Testosterone protects mice against zika virus infection and suppresses the inflammatory response in the brain

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Highlights

- Testosterone treatment reduces the mortality of ZIKV-infected mice
- Testosterone treatment attenuates ZIKV-induced testicular damage and encephalitis in mice
- Testosterone treatment reduces CD8+ T cell infiltration into the brains of ZIKV-infected mice

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Testosterone protects mice against zika virus infection and suppresses the inflammatory response in the brain

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SUMMARY
Testosterone is essential to human growth and development as well as immune regulation. Zika virus (ZIKV), an emerging arbovirus associated with neurological complications including neuroinflammation, can also cause testicular damage and decrease testosterone secretion. However, whether the dysregulation of testosterone plays a role in the process of neuroinflammation during ZIKV pathogenesis is still unclear. In this study, we found that ZIKV infection caused testicular damage and decreased testosterone secretion in male mice, and testosterone supplementation after ZIKV infection reduced their mortality and attenuated the pathological symptoms. Further investigation revealed that testosterone treatment after ZIKV infection alleviated inflammation and nerve injury in the mouse brain. Additionally, reduced CD8+ T cell infiltration and interferon-gamma production were observed in brains of testosterone-treated mice. Overall, our results demonstrated that testosterone plays a protective role in ZIKV-infected mice, and thus it can be developed as a potential therapeutic drug against ZIKV infection.

INTRODUCTION
ZIKV, as a member of the Flavivirus genus, contains a positive-stranded RNA genome that encodes 3 structural proteins (capsid (C), pre-membrane (prM), and envelope (E)) and 7 non-structural proteins (NS1, NS2A, NS3, NS4A, NS4B, and NS5) (Pierson and Diamond, 2018). Over the half century since its discovery, ZIKV infection has been reported only sporadically until an outbreak in Yap Island in 2007. About three-quarters of Yap residents older than three years of age were infected with ZIKV (Lanciotti et al., 2008). ZIKV massively spread into the Americas in 2015 (Krow-Lucal et al., 2018), and 2016–2017 witnessed the peak of the ZIKV epidemic. A total of 216,207 ZIKV cases were reported in Brazil including 8,604 babies born with deformities during 2016–2017 (Bogoch et al., 2016; Narasimhan et al., 2020).

ZIKV has obvious tissue tropism, and it mainly damages the nervous system and reproductive system (Shahrizaila et al., 2021; Stassen et al., 2018). The increasing reports on the ZIKV infection-related microcephalic births and serious neurological complications draw extensive concern such as Guillain-Barre’ syndrome (GBS) which is an acute inflammatory immune-mediated polyradiculoneuropathy characterized by tingling, progressive weakness, autonomic nervous disorder, and pain (Rodriguez et al., 2018). Non-vector transmission of ZIKV infection can occur via mother-to-child, organ transplantation, blood transfusion, and sexual contact (Kazmi et al., 2020). Recent studies have found that ZIKV can cause hematosperrmia in male patients (Torres et al., 2016), and ZIKV RNA shedding in semen can still be detectable at day 181 after symptom onset (Barzon et al., 2016), which is a potential threat to infected men and their partners. The establishment of type-1 interferon receptor-deficient (A129) mice infection model revealed that ZIKV infection caused testicular damage by inducing the inflammation of the testis and epididymis and reduced testosterone level (Govero et al., 2016; Ma et al., 2016).

Testosterone is a steroid hormone produced by the testis of male or the ovaries of female. Leydig cells are the main testosterone-producing cells (Hall et al., 1969). Testosterone can affect growth and development and regulate the body’s immune response (Trigunaite et al., 2015). One previous study has revealed that sex hormone differences are the reason why women are more likely to develop autoimmune diseases.
than men (Whitacre, 2001). For example, an animal study of systemic lupus erythematosus (SLE), an autoimmune inflammatory disease, has indicated that female mice treated with testosterone have been found to have reduced disease severity and longer survival (Roubinian et al., 1977). Rheumatoid arthritis (RA) is a systemic disease of articular and extrarticular co-existence caused by an autoimmune response mainly in women. Testosterone prevents the development of joint and lung disease in male SKG mice (a mouse model of rheumatoid arthritis) (Keith et al., 2013). Additionally, testosterone inhibits tumor development through immunosuppression. For instance, in follicular thyroid cancer, testosterone has been reported to influence tumor progression by inhibiting CD8+ T cells and M1 macrophages (Zhang et al., 2015). Testosterone also plays an important role in the CNS and can improve cognitive performance by enhancing synaptogenesis (Frye et al., 2004). Treatment with testosterone reduces the rate of whole-brain atrophy (Sicotte et al., 2007) and preserves excitatory synaptic transmission during autoimmune demyelination (Ziehn et al., 2012). However, there are few reports on the role of testosterone in ZIKV pathogenesis.

In this study, we found that ZIKV infection caused testicular damage and decreased testosterone secretion. Testosterone treatment reduced the mortality of ZIKV-infected mice. Further analysis revealed that testosterone treatment inhibited the activation of CD8+ T cells in spleen and their infiltration into brain and reduced the expression of inflammatory cytokines in brain tissue. These results suggest that testosterone plays a protective role in ZIKV infection, indicating it may be a potential candidate drug for the treatment of ZIKV infection.

RESULTS
Zika virus infection causes testicular damage and testosterone decline in mice
ZIKV has obvious tissue tropism, and previous studies have shown that the brain and testis are the main target organs of the virus in male mice (Christian et al., 2019; Ma et al., 2016). In this study, to investigate the effect of ZIKV on testicular injury, the testis of male A129 mice were pathologically analyzed at 8 days post-infection (dpi) with ZIKV. Compared with the uninfected mice, the ZIKV-infected mice showed obvious hyperemia in vas deferens and testis (Figures 1A and 1B). The original structure of the seminiferous tubules of ZIKV-infected mice was damaged, and thus a large number of spermatogonial cells were detached from the seminiferous tubule wall and entered into the spermatogenic tubule center (Figure 1C). Testosterone is mainly produced by the testes. To determine whether testicular damage caused by ZIKV infection could affect testosterone secretion, the concentrations of testosterone in serum and testis were determined on day 8 post-ZIKV infection. The results showed that ZIKV infection reduced the levels of testosterone in both serum and testis (Figures 1Da and 1E).

Testosterone supplementation decreases Zika virus-induced mouse lethality
Previous studies have shown that ZIKV can target the mouse brain and cause severe neuroinflammation (Figueiredo et al., 2019). Considering the immunosuppressive effect of testosterone, we speculated that the decreased secretion of testosterone may play a role in ZIKV pathogenesis. To this end, ZIKV-infected A129 mice were intraperitoneally injected with testosterone or the same dose of sesame oil at 2 and 4 dpi (Figure 2A). At 8 dpi, the serum testosterone concentration was measured. The results showed that testosterone level was largely increased in testosterone-treated mice compared with that in sesame oil-treated mice (Figure 2B). By observing the survival rate of mice, we found that all the mice in the testosterone group or control group survived, while mice in ZIKV + sesame oil group and in ZIKV + testosterone group started to display morbidity on 8 and 10 dpi, respectively. After ZIKV infection, a high mortality rate (90%) of mice was observed in the ZIKV + sesame oil group, while the mortality rate of mice in the ZIKV + testosterone group was 50% (Figure 2C). In addition, the body weight and clinical scores (Figure 2E) of mice in each group were analyzed. The results showed that although testosterone treatment did not affect the weight loss upon ZIKV infection (Figure 2D), it alleviated the occurrence of disease in ZIKV-infected mice.

Testosterone treatment reduces Zika virus-induced testicular damage and inflammatory response in mouse brain tissues
To evaluate the role of testosterone on ZIKV-induced testicular injury, the morphologic and pathologic changes of the reproductive tract in ZIKV-infected male mice after testosterone treatment were observed at 8 dpi. The results showed that testosterone treatment following ZIKV infection reduced the hyperemia of male mice reproductive tract (Figure 3A), and alleviated testicular lesions (Figure 3B),
suggesting the testosterone treatment attenuated the ZIKV-induced damage of the reproductive tract of male mice.

Brain is one of the main target organs for ZIKV, and the invasion of ZIKV into the brain of fetuses and adults can cause encephalitis and meningoencephalitis (Carteaux et al., 2016; de Almeida Oliveira Evangelista et al., 2021). Given the role of testosterone in reducing ZIKV-induced mouse mortality, we investigated the effect of testosterone on the neuroinflammation caused by ZIKV infection. To this end, H&E staining was performed for histopathological changes analysis of the brain sections at 8 dpi. Perivascular cuffing was observed in ZIKV-infected mice at 8 dpi, but this symptom was obviously alleviated after ZIKV-infected

Figure 1. ZIKV infection causes testicular damage and reduces serum testosterone level in mice
A129 mice were intraperitoneally injected with 10^3 PFU of ZIKV H/PF/2013 strain in 100 μL of DMEM or equal volume of DMEM. Male reproductive system and serum samples were collected on 8 dpi.
(A and B) Comparison of male reproductive system (A) and testicular appearance (B) between ZIKV-infected and uninfected mice.
(C) H&E staining of mouse testis samples. After ZIKV infection, the structure of seminiferous tubules was disordered with numerous shed spermatogonial cells in the center of seminiferous tubules (black arrow). Scale bars, 500 μm.
(D and E) Testosterone levels in serum and testis of mice with or without ZIKV infection were determined by ELISA. Data were expressed as mean ± SEM of three independent experiments (n = 3 mice). *p < 0.05 was considered statistically significant.
mice received testosterone treatment (Figure 3C). In addition, testosterone treatment relieved meningitis (Figure 3D) and hemorrhage around the hippocampus (Figure 3E) caused by ZIKV infection. Furthermore, RT-qPCR results showed that testosterone treatment inhibited the up-regulation of proinflammatory cytokines IL-6 and TNF-α induced by ZIKV infection (Figures 3F and 3G). Then the viral RNA level was measured, and the results showed that testosterone treatment reduced viral load in mouse brain (Figure 3H). Taken together, our results demonstrated that testosterone treatment reduced inflammation and pathological changes in mouse brain caused by ZIKV infection.

Testosterone treatment has no effect on viral replication in A549 and TM4 cells

To investigate whether testosterone treatment alleviated the mouse neuroinflammation by interfering with viral replication, A549 cells and TM4 cells, which were known to express testosterone receptor (Deng et al., 2017; Kaiser et al., 1996), were used to evaluate the effect of testosterone on ZIKV replication in vitro. The cell viability assay was performed to examine the cytotoxic effect of testosterone on A549 cells and TM4 cells, and the results revealed that 10 μg/mL testosterone exhibited no cytotoxic effect, while a
Figure 3. Testosterone treatment reduces ZIKV-induced testicular damage and inflammatory response in mouse brain

ZIKV-infected A129 mice were intraperitoneally injected with testosterone or the same dose of sesame oil at 2 and 4 dpi. Tissue samples were collected on 8 dpi.

(A) Comparison of the male reproductive system appearance between testosterone-treated and untreated mice after ZIKV infection.

(B) H&E staining of mouse testis samples. Scale bars, 500μm.

(C–E) Pathological changes in brain tissues were analyzed by H&E staining. Testosterone treatment reduced the infiltration of inflammatory cells and the formation of perivascular cuffing (C), attenuated meningitis (D), and decreased the number of red blood cells around the hippocampus (E) in brain tissues of ZIKV-infected mice. Scale bars, 500μm.

(F and G) mRNA expression levels of IL-6 (F) and TNF-α (G) were determined by real-time RT-qPCR (n = 6 mice).

(H) Viral RNA levels in mouse brain were determined by real-time RT-qPCR (n = 6 mice). *p < 0.05, **p < 0.001.
100 μg/mL concentration displayed significant cytotoxicity (Figure 4A). Thus, 1 × 10^5 A549 cells and TM4 cells were treated with different concentrations of testosterone in DMSO. After 48 h, cytotoxic effect of testosterone on A549 cells and TM4 cells was determined by using luminescence-based cell viability assay. (B and C) 1 × 10^5 A549 cells and TM4 cells were seeded on a 24-well cell culture plate and then infected with 0.5 MOI ZIKV meanwhile maintaining cells in 10 μg/mL testosterone diluted in 1μL DMSO or equal volume of DMSO. At 24, 36, and 48 h post-infection (hpi), cells were collected, and the viral RNA levels (B) and titers (C) were determined by RT-qPCR and plaque assay respectively.

Testosterone treatment inhibits inflammatory response rather than type-I interferon response in Zika virus-infected A549 cells

Since type-I interferon (IFN-I) receptor-deficient mice were used in this study, the in vivo experiments could not evaluate the effect of testosterone on IFN-I response during ZIKV infection. Therefore, A549 cells were infected with ZIKV followed by testosterone or mock treatment. Cells were collected at 24 hpi, and mRNA levels of IFN-β and ISGs, including ISG15, OAS1, and MX1, were measured by RT-qPCR. The results showed that testosterone treatment did not affect the transcription of IFN-β and its downstream ISGs during ZIKV infection (Figures 5A–5D).
To further explore the effect of testosterone on inflammatory response caused by ZIKV infection, expression of TNF-α and IL-6 were examined. The results showed that testosterone treatment significantly reduced the mRNA levels of TNF-α (Figure 5E); however, no obvious difference in IL-6 expression was shown in testosterone-treated cells (Figure 5F), suggesting a selective inhibitory effect of testosterone on the expression of inflammatory cytokines during ZIKV infection in vitro.

**Figure 5. Testosterone inhibits inflammatory response rather than IFN-I response in ZIKV-infected A549 cells**

A549 cells were infected with ZIKV at 0.5 MOI followed by the treatment of testosterone or equal concentration of DMSO. Total cellular RNA was extracted at 24 hpi. mRNA levels of IFN-β (A), ISG15 (B), OAS1 (C), and MX1 (D), TNF-α (E), IL-6 (F) were determined by RT-qPCR. All experiments were performed in three independent biological replicates, with each experiment being performed three times *p < 0.05, **p < 0.01, ***p < 0.001. To further explore the effect of testosterone on inflammatory response caused by ZIKV infection, expression of TNF-α and IL-6 were examined. The results showed that testosterone treatment significantly reduced the mRNA levels of TNF-α (Figure 5E); however, no obvious difference in IL-6 expression was shown in testosterone-treated cells (Figure 5F), suggesting a selective inhibitory effect of testosterone on the expression of inflammatory cytokines during ZIKV infection in vitro.
Testosterone inhibits Zika virus-induced CD8+ T cell activation in spleen

Since no effect of testosterone on viral replication and IFN-I response was observed, we further evaluate its effect on adaptive immunity during ZIKV infection. Firstly, the antibody levels in the serum of ZIKV-infected mice with or without the treatment of testosterone were determined. Serum samples were collected on 8 dpi, and the antibody level against ZIKV E protein was detected by ELISA. The serum of mice immunized with E protein was used as positive control and uninfected mouse serum was used as a negative control. No significant difference in antibody levels was observed between testosterone-treated and untreated-mice after ZIKV infection (Figure 6A). These results suggested that testosterone did not regulate the level of binding antibodies against ZIKV E protein during ZIKV infection.

To further investigate how testosterone participated in the pathogenic process of ZIKV infection, the distribution of CD4+ T cells and CD8+ T cells in spleen was observed by immunohistochemistry. As shown in Figures 6B and 6C, CD4+ T cells and CD8+ T cells were mainly present in splenic corpuscles. After ZIKV infection, a large number of CD8+ T cells were observed outside the splenic corpuscles (black arrow); However, testosterone injection reduced the number of CD8+ T cells outside the splenic corpuscles. Then, we analyzed the proportion of CD4+ T cells and CD8+ T cells in the spleen of mice by flow cytometry. The results showed that ZIKV infection led to a significant increase in the proportion of CD3+ T cells in spleen, but testosterone injection inhibited the increase of CD3+ T cells induced by ZIKV infection (Figures 6D and 6E). Although testosterone treatment did not significantly change the proportion of CD8+ T cells in CD3+ T cells, it significantly reduced the proportion of CD8+ T cells in total spleen cells (Figures 6D, 6F, and 6G). However, infection with ZIKV did not change the proportion of CD4+ T cells in CD3+ T cells and total spleen cells (Figures 6D, 6H, and 6I). These results suggested that testosterone treatment could suppress the CD8+ T activation during ZIKV infection.

Testosterone treatment reduces CD8+ T cell infiltration into the brain of mice infected with Zika virus

ZIKV infection can cause infiltration of CD8+ T cells into brain and produce inflammatory response (Huang et al., 2017). To determine the effect of testosterone on the infiltration of CD8+ T cells into mouse brain after ZIKV infection, the immunohistochemical assay was performed to examine the number of CD8+ T cells. The results showed that there were more CD8+ T cells in the ZIKV-infected mouse brain than those in the uninfected mouse brain, while testosterone treatment reduced the CD8+ T cell infiltration caused by ZIKV infection (Figure 7A). To further verify this result, the mouse brain cells were isolated for flow cytometry assay (Figure 7B). Consistently, the results showed that the number of CD8+ T cells increased significantly after ZIKV infection, while testosterone supplementation significantly reduced the CD8+ T cell number in mouse brain (Figures 7C and 7D). These results suggested that testosterone treatment could suppress the infiltration of CD8+ T cells into the the brain of ZIKV-infected mice.

Testosterone treatment reduces the expression of interferon-γ and chemokine in the brain of Zika virus-infected mice

IFN-γ, mainly expressed by T cells, plays an important role in the regulation of immune response, and testosterone has been reported to inhibit IFN-γ secretion (Ho et al., 2017). Thus, we detected the levels of IFN-γ in mouse serum and brain on day 8 after ZIKV infection by ELISA. The results showed that the concentrations of IFN-γ in the brain and serum were significantly increased after ZIKV infection, and the increase was inhibited by testosterone treatment (Figures 8A and 8B). Considering that chemokines play important role in recruiting T cells, the mRNA levels of chemokines such as CCL2 and CCL5 in mouse brain were analyzed by RT-qPCR. The results indicated that testosterone treatment inhibited the upregulation of chemokines caused by ZIKV infection (Figures 8C and 8D). To further verify this result, A549 cells were infected with ZIKV followed by the treatment of testosterone, and the mRNA levels of CCL2 and CCL5 were examined. As expected, testosterone treatment significantly reduced the expression of CCL2 and CCL5 after ZIKV infection (Figures 8E and 8F). These results revealed that testosterone may inhibit the infiltration of CD8+ T cells into the mouse brain by reducing the chemokine production during ZIKV infection.

DISCUSSION

ZIKV infection usually leads to mild clinical symptoms, but some patients also develop inflammation in the CNS, ending up with neurological sequelae (Barbi et al., 2018). In this study, we found severe inflammation in the brain of mice infected with ZIKV, which might be attributed to the infiltration of activated CD8+ T cells.
into the brain. CD8+ T cells are important immune cells that can release inflammatory factors and kill target cells directly. One previous study has reported that CD8+ T cells can prevent ZIKV infection in the CNS (Huang et al., 2017). However, some other studies have indicated that the infiltration of a large number of CD8+ T into the brain would lead to segmental apoptotic neurodegeneration, astrocyte proliferation,
and microglial activation, and eventually result in encephalitis and even death of the organism (Melzer et al., 2009; Pitsch et al., 2021). In this study, neurologic symptoms such as hindlimb paralysis were observed in mice infected with ZIKV, but whether these neurologic symptoms were related to the infiltration of CD8+ T cells into the brain remains to be further investigated.

Inflammation is a special function of the immune system, and it can resist the invasion of pathogens. However, some pathogens cause an excessive storm of inflammation, thus damaging the organism. After
SARS-CoV-2 infection, macrophages and monocytes are recruited to release cytokines and initiate adaptive T and B cell immunity in response to infection, and this response process usually clears up viral infection, but in some patients, a dysfunctional immune response can trigger a cytokine storm, thus mediating widespread lung inflammation (Tay et al., 2020). It has been reported that the levels of IL-2, IL-7, IL-10, IP-10, MCP1, and TNF-α in blood plasma were significantly increased after patients were severely infected with SARS-CoV-2 (Huang et al., 2020). Therefore, the suppression of inflammation caused by viral infection...
is an important treatment strategy against COVID-19 (Yuin Ho et al., 2020). ZIKV infection can also lead to the inflammation of the brain, thus resulting in sequelae and even death of the host. Similarly, the regulation of inflammation intensity might also be a potential treatment strategy after the ZIKV infection.

Multiple studies have found that there are some obvious differences in immune response between men and women. For example, women are more prone to autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Pikwer et al., 2014; Roubinian et al., 1977). These differences might be owing to sex hormone differences. The gender statistics of patients with ZIKV reveal that the proportion of female patients is higher than that of male patients (Bastos and Coelho, 2018). The two possible explanations are as follows: One is that ZIKV can be sexually transmitted from male to female, resulting in an increase in the number of female patients. The other one is that female patients tend to seek medical treatment owing to pregnancy and other factors, thus resulting in an increase in the number of statistics. In this study, based on our data, we speculated that different sex hormones might lead to more female patients infected with ZIKV than male ones, and thus supplementing or antagonizing hormones might be a strategy to regulate immune responses, treat autoimmune diseases, or inhibit virus-induced inflammatory storms.

Testosterone, as a sex hormone produced in the body, has been used to treat sexual dysfunction and osteoporosis in both men and women (Golds et al., 2017; Ingram et al., 2020). The advantage of treatment with testosterone over other drugs is its safety in humans (Salter and Mulhall, 2019). In recent years, many studies of testosterone treatment have reported that testosterone can inhibit interferon, inflammatory factors, and other immune responses (Tuku et al., 2020; Vom Steeg et al., 2020). Although the immune response including inflammation can protect the body from pathogens, excessive inflammation can cause damage to the body itself, and the application of testosterone may balance the resistance to pathogens and the damage to the body itself. This study provides evidence that testosterone plays an important role in the regulation of ZIKV-induced neuroinflammatory response. Our in vitro and in vivo experiments have shown that testosterone treatment has a protective effect on mice infected with ZIKV by reducing CD8+ T cells, IFN-γ, and inflammatory factors in the mouse brain. These results indicate that testosterone may be used as a potential drug for the treatment of ZIKV-associated diseases.

Limitations of the study
In our study, testosterone was found to protect mice against ZIKV infection. Testosterone injection after ZIKV infection increased the survival rate of mice by 40% and attenuated inflammatory response in mouse brain. The protection efficiency of testosterone in mice infected with ZIKV may be further improved by exploring the timing, route, and dose of testosterone treatment. In addition, only the male mouse model was used in our experiment, since ZIKV infection could lead to the reduction of testosterone release in male. Although women can be treated with testosterone clinically, the protective effect of testosterone on female and adverse fetal outcomes remains to be further studied.

STAR METHODS
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AUTHOR CONTRIBUTIONS

ZBH, CSB, SYH, and YJ designed research; ZBH, SJJ, LHR, and YLE performed experiments; ZBH, LQ, and ZLP analyzed data; ZBH and SJJ organized the data; ZBH, CSB, SYH, and YJ wrote the article. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| CD3-FITC            | BD     | Cat#555274 |
| CD4-PE              | BD     | Cat#553730 |
| CD8-APC             | BD     | Cat# 553035 |
| DAPI                | Invitrogen | Lot 2116139 |
| Bacterial and virus strains |        |            |
| ZIKV H/PF/2013 strain | Provided by Dr. Bo Zhang, Wuhan Institute of Virology | GenBank: KJ776791 |
| Chemicals, peptides, and recombinant proteins | | |
| Testosterone        | Aladdin | Lot#G2030170 |
| Critical commercial assays | | |
| Mouse IFN-gamma ELISA Kit | ABclonal | RK00019 |
| Testosterone ELISA   | abcam  | Ab108666  |
| Trizol reagent       | Invitrogen | 15596026 |
| 2X Universal SYBR Green Fast qPCR Mix | ABclonal | RK21203 |
| ABScript II cDNA First-Strand Synthesis Kit | ABclonal | RK20400 |
| Experimental models: Cell lines | | |
| A549                 | ATCC   | CCL-185    |
| TM4                 | ATCC   | CRL-1715   |
| Vero                | ATCC   | CCL-81     |
| Experimental models: Organisms/strains | | |
| A129                | (Dowall et al., 2016) | https://pubmed.ncbi.nlm.nih.gov/27149521/ |
| Software and algorithms | | |
| FlowJo              | BD     |            |
| GraphPad Prism      | GraphPad |            |
| QuantStudio™ Real-Time PCR Software | Thermo Fisher | https://www.thermofisher.cn/cn/zh/home/global/forms/life-science/quantstudio-3-5software.html |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jing Ye (yej@mail.hzau.edu.cn).

Materials availability
This study did not generate new unique reagents. All unique/stable reagents used in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability
- This paper does not report original code.
- All data reported in this paper will be shared by the lead contact upon request.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal experiments
All mouse experimental procedures were approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University with ID Number of HZAUOMO-2021-0164. Adult male 8-week-old A129 mice were purchased from the Laboratory Animal Center of Huazhong Agricultural University, Wuhan, China. The mice were randomly divided into four groups. Group 1 (control group) was treated with sesame oil; Group 2 (testosterone group) was treated only with testosterone; Group 3 (ZIKV + sesame oil group) was infected with ZIKV followed by treatment with sesame oil; and Group 4 (ZIKV + testosterone group) was infected with ZIKV followed by treatment with testosterone. Mice in ZIKV infection group and ZIKV + testosterone groups were intraperitoneally injected with 10^3 PFU of ZIKV H/PF/2013 strain in 100 μL of DMEM, and mice in other groups were injected with equal volume of DMEM. On day 2 and 4 after ZIKV infection, the mice in the testosterone group and ZIKV + testosterone group were intraperitoneally injected with 0.05mg testosterone in 100 μL of sesame seed oil, and mice in other groups were injected with equal volume of sesame seed oil.

Cell culture and virus
TM4 (mouse Sertoli cell line) cells, Vero (African green monkey kidney cell line) cells, and A549 (human alveolar epithelium cell line) cells were maintained in Dulbecco modified Eagle medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C in a 5% CO2 atmosphere. ZIKV H/PF/2013 strain (GenBank: KJ776791) was kindly provided by Dr. Bo Zhang, Wuhan Institute of Virology, Chinese Academy of Sciences, propagated, and titrated on Vero cells.

METHOD DETAILS

Virus infection
TM4 cells or A549 cells were cultured in 24-well plates. After being washed with DMEM, cells were infected with ZIKV H/PF/2013 strain at multiplicity of infection (MOI) of 0.5. The mock-infected cells were prepared using the same procedures without virus infection.

Testosterone
Testosterone purchased from Aladdin was dissolved in chloroform at a concentration of 100 mg/mL for storage, and it was diluted with dimethyl sulfoxide (DMSO) or sesame oil for in vitro and in vivo experiments respectively.

Cell viability assay
CellTiter-Glo® One Solution Assay kit (Promega) was used to measure cell viability. A549 cells and TM4 cells were seeded on a 96-well cell culture plate at a density of 2x10^3 cells/mL, and incubated at 37°C with 5% CO2 for 24 h. The medium was replaced with that containing different concentrations of testosterone in DMSO. After 48 h, the cells were washed with phosphate buffered saline (PBS), and 100 μL CellTiter-Glo reagent was added to each well. The cells were stirred in a shaker for 2 min and then incubated at room temperature for 10 min for full cell lysis. The luminescence signal under each condition was measured using a multi-template reader, and the luminescence value of treatment groups was compared with that of control group.

RNA extraction and quantitative real-time PCR (RT-qPCR)
Total RNA of cells was extracted with Trizol reagent (Invitrogen), and 1μg of RNA was used to synthesize cDNA using a ABscript1 cDNA First Strand synthesis kit (ABclonal Technology). The RT-qPCR was performed in a 7500 real-time PCR system (Applied Biosystems) with 2X Universal SYBR Green Fast qPCR Mix (ABclonal Technology). The data were normalized to the level of β-actin expression in each sample. The primers used for RT-qPCR were as follows ( M: mouse; H: human ):

TNF-α-F (M): 5’-CAGGCGGTGCTATGTCTC -3’;
TNF-α-R (M): 5’-CGATCACCCTGGAAGTACGTA -3’;
CCL-5-F (M): 5’-TGCCCCAGTCAAGGATATTTC -3’;

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CCL-5-R (M): 5’-AACCCACTTCTCTCTGGGTTG-3’;
IL-6-F (M): 5’-CATGTTCTCTGGGAAATCGTG-3’;
IL-6-R (M): 5’-TCCAGTTTGTAGCATCCATC-3’;
CCL-2- (M)F: 5’-CGGCGAGATCAGAACCTACAAC-3’;
CCL-2- (M)R: 5’-GGCACTGTCACACTGGTCACTC-3’;
β-actin-F (M): 5’-CACTGCCGCATCCTCTTCCTCCC-3’;
β-actin-R (M): 5’-CAATAGTGATGACCTGGCCGT-3’;
ZIKV-F: 5’-AACATGGCGGAGGTAAGA-3’;
ZIKV-R: 5’-TGTCTGATTGCTTGTCAAGGT-3’;
OAS1-F (H): 5’-CTGGATTCCTGCTGGCTGAAAG,
OAS1-R (H): 5’-CTGGAGTGTGCTGGGTCTATG;
MX1-F (H): 5’-GGGCTTGTGAATTCTGTGGC,
MX1-R (H): 5’-CCTTGGAAATGGTGCTGGAT;
IFN-β-F (H): 5’-TGCTCTGGCACAACAGGTAG,
IFN-β-R (H): 5’-AGCCTCCCATCAATTGCCA;
ISG15-F (H): 5’-ACAGCCATGGGCTGGGA,
ISG15-R (H): 5’-GATCTGCCTCTCTAGCCT;
β-actin-F (H): 5’-AGCGGGAAATCGTGCGTGAC-3’;
β-actin-R (H): 5’-GGAAGGAAGGCTGGAAGAGTG-3’;
TNF-α-F (H): 5’-GCTGCATTTGGAGTGATCG-3’;
TNF-α-R (H): 5’-GAGGGTTTGCTACAACATGG-3’;
IL-6-F (H): 5’-TCAATGAGGAGACTTGCTGG-3’;
IL-6-R (H): 5’-GGGTCAGGGGTGGTTATTGC-3’;
CCL-2- (H)F: 5’-CAATCAATGCCCCAGTCACC-3’;
CCL-2- (H)R: 5’-CCTGAACCCACTTCTGCTTG-3’;
CCL-5-F (H): 5’-GTGCCAACCCAGAGAAGAAGT-3’;
CCL-5-R (H): 5’-GAGCAAGCAATGACAGGAAGAAG-3’;

**Enzyme-linked immunosorbent assay (ELISA)**

The mice brain tissues were homogenized in PBS and centrifuged at 5000×g to obtain the supernatants. IFN-γ level in the brain was determined according to the instructions of ELISA kit purchased from ABclonal.
Technology. After the mice blood was placed at 37°C for 30 min, the serum was separated by centrifugation at 1000 × g for 5 min. Serum testosterone level were detected using an ELISA kit purchased from Abcam.

Flow cytometry
The mice spleens were separated, added with PBS, ground, and filtered with a 40 μm cell strainer (FALCON), followed by centrifugation at 1600 RPM at 4°C for 5 min. After the supernatants were removed, 2 mL red blood cell lysis solution was added to re-suspend the cells, and the cells were incubated at room temperature for 5 min for full lysis of red blood cells, followed by adding 8 mL PBS to stop the lysis. After centrifugation of the lysis solution, the supernatant was discarded, and the cells were suspended by 0.2% BSA. The 10^6 cells were incubated with antibodies CD3-FITC, CD4-PE, and CD8-APC purchased from BD BioSciences. Cell nuclei were stained with 6-diamidino-2-phenylindole (DAPI, Invitrogen) to distinguish dead cells. The numbers of CD8\(^+\) and CD4\(^+\) T cells were detected and analyzed by flow cytometry.

QUANTIFICATION AND STATISTICAL ANALYSIS
All experiments were conducted at least three times under similar conditions. The data were analyzed using GraphPad Prism version 5 and presented as mean ± standard error (SEM). The statistical analysis of differences between two groups was performed using two-tailed Student’s t-test. Two-way ANOVA was conducted for multiple comparisons and Bonferroni posttest was used to determine statistical differences between groups. In all tests, p < 0.05 was considered as statistically significant.