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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.
INTRODUCTION

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

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

Here, by infecting with ZIKV testicular tissue explants from healthy donors, we show that ZIKV replicates and produces infectious viral particles in human testis. We evidence infection of a broad range of testicular cell types, including resident macrophages and the germ cell line, and confirm the latter in patients’ semen. Infection had no effect on basal testosterone and inhibin B production or overall cell viability ex vivo. ZIKV triggered a wide range of antiviral genes in human testes, but up-regulation of types I, II and III IFN was not observed and pro-inflammatory
response was minimal. Finally, our data on IFNAR-/− mice points at similarities and differences between mouse and human testis to ZIKV infection.
RESULTS

ZIKV replicates in human testicular tissue

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

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

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

ZIKV infects somatic and germ cells in human testis explants

To determine ZIKV’s the target cells in the human testis, mock or ZIKV-infected testis explants were submitted to RNAscope in situ hybridization (ISH) using probes specific for ZIKV RNA (Figure 2A-H, controls in Figures 2A and S3), and to immunohistochemistry using an antibody against the non-structural NS1 viral protein (Figure 2I-M)figure Figure Figure Figure. Infected testes
displayed strong vRNA staining of the interstitial tissue cells and within the extracellular matrix bordering the seminiferous tubules, along with a more diffuse staining in some interstitial areas (Figure 2B-F). A weaker dotty staining was also observed inside a few seminiferous tubules (Figure 2G-H), suggestive of association of the ZIKV with germ cells (Figure 2G) and Sertoli cells (Figure 2H). NS1 antibody (Figure 2I-M) similarly labeled cells within the seminiferous tubule wall (Figure 2I) and the interstitium (Figure 2J), demonstrating ZIKV replication in these target cells. Within the tubules, different germ cell categories including spermatogonia (identified based on their position in the seminiferous epithelium, nucleus size and distinctive morphological features) (Figure 2K) and a few Sertoli cells (identified based on distinctive nucleus shape) (Figure 2L), stained positive for NS1. Infected cells (vRNA+ or NS1+) displayed similar localization at the different time points of infection (days 3, 6 and 9) for the two ZIKV strains tested (Figure S4 and data not shown).

To further identify the nature of the infected cells, we combined ISH for vRNA with fluorescent immuno-labelling for specific cell markers, and undertook quantification of infected cells in testicular tissue from 4 donors. Interstitial infected cells were primarily CD68/CD163+ testicular macrophages (median 12.7 cells/mm²), and to a lesser extent CYP11A1+ Leydig cells (median 3 cells/mm²) (Figure 3A, B, G). Staining for α smooth actin (αSMA) demonstrated the infection of myoid peritubular cells bordering the seminiferous tubules (median 10 cells/mm²) (Figure 3C, G). Within the tubules, dotty fluorescent ZIKV staining close to the lumen histologically co-localized with late germ cells (Figure 3D). Such staining was also present at the base of the tubules, where co-labeled DDX4+ early germ cells were evidenced (DDX4 being a specific marker expressed by most of the germ cells) (Figure 3E). Staining was never observed when using a vRNA probe on mock-infected negative controls (Figure 3F). Infected cells in seminiferous tubules were mostly germ cells (median 11 cells/mm²), while infected Sertoli cells represented a median of 3.5 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 $2.82 \times 10^3$ to $2.09 \times 10^7$ copies/µg 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 $1.26 \times 10^4$ 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$_{50}$/ml and maximum titer of $4 \times 10^6$ TCID$_{50}$/ml (Figure 4C). ZIKV replicated in DDX4+ germ cells, FSH receptor+ Sertoli cells and αSMA+ 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/µg 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$_{50}$/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
the testis 7 days and 11 days post-symptoms onset (Figure 4E). A subset of spermatozoa also labelled for ZIKV-E (Figure S6).

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

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

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

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

To investigate the immune response to ZIKV infection, we assessed the concentration of a panel of antiviral and pro-inflammatory cytokines (IFNβ, IFNα2, IFN λ.1, IFN λ.2/3, IFNγ, IL1β, IL6, TNFα, IL8, IL-12p70, CXCL10, IL-10 and GM-CSF) in testis explant supernatants. Type I, II and III IFN concentrations in testis culture supernatants were unchanged by the infection at days 3 and 6 p.i. in 7 independent testis cultures tested (Figure 6A and S8). Among pro-inflammatory cytokines, only CXCL10 was significantly increased (Figure 6A and S8), and its induction positively correlated with vRNA load (Figure 6B).

We then analysed the transcripts of 11 of these cytokines (IFNβ, IFNα1, IFNα2, IFNα4, IFNλ.1, IFNλ.2, IFNλ.3, IL-1β, IL-6, TNFα, and CXCL10) by RT-qPCR on ZIKV-infected versus mock-infected testes. Type I, II and III IFN transcripts in uninfected testis tissues were below the measurement threshold irrespective of infection (data not shown), while CXCL10 was increased (median fold change (FC) 43.4 at day 9, range 7.7-227.8) in the testis from 4 out of 6 donors (Figure 6C).

Extending the analysis to a wider range of genes involved in pathogen sensing (RIG-I, MDA5), antiviral response (IFNε, IFI27, IFIT1, IFITM1, IRF7, ISG15, Mx1, Mx2, OAS1, OAS2, RSAD2), inflammation (CCR7, CD14, CD64, HLA-DR, MCSF), chemoattraction (CCL2, CCL5, CXCL1, CXCL2.), and control of inflammation (IL-10, TGFβ, CD163, SOCS1, SOCS3), we observed the induction of a broad range of antiviral genes from day 3 onwards in the testis from 3 out of 6 donors and at day 9 in one other donor (Figure 6C).
A strong induction of ISG15 (FC 12.2, range 5.2-45.6), IFIT-1 (FC 12.8, range 9.7-29.6), OAS1 (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 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, along with a relatively more moderate induction of IFI27 (FC 3.1, range 2.2-10.6), IFITM1 (FC 1.8, 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, range 1.8 to 9.4) (Figure 6C).

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

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

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

**Innate immune response to ZIKV infection in the testis from IFNAR<sup>−/−</sup> mouse**

To support our hypothesis of a type I IFN independent antiviral response induced by ZIKV in testis and compare our findings on ZIKV tropism and initial antiviral/pro-inflammatory responses in human testis explants versus that in a widely-used animal model, we analysed the testis of type I IFN receptor-defective (IFNAR<sup>−/−</sup>) mice using similar techniques and viral strain.

ZIKV RNA measurement in testes from IFNAR<sup>−/−</sup> mice infected for 5 and 9 days showed high viral loads in this organ (Figure 7A). Despite differences in intensity and sequence of infection, ZIKV tropism in the mouse testis in vivo was comparable overall to that in human testis ex vivo. At day 5,
testicular infection localized primarily within the interstitial tissue and the peritubular cells, while seminiferous tubules were spared (Figure 7B). Co-labeling of ZIKV RNA with cell markers showed infection of STAR+Leydig cells and F4/80+ macrophages (Figure 7C). At day 9 p.i. (a time at which some mice started to die), strong labeling for vRNA became prominent within Sertoli and germ cells (Figure 7B). We did not observe modifications of testicular morphology at these early time points, in agreement with previous studies (12, 22).

We next examined the induction of genes involved in antiviral response and inflammation in mouse testis (Figure 7D). Similar to human testis explants, and despite a lack of type I IFN signaling, a 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), 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 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 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 7D). In contrast to human testis, Mx1, MDA5 and RIG-1 were not induced at either day 5 or day 9 (Figure 7D). These results suggest that ZIKV induces a type I IFN signaling-independent antiviral response in both humans and mice. In contrast to human testis, in which CXCL10 was the only pro-inflammatory gene increased by the infection, TNFα (median fold change 48.0, range 32.6-122.6 at day 5, and median 6.9, range 2.6-12.3 fold at day 9), IL-1β (median 7.7, range 1.8-13.8 fold at day 5 only) and IL-6 (median 10.7, range 4.9-27.5 fold at day 5 only) were up-regulated in infected mouse testis (Figure 7D), while IFNγ (produced by NK and T cells) was maximally increased at day 9 (median 11.2, range 6.2-30.8 fold) over day 5 (median 6.2, range 4.0-12.7 fold). IFNβ was the most dramatically-stimulated innate immune gene at day 5 (median 236.7, range 130.2-353.4 fold), while IFNα1, 2, 4 genes were modestly and transiently up-regulated at day 5 and IFNλ2/3 mRNAs levels were unchanged (Figure 7D). When looking at markers of immune cell subtypes, a transient increase in transcripts encoding the myeloid cell marker CD14 was observed at day 5, whereas transcripts encoding CD3 (T cell marker) and CD8 (cytotoxic T cell marker) were maximally up-
regulated at day 9, in line with the IFNγ expression pattern. The markers for B cells (CD19 and CD20), regulatory T cells (FoxP3) and macrophages (CD68) were unchanged, while CD4 (expressed by T helper and myeloid cells) was down-regulated (Figure 7D). The infiltration of T lymphocytes in infected mouse testis was confirmed by CD3 immunostaining and quantification of positive cells (Figure S11), further demonstrating mouse testis inflammation. Overall, the induction of an antiviral response in human and IFNAR−/− mice testis supports the existence of a type I IFN signaling independent response to ZIKV infection in testis.
We show that Asian ZIKV replicates in the human testis ex vivo. Infected somatic cells within testis explants were mostly macrophages and peritubular cells, while a lower number of Leydig cells and Sertoli cells were observed. Considering the relative proportion of these different cell types in human testicular tissue (approximately one macrophage for 10 Leydig cells, 36 peritubular cells, 40 Sertoli cells and 400 germ cells) (23, 24), macrophages are likely the cell type most susceptible to ZIKV infection within the testis. Importantly, we demonstrate that ZIKV replicates within testicular germ cells, from stem cell (spermatogonia) to spermatozoa precursors (spermatids). In agreement, Robinson et al. lately reported on the infection of male germ cells after 3 days exposure of human seminiferous tubules to African ZIKV (25). Our detection of infected germ cells in semen from ZIKV-infected men confirms these in vitro and ex vivo findings. The presence of ZIKV in ejaculated spermatozoa adds to previous findings of ZIKV antigen, RNA and infectious particles in spermatozoa (19) (26). Spermatozoa and immature germ cells may be infected during epididymal transit (duration 1 to 21 days) or within the testis. To infect these cells, the virus has to cross the blood testis barrier formed by the Sertoli cell tight junctions. Direct infection of the Sertoli cells is supported by our results in primary cells and that of other authors in commercial Sertoli cells (27, 28). Sertoli cells were showed to release ZIKV particles on their adluminal side, whereas the tight and adherens junction protein expression was not altered by infection (27). In agreement with these data, the ZO-1 labelling we observed in human explants suggested intact Sertoli cell barrier despite infection. In contrast, Sertoli cell exposure to inflammatory mediators produced by ZIKV-infected blood-derived macrophages (which phenotype differs from that of the anti-inflammatory testicular macrophages) altered their barrier function (27). Thus, the alteration of the blood-testis-barrier by testis-infiltrating macrophages may provide an additional way for the virus to reach seminal lumen and late germ cells. Altogether, our data show
that ZIKV replicates in germ cells and suggest that the virus might be able to bypass the blood testis barrier by infecting Sertoli cells.

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

Our findings suggest that ZIKV could affect sperm production. Beside germ cell infection, somatic cell infection might disrupt the paracrine control of spermatogenesis (31, 32), and the infection of the contractile peritubular cell (33) might decrease the expulsion of spermatozoa from tubules into the epididymis. Infected men showed a decrease in sperm count and an increase in spermatozoa abnormalities during the 2 months post clinical onset (19, 34). Our results suggest that direct infection of germ and/or testicular somatic cells might be involved in such altered sperm parameters, although fever and/or immune response could be involved (35, 36).
The infection had no significant effects on testis explants morphology nor viability. This is in contrast to findings in mouse models where damaging effects of ZIKV infection on testis became evident after leukocytes’ infiltration (12–14). Since the ex vivo testis model lacks the presence of an intact immune system, we cannot rule out an effect of acquired immunity on the testis from ZIKV-infected men. However, testicular atrophy or orchitis have never been reported in clinical cases or non-human primate studies, and immune infiltrations were not observed in the latter (16–18), suggesting the absence of massive inflammation. The lack of cell death induction in our ex vivo model could be linked to culture conditions (e.g. viral strains/doses used, duration of infection) or to the limited number of infected cells. However, it might also reflect the ability of the virus to replicate in testicular cells in a non-cytopathic manner. Non or minimal cytopathic infection and persistence of ZIKV has been reported in different cell types, including human placental macrophages (37), brain microvascular endothelial cells (38), and lately, male mouse germ cells (25). Absence of cytopathic effect was also reported in Sertoli cells infected with ZIKV (27, 39). In contrast, high cytotoxicity was reported in ZIKV-infected human testis organoids (40). However, in this organoid system, the architecture of the testis is not preserved and physiological cellular interactions lost. Altogether, we hypothesize that non-lytic infection, in combination with evasion from immune responses, may allow viral persistence in the human testis.

A broad range of antiviral genes was induced by ZIKV in testis explants. Several of these classically-defined Interferon-Stimulated Genes (ISGs), such as ISG15 (41), RSAD2 (42), IFITM1 (43), OAS1 (44), Mx1 (45) and IFIT gene family members (46) have an inhibiting activity on flaviviruses and/or ZIKV replication. Most interestingly, ZIKV did not increase type I, II or III IFNs secretion by testicular explants, and their transcripts consistently remained below detection at all time points. A level of IFN production below the sensitivity of our assays, or an active inhibition of IFN production by ZIKV, could explain these results. Thus, ZIKV non-structural proteins inhibit different steps of type I IFN induction cascade (47, 48). Alternatively, the absence of IFN up-
regulation in testis explants might reflect a specificity of this immune-suppressed organ, in which sustained high concentrations of type I IFN trigger germ cell apoptosis and sterility (49). Detection of ISGs over-expression in the testis from ZIKV-infected IFNAR-/- mice further suggest IFN-independent induction of ISGs in the testis. The increased level of RIG-1 mRNA in infected testis suggests it may be involved in the direct induction of ISGs, although other effectors could be in play (50). However, the broad induction of ISGs may fail to control ZIKV replication in the testis in the absence of increased type I IFN secretion to amplify and stabilize the antiviral response. In contrast to antiviral genes, ZIKV infection of the human testis did not affect any of the classic pro-inflammatory cytokines, except for CXCL10 which secretion was modestly increased. Interestingly, the level of antiviral transcripts at day 9 p.i. positively correlated with the level of infection at days 3 and 6, suggesting that the initial level of infection influenced the intensity of the antiviral response. Accordingly, antiviral genes were not induced in explants showing lower levels of infection. Thus host factors other than those we have studied may play a role in susceptibility to ZIKV. Altogether, our results indicate that ZIKV induces a broad induction of antiviral effectors but no IFN up-regulation and minimal pro-inflammatory response in ex vivo infected human testis. We hypothesize that such innate immune response, along with a lack of cytopathic effect, might facilitate the persistence of ZIKV for extended periods in the testis, and contribute to the prolonged release of ZIKV in semen.

Animal models are crucial for mechanistic studies and the in vivo testing of antiviral strategies. Cross-validation with human data is essential to assess their similarities and differences. Discrepant results on the interstitial (13, 22, 51) and/or intratubular localization of ZIKV (15, 17, 67, 68, 69) were reported in mouse testis from IFN-signaling deficient mice. Our results in the IFNAR +/- mouse model reconciled these results since ZIKV infection was exclusively located in interstitial cells and peritubular cells at day 5 p.i., whereas by day 9 the infection had progressed to 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
cells but not in immunocompetent mouse cells), or ex vivo/in vivo differences (e.g. infiltrating cells pro-inflammatory activity) requires further investigation.

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

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

In conclusion, we demonstrated that ZIKV replicates in the human testis ex vivo and infects a range of somatic cells and germ cells. Replication of ZIKV in testicular germ cells was evidenced in semen from ZIKV-infected men, along with ZIKV association with spermatozoa. ZIKV had no major deleterious effect on the morphology and hormonal production of the human testis in culture. Despite a broad induction of antiviral genes, the absence of IFN up-regulation and minimal pro-
inflammatory response of the human testis ex vivo, along with the lack of ZIKV cytopathic effect on testicular cells, might favour the prolonged ZIKV infection observed in this organ, and account for the absence of orchitis in men infected by ZIKV. Overall, our results suggest that ZIKV infection of the human testis may be involved in the persistence of the virus in semen and in altered semen parameters. These results call for further investigation on the impact of ZIKV on the reproductive health of ZIKV-infected men and warn against the potential horizontal and vertical transmission of ZIKV through the infected germ line. Finally, the ex vivo model of ZIKV infection of the human testis we developed provides a valuable tool for the testing of antiviral agents.
MATERIAL AND METHODS

Cells lines and viruses
Asian Zika virus strains isolated during the 2015 outbreak in the French Caribbean (MRS_OPY_Martinique_PaRi-2015, passaged once in Vero cells) and during the 2013 outbreak in Polynesia (H/PF/2013, passaged three times in Vero cells) were obtained from the European Virus Archive (EVA) and further propagated in VeroE6 cells for 2 additional passages. VeroE6 cells (African green monkey kidney epithelial cells) were maintained in DMEM supplemented with 10% FCS, Glutamine (2mM) and 1% penicillin/streptomycin at 37°C with 5% CO₂ (all reagents from GIBCO). To produce viral stocks, VeroE6 cells were infected at an MOI of 0.01 in serum-free medium for 2 hours, then complete medium was added to reach a final serum concentration of 5%. When cytopathic effect was evident, supernatant was centrifuged, filtered (0.45 µm), aliquoted and frozen at -80°C. The human testicular germ cell tumor (seminoma) cell line Tcam-2 (66) was kindly provided by Dr Janet Shipley (The Institute of Cancer Research, London).

Organotypic culture of human testis explants and infection
Testes were dissected into 3 mm³ sections transferred onto 24 well plates (2 sections/well) containing 500µl of medium (DMEM/F12 supplemented with 1X nonessential amino acids, 1X ITS, 100U/ml penicillin, 100µg/ml streptomycin, 10% FCS, all from GIBCO) in the presence or absence of 10⁵ TCID₅₀ of ZIKV (corresponding to 2.2.10⁷ to 2.9.10⁷ vRNA copies for MRS_OPY_Martinique_PaRi-2015 and 8.10⁷ vRNA copies for H/PF/2013). After overnight incubation, tissue fragments were washed 3 times with PBS and transferred onto a polyethylene terephthalate insert (3µm high density pores) in 12 well plates containing 1 ml of medium. 8 hours later, the medium was changed again to further wash away potential residual virus input (time 0 for sample collection). For each experimental condition, a minimum of two wells were tested. The
culture was maintained up to 9 day p.i. in a humidified atmosphere containing 5% CO₂ at 37°C with medium collected and fully changed every 3 days, in order to thoroughly wash input virus and assess viral production dynamic. Media were stored frozen at −80°C for vRNA and titer measurement. Tissue fragments were either fixed in neutral buffered 4% formaldehyde or frozen and stored at −80°C.

**Isolation and infection of testicular cells**

Testis fragments were incubated in digesting medium (2mg/ml hyaluronidase, 2mg/ml collagenase I, 20µg/ml in DMEM/F12) for 60 minutes at 37°C under agitation (110 rpm) to dissociate interstitial tissue from seminiferous tubules. After centrifugation, the seminiferous tubule pellet digested by trypsin (0.25%, 5ml/g, 20 minutes at 37°C). Trypsin was inactivated and cells filtered (60µm) and cultured overnight in DMEM/F12 medium supplemented with 1X nonessential amino acids, 1X ITS (human insulin, human transferrin, and sodium selenite), 100U/ml penicillin, 100µg/ml streptomycin and 10% FCS (all from GIBCO). Primary TGCs and Tcam-2 cells were incubated with ZIKV diluted in serum-free medium at a multiplicity of infection (MOI) of 1 (corresponding to 1.43 x 10⁶ TCID₅₀/million cells) for 2 hours at 37°C 5% CO₂. Virus was removed by washing and trypsin treatment for 5 minutes at 37°C. Primary testicular cells were cultured at a density of 0.5 million cells/ml in supplemented StemPro-34 (Invitrogen) as described elsewhere (67). T-cam2 cells were cultured at a density of 0.1 million cells/ml in RPMI1640 supplemented with P/S, Glutamine (2mM) and 10% FCS (all reagents from GIBCO).

**Semen samples**

Semen was liquefied at 37°C for 30 minutes and 10µl spread on a glass slide and dried at room temperature. Smears were fixed in 4% formaldehyde and stored at -20°C. Viral loads for ZIKV in seminal plasma and seminal cells were 7,25 log copies/ml and 6,7 log copies/µg total RNA respectively for the donor at day 7, and 7,8 log copies/ml and 7,8 log copies/2x10⁶ cells respectively.
for the donor at day 11. Patients’ serology for dengue was negative and semen samples tested negative for dengue in RT-PCR.

**Mice**

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

**Real-time quantitative RT-PCR**

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

Primers for innate immune response effector genes (Supplementary Table 1) were designed using Primer-BLAST tool (70). Total RNA was reverse-transcribed using the Iscript cDNA Synthesis Kit.
QPCR reactions were performed on a Bio-Rad Laboratories CFX384 instrument using iTaq SYBR green mix (BioRad) and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, followed by melt-curve analysis. Gene expression fold changes were calculated with the 2^{-\Delta\Delta C_{t}} method normalized to beta-actin and mock-infected samples expression levels.

**Determination of viral titer**

Vero E6 cells seeded in opaque-walled 96 well plates at a final concentration of 1.5x 10^{5} cells/ml in DMEM with 5% FCS were put in contact the next day with serial dilutions of supernatant. Median tissue culture infective dose (TCID_{50})/ml was measured at day 5 post-infection using the Viral ToxGlo Assay (Promega).

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

Tissues or cell pellets were fixed in 4% formaldehyde and embedded in paraffin. RNA ISH was performed using RNAscope 2.5 (Advanced Cell Diagnostics) according to the manufacturer’s instructions, as previously described (15, 87). RNAscope ISH is a highly specific and sensitive technique, with the ability to detect single molecules (73). Formaldehyde fixed paraffin-embedded tissue sections or cell pellets were deparaffinized in xylene and dehydrated in ethanol. with for 10 min at room temperature. Slides deparaffinized and H_{2}O_{2} quenched for endogenous peroxidases were boiled in RNAscope Target Retrieval Reagents (citrate buffer10mM, pH6, 15 minutes) and incubated in RNAscope Protease Plus (40°C, 20 minutes), prior to probe hybridization. Sections were incubated with target probes (2 hours, 40°C), washed in buffer and incubated with amplification reagents. Chromogenic detection was performed using Fast Red as substrate for alkaline phosphatase to generate red signal. Slides were counterstained with hematoxylin and mounted in Eukitt (O. KINDLER) before observation using bright-field microscopy. The “double Z” probes targeting ZIKV RNA (consensus sequence, target region 219-5443, catalog #467771), 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. Sections 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- αSMA (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, αSMA+ peritubular cells, germ cells and Sertoli cells (identified on morphological criteria in light
microscopy) in testis explants was assessed in 4 donors and at least 3 whole testis tissue sections/donors (corresponding to about 12 mm²/testis donor). Quantification of CD3+ cells in mouse testis was performed in 3 mock-infected animals, 4 animals at day 5 p.i. and 4 animals at day 9 p.i., in at least 5 mm²/testis. Slides were scanned with a NanoZoomer slide scanner (Hamamatsu Photonics, France, at Plateforme H2P2, Biosit, Rennes, France). Immunostained positive cells were counted with ImageJ free software.

**Immunocytofluorescence**

Semen smears from donors and cell pellets from testicular cell cultures put onto polylysine-coated glass coverslips were fixed in 4% PAF for 20 minutes at RT. After permeabilization (0.2% Triton X-100, 10 minutes), the slides were incubated in blocking buffer (0.2% Triton X-100, 1% goat serum, 2 hours) and stained with antibodies against ZIKV NS1 (1:1000, Biofront Technologies) or flavivirus envelope Ab 4G2 (1:1000, Millipore). NS1 antibody was either directly coupled to Alexa Fluor 647 (Zenon labeling kit, Molecular Probes) or revealed using Alexa-fluor 555 goat anti-mouse (Life Technologies). Infected cell characterization was performed using rabbit anti-DDX4 (5µg/ml, Abcam), anti-Stra8 (9,6µg/ml, ThermoFisher Scientific) and anti-FSHR (10µg/ml, Origene), detected using Alexa-fluor 488 (Invitrogen) or Alexa-fluor 647 goat anti-rabbit (Jackson Immunoresearch), mouse anti-MageA4 (clone 57B, 4µg/ml) coupled to Alexa Fluor 647 (Zenon labeling kit, Molecular Probes), and mouse anti-αSMA (clone 1A4, 0,5µg/ml, Dako) detected using Alexa-fluor 555 goat-anti mouse antibody. Isotype control antibodies or non-infected cells were used as negative controls. Slides were counterstained with Prolong medium containing DAPI (Molecular Probes). Images were acquired with the SP8 confocal system (Leica) connected to LAS software or with DMRXA wide field microscope (Applied Precision), and analyzed using Fiji software.
Testosterone and inhibin B immunoassays

Testosterone was assayed using a specific radioimmunoassay (Immunotech, Beckman Coulter).

Inhibin B was assayed using a commercial enzyme-linked immunosorbent assay (ELISA) kit (DSL-10-84100 Active, Beckman Coulter).

Viability assay

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

Cytokine release measurement

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

Statistics

Data were analyzed with non-parametric paired Friedman-Dunn's or unpaired Kruskal-Wallis-Dunn's test when more than 2 sets of samples were compared, as specified in figure legends. The non-parametric Mann-Whitney test was used to analyze differences in viral load of mice testis at day 5 and 9 post infection. Correlations were calculated using the Spearman test. Values were considered significant when P<0.05. Statistical analyses were performed using commercially-available software (GraphPadPrism 6, GraphPad Software, Inc., La Jolla, California, USA).

Study approval

Normal testes were obtained either after orchidectomy from prostate cancer patients who had not received any hormone therapy or at autopsy, and processed within 2 hours of surgery. The
procedure was approved by a local ethics committee (authorization #DC-2016-2783) and the French national agency for biomedical research (authorization #PF S09-015).

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

Mice were housed at the Institut Pasteur Animal Facility accredited by the French Ministry of Agriculture for performing experiments on live rodents. Work on animals was performed in compliance with French and European regulations on care and protection of laboratory animals (EC Directive 2010/63, French Law 2013-118, February 6th, 2013). All experiments with IFNAR−/− mice were approved by the Ethics Committee #89 and registered under the reference #2016-0018.
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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Figure 1 ZIKA virus replicates in human testicular tissue. Human testis explants from 8 donors were ex vivo infected overnight with $10^5$ TCID$_{50}$ (corresponding to $2.2 \times 10^7$ to $2.9 \times 10^7$ vRNA copies) from a low-passage ZIKV strain isolated in 2015 in the French Caribbean (MRS_OPY_Martinique_PaRi-2015). Explants were thoroughly washed and cultured on inserts in 1 ml of medium/well for 9 days, with media fully removed and changed every 3 days. Each of the time points (day 3, day 6, day 9) represent de novo viral release over a 3-day-culture period. A) ZIKV RNA release over a 3-day-culture period at day 3, day 6 and day 9 detected by RT-qPCR; B) Viral titers determined by infectivity assay of 3-day-culture period tissue supernatants on VeroE6 cells. Each symbol represents a different donor (same symbol/donor throughout the manuscript figures). Dotted lines represent the detection limit of the assays. Mock-infected explants were always below detection level. Bars represent median. *$P<0.05$; **$P<0.01$ (Friedman-Dunns non-parametric comparison).
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.
Figure 3 Characterization and quantification of ZIKV-infected human testicular cells ex vivo.

RNAscope in situ hybridization for vRNA coupled with immunofluorescence for cell markers identified ZIKV RNA in CD68/CD163+ macrophages (A), Cyp11A1+ Leydig cells (B), αSMA+ peritubular cells (C), late germ cells localized near the lumen in seminiferous tubules (white arrows: round spermatids; red arrows: elongated spermatids) (D) and DDX4+ early germ cells (E). Staining for ZIKV was never observed in mock-infected testis (F). Nuclei are stained in blue. Scale bars=20μm. (G) Infected cells were quantified in at least 3 whole tissue sections from 4 testis donors (each represented by a different symbol) at day 9 p.i. MΦ: macrophages; P: peritubular cells; L: Leydig cells; S: Sertoli cells; GC: germ cells. Bars represent median. *P<0.05 (Friedman-Dunns non parametric comparison).
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$_{50}$ 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 (αSMA). 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=20µm.
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 (αSMA) 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=50μm. 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=100um. 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=20um. 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.