1 **Zika virus causes placental pyroptosis and associated adverse fetal outcomes by activating**

2 **GSDME**

3

4 **Authors**

5 Zikai Zhao [a,b,c], Qi Li [a,b,c], Usama Ashraf [b], Mengjie Yang [a,b,c], Wenjing Zhu [a,b,c], Zheng Chen [a,b,c],

6 Shengbo Cao [a,b,c]*, Jing Ye [a,b,c]*

7

8 **Affiliations**

9 [a] State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan

10 430070, China

11 [b] Key Laboratory of Preventive Veterinary Medicine in Hubei Province, College of Veterinary

12 Medicine, Huazhong Agricultural University, Wuhan 430070, China

13 [c] The Cooperative Innovation Center for Sustainable Pig Production, Huazhong Agricultural

14 University, Wuhan 430070, China

15

16 *** Corresponding author:**

17 Jing Ye

18 Email: yej@mail.hzau.edu.cn

19 and

20 Shengbo Cao

21 Email: sbcao@mail.hzau.edu.cn

22

23

Abstract

Zika virus (ZIKV) can be transmitted from mother to fetus during pregnancy, causing adverse fetal outcomes. Several studies have indicated that ZIKV can damage the fetal brain directly; however, whether the ZIKV-induced maternal placental injury contribute to adverse fetal outcomes are sparsely defined. Here, we discovered that ZIKV causes the pyroptosis of placental cells by activating the executor Gasdermin E (GSDME) *in vitro* and *in vivo*. Mechanistically, caspase-8 undergoes activation upon the recognition of 5' untranslated region of viral RNA by RIG-I, followed by the stimulation of caspase-3 to ultimately escalate the GSDME cleavage. Further analyses revealed that the ablation of GSDME in ZIKV-infected pregnant mice attenuates placental pyroptosis, which consequently confers protection against adverse fetal outcomes. In conclusion, our study unveils a novel mechanism of ZIKV-induced adverse fetal outcomes via causing placental cell pyroptosis, which could be employed for developing new therapies for ZIKV-associated diseases.

Significance statement

Several studies have elucidated the link between ZIKV infection and congenital ZIKV syndroms (CZS), but the pathogenesis yet needs further study. Here, we reported a novel pathogenic mechanism of ZIKV which leads to pyroptosis of placental cells through activating the pyroptotic executor GSDME, rather than GSDMD. Upon ZIKV infection, GSDME-mediated pyroptosis damages the structure and function of the placenta, thereby affecting the development of the fetus and contributing to the adverse fetal outcomes. Our study highlights the importance of pyroptotic executor GSDME in regulate ZIKV pathogenicity and further confirms that placental injury caused by ZIKV infection is a key factor for CZS.

Keyword: Zika virus (ZIKV); pyroptosis; Gasdermin E (GSDME); placental injury

Introduction

Zika virus (ZIKV), a mosquito-borne flavivirus, was initially identified in 1947 but received little concern until it posed a serious public health threat in the Pacific from 2007 to 2015(1). ZIKV exhibits obvious tropism and infects several immunologically privileged regions that include male and female reproductive organs, adult and fetal central/peripheral nervous system, urinary tract, and the structural and neural portions of the eye (2). Clinically, most ZIKV cases are asymptomatic or mild with low-grade fever, rash, arthralgia, myalgia, and conjunctivitis (3, 4). Nevertheless, the ZIKV infection of pregnant women still remains a major concern because the placental infection and the vertical transmission of the virus can cause adverse effects on the fetus, such as congenital ZIKV syndrome (CZS) which is characterized by microcephaly, intrauterine growth restriction, spontaneous abortion, and developmental abnormalities (5).

Although several studies have explicitly indicated a causal relationship between ZIKV infection and CZS (6-8), the underlying mechanism is not completely elucidated. On the one hand, ZIKV can infect the fetus through the transplacental route, which can lead to CZS in all trimesters of pregnancy (6, 9, 10). Studies using the brain organoids, neurospheres, and human pluripotent stem cell-derived brain cells have identified that neural stem and progenitor cells can undergo growth and developmental aberrations upon ZIKV infection, thereby resulting in microcephaly (11-13). On the other hand, the ZIKV infection-caused placental injury also likely contributes to adverse fetal outcomes. Several studies have demonstrated that ZIKV infects placental macrophages and trophoblasts, which leads to placental insufficiency and pathology (14-16). Using the mouse pregnancy models, it has been shown that ZIKV infection disrupts the architecture and function of the placenta by triggering trophoblast apoptosis and vascular endothelial cell damage (5, 8, 17, 18). Considering that placenta is infected prior to the fetus and that placenta is indispensable for the maternal-fetal nutrient exchange, it is reasonable to speculate that the placental damage during ZIKV infection could be more decisive for adverse fetal outcomes.

3

During ZIKV infection, the placenta can suffer from apoptosis (5, 18), which is a prototype of programmed cell death (PCD) and is crucial for reproduction, embryonic development, immunity, and viral infections. Therefore, it is meaningful to explore whether other PCD processes, such as pyroptosis, are involved in the ZIKV-induced placental damage. Pyroptosis is an innate immune mechanism against intracellular pathogens, and is featured by cell swelling and the emergence of large bubble-like structures from the plasma membrane that differentiate it from other PCDs (19, 20). Recent studies have identified gasdermin D (GSDMD) as a key pyroptosis executor, whose N-terminus domain is capable of binding to membrane lipids, phosphoinositides, and cardiolipin in order to perforate the membrane, resulting in the release of cytosolic content (21-23). Gasdermin E (GSDME), also known as DFNA5, belongs to the same superfamily as GSDMD, and was originally identified as a gene related to hearing impairment (24). GSDME is considered as a potential tumor suppressor gene in several types of cancers, such as gastric and hepatocellular carcinomas (25-27) Recently, it has also been demonstrated to mediate pyroptosis via its activated N-terminus (GSDME-N) domain that is specifically cleaved by caspase-3 and granzyme B (27, 28). GSDME can switch the caspase-3-mediated apoptosis to pyroptosis or can mediate secondary necrosis after apoptosis (29), indicating an extensive cross-talk between apoptosis and GSDME-mediated pyroptosis.

In the context of viral infections, pyroptosis is one of the imperative pathogenic mechanisms that occur during flavivirus infections. For instance, dengue virus can induce the NLRP3 inflammasome-dependent pyroptosis in human macrophages via the C-type lectin 5A, which is critical for dengue hemorrhagic fever (30, 31). Japanese encephalitis virus enhances the pyroptosis of peritoneal macrophages to raise the serum interleukin-1α level, which in turn promotes viral neuroinvasion and blood-brain-barrier disruption (32). Particularly, a recent study demonstrated the effect of ZIKV in inducing the caspase-1- and GSDMD-mediated pyroptosis of neural progenitor

4

98   cells that contributes to CZS (33). In view of all these studies, it is worth exploring whether

99   pyroptosis participates in the ZIKV-induced placental damage and adverse fetal outcomes.

100   Here, we report that ZIKV infection activates caspase-3 via the caspase-8-mediated extrinsic

101   apoptotic pathway, which in turn activates GSDME to induce the pyroptosis of placental

102   trophoblasts. This phenomenon was found to associate with the ZIKV 5'UTR, but not with the

103   ZIKV structural or nonstructural proteins, upon its recognition by the RIG-I sensor. By establishing

104   a mouse model of ZIKV infection, we further showed that the deletion of GSDME leads to the

105   reduction of placental damage and associated adverse fetal outcomes in infected pregnant mice.

106   This study provides novel insights into the mechanism of ZIKV-induced placental damage and CZS;

107   therefore, it may lead to the development of new therapeutic options to avert adverse fetal outcomes

108   and/or miscarriages during ZIKV infection.

109

110   **Results**

111   **ZIKV infection induces the GSDME-mediated pyroptosis in JEG-3 cells**

112   Previous studies have shown that ZIKV infection during the first trimester of pregnancy leads to a

113   higher chance of CZS, but the pathogenesis yet to be further studied (4, 34). Since the ZIKV-

114   infected placenta shows obvious apoptosis of trophoblasts (5), it prompted us to hypothesize

115   whether other routes of cell death are triggered in response to ZIKV infection. To this end, we chose

116   a ZIKV-permissive human choriocarcinoma cell line JEG-3, which represents the features of

117   trophoblasts and is considered a suitable in vitro model to study the first-trimester placental function

118   (35). Upon ZIKV infection, JEG-3 cells showed evident cell swelling with balloon-like structures

119   originating from the plasma membrane that appears to be distinct from the apoptotic blebbing

120   (Figure 1A), displaying a characteristic pyroptotic cell morphology. Along with decreased cell

121   viability, the swelling cells were also found to release lactate dehydrogenase (LDH, an indicator of

122   the pyroptotic cell cytotoxicity) in an infection time-dependent manner, which depicts the rupture

5

123   and leakage of the plasma membrane (Figure 1B). We next determined whether the pyroptosis

124   executors, GSDMD and GSDME, were activated during the infection of JEG-3 cells with ZIKV.

125   To this end, the infected cells were subjected to Western blot analysis, and the results revealed that

126   infection led to the release of the N-terminal domain of GSDME (GSDME-N), whereas GSDMD

127   exhibited no effect (Figure 1C), thereby implying that ZIKV infection may induce pyroptosis

128   through GSDME. To further confirm this observation, we generated the GSDME-knockout (KO)

129   JEG-3 cell line by employing the CRISPR/Cas9 system (Figure 1D). As expected, deletion of

130   GSDME significantly suppressed the ZIKV-induced cell swelling (Figure 1E) together with

131   diminished LDH release (Figure 1F). Overall, these findings demonstrate that ZIKV infection

132   induces the pyroptosis of JEG-3 cells in the GSDME-mediated manner.

133   **Both the cellular GSDME abundance and the susceptibility to ZIKV infection determine the**

134   **occurrence of pyroptosis**

135   Since there is no previous study reporting that ZIKV can induce GSDME-mediated pyroptosis, we

136   asked whether it is a common cellular process during ZIKV infection. We infected five different

137   human tissue-derived cell lines, including Huh-7 (liver), A549 (lung), HeLa (cervix), HEK-293T

138   (kidney) and SH-SY5Y (neuroblasts) with ZIKV, and subsequently examined their cytopathies. It

139   was observed that Huh-7 and A549 cells showed distinct pyroptotic cell death, elevated LDH levels,

140   and GSDME cleavage upon ZIKV infection, whereas HeLa, HEK-293T, and SH-SY5Y cells

141   exhibited no obvious cytopathic effects even at 72 h post-infection (Figures 2A to C). The GSDME-

142   mediated pyroptosis is closely related to the GSDME abundance (29). We noticed that infected SH-

143   SY5Y cells did not undergo pyroptosis despite expressing the high level of GSDME, but the

144   relatively lower GSDME-expressing Huh-7 and A549 cells presented the different levels of

145   pyroptosis and GSDME activation upon infection (Figures 2A-2C, and S1A), suggesting that other

146   factors are involved in ZIKV-induced pyroptosis.

6

147 In the context of infection, all these cells showed marked discrepancies in terms of susceptibility to

148 ZIKV (36, 37). Among them, JEG-3, Huh-7 and A549 cells displayed the highest susceptibility to

149 ZIKV infection, as assessed by the immunofluorescence assay and the plaque assay (Figure S1B

150 and S1C). The observed positive correlation between the cellular susceptibility to infection and the

151 increased cytopathic effects indicates that the cellular susceptibility to ZIKV infection is probably

152 another key factor that determines the occurrence of pyroptosis (Figure 2D). We then infected JEG-

153 3 cells with ZIKV at an increasing multiplicity of infection (MOI) to investigate whether more viral

154 particles could lead to graver pyroptosis. At 24 h post-infection, the ZIKV-induced LDH release

155 (Figure 2E) and GSDME activation (Figure 2F) were found to be enhanced in a virus dose-

156 dependent manner, which implicit that the productive infection or the pathogen-associated

157 molecular patterns of ZIKV are critical to its pathogenesis. Collectively, these results indicate that

158 the ZIKV-GSDME-mediated pyroptosis is more likely to occur in those cells that are susceptible

159 to ZIKV infection, and with relatively higher GSDME abundance.

160 **ZIKV infection activates GSDME via extrinsic apoptotic pathway**

161 It is known that GSDME is cleaved and activated specifically by caspase-3, which consequently

162 results in pyroptosis (28, 29). Hence, we explored the activation of caspase-3 upon ZIKV infection.

163 At 24 h post-infection, caspase-3 underwent activation and obvious cleavage of GSDME was

164 noticed in infected JEG-3 cells (Figure 3A). To verify if other cell death pathways are involved in

165 the ZIKV-induced pyroptosis, ZIKV-infected JEG-3 cells were incubated with caspase-3 inhibitor

166 (Z-DEVD-FMK), caspase-1 inhibitor (VX-765), pan-caspase inhibitor (Z-VAD-FMK) or necrosis

167 inhibitor (GSK872). As the results are shown in Figure 3B and 3C, ZIKV infection caused the

168 induction of pyroptotic morphological features, and the cell death was partially prevented in the

169 presence of caspase-3 or pan-caspase inhibitors. In line with these findings, the Western blot

170 analysis revealed that caspase-3 or pan-caspase inhibitors significantly attenuated the concomitant

171 cleavage of caspase-3 and GSDME (Figure 3D). In contrast, the treatment of cells with caspase-1

7

172 inhibitor and necrosis inhibitor showed no effect on the cell death caused by ZIKV (Figures 3B and

173 3D), which suggests that necrosis and GSDMD-mediated pyroptosis were not involved in it.

174 Altogether, these data indicate that ZIKV infection facilitates the caspase-3-dependent cleavage of

175 GSDME to elicit pyroptotic cell death.

176 Before the caspase-3 was reported as a key regulator of the GSDME-mediated pyroptosis, it was

177 considered an essential effector at the end of apoptotic cascades. During apoptosis, caspase-3 can

178 be activated by intrinsic and/or extrinsic pathways: the former refers to the permeabilization of the

179 mitochondrial membrane and the assembly of apoptosome resulting in the activation of caspase-9,

180 and the latter involves the activation of death receptors and caspase-8 (38, 39). It has been

181 previously demonstrated that the GSDME-dependent pyroptosis undergoes activation downstream

182 of the intrinsic apoptotic pathway and can potentially be activated downstream of the extrinsic

183 apoptosis pathway (17, 40, 41). However, the engagement of intrinsic and/or extrinsic pathways in

184 order to regulate the GSDME-dependent pyroptosis during ZIKV infection, remains to be studied.

185 To delineate the molecular mechanism by which cellular pyroptosis was instigated under ZIKV

186 infection, we detected the activation of caspase-8 and caspase-9 in infected JEG-3 cells by the

187 Western blot analysis. It is not unexpected to find that ZIKV infection triggered the activation of

188 both caspase-8 and caspase-9 (Figure 3E), considering the fact that various biological processes are

189 triggered during ZIKV infection. Next, the ZIKV-infected cells incubated with caspase-8 inhibitor

190 (Z-IETD-FMK) or caspase-9 inhibitor (Z-LEHD-FMK), and subsequently were subjected to the

191 analysis of pyroptotic cell parameters. It was found that caspase-8 inhibitor remarkably inhibited

192 the cell membrane rupture, LDH release, and GSDME cleavage in infected cells (Figures 3F and3

193 H). Interestingly, the caspase-8 inhibitor treatment also reduced the activation of caspase-9, and the

194 caspase-9 inhibitor treatment slightly inhibited the activation of GSDME without exerting any

195 impact on the ZIKV-induced pyroptotic cell death (Figures 3F and 3H). These findings urged us to

196 reflect on whether caspase-8 was activated before caspase-9 in the GSDME-mediated signaling

8

197 pathway. As recent studies demonstrate that the GSDME-N can permeabilize the mitochondrial

198 membrane and activate the intrinsic apoptotic pathway (42), it implies the possibility that during

199 the activation of GSDME upon ZIKV infection the caspase-8-associated extrinsic apoptosis may

200 have activated first, which consequently targets mitochondria to activate the caspase-9-associated

201 intrinsic apoptosis, thereby creating a self-amplifying feed-forward loop through consecutively

202 activating caspase-3. Given that the activation of death receptors upon ligand binding is a crucial

203 step in the extrinsic apoptosis pathway, we then evaluated the influence of ZIKV infection on the

204 expression of tumor necrosis factor α (TNF-α). ZIKV infection significantly enhanced the

205 expression of TNF-α in infected JEG-3 cells, as determined by quantitative real-time reverse

206 transcription PCR (qRT-PCR) (Figure S2). Despite elusive mechanistic underpinnings, these data

207 suggest that the extrinsic apoptotic pathway plays a key role in mediating the ZIKV-induced

208 pyroptosis, and that the potential crosstalk between pyroptosis and apoptosis during the ZIKV

209 pathogenesis could not be neglected.

210 **The genomic RNA of ZIKV activates the GSDME-dependent pyroptosis through RIG-I**

211 **pathway**

212 In order to explore which part of the ZIKV is responsible for inducing pyroptosis, we first infected

213 JEG-3 cells with ZIKV or UV-inactivated ZIKV to test the contribution of viral structural proteins

214 in inducing the pyroptosis, and found that ZIKV structural proteins are not functional pyroptosis

215 agonists (Figures S3A and S3B). Next, we transfected JEG-3 cells with individual viral non-

216 structural (NS) proteins fused with the N-terminal 3XFlag-tag to find which NS proteins are capable

217 of activating pyroptosis. The results showed that all NS proteins had no effect on pyroptosis

218 (Figures 4A and S3C), hence guiding us to speculate that the viral RNA may participate in

219 orchestrating this phenomenon.

220 For this purpose, we obtained the 5' and 3' untranslated region (UTR) RNA of the ZIKV through

221 the in vitro transcription assay to mimic the viral genome, and subsequently transfected them into

9

222 JEG-3 cells. As the results shown in Figures 4B and 4C, the UTRs induced the distinct pyroptotic

223 cell death and LDH release. In consistent with these results, the Western blot analysis exhibited that

224 the ZIKV UTRs caused the activation of GSDME via the same signaling pathway as induced by

225 ZIKV infection (Figure 4D). It is worth noting that, compared with the ZIKV 5'UTR, the 3' UTR

226 caused relatively a weaker effect on inducing pyroptosis (Figures 4B and 4C), hinting that the

227 recognition of specific viral RNA motifs may aid the GSDME-dependent pyroptosis.

228 Similar to other flaviviruses, ZIKV contains a single-stranded (ss), positive-sense RNA genome.

229 Upon infection of the host cell by RNA viruses, cellular pattern recognition receptors (PRRs) play

230 a decisive role in detecting viral RNAs and restricting viruses' replication. Among the PRRs, the

231 retinoic acid-inducible gene I (RIG-I) and Toll-like receptor (TLR) 7 and 8 are responsible for

232 sensing the viral ssRNA genome (43, 44). Based on the above-mentioned results that the ZIKV

233 RNA genome stimulates pyroptosis, whether RIG-I, TLR7 or TLR8 contributes to the onset of this

234 process was evaluated. The RIG-I-knockdown (KD), TLR7-KD, or TLR8-KD JEG-3 cell lines

235 were generated using the CRISPR/Cas9 system (Figure S4A). ZIKV infection of engineered or

236 wild-type (WT) cells revealed that the KD of RIG-I, but not TLR7 and TLR8, conspicuously

237 attenuated the ZIKV-induced pyroptotic cell death and GSDME cleavage, when compared to that

238 in the WT cells (Figures 4E and 4F). In addition, the KD of RIG-I suppressed the ZIKV-induced

239 production of TNF-α, and the activation of pyroptosis caused by ZIKV 5' UTR (Figures S4B and

240 S4C), suggesting that RIG-I-mediated recognition of ZIKV genome is important for launching

241 pyroptosis. Taken together, these results manifest that the ZIKV genome acts as a pivotal factor in

242 inducing pyroptosis through RIG-I pathway.

243 **ZIKV infection of pregnant immunocompetent WT C57BL/6N mice results in placental**

244 **pyroptosis and CZS**

245 To verify whether ZIKV infection lead to pyroptosis of placental tissue in vivo, WT C57BL/6N

246 mice were infected intravenously with 1×10^6 PFU of ZIKV French Polynesia strain H/PF/2013 or

247 with an equal volume of Vero cell culture supernatant at embryonic day 9.5 (E9.5) of pregnancy.

248 At E16.5, mice were intravenously administrated with propidium iodide (PI) to evaluate cell

249 viability, and were sacrificed 15 min later. Placentas and individual fetuses were evaluated for

250 morphological appearance (scheme outlined in Figure 5A). Notably, most of dams infected with

251 ZIKV showed abnormal pregnancies (Figure S5A), and the fetuses of mock-infected mice

252 displayed apparent differences compared to the ZIKV-infected mice, which contained fetuses

253 showing a variable degree of CZS (Figure 5B). Among all 14 ZIKV-infected dams, only 2 dams

254 displayed normal pregnancies. The remaining 12 dams exhibited abnormal pregnancies, with 4

255 dams containing only placental residues and embryonic debris as all fetuses had undergone

256 resorption. Other 8 infected dams carried at least one or more placental residues and

257 morphologically abnormal fetuses. Out of 109 implantation sites carried by all 14 infected dams,

258 67 (61%) sites were affected, among which 52 showed fetal resorptions and 15 showed growth

259 restriction and malformation (Figures S5C and S5B). These data indicate that ZIKV infection

260 indeed leads to CZS in our experimental model.

261 Furthermore, the general histological examination of the placentas was conducted in order to

262 observe the pathological changes associated with ZIKV infection. Mock-infected placentas

263 presented normal features of the embryo-derived junctional and labyrinth zone, a place where the

264 nutrient exchange occurs (Figure 5D). In infected placentas, obvious abnormal morphological

265 changes were noticed, including hyperplasia, necrosis and thrombi. Specifically, the hyperplastic

266 trophoblast labyrinth showed denser cellularity and less vascular spaces, suggesting that the

267 placental and embryonic blood circulation may have been compromised. Otherwise, abnormal

268 spheroid structures were found in the junctional and labyrinth zone, which contained necrotic

269 trophoblast cells, and thrombi were observed in the labyrinth zone. Hence, these observed changes

270 indicate that ZIKV infection contributes to placenta pathology.

11

271 As previous studies reported that the placental pathology during ZIKV infection corresponds to

272 adverse fetal outcomes (5, 15), we asked whether the GSDME-mediated placental pyroptosis is

273 involved in regulating this phenomenon. We first tested the activation of caspase-3 (a key signaling

274 molecule upstream of GSDME), and the immunohistochemistry assay showed strong activation of

275 it in the trophoblast labyrinth (Figure 5E). Moreover, we conducted the PI staining assay by

276 considering the fact that pyroptotic cells are permeable to small molecular weight, membrane-

277 impermeable dyes such as 7-aminoactinomycin D, ethidium bromide, and PI (20). No PI-positive

278 cells were visualized in the placentas of mock-infected dams. In contrast, in the placentas of ZIKV-

279 infected dams, we detected PI-positive signal in the decidua and labyrinth zone, suggesting the

280 occurrence of pyroptosis (Figure 5F). Taken together, these findings emphasize the importance of

281 ZIKV-induced placental injury in developing CZS, and establish a link between placental injury

282 and pyroptosis.

283 **GSDME contributes to the ZIKV-induced CZS through mediating placental pyroptosis**

284 To determine the role of GSDME in contributing to the ZIKV-induced placental damage and

285 adverse fetal outcomes, Gsdme$^{-/-}$ mice were used. WT or Gsdme$^{-/-}$ mice were infected intravenously

286 with 1×10^6 PFU of ZIKV French Polynesia strain H/PF/2013 or with an equal volume of Vero

287 cell culture supernatant at E9.5 of pregnancy, and were sacrificed 7 days later at E16.5 (scheme

288 outlined in Figure 5A). Although ZIKV-infected Gsdme$^{-/-}$ dams also showed a certain degree of

289 abnormal pregnancies, the number and severity of affected dams and fetuses were markedly

290 decreased compared to the WT dams (Figures 6A-6B, and S6). Among all 10 ZIKV-infected

291 Gsdme$^{-/-}$ dams, 4 dams showed abnormal pregnancies with a mixture of placental residues and

292 stunted embryonic growth. The case in which all fetuses were resorbed in a dam vanished, and all

293 fetuses carried by the rest of 6 dams showed no morphological abnormalities. While 67 (61%)

294 fetuses carried by the ZIKV-infected WT dams were affected, the affected fetuses in the ZIKV-

295 infected Gsdme$^{-/-}$ dams were only 18 (20%), among which 14 showed fetal resorptions and 4

296 showed growth restriction and malformation. Thus, these data indicate that GSDME is associated

297 with ZIKV-induced adverse fetal outcomes.

298 We next performed the histological examination of placentas regardless of whether their

299 corresponding fetuses were affected or not. Compared to the placentas from ZIKV-infected WT

300 dams, trophoblastic hyperplasia, reduced vascular spaces, abnormal spheroid structures, and

301 necrotic cells were not seen in the placentas from ZIKV-infected Gsdme$^{-/-}$ dams (Figure 6C). In

302 addition, the PI staining assay revealed relatively fewer PI-positive signals in the placentas from

303 ZIKV-infected Gsdme$^{-/-}$ dams, implying that the deficiency of GSDME inhibits the pyroptosis of

304 placentas (Figure 6D).

305 In order to rule out the possibility that the variable infection status of ZIKV in WT and Gsdme$^{-/-}$

306 mice may confer variable fetal outcomes, we measured the ZIKV RNA levels in maternal tissues

307 (spleen, brain, and serum), placentas, and fetuses (fetal brain). Comparable ZIKV RNA levels were

308 detected in the spleens of these two types of mice, but the viral RNA remained undetectable in their

309 brains and serum (Figure 6E). No significant differences were found in viral copies and infection

310 ratio between the placentas and fetal brains from ZIKV-infected WT and Gsdme$^{-/-}$ dams (Figure

311 6F). Thereby, based on these data, we conclude that maternal GSDME does not affect ZIKV

312 infection, but the GSDME in placenta promotes CZS by mediating placental pyroptosis.

313

314 **Discussion**

315 In this study, we identified a novel mechanism that ZIKV utilizes GSDME to induce the pyroptosis

316 of placental cells in vitro and in vivo, which consequently associates with adverse fetal outcomes

317 in infected mice. These data established a potential relevance of the placental pyroptotic process

318 with the ZIKV-associated adverse fetal outcomes, thus suggesting that placental pyroptosis is a

319 vital feature of ZIKV pathogenesis.

320   Previously, it has been reported that ZIKV infection activates inflammatory pathways and

321   facilitates the secretion of pro-inflammatory cytokine interleukin-1β (IL-1β) in vitro and in vivo

322   (45, 46). ZIKV NS5 and NS1 proteins, by interacting with NLRP3 and by stabilizing caspase-1,

323   respectively, promote the NLRP3 inflammasome assembly/activation to augment the IL-1β

324   secretion (47, 48). Since the NLRP3 inflammasome functions upstream of pyroptosis executor

325   GSDMD and IL-1β is passively released during cell lysis (20), it can be assumed that pyroptosis

326   may confer ZIKV pathogenesis. Interestingly, other than the mechanisms mentioned above, our

327   results demonstrate that ZIKV triggered pyroptosis through activating GSDME in placenta. So far,

328   researches on GSDME have mainly focused on its anti-cancer effects, but little is known about its

329   function in the pathogenesis of virus. Our research not only identifies for the first time that GSDME

330   can be activated by ZIKV, but also hints the important role of GSDME in viral pathogenesis.

331   Different from a recent study indicating that ZIKV induces the GSDMD-mediated pyroptosis of

332   neural progenitor cells (33), our data revealed a previously unrecognized mechanism of ZIKV-

333   induced fetal adverse outcomes, which is indirectly caused by GSDME-mediated pyroptosis.

334   Unlike the GSDMD-mediated pyroptosis driven by caspase-1 and caspase-4/5/11, we found that

335   GSDME undergoes activation by caspase-8 and caspase-3, which is partially consistent with

336   published studies, wherein GSDME was subjected to be cleaved and activated by caspase-3 and

337   granzyme B (22, 23, 29, 41).

338   In our study, pyroptosis was not found as a universal phenomenon upon ZIKV infection, despite

339   the fact that ZIKV can infect multiple cell lines. For instance, ZIKV-infected HeLa, HEK293T, and

340   SH-SY5Y cells exhibited no apparent pyroptotic morphological changes, albeit some of them

341   express GSDME in abundance. By evaluating the susceptibility of low GSDME-expressing HEK-

342   293T and Huh-7 cells to ZIKV infection, we found that the infection susceptibility is an additional

343   decisive factor, as Huh-7 cells, which are highly susceptible to ZIKV, were prone to pyroptosis

344   (Figures 2A-2C and EV1). These findings not only highlight the important role of GSDME in

14

345　pathogenesis of ZIKV, but also raise the question of whether other ZIKV-induced symptoms are

346　associated with GSDME-mediated pyroptosis, which could be alluring to investigate in future

347　studies.

348　ZIKV-induced apoptosis has been widely described in the central nervous system and the female

349　reproductive system, in which caspase-3 plays a crucial role (5, 17). Given that caspase-3 is

350　downstream of caspase-8 and caspase-9, which individually correspond to extrinsic and intrinsic

351　apoptosis pathways, caspase-8- and caspase-9-specific inhibitors were used to assess their

352　respective effects on activating the caspase-3-GSDME-mediated pyroptosis during ZIKV infection

353　of JEG-3 cells. Inhibition of caspase-8 attenuated the pyroptotic cell features and GSDME cleavage

354　in infected cells, whereas caspase-9 inhibition caused no such effects in a significant manner. Our

355　observation of increased TNF-α production in ZIKV-infected cells also supports this conclusion,

356　as TNF-α is a vital ligand to commence the caspase-8-mediated signaling (49). Caspase-8 is a

357　multifunctional effector protein related to various signaling pathways, and acts as a molecular

358　switch for apoptosis, necrosis, and pyroptosis (50). In the case of pyroptosis, caspase-8 is capable

359　of inducing the GSDMD- and GSDME-mediated pyroptosis in murine macrophages (51-53).

360　However, our data showed that GSDMD was not activated by caspase-8 in ZIKV-infected JEG-3

361　cells, inferring the possibility that some specific conditions are required for inducing the GSDMD-

362　mediated pyroptosis. In addition, caspase-8 inhibits necrosis regulated by receptor-interacting

363　serine/threonine kinase 3 (49), which fortifies our conclusion that necrosis did not occur in ZIKV-

364　infected JEG-3 cells.

365　During the viral infection of host cells, PRRs are vital for detecting the viral genomes and limiting

366　the spread of viruses (54). For instance, RIG-I and TLR7 recognize the genomic RNAs of hepatitis

367　C and West Nile viruses, respectively, to induce host defense m(55, 56). Based on our findings that

368　the ZIKV RNA genome, rather than the ZIKV-encoded proteins, spurs pyroptosis, we asked if PPRs,

369　including TLR7, TLR8, and RIG-I were involved in the occurrence of this cell death process. Using

15

370 the CRISPR-Cas9-engineered respective KD JEG-3 cells, we observed the necessity of RIG-I to

371 induce the GSDME-mediated pyroptosis via recognizing the ZIKV RNA genome, with a piece of

372 further evidence that the ZIKV 5'UTR was more effective than the 3'UTR in initiating pyroptosis.

373 These data are in concordant with a recent study which demonstrated that RIG-I recognizes the

374 ZIKV 5'UTR in human cells and the enrichment of viral RNA on RIG-I decreases along with the

375 genome toward the 3'UTR (57), hence proposing a possibility that the activation degree of RIG-I

376 determines the outcome of pyroptosis.

377 Several studies by using immunocompromised mouse models have demonstrated the detection of

378 ZIKV in the placentas and embryos, resulted in trophoblast apoptosis, vascular damage, loss of

379 placental structure, and reduced neonatal brain cortical thickness (5, 15). Under natural conditions,

380 mice are resistant to ZIKV due to the activation of type-I interferon and research has illustrated that

381 type-I interferon signaling may play a vital role in mediating fetal demise after ZIKV infection by

382 causing abnormal placental development (18), implying that maternal immune is response to

383 undertake the antiviral role and also promotes the occurrence of pathologies. In this context, to

384 simulate the pathological changes triggered by ZIKV-infection in the natural state as much as

385 possible, we employed immunocompetent mice for experiments. It is worth noting that regardless

386 of whether placental lesions and fetal resorption occurred or not, the ZIKV RNA was infrequently

387 detected, which suggests that placental function may be affected under a negligible quantity of viral

388 particles, or the virus had been eliminated at this stage. As the placenta acts as a crucial tissue

389 between the gravida and embryo, preventing pathogen transmission during pregnancy and

390 mediating the exchange of nutrients imply that any inappreciable placental damage can lead to

391 devastating consequences.

392 In summary, our study identified a novel mechanism of ZIKV pathogenesis that causes placental

393 pyroptosis in vitro and in vivo, triggered upon the recognition of the viral genome by RIG-I

394 followed by the caspase-8- and caspase-3-mediated activation of pyroptosis executor GSDME in

16

395   placental cells. The ablation of GSDME in infected pregnant mice attenuated placental pyroptosis

396   and adverse fetal outcomes. These findings may provide opportunities to establish therapies that

397   might halt placental damage and subsequent adverse fetal outcomes during ZIKV infection.

398

Materials and Methods

Ethics statement

401   All animal studies were conducted in strict accordance with the Guide for Care and Use of

402   Laboratory Animals of Laboratory Animal Centre, Huazhong Agriculture University, and all

403   experiments conform to the relevant regulatory standards. The experiments and protocols were

404   approved by the Animal Management and Ethics Committee of Huazhong Agriculture University

405   (Assurance number HZAUMO-2020-0053).

406

Reagents and antibodies

408   Mouse monoclonal anti-ZIKV NS5 antibody was generated in our lab. Rabbit monoclonal anti-

409   GSDME (ab215191) antibody was purchased from Abcam. Mouse monoclonal anti-GAPDH

410   (AC002), mouse monoclonal anti-Flag (AE005), rabbit monoclonal anti-TLR7 (A19126), rabbit

411   polyclonal anti-TLR8 (A12906), and rabbit monoclonal anti-caspase-3 (A19654) antibodies were

412   purchased from ABclonal Technology. Rabbit polyclonal anti-GSDMD (96458), mouse

413   monoclonal anti-caspase-8 (9746), and rabbit polyclonal anti-caspase-9 (9502) antibodies were

414   purchased from Cell Signaling Technology. Rabbit polyclonal anti-RIG-I (20566-1-AP) was

415   purchased from Proteintech. Horseradish peroxidase-labeled goat anti-mouse (AS003) or anti-

416   rabbit (AS014) secondary antibodies were obtained from ABclonal Technology.

417   The chemical reagents DAPI (D8417), PI (P4170) and Hoechst (B2261) were purchased from

418   Sigma-Aldrich. The inhibitors specific to pan-caspase (zVAD-FMK), caspase-1 (VX-765),

17

419 caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK), caspase-9 (Z-LETD-FMK), and necrosis

420 (GSK-872) were purchased from Selleck.

421 Cell culture, viruses and plaque assay

422 All cell lines were obtained from the American Type Culture Collection (ATCC). Human

423 embryonic kidney (HEK-293T), human alveolar epithelial adenocarcinoma (A549), human

424 hepatocellular carcinoma (Huh-7), and African green monkey kidney (Vero) cells were cultured in

425 Dulbecco's modifed Eagle's medium (DMEM; Sigma) supplemented with 100 U/ml penicillin, 100

426 g/ml streptomycin, and 10% fetal bovine serum (FBS, GIBCO). Aedes albopictus mosquito (C6/36)

427 and human cervical cancer (HeLa) cells were cultured in RPMI-1640 medium (Hyclone)

428 supplemented with 10% FBS and 2 mM L-glutamine. Human neuroblastoma (SH-SY5Y) and

429 human choriocarcinoma/trophoblastic cancer (JEG-3) cells were grown in DMEM supplemented

430 with 10% FBS and MEM Non-essential Amino Acid Solution (Sigma). C6/36 cells were grown at

431 28°C in a 5% CO2 incubator, whereas all other cells were grown at 37°C in a 5% CO2 incubator.

432 The ZIKV strain H/PF/2013 was propagated in Vero cells and were titrated by plaque assays on

433 Vero cells. In brief, relevant cells were inoculated with ZIKV at a MOI of 0.1, and the culture

434 supernatants were harvested at 12-72 h post-infection. The supernatants were then serially diluted

435 and inoculated onto the monolayers of Vero cells. After 1 h of absorption, cells were washed with

436 serum-free DMEM and cultured in DMEM containing 3% FBS and 1.5% sodium carboxymethyl

437 cellulose (Sigma-Aldrich). Visible plaques were counted, and viral titers were calculated after 5

438 days of incubation.

439

440 **Cytotoxicity assay**

441 Cell death was assessed by the LDH assay using the CytoTox 96 Non-Radioactive Cytotoxicity

442 Assay kit (Promega). Cell viability was determined by the CellTiter-Glo Luminescent Cell Viability

18

443 Assay kit (Promega). All the relevant experiments were performed following the manufacturer's

444 instructions.

445

446 **Western blot analysis**

447 Total cell lysates were prepared using the RIPA buffer (Sigma) containing protease inhibitors

448 (Roche). After sonication, the protein concentration in each sample was determined by using the

449 BCA protein assay kit (Thermo Fisher Scientific) and boiled at 95°C for 10 min. Equivalent

450 amounts of protein samples were separated by SDS-PAGE and electroblotted onto a polyvinylidene

451 fluoride membrane (Roche) using a Mini Trans-Blot Cell (Bio-Rad). The membranes were blocked

452 at room temperature (RT) for 2 h in PBS containing 3% bovine serum albumin (BSA), followed by

453 incubation with the indicated primary antibodies overnight at 4°C. After washing three times with

454 TBS-Tween (50 mM Tris-HCl, 150 mM NaCl, and 0.1% [v/v] Tween 20, pH 7.4), the membranes

455 were incubated with secondary antibodies at RT for 45 min. Finally, the membranes were visualized

456 with a chemiluminescence system (Bio-Rad) after three times of wash.

457

458 **Microscopy and immunofluorescence analysis**

459 To examine the morphology of pyroptotic cells, relevant cells were seeded in 6-well plates at ~40%

460 confluency and then subjected to indicated treatments. Phase contrast cell images were captured by

461 the NIKON Ti-U microscope.

462 For immunofluorescence analysis, relevant cells were seeded in 12-well plates and infected with

463 ZIKV at a MOI of 0.1. At indicated time post-infection, cells were fixed with pre-cold methanol

464 for 10 min at −20°C. Subsequently, cells were washed three times by PBS and were blocked in PBS

465 containing 1% BSA for 1 h at RT. Thereafter, cells were incubated with anti-ZIKV NS5 antibody

466 for 2 h at RT., followed by washing three times with PBS and incubation with Alexa Fluor 488-

467 conjugated goat anti-mouse (Invitrogen) for 45 min. Cells were then washed and incubated with

19

468 DAPI for another 10 min at RT. After final washing, cells were visualized using the EVOS FL auto

469 (Thermo Fisher Scientific).

470

471 **Plasmid construction and transfection**

472 Using the ZIKV cDNA as template, NS1, NS2A, NS2B3P, NS3H, NS4A, NS4B, and NS5 were

473 separately cloned into the p3XFLAG-CMV-10 using the PCR/restriction digest-based cloning

474 method, and were finally verified by sequencing. Transfections were performed using the

475 Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

476

477 **CRISPR-Cas9 knockdown and knockout cells**

478 Two guide RNAs (gRNAs) target to each desired gene were cloned into the lentiviral vector

479 lentiCRISPR v2. About 800 ng lentiviral vector, 400 ng packaging plasmid pMD2.G, and 800 ng

480 pSPAX2 were co-transfected into HEK 293T cells using the FuGENE HD Transfection Reagent

481 (Promega) according to the manufacturer's instructions. At 48 h post-transfection, viral

482 supernatants were collected, and then inoculated to 4×105 JEG-3 cells for another 48 h. For RIG-

483 I, TLR-7, and TLR-8 knockdown in JEG-3 cells, the gRNA-expressing cells were selected with 1.5

484 µg/ml puromycin, and then plated into 12-well plates for further experiments. For GSDME

485 knockout in JEG-3 cells, the gRNA-expressing cells were selected with 1.5 µg/ml puromycin

486 following the cell sorting using a flow cytometry system. The single-cell clones were cultured in

487 96-well plates for another 10 days or longer. The immunoblotting was used to screen for GSDME-

488 deficient clones and to verify the knockdown efficiency. The genotyping of the knockout cells was

489 determined by sequencing.

490 The sequences of gRNAs were as follows:

491 gRNA-GSDME-1, 5'-TAAGTTACAGCTTCTAAGTC-3';

492 gRNA-GSDME-2, 5'-CAGTTTTTATCCCTCACCCT-3';

493   gRNA-RIG-I-1, 5'-GGATTATATCCGGAAGACCC-3';

494   gRNA-RIG-I-2, 5'-TCCTGAGCTACATGGCCCCC-3';

495   gRNA-TLR-7-1, 5'-GGTGAGGTTCGTGGTGTTCG-3';

496   gRNA-TLR-7-2, 5'-CCTGCGGTATCTCTAGTAGC-3';

497   gRNA-TLR-8-1, 5'-GTGCAGCAATCGTCGACTAC-3';

498   gRNA-TLR-8-2, 5'-AATCCCGGTATACAATCAAA-3'.

499

Mice and infections

501   C57BL/6N WT mice were purchased from Vital River Laboratory Animal Technology Co.

502   Gsdme$^{-/-}$ mice were kindly provided by Prof. Feng Shao (National Institute of Biological Sciences,

503   Beijing). Mice were set up for timed matings, and were injected intravenously with 50 μL Vero cell

504   culture supernatant or 1×10^6 PFU of ZIKV in a volume of 50 μL at the E9.5. Mice were then

505   sacrificed at E16.5, and placentas, fetuses, and maternal tissues were harvested for the subsequent

506   analyses.

507

Measurement of viral burden

509   ZIKV-infected pregnant mice were euthanized at E16.5. Maternal tissues, fetuses, and their

510   corresponding placentas were harvested. Samples were weighed and homogenized with stainless

511   steel beads in 1mL of DEME supplemented with 2% heat-inactivated FBS. All homogenized tissues

512   were stored at -80°C until virus titration was performed. Some placentas were divided equally into

513   two parts, and one part was fixed in 4% paraformaldehyde for histological examination.

514   Total RNA in homogenized samples and serum were extracted using the TRIzol Reagent

515   (Invitrogen) according to the manufacturer's instructions, and were reverse-transcribed to cDNA

516   with random hexamers. ZIKV RNA levels were determined by quantitative real-time PCR on a

517   7500 Real-time PCR system (Applied Biosystems). Viral burden was expressed on a log10 scale

21

518 as viral RNA equivalents per g or per mL after comparison with a standard curve produced using

519 the serial 10-fold dilutions of ZIKV RNA. The ZIKV-specific primer set and probe were used as

520 published previously (58): forward primer, 5'-CCGCTGCCCAACACAAG-3'; reverse primer, 5'-

521 CCACTAACGTTCTTTTGCAGACAT-3'; and Probe, 5'-/56-

522 FAM/AGCCTACCT/ZEN/TGACAAGCAATCAGACACTCAA/3IABkFQ/-3' (Sangon).

523

Histological and immunohistochemistry staining

525 For histological staining, placentas were fixed in 4% paraformaldehyde overnight at 4°C and

526 embedded in paraffin. At least three placentas from different litters were sectioned and stained with

527 hematoxylin and eosin.

528 For immunohistochemistry staining, ~5 μm thick paraffin sections were placed in 3% H2O2 for 30

529 min to quench the endogenous peroxidase activity, and then the sectioned slides were incubated in

530 citrate buffer at 96°C for 30 min for antigen retrieval. After washing with PBS containing 0.1%

531 Tween 20, the sections were blocked in 5% BSA for 1 h and incubated overnight at 4°C with rabbit

532 anti-cleaved-caspase-3 primary antibody (Servicebio) diluted in PBS with 0.1% Tween 20. After

533 washing, the sections were incubated with secondary antibody (horseradish peroxidase-labeled

534 sheep anti-rabbit IgG, Beijing ZSGB-BIO Co., Ltd.) for 45 min. Finally, the slides were developed

535 using the 3,3'-diaminobenzidine (DAB), and hematoxylin was used for counterstaining. All

536 immunohistochemical sections were scanned with a Leica Apero CS2 slide scanning system.

537

In vivo propidium iodide (PI) staining assay of placental pyroptosis

539 Mock- or ZIKV-infected mice were administered PI (1.5 mg/kg) via the intravenous route at E16.5

540 before subjecting them to sacrifice. About 15 min later, mice were euthanized, and the placentas

541 were collected and embedded in the O.C.T compound (SAKURA, 4583). After slicing, the slides

542 were stained with Hoechst and scanned on EVOS FL auto (Thermo Fisher Scientific).

543

In vitro RNA transcription and transfection

The DNA fragments corresponding to the 5' UTR and 3' UTR of ZIKV genome were cloned using the cDNAs from viral stocks. The 5' and 3' UTR fragments were amplified by PCR using the following primers: 5' UTR forward primer 5'-CGGGGTACCAGTTGTTGATCTGTGTGAATCAGA-3', 5' UTR reverse primer 5'-CCGCTCGAGGACCAGAAACTCTCGTTTCCAAA-3', 3' UTR forward primer 5'-CGGGGTACCGCACCAATCTTAGTGTTGTCAGGCC-3', and 3' UTR reverse primer 5'-CCGCTCGAGAGACCCATGGATTTCCCCAC-3'. Amplified fragments were digested with KpnI and XhoI and cloned into the pre-cut pcDNA4/myc-His A vector (Invitrogen) by using the DNA ligation Kit (Takara). Plasmid DNA was then linearized with the restriction enzyme XhoI, and used as a template for T7 in vitro transcription using the mMESSAGE mMACHINE T7 Transcription Kit (Ambion). The RNA was precipitated with lithium chloride and quantified by spectrophotometry. Transfections were performed using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions

558

RNA extraction and quantitative real-time PCR

Total RNA in treated cells was extracted using TRIzol Reagent (Invitrogen), and 1 µg RNA was used to synthesize cDNA using a first-strand cDNA synthesis kit (TOYOBO). Quantitative real-time PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems) and SYBR Green PCR Master Mix (TOYOBO). Data were normalized to the level of β-actin expression in each sample. The primer pairs used were as follows: β-actin: forward 5'-AGCGGGAAATCGTGCGTGAC-3', reverse 5'-GGAAGGAAGGCTGGAA GAGTG-3'; TNF-α: forward 5'-CCTCTCTAATCAGCCCTCTG-3', reverse 5'-GAGGACCTGGGAGTAGATGAG-3'.

23

568

Statistical analysis

All data were analyzed with the GraphPad Prism software. For viral burden analysis, the log titers

and levels of viral RNA were analyzed by nonparametric Mann-Whitney tests. Contingency data

were analyzed by Fisher's exact test using numbers in each group. The statistical analysis of

differences between two groups was performed using two-tailed Student's t-test. For all statistical

significance indications in this manuscript, ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p <$

0.05 and ns, no significance.

576

589

Author contributions:

J.Y. and S.C. conceived and supervised the experiments. Z.Z., Q.L., M.Y., W.Z. and Z.C.

performed the experiments. Z.Z. and J.Y. planned experiments and analyzed results. Z.Z., J.Y. and

593   U.A. wrote the manuscript. J.Y. and S.C. checked and finalized the manuscript. All authors read

594   and approved the final manuscript.

595

596   **Competing interests:**

597   The authors declare that they have no competing interests.

598

References

1. Petersen LR, Jamieson DJ, Powers AM, Honein MA. 2016. Zika Virus. N Engl J Med 374:1552-63.

2. Zimmerman MG, Wrammert J, Suthar MS. 2020. Cross-Reactive Antibodies during Zika Virus Infection: Protection, Pathogenesis, and Placental Seeding. Cell Host Microbe 27:14-24.

3. Duffy MR, Chen TH, Hancock WT, Powers AM, Kool JL, Lanciotti RS, Pretrick M, Marfel M, Holzbauer S, Dubray C, Guillaumot L, Griggs A, Bel M, Lambert AJ, Laven J, Kosoy O, Panella A, Biggerstaff BJ, Fischer M, Hayes EB. 2009. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med 360:2536-43.

4. Musso D, Gubler DJ. 2016. Zika Virus. Clin Microbiol Rev 29:487-524.

5. Miner JJ, Cao B, Govero J, Smith AM, Fernandez E, Cabrera OH, Garber C, Noll M, Klein RS, Noguchi KK, Mysorekar IU, Diamond MS. 2016. Zika Virus Infection during Pregnancy in Mice Causes Placental Damage and Fetal Demise. Cell 165:1081-1091.

6. Brasil P, Pereira JP, Jr., Moreira ME, Ribeiro Nogueira RM, Damasceno L, Wakimoto M, Rabello RS, Valderramos SG, Halai UA, Salles TS, Zin AA, Horovitz D, Daltro P, Boechat M, Raja Gabaglia C, Carvalho de Sequeira P, Pilotto JH, Medialdea-Carrera R, Cotrim da Cunha D, Abreu de Carvalho LM, Pone M, Machado Siqueira A, Calvet GA, Rodrigues Baiao AE, Neves ES, Nassar de Carvalho PR, Hasue RH, Marschik PB, Einspieler C, Janzen C, Cherry JD, Bispo de Filippis AM, Nielsen-Saines K. 2016. Zika Virus Infection in Pregnant Women in Rio de Janeiro. N Engl J Med 375:2321-2334.

7. Mlakar J, Korva M, Tul N, Popovic M, Poljsak-Prijatelj M, Mraz J, Kolenc M, Resman Rus K, Vesnaver Vipotnik T, Fabjan Vodusek V, Vizjak A, Pizem J, Petrovec M, Avsic Zupanc T. 2016. Zika Virus Associated with Microcephaly. N Engl J Med 374:951-8.

8. Yockey LJ, Varela L, Rakib T, Khoury-Hanold W, Fink SL, Stutz B, Szigeti-Buck K, Van den Pol A, Lindenbach BD, Horvath TL, Iwasaki A. 2016. Vaginal Exposure to Zika Virus during Pregnancy Leads to Fetal Brain Infection. Cell 166:1247-1256 e4.

9. Hoen B, Schaub B, Funk AL, Ardillon V, Boullard M, Cabie A, Callier C, Carles G, Cassadou S, Cesaire R, Douine M, Herrmann-Storck C, Kadhel P, Laouenan C, Madec Y, Monthieux A, Nacher M, Najioullah F, Rousset D, Ryan C, Schepers K, Stegmann-Planchard S, Tressieres B, Volumenie JL, Yassinguezo S, Janky E, Fontanet A. 2018. Pregnancy Outcomes after ZIKV Infection in French Territories in the Americas. N Engl J Med 378:985-994.

10. Reynolds MR, Jones AM, Petersen EE, Lee EH, Rice ME, Bingham A, Ellington SR, Evert N, Reagan-Steiner S, Oduyebo T, Brown CM, Martin S, Ahmad N, Bhatnagar J, Macdonald J, Gould C, Fine AD, Polen KD, Lake-Burger H, Hillard CL, Hall N, Yazdy MM, Slaughter K, Sommer JN, Adamski A, Raycraft M, Fleck-Derderian S, Gupta J, Newsome K, Baez-Santiago M, Slavinski S, White JL, Moore CA, Shapiro-Mendoza CK, Petersen L, Boyle C, Jamieson DJ, Meaney-Delman D, Honein MA, Collaboration USZPR. 2017. Vital Signs: Update on Zika Virus-Associated Birth Defects and Evaluation of All U.S. Infants with Congenital Zika Virus Exposure - U.S. Zika Pregnancy Registry, 2016. MMWR Morb Mortal Wkly Rep 66:366-373.

11. Tang H, Hammack C, Ogden SC, Wen Z, Qian X, Li Y, Yao B, Shin J, Zhang F, Lee EM, Christian KM, Didier RA, Jin P, Song H, Ming GL. 2016. Zika Virus Infects Human Cortical Neural Progenitors and Attenuates Their Growth. Cell Stem Cell 18:587-90.

12. Cugola FR, Fernandes IR, Russo FB, Freitas BC, Dias JL, Guimaraes KP, Benazzato C, Almeida N, Pignatari GC, Romero S, Polonio CM, Cunha I, Freitas CL, Brandao WN, Rossato C, Andrade DG, Faria Dde P, Garcez AT, Buchpigel CA, Braconi CT, Mendes E, Sall AA, Zanotto PM, Peron JP, Muotri AR, Beltrao-Braga PC. 2016. The Brazilian Zika

26

648   virus strain causes birth defects in experimental models. Nature 534:267-71.

649 13. Garcez PP, Loiola EC, Madeiro da Costa R, Higa LM, Trindade P, Delvecchio R,
650   Nascimento JM, Brindeiro R, Tanuri A, Rehen SK. 2016. Zika virus impairs growth in
651   human neurospheres and brain organoids. Science 352:816-8.

652 14. Quicke KM, Bowen JR, Johnson EL, McDonald CE, Ma H, O'Neal JT, Rajakumar A,
653   Wrammert J, Rimawi BH, Pulendran B, Schinazi RF, Chakraborty R, Suthar MS. 2016.
654   Zika Virus Infects Human Placental Macrophages. Cell Host Microbe 20:83-90.

655 15. Szaba FM, Tighe M, Kummer LW, Lanzer KG, Ward JM, Lanthier P, Kim IJ, Kuki A,
656   Blackman MA, Thomas SJ, Lin JS. 2018. Zika virus infection in immunocompetent
657   pregnant mice causes fetal damage and placental pathology in the absence of fetal infection.
658   PLoS Pathog 14:e1006994.

659 16. Brown JA, Singh G, Acklin JA, Lee S, Duehr JE, Chokola AN, Frere JJ, Hoffman KW,
660   Foster GA, Krysztof D, Cadagan R, Jacobs AR, Stramer SL, Krammer F, García-Sastre A,
661   Lim JK. 2019. Dengue Virus Immunity Increases Zika Virus-Induced Damage during
662   Pregnancy. Immunity 50:751-762.e5.

663 17. Ribeiro MR, Moreli JB, Marques RE, Papa MP, Meuren LM, Rahal P, de Arruda LB, Oliani
664   AH, Oliani D, Oliani SM, Narayanan A, Nogueira ML. 2018. Zika-virus-infected human
665   full-term placental explants display pro-inflammatory responses and undergo apoptosis.
666   Arch Virol 163:2687-2699.

667 18. Yockey LJ, Jurado KA, Arora N, Millet A, Rakib T, Milano KM, Hastings AK, Fikrig E,
668   Kong Y, Horvath TL, Weatherbee S, Kliman HJ, Coyne CB, Iwasaki A. 2018. Type I
669   interferons instigate fetal demise after Zika virus infection. Sci Immunol 3.

670 19. Shi J, Gao W, Shao F. 2017. Pyroptosis: Gasdermin-Mediated Programmed Necrotic Cell
671   Death. Trends Biochem Sci 42:245-254.

672 20. Jorgensen I, Miao EA. 2015. Pyroptotic cell death defends against intracellular pathogens.
673   Immunol Rev 265:130-42.

674 21. Ding J, Wang K, Liu W, She Y, Sun Q, Shi J, Sun H, Wang DC, Shao F. 2016. Pore-forming
675   activity and structural autoinhibition of the gasdermin family. Nature 535:111-6.

676 22. Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, Zhuang Y, Cai T, Wang F, Shao F. 2015.
677   Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature
678   526:660-5.

679 23. Kayagaki N, Stowe IB, Lee BL, O'Rourke K, Anderson K, Warming S, Cuellar T, Haley B,
680   Roose-Girma M, Phung QT, Liu PS, Lill JR, Li H, Wu J, Kummerfeld S, Zhang J, Lee WP,
681   Snipas SJ, Salvesen GS, Morris LX, Fitzgerald L, Zhang Y, Bertram EM, Goodnow CC,
682   Dixit VM. 2015. Caspase-11 cleaves gasdermin D for non-canonical inflammasome
683   signalling. Nature 526:666-71.

684 24. Van Laer L, Huizing EH, Verstreken M, van Zuijlen D, Wauters JG, Bossuyt PJ, Van de
685   Heyning P, McGuirt WT, Smith RJ, Willems PJ, Legan PK, Richardson GP, Van Camp G.
686   1998. Nonsyndromic hearing impairment is associated with a mutation in DFNA5. Nat
687   Genet 20:194-7.

688 25. Akino K, Toyota M, Suzuki H, Imai T, Maruyama R, Kusano M, Nishikawa N, Watanabe
689   Y, Sasaki Y, Abe T, Yamamoto E, Tarasawa I, Sonoda T, Mori M, Imai K, Shinomura Y,
690   Tokino T. 2007. Identification of DFNA5 as a target of epigenetic inactivation in gastric
691   cancer. Cancer Sci 98:88-95.

692 26. Wang CJ, Tang L, Shen DW, Wang C, Yuan QY, Gao W, Wang YK, Xu RH, Zhang H. 2013.
693   The expression and regulation of DFNA5 in human hepatocellular carcinoma DFNA5 in
694   hepatocellular carcinoma. Mol Biol Rep 40:6525-31.

695 27. Zhang Z, Zhang Y, Xia S, Kong Q, Li S, Liu X, Junqueira C, Meza-Sosa KF, Mok TMY,
696   Ansara J, Sengupta S, Yao Y, Wu H, Lieberman J. 2020. Gasdermin E suppresses tumour
697   growth by activating anti-tumour immunity. Nature 579:415-420.

28. Rogers C, Fernandes-Alnemri T, Mayes L, Alnemri D, Cingolani G, Alnemri ES. 2017. Cleavage of DFNA5 by caspase-3 during apoptosis mediates progression to secondary necrotic/pyroptotic cell death. Nat Commun 8:14128.

29. Wang Y, Gao W, Shi X, Ding J, Liu W, He H, Wang K, Shao F. 2017. Chemotherapy drugs induce pyroptosis through caspase-3 cleavage of a gasdermin. Nature 547:99-103.

30. Wu MF, Chen ST, Yang AH, Lin WW, Lin YL, Chen NJ, Tsai IS, Li L, Hsieh SL. 2013. CLEC5A is critical for dengue virus-induced inflammasome activation in human macrophages. Blood 121:95-106.

31. Chen ST, Lin YL, Huang MT, Wu MF, Cheng SC, Lei HY, Lee CK, Chiou TW, Wong CH, Hsieh SL. 2008. CLEC5A is critical for dengue-virus-induced lethal disease. Nature 453:672-6.

32. Wang ZY, Zhen ZD, Fan DY, Qin CF, Han DS, Zhou HN, Wang PG, An J. 2020. Axl Deficiency Promotes the Neuroinvasion of Japanese Encephalitis Virus by Enhancing IL-1alpha Production from Pyroptotic Macrophages. J Virol 94.

33. He Z, An S, Chen J, Zhang S, Tan C, Yu J, Ye H, Wu Y, Yuan J, Wu J, Zhu X, Li M. 2020. Neural progenitor cell pyroptosis contributes to Zika virus-induced brain atrophy and represents a therapeutic target. Proc Natl Acad Sci U S A 117:23869-23878.

34. Jagger BW, Miner JJ, Cao B, Arora N, Smith AM, Kovacs A, Mysorekar IU, Coyne CB, Diamond MS. 2017. Gestational Stage and IFN-lambda Signaling Regulate ZIKV Infection In Utero. Cell Host Microbe 22:366-376 e3.

35. Zachariades E, Foster H, Goumenou A, Thomas P, Rand-Weaver M, Karteris E. 2011. Expression of membrane and nuclear progesterone receptors in two human placental choriocarcinoma cell lines (JEG-3 and BeWo): Effects of syncytialization. Int J Mol Med 27:767-74.

36. Ramos da Silva S, Cheng F, Huang IC, Jung JU, Gao SJ. 2019. Efficiencies and kinetics of infection in different cell types/lines by African and Asian strains of Zika virus. J Med Virol 91:179-189.

37. Chan JF, Yip CC, Tsang JO, Tee KM, Cai JP, Chik KK, Zhu Z, Chan CC, Choi GK, Sridhar S, Zhang AJ, Lu G, Chiu K, Lo AC, Tsao SW, Kok KH, Jin DY, Chan KH, Yuen KY. 2016. Differential cell line susceptibility to the emerging Zika virus: implications for disease pathogenesis, non-vector-borne human transmission and animal reservoirs. Emerg Microbes Infect 5:e93.

38. Man SM, Kanneganti TD. 2016. Converging roles of caspases in inflammasome activation, cell death and innate immunity. Nat Rev Immunol 16:7-21.

39. Nagata S. 2018. Apoptosis and Clearance of Apoptotic Cells. Annu Rev Immunol 36:489-517.

40. Yu J, Li S, Qi J, Chen Z, Wu Y, Guo J, Wang K, Sun X, Zheng J. 2019. Cleavage of GSDME by caspase-3 determines lobaplatin-induced pyroptosis in colon cancer cells. Cell Death Dis 10:193.

41. Lu H, Zhang S, Wu J, Chen M, Cai MC, Fu Y, Li W, Wang J, Zhao X, Yu Z, Ma P, Zhuang G. 2018. Molecular Targeted Therapies Elicit Concurrent Apoptotic and GSDME-Dependent Pyroptotic Tumor Cell Death. Clin Cancer Res 24:6066-6077.

42. Rogers C, Erkes DA, Nardone A, Aplin AE, Fernandes-Alnemri T, Alnemri ES. 2019. Gasdermin pores permeabilize mitochondria to augment caspase-3 activation during apoptosis and inflammasome activation. Nat Commun 10:1689.

43. Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G. 2006. 5'-Triphosphate RNA is the ligand for RIG-I. Science 314:994-7.

44. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor

7 and 8. Science 303:1526-9.

45. Tappe D, Perez-Giron JV, Zammarchi L, Rissland J, Ferreira DF, Jaenisch T, Gomez-Medina S, Gunther S, Bartoloni A, Munoz-Fontela C, Schmidt-Chanasit J. 2016. Cytokine kinetics of Zika virus-infected patients from acute to reconvalescent phase. Med Microbiol Immunol 205:269-73.

46. He Z, Chen J, Zhu X, An S, Dong X, Yu J, Zhang S, Wu Y, Li G, Zhang Y, Wu J, Li M. 2018. NLRP3 Inflammasome Activation Mediates Zika Virus-Associated Inflammation. J Infect Dis 217:1942-1951.

47. Wang W, Li G, De W, Luo Z, Pan P, Tian M, Wang Y, Xiao F, Li A, Wu K, Liu X, Rao L, Liu F, Liu Y, Wu J. 2018. Zika virus infection induces host inflammatory responses by facilitating NLRP3 inflammasome assembly and interleukin-1beta secretion. Nat Commun 9:106.

48. Zheng Y, Liu Q, Wu Y, Ma L, Zhang Z, Liu T, Jin S, She Y, Li YP, Cui J. 2018. Zika virus elicits inflammation to evade antiviral response by cleaving cGAS via NS1-caspase-1 axis. EMBO J 37.

49. Tummers B, Green DR. 2017. Caspase-8: regulating life and death. Immunol Rev 277:76-89.

50. Fritsch M, Gunther SD, Schwarzer R, Albert MC, Schorn F, Werthenbach JP, Schiffmann LM, Stair N, Stocks H, Seeger JM, Lamkanfi M, Kronke M, Pasparakis M, Kashkar H. 2019. Caspase-8 is the molecular switch for apoptosis, necroptosis and pyroptosis. Nature 575:683-687.

51. Orning P, Weng D, Starheim K, Ratner D, Best Z, Lee B, Brooks A, Xia S, Wu H, Kelliher MA, Berger SB, Gough PJ, Bertin J, Proulx MM, Goguen JD, Kayagaki N, Fitzgerald KA, Lien E. 2018. Pathogen blockade of TAK1 triggers caspase-8-dependent cleavage of gasdermin D and cell death. Science 362:1064-1069.

52. Sarhan J, Liu BC, Muendlein HI, Li P, Nilson R, Tang AY, Rongvaux A, Bunnell SC, Shao F, Green DR, Poltorak A. 2018. Caspase-8 induces cleavage of gasdermin D to elicit pyroptosis during Yersinia infection. Proc Natl Acad Sci U S A 115:E10888-E10897.

53. Zeng CY, Li CG, Shu JX, Xu LH, Ouyang DY, Mai FY, Zeng QZ, Zhang CC, Li RM, He XH. 2019. ATP induces caspase-3/gasdermin E-mediated pyroptosis in NLRP3 pathway-blocked murine macrophages. Apoptosis 24:703-717.

54. Brennan K, Bowie AG. 2010. Activation of host pattern recognition receptors by viruses. Curr Opin Microbiol 13:503-7.

55. Saito T, Owen DM, Jiang F, Marcotrigiano J, Gale M, Jr. 2008. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. Nature 454:523-7.

56. Town T, Bai F, Wang T, Kaplan AT, Qian F, Montgomery RR, Anderson JF, Flavell RA, Fikrig E. 2009. Toll-like receptor 7 mitigates lethal West Nile encephalitis via interleukin 23-dependent immune cell infiltration and homing. Immunity 30:242-53.

57. Chazal M, Beauclair G, Gracias S, Najburg V, Simon-Loriere E, Tangy F, Komarova AV, Jouvenet N. 2018. RIG-I Recognizes the 5' Region of Dengue and Zika Virus Genomes. Cell Rep 24:320-328.

58. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, Stanfield SM, Duffy MR. 2008. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis 14:1232-9.

Figure legend

Figure 1. ZIKV infection induces the GSDME-mediated pyroptosis in JEG-3 cells. JEG-3 or GSDME-KO JEG-3 cells were infected with ZIKV at a MOI of 1. At indicated time post-infection, cells were subjected to microscopy, cytotoxicity and Western blot analyses.

(A) JEG-3 cells were infected with ZIKV for 24 h. Representative cell morphology was shown. Scale bar, 50 μm.

(B) LDH levels in supernatant and cell viability were measured at indicated time post-infection.

(C) Immunoblot analyses of GSDME-FL, GSDME-N and GSDMD-FL in ZIKV-infected JEG-3 cells at indicated time post-infection.

(D to F) JEG-3 and GSDME-KO JEG-3 cells were infected with ZIKV for 24 h. Immunoblot analyses of GSDME-FL and GSDME-N by Western blot (D). Representative cell morphology was shown. Scale bar, 50 μm (E). LDH levels in supernatant and cell viability were measured (F).

All data are presented as the mean ± SEM of at least three independent experiments. GSDME-FL, full-length GSDME; GSDME-N, the N- terminal cleavage products of GSDME; ***, P<0,001; ****, P < 0.0001; ns, no significance.

Figure 2. Both the cellular GSDME abundance and the susceptibility to ZIKV infection determine the occurrence of pyroptosis.

(A to C) Relevant cells were infected with ZIKV at a MOI of 1. At indicated time post-infection, cells were subjected to microscopy, cytotoxicity and Western blot analyses. Phase-contrast images of ZIKV-induced pyroptotic cell death in HeLa, HEK 293T, Huh-7, A549 and SH-SY5Y cells at 72 h post-infection. Scale bar, 50 μm (A). Comparison of ATP cell viability, LDH release-based cell death (B) and GSDME cleavage (C) in ZIKV-infected relevant cells.

(D) Table summarizing results shown in Figure 2A, B, C and Figure EV1 from ZIKV infection experiments with relevant cell lines.

820   (E and F) Analyses of ATP cell viability, LDH release-based cell death (E) and GSDME cleavage

821   (F) in JEG-3 cells at 36 hours post-infection. Unpaired t test versus mock.

822   All data are presented as the mean ± SEM of at least three independent experiments. *, P<0.05; **,

823   P < 0.01; ****, P<0.001; ****, P < 0.0001; ns, no significance.

824

825   **Figure 3. ZIKV infection activates GSDME via extrinsic apoptotic pathway. JEG-3 cells were**

826   **infected with ZIKV at a MOI of 1 then subjected to indicated treatments. At 24 h post-**

827   **infection, cells were subjected to microscopy, cytotoxicity and Western blot analyses.**

828   (A) Immunoblot analyses of GSDME-FL, GSDME-N, and casepase-3 in ZIKV-infected JEG-3

829   cells.

830   (B to D) ZIKV-infected JEG-3 cells were incubated with either 10 μM VX-765, 25 μM Z-DEVD-

831   FMK, 25 μM Z-VAD-FMK, or 10 μM GSK872 for 24 h and then the cells were subjected to

832   microscopy (B), cytotoxicity (C) and Western blot analyses (D). Scale bar, 50 μm.

833   (E) Immunoblot analyses of GSDME-FL, GSDME-N, caspase-9 and casepase-8 in ZIKV-infected

834   JEG-3 cells.

835   (F to H) ZIKV-infected JEG-3 cells were incubated with either 25 μM Z-IETD-FMK or 25 μM Z-

836   LEHD-FMK for 24 h and then the cells were subjected to microscopy (F), cytotoxicity (G) and

837   Western blot analyses (H). Scale bar, 50 μm.

838   All data are presented as the mean ± SEM of at least three independent experiments. **, p < 0.01;

839   ***, p < 0.001; ****, p < 0.0001; ns, no significance.

840

841   **Figure 4. The genomic RNA of ZIKV activates the GSDME-dependent pyroptosis through**

842   **RIG-I-caspase-8-caspase-3 pathway.**

843 (A) JEG-3 cells were seeded in 6-well plate, and transfected with 2 μg indicated plasmids. At 24 h

844 post-transfection, cells were subjected to analyze for GSDME-FL, GSDME-N and Flag by Western

845 blot. Asterisks indicate specific bands.

846 (B to D) JEG-3 cells were seeded in 6-well plate and were transfected with 1 μg ZIKV 5' UTR or

847 3' UTR. At 24 h post-transfection, cells were subjected to microscopy (B), cytotoxicity (C) and

848 Western blot analyses. Scale bar, 50 μm.

849 (E to G) Relevant cell lines were infected with ZIKV at a MOI of 1. At 24 h post-infection, cells

850 were subjected to microscopy (E), cytotoxicity (F) and analyzed for GSDME-FL, GSDME-N,

851 caspase-8, caspase-9 and caspase-3 by Western blot (G). Scale bar, 50 μm.

852 All data are presented as the mean ± SEM of at least three independent experiments. **, $p < 0.01$;

853 ***, $p < 0.001$; ****, $p < 0.0001$; ns, no significance.

854

855 **Figure 5. ZIKV infection of pregnant immunocompetent WT C57BL/6N mice results in**

856 **placental pyroptosis and CZS.** Pregnant dams at E9.5 were infected with 1×106 PFU of ZIKV

857 by intravenous route. Mock mice were inoculated with Vero cell culture supernatant. Mice were

858 sacrificed 7 days post-infection at E16.5, and maternal tissues, placentas and fetuses were harvested

859 and analyzed.

860 (A) Scheme of infection and the follow up analyses.

861 (B) Representative images of fetuses at E16.5 are shown.

862 (C) Impact of ZIKV infection on fetuses at E16.5. The percentage of fetuses that were affected (i.e.

863 had undergone resorption, or exhibited any sign of growth restriction, malformation) is shown.

864 Numbers on bars indicate normal fetuses (top) or affected fetuses (bottom).

865 (D) Representative hematoxylin and eosin staining showed pathological features of placentas at

866 E16.5. The asterisks indicate abnormal spheroid structure. Arrows indicate necrotic trophoblast

867 cells. Arrowheads indicate thrombi. Scale bar, 50 μm.

32

868 (E) Representative images of immunohistochemistry of placenta sections stained for Cleaved-

869 caspase-3. Scale bar, 100 μm.

870 (F) Propidium iodide was intravenously injected into the mice before assay. Representative placenta
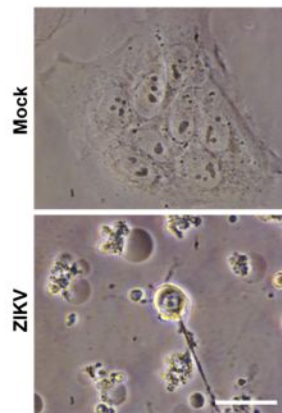
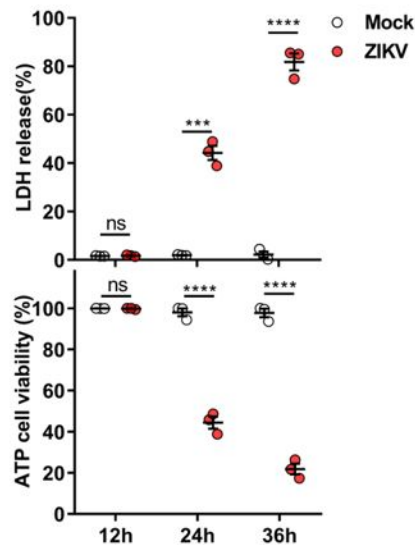871 section images are shown. Scale bar, 400 μm. Number of samples in each group is listed.

872 Data for all panels are pooled from 3–5 independent experiments. For (C), significance was

873 determined by Fisher's exact test. Data shown are median with interquartile range. ****, $p < 0.0001$;

874 ns, no significance.

875

876 **Figure 6. GSDME contributes to the ZIKV-induced CZS through mediating placental**

877 **pyroptosis. Scheme of infection and analyses were shown in Figure 5A.** The WT Mock and WT

878 ZIKV groups were the same groups as shown in Figure 5.

879 (A) Representative images of fetuses from WT and Gsdme$^{-/-}$ dams at E16.5 are shown.

880 (B) Impact of ZIKV infection on fetuses from WT and Gsdme$^{-/-}$ dams at E16.5. The percentage of

881 fetuses that were affected (i.e. had undergone resorption, or exhibited any sign of growth restriction,

882 malformation) is shown. Numbers on bars indicate normal fetuses (top) or affected fetuses (bottom).

883 (C) Representative hematoxylin and eosin staining showed pathological features of placentas at

884 E16.5. The asterisks indicate abnormal spheroid structure. Arrows indicate necrotic trophoblast

885 cells. Arrowheads indicate thrombi. Scale bar, 50 μm.

886 (D) Propidium iodide was intravenously injected into the mice before assay. Representative

887 placenta section images are shown. Scale bar, 400 μm.

888 (E) ZIKV RNA levels of maternal spleens, serum and brains of WT and Gsdme$^{-/-}$ dams infected

889 with ZIKV.

890 (F) ZIKV RNA levels of all placentas and fetal heads carried by WT and Gsdme$^{-/-}$ dams infected

891 with ZIKV. Number of samples in each group is listed.

33

892   Data for all panels are pooled from 3–5 independent experiments. For (B), significance was

893   determined by Fisher's exact test. For (E) and (F), the Mann-Whitney test was used to calculate

894   significance. Data shown are median with interquartile range, and the dotted line depicts the limit

895   of detection. ****, $p < 0.0001$; ns, no significance.

896

Figure 1

A

Mock

ZIKV

B

Mock ○
ZIKV ●

LDH release(%)

100
80
60
40
20
0

ns   ***   ****

ATP cell viability (%)

100
80
60
40
20
0

ns   ****   ****

12h   24h   36h

C

	12h	24h	36h	kDa
ZIKV	− +	− +	− +	
NS5				100
GSDME-FL				50
GSDME-N				25
GSDMD-FL				50
GAPDH				35

D

	WT	GSDME-KO	kDa
ZIKV	− +	− +	
NS5			100
GSDME-FL			50
GSDME-N			25
GAPDH			35

E

JEG-3   GSDME-KO

Mock

ZIKV

F

Mock ○
ZIKV ●

LDH release (%)

60

40

20

0

****

ATP cell viability (%)

100
80
60
40
20
0

***

WT   GSDME-KO

Figure 2

A

	HeLa	HEK-293T	Huh-7	A549	SH-SY5Y
Mock					
ZIKV					

B

Hela · HEK 293T · Huh-7 · A549 · SH-SY5Y

LDH release (%) / ATP cell viability (%) at 24h, 48h, 72h

○ Control ● ZIKV

C

ZIKV: 24h 48h 72h (− +) for each cell line

NS5 — 100 kDa
GSDME-FL — 50 kDa
GSDME-N — 25 kDa
GAPDH — 35 kDa

HeLa · HEK-293T · Huh-7 · A549 · SH-SY5Y

D

	GSDME abundance	Susceptibility to ZIKV	Pyroptosis
JEG-3	Medium	High	Severe
HeLa	High	Low	None
HEK-293T	Low	Low	None
Huh-7	Low	High	Moderate
A549	Medium	High	Severe
SH-SY5Y	High	Low	None

E

LDH release (%): Mock, 0.1, 0.5, 1, 2, 5
ATP cell viability (%): Mock, 0.1, 0.5, 1, 2, 5 (moi)

F

MOI: Mock 0.1 0.5 1 2 5

NS5 — 100 kDa
GSDME-FL — 50 kDa
GSDME-N — 25 kDa
GAPDH — 35 kDa
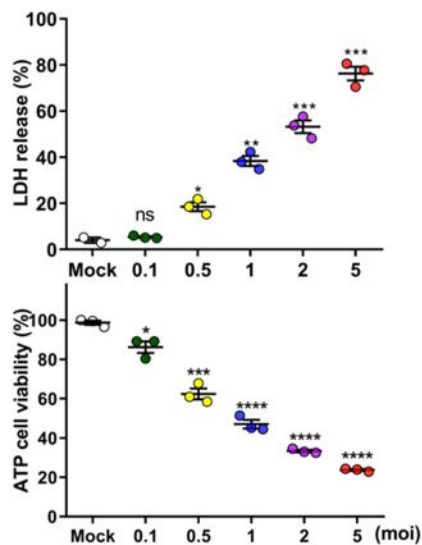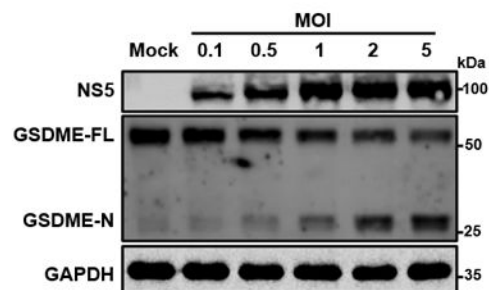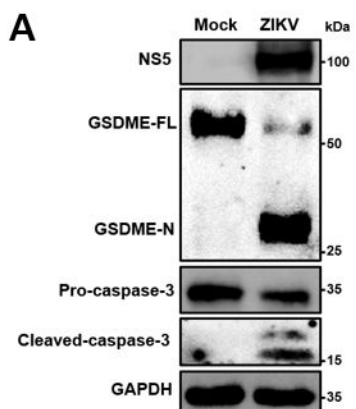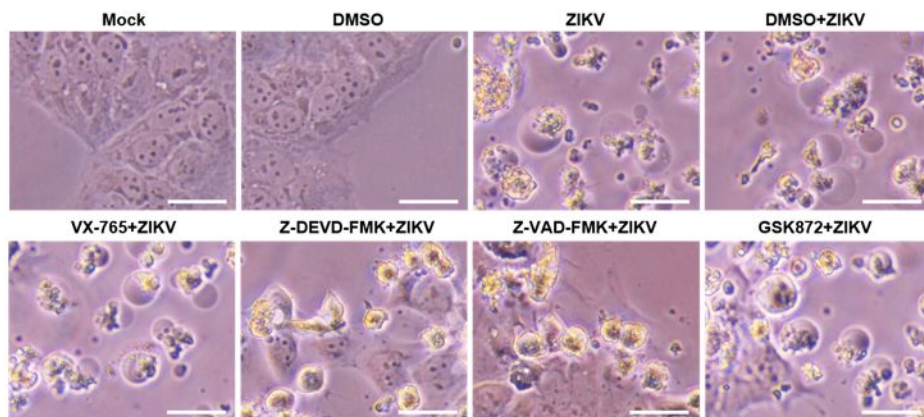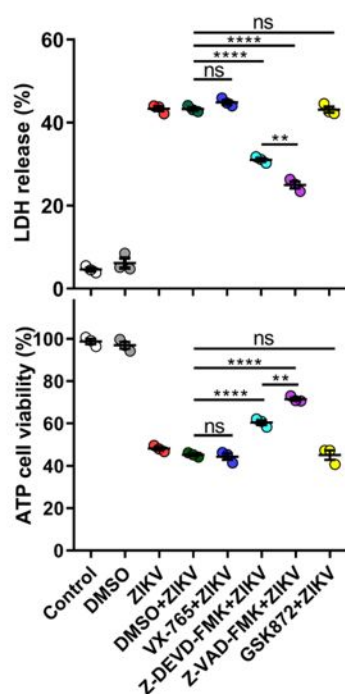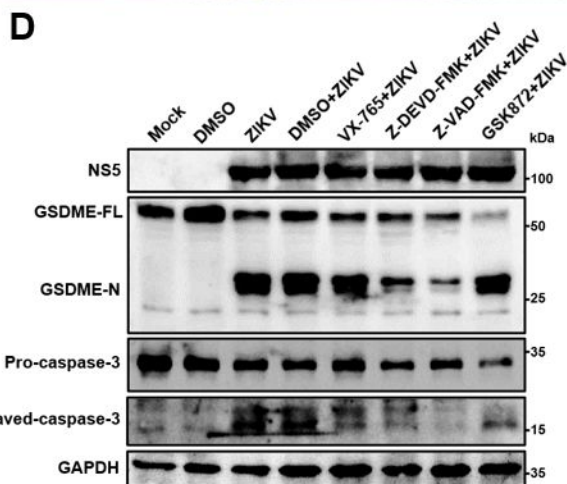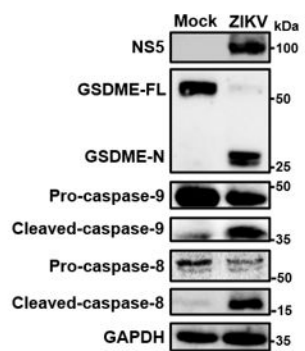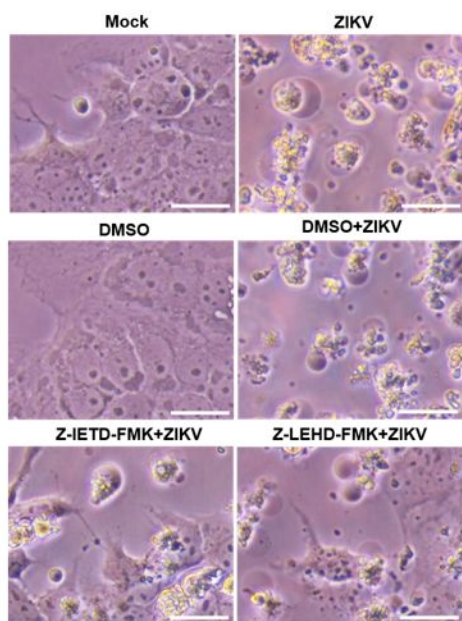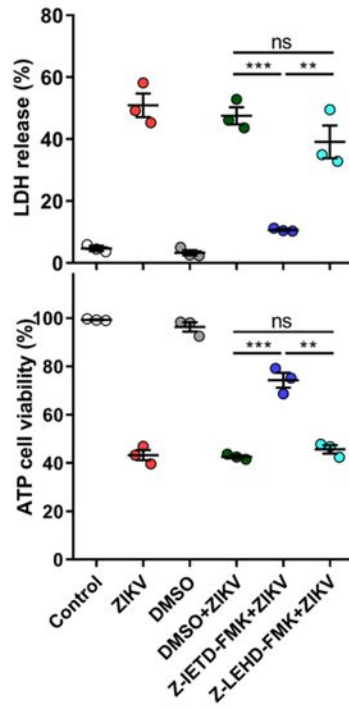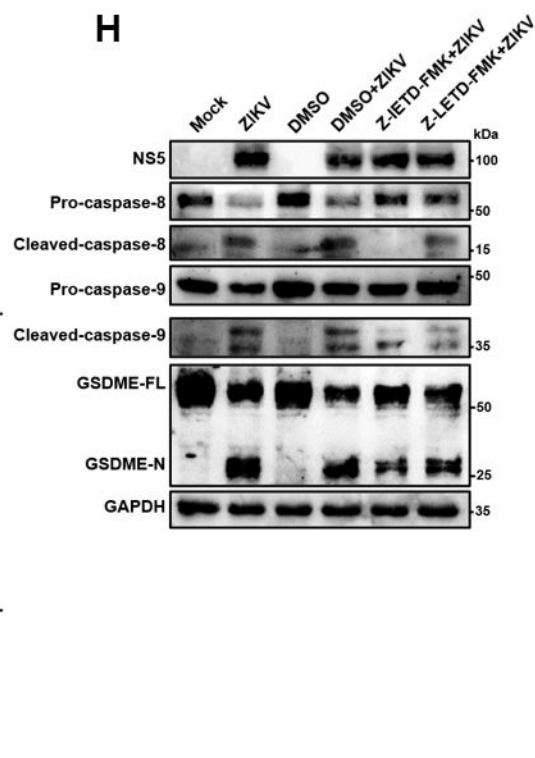
Figure 3

Figure 4

Figure 5

Figure 6

A C57BL/6N Gsdme⁻/⁻

Mock

ZIKV

B Fetus

C Placental junctional zone

Mock ZIKV

WT

Gsdme⁻/⁻

Mock ZIKV Placental labyrinth

WT

Gsdme⁻/⁻

D PI Hoechst Merge

WT Mock

WT ZIKV
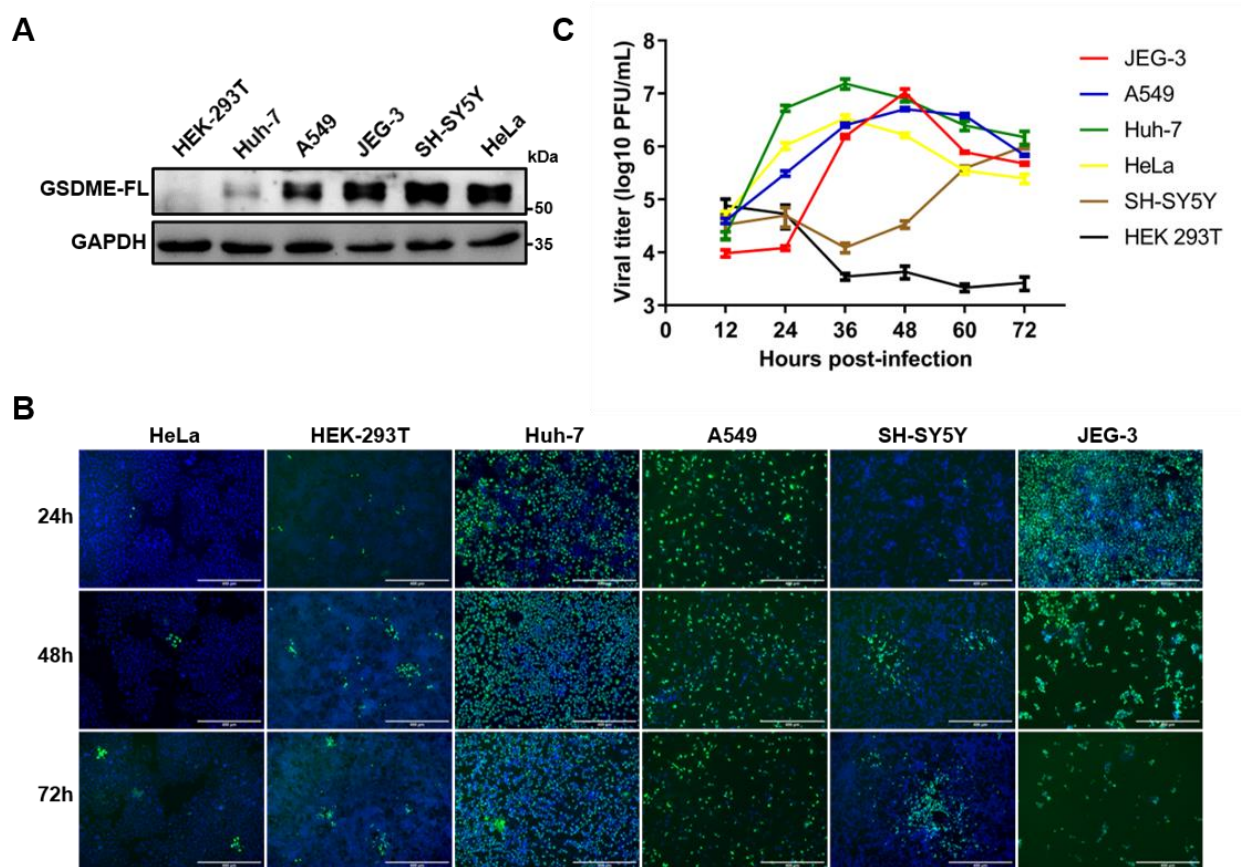
Gsdme⁻/⁻ Mock

Gsdme⁻/⁻ ZIKV

E Spleen Serum Brain

F Placenta Fetal brain
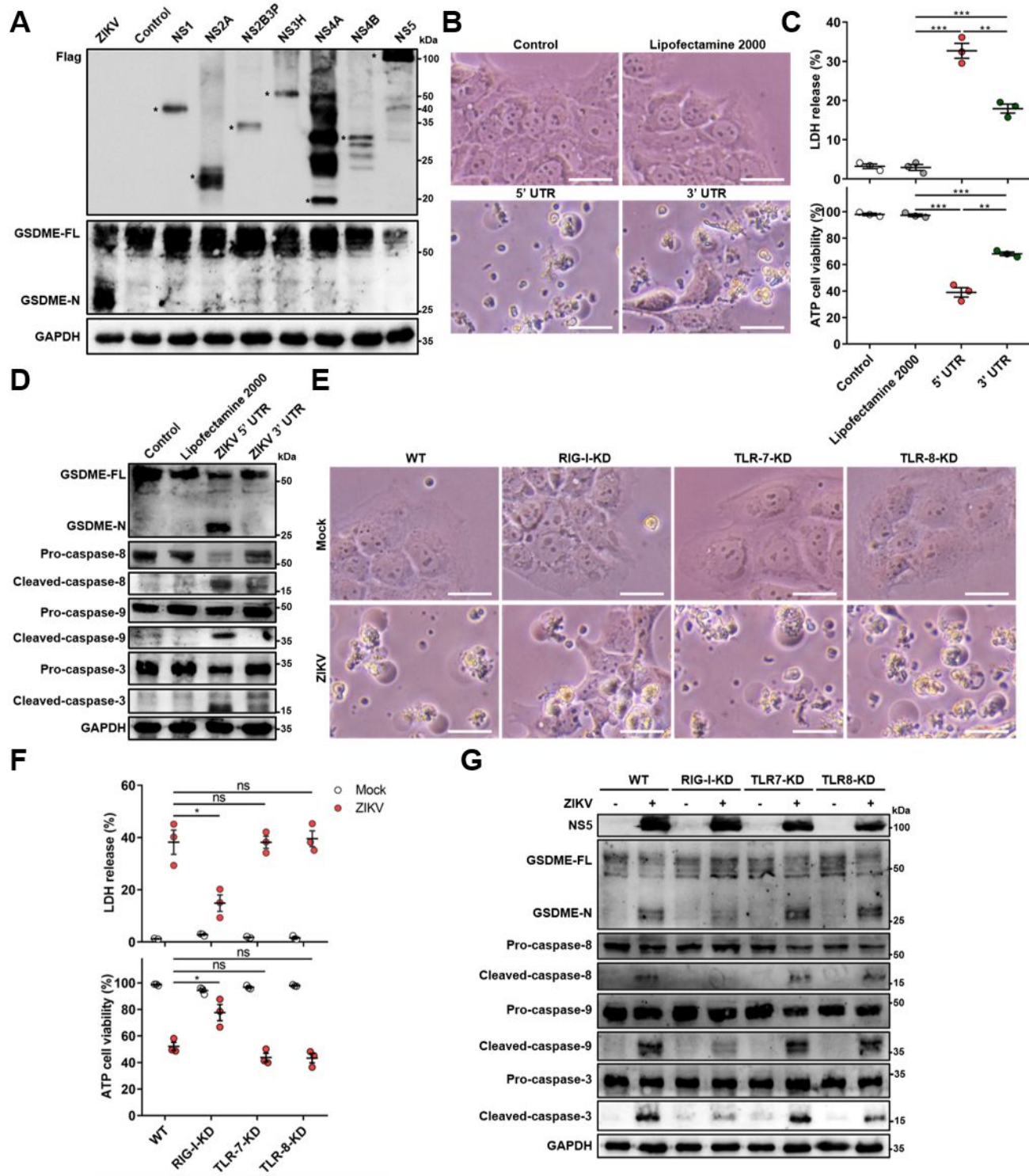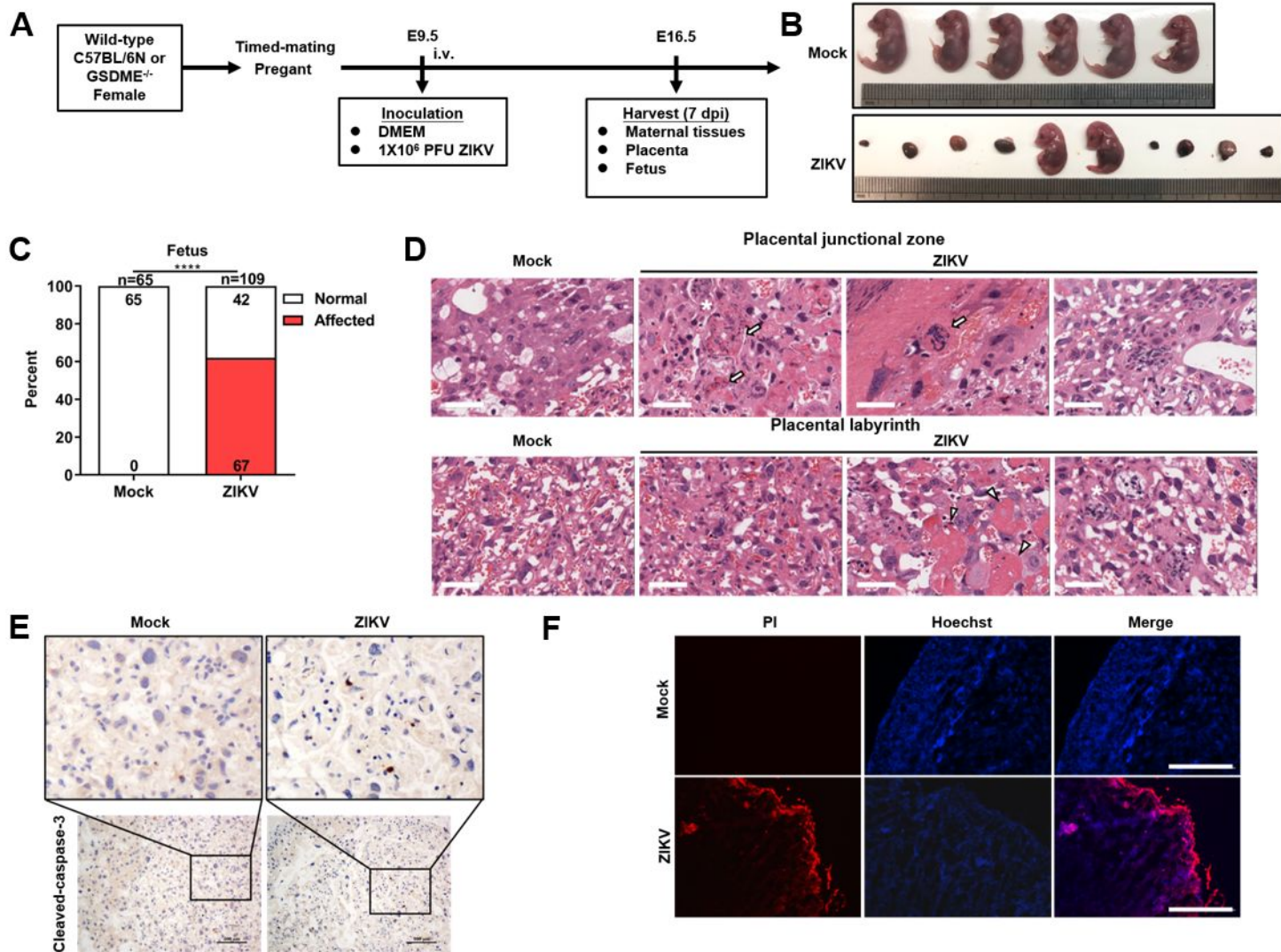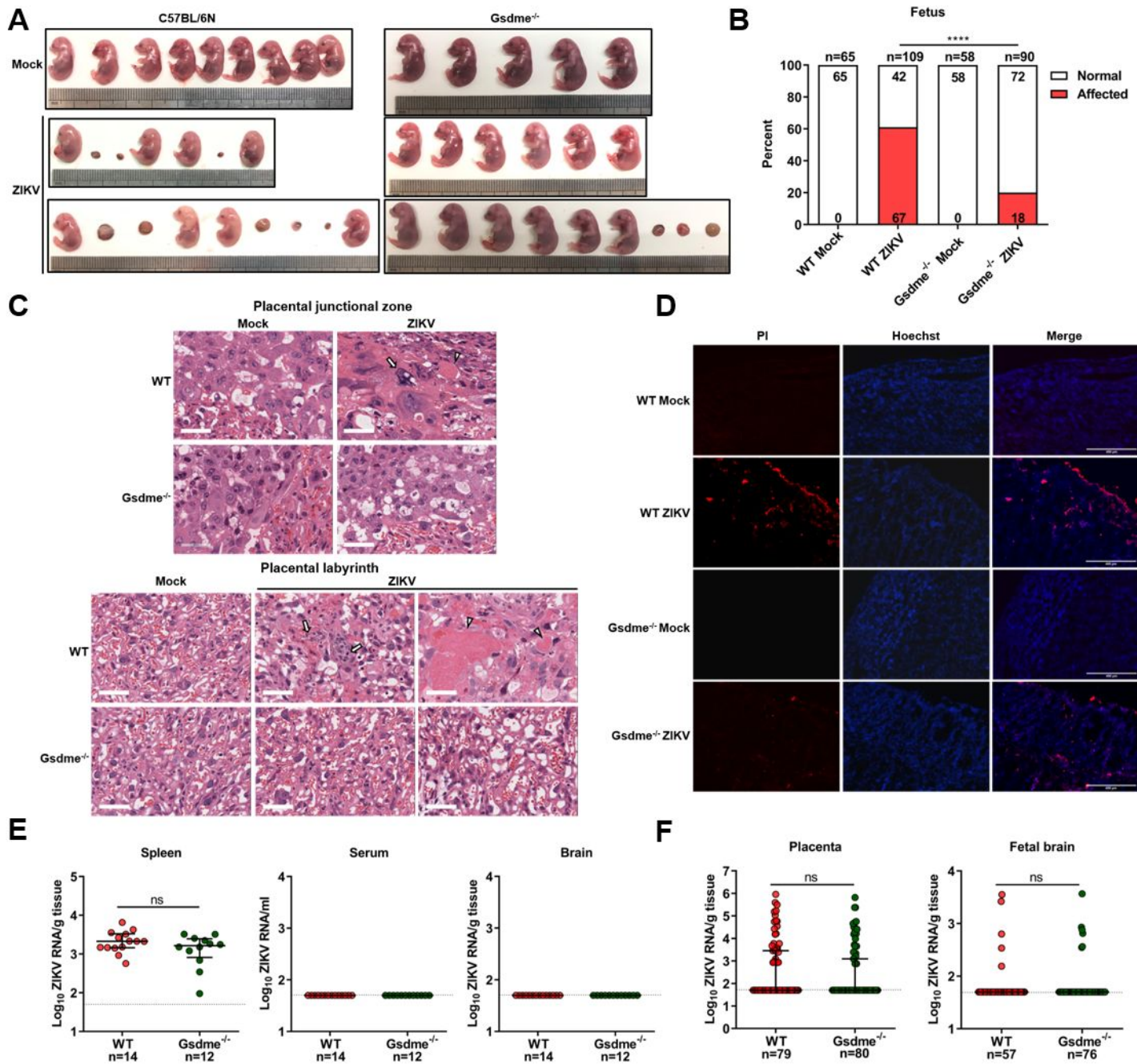
Fig. S1

Fig. S1. The GSDME abundance and the susceptibility to ZIKV infection of relevant cell lines. (A) Immunoblot analyses of GSDME-FL in indicated cell lines. **(B to C)** Relevant cells were infected with ZIKV at a MOI of 0.1. At indicated time post-infection, cells were subjected to immunofluorescence analysis (B). Scale bar, 400 μm. For plaque assay, the supernatants of ZIKV-infected cells were harvested at 12-72 h post-infection, and the titration was performed on Vero cells (C).

Fig. S2
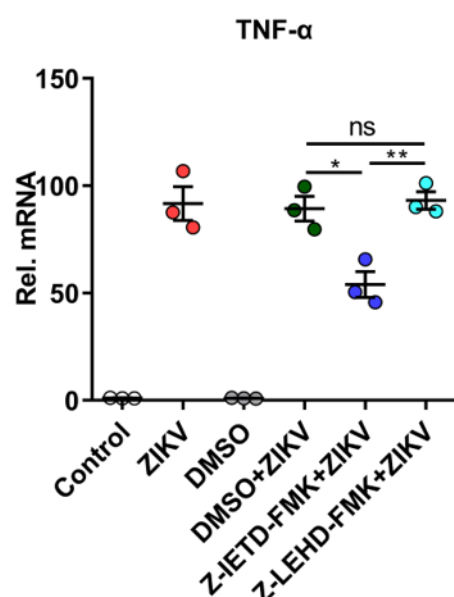
**Fig. S2. ZIKV infection significantly enhances the expression of TNF-α in infected JEG-3 cells.** JEG-3 cells were infected with ZIKV at a MOI of 1, and were subsequently incubated with either 25 μM Z-IETD-FMK or 25 μM Z-LEHD-FMK for 24 h. Then the cells were harvested for RNA extraction and subjected to detect mRNA level of TNF-α by qRT-PCR. Data were normalized to the level of β-actin expression in each sample. All data are presented as the mean ± SEM of at least three independent experiments. **, p< 0.5; **, p < 0.01; ns, no significance.

Fig. S3
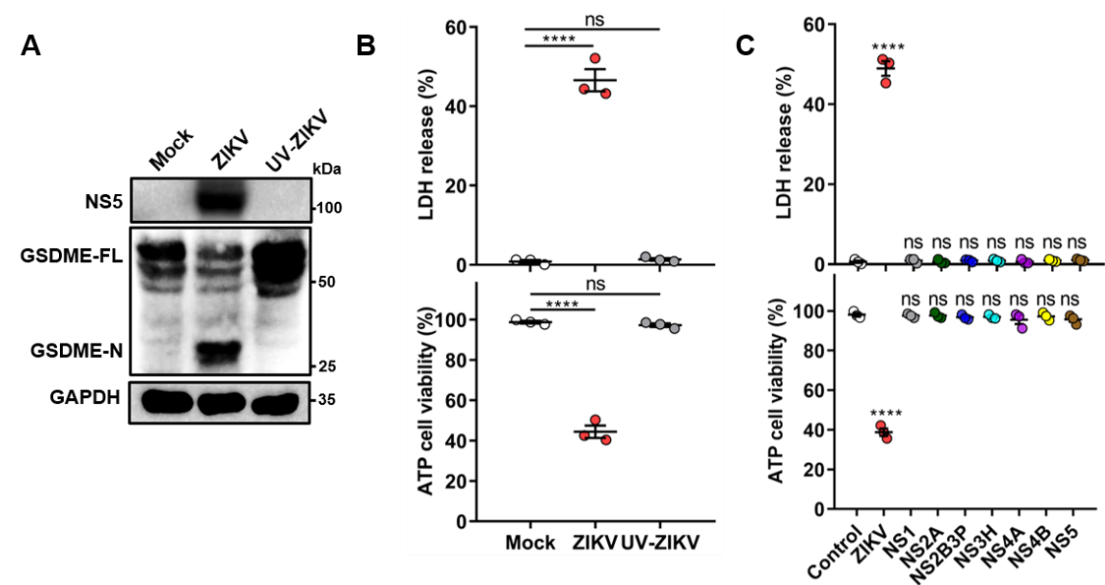
Fig. S3. The structural and non-structural proteins of ZIKV are not capable of inducing pyroptosis. (A to B) JEG-3 cells were infected with ZIKV or UV-inactivated ZIKV at a MOI of 1. At 24 h post-infection, cells were subjected to western blot (A) and cytotoxicity analyses (B). **(C)** JEG-3 cells were seeded in 6-well plate and transfected with 2 μg indicated plasmids. At 24 h post-transfection, cells were subjected to cytotoxicity analyses. Unpaired t test versus Control. All data are presented as the mean ± SEM of at least three independent experiments. ****, p < 0.0001; ns, no significance.

Fig. S4
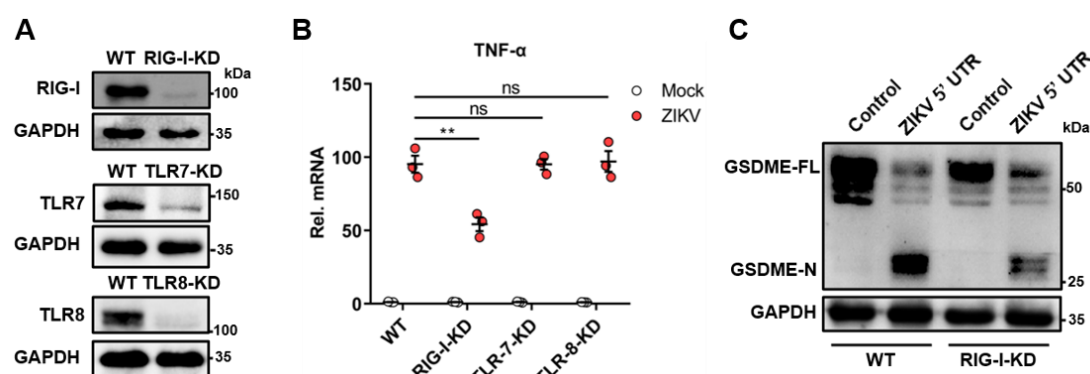
Fig. S4. The KD of RIG-I suppresses the ZIKV or ZIKV 5' UTR-induced activation of pyroptosis. (A) Verification of RIG-I, TLR7and TLR8 knockdown efficiency by immunoblotting. **(B)** JEG-3 WT or RIG-I KD cells were seeded in 6-well plate and transfected with 1 μg ZIKV 5' UTR. At 24 h post-transfection, the cells were harvested for western blot analyses. **(C)** Relevant cells were infected with ZIKV at a MOI of 1. At 24 h post-infection, the cells were harvested for RNA extraction and subjected to detect mRNA level of TNF-α by qRT-PCR. Data were normalized to the level of β-actin expression in each sample. All data are presented as the mean ± SEM of at least three independent experiments. **, $p < 0.01$; ns, no significance.

Fig. S5
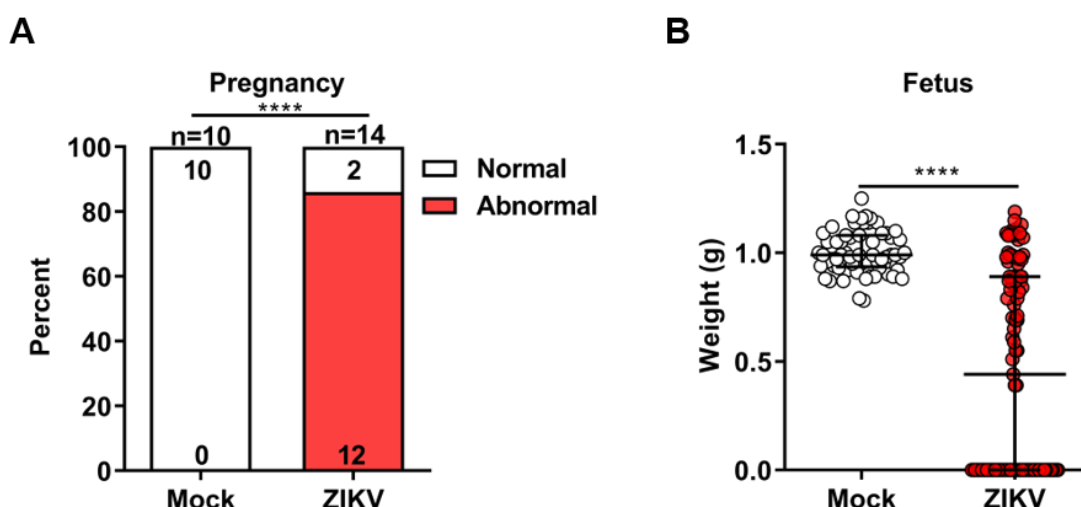
Fig. S5. Impact of ZIKV on dams and fetuses at E16.5. Pregnant dams were infected with ZIKV or Vero cell culture supernatant (Mock) as described in Fig. 5A. **(A)** The percentage of dams showing abnormal pregnancy (at least one fetus showed morphological abnormality or suffered demise/resorption). Numbers on bars indicate normal pregnancy (top) or abnormal pregnancy (bottom). Significance was determined by Fisher's exact test. ****, $p<0.0001$. **(B)** The weight of fetuses. Data shown are median with interquartile range, and Mann-Whitney test was used to calculate significance. ****, $p < 0.0001$. Data are pooled from 3-5 independent experiments.

Fig. S6
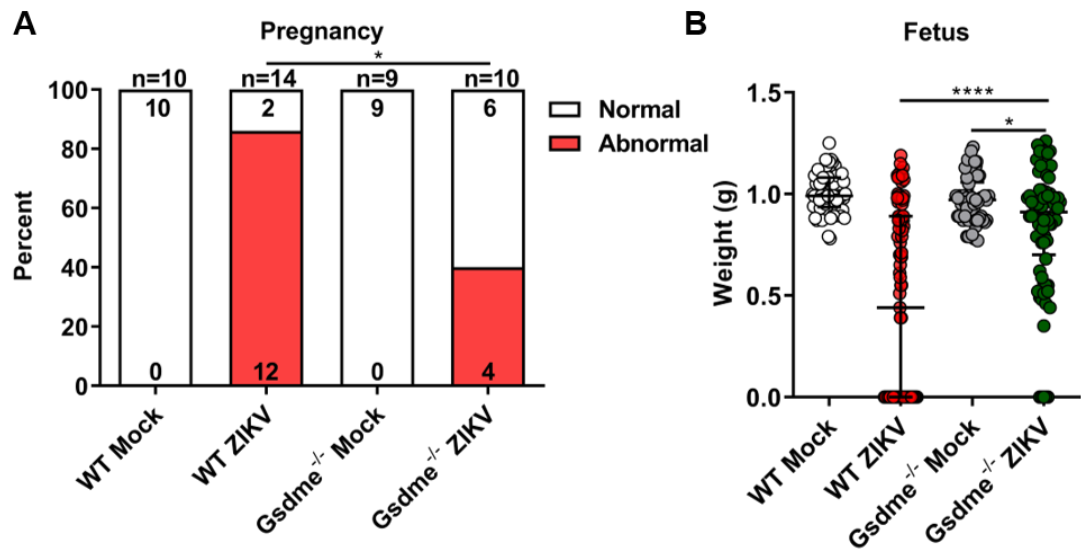
Fig. S6. GSDME deletion restrains ZIKV-induced abnormal pregnancy and adverse fetal outcomes. Pregnant WT and Gsdme$^{-/-}$ dams were infected with ZIKV or Vero cell culture supernatant (Mock) as described in Fig. 5A. **(A)** The percentage of dams showing abnormal pregnancy (at least one fetus showed morphological abnormality or suffered demise/resorption). Numbers on bars indicate normal pregnancy (top) or abnormal pregnancy (bottom). Significance was determined by Fisher's exact test. *, p<0.05. **(B)** The weight of fetuses. Data shown are median with interquartile range, and Mann-Whitney test was used to calculate significance. *, p<0.05; ****, p < 0.0001. Data are pooled from 3-5 independent experiments.