Although little is known about the function of
753 ABC transporters in the Jz, our data highlight the need for further studies investigating
754 the biological importance of ABC transporters in the placental endocrine and structural
755 zones of the rodent hemochorial placenta under normal and infective conditions.

756 ZIKV impaired Lz Bcrp and Abca1 expression in ICompetent (high) and
757 ICompromised (low) mice. However, no effects were observed in ICompetent animals
758 at a low ZIKV titer or in Abcg1 in any experimental setting. Thus, ZIKV also likely
759 increases fetal accumulation of Bcrp substrates (antibiotics, antiretrovirals,
760 sulfonylureas, folate, mercuric species, estrogenic mycotoxins, carcinogens and
761 phototoxic compounds, among others) and disrupts placental lipid homeostasis (lipids,
762 cholesterol, and cytotoxic oxysterols) by reducing placental Abca1 expression (26,73–
763 76). We can speculate that the increased fetal accumulation of the P-gp, Bcrp and
764 Abca1 substrates during ZIKV infection may contribute to the establishment of
765 congenital Zika syndrome, although additional studies are clearly required to answer
766 this important question. The present data are in agreement with previous publications
767 showing that bacterial, viral and protozoan inflammation alters the expression and/or
768 function of P-gp, Bcrp and Abca1 in biological barriers, such as the placenta, yolk sac
769 and blood-brain barriers (26,27,36,64,77–79).

770 **5. Conclusion**

771 Our data show that gestational ZIKV impacts the fetal phenotype independently
772 of term fetal viremia. Abnormal placental cell turnover, ultrastructure and transporter
773 expression may result from specific proinflammatory responses that depend on the
774 ZIKV infective load and maternal immune status. Fetal accumulation of drugs,

environmental toxins and lipids within the fetal compartment may potentially be increased in ZIKV-infected pregnancies due to altered levels of key ABC transporters.

Conflict of Interest:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

CBVA, FFB, EB, LBA and TMOC conceived and designed the experiments. CBVA, VRSM, SVAC, HRG, RPCS and VMON performed the experiments. CBVA, SVAC, FFB, EB, SGM, LBA and TMOC analyzed the data. CBVA, VRSM, EB, LBA and TMOC wrote the paper and edited the manuscript. All authors contributed to the article and approved the submitted version.

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