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Zika virus modulates blood-brain barrier of brain microvascular endothelial cells

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Abstract. Zika virus (ZIKV) is a mosquito-borne *Flaviviruses*. ZIKV is known to cause birth defect in pregnant women, especially microcephaly in the fetus. Hence, more study is required to understand the infection of Zika virus towards human brain microvascular endothelial cells (MECs). In this study, brain MECs were infected with ZIKV at MOI of 1 and 5 *in vitro*. The changes in barrier function and membrane permeability of ZIKV-infected brain MECs were determined using electric cell-substrate impedance sensing (ECIS) system followed by gene expression of ZIKV-infected brain MECs at 24 hours post infection using one-color gene expression microarray. The ECIS results demonstrated that ZIKV infection enhances vascular leakage by increasing cell membrane permeability via alteration of brain MECs barrier function. This was further supported by high expression of proinflammatory cytokine genes (lnc-IL6-2, TNFAIP1 and TNFAIP6), adhesion molecules (CERCAM and ESAM) and growth factor (FIGF). Overall, findings of this study revealed that ZIKV infection could alter the barrier function of brain MECs by altering adhesion molecules and inflammatory response.

INTRODUCTION

Zika virus (ZIKV) is a vector-borne single-stranded RNA virus mainly transmitted by female *Aedes* mosquito bite (Hamel *et al.*, 2015). It has been reported that this single-stranded RNA virus also targets skin immune cells, including dermal fibroblasts, epidermal keratinocytes and immature dendritic cells (Hamel *et al.*, 2015) and infects human radial glial cells, astrocytes, endothelial cells, microglia in developing human cortex and developing retina cells (Nowakowski *et al.*, 2016).

Clinically ZIKV causes a mild self-limiting fever, skin rash, joint pains and occasionally the inflammation of the conjunctiva (Petersen, Jamieson, Powers & Honein, 2016). Although the clinical manifestations of ZIKV infection are typically mild, there is evidence showing the ability of ZIKV to cause neurological damage, such as Guillain-Barré syndrome in adults (Cao-Lormeau *et al.*, 2016) and congenital birth defects such as microcephaly in fetus (Calvet *et al.*, 2016; Rubin, Greene & Baden, 2016). Birth defects are not the only symptoms caused by this viral infection as hemorrhagic fever and plasma leakage manifestations were also observed in lethal acute ZIKV infections (Zonneveld *et al.*, 2016). These characteristics could be involved in severe ZIKV cases as scientists recently evidenced blood-brain barrier (BBB) leakage (Shao *et al.*, 2016).

Thus, the mechanism and outcome of ZIKV infection, especially in blood-brain barrier need further documentation. Therefore, in this study, we aimed to investigate the effect of ZIKV infection on human brain microvascular endothelial cells *in vitro*.

MATERIALS AND METHODS

Cell culture and ZIKV propagation

Transfected human brain microvascular endothelial cells (THBMEC) (ATCC, Manassas, Virginia, USA), was culture using Medium 199 (ThermoFisher, Waltham, Massachusetts, USA) (Nag, 2003). The cell line was maintained at 37°C in humidified air containing 5% CO_2. Zika virus (strain: PRVABC59) was propagated in Vero C1008 cells (ATCC) using Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher) supplemented with 2% fetal bovine serum (FBS). The virus stocks were titrated with a Foci Forming Unit Assay using immuno-staining as described (Zandi *et al.*, 2012).

Detection of ZIKV RNA and RT-qPCR

The THBMEC were infected with ZIKV at MOIs of 1 and 5. The cells and supernatant were collected at 24 hpi and the viral RNA was extracted from supernatant and cells using High Pure Viral RNA Kit (Roche, Germany) and RNeasy Mini Kit (QIAgen, Hilden, Düsseldorf, DE) respectively following the manufacturer's protocol. ZIKV was detected using a commercial kit (DOUBLE CHECK Kit, Genekam Biotechnology, Germany) on a CFX96 Real-Time PCR System (BIO-RAD). The standard curve was created from ten-fold serial dilutions of the known copy number of ZIKV RNA. Data was interpreted using CFX manager version 3.1 software (Bio-Rad, USA).

ECIS measurement of ZIKV-infected THBMEC

Electric Cell-substrate Impedance Sensing (ECIS) with 16 wells array station (ECIS Zθ) (Applied Biophysics) was used to measure the changes in complex impedance spectrum (resistance and capacitance) changes induced in THBMEC before and after ZIKV infection at MOI of 1 and 5. The brain MECs were grown to a confluent monolayer before infected with ZIKV in eight-well electrode array (8W10E) (Applied Biophysics, Troy, New York, USA). ECIS can be modelled to study of cell morphology in three different parameters which includes (a) cell-to-cell interaction exhibiting as brain function (Rb), (b) cell-to-matrix interaction demonstrating cell-substrate adhesion (α) and (c) the membrane permeability representing as cell membrane capacitance (Cm) (Mazen M. Jamil Al-Obaidi, 2017; Robert Szulcek, 2014; Soe *et al.*, 2017). The real time changes of brain MECs after infection with ZIKV at MOI of 5 were monitored for 24 hours post infection (hpi). The resistance changes were detected at low alternative current (AC) with a frequency of 2000 Hz. To measure the current flowing through the insulating cell membrane by trans cellular pathways a higher AC frequency (40,000 Hz) was used. The raw data was normalized to the uninfected brain MECs to compare the effects of ZIKV in the infected brain MECs. The results presented as standard error of mean between the duplicate at a regular interval.

Gene expression analysis of ZIKV-infected brain MECs

THBMEC were grown into confluent monolayer in 6 well plates before infected with ZIKV at MOI 5 within 24 hours. The cells were collected and subjected for microarray gene expression analysis. The cells RNA was extracted using RNeasy Mini Kit (QIAgen, Hilden, Düsseldorf, DE) respectively following the manufacturer's protocol. Gene expression analysis of ZIKV-infected brain MECs was conducted using One-Color Microarray-Based Gene Expression (Agilent Technologies). The data was normalized to the uninfected THBMEC to determine the gene expressed in ZIKV-infected brain MECs. The data was presented as normalized fold-change with a cut-off value of fold-change > 2.0.

Statistics

All data are presented as mean with error bars representing the standard error of mean. Statistical analyses of the experimental data were performed with GraphPad Prism 7 Statistical software for Mac OS (GrapPad Software, Inc., La Jolla, CA, USA). Student t-test analyis was used to determine the differences in the resistance and capacitance readings between the infected and uninfected THBMEC.

RESULTS

ZIKV is permissive towards THBMEC

To determine the infection and replication efficiency of ZIKV towards THBMEC, the cells were infected with ZIKV at MOIs of 1 and 5. The total RNA (intracellular and extracellular) were measured using qRT-PCR at 24 hpi (Figure 1). Our data showed that ZIKV was able to infect and replicate in brain MECs at both MOIs. The highest replication rate was detected in the cells at MOI of 5 (Figure 1). The total RNA copy number range between 5.63E+12 (extracellular) to 6.32E+12 (intracellular) in MOI of 5 as for MOI of 1, the RNA copy number range between 3.61E+12 (extracellular) to 4.48E+12 (intracellular). Nevertheless, regardless of MOIs, ZIKV actively replicated in THBMEC.

ZIKV modulated THBMEC barrier function and membrane permeability

The changes in resistance and capacitance reading were commonly used to interpret the changes in barrier function and cell membrane permeability. Resistance reading described the cells barrier function and quality (Figure 2a) while capacitance described cell membrane permeability (Figure 2b). It was observed that upon infection to ZIKV virus, the barrier function of THBMEC is modulated at both MOI of 1 and 5. The resistance reading for both MOIs significantly dropped from 2 hpi and plateau with no resistance changes after 24 hpi compared to control. The barrier modulation started with barrier tightening at 1 hpi followed by loss in barrier function at 2 hpi. Generally, the capacitance reading showed a significant increase in cell membrane permeability at 2 hpi and continued with no significant changes after that. However, MOI 5 showed higher membrane permeability compared to MOI 1.

In addition, the impedance reading can be further modelled into barrier function (Rb), cell-substrate adhesion (α) and cell membrane capacitance (Cm) which representing cell-to-cell interaction, cell-to-matrix interaction and membrane permeability (Figure 3 a-c). It was observed that upon infection to ZIKV virus, brain MECs were modulated from 2 hpi. This modulation is mainly due to alteration in cell-to-cell interactions (Rb) and increased in cell membrane permeability (Cm), through alteration in both paracellular pathway and trans cellular pathway (Figure 3a and 3b). There was slight modulation seen in the cell-to-matrix interaction (α) in THBMEC upon ZIKV infection indicating the weakening in adhesive bonding between cells and its extracellular matrix. Therefore, modelling of these parameters indicated that ZIKV infection altered barrier function of THBMEC whereby reduction in impedance was first observed indicating weakening of cell-to-cell interactions and followed by increased in membrane permeability of MECs at early stage of infection (Figure 3).

Genes expression of ZIKV-infected brain MECs

Our microarray data revealed that ZIKV infection induces inflammatory function related genes in THBMEC as shown in Figure 4. The genes upregulated were LNCipedia lincRNA (lnc-IL6-2), Homo sapiens tumor necrosis factor alpha-induced protein 1 (TNFAIP1), Homo sapiens tumor necrosis factor alpha-induced protein 6 (TNFAIP6), Homo sapiens cerebral endothelial cell adhesion molecule (CERCAM), Homo sapiens junctional adhesion molecule 3 (JAM3), Homo sapiens endothelial cell adhesion molecule (ESAM) and Homo sapiens c-fos induced growth factor (vascular endothelial growth factor D) (FIGF) (Figure 4). Table 1 and 2 shows upregulated and downregulated genes that were normalized against untreated THBMEC. The expression of these genes, however, needs further validation against housing keeping gene (GADPH) using qRT-PCR analysis.

DISCUSSION

Cellular tropism is an important factor for virus survival (Nomaguchi, Fujita, Miyazaki, & Adachi, 2012). In the case of ZIKV infection, cellular tropism play an important role in ZIKV association with microcephaly as ZIKV

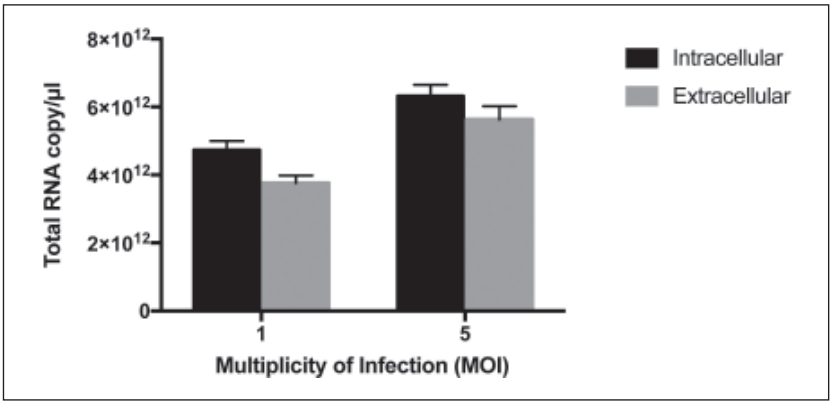

Figure 1. Total RNA copy/ul (intracellular and extracellular) of ZIKV-infected THBMEC at MOI 1 and 5 over 24 hour post infection.

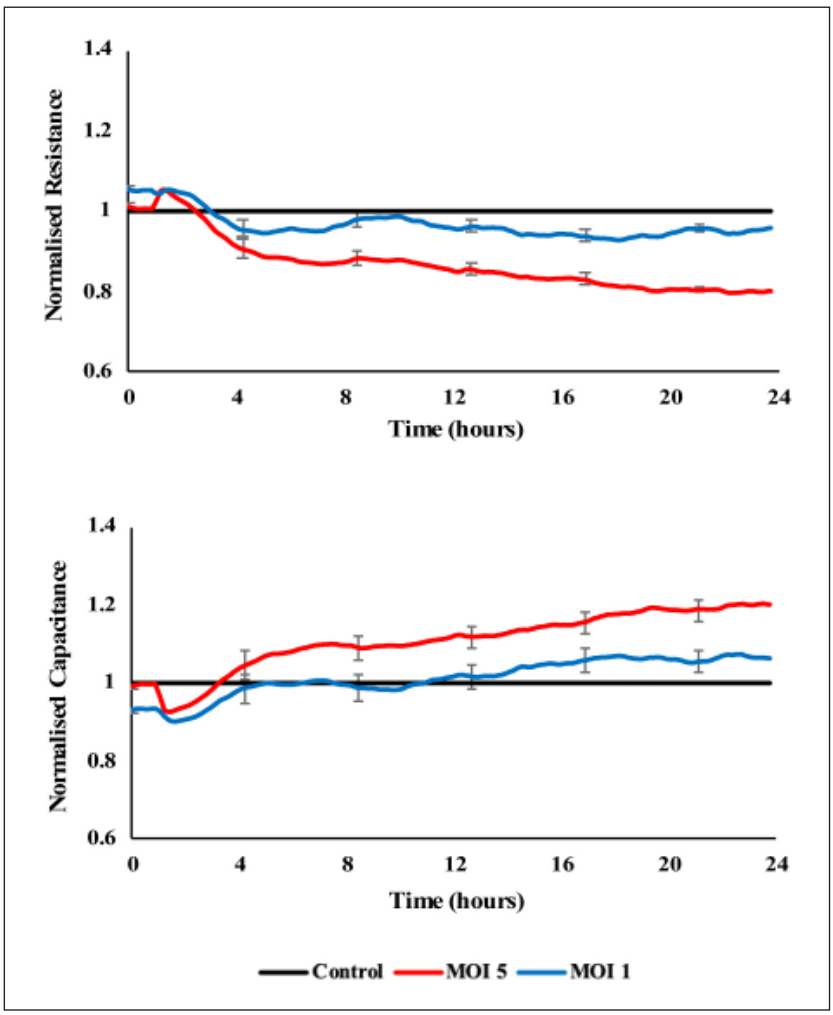

Figure 2. Normalized real time changes of a) resistance and b) capacitance in ZIKV-infected THBMEC at MOI of 1 and 5. The measured resistances and capacitance of ZIKV-infected THBMEC were normalized to uninfected cells within 24 hpi.

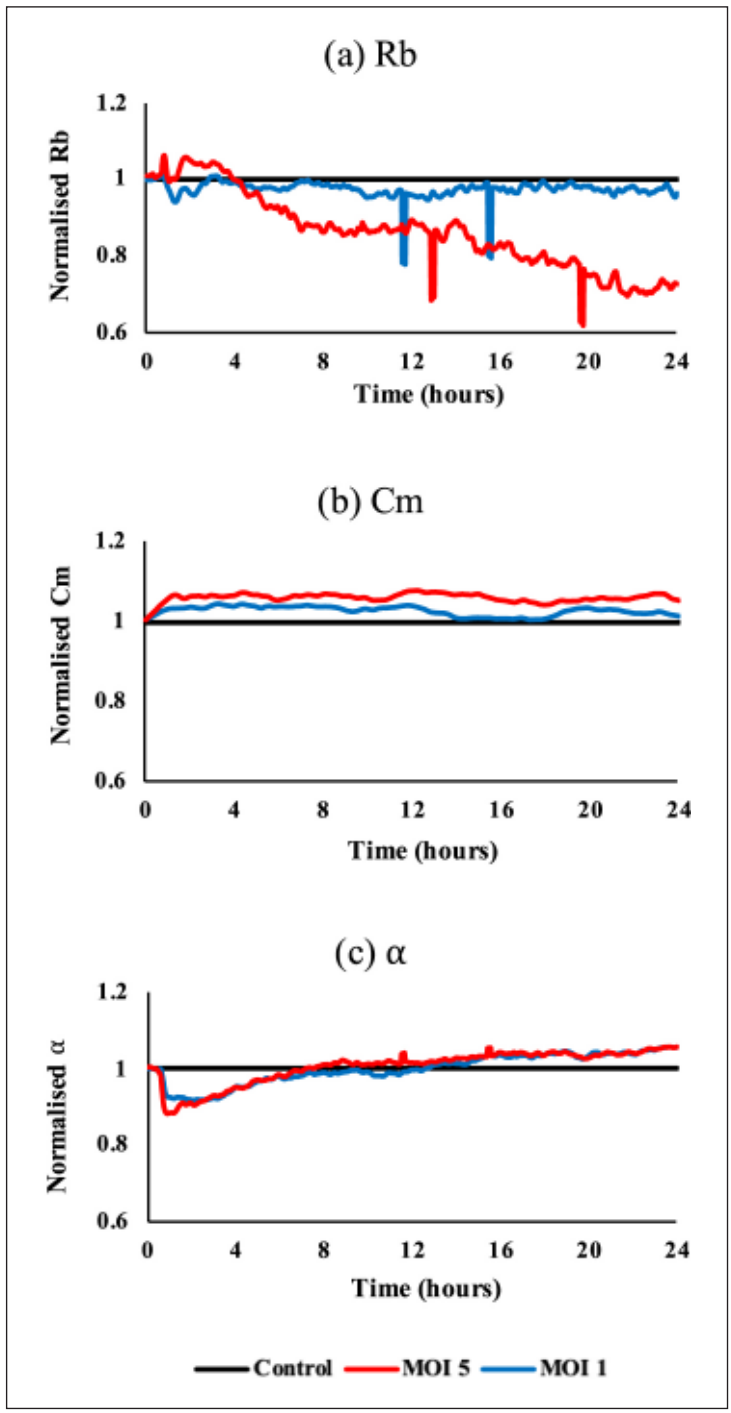

Figure 3. ECIS modelling of ZIKV-infected THBMEC compared to uninfected cells (control) at 24 hour post infection (hpi). The diagram described the changes in cell behavior in three parameters: normalized (a) barrier function (Rb), (b) cell membrane capacitance (Cm) and (c) cell-substrate interaction (α) in ZIKV-infected THBMEC.

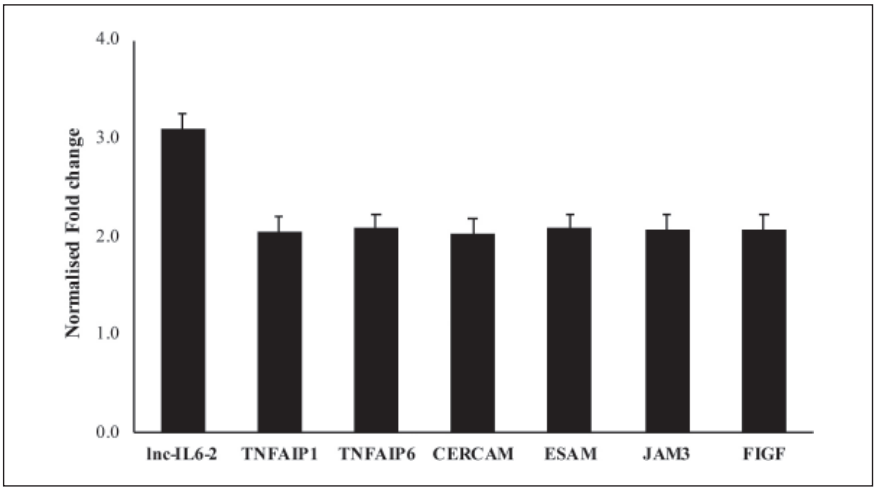

Figure 4. Normalised fold-change of gene expressed by ZIKV-infected THBMEC at MOI of 5 over 24 hpi.

Table 1. Upregulated genes in ZIKV-infected brain MECs at 24h

Genes	Fold-change
lnc-IL6-2	3.092429
TNFAIP1	2.044019
TNFAIP6	2.0756152
CERCAM	2.0239623
ESAM	2.075584
JAM3	2.0627003
FIGF	2.0695245

Table 2. Downregulated genes in ZIKV-infected brain MECs at 24h

Genes	Fold-change
HEPACAM	-4.30
IL13RA1	-3.7796383
IL5	-2.888881
FGF18	-2.5544252
IL1RL2	-2.5534678
lnc-IL1R2-1	-2.4059527
lnc-IL15RA-1	-2.3948574

has the preponderance towards brain cells. Past studies have reported on the cell tropism of ZIKV using few types of brain cells. A study of ZIKV-infected human neural progenitor cells (hNPCs) has illustrated that ZIKV infection was able to dysregulate the progression of cell cycle and increase caspase-3-mediated apoptosis causing attenuation in hNPCs (Tang *et al.*, 2016). Growth defects and cell death induction of induced pluripotent stem cells (iPSC) derived from neural stem cells in response to ZIKV infection led to impairment of formation of neurospheres (Garcez *et al.*, 2016). ZIKV infected human fetal neural stem cells (fNSCs) also caused the inhibition of neurosphere growth and neurogenic differentiation potential, as well as the induction of autophagy (Liang *et al.*, 2016). Severe cytopathic effects with persistent infection has been shown in the studies on hNPCs to ZIKV infection. These findings imply that neural progenitors in the developing human fetus can be direct targets of detrimental ZIKV-induced pathology (Hanners *et al.*, 2016). Neural progenitors as a potential direct target indicated the importance of developing efficient therapeutics for controlling the infection. Despite all these studies, ZIKV virus infection especially on brain MECs is still under explored. Hence, the present study provides a basic understanding of potential mechanism used by ZIKV in causing vascular leakage in brain MECs.

Our current study has shown that brain MEC is permissive towards ZIKV infection by the detection of ZIKV total RNA copy number in both intracecullar and extracellular. However, infectivity of

ZIKV towards brain MECs should be incorporated with foci-forming assay in order to confirm the presence of infectious virus particles. Previous studies also reported the permissiveness of ZIKV towards specific human organs by the detection of AXL receptors in the human brain, retinal and dermal cells (Hamel *et al.*, 2015; Nowakowski *et al.*, 2016). AXL is one of the receptors implicated in cell entry of enveloped viruses including ZIKV. Shao *et al.* (2016) reported using a mouse model that neuronal cell death correlated with ZIKV infection led to microcephaly. Injection of virus intra-cerebrally into embryonic mouse brains with ZIKV lead to abnormal vasculature with leaky blood brain barrier, extensive neuronal cell death, astrogliosis and brain injury (Shao *et al.*, 2016). In a human case study, fetal brain abnormalities were discovered to be associated with ZIKV infection at high viral loads and viral particles were detected in fetal brain (Driggers *et al.*, 2016).

To date modulation of vascular barrier function in different studies occurred only at high viral load, where severe clinical symptoms were reported to be associated with the level of viral load in other Flavivirus family, especially dengue infected patients (de Melo Iani *et al.*, 2016; Soe *et al.*, 2017). Cohort studies across the decade demonstrated that higher viral load was detected in patients with hemorrhagic manifestations during dengue infection (de Melo Iani *et al.*, 2016; Pal, Dutta, Mandal, Saha & Tripathi, 2014; Wang *et al.*, 2003). The association of ZIKV infection with vascular leakage, however is still under further investigation. But the case of deadly ZIKV infection of a teenage girl with sickle cell disease and three cases of elderly male death with a history of hypertension, diabetes mellitus (DM) and chronic pulmonary disease suggested an association of a fatal outcome with vascular leakage in immune-compromised patients that probably exacerbated the symptoms of ZIKV infection (Arzuza-Ortega *et al.*, 2016; Zonneveld *et al.*, 2016).

Previous study conducted showed the role of endothelial cells (ECs) in modulating immune system and inflammatory response (Gimbrone, Cotran & Folkman, 1974). Immune regulation by ECs hence, is influenced by the tissue microenvironment through tissue-specific endothelial cell-leukocyte interactions and inflammation-activation (Danese, Dejana & Fiocchi, 2007). In one *in vitro* study conducted in 2015, researchers observed that dengue virus (DENV) NS1 triggers endothelial barrier dysfunction, causing increased permeability in human endothelial cell monolayers leading to vascular leakage (Beatty *et al.*, 2015). Recently, scientists found the existence of similar structural features of DENV NS1 in ZIKV which could provide evidence that ZIKV can potentially induce plasma leakage in brain microvascular endothelial cells (MECs) used in this study (Xu *et al.*, 2016). Several evidences indicate that dengue virus stimulated immune response play a crucial part in physiological cascade leading to vascular leakage (Beatty *et al.*, 2015; de Melo Iani *et al.*, 2016; Soe *et al.*, 2017). Virus infection therefore, induces plasma leakage via inappropriate cytokines productions leading to inflammation (Danese *et al.*, 2007). This was further supported by several studies done on gene expression profiling of dengue virus infection in various cell lines and patients blood samples. Fink *et al.* has evidenced genes involved in three major pathway (NF-κB, IFN and ubiquitin proteosome) has been significantly upregulated in both cell lines and patient samples. Amongst the highest upregulated genes were chemokines IP-10 and I-TAC in patients peripheral blood at early onset of dengue infection. High expression of viperin (IFN pathway) and MG-132 and ALLN (ubiquitn-proteosome pathway) results in significant reduce of viral replication in cell line used (Fink *et al.*, 2007). This implicates that identification of these host genes associated with dengue infection provides important knowledge that these genes therefore can be used as potential targeted biomarker for dengue virus pathogenesis.

The barrier dysfunction of brain MECs seen in our ECIS data may potentially be modulated by inappropriate regulation of genes responsible for cytokine production

and tight junctional proteins. This aspect neccessitates the microarray analysis on MECs exposed to ZIKV infection. Recent study demonstrated a drastic downregulation of of microRNAs (miRNAs) particularly miR-155, miR-203, miR-29a, and miR-124-3p in ZIKV-infected primary mouse neuron. These miRNAs are responsible in flavivirus infection, antiviral, immunity and brain damage (Azouz *et al.*, 2019). These genes play an important role in regulation of pathway related to neurological development and neuroinflammatory responses. Furthermore, another study done on ZIKV infection in liver (HepG2), lung (A549) and kidney (MA104) cells modulated miRNA levels with high expression of the genes encoding the miRNA-related proteins DGCR8, Ago1, and Ago3 in HepG2 cells and Drosha, Dicer, Ago2, and Ago3 in A549 and MA104 cells. These results provide an additional information on mechanism of host cell dysfunction in these organs (Ferreira *et al.*, 2018). These genes can be used as therapeutic biomarkers for the management of ZIKV in neurological disease.

In present study, microarray analysis on brain MECs exhibited upregulation of genes responsible in the modulation of inflammatory effect. The genes identified were related to the production of pro-inflammatory cytokines IL-6 (lnc-IL6-2), TNF-α (TNFAIP1 and TNFAIP6), adhesion molecules (CERCAM, JAM3 and ESAM) and growth factor (FIGF). Proinflammatory genes expressed contribute to vascular dysfunction by modulation of proinflammatory cytokines IL-6 and TNF-α into microvasculature by increasing leukocyte recruitment at the site of infection. IL-6 and TNF-α were also known to cause neuroinflammation in ZIKV-infected patients (Kam *et al.*, 2017; Lum *et al.*, 2017; Naveca *et al.*, 2018). Similar observation has been reported in gene expression study of acute dengue hemorrhagic fever, whereby significant upregulation of TNFAIP6 observed in dengue patient peripheral blood sample suggesting the correlation of viral replication rate and attenuation of host innate immune response (Simmons *et al.*, 2007). The present study have also evidenced upregulation of adhesion molecules genes (CERCAM, JAM3 and ESAM) and endothelial growth factor gene (FIGF) upon ZIKV infection. Overall, these gene regulations implicate that ZIKV infection could enhance vascular leakage by disrupting cytoskeleton and tight junctional proteins arrangement of brain MECs. However, future studies should include validation on these gene expressions using qRT-PCR in order to confirm their role in causing vascular leakage.

In conclusion, this study reveals the association of THBMEC and permeability towards ZIKV infection. ZIKV infection enhance vascular leakage potentially via the modulation of inflammatory response and altering cell adhesion molecules. Despite ZIKV being highly homologous to dengue virus, there is limited evidence of ZIKV causing hemorrhagic manifestations (Lazear & Diamond, 2016). Knowledge on how Zika virus controls and repairs the MECs may enable understanding towards management of vascular leakage in dengue. Hence, future studies targeting different MECs originated from other human organs involving several time-points of post infection is necessary. Besides this, as high expression of pro-inflammatory cytokine genes, adhesion molecules and growth factor has been observed in current study, future studies on cytokine profiling of Zika virus-infected MECs should also be carried out.

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