ZIKV disrupts fetal outcome, placental ultrastructure and drug transporter expression in mice

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Abstract

Congenital Zika virus (ZIKV) infection can induce fetal brain abnormalities. Here, we investigated whether maternal ZIKV infection may affect placental physiology and metabolic transport potential, and impact the fetal outcome, regardless of viral presence in the fetus at term. Low (103 PFU-ZIKVPE243; low-ZIKV) and high ($5x10^7$ PFU-ZIKVPE243; high-ZIKV) virus titers were injected into immunocompetent (ICompetent C57BL/6) and immunocompromised (ICompromised A129) mice at gestational day (GD)12.5 for tissue collection at GD18.5 (term). High-ZIKV elicited fetal death rates of 66% and 100%, whereas low-ZIKV induced fetal death rates of 0% and 60% in C57BL/6 and A129 dams, respectively. All surviving fetuses exhibited intrauterine growth restriction (IUGR) and decreased placental efficiency. High-ZIKV infection in C57BL/6 and A129 mice resulted in virus detection in maternal spleens and placenta, but only A129 fetuses presented virus RNA in the brains. Nevertheless, pregnancies of both strains produced fetuses with decreased head sizes ($p<0.05$). Low-ZIKV-A129 dams had higher IL-6 and CXCL1 levels ($p<0.05$) and their placentas showed increased CCL-2 and CXCL-1 contents ($p<0.05$). In contrast, low-ZIKV-C57BL/6 dams had an elevated CCL2 serum level, and increased type I and II IFN expression in the placenta. Notably, increased apoptotic rates, less abundant microvilli and mitochondrial degeneration were evidenced in the placental labyrinth (Lz) of ICompromised and high-ZIKV-ICompetent mice, but not in low-ZIKV-C57BL/6. In addition, decreased placental expression of the drug P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and the lipid ABCA1 transporters was detected in all ZIKV-infected groups, but BCRP and ABCA-1 were only reduced in ICompromised and high-ZIKV ICompetent mice. Our data indicate that gestational ZIKV infection triggers specific proinflammatory responses and affects placental turnover and transporter expression, in a way dependent on virus concentration

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

1. Introduction

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

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

and Hofbauer cells are permissive to ZIKV, and placentae from ZIKV-infected women had chorionic villi with a higher mean diameter (11–14). However, at present, there is limited information on the precise mechanism of maternal-fetal ZIKV transmission.

Many mouse models have been developed to identify how ZIKV overcomes placental defenses. First, limited information was obtained due to the apparent inability of the virus to infect wild-type (WT) mice (15). ZIKV NS5 targets the interferon signaling pathway in humans but not in mice (16). As such, WT mice develop no clear evidence of clinical disease (16,17) and are of limited use in modeling the disease. However, mice lacking an interferon signaling response show evidence of disease and have been widely used to investigate ZIKV infection during pregnancy (8,16,18).

The interferon system, especially type III interferon, is one of the key mechanisms of host defense and viral targets for immune evasion (19). Type III interferons have a role in protection against ZIKV infection in human syncytiotrophoblasts from term placenta (20). Luo et al. have shown that the inhibition of Toll-like receptors 3 and 8 inhibits the cytokine output of ZIKV-infected trophoblasts (21). In addition, viral replication coincides with the induction of proinflammatory cytokines such as IL-6. This cytokine has a crucial role in inflammation and affects the homeostatic processes related to tissue injury and activation of stress-related responses (22,23). ZIKV infection can trigger an inflammatory response with IL-6 release (11,24).

Maternal infection has profound effects on placental permeability to drugs and environmental toxins. Changes in the expression and function of specific ABC transporters in the placenta and yolk sac following infective and inflammatory stimuli have been demonstrated (25–29). ABC transporters are efflux transporters that control the biodistribution of several endogenous and exogenous substrates, including xenobiotics (antiretrovirals, synthetic glucocorticoids), steroid hormones (estrogens,

androgens), nutrients (folate, cholesterol) and immunological factors (chemokines and cytokines) within the maternal-fetal interface (30). The best described ABC transporters in the placenta are the multidrug resistance P-glycoprotein (P-gp), the breast cancer resistance protein (Bcrp) and the lipid Abca1 and Abcg1 transporters. P-gp and BCRP transporters are responsible for preventing fetal accumulation of xenobiotics and environmental toxins that may be present in the maternal circulation, whereas ABCA1 and ABCG1 control the placental exchange of cytotoxic oxysterol and lipid permeability throughout pregnancy; therefore, they play an important role in fetal protection and placental lipid homeostasis (25).

Despite a limited number of studies showing ZIKV infection in immunocompetent mice, it was previously demonstrated that intrauterine inoculation of high virus titer resulted in decreased fetal viability, with worse outcomes following infection in early gestation (31). In another report, intravenous infection at very early embryonic day resulted in fetal demise even though the virus was not found in the fetal compartment in most treated animals (32). In the present study, we hypothesize that maternal exposure to ZIKV affects placental function, including placental ultrastructure and ABC transporter (P-gp, Bcrp, Abca1 and Abcg1) protein expression, even in the absence of vertical transmission and that these effects are dependent on viral infective titers and maternal immune status.

2. Materials and methods

2.1 Virus preparation and storage

The Brazilian $ZIKV_{PE243}$ (GenBank ref. number KX197192) strain was isolated from a febrile case during the ZIKV outbreak in the state of Pernambuco, Brazil and was kindly provided by Dr Ernesto T. Marques Jr. (Centro de Pesquisa Aggeu Magalhães, FIOCRUZ, PE). Viruses were propagated in C6/36 cells with viral titers determined by

129 plaque assays in Vero cells, as previously described (33). Supernatants of noninfected
130 C6/36 cells cultured under the same conditions were used as mock controls.

131 ***2.2 Animal experimentation and study design***

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

148 On GD12.5, pregnant mice (ICompetent and ICompromised pregnancies) were
149 injected with a single intravenous (i.v.) titer of ZIKV or mock control. ICompetent and
150 ICompromised pregnant mice were randomly subdivided into three experimental groups:
151 the mock (control) group, which received supernatants of noninfected C6/36 cell injection
152 (ICompetent mock and ICompromised mock); the high-ZIKV-titer group, inoculated
153 with $5x10^7$ plaque-forming units (PFU) of $ZIKV_{PE243}$ (ICompetent high and

154 ICompromised high); and the ZIKV low-titer group, injected with 10^3 PFU of $ZIKV_{PE243}$
155 (ICompetent low and ICompromised low).

156 On the morning of GD18.5, all animals were euthanized with a sodium
157 phenobarbital overdose of 300 mg/kg. Maternal blood was collected by cardiac puncture,
158 centrifuged (10 min, 4000 g) and stored at -20°C. The maternal brain and spleen, all
159 placentae and all fetuses were dissected, collected and weighed, followed by fetal head
160 isolation and measurement. The three placentae closest to the mean weight in a litter were
161 selected for further analysis and cut in half using umbilical cord insertion as a reference
162 (34–36). One-half of the placental disk was frozen in liquid nitrogen for qPCR, and the
163 other half was fixed overnight in buffered paraformaldehyde (4%, Sigma-Aldrich, Brazil)
164 for ultrastructural and protein expression/localization analysis. Matched fetal heads,
165 maternal brains and spleens were frozen in liquid nitrogen for qPCR.

166

167 ***2.3 ZIKV RNA quantification by qPCR***

168 ZIKV load was evaluated in maternal blood, brains and spleens and in the
169 placentae and fetal heads. Brains, spleens, placentae and fetal heads were macerated in
170 RPMI medium (Gibco™ RPMI 1640 Medium) normalized by the ratio of 0.2 mg of tissue
171 to every 1 µl of medium and plotted per gram of tissue. The macerated volume was
172 centrifuged at 4500 g for 5 min to remove tissue residues, and then 500 µl of the
173 centrifuged volume was used for RNA extraction using 1 mL of TRIzol reagent (Life
174 Technologies, Thermo Fischer, USA). Treatment with DNase I (Ambion, Thermo
175 Fischer, USA) was performed to prevent contamination by genomic DNA. cDNA was
176 synthesized using a cDNA High Capacity Kit (Applied Biosystems, Thermo Fischer,
177 USA) according to the manufacturer's instructions by subjecting the samples to the
178 following cycle: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. qPCR was

179 performed using a StepOnePlus Real-Time PCR system and TaqMan Master Mix
180 Reagents (Applied Biosystems, Thermo Fischer, USA) using primers and probes specific
181 for the protein E sequence (37). Samples were then subjected to the following cycle: 50°C
182 for 2 min, 40x (95°C for 10 min, 95°C for 15 sec, 60°C for 1 min).

183

184 ***2.3.1 qPCR***

185 The placenta was macerated in 1.5 mL of TRIzol reagent (Life Technologies,
186 Thermo Fischer, USA). RNA extraction was performed following the manufacturer's
187 protocol. cDNA was prepared using Power SYBR Green PCR Master Mix (Life
188 Technologies, Thermo Fisher, USA). The reaction was carried out for selected genes
189 using intron-spanning primers (Table 1) and the StepOnePlus Real-Time PCR system
190 (Life Technologies, Thermo Fischer, USA). Samples were subjected to the following
191 cycle: 95°C for 10 min followed by 40 amplification cycles consisting of DNA
192 denaturation for 30 sec at 95°C and annealing of primers for 30 sec at 60°C. The threshold
193 cycles (Ct) for each gene of interest and for the reference gene glycerol 3-phosphate
194 dehydrogenase (*Gapdh*) and RNA Polymerase II Subunit A (*Polr2a)* were determined.
195 The relative expression for each gene was calculated using $2^{-\Delta\Delta CT}$ (38) and graphically
196 expressed as the fold-increase. The efficiency was calculated using the standard curve
197 method. The melting curves were analyzed for each sample.

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

Gene	Primer sequences	GenBank accession no.
ZIKV	5'CCGCTGCCCAACACAAG3' 5'CCACTAACGTTCTTTTGCAGACAT3'	
mIL-6	5'TCATATCTTCAACCAAGAGGTA3'	NM_031168.2

	5'CAGTGAGGAATGTCCACAAACTG3'	
mIL-1b	5'GTAATGAAAGACGGCACACC3'	XM_006498795.4
	5'ATTAGAAACAGTCCAGCCCA3'	
mIL-10	5'TAAGGGTTACTTGGGTTGCCAAG3'	NM_010548.2
	5'CAAATGCTCCTTGATTTCTGGGC3'	
mIFN-γ	5'AGCAACAGCAAGGCGAAAA3'	NM_008337.4
	5'CTGGACCTGTGGGTTGTTGA3'	
m-IFN-I	5'CTGGAGCAGCTGAATGGAAAG3'	NM_010510.1
	5'CTTGAAGTCCGCCCTGTAGGT3'	
mGAPDH	5'CTTTGTCAAGCTCATTTCCTGG3'	XM_017321385.2
	5'TCTTGCTCAGTGTCCTTGC3'	
TNF-a	5'CCTCACACTCAGATCATCTTCTCA3'	NM_013693.3
	5'TGGTTCTCTTTGAGATCCATGC3'	
Polr2a	5'TCTGCCAAGAATGTGACGCT3'	NM_001291068.1
	5'CCAAGCGGCAAAGAATGTCC3'	

2.4 Detection of cytokines and chemokines in the maternal serum and 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 the Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Analysis of the cytokines IL-6 and IL1-β 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 the 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 MAGPIX® System (Merck Millipore, Germany). The analyses were performed using the Luminex xPonent® for MAGPIX® v software. 4.2 (Luminex Corp., USA). For each reaction well, the MAGPIX Luminex® platform reports the median fluorescence intensity (MFI) for each of the analytes in the samples. The levels of each analyte were then calculated against the standard curve. The ratio between the value obtained and the protein quantification for each sample was determined and plotted.

2.5 Virus titration by plaque assays

Blood from mock- and $ZIKV_{PE243}$-infected mice was collected from the base of the tail at 4 hours, 48 hours and 144 hours following the appropriate treatments and subsequently centrifuged at 400 g for 30 min for plasma separation. Samples obtained at different periods post infection were titrated by a plaque assay. Vero cells (obtained from ATCC® CCL81™) (African green monkey kidney epithelial cell line) were plated in 24-well plates at $4x10^4$ cells per well in DMEM (GIBCO, Thermo Fisher, USA) supplemented with 5% FBS (GIBCO, Thermo Fisher, USA) and 1% gentamicin (10 µg/ml) (GIBCO, Thermo Fisher, USA) and cultured overnight for complete adhesion at 37°C with 5% CO_2. Then, the medium was removed, and the cells were washed with 1x PBS and incubated with serial (base 10) dilutions of virus in FBS-free medium. After 90 min of incubation under gentle shaking, the medium was removed, and the cells were washed with 1x PBS and cultured with 1.5% carboxymethylcellulose (CMC) supplemented with 1% FBS (GIBCO, Thermo Fisher, USA). After 5 days, the cells were

fixed overnight with 4% formaldehyde and stained with 1% crystal violet in 20% methanol (ISOFAR, Brazil) for 1 hour. Plaques were counted, and the virus yield was calculated and expressed as plaque-forming units per milliliter (PFU/ml).

2.6 Histological, immunohistochemistry and TUNEL analyses of the placenta

Placental fragments were fixed overnight and subjected to dehydration (increasing ethanol series, ISOFAR, Brazil), diaphanization with xylol (ISOFAR, Brazil) and paraffin (Histopar, Easypath, Brazil). Sections (5 μm) were prepared using the Rotatory Microtome CUT 5062 (Slee Medical GmbH, Germany) and subjected to immunohistochemistry and TUNEL analyses.

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

Brazil), dehydrated, diaphanized and mounted with coverslip and entellan (Merck, Germany).

For analysis of apoptotic nuclei, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed using the ApopTag® In Situ Peroxidase Detection Kit (S7100, Merck Millipore, USA) according to the manufacturer's recommendations and as previously described (35). All negative controls were performed with the omission of the primary antibody.

Image acquisition was performed using a high-resolution Olympus DP72 (Olympus Corporation, Japan) camera coupled to an Olympus BX53 light microscope (Olympus Corporation, Japan). For nuclear quantification of Ki-67 and TUNEL immunolabeling, Stepanizer software (39) was used. For this analysis, we evaluated 15 images from different random fields of the Lz and Jz for each animal, in a total of five animals from each ICompetent group and three animals from each ICompromised group. A total of 360 digital images per placental region (Lz and Jz) were evaluated in each experimental group. Total numbers of immunolabeled Ki-67 or TUNEL nuclei from each image were normalized by the total image area to obtain an index of estimated numbers of proliferative and apoptotic nuclei in the whole histological section analyzed. Analysis was undertaken by two investigators blinded to the treatment.

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

2.7 Transmission electron microscopy (TEM)

Sections of placental Lz and Jz were fixed in paraformaldehyde (4%, Sigma-Aldrich, Brazil) for 48 hours, postfixed with osmium tetroxide (Electron Microscopy Sciences, USA) and potassium ferrocyanide (Electron Microscopy Sciences, USA) for 60 min and dehydrated with an increasing series of acetone (30%, 50%, 70%, 90% and two of 100%) (ISOFAR, 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 Lz and Jz from different groups was performed by investigating the ultrastructural characteristics of the mitochondria as well as the ER cisterns. The qualitative evaluation consisted of analyzing the disruption of the mitochondrial membranes, mitochondrial morphology, preservation of mitochondrial cristae and matrix intensity (40). Ultrastructural analysis of nuclear morphology and the presence of microvilli in trophoblast sinusoidal giant cells was also undertaken. The analysis of the cisterns of the ER was performed by evaluating the dilation of their lumen (41).

2.8 Statistical analysis

GraphPad Prism 8 software (GraphPad Software, Inc., USA) was used for statistical analysis. The D'Agostino & Pearson normality test was used to evaluate normal distribution, and outliers were identified using the Grubbs test. Data are expressed as the mean ±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 nonparametric Mann-Whitney test was performed between 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. "n" represents the number of litters in Figures 1 and 2 (34–36).

3. Results

3.1 Weight gain across 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 ($5x10^7$ PFU) and low (10^3 PFU) virus inoculum titers. As shown in Table 2, ICompetent C57BL/6 mice from all groups and ICompromised A129 dams inoculated with mock and low 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.

Table 2: Maternal weight gain throughout pregnancy

	ICompetent			ICompromised		
Maternal weight (g)	**Mock (n=11)**	**High (n=15)**	**Low (n=9)**	**Mock (n=3)**	**High (n=3)**	**Low (n=3)**
GD0.5	22.4±0.7[a]	23.0±0.6[a]	21.9±0.7[a]	21.7±1.9[a]	21.7±0.9[a,b]	25.0±1.0[a]
GD12.5	25.5±0.6[b]	26.2±0.8[b]	25.1±0.5[b]	25.3±1.2[a]	26.0±1.0[a]	28.0±0.6[a]
GD18.5	31.6±0.7[c]	30.3±0.9[c]	31.4±1.1[c]	32.0±1.5[b]	20.0±1.5[b]	30.7±0.3[b]
ΔGD12.5-0.5 (Δ)	4	3	3	3	4	3
ΔGD18.5-0.5 (Δ)	9	7	10	10	-2	5

GD=gestational day; Δ=delta; values are expressed as the mean±standard error of the mean (SEM). One-way ANOVA followed by Tukey's test analyzed differences among GD0.5, 12.5 and 18.5 in each group; different letters indicate $p<0.05$.

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 mates 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 1B). In the low-ZIKV groups, C57BL/6 mice had no (0%) fetal death, while A129 mice exhibited a 42% fetal death rate (Figure 1B). 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 C-F). However, no changes in fetal weight and fetal head sizes were observed when the mice were infected with the lower ZIKV titer (Figure 1 C-F).

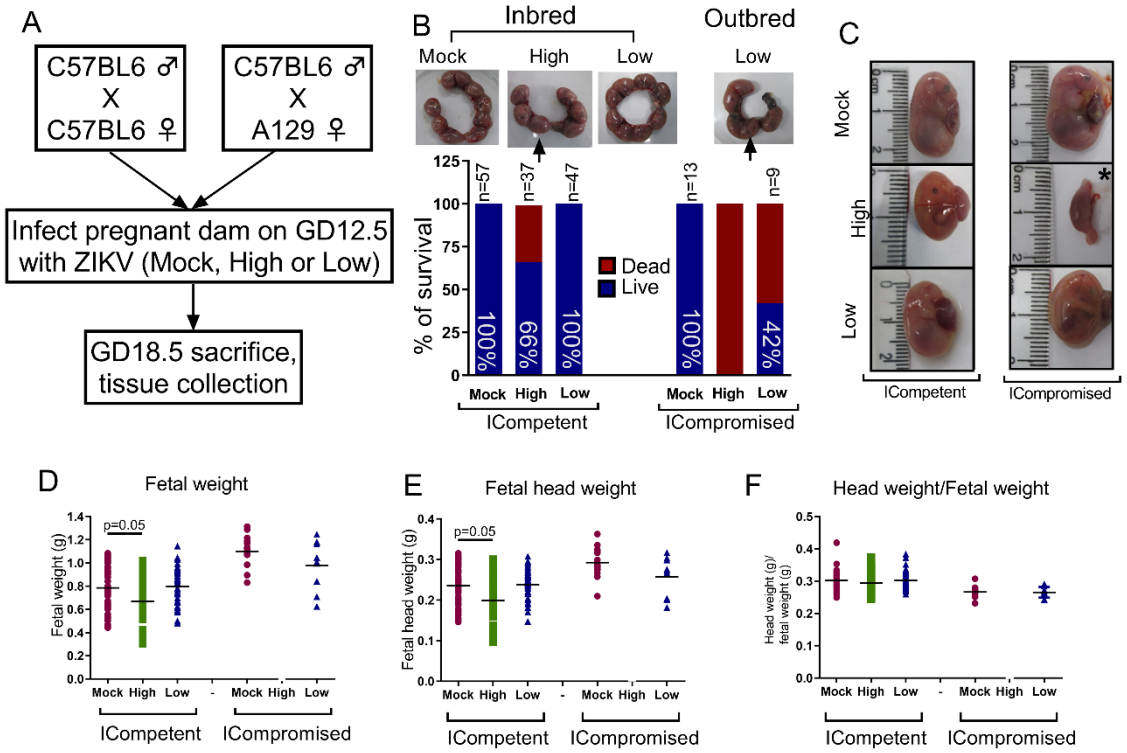


Figure 1: ZIKV infection induced fetal changes during pregnancy. A) Experimental schematic. The fetuses from C57BL/6 dams and sires were termed ICompetent. The fetuses from the mates of A129 dams and C57BL/6 sires were termed ICompromised. B) Uterine horn images and survival images (arrows show resorption sites). C) Fetal/reabsorption images. Measurement of fetal biometric parameters at GD18.5. D) Fetal weight, (E) fetal head weight and (F) fetal head weight/fetal weight ratio in ICompetent (n=11/15/9 dams in the mock, high and low groups, respectively) and ICompromised (n=3 dams/group). * example reabsorption. The values are plotted as individual values, and the bar represents the mean. Unpaired Student's t-test or nonparametric Mann-Whitney test was used for ICompromised groups, and one-way ANOVA followed by Tukey's post-test was used for ICompetent comparisons between different groups.

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 was also detected in the majority of placentae of those mice (Figure 2A-B) but not in the maternal and fetal C57BL/6 brains (Figure 2A-B), suggesting that the virus was not transmitted to the fetuses. Although low ZIKV inoculation resulted in virus detection in

the spleens of pregnant C57BL/6 mice, infection was not evidenced in the placentae or fetal brains. In contrast, ZIKV RNA was detected in all analyzed organs from ICompromised A129 dams, including the maternal brain and spleen and the placenta and fetal brain (Figure 2A-B). Viremia in maternal serum was evaluated at 4, 48 and 144 hours after infection. Within 4 hours, the presence of the virus was verified in the serum (high ICompetent = 637.5 PFU/mL, low ICompetent = 740 PFU/mL and low ICompromised = 325 PFU/mL), indicating that the virus was correctly inoculated. Afterwards, ZIKV RNA was detected in ICompromised dams at 48 and 144 hours postinoculation but not in ICompetent dams.

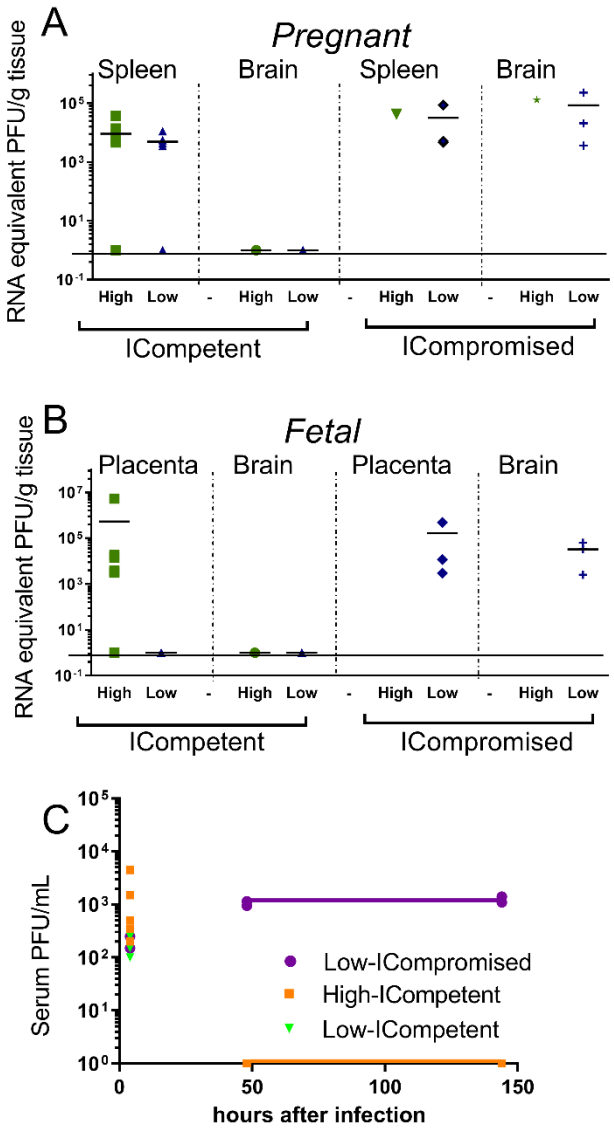


Figure 2: Viral load present in the spleen, placenta, fetal and maternal brains after ZIKV infection. A) Maternal spleen and brain viral load and B) placenta and fetal brain viral load of ZIKV-infected dams. C) Animal viral serology of all groups after 4, 48 and 144 infection hours. ICompetent (n=11/15/9 dams in the mock, high and low ZIKV titer groups, respectively) and ICompromised (n=3 dams/group).

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 (42–44) 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 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 cytokines and chemokines expression at the placenta demonstrated that CXCL1 and CCL2 chemokines were also upregulated in the ICompromised, but not of ICompetent mice (Figure 3 D-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 nor ICompromised mice (Figure 3D-F).

We also assessed the placental mRNA expression of a range of cytokines related to placental infective responses: Ifn-γ, Il-6, Ifn-1, Tnf-α, Il-1β, and Il-10. All the infected mice showed a significantly increase in TNF- α mRNA expression, with higher levels detected in ICompetent mice (Figure 3G-I). The low-ZIKV ICompetent mice presented a modest, but significant type I IFN expression, which was not detected in the high-ZIKVgroup (Figure 3G). Additionally, both low-ZIKV infected groups (ICompetent and ICompromised) presented increased Ifn-γ mRNA expression (Figure 3G and 3I, $p< 0.05$). Interestingly, placental Il-6 mRNA levels were only elevated in ICompromised pregnancies compared to the mock ($p=0.05$) (Figure 3I). Il-1b and IL-10 remained unchanged in all groups analyzed (Figure 3 G-I).

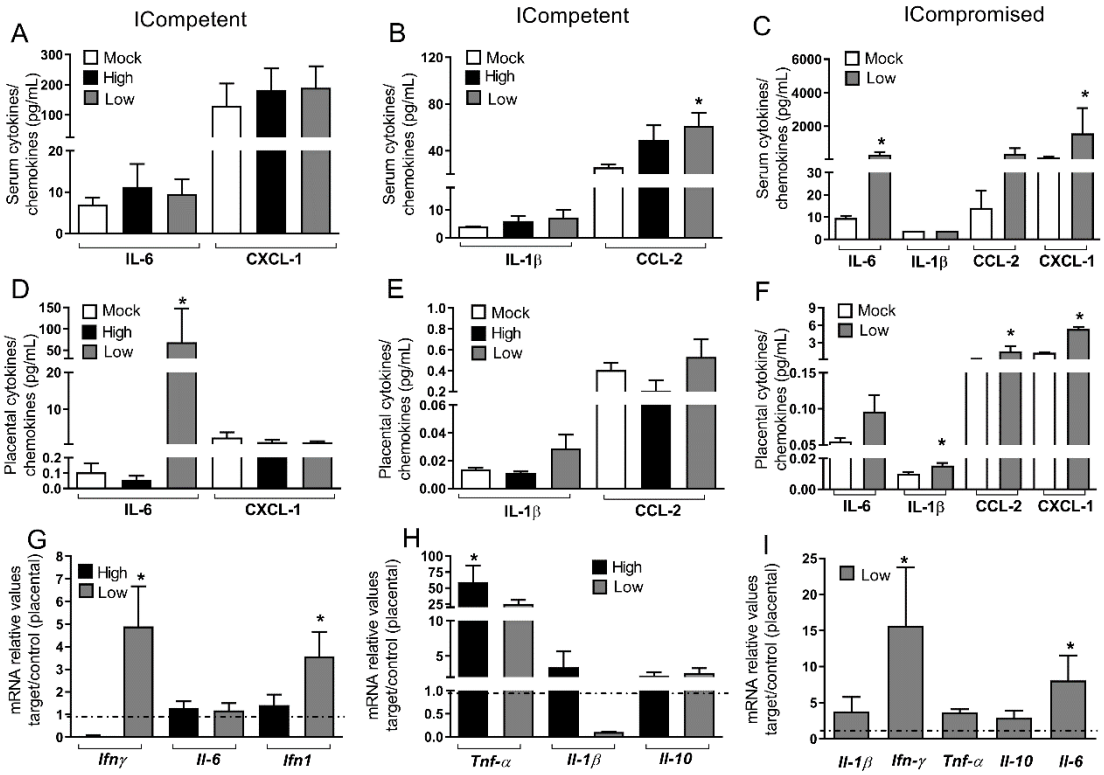


Figure 3: ZIKV promotes an inflammatory response in the placenta, maternal spleen and serum during pregnancy.
A) Cytokine and chemokine serum levels at GD18.5 in the ICompetent mock (n=9 dams), high (n=8) and low (n=9) and ICompromised mock and low (n=3/groups) ZIKV titer groups. A-C) IL-1β, IL-6, CCL-2 and CXCL-1 maternal serum levels. D-F) Placental IL-1β, IL-6, CCL-2 and CXCL-1 levels. G-I) Placental RNA levels of Ifnγ, *Ifn1*, *Il-1β, Il-6 and Tnf-α*. The broken line shows the expression levels of both lineages in the mock group. The values are expressed as the mean ± SEM. Unpaired Student's t-test or nonparametric Mann-Whitney test was used for comparisons between ICompetent groups, and one-way ANOVA followed by Tukey's posttest was used for comparisons between different ICompetent groups. * $p<0.05$.

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

We did not observe changes in placental weight or in the fetal:placental weight ratio in any of the groups investigated (data not shown). However, since we detected the

presence of ZIKV RNA in the placentas, we investigated the cellular proliferation (Ki-67$^+$ cells) and apoptotic ratio in the labyrinth zone (Lz) and junctional zone (Jz) of the mouse placenta. Increased Ki-67 staining was observed in the Lz of high- and low-ZIKV-treated ICompetent animals compared to mock animals (Figure 4A, p=0.005 and p=0.015), whereas the Lz apoptotic ratio was increased in high- and decreased in low-ZIKV dams (p=0.01, Figure 4B). A similar apoptotic pattern was detected in the Jz of ICompetent pregnancies (p=0.008 and p=0.004, respectively; Figure 5B), although no differences were observed in Ki67 staining (Figure 5A). In contrast, in ICompromised dams, Lz Ki-67 staining was decreased (p=0.001; Figure 4C), while the apoptotic reaction was increased in ZIKV-infected animals (p=0.05; Figure 5D). Jz from ICompromised offspring exhibited increased Ki-67 labeling and no differences in the apoptotic reaction (p=0.001; Figure 5 C-D).

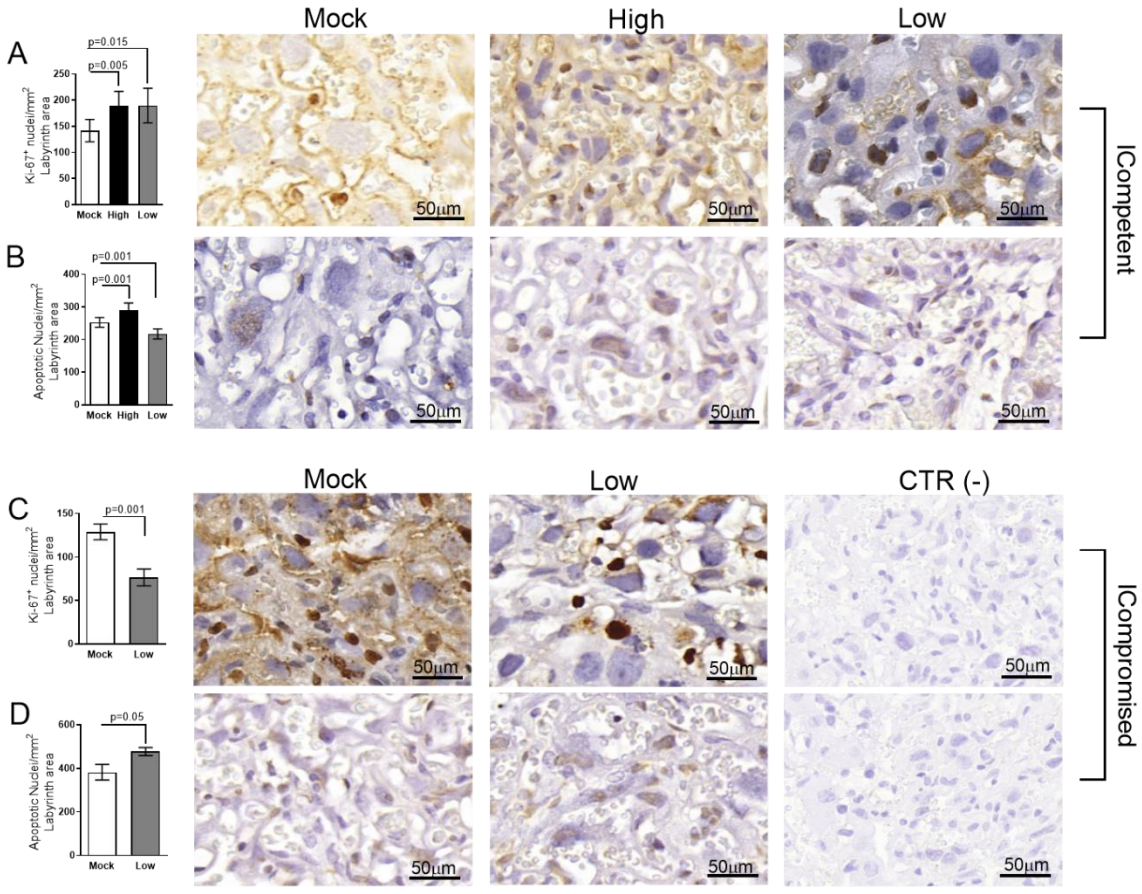


Figure 4: Placental remodeling of ICompetent groups induced by ZIKV infection. A) Quantification of Ki-67$^+$ nuclei in the mouse placenta Lz and representative images of nuclei immunostained by Ki-67$^+$-positive nuclei in the ICompetent group (n=6/group). B) The image shows the apoptotic nuclei score in the Lz and representative images of nuclei immunostained by TUNEL in the ICompetent group (n=5/group). C) Quantification of Ki-67+ nuclei in the mouse placenta Lz and representative images of nuclei immunostained by Ki-67$^+$ nuclei in the ICompromised group (n=3/group). D) The image shows the apoptotic nuclei score in the LZ and representative images of nuclei immunostained by TUNEL in the ICompromised group (n=3/group). The values are expressed as the mean ± SEM. One-way ANOVA followed by Tukey's post hoc test was used for comparisons between different ICompetent groups. Unpaired Student's t-tests or nonparametric Mann-Whitney tests were used for the ICompromised groups. CTR(-)=All negative controls were performed with the omission of the primary antibody. Scale bar = 50 µm.

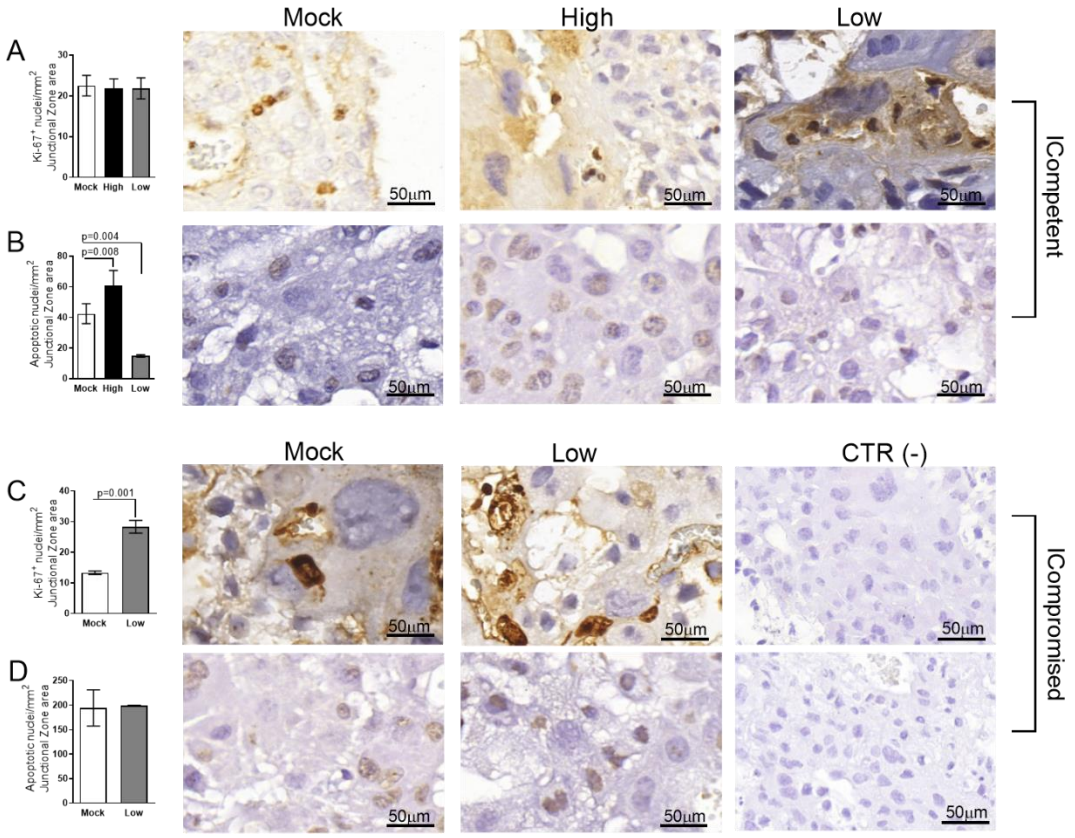


Figure 5: ZIKV induces hyperproliferation of the JZ area in infected placentas of ICompetent groups. A) Quantification of Ki-67+ nuclei in the placental Jz of mice and representative images of immunostained nuclei in the ICompetent (n=6/group). B) The image shows apoptotic nuclei quantification in the Jz area and representative images of nuclei immunostained by TUNEL in the ICompetent (n=5/group). C) Quantification of Ki-67+ nuclei in the placental Jz area and representative images of immunostained nuclei in the ICompromised group (n=3/group). D) The image shows apoptotic nuclei quantification in the Jz area and representative images of nuclei immunostained by TUNEL in the ICompromised (n=3/group). The values are expressed as the mean ± SEM.

One-way ANOVA followed by Tukey's post hoc test was used for comparisons between different ICompetent groups. Unpaired Student's t-tests or nonparametric Mann-Whitney tests were used for the ICompromised groups. CTR(-)=All negative controls were performed with the omission of the primary antibody. Scale bar = 50 µm.

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

Ultrastructural analyses in the Lz 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 6D). In sharp contrast, high-ZIKV ICompetent (Figure 6B) and 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. The sinusoidal giant trophoblastic cells from the low-ZIKV ICompetent (Figure 6C) also had fewer villi and degenerated mitochondria than the mock placentae, but there and had no effect on the ER or euchromatic nuclei.

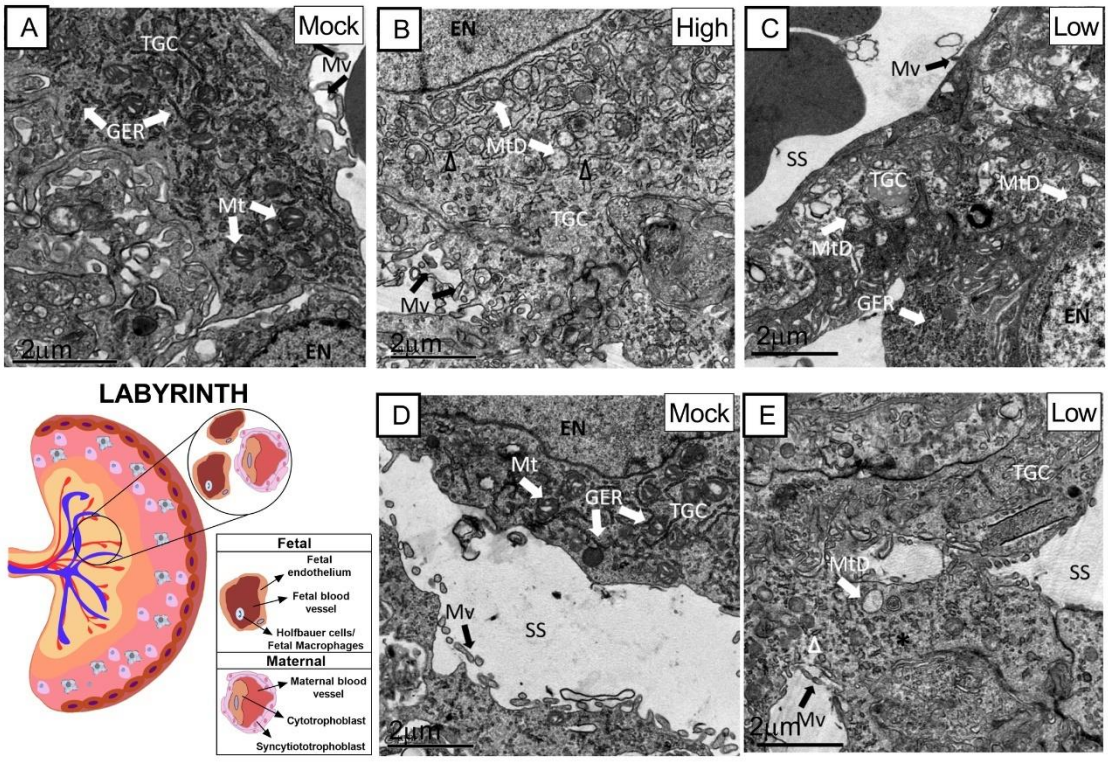


Figure 6: Associated ultrastructural changes in the placental LZ after ZIKV infection. Transmission electron photomicrographs of ICompetent mock (A); ICompetent high (B); ICompetent low (C); ICompromised mock (D) and ICompromised low (E) groups (N=5 dams/group). We observed dilatation in the ER cisterns of ICompetent high placentas. Additionally, there was a reduction in the microvilli from both 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; ⊗= Dilated Granular Endoplasmic Reticulum; *= Fragmented Granular Endoplasmic Reticulum; Mt= Mitochondria; MtD= Degenerate Mitochondria; Mv= Microvilli; EN= Euchromatic Nuclei; SS= Sinusoidal Space; TGC= Trophoblastic Giant Cell. Scale bar = 2 µm.

The Jz of mock ICompetent and ICompromised (Figure 7A and 7D) and of low-ZIKV ICompetent (Figure 7C) placentae exhibited euchromatic nuclei, with evident heterochromatin, preserved mitochondria and narrow cisternae granular ER. High-ZIKV ICompromised and low-ZIKV ICompromised placentae had degenerated mitochondria, granular ER with dilated cisterns and euchromatic nuclei (Figure 7B and 7E).

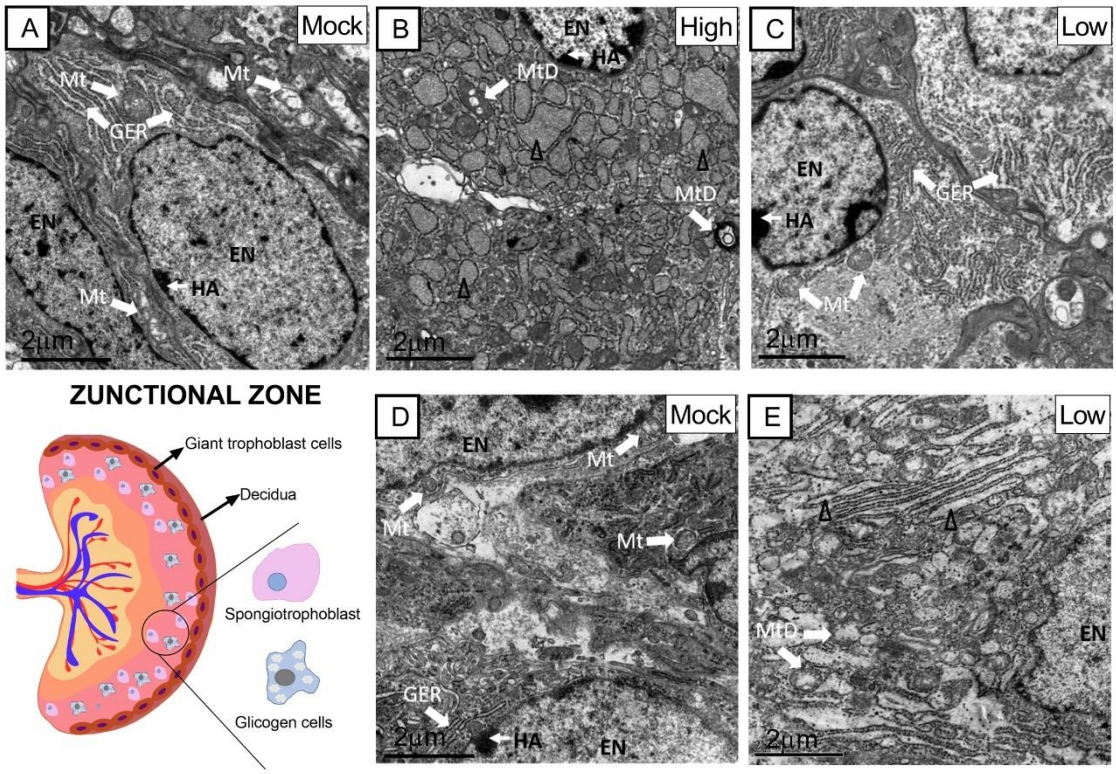
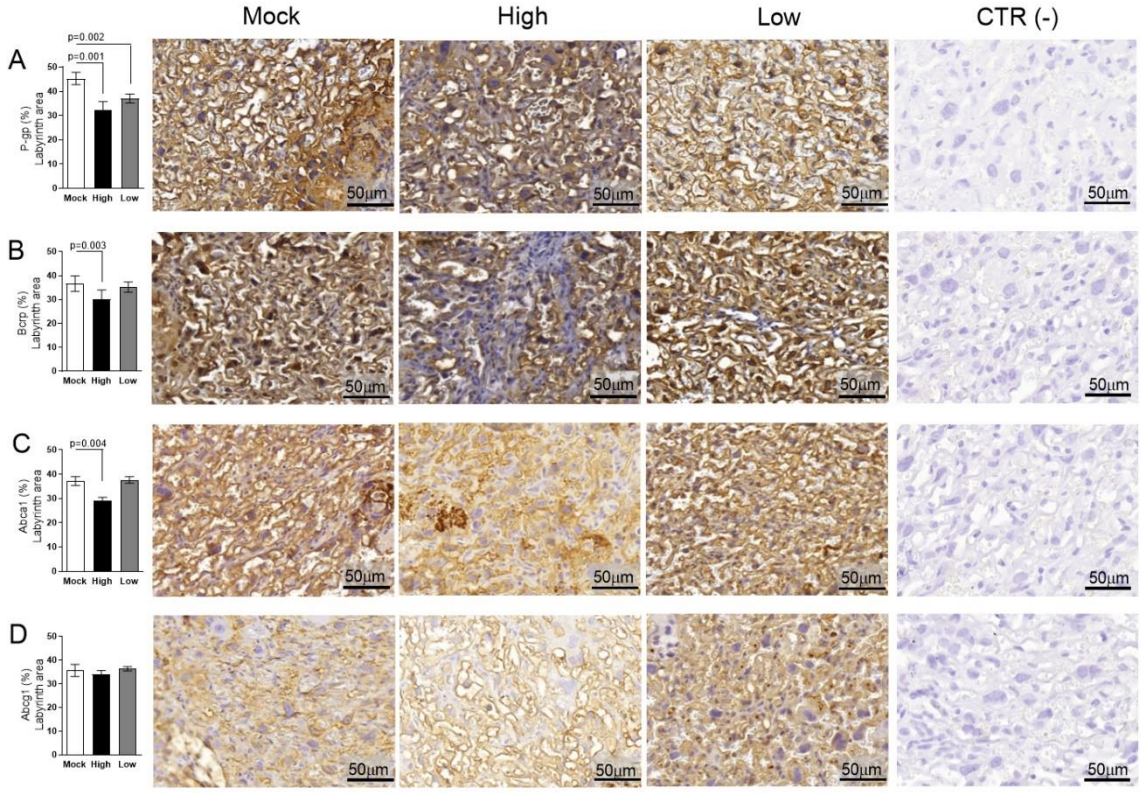


Figure 7: Associated ultrastructural changes in the placental JZ after ZIKV infection. Transmission electron photomicrographs of ICompetent mock (A); ICompetent high (B); ICompetent low (C); ICompromised mock (D) and ICompromised low (E) groups (N=5 dams/group). We found deteriorating mitochondria and dilated reticulum endoplasmic cisterns in both the high ICompetent and low ICompromised groups. GER= Granular Endoplasmic Reticulum; ⊗= Dilated Granular Endoplasmic Reticulum; Mt= Mitochondria; MtD= Degenerate Mitochondria; Mv= Microvilli; EN= Euchromatic Nuclei; SS= Sinusoidal Space; TGC= Trophoblastic Giant Cell. White arrows HA = Heterochromatin Area. Scale bar = 2 µm.

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

Evaluation of key ABC transporters in the Lz of mock and ZIKV-infected ICompetent and ICompromised placentae revealed that immunolabeling of the drug P-gp and Bcrp efflux transporter systems was primarily present at the cellular membranes of the sinusoidal trophoblastic giant cells, with diffuse cytoplasmic Bcrp staining. Labeling of the Abca1 and Abcg1 lipid efflux transporters was moderately and heterogeneously distributed within the Lz. Less Lz-P-gp was observed in ICompetent mice infected with

519 both high- and low-ZIKV infective regimens, in comparison to mock-treated animals
520 (p=0.001 and p=0.002, respectively; Figure 8A), whereas Bcrp and Abca1 staining was
521 only reduced was observed in high-ZIKV-infected mice (p=0.003 and p=0.004,
522 respectively; Figure 8B-C). P-gp, Bcrp and Abca1 transporter immunostaining was
523 downregulated in ICompromised low ZIKV-treated animals (p=0.001, p=0.05 and
524 p=0.05, respectively; Figure 9A-C). No changes in Abcg1 were observed in any of the
525 experimental groups (Figure 8D and Figure 9D).



526

527 **Figure 8: ZIKV infection decreases P-gp, Bcrp and Abca1 expression in the**
528 **placental LZ of infected mice in the ICompetent groups.** Immunostaining
529 quantification and representative immunohistochemistry images of A) P-gp, B) Bcrp, C)
530 Abca1 and D) Abcg1. The images represent the percentage of stained cells in each
531 analyzed field. The values are expressed as the mean ± SEM. One-way ANOVA followed
532 by Tukey's post hoc test was used for comparisons between different ICompetent groups.
533 Scale bar = 50 µm. N=6/ group. CTR(-)=All negative controls were performed with the
534 omission of the primary antibody.

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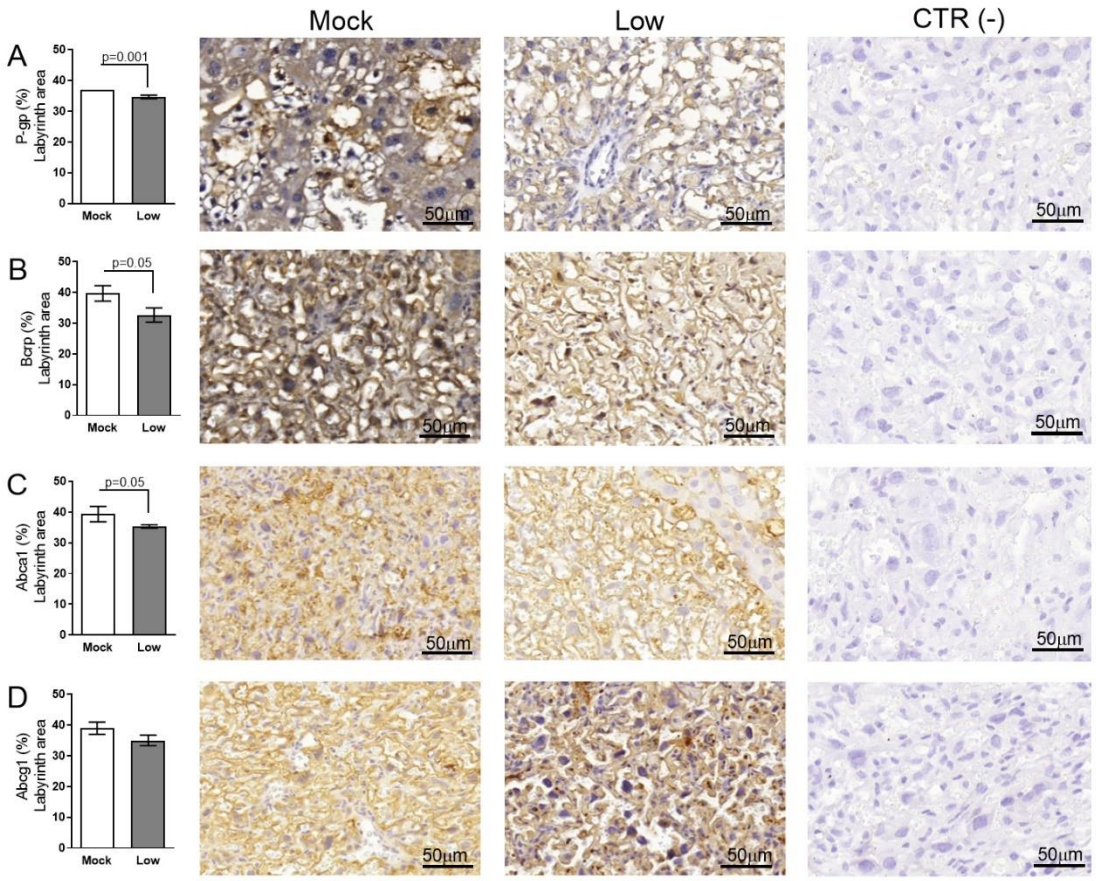

Figure 9: ZIKV infection decreases P-gp, Bcrp and Abca protein expression in the placental LZ of infected mice in the ICompromised groups. Immunostaining quantification and representative immunohistochemistry images of A) P-gp, B) Bcrp, C) Abca1 and D) Abcg1. The images represent the percentage of stained cells in each analyzed field. The values are expressed as the mean ± SEM. Unpaired Student's t-tests or nonparametric Mann-Whitney tests were used for the ICompromised groups. Scale bar = 50 µm. N=3 for each group. CTR(-)=All negative controls were performed with the omission of the primary antibody.

Next, the impact of ZIKV on ABC transporters in the Jz layer (structural and endocrine layers of the mouse placenta) was assessed. P-gp and Bcrp were predominantly localized at the cellular membranes of spongiotrophoblast cells, whereas 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-D and Figure 11A-D).

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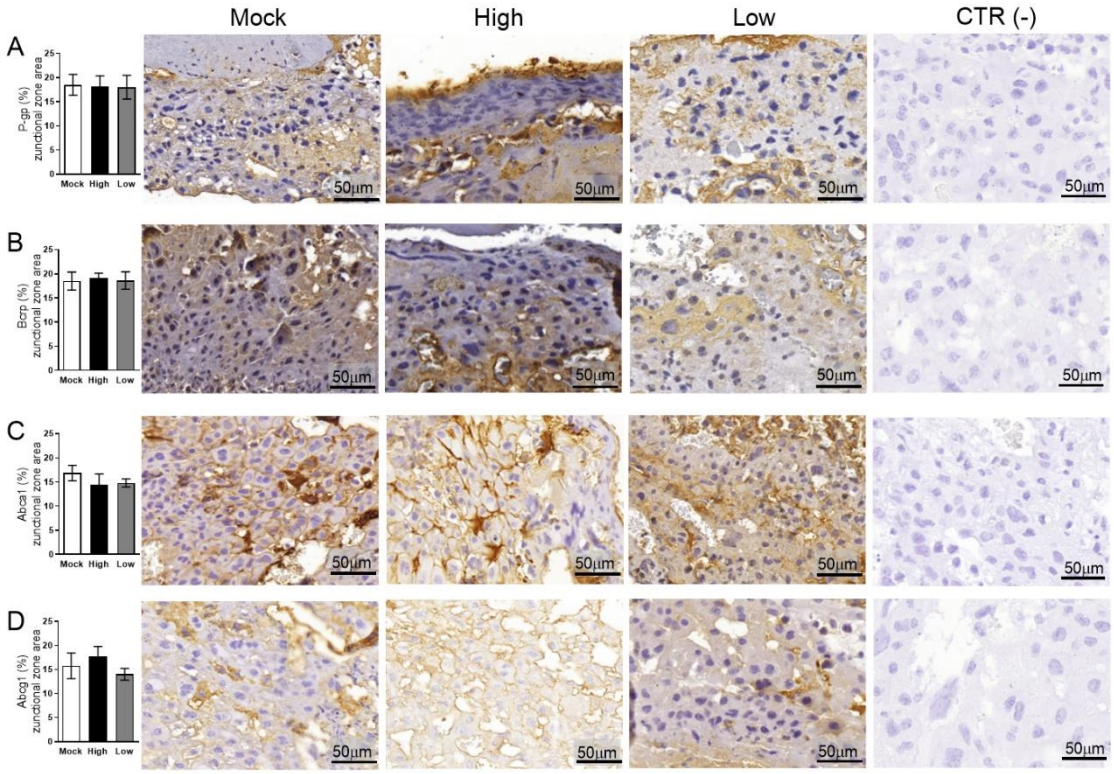

553

554 **Figure 10: ZIKV infection did not impact P-gp, Bcrp, Abca1 or ABcg1 expression**
555 **in the JZ area in infected placentas of ICompetent groups.** Immunostaining
556 quantification and representative immunohistochemistry images of A) P-gp, B) Bcrp, C)
557 Abca1 and D) Abcg1. The images represent the percentage of stained cells/viable tissue.
558 The values are expressed as the mean ± SEM. One-way ANOVA followed by Tukey's
559 post hoc test was used for comparisons between different ICompetent groups. Scale bar
560 = 50 µm. N=6/each group. CTR(-)=All negative controls were performed with the
561 omission of the primary antibody.

562

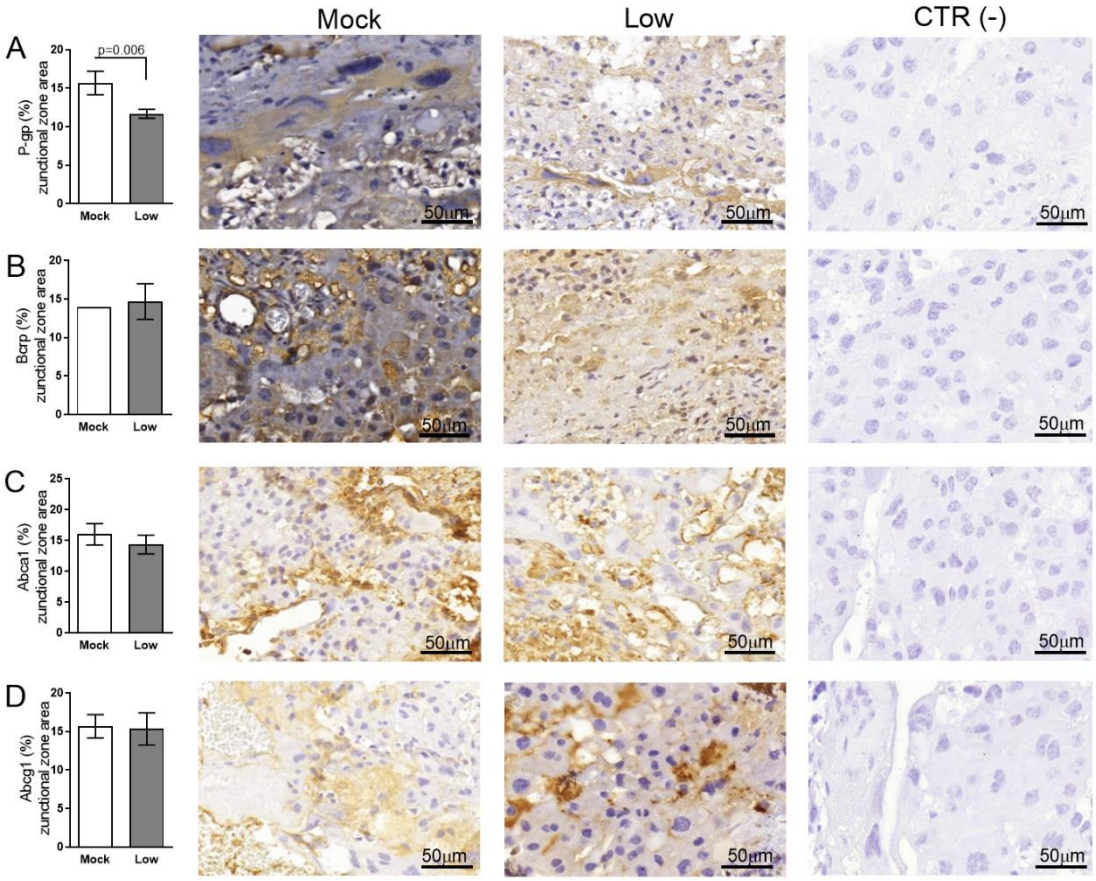

Figure 11: ZIKV infection decreases Bcrp expression in the JZ area in infected placentas of the ICompromised group. Immunostaining quantification and representative immunohistochemistry images of A) P-gp, B) Bcrp, C) Abca1 and D) Abcg1. The images represent the percentage of stained cells/viable tissue. The values are expressed as the mean ± SEM. Unpaired Student's t-tests or nonparametric Mann-Whitney tests were used for the ICompromised groups. Scale bar = 50 µm. N=3 for each group. CTR(-)=All negative controls were performed with the omission of the primary antibody.

4. Discussion

In this study, we investigated several fetuses and placentas feature at term (GD18.5) in ICompetent (C57BL/6) and ICompromised (A129) mice exposed to ZIKV at mid-pregnancy (GD12.5). Fetal survival rates, systemic and placental inflammatory responses, placental ultrastructure and cell turnover, as well as the expression of key drug (P-gp and Bcrp) and lipid (Abca1) efflux transporter systems in the placenta, were consistently impacted by ZIKV in both strains. The magnitude of the effects was is clearly

related to the infective titer (high and low) of ZIKV and maternal immune status (ICompetent-C57BL/6 x and ICompromised-A129) and fetal alterations were not exclusively dependent on the virus detection in the fetuses.

Infection of ICompetent mice with ZIKV did not result in viremia in the initial postinoculation phase, although viral RNA was detected in the maternal spleen in both the high- and low-ZIKV groups, confirming systemic infection. This is consistent with a previous report (45). Our data demonstrate that pregnant ICompetent C57BL/6 mice were more susceptible to higher ZIKV than to lower ZIKV infective titers. Since viral RNA was only detected in the placentae of high ZIKV, and fetal survival rates and weight were impacted to a greater extent in those mice. Strikingly, even though the virus was not present in the fetal brain (at least at term),fetal and fetal head weights were lower in the high-ZIKV titer regimen, suggesting that high infective viral load in mid-pregnancy, even in ICompetent individuals, can induce IUGR and lower fetal head weight despite a lack of transmission to the fetal brain (46). On the other hand, ICompromised placentae and fetal brains had detectable viral transcripts, with no changes in weight, which is consistent with previous data (18). In fact, in our models, the presence (ICompromised) or the absence (ICompetent) of the virus in the fetal brain did not correspond to fetal head size (decreased in only high ICompetent). The data from ICompetent and ICompromised placentae demonstrate how important the maternal immunological status is to control viremia, fetal survival and accessibility of the virus into 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 of 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.

604 A distinct inflammatory profile was also detected in the three analyzed groups. At
605 the protein level, low-ZIKV-ICompromised dams exhibited increased maternal IL-6 and
606 CXCL-1 and placental CCL-2 and CXCL-1, whereas low-ZIKV-ICompetent dams had
607 increased maternal CCL2 and placental IL-6 levels. CCCL-2 and CXCL-1 are related to
608 fetal death and preterm delivery (42–44) and could be associated with pronounced fetal
609 injure detected upon ICompromised pregnancy. In addition, it was previously
610 demonstrated (47) that IL-6 is related to fetal response syndrome, characterized by
611 activation of the fetal immune system. This syndrome is known to increase fetal morbidity
612 and affect several organs, such as the adrenal gland, brain and heart (31,47–50). At the
613 mRNA level, IL6 expression was only detected in ICompromised placentae at term and
614 may indicate a sustained harmful response in these mice until term. IFN signaling
615 pathway may be triggered by ZIKV (16) and represents one of the key mechanisms of
616 host defense and viral targets for immune evasion (19), but we did only detected a slight
617 increase in type I IFN in low-ZIKV ICompentent mice at term. However, we cannot rule
618 out the possibility that these cytokines might have been produced earlier. IFN-γ
619 expression was significantly enhanced in both ICompetent and ICompromised low-ZIKV
620 derived placentas, but not in the high-ZIKV infected mice. Although we could not assess
621 cytokine expression in high-ZIKV ICompromised placentas, one may extrapolate that
622 low-ZIKV infection could result in stimulation of IFN-γ-producing cells, which has been
623 previously showed to be protective for ZIKV-infected mice (51).

624 Both ICompromised and ICompetent mice showed increased expression of
625 placental *Tnf-α* mRNA, which has been demonstrated to be is directly related to placental
626 damage, abortion and premature birth (REF). In addition increased TNF-*α* response is
627 relatated to impaired placental hormone production and trophoblastic invasion and
628 increased apoptosis in pregnancy (52,53). Although we did not assessed TNF-α protein

629 levels in the placenta and maternal blood, this response could be implicated in the overall
630 damage detected.

631 Although differences in placental weight were not observed, ZIKV infection mid-
632 pregnancy had a profound effect on placental cellular turnover, dependent on titer, strain
633 and/or placental compartment. Lz is responsible for fetal and maternal nutrient, gas and
634 waste exchange, while Jz provides structural support, nutrient storage and hormone
635 synthesis (34). ZIKV induced a consistent increase in Lz proliferation in all groups.
636 However, the Lz apoptotic rate was increased only in the high-ZIKV-ICompetent and
637 ICompromised groups and decreased in low-ZIKV-ICompetent mice. The mechanisms
638 underlying these differences are unknown but may be related to the distinct maternal and
639 placental proinflammatory responses and/or to the direct effect of the virus on the
640 placenta (54). Increased Lz apoptosis in the high-ICompetent-ZIKV group may be one of
641 the mechanisms driving the lower fetal and fetal head weight detected in this group. In
642 this context, changes in placental turnover can determine placental maturation and
643 function and lead to fetal distress and developmental abnormalities (55). An increase in
644 the Lz apoptotic ratio may signify damage to this placental layer, which is consistent with
645 the fact that diverse pathological lesions associated with congenital disorders were
646 described in placentae from women infected by ZIKV at different stages of pregnancy
647 (56). Conversely, no proliferative changes were observed in Jz in high-ZIKV and low-
648 ZIKV ICompetent mice, while increased and decreased apoptotic rates were detected. It
649 follows that the lack of Jz-Ki-67 induction may suggest that this layer is less capable of
650 restoring proliferation in response to high-ZIKV challenge, and this may be related to
651 decreased fetal growth.

652 Our placental ultrastructural analysis detected consistent differences across
653 ZIKV-exposed groups. The Lz and Jz layers from both strains exhibited signs of ER

654 stress, i.e., dilated ER cisterns or fragmented ER granular structures. These alterations
655 may result from the accumulation of folded or poorly folded viral proteins in the ER
656 lumen (41,57,58). It has been demonstrated that the *Flaviridae* family uses the ER to
657 replicate (59), and according to Offerdahl et al. (2017)(58), there is evidence of ZIKV
658 interacting with this organelle, promoting an increased release of Ca^{+2} from the ER to the
659 cell cytoplasm, thereby causing an increase in the production of reactive oxygen species
660 (ROS) (60,61).

661 The mitochondrial ultrastructure in the Lz and Jz layers was severely impacted by
662 ZIKV exposure. We found evidence of mitochondrial degeneration, i.e., mitochondrial
663 membrane rupture, absence of mitochondrial ridges and less electron-dense
664 mitochondrial matrix in all treated groups. Placental mitochondrial dysfunction is
665 associated with IUGR (62,63) and may be related, at least in part, to the lower fetal weight
666 observed in high-ZIKV-ICompetent fetuses along with the altered placental apoptotic and
667 proliferative patterns. Furthermore, mitochondrial dysfunction together with ER stress is
668 likely to modify the placental ROS balance and generate local oxidative stress (64), which
669 is associated with impaired fetal development (65). Of importance, associations between
670 mitochondrial disruption, ER stress and placental cell senescence have been reported.
671 Senescence is characterized as an irreversible interruption of the cell cycle and acquisition
672 of a senescence-associated secretory phenotype (SASP) that promotes the release of
673 cytokines such as Il-1, Il-6, Il-8 and proinflammatory proteases (65). Therefore, the
674 increased expression of IL-6 detected in the placentas of ICompromised mice suggest a
675 SASP profile, which may be related to changes in the ER and mitochondrial
676 ultrastructure, accompanied by important changes in apoptosis and cell proliferation. The
677 interactions between mitochondria and the ER are critical for homeostasis and cell
678 signaling (66). In conjunction with the ER, mitochondria can regulate cell death mediators

679 in response to hypoxia and inflammation (67). The increase in apoptosis observed in the
680 high-titer ICompetent groups and the low-titer ICompromised group may be related to
681 the mitochondrial damage and ER stress observed. In fact, we observed an important
682 decrease in microvillus abundance in sinusoidal giant trophoblast cells. Previously, we
683 observed a decrease in microvillus density in the Lz of pregnancies exposed to malaria in
684 pregnancy (MiP) (35). Together, our data show that different gestational infective stimuli
685 (MiP and ZIKV) are capable of damaging placental microvillus abundance and impairing
686 proper fetal-maternal exchange function and fetal growth/survival.

687 Next, to investigate whether maternal ZIKV exposure may influence fetal
688 protection, we evaluated the placental localization and expression (semiquantitative) of
689 the ABC efflux transporter systems P-gp, Bcrp, Abca1 and Abcg1, which are highly
690 enriched in labyrinthine microvilli and in human syncytiotrophoblasts. These efflux
691 transporters exchange drugs, environmental toxins, cytotoxic oxysterols and lipids within
692 the maternal-fetal interface (25). We found a consistent decrease in labyrinthine P-gp
693 expression in all ZIKV-exposed groups, demonstrating that ZIKV infection during
694 pregnancy has the potential to increase fetal exposure to P-gp substrates such as synthetic
695 glucocorticoids, antibiotics, antiretrovirals, antifungals, stomach-protective drugs, and
696 nonsteroidal anti-inflammatory drugs (25). Furthermore, Jz-P-gp was decreased in
697 ICompromised placentae. Although little is known about the Jz function of ABC
698 transporters, our data highlight the need for further studies investigating the biological
699 importance of ABC transporters in the placental endocrine and structural zones of the
700 rodent hemochorial placenta under normal and infective conditions.

701 ZIKV impaired Lz Bcrp and Abca1 expression in ICompetent (high) and
702 ICompromised (low) mice. However, no effects were observed in ICompetent animals at

a low ZIKV titer or in Abcg1 in any experimental setting. As such, ZIKV also likely increases the fetal accumulation of Bcrp substrates (antibiotics, antiretrovirals, sulfonylureas, folate, mercuric species, estrogenic mycotoxins, carcinogens phototoxic compounds, among others) and disrupts placental lipid homeostasis (lipids, cholesterol, cytotoxic oxysterols) by reducing placental Abca1 expression (25,68–71). We can speculate that the increased fetal accumulation of the P-gp, Bcrp and Abca1 substrates during ZIKV infection may contribute to the establishment of congenital Zika syndrome, although additional studies are clearly required to answer this important question. The current data are in agreement with previous publications showing that bacterial, viral and protozoan inflammation alters the expression and/or function of P-gp, Bcrp and Abca1 in biological barriers such as the placenta, yolk sac and blood-brain barriers (25,26,35,59,72–74).

5. Conclusion

Our data show that gestational ZIKV impacts the fetal phenotype independently of term fetal viremia. Abnormal placental cell turnover, ultrastructure and transporter expression may result from the specific proinflammatory responses that depend on the ZIKV infective load and maternal immune status. Fetal accumulation of drugs, environmental toxins and lipids within the fetal compartment may be potentially 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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