



## **Zika virus infects human testicular tissue and germ cells**

Giulia Matusali, Laurent Houzet, Anne-Pascale Satie, Dominique Mahé,  
Florence Aubry, Therese Couderc, Julie Frouard, Salomé Bourgeau, Karim  
Bensalah, Sylvain Lavoué, et al.

### **► To cite this version:**

Giulia Matusali, Laurent Houzet, Anne-Pascale Satie, Dominique Mahé, Florence Aubry, et al.. Zika virus infects human testicular tissue and germ cells. *Journal of Clinical Investigation*, 2018, 128 (10), pp.4697-4710. 10.1172/JCI121735 . inserm-01868794

**HAL Id: inserm-01868794**

**<https://inserm.hal.science/inserm-01868794>**

Submitted on 5 Sep 2018

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Zika virus infects human testicular tissue and germ cells

Giulia Matusali, ... , Anna Le Tortorec, Nathalie Dejucq-Rainsford

*J Clin Invest.* 2018. <https://doi.org/10.1172/JCI121735>.

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.

**Find the latest version:**

<http://jci.me/121735/pdf>



# **Zika virus infects human testicular tissue and germ cells**

Giulia Matusali<sup>1</sup>, Laurent Houzet<sup>1</sup>, Anne-Pascale Satie<sup>1</sup>, Dominique Mahé<sup>1</sup>, Florence Aubry<sup>1</sup>,  
Thérèse Couderc<sup>2,3</sup>, Julie Frouard<sup>1</sup>, Salomé Bourgeau<sup>1</sup>, Karim Bensalah<sup>4</sup>, Sylvain Lavoué<sup>5</sup>,  
Guillaume Joguet<sup>6</sup>, Louis Bujan<sup>7</sup>, André Cabié<sup>8</sup>, Gleide Avelar<sup>9</sup>, Marc Lecuit<sup>2,3,10</sup>, Anna Le  
Tortorec<sup>1</sup>, Nathalie Dejucq-Rainsford<sup>1\*</sup>

<sup>1</sup> Univ Rennes, Inserm, EHESP, Irset (Institut de recherche en santé, environnement et travail) -  
UMR\_S1085, F-35000 Rennes, France

<sup>2</sup> Institut Pasteur, Biology of Infection Unit, Paris, France

<sup>3</sup> Inserm U1117, Paris, France

<sup>4</sup> Service d'Urologie, Centre Hospitalier Universitaire de Rennes, F-35000 Rennes, France

<sup>5</sup> Unité de coordination hospitalière des prélèvements d'organes et de tissus, Centre Hospitalier  
Universitaire de Rennes, F-35000 Rennes, France

<sup>6</sup> Centre Caribéen de Médecine de la Reproduction-CECOS CHU de Pointe à Pitre Guadeloupe,  
France

<sup>7</sup> Research Group on Human Fertility EA 3694, University Paul Sabatier Toulouse III-CECOS,  
Hôpital Paule de Viguier, CHU Toulouse, Toulouse, France

<sup>8</sup> Inserm Centre d'Investigation Clinique 1424, Centre Hospitalier Universitaire de Martinique,  
France; Service de maladies infectieuses, Centre Hospitalier Universitaire de Martinique, France

<sup>9</sup> Department of morphology, Federal University of Minas Gerais, Belo Horizonte, Brazil

<sup>10</sup> Paris-Descartes University, Department of Infectious Diseases and Tropical Medicine,  
Necker-Enfants Malades University Hospital, Paris, France

\*Corresponding author:

Nathalie DEJUCQ-RAINSFORD

IRSET-Inserm U1085

9 avenue du Pr Léon Bernard

F-35000 Rennes, France

E-mail : [nathalie.dejucq-rainsford@univ-rennes1.fr](mailto:nathalie.dejucq-rainsford@univ-rennes1.fr)

Phone : +33 2 2323 5069

The authors declare no conflict of interest.

Word count: 10686

## 38    **ABSTRACT**

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

50

51

52

53

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

57

## 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<sup>-/-</sup> 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  $5.85 \times 10^7$  copies/ml) and 6-9 (median  $8.28 \times 10^7$   
 93 copies/ml) compared to days 0-3 p.i. (median  $5.29 \times 10^6$  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  $3 \times 10^2$   
 97 TCID<sub>50</sub>/ml) and days 6-9 p.i. (median  $7.50 \times 10^4$  TCID<sub>50</sub>/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  $2 \times 10^6$  TCID<sub>50</sub>/ml (Figure S1), with a median of  $3.16 \times 10^5$  TCID<sub>50</sub>/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/mm<sup>2</sup>), and to a lesser extent CYP11A1+ Leydig cells (median 3  
 125 cells/mm<sup>2</sup>) (Figure 3A, B, G). Staining for  $\alpha$  smooth actin ( $\alpha$ SMA) demonstrated the infection of  
 126 myoid peritubular cells bordering the seminiferous tubules (median 10 cells/mm<sup>2</sup>) (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/mm<sup>2</sup>), while infected Sertoli cells represented a median of 3.5  
 133 cells/mm<sup>2</sup> (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  $2.82 \times 10^3$  to  $2.09 \times 10^7$  copies/ $\mu$ g total RNA between 6h and 120h p.i. (Figure 4A). ZIKV RNA in culture supernatants significantly increased of about 4 log<sub>10</sub> between 6h and 120h p.i. (median values of  $1.26 \times 10^4$  and  $5.01 \times 10^7$  copies/ml respectively) (Figure 4B). Infectious virus titers also rose between 48h and 120h, reaching a median of  $4 \times 10^5$  TCID<sub>50</sub>/ml and maximum titer of  $4 \times 10^6$  TCID<sub>50</sub>/ml (Figure 4C). ZIKV replicated in DDX4+ germ cells, FSH receptor+ Sertoli cells and  $\alpha$ 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  $6.31 \times 10^5$  copies/ $\mu$ g total RNA at 72h p.i., reaching up to  $1 \times 10^6$  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  $8.2 \times 10^3$  TCID<sub>50</sub>/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 $\beta$ , IFN $\alpha$ 2, IFN  $\lambda$ 1, IFN  $\lambda$ 2/3, IFN $\gamma$ , IL1 $\beta$ , IL6,  
 191 TNF $\alpha$ , 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 $\beta$ , IFN $\alpha$ 1, IFN $\alpha$ 2, IFN $\alpha$ 4, IFN $\lambda$ 1,  
 197 IFN $\lambda$ 2, IFN $\lambda$ 3, IL-1 $\beta$ , IL-6, TNF $\alpha$ , 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 $\epsilon$ , 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 $\beta$ , 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<sup>-/-</sup> 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<sup>-/-</sup>) [mice using similar techniques and viral strain](#).

230 ZIKV RNA measurement in testes from IFNAR<sup>-/-</sup> 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 $\alpha$  (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 $\beta$  (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 $\gamma$  (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 $\beta$  was the  
 253 most dramatically-stimulated innate immune gene at day 5 (median 236.7, range 130.2-353.4 fold),  
 254 while IFN $\alpha$ 1, 2, 4 genes were modestly and transiently up-regulated at day 5 and IFN $\lambda$ 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 $\gamma$  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<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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% CO<sub>2</sub> (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 mm<sup>3</sup> 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 10<sup>5</sup> TCID<sub>50</sub> of ZIKV (corresponding to 2.2.10<sup>7</sup> to 2.9.10<sup>7</sup> vRNA copies for  
442 MRS\_OPY\_Martinique\_PaRi-2015 and 8.10<sup>7</sup> 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% CO<sub>2</sub> 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  $1.43 \times 10^6$  TCID<sub>50</sub>/million cells) for 2 hours at 37°C 5% CO<sub>2</sub>. 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/2x10<sup>6</sup> 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<sup>-/-</sup> mice). 7-week-old male IFNAR<sup>-/-</sup> mice were infected through  
478 the intra-peritoneal route with 10<sup>4</sup> TCID<sub>50</sub>/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  $1.5 \times 10^5$  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 (TCID<sub>50</sub>)/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 H<sub>2</sub>O<sub>2</sub> 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-  $\alpha$ 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,  $\alpha$ 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 mm<sup>2</sup>/ 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 mm<sup>2</sup>/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<sup>-/-</sup>  
609 mice were approved by the Ethics Committee #89 and registered under the reference #2016-0018.

610

611

## 612 **AUTHOR CONTRIBUTIONS**

613 GM performed experiments, analyzed data, co-wrote the paper and contributed to data  
 614 interpretation and study design. LH, APS, DM, FA and ALT performed experiments, analyzed data  
 615 and contributed to writing the paper. LH designed primers and interpreted PCR array results. JF and  
 616 SB performed experiments. GA interpreted data. KB and SL contributed testis tissues. GJ, LB and  
 617 AC contributed semen samples. TC and ML performed IFNAR mouse infection and tissue  
 618 collection. NDR designed experiments, interpreted the data and wrote the paper.  
 619 All authors read, edited and approved the manuscript.

620

## 621 **ACKNOWLEDGEMENTS**

622 This project received funding from the European Union's Horizon 2020 research and innovation  
 623 program under grant agreement no. 733176, ZIKAPLAN (grant agreement no. 734584) and  
 624 ZIKALLIANCE (grant agreement no. 734548) and was funded in part by the LabEx IBEID, Institut  
 625 Pasteur and Inserm. GM received support from REACTing. GA and NDR received funding from  
 626 CAPES-COFECUB. GJ and LB received funding from Agence de la Biomédecine and Agence  
 627 Régionale de santé Guadeloupe for the collection of semen from infected men. AC received funding  
 628 from the French Ministry of Health (Soutien Exceptionnel à la Recherche et à l'Innovation) and  
 629 support from REACTing. Experiments were conducted in part on L3, MRic and H2P2 platforms at  
 630 Biosit federative structure (Univ Rennes, CNRS, Inserm, BIOSIT [(Biologie, Santé, Innovation  
 631 Technologique de Rennes)] - UMS 3480, US\_S 018). This publication was supported by the  
 632 European Virus Archive goes Global (EVAg) project that has received funding from the European  
 633 Union's Horizon 2020 research and innovation program under grant agreement N°653316. We  
 634 thank Xavier Montagutelli for his help with animal experiment and Laurianne Lesné and Christèle  
 635 Desdoits for their assistance in hormonal assays.

636

637

## REFERENCES

1. Coelho FC et al. Higher incidence of Zika in adult women than adult men in Rio de Janeiro suggests a significant contribution of sexual transmission from men to women. *Int. J. Infect. Dis.* 2016;51:128–132.
2. Yakob L, Kucharski A, Hue S, Edmunds WJ. Low risk of a sexually-transmitted Zika virus outbreak. *Lancet Infect. Dis.* 2016;16(10):1100–1102.
3. Moreira J, Peixoto TM, Siqueira AM, Lamas CC. Sexually acquired Zika virus: a systematic review. *Clin. Microbiol. Infect.* 2017;23(5):296–305.
4. Duggal NK et al. Frequent Zika Virus Sexual Transmission and Prolonged Viral RNA Shedding in an Immunodeficient Mouse Model. *Cell Rep.* 2017;18(7):1751–1760.
5. Haddow AD et al. High Infection Rates for Adult Macaques after Intravaginal or Intrarectal Inoculation with Zika Virus. *Emerg. Infect. Dis.* 2017;23(8):1274–1281.
6. Carroll T et al. Zika virus preferentially replicates in the female reproductive tract after vaginal inoculation of rhesus macaques. *PLOS Pathog.* 2017;13(7):e1006537.
7. Tang WW et al. A Mouse Model of Zika Virus Sexual Transmission and Vaginal Viral Replication. *Cell Rep.* 2016;17(12):3091–3098.
8. Duggal NK, McDonald EM, Ritter JM, Brault AC. Sexual transmission of Zika virus enhances in utero transmission in a mouse model. *Sci. Rep.* 2018;8(1):4510.
9. Epelboin S et al. Zika virus and reproduction: facts, questions and current management. *Hum. Reprod. Update* 2017;23(6):629–645.
10. Barzon L et al. Virus and Antibody Dynamics in Travelers With Acute Zika Virus Infection. *Clin. Infect. Dis.* 2018;66(8):1173–1180.
11. Mead PS et al. Zika Virus Shedding in Semen of Symptomatic Infected Men. *N. Engl. J. Med.* 2018;378(15):1377–1385.
12. Govero J et al. Zika virus infection damages the testes in mice. *Nature* 2016;540(7633):438–442.
13. Ma W et al. Zika Virus Causes Testis Damage and Leads to Male Infertility in Mice. *Cell* 2016;167(6):1511–1524 e10.
14. Griffin BD et al. DNA vaccination protects mice against Zika virus-induced damage to the testes. *Nat. Commun.* 2017;8:15743.
15. Shan C et al. A single-dose live-attenuated vaccine prevents Zika virus pregnancy transmission and testis damage. *Nat. Commun.* 2017;8(1):676.
16. Osuna CE et al. Zika viral dynamics and shedding in rhesus and cynomolgus macaques. *Nat. Med.* 2016;22(12):1448–1455.
17. Hirsch AJ et al. Zika Virus infection of rhesus macaques leads to viral persistence in multiple tissues. *PLOS Pathog.* 2017;13(3):e1006219.
18. Koide F et al. Development of a Zika Virus Infection Model in Cynomolgus Macaques. *Front. Microbiol.* 2016;7:2028.
19. Joguet G et al. Effect of acute Zika virus infection on sperm and virus clearance in body fluids: a prospective observational study. *Lancet Infect. Dis.* 2017;17(11):1200–1208.
20. Roulet V et al. Human testis in organotypic culture: application for basic or clinical research. *Hum Reprod* 2006;21(6):1564–1575.
21. Young JC et al. TCam-2 seminoma cell line exhibits characteristic foetal germ cell responses to TGF-beta ligands and retinoic acid. *Int. J. Androl.* 2011;34(4pt2):e204–e217.
22. Uraki R et al. Zika virus causes testicular atrophy. *Sci. Adv.* 2017;3(2):e1602899.
23. Xia Y, Zhu WJ, Hao SF, Liang WB, Li J. Stereological analysis of age-related changes of testicular peritubular cells in men. *Arch. Gerontol. Geriatr.* 2012;55(1):116–119.
24. Nieschlag E, Behre HM (eds). *Andrology*. Verlag Berlin Heidelberg: Springer; 2010.
25. Robinson CL et al. Male germ cells support long-term propagation of Zika virus. *Nat. Commun.* 2018;9(1):2090.

- 688 26. Mansuy JM et al. Zika virus in semen and spermatozoa. *Lancet Infect. Dis.* 2016;16(10):1106–  
689 1107.
- 690 27. Siemann DN, Strange DP, Maharaj PN, Shi P-Y, Verma S. Zika Virus Infects Human Sertoli  
691 Cells and Modulates the Integrity of the *In Vitro* Blood-Testis Barrier Model. *J. Virol.*  
692 2017;91(22):e00623-17.
- 693 28. Kumar A et al. Human Sertoli cells support high levels of Zika virus replication and persistence.  
694 *Sci. Rep.* 2018;8(1):5477.
- 695 29. Kristensen DM et al. Ibuprofen alters human testicular physiology to produce a state of  
696 compensated hypogonadism. *Proc. Natl. Acad. Sci.* 2018;115(4):E715–E724.
- 697 30. Adamopoulos DA, Lawrence DM, Vassilopoulos P, Contoyiannis PA, Swyer GL. Pituitary-  
698 testicular interrelationships in mumps orchitis and other viral infections. *Br-Med-J*  
699 1978;1(6121):1177–1447.
- 700 31. Potter SJ, DeFalco T. Role of the testis interstitial compartment in spermatogonial stem cell  
701 function. *Reproduction* 2017;153(4):R151–R162.
- 702 32. Cheng CY, Wong EW, Yan HH, Mruk DD. Regulation of spermatogenesis in the  
703 microenvironment of the seminiferous epithelium: new insights and advances. *Mol Cell Endocrinol*  
704 2010;315(1–2):49–56.
- 705 33. Mayerhofer A. Human testicular peritubular cells: more than meets the eye. *Reproduction*  
706 2013;145(5):R107–R116.
- 707 34. Huits R et al. Zika virus in semen: a prospective cohort study of symptomatic travellers  
708 returning to Belgium. *Bull. World Health Organ.* 2017;95(12):802–809.
- 709 35. Sergerie M, Mieusset R, Croute F, Daudin M, Bujan L. High risk of temporary alteration of  
710 semen parameters after recent acute febrile illness. *Fertil. Steril.* 2007;88(4):970.e1-7.
- 711 36. Evenson DP, Jost LK, Corzett M, Balhorn R. Characteristics of human sperm chromatin  
712 structure following an episode of influenza and high fever: a case study. *J. Androl.* 2000;21(5):739–  
713 46.
- 714 37. Quicke KM et al. Zika Virus Infects Human Placental Macrophages. *Cell Host Microbe*  
715 2016;20(1):83–90.
- 716 38. Mladinich MC, Schwedes J, Mackow ER. Zika Virus Persistently Infects and Is Basolaterally  
717 Released from Primary Human Brain Microvascular Endothelial Cells. *MBio* 2017;8(4):e00952-17.
- 718 39. Strange DP, Green R, Siemann DN, Gale M, Verma S. Immunoprofiles of human Sertoli cells  
719 infected with Zika virus reveals unique insights into host-pathogen crosstalk. *Sci. Rep.*  
720 2018;8(1):8702.
- 721 40. Strange DP et al. Human testicular organoid system as a novel tool to study Zika virus  
722 pathogenesis. *Emerg. Microbes Infect.* 2018;7(1):82.
- 723 41. Singh PK et al. Zika virus infects cells lining the blood-retinal barrier and causes chorioretinal  
724 atrophy in mouse eyes. *JCI insight* 2017;2(4):e92340.
- 725 42. Van der Hoek KH et al. Viperin is an important host restriction factor in control of Zika virus  
726 infection. *Sci. Rep.* 2017;7(1):4475.
- 727 43. Savidis G et al. The IFITMs Inhibit Zika Virus Replication. *Cell Rep.* 2016;15(11):2323–2330.
- 728 44. Perelygin AA et al. Positional cloning of the murine flavivirus resistance gene. *Proc. Natl.*  
729 *Acad. Sci.* 2002;99(14):9322–9327.
- 730 45. Chen J et al. Outcomes of Congenital Zika Disease Depend on Timing of Infection and  
731 Maternal-Fetal Interferon Action. *Cell Rep.* 2017;21(6):1588–1599.
- 732 46. Schoggins JW et al. A diverse range of gene products are effectors of the type I interferon  
733 antiviral response. *Nature* 2011;472(7344):481–485.
- 734 47. Ngono AE, Shresta S. Immune Response to Dengue and Zika. *Annu. Rev. Immunol.*  
735 2018;36(1):annurev-immunol-042617-053142.
- 736 48. Xia H et al. An evolutionary NS1 mutation enhances Zika virus evasion of host interferon  
737 induction. *Nat. Commun.* 2018;9(1):414.
- 738 49. Satie AP et al. Excess type I interferon signaling in the mouse seminiferous tubules leads to

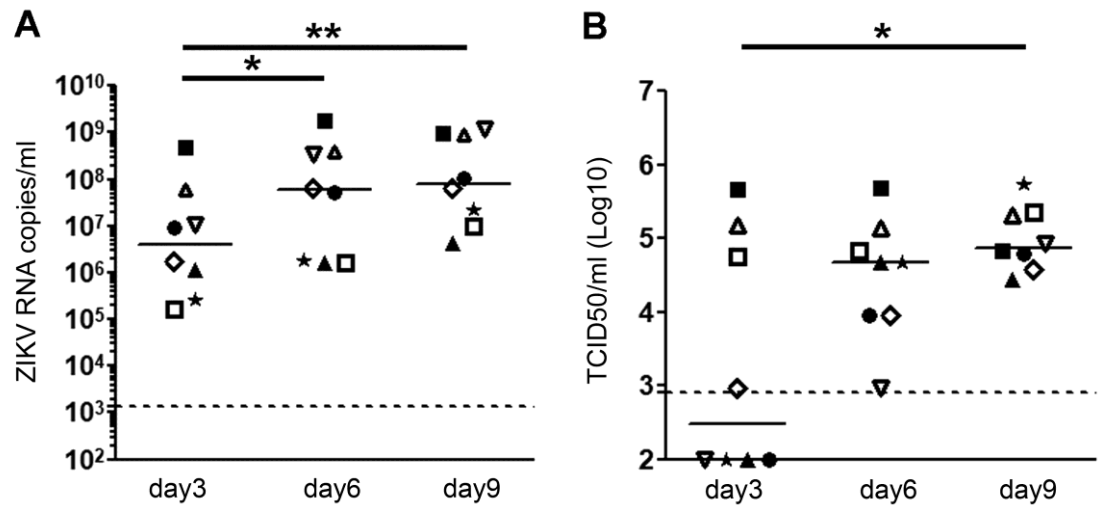
- germ cell loss and sterility. *J Biol Chem* 2011;286(26):23280–23295.
50. Hasan M et al. Trex1 regulates lysosomal biogenesis and interferon-independent activation of antiviral genes. *Nat. Immunol.* 2012;14(1):61–71.
51. Sheng Z-Y et al. Sertoli Cells Are Susceptible to ZIKV Infection in Mouse Testis. *Front. Cell. Infect. Microbiol.* 2017;7:272.
52. Chan JF-W et al. Zika Virus Infection in Dexamethasone-immunosuppressed Mice Demonstrating Disseminated Infection with Multi-organ Involvement Including Orchitis Effectively Treated by Recombinant Type I Interferons. *EBioMedicine* 2016;14:112–122.
53. Winkler CW et al. Adaptive Immune Responses to Zika Virus Are Important for Controlling Virus Infection and Preventing Infection in Brain and Testes. *J. Immunol.* 2017;198(9):3526–3535.
54. Dejuqc N, Lienard MO, Guillaume E, Dorval I, Jegou B. Expression of interferons-alpha and -gamma in testicular interstitial tissue and spermatogonia of the rat. *Endocrinology* 1998;139(7):3081–3087.
55. Le Tortorec A et al. Antiviral responses of human Leydig cells to mumps virus infection or poly I:C stimulation. *Hum Reprod* 2008;23(9):2095–2103.
56. Dejuqc N, Lienard MO, Jegou B. Interferons and interferon-induced antiviral proteins in the testis. *J Reprod Immunol* 1998;41(1–2):291–300.
57. Dejuqc N, Chousterman S, Jegou B. The testicular antiviral defense system: localization, expression, and regulation of 2'5' oligoadenylate synthetase, double-stranded RNA-activated protein kinase, and Mx proteins in the rat seminiferous tubule. *J Cell Biol* 1997;139(4):865–873.
58. Lalle E et al. Prolonged detection of dengue virus RNA in the semen of a man returning from Thailand to Italy, January 2018. *Eurosurveillance* 2018;23(18). doi:10.2807/1560-7917.ES.2018.23.18.18-00197
59. Barbosa CM et al. Yellow Fever Virus DNA in Urine and Semen of Convalescent Patient, Brazil. *Emerg. Infect. Dis.* 2018;24(1):176–178.
60. Bandeira AC et al. Prolonged shedding of Chikungunya virus in semen and urine: A new perspective for diagnosis and implications for transmission. *IDCases* 2016;6:100–103.
61. Armah HB et al. Systemic distribution of West Nile virus infection: postmortem immunohistochemical study of six cases. *Brain Pathol.* 2007;17(4):354–62.
62. Guérin B, Pozzi N. Viruses in boar semen: detection and clinical as well as epidemiological consequences regarding disease transmission by artificial insemination. *Theriogenology* 2005;63(2):556–72.
63. Stassen L, Armitage C, van der Heide D, Beagley K, Frentiu F. Zika Virus in the Male Reproductive Tract. *Viruses* 2018;10(4):198.
64. Spencer JL et al. Replication of Zika Virus in Human Prostate Cells: A Potential Source of Sexually Transmitted Virus. *J. Infect. Dis.* 2018;217(4):538–547.
65. Houzet L et al. Seminal SIV in chronically-infected cynomolgus macaques is dominated by virus originating from multiple genital organs. *J. Virol.* 2018;JVI.00133-18.
66. Mizuno Y, Gotoh A, Kamidono S, Kitazawa S. [Establishment and characterization of a new human testicular germ cell tumor cell line (TCam-2)]. *Nihon Hinyokika Gakkai Zasshi.* 1993;84(7):1211–8.
67. Sadri-Ardekani H et al. Propagation of Human Spermatogonial Stem Cells In Vitro. *JAMA* 2009;302(19):2127.
68. Müller U et al. Functional role of type I and type II interferons in antiviral defense. *Science* 1994;264(5167):1918–21.
69. Lanciotti RS et al. Genetic and Serologic Properties of Zika Virus Associated with an Epidemic, Yap State, Micronesia, 2007. *Emerg. Infect. Dis.* 2008;14(8):1232–1239.
70. Ye J et al. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 2012;13(1):134.
71. Wang F et al. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J. Mol. Diagnostics* 2012;14(1):22–29.

- 790 72. Deleage C et al. Defining HIV and SIV Reservoirs in Lymphoid Tissues. *Pathog. Immun.*  
791 2016;1(1):68–106.
- 792 73. Deleage C, Chan CN, Busman-Sahay K, Estes JD. Next-generation in situ hybridization  
793 approaches to define and quantify HIV and SIV reservoirs in tissue microenvironments.  
794 *Retrovirology* 2018;15(1):4.
- 795 74. Matusali G et al. Detection of Simian Immunodeficiency Virus in Semen, Urethra, and Male  
796 Reproductive Organs during Efficient Highly Active Antiretroviral Therapy. *J Virol*  
797 2015;89(11):5772–5787.
- 798 75. Roulet V et al. Susceptibility of human testis to human immunodeficiency virus-1 infection in  
799 situ and in vitro. *Am J Pathol* 2006;169(6):2094–2103.
- 800

801

802 **FIGURES AND FIGURE LEGENDS**

803



804

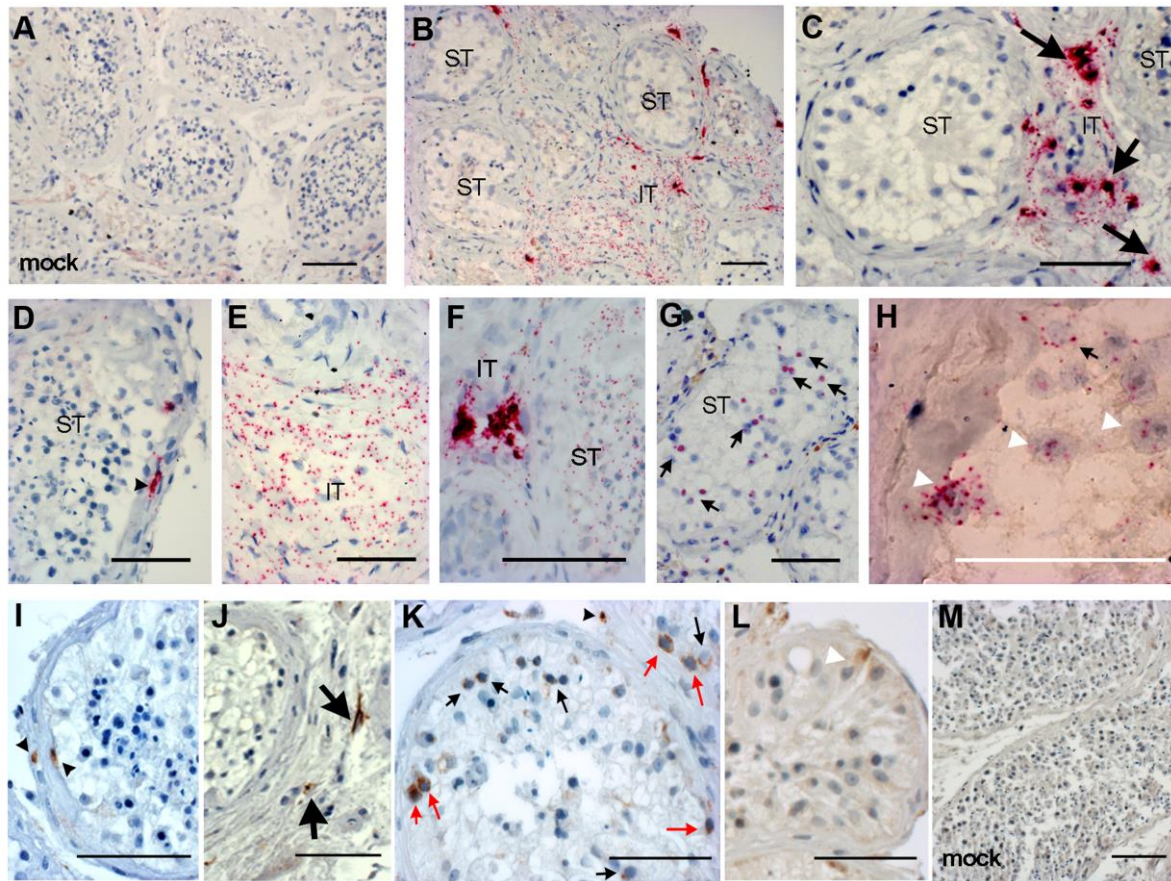
805

806 **Figure 1 ZIKA virus replicates in human testicular tissue.** Human testis explants from 8 donors  
 807 were ex vivo infected overnight with  $10^5$  TCID<sub>50</sub> (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).

818

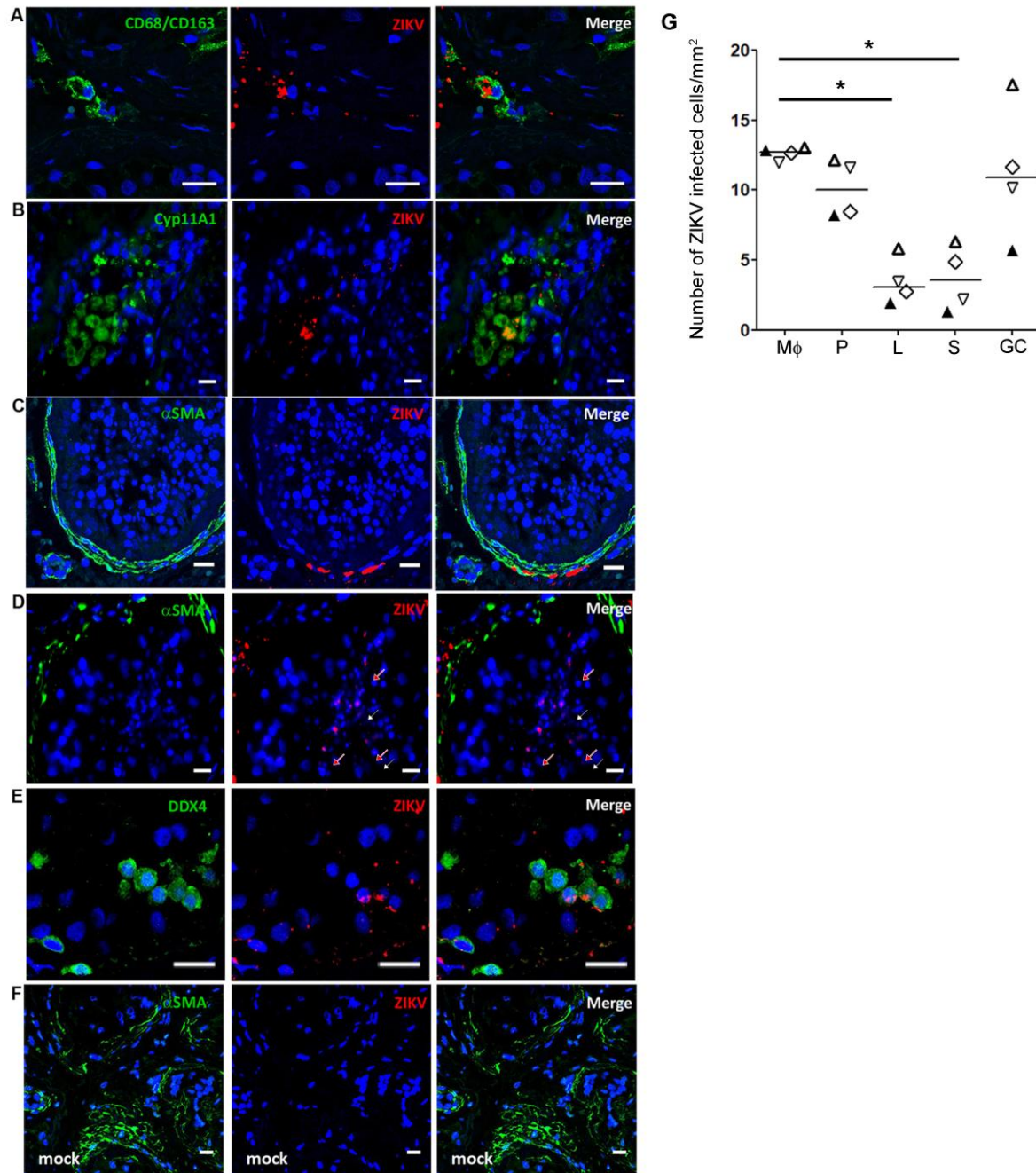
819

820



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

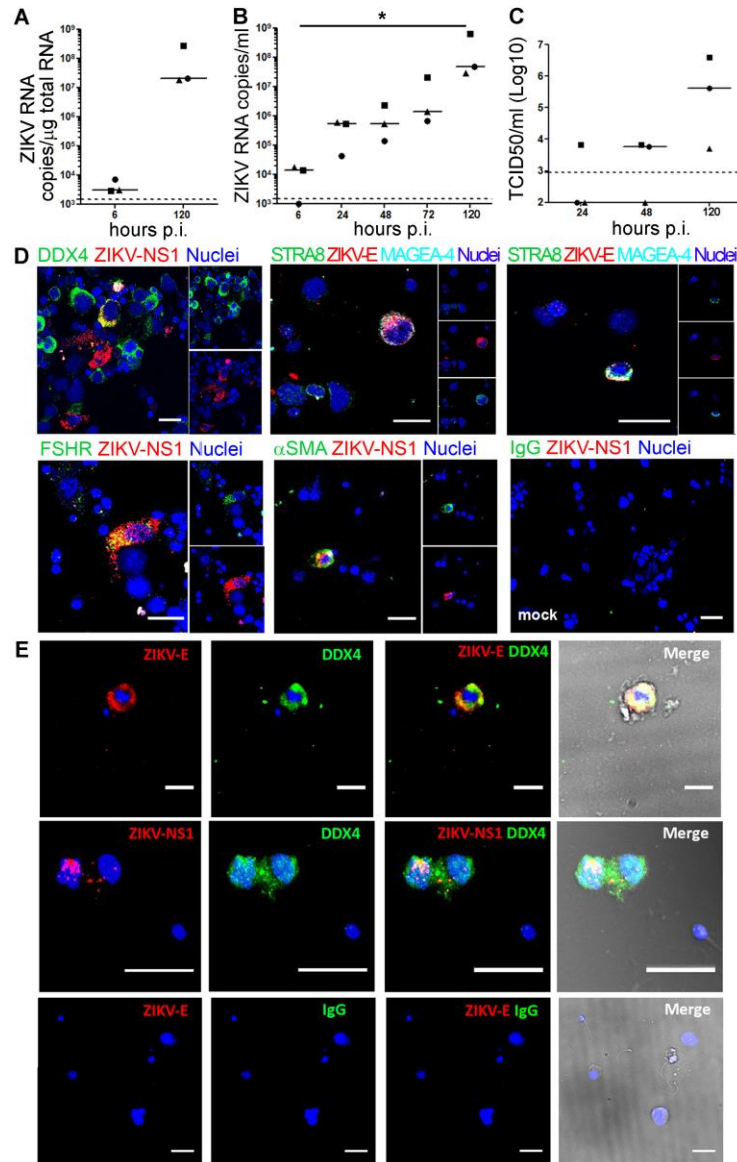
A-H) Representative images of RNAscope in situ hybridization for ZIKV RNA in control mock-infected (A) and ZIKV-infected testis explant (n=8 independent donors) after 6 days of culture (B-H). ZIKV RNA labeling was observed in the interstitial tissue (IT) of testis explants (B, C, E, F), in cells bordering 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=100µm; White bar=50µm.



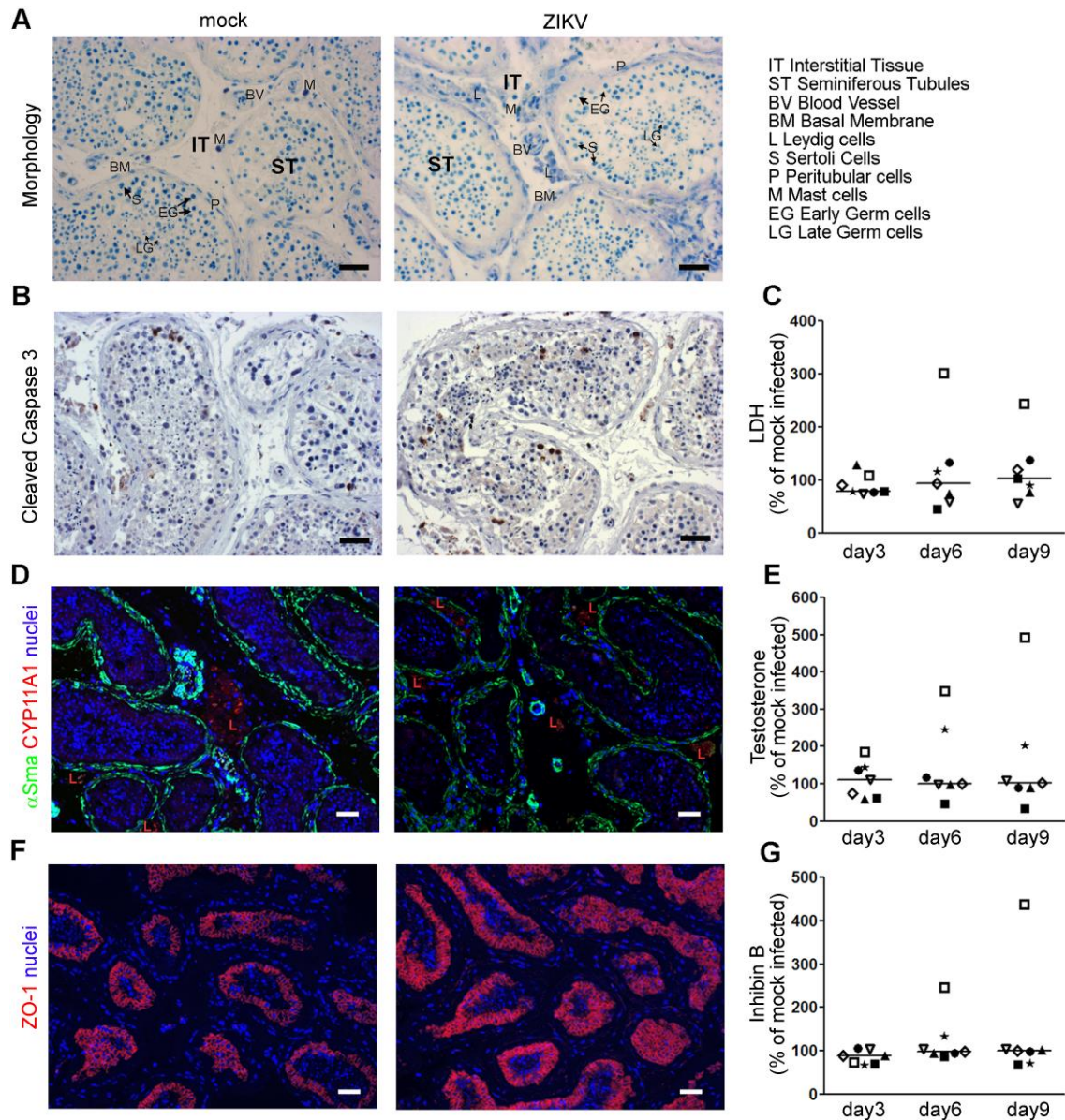
835

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),  $\alpha$ 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 $\mu$ 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).  
 846

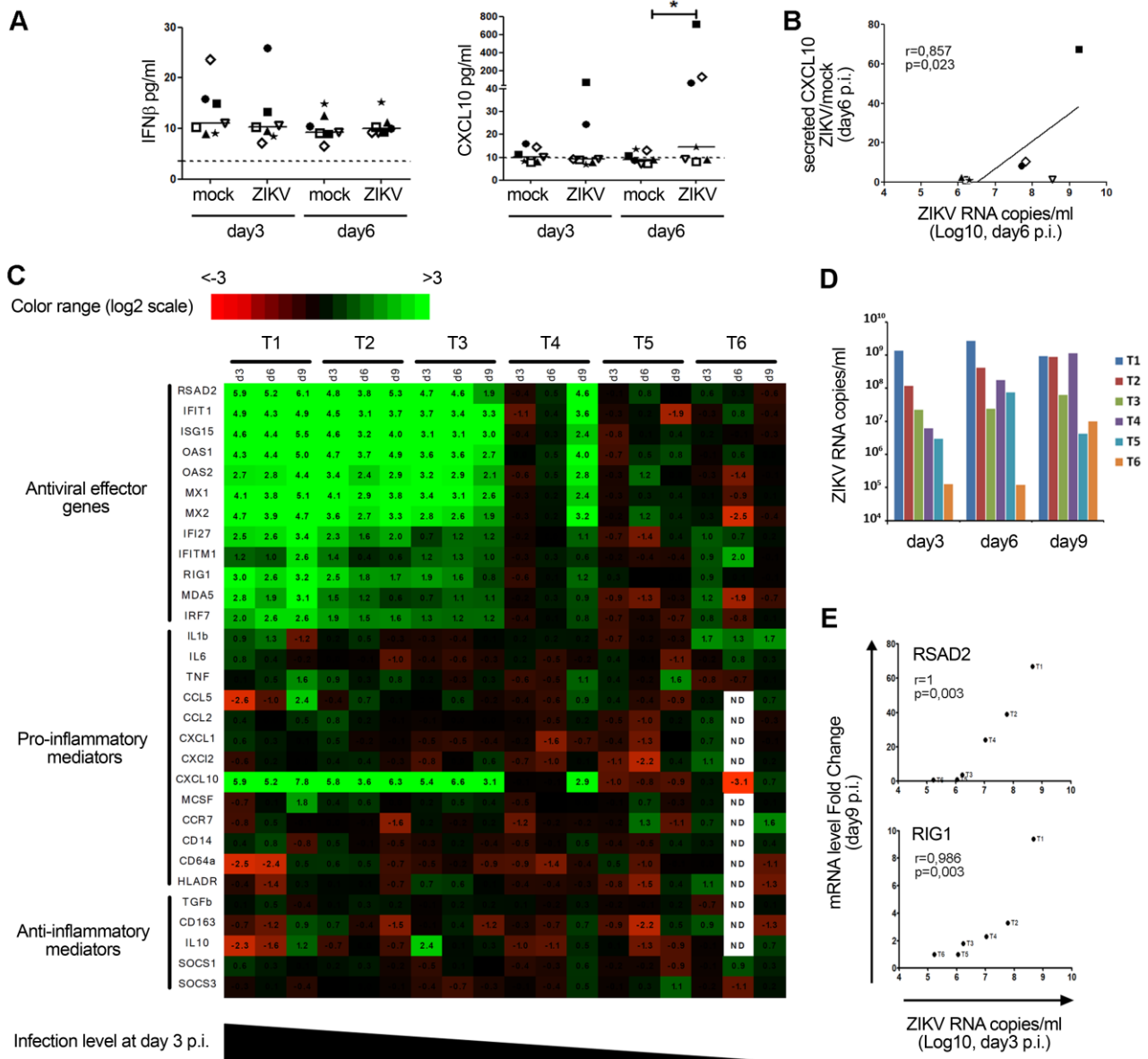
847



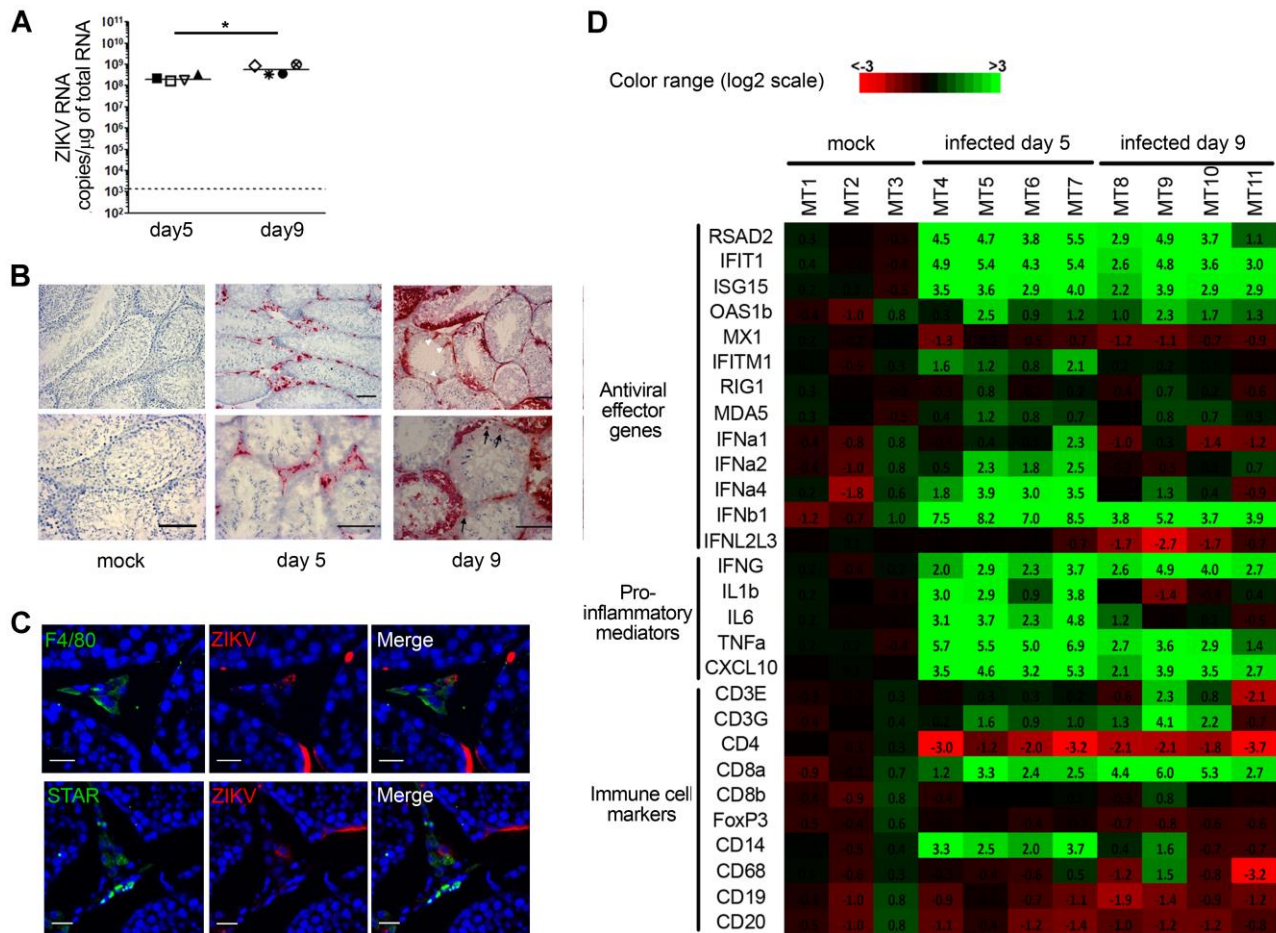
**Figure 4 ZIKV replicates in human testicular germ cells in vitro and in vivo.** A-C) Primary testicular cells were infected with ZIKV (MOI 1, corresponding to  $7.15 \times 10^5$  TCID<sub>50</sub> units/ ml/ 0.5 million cells). ZIKV RNA detected by RT-qPCR in cells (A) and culture supernatants (B). 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 ( $\alpha$ 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 $\mu$ 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 ( $\alpha$ 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 $\mu$ 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- $\beta$  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<sup>-/-</sup> 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.