Viral Retinopathy in Experimental Models of Zika Infection

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With formidable teratogenic effects, Zika virus (ZIKV) has gained global attention since the first American outbreak in Brazil.1 Vertical transmission of ZIKV during pregnancy can cause a myriad of malformations exemplified by microcephaly. Ocular abnormalities, including microphthalmia, as well as chorioretinal atrophy and gross pigmentation, have been identified in 34% to 55% of infants with microcephaly.2,3 Eye signs and symptoms in adult ZIKV infection have also been reported, with viral RNAs detected in both serum and aqueous humor of the patient.4 ZIKV infection in the eye can affect any part of the uveal tract (iris, ciliary body, retina, and choroid), while most clinical cases have defects in the posterior segment.5–7 There was a case report of severe vision decline while most clinical cases have defects in the posterior part of the uveal tract (iris, ciliary body, retina, and choroid), while most clinical cases have defects in the posterior segment.5–7 There was a case report of severe vision decline in permissive retinal cells were assessed in cultured retinal pigment epithelial (RPE) and Müller cells. Results. ZIKV-infected mice developed a spectrum of ocular pathologies that affected multiple layers of the retina. A primary target of ZIKV in the eye was Müller glial cells, which displayed decreased neurotrophic function and increased expression of proinflammatory cytokines after infection. ZIKV also infected RPE; and both the RPE and Müller cells expressed viral entry receptors TYRO3 and AXL. Retinitis, focal retinal degeneration, and ganglion cell loss were observed after the clearance of viral particles. Conclusions. Our data suggest that ZIKV can infect infant eyes with immature blood–retinal barrier and cause structural damages to the retina. The ocular findings in microcephalic infants may not be solely caused by ZIKV-induced impairment of neurodevelopment.

Keywords: Zika virus, AXL, Tyro 3, Müller cell, RPE

Purpose. Emerging evidence has shown that both congenital and adult Zika virus (ZIKV) infection can cause eye diseases. The goals of the current study were to explore mechanisms and pathophysiology of ZIKV-induced eye defects.

Methods. Wild-type or A129 interferon type I receptor-deficient mice were infected by either FSS13025 or Mex1-7 strain of ZIKV. Retinal histopathology was measured at different time points after infection. The presence of viral RNA and protein in the retina was determined by in situ hybridization and immunofluorescence staining, respectively. Growth curves of ZIKV in permissive retinal cells were assessed in cultured retinal pigment epithelial (RPE) and Müller glial cells.

Results. ZIKV-infected mice developed a spectrum of ocular pathologies that affected multiple layers of the retina. A primary target of ZIKV in the eye was Müller glial cells, which displayed decreased neurotrophic function and increased expression of proinflammatory cytokines after infection. ZIKV also infected RPE; and both the RPE and Müller cells expressed viral entry receptors TYRO3 and AXL. Retinitis, focal retinal degeneration, and ganglion cell loss were observed after the clearance of viral particles.

Conclusions. Our data suggest that ZIKV can infect infant eyes with immature blood–retinal barrier and cause structural damages to the retina. The ocular findings in microcephalic infants may not be solely caused by ZIKV-induced impairment of neurodevelopment.
Medical Branch (UTMB). All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A129 mice were obtained from a colony at UTMB and were maintained under pathogen-free caging and reared until the desired age. Wild-type C57BL/6J mice were initially purchased from Jackson Lab (Bar Harbor, ME, USA). Mice were inoculated with 100 μL ZIKV diluted to 1 × 10^6 plaque-forming units (PFU)/mL in phosphate-buffered saline (PBS) via the intraperitoneal route. Daily checks were performed on mice to monitor for signs of illness and morbidity. All mice meeting moribund criteria (tremors, paralysis, severe lethargy, and/or >20% weight loss) were euthanized. At the time of necropsy, mice were euthanized by CO₂ asphyxiation and exsanguinated by intracardiac puncture. One eye was harvested for viral titration and the other was fixed in 4% paraformaldehyde for at least 24 hours prior to histology.

**Viruses**

The ZIKV Mex1-7 strain (ZIKVMEX) was isolated from a pool of Aedes mosquitoes from Mexico in 2015 and passed three times in Vero cells prior to use. The FSS13025 strain of ZIKV (ZIKVFSS) was obtained from the World Reference Collection for Emerging Viruses and Arboviruses (WRCEVA) cultivated at UTMB. ZIKVFSS was passaged in the following cell lines prior to use: 1X AP-1, 1X G6/36, and 5X Vero 2.

**Cell Culture**

Primary cultures of mouse retinal Müller cells were established from neonatal mice (postnatal day [P]3–P5), following established methods with modifications. Briefly, retina tissues were dislodged into single-cell suspension after collagenase (Worthington Biochemical, Lakewood, NJ, USA) digestion, filtered through 40-μm nylon strainer (Thermo Fisher Scientific, Waltham, MA, USA), and collected by centrifugation. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 20% fetal bovine serum (FBS) (Sigma-Aldrich Corp., St. Louis, MO, USA), 20% L929 cell-conditioned medium, 2 mM Glutamax (Thermo Fisher Scientific, 100 U/mL penicillin and streptomycin. After 10 to 14 days in culture, cells were treated with 0.05% trypsin-EDTA for 1 minute at 37°C. Microglia remained attached to the plate. The majority of dissociated cells were glutamine synthetase (GS)-positive Müller cells and were used for the in vitro infection experiments without further passing.

Primary cultures of human fetal RPE (hfRPE) cells were established as previously described. Cells were grown and passaged in alpha-modified Eagle's medium (α-MEM) containing 10% FBS and N1 supplements (Sigma-Aldrich Corp.). Before seeding, the plates and wells were coated with collagen (STEMCELL Technologies, Vancouver, BC, Canada). Cells between passages 3 and 6 were used for experiments. Viral infection was performed in growth media at 10:1 multiplicity of infection (MOI) for 1 hour. Afterward the viral inoculation was removed and cells were replenished with fresh medium.

**Titrations of Tissues and Serum Viral Load**

Upon necropsy, one eye with the optic nerve was collected and homogenized with TissueLyser II (QIAGEN, Hilden, Germany). Blood from the sample animal was spun at 3000 g for 5 minutes and serum was transferred to a separate tube. All titrations were performed as previously described. Briefly, Vero cell monolayers were infected with 10-fold serial dilutions of samples for 1 hour, followed by overlaying of semisolid 4% methylcellulose in DMEM. Cultures were incubated for 3 days prior to removing the overlay, washed once with PBS, and fixed with a 50:50 vol/vol mixture of methanol and acetone for 30 minutes. ZIKV-infected foci were visualized by immunohistochemistry. Final titers were reported as PFU/mL serum or PFU/g tissue. The average eye weight of 0.016 g was used for calculations.

**Histology and Immunofluorescence Microscopy**

Paraffin sections of posterior eyes were prepared as described previously. Sagittal sections of 4-μm thickness were cut from cornea to optic nerve and stained with hematoxylin and eosin (H&E). For immunofluorescence labeling, an antigen retrieval step was performed by boiling sections in 10 mM sodium citrate buffer (pH 6.2) (Thermo Fisher Scientific) for 20 minutes. The sources of antibodies used for the study are listed in Supplementary Table S1. Fluorescence images were acquired on a Carl Zeiss Observer Z1 microscope (Thornwood, NY, USA) equipped with Apopome and ZEN imaging software.

**Dual Fluorescent RNA In Situ Hybridization**

In situ hybridization was performed with the ViewRNA ISH tissue assay kit (Affymetrix, Cleveland, OH, USA), following the manufacturer's recommendations. Sections of 8-μm thickness were deparaffinized and digested with protease at 40°C for 15 minutes to unmask the RNA targets. Two sets of RNA probes, targeting either ZIKV polyprotein (VF1-19981-06) or GS (VB6-16850-06), were hybridized with the samples for 2 hours at 40°C. Alkaline phosphatase-conjugated detection probes and signal amplifiers were used in sequential reactions to develop signals from gene-specific probes. ZIKV polyprotein was visualized after chromogenic reaction with fast red substrate, which showed red color in bright-field and fluoresced in Cy3 channel. GS staining was visualized as blue in bright-field and fluorescent in the far red channel.

**Magnetic Activated Cell Sorting (MACS)**

Müller cells were isolated using the MACS cell separation system (Miltenyi Biotec, San Diego, CA, USA). ZIKVFSS-infected retina tissues were harvested at 6 days post infection (dpi) and digested with collagenase. The cell suspension was first labeled with FITC-conjugated anti-CD44 antibody (eBioscience, San Diego, CA, USA), followed by binding to magnetic bead-conjugated anti-FITC antibody. Tagged cells were isolated with a MidiMACS column (Miltenyi Biotec, San Diego, CA, USA). Both Müller cells and flow-through fractions were collected for downstream RT-PCR analyses.

**RNA Isolation, cDNA Synthesis, and Quantitative RT-PCR**

Total RNA was isolated with TRIzol reagent (Thermo Fisher Scientific) and treated with DNase (Ambion, Austin, TX, USA) to remove residual trace of genomic DNA. Complementary DNA (cDNA) was synthesized from 0.2 to 1 μg total RNA using random hexamer (Promega, Madison, WI, USA). Gene-specific primers used for RT-PCR amplification are listed in Supplementary Table S2. Quantitative RT-PCR was performed on an ABS 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Results were presented as fold change after normalization to the expression level of β-actin.
are listed in Supplementary Table S3. Cells were transfected with 100 pmol siRNA duplexes by Lipofectamine 2000 (Thermo Fisher Scientific). Forty-eight hours after transfection, cells were used for ZIKV infection at MOI of 10:1. Viral RNA expression was measured by quantitative RT-PCR 24 hours thereafter.

**Western Blot Analyses**

Cells were lysed in buffer containing CellLyticM cell lysis reagent (Sigma-Aldrich Corp.) and 2X Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) at 1:1 ratio, 10 mM glycerophosphate, 10 mM pyrophosphate, 1 mM NaF; 1 mM Na3VO4, and protease inhibitor cocktails. After sonication, samples were resolved on SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were probed with antibodies against ZIKV envelope protein (ENV) (BioFront Technologies, Tallahassee, FL, USA), AXL, TYRO5 (Cell Signaling Technology, Danvers, MA, USA), and β-actin (Sigma-Aldrich Corp.). Signals were detected by an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA).

**Statistical Analyses**

All experiments were repeated at least three times. Data were analyzed with the GraphPad Software (La Jolla, CA, USA) and presented as mean ± SEM. The significance of differences between two groups was evaluated using a Student’s t-test. For multiple comparisons, 1-way ANOVA followed by Tukey’s multiple comparison tests was used. Differences were considered statistically significant when the P value was less than 0.05.

**RESULTS**

**ZIKV Retinopathy in Young A129 Mice**

We employed two strains of ZIKV to study the mechanism of viral pathogenesis in the eye. ZIKV<sub>FSS</sub> is a member of the Asian lineage and shares >98% nucleotide sequence identity with all American strains. ZIKV<sup>MEX</sup> was isolated from a pool of Aedes aegypti mosquitoes from the first outbreak in Mexico in 2015. When A129 mice were infected by ZIKV<sub>FSS</sub> at the time of weaning (3 weeks), viral ENV was detected at 4 dpi around the blood–ocular barrier sites in the ciliary body, major retinal membranes (Bio-Rad). ENV protein (GFAP), a marker protein of astrocytes and activated Müller cells (Figs. 2B, 2C). This was further validated with co-staining of GS, a Müller cell–specific marker (Fig. 2D). Müller gliosis was preceded by the appearance of ZIKVMEX ENV protein (Fig. 2C). ENV staining was also detected in the RPE (Fig. 2G), consistent with the previously reported focal RPE change in both congenital and acquired cases of human eye infection. 7,25

**Müller Cells and RPE as ZIKV Host Cells in the Retina**

After confirming the presence of ZIKV in the retinal tissue, we next examined the viral tropism in major retinal cells. The branching staining pattern of ZIKV in the inner retina first prompted us to focus on the astrocytes and Müller cells (Fig. 2A). Immunostaining of retinal sections, both ZIKV<sub>FSS</sub> and ZIKV<sup>MEX</sup> ENV showed colocalization with glial fibrillary acidic protein (GFAP), a marker protein of astrocytes and activated Müller cells. In A129 mice infected with the contemporary ZIKV<sup>MEX</sup> strain, the branching staining pattern of ZIKV in the inner retina first prompted us to focus on the astrocytes and Müller cells (Fig. 2A). Immunostaining (Figs. 4A, 4B, Supplementary Figs. S2A, S2B) and Western blot analyses (Fig. 4C) showed the presence of replication-competent ZIKV in culture supernatant fractions of infected cells was further determined by a standard focus forming assay in Vero cells. As shown in Figure 4D, viral titers had a time-dependent increase in supernates from RPE cells infected with either ZIKV<sub>FSS</sub> or ZIKV<sup>MEX</sup>, indicating that RPE cells were highly permissive to productive infection. Of note, there was no significant time-dependent increase of viral titer in the supernates of ZIKV-inoculated Müller cells.

Previous studies reported that entry of ZIKV into cells was dependent on cell surface receptors including CD209/DC-SIGN, TIM1, TYRO5, and AXL. Both RPE and Müller cells
expressed TYRO3 and AXL (Figs. 4C, 4E). While TIM1 is considered as an entry coreceptor for ZIKV, we did not detect TIM1 transcripts in examined tissues (Fig. 4E), indicating low, if any, expression in the posterior eye. There was weak expression of CD209, a C-type lectin receptor, in RPE tissues (Fig. 4E). Cultured retinal microglia, however, did not express the TAM family receptors (Fig. 4E), which is consistent with their lack of ZIKV infection in vivo (Fig. 2E). Knockdown of both TYRO3 and AXL by siRNA suppressed the amount of viral RNA in cultured Müller or RPE cells infected by ZIKV (Fig. 4F). The average efficiency of gene knockdown by siRNA was approximately 40%. Altogether, these data support-
FIGURE 2. Cell type specificity of ZIKV infection in the retina. (A) Retinal structure outlined by a schematic illustration. AS, astrocyte; RGC, retinal ganglion cell; Mü, Müller cell; MG, microglia; BP, bipolar cell; PR, photoreceptors. (B–F) Dual immunostaining of ZIKV ENV with cell type–specific markers, including GFAP for astrocytes and activated Müller cells (B, C), GS for total Müller cells (D), IBA1 for microglia (E), and RBPMS for ganglion cells (F). (G) ENV staining in the RPE. Quantification of infiltrated microglia was performed at different time points after infection. Each time point represents at least three infected animals. *P < 0.05; **P < 0.01. 1-way ANOVA and Bonferroni post hoc test. Scale bars: 50 μm.
FIGURE 3. ZIKV RNA in retinal Müller cells. (A) Dual staining of ZIKV polyprotein (red) and GS (blue) RNA by in situ hybridization, in retina of a 3-week-old A129 mice infected by ZIKV MEX at 17 dpi. Fluorescent images of the boxed area were enlarged to illustrate the colocalization. (B) Schematic illustration of magnetic activated cell sorting of Müller cells from ZIKV-infected retina. RT-PCR analyses of ZIKV RNA and Müller cell marker in both isolated Müller cells and flow-through fraction. RNA was isolated from A129 mice infected by ZIKV FSS at 6 dpi. Scale bars: 200 μm (A); inset = 50 μm.
ed RPE and Müller as major target cells for ZIKV infection. RPE is highly permissive to ZIKV and could be a major source of active viral production at early time points. Müller cells can be chronically infected but probably do not support productive infection.

**ZIKV-Induced Müller Cell Dysfunction and Neuronal Loss**

Müller glia have essential neural supporting functions, such as secretion of neurotrophic factors and removal of excessive...

**Figure 4.** ZIKV infection of cultured retinal cells. (A) Immunostaining of viral ENV or NS1 in cultured human fetal RPE cells at 2 dpi by ZIKV<sub>FSS</sub>. **Bottom:** reconstructions of series of Z-stack images at higher magnification. Blue: nuclei. (B) Immunostaining of primary retinal Müller cells infected with ZIKV<sub>FSS</sub>. (C) Western blot analyses of expression of viral proteins, AXL and TYRO3 receptors in infected cells. (D) Growth curves of ZIKV<sub>FSS</sub> or ZIKV<sub>MEX</sub> in RPE and Müller cells. (E) RT-PCR analyses of mRNA expression of potential viral entry receptors in retinal and RPE tissues from 1-month-old A129 mice (top), or cultured primary mice retinal Müller and microglial cells (bottom). (F) Quantitative RT-PCR analyses of viral RNA in Müller cells transfected with siRNA targeting TYRO3 and/or AXL. Data presented are the average from three separate experiments. Scale bars: 20 μm (A, upper), 5 μm (A, lower), 50 μm (B).
FIGURE 5. Functional consequences of ZIKV infection of the retina. (A, B) Quantitative RT-PCR analyses of neurotrophic and proinflammatory gene expression in cultured Müller cells infected with either ZIKV FSS or ZIKV MEX (n = 4). (C) H&E-stained retinal histology sections from infected A129 mice. The age of the animals was either 3 or 11 weeks at time of viral inoculation. V, vitreous cavity. Arrows: intravitreal infiltrates; open arrows: retinitis with cell infiltration; asterisks: outer nuclear layer damage; arrowheads: subretinal inflammatory cells; open arrowheads: RPE defects. (D) H&E-stained sections of the optic nerve head (ONH). Immediately neighboring sections were stained for viral ENV and IBA1+ cell infiltration. Arrowheads: necrotic lesion; arrows: mononuclear cell infiltrate; asterisk: same blood vessel on adjacent sections. (E) Representative micrograph
glutamate to prevent excitotoxicity.\textsuperscript{30,31} Active Müller gliosis is an early sign of retinal stress and insults.\textsuperscript{30} To explore the functional consequences of ZIKV infection, we measured the expression of neurotrophic factors, glutamate transporter, and inflammatory markers in cultured Müller cells. ZIKV FSS decreased the expression of brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), insulin-like growth factor 1 (IGF1), and glutamate transporter L-glutamate/L-aspartate transporter (GLAST) (Fig. 5A). Similar changes occurred in ZIKV MEX-infected Müller cells, although to a lesser extent. Expression of proinflammatory genes and cytokines, including iNOS, IL6, and CCL2, was upregulated by ZIKV FSS but not ZIKV MEX (Fig. 5B, Supplementary Fig. S2C).

Histopathologic examinations showed that eyes of A129 mice infected by ZIKV FSS mainly displayed signs of posterior uveitis, including intravitreal and retinal infiltrates and focal RPE defects (Fig. 5C, Supplementary Fig. S3). A129 mice at 11 weeks of age were resistant to ocular ZIKV FSS infection as their eyes showed no sign of abnormality (Fig. 5C). ZIKV MEX induced more severe ocular pathologic changes with heavy infiltrates, retinitis, photoreceptor loss, and RPE defects at both 9 and 17 dpi of ZIKV MEX (Fig. 5C, Supplementary Figs. S2B, S2C). Optic nerve viral infiltration and axonal neuropathy were observed in 17-dpi samples with extensive IBA1\textsuperscript{32} infiltrating cells on the immediate adjacent section (Fig. 5D). Caspase-3 activation was detected in the nerve fiber layer and inner nuclear layer peaking at 9 dpi, indicating apoptosis of astrocytes or infiltrated macrophages in the vitreous (Fig. 5E).

The long-term sequelae of compromised neural support by Müller cells and RPE, and the proinflammatory microenvironment in the retina, resulted in significant loss of cone photoreceptors and ganglion cells after ZIKV infection, most prominent in the peripheral sites in 60- and 70-dpi samples (Figs. 5E, 5G, Supplementary Fig. S4). Focal degeneration of the outer nuclear layer was likely caused by RPE infection (Fig. 5H). Aside from significant RGC loss, most retinal inflammation regressed at 60 dpi (75\% ZIKV-infected mice) with residual intravitreal cells and positive viral staining only around ciliary bodies (Supplementary Fig. S4). In one animal, delayed viral clearance was associated with severe retinal damage at 70 dpi (Supplementary Fig. S4C). The findings suggest that permanent retinal structural damage can occur after neonatal ZIKV infection.

**DISCUSSION**

In recent years, numerous clinical studies have reported retinal infection and posterior uveitis caused by emerging and re-emerging viruses,\textsuperscript{32} such as Ebola,\textsuperscript{33,34} West Nile,\textsuperscript{35,36} Chikungunya,\textsuperscript{37} dengue,\textsuperscript{12} and ZIKV.\textsuperscript{6,38} Viral persistence in the ocular tissue is a potential threat of disease transmission,\textsuperscript{15,52} and can lead to long-term irreversible damages to the neural retina. ZIKV infection is congenital. However, a recent study showed that in mice with intrauterine infection, viral RNA was detectable in brain 1 week after birth, and the well-developed retina of these animals still showed progressive degeneration during the first 2 months after birth.\textsuperscript{38} A recent clinical study also reported that some of the ZIKV-infected infants showed microcephaly around 1 year after birth.\textsuperscript{39} Therefore, it is likely that, in addition to the developmental defects, neuroinflammation and degeneration in adult retina and brain will contribute to ZIKV-induced pathology and functional defects.

Data from our current study show that, before the blood-retinal barrier and host immunity are well developed,\textsuperscript{40} ZIKV may enter the retinal tissue and establish an infection of susceptible cell populations (Figs. 1–3) and eventually lead to irreversible retinal injury (Fig. 5). ZIKV preferentially infects RPE and Müller cells (Figs. 2, 4), which are key supporting cells for neuronal survival, function, and injury repair in the retina.\textsuperscript{50} Müller cell ablation causes neuronal and vascular pathologic effects resembling ocular features of congenital ZIKV eye disease.\textsuperscript{31} Viral RNA was detected in Müller cells by in situ hybridization and RT-PCR (Fig. 3), suggesting that the presence of ZIKV ENV was not due to engulfing neighboring infected cell debris.

Organisms with immune privilege, such as brain, testis, placenta, and the posterior eye, tend to have higher viral loads and are more prone to ZIKV-induced tissue damage in A129 mice.\textsuperscript{52} Initial viral entry appeared through both the RPE (Fig. 2G) and the pigmented ciliary epithelium (Fig. 1A); viral spread may involve the breakdown of the blood-retinal barrier or blood-aqueous barrier at either site. The eyes of 11-week-old A129 mice were not infected by ZIKV (Supplementary Fig. S1C), suggesting that the ocular barrier exerted protection in adult mice. On the other hand, the IFN responses in mice are much more robust than those in primates, due to the differences in viral protease-mediated cleavage of STING, a viral RNA sensor that initiates the IFN responses.\textsuperscript{42} Future studies will be needed to further define whether similar mechanisms of ZIKV pathogenesis are applicable to humans or nonhuman primates. Previous studies have reported retinal infection and posterior uveitis caused by emerging and re-emerging viruses,\textsuperscript{32} such as Ebola,\textsuperscript{33,34} West Nile,\textsuperscript{35,36} Chikungunya,\textsuperscript{37} dengue,\textsuperscript{12} and ZIKV.\textsuperscript{6,38} Viral persistence in the ocular tissue is a potential threat of disease transmission,\textsuperscript{15,52} and can lead to long-term irreversible damages to the neural retina. ZIKV infection is congenital. However, a recent study showed that in mice with intrauterine infection, viral RNA was detectable in brain 1 week after birth, and the well-developed retina of these animals still showed progressive degeneration during the first 2 months after birth.\textsuperscript{38} A recent clinical study also reported that some of the ZIKV-infected infants showed microcephaly around 1 year after birth.\textsuperscript{39} Therefore, it is likely that, in addition to the developmental defects, neuroinflammation and degeneration in adult retina and brain will contribute to ZIKV-induced pathology and functional defects.

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explore the mechanisms of viral pathogenesis in susceptible retinal host cells of ZIKV in the retina. A better understanding of the virus will provide insightful details as to the progression of long-term ocular ZIKV sequelae.

In summary, our studies have shown that ZIKV can infect the retina in both immunodeficient and immunocompetent mice. Infection with the ZIKV (AH3) strain caused structural damage to the neuronal tissues in the retina that likely has long-term effects on visual function. Müller and RPE cells are the main host cells of ZIKV in the retina. A better understanding of the mechanisms of viral pathogenesis in susceptible retinal cells, and further characterization of the strain differences, will benefit the development of novel target-based therapy against ZIKV.

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