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p38MAPK plays a critical role in induction of a pro-inflammatory phenotype of retinal Müller cells following Zika virus infection

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Abstract

Zika virus (ZIKV) infection has been associated with ocular abnormalities such as chorioretinal atrophy, optic nerve abnormalities, posterior uveitis and idiopathic maculopathy. Yet our knowledge about ZIKV infection in retinal cells and its potential contribution to retinal pathology is still very limited. Here we found that primary Müller cells, the principal glial cells in the retina, expressed a high level of ZIKV entry cofactor AXL gene and were highly permissive to ZIKV infection. In addition, ZIKV-infected Müller cells exhibited a pro-inflammatory phenotype and produced many inflammatory and growth factors. While a number of inflammatory signaling pathways such as ERK, p38MAPK, NF- κ B, JAK/STAT3 and endoplasmic reticulum stress were activated after ZIKV infection, inhibition of p38MAPK after ZIKV infection most effectively blocked ZIKV-induced inflammatory and growth molecules. In comparison to ZIKV, Dengue virus (DENV), another Flavivirus infected Müller cells more efficiently but induced much lower pro-

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inflammatory responses. These data suggest that Müller cells play an important role in ZIKV-induced ocular pathology by induction of inflammatory and growth factors in which the p38MAPK pathway has a central role. Blocking p38MAPK may provide a novel approach to control ZIKV-induced ocular inflammation.

Keywords

zika virus; ocular abnormalities; Müller cells; inflammation; p38MAPK

1. Introduction

Zika virus (ZIKV) is an arthropod-borne virus (arbovirus) of the *Flavivirus* genus. It is transmitted by *Aedes* mosquitoes and is structurally related to Dengue (DENV), West Nile (WNV), Japanese encephalitis (JEV), and yellow fever (YFV) viruses (Jampol and Goldstein, 2016). ZIKV was initially isolated from a rhesus monkey in the Zika Forest of Uganda in 1947 (Dick et al., 1952), and has caused outbreaks in Asia, the Pacific island and more recently in South and Central America (Chen and Hamer, 2016; Samarasekera and Triunfol, 2016). Although symptomatic infection of ZIKV in humans normally results in a mild and self-limiting febrile disease, it has been linked to neurological autoimmune disorder Guillain-Barré syndrome in adults and microcephaly in fetuses and infants born to mothers infected with ZIKV during pregnancy particularly during the first or second trimester (Jampol and Goldstein, 2016; Li et al., 2016; Wikan and Smith, 2016). ZIKV has been detected in human fetal brain tissue of microcephalic infants and the amniotic fluid of pregnant women with microcephalic fetuses (Li et al., 2016). Studies using neural progenitor cells (NPCs) and mice further show that ZIKV may disrupt the development of and induce the death in NPCs, which leads to microcephaly (Dang et al., 2016; Li et al., 2016). Currently, little is known about ZIKV pathogenesis and there is no approved antiviral therapy or licensed human vaccines, though several groups have identified potential antiviral targets or candidate vaccines in experimental models (Abbink et al., 2016; Barrows et al., 2016; Richner et al., 2017; Xie et al., 2017; Xu et al., 2016).

The retina is an extension of the brain and often shares many of the pathological changes seen in the central nervous system (CNS). Infants with microcephaly due to ZIKV infection are often associated with a high rate of ocular abnormalities in which the most common lesions are chorioretinal atrophy and optic nerve abnormalities (de Paula Freitas et al., 2016; Ventura et al., 2016). Retinopathy in ZIKV-infected adults is less appreciated, but a few reports suggest posterior uveitis and idiopathic maculopathy in ZIKV patients (Kodati et al., 2017; Parke et al., 2016; Wong et al., 2017). Moreover, several groups reported ocular pathological changes in ZIKV-infected mice (Cui et al., 2017; Miner et al., 2016; Singh et al., 2017; van den Pol et al., 2017). These studies provide direct evidence that ZIKV is present in retinal cells upon systemic or local infection and ZIKV infection causes conjunctivitis, panuveitis and chorioretinal atrophy. Nevertheless, our knowledge of ZIKV infection in retinal cells and its potential contribution to retinal pathology is still very limited.

Müller cells are specialized neuroglial cells in the retina (Newman and Reichenbach, 1996; Reichenbach and Bringmann, 2013). Their cell bodies are located in the inner nuclear layer (INL), with processes extending from the outer to the inner limiting members. Müller cells form an architectural support structure across the whole retina and provide homeostatic and metabolic support to retinal neurons, which are assumedly carried out by astrocytes, oligodendrocytes and ependymal cells in other regions of the CNS (Newman and Reichenbach, 1996; Reichenbach and Bringmann, 2013). Under pathological conditions, Müller cells are activated and produce inflammatory cytokines and growth factors that lead to retinal inflammation, vascular leakage and neuronal degeneration in retinopathies including diabetic retinopathy, age-related macular degeneration and uveitis (de Hoz et al., 2016; Sauter and Brandt, 2016; Wang et al., 2015b; Zhong et al., 2012). Since ZIKV has been detected in cells located in the INL, including those with the morphology of Müller cells (Miner et al., 2016), here, we investigated the effects of ZIKV infection on primary mouse retinal Müller cells.

2. Materials and methods

2.1 Animals

C57BL/6 wild type mice used for isolation of Müller cells were purchased from the Jackson Laboratory (Bar Harbor, ME) and were bred in a pathogen-free mouse facility at the University of Texas Medical Branch (UTMB). The experimental procedures and use of animals were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and all protocols including isolation of Müller cells from mice were approved by the Institutional Animal Care and Use Committee at UTMB.

2.2 Cell culture

Primary Müller cells were isolated from mouse retina as described previously (Wang et al., 2015a) and maintained in Dulbecco's Modification of Eagle's Medium (DMEM) (Corning, Corning, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah) and 1% penicillin/streptomycin (Cellgro, Herndon, VA). Human retinal microvascular endothelial cells (HRMECs) and mouse photoreceptor cell line 661W were cultured as described previously (Ameri et al., 2014; Zhu et al., 2017). Vero cells were cultured in Minimum Essential Medium Eagle medium (MEM, Cellgro) supplemented with 8% FBS. Cells were incubated at 37°C with 5% CO₂.

2.3 ZIKV and DENV2 infection

The Asian lineage FSS13025 of ZIKV was obtained from Dr. Robert Tesh at the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA, Galveston, TX) and was amplified once in Vero cells. DENV2 strain 16681 was provided by Dr. Alan D. Barrett (Department of Pathology, University of Texas Medical Branch, Galveston, TX). Müller cells, HRMECs or 661W cells were cultured at a density of 5×10^4 /well in 24-well plates and were infected with ZIKV at a multiplicity of infection (MOI) of 0.02 or 1 (for Müller cells) or 0.2 (for HRMECs or 661W). Müller cells were infected with DENV2 at a MOI of 0.02. At various times post infection, cells and supernatant were collected for measurement

of viral load and cytokine production. In some experiments, inhibitors were added at day 3 post infection. The concentration of different inhibitors is listed as follows: PD98059 (20 μ M), SB202190 (10 μ M), PDTC (1 μ M), Stattic (3 μ M), AG490 (10 μ M), TUDCA (500 μ M), PBA (4 mM), GSK2606414 (300 nM), 4 μ 8C (25 μ M), Apocynin (1 mM).

2.4 Focus-forming assay

Vero cell monolayers were incubated with sample dilutions for 1 hour. A semisolid overlay containing 0.8% methylcellulose (Sigma-Aldrich, St. Louis, MO), 3% FBS, 1% penicillin/streptomycin, and 1% L-glutamine (Life Technologies, Rockville, MD) was then added. At 48 hours, the semisolid overlay was removed, and cell monolayers were washed with PBS, air dried and fixed with 1:1 acetone: methanol solution for at least 30 minutes at -20°C . Cells were next subjected to immunohistochemical (IHC) staining with either a ZIKV hyperimmune mouse ascitic fluid (obtained through the WRCEVA) or 4G2 antibody followed by goat anti-rabbit HRP-conjugated IgG (KPL, Gaithersburg, MD) at room temperature for 1 hour respectively. After secondary antibody, cells were incubated with a peroxidase substrate (Vector Laboratories, Burlingame, CA) until color developed. The number of foci was determined and used to calculate viral titers expressed as FFU/ml.

2.5 Quantitative PCR (qPCR)

Total RNA was extracted using Trizol (Life Technologies) or RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's standard protocol. cDNA was synthesized using a qScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was conducted with SYBR Green PCR Master Mix (Bio-Rad Laboratories) on a Bio-Rad CFX Connect Real-Time system and a Ct-value of 45 was chosen as the cut-off. Primer sequences for mouse transcripts were as follows and as described previously (Klein et al., 2005; Lanciotti et al., 2008; Santiago et al., 2013; Waggoner and Pinsky, 2016; Wang et al., 2004): HPRT For-5'-GAA AGA CTT GCT CGA GAT GTC ATG-3'; HPRT Rev-5'-CAC ACA GAG GGC CAC AAT GT-3'; TYRO3 For-5'-CCC CAT TCC CAT GGT CAT C-3'; TYRO3 Rev-5'-GCA GAA AGG CGT GCA AGT CT-3'; AXL For-5'-CCC AGC ACA GTC TGC AAA CTC-3'; AXL Rev-5'-TGG GCT TCA CAT GAG AAA GAA G-3'; MERTK For-5'-ACG GCA GAA GTT CAC GAG AAC-3'; MERTK Rev-5'-CAT TGC CCC CAT TCA AGA TG-3'; ZIKV prM gene For-5'-TTG GTC ATG ATA CTG CTG ATT GC-3'; ZIKV prM gene Rev-5'-CCT TCC ACA AAG TCC CTA TTG C-3' (Lanciotti et al., 2008; Waggoner and Pinsky, 2016); DENV2 E gene For-5'-CAG GTT ATG GCA CTG TCA CGA T-3'; DENV2 E gene Rev-5'-CCA TCT GCA GCA ACA CCA TCT C-3' (Santiago et al., 2013); ICAM-1 For-5'-CAG TCC GCT GTG CTT TGA GA-3'; ICAM-1 Rev-5'-CGG AAA CGA ATA CAC GGT GAT-3'; CXCL1 For-5'-GCA GAC CAT GGC TGG GAT T-3'; CXCL1 Rev-5'-CCT GAG GGC AAC ACC TTC AA-3'; CCL5 For-5'-TCC AAT CTT GCA GTC GTG TTT G-3'; CCL5 Rev-5'-TCT GGG TTG GCA CAC ACT TG-3'; VEGF For-5'-TAC CTC CAC CAT GCC AAG TG-3'; VEGF Rev-5'-TCA TGG GAC TTC TGC TCT CCT T-3'. Primer sequences for human transcripts were as follows: GAPDH For-5'-CTC AAG ATC ATC AGC AAT GCC T-3'; GAPDH Rev-5'-AAG TTG TCA TGG ATG ACC TTG G-3'. Data obtained from RT-qPCR reaction was analyzed by using the formula $2^{-[\text{CT}(\text{target gene}) - \text{CT}(\text{GAPDH or HPRT or } \beta\text{-actin gene})]}$ as described before

or the CT method with HPRT, β -actin or GAPDH used as the reference gene for normalization.

2.6 Immunofluorescence staining (IF)

Primary Müller cells were seeded at a density of 2×10^4 /well in 24-well plate. After 24 hours, cells were infected with ZIKV at a MOI of 0.02. After washing, cells were supplemented with fresh complete medium and incubated at 37°C. At 4 days after infection, cells were fixed with 4% paraformaldehyde (PFA) in PBS, briefly rinsed and blocked with PowerBlock (Biogenx, San Ramon, CA) for 1 hour. Subsequently, cells were incubated with primary antibody against ZIKV (ZKN-7, 1:1000, FutraTech Inc., San Diego, CA) overnight at 4°C. After rinsing, cells were incubated with Alexa Fluor 488-labeled goat anti-mouse secondary antibody (1:1000; Life Technologies) and mounted with Fluoroshield™ with DAPI histology mounting medium (Sigma-Aldrich). Cells were examined by an Olympus 1×71 epifluorescence microscope (Olympus, Waltham, MA).

2.7 Western blot

Protein from cells was dissolved in 1X SDS loading buffer and separated on SDS-PAGE gels. Subsequently, proteins were electroblotted onto PVDF membranes (Bio-Rad Laboratories), probed with primary and secondary antibodies, and detected using the enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL). The primary antibodies included the following: Phospho-ERK (Thr202/Tyr204), Phospho-p38 (Thr180/Tyr182), Phospho-p65 (Ser 468), Phospho-STAT3 (Tyr705), Phospho-JAK2 (Tyr1007/Tyr1008), Phospho-JNK (Thr183/Tyr185), p38, Phospho-MKK3/6 (Ser189/Ser207), Phospho-ATF2 (Thr71), Phospho-HSP27 (Ser81), Phospho-MAPKAPK2 (Thr334), and Phospho-MSK1 (Thr581) from Cell Signaling Technology (Beverly, MA); phospho-JAK1 (Thy1022/Tyr1023) from EMD Millipore (Billerica, MA); ZIKV Envelope protein (ZENV11-S) from Alpha Diagnostic Intl. Inc (San Antonio, TX); mouse monoclonal anti- α -Tubulin from Sigma-Aldrich was used as loading control. Densitometry analysis was conducted using ImageJ software.

2.8 MTT assay

The cytotoxicity and cell viability were determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay (Stockert et al., 2012; Zhu et al., 2017). Cells were seeded at a density of 1×10^4 cells/well in 96-well plates and treated with inhibitors at day 3. At day 4, 20 μ l of 5 mg/ml MTT was added to each well and plates were incubated at 37 °C in cell culture incubator for 1 hour. After media were sucked off, 100 μ l of DMSO was added to each well and plates were shaken at room temperature for 10 minutes to dissolve intracellular MTT formazan crystals, followed by measurement of absorbance at 540 nm. Value of the blank wells with media only was deducted from the measured value.

2.9 Statistical analysis

All experiments were repeated at least three times. Data were presented as mean $\pm$ SEM. Results were analyzed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Comparison between experimental groups was made by Student's *t*-test and one-way

ANOVA followed by *post hoc* Student' *t*-test using the Student–Newman–Keuls method. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Müller cells are highly susceptible to ZIKV infection

TAM receptors, including TYRO3, AXL and MERTK, are the putative entry receptors for some flaviviruses (Miner et al., 2016) and ZIKV is known to utilize these cell surface receptors to infect various cell types (Pagani et al., 2017; Richard et al., 2017; Savidis et al., 2016). To determine if primary Müller cells are permissive to ZIKV infection, we first examined the expressions of TAM receptors by quantitative PCR (qPCR). As expected, all three receptors are expressed in Müller cells (Fig. 1A). Among them, AXL, which is recently shown to be the primary ZIKV entry cofactor on human umbilical vein endothelial cells (Richard et al., 2017), was abundantly expressed in Müller cells and its relative expression level was about 60% of the internal control HPRT gene. Next, we infected Müller cells with the Asian lineage ZIKV strain FSS13025 at a multiplicity of infection (MOI) of 0.02 for 1 hour and assessed viral RNA at 1 day and 4 days after infection. ZIKV RNA was detected at 1 day after infection, which was dramatically increased at day 4 (Fig. 1B). Correlating with the increased viral RNA level, the focus-forming assay suggested that ZIKV was released to Müller cell culture supernatant at day 1 and its titer was increased at day 4 (Fig. 1C). Analysis of ZIKV-infected Müller cells by immunofluorescence staining (IF) also confirmed the presence of ZIKV protein in a portion of Müller cells (Fig. 1D). Furthermore, we detected ZIKV Envelope protein by western blot in cells infected with ZIKV at a MOI of 1 (Fig. 1E). These data indicate that ZIKV can effectively infect and replicate in Müller cells. As a comparison, we infected human retinal microvascular endothelial cells (HRMECs) and mouse retinal photoreceptor cells (661W cells (Zhu et al., 2017)) at a higher MOI of 0.2. Similar to Müller cells, ZIKV viral RNA in HRMECs was detected at day 1 and increased at day 4 after infection (Fig. 1F). In contrast, although a high level of viral RNA was detected in photoreceptor cells at day 1 after infection, the infection rate dramatically decreased at day 4 (Fig. 1G), suggesting photoreceptor cells poorly support ZIKV replication.

3.2 Müller cells exhibit a pro-inflammatory phenotype after ZIKV infection

ZIKV infection in the eye causes inflammation and injury (Kodati et al., 2017; Miner et al., 2016; Parke et al., 2016; Singh et al., 2017; Wong et al., 2017). To determine the potential role of Müller cells in ZIKV-induced retinal inflammation, we assessed the levels of a number of key pro-inflammatory cytokines, chemokines, adhesion molecules and growth factors in Müller cells after ZIKV infection (Fig. 2A). At day 1, only CXCL1, CXCL10 and CCL2 were slightly increased by ZIKV infection. At day 4, ZIKV infection induced significant increases in IL-6 (a potent pro-inflammatory cytokine), ICAM-1 (an adhesion molecule), CXCL1 (a chemokine for neutrophil recruitment), CXCL10 (a chemokine for T lymphocyte recruitment), CCL2 and CCL5 and CCL7 (chemokines for monocyte recruitment), and VEGF (vascular endothelial growth factor/vascular permeability factor) (Fig. 2A). Levels of TNF α , IFN α and IFN β , however, were not changed. The lack of induction of IFN α and IFN β suggest that Müller cells do not elicit type I IFN response to

protect infected and uninfected cells. We further performed linear regression analyses to investigate the association between ZIKV RNA and induction of inflammatory factors using samples from independent experiments (Fig. 2B). ZIKV-induced production of the tested cytokines was not proportionally correlated with ZIKV viral load, suggesting a minimal amount of ZIKV is needed to induce the pro-inflammatory phenotype of Müller cells.

3.3 p38MAPK is a key mediator of ZIKV-induced inflammation

To understand the potential mechanism of ZIKV-induced upregulation of the aforementioned inflammatory and growth factors, we investigated whether ZIKV induces activation of MAPKs, NF- κ B and JAK/STAT3, which play critical roles in induction of these factors. At day 3 and day 4 after ZIKV infection, the levels of phosphorylated MAPKs (ERK and p38MAPK), NF- κ B p65 and STAT3 were all significantly enhanced (Fig. 3).

Phosphorylation of JAK1 and JAK2 was significantly increased at day 3, but not at day 4 after ZIKV infection. In contrast, JNK, another member of the MAPK family, was slightly decreased. These data suggest that ERK, p38MAPK, NF- κ B p65, JAK1/2, and STAT3 are potentially involved in the ZIKV-induced pro-inflammatory phenotype of Müller cells. Therefore, we next treated cells at day 3 post infection with the inhibitors for ERK (PD98059), p38MAPK (SB202190), NF- κ B (PDTC), STAT3 (Stattic) and JAK1/JAK2 (AG490), and determined their effects on ZIKV-induced inflammatory and growth factors. Since endoplasmic reticulum (ER) stress and NADPH oxidase have been shown to regulate the activation of these kinases and transcription factors in response to different stimuli (Valadao et al., 2016; Zhang et al., 2009; Zhu et al., 2017), the inhibitors for ER stress and NADPH oxidase were also included: TUDCA and PBA—chemical chaperones blocking ER stress, GSK2606414—inhibitor of ER stress branch PERK, 4 μ 8C—inhibitor of ER stress branch IRE1, and apocynin—inhibitor of NADPH oxidase. Most inhibitors were not toxic to cells, while PBA, 4 μ 8C and Apocynin slightly reduced Müller cell viability as determined by MTT assay (Supplementary Figure 1). Although some of these inhibitors affected the basal level of inflammatory and growth factors to some extent in non-infected cells (Supplementary Figure 2), their alterations were different from those in inhibitor-treated ZIKV-infected cells (Fig. 4A & Table 1), suggesting that tested inhibitors are specifically blocking/enhancing ZIKV-induced signaling rather than altering basal signaling. Among these inhibitors, the p38MAPK inhibitor significantly attenuated ZIKV-induced expression of all of these factors except for CXCL10 and CCL5; while other inhibitors did not (Fig. 4A). To determine whether inhibitors affect ZIKV infection, we measured viral load in vehicle and inhibitor-treated cells. We found no significant difference between inhibitor-treated group and vehicle-treated group though there was a trend of increase of ZIKV RNA after blocking ERK, NF- κ B, JAK2/3, IRE1 and NADPH oxidase, and a trend of slight decrease after blocking p38MAPK (Fig. 4B).

The finding that blocking p38MAPK effectively reduced the inflammatory phenotype of Müller cells after ZIKV-infection has prompted us to further explore the p38MAPK pathway after ZIKV infection. Total level of p38MAPK protein was not altered by ZIKV infection. Nevertheless, levels of active forms of p38MAPK, its upstream activator MKK3/6 and multiple downstream effectors including ATF2, HSP27, MSK1 and MAPKAPK2 were all significantly increased (Fig. 5A). To test whether the p38MAPK inhibitor is functional, we

treated non-infected and ZIKV-infected Müller cells with vehicle or p38MAPK inhibitor SB202190 at day 3 after infection and determined levels of phosphorylated p38MAPK, total p38MAPK and phosphorylated MSK1 at day 4 (Fig. 5B). We found basal and ZIKV-induced MSK1 phosphorylation was attenuated by SB202190, indicating the inhibitor is functional in blocking p38MAPK activity. Although p38MAPK levels were not changed, phosphorylated p38MAPK was markedly increased after treatment with p38 inhibitor in both non-infected and infected cells. As SB202190 inhibits p38MAPK activity by preventing ATP binding to the active kinase but not by inhibiting its phosphorylation, the increase of the active form of p38MAPK reflects a compensatory regulation of p38MAPK activity after inhibitor treatment, as observed by others (Green et al., 2011; Keshari et al., 2013). All together, these data suggest that there are likely multiple innate signaling pathways involved in induction of the pro-inflammatory phenotype during ZIKV infection and p38MAPK pathway has a central role in this process.

3.4 DENV induces overlapped but distinct cellular responses in Müller cells

DENV is another Flavivirus that is structurally related to ZIKV. We then extended our study to determine whether DENV also infects Müller cells and induces the same responses as ZIKV does. We infected Müller cells with DENV2 at a MOI of 0.02, followed by treatment with vehicle or p38MAPK inhibitor SB202190 at day 3 after infection. DENV2 RNA and the expression of inflammatory and growth factors were measured by qPCR at day 4 (Fig. 6). The level of DENV2 RNA was much higher than that of ZIKV ($\text{DENV2}/\beta\text{-actin}=1046 \times 10^{-4}$; $\text{ZIKV}/\beta\text{-actin}=16.6 \times 10^{-4}$). However, DENV2-induced increases in CXCL1, CCL2, CCL7 and VEGF were much less than those induced by ZIKV (Fig. 6B), suggesting DENV2 is a weaker inducer of proinflammatory responses in Müller cells compared to ZIKV. Although treatment with the p38MAPK inhibitor also significantly attenuated DENV2-induced production of inflammatory factors (Fig. 6B), the magnitude of inhibition on CCL2 and CCL7 expression was reduced during DENV infection (Fig. 4 & 6, Table 2). Overall, our results suggest that ZIKV and DENV induce overlapped but distinct cellular responses in Müller cells.

4. Discussion

ZIKV has been shown to effectively infect neural progenitor cells and glial cells such as astrocytes in the brain (Li et al., 2016; van den Pol et al., 2017). Müller cells are derived from retinal neuronal progenitor cells and serve as the principal glial cells in the retina. Here, we provide compelling evidence that primary Müller cells express a high level of the ZIKV entry factor AXL gene and are permissive to infection. In particular, even with a low infection rate (MOI of 0.02), we have shown that ZIKV induces productive replication in these cells. Our study is the first to prove ZIKV infection in primary Müller cells though two other groups recently tested ZIKV infection in MIO-M1 cells (Roach and Alcendor, 2017; Singh et al., 2017), an immortalized Müller cell line that is highly proliferative and expresses markers of both glial and photoreceptor cells (Hollborn et al., 2011). In these two studies, MIO-M1 cells were infected with ZIKV for a longer time period (1–4 days) without washing and at higher infection levels (MOI of 0.1 and 1). Further, the results from these two studies seem conflicting. One group showed that Müller cells are permissive to ZIKV

infection (Singh et al., 2017); while the other suggests the opposite (Roach and Alcendor, 2017). Given that Müller cells are widely spread in the whole retina and are in contact with other retinal cells, our data suggest that they may facilitate ZIKV dissemination throughout the retina. Similarly, primary retinal endothelial cells which were also found to be permissive to ZIKV infection in our studies, could potentially promote ZIKV spreading from blood to retinal tissues. In contrast, 661W cells, a photoreceptor cell line, do not support ZIKV replication. Therefore, our studies demonstrate that there are significant differences in ZIKV susceptibility among different retinal cell types.

As an important supporting glial cell in the retina, Müller cell abnormal function has been linked to many retinal diseases (de Hoz et al., 2016; Sauter and Brandt, 2016; Wang et al., 2015b; Zhong et al., 2012). We found that ZIKV not only replicates productively in Müller cells but also induces a robust inflammatory response. Since inflammation is a key player in retinal neuronal and vascular injury, and Müller cell-derived VEGF has an essential role in inducing blood-retina barrier breakdown (Wang et al., 2015b), our data suggest that ZIKV-induced pro-inflammatory phenotype of Müller cell likely contributes to the ocular pathological changes in uveitis, chorioretinal atrophy and optic nerve abnormalities. We noted that the levels of induction of inflammatory factors in ZIKV-infected Müller cells are not linearly correlated with viral load. These results indicate that ZIKV is likely to cause retinal injury even at a low infection rate in Müller cells. Additionally, ZIKV induced significant inflammatory reactions at 3–4 days after infection despite that viral RNA was already high at day 1 post infection. This delayed response suggests a “window period” between ZIKV infection and manifestation of retinal pathological changes.

ZIKV-induced changes in gene expression are also noted in other cell types (McGrath et al., 2017; Roach and Alcendor, 2017; Singh et al., 2017). However, the specific intracellular signaling pathways that mediate ZIKV-induced cellular responses remain unknown. In this study, we provide the first evidence that ZIKV activates diverse intracellular pathways, including ERK, p38MAPK, NF- κ B, STAT3, and ER stress, which regulate ZIKV-induced expression of inflammatory and growth factors differently. Among them, we demonstrate that inhibition of p38MAPK markedly blocks the expression of most of inflammatory and growth factors induced by ZIKV. In particular, we added the p38MAPK inhibitor at 3 days post ZIKV infection, a time when many inflammatory pathways, including p38MAPK, had already been activated. Thus, the potent anti-inflammatory effects of p38MAPK inhibitor on ZIKV-infected Müller cells are considered as “treatment” but not “prevention”. Since a number of p38MAPK inhibitors are in clinical development for treating diseases such as arthritis and chronic obstructive pulmonary disease (Norman, 2015), our study warrants further evaluation of the effects of these inhibitors in other ZIKV-infected *in vitro* and *in vivo* models as well as in human subjects, which may lead to a novel therapy for ZIKV-induced pathological changes. This possibility is supported by two related studies showing that administration of p38MAPK inhibitor limits inflammation, prevents hematocrit rise, lymphopenia and liver injury, and improves survival rate in mice infected by DENV (Fu et al., 2014; Sreekanth et al., 2016), another flavivirus that induces overlapped but distinct cellular responses with ZIKV.

It has been reported that flavivirus infection activates multiple pathogen recognition receptor (PRR)s, including RIG-I-like receptors (RLRs) and toll like receptor (TLR) 3 and 7. These receptors signal through different adaptor molecules, to activate many signaling molecules including MAPK in order to regulate the expression of genes involved in control of virus infection, inflammation, cell stress, death or survival (Jiang et al., 2014; Suthar et al., 2013; Valadao et al., 2016). Both TLR3 and RIG-I are involved in JEV-induced p38MAPK activation in microglial cells (Jiang et al., 2014). Furthermore, DENV NS1 was shown to activate TLR4 signaling in primary human myeloid cells to secrete pro-inflammatory cytokines for induction of vascular leakage (Puerta-Guardo et al., 2016). Thus, future studies will be focused on identifying PRRs involved in activation of p38MAPK in ZIKV-infected Müller cells.

In summary, we found that Müller cells are highly permissive to ZIKV infection and exhibit a pro-inflammatory phenotype after ZIKV infection. While multiple pathways are involved in ZIKV-induced inflammatory reactions, p38MAPK is a key mediator for ZIKV-induced expression of inflammatory and growth factors. Although innate inflammation is an important step for the host to fight against viral infection, uncontrolled or excessive inflammation causes tissue injury and leads to diseases. Further studies to address the specific contribution of ZIKV-infected Müller cells to retinal inflammation and injury as well as the therapeutic potential of p38MAPK inhibitors may facilitate understanding ZIKV-induced retinopathy and developing novel interventions for retinal diseases associated with ZIKV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Glossary

CNS	central nervous system
DENV	Dengue virus
HRMECs	human retinal microvascular endothelial cells
IF	immunofluorescence staining
INL	inner nuclear layer
JEV	Japanese encephalitis virus
NPCs	neural progenitor cells

PRR	pathogen recognition receptor
qPCR	quantitative PCR
RLR	RIG-I-like receptor
TLR	toll like receptor
WNV	West Nile virus
YFV	yellow fever virus
ZIKV	Zika virus

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Highlights

- Primary Müller cells were highly permissive to ZIKV infection.
- ZIKV-infected Müller cells exhibited a pro-inflammatory phenotype.
- The p38MAPK pathway has a central role in ZIKV-induced production of inflammatory and growth factors in Müller cells.

Figure 1. ZIKV infects primary mouse retinal Müller cells

(A) The mRNA expression of TAM receptors (TYRO3, AXL, and MERTK) in Müller cells was examined by qPCR. N=6; *P<0.05 compared with TYRO3; #P<0.05 compared with MERTK. (B) Zika viral RNA was detected in Müller cells by qPCR at day (D) 1 and day 4 after infection. MOI=0.02. NI: non-infected control. N=6. *P<0.05 compared with relevant non-infected control. #P<0.05 compared with ZIKV infection at day 1. (C) Virus concentration in Müller cell culture supernatant at day 1 or day 4 after infection was determined by focus-forming assay and calculated as FFU/ml. *P<0.05 compared with day 1. MOI=0.02. (D) Representative fluorescent images stained for ZIKV (green) and cell nucleus (DAPI, blue) showing co-localization of virus and cells at day 4 after infection. MOI=0.02. 200x. (E) ZIKV Envelope (Env) protein was determined by Western blot at day 4 after infection. MOI=1. α -Tubulin was used as loading control. (F, G) Relative levels of viral

RNA in HRMECs and 661W cells at day 1 and day 4 after infection. MOI=0.2. GAPDH and β -actin serve as internal controls respectively. N=3; *P<0.05 compared with relevant non-infected control. #P<0.05 compared with ZIKV infection at day 1.

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Figure 2. ZIKV induces a pro-inflammatory phenotype of Müller cells

(A) Relative mRNA levels of inflammatory genes in Müller cells at day (D) 1 and day 4 after infection. MOI=0.02. NI: non-infected control. N=6; * P <0.05 compared with relevant non-infected control. (B) Linear regression analyses for the association between ZIKV viral RNA and induction of inflammatory factors at 4 days after infection. N=6.

Figure 3. ZIKV infection activates various signaling pathways in Müller cells

Müller cells were infected with ZIKV at a MOI of 0.02, proteins were collected at day 3 or day 4 after infection and MAPKs, NF- κ B and JAK/STAT3 signaling pathways were analyzed by Western blot. Samples collected from three independent experiments were blotted in the same gel. Bar graph represents the densitometric analysis of western blot data. NI: non-infected control. *P<0.05 compared with relevant non-infected control.

Figure 4. The effects of various inhibitors on ZIKV-induced inflammatory response in Müller cells

Müller cells were infected with ZIKV at a MOI of 0.02, and different inhibitors were added at day 3. Cells were harvested at day 4 after infection and inflammatory genes (A) and Zika viral RNA (B) were analyzed by qPCR. Cells treated with vehicle (0.1 % DMSO) were used as reference. N=4; *P<0.05 compared with ZIKV-infected vehicle-treated control.

Figure 5. ZIKV activates p38MAPK pathway in Müller cells

(A) Müller cells were infected with ZIKV at a MOI of 0.02, and proteins were collected at day 4 after infection. The protein kinases associated with the p38MAPK signaling pathway were then analyzed by Western blot. Samples collected from three independent experiments were blotted in the same gel. Bar graph represents the densitometric analysis of western blot data. NI: non-infected control. * $P < 0.05$ compared with relevant non-infected control. (B) Müller cells were treated with p38MAPK inhibitor SB202190 at day 3 after ZIKV infection and phosphorylated p38MPAK, total p38MAPK and phosphorylated MSK1 were determined at day 4. Bar graph represents the densitometric analysis of western blot data. $N = 3$; * $P < 0.05$ vs relevant non-infected control treated with vehicle (0.1 % DMSO); # $P < 0.05$ vs relevant ZIKV-infected samples treated with vehicle.

Figure 6. DENV induces a pro-inflammatory phenotype of Müller cells

Müller cells were infected with DENV2 at a MOI of 0.02, vehicle (0.1 % DMSO) and inhibitor SB202190 (SB) were added at day 3, and cells were harvested at day 4 after infection. Envelope gene of DENV2 (A) and inflammatory genes (B) were analyzed by qPCR. NI: non-infected control. N=4; *P<0.05 vs relevant non-infected control treated with vehicle; #P<0.05 vs relevant DENV2-infected samples treated with vehicle.

Table 1
Summary of the effects of various inhibitors on ZIKV-induced inflammatory response in Müller cells

Compared with vehicle-treated control, inhibitor treatment induced percentage change: (–) no change; (↑) 0–30% increase; (↑↑) 31–200% increase; (↑↑↑) 201–400% increase; (↑↑↑↑) more than 400% increase; (↓) 0–25% decrease; (↓↓) 26–50% decrease; (↓↓↓) 51–75% decrease; (↓↓↓↓) more than 75% decrease. The top blue arrows indicate the effect of various inhibitors on inflammatory response in non-infected Müller cells, compared with vehicle-treated non-infected Müller cells; the bottom red arrows indicate the effect of various inhibitors on ZIKV-induced inflammatory response in ZIKV-infected Müller cells, compared with vehicle-treated ZIKV-infected Müller cells.

	IL-6	ICAM1	CXCL1	CXCL10	CCL2	CCL5	CCL7	VEGF
PD98059	- ↑↑	↑↑ ↑↑	- ↑	- ↑↑↑↑	↓↓ ↓	↑↑ ↑	- -	↑ ↑
SB202190	↓↓ ↓↓↓	↑↑ ↓↓	- ↓↓↓	- -	↓↓ ↓↓↓	↓↓↓ -	↑ ↓↓↓	↓↓ ↓↓
PDTC	↑↑ -	- -	↑↑ -	↑↑↑ ↓↓	↑↑↑ -	↓↓↓ -	↑↑↑ -	↓↓ ↑↑
Stattic	- ↓↓	- ↓↓	- ↑	- ↓↓	- -	- ↓	- -	- -
AG490	↑↑ -	↓ -	- ↓↓	- -	- ↓↓	↓↓↓ -	- ↓↓	↑ ↑
TUDCA	↑↑ -	- ↑↑	- -	- -	- ↓	↓↓ -	- ↓	- -
PBA	↑↑ ↑↑	↑↑↑↑ ↑↑	↑↑ -	- ↑↑↑↑	- -	- -	- ↓	- -
GSK2606414	- ↑↑	↑↑ ↑↑	- ↑	- ↑↑↑	- ↓	↓↓ ↑↑	- -	↑↑ ↑
4μ8C	↑↑ ↓↓	↑↑ ↑↑	↑↑ ↓↓	- ↑↑↑	- ↓↓	↓↓ -	- ↓↓	- -
Apocynin	- ↓	↓↓ -	- ↓	- -	↓↓ ↓	↓↓↓ -	- -	↓↓ ↑↑

Comparison of the inflammatory response of ZIKV-infected and DENV2-infected Müller cells following blockade of p38MAPK by SB202190

Table 2

Compared with vehicle-treated control, inhibitor treatment induced percentage change: (–) no change; (↓) 0–25% decrease; (↓↓) 26–50% decrease; (↓↓↓) 51–75% decrease; (↓↓↓↓) more than 75% decrease.

	IL-6	ICAM1	CXCL1	CXCL10	CCL2	CCL5	CCL7	VEGF
ZIKV	↓↓↓	↓↓	↓↓↓	-	↓↓↓	-	↓↓↓	↓↓
DENV2	↓↓↓	↓↓	↓↓↓	-	↓	↓ ↓	↓	↓↓