

HEALTH AND MEDICINE

Zika virus causes testicular atrophy

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Zika virus (ZIKV) is an emerging mosquito-borne flavivirus that has recently been found to cause fetal infection and neonatal abnormalities, including microcephaly and neurological dysfunction. ZIKV persists in the semen months after the acute viremic phase in humans. To further understand the consequences of ZIKV persistence in males, we infected *Ifnar1*^{-/-} mice via subcutaneous injection of a pathogenic but nonlethal ZIKV strain. ZIKV replication persists within the testes even after clearance from the blood, with interstitial, testosterone-producing Leydig cells supporting virus replication. We found high levels of viral RNA and antigen within the epididymal lumen, where sperm is stored, and within surrounding epithelial cells. Unexpectedly, at 21 days post-infection, the testes of the ZIKV-infected mice were significantly smaller compared to those of mock-infected mice, indicating progressive testicular atrophy. ZIKV infection caused a reduction in serum testosterone, suggesting that male fertility can be affected. Our findings have important implications for nonvector-borne vertical transmission, as well as long-term potential reproductive deficiencies, in ZIKV-infected males.

INTRODUCTION

Zika virus (ZIKV), a positive-stranded RNA virus belonging to the Flavivirus family, has recently been associated with several unexpected viral characteristics (1, 2). Although the main mode of ZIKV transmission is thought to be mosquito-mediated, as in other flaviviruses (3, 4), ZIKV has additionally been found to use both maternal-fetal (5) and sexual transmission (6) as a means of virus spread. Upon systemic infection, virus replication can be detected in most organs; it has also been found within several immune-privileged sites, including the brain (7–18), placenta (19, 20), eyes (21), ovaries (22), and testes (23, 24). In humans, persistent shedding of infectious virus has been found in vaginal secretions (25) and semen (26–33) at times well past the acute viremic and symptomatic stage of virus infection.

RESULTS

To understand the pathology associated with persistence of ZIKV shedding within the testes, we used a murine infection model that leads to high levels of systemic virus replication but does not ultimately result in death (*Ifnar1*^{-/-} mice challenged with ZIKV^{MEX}). ZIKV^{MEX}-infected *Ifnar1*^{-/-} mice demonstrated relatively mild body weight loss (Fig. 1A) and developed peak viremia at 5 days post-infection (dpi) (Fig. 1B). We were surprised to find that the ZIKV^{MEX} strain, which has not yet been published in mouse models, did not result in the lethal pathology of previous strains (22, 24). This allowed us to monitor the effects of ZIKV infection of a pandemic strain at later time points of infection. Consistent with these findings, mice sacrificed at 5 dpi were found to have high levels of viral RNA (Fig. 1C) and infectious virus (Fig. 1D) within the brain, testes, and blood. At 9 dpi, ZIKV was essentially cleared from the bloodstream and was undetectable at 21 dpi (Fig. 1B).

To decipher the cellular tropism within the testes, we performed immunohistochemistry (IHC) using a mouse monoclonal antibody

recognizing ZIKV NS1 antigen on testes and epididymis of ZIKV^{MEX}-infected *Ifnar1*^{-/-} mice at 5 dpi. As shown in Fig. 2A, viral antigens were mainly detected within the interstitial cells of the testes. These regions are mainly composed of Leydig cells, a testicular cell type that supports sperm production through the generation of testosterone (34). We did not detect ZIKV within the seminiferous tubules in ZIKV^{MEX}-infected *Ifnar1*^{-/-} mice at 5 dpi (Fig. 2A, a and b). Because the epididymis is a highly convoluted tube that stores and carries sperm (35), we also examined epididymal tissue of ZIKV^{MEX}-infected *Ifnar1*^{-/-} mice for ZIKV antigen and found an overwhelming amount of antigen associated with the sperm mass within the epididymal duct lumen (Fig. 2A, d). In addition, we also observed several epithelial cells lining the lumen that were positive for viral antigen (red arrows in Fig. 2A, d). In further support of ZIKV infection of testes, RNA analysis of several cytokines indicated that an innate immune response was elicited at 5 dpi (Fig. 2B).

Because IHC analysis suggested that Leydig cells, which are responsible for the supply of testosterone in testes, are targets for ZIKV infection, we next determined the expression level of several genes related to testosterone synthesis (36) in ZIKV^{MEX}-infected *Ifnar1*^{-/-} mice testes and found a consistent reduction among all genes tested as compared in testes of ZIKV^{MEX}-infected *Ifnar1*^{-/-} mice (Fig. 2C). To confirm the susceptibility of Leydig cells to ZIKV, we infected isolated WT or *Ifnar1*^{-/-} Leydig cells with ZIKV^{MEX} in vitro. As shown in Fig. 2D, Leydig cells were able to productively support ZIKV replication, indicating that Leydig cells could serve as a target and a reservoir cell type for ZIKV within the testes.

Strikingly and rather unexpectedly, at 21 dpi, the testes of ZIKV^{MEX}-infected *Ifnar1*^{-/-} mice were significantly reduced in size as compared to those of uninfected mice (Fig. 3A), as determined by both weight (Fig. 3B) and length (Fig. 3C). These findings suggest that persistent ZIKV infection may lead to hypofertility. Because IHC, in vitro infection, and the decreased expression of genes related with testosterone synthesis indicated Leydig cells as putative targets for ZIKV infection within the testes, we next set to determine whether atrophy could occur as the result of decreased testosterone levels in ZIKV-infected mice. We examined levels of testosterone within serum from ZIKV^{MEX}-infected *Ifnar1*^{-/-} mice at 5 and 21 dpi as compared with mock-infected *Ifnar1*^{-/-} mice and found a significant decrease in testosterone at 5 dpi with a consistent reduction at 21 dpi (Fig. 3D). Notably, the testosterone levels

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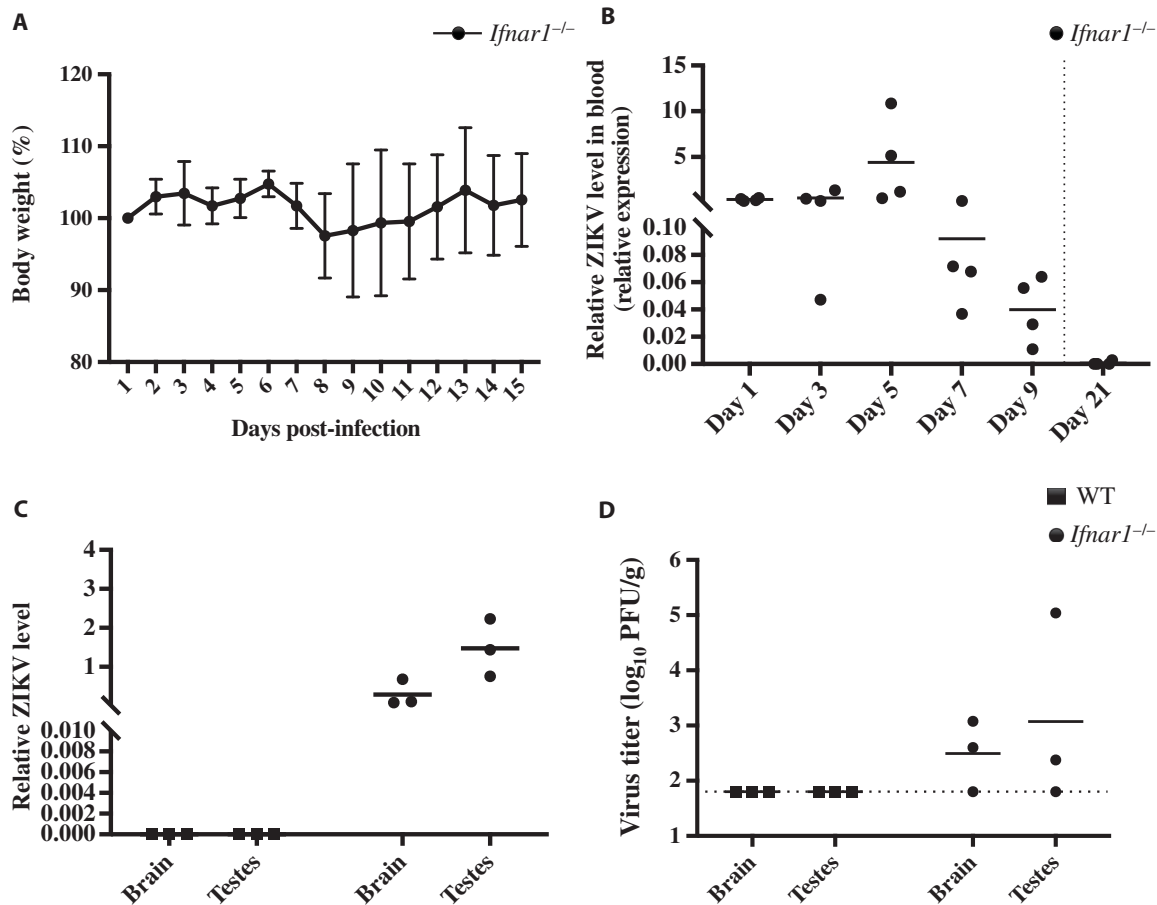


Fig. 1. Characterization of ZIKV in mice. *Ifnar1*^{-/-} mice (*n* = 4) were infected with 10⁵ plaque-forming units (PFU) of ZIKV^{MEX} via subcutaneous route. (A) Weights were monitored for 15 days after virus infection. The values represent average scores of body weight compared with initial body weight ± SD from four mice. (B) Blood samples were collected at 1, 3, 5, 7, 9, and 21 dpi, and the virus loads were examined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Three wild-type (WT) and *Ifnar1*^{-/-} mice were infected subcutaneously with 10⁵ PFU of ZIKV^{MEX}. Multiple organs were collected from mice (*n* = 3) after challenge on day 5 and subjected to virus loads by qRT-PCR (C) and by plaque assays in Vero cells (D). The results are expressed as the mean titers. The detection limit of plaque assays is 1.8 log₁₀ PFU/g.

at 5 dpi (six of seven mice) and at 21 dpi (two of seven mice) were lower than the assay detection limit. These findings support other data documenting ZIKV infection of Leydig cells (Fig. 2, A and D) and suggest a potential mechanism for ZIKV-induced testicular atrophy. Viral RNA was noticeably higher in the epididymis compared to testes (Fig. 3E), consistent with the copious amount of viral antigen detected by IHC within the epididymal lumen (Fig. 2A). It remains conceivable that multiple cell types within the reproductive tract produce virus, and the cell-free virus becomes stored and concentrated in the epididymis before physical expulsion upon sexual activity.

DISCUSSION

Because humans with a fully functioning immune system also demonstrate persistent ZIKV infection of the testes, these findings have major implications for the fertility of men who have been exposed to ZIKV. It will be important to monitor the fertility of men who have been infected with ZIKV to better understand the impact in humans. We hypothesize that active infection of Leydig cells within the interstitial regions of the testes could cause decreased testosterone production, which ultimately results in testicular atrophy. There is precedence for deleterious effects

on the production of testosterone and spermatogenesis caused by infection with mumps virus, which also targets Leydig cells as a site of replication within the testes (37–39). Alternatively, or in conjunction, because inflammation is known to also lead to testicular atrophy, the phenotype may be caused indirectly, or enhanced by, the immune response to detection of ZIKV. Because all experiments were carried out in *Ifnar1*^{-/-} mice, our data rule out the possibility of type I interferon signaling in testicular atrophy caused by ZIKV.

RNA analysis of testes samples of ZIKV^{MEX}-infected *Ifnar1*^{-/-} mice found ZIKV to be present within the testes and epididymis at 5 dpi in all mice and at 21 dpi in most mice. Because there is no detectable ZIKV RNA within the blood of ZIKV^{MEX}-infected *Ifnar1*^{-/-} mice at 21 dpi (Fig. 1B), this observation recapitulates the human presentation of persistent ZIKV replication within the testes. For ZIKV^{MEX}-infected *Ifnar1*^{-/-} mice sacrificed at 35 dpi, when the epididymis was measured to be ZIKV-negative, some of the testes were still reduced in size (15 to 45% shorter in length) as compared to testes of mock-infected mice. Determination of the possibility of recovery from testicular atrophy over greater time periods is warranted. Recently, two other groups also reported that ZIKV can cause long-term testicular damage; however, the infected cell types found in these studies (spermatogonia, primary spermatocytes, Sertoli cells, and/or peritubular myoid cells) differ from those reported here, indicating that further

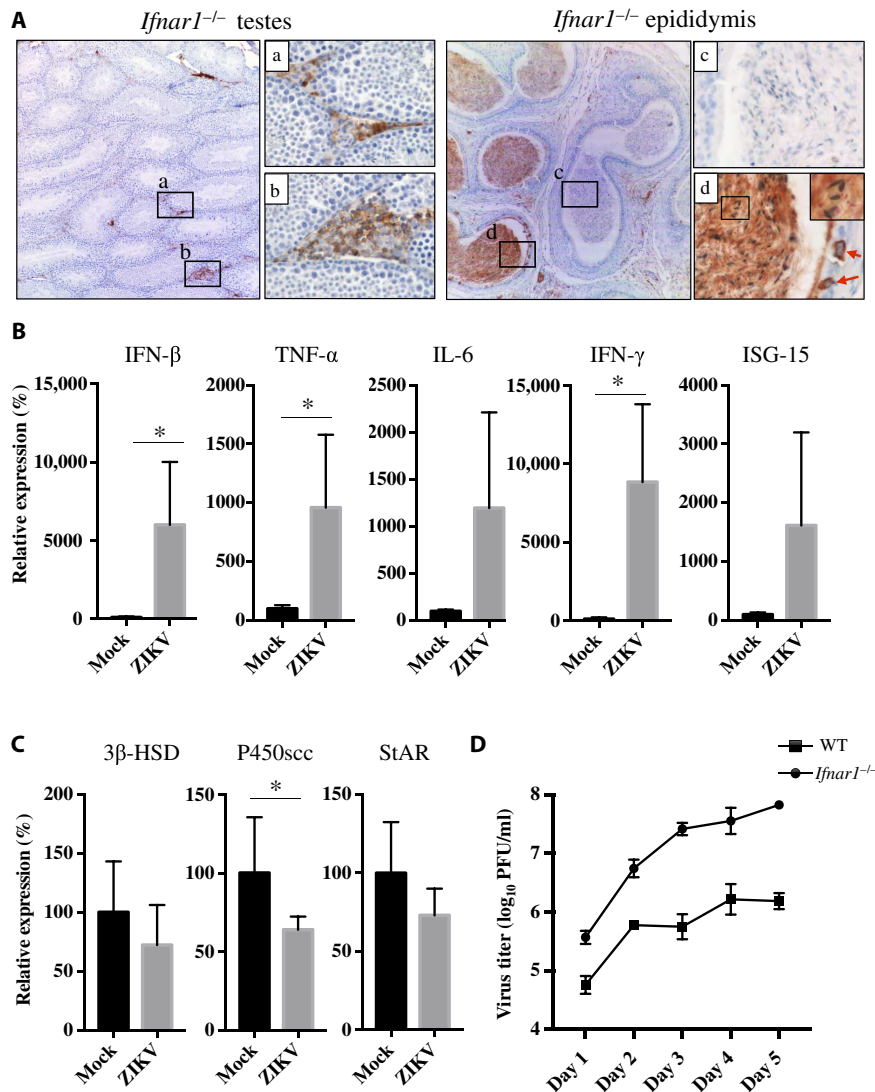


Fig. 2. ZIKV infection in testes and epididymis. *Ifnar1*^{-/-} mice were infected with 10⁵ PFU of ZIKV^{MEX} via subcutaneous route. (A) IHC was performed using an anti-ZIKV NS1 antibody. Left panels (low magnification) show insets shown on the right (higher magnification). Red arrowheads indicate infected epithelial cells. (B and C) At 5 dpi, testes were collected, and total RNA was extracted for qRT-PCR of interferon-β (IFN-β), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IFN-γ, and interferon-stimulated gene 15 (ISG-15) (B). The testicular mRNA expression levels of testosterone-synthetic genes [steroidogenic acute regulatory factor (StAR), cytochrome P-450 side-chain cleavage enzyme (P450_{scc}), and 3β-hydroxysteroid dehydrogenase (3β-HSD)] were examined by qRT-PCR (C). Data are normalized to mouse β-actin (**P* < 0.05). (D) Isolated Leydig cells from WT and *Ifnar1*^{-/-} mice were infected with ZIKV at a multiplicity of infection of 10. At the indicated times after infection, virus titers in the supernatant were determined with Vero cells. The reported values are means ± SD.

studies are required to examine the temporal and molecular details of ZIKV tropism within the reproductive tract (40, 41). In addition, further work is necessary to assess the relative contributions of the innate immune response and/or testosterone reduction in ZIKV-induced testicular atrophy. Regardless, the hypofertility implications of testicular atrophy call for an urgent global need to develop vaccines and antiviral therapeutics.

MATERIALS AND METHODS

Ethics statement

This study was performed in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine.

Cells

C6/36 *Aedes albopictus* cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and 1% tryptose phosphate broth at 30°C in 5% CO₂. Vero cells (African green monkey kidney epithelial cells) were maintained in DMEM with 10% FCS at 37°C in 5% CO₂. C6/36 cells were used for generating ZIKV stocks, and Vero cells were used for plaque assays, as described below.

Virus

An American-derived ZIKV (MEX2-81 strain, referred to as ZIKV^{MEX}) isolated in 2016 was used in this study (19, 42). C6/36 cells were infected with ZIKV^{MEX} and maintained up to 10 days. Cell-free supernatants were collected and stored at -80°C.

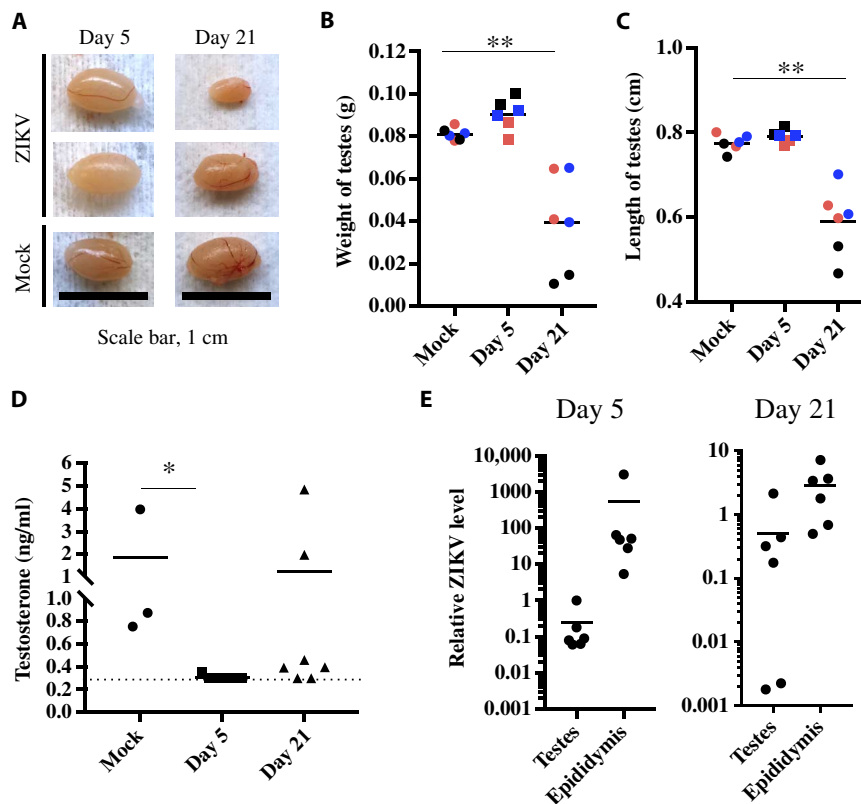


Fig. 3. Testicular abnormalities after ZIKV infection. The testes of *Ifnar1*^{-/-} mice infected with 10⁵ PFU of ZIKV^{MEX} via subcutaneous route and of mock-infected mice were harvested at 5 and 21 dpi. (A) The pictures compare the testes of infected mice. Scale bar, 1 cm. (B and C) Weight and length of whole testes from infected animals were measured ($n = 6$ testes comprising three mice; $**P < 0.01$). Same color indicates the same mice. (D) The levels of testosterone in blood of infected mice were investigated by enzyme-linked immunosorbent assay ($*P < 0.05$). The detection limit is 0.3 ng/ml. (E) The relative viral RNA levels in testes and epididymis at 5 and 21 dpi were examined by qRT-PCR. Data are normalized to mouse β -actin.

Plaque assays

Vero cells were seeded in 12-well plates 24 hours before infection. Serial-diluted ZIKV was incubated for 1 hour at 37°C in 5% CO₂. Then, cells were overlaid with 2% agarose and 2× medium. At 3 to 4 dpi, cells were fixed by 10% formalin and stained with 0.005% amido black, and PFU were counted.

Mouse experiments

Four- to six-week-old *Ifnar1*^{-/-} (C57BL/6 background) and WT C57BL/6 mice were analyzed in this study. Mice were bred in a specific pathogen-free facility at Yale University or purchased from The Jackson Laboratory (WT C57BL/6). Mice were inoculated with ZIKV via subcutaneous injection (footpad; a volume of 50 μ l) with 10⁵ PFU of ZIKV. Survivals and weights were monitored every day up to 15 dpi. Mice exhibiting a weight loss of >20% of initial body weight or neurological disease were euthanized. To examine the viremia, we collected blood samples at 1, 3, 5, 7, 9, and 21 dpi and performed real-time PCR. To assess virus growth or host responses in organs, we euthanized three mice at 5 or 21 dpi, and their organs were collected, homogenized with DMEM with 10% FCS or TRIzol, and titrated using plaque assays or real-time PCR.

RNA extraction, real-time PCR, and PCR

The homogenized organ and blood samples from ZIKV-infected mice were transferred to a fresh 1.5-ml tube to which chloroform was added. The tubes were vortexed well and centrifuged for 10 min at

14,000 rpm at 4°C. The aqueous layers were mixed with 100% ethanol and then subjected to RNA extraction using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Extracted RNA was reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Gene expressions of samples were queried using iQ SYBR Green Supermix. ZIKV RNA or target gene mRNA levels were normalized to mouse β -actin RNA levels according to the $2^{-\Delta\Delta Ct}$ calculations. The qRT-PCR primer sequences are available upon request.

Detection of testosterone in serum

The concentration of testosterone in serum of ZIKV-infected *Ifnar1*^{-/-} mice was performed by using the Mouse Testosterone ELISA Kit (Abcam) according to the manufacturer's instructions.

Isolation of Leydig cells

Leydig cells were isolated from WT and *Ifnar1*^{-/-} mice according to previous studies (36, 43). In brief, the testes of two mice were decapsulated and incubated with collagenase (0.5 mg/ml) in F12/DMEM (Life Technologies) supplemented with 10% FCS at room temperature for 15 min with gentle inverting. The suspensions were filtered through 70- μ m mesh to separate the interstitial cells from seminiferous tubules. The interstitial cells were cultured in F12/DMEM supplemented with FCS. After 24 hours, Leydig cells were detached by 0.125% trypsin treatment for 5 min and resuspended to dish.

Histopathological analysis

The testes of mice were preserved in 4% paraformaldehyde/phosphate-buffered saline for pathological examination. The fixed samples were processed for paraffin embedding. Sections were processed for immunohistological staining with an anti-ZIKV NS1 antibody.

Data analysis

Data analysis was performed using GraphPad Prism and Microsoft Excel. One-tailed Student's *t* test was used to determine the significance of qRT-PCR values for host responses. One-way analysis of variance (ANOVA) was used to compare the weight and length of testes and testosterone levels.

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Zika virus causes testicular atrophy

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