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Research

Virology

Zika virus (ZIKV) is a teratogenic mosquito-borne flavivirus which can be sexually transmitted from man to woman. High viral loads and prolonged viral shedding in semen suggest that ZIKV replicates within the human male genital tract, but its target organs are unknown. Using ex vivo infection of organotypic cultures, we demonstrated here that ZIKV replicates in human testicular tissue and infects a broad range of cell types, including germ cells, which we also identified as infected in the semen from ZIKV-infected donors. ZIKV had no major deleterious effect on the morphology and hormonal production of the human testis explants. Infection induced a broad antiviral response but no interferon up-regulation and minimal pro-inflammatory response in testis explants, with no cytopathic effect. Finally, we studied ZIKV infection in mouse testis, and compared it to human infection. This study provides key insights into how ZIKV may persist in semen and alter semen parameters, as well as a valuable tool for testing antiviral agents.

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Zika virus infects human testicular tissue and germ cells

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ABSTRACT

Zika virus (ZIKV) is a teratogenic mosquito-borne flavivirus which can be sexually transmitted from man to woman. High viral loads and prolonged viral shedding in semen suggest that ZIKV replicates within the human male genital tract, but its target organs are unknown. Using ex vivo infection of organotypic cultures, we demonstrated here that ZIKV replicates in human testicular tissue and infects a broad range of cell types, including germ cells, which we also identified as infected in the semen from ZIKV-infected donors. ZIKV had no major deleterious effect on the morphology and hormonal production of the human testis explants. Infection induced a broad antiviral response but no interferon up-regulation and minimal pro-inflammatory response in testis explants, with no cytopathic effect. Finally, we studied ZIKV infection in mouse testis, and compared it to human infection. This study provides key insights into how ZIKV may persist in semen and alter semen parameters, as well as a valuable tool for testing antiviral agents.

Keywords: Zika virus; sexual transmission; semen; human testis; organotypic culture; tropism; germ cells; macrophages; Sertoli cells; peritubular cells; Leydig cells; innate immune response; interferons; inflammation; mouse testis.

58 INTRODUCTION

59 Zika virus (ZIKV) is a teratogenic arthropod-borne flavivirus, which recently emerged in the Pacific
60 (2007), Oceania (2013) and the Americas (2015).

61 While ZIKV's primary mode of transmission is through mosquito bites, male-to-female
62 sexual transmission has been reported by cohort studies (1, 2), and by case reports in non-endemic
63 countries (3). Importantly, male-to-female sexual transmission in animal models was found to
64 enhance viral dissemination in the female genital tract and transmission to the fetus (4–8). In
65 humans, high viral loads and prolonged shedding of viral RNA (vRNA) and infectious virus (up to
66 1 year and 69 days, respectively) in the absence of viremia have been found in semen (9–11),
67 strongly suggesting a tropism of ZIKV for the male genital tract. Studies in immunodeficient mice
68 evidenced high levels of ZIKV infection within the testis, leading to orchitis and impaired
69 testosterone and sperm production (12–15). However, these mouse models do not reflect the
70 pathophysiology in humans: unlike humans, mice only become infected following abrogation of
71 type I interferon (IFN) signaling and die of infection in most cases. This defective antiviral response
72 may enhance the susceptibility and pathogenicity of ZIKV. In sharp contrast, ZIKV infection in
73 macaque models either spared the testis or led to a moderate infection, with no deleterious effects
74 observed (16–18). Interestingly, a recent study on 15 ZIKV-infected men reported a lower total
75 sperm count at day 30 post symptoms onset compared with day 7, suggesting an effect of the
76 infection on the testis or epididymis (19).

77 Here, by infecting with ZIKV testicular tissue explants from healthy donors, we show that
78 ZIKV replicates and produces infectious viral particles in human testis. We evidence infection of a
79 broad range of testicular cell types, including resident macrophages and the germ cell line, and
80 confirm the latter in patients' semen. Infection had no effect on basal testosterone and inhibin B
81 production or overall cell viability ex vivo. ZIKV triggered a wide range of antiviral genes in
82 human testes, but up-regulation of types I, II and III IFN was not observed and pro-inflammatory

83 response was minimal. Finally, our data on IFNAR^{-/-} mice points at similarities and differences
84 between mouse and human testis to ZIKV infection.

85

86 RESULTS

87 ZIKV replicates in human testicular tissue

88 Testis explants from 8 uninfected donors were exposed to ZIKV ex vivo and maintained in culture
 89 medium as previously described (20). We first assessed the replication rate of a ZIKV strain derived
 90 from the 2015 outbreak in the Americas, by measuring viral release over 3-day-culture periods at
 91 day 3, day 6 and day 9 post-infection (p.i.). A significant increase in the levels of vRNA release rate
 92 was observed between days 3-6 (median 5.85×10^7 copies/ml) and 6-9 (median 8.28×10^7
 93 copies/ml) compared to days 0-3 p.i. (median 5.29×10^6 copies/ml) (Figure 1A), while vRNA was
 94 below the detection threshold in mock-infected testes (not shown).

95 Testis ability to produce infectious ZIKV particles was tested on reporter VeroE6 cells. A
 96 significant increase in supernatants infectivity was observed between days 0-3 (median 3×10^2
 97 TCID₅₀/ml) and days 6-9 p.i. (median 7.50×10^4 TCID₅₀/ml), demonstrating the infectivity of viral
 98 progeny (Figure 1B). The highest cumulative titer at day 9 (i.e. reflecting infectious viral production
 99 throughout culture) was 2×10^6 TCID₅₀/ml (Figure S1), with a median of 3.16×10^5 TCID₅₀/ml.
 100 Similarly, vRNA and infectious virion release rates increased during the culture of testis explants
 101 exposed to another ZIKV strain isolated during the 2013 outbreak in French Polynesia (Figure S2).

102 Altogether, these data demonstrate that ZIKV efficiently infects and replicates in the human testis
 103 ex vivo, producing infectious viral particles.

104

105 ZIKV infects somatic and germ cells in human testis explants

106 To determine ZIKV's target cells in the human testis, mock or ZIKV-infected testis explants
 107 were submitted to RNAscope in situ hybridization (ISH) using probes specific for ZIKV RNA
 108 (Figure 2A-H, controls in Figures 2A and S3), and to immunohistochemistry using an antibody
 109 against the non-structural NS1 viral protein (Figure 2I-M)Figure FigureFigure. Infected testes

110 displayed strong vRNA staining of the interstitial tissue cells and within the extracellular matrix
 111 bordering the seminiferous tubules, along with a more diffuse staining in some interstitial areas
 112 (Figure 2B-F). A weaker dotted staining was also observed inside a few seminiferous tubules (Figure
 113 2G-H), suggestive of association of the ZIKV with germ cells (Figure 2G) and Sertoli cells (Figure
 114 2H). NS1 antibody (Figure 2I-M) similarly labeled cells within the seminiferous tubule wall (Figure
 115 2I) and the interstitium (Figure 2J), demonstrating ZIKV replication in these target cells. Within the
 116 tubules, different germ cell categories including spermatogonia (identified based on their position in
 117 the seminiferous epithelium, nucleus size and distinctive morphological features) (Figure 2K) and a
 118 few Sertoli cells (identified based on distinctive nucleus shape) (Figure 2L), stained positive for
 119 NS1. Infected cells (vRNA+ or NS1+) displayed similar localization at the different time points of
 120 infection (days 3, 6 and 9) for the two ZIKV strains tested (Figure S4 and data not shown).
 121 To further identify the nature of the infected cells, we combined ISH for vRNA with fluorescent
 122 immuno-labelling for specific cell markers, and undertook quantification of infected cells in
 123 testicular tissue from 4 donors. Interstitial infected cells were primarily CD68/CD163+ testicular
 124 macrophages (median 12.7 cells/mm²), and to a lesser extent CYP11A1+ Leydig cells (median 3
 125 cells/mm²) (Figure 3A, B, G). Staining for α smooth actin (α SMA) demonstrated the infection of
 126 myoid peritubular cells bordering the seminiferous tubules (median 10 cells/mm²) (Figure 3C, G).
 127 Within the tubules, dotted fluorescent ZIKV staining close to the lumen histologically co-localized
 128 with late germ cells (Figure 3D). Such staining was also present at the base of the tubules, where
 129 co-labeled DDX4+ early germ cells were evidenced (DDX4 being a specific marker expressed by
 130 most of the germ cells) (Figure 3E). Staining was never observed when using a vRNA probe on
 131 mock-infected negative controls (Figure 3F). Infected cells in seminiferous tubules were mostly
 132 germ cells (median 11 cells/mm²), while infected Sertoli cells represented a median of 3.5
 133 cells/mm² (Figure 3G).

Collectively these data indicate that ZIKV has a tropism for germ cells and somatic cells within the human testis.

ZIKV replicates in human testicular germ cells in vitro and in vivo

We exposed freshly-isolated seminiferous tubules cells to ZIKV to investigate their ability to produce infectious viral particles which might be released into semen. In 3 independent primary cultures, vRNA increased in cells from a median of 2.82×10^3 to 2.09×10^7 copies/ μ g total RNA between 6h and 120h p.i. (Figure 4A). ZIKV RNA in culture supernatants significantly increased of about 4 log₁₀ between 6h and 120h p.i. (median values of 1.26×10^4 and 5.01×10^7 copies/ml respectively) (Figure 4B). Infectious virus titers also rose between 48h and 120h, reaching a median of 4×10^5 TCID₅₀/ml and maximum titer of 4×10^6 TCID₅₀/ml (Figure 4C). ZIKV replicated in DDX4+ germ cells, FSH receptor+ Sertoli cells and α SMA+ peritubular cells (Figure 4D). ZIKV envelope (ZIKV-E) was detected in undifferentiated spermatogonia (MAGEA4+ Stra8-) and in MAGEA4+ Stra8+ cells, corresponding to differentiated spermatogonia up to preleptotene spermatocytes stage (Figure 4D).

To further explore the germ cells' productive infection and since primary testicular germ cells cannot be cultured without somatic support, we used the seminoma-derived germ cell line T-cam2, which displays characteristics of fetal germ cells (21). In 3 independent experiments, vRNA in T-cam2 cells rose from below detection at 6h to a median of 6.31×10^5 copies/ μ g total RNA at 72h p.i., reaching up to 1×10^6 copies/ml in culture supernatants (Figure S5A, B). ZIKV-E was evidenced in T-cam2 by immunofluorescence (Figure S5C). The production of infectious viral particles was evidenced in the two cultures showing the highest viral loads, with a maximum titer of 8.2×10^3 TCID₅₀/ml (Figure S5D).

These findings were corroborated in vivo by analyzing the semen cell smear from two ZIKV-infected donors, in which we revealed the presence of ZIKV-E or NS1+ germ cells exfoliated from

158 the testis 7 days and 11 days post-symptoms onset (Figure 4E). A subset of spermatozoa also
 159 labelled for ZIKV-E (Figure S6).

160 Altogether, these data indicate that ZIKV replicates in human germ cells at different stages of
 161 differentiation and infects testicular germ cells in ZIKV-infected men.

162

163 **ZIKV infection ex vivo has no major impact on human testis morphology or hormonal** 164 **production**

165 We next assessed the impact of ZIKV on human testis morphology, viability and function during
 166 the ex vivo culture time frame. The tissue architecture and histology of infected testes were similar
 167 to that of mock-infected testes all throughout the culture period (Figure 5). In both infected and
 168 mock-infected testes we observed conserved interstitial tissue (comprising groups of Leydig cells,
 169 mast cells, and blood vessels), seminiferous basement membrane of similar thickness (increased
 170 thickness being a sign of injury), and seminiferous tubules encompassing Sertoli cells and early and
 171 late germ cells (Figure 5 A, D). Caspase-dependent apoptosis evidenced by cleaved caspase 3
 172 immunostaining was similar in infected and mock-infected testis and, as expected, primarily
 173 affected isolated germ cells (Figure 5B). The measurement of LDH release confirmed that the
 174 overall viability of the organ was not affected by the infection (Figure 5C). Testosterone
 175 concentrations were not different in infected versus mock-infected testes (Figure 5E), and the
 176 expression of genes encoding steroidogenic enzymes unmodified by ZIKV (Figure S7 A). Sertoli
 177 cells positively stained for the tight junction marker protein ZO-1 in both infected and mock-
 178 infected testis until day 9 p.i., suggesting an intact barrier (Figure 5 F). Inhibin B (a marker of
 179 Sertoli cell function) protein and mRNA levels showed no significant differences between infected
 180 and mock-infected testis up to day 9 p.i. (Figure 5G and Figure S7 B). Finally, the levels of
 181 peritubular cells (Acta2), early meiotic (PGK2) and late post-meiotic (PRM2) germ cells transcripts
 182 were unchanged by the infection (Figure S7 B).

183 Overall, although actively replicating within the testis, ZIKV did not appear to affect testis
 184 morphology, induce cell death, or trigger any drastic effect on the testis functions during the 9-day
 185 culture.

186

187 **ZIKV triggers a broad antiviral response but no IFN-up-regulation and a minimal pro-**
 188 **inflammatory response in human testicular tissue**

189 To investigate the immune response to ZIKV infection, we assessed the concentration of a panel of
 190 antiviral and pro-inflammatory cytokines (IFN β , IFN α 2, IFN λ 1, IFN λ 2/3, IFN γ , IL1 β , IL6,
 191 TNF α , IL8, IL-12p70, CXCL10, IL-10 and GM-CSF) in testis explant supernatants. Type I, II and
 192 III IFN concentrations in testis culture supernatants were unchanged by the infection at days 3 and 6
 193 p.i. in 7 independent testis cultures tested (Figure 6A and S8). Among pro-inflammatory cytokines,
 194 only CXCL10 was significantly increased (Figure 6A and S8), and its induction positively
 195 correlated with vRNA load (Figure 6B).

196 We then analysed the transcripts of 11 of these cytokines (IFN β , IFN α 1, IFN α 2, IFN α 4, IFN λ 1,
 197 IFN λ 2, IFN λ 3, IL-1 β , IL-6, TNF α , and CXCL10) by RT-qPCR on ZIKV-infected versus mock-
 198 infected testes. Type I, II and III IFN transcripts in uninfected testis tissues were below the
 199 measurement threshold irrespective of infection (data not shown), while CXCL10 was increased
 200 (median fold change (FC) 43.4 at day 9, range 7.7-227.8) in the testis from 4 out of 6 donors
 201 (Figure 6C).

202 Extending the analysis to a wider range of genes involved in pathogen sensing (RIG-I, MDA5),
 203 antiviral response (IFN ϵ , IFI27, IFIT1, IFITM1, IRF7, ISG15, Mx1, Mx2, OAS1, OAS2, RSAD2),
 204 inflammation (CCR7, CD14, CD64, HLA-DR, MCSF), chemoattraction (CCL2, CCL5, CXCL1,
 205 CXCL2), and control of inflammation (IL-10, TGF β , CD163, SOCS1, SOCS3), we observed the
 206 induction of a broad range of antiviral genes from day 3 onwards in the testis from 3 out of 6 donors
 207 and at day 9 in one other donor (Figure 6C).

208 A strong induction of ISG15 (FC 12.2, range 5.2-45.6), IFIT-1 (FC 12.8, range 9.7-29.6), OAS1
 209 (FC 22.9, range 6.4-32.5), OAS2 (FC 7.1, range 4.2-21.6), Mx1 (FC 9.8, range 5.1-35.1), Mx2 (FC
 210 9.5, range 3.7-25.3) and RSAD2 (FC 31.5, range 3.7-66.8) was measured at day 9 in these 4 donors,
 211 along with a relatively more moderate induction of IFI27 (FC 3.1, range 2.2-10.6), IFITM1 (FC 1.8,
 212 range 1.5-6.2), IRF7 (FC 2.7, range 1.8-6), MDA5 (FC 1.9, range 1.6-8.5) and RIG-1 (FC 2.8,
 213 range 1.8 to 9.4) (Figure 6C).

214 The fold change of these genes at day 9 correlated one to another, except for IFITM1 and MDA5
 215 (Figure S9). The fold increase at day 9 of a number of genes involved in the antiviral response
 216 (RSAD2, IFIT1, ISG15, OAS1, OAS2, Mx1, Mx2, IFI27, IRF7), pathogen sensing (RIG1), and
 217 CXCL10 positively correlated with the level of infection (shown in Figure 6B) in the corresponding
 218 testis supernatant at days 3 and 6 (Figure 6E and Figure S9).

219 Finally, we assessed expression levels of all transcripts, including type I, II and III IFN, at earlier
 220 time points (4h, 18h, 48h p.i.) in two testis explants, and did not observe any up-regulation (Figure
 221 S10 and data not shown).

222 Altogether, these results are consistent with ZIKV infection inducing a broad antiviral and minimal
 223 pro-inflammatory response in the absence of detectable IFN stimulation in human testis explants.

224

225 **Innate immune response to ZIKV infection in the testis from IFNAR^{-/-} mouse**

226 To support our hypothesis of a type I IFN independent antiviral response induced by ZIKV in testis
 227 and compare our findings on ZIKV tropism and initial antiviral/pro-inflammatory responses in
 228 human testis explants versus that in a widely-used animal model, we analysed the testis of type I
 229 IFN receptor-defective (IFNAR^{-/-}) [mice using similar techniques and viral strain](#).

230 ZIKV RNA measurement in testes from IFNAR^{-/-} mice infected for 5 and 9 days showed high viral
 231 loads in this organ (Figure 7A). Despite differences in intensity and sequence of infection, ZIKV
 232 tropism in the mouse testis in vivo was comparable overall to that in human testis ex vivo. At day 5,

233 testicular infection localized primarily within the interstitial tissue and the peritubular cells, while
 234 seminiferous tubules were spared (Figure 7B). Co-labeling of ZIKV RNA with cell markers showed
 235 infection of STAR+Leydig cells and F4/80+ macrophages (Figure 7C). At day 9 p.i. (a time at
 236 which some mice started to die), strong labeling for vRNA became prominent within Sertoli and
 237 germ cells (Figure 7B). We did not observe modifications of testicular morphology at these early
 238 time points, in agreement with previous studies (12, 22).

239 We next examined the induction of genes involved in antiviral response and inflammation in mouse
 240 testis (Figure 7D). Similar to human testis explants, and despite a lack of type I IFN signaling, a
 241 strong induction of ISG15 (FC 11.7, range 7.7-15.6 at day 5 and FC 7.4, range 4.7-15.1 at day 9),
 242 RSAD2 (FC 24.6, range 13.7-45.6 at day 5, and FC 10.1, range 2.21-30.39 at day 9), IFIT1 (FC
 243 36.0, range 19.5-42.6 at day 5, and FC 10.1, range 6.2-27.3 at day 9) and CXCL10 (FC 17.5, range
 244 9.4-39.2 at day 5, and FC 9.0, range 4.2-14.6 at day 9) was measured in infected mice testis (Figure
 245 7D). In contrast to human testis, Mx1, MDA5 and RIG-1 were not induced at either day 5 or day 9
 246 (Figure 7D). These results suggest that ZIKV induces a type I IFN signaling-independent antiviral
 247 response in both humans and mice. In contrast to human testis, in which CXCL10 was the only pro-
 248 inflammatory gene increased by the infection, TNF α (median fold change 48.0, range 32.6-122.6 at
 249 day 5, and median 6.9, range 2.6-12.3 fold at day 9), IL-1 β (median 7.7, range 1.8-13.8 fold at day
 250 5 only) and IL-6 (median 10.7, range 4.9-27.5 fold at day 5 only) were up-regulated in infected
 251 mouse testis (Figure 7D), while IFN γ (produced by NK and T cells) was maximally increased at day
 252 9 (median 11.2, range 6.2-30.8 fold) over day 5 (median 6.2, range 4.0-12.7 fold). IFN β was the
 253 most dramatically-stimulated innate immune gene at day 5 (median 236.7, range 130.2-353.4 fold),
 254 while IFN α 1, 2, 4 genes were modestly and transiently up-regulated at day 5 and IFN λ 2/3 mRNAs
 255 levels were unchanged (Figure 7D). When looking at markers of immune cell subtypes, a transient
 256 increase in transcripts encoding the myeloid cell marker CD14 was observed at day 5, whereas
 257 transcripts encoding CD3 (T cell marker) and CD8 (cytotoxic T cell marker) were maximally up-

258 regulated at day 9, in line with the IFN γ expression pattern. The markers for B cells (CD19 and
259 CD20), regulatory T cells (FoxP3) and macrophages (CD68) were unchanged, while CD4
260 (expressed by T helper and myeloid cells) was down-regulated (Figure 7D). The infiltration of T
261 lymphocytes in infected mouse testis was confirmed by CD3 immunostaining and quantification of
262 positive cells (Figure S11), further demonstrating mouse testis inflammation. Overall, the induction
263 of an antiviral response in human and IFNAR^{-/-} mice testis supports the existence of a type I IFN
264 signaling independent response to ZIKV infection in testis.

265

266 DISCUSSION

267 We show that Asian ZIKV replicates in the human testis ex vivo. . Infected somatic cells
268 within testis explants were mostly macrophages and peritubular cells, while a lower number of
269 Leydig cells and Sertoli cells were observed. Considering the relative proportion of these different
270 cell types in human testicular tissue (approximately one macrophage for 10 Leydig cells, 36
271 peritubular cells, 40 Sertoli cells and 400 germ cells) (23, 24), macrophages are likely the cell type
272 most susceptible to ZIKV infection within the testis. Importantly, we demonstrate that ZIKV
273 replicates within testicular germ cells, from stem cell (spermatogonia) to spermatozoa precursors
274 (spermatids). In agreement, Robinson et al. lately reported on the infection of male germ cells after
275 3 days exposure of human seminiferous tubules to African ZIKV (25). Our detection of infected
276 germ cells in semen from ZIVK-infected men confirms these in vitro and ex vivo findings.. The
277 presence of ZIKV in ejaculated spermatozoa adds to previous findings of ZIKV antigen, RNA and
278 infectious particles in spermatozoa (19) (26). Spermatozoa and immature germ cells may be
279 infected during epididymal transit (duration 1 to 21 days) or within the testis. To infect these cells,
280 the virus has to cross the blood testis barrier formed by the Sertoli cell tight junctions. Direct
281 infection of the Sertoli cells is supported by our results in primary cells and that of other authors in
282 commercial Sertoli cells (27, 28). Sertoli cells were showed to release ZIKV particles on their
283 adluminal side, whereas the tight and adherens junction protein expression was not altered by
284 infection (27). In agreement with these data, the ZO-1 labelling we observed in human explants
285 suggested intact Sertoli cell barrier despite infection. In contrast, Sertoli cell exposure to
286 inflammatory mediators produced by ZIKV-infected blood-derived macrophages (which phenotype
287 differs from that of the anti-inflammatory testicular macrophages) altered their barrier function (27).
288 Thus, the alteration of the blood-testis-barrier by testis-infiltrating macrophages may provide an
289 additional way for the virus to reach seminal lumen and late germ cells. Altogether, our data show

290 that ZIKV replicates in germ cells and suggest that the virus might be able to bypass the blood testis
291 barrier by infecting Sertoli cells.

292 In our ex vivo model, testosterone and inhibin B release were not modified by infection, nor
293 were their related gene expression. This is not a limitation of our culture system since it is
294 successfully used to assess the hormonal production of human testis (29). This lack of effect might
295 be linked to low infection levels of Leydig and Sertoli cells ex vivo, as supported by relatively low
296 number of infected cells. In ZIKV-infected mouse testis, testosterone and inhibin B levels were
297 preserved at day 7 p.i. while testis integrity was still maintained, whereas they decreased after
298 orchitis (12, 13), suggesting that inflammation rather than testis infection caused altered hormone
299 secretion. In a 4-month follow-up of a cohort of 15 ZIKV-infected men, testosterone levels were not
300 significantly affected, whereas slightly lower inhibin B levels were reported at day 7 post-symptoms
301 onset compared with later time points (19). Such transient imbalance of reproductive hormones can
302 be related to fever or other systemic effects (30). However, we did not study the effect of ZIKV on
303 LH- and FSH-stimulated hormone secretion, and a systemic impact on testicular hormones in vivo
304 cannot be ruled out. Moreover, we cannot exclude effects at the single cell level that cannot be
305 detected when analyzing the whole tissue.

306 Our findings suggest that ZIKV could affect sperm production. Beside germ cell infection,
307 somatic cell infection might disrupt the paracrine control of spermatogenesis (31, 32), and the
308 infection of the contractile peritubular cell (33) might decrease the expulsion of spermatozoa from
309 tubules into the epididymis, . Infected men showed a decrease in sperm count and an increase in
310 spermatozoa abnormalities during the 2 months post clinical onset (19, 34). Our results suggest that
311 direct infection of germ and/or testicular somatic cells might be involved in such altered sperm
312 parameters, although fever and/or immune response could be involved (35, 36).

313 The infection had no significant effects on testis explants morphology nor viability. This is
 314 in contrast to findings in mouse models where damaging effects of ZIKV infection on testis became
 315 evident after leukocytes' infiltration (12–14). Since the ex vivo testis model lacks the presence of an
 316 intact immune system, we cannot rule out an effect of acquired immunity on the testis from ZIKV-
 317 infected men. However, testicular atrophy or orchitis have never been reported in clinical cases or
 318 non-human primate studies, and immune infiltrations were not observed in the latter (16–18),
 319 suggesting the absence of massive inflammation. The lack of cell death induction in our ex vivo
 320 model could be linked to culture conditions (e.g. viral strains/doses used, duration of infection) or to
 321 the limited number of infected cells. However, it might also reflect the ability of the virus to
 322 replicate in testicular cells in a non-cytopathic manner. Non or minimal cytopathic infection and
 323 persistence of ZIKV has been reported in different cell types, including human placental
 324 macrophages (37), brain microvascular endothelial cells (38), and lately, male mouse germ cells
 325 (25). Absence of cytopathic effect was also reported in Sertoli cells infected with ZIKV (27, 39). In
 326 contrast, high cytotoxicity was reported in ZIKV-infected human testis organoids (40). However, in
 327 this organoid system, the architecture of the testis is not preserved and physiological cellular
 328 interactions lost. Altogether, we hypothesize that non-lytic infection, in combination with evasion
 329 from immune responses, may allow viral persistence in the human testis.

330 . A broad range of antiviral genes was induced by ZIKV in testis explants. Several of these
 331 classically-defined Interferon-Stimulated Genes (ISGs), such as ISG15 (41), RSAD2 (42), IFITM1
 332 (43), OAS1 (44), Mx1 (45) and IFIT gene family members (46) have an inhibiting activity on
 333 flaviviruses and/or ZIKV replication. Most interestingly, ZIKV did not increase type I, II or III
 334 IFNs secretion by testicular explants, and their transcripts consistently remained below detection at
 335 all time points. A level of IFN production below the sensitivity of our assays, or an active inhibition
 336 of IFN production by ZIKV, could explain these results. Thus, ZIKV non-structural proteins inhibit
 337 different steps of type I IFN induction cascade (47, 48). Alternatively, the absence of IFN up-

338 regulation in testis explants might reflect a specificity of this immune-suppressed organ, in which
 339 sustained high concentrations of type I IFN trigger germ cell apoptosis and sterility (49). Detection
 340 of ISGs over-expression in the testis from ZIKV-infected IFNAR^{-/-} mice further suggest IFN-
 341 independent induction of ISGs in the testis. The increased level of RIG-1 mRNA in infected testis
 342 suggests it may be involved in the direct induction of ISGs, although other effectors could be in
 343 play (50). However, the broad induction of ISGs may fail to control ZIKV replication in the testis in
 344 the absence of increased type I IFN secretion to amplify and stabilize the antiviral response. In
 345 contrast to antiviral genes, ZIKV infection of the human testis did not affect any of the classic pro-
 346 inflammatory cytokines, except for CXCL10 which secretion was modestly increased. Interestingly,
 347 the level of antiviral transcripts at day 9 p.i. positively correlated with the level of infection at days
 348 3 and 6, suggesting that the initial level of infection influenced the intensity of the antiviral
 349 response. Accordingly, antiviral genes were not induced in explants showing lower levels of
 350 infection. Thus host factors other than those we have studied may play a role in susceptibility to
 351 ZIKV. Altogether, our results indicate that ZIKV induces a broad induction of antiviral effectors but
 352 no IFN up-regulation and minimal pro-inflammatory response in ex vivo infected human testis. We
 353 hypothesize that such innate immune response, along with a lack of cytopathic effect, might
 354 facilitate the persistence of ZIKV for extended periods in the testis, and contribute to the prolonged
 355 release of ZIKV in semen.

356 Animal models are crucial for mechanistic studies and the in vivo testing of antiviral
 357 strategies. Cross-validation with human data is essential to assess their similarities and differences.
 358 Discrepant results on the interstitial (13, 22, 51) and/or intratubular localization of ZIKV (15, 17,
 359 67, 68, 69) were reported in mouse testis from IFN-signaling deficient mice. Our results in the
 360 IFNAR^{-/-} mouse model reconciled these results since ZIKV infection was exclusively located in
 361 interstitial cells and peritubular cells at day 5 p.i., whereas by day 9 the infection had progressed to
 362 the seminiferous tubules where it became prominent. In human testis, seminiferous tubule cell

infection was consistently weaker than that in the mouse and that in human interstitial cells.. This was not modified when increasing the infective viral dose 10 times (not shown). The difference in seminiferous tubule infection level in mice versus humans might reflect differences in the susceptibility of mouse-versus-human Sertoli and germ cells to ZIKV infection and/or in their innate immune response.

Like in human testis, we evidenced several ISG mRNA induction in the testis from IFNAR^{-/-} mice, indicating a type I IFN-signaling independent induction that corroborates our results in human testis explants. Interestingly, in contrast to the human testis, the pathogen sensor RIG-1 was not up-regulated in the mouse testis, which may suggest different sensing mechanism. Unlike human testis also, type I IFNs and a number of pro-inflammatory genes were upregulated in mice. We previously showed that unlike their rodent counterparts, which produced large amounts of IFN, primary human Leydig cells did not produce IFN in response to paramyxovirus infection or double strand RNA stimulation (54, 55). This key difference between mouse and human testis in terms of IFN production may explain why in IFN-signaling competent mice, ZIKV tropism has been reported as essentially restricted to germ cells (25), whereas a broad tropism for both somatic and germ cells is observed in human testis explants and in IFNAR^{-/-} mice. Indeed, we previously showed in rodents that meiotic and post-meiotic male germ cells lack the functional type I IFN receptor (49), and do not express ISGs after viral or IFN stimulation, unlike testicular somatic cells (56, 57). Differences in antiviral (e.g. sensing pathways and ISG induction patterns) and pro-inflammatory immune responses in human versus mouse testis may explain the testis pathogenicity observed in type I IFN-signaling deficient mouse models (along with differences in infection levels). Differences in type I IFN up-regulation following ZIKV infection may also explain the restricted tropism of ZIKV in the testis from immunocompetent mice (25) when compared to human testis explants. Whether these differences derive from intrinsic differences between human and mouse testicular cells, differential escape mechanisms mediated by ZIKV (e.g. specific counteracting of type I IFN by ZIKV in human

388 cells but not in immunocompetent mouse cells), or ex vivo/in vivo differences (e.g. infiltrating cells
389 pro-inflammatory activity) requires further investigation.

390 To date, ZIKV is the only arbovirus known to be sexually transmitted within the human
391 population. RNA from other arboviruses such as dengue, yellow fever and chikungunya viruses
392 were recently evidenced in semen from infected men for a prolonged period (58–60), but no cases
393 of sexual transmission have been documented so far. Dengue virus did not productively infect/alter
394 testicular cells in mouse models (12, 13, 25, 51) and poorly infected human Sertoli cells. Although
395 the neuro-tropic West Nile virus (WNV) replicated to levels similar to ZIKV in a Sertoli cell line
396 (27), testis from men with neuro-invasive WNV tested negative, except for one immune-suppressed
397 patient (61). Interestingly, Japanese encephalitis virus, another mosquito-borne neuro-tropic
398 flavivirus, infects the testis of boars and their semen for a long period of time, disrupts
399 spermatogenesis and can be transmitted through semen (62).

400 Of note, other male genital organs may be involved in ZIKV shedding in human semen, as
401 suggested by ZIKV prolonged sexual transmission from vasectomized men (63), and by ZIKV
402 replication in human prostate cell lines and cell lines based organoids (64). Interestingly, we
403 recently demonstrated in SIV-infected cynomolgus macaques that depending on the individuals,
404 different male genital organs may be the source of the virus in semen (65). Nevertheless, the
405 significant reduction of ZIKV titers and shorter infectivity window in semen from vasectomized
406 mice indicates the importance of testis/epididymis contributions to infectious virus shedding (4).

407 In conclusion, we demonstrated that ZIKV replicates in the human testis ex vivo and infects
408 a range of somatic cells and germ cells. Replication of ZIKV in testicular germ cells was evidenced
409 in semen from ZIKV-infected men, along with ZIKV association with spermatozoa. ZIKV had no
410 major deleterious effect on the morphology and hormonal production of the human testis in culture.
411 Despite a broad induction of antiviral genes, the absence of IFN up-regulation and minimal pro-

412 inflammatory response of the human testis ex vivo, along with the lack of ZIKV cytopathic effect
413 on testicular cells, might favour the prolonged ZIKV infection observed in this organ, and account
414 for the absence of orchitis in men infected by ZIKV. Overall, our results suggest that ZIKV
415 infection of the human testis may be involved in the persistence of the virus in semen and in altered
416 semen parameters. These results call for further investigation on the impact of ZIKV on the
417 reproductive health of ZIKV-infected men and warn against the potential horizontal and vertical
418 transmission of ZIKV through the infected germ line. Finally, the ex vivo model of ZIKV infection
419 of the human testis we developed provides a valuable tool for the testing of antiviral agents.

420

421

422 MATERIAL AND METHODS

423

424 Cells lines and viruses

425 Asian Zika virus strains isolated during the 2015 outbreak in the French Caribbean
426 (MRS_OPY_Martinique_PaRi-2015, passaged once in Vero cells) and during the 2013 outbreak in
427 Polynesia (H/PF/2013, passaged three times in Vero cells) were obtained from the European Virus
428 Archive (EVA) and further propagated in VeroE6 cells for 2 additional passages. VeroE6 cells
429 (African green monkey kidney epithelial cells) were maintained in DMEM supplemented with 10%
430 FCS, Glutamine (2mM) and 1% penicillin/streptomycin at 37°C with 5% CO₂ (all reagents from
431 GIBCO). To produce viral stocks, VeroE6 cells were infected at an MOI of 0.01 in serum-free
432 medium for 2 hours, then complete medium was added to reach a final serum concentration of 5%.
433 When cytopathic effect was evident, supernatant was centrifuged, filtered (0,45 µm), aliquoted and
434 frozen at -80°C. The human testicular germ cell tumor (seminoma) cell line Tcam-2 (66) was kindly
435 provided by Dr Janet Shipley (The Institute of Cancer Research, London).

436

437 Organotypic culture of human testis explants and infection

438 Testes were dissected into 3 mm³ sections transferred onto 24 well plates (2 sections/well)
439 containing 500µl of medium (DMEM/F12 supplemented with 1X nonessential amino acids, 1X
440 ITS, 100U/ml penicillin, 100µg/ml streptomycin, 10% FCS, all from GIBCO) in the presence or
441 absence of 10⁵ TCID₅₀ of ZIKV (corresponding to 2.2.10⁷ to 2.9.10⁷ vRNA copies for
442 MRS_OPY_Martinique_PaRi-2015 and 8.10⁷ vRNA copies for H/PF/2013). After overnight
443 incubation, tissue fragments were washed 3 times with PBS and transferred onto a polyethylene
444 terephthalate insert (3µm high density pores) in 12 well plates containing 1 ml of medium. 8 hours
445 later, the medium was changed again to further wash away potential residual virus input (time 0 for
446 sample collection). For each experimental condition, a minimum of two wells were tested. The

447 culture was maintained up to 9 day p.i. in a humidified atmosphere containing 5% CO₂ at 37°C with
 448 medium collected and fully changed every 3 days, in order to thoroughly wash input virus and
 449 assess viral production dynamic. . Media were stored frozen at -80°C for vRNA and titer
 450 measurement. Tissue fragments were either fixed in neutral buffered 4% formaldehyde or frozen
 451 and stored at -80°C.

452

453 **Isolation and infection of testicular cells**

454 Testis fragments were incubated in digesting medium (2mg/ml hyaluronidase, 2mg/ml collagenase
 455 I, 20µg/ml in DMEM/F12) for 60 minutes at 37°C under agitation (110 rpm) to dissociate
 456 interstitial tissue from seminiferous tubules. After centrifugation, the seminiferous tubule pellet
 457 digested by trypsin (0.25%, 5ml/g, 20 minutes at 37°C). Trypsin was inactivated and cells filtered
 458 (60µm) and cultured overnight in DMEM/F12 medium supplemented with 1X nonessential amino
 459 acids, 1X ITS (human insulin, human transferrin, and sodium selenite), 100U/ml penicillin,
 460 100µg/ml streptomycin and 10% FCS (all from GIBCO). Primary TGCs and Tcam-2 cells were
 461 incubated with ZIKV diluted in serum-free medium at a multiplicity of infection (MOI) of 1
 462 (corresponding to 1.43×10^6 TCID₅₀/million cells) for 2 hours at 37°C 5% CO₂. Virus was
 463 removed by washing and trypsin treatment for 5 minutes at 37°C. Primary testicular cells were
 464 cultured at a density of 0.5 million cells/ml in supplemented StemPro-34 (Invitrogen) as described
 465 elsewhere (67). T-cam2 cells were cultured at a density of 0.1 million cells/ml in RPMI1640
 466 supplemented with P/S, Glutamine (2mM) and 10% FCS (all reagents from GIBCO).

467 **Semen samples**

468 Semen was liquefied at 37°C for 30 minutes and 10µl spread on a glass slide and dried at room
 469 temperature. Smears were fixed in 4% formaldehyde and stored at -20° C. Viral loads for ZIKV in
 470 seminal plasma and seminal cells were 7,25 log copies/ml and 6,7 log copies/µg total RNA
 471 respectively for the donor at day 7, and 7,8 log copies/ml and 7,8 log copies/2x10⁶ cells respectively

472 for the donor at day 11. Patients' serology for dengue was negative and semen samples tested
 473 negative for dengue in RT-PCR.

474

475 **Mice**

476 Mice lacking the type 1 interferon receptor (68) were backcrossed >10 times onto the C57BL/6
 477 background (referred to as IFNAR^{-/-} mice). 7-week-old male IFNAR^{-/-} mice were infected through
 478 the intra-peritoneal route with 10⁴ TCID₅₀/100 µl of ZIKV (H/PF/2013) or with PBS. 5 and 9 days
 479 after infection, mice were sacrificed with carbon dioxide and collected tissue either frozen at -80°C
 480 or fixed in PFA 4%.

481

482 **Real-time quantitative RT-PCR**

483 Total RNA was extracted using the QIAamp vRNA (for supernatants) or RNeasy isolation kit (for
 484 tissue/cells) and treated with DNase (all from Qiagen). Extracted RNA from explant supernatants
 485 was subjected to RT-qPCR using GoTaq Probe 1-step RT-qPCR System (Promega). Primers and
 486 probes for ZIKV described in (69) were adapted as follows: ZIKV primer forward
 487 ccgctgccaacacaag, ZIKV primer reverse ccactaacgttctttgcagacat, ZIKV probe
 488 agcctaccttgacaagcaatcagacactcaa. A standard curve with serial dilution of a known number of
 489 copies of vRNA was systematically run. The relative quantification of steroidogenesis enzymes
 490 mRNA (STAR, steroidogenic acute regulatory protein; CYP11A1, cytochrome P450 family 11
 491 subfamily A member 1; HSD3B2, hydroxy-delta-5-steroid dehydrogenase 3 beta- and steroid delta-
 492 isomerase 2; HSD17B3, hydroxysteroid 17-beta dehydrogenase 3; CYP17A1, cytochrome P450
 493 family 17 subfamily A member 1) and testicular cells markers mRNA (Inhibin B; Acta2, actin alpha
 494 2 smooth muscle aorta; PGK2, phosphoglycerate kinase 2; PRM2, protamine 2) was performed as
 495 previously described (29).

496 Primers for innate immune response effector genes (Supplementary Table 1) were designed using
 497 Primer-BLAST tool (70). Total RNA was reverse-transcribed using the Iscript cDNA Synthesis Kit

498 (Bio-Rad). QPCR reactions were performed on a Bio-Rad Laboratories CFX384 instrument using
 499 iTaq SYBR green mix (BioRad) and 40 cycles of 15 seconds at 95°C and 1 minute at 60° C,
 500 followed by melt-curve analysis. Gene expression fold changes were calculated with the $2^{-\Delta\Delta C_t}$
 501 method normalized to beta-actin and mock-infected samples expression levels.

502

503 **Determination of viral titer**

504 Vero E6 cells seeded in opaque-walled 96 well plates at a final concentration of 1.5×10^5 cells/ml in
 505 DMEM with 5% FCS were put in contact the next day with serial dilutions of supernatant . Median
 506 tissue culture infective dose (TCID₅₀)/ml was measured at day 5 post-infection using the Viral
 507 ToxGlo Assay (Promega).

508

509 **Histology, RNAscope in situ hybridization (ISH) and immunohistochemistry (IHC)**

510 Tissues or cell pellets were fixed in 4% formaldehyde and embedded in paraffin. RNA ISH was
 511 performed using RNAscope 2.5 (Advanced Cell Diagnostics) according to the manufacturer's
 512 instructions, as previously described (15, 87). RNAscope ISH is a highly specific and sensitive
 513 technique, with the ability to detect single molecules (73). Formaldehyde fixed paraffin-embedded
 514 tissue sections or cell pellets were deparaffinized in xylene and deshydrated in ethanol. with for 10
 515 min at room temperature. Slides deparaffinized and H₂O₂ quenched for endogenous peroxidases
 516 were boiled in RNAscope Target Retrieval Reagents (citrate buffer 10mM, pH6, 15 minutes) and
 517 incubated in RNAscope Protease Plus (40°C, 20 minutes), prior to probe hybridization. Sections
 518 were incubated with target probes (2 hours, 40°C), washed in buffer and incubated with
 519 amplification reagents. Chromogenic detection was performed using Fast Red as substrate for
 520 alkaline phosphatase to generate red signal. Slides were counterstained with hematoxylin and
 521 mounted in Eukitt (O. KINDLER) before observation using bright-field microscopy. The “double
 522 Z” probes targeting ZIKV RNA (consensus sequence, target region 219-5443, catalog #467771),
 523 positive (targeting the 2514-3433 region of human *POLR2A* gene, catalog #310451) and negative

(targeting the 414-862 region of bacterial *dapB* gene, catalog #310043) control probes were all obtained from Advanced Cell Diagnostics. Staining specificity was verified as showed in Figure S3. FigureSections of ZIKV-infected Vero cell pellets and mock-infected testis tissues were systematically used as positive and negative controls.

Dual fluorescent ISH-IHC experiments was performed essentially as we previously described (74). Briefly, tissue sections were first submitted to ISH, then blocked in PBS/BSA 2% and incubated overnight at 4°C with primary antibody, Sections were washed, incubated with either anti-mouse or anti-rabbit Alexa-488 fluorescent secondary antibodies diluted 1/500 (chicken anti-mouse 488 ref A21200, chicken anti-rabbit 488 ref A21441, Life Technologies), and counterstained with Prolong medium containing DAPI before observation with a Zeiss Axio Imager M1 fluorescence microscope connected to a digital camera (Carl Zeiss) using Zen software. Fluorescent Fast Red signal was read at 550 nm.

Single immunohistochemistry was performed as described (75). For immunofluorescence experiments, Alexa 488 or 594-coupled secondary antibodies diluted 1/500 (goat anti-mouse 594 ref A11032, chicken anti-rabbit 594 ref A21442, donkey anti-rat 488 ref A21208, all from Life Technologies) were used and sections mounted with Prolong DAPI to stain the nuclei. Cell staining was never observed for isotypic controls or mock-infected samples. Primary antibodies used : mouse anti-NS1 (Biofront Technologies, clone 0102136, 4µg/ml), anti-CD68 (DAKO, clone KP1, 1,85µg/ml), anti-CD163 (LEICA Novocastra, clone 10D6, 1/100), anti- α SMA (DAKO, clone 1A4, 1,4µg/ml), anti-DDX4 (GENETEX, clone 2F9H5, 1/200), anti-ZO-1 (THERMO FISHER, clone ZO1-1A12, 10µg/ml); rabbit anti-Cyp11A1 (Sigma, 1/250), anti-cleaved caspase 3 (Cell Signaling, Asp175, 1/50), anti-DDX4 (Abcam, 2µg/ml), anti-STAR (Cell signalling, 1/200), anti-CD3 (Dako, #A0452, 10 µg/ml); rat anti-mouse F4/80 (Abcam, clone BM8, 1/20).

The number of ZIKV RNA+ CD68/CD163+ macrophages, CYP11A+ Leydig cells, α SMA+ peritubular cells, germ cells and Sertoli cells (identified on morphological criteria in light

549 microscopy) in testis explants was assessed in 4 donors and at least 3 whole testis tissue
550 sections/donors (corresponding to about 12 mm²/ testis donor). Quantification of CD3+ cells in
551 mouse testis was performed in 3 mock-infected animals, 4 animals at day 5 p.i. and 4 animals at day
552 9 p.i., in at least 5 mm²/testis. Slides were scanned with a NanoZoomer slide scanner (Hamamatsu
553 Photonics, France, at Plateforme H2P2, Biosit, Rennes, France). Immunostained positive cells were
554 counted with ImageJ free software.

555

556 **Immunocytofluorescence**

557 Semen smears from donors and cell pellets from testicular cell cultures put onto polylysine-coated
558 glass coverslips were fixed in 4% PAF for 20 minutes at RT. After permeabilization (0,2% Triton
559 X-100, 10 minutes), the slides were incubated in blocking buffer (0,2% Triton X-100, 1% goat
560 serum, 2 hours) and stained with antibodies against ZIKV NS1 (1:1000, Biofront Technologies) or
561 flavivirus envelope Ab 4G2 (1:1000, Millipore). NS1 antibody was either directly coupled to Alexa
562 Fluor 647 (Zenon labeling kit, Molecular Probes) or revealed using Alexa-fluor 555 goat anti-
563 mouse (Life Technologies). Infected cell characterization was performed using rabbit anti-DDX4
564 (5µg/ml, Abcam), anti-Stra8 (9,6µg/ml, ThermoFisher Scientific) and anti-FSHR (10µg/ml,
565 Origene), detected using Alexa-fluor 488 (Invitrogen) or Alexa-fluor 647 goat anti-rabbit (Jackson
566 Immunoresearch), mouse anti-MageA4 (clone 57B, 4µg/ml) coupled to Alexa Fluor 647 (Zenon
567 labeling kit, Molecular Probes), and mouse anti-αSMA (clone 1A4, 0,5µg/ml, Dako) detected using
568 Alexa-fluor 555 goat-anti mouse antibody. Isotype control antibodies or non- infected cells were
569 used as negative controls. Slides were counterstained with Prolong medium containing DAPI
570 (Molecular Probes). Images were acquired with the SP8 confocal system (Leica) connected to LAS
571 software or with DMRXA wide field microscope (Applied Precision), and analyzed using Fiji
572 software.

573

574 **Testosterone and inhibin B immunoassays**

575 Testosterone was assayed using a specific radioimmunoassay (Immunotech, Beckman Coulter).
576 Inhibin B was assayed using a commercial enzyme-linked immunosorbent assay (ELISA) kit (DSL-
577 10-84100 Active, Beckman Coulter).

578

579 **Viability assay**

580 Cell viability was assessed by measuring the release of lactate dehydrogenase (LDH) using the
581 enzymatic fluorimetric assay CytoTox-ONE™ Homogeneous Membrane Integrity Assay
582 (Promega).

583

584 **Cytokine release measurement**

585 A bead-based multiplex flow cytometry Legendplex assay (Biolegend, Ozyme) was used.
586 Fluorescence was read using the BD LSRII Fortessa flow cytometer.

587

588 **Statistics**

589 Data were analyzed with non-parametric paired Friedman-Dunn's or unpaired Kruskal-Wallis-
590 Dunn's test when more than 2 sets of samples were compared, as specified in figure legends. The
591 non-parametric Mann-Whitney test was used to analyze differences in viral load of mice testis at
592 day 5 and 9 post infection. Correlations were calculated using the Spearman test. Values were
593 considered significant when $P < 0,05$. Statistical analyses were performed using commercially-
594 available software (GraphPadPrism 6, GraphPad Software, Inc., La Jolla, California, USA).

595

596 **Study approval**

597 Normal testes were obtained either after orchidectomy from prostate cancer patients who had not
598 received any hormone therapy or at autopsy, and processed within 2 hours of surgery. The

599 procedure was approved by a local ethics committee (authorization #DC-2016-2783) and the French
600 national agency for biomedical research (authorization #PF S09-015).

601 Semen samples were obtained by masturbation from two ZIKV-infected donors living in the French
602 Caribbean at 7 and 11 days post-symptoms onset respectively, after informed consent was obtained,
603 in the French Cohort of Patients Infected by an Arbovirus (CARBO; ClinicalTrials.gov identifier
604 NCT01099852).

605 Mice were housed at the Institut Pasteur Animal Facility accredited by the French Ministry of
606 Agriculture for performing experiments on live rodents. Work on animals was performed in
607 compliance with French and European regulations on care and protection of laboratory animals (EC
608 Directive 2010/63, French Law 2013-118, February 6th, 2013). All experiments with IFNAR^{-/-}
609 mice were approved by the Ethics Committee #89 and registered under the reference #2016-0018.

610

611

AUTHOR CONTRIBUTIONS

GM performed experiments, analyzed data, co-wrote the paper and contributed to data interpretation and study design. LH, APS, DM, FA and ALT performed experiments, analyzed data and contributed to writing the paper. LH designed primers and interpreted PCR array results. JF and SB performed experiments. GA interpreted data. KB and SL contributed testis tissues. GJ, LB and AC contributed semen samples. TC and ML performed IFNAR mouse infection and tissue collection. NDR designed experiments, interpreted the data and wrote the paper.

All authors read, edited and approved the manuscript.

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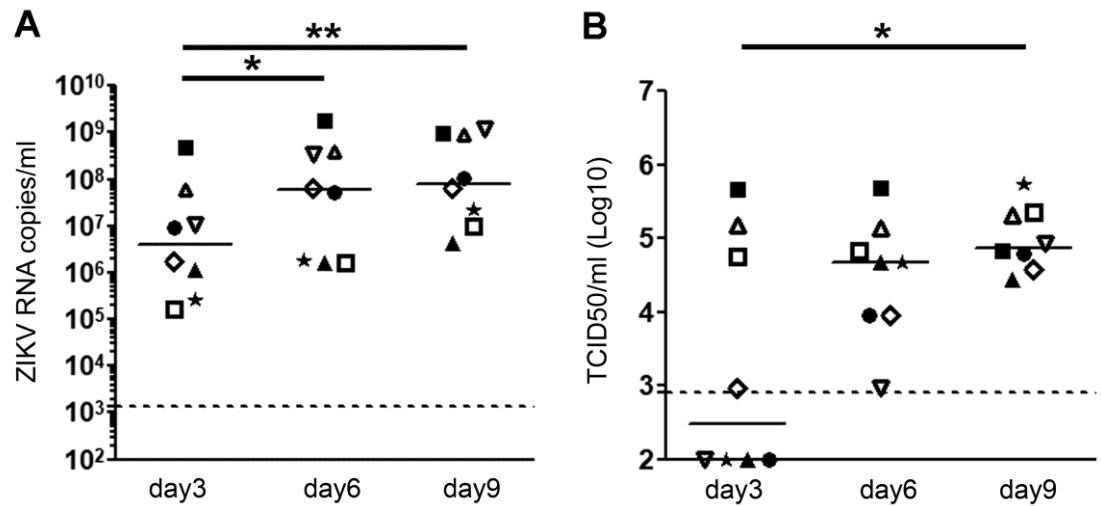
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802 **FIGURES AND FIGURE LEGENDS**

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806 **Figure 1 ZIKA virus replicates in human testicular tissue.** Human testis explants from 8 donors
 807 were ex vivo infected overnight with 10^5 TCID₅₀ (corresponding to $2.2 \cdot 10^7$ to $2.9 \cdot 10^7$ vRNA copies)
 808 from a low-passage ZIKV strain isolated in 2015 in the French Caribbean
 809 (MRS_OPY_Martinique_PaRi-2015). Explants were thoroughly washed and cultured on inserts in
 810 1 ml of medium/well for 9 days, with media fully removed and changed every 3 days. Each of the
 811 time points (day 3, day 6, day 9) represent de novo viral release over a 3-day-culture period. A)
 812 ZIKV RNA release over a 3-day-culture period at day 3, day 6 and day 9 detected by RT-qPCR; B)
 813 Viral titers determined by infectivity assay of 3-day-culture period tissue supernatants on VeroE6
 814 cells. Each symbol represents a different donor (same symbol/donor throughout the manuscript
 815 figures). Dotted lines represent the detection limit of the assays. Mock-infected explants were
 816 always below detection level. Bars represent median. * $P < 0,05$; ** $P < 0,01$ (Friedman-Dunns non-
 817 parametric comparison).

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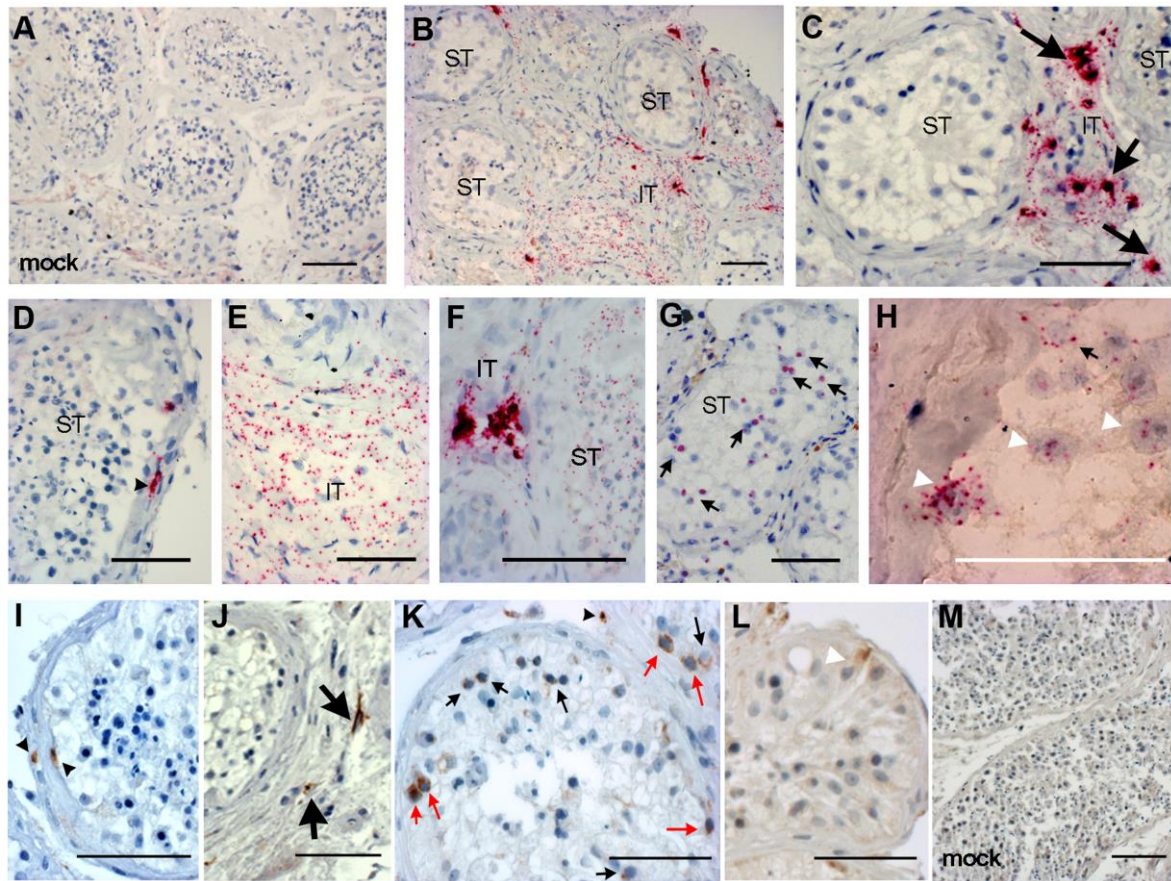
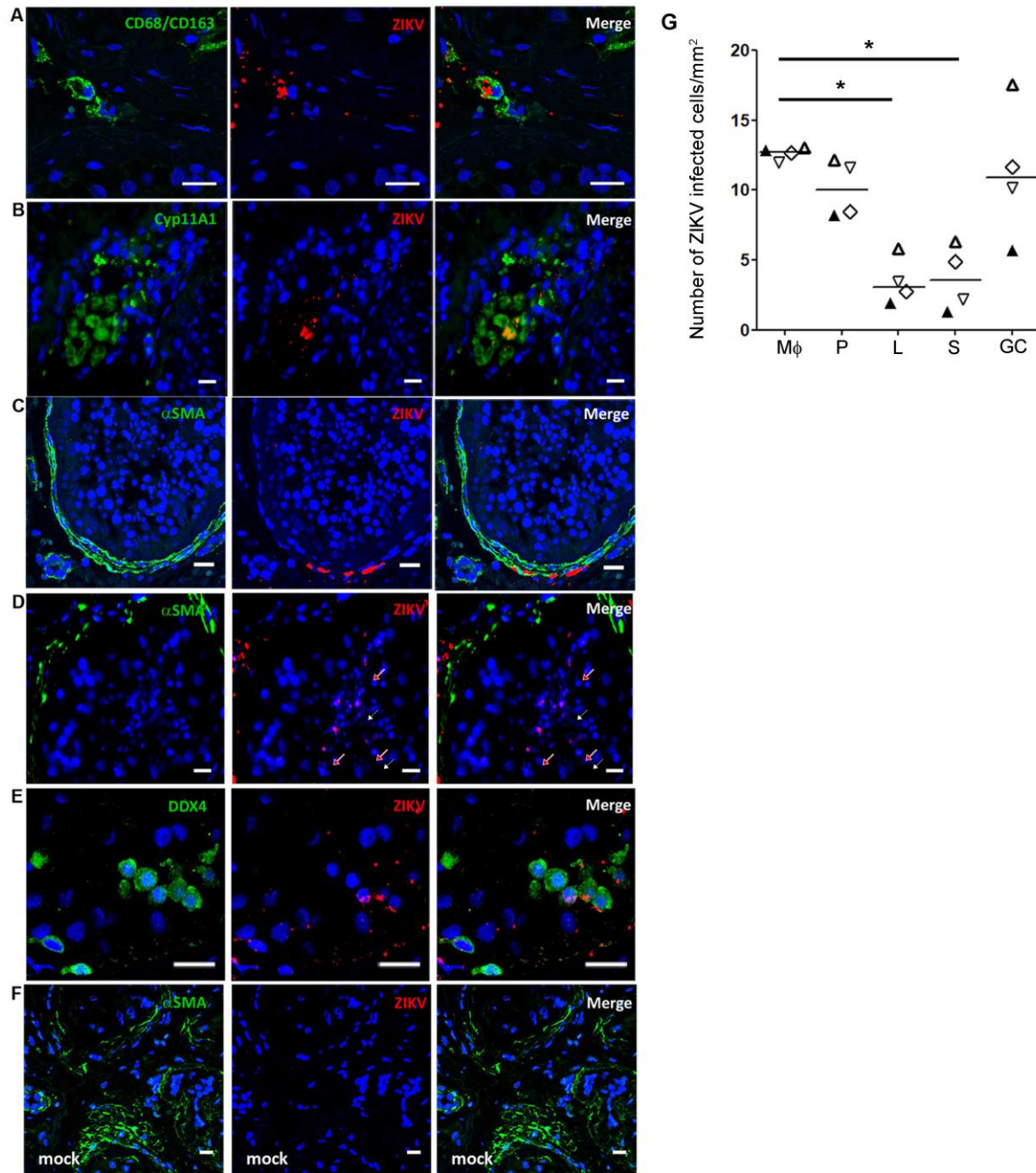


Figure 2 ZIKV infects somatic and germ cells in human testis explants.

A-H) Representative images of RNAscope in situ hybridization for ZIKV RNA in control mock-infected (A) and ZIKV-infected testis explant (n=8 independent donors) after 6 days of culture (B-H). ZIKV RNA labeling was observed in the interstitial tissue (IT) of testis explants (B, C, E, F), in cells bordering the seminiferous tubules (ST) (B, D) and within seminiferous tubules (F,G,H). I-M) Representative images of immunohistochemistry staining of NS1-ZIKV performed on ZIKV-infected (I-L) and mock-infected (M) testis explants in culture for 6 days (n=8 independent donors). Black arrow heads point at infected cells in the extracellular matrix surrounding the seminiferous tubules. Thick arrows point at infected cells in the interstitial tissue. Thin black arrows point at infected germ cells. Thin red arrows point at infected spermatogonia. White arrow heads point at Sertoli cell nucleus. Black scale bars=100 μ m; White bar=50 μ m.



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836 **Figure 3 Characterization and quantification of ZIKV-infected human testicular cells ex vivo.**
 837 RNAscope in situ hybridization for vRNA coupled with immunofluorescence for cell markers
 838 identified ZIKV RNA in CD68/CD163+ macrophages (A), Cyp11A1+ Leydig cells (B), α SMA+
 839 peritubular cells (C), late germ cells localized near the lumen in seminiferous tubules (white arrows:
 840 round spermatids; red arrows: elongated spermatids) (D) and DDX4+ early germ cells (E). Staining
 841 for ZIKV was never observed in mock-infected testis (F). Nuclei are stained in blue. Scale
 842 bars=20 μ m. (G) Infected cells were quantified in at least 3 whole tissue sections from 4 testis
 843 donors (each represented by a different symbol) at day 9 p.i. Mφ: macrophages; P: peritubular cells;
 844 L: Leydig cells; S: Sertoli cells; GC: germ cells. Bars represent median. *P<0,05 (Friedman-Dunns
 845 non parametric comparison).
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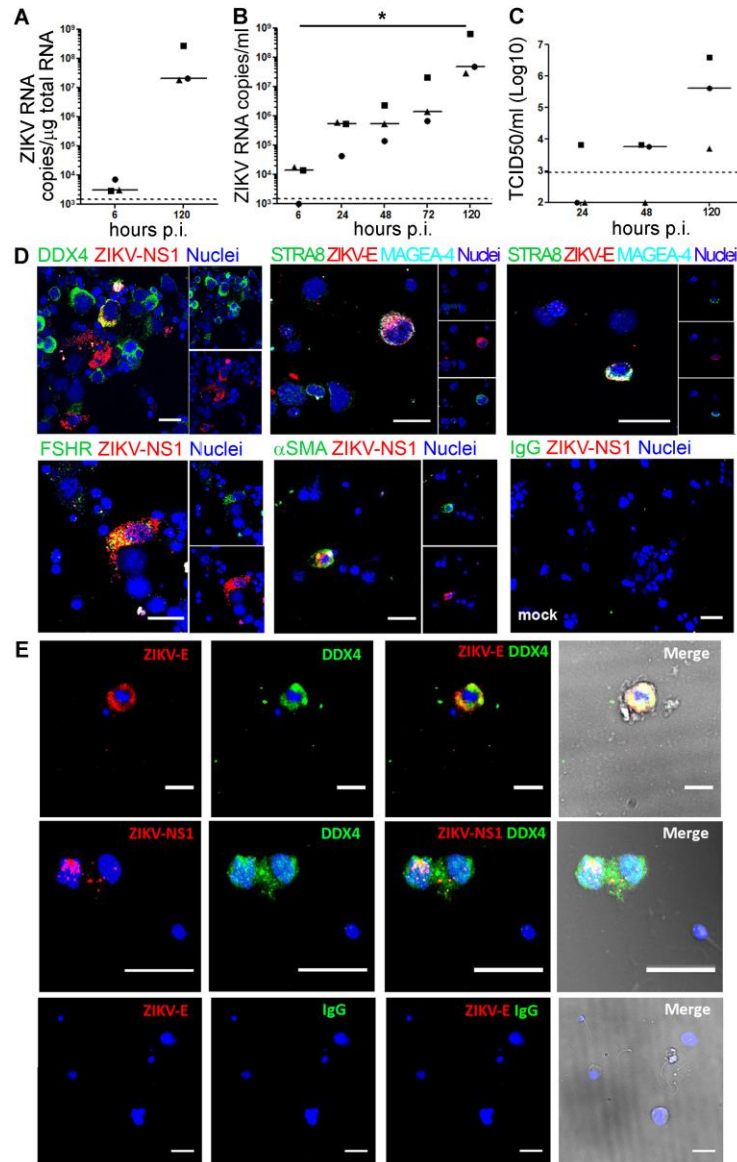


Figure 4 ZIKV replicates in human testicular germ cells in vitro and in vivo. A-C) Primary testicular cells were infected with ZIKV (MOI 1, corresponding to 7.15×10^5 TCID₅₀ units/ ml/ 0.5 million cells). ZIKV RNA detected by RT-qPCR in cells (A) and culture supernatants (B). C) Viral titers determined by infectivity assay of tissue supernatants on VeroE6 cells. Each dot represents independent donors. Bars represent median values. Dotted lines indicate detection limit. * $P < 0.05$ (Friedman-Dunns non-parametric comparison). D) Immunofluorescence against ZIKV NS1 or envelope (ZIKV-E) proteins combined with cell markers for all germ cells (DDX4) or specific germ cell types (STRA8, MAGEA4), Sertoli cells (FSH-R) and peritubular cells (α SMA). Nuclei are stained in blue. E) Detection of infected germ cells in semen from ZIKV-infected men. Immunofluorescent labelling of semen cell smears from two ZIKV-infected patients, one at day 7 (top panel) and one at day 11 (middle panel) post-symptoms onset. ZIKV Envelope (ZIKV-E) or NS1 protein co-labelled with the germ cell marker DDX4. Bottom panels show semen from a healthy individual stained with anti- ZIKV-E antibody and IgG isotype as a negative control. Nuclei are stained in blue. In the merge panels, brightfield images are included to visualize the cell's morphology. Scale bars=20 μ m.

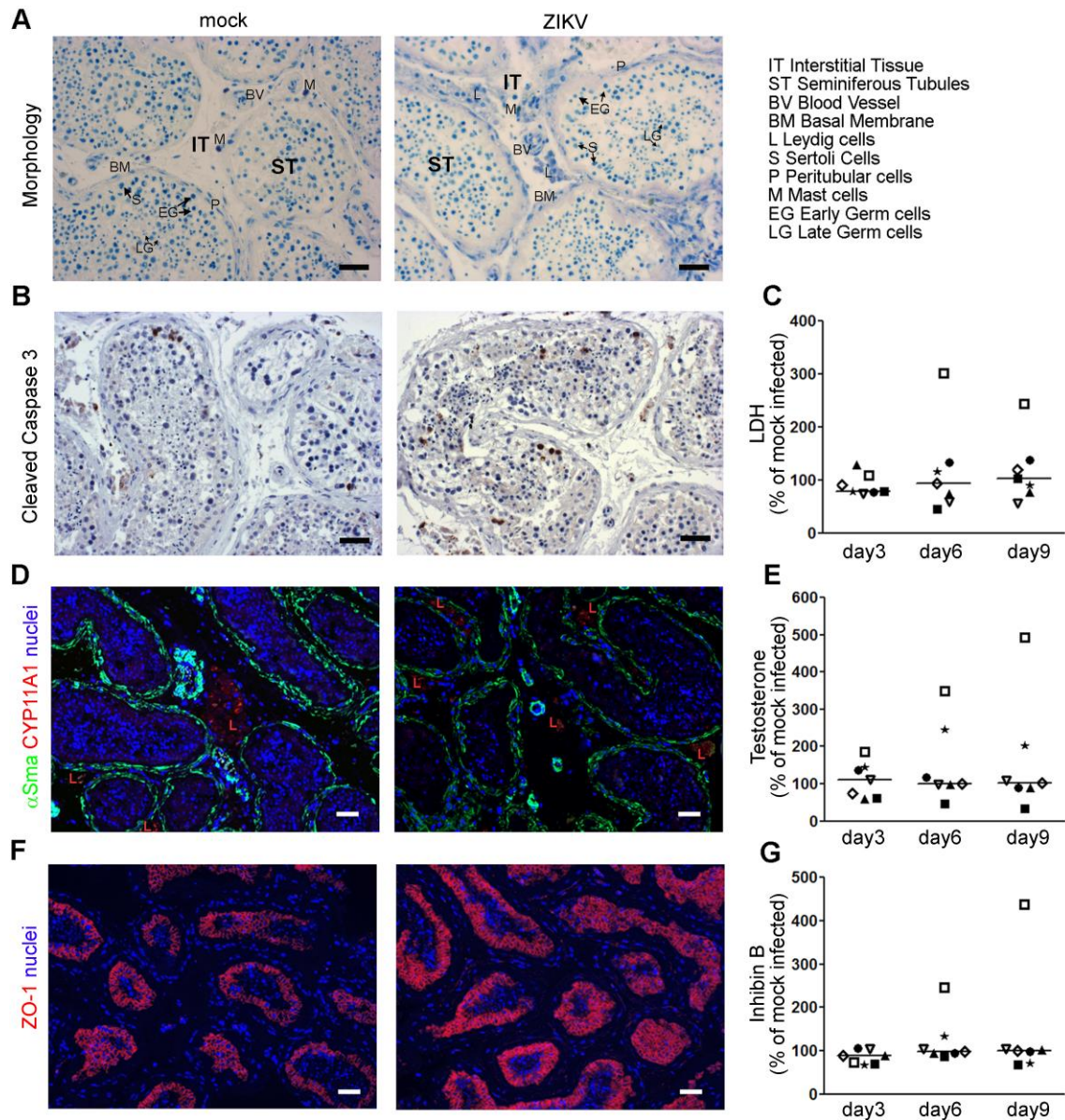


Figure 5 ZIKV infection ex vivo does not alter human testis explant morphology, cell viability or hormonal production. A) Toluidine histological staining of testis explants, shown here for mock- infected (left panel) and ZIKV-infected (right panel) testis explants at day 6 post-infection (p.i.). B) Cleaved caspase 3 IHC to detect apoptotic cells in mock (left panel) and ZIKV-infected (right panel) testis explants, shown here for day 6 p.i. C) Lactate dehydrogenase (LDH) release in testis supernatant expressed as % of mock-infected explants at the corresponding day of culture. D) Immuno-fluorescent co-labeling of peritubular (α SMA) and Leydig (Cyp11A1) cells, shown on tissue sections at day 6 p.i. for mock (left panel) and ZIKV-infected (right panel) explants. Nuclei are stained in blue. E, G) Testosterone and inhibin B release in testis supernatants expressed as % of mock-infected explants at the corresponding day of culture. F) Immuno-fluorescent labeling of Sertoli cells tight junctions associated protein ZO-1 in tissues sections for mock (left panel) and ZIKV-infected (right panel) explants, shown at day 6 p.i. Nuclei are stained in blue. Bars=50 μ m. C, E, G: each symbol represents a different donor; horizontal bars represent median values.

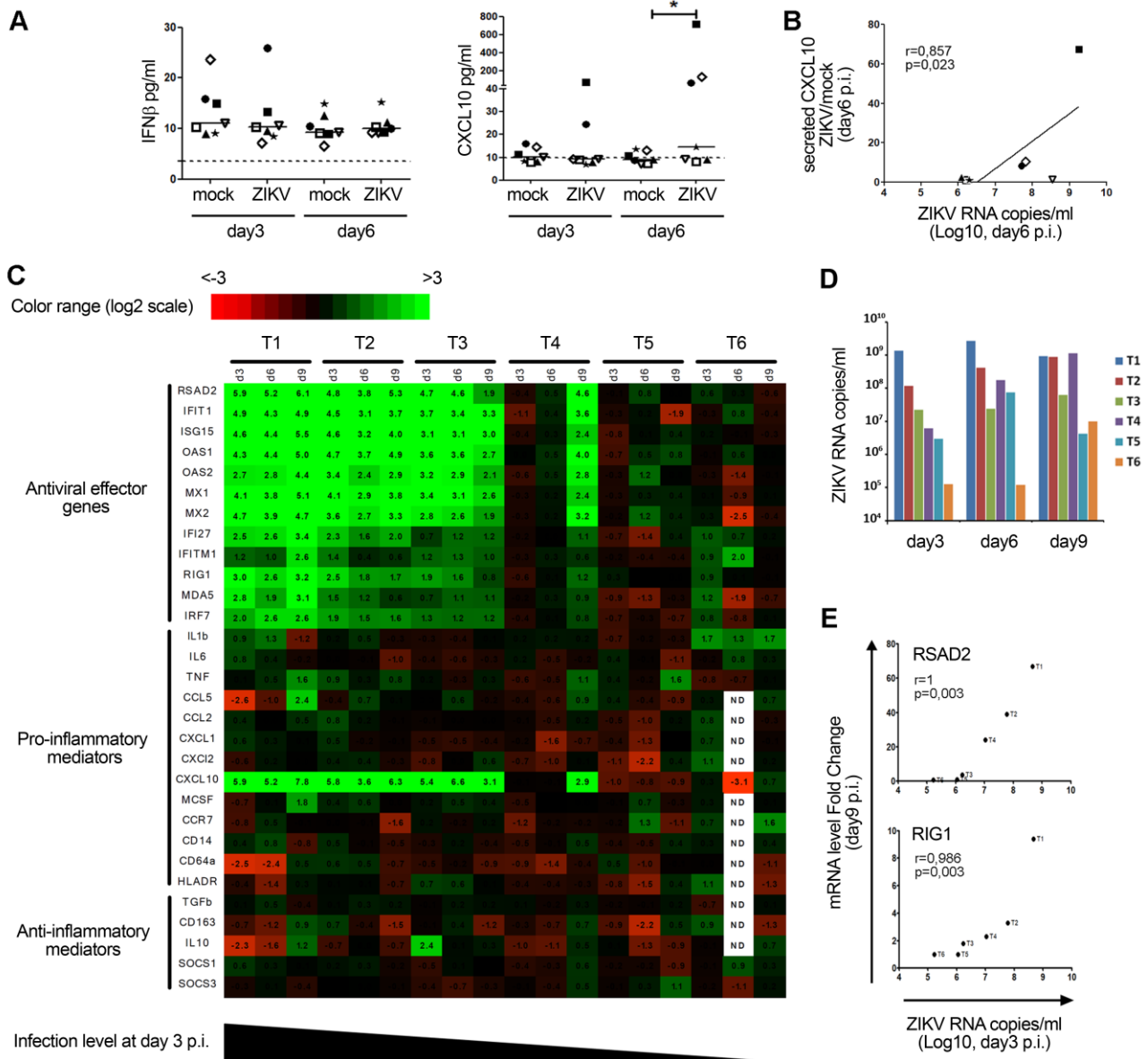


Figure 6 ZIKV triggers a broad antiviral response but no IFN-up-regulation and a minimal pro-inflammatory response in human testicular tissue. A) Levels of IFN- β and CXCL10 measured by flow-cytometer based multiplex assay in mock-infected and ZIKV-infected human testis explant supernatants. Each symbol represents a different donor. Bars represent median values. * $P < 0.05$ (Friedman-Dunns non-parametric comparison). B) Correlation between secreted CXCL10 induction in ZIKV-infected versus mock-infected explants and ZIKV RNA level in culture supernatant at day 6 post-infection (p.i.) (Spearman non-parametric test). C) Innate immune gene expression determined by RT-qPCR in the testis explants from 6 donors (T1-T6) infected with ZIKV for 3, 6 and 9 days (d3, d6, d9). Heat-map shows log2 transformed expression ratios between ZIKV-infected and time-matched mock-infected controls. Green indicates up-regulation and red down-regulation of mRNA compared to controls. Type I and II IFN mRNAs were below the quantification threshold (not shown). D) Viral loads in supernatants of the testis explants analyzed in (C). E) Examples of correlation between gene expression fold at day 9 and the level of infection at day 3 p.i. (Spearman non-parametric test). Other correlations are shown in Figure S9.

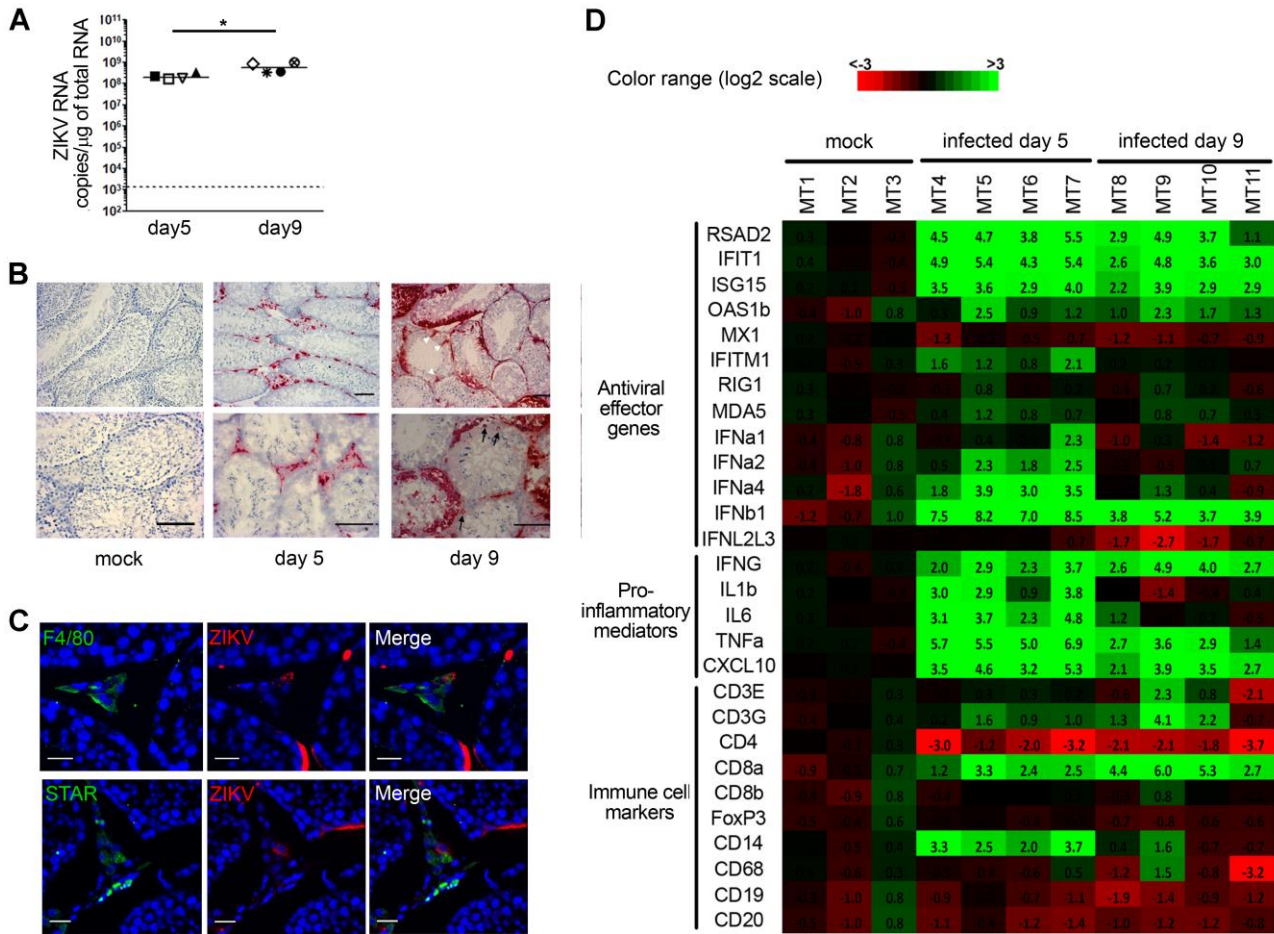


Figure 7 Innate immune response to ZIKV infection in the testis from IFNAR^{-/-} mouse.

A) vRNA measured by RT-qPCR in the testis from mice infected with ZIKV for 5 or 9 days (4 animals/group). Each dot represents one animal and horizontal bars represent the median. The dotted line indicates the limit of detection. Mock-infected (n=3) were below the detection threshold (not shown). * P<0,05 (Mann-Whitney test, non-parametric comparison). B) Detection of ZIKV RNA by RNAscope in situ hybridization in testis tissue sections from mice mock-infected or at day 5 or day 9 post-infection. White arrow heads point at Sertoli cells, thin black arrows point at germ cells. Scale bars=100μm. C) RNAscope in situ hybridization for ZIKV RNA coupled with immunofluorescence for cell markers identified ZIKV RNA in F4/80+ macrophages and STAR+ Leydig cells. Nuclei are stained in blue. Scale bars=20μm. D) Expression of a range of innate immune genes and of genes encoding immune cell markers was determined by RT-qPCR in testis from 3 mock-infected mice (mouse testis MT1 to MT3) and 4 ZIKV-infected mice at day 5 (MT5 to MT7) and day 9 (MT8 to MT11) post-infection. Fold induction is presented as a heat-map of log2 transformed expression ratios to the average expression level in mock-infected mice. On the scale bar, green indicates up-regulation and red, down-regulation.