

1 **Gas6 drives Zika virus-induced neurological complications in humans and**
2 **congenital syndrome in immunocompetent mice**

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75 **ABSTRACT**

76 Zika virus (ZIKV) has the ability to cross placental and brain barriers, causing
77 congenital malformations in neonates and neurological disorders in adults. However, the
78 pathogenic mechanisms of ZIKV-induced neurological complications in adults and congenital
79 malformations remain unknown. Gas6 is a soluble TAM receptor ligand able to promote
80 flavivirus internalization and downregulation of immune responses. Here we demonstrate high
81 Gas6 levels in the serum of patients with neurological complications which correlated with
82 downregulation of genes associated with the type I IFN responses as consequence of *Socs1*
83 upregulation. Gas6 gamma-carboxylation is essential for ZIKV replication in monocytes, the
84 main source of this protein. Gas6 also facilitates ZIKV replication in adult immunocompetent
85 mice enabled susceptibility to transplacental infection and congenital malformations. Our data
86 thus indicate that ZIKV promotes the upregulation of its ligand Gas6, which contributes to viral
87 infectivity and drives the development of severe adverse outcomes during ZIKV infection.

88

89 **KEYWORDS:** Zika virus, TAM receptors, type I Interferon, Socs1, congenital infection

90 INTRODUCTION

91

92 In 2016, Zika virus (ZIKV) emerged as an important global health problem, starting in
93 South America, and then spreading to more than 94 countries worldwide. First discovered in
94 1947 in Uganda, Africa, it has not been considered a threat to human health, until the outbreaks
95 in Yap, Micronesia (2007) and French Polynesia (2013) [1]. Most individuals are
96 asymptomatic or develop a benign febrile disease characterized by cutaneous rash and
97 conjunctivitis. However, it was further shown that ZIKV can cross the placental barrier and
98 reach foetal tissues causing the congenital ZIKV syndrome (CZS), that may range from foetal
99 growth restriction and microcephaly to severe retinal damage and arthrogryposis [1-3]. This
100 was unprecedented and demanded great efforts of the scientific community to understand the
101 underlying mechanisms involved in host-virus interaction, mainly those related to
102 susceptibility and pathogenicity.

103 Central nervous system (CNS) manifestations after congenital infection, such as brain
104 calcifications, lissencephaly, ventricular hypertrophy and microcephaly, are among the most
105 notable and concerning outcomes of ZIKV infection in newborns [4-6]. However, severe ZIKV
106 infection is not limited to newborns: neurological manifestations as acute myelitis,
107 encephalitis, meningoencephalitis and Guillain-Barré syndrome can also occur in adults [7-9].
108 This raises not only the question on what are the genes that confer susceptibility to ZIKV
109 neuropathology, but also, what are the cellular and molecular mechanisms orchestrating such
110 phenomenon. In this context, we believe the interaction between viral particles with host cells,
111 the first step of infection, may be of pivotal relevance. This interaction dictates more than the
112 viral tissue tropism, but also triggers a diversity of intracellular pathways that may greatly
113 account for either failure or success of infection [10, 11].

114 Growth arrest-specific 6 (Gas6) is a 75 KDa secreted protein composed of an N-terminal
115 Gla domain, followed by four epidermal growth factor (EGF)-like domains and a C-terminal
116 SHBG domain [12]. Upon γ -carboxylation of the Gla domain, Gas6 is able to interact with
117 TAM (Tyro3, Axl and Mer) receptors and phosphatidylserine (PtdSer) promoting phagocytic
118 internalization of the apoptotic bodies [12]. TAM activation triggers different signalling
119 pathways involved in cell survival, mainly orchestrated by PI3K and Akt [12, 13].
120 Interestingly, PtdSer containing viruses, such as flaviruses and filoviruses, may also bind to
121 Gas6, activating clathrin-mediated phagocytic internalization and subverting cellular immune
122 response by activating negative regulators of anti-viral cytokines, as SOCS-1 and SOCS-3 [10,

123 11, 14], negatively regulating type I interferon receptor (IFNAR) signalling pathway [12, 13-
124 18]. However, the overall interplay of viral and host factors, such as GAS6, to orchestrate the
125 clinical course of ZIKV infection is not fully understood.

126 Here we investigated the role of the PtdSer ligand Gas6 in the pathogenesis of ZIKV
127 infection. We observed that circulating levels of Gas6 are upregulated in the serum of ZIKV-
128 infected patients, including pregnant women, and a further increase occurs in patients with
129 neurological complications. Concurrently, there is a reduced transcriptional expression of
130 genes associated with type I IFN responses and other immune signatures, probably as
131 consequence of *Socs1* upregulation in peripheral blood cells. ZIKV infected monocytes were
132 an important source of Gas6 production and Gas6 gamma-carboxylation was essential for
133 ZIKV replication. Conversely, we also demonstrate that Gas6 is upregulated in ZIKV-infected
134 pregnant mice and that pre-incubation of ZIKV with recombinant Gas6 facilitates ZIKV
135 replication and rendered them susceptible to transplacental infection and congenital
136 malformations. We thus propose that Gas6 dampens antiviral immune response in the
137 periphery, promoting viral replication and facilitating severe clinical outcome. Collectively,
138 our data brings novelty to the role of Gas6 on the understanding of ZIKV pathogenesis, as a
139 relevant host factor driving severe outcomes, such as neurological complications. Addressing
140 this issue could help develop predictive approaches for early diagnostics and open new
141 possibilities to develop effective treatment against severe complications.

142 **RESULTS**

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144 **Patients**

145 In this cross-sectional study, peripheral blood, serum, and urine samples were collected
 146 from 90 patients and 13 healthy donors during ZIKV epidemics in Brazil. These samples were
 147 originally collected between February 2016 and June 2017 in different hospitals in the city of
 148 Campinas. Included participants were recruited based on their clinical symptoms during
 149 hospital admission and according to the results of ZIKV laboratorial tests [9]. In brief, we
 150 included all patients with neurological complications suspected of arbovirus infection that we
 151 had access to during the period (inclusion and exclusion criteria are described in star methods
 152 section). In addition, we included samples from 57 patients with a mild disease, all positive for
 153 ZIKV by RT-qPCR (Non-Neuro^{ZIKV}). Of the 33 patients with neurological complications, 19
 154 (60%) were positive for ZIKV in previous tests or during hospitalization (Neuro^{ZIKV}) and 14
 155 were negative for ZIKV (Neuro^{NON-ZIKV}). Neurological complications started between 2 and
 156 15 days after onset of acute symptoms (mean of 4 days) in ZIKV patients. At the day of sample
 157 collection, there was no difference in ZIKV RNA load in the peripheral blood samples from
 158 the Non-Neuro^{ZIKV} and Neuro^{ZIKV} patients (median near of 2000 copies/mL of the RNA viral
 159 in both groups of patients) (Table 1 summarizes the demographic data and clinical
 160 manifestations).

161

162 **Table 1.** Sociodemographic, clinical and laboratorial findings in the considered groups
 163 included in this study

| Characteristic | Patients No (%) | | | |
|---|-------------------------------------|---------------------------------|-------------------------------------|--------------|
| | Non-Neuro ^{ZIKV} (n=57) | Neuro ^{ZIKV} (n=19) | Neuro ^{NON-ZIKV} (n=14) | HD (n=13) |
| Demographics | | | | |
| Age, median (range) in years | 31 (1 – 69) | 32 (1 – 82) | 26 (1 – 74) | 29 (18 – 45) |
| Female sex | 42 (73.7) | 11 (57.8) | 6 (42.8) | 8 (61.5) |
| Preceding symptoms | | | | |
| Fever | 35 (61.5) | 10 (52.6) | 7 (50.0) | NA |
| Rash | 37 (65) | 8 (42.1) | 4 (28.5) | NA |
| Conjunctivitis | 24 (42.1) | 7 (36.8) | 2 (14.3) | NA |
| Headache | 22 (38.6%) | 5 (26.3) | 3 (21.4) | NA |
| Arthralgias | 12 (21%) | 3 (15.7) | 3 (21.4) | NA |
| Myalgias | 19 (33.3%) | 2 (10.5) | 1 (7.2) | NA |
| Time from onset of viral symptoms to ZIKV diagnosis, median (range) in days | 3 (0 – 10) | 4 (1 – 10) | NA | NA |
| Laboratory tests | | | | |
| ZIKV viral load in urine (median of copies/mL) | 2079 | 2049 | NA | NA |

| | | | | |
|--|---------|------------|----------|---------|
| NS1/IgM DENV detection | 0 (0.0) | 0 (0.0) | 3 (21.4) | 0 (0.0) |
| DENV qRT-PCR | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| CHIKV qRT-PCR | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| OROV qRT-PCR | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Neurological diagnosis | | | | |
| Guillain-Barré syndrome | NA | 5 (26.3) | 3 (21.4) | NA |
| Encephalitis | NA | 6 (26.3) | 7 (50) | NA |
| Meningitis | NA | 4 (31.6) | 2 (14.2) | NA |
| Meningoencephalitis | NA | 3 (15.8) | 2 (14.2) | NA |
| Transverse myelitis | NA | 1 (2.6) | 0 (0) | NA |
| Time from onset of viral symptoms to neurological symptoms, median (range) in days | NA | 4 (2 – 15) | 7 (1-29) | |

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Samples of 8 ZIKV-infected pregnant women and 6 infants born of these women were also analysed in this study. Two of these infants had growth restriction and CNS alterations compatible with CZS. Due the small number of patients, no clinical comparison was made between these two groups.

169 **Increased Gas6 expression in ZIKV adult patients with neurological complications**

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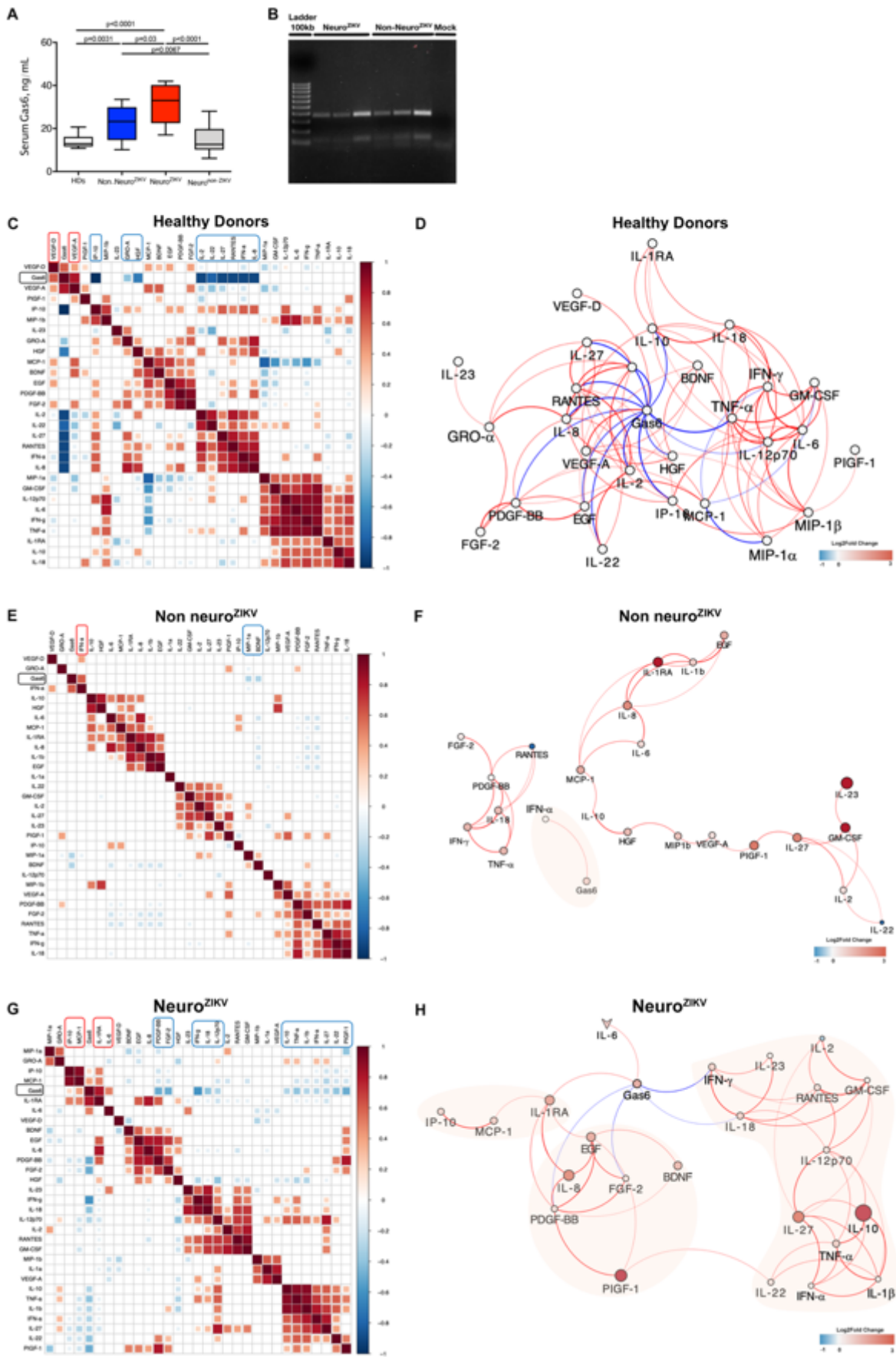
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To correlate Gas6 levels to the pathogenesis of ZIKV-associated neurological complications, we first determined Gas6 levels in patients' serum by ELISA. Figure 1A shows that circulating levels of Gas6 are significantly increased in ZIKV-infected patients compared to healthy donors (Non-Neuro^{ZIKV}: 22.56 ng/mL [25-75 interquartile range (IQ) 14.6-29.9] versus 13.36 ng/mL [25-75 IQ 11.6-16.1] in HDs, $p = 0.0062$), whose circulating Gas6 levels were comparable to previous studies [19-21]. Importantly, Neuro^{ZIKV} patients showed a further increase of serum Gas6 in comparison to Non-Neuro^{ZIKV} patients (33.05ng/mL [25-75IQ 22.5-40.2], $p = 0.0289$) (Figure 1A). To rule out the possibility of co-infections influencing Gas6 levels observed in these patients, we used a high-throughput screening (HTS) virus metagenomic approach to identify viral co-infections that were not detected by RT-qPCR or diagnosed by laboratory tests. ZIKV mono-infections in the Neuro^{ZIKV} patients were confirmed in 9 out of 10 patients tested. In one patient, the complete genome of a strain of Pegivirus C (*Pegivirus* genus, *Flaviviridae* family) was sequenced (Supp Fig 1). Noteworthy, this virus has not been associated with neurological complications in humans. Finally, unchanged Gas6 levels in the serum of 14 patients with neurological diseases non-related to acute ZIKV infection (Neuro^{NON-ZIKV}) revealed that Gas6 upregulation is a ZIKV-specific response (Figure 1A). In addition, genotyping analysis revealed no difference in *GAS6* haplotypes between the ZIKV-infected patient groups, both showing a predominant frequency of the c.834 + 7G>A AA genotype (Figure 1B).

190 **Gas6 upregulation suppresses antiviral IFN response in ZIKV adult patients with**
191 **neurological complications**

192 To determine the mechanisms by which ZIKV-induced Gas6 upregulation contributes
193 to the pathogenesis of neurological complications in adult patients, we used network analysis
194 of biomarkers and transcriptional profiling to determine how Gas6 orchestrates with a specific
195 signature of immune mediators associated with ZIKV infection, previously determined in our
196 cohort [9]. In accordance with its role as pleiotropic inhibitor of innate immune responses [12,
197 13, 16], Gas6 negatively correlates with several pro-inflammatory cytokines/chemokines, such
198 as IL-2, IL-8, IL-27, RANTES, IP-10 and TNF- α ($r > 0.7$; $p < 0.05$) in healthy donors (Figure
199 1C, D). Interestingly, a striking change in the pattern of interactions between all biomarkers
200 appeared in Non-Neuro^{ZIKV} patients. In these patients, Gas6 significantly and positively
201 correlates only with IFN- α ($r = 0.60$; $p < 0.05$), apart from other 2 functional clusters of
202 significant interactions between the measured biomarkers (Figures 1E, F). In addition, the
203 network graph of Non-Neuro^{ZIKV} patients is more heterogeneous and shows a decentralized
204 topology with lower complexity and connectivity between the immune mediators when
205 compared to the highly dense, homogenous and centralized graph of HDs (Non-Neuro^{ZIKV} vs
206 HDs network density: 0.120 vs 0.224; network centralization: 0.107 vs 0.335; network
207 diameter: 10 vs 5) (Figure 1D, F). Interestingly, a further increase on Gas6 levels above certain
208 threshold (estimated to be above 30ng/mL), as observed in Neuro^{ZIKV} patients (Supp Fig 2),
209 also changes the patterns of interactions. In these patients, Gas6 displays positive correlation
210 with IL-1RA, IL-6, MCP-1 and IP-10 ($r = 0.8$; 0.5, 0.4 and 0.3, respectively; $p < 0.05$) and
211 negative correlations with IFN- α , IFN- γ and IL-18 ($r = -0.4$; -0.7 and -0.5, respectively;
212 $p < 0.05$) (Figures 1G, H). In addition, as shown in the network graph (Figure 1H), because IFN-
213 γ forms a functional cluster of interactions with a variety of cytokines with key roles in the
214 antiviral response, Gas6 also displays indirect negative correlations with TNF- α , IL-1 β , IL-12,
215 IL-22 and IL-27 (r between -0.3 and -0.5; $p < 0.05$), as shown in Figure 1G.

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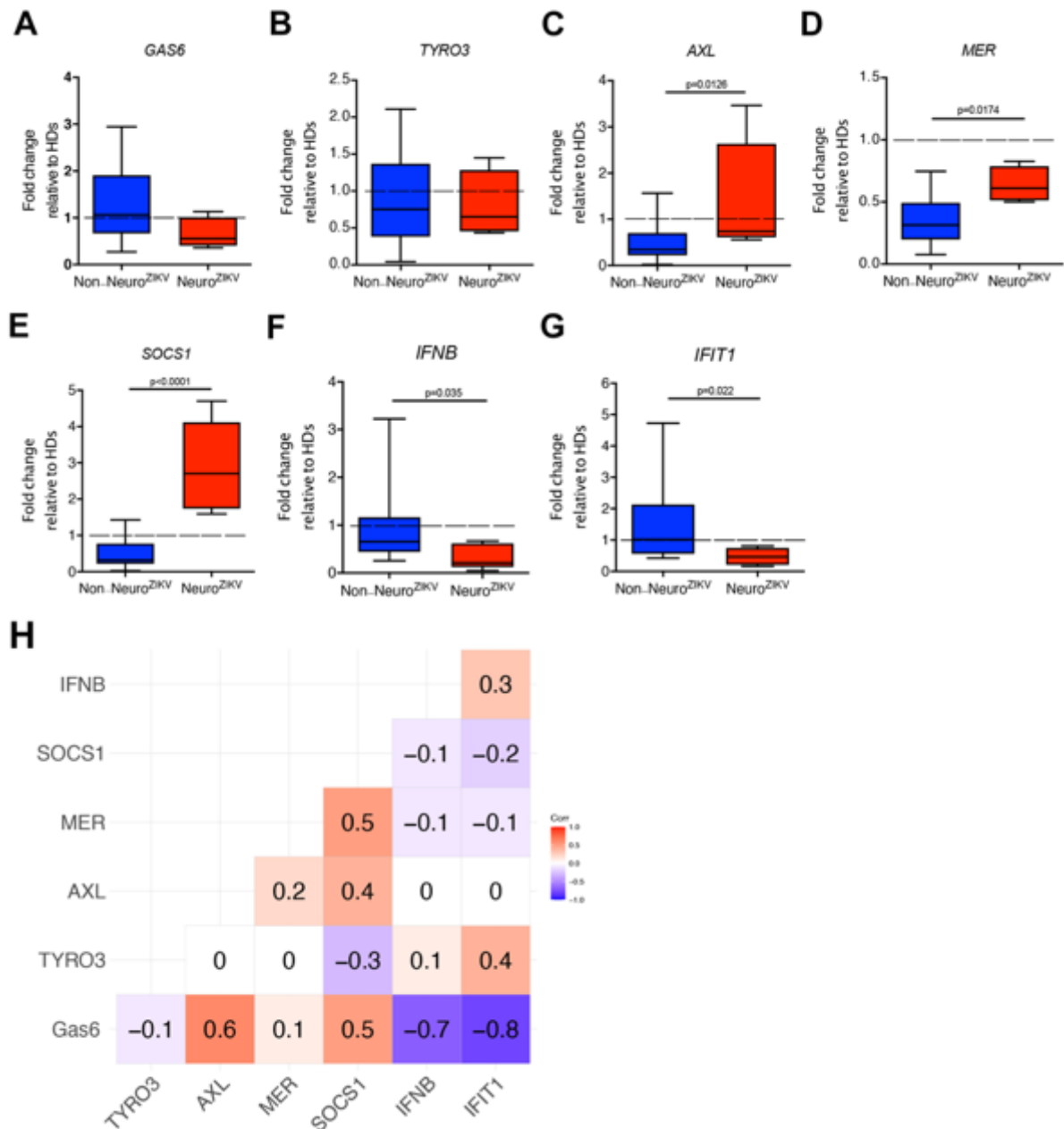


218 **Figure 1: Elevate Gas6 levels in ZIKV patients is a ZIKV-specific response associated with immune**
219 **signatures related to disease manifestation.** (A) Levels of Gas6 in acute-phase patient serum samples of Zika
220 virus (ZIKV) cross-sectional cohort in Campinas, Brazil, were determined by ELISA: Healthy donors (HDs, n =
221 13), ZIKV patients without neurological complications (Non-Neuro^{ZIKV}; n = 57), patients with neurological
222 complications (Neuro^{ZIKV}; n = 19) and patients presenting neurological complications unrelated to ZIKV infection
223 (Neuro^{NON-ZIKV}; n = 14). Gas6 concentration is depicted as Tukey box plots. *P* values were determined using
224 Kruskal-Wallis test with post hoc correction for multiple testing using the original FDR method of Benjamini and
225 Hochberg. (B) Identification of *GAS6* 834 + 7AA genotypes in ZIKV-infected patient groups by agarose gel
226 electrophoresis. Lane 1: Ladder 100kb size marker. Lanes 2-4: Neuro^{ZIKV}; Lanes 5-7: Non-Neuro^{ZIKV}; Lane 8:
227 Mock. AA homotype showing 345 and 136 bp bands. (C, E and G) Representative images of Pearson's correlation
228 matrix calculated for each clinical group. A reduced complexity model was established by focusing on significant
229 interactions between ZIKV-specific immune signatures and Gas6 determined by Pearson's correlation
230 coefficients. Only correlations with associated *p*-value <0.05 are shown and hierarchical clustering was applied.
231 (D, F and H). Representative images of the networks of interactions (prefuse-force layout) determined by
232 Pearson's correlation coefficients. Each connecting line (edge) represents a significant interaction (*p* < 0.05 and
233 absolute Pearson's *r* ≥ 0.5) calculated by the network analysis using the R software. Edge weights and colour are
234 defined as the correlation strength; positive correlations are represented by red edges; negatives correlations are
235 represented by blue edges. Node colour and size represent Log₂ Fold Change of each biomarker normalized by
236 baseline levels in healthy donors.

237

238 To confirm whether Gas6 drives suppression of antiviral response, we conducted
239 quantitative mRNA expression analysis in peripheral blood cells isolated from healthy donors
240 and patients with and without neurological complications. As shown in Figure 2, although Gas6
241 mRNA expression did not change (Figure 2A), mRNA expression of Axl and Mer is
242 upregulated in the peripheral blood cells from Neuro^{ZIKV} patients (Figure 2C, D), whereas
243 Tyro3 did not change (Figure 2B). In accordance with TAM-induced SOCS upregulation [13,
244 22], we observed a significant increase of SOCS1 mRNA in circulating cells from Neuro^{ZIKV}
245 patients (Figure 2E). Conversely, mRNA expression of IFN-β and IFIT-1 is significantly
246 reduced in Neuro^{ZIKV} patients (Figure 2F, G). Thus, in ZIKV adult patients, increased
247 circulating Gas6 levels shows a negative correlation with *IFNB* and *IFIT1* gene expression and
248 positively correlates with *AXL* and *SOCS1* expression in peripheral blood cells (Figure 2H).

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250

251 **Figure 2: Transcriptional landscape of peripheral blood cells from ZIKV adult patients.** Quantitative mRNA

252 expression was determined in peripheral blood cells isolated from healthy donors (HDs) and ZIKV-infected adult

253 patients with (Neuro^{ZIKV}) and without neurological complications (Non-Neuro^{ZIKV}) by qRT-PCR. (A) *GAS6*; (B)

254 *TYRO*; (C) *AXL*; (D) *MER*; (E) *SOCS1*; (F) *IFNB*; (G) *IFIT1*. Graphs depict fold change calculated relative to

255 healthy donor samples (dashed line) as Tukey box plots, after results were normalized to GAPDH housekeeping

256 gene expression. *P* values were determined using student's t-test was used to compare groups with normally

257 distributed data or Mann-Whitney test to compare groups with non-normal distributions; **p* < 0.05; ***p* < 0.01.

258 (H) Spearman's Rank Correlations were determined to assess the association between levels of Gas6 in the serum

259 and expression levels of the transcripts quantified in the matched peripheral blood cells form ZIKV-infected adult

260 patients.

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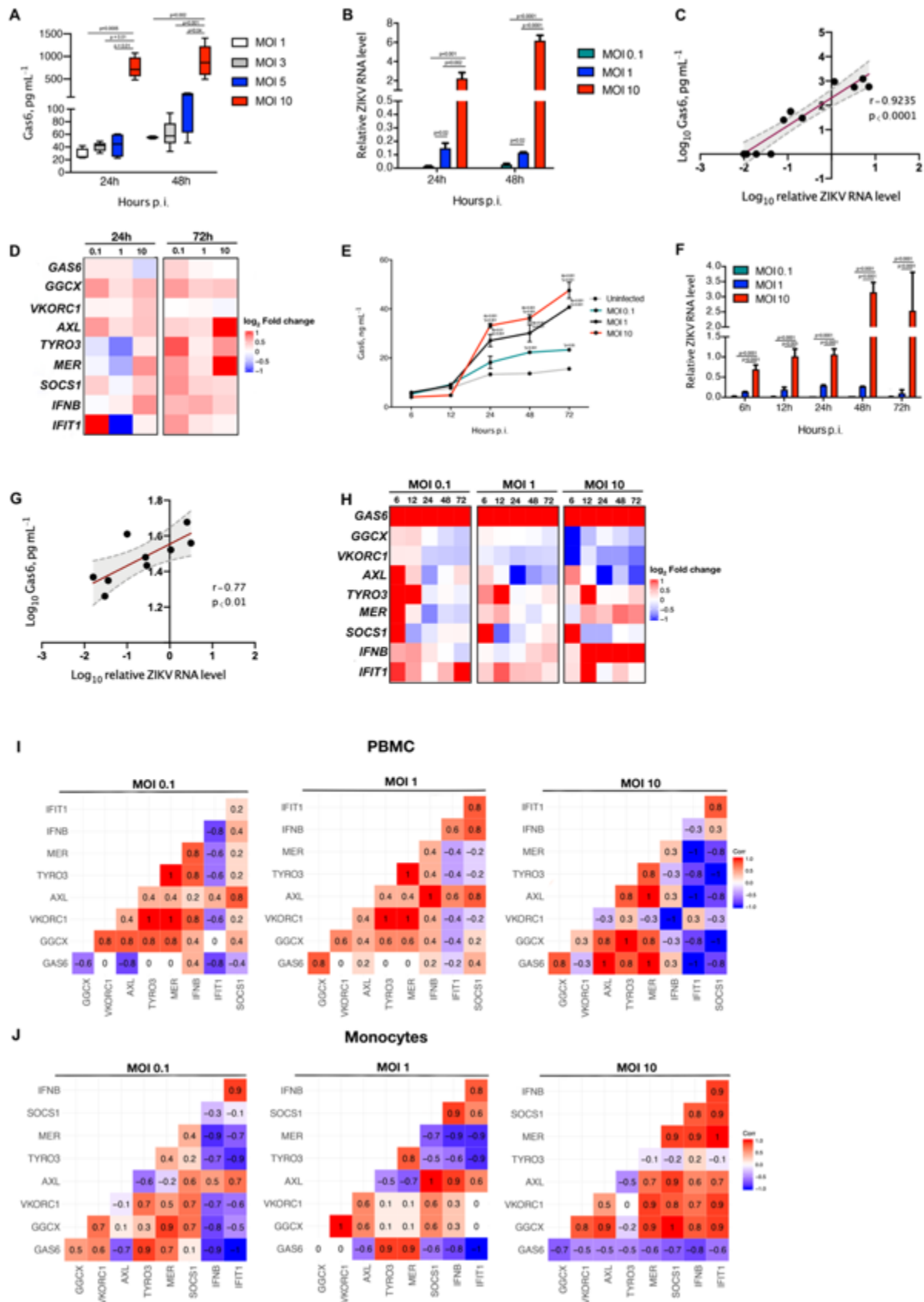
262 **Gas6-producing monocytes dampen the antiviral response during ZIKV infection**

263 Next, we sought to investigate the peripheral cellular sources of Gas6 contributing to
264 its increased levels in the circulation during infection. For that, we cultured and infected
265 PBMCs isolated from healthy donors with ZIKV (Brazilian strain BeH823339) at multiplicities
266 of infection (MOI) 0.1, 1 and 10. Cell pellets and their respective supernatants were collected
267 at different time points post-infection and Gas6 levels were determined by ELISA and cell
268 pellets were used for mRNA quantification by qRT-PCR. PBMCs show an interesting ZIKV
269 viral load-dependent increase of Gas6 production in the supernatants, both at 24 and 48 h post-
270 infection (Figures 3A-C). As neurological complications of ZIKV infection may correlate with
271 BBB disruption, we also evaluated (hBMEC). As shown in Supp Fig 3B, ZIKV infection does
272 not stimulate Gas6 production by hBMECs, indicating that blood mononuclear cells might be
273 the main source of circulating Gas6 during ZIKV infection.

274 To determine whether increased Gas6 production correlates with transcriptional
275 changes induced by infection, we first verified that Gas6 mRNA is not significantly increased
276 at 24h and 48h p.i., as represented in the heatmap of log₂ Fold change in Figure 3D. This is
277 consistent with the analysis *ex vivo*, as shown in Figure 2A. As γ -glutamyl carboxylation of
278 Gas6 via vitamin-K-dependent enzyme γ -glutamyl carboxylase (GGCX) and Vitamin-K
279 epoxide reductase enzyme complex 1 (VKORC1) [23] is needed for Gas6 biological activity
280 and to bridge enveloped viruses to TAM receptors [23-27], we checked the expression of
281 related molecules. Upregulation of GGCX mRNA expression is observed 24h and 72h p.i in
282 infected cells at all MOIs, while VKORC1 mRNA expression was not affected by infection
283 (Figure 3D, Supp Fig 4A). In agreement with a previous study [28], ZIKV infection induces
284 upregulation of Axl, Tyro and Mer transcripts at higher MOI 10 (Figure 3D, Supp Fig 4A).
285 SOCS-1 is upregulated in ZIKV-infected PBMCs at all MOIs (Figure 3D, Supp Fig 4A), except
286 for MOI 1 at 24 h. Interestingly, although IFN- β mRNA is upregulated later during infection
287 (72h p.i.), the cells seem irresponsive, once IFIT-1 mRNA expression does not change in
288 comparison to uninfected cells at MOIs 1 and 10 (Figure 3D, Supp Fig 4A). Only at the lower
289 viral load (MOI 0.1) at 24h p.i. a significant upregulation of IFIT-1 mRNA expression is
290 observed (Figure 3D, Supp Fig 4A), which later decreases at 72h.

291 Monocytes are the dominant cell type infected by ZIKV among peripheral blood cells
292 [29, 30]. To further understand the role of Gas6 in facilitating ZIKV replication, we used
293 cultured human monocytes (THP-1 cells) infected with ZIKV at MOI 0.1, 1 and 10. Cell pellets
294 and supernatants were collected at different time points after infection. Similarly to PBMCs,
295 Gas6 production by THP-1 cells is increased in a time-dependent and viral load-dependent

296 manner, with a significant increase observed after 24hp.i. (Figure 3E-G). Interestingly, as
297 shown in the heatmap of log₂ Fold change in comparison to mock cells (uninfected) in the
298 Figure 3H, Gas6 mRNA is upregulated in THP-1 cells as soon as (6h p.i.) and remains
299 increased throughout the experiment (Supp Fig 4B). GGCX and VKORC1 mRNA expression
300 were not significantly altered, although it tends to decrease at higher MOI 10 (Figure 3H, Supp
301 Fig 4B). Importantly, Axl, Mer, Tyro3 and SOCS-1 mRNA expression was increased early
302 after infection (6-12h p.i.), in particular at lower MOI 0.1 (Figure 3H, Supp Fig 4B). IFN-β is
303 upregulated only at MOI 10 (Figure 3H, Supp Fig 4B), but the cells are irresponsive, once
304 IFIT-1 expression does not significantly change in comparison to uninfected cells (Figure 3H).
305 Similar to PBMCs, a late significant increase (72h p.i.) of IFIT-1 mRNA expression is observed
306 only at the lower MOI 0.1 (Figure 3H, Supp Fig 4B). Interestingly, in PBMCs, Gas6 expression
307 negatively correlates with IFIT-1 at all viral loads (Figure 3I) while in monocytes THP-1 cells,
308 Gas6 negatively correlates with IFN-β and IFIT-1 at all viral loads (Figure 3J). Altogether,
309 these data show that ZIKV infection induces a significant upregulation of Gas6 production in
310 monocytes, which in turn facilitates viral replication by suppressing type I IFN antiviral
311 response.
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313

314

Figure 3: ZIKV Infection stimulates Gas6 upregulation and leads to downregulation of type I IFN response

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in peripheral blood mononuclear cells (PBMCs) and monocytes. (A) Gas6 levels were determined by ELISA

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in the supernatant of PBMCs from healthy donors collected at 24h and 48h after *in vitro* infection with ZIKV at

317 different multiplicities of infections (MOI 0.1, 1, 3, 5 and 10). Gas6 concentration is depicted as Tukey box plots.
318 Gas6 was not detected in uninfected (Mock) and ZIKV-infected PBMCs at MOI 0.1, so these conditions are not
319 represented in the graph. The data shown are representative images of two independent experiments using cells
320 from different donors. P values were determined for comparisons between MOIs at the corresponding time point
321 using Kruskal-Wallis test with post hoc correction for multiple testing using the original FDR method of
322 Benjamini and Hochberg. (B, D, F and H) PBMCs and THP-1 monocytes were challenged with ZIKV (MOI 0.1,
323 1 and 10), respectively. Total cellular RNA was extracted at different time points after infection as indicated in
324 the graphs, and relative viral RNA levels, *GAS6*, *GGCX*, *VKORC1*, *AXL*, *TYRO3*, *MER*, *SOC31*, *IFNB* AND
325 *IFIT1* mRNA levels were determined by real-time quantitative PCR. (B, F) The data shown are mean \pm SEM
326 representative of three independent experiments using cells from different donors. P values were determined for
327 comparisons between conditions at the corresponding time point using One-way analyses of variance statistical
328 test with Bonferroni-corrected multiple comparisons test. (D, H) Heatmap reflecting expression intensity (\log_2
329 Fold change), in comparison to mock (uninfected cells), of genes in PBMCs or in THP-1 monocytes at different
330 time points (from 6 up to 72h) after *in vitro* infection with ZIKV at different multiplicities of infections (MOI 0.1,
331 1 and 10). (E) Gas6 levels were determined by ELISA in the supernatant of THP-1 monocytes at different time
332 points (from 6 up to 72h) after *in vitro* infection with ZIKV at different multiplicities of infections (MOI 0.1, 1
333 and 10). Gas6 concentration is depicted as mean \pm SEM. The data shown are representative of three independent
334 experiments. P values were determined using Two-way analyses of variance with Bonferroni-corrected multiple
335 comparisons test. (C, G) Correlation plots comparing logarithmically transformed ZIKV RNA levels with Gas6
336 concentration within matched cell and supernatant from PBMCs or THP-1 monocytes, respectively, after *in vitro*
337 infection with ZIKV. Regression line indicated in red with 95% confidence area shown in shaded gray. (C)
338 Spearman's Rank and (G) Pearson's correlation coefficient and associated p-values are shown. (I, J)
339 Representative images of Spearman's Rank Correlations matrixes. Correlations were determined to assess the
340 association between transcripts quantified in the PBMCs or in THP-1 monocytes, respectively, after *in vitro*
341 infection with ZIKV at different multiplicities of infections (MOI 0.1, 1 and 10). Relative gene expression levels
342 at different time points (from 6 up to 72h) in the corresponding MOI condition were used as input.

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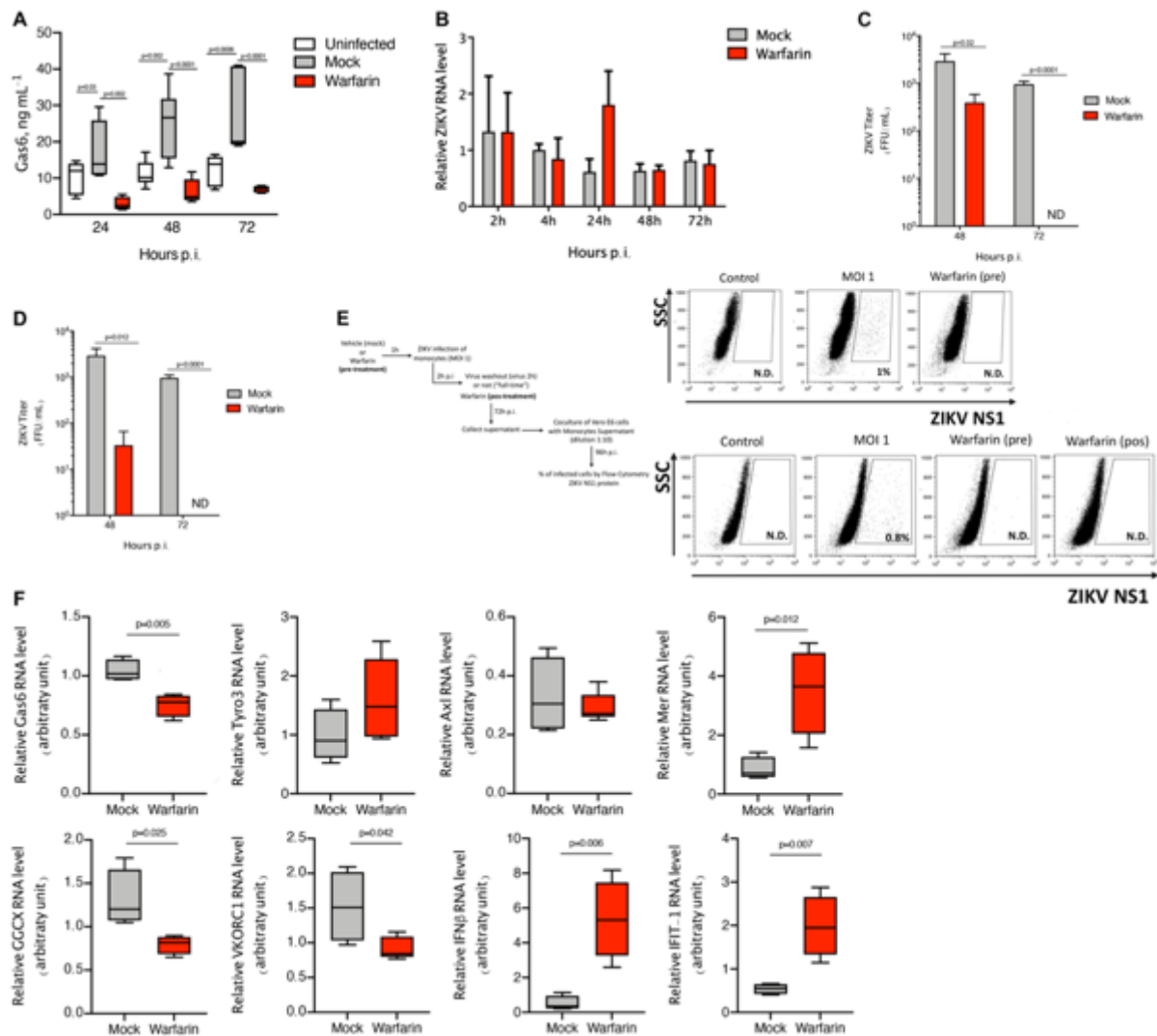
344 **Inhibition of Gas6 γ -glutamic acid carboxylation (Gla domain) restores antiviral response**

345 To further determine the mechanistic link between Gas6 production, ZIKV replication and
346 suppression of antiviral response, we tested whether inhibiting Vitamin-K-dependent γ -
347 carboxylation of Gas6 glutamic acid residues (Gla domain) restores antiviral immune response
348 and controls viral replication. We used low-dose warfarin, which specifically block γ -
349 carboxylation of Gla domain in Gas6, resulting in decreased TAM receptor activation [23, 27,
350 31]. In parallel, we used R428, a well-known inhibitor of Axl tyrosine kinase activity tested in
351 ZIKV infection [16]. THP-1 cells were infected with ZIKV at MOI 1 and treated with 2 μ M of
352 warfarin or 2 μ M R428 throughout the course of infection (up to 72h). Treatments started at the
353 moment of infection or 2h p.i. (after virus entry), and cell pellets and their respective
354 supernatants were collected at different time points. Warfarin treatment blocked Gas6

355 upregulation at the protein (Figure 4A) and mRNA levels (Figure 4F), although ZIKV RNA
356 level did not change (Figure 4B). Warfarin also decreased GGCX and VKORC1 mRNA
357 expression (Figure 4F). Corroborating this, R428 treatment potently reduced ZIKV-induced
358 Gas6 production (Supp Fig 5A).

359 Interestingly, incubation of ZIKV -permissive Vero cell line with the supernatant from the
360 ZIKV-infected monocytes revealed that warfarin treatment, at the moment of infection or 2h
361 p.i., significantly decreases the production of viable viral particles at 48h p.i. (Figures 4C, D).
362 At 72h p.i., the drug completely blocked viral production (Figures 4C, D). R428 did not change
363 ZIKV RNA levels but showed a modest but significant effect on decreasing viral production
364 at 72h p.i. (Supp Fig 5B, C). Accordingly, flow cytometry analysis of ZIKV NS1 protein in
365 Vero cells showed that infection was not detected when cells were exposed to supernatants
366 from monocytes treated with warfarin, regardless whether the initial virus inoculum was
367 washed-out 2h p.i. or not or whether warfarin treatment started 2h p.i. (pos-treatment approach)
368 or at the moment of infection (pre-treatment approach) (Figure 4E). Interestingly, warfarin
369 induced a potent upregulation of IFN β and IFIT-1 expression (Figure 4F). Meanwhile, R428
370 increased the expression of Axl and Mer (Supp Fig 5D). Consistent with the blockade of type
371 I IFN by Axl signalling pathway, R428 allowed the increase of IFN- β and IFIT-1 (Supp Fig
372 5D), while it did not significantly affect GGCX or VKORC1 mRNA expression (Sup Fig 5D).
373 Thus, blockage of Gas6 activity by inhibition of γ -carboxylation of Gla domain restores type I
374 IFN antiviral response sufficient to block the production of infective virus particles.
375 Collectively, these data show the mechanism by which Gas6 upregulation contributes to a
376 higher ZIKV infection.

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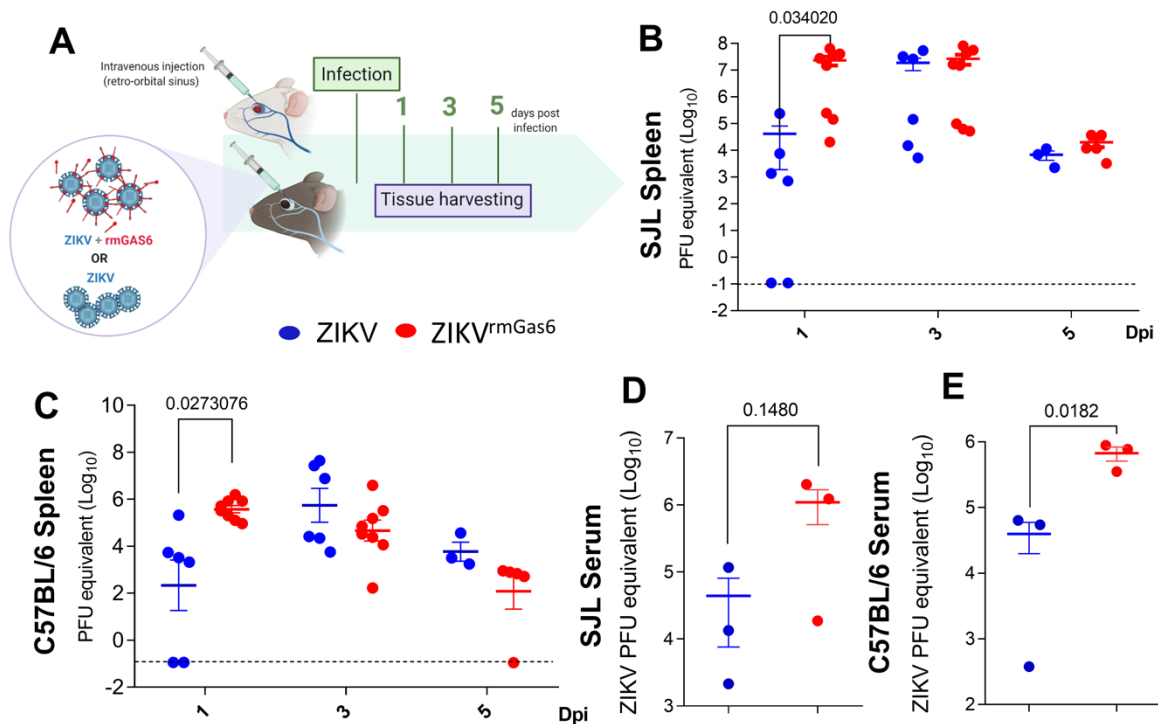
Figure 4: Inhibition of Gas6 γ -glutamic acid carboxylation (Gla domain) by warfarin treatment restores antiviral response. (A) Gas6 levels were determined by ELISA in the supernatant of THP-1 monocytes collected at 24h, 48h and 72h after *in vitro* infection with ZIKV (MOI 1). Infected cells were treated or not (mock) with 2 μ M warfarin throughout the course of infection. Gas6 concentration is depicted as Tukey box plots. The data shown are representative images of three independent experiments. P values were determined for comparisons between conditions at the corresponding time point using One-way analyses of variance statistical test with Bonferroni-corrected multiple comparisons test. (B, F) THP-1 monocytes were challenged *in vitro* with ZIKV (MOI 1) and treated or not (mock) with 2 μ M warfarin throughout the course of infection. Total cellular RNA was extracted at different time points after infection. Relative viral RNA levels, *GAS6*, *GCGX*, *VKORC1*, *AXL*, *TYRO3*, *MER*, *SOCS1*, *IFNB* AND *IFIT1* mRNA levels were determined by real-time quantitative PCR. The data shown are representative images of three independent experiments. (F) Graphs depict relative RNA expression as Tukey box plots after results were normalized to GAPDH housekeeping gene expression. P values were determined using student's t-test to compare groups with normally distributed data or Mann-Whitney test to compare groups with non-normal distributions. (C, D) ZIKV titer (FFU/mL) was determined by focus-forming assay after incubation of ZIKV-permissive Vero E6 cell line with the 48h or 72h supernatant from monocytes after infection *in vitro* with ZIKV (MOI 1), treated or not (mock) with 2 μ M warfarin at the moment of infection

395 (pre-treatment) or 2h after infection (post-treatment), respectively. The data shown are representative images of
396 three independent experiments. P values were determined using Mann-Whitney test comparing conditions at the
397 corresponding time point. (E) Vero E6 cells were incubated for 4 days with 72h p.i. supernatants from THP-1
398 monocytes infected *in vitro* with ZIKV MOI 1, treated or not (mock) with 2 μ M warfarin 1h before infection (pre-
399 treatment) or 2h after infection (pos-treatment). The virus was left throughout the course of the experiment, up to
400 72h in the monocyte culture (upper panel), or removed 2h after infection by washing the cells three times with
401 PBS (virus washout; lower panel). The percentage of infected cells was determined by flow cytometry analysis
402 of ZIKV NS1 protein expressed in Vero cells. The data shown are representative images of three independent
403 experiments.

404

405 **Gas6 facilitates ZIKV replication in immunocompetent mice**

406 So far, our data show that Gas6 expression is stimulated during ZIKV infection and it
407 is associated with neurological complications in human patients. The underlying pathogenic
408 mechanism of Gas6 involves attachment and invasion and facilitation of viral replication by
409 suppressing the antiviral response. Although we prove a correlation between Gas6 levels,
410 ZIKV RNA load and production of infecting particles in infected cells *in vitro*, we could not
411 find the same association in our cross-sectional cohort. To confirm that upregulation of Gas6
412 favours viral replication *in vivo*, immunocompetent adult C57BL/6 and SJL mice were
413 intravenously infected with 10² pfu ZIKV (BeH815744) previously incubated or not with
414 1 μ g/mL recombinant mouse Gas6 (ZIKV^{rmGas6}) (Figure 5A). Viral load was determined at 1,
415 3 and 5 d.p.i. in the spleen and serum (Figure 5A). Interestingly, infection with Gas6-coated
416 ZIKV resulted in increased viral RNA in the spleen (Figure 5B-C) and serum (Figure 5D-E) in
417 both strains at 1 d.p.i. After 3 and 5 days of infection, differences were no longer detected.
418 Similar to *in vitro* infection of cultured human cells, these data corroborate the role of Gas6 in
419 favouring ZIKV infection and replication.



420

421 **Figure 5: Gas6 facilitates ZIKV replication in immunocompetent mice.** SJL and C57BL/6 mice were infected
 422 by intravenous route with 10^2 pfu of pure ZIKV (BeH815744) or ZIKV previously incubated with rmGas6
 423 ($1\mu\text{g}/\text{mL}$) (ZIKV^{rmGas6}) (A). After 1-, 3- and 5-days post-infection the viral load was analysed by qPCR in spleen
 424 (B, C) and serum (D, E) of both mice strains. *P* values were determined using a Student's *t*-test. Graph bars are
 425 shown as mean \pm SEM and are representative of three independent experiments; 1 and 3 dpi: ZIKV ($n = 6$),
 426 ZIKV^{rmGas6} ($n = 8$); 5 dpi: ZIKV ($n = 3$), ZIKV^{rmGas6} ($n = 5$).

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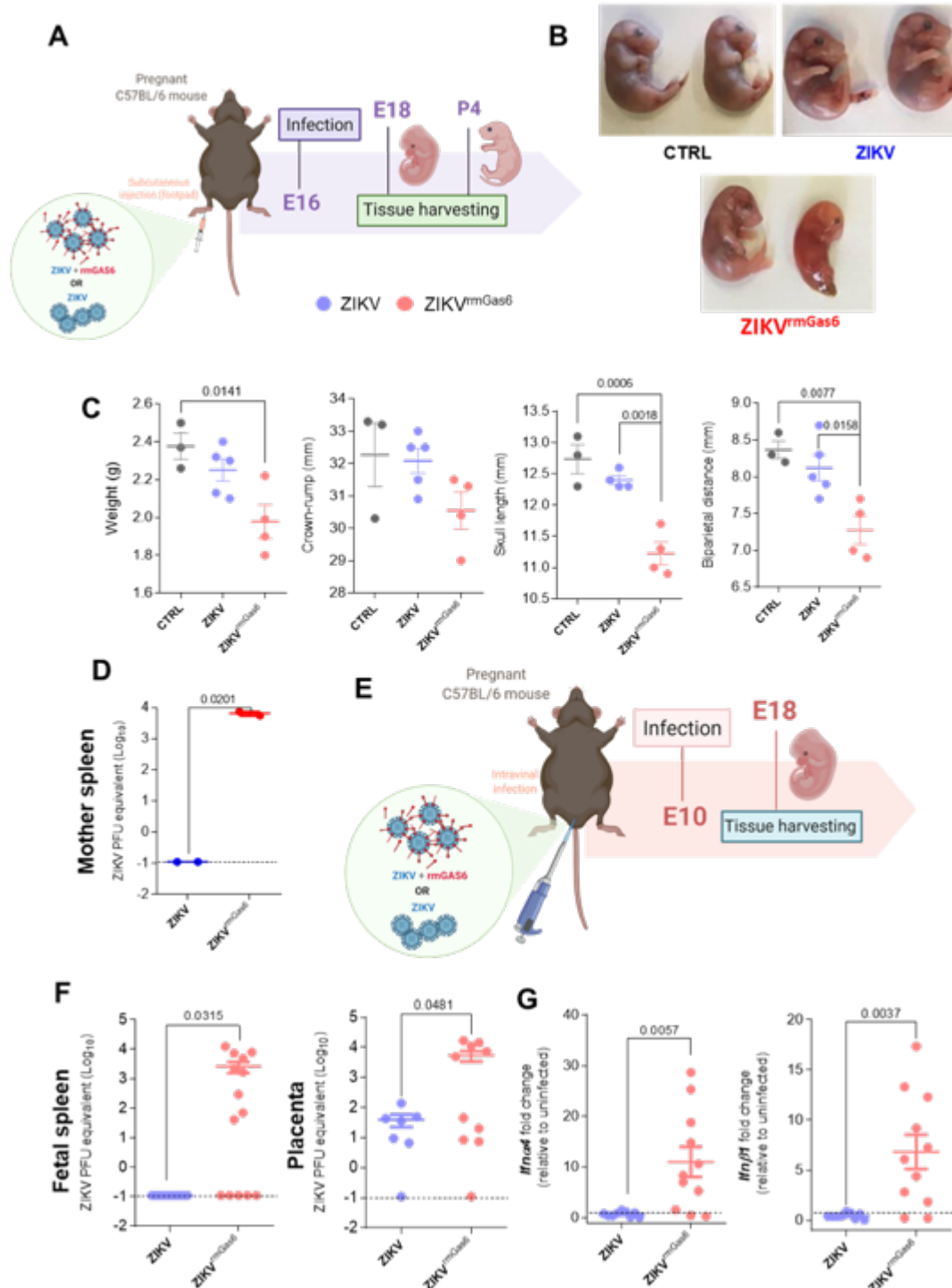
428 **Gas6 promotes congenital syndrome and transplacental infection in ZIKV-infected**
 429 **immunocompetent mice**

430 To further evaluate whether Gas6 drives disease severity during ZIKV infection, we
 431 evaluated its association with transplacental infection and development of congenital zika
 432 syndrome (CZS). Determination of Gas6 levels in the serum of 8 ZIKV-infected pregnant
 433 women revealed a significant increase during acute phase of infection (18.05 ± 1.97 ng/mL
 434 versus 13.36 ± 1.23 ng/mL) (Supp Figure 6A). On the other hand, we did not detect a difference
 435 in Gas6 levels in the serum of babies with ($n = 2$) or without ($n = 4$) CZS (Supp Figure 6B).

436 As the number of ZIKV-infected pregnant women with fetal growth-associated
 437 malformations included in this study was limited to two patients, we decided to evaluate the
 438 effect of Gas6 using a mouse model of CZS. For this, we subcutaneously injected pregnant
 439 C57BL/6 mice with Gas6-coated ZIKV (ZIKV^{rmGas6}) at embryonic day (E) 16 and performed

440 analysis at E18 or postnatal day (P) 4 (Figure 6A and Supp Figure 6). Importantly, infection of
441 pregnant C57BL/6 mice with ZIKV^{rmGas6} rendered their offspring susceptible to fetal
442 malformations (Figure 6B). We evidenced macroscopic changes at E18 and growth delay at
443 P4 (Figure 6B, C). Significant differences in biparietal distance, skull length and weight of the
444 ZIKV^{rmGas6} newborns were observed (Figure 6C). No difference in viral load between ZIKV
445 and ZIKV^{rmGas6} groups was found in the placenta and fetal spleen at E18 (Supp Figure 7A) as
446 well as in the spleen of P4 newborns (Supp Figure 7B). However, a higher viral load was
447 observed in the spleen of ZIKV^{rmGas6}-infected pregnant mice as long as 8 days post-infection
448 (day 0 at E16, and day 8 at P4) (Figure 6D).

449 Since *Axl* is expressed in decidua, Hoffbauer, trophoblast and fibroblasts cells of
450 human placenta² and ZIKV has been considered a sexually transmitted disease (STD), we
451 sought to verify whether *Gas6* facilitates intra-uterine ZIKV infection. For that, C57BL/6
452 pregnant mice were intravaginally infected at E10 with ZIKV or ZIKV^{rmGas6} and viral load was
453 determined at E18 (Figure 6E). Contrasting with the subcutaneous infection, no difference in
454 fetal size was observed (Supp Figure 7C, D), but there is a significant increase in ZIKV load
455 in the fetal spleen and in the placenta of ZIKV^{rmGas6}-infected pregnant mice (Figure 6F).
456 Although we see no change in *Stat1* and *Socs1* mRNA expression, *Ifna4* and *Ifnb1* expression
457 were unexpectedly increased in the placenta of ZIKV^{rmGas6}-infected pregnant mice (Figure 6G).
458



459

460 **Figure 6: Gas6 promotes ZCS and transplacental infection in ZIKV-infected resistant immunocompetent**

461 **mice.** Pregnant mice were infected subcutaneously with pure ZIKV (10^5) or ZIKV previously incubated with

462 rmGas6 ($1\mu\text{g}/\text{mL}$) (ZIKV^{rmGas6}) on E (embryonic day) 16. The organs were harvested on E18 or postpartum day

463 (P) 4 (A). The viral load was analysed by qPCR. Foetus's pictures at E18 (B). Dimension analyses at P4 (C). *P*

464 values were determined using a one-way ANOVA followed by Tukey's post hoc test. Mother's spleen viral load

465 (D). Pregnant mice were also infected intravaginally on E10 with pure ZIKV (10^5) or ZIKV previously incubated

466 with rmGas6 ($1\mu\text{g}/\text{mL}$). Tissues were harvested at E18 (E). Viral load in spleen and placenta (F). Gene expression

467 in placenta (G). Fold change was calculated between uninfected and infected groups. The significances between

468 the groups were performed by Student *t*-test (D, F and G). Graph bars are shown as mean \pm SEM and are
469 representative of two independent experiments. Numbers of experimental groups: (B) Control n=6; ZIKV n=11;
470 ZIKV^{rmGas6} n=14. (C) Control n=3; ZIKV n=5; ZIKV^{rmGas6} n=4. (D) ZIKV n=2; ZIKV^{rmGas6} n=2. (F) ZIKV n=7;
471 ZIKV^{rmGas6} n=10.
472

473 DISCUSSION

474

475 The ZIKV epidemic that occurred in 2016 was a great public health problem. ZIKV is
476 able to cross the blood-brain and placental barriers, infecting the central nervous system (CNS)
477 of adults and developing fetuses, greatly increasing the severity of the disease and the impact
478 of this infection [32-39]. In fact, studies with *ex vivo* slices from human cortical tissue and
479 adult mice demonstrated that ZIKV is able to infect and replicate in mature neurons, causing
480 local inflammation and damage in hippocampal synapses, resulting in impairment of synaptic
481 function and memory [40]. These findings show that contemporary occurring ZIKV strains are
482 highly neurotropic and detrimental to both mature as well as to the developing central nervous
483 system (CNS) [33, 41]. Although studies have raised the contribution of several factors in the
484 development of these conditions [34, 42, 43], the mechanisms by which the virus reaches the
485 brain of adult individuals, is not fully understood. In this context, here we demonstrate that
486 circulating levels of Gas6 are upregulated in ZIKV-infected patients showing neurological
487 complications. This upregulation is ZIKV-specific and could be influenced by intrinsic host
488 features such as allelic variability of the human *GAS6* gene. Elucidation of the human *GAS6*
489 gene structure and allelic variants revealed the presence of eight different variants confirmed
490 to be single nucleotide polymorphisms (SNPs). The SNP in the intron 8 (c.834+7G>A;
491 genotypes GG, AG, and AA) controls circulating levels of Gas6 and plays a key role in the
492 pathogenesis of different circulatory disease, such as preeclampsia, acute coronary syndrome
493 and stroke [21, 44-46]. We observed no difference in *GAS6* haplotypes, as both groups of
494 patients showed the predominant frequency of the c.834 + 7G>A;AA genotype, indicating that
495 higher Gas6 levels observed in Neuro^{ZIKV} patients correlate to ZIKV infection.

496 Different from hBMECs, monocytes were more responsive to ZIKV infection by
497 upregulating transcriptional and translational machinery resulting in the increase of Gas6
498 expression. This indicates that monocytes might be the main cellular source and contributing
499 to increased Gas6 levels in the plasma of ZIKV-infected patients. It is worth mentioning that
500 this is consistent with the fact that circulating CD14⁺CD16⁺ monocytes carry ZIKV particles
501 during infection [29, 30]. Moreover, in immune cells, engagement of TAM receptors by Gas6
502 results in the inhibition of inflammatory responses driven by Toll-like receptors (TLR) and
503 type I IFN signalling pathways [12-14, 22, 47]. The mechanism depends on the expression of
504 SOCS, resulting in an autocrine and paracrine inhibition of both signalling pathways during
505 ZIKV infection *in vivo*. This response in leukocytes, in particularly in monocytes, and
506 microvascular endothelial cells might contribute to virus spread and replication in the periphery

507 and potentially in target tissues, such as the CNS. Therefore, our network analysis of
508 associations between Gas6 and ZIKV-specific immune signatures and transcriptional profiling
509 of peripheral blood cells further elucidate the pathogenic mechanism of action of Gas6.
510 Different from healthy donors, the increase of Gas6 levels in Non-Neuro^{ZIKV} patients changes
511 its patterns of correlations, and we have found a significant positive correlation between Gas6
512 and IFN- α . This along with increased circulating levels of proinflammatory cytokines,
513 chemokines and growth factors suggests that an active production of a network of immune
514 mediators could provide a strong antiviral environment during the acute phase of disease,
515 resulting in a milder clinical outcome. Conversely, in Neuro^{ZIKV} patients, the further increase
516 of Gas6 expression changes its pattern of interactions. Gas6 displays a positive correlation with
517 IL-6, MCP-1, IP-10, IL-1RA and negative correlations with IFN- α , IFN- γ and IL-18. In
518 addition, as shown in the network graph, as IFN- γ forms a functional cluster with a variety of
519 important mediators in host defense against infection [9, 48], Gas6 also displays indirect
520 negative correlations with TNF- α , IL-1 β , IL-12, IL-22 and IL-27. This pattern of interactions
521 reveals that elevation of circulating Gas6 levels above a certain threshold, which we estimate
522 to be higher than 30ng/mL, dampens the protective immune response that provide a strong
523 antiviral environment and a milder clinical outcome. In support, transcriptional analysis in
524 peripheral blood cells from ZIKV patients with neurological complications indicate that this
525 response is achieved by a ZIKV–Gas6–TAM receptor interaction that ultimately induces
526 downregulation of type I IFN genes modulated by SOCS1. This mechanism was corroborated
527 in cultured monocytes, where ZIKV-induced Gas6 expression also correlated with suppression
528 of type I IFN response.

529 The antiviral response also involves recruitment and coordination of specific subsets of
530 immune cells orchestrated primarily by chemokines. Gas6 can also function as an
531 inflammatory molecule by inducing leukocyte adhesion on endothelial cells surface and
532 extravasation through a P-selectin–dependent mechanism [49]. Moreover, Axl and Mer, in
533 cooperation with IFNAR signalling, have been described as key molecules for maintenance of
534 the blood-brain barrier (BBB) and protection to WNV and La Crosse virus infection [50].
535 Accordingly, *in vivo* studies of ZIKV infection in immunocompromised IFNAR-KO mice
536 lacking Axl have shown protection from ZIKV neuropathogenesis and severe infection [18].
537 In this context, suppression of IFNAR signalling due to increased amounts of circulating Gas6
538 in Neuro^{ZIKV} patients potentially impairs BBB homeostasis and integrity. This may allow viral
539 and cellular extravasation to CNS through loose endothelial cells junctions. Noteworthy, in

540 Neuro^{ZIKV} patients, Gas6 positively correlates with IL-6, IP-10 and MCP-1, suggesting that
541 there might be a parallel between ZIKV and cytokine release syndrome (CRS). In addition, our
542 data reveal a unique and inappropriate inflammatory response in Neuro^{ZIKV} patients. This
543 response is defined by a failed type IFN-I response in the periphery, juxtaposed to elevated
544 chemokines levels and high expression of IL-6, leading to recruitment and infiltration of
545 effector immune cells in deep tissues. Thus, infiltration of cells, such as CD4 and CD8 T cells,
546 along with infection of mature neurons could induce a local inflammatory response in the CNS,
547 potentially resulting in neurological complications [40]. We propose that Gas6 mediated
548 reduced innate antiviral defences coupled with exuberant inflammatory cytokine production
549 are driving features of severe clinical outcome in ZIKV adult patients.

550 It has been shown that inhibition of Axl signalling by different pharmacological or
551 genetic approaches decrease ZIKV replication *in vitro* in CNS cells, endothelial cells and
552 dendritic cells as well as reduce brain pathology in experimental models [16, 18, 51-53]. TAM
553 receptors activation by Gas6 is highly dependent on its γ -carboxyglutamic acid-rich (Gla)
554 domain, required for its biological activity and to bridge enveloped viruses to bind and activate
555 TAM receptors [23-27]. After translation, Gas6 is activated by γ -glutamyl carboxylation via
556 the Vitamin-K cycle and cycle [23]. Vitamin-K epoxide reductase enzyme complex 1
557 (VKORC1) recycles Vitamin-K Epoxide back to Vitamin-K Hydroquinone, which in turn
558 serves as a co-factor in the γ -carboxylation of Gas6 induced by Vitamin-K-dependent enzyme
559 γ -glutamyl carboxylase (GGCX). Low-dose of warfarin functions as a direct VKORC1
560 inhibitor, preventing γ -carboxylation of Gas6 and TAM receptor activation [23, 31]. Thus, we
561 used low-dose warfarin to further determine the mechanistic link between Gas6 production,
562 ZIKV replication and suppression of antiviral response. It is important to highlight that one
563 could argue that decreased Gas6 production after warfarin treatment could be a result of
564 decreased binding of anti-Gas6 capture antibody in the ELISA due to restricted recognition of
565 γ -carboxylated residues (amino acids 53-92) in Gas6 protein. However, the capture antibody
566 recognizes the residues 118-678, which are not in the Gla-domain. In addition, Gas6 can be
567 transcriptionally upregulated by Axl-mediated autocrine mechanisms [23], which could
568 explain warfarin-induced downregulation of Gas6 expression. In our experiments, this
569 mechanism was confirmed by restoration of antiviral response and complete blockage of
570 production of infective viral particles when monocytes were treated with warfarin. These
571 findings implicate that, by tethering ZIKV to TAM receptors, Gas6 mediates the pivotal
572 suppression of type I interferon receptor (IFNAR) signalling, thereby favouring ZIKV evasion

573 from antiviral immunity and sustained replication, pointing out how interactions with
574 membrane receptors go beyond attachment and internalization of viral particles [54].

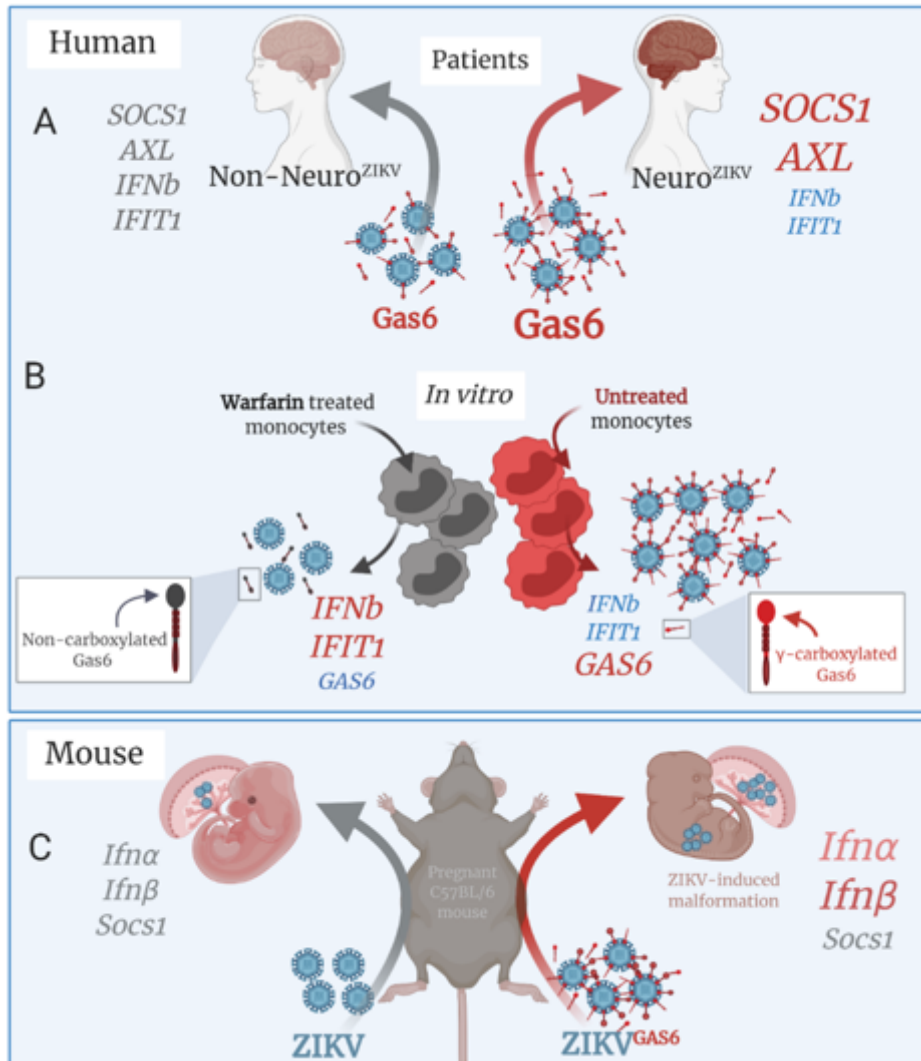
575 We demonstrate a correlation between Gas6 protein expression, ZIKV RNA load and
576 production of infecting particles associated with suppression of type I IFN response in human
577 cells infected *in vitro*. Although we did not detect difference in ZIKV RNA load in the
578 peripheral blood specimens in our cross-sectional cohort, this could probably be due to the
579 moment blood sampling was performed. When immunocompetent adult C57BL/6 and SJL
580 mice were infected with Gas6-coated ZIKV (ZIKV^{rmGas6}), we observed that upregulation of
581 Gas6 increases viral load very early after infection, at 1 d.p.i. After 3 and 5 days of infection,
582 differences were no longer detected. This might explain our observation in patients, as the
583 median interval between illness onset and sampling was 4 days, ranging from 1 to 6 days. Thus,
584 similar to *in vitro* infection of cultured human cells these data corroborate the role of Gas6 in
585 increasing ZIKV replication *in vivo*. Intriguingly, some studies that also used *in vivo*
586 experimental approaches have demonstrated that the presence of TAM receptors are not
587 required for ZIKV infection in the testis, eye, brain and neither for transplacental infection [55-
588 57]. Nevertheless, our data are the first to describe that Gas6 facilitates ZIKV replication in
589 adult immunocompetent mice model acting as a gatekeeper for viral entry. In addition, these
590 observations corroborate *in vitro* studies of ZIKV and DENV infection [16, 24].

591 We had previously evidenced the resistance of C57BL/6 mice to congenital
592 malformations caused by ZIKV [34]. Here, we demonstrated that rmGas6 could render
593 C57BL/6 susceptible to ZIKV induced growth restriction and viral transplacental passage
594 associated with increased placenta *Ifna* and *Ifnβ* expression. Axl is expressed in decidua,
595 Hoffbauer, trophoblast and fibroblasts cells of human placenta, suggesting as the receptor viral
596 transplacental passage [58]. Interestingly, recent studies, one in preprint, have demonstrated
597 that CZS is associated with exacerbated type I IFN and insufficient type III IFN in placenta at
598 term, probably without modulation of TAM or TIM receptor mRNA expression in placental
599 sites infected with ZIKV [59, 60]. In addition, human babies carrying CG/CC genotypes of
600 rs2257167 in IFNAR1 presented higher risk of developing CZS [59]. However, the correlation
601 between TAM receptors and Gas6 expression in susceptibility to congenital ZIKV syndrome
602 has not been established. Our data demonstrate that intravaginal infection with Gas6-coated
603 ZIKV leads to increased viral load in the placenta of pregnant mice and in the spleen of their
604 foetuses, pointing out the role of Gas6-TAM receptors for transplacental passage. Supporting
605 these results, previous studies have demonstrated that placental viral infection is capable to
606 elicit morphological changes in the foetal brain associated with IFN-β immune response at the

607 maternal-foetal interface [58]. Interestingly, deficient *Ifnar* mice, despite higher viral load, do
608 not present significant impairment in the offspring development. However, those whom type I
609 IFN response was present (*Ifnar*^{+/-}), despite lower viral load, demonstrated placental spiral
610 arteries apoptosis, foetal hypoxia and prominent growth restriction. In the same study,
611 intraperitoneal injection of poly: IC, a major TLR3 agonist which is also activated by ZIKV
612 [61], led to resorption of all foetuses in an IFNAR-dependent manner [58]. Accordingly, the
613 hypothesis for our findings would be that the immunocompetence described in C57BL/6
614 animals may be beneficial while low amounts of pathogen are present. However, if viral load
615 is increased, as it happens due to infection with ZIKV^{tmGas6}, the exacerbated immune response
616 in the pregnant mother could result in detrimental changes to foetal development probably
617 resulting from increased placental damage.

618 Together, our observations in ZIKV-infected adult patients and *in vitro* infections
619 identify a relevant pathogenic mechanism associated to the development of severe outcomes
620 during ZIKV infection, where the virus promotes upregulation of its own ligand Gas6, which
621 contributes to viral infectivity by suppressing an efficient type I IFN antiviral response (Figure
622 7A and B). Our *in vivo* findings in immunocompetent mice models corroborate the role of Gas6
623 for favouring ZIKV infection by acting as a gatekeeper in addition to its association with the
624 pathogenesis of congenital syndrome (Figure 7C). Given the novelty of our findings, the
625 relevance to other fields such as the correlation of Gas6 plasma levels and disease severity that
626 until now has been described in lupus [62], liver fibrosis [63] and preeclampsia [64], the
627 relevance of the immunocompetent mouse model for studies with ZIKV and the potential for
628 development of new therapeutic agents that may target Gas6, our data open avenues for
629 research on the factors that drive neurological complications in adults and newborns by this
630 important emerging virus.

631



632

633 **Figure 7: Gas6 is associated with neurological complications in humans and drives ZIKV-**
634 **congenital malformations in immunocompetent mice.** (A) Elevated Gas6 levels in the serum of ZIKV patients
635 with neurological complications (Neuro ZIKV) is associated with upregulation of AXL and SOCS1 and
636 downregulation of IFN β and IFIT1. (B) In vitro treatment with Warfarin reduces Gas6 levels and restores type I
637 IFN antiviral response by inhibiting Gas6 γ -glutamic acid carboxylation thus decrease ZIKV infection. (C) The
638 mouse infection with ZIKV pre-treated with recombinant Gas6 (ZIKV Gas6) facilitates Zika virus infection and
639 leads to malformations in the offspring which is associated with upregulation of Ifn α and Ifn β . Created with
640 BioRender.com.

641

642 MATERIALS AND METHODS

643

644 Ethical Approval: The present study was conducted according to the Declaration of Helsinki
645 principles after approval by the Research Ethics Committee of the University of Campinas
646 (CAAE: 56793516.0.0000.5404) [9]. Written informed consent was obtained from all
647 participants or from participants' parents / legal guardians. In addition, animal experiments
648 were carried out in accordance with the recommendations of the IACUC (Institutional Animal
649 Care and Use Committee). The protocols were approved by Ethics Committee for Animal
650 Research of University of Sao Paulo (CEUA - 63/2016) and all efforts were made to minimize
651 animal suffering.

652

653 Patients: This was a cross-sectional study that enrolled 90 patients and 13 healthy donors during
654 the Zika virus (ZIKV) epidemic in Brazil. The patients were admitted to hospitals through
655 emergency departments (ED) in the city of Campinas, Southeast of Brazil, during the ZIKV
656 outbreak (February 2016 to June 2017). 57 of these 90 patients had a mild self-limited illness
657 by ZIKV, characterized by the presence of the following symptoms: fever, rash, conjunctivitis,
658 myalgia, headache, arthralgia and periarticular edema, while 33 patients had clinical signs
659 compatible with neurological syndromes, 19 with diagnosis of ZIKV and 14 without ZIKV
660 detection. This study included patients presenting clinical diagnosis of encephalitis,
661 meningoencephalitis, transverse myelitis and Guillain-Barré syndrome of undetermined origin
662 that had preceding symptoms compatible with arbovirus infection up to 60 days before the
663 onset of the neurological condition. The clinical classification was done following international
664 clinical criteria [65, 66] and patients with history of a prior motor neuropathy or spinal cord
665 disease were excluded of the study. Clinical data were retrospectively retrieved from medical
666 records and the clinical and demographics data are summarized in the Table 1 in the main text.
667 Patients included in this study were grouped as follows:

668 *Patients ZIKV⁺ with mild symptoms (Non-Neuro^{ZIKV}):* 57 patients presenting mild
669 symptoms of infection, such as low fever, rash, myalgia and conjunctivitis. ZIKV infection
670 was confirmed by qRT-PCR.

671 *Patients ZIKV⁺ with neurological complications (Neuro^{ZIKV}):* 19 patients presenting
672 neurologic complications secondary to ZIKV were diagnosed with Guillain-Barré syndrome,
673 encephalitis, meningitis, meningoencephalitis or transverse myelitis according to clinical
674 criteria. Neurological complications started at a median of 4 days after onset of ZIKV acute
675 symptoms. ZIKV infection was confirmed by qRT-PCR and/or specific IgM detection,

676 *Patients ZIKV with neurological symptoms (Neuro^{NON-ZIKV}):* 14 patients presenting
677 neurologic complications as described above but were negative for ZIKV by qRT-PCR and/or
678 specific IgM detection. Although three of these patients were positive for DENV by NS1/IgM
679 Rapid immunochromatographic tests, the pathological origin of these neurological symptoms
680 was undetermined at the moment of sample collection. All these patients were negative for
681 DENV, CHIKV and OROV by qRT-PCR.

682 *Healthy donors (HDs):* 13 age-matched individuals without signs of infection within
683 30 days prior to sample collection. They were included and pre-screened for presence of ZIKV
684 RNA and ZIKV-specific antibodies.

685 Additionally, not included in the 90 described patients, we analysed acute-phase serum
686 samples of 8 ZIKV-infected pregnant women and 6 infants born of these women. Of these
687 infants, two had CNS abnormalities associated to Congenital Zika Syndrome (CZS), while 4
688 infants were healthy, without congenital zika syndrome (Non CZS). All participants were
689 tested for a series of arboviruses by qRT-PCR. All patients and healthy donors were negative
690 to Dengue (DENV), Chikungunya viruses (CHKV) and Oropouche were negative as
691 determined by RT-qPCR. Samples were collected after consent of the patients.

692

693 Serum Collection and Processing: Peripheral blood and/or urine specimens were collected at a
694 median of 3 days post-illness onset. Serum was obtained from 10 mL of peripheral blood
695 collected in a dry heparinized tube after peripheral venepuncture. All samples were transported
696 and processed as previously described [9]. Positivity ZIKV RNA in the blood, serum and urine
697 samples was verified by real-time quantitative RT-PCR (qRT-PCR) [9, 67]. In addition, the
698 presence of ZIKV-specific IgM and IgG antibodies in the serum was determined by enzyme-
699 linked immunosorbent assay (ELISA), as previously described [9, 67].

700

701 Cells: This study was conducted using the following cells lines: Vero E6 (ATCC®,
702 (Manassas, Virginia, USA) CRL-1586™), C6/36 (ATCC® CRL-1660™), THP-1 (ATCC®
703 TIB-202™) and hBMECs (previously described here [68] and kindly provided by Dr. Julio
704 Scharfstein – Instituto de Biofísica Carlos Chagas Filho, UFRJ). VeroE6 were cultured in
705 DMEM medium (high glucose) supplemented with 10% fetal bovine serum (FBS). mosquito
706 cell line were cultured in Leibovitz's L-15 medium supplemented with 10% FBS and 1%
707 penicillin/streptomycin. Human brain microvascular endothelial cells (hBMEC) were cultured
708 in DMEM high glucose, supplemented with 1% L-glutamine, 1% non-essential aminoacids,
709 and 10% FBS [19]. Peripheral blood mononuclear cells (PBMC) from healthy donors were

710 obtained after centrifugation of buffy coat samples over ficoll-hypaque gradient. Subsequently,
711 the mononuclear cell ring was collected and washed with 1x PBS, followed by centrifugation.
712 Then, the pellet was resuspended in complete RPMI-1640 (supplemented with 10% FBS and
713 1% antibiotics). Cells were then counted for determination of cell viability by Trypan Blue.
714 PBMCs and THP-1 human monocytic cell line were maintained in a complete RPMI-1640
715 medium supplemented with 10% FBS, 2mM L-Glutamine (Corning), and 1x Penicillin-
716 Streptomycin Solution at 37°C in a fully humidified atmosphere containing 5% CO₂.

717

718 Virus strains: For *in vitro* studies in cultured human cells, Brazilian ZIKV strain (BeH823339,
719 GenBank KU729217), originally isolated from a patient in Ceará, Brazil in 2015, was provided
720 by Professor Edison Durigon (Biomedical Sciences Institute, University of São Paulo, Brazil).
721 Virus stocks were produced by inoculating Vero CCL81 cells (ATCC) with ZIKV in minimum
722 essential medium (MEM) for 2h at 37°C and 5% CO₂. Further, the supernatant was removed,
723 and MEM supplemented with 2% FBS, 1% penicillin and streptomycin was added. The cells
724 were incubated for 4 days until 70% of cytopathic effect. Supernatant was then collected,
725 centrifuged for 5 min at 10,000g, 4°C and snap-frozen at -80°C until use.

726

727 For *in vivo* studies in mice, Brazilian ZIKV (BeH815744, GenBank KU365780) was provided
728 by Evandro Chagas Institute in Belém, Pará, Brazil and propagated in C6/36 cells. Cultures
729 were infected for 1h at 27°C in the absence of CO₂. Further, 45mL of complete medium was
730 added (2% of FBS + 1% of Pen/Strep) and cultures were followed until reaching cytopathic
731 effect. At this time, supernatants were harvested and centrifuged at 3200rpm for 10min at 4°C
732 to remove any detached cell. ZIKV culture supernatants were further precipitated with 50% of
733 PEG (polyethylene glycol) for 18h at 4°C. Precipitated virus supernatants were centrifuged
734 (30', 3200 g, 4°C), and the pellet was diluted in DMEM with 25Mm HEPES quantified by
735 PFU assay in VERO cells and used as necessary.

736

737 Mice: SJL and C57BL/6 mice were bred under specific-pathogen-free conditions at University
738 of São Paulo animal facility of the Department of Immunology - ICB. 8-week-old non-pregnant
739 or 11-week-old pregnant female mice were used. All animals were maintained in accordance
740 with institutional guidelines for animal welfare after approval by the Institutional Animal Care
741 and Use Committee at University of São Paulo, as described above.

742

743 ZIKV real-time quantitative RT-PCR: Viral RNA from blood, serum or urine samples was
744 extracted using the easyMAG mated extractor (BioMerieux, Quebec, Canada) or QIAamp viral
745 RNA mini kit (Qiagen, Hilden, USA), according to manufacturer's instructions. Estimation of
746 viral RNA copy number in patients' samples was performed using real-time RT-PCR (TaqMan
747 RNA to-Ct 1-Step Kit; Applied Biosystems) with primers and probes, as previously described
748 [9, 65]. qRT-PCRs with cycle threshold (Ct) values higher than 40 cycles were considered
749 negative. Quantitative assay was performed using a standard curve produced with serial 10-
750 fold dilutions of ZIKV RNA expressed on a log₁₀ scale as genome equivalents/sample.

751

752 Viral RNA Sequencing and Assembly: To evaluate the quantity and quality of the RNA
753 extracted, a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, USA) and an Agilent 2100
754 Bioanalyzer (Agilent Technologies, Santa Clara, USA) were used, respectively. Synthesis of
755 cDNA was performed using SuperScript II and random hexamer primers according to the
756 manufacturer's recommendations (Invitrogen, Carlsbad, USA). Nucleotide sequencing was
757 performed using the TruSeq RNA sample preparation kit in an Illumina HiSeq 2500 instrument
758 (Illumina, San Diego, USA) with a paired-end and 150-base-read protocol in *RAPID* module.
759 The sequencing reads were assembled *de novo* using the metaViC pipeline (available on
760 <https://github.com/sejmodha/MetaViC>), as previously described [69].

761

762 Viral quantitation by focus forming unit assay and plaque forming unit assay: The viral titer
763 of viral isolates was determined by both focus forming unit (FFU) and plaque forming unit
764 (PFU) assays in Vero E6 cells. The quantitation of viral load during in vitro experiments were
765 performed by FFU assay, while the viral load in different tissues of animals were determined
766 by PFU assay. For FFU quantification, samples were clarified by centrifugation (2,000 x g at
767 4°C for 10 min) and diluted serially prior to infection. These dilutions were added to Vero E6
768 cells in 96-well plates for 2 h for viral adsorption and, after supernatants removal, cells were
769 maintained with MEM + 1% CMC medium (final concentration) supplemented with 5% FBS
770 and 1% penicillin/streptomycin for 48 hours at 37°C and 5% CO₂. The overlay was removed,
771 and cells were fixed overnight with 1% paraformaldehyde (PFA) in PBS. To detect infected
772 cell foci, cells were permeabilized with Triton buffer (PBS 0.15M, 0.1% BSA and 0.1%
773 Triton X-100) and foci were detected after incubation with a mouse anti-ZIKV NS1 antibody
774 in a volume of 50 µl for 2 h at room temperature. After three washes with 300 µl of
775 permeabilization-wash buffer (P-W; PBS, 0.1% saponin, and 0.1% bovine serum albumin

776 [BSA]), the samples were incubated with 50 μ l of a 1:2,000 dilution of horseradish peroxidase
777 (HRP)-conjugated goat anti-mouse IgG for 1 h at room temperature. Focci were stained by the
778 addition of the TrueBlue detection reagent (KPL), and the blue spots were counted after three
779 washes with distilled water.

780 For PFU quantification, Vero E6 cells (1×10^5 /well) distributed in 24-well plate were
781 culture with clarified samples from infected mice. These samples were diluted ($10^5 - 10^1$) in
782 RPMI-16-40 medium and used to infect VeroE6 cells for 1h at 37°C. Further, supernatants
783 were removed and DMEM medium (2% FBS + 1% penicillin/streptomycin) + 1.5% CMC
784 (Carboxymethyl cellulose) were added in each well. Cells were cultured in 37°C for 5 days and
785 then stained with crystal violet for plaque counting. Viral titer of the stock sample was
786 determined by: [average number of plaques/ (dilution factor of well)] x (volume of inoculum
787 per plate).

788

789 ZIKV infection and treatment: For *in vitro* infection, PBMCs, THP-1 monocytes and hBMECs
790 were infected with ZIKV MOIs (multiplicity of infection) ranging from 0.1 to 10, depending
791 on the experimental design. In some experiments, cells were submitted to starvation (serum
792 free medium) from 4 to 18 hours prior to infection with the virus for 2 h at 37°C. Afterwards,
793 cells were washed four times with PBS. Culture medium was added to each well and the cells
794 were incubated at 37°C and 5% CO₂ for the duration of the experiment. For Gas6 γ -
795 carboxylation and Axl kinase blockade experiments, cells were incubated or not (mock) with
796 2 μ M warfarin or 2 μ M R428 (BerGenBio), respectively. Cells were harvested after 1-, 2-, 3-,
797 4-, 6-, 9-, 12-, 24-, 48- or 72-hours post infection depending on the experimental design.
798 Medium was harvested at specified time points for determination of Gas6 production, ZIKV
799 titers and replication either by PFU or flow cytometry.

800

801 Mice infection: For *in vivo* experiments in mice, the BeH815744 virus was incubated or not
802 with 1 μ g/mL of recombinant mouse Gas6 (rmGas6) in μ l for 4 hours at 37°C prior to mice
803 inoculation [20]. 8-week-old SJL or C57BL/6 WT mice were inoculated with 10^2 ZIKV pfu
804 diluted in 100 μ L of PBS or pre-incubated with 1 μ g/mL of recombinant murine Gas6
805 (ZIKV^{rmGas6}) by intravenous route (retroorbital sinus). Mice were euthanized at days 1, 3 or 5
806 post infection depending on the experimental design. To study congenital infection, 11-week-
807 old C57BL/6 WT pregnant mice were infected with ZIKV 10^5 pfu in 30 μ L of PBS pre-
808 incubated or not with 1 μ g/mL rmGas6 at embryonic day E10 or E16 intravaginally or

809 subcutaneously (footpad), respectively. Tissues were harvested at E18 or postnatally (P) at day
810 4.

811

812 Relative quantitation by Real-time quantitative RT-PCR: For *in vitro* analysis, RNA was
813 extracted using the miRVana miRNA Extraction kit (Ambion) according to the manufacturer's
814 instructions. RNA quantity was determined by NanoDrop spectrometric dosing. Total RNA
815 samples (up to 1 µg) were reverse transcribed using the oligo(dT) primer from the High
816 Capacity cDNA Reversion Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA).
817 mRNA expression of the TAM receptors Tyro-3 (*Tyro-3*), Axl (*Axl*) and Mer (*Mer*),
818 Suppressor of cytokine signalling-1/2 (*SOCS1*), Interferon-β (*IFN-β*), Interferon-induced
819 protein with tetratricopeptide repeats-1 (*IFIT-1*), Gas6 (*Gas6*), Gamma-glutamyl carboxylase
820 (*GGCX*), Vitamin K Epoxide Reductase Complex subunit 1 (*VKORC1*) and the housekeeping
821 gene Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were determined by qRT-PCR,
822 using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Cycling
823 conditions were the following: 2 minutes at 95 °C and 35 cycles of 15 seconds at 95 °C and
824 60°C for 1 minute; 31 seconds at 65 °C and 60 cycles of 65 °C for 5 s (+0.5 °C/cycle; ramp 0.5
825 °C/s). The oligonucleotides used are described in Supplemental Table S1. Cycling conditions
826 were the following: 95 °C for 5 min; 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. The
827 median cycle threshold (C_t) value and $2^{-\Delta C_t}$ method were used for relative quantification
828 analysis and all C_t values were normalized to GAPDH. Results were expressed as means and
829 SEM of biologic replicates. All qRT-PCR assays were performed on the CFX96 Touch™ Real-
830 Time PCR Detection System (BioRad, Hercules, USA).

831 For *in vivo* analysis, RNA extraction from mice organs was performed according to the
832 protocol provided by manufacturer TRIzol™ Reagent (Cat.No. 15596026). From the obtained
833 RNA, we performed qRT-PCR according to the protocol provided by the manufacturer
834 (Applied Biosystems Cat. No 4368813). The PCR reactions were done using TaqMan® gene
835 expression assay with the following cycles 95°C for 2 minutes and 45 cycles of 95°C for 15
836 seconds and 60°C for 1 minute. Amplification was normalized by beta actin expression. Gene
837 expression was analyzed by $2^{-\Delta\Delta C_t}$ method. Results were expressed as means and SEM of
838 biologic replicates.

839

840 Flow Cytometry: Vero E6 cells were incubated for 4 days with 72h p.i. supernatants from THP-
841 1 monocytes infected *in vitro* with ZIKV MOI 1, submitted or not to the different

842 pharmacological treatments (mock, pre- or 2h p.i. treatment with 2uM warfarin). Cells were
843 fixed/permeabilized with Cytotfix/Cytoperm (BD Biosciences, Franklin Lakes, New Jersey,
844 U.S.) according to manufacturer's instructions and incubated with 1:100 mouse anti-ZIKV
845 NS1 antibody at 4°C for 30 minutes. Further, cells were washed with Permwash (BD
846 Biosciences) and incubated with 1:100 Alexa Fluor 488-conjugated goat anti-mouse IgG H&L,
847 washed and subjected to flow cytometry using a FACS Calibur (BD Biosciences). Flow data
848 was analyzed with FlowJo V10.

849

850 ELISA for Gas6 quantification: To measure Growth Arrest-Specific Protein 6 (Gas6) in
851 patients' serum samples and supernatants from cells *in vitro*, ELISA was performed using
852 Human Gas6 DuoSet ELISA kit according to the manufacturer's instructions (R&D Systems,
853 Minneapolis, Minnesota, USA). Briefly, 96-well microplate was coated with the goat anti-
854 human Gas6 capture antibody diluted to the working concentration in PBS and incubated
855 overnight at room temperature. The plate was washed and incubated with blocking buffer
856 (reagent diluent) at room temperature for 1h After washing, samples were added (dilution 1:50
857 for serum samples and dilution 1:25 for cell supernatant samples) and incubated for 2h. Next,
858 plates were washed and biotinylated goat anti-human Gas6 detection antibody, diluted in
859 blocking buffer was incubated for 2h at room temperature, followed by streptavidin-HRP
860 incubation for 20 minutes at room temperature. Plates were washed and incubated for 20 min
861 with substrate solution. Optical density was determined at 450 nm followed by wavelength
862 correction at 540 nm.

863

864 Single-nucleotide polymorphism of Gas6 (SNP): Total DNA from patients' peripheral blood
865 was extracted using QIAmp DNABlood Mini Kit (Qiagen, Germantown, Maryland, USA)
866 following manufacture's instruction. Extracted DNA was used to perform PCR and
867 electrophoresis analysis of the GAS6 Intron 8 c.834+7G>A SNP, as previously described [21].
868 Reactions were performed using Taq DNA Polymerase Recombinant kit (Thermo Fisher
869 Scientific, Waltham, Massachusetts, USA) following manufacture's recommendations.
870 Thermocycling was as follows: 5 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 30 seconds
871 at 55°C and 45 seconds at 72°C, then followed by 5 minutes at 72°C and 4°C until digestion.
872 Afterwards, amplicons were digested with restriction enzyme HhaI (New England Biolabs,
873 Ipswich, Massachusetts, USA) at 37°C for 4 hours and separated in 1.5% agarose gel with
874 ethidium bromide at 80V for 90 minutes. UV documentation in Gel Doc XR+ System (BioRad,
875 Hercules, California, USA).

876

877 Correlation coefficients and network analysis: Pearson's or Spearman's Rank correlation
878 coefficients were determined to assess the association between Gas6 and ZIKV-specific
879 immune signatures determined in the serum samples from healthy donors and ZIKV-infected
880 patients by Multiplex Microbead-Based Immunoassay, as previously described [9]. Correlation
881 coefficients were also determined to assess the association between Gas6 levels in the serum
882 and gene expression in the matched peripheral blood cells from ZIKV-infected adult patients.
883 Correlations between transcripts were also quantified in the cells infected *in vitro* with ZIKV.
884 For the network analysis, the systemic level of each biomarker was an input in the R software
885 (v. 3.4.3). Along with the rank-order correlation coefficient, the p-value to test for non-
886 correlation was evaluated using $p < 0.05$ as a cutoff. Next, correlation networks were generated
887 by the analysis of relationships among each biomarker in the serum samples. Based on the
888 correlation coefficients, the same software was applied to identify links (edges) of interactions
889 between the biomarkers (nodes). The correlation strength is represented by edge transparency
890 and width; positive correlations are represented by red edges, and negatives correlations are
891 represented by blue edges. Following this approach, each biomarker was selected as a target,
892 and the R software was used to perform a search within the other biomarkers for those that
893 were associated with the target, in terms of correlation strength. As a result, the features related
894 to the selected target were linked. This process was repeated for each biomarker, and the result
895 was the inferred network among the input values. To analyze the structure of the networks, the
896 graphs for the network analysis were customized in the Cytoscape software (v 3.5.1) using the
897 pre-fuse force-directed layout. This layout follows an algorithm that in the equilibrium state
898 for the system of forces, determined by the correlation strength, the edges tend to have uniform
899 length, and nodes that are not connected by an edge tend to be drawn further apart

900

901 Statistical Analysis: Data normality was checked by the Shapiro-Wilk test. Two-tailed
902 Student's t-test or One-way analyses of variance statistical test with Bonferroni-corrected
903 multiple comparisons test were used to compare means between groups with normally
904 distributed data. Data sets with non-parametric distributions were compared using the Mann-
905 Whitney test or Kruskal-Wallis test with post hoc correction for multiple testing using the
906 original FDR method of Benjamini and Hochberg, with $p < 0.05$ considered significant. Data
907 are presented as Tukey box plots or means and SEM, unless otherwise stated, of 2–3

908 representative and independent experiments with similar results. Analysis were performed and
909 the graphs generated in GraphPad Prism 8 and R software. $p < 0,05$ were considered relevant.

910

911 **NOTES**

912

913 **Author contributions**

914 Conceptualization, J.L.S.F, L.G.O, F.T.M.C., J.P.S.P. and J.L.P.-M.; Methodology, J.L.S.F.,
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919 Original Draft, J.L.S.F. and L.G.O; Review & Editing, W.M.S., L.F.P.N., C.V.R, F.T.M.C.,
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922 M.L.M; Funding Acquisition, F.T.M.C, J.P.S.P. and J.L.P.-M.; Resources, L.F.P.N., C.V.R.,
923 F.T.M.C, J.P.S.P., J.L.P.-M.; Supervision, F.T.M.C, J.P.S.P. and J.L.P.-M.

924

925 **Acknowledgments**

926 We thank the study participants and healthy volunteers for their participation and the clinical
927 staffs from University of Campinas Hospitals and other hospitals of this city for assistance in
928 patient enrolment and healthcare, blood sample preparation, study coordination, and data entry.
929 We thank the staff of the Life Sciences Core Facility (LaCTAD) from Unicamp for the High-
930 throughput sequencing.

931

932 **Financial support**

933 This study was financially supported by the São Paulo Research Foundation (FAPESP
934 2016/00194-8; 2017/26170-0 and 2017/22054-1); Biomedical Research Council (BMRC; core
935 research grants provided to the Singapore Immunology Network); the BMRC A*STAR-led
936 Zika Virus Consortium Fund (project 15/1/82/27/001); the Agency for Science, Technology
937 and Research (A*STAR), Singapore. J.L.S.F., L.G.O., L.M., P.L.P., N.G.Z., C.M.P., D.A.T.-
938 T., W.M.S., N.B., J.F., S.P.M., G.F.S. and K.B.-d.-S., received scholarship from FAPESP,
939 grant numbers 2016/12855-9; 2016/21259-0; 2018/13866-0; 2017/02402-0, 2016/07371-2;
940 2017/11828-0; 2017/26908-0, 2017/22062-9, 2018/13645-3, 2018/10224-7, 2020/02159-0 and
941 2020/02448-2, respectively. National Council for Scientific and Technological Development

942 (CNPq) supported D.A.T.-T. and M.C.M., grant numbers 141844/2019-1 and 421724/2017-0.

943 Fundo de Apoio ao Ensino, Pesquisa e Extensão (FAEPEX) supported M.R.A., grant number

944 208/17.

945 **Disclaimer.** The funders had no role in study design, data collection and analysis, decision to

946 publish, or preparation of the manuscript.

947

948 **Declaration of Interests**

949 There are no conflicts of interest.

950 **REFERENCES**

- 951 1. Ioo, S., et al., *Current Zika virus epidemiology and recent epidemics*. *Med Mal Infect*,
952 2014. **44**(7): p. 302-7. Available from: 10.1016/j.medmal.2014.04.008
- 953 2. Lupton, K., *Zika virus disease: a public health emergency of international concern*. *Br*
954 *J Nurs*, 2016. **25**(4): p. 198, 200-2. Available from: 10.12968/bjon.2016.25.4.198
- 955 3. Duffy, M.R., et al., *Zika virus outbreak on Yap Island, Federated States of Micronesia*.
956 *N Engl J Med*, 2009. **360**(24): p. 2536-43. Available from: 10.1056/NEJMoa0805715
- 957 4. Mlakar, J., et al., *Zika Virus Associated with Microcephaly*. *N Engl J Med*, 2016.
958 **374**(10): p. 951-8. Available from: 10.1056/NEJMoa1600651
- 959 5. França, G.V., et al., *Congenital Zika virus syndrome in Brazil: a case series of the first*
960 *1501 livebirths with complete investigation*. *Lancet*, 2016. **388**(10047): p. 891-7. Available
961 from: 10.1016/S0140-6736(16)30902-3
- 962 6. Ventura, C.V., et al., *Ophthalmological findings in infants with microcephaly and*
963 *presumable intra-uterus Zika virus infection*. *Arq Bras Oftalmol*, 2016. **79**(1): p. 1-3. Available
964 from: 10.5935/0004-2749.20160002
- 965 7. Cao-Lormeau, V.M., et al., *Guillain-Barré Syndrome outbreak associated with Zika*
966 *virus infection in French Polynesia: a case-control study*. *Lancet*, 2016. **387**(10027): p. 1531-
967 1539. Available from: 10.1016/S0140-6736(16)00562-6
- 968 8. Waggoner, J.J., et al., *Viremia and Clinical Presentation in Nicaraguan Patients*
969 *Infected With Zika Virus, Chikungunya Virus, and Dengue Virus*. *Clin Infect Dis*, 2016. **63**(12):
970 p. 1584-1590. Available from: 10.1093/cid/ciw589
- 971 9. Kam, Y.W., et al., *Specific Biomarkers Associated With Neurological Complications*
972 *and Congenital Central Nervous System Abnormalities From Zika Virus-Infected Patients in*
973 *Brazil*. *J Infect Dis*, 2017. **216**(2): p. 172-181. Available from: 10.1093/infdis/jix261
- 974 10. Moller-Tank, S. and W. Maury, *Phosphatidylserine receptors: enhancers of enveloped*
975 *virus entry and infection*. *Virology*, 2014. **468-470**: p. 565-580. Available from:
976 10.1016/j.virol.2014.09.009
- 977 11. Laureti, M., et al., *Flavivirus Receptors: Diversity, Identity, and Cell Entry*. *Front*
978 *Immunol*, 2018. **9**: p. 2180. Available from: 10.1016/j.virol.2014.09.009
- 979 12. van der Meer, J.H., T. van der Poll, and C. van 't Veer, *TAM receptors, Gas6, and*
980 *protein S: roles in inflammation and hemostasis*. *Blood*, 2014. **123**(16): p. 2460-9. Available
981 from: 10.1182/blood-2013-09-528752
- 982 13. Lemke, G. and C.V. Rothlin, *Immunobiology of the TAM receptors*. *Nat Rev Immunol*,
983 2008. **8**(5): p. 327-36. Available from: 10.1038/nri2303
- 984 14. Rothlin, C.V., et al., *TAM receptors are pleiotropic inhibitors of the innate immune*
985 *response*. *Cell*, 2007. **131**(6): p. 1124-36. Available from: 10.1016/j.cell.2007.10.034

- 986 15. Chen, J., Yang, Y., Yang, Y. *et al.* *AXL promotes Zika virus infection in astrocytes by*
987 *antagonizing type I interferon signalling.* *Nat Microbiol*, 2018(3): p. 302–309. Available from:
988 10.1038/s41564-017-0092-4
- 989 16. Meertens, L., et al., *Axl Mediates ZIKA Virus Entry in Human Glial Cells and*
990 *Modulates Innate Immune Responses.* *Cell Rep*, 2017. **18**(2): p. 324-333. Available from:
991 10.1016/j.celrep.2016.12.045
- 992 17. Hamel, R., et al., *Biology of Zika Virus Infection in Human Skin Cells.* *J Virol*, 2015.
993 **89**(17): p. 8880-96. Available from: 10.1128/JVI.00354-15
- 994 18. Hastings, A.K., et al., *Loss of the TAM Receptor Axl Ameliorates Severe Zika Virus*
995 *Pathogenesis and Reduces Apoptosis in Microglia.* *iScience*, 2019. **13**: p. 339-350. Available
996 from: 10.1016/j.isci.2019.03.003
- 997 19. Papa, M.P., et al., *Zika Virus Infects, Activates, and Crosses Brain Microvascular*
998 *Endothelial Cells, without Barrier Disruption.* *Front Microbiol*, 2017. **8**: p. 2557. Available
999 from: 10.3389/fmicb.2017.02557
- 1000 20. Morizono, K., et al., *The soluble serum protein Gas6 bridges virion envelope*
1001 *phosphatidylserine to the TAM receptor tyrosine kinase Axl to mediate viral entry.* *Cell Host*
1002 *Microbe*, 2011. **9**(4): p. 286-98. Available from: 10.1016/j.chom.2011.03.012
- 1003 21. Ozakpinar, O.B., et al., *Association between the growth arrest-specific 6 (Gas6) gene*
1004 *polymorphism c.834 + 7G>A and preeclampsia.* *J Matern Fetal Neonatal Med*, 2016. **29**(7): p.
1005 1149-53. Available from: 10.3109/14767058.2015.1038516
- 1006 22. Alciato, F., et al., *TNF-alpha, IL-6, and IL-1 expression is inhibited by GAS6 in*
1007 *monocytes/macrophages.* *J Leukoc Biol*, 2010. **87**(5): p. 869-75. Available from:
1008 10.1189/jlb.0909610
- 1009 23. Davra, V., et al., *Ligand Activation of TAM Family Receptors-Implications for Tumor*
1010 *Biology and Therapeutic Response.* *Cancers (Basel)*, 2016. **8**(12). Available from:
1011 10.3390/cancers8120107
- 1012 24. Meertens, L., et al., *The TIM and TAM families of phosphatidylserine receptors mediate*
1013 *dengue virus entry.* *Cell Host Microbe*, 2012. **12**(4): p. 544-57. Available from:
1014 10.1016/j.chom.2012.08.009
- 1015 25. Bhattacharyya, S., et al., *Enveloped viruses disable innate immune responses in*
1016 *dendritic cells by direct activation of TAM receptors.* *Cell Host Microbe*, 2013. **14**(2): p. 136-
1017 47. Available from: 10.1016/j.chom.2013.07.005
- 1018 26. Perera-Lecoin, M., et al., *Flavivirus entry receptors: an update.* *Viruses*, 2013. **6**(1): p.
1019 69-88. Available from: 10.3390/v6010069
- 1020 27. Nakano, T., et al., *Requirement of gamma-carboxyglutamic acid residues for the*
1021 *biological activity of Gas6: contribution of endogenous Gas6 to the proliferation of vascular*
1022 *smooth muscle cells.* *Biochem J*, 1997. **323** (Pt 2): p. 387-92. Available from:
1023 10.1042/bj3230387

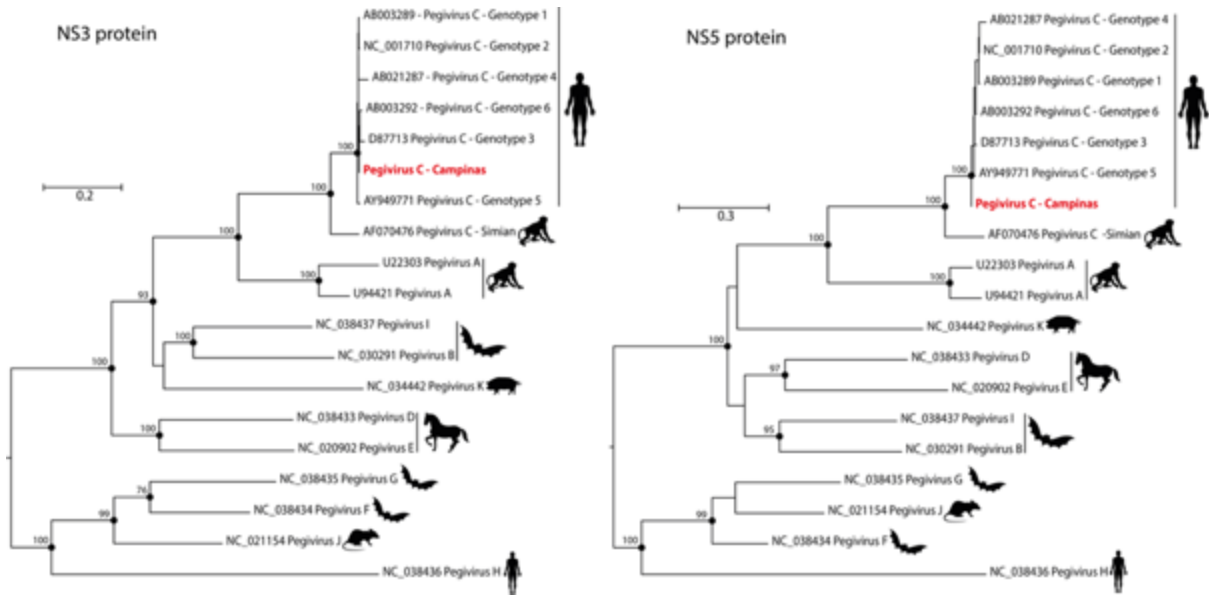
- 1024 28. Sun, X., et al., *Transcriptional Changes during Naturally Acquired Zika Virus Infection*
1025 *Render Dendritic Cells Highly Conducive to Viral Replication*. Cell Rep, 2017. **21**(12): p.
1026 3471-3482. Available from: 10.1016/j.celrep.2017.11.087
- 1027 29. Foo, S.S., et al., *Asian Zika virus strains target CD14(+) blood monocytes and induce*
1028 *M2-skewed immunosuppression during pregnancy*. Nat Microbiol, 2017. **2**(11): p. 1558-1570.
1029 Available from: 10.1038/s41564-017-0016-3
- 1030 30. Michlmayr, D., et al., *CD14(+)CD16(+) monocytes are the main target of Zika virus*
1031 *infection in peripheral blood mononuclear cells in a paediatric study in Nicaragua*. Nat
1032 Microbiol, 2017. **2**(11): p. 1462-1470. Available from: 10.1038/s41564-017-0035-0
- 1033 31. Kirane, A., et al., *Warfarin Blocks Gas6-Mediated Axl Activation Required for*
1034 *Pancreatic Cancer Epithelial Plasticity and Metastasis*. Cancer Res, 2015. **75**(18): p. 3699-
1035 705. Available from: 10.1158/0008-5472.CAN-14-2887-T
- 1036 32. Miner, J.J. and M.S. Diamond, *Zika Virus Pathogenesis and Tissue Tropism*. Cell Host
1037 Microbe, 2017. **21**(2): p. 134-142. Available from: 10.1016/j.chom.2017.01.004
- 1038 33. Yuan, L., et al., *A single mutation in the prM protein of Zika virus contributes to fetal*
1039 *microcephaly*. Science, 2017. **358**(6365): p. 933-936. Available from:
1040 10.1126/science.aam7120
- 1041 34. Cugola, F.R., et al., *The Brazilian Zika virus strain causes birth defects in experimental*
1042 *models*. Nature, 2016. **534**(7606): p. 267-71. Available from: 10.1038/nature18296
- 1043 35. Russo, F.B., P. Jungmann, and P.C.B. Beltrão-Braga, *Zika infection and the*
1044 *development of neurological defects*. Cell Microbiol, 2017. **19**(6). Available from:
1045 10.1111/cmi.12744
- 1046 36. Rasmussen, S.A., et al., *Zika Virus and Birth Defects--Reviewing the Evidence for*
1047 *Causality*. N Engl J Med, 2016. **374**(20): p. 1981-7. Available from: 10.1056/NEJMSr1604338
- 1048 37. de Araújo, T.V.B., et al., *Association between microcephaly, Zika virus infection, and*
1049 *other risk factors in Brazil: final report of a case-control study*. Lancet Infect Dis, 2018. **18**(3):
1050 p. 328-336. Available from: 10.1016/S1473-3099(17)30727-2
- 1051 38. Soares de Oliveira-Szejnfeld, P., et al., *Congenital Brain Abnormalities and Zika Virus:*
1052 *What the Radiologist Can Expect to See Prenatally and Postnatally*. Radiology, 2016. **281**(1):
1053 p. 203-18. Available from: 10.1148/radiol.2016161584
- 1054 39. Proenca-Modena, J.L., et al., *Zika virus: lessons learned in Brazil*. Microbes Infect,
1055 2018. **20**(11-12): p. 661-669. Available from: 10.1016/j.micinf.2018.02.008
- 1056 40. Figueiredo, C.P., et al., *Zika virus replicates in adult human brain tissue and impairs*
1057 *synapses and memory in mice*. Nat Commun, 2019. **10**(1): p. 3890. Available from:
1058 10.1038/s41467-019-11866-7
- 1059 41. Souza, I.N.O., et al., *Late Neurological Consequences of Zika Virus Infection: Risk*
1060 *Factors and Pharmaceutical Approaches*. Pharmaceuticals (Basel), 2019. **12**(2). Available
1061 from: 10.3390/ph12020060

- 1062 42. van den Pol, A.N., et al., *Zika Virus Targeting in the Developing Brain*. J Neurosci, 1063 2017. **37**(8): p. 2161-2175. Available from: 10.1523/JNEUROSCI.3124-16.2017
- 1064 43. Wu, K.Y., et al., *Vertical transmission of Zika virus targeting the radial glial cells* 1065 *affects cortex development of offspring mice*. Cell Res, 2016. **26**(6): p. 645-54. Available from: 1066 10.1038/cr.2016.58
- 1067 44. Jiang, L., et al., *Plasma level of growth arrest-specific 6 (GAS6) protein and genetic* 1068 *variations in the GAS6 gene in patients with acute coronary syndrome*. Am J Clin Pathol, 2009. 1069 **131**(5): p. 738-43. Available from: 10.1309/AJCP3CX3AUVRBHCF
- 1070 45. Muñoz, X., et al., *Human vitamin K-dependent GAS6: gene structure, allelic variation,* 1071 *and association with stroke*. Hum Mutat, 2004. **23**(5): p. 506-12. Available from: 1072 10.1002/humu.20025
- 1073 46. Muñoz, X., et al., *Association of specific haplotypes of GAS6 gene with stroke*. Thromb 1074 Haemost, 2007. **98**(2): p. 406-12.
- 1075 47. Zhao, G.J., et al., *Growth Arrest-Specific 6 Enhances the Suppressive Function of CD4*. 1076 Mediators Inflamm, 2017. **2017**: p. 6848430. Available from: 10.1155/2017/6848430
- 1077 48. Chaix, J., et al., *Cutting edge: Priming of NK cells by IL-18*. J Immunol, 2008. **181**(3): 1078 p. 1627-31. Available from: 10.4049/jimmunol.181.3.1627
- 1079 49. Tjwa, M., et al., *Gas6 promotes inflammation by enhancing interactions between* 1080 *endothelial cells, platelets, and leukocytes*. Blood, 2008. **111**(8): p. 4096-105. Available from: 1081 10.1182/blood-2007-05-089565
- 1082 50. Miner, J.J., et al., *The TAM receptor Mertk protects against neuroinvasive viral* 1083 *infection by maintaining blood-brain barrier integrity*. Nat Med, 2015. **21**(12): p. 1464-72. 1084 Available from: 10.1038/nm.3974
- 1085 51. Richard, A.S., et al., *AXL-dependent infection of human fetal endothelial cells* 1086 *distinguishes Zika virus from other pathogenic flaviviruses*. Proc Natl Acad Sci U S A, 2017. 1087 **114**(8): p. 2024-2029. Available from: 10.1073/pnas.1620558114
- 1088 52. Liu, S., et al., *AXL-Mediated Productive Infection of Human Endothelial Cells by Zika* 1089 *Virus*. Circ Res, 2016. **119**(11): p. 1183-1189. Available from: 1090 10.1161/CIRCRESAHA.116.309866
- 1091 53. Bowen, J.R., et al., *Zika Virus Antagonizes Type I Interferon Responses during* 1092 *Infection of Human Dendritic Cells*. PLoS Pathog, 2017. **13**(2): p. e1006164. Available from: 1093 10.1371/journal.ppat.1006164
- 1094 54. Oliveira, L.G. and J.P.S. Peron, *Viral receptors for flaviviruses: Not only gatekeepers*. 1095 J Leukoc Biol, 2019. **106**(3): p. 695-701. Available from: 10.1002/JLB.MR1118-460R
- 1096 55. Ma, W., et al., *Zika Virus Causes Testis Damage and Leads to Male Infertility in Mice*. 1097 Cell, 2017. **168**(3): p. 542. Available from: 10.1016/j.cell.2016.11.016

- 1098 56. Miner, J.J., et al., *Zika Virus Infection in Mice Causes Panuveitis with Shedding of*
1099 *Virus in Tears*. Cell Rep, 2016. **16**(12): p. 3208-3218. Available from:
1100 10.1016/j.celrep.2016.08.079
- 1101 57. Hastings, A.K., et al., *TAM Receptors Are Not Required for Zika Virus Infection in*
1102 *Mice*. Cell Rep, 2017. **19**(3): p. 558-568. Available from: 10.1016/j.celrep.2017.03.058
- 1103 58. Yockey, L.J., et al., *Type I interferons instigate fetal demise after Zika virus infection*.
1104 Sci Immunol, 2018. **3**(19). Available from: 10.1126/sciimmunol.aao1680
- 1105 59. Azamor, T., et al., *Congenital Zika Syndrome is associated with interferon alfa receptor*
1106 *I*. BioRxiv preprint, 2020. doi: <https://doi.org/10.1101/715862>. Available from:
1107 doi.org/10.1101/715862
- 1108 60. Nobrega, G.M., et al., *TAM and TIM receptors mRNA expression in Zika virus infected*
1109 *placentas*. Placenta, 2020. **101**: p.204–207. Available from: 10.1016/j.placenta.2020.09.062
- 1110 61. Dang, J., et al., *Zika Virus Depletes Neural Progenitors in Human Cerebral Organoids*
1111 *through Activation of the Innate Immune Receptor TLR3*. Cell Stem Cell, 2016. **19**(2): p. 258-
1112 265. Available from: 10.1016/j.stem.2016.04.014
- 1113 62. Gong, S., et al., *Plasma sMer, sAxl and GAS6 levels correlate with disease activity and*
1114 *severity in lupus nephritis*. Eur J Clin Invest, 2019. **49**(3): p. e13064. Available from:
1115 10.1111/eci.13064
- 1116 63. Smirne, C., et al., *Gas6/TAM Signaling Components as Novel Biomarkers of Liver*
1117 *Fibrosis*. Dis Markers, 2019. **2019**: p. 2304931. Available from: 10.1155/2019/2304931
- 1118 64. Peng, S., et al., *Plasma levels of TAM receptors and ligands in severe preeclampsia*.
1119 Pregnancy Hypertens, 2018. **13**: p. 116-120. Available from: 10.1016/j.preghy.2018.05.012
- 1120 65. Kalita, J., U.K. Misra, and M. Das, *Neurophysiological criteria in the diagnosis of*
1121 *different clinical types of Guillain-Barre syndrome*. J Neurol Neurosurg Psychiatry, 2008.
1122 **79**(3): p. 289-93. Available from: 10.1136/jnnp.2007.118000
- 1123 66. Venkatesan, A., et al., *Case definitions, diagnostic algorithms, and priorities in*
1124 *encephalitis: consensus statement of the international encephalitis consortium*. Clin Infect Dis,
1125 2013. **57**(8): p. 1114-28. Available from: 10.1093/cid/cit458
- 1126 67. Judice, C.C., et al., *Efficient detection of Zika virus RNA in patients' blood from the*
1127 *2016 outbreak in Campinas, Brazil*. Sci Rep, 2018. **8**(1): p. 4012. Available from:
1128 10.1038/s41598-018-22159-2
- 1129 68. Nikolskaia, O.V., et al., *Blood-brain barrier traversal by African trypanosomes*
1130 *requires calcium signaling induced by parasite cysteine protease*. J Clin Invest, 2006. **116**(10):
1131 p. 2739-47. Available from: 10.1172/JCI27798
- 1132 69. Souza, W.M., et al., *Viral diversity of Rhipicephalus microplus parasitizing cattle in*
1133 *southern Brazil*. Sci Rep, 2018. **8**(1): p. 16315. Available from: 10.1038/s41598-018-34630-1

1135 SUPPLEMENTAL FIGURES

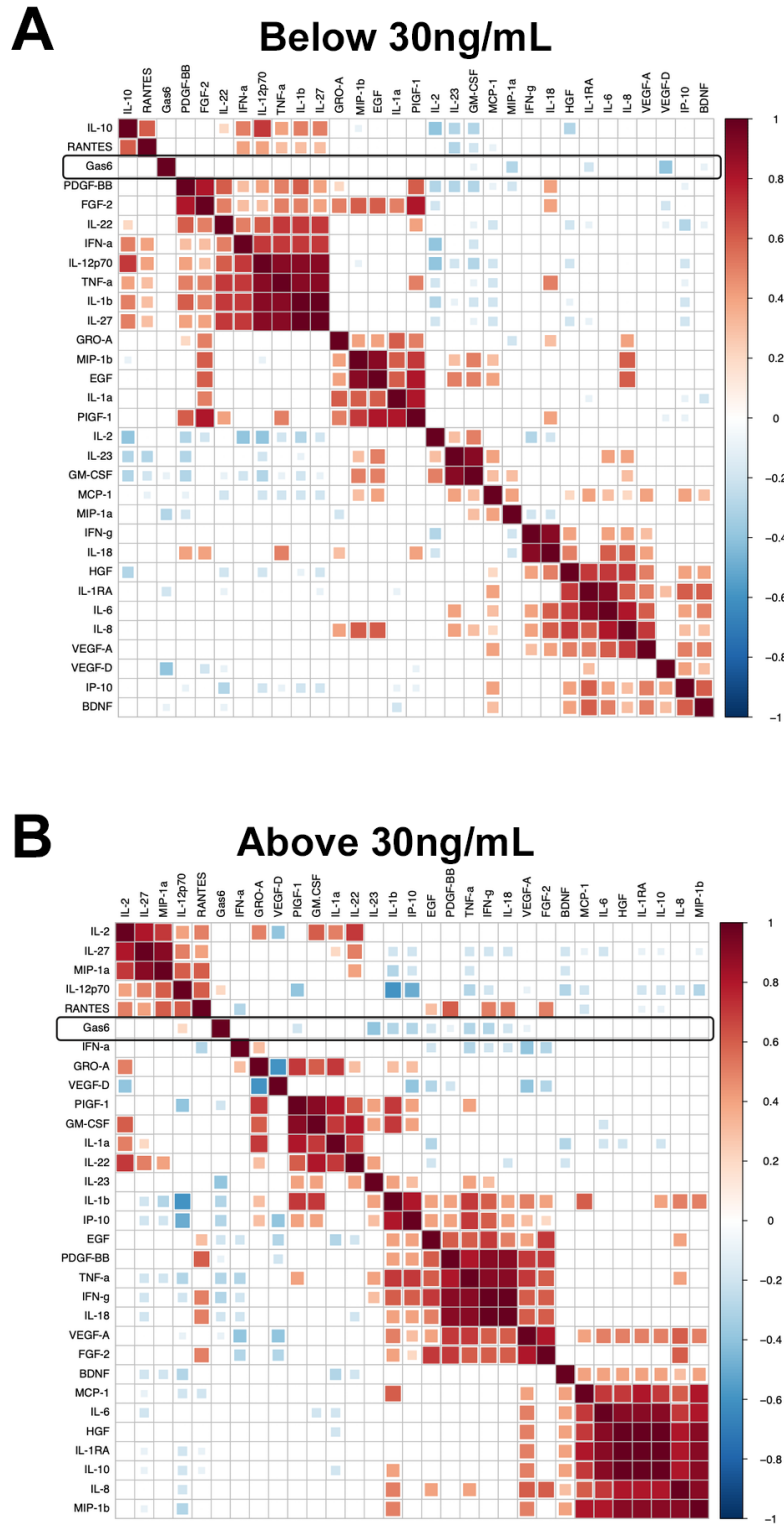
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1138 **Supplementary figure 1: The virus detected by high-throughput sequencing in the Neuro^{ZIKV} patient is a**
1139 **human pegivirus.** Phylogenetic trees of maximum likelihood showing the evolutionary relationships of the
1140 detected human pegivirus (named of human pegivirus strain Campinas, highlighted in red) with representative
1141 members of the *Pegivirus* genus according to the alignment of the NS3 and NS5 genes. Bootstrap values are
1142 indicated inside black circles, and hosts are indicated by figures.

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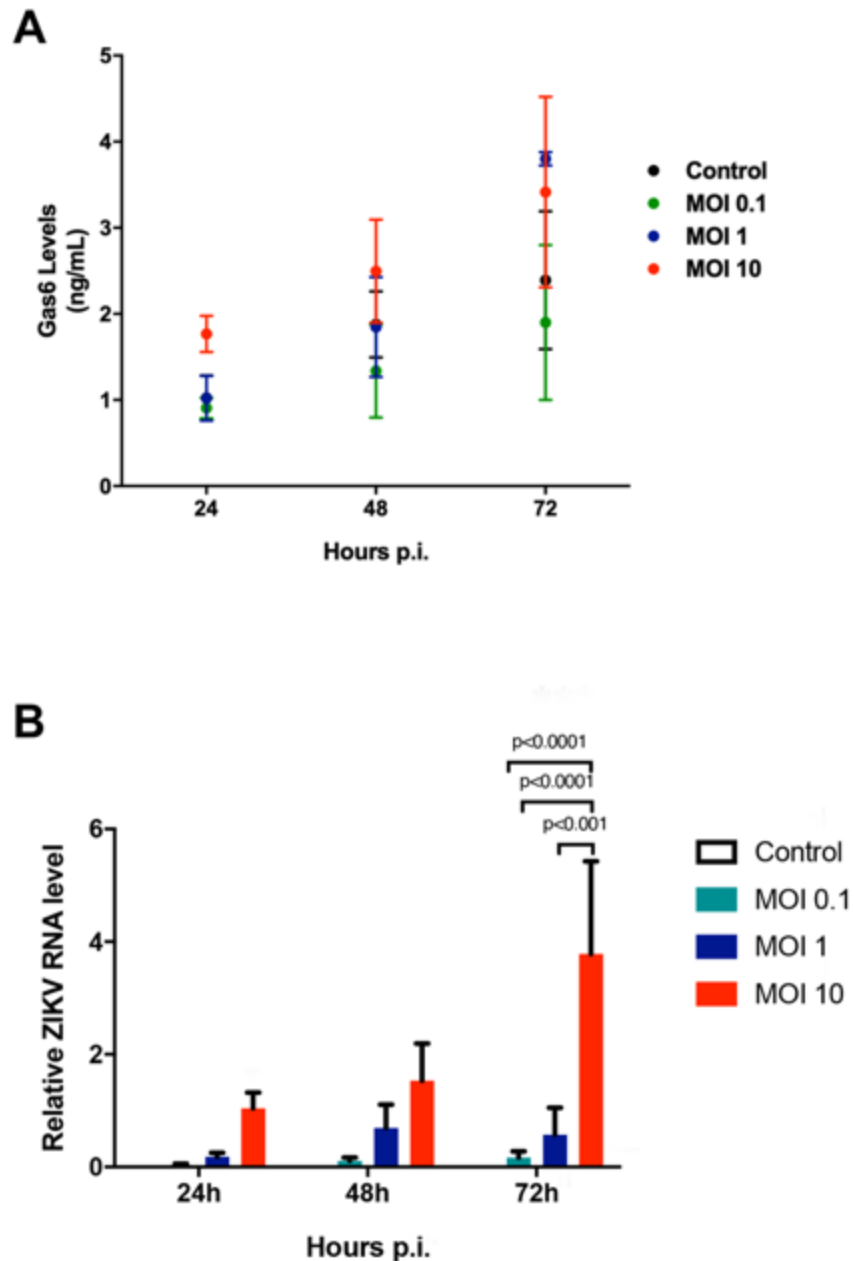
1145 **Supplementary figure 2: Estimation of Gas6 threshold changing correlations with ZIKV-specific immune**

1146 **signatures.** A reduced complexity model was established by focusing on informative interactions between ZIKV-

1147 specific immune signatures and Gas6 determined by Pearson's correlation coefficients based on Gas6 levels. (A)

1148 Below 30ng/mL; (B) Above 30ng/mL Only correlations with associated p -value <0.05 are shown.

1149



1150

1151 **Supplementary figure 3: Gas6 production by human brain microvascular endothelial cells (hBMECs) *in***

1152 ***vitro* is not affected by ZIKV infection.** (A) Gas6 levels in the supernatant of hBMEC at different time points

1153 (24, 48 and 72h) after *in vitro* infection with ZIKV at different multiplicities of infections (MOI 0.1, 1 and 10).

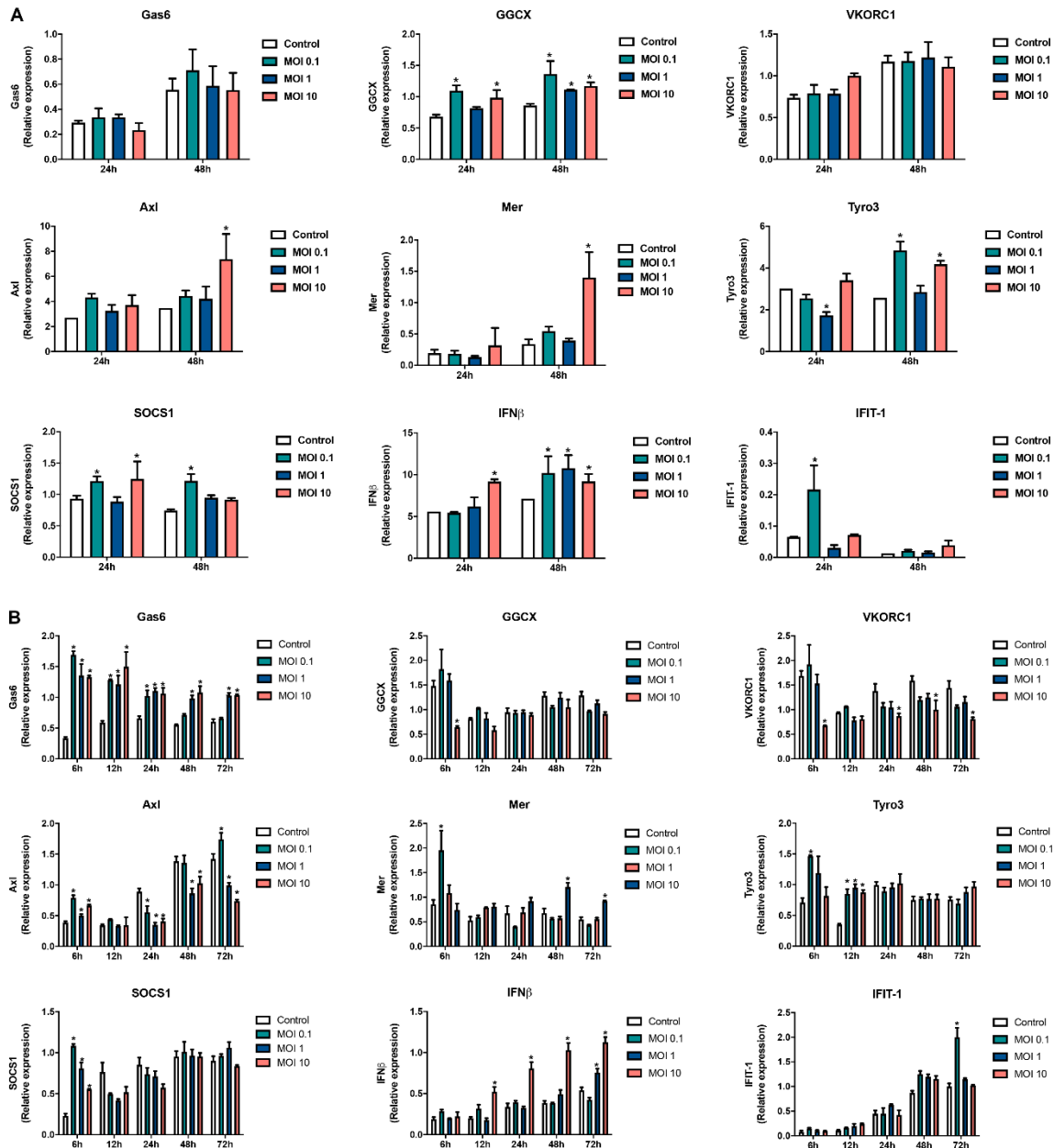
1154 (B) Total cellular RNA was extracted at different time points after infection as indicated in the graphs, and relative

1155 viral RNA levels were determined by real-time quantitative PCR. The data shown are representative images of 3

1156 independent experiments. P values were determined using one-way ANOVA statistical test with for each time

1157 point with Bonferroni-corrected multiple comparisons test.

1158



1159

1160

Supplementary figure 4: ZIKV Infection stimulates Gas6 upregulation and downregulates type I IFN

1161

response in peripheral blood mononuclear cells (PBMCs) and monocytes. (A) PBMCs and (B) THP-1

1162

monocytes were challenged with ZIKV (MOI 0.1, 1 and 10), respectively. Total cellular RNA was extracted at

1163

different time points after infection as indicated in the graphs, and relative viral RNA levels, *GAS6*, *GGCX*,

1164

***VKORC1*, *AXL*, *TYRO3*, *MER*, *SOCS1*, *IFNB* AND *IFIT1* mRNA levels were determined by real-time quantitative**

1165

PCR. (A) The data shown are mean \pm SEM representative of three independent experiments using cells from

1166

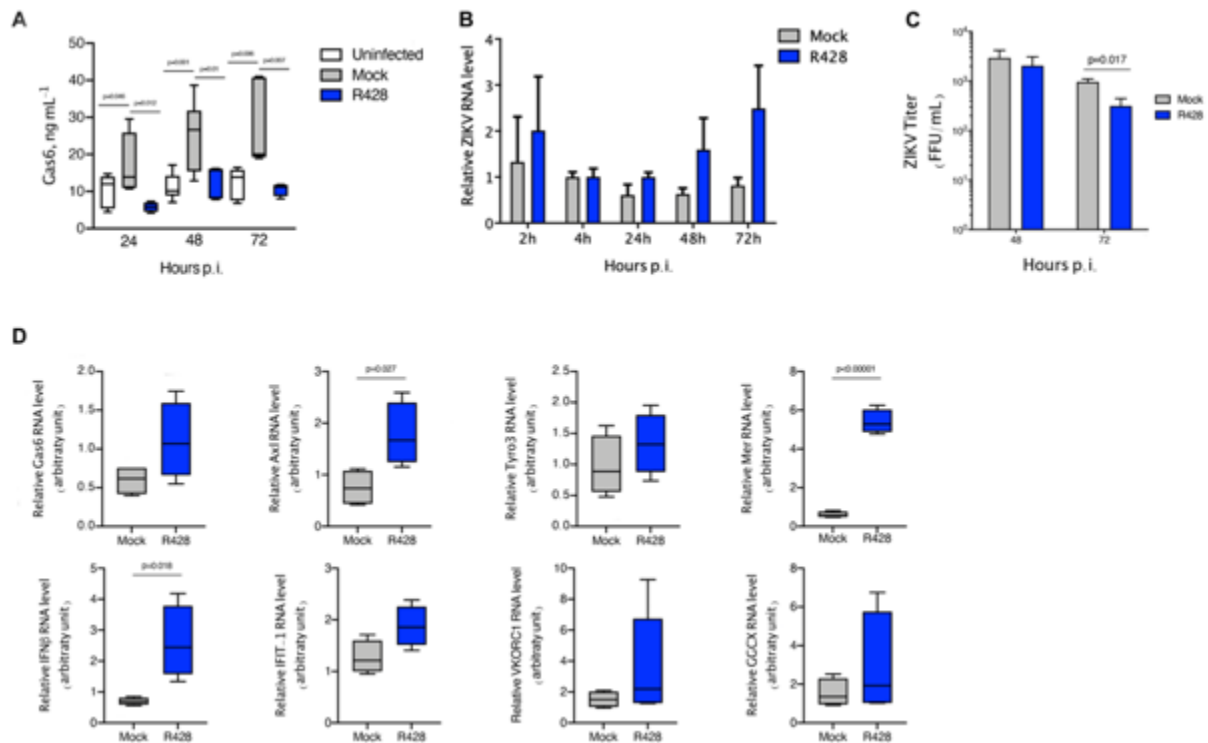
different donors. P values were determined for comparisons between conditions at the corresponding time point

1167

using Two-way analyses of variance statistical test with Tukey-corrected multiple comparisons test. * $p < 0.05$ vs

1168

control (uninfected cells).



1169

1170

Supplementary figure 5: R428 treatment, inhibitor of Axl tyrosine kinase activity, restores antiviral

1171

response. (A) Gas6 levels were determined by ELISA in the supernatant of THP-1 monocytes collected at 24h,

1172

48h and 72h after *in vitro* infection with ZIKV (MOI 1). Infected cells were treated or not (mock) with 2μM R428

1173

throughout the course of infection. Gas6 concentration is depicted as Tukey box plots. The data shown are

1174

representative images of three independent experiments. P values were calculated for comparisons between

1175

conditions at the corresponding time point using One-way analyses of variance statistical test with Bonferroni-

1176

corrected multiple comparisons test. (B, D) THP-1 monocytes were challenged *in vitro* with ZIKV (MOI 1) and

1177

treated or not (mock) with 2μM R428 throughout the course of infection. Total cellular RNA was extracted at

1178

different time points after infection. Relative viral RNA levels, *GAS6*, *GGCX*, *VKORC1*, *AXL*, *TYRO3*, *MER*,

1179

SOCS1, *IFNB* AND *IFIT1* mRNA levels were determined by real-time quantitative PCR. The data shown are

1180

representative images of three independent experiments. (B) The data shown are mean ± SEM representative of

1181

three independent experiments. (D) Graphs depict relative RNA expression as Tukey box plots after results were

1182

normalized to GAPDH housekeeping gene expression. P values were calculated using student's t-test to compare

1183

groups with normally distributed data or Mann-Whitney test to compare groups with non-normal distributions.

1184

(C, D) ZIKV titer (FFU/mL) was determined by plaque forming assay after incubation of ZIKV-permissive Vero

1185

E6 cell line with the 48h or 72h supernatant from monocytes after infection *in vitro* with ZIKV (MOI 1), treated

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or not (mock) with 2μM R428 at the moment of infection (pre-treatment). The data shown are representative

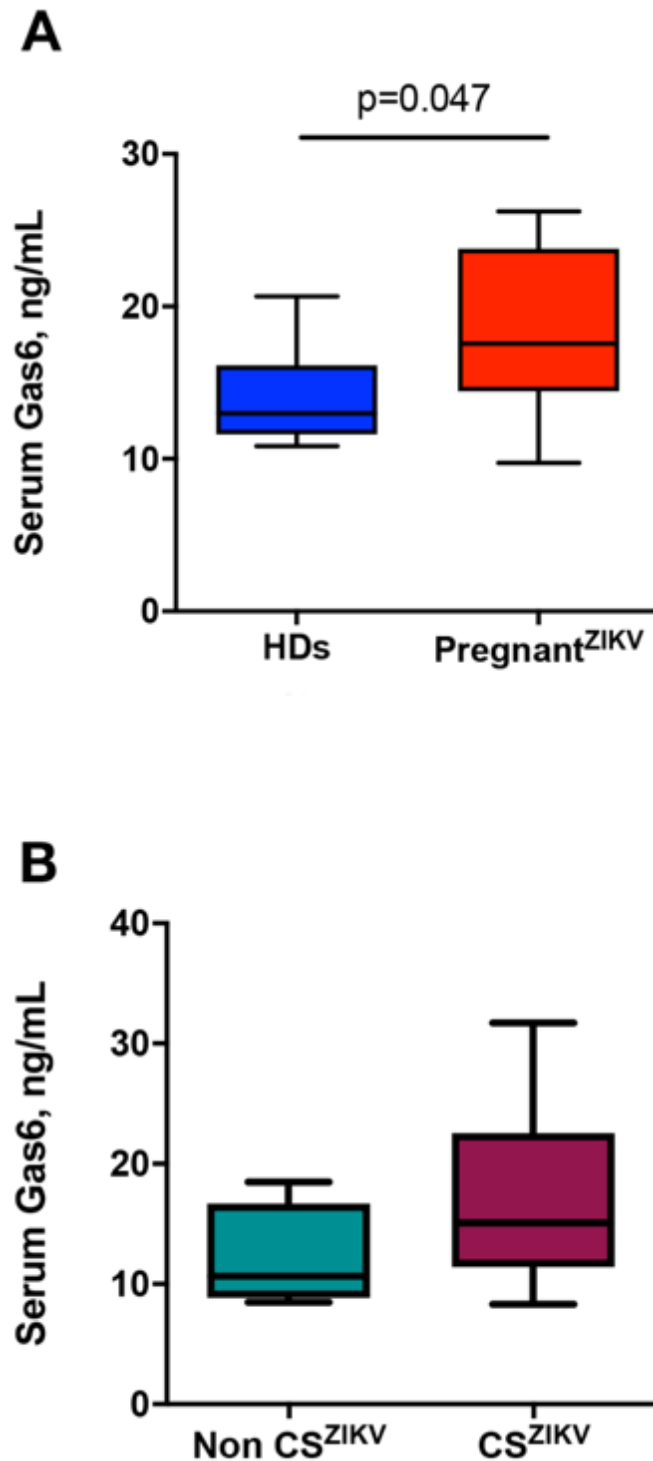
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images of three independent experiments. Mann-Whitney test was used to compare conditions at the

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corresponding time point.

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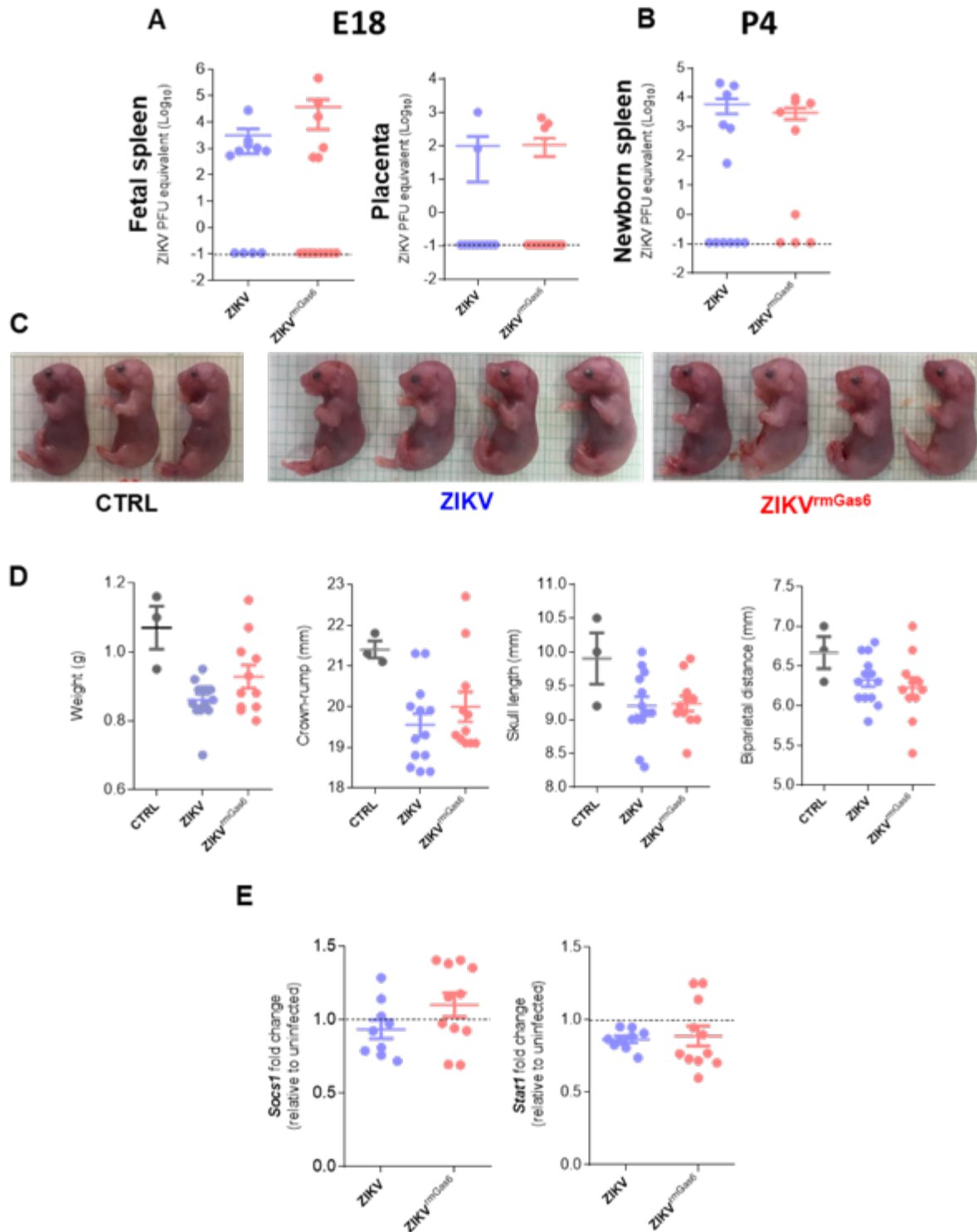
1191 **Supplementary figure 6: Gas6 expression is increased in the circulation of ZIKV-infected pregnant women.**

1192 (A) Levels of Gas6 in acute-phase serum samples of 8 Zika virus (ZIKV)- infected pregnant women and 6 infants

1193 born to women with Zika virus (ZIKV) infection, were determined by ELISA. (B) Two infants were born with

1194 CNS abnormalities associated to ZIKV congenital syndrome (CS^{ZIKV}) and 4 without congenital syndrome (Non

1195 CS^{ZIKV}). Gas6 concentration is depicted as Tukey box plots. P values were determined using student's t-test.



1196

1197 **Supplementary figure 7: Dimension analysis and ZIKV viral load in C57BL/6 offspring.** Pregnant mice were

1198 infected subcutaneously with pure ZIKV (10^5) or ZIKV previously incubated with rmGas6 (1 μ g/mL)

1199 (ZIKV^{rmGas6}) on E (embryonic day) 16. The organs were harvested on E18 or postpartum day (P) 4. The viral load

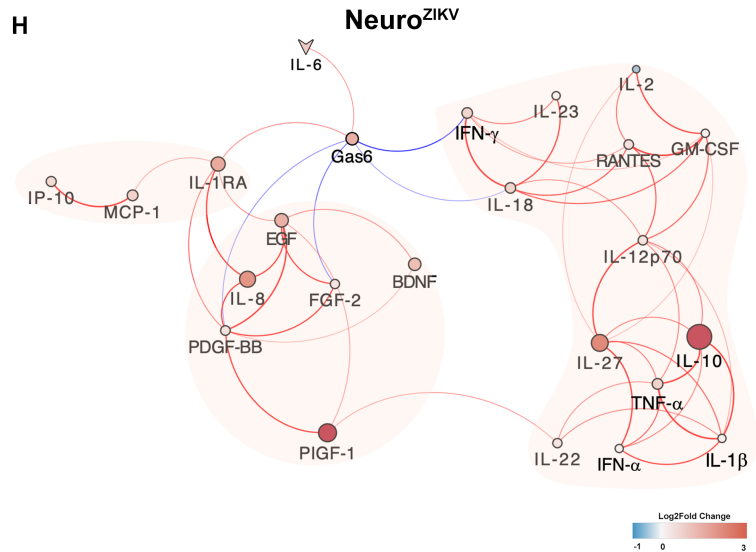
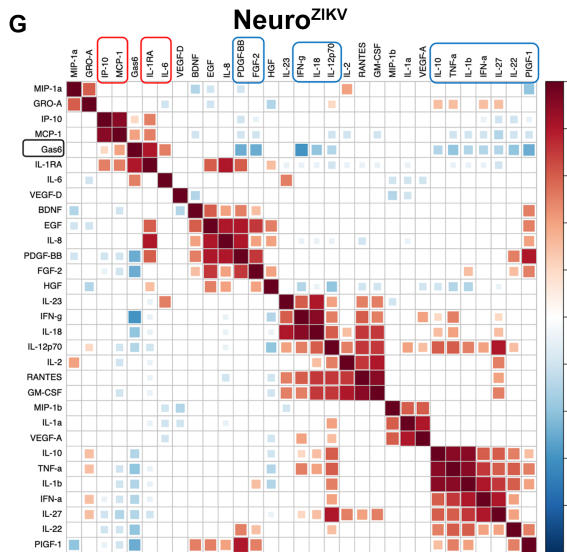
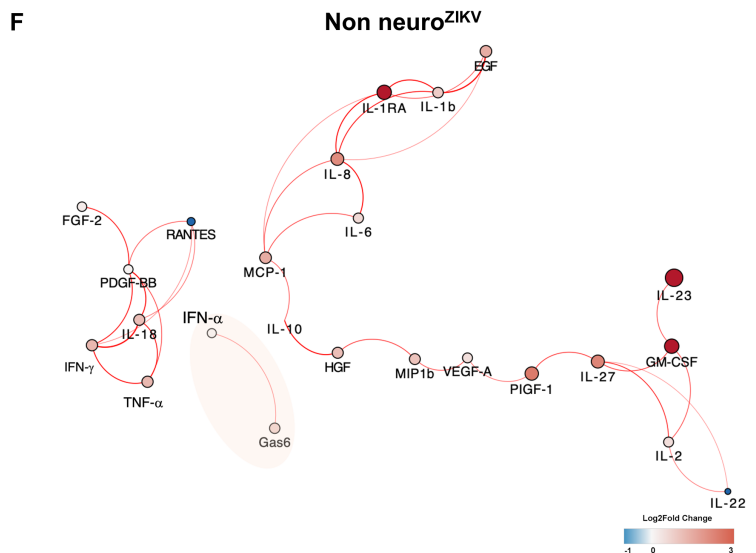
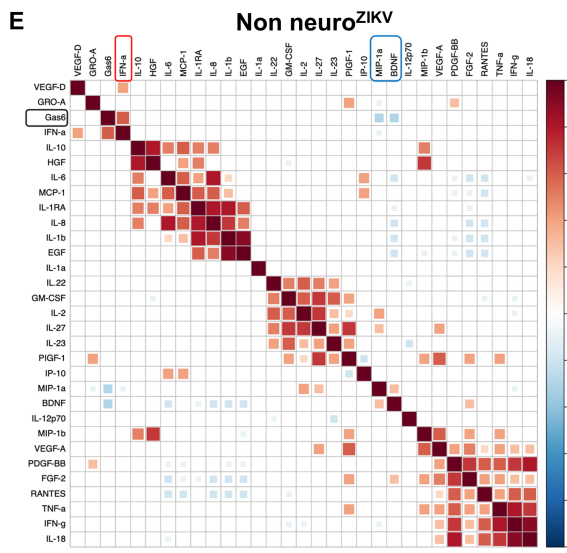
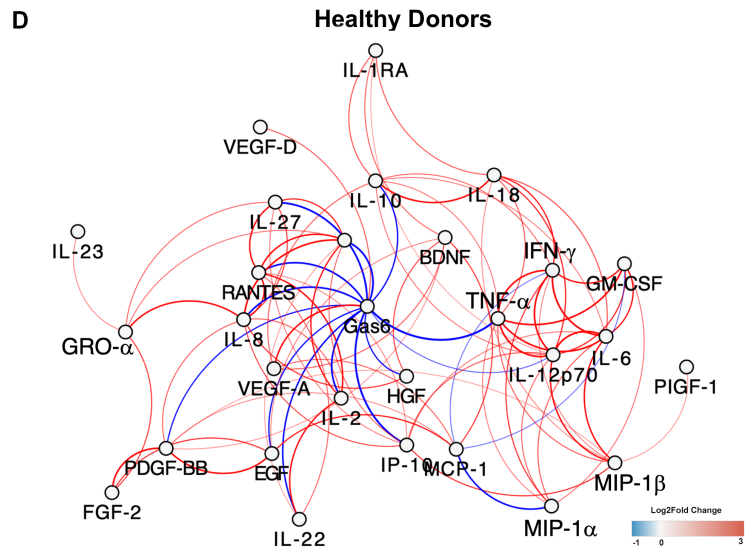
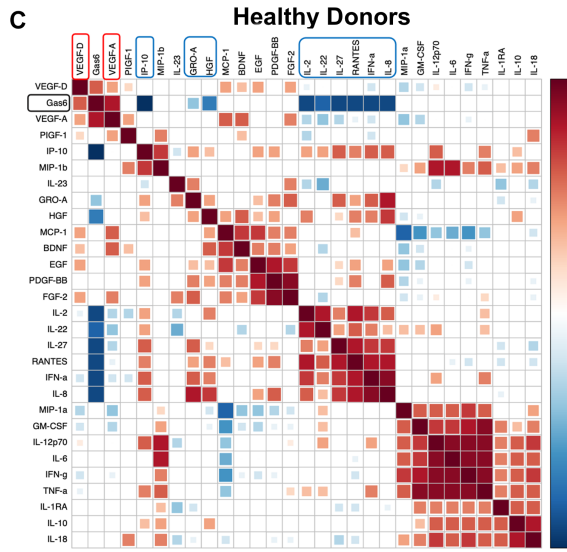
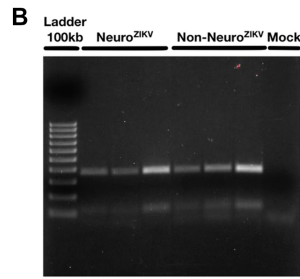
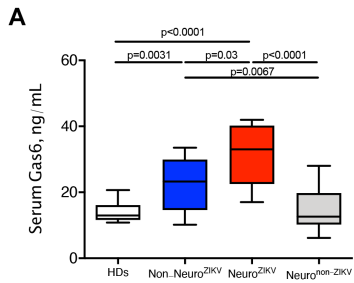
1200 was analyzed by qPCR. Viral load on E18 (A) and P4 (B). Pregnant mice were also infected intravaginally on

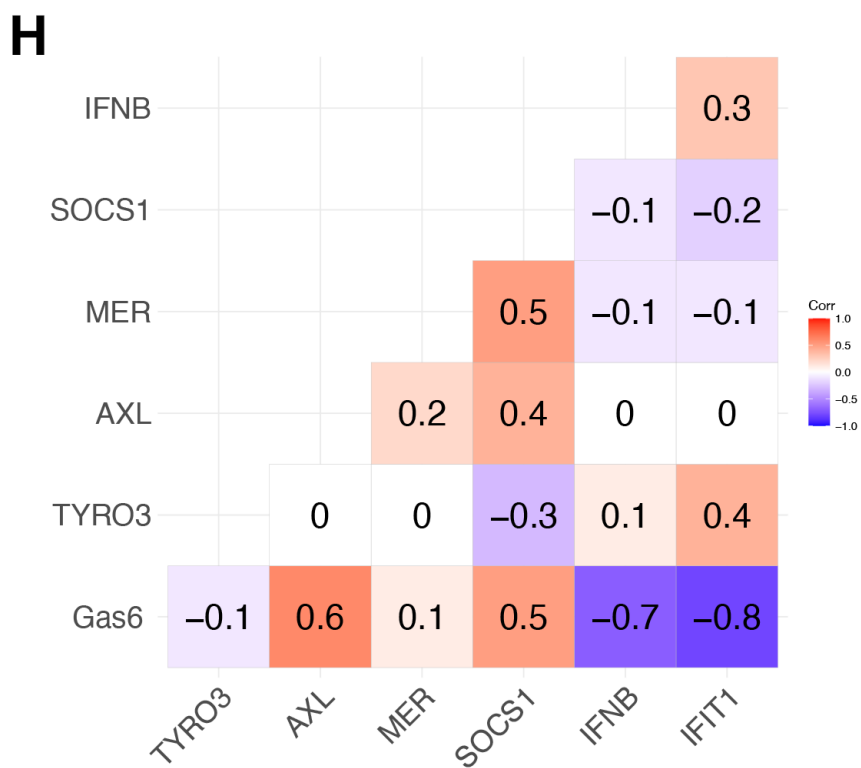
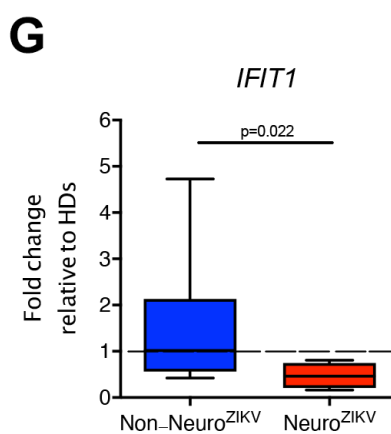
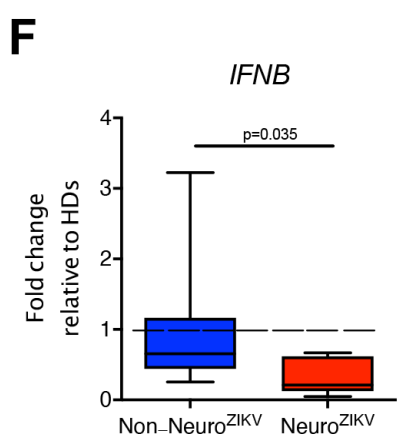
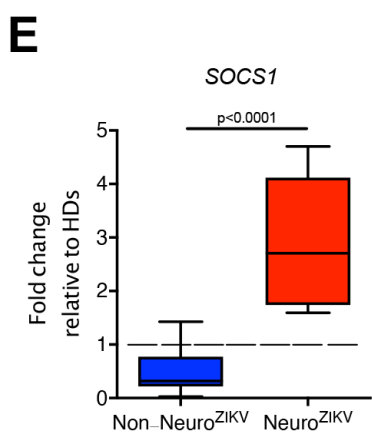
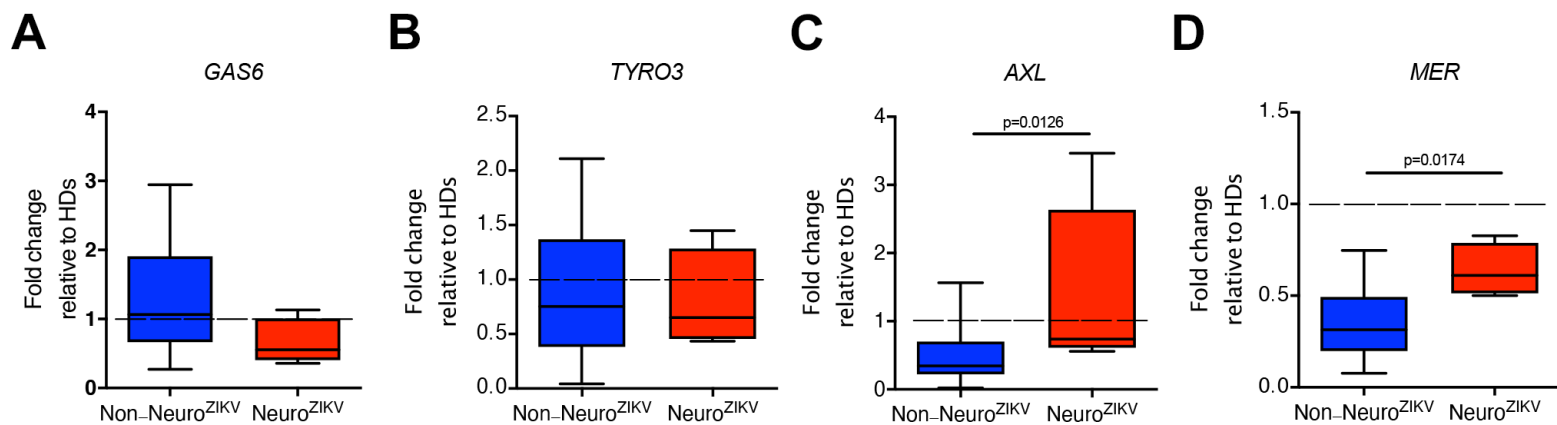
1201 E10 with pure ZIKV (10^5) or ZIKV previously incubated with rmGas6 (1 μ g/mL). Tissues were harvested at E18.

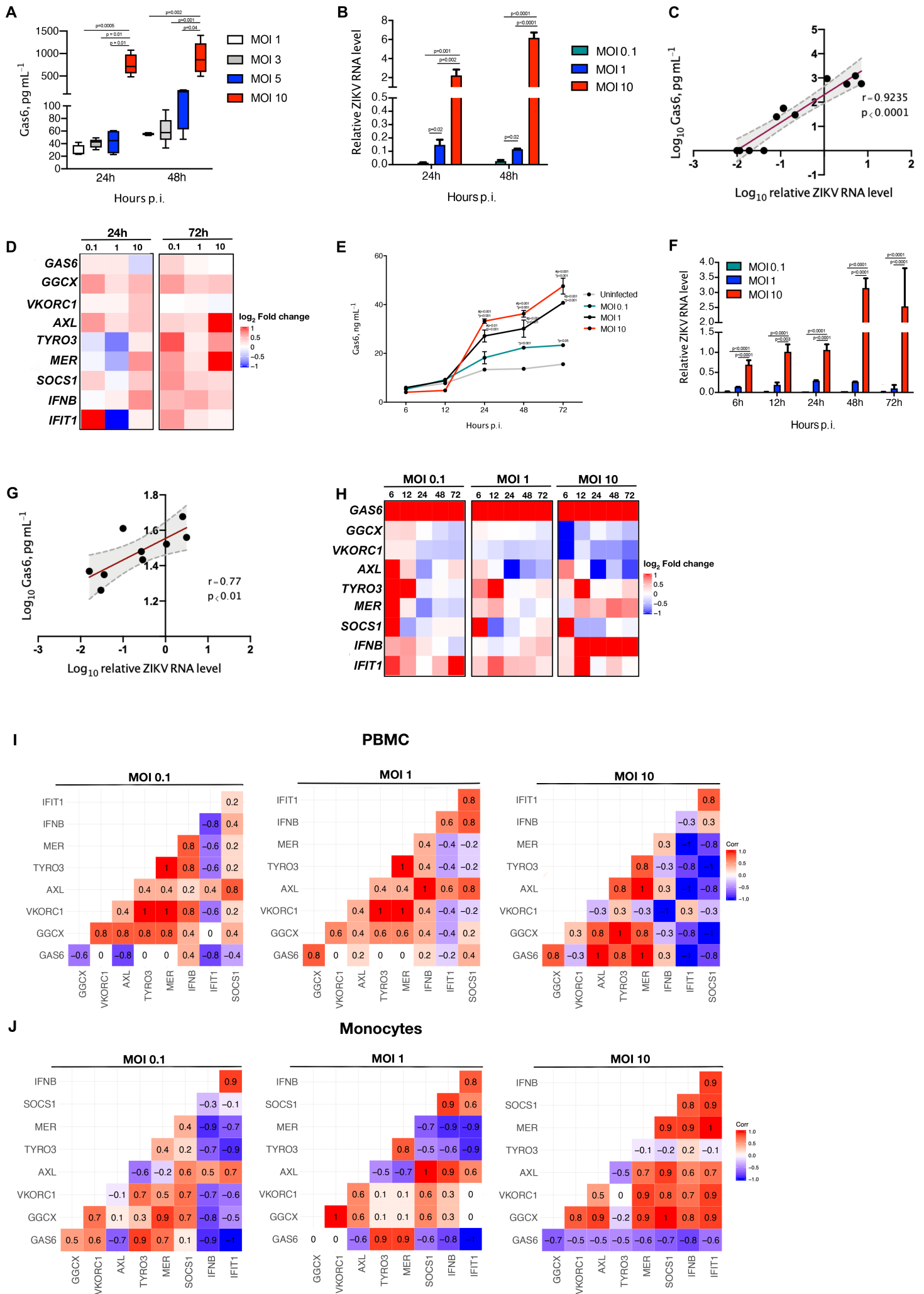
1202 Foetus pictures (C). Dimension analyses (D). Placenta gene expression (E). Fold change was calculated between

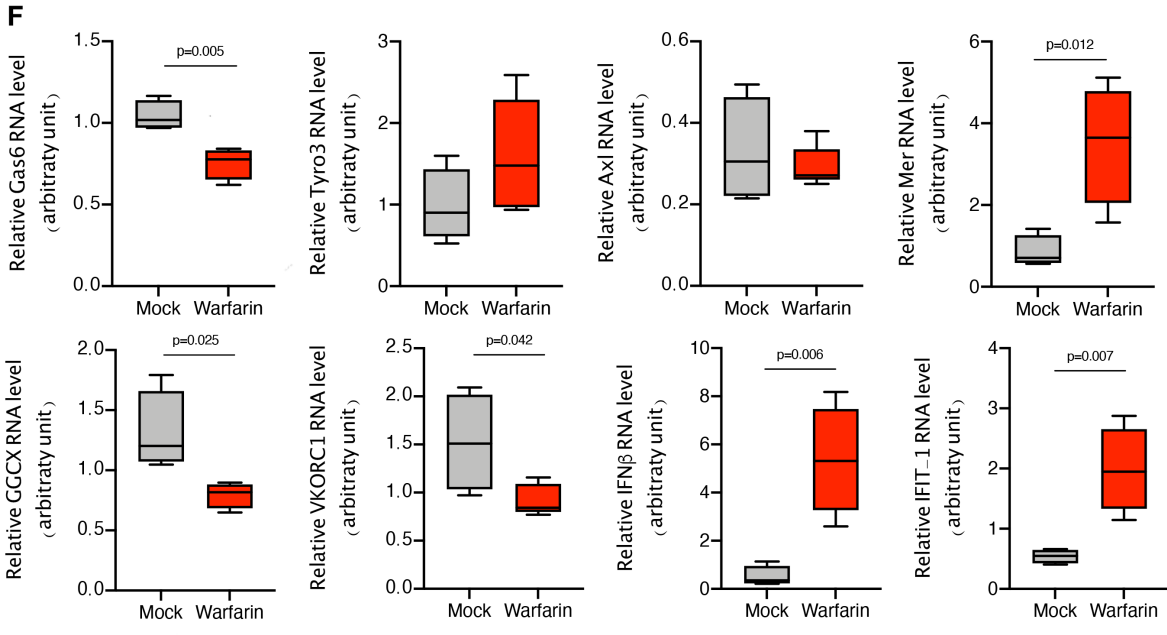
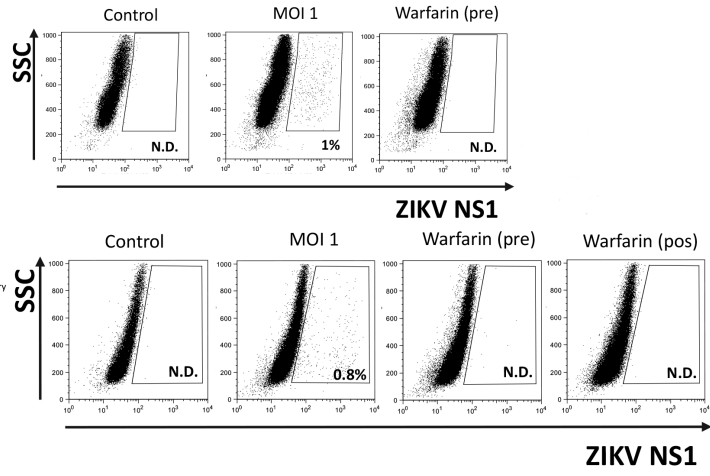
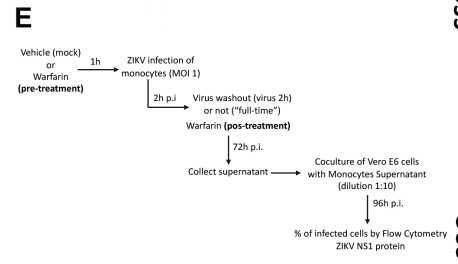
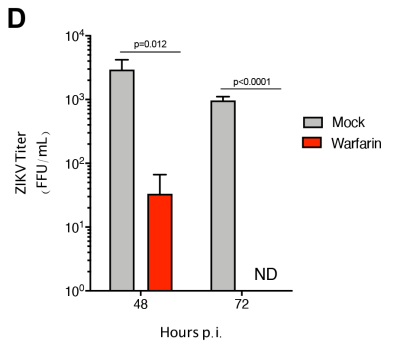
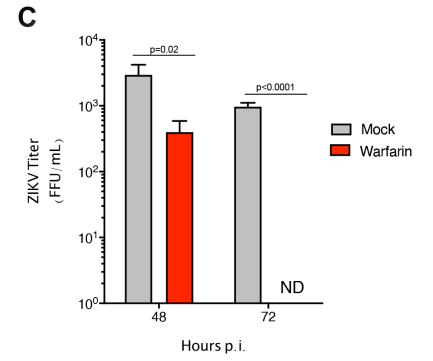
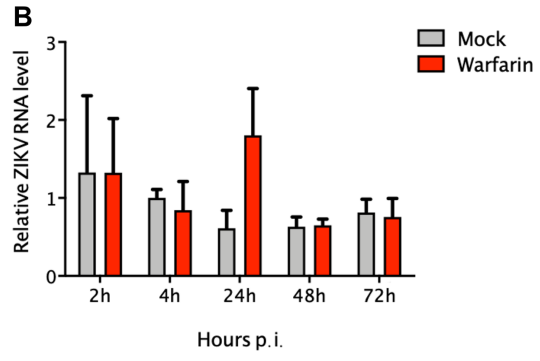
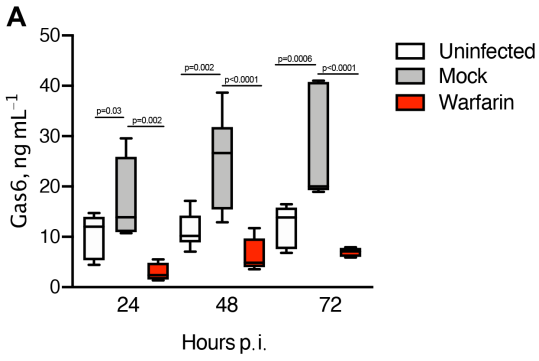
1203 uninfected and infected groups. Graph bars are shown as mean \pm SEM and are representative of two independent

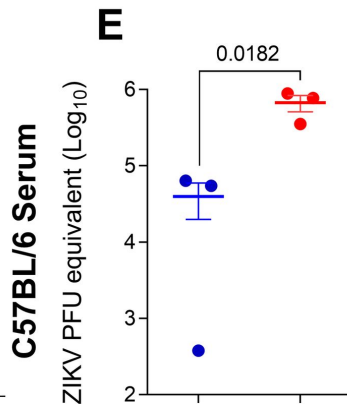
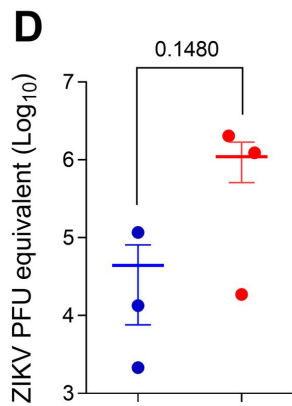
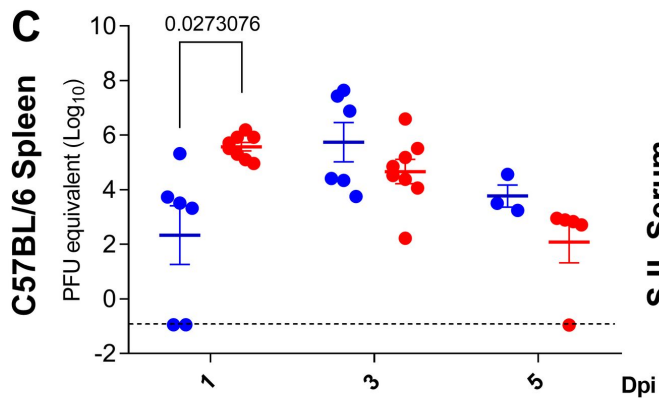
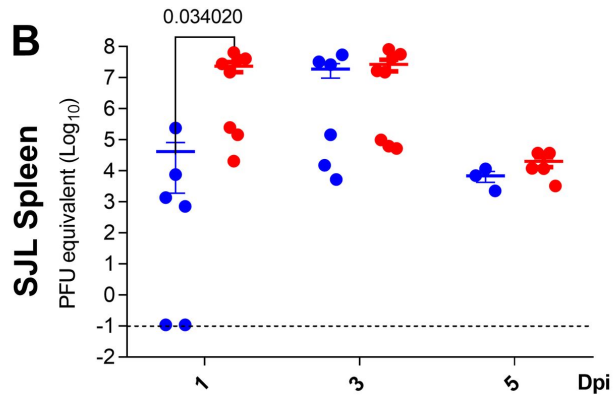
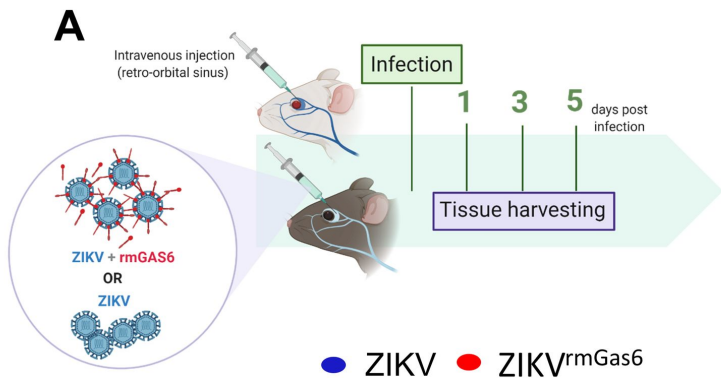
1204 experiments. Numbers of experimental groups: (A) Control n=6 ZIKV n=11; ZIKV^{rmGas6} n=14. (B) Control n=6
1205 ZIKV n=12; ZIKV^{rmGas6} n=9. (C and D) Control n=3 ZIKV n=13; ZIKV^{rmGas6} n=11. (E) Control n=3; ZIKV n=9;
1206 ZIKV^{rmGas6} n=11.
1207

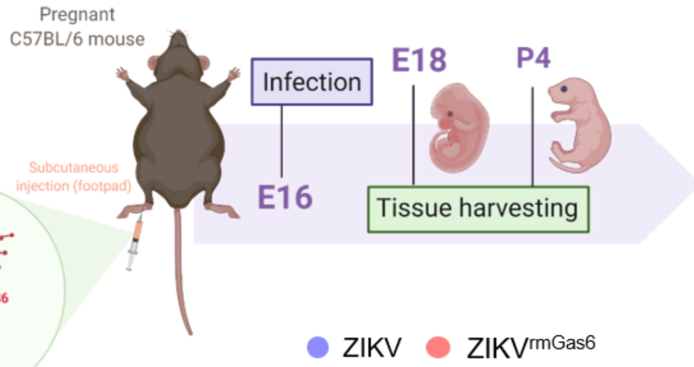
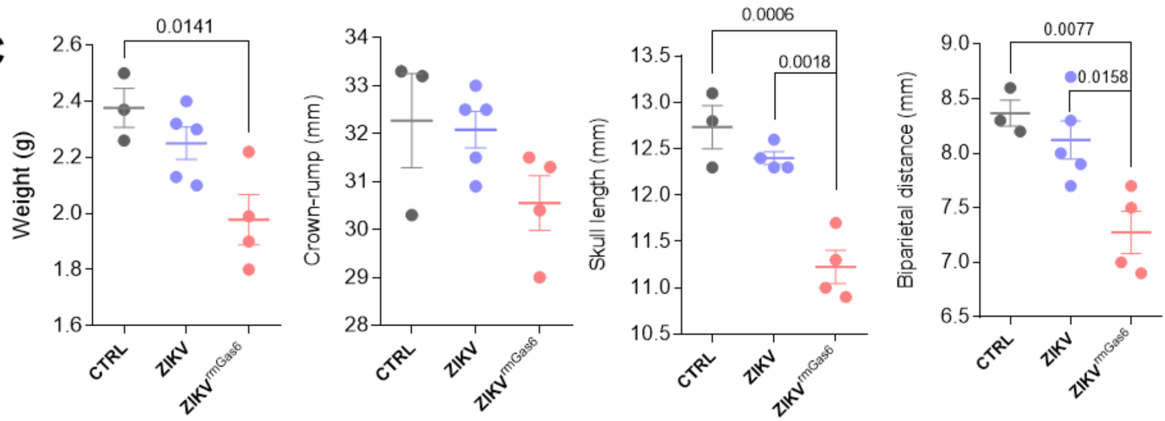
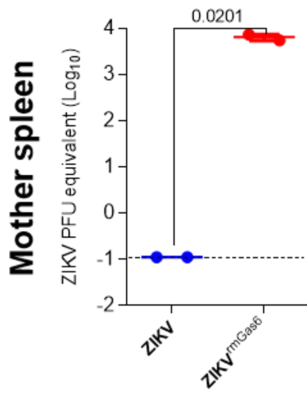
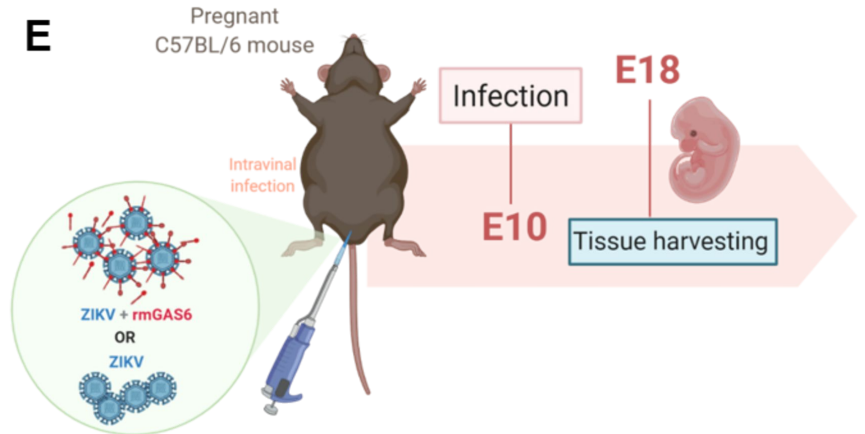
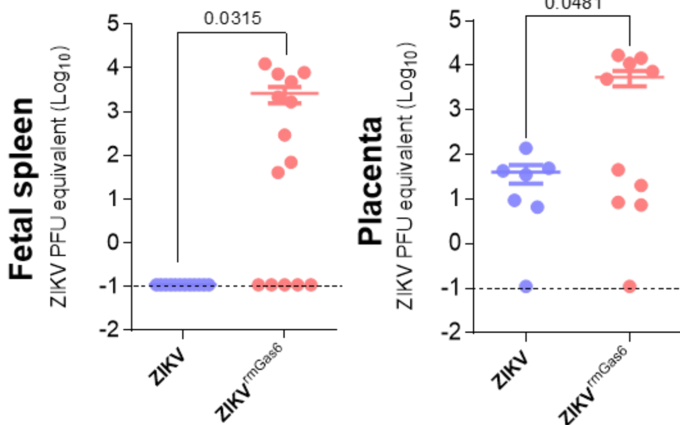
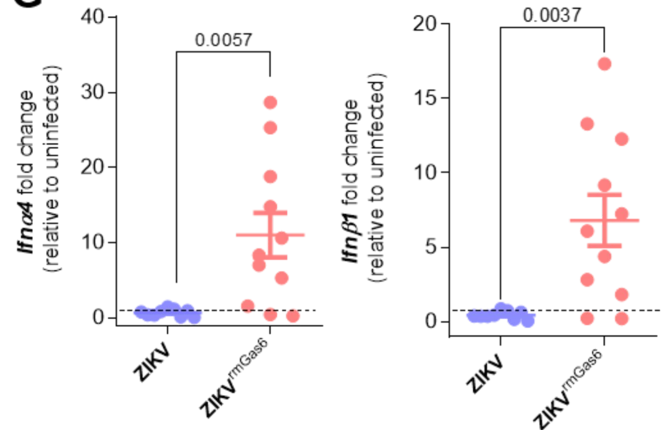










A**B****C****D****E****F****G**

Human

A

SOCS1
AXL
IFN β
IFIT1

Non-Neuro^{ZIKV}

Patients

Neuro^{ZIKV}

SOCS1
AXL
IFN β
IFIT1

Gas6

Gas6

B

Warfarin treated
monocytes

In vitro

Untreated
monocytes

Non-carboxylated
Gas6

IFN β
IFIT1
GAS6

IFN β
IFIT1
GAS6

γ -carboxylated
Gas6

Mouse

C

Ifn α
Ifn β
Socs1

Pregnant
C57BL/6
mouse

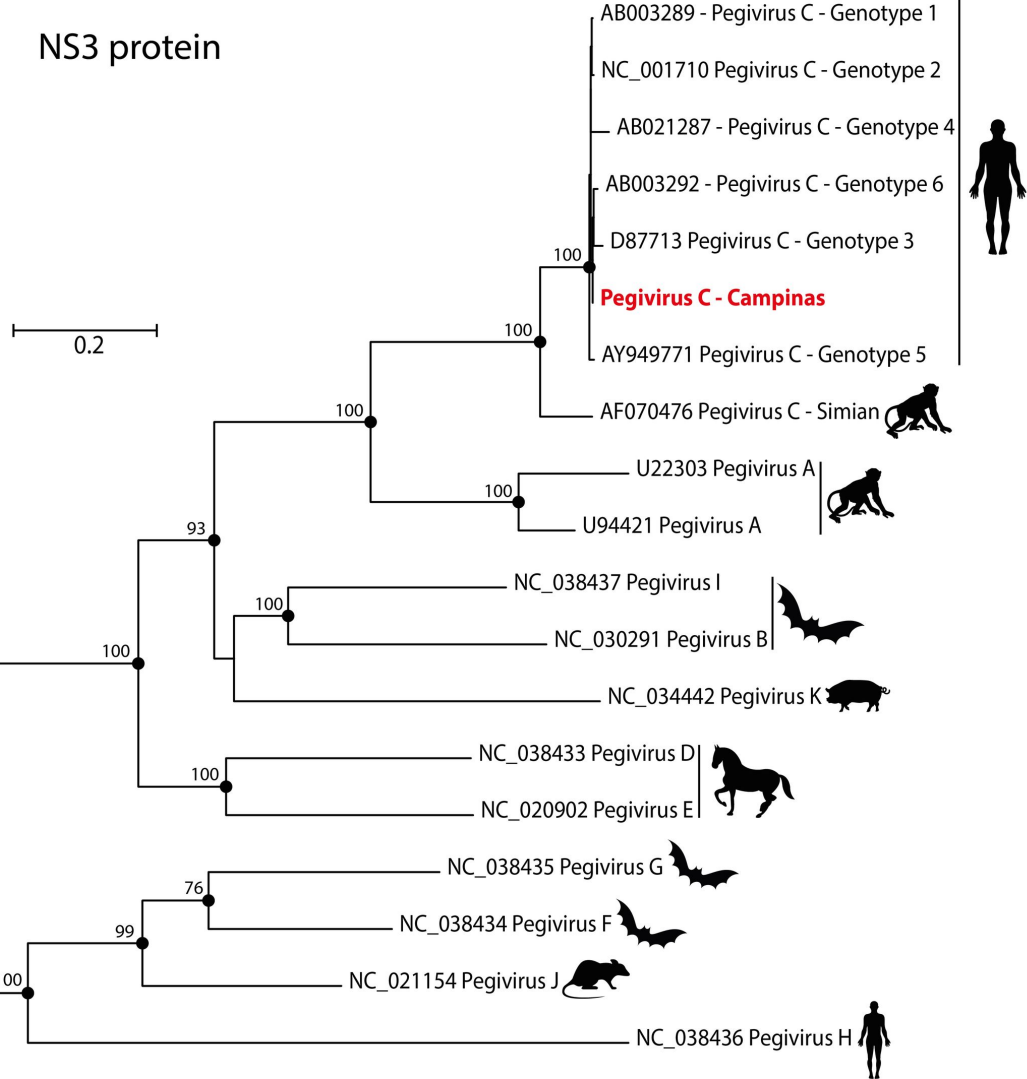
ZIKV-induced
malformation

Ifn α
Ifn β
Socs1

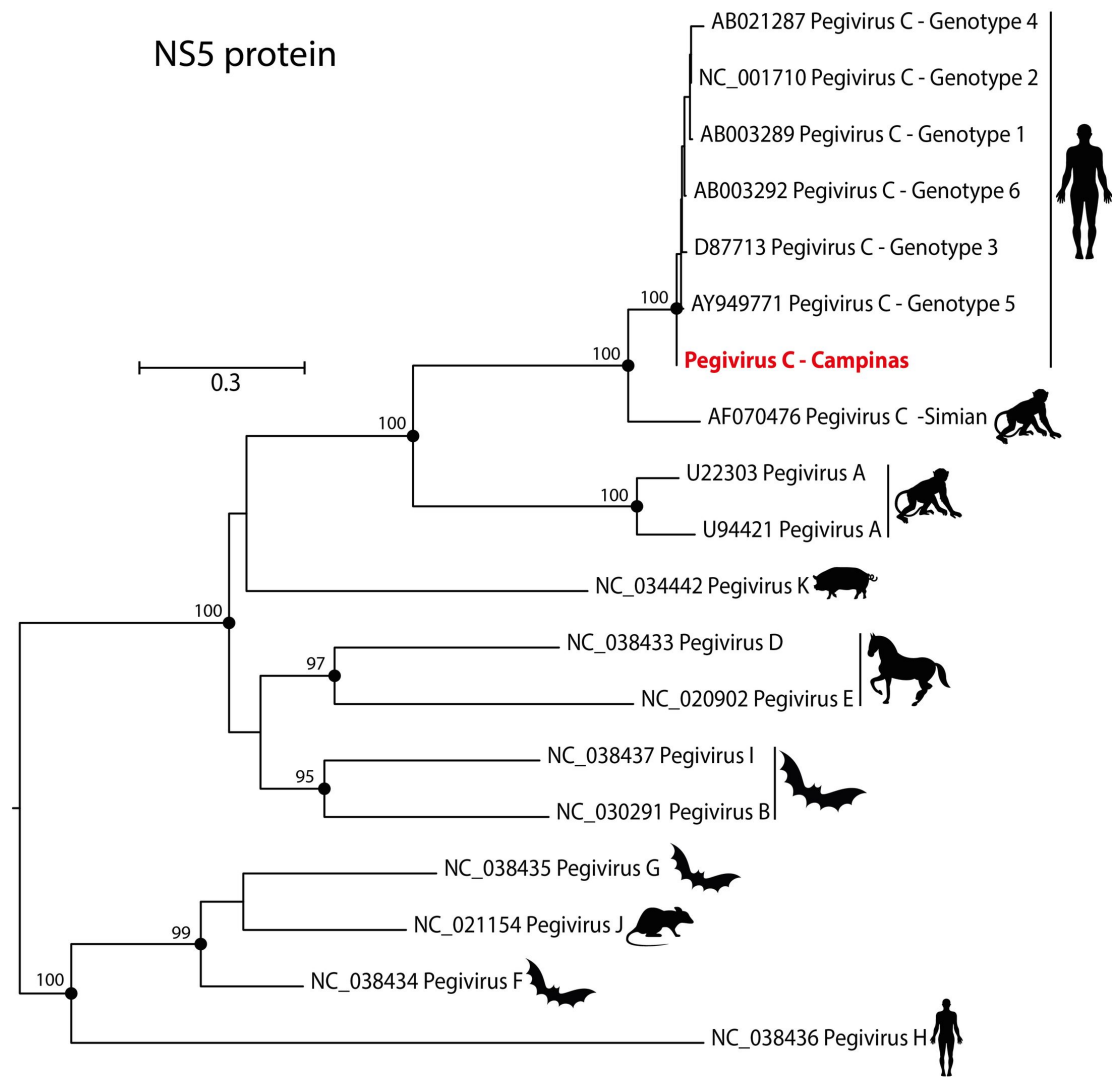
ZIKV

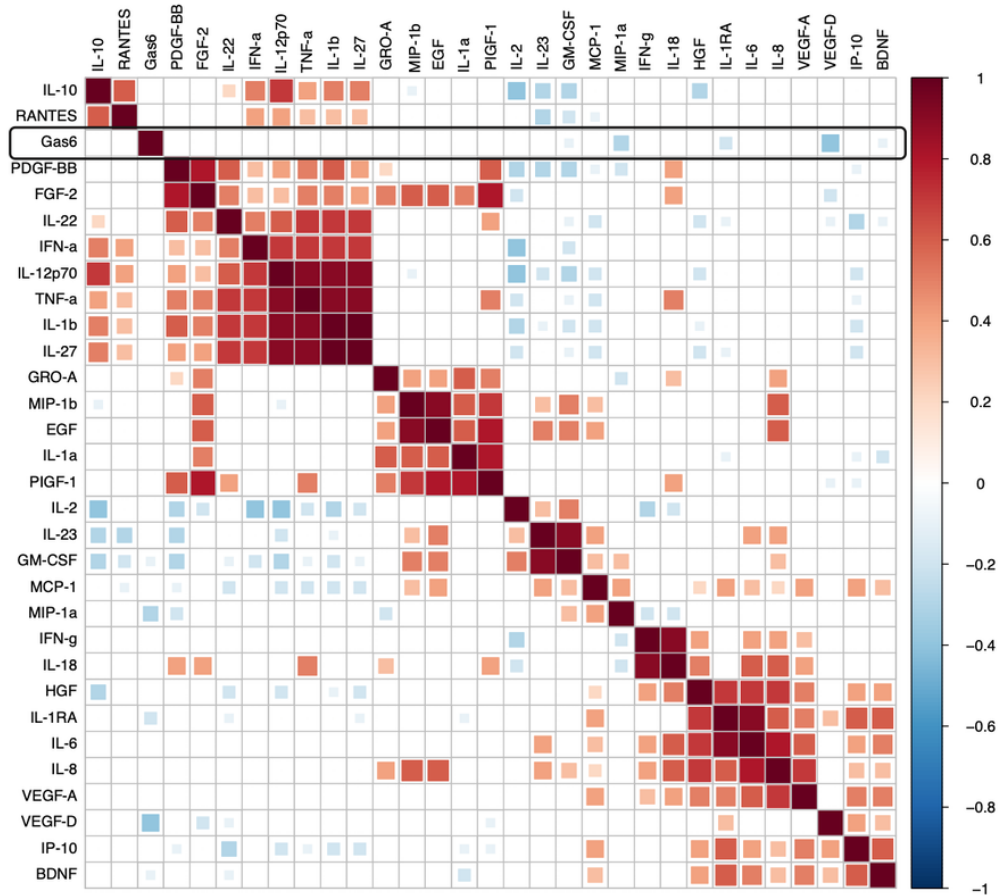
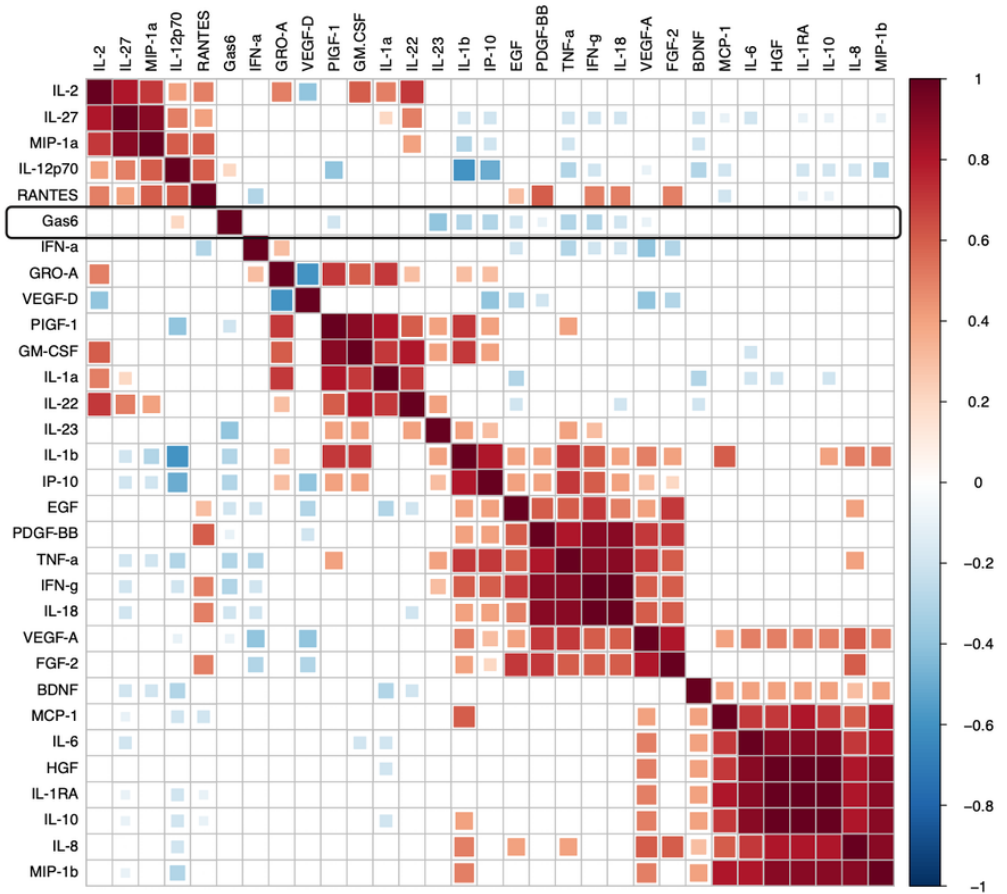
ZIKV^{GAS6}

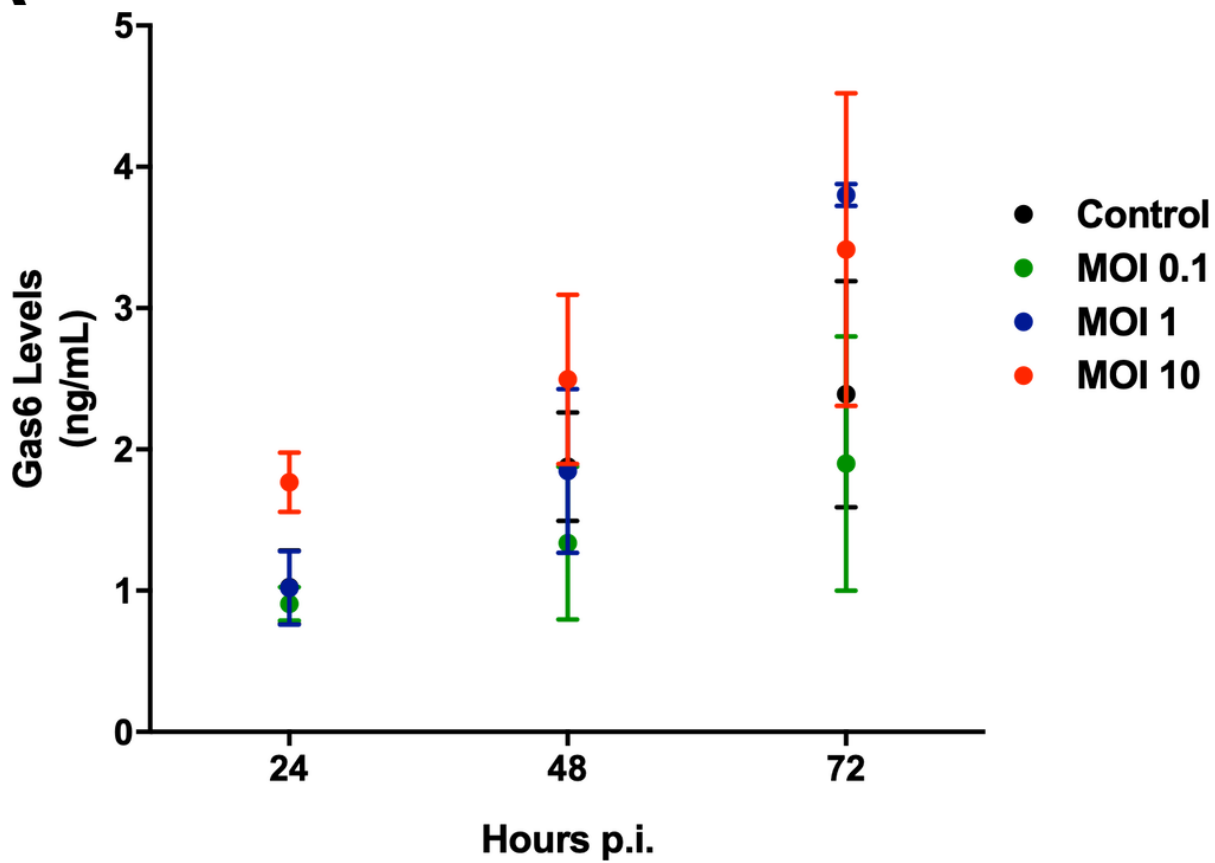
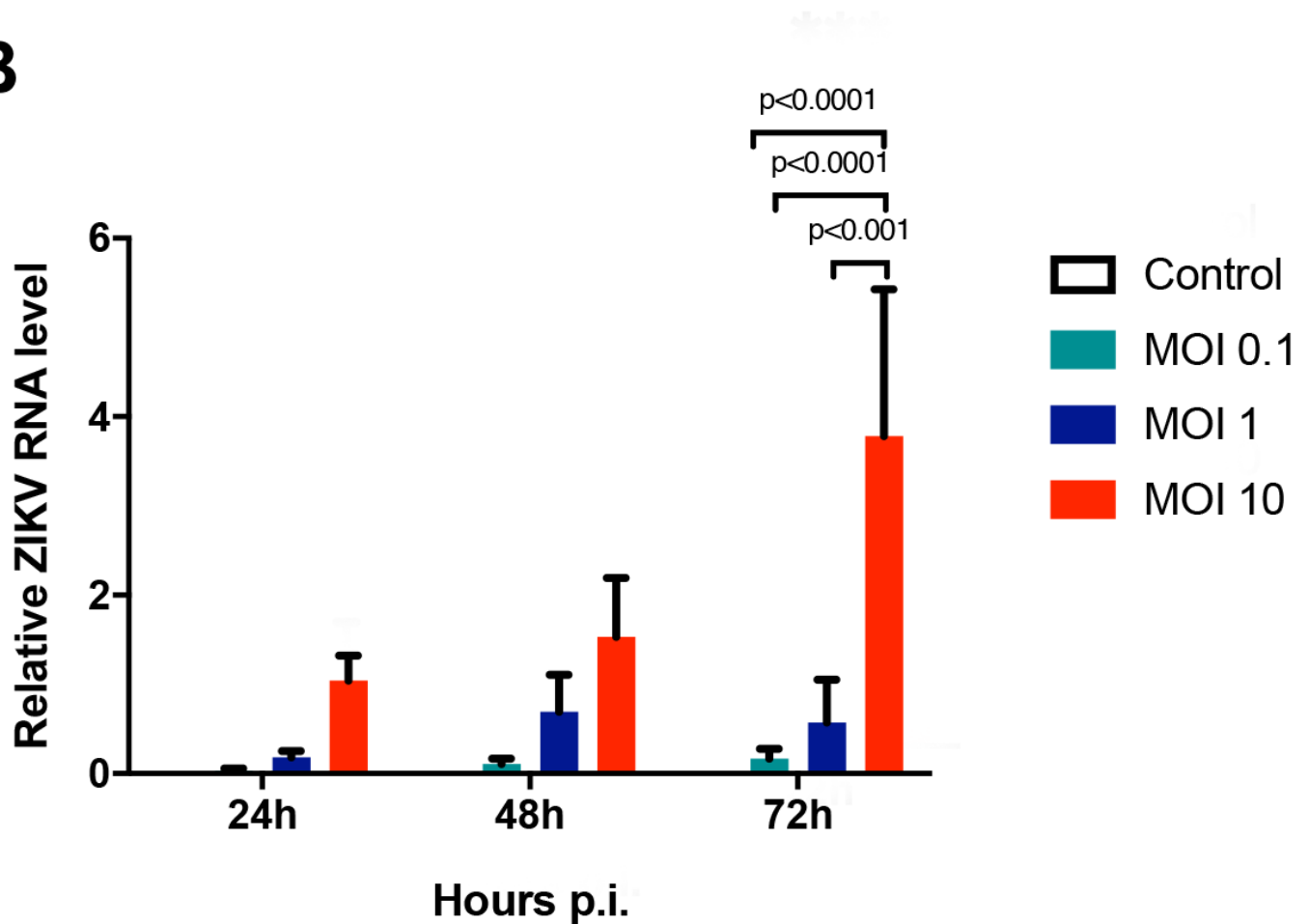
NS3 protein

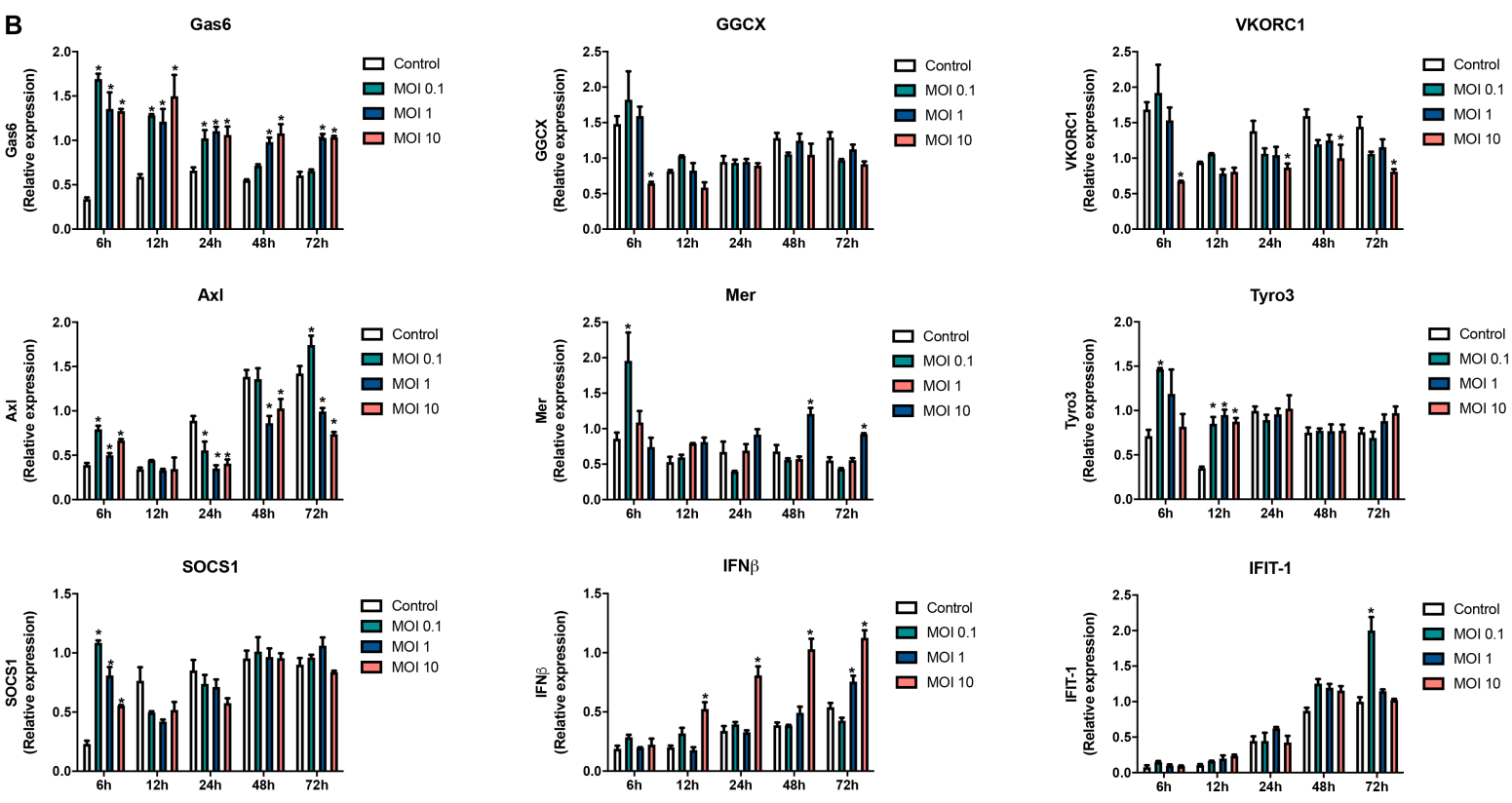
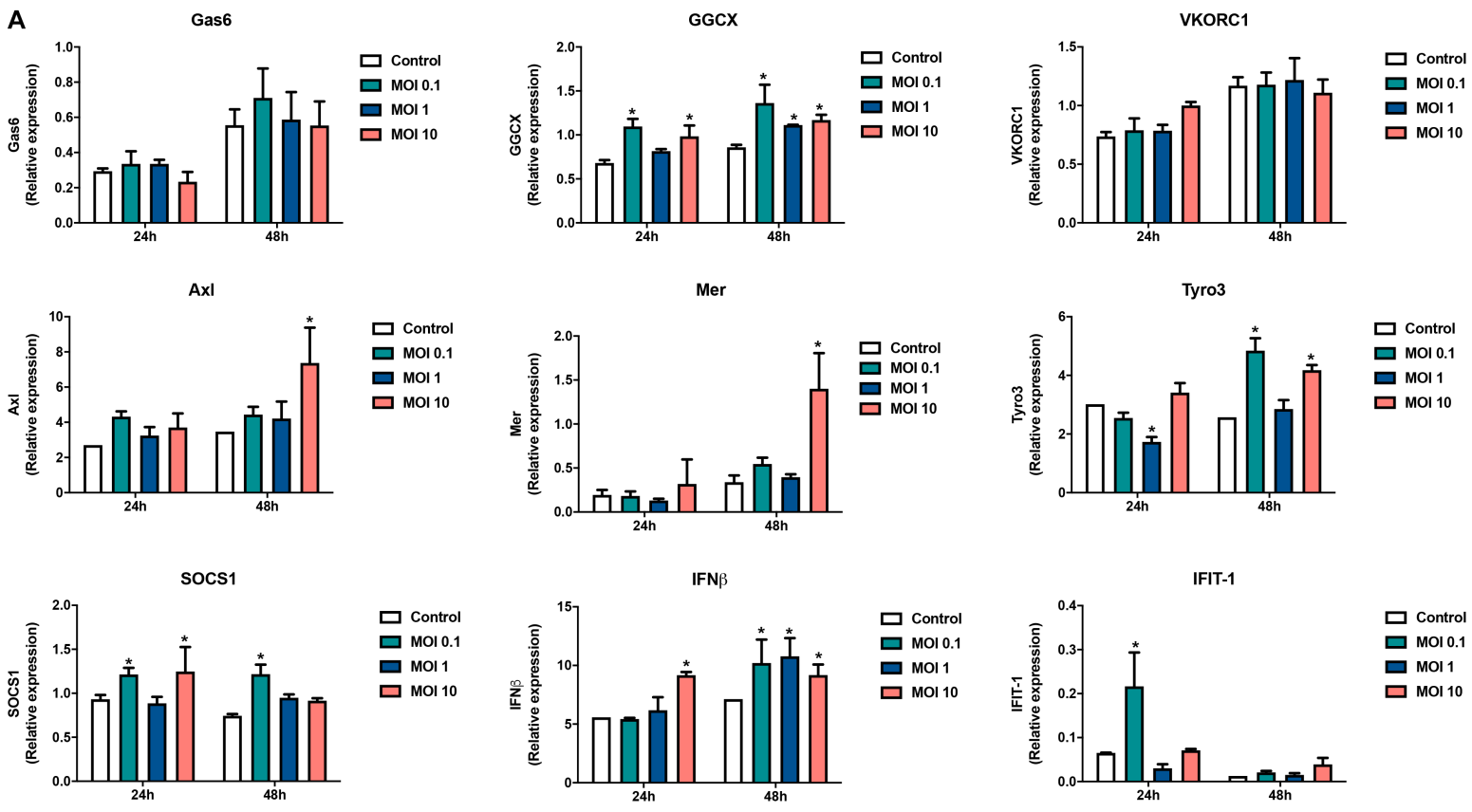


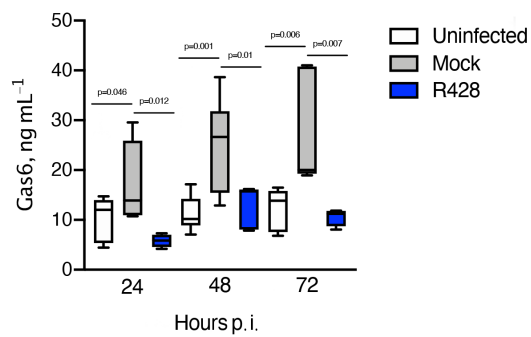
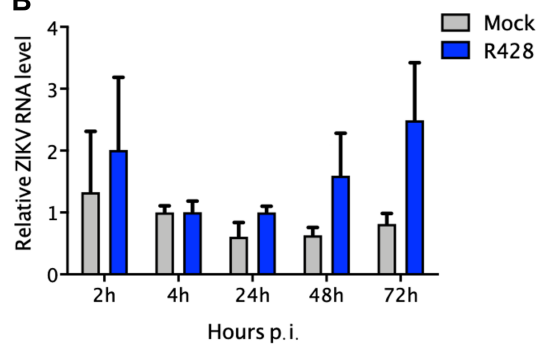
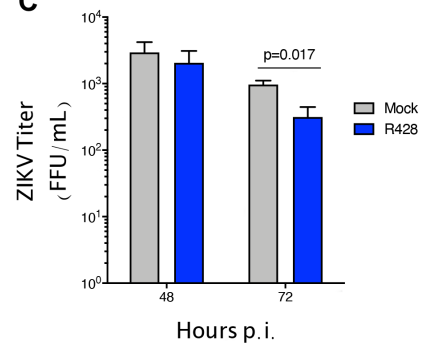
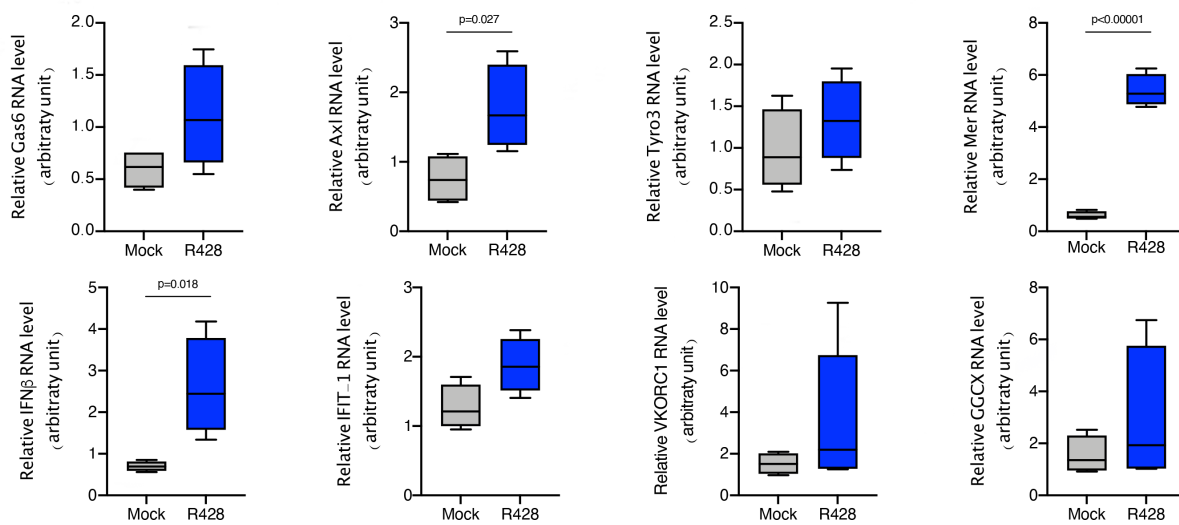
NS5 protein

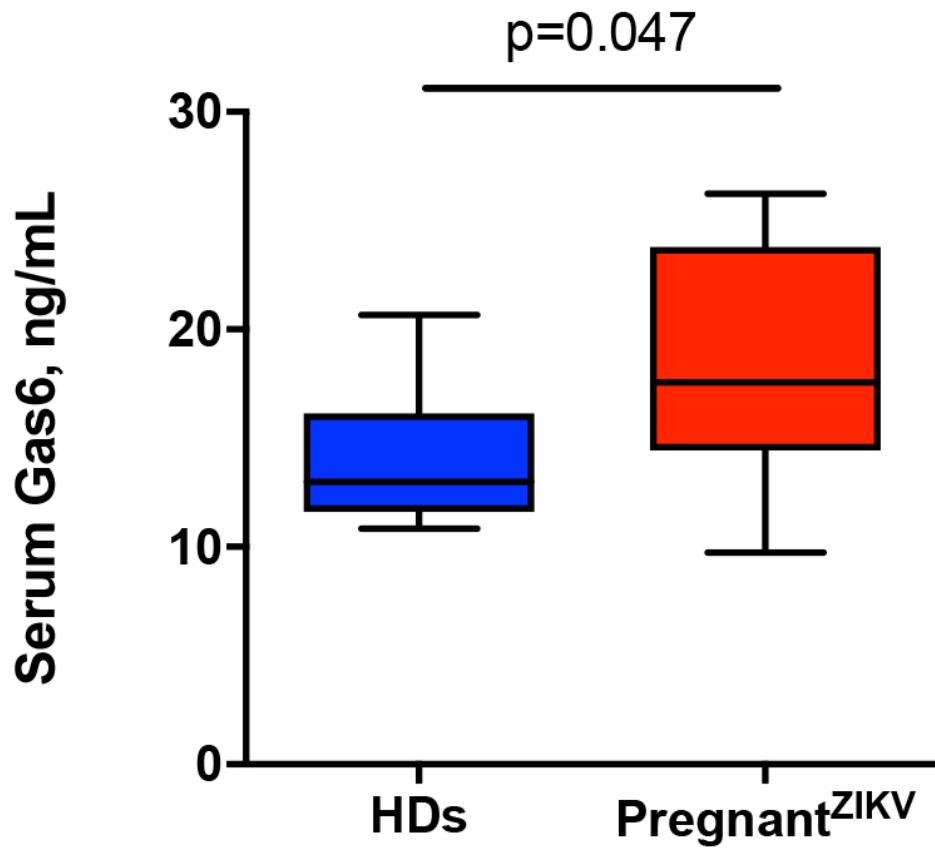
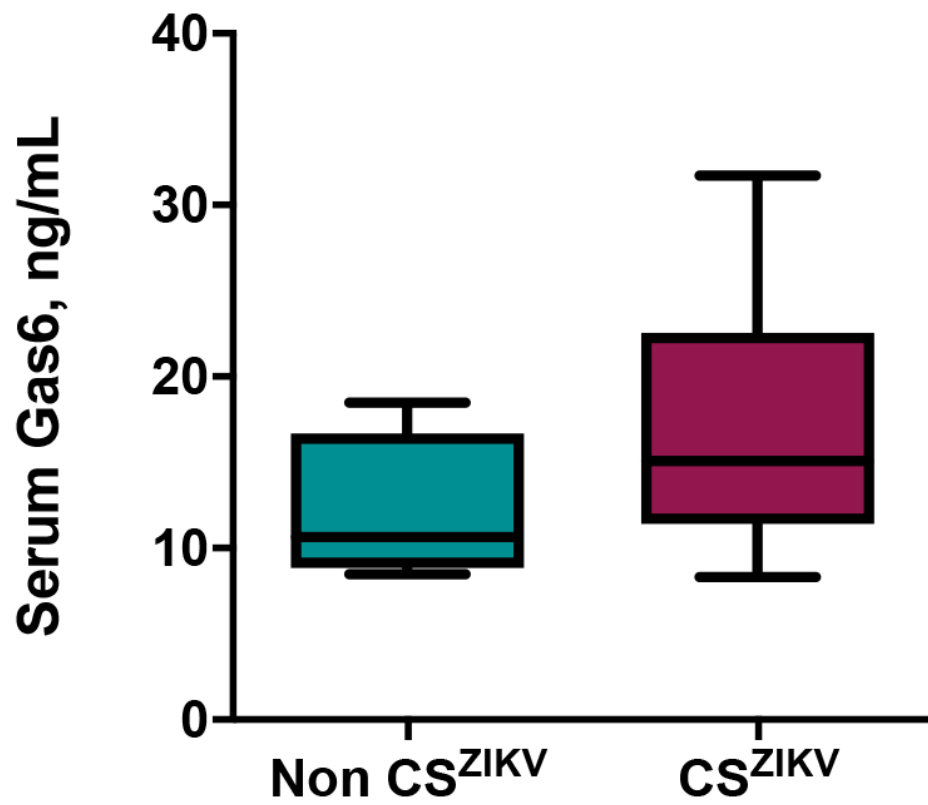


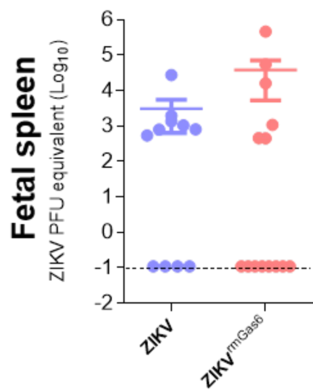
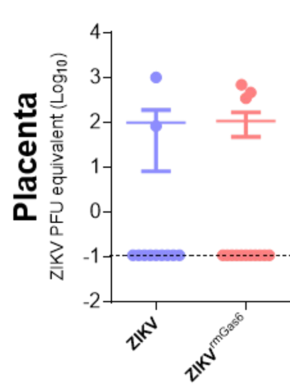
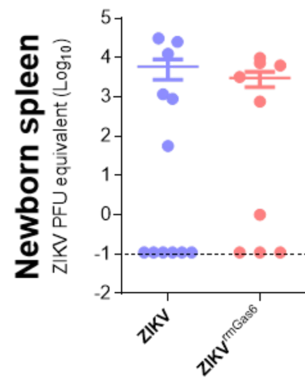
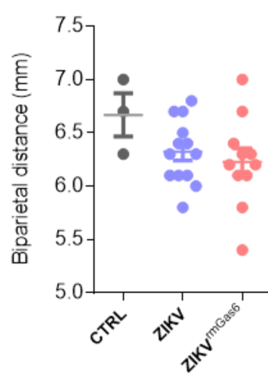
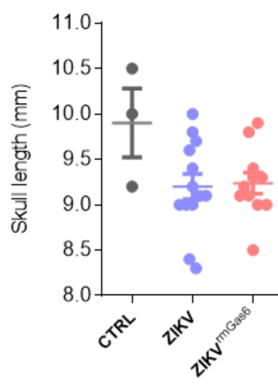
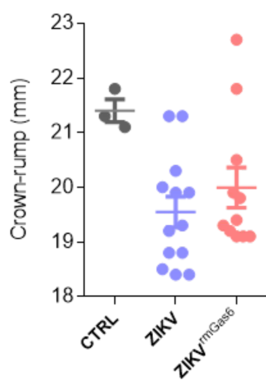
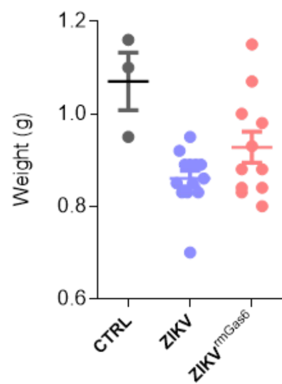
A**Below 30ng/mL****B****Above 30ng/mL**

A**B**



A**B****C****D**

A**B**

A**E18****B****C****CTRL****ZIKV****ZIKV^{rmGas6}****D****E**