Zika virus induces inflammasome activation in the glial cell line U87-MG

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ARTICLE INFO

Keywords:
ZIKV
U87 glial cell
Neuroinflammation
Inflammasome NLRP3
Oxidative stress
IL-1β

ABSTRACT

In the last years, neurological complications related to Zika virus (ZIKV) infection have emerged as an important threat to public health worldwide. ZIKV infection has been associated to neurological disorders such as congenital microcephaly in newborns and Guillain-Barré syndrome, myelopathy and encephalitis in adults. ZIKV is characterized by neurotropism and neurovirulence. Several studies have identified microglial nodules, gliosis, neuronal and glial cells degeneration and necrosis in the brain of ZIKV infected infants, suggesting that ZIKV could play a role in these neurological disorders through neuroinflammation and microglial activation.

Little information is available about neuroinflammation and ZIKV-related neurological disorders. Therefore, we investigated if ZIKV is able to infect a glial cell line (U87-MG) and how the glial cell line responds to this infection in terms of inflammation (IL-1β, NLRP-3 and CASP-1), oxidative stress (SOD2 and HemeOX) and cell death.

We observed a significant increase of ZIKV load in both cells and supernatants after 72 h, compared to 48 h of infection. We found that ZIKV infection induces an increase of *IL-1β*, *NLRP-3* and *CASP-1* genes expression. Significant increase of IL-1β and unchanged pro-IL-1β protein levels have also been detected. Moreover, we observed *SOD2* and *HemeOX* increased gene expression mainly after 72 h post ZIKV infection. Subsequently, we found a decrease of U87-MG cell viability, after both 48 h and 72 h of ZIKV infection.

Our results show that U87-MG cells are susceptible to ZIKV infection. ZIKV is able to successfully replicate in infected cells causing oxidative stress, NLRP3 inflammasome activation and subsequent release of mature IL-1β; this process culminates in cell death. Thus, considering the central role of neuroinflammation in neurological disorders, it is important to comprehend every aspect of this mechanism in order to better understand the pathogenesis of ZIKV infection and to identify possible strategies to fight the virus by rescuing cell death.

1. Introduction

The rapid spread of Zika virus (ZIKV) and its association with congenital brain defects are considered an important threat to public health worldwide. ZIKV is an emerging Arbovirus belonging to the Flaviviridae family and Flavivirus genus, which includes, among others, Dengue, West Nile, Yellow Fever and Japanese encephalitis viruses. Like other members of this genus, ZIKV has a positive-sense, single-stranded RNA genome [1].

ZIKV infection was reported sporadically in different regions of Africa, as well as in some countries in Asia; in the Yap Islands, an island chain in the Federated States of Micronesia, had represented the first major outbreak of Zika virus. Subsequently, ZIKV has been identified in French Polynesia, Easter Island (Chile), the Cook Islands, New Caledonia and more recently in Central and South America [2,3].

Phylogenetic analyses disclose two important lineages, African and Asian, originating from a single ancestor. The African lineage has never been associated with congenital malformations or

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neurological complications, as observed for the Asian lineage. The Asian lineage has been involved in the epidemics in the Pacific Islands and more recently in Brazil, South and Central Americas [4,5].

The clinical manifestations are usually not specific and characterized by fever, maculopapular rash, headache, conjunctivitis, myalgia, and arthralgia. Major complications include neurological disorders such as congenital microcephaly in newborns and Guillain-Barré syndrome (GBS), myelopathy and encephalitis in adults [1,6].

Mother to-child transmission of ZIKV, occurring in utero or perinatally, has already been described. The association between this infection during pregnancy and foetal malformations was determined after the increase in microcephaly cases in newborns in the northeastern region of Brazil in 2015, during ZIKV outbreak [7,8].

To date, several types of foetal malformations have been described as associated with ZIKV intrauterine infection, currently referred to as *congenital ZIKV syndrome* (CZS), which includes not only microcephaly and foetal brain damage, but also a number of abnormalities of the musculoskeletal development, ocular, craniofacial, genitourinary and intrauterine growth retardation [9,10].

Several studies have shown the presence of viral RNA and antigen in the brain, but not in other organs, of congenitally infected foetuses and newborns [6,9,11]. In fact, ZIKV targets neural progenitor cells, leading to damage and consequent loss of these cells as well as reduced generation of new neurons in these regions [12]. Autopsy and pathological examination of the brain of infants with microcephaly, who had died less than 24 h after birth, revealed microglial nodules, gliosis, cellular degeneration and necrosis that preferentially targeted neuronal and glial cells [6,13].

The ZIKV entry into a host cell is mediated by the viral envelope protein, which interacts with several cell surface receptors and attachment factors. Nowakowski TJ and collaborators identified AXL as a possible candidate ZIKV entry receptor, highly expressed in central nervous system (CNS) cells such as microglial cells, astrocytes and radial glial cells [14].

ZIKV induces an innate antiviral response in mammalian cells, such as primary human skin fibroblasts, producing type I interferons in infected cells [15,16]. Furthermore, markers associated with oxidative stress responses have been observed in patients with severe Dengue virus infection. Olagnier and collaborators have demonstrated a relationship between oxidative stress and viral pathogenesis using human dendritic cells (DC), the primary target of DENV infection; actually, oxidative stress triggers innate antiviral immune responses and induces apoptosis [17]. Flavivirus replication may result in oxidative stress that in turn might induce the inflammasome activation. NLRP3 is the best-known Nod-like receptor (NLR) associated with inflammasome activation. NLRP3 activation is also correlated to oxidative stress, being oxidative stress observed upon treatment with many NLRP3 activators [18,19]. Chen and collaborators reported that HCV RNA, in human myeloid cells, activates the NLRP3 inflammasome in a ROS-dependent manner, confirming the close relationship between oxidative stress and inflammasome activation [20]. NLRP3 a cytosolic multiprotein complex, through the activation of pro-caspase-1, induces proteolysis and release of pro-inflammatory cytokines, such as IL-1β; this release could prompt pyroptotic cell death [21,22]. Wu and collaborators observed NLRP3 inflammasome activation and IL-1β release in macrophages infected with Dengue virus. These events lead ultimately to cell death via pyroptosis [23]. Several studies reported NLRP3 inflammasome activation and IL-1β release during infection of flaviviruses associated to CNS damage [24,25].

To date little information regarding ZIKV, inflammasome activation and inflammation especially in CNS are available. For these reasons we decided to investigate if ZIKV is able to infect the U87-MG glial cell line and to study how U87-MG responds to this infection in terms of inflammation (IL-1β, inflammasome NLRP3 and CASP1), oxidative stress (SOD2 and HemeOX) and cell death.

2. Materials and methods

2.1. Cell culture and ZIKV infection

U87-MG cells (human glioblastoma cell line), generously provided by Prof. Del Sal G. (University of Trieste, Italy) were cultured in Minimum Essential Medium Eagle (MEME, Sigma, Italy), supplemented with 10% fetal bovine serum (FBS, Euroclone, Italy), 4 mM glutamine and 2 mM penicillin streptomycin anphotericin B 1 × solution (Sigma, St. Louis, MO) and used between passages 4 to 10 to avoid cellular senescence.

ZIKV, MRS ZKV CTA 723 strain has been kindly provided by the Italian Istituto Superiore di Sanità. U87-MG were seeded in 6-well or 96 multi-well plate and after 24 h were infected at MOI 4.5 and incubated 24 h at 37 °C and 5% CO_2. After 24 h the medium was removed and cells were washed once with PBS. Culture medium was added to each well, and cells were incubated at 37 °C and 5% CO_2 for further 24 h and 48 h.

2.2. Detection of ZIKV

Culture supernatants and cells collected 48 h and 72 h post infection. Nucleic acids were extracted from supernatants and cells with QIAamp Viral RNA Mini Spin Kit (Qiagen) according to the manufacturer's protocol and were eluted in 70 μL. ZIKV RNA was detected by a qualitative real-time RT-PCR, amplifying a 76-nucleotide segment of the envelope gene, as described by Lanciotti et al. [26] (Tab. 1). The amplification were carried out in a 7300 Real-time PCR System (Applied Biosystems, Thermo Fisher Scientific) by using SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase kit (Invitrogen, Life Technologies). We performed ZIKV quantification using the Zika MR766 molecular standard, kindly provided by the Robert Koch Institute (Berlin, Germany).

2.3. RNA isolation and real time-PCR

Total RNA was reverse transcribed into cDNA with a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems®, Monza, Italy). Semi-quantitative real-time PCR was performed using Taqman Gene Expression Assays for human ACTB (Hs99999903_m1), IL-1β (Hs01555410_m1), NLRP3 (Hs00366465_m1), CASP1 (Hs00354836_m1), SOD2 (Hs00167309_m1) and HemeOH (Hs01110250_m1) genes (Applied Biosystems® Thermo Fisher, Monza, Italy) with the ABI 7500 Fast Real-Time PCR platform (Applied Biosystems® Thermo Fisher, Monza, Italy). The PCR amplification cycle was "standard mode": after denaturation at 96 °C for 10 min, 40 PCR cycles were performed composed by 15 s at 95 °C and a final melting step for 1 min at 60 °C. All samples were analysed in triplicate using the SDS 1.4 software (Applied Biosystems® Thermo Fisher, Monza, Italy). Results were normalized to *ACTB* expression and then to untreated cells according to $2^{-\Delta\Delta Ct}$ method [27].

2.4. Western blot

U87-MG cells were lysed in RIPA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Triton X-100, 140 mM NaCl). Cellular lysates were quantified using Bradford assay (Biorad, Hercules, USA); equal amounts of protein were loaded on 4–20% Tris-Tricine gels and

transferred on nitrocellulose membrane (Biorad, Hercules, USA). Membranes were then incubated with primary antibodies and then developed with HRP-conjugated secondary antibodies and Clarity™ substrate (Biorad Hercules, USA). Primary antibodies used in this study were: anti- IL-1β (Abcam, UK), and -HSP-90 (Santa-Cruz, Dallas, USA). Relative quantification of pro-IL-1β and IL-1β was normalized to HSP-90 optical density.

2.5. MTT cell viability assay

Cell viability was determined by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Trevigen, Gaithersburg, MD). Absorbance was measured in GloMax®-Multi Detection System (Promega, Fitchburg, WI).

2.6. Data analysis

Statistical significance was calculated using paired *t*-test or one-way analysis of variance (ANOVA) and Bonferroni post-test correction in case of multiple comparisons. Analysis was performed using GraphPad Prism software (version 5.0).

3. Results

3.1. ZIKV replicates in U87-MG

In order to assess infectivity of ZIKV in U87-MG cells, the quantification of ZIKV in supernatants and U87-MG cells exposed to ZIKV was evaluated 48 and 72 h after the infection.

The ZIKV load increased both in U87-MG cells and in supernatant; in fact, intracellular ZIKV load augmented significantly after 72 h of infection compared to the viral load in cells after 48 h of infection (ZIVK load copies/μL: ZIKV 72 h $6.21 \times 10^9 \pm 1.73 \times 10^9$, $p = 0.006$.; ZIKV 48 h $5.52 \times 10^8 \pm 6.5 \times 10^8$) (Fig. 1a). Also in supernatant ZIKV load was significantly higher after 72 h of infection than after 48 h of infection (Fig. 1b) (ZIVK load copies/μL: ZIKV 72 h $2.78 \times 10^6 \pm 5.62 \times 10^5$, $p = 0.0024$; ZIKV 48 h $5.3 \times 10^5 \pm 8.35 \times 10^4$) (Fig. 1b).

3.2. IL-1β and NLRP3 inflammasome activation after ZIKV infection

The ZIKV induction of inflammation associated to NLRP3 inflammasome activation and subsequent IL-1β production in U87-MG was investigated. In addition to *IL-1β*, also the gene expression of *NLRP-3* and *CASP-1*, the main components of the inflammasome, was investigated.

We observed a 2.07 ± 0.25 fold *IL-1β* expression after 48 h and a further significant increase of 3.62 ± 1.75 times after 72 h compared to the control cells (NT) ($p = 0.04$) (Fig. 2a).

ZIKV in U87-MG induced an increase of 3.86 ± 1.07 and 3.17 ± 1.63 times for *NLRP3* (Fig. 2b) and of 2.93 ± 0.86 and 3.11 ± 1.87 for *CASP-1* (Fig. 2c) after 48 and 72 h respectively when compared to untreated condition; however the increase was not statistically significant.

To double-check the *IL-1β* gene expression results, we also measured IL-1β protein levels through western blot analysis. We confirmed the same trend observed when analysing *IL-1β* expression: ZIKV caused after 72 h a significant increase of IL-1β protein levels with a relative quantification of 0.57 ± 0.5 fold and 1.76 ± 0.01 fold increase in infected vs uninfected cells after 48 and 72 h, respectively (72 h $p = 0.02$) (Fig. 2d). Instead, pro-IL-1β protein levels did not vary, confirming that the majority of the pro-IL-1β protein produced is cleaved in IL-1β active form (pro-IL-1β relative quantification in ZIKV infected cells was 0.79 ± 0.47 after 48 h and 1.00 ± 0.27 after 72 h when compared to control cells) (Fig. 2e).

3.3. Oxidative stress after ZIKV infection

In order to verify whether inflammasome NRLP3 activation is connected to oxidative stress, we evaluated the gene expression of *SOD2* and *HemeOX*, two important markers of oxidative stress.

ZIKV infection in U87-MG induces oxidative stress, especially after 72 h. We observed a 4.29 ± 0.87 fold increase of *SOD2* expression after 48 h and further 5.21 ± 3.53 fold increase after 72 h when compared to untreated condition, however the increase was not statistically significant (Fig. 3a). The *HemeOX* expression increased of 3.02 ± 1.06 times after 48 h and then significantly augmented 4.42 ± 1.72 times after 72 h of ZIKV infection, when compared to control cells ($p = 0.03$) (Fig. 3b).

3.4. ZIKV alters U87-MG cell viability

Considering the important inflammatory effect of ZIKV in U87-MG, we evaluated this effect on cell viability. A significant decrease of cell viability in ZIKV infected cells was observed, both after 48 and 72 h, when compared to untreated condition (% Survival: ZIKV 48 h 84.11 ± 2.33, $p = 0.0003$; ZIKV 72 h 76.68 ± 5.46, $p = 0.00001$; NT 100) (Fig. 4).

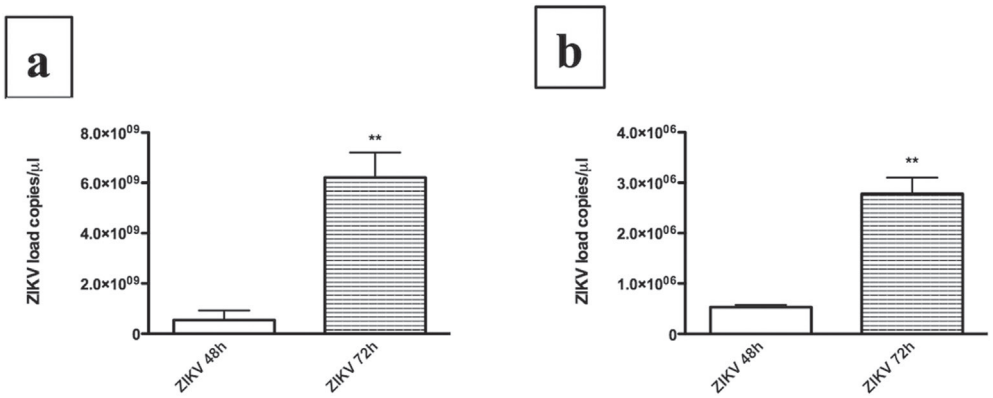

Fig. 1. ZIKV has been quantified in U87-MG cells and supernatants. U87-MG cells were infected with ZIKV for 48 and 72 h. At the end of the incubation periods we separated and collected supernatants and cells. ZIKV RNA was semi-quantitatively detected by a real time quantitative polymerase chain reaction (PCR), amplifying a 76-nucleotide segment of the envelope gene, according to Lanciotti et al. (2008). ZIKV load copies/μL was performed using Zika virus PCR-standard **(a)** in U87-MG cells and **(b)** in supernatant. Results are representative of three independent experiments. Data analyses were performed with paired *t*-test comparing 48 h with 72 h after infection; **$p < 0.01$.

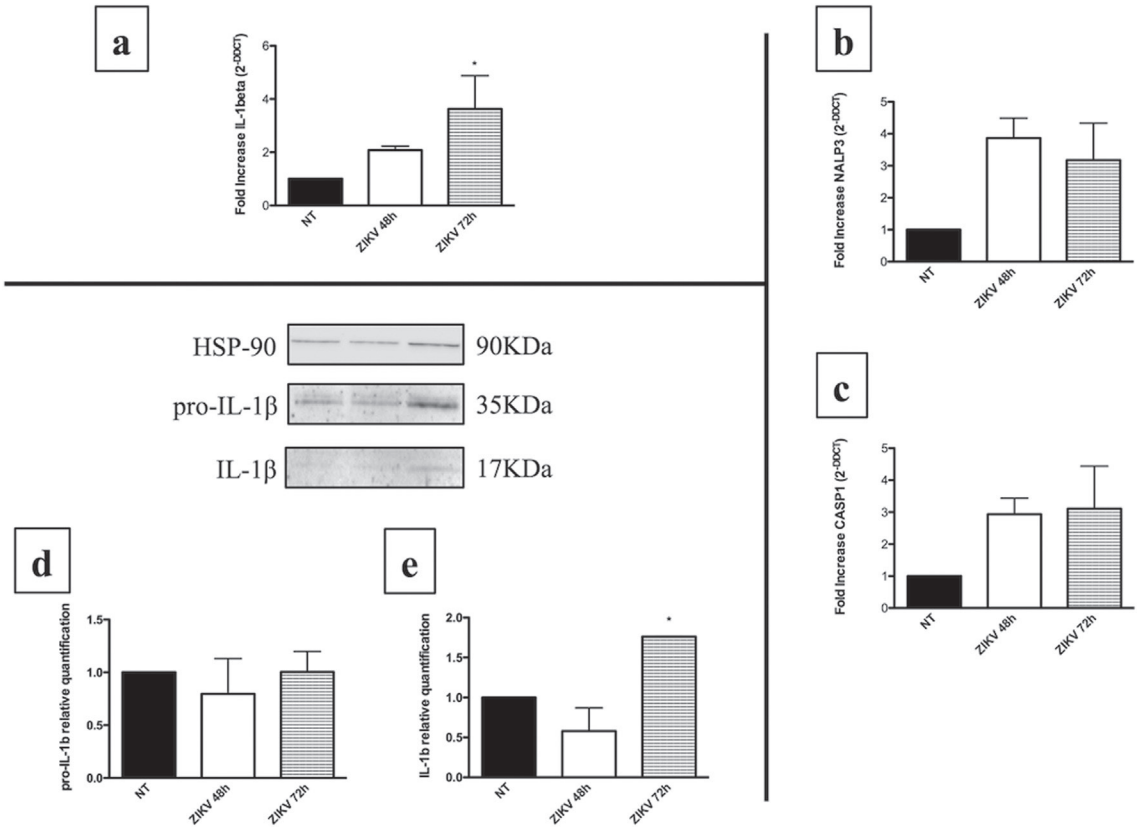

Fig. 2. ZIKV induces the increase of IL-1β and inflammasome activation. U87-MG cells were infected with ZIKV for 48 and 72 h. **(a)** Expression of *IL-1β*, **(b)** *NLRP3* and **(c)** *CASP1* (the main components of inflammasome) were measured after incubation periods. Analyses were performed using real time quantitative polymerase chain reaction (PCR), and results were normalized to *ACTB* expression. Expression of untreated cells was normalized to 1. Expression data for the three independent experiments are reported as $2-\Delta\Delta Ct$ average ± SD, in which $\Delta\Delta Ct = \Delta Ct\_stimulated\ HC - \Delta Ct\_RHC$. Data analyses were performed with one-way ANOVA and Bonferroni correction comparing NT (not treated) cells with the other experimental conditions; $^{*}p < 0.05$. **(c)** Representative Western blots showing expression of pro-IL-1β, IL-1β, and HSP-90, the latter used as the reference protein, NT = not transfected cells. Then quantification of **(d)** pro-IL-1β and **(e)** IL-1β increase normalized to HSP-90 optical density in U87-MG cells. Results are representative of three independent experiments. Data analyses were performed with one-way ANOVA and Bonferroni correction comparing NT cells with the other experimental conditions; $^{*}p < 0.05$.

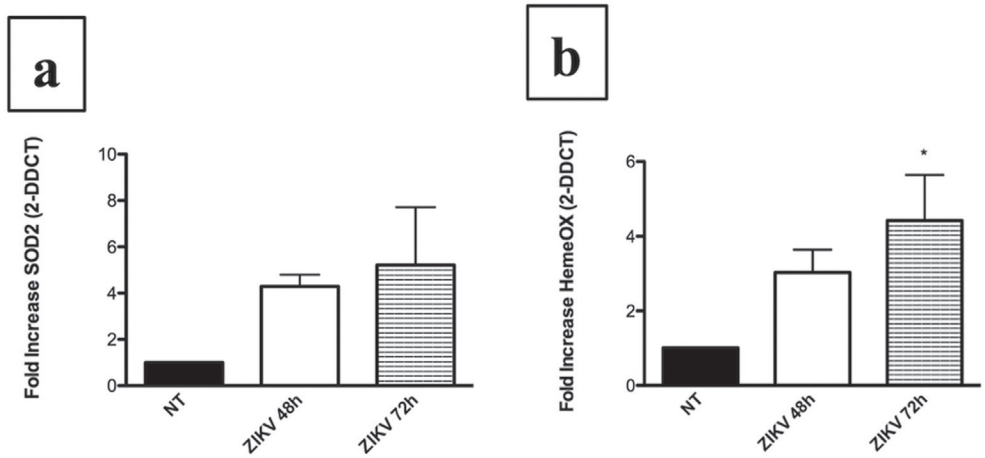

Fig. 3. ZIKV induces oxidative stress increasing *SOD2* and *HemeOX* genes expression. U87-MG cells were infected with ZIKV for 48 and 72 h. Oxidative stress was assessed evaluating the expression of **(a)** *SOD2* and **(b)** *HemeOX* genes. Analyses were performed using real time quantitative polymerase chain reaction (PCR), and results normalized to *ACTB* expression. Expression of untreated cells was normalized to 1. Expression data for the three independent experiments are reported as $2-\Delta\Delta Ct$ average ± SD, in which $\Delta\Delta Ct = \Delta Ct\_stimulated\ HC - \Delta Ct\_RHC$. Data analyses were performed with one-way ANOVA and Bonferroni correction comparing NT (not treated) cells with the other experimental conditions; $^{*}p < 0.05$.

4. Discussion

To date, ZIKV infection has been associated to neurological disorders such as congenital microcephaly in newborns and Guillain-Barré syndrome (GBS), myelopathy and encephalitis in adults [8]. ZIKV is characterized by neurotropism and neurovirulence; in fact, in many registered cases ZIKV was identified mainly in cerebrospinal fluid (CSF) both in newborns and adults

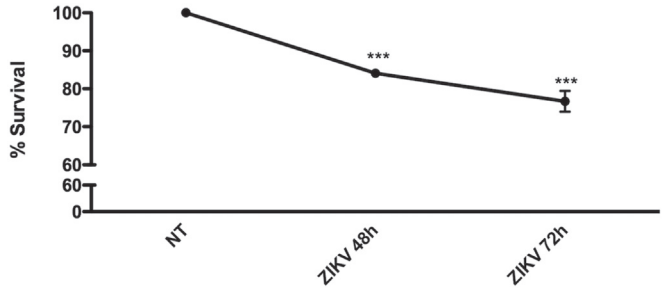

Fig. 4. ZIKV induces the decrease of U87-MG cell viability. U87-MG cells were infected with ZIKV for 48 and 72 h. Cell viability, expressed in % Survival, was evaluated by MTT assay. Data are shown as the percentage of levels compared with NT, considered as 100% of survival in the respective experimental setting. Results are representative of three independent experiments. Data analyses were performed with one-way ANOVA and Bonferroni correction comparing NT (not treated) cells with the other experimental conditions; ***$p < 0.001$.

[9,11,16,28]. Several studies identified microglial nodules, gliosis, neuronal and glial cells cellular degeneration and necrosis in brain of ZIKV + infants [11,13], suggesting that neuroinflammation and microglial activation could have a role in these neurological disorders.

To date, little information is available about neuroinflammation and ZIKV neurological disorders; so we decided to study ZIKV infection in glial cell (U87-MG), the cells mainly involved in the inflammatory response and immune surveillance in CNS.

Firstly, we investigated if ZIKV is able to infect U87-MG cells: we found that ZIKV is able to infect U87-MG and replicate in this cell line (Fig. 1). These results are in accordance with a study by Nowakowski and collaborators, that identified AXL, a possible candidate ZIKV entry receptor, highly expressed in glial cells [14].

Then, we studied how U87-MG responded to ZIKV infection in terms of inflammation, evaluating IL-1β gene expression and protein production as well as gene expression of the two main components of inflammasome, *NLRP-3* and *CASP-1*. We found that ZIKV infection, mainly after 48 h, induces the increase of *IL-1β*, *NLRP-3* and *CASP-1* expression; these findings have been confirmed by the significant increase of IL-1β protein levels and by unchanged pro-IL-1β protein levels (Fig. 2). Therefore, ZIKV in U87-MG induces inflammation connected to NLRP3 inflammasome that, through the activation of pro-caspase-1, induces proteolysis of pro-IL-1β and release of mature IL-1β. Several studies observed NLRP3 inflammasome activation and IL-1β release after infection by flavivirus, such as Dengue, West Nile, Yellow Fever and Japanese encephalitis viruses [23–25]. In most case, inflammasome and caspase-1 activation led to pyroptosis cell death, which might contribute to the amplification of inflammatory response [21,23]. Inflammasome NLRP3 is very sensitive to oxidative stress possibly generated by flavivirus infection; in fact, Chen W. and collaborators observed that HCV RNA, in human myeloid cells, activates the NLRP3 inflammasome in a ROS-dependent manner, confirming the close relationship between oxidative stress and inflammasome activation [20].

In agreement with these evidences, we observed an increased gene expression for two important antioxidant enzymes commonly used to assess oxidative stress, the superoxide dismutase 2 (*SOD2*) and heme oxygenase (*HemeOX*), mainly after 48 h of ZIKV infection (Fig. 3). SOD2 is the major antioxidant enzyme localized within mitochondria and it efficiently eliminates the superoxide generated from molecular oxygen in the respiratory chain. In addition to having a role in oxidative stress, *SOD2* expression in CNS is often associated with neuroinflammation [29,30]. Instead, HemeOX is a major endoplasmic reticulum associated heme protein, and it is known to have an important antioxidant role in tissue injury, attenuating the overall production of ROS in disease states. In addition, *HemeOX* overexpression may be a trigger of mitochondrial dysfunction characteristic of many neurodegenerative disorders [31,32]. So, our results show that ZIKV infection in U87-MG is able to increase the oxidative stress that could be the cause of NLRP3 inflammasome activation. Oxidative stress has been shown following many triggers including viral infection. This stress may contribute both to the activation of the inflammatory response and to viral pathogenesis, regulation of viral replication and modulation of cellular response [33]. Moreover, Liao and collaborators observed that oxidative stress of glial cells infected with JEV is implicated in inflammation and also in cell death [34].

Finally we found a decrease of U87-MG cell viability, after both 48 h and 72 h of ZIKV infection (Fig. 4). Therefore, our results indicate that U87-MG cells are susceptible to the ZIKV infection; the virus is able to successfully replicate in infected cells causing oxidative stress, NLRP3 inflammasome activation and subsequent release of IL-1β mature. This process culminates in cell death that could hypothesize as occurring through pyroptosis, a caspase-1 dependent programmed cell death that involves NLRP3 inflammasome and IL-1β [35].

So, considering the central role of neuroinflammation in neurological disorders, it is important to comprehend every aspect of this mechanism in order to better understand the pathogenesis of ZIKV infection. Unfortunately to date, the pathogenesis of ZIKV is partly unknown, but the balance between stress, inflammation and cellular responses is the basis of the control of viral replication, and then the development of the disease itself. Hence, the knowledge of these molecular mechanisms is essential also to identify novel strategies to fight the virus as well as to rescue the cell death in neuronal cells.

Funding

This work was supported by a grant from the Institute for Maternal and Child Health IRCCS "Burlo Garofolo" (RC 42/11) and by a grant from the Brazilian Minister of Health (Chamada MCTIC/FNDCT-CNPq/MEC-CAPES/MS-Decit/No 14/2016 Project no 440371/2016-3).

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

Prof. Del Sal G. (University of Trieste, Italy) generously providing U87-MG cells (human glioblastoma cell line); Robert Koch Institute (Berlin, Germany), supported by the European Virus Archive goes Global (EVAg) project that has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 653316, generously providing the quantified Zika MR766 molecular standard.

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