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In situ inflammasome activation results in severe damage to the central nervous system in fatal Zika virus microcephaly cases

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ABSTRACT

Zika virus (ZIKV) has caused substantial concern worldwide owing to its association with severe birth defects, such as microcephaly and other congenital malformations. Inflammasomes, i.e., multi-protein complexes that induce inflammation and pyroptosis, are predicted to contribute to the immune response to this flavivirus. Accordingly, in this study, the *in situ* inflammasome response was evaluated in fatal cases of ZIKV-linked microcephaly. Brain tissue samples were collected from eight babies, including four ZIKV-positive microcephalic neonates who died after birth and four flavivirus-negative neonatal controls who died of other causes and whose central nervous system (CNS) architecture was preserved. In the ZIKV-positive newborn/stillbirth babies, the major histopathological alterations included atrophy of the cortical layer, a predominance of mononuclear cell infiltration in the Virchow–Robin space, neuronal necrosis, vacuolization and neuronal degeneration, neuronophagy, and gliosis. An immunohistochemical analysis of tissues in the neural parenchyma showed significantly higher expression of the receptors NLRP1, NLRP3, and AIM2, cytokines IL-1β, IL-18, and IL-33, and enzymes caspase 1, iNOS, and arginase 1 in ZIKV-positive microcephaly cases than in flavivirus-negative controls. These results suggest that inflammasome activation can aggravate the neuroinflammatory response and consequently increase CNS damage in neonates with fetal neural ZIKV infection and microcephaly.

1. Introduction

Zika virus (ZIKV) is an arbovirus belonging to the family *Flaviviridae* and genus *Flavivirus* [1]. It is mainly transmitted through the bite of an infected *Aedes aegypti* mosquito, but also through blood transfusion, vertical transmission, and sexual contact [2]. ZIKV was initially isolated in 1947 from the serum of a febrile sentinel rhesus monkey (*Macaca mulatta*) in Zika Forest, Uganda [3,4]. ZIKV is enveloped and icosahedral and possesses single-stranded, positive-sense, non-segmented RNA that yields three structural and seven non-structural proteins, similar to other flaviviruses, e.g., dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), and Japanese encephalitis virus (JEV) [5–7]. It infects various cells and organs. In the placenta, ZIKV infects Hofbauer cells, trophoblasts, and endothelial cells (human, mice, and human-non-primates). In the reproductive tract, ZIKV infects Leydig cells, Sertoli cells, spermatogonia (in the testis of mice), vaginal epithelial cells (mice), and uterine fibroblasts (*in vitro* infection of human samples).

In the brain, ZIKV infects neural progenitors, mature neurons, and glial cells (human, mice, and human-non-primates). This pantropism results in ZIKV detection in multiple cells and also in different body fluids, including conjunctival fluid or tears (mice and human), saliva,

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Table 1
Summary of four fatal Zika virus (ZIKV) microcephaly cases and four control samples according to category, lifetime, sex, and ZIKV immunohistochemistry **(IHC)** and real-time PCR **(RT-qPCR)** results**. M:** male; **F:** female; **IHC:** immunohistochemistry assay; **RT-qPCR:** quantitative real-time reverse transcription polymerase chain reaction; **Pos:** positive; **Neg**: negative; **NA**: not available; **NP:** not performed.

Case	Category	Lifetime	Sex	Case information	IHC (ZIKV)	RT-qPCR (ZIKV)
Microcephaly						
1	Newborn	2 h	M	Microcephaly and other malformations(nasopalatine palate and syndactyly, club foot)	Pos	Neg
2	Newborn	27 days	M	Microcephaly and other congenital malformations	Pos	Pos
3	Newborn	2 days	M	Microcephaly	Pos	Neg
4	Stillbirth	NA	F	Microcephaly detected at birth	Pos	NP
Control						
5	Newborn	10 days	F	No microcephaly. Death ten days after birth but no evidence of ZIKV infection	Neg	Neg
6	Stillbirth	NA	M	Microcephaly case no ZIKV infection	Neg	Neg
7	Stillbirth	NA	F	Microcephaly case no ZIKV infection	Neg	NP
8	Newborn	19 h	F	Microcephaly case and no evidence of ZIKV infection. Mother with hyperthyroidism and pregnancy-induced hypertension	Neg	Neg

semen (mice, human-non-primates, and humans), cervical mucus (human), vaginal washings (mice and human), and urine (human-non-primates and human) [8–11].

ZIKV infection has become a global health threat, with an increase in epidemics, rapid dissemination, unusual routes of transmission, and severe sequelae, especially in newborns. According to the World Health Organization (WHO), as of November 2016, the virus has been reported in over 69 countries and territories worldwide [12]. An explosive epidemic in Brazil sparked particular concern owing to hundreds of cases of microcephaly in neonates [13,14]. In February 2016, the WHO declared that the ZIKV epidemic was a public health emergency of international concern [14].

Microcephaly and other congenital malformations resulting from ZIKV infection are associated with various underlying mechanisms that modulate the host immune response in the central nervous system (CNS) [15,16]. Experimental studies with mice and *in vitro* have shown that astrocytes, glial cells, and mature neurons in the cortical layer of the brain induce the production of TAM receptors that facilitate ZIKV entry into these cells [17–19]. Microcephaly can likely be attributed at least in part to cell death, and studies have shown that the elevated expression of caspase 3 in cortical neurons contributes to neuronal apoptosis [20,21].

The neuroinflammatory process, including the inflammasome response, against ZIKV is essential for inducing the activation of the glial cell line U87-MG, as well as the expression of NLRP3, caspase 1, and IL-1β [22]. Inflammasomes are multi-protein complexes that induce inflammation and increase the expression of NLRP1, NLRP3, NLRC4, AIM2, and caspase 1, which cleaves the cytokines pro-IL-1β, pro-IL-18, and pro-IL-33, and provokes cell death by pyroptosis [22–25].

In situ immune responses are not yet understood, but inflammasome activation in the neural parenchyma is hypothesized to play a role in the development of microcephaly in response to ZIKV infection. Accordingly, we investigated the *in situ* inflammasome response in fatal cases of microcephaly caused by ZIKV.

2. Material and methods

2.1. 1 Ethics statement

Patient samples were obtained and processed as part of surveillance response measures for the ZIKV epidemic in Brazil on an emergency basis, as defined by the Ministry of Health. This study was approved (no. 1.888.946) by the Research Ethics Board (CEP) of the Evandro Chagas Institute (IEC). All methods were performed in accordance with the relevant guidelines and regulations approved by CEP/IEC and the rules and regulations of the Brazilian Ministry of Health for studies using biological samples.

2.2. Patients, samples, and diagnosis of ZIKV infection

Brain tissue samples were collected from eight patients, four of which were newborns/stillbirths with microcephaly and ZIKV positive confirmatory analysis laboratory and four of which were controls. All tissue samples were obtained during an investigation of deaths suspected to be caused by ZIKV during the microcephaly epidemic. The four selected controls presented CNS architecture preserved and were negative to ZIKV and for the most common flaviviruses circulating in Brazil (DENV and YFV). In the screening of these patients, prenatal follow-up and the request of tests to TORCHS group (syphilis, toxoplasmosis, rubella, cytomegalovirus, and herpes simplex) ruled out the possibility of infection. More detailed information on the patients included in this study can be found in Table 1.

2.3. Real-time quantitative RT-PCR (RT-qPCR) and virus isolation

We attempted to isolate the virus in cultures of C6/36 *Aedes albopictus* cells [26]. In brief, blood and tissue samples were titrated in phosphate-buffered saline (PBS) containing 10% fetal bovine serum and antibiotics. After 10 min of centrifugation at 2100*g* at 4 °C, 100 μL of the clarified supernatant was inoculated into a 25 cm^3 tissue culture flask with a monolayer of mosquito cells. After incubation for 2 h at 28 °C, medium was added, and the cells were incubated at room temperature (25 °C) for 10 days. The cells were initially examined by indirect immunofluorescent assay (IFA) using a flavivirus group hyperimmune polyclonal mouse antibody. The hyperimmune polyclonal mouse antibody was produced in the IEC from of an purified viral suspension containing the most prevalent flavivirus circulating in the country and that was produced in mice BALBc to obtain hyperimmune mouse ascitic fluid for antibody analyses. Methodology of execution of the experiments was based and adapted according of the standardization protocol as described elsewhere [27,28]. Subsequently, IFA-positive cells culture were tested by RT-qPCR [29,30].

RNA was extracted from supernatants of tissue homogenates and from cells culture using a TRIzol Plus RNA Extraction Kit (Ambion, Foster City, CA, USA) according to the manufacturer's instructions. RT-qPCR was performed using the 7500 Real Time PCR System (Applied