Influence of Zika virus on the cytotoxicity, cell adhesion, apoptosis and inflammatory markers of glioblastoma cells

DANIEL RODRIGO MARINOWIC[1,2], GABRIELE GOULART ZANIRATI[1,3], PAMELLA NUNES AZEVEDO[1,2], ÂNGELA ZANATTA[1,2], ISMAEL PLENTZ[3], ALLAN MARINHO ALCARÁ[1,3], FERNANDA BUENO MORRONE[2,4,5], THAMIRIS BECKER SCHEFFEL[4,5], ANGÉLICA REGINA CAPPELLARI[4,5], PAULO MICHEL ROEHE[6], ANA PAULA MUTERLE VARELA[6], DENISE CANTARELLI MACHADO[1,2], FABIANA SPILLARI VIOLA[1,2] and JADERSON COSTA DA COSTA[1-3]

[1]Brain Institute of Rio Grande do Sul, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul 90610-000; Graduate Programs in [2]Medicine and Health Sciences, and [3]Medicine, Pediatrics and Child Health, School of Medicine; [4]Applied Pharmacology Laboratory; [5]Graduate Program in Molecular and Cellular Biology, School of Health and Life Sciences, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul 90619-900; [6]Laboratory of Virology, Department of Microbiology, Immunology and Parasitology, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul 90040-060, Brazil

Received June 24, 2022; Accepted August 8, 2023

DOI: 10.3892/ol.2024.14309

Abstract. Glioblastoma (GBM) is one of the most common types of brain tumor in adults. Despite the availability of treatments for this disease, GBM remains one of the most lethal and difficult types of tumors to treat, and thus, a majority of patients die within 2 years of diagnosis. Infection with Zika virus (ZIKV) inhibits cell proliferation and induces apoptosis, particularly in developing neuronal cells, and thus could potentially be considered an alternative for GBM treatment. In the present study, two GBM cell lines (U-138 and U-251) were infected with ZIKV at different multiplicities of infection (0.1, 0.01 and 0.001), and cell viability, migration, adhesion, induction of apoptosis, interleukin levels and CD14/CD73 cell surface marker expression were analyzed. The present study demonstrated that ZIKV infection promoted loss of cell viability and increased apoptosis in U-138 cells, as measured by MTT and triplex assay, respectively. Changes in cell migration, as determined by wound healing assay, were not observed; however, the GBM cell lines exhibited an increase in cell adhesion when compared with non-tumoral cells (Vero). The Luminex immunoassay showed a significant increase in the expression levels of IL-4 specifically in U-251 cells (MOI 0.001) following exposure to ZIKV. There was no significant change in the expression levels of IFN-γ upon ZIKV infection in the cell lines tested. Furthermore, a marked increase in the percentage of cells expressing the CD14 surface marker was observed in both GBM cell lines compared with in Vero cells; and significantly increased CD73 expression was observed particularly in U-251 cells, when compared with uninfected cells. These findings indicate that ZIKV infection could lead to reduced cell viability, elevated CD73 expression, improved cellular adherence, and higher rates of apoptosis in glioblastoma cells. Further studies are required to explore the potential use of ZIKV in the treatment of GBM.

Correspondence to: Dr Daniel Rodrigo Marinowic, Brain Institute of Rio Grande do Sul, Pontifical Catholic University of Rio Grande do Sul, Building 63, Avenida Ipiranga 6690, Porto Alegre, Rio Grande do Sul 90610-000, Brazil
E-mail: daniel.marinowic@pucrs.br

Key words: Zika virus, apoptosis, cell adhesion, glioblastoma, viral therapy

Introduction

Glioblastoma (GBM) is one of the most common and aggressive primary brain tumors in adults (1). Despite the availability of established treatments for GBM, including surgery, radiotherapy and chemotherapy, the median survival time of patients with GBM is ~1 year following diagnosis (2). GBM is an invasive and highly recurrent tumor type. The cellular heterogeneity exhibited by GBM, in addition to the limited ability of treatments to penetrate the blood-brain barrier, renders these tumors resistant to treatment (3). Based on current knowledge on the complexity of this neoplasia, treatment strategies require a multidisciplinary approach, which involves the pathophysiological understanding of tumor invasion, migration and treatment resistance mechanisms (4).

Among the current alternatives for treatment of this disease, viral therapy using Zika virus (ZIKV) has emerged as a novel strategy for the treatment of GBM, as there is evidence that ZIKV is capable of infecting and killing neural stem cells (5,6). Previous studies have reported the presence of viral RNA ~24 h after viral exposure in pluripotent and neural stem cells, neurospheres and brain organoid cell cultures, in which

morphological abnormalities and the induction of apoptosis were reported (7,8). In an embryonic brain tumor cell line and animal models of brain tumors, ZIKV infection induced cell death and tumor regression (9).

Kaid *et al* (9) assessed the susceptibility of human cell lines to ZIKV infection. Human neural progenitor cells were identified to be a direct target of the virus and to exhibit a reduction in cell viability, increased cell death and dysregulation of the cell cycle as a consequence of ZIKV infection (8,10). A previous preclinical study using microglial cells subjected to ZIKV exposure reported that ZIKV infected microglia and could induce an inflammatory response (11). Consequently, ZIKV infection of microglia was reported to induce a reduction in neurosphere size and growth rate, in addition to inhibiting neuronal precursor cell proliferation. Together, these data suggest that microglia are susceptible to ZIKV infection, which induces neuroinflammation and the production of neurotoxic factors that are detrimental to the proliferation and differentiation of neuronal precursor cells (12).

Previous studies involving ZIKV-infected tumor stem cells have reported that >60% of tumor stem cells were infected with the virus within 48 h of viral exposure (5). Additionally, tumor stem cells isolated following surgical resection were reported to be infected with the virus upon exposure to ZIKV, whilst healthy neuronal cells were not infected (5). A previous study using animal models of GBM reported a reduction in tumor growth upon ZIKV infection, which suggests that this virus could serve as a potential candidate for viral therapy of GBM (5).

The features of GBM are implicated in its aggressiveness. Tumor migration and invasiveness are the main causes of issues with GBM treatment, as these processes are involved in tumor recurrence (13). CD73 is an ectoenzyme of the purinergic system, is present in the extracellular space and is the main source of adenosine (14). CD73 expression has been reported to be upregulated in several types of cancer, including GBM, and CD73 serves as a membrane protein assisting cell adhesion and migration processes (15). CD14 is a cell surface marker expressed preferentially by the cells of the immune system. In certain types of cancer, CD14 expression is required for increased cytokine production and tumor growth (16).

The neurotoxic effects reported in cases of microcephaly caused by ZIKV infection, in addition to data reporting the ZIKV tropism for immature neuronal cells (17), indicate that ZIKV-based therapy may potentially be a promising future treatment strategy for GBM (6). In the present study, the effect of ZIKV infection on migration, adhesion and expression levels of inflammatory markers in human GBM cell lines was analyzed.

Materials and methods

Cell lines. Human GBM cell lines (U-138 and U-251) and the Vero E6 (Vero) cell line were purchased from American Type Culture Collection. Vero cells (African green monkey kidney epithelial cells) were used as a non-malignant control (18). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, 100 U/ml streptomycin and 100 μg/ml gentamicin with 5% CO_2 at 37˚C.

Ethical approval. The present study was approved by the Scientific Committee of the Brain Institute of Pontifical Catholic University of Rio Grande do Sul (approval no. 8249; Porto Alegre, Brazil) and by the National System for the Management of Genetic Heritage and Associated Traditional Knowledge approval no. A708680; Brasilia, Brazil).

ZIKV infection. The Brazilian ZIKV strain 17 was previously isolated from a 33-year-old patient with clinical features suggestive of ZIKV infection in 2016 (19). The isolated virus was used in the present study according to the Brazilian National Research Council after written informed consent was provided for the previous study (19). ZIKV infection of the patient was confirmed using an ELISA kit with IgG and IgM antibodies specific for ZIKV. Reverse transcription-quantitative PCR was performed to detect RNA from ZIKV, Dengue virus and Chikungunya virus (19). ZIKV was propagated in Vero cells and titrated using a standard plaque assay (20,21), and viral stocks were maintained at -80˚C. U-138, U-251 human GBM and Vero cells were infected with ZIKV at multiplicities of infection (MOIs) of 0.1, 0.01 and 0.001 for 2, 12 or 24 h at 37˚C. The control group contained the same uninfected cells.

Cell viability assay. To assess the viability of U-138, U-251 and Vero cells infected with ZIKV, cells ($4.5x10^5$ cells/well in 200 μl DMEM were plated into 96-well plates (TPP Techno Plastic Products AG). Cells were cultured for 24 h and then infected with ZIKV at different MOIs for 2 h at 37˚C. Cells were washed with Dulbecco's phosphate-buffered saline (DPBS), followed by the addition of 200 μl MTT solution (solubilized in 200 μl DMSO) at a concentration of 5 mg/ml in supplemented DMEM. Cells were incubated for 4 h at 37˚C in a 5% CO_2 incubator. Subsequently, the optical density of the samples was measured at a wavelength of 570 nm using an ELISA reader. Absorbance values of uninfected cells served as the control for the calculation of the percentage of viable cells: (optical density of infected cells x100/optical density of uninfected cells).

Triplex assay. Following ZIKV infection of GBM and Vero cell lines for 2, 12 and 24 h at a range of MOIs (0.1, 0.01 and 0.001), cells were trypsinized and cultured in a 96-well plate at a density of $2x10^4$ cells/well in triplicate. To quantify the viability/cytotoxicity of infected cells, the ApoTox-Glo™ Triplex Assay (cat. no. G6320; Promega Corporation) was used. Briefly, 20 μl Viability/Cytotoxicity Reagent was added to each well and the cells were placed on an orbital shaker at 130 x g at room temperature for 30 sec, followed by incubation at 37˚C for 30 min. Viability (excitation wavelength, 400 nm; emission wavelength, 505 nm) and cytotoxicity (excitation wavelength, 485 nm; emission wavelength, 520 nm) of samples was then measured using the SpectraMax® M plate reader (Molecular Devices, LLC). A quantity of 100 μl Caspase-Glo® 3/7 reagent was added to each well and samples were incubated at 37˚C for 30 min before luminescence was measured. All measurements were taken using the SpectraMax® M plate reader.

Wound healing assay. Evaluation of cell migration was performed as previously described by Valster *et al* (22). U-251, U-138 and Vero cells were seeded in 12-well plates (TPP Techno Plastic Products AG) and cultured until they reached 80% confluence, as observed using the EVOS microscope (Thermo Fisher Scientific, Inc.). Cells were subjected to a progressive reduction in FBS concentration from 10 to 5%, and then to the final FBS concentration of 0.5%; cells were incubated with each concentration of FBS for 24 h. Subsequently, cells were exposed to ZIKV at a range of MOIs for 2 h. Cells were washed with DPBS and a scratch wound were created on the cell monolayers using a P200 pipette tip. DPBS was removed and cells were cultured in DMEM supplemented with 0.5% FBS. Images were captured at 0, 12 and 24 h. The images were analyzed using Image-Pro Plus software (version 7; Media Cybernetics, Inc.) and the cell migration distance was determined as follows: (Initial width of the wound x0.5)-(width of the wound/2).

Cell adhesion assay. Following exposure to ZIKV at three different MOIs for 2 h at 37˚C, the GBM and Vero cell lines were trypsinized and seeded in triplicate in 96-well plates at $1x10^4$ cells/well in 100 μl DMEM supplemented with 10% FBS. After 2 h of incubation, the culture medium was aspirated, and cells were washed with DPBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were washed with DPBS and 50 μl 0.5% crystal violet (dissolved in 20% methanol) was added to each well at room temperature. After 10 min, the dye was aspirated and eluted with 50 μl acetic acid (10%) at room temperature. Cell adhesion was measured using a plate reader at a wavelength of 570 nm.

Luminex immunoassay. The Luminex immunoassay (ProcartaPlex™ Hu HS 9 Plex; cat. no. EPXS090-12199-901; Thermo Fisher Scientific, Inc.) was performed according to the manufacturer's protocol to measure the levels of IFN-γ and IL-4 in the culture supernatant of GBM and Vero cell lines ($2x10^4$ cells/well) following exposure to ZIKV at a range of MOIs (0.1, 0.01 and 0.001) for 2 h. Magnetic beads (50 μl) corresponding to each analyte were added to the 96-well plate and then washed with Wash Buffer Concentrate (10X). Subsequently, 50 μl standards or samples were added; for background wells, 50 μl cell culture medium was added. The plate containing cells was subsequently shaken on a platform at 130 x g rpm for 2 h at room temperature and washed using Wash Buffer Concentrate (10X). Then, 25 μl Detection Antibody Mix (1X) was added. The plate was shaken at 130 x g for 30 min at room temperature, followed by the addition of 50 μl streptavidin to each sample with shaking for 30 min at room temperature. Samples were washed using wash buffer and 50 μl Amplification Reagent 1 was added to the sample with shaking for 30 min at room temperature, followed by the addition of 50 μl Amplification Reagent 2 with shaking for 30 min at room temperature. Finally, 120 μl Reading Buffer was added with shaking for 5 min at room temperature and the cytokine levels were measured using the MAGPIX® (Luminex Corporation).

Flow cytometry. Following exposure to ZIKV for 2 h (MOI 0.1, MOI 0.01 and MOI 0.001) with 5% CO_2 at 37˚C, GBM and Vero cell lines at a density of $2x10^4$ cells/well were incubated for 2 h at room temperature with anti-CD14 antibodies (1:200; cat. no. 561712; BD Biosciences) conjugated to FITC and anti-CD73 antibodies (1:200; cat. no. 550257; BD Biosciences) conjugated to PE. Cells were then washed with DPBS and the labeled cell populations were analyzed by flow cytometry using the FACSCalibur™ flow cytometer (BD Biosciences) and BD CellQuest Pro software (version 5.1; BD Biosciences).

Statistical analysis. Statistical analysis was performed using GraphPad Prism software version 6.0 (GraphPad Software; Dotmatics). All experiments were independently repeated two times in triplicate. Triplex assay, cell migration and flow cytometry results were analyzed using two-way ANOVA with Bonferroni's post hoc test. Cell viability, adhesion and Luminex assays were analyzed using one-way ANOVA with Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ZIKV infection promotes loss of cell viability and induced apoptosis in GBM cells. To assess the effect of a range of ZIKV MOIs on GBM cell viability, an MTT assay was performed. Cell viability remained close to 100% at all MOIs examined for U-251 and Vero cells. However, a small fluctuation in cell viability of U-138 and Vero cells was detected following 2 h of ZIKV infection (Fig. 1A-C). U-138 cells exhibited sensitivity to ZIKV infection, as infection of U-138 cells with ZIKV at an MOI of 0.01 and 0.001 significantly decreased cell viability by ~20 and 60%, respectively, when compared with the control (Fig. 1A). However, ZIKV infection did not appear to significantly impact the viability of U-251 cells. The triplex assay results demonstrated that there was no significant change in the cell viability or cytotoxicity of GBM cell lines following ZIKV infection at any time point or MOI tested (Fig. 1D). The cytotoxicity of the Vero cell line was markedly reduced at the 12 h time point of ZIKV infection at a MOI of 0.1, 0.01 and 0.001 compared with in uninfected control cells. A marked increase in the apoptosis of U-251 and U-138 cells was observed upon exposure to ZIKV at a MOI of 0.1 for 2 h compared with that of uninfected cells (controls) but this was not statistically significant. The apoptosis rate of U-251 cells was demonstrated to be comparable to non-ZIKV infected cells at a MOI of 0.1 at the 12-h time point. U-138 cells exhibited a marked increase in the rate of apoptosis following 12 h of ZIKV exposure compared with uninfected controls at a MOI of 0.1. Exposure of U-138 cells to ZIKV at a MOI of 0.01 at both 2 and 12 h was associated with similar rates of apoptosis. U-251 cells exhibited a marked decrease in the rate of apoptosis 12 h after infection with ZIKV at a MOI of 0.01 when compared with uninfected cells.

Effects of ZIKV exposure on cell migration and adhesion. The wound healing assay demonstrated that there was no statistically significant difference in the migration of cells across the wound for any of the ZIKV MOIs tested over a 12 and 24 h infection period compared with that of uninfected control cells (Fig. 2A). None of the ZIKV MOIs tested altered the migratory behavior of the cell lines

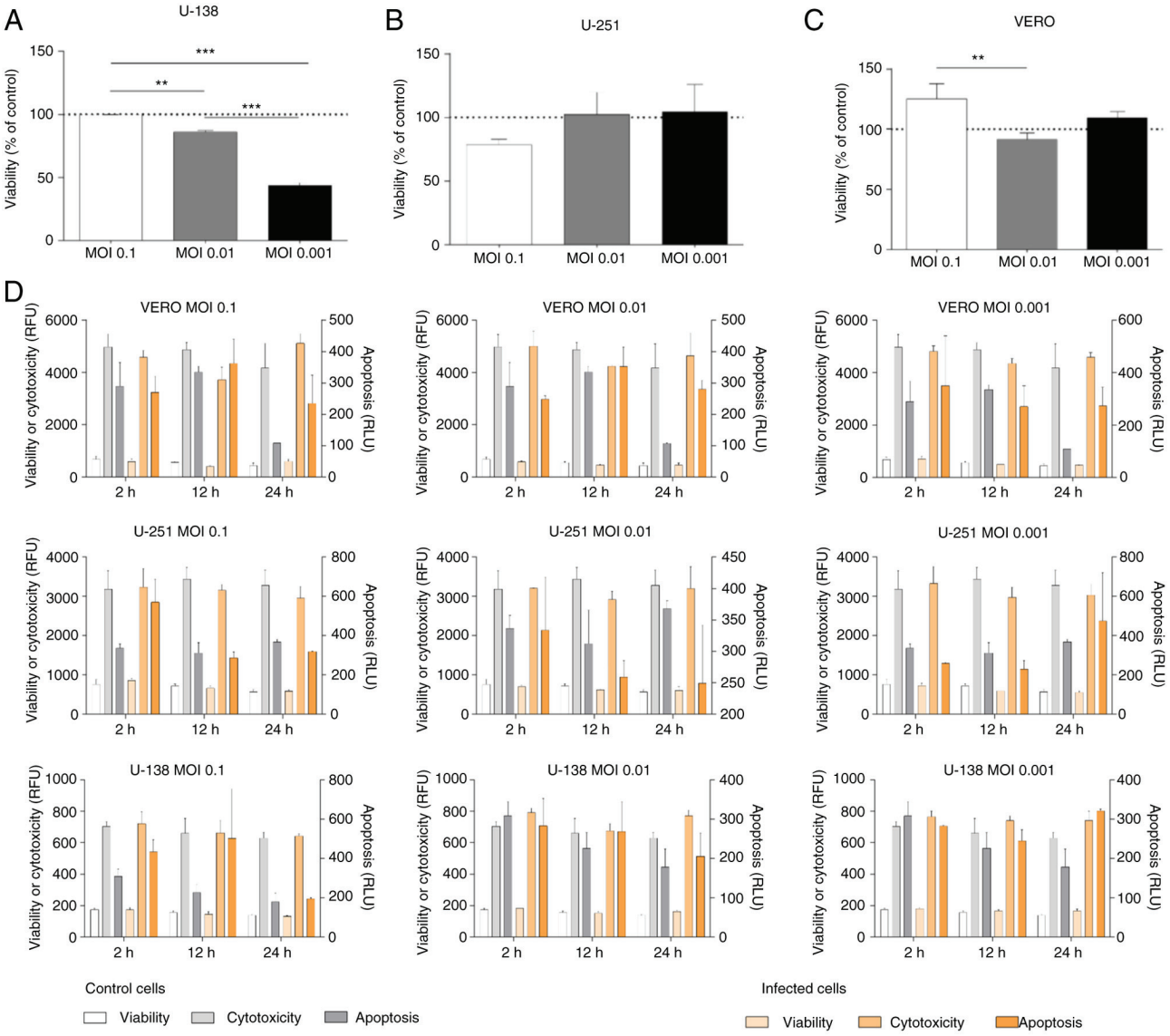

Figure 1. Viability of U-138, U-251 and Vero cells following infection by ZIKV. An MTT assay was performed following 2 h of infection by ZIKV at a range of MOIs in (A) U-138, (B) U-251 and (C) Vero cells. Data are presented as the mean ± standard deviation relative to the percentage of viable cells not exposed to ZIKV. (D) Cell viability, cytotoxicity and apoptosis analysis of U-251, U-138 and Vero cells using a Triplex assay following ZIKV exposure at a range of MOIs for 2, 12 and 24 h. Data are presented as the mean ± standard deviation. **P<0.005; ***P<0.001. ZIKV, Zika virus; MOI, multiplicity of infection; RFU, relative fluorescence units; RLU, relative luminescence units.

examined. ZIKV exposure increased the cell adhesion properties of the U-138 and U-251 GBM cell lines for all ZIKV MOIs tested (Fig. 2B). ZIKV infection of Vero cells did not impact cell adhesion following exposure to three different MOIs. In the GBM cell lines tested, 2 h of ZIKV infection was demonstrated to increase cell adhesion at each MOI of ZIKV analyzed. There was no statistically significant difference observed among the treatment groups analyzed (MOI of 0.1, 0.01 and 0.001). The U-251 cell line exhibited a 32% increase in cell adhesion upon ZIKV infection at an MOI of 0.1 and a 42% increase upon ZIKV infection at an MOI of 0.01, when compared with uninfected control cells. In the U-138 cell line, a 40% increase in cell adhesion was observed when cells were infected with ZIKV at an MOI of 0.1 and a 39% increase in cell adhesion was observed upon ZIKV infection at an MOI of 0.01, when compared with uninfected control cells (Fig. 2B).

ZIKV infection may increase the expression levels of CD14 and CD73 cell surface markers, and inflammation-related markers. The production of certain cell surface and inflammatory markers in GBM cell lines was investigated to determine the effect of ZIKV infection on immune signaling (Fig. 3). U-138 and U-251 cells infected with ZIKV exhibited increased CD14 expression compared with infected Vero cells (Fig. 3A). U-138 and U-251 cells exhibited similar expression of cell surface markers 2 h after exposure to ZIKV. The baseline percentage of U-138 cells expressing CD14 was 1.78%, which rose to 2.82% following 2 h of ZIKV infection at a MOI of 0.01. U-251 cells infected with ZIKV at a MOI of 0.001 exhibited a doubling in CD14 expression compared with uninfected cells, from 1.80 to 3.55% of cells. A statistically significant increase in CD73 expression in U-251 cells was observed for the three ZIKV MOIs analyzed, when compared with uninfected cells (Fig. 3B). The percentage of CD73-labeled cells

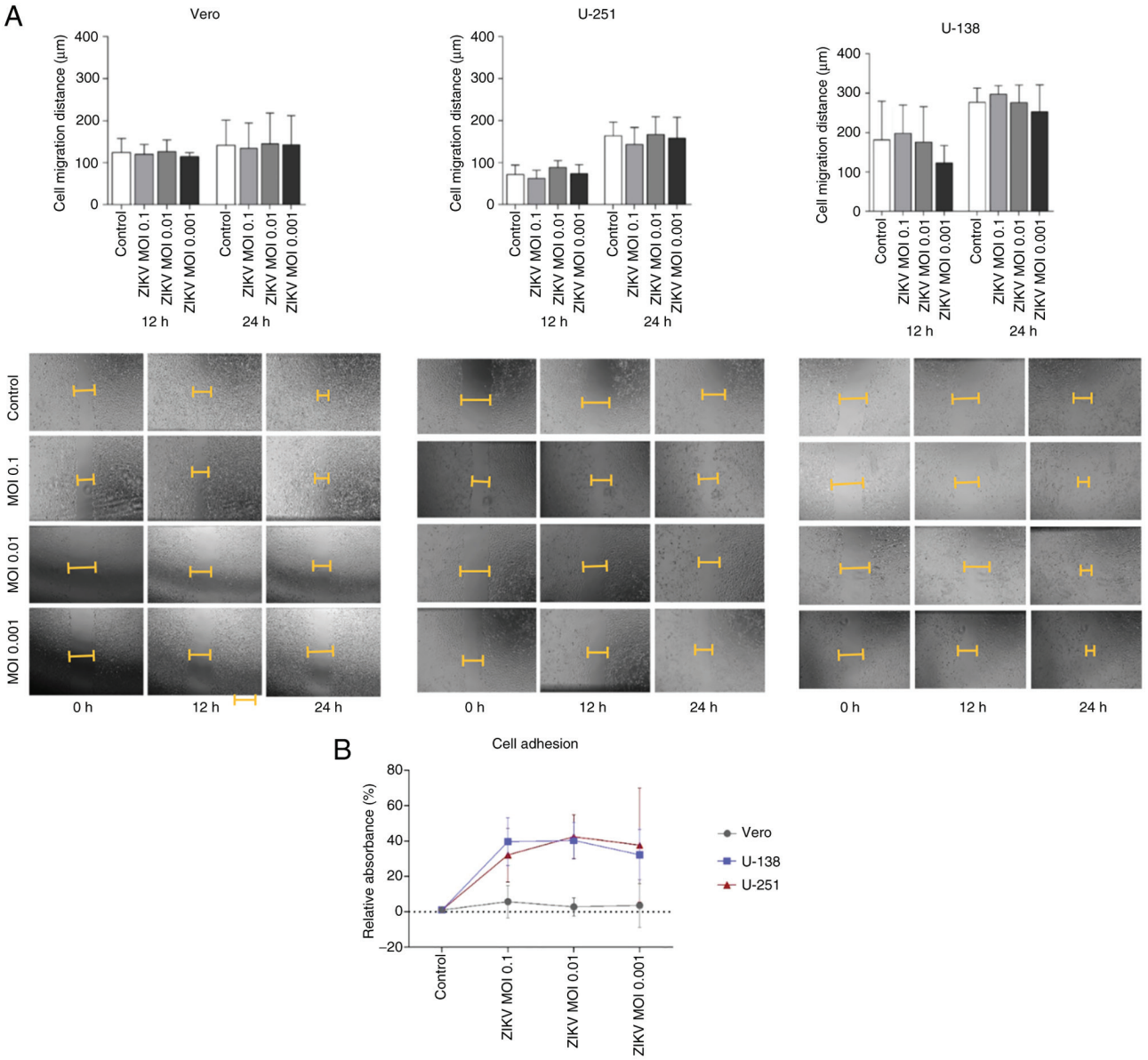

Figure 2. Wound healing and adhesion assays of Vero, U-251 and U-138 cells infected with a range of MOIs of ZIKV. (A) Cell migration distance (μm) across the wound is presented over 24 h. Magnification, x200. (B) Cell adhesion assay of Vero, U-138 and U-251 cells infected with a range of MOIs of ZIKV. Data are presented as the relative absorbance of cells stained with 0.5% crystal violet that adhered to plates compared with uninfected controls. Data are presented as the mean ± standard deviation. MOI, multiplicity of infection; ZIKV, Zika virus.

in the uninfected cells group was 14.41%, which increased to 25.8% following exposure to ZIKV at a MOI of 0.1 and 29.3% following exposure to ZIKV at a MOI of 0.01. In U-138 cells, there was no significant increase in the percentage of CD73-labeled cells following exposure to different concentrations of ZIKV.

A previous study reported that the expression of the cell surface marker CD14 was inversely associated with the expression of inflammatory cytokines (23). In order to further investigate the mechanisms involved in the effects of ZIKV treatment, the expression levels of inflammatory markers were analyzed. ZIKV infection markedly reduced the levels of IFN-γ and IL-4 in the Vero cell line after 2 h of exposure to the virus compared with those in uninfected cells, but this was not statistically significant (Fig. 3C and D). Infection of U-138 and U-251 cells with ZIKV at any MOI tested for 2 h did not significantly impact the expression levels of IFN-γ. By contrast, U-251 cells exhibited a significant reduction in the expression levels of IL-4 when exposed to MOI 0.001 compared with MOI 0.1.

Discussion

In the present study, U-251 and U-138 human GBM cell lines were used to evaluate the effects of infection with ZIKV at a range of different MOIs and exposure times. As demonstrated using the MTT assay, 2 h of exposure to ZIKV caused a reduction in the viability of U-138 cells at the lowest MOIs of ZIKV tested (MOIs of 0.01 and 0.001) compared with in uninfected cells. A recent study by Shang *et al* (24) reported that differences in the MOI of severe acute respiratory syndrome coronavirus 2 infecting the Vero cell line (MOIs of

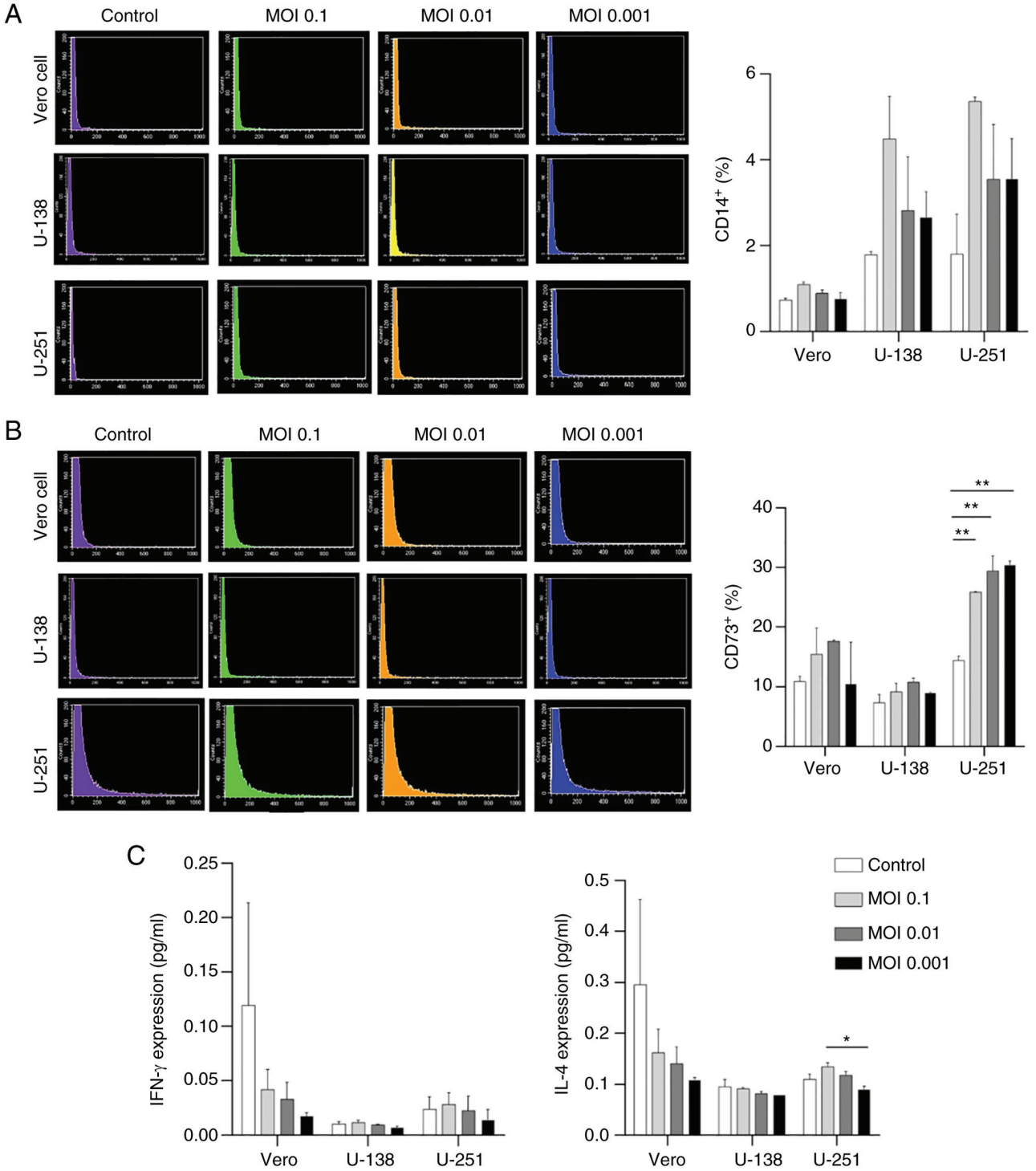

Figure 3. Immunophenotypic profile and interleukins. Expression profile of (A) CD14 and (B) CD73 cell surface markers presented as the percentage of Vero, U-138 or U-251 cells positive for each cell surface marker following infection with ZIKV at a range of MOIs. Expression levels of cytokines (C) IFN-γ and IL-4 in Vero, U-138 and U-251 cells infected with ZIKV at a range of MOIs. Data are presented as the mean ± standard deviation. *P<0.05; **P<0.005. ZIKV, Zika virus; MOI, multiplicity of infection.

0.00032 and 0.0016) did not impact the viability of infected cells. Furthermore, MOIs of 0.008, 0.040, 0.200 and 1.000 of SARS-CoV-2 were also tested, and were reported to have no impact on cytotoxicity, and thus, cell viability upon infection with a virus may not be dependent of the titration of virus (24). In the present study, a Triplex assay was used to analyze the potential impact of different MOIs of ZIKV on cell viability. No statistically significant differences were observed in the cell lines following exposure to ZIKV when compared to uninfected cells. In addition, ZIKV infection of U-138 and U-251 cells did not significantly impact the migratory potential of cells. However, it is difficult to quantitatively translate these data through parameters that identify the variable kinetics of viral infection. Mathematical models based on different viral parameters can be used to identify the main behavioral differences among particular viruses, and to understand their

virulence over different time courses and the number of harmful post-infection viral particles (19).

The present study demonstrated an increase in the expression levels of caspase 3/7 in U-251 and U-138 cells following 2 h of infection with a MOI 0.1 of ZIKV, compared with uninfected control cells. Following 12 h of ZIKV infection at a MOI of 0.1, there was no statistically significant difference in caspase 3/7 expression in U-251 cells compared with the control. U-138 cells exhibited an increase in the rate of apoptosis following 12 h of ZIKV exposure at a MOI of 0.1. Therefore, induction of ZIKV-mediated apoptosis may begin in the early stages of viral infection and last for long periods, and this effect also does not appear to be dependent on high viral concentrations, due to the fact that the values related to the rate of apoptosis did not change throughout our analysis. The genetic and phenotypic variations among the studied cell lines may cause different responses to ZIKV infection, which may involve the activation of different cell pathways and induction of apoptotic cell death. A previous study by Kaid *et al* (9) reported the impact of ZIKV infection on three different brain tumor cell lines, and also indicated varying levels of ZIKV efficacy in reducing cell viability. Furthermore, the same study also reported increased ZIKV infection efficiency in tumor cells compared with neural progenitor cells, which suggests that ZIKV may preferentially infect cancer cells and promote cell death in different cell types at different rates (9).

In addition to the increased cell proliferation that is characteristic of GBM, its highly invasive and infiltrative profile makes GBM a difficult malignancy to treat. GBM tumors are characterized by cell heterogeneity, high abundance of tumor stem cells and genetic alterations, such as mutations, deletions and epigenetic changes, which interfere with certain intracellular signaling processes (25). GBM tumors can also cause alterations in the host immune system, and thus, to improve the treatment success of GBM, the mechanisms involved in maintenance of the malignant phenotype should be considered (3).

In the present study, the ability of ZIKV to modulate invasive processes in human GBM cell lines was assessed. The present study demonstrated that infection of U-138 and U-251 cells with ZIKV at all MOIs used led to an increase in cell adhesion. Cellular adhesion and migration are processes involved in cell motility. The sequential binding of integrins to extracellular matrix proteins associated with cytoskeletal protrusions favors the migration of individual cells, a phenotypic characteristic of mesenchymal and tumor cells, such as those of GBM (26). Thus, the ability to ensure that tumor cells remain adhered to each other would hinder the process of local invasion, which is characteristic of this disease. A previous study reported that ZIKV treatment reduced spinal cord metastasis levels by 60% following brain tumor implantation in an orthotopic implant xenograft model (9). Therefore, it could be suggested that ZIKV exposure reduces GBM cell invasiveness by increasing cell adhesion.

In addition to integrins, numerous other types of molecules participate in the cell adhesion process (26). Previous studies have reported that the CD73 protein is a regulatory molecule involved in cell proliferation, migration and invasion (15,27). Furthermore, one previous *in vitro* study reported that the dysregulation of CD73 activity caused reduced adhesion in the U-138 GBM cell line (28). Therefore, the increase in CD73 expression in the infected U-251 cell line compared with that in uninfected U-251 cells, demonstrated in the present study may cause an increase in cell adhesion following exposure of GBM cells to a range of ZIKV concentrations. CD73 is widely expressed in highly undifferentiated cells, and it is currently used as a marker of mesenchymal cells (29). Certain types of tumors, such as breast cancer, melanoma, GBM and multiform GBM, exhibit high levels of CD73 expression and activity. The association of CD73 with the proliferative and invasive capacity of these types of tumors enables this membrane protein to act as a marker for a high degree of tumor malignancy (30).

In the present study, increased CD14 expression in GBM cell lines was demonstrated, when compared with uninfected cells. This may be a consequence of immune system stimulation. However, a previous study showed that GBM was a type of tumor the immune system is non-reactive to, which has hindered treatment using immunotherapy, a method which is successful against other types of cancer (16). ZIKV stimulation of the immune system in the tumor microenvironment may be promising for the treatment of GBM, as combining oncolytic viruses with immunotherapy as a cancer treatment has previously been reported to induce an antitumor immune response (31). A non-significant increase in CD14 expression was demonstrated in the U-138 and U-251 cell lines when cells were exposed to ZIKV, as opposed to no notable difference in CD14 expression in Vero cells exposed to ZIKV. In the present study, no significant difference was observed in the expression levels of CD73 in Vero and U-138 cell lines. Infection of Vero cells with ZIKV alters several signaling pathways. A previous proteomics study reported marked changes in the expression levels of 125 proteins in Vero cells infected by ZIKV, and the main functions of these proteins were related to the inflammatory response and cell signaling cascades involved in immunity, cell division and cell death (32).

In the present study, 2 h of exposure to ZIKV (MOI 0.1) increased the expression levels of IFN-γ in the U-251 cell line compared with uninfected controls, although this was not statistically significant and this change in expression decreased with increasing ZIKV concentration. There was no difference in the expression levels of IFN-γ in the U-138 cell line following ZIKV infection compared with those in uninfected controls. A previous study by Kane and Yang (27) reported that increased IFN-γ expression disrupted GBM proliferation, induced apoptosis, increased tumor immunogenicity and inhibited neovascularization. A potential reduction in cell viability and a consequent decrease in the number of cells following ZIKV exposure may influence the expression levels of interleukins secreted by cells. In the present study, as a reduction in cell viability upon ZIKV infection was demonstrated in the U-138 and Vero cell lines, the reduction in the expression levels of cytokines may not be related to cytotoxicity. Cell death, as measured using a cytotoxicity assay, can be a major factor in the alteration of tissue homeostasis. Therefore, the relationship between cell viability and the secretion of interleukins may be proportional (33). As demonstrated in the present study, the lowest concentration of ZIKV used had a larger effect on the reduction of IL-4 expression in U-251 and Vero cells, when compared with uninfected cells of the same lineage.

Due to the complexity of the tumor microenvironment, current available treatments for GBM have a low efficacy due

to the absence of available tumor molecular targets, as well as a lack of a response of the tumor to immunotherapy (34). Understanding of the function of the tumor microenvironment, gene expression and molecular alterations exhibited by GBM tumor cells are crucial for exploring novel treatment modalities. An improved knowledge regarding the use of oncolytic viruses for cancer treatment would enable the further exploration of this type of treatment, used either alone or in combination with chemotherapy or immunotherapy agents.

ZIKV has certain features that may potentially justify the consideration of its use for treating brain tumors, such as GBM, as it can induce apoptosis, reduce the migratory capacity of neuronal and developing cells, and potentially induce the host immune system (6). The present study demonstrated that 24 h of ZIKV exposure reduced cell viability, elevated CD73 expression, improved cellular adherence and higher rates of apoptosis, indicating that novel mechanisms are involved in the interaction between ZIKV and GBM tumor cells. Further studies need to be conducted using animal models to address unanswered questions concerning the safety of the ZIKV dose to be used, the most effective route of exposure to the virus and if the genetic modification of ZIKV could reduce the systemic effects of viral infection. These studies may potentially enable ZIKV to be developed into a feasible and safe approach for the treatment of brain tumors.

The findings of the present study may indicate that ZIKV infection could cause GBM tumors to become static, due to increased expression of CD73, reducing their invasive capacity and potentially leading to an increase in tumor susceptibility to chemotherapy, radiotherapy or surgery. Further studies are required to investigate if ZIKV can effectively inhibit the proliferation of GBM cells. However, if the safety and efficacy of ZIKV therapy for GBM is demonstrated, this may be explored as a future potential treatment option for GBM. In addition, for an improved understanding of the reported processes, performing future experiments with a reconstructed virus is important.

Acknowledgements

Not applicable.

Funding

Funding was received from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brazil (finance code 001) and Conselho Nacional de Desenvolvimento Científico e Tecnológico.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FSV, DRM and JCDC conceived and designed the study. DRM, FBM, GGZ, PNA, AZ, IP, AMA, TBS and ARC analyzed and interpreted the data. DRM, FSV, JCDC, PMR, APMV and DCM analyzed and interpreted the data. DRM, JCDC, GGZ and TBS revised the manuscript for intellectual content. All authors agree to be accountable for all aspects of the work. All authors read and approved the final version of the manuscript. DRM and DCM confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Scientific Committee of the Brain Institute of Pontifical Catholic University of Rio Grande do Sul (approval no. 8249; Porto Alegre, Brazil) and the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (Brasilia, Brazil; approval no. A708680).

Patient consent for publication

Not applicable.

Competing interests

JCDC is a researcher of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Federal District, Brazil), which provided funding for the present study. The other authors declare that they have no competing interests.

References

1. Lapointe S, Perry A and Butowski NA: Primary brain tumours in adults. Lancet 392: 432-446, 2018.
2. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, *et al*: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352: 987-996, 2005.
3. Cai X and Sughrue ME: Glioblastoma: New therapeutic strategies to address cellular and genomic complexity. Oncotarget 9: 9540-9554, 2017.
4. Dunwoodie LJ, Poehlman WL, Ficklin SP and Feltus FA: Discovery and validation of a glioblastoma co-expressed gene module. Oncotarget 9: 10995-11008, 2018.
5. Zhu Z, Gorman MJ, McKenzie LD, Chai JN, Hubert CG, Prager BC, Fernandez E, Richner JM, Zhang R, Shan C, *et al*: Zika virus has oncolytic activity against glioblastoma stem cells. J Exp Med 214: 2843-2857, 2017.
6. Francipane MG, Douradinha B, Chinnici CM, Russelli G, Conaldi PG and Iannolo G: Zika Virus: A new therapeutic candidate for glioblastoma treatment. Int J Mol Sci 22: 10996, 2021.
7. Muffat J, Li Y, Omer A, Durbin A, Bosch I, Bakiasi G, Richards E, Meyer A, Gehrke L and Jaenisch R: Human induced pluripotent stem cell-derived glial cells and neural progenitors display divergent responses to Zika and dengue infections. Proc Natl Acad Sci 115: 7117-7122, 2018.
8. Tang H, Hammack C, Ogden SC, Wen Z, Qian X, Li Y, Yao B, Shin J, Zhang F, Lee EM, *et al*: Zika virus infects human cortical neural progenitors and attenuates their growth. Cell Stem Cell 18: 587-590, 2016.
9. Kaid C, Goulart E, Caires-Júnior LC, Araujo BHS, Soares-Schanoski A, Bueno HMS, Telles-Silva KA, Astray RM, Assoni AF, Júnior AFR, *et al*: Zika virus selectively kills aggressive human embryonal cns tumor cells in vitro and in vivo. Cancer Res 78: 3363-3374, 2018.
10. Komarasamy TV, Adnan NAA, James W and Balasubramaniam VRMT: Zika virus neuropathogenesis: The different brain cells, host factors and mechanisms involved. Front Immunol 13: 773191, 2022.
11. Diop F, Vial T, Ferraris P, Wichit S, Bengue M, Hamel R, Talignani L, Liegeois F, Pompon J, Yssel H, *et al*: Zika virus infection modulates the metabolomic profile of microglial cells. PLoS One 13: e0206093, 2018.

12. Wang J, Liu J, Zhou R, Ding X, Zhang Q, Zhang C and Li L: Zika virus infected primary microglia impairs NPCs proliferation and differentiation. Biochem Biophys Res Commun 497: 619-625, 2018.
13. Liu CA, Chang CY, Hsueh KW, Su HL, Chiou TW, Lin SZ and Harn HJ: Migration/Invasion of malignant gliomas and implications for therapeutic treatment. Int J Mol Sci 19: 1115, 2018.
14. Yegutkin GG: Enzymes involved in metabolism of extracellular nucleotides and nucleosides: Functional implications and measurement of activities. Crit Rev Biochem Mol Biol 49: 473-497, 2014.
15. Azambuja JH, Gelsleichter NE, Beckenkamp LR, Iser IC, Fernandes MC, Figueiró F, Battastini AMO, Scholl JN, de Oliveira FH, Spanevello RM, *et al*: CD73 downregulation decreases in vitro and in vivo glioblastoma growth. Mol Neurobiol 56: 3260-3279, 2019.
16. Cheah MT, Chen JY, Sahoo D, Contreras-Trujillo H, Volkmer AK, Scheeren FA, Volkmer JP and Weissman IL: CD14-expressing cancer cells establish the inflammatory and proliferative tumor microenvironment in bladder cancer. Proc Natl Acad Sci USA 112: 4725-4730, 2015.
17. Oliveira MDS, Cassiano LMG, Pioline J, de Carvalho KRA, Salim ACM, Alves PA, Fernandes GDR, Machado AMV and Coimbra RS: Organotypic hippocampal culture model reveals differential responses to highly similar Zika virus isolates. J Neuroinflammation 20: 140, 2023.
18. Yang L, Qin X, Liu H, Wei Y, Zhu H and Jiang M: Design, synthesis and biological evaluation of a series of new resveratrol analogues as potential anti-cancer agents. R Soc Open Sci 6: 190125, 2019.
19. Oliveira DBL, Durigon GS, Mendes ÉA, Ladner JT, Andreata-Santos R, Araujo DB, Botosso VF, Paola ND, Neto DFL, Cunha MP, *et al*: Persistence and intra-host genetic evolution of zika virus infection in symptomatic adults: A special view in the male reproductive system. Viruses 10: 615, 2018.
20. Iwami S, Sato K, De Boer RJ, Aihara K, Miura T and Koyanagi Y: Identifying viral parameters from in vitro cell cultures. Front Microbiol 3: 319, 2012.
21. Fontes-Garfias CR, Shan C, Luo H, Muruato AE, Medeiros DBA, Mays E, Xie X, Zou J, Roundy CM, Wakamiya M, *et al*: Functional analysis of glycosylation of zika virus envelope protein. Cell Rep 21: 1180-1190, 2017.
22. Valster A, Tran NL, Nakada M, Berens ME, Chan AY and Symons M: Cell migration and invasion assays. Methods 37: 208-215, 2005.
23. Rey Nores JE, Bensussan A, Vita N, Stelter F, Arias MA, Jones M, Lefort S, Borysiewicz LK, Ferrara P and Labéta MO: Soluble CD14 acts as a negative regulator of human T cell activation and function. Eur J Immunol 29: 265-276, 1999.
24. Shang C, Zhuang X, Zhang H, Li Y, Zhu Y, Lu J, Ge C, Cong J, Li T, Tian M, *et al*: Inhibitors of endosomal acidification suppress SARS-CoV-2 replication and relieve viral pneumonia in hACE2 transgenic mice. Virol J 18: 46, 2021.
25. Fabian C, Han M, Bjerkvig R and Niclou SP: Novel facets of glioma invasion. Int Rev Cell Mol Biol 360: 33-64, 2021.
26. Friedl P and Wolf K: Plasticity of cell migration: A multiscale tuning model. J Cell Biol 188: 11-19, 2010.
27. Kane A and Yang I: Interferon-gamma in brain tumor immunotherapy. Neurosurg Clin N Am 21: 77-86, 2010.
28. Cappellari AR, Vasques GJ, Bavaresco L, Braganhol E and Battastini AM: Involvement of ecto-5'-nucleotidase/CD73 in U138MG glioma cell adhesion. Mol Cell Biochem 359: 315-322, 2012.
29. Calloni R, Cordero EA, Henriques JA and Bonatto D: Reviewing and updating the major molecular markers for stem cells. Stem Cells Dev 22: 1455-1476, 2013.
30. Soleimani A, Taghizadeh E, Shahsavari S, Amini Y, Rashidpour H, Azadian E, Jafari A, Parizadeh MR, Mashayekhi K, Soukhtanloo M and Jaafari MR: CD73; a key ectonucleotidase in the development of breast cancer: Recent advances and perspectives. J Cell Physiol 234: 14622-14632, 2019.
31. Lawler SE, Speranza MC, Cho CF and Chiocca EA: Oncolytic viruses in cancer treatment: A review. JAMA Oncol 3: 841-849, 2017.
32. Glover KKM, Gao A, Zahedi-Amiri A and Coombs KM: Vero cell proteomic changes induced by zika virus infection. Proteomics 19: e1800309, 2019.
33. Méry B, Guy JB, Vallard A, Espenel S, Ardail D, Rodriguez-Lafrasse C, Rancoule C and Magné N: In vitro cell death determination for drug discovery: A landscape review of real issues. J Cell Death 10: 1179670717691251, 2017.
34. Bikfalvi A, da Costa CA, Avril T, Barnier JV, Bauchet L, Brisson L, Cartron PF, Castel H, Chevet E, Chneiweiss H, *et al*: Challenges in glioblastoma research: Focus on the tumor microenvironment. Trends Cancer 9: 9-27, 2023.