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Article

The ZIKA Virus Controls Cell Death through the Anti-Apoptotic Bcl-2 Family Proteins

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Abstract: Zika virus (ZIKV) is an emerging human mosquito-transmitted pathogen of global concern, known to cause severe complications such as congenital defects and neurological disorders in adults. ZIKV infection is associated with cell death. However, previous studies suggest that the virally-induced apoptosis occurs at a slower rate compared to the course of viral production. In this present study, we investigated the capacity of ZIKV to delay host cell apoptosis. We provide evidence that ZIKV has the ability to control programmed cell death whether it is intrinsically or extrinsically induced. In cells expressing viral replicon-type constructions, we show that this control is achieved through replication. Finally, our work highlights an important role for anti-apoptotic Bcl-2 family protein in the ability of ZIKV to control apoptotic pathways, avoiding premature cell death and thereby promoting virus replication in the host-cell.

Keywords: Zika virus; programmed cell death; viral replication; Bcl-2 protein family

1. Introduction

Zika virus (ZIKV) which is a flavivirus belonging to the *Flaviviridae* family, like Dengue virus (DENV) yellow fever virus (YFV) or West Nile virus (WNV) has become a major medical problem worldwide. The human disease known as Zika fever is characterized by mild flu-like symptoms including fever, maculopapular rash, headache and sometimes conjunctivitis, arthralgia, and myalgia. Symptoms are usually resolved within a week [1]. However, during the latest outbreaks, serious pathological features of the disease have been reported. Complications such as microcephaly in newborns or Guillain-Barré Syndrome (GBS) in adults were documented during the French Polynesia outbreak in 2013 and in Brazil in 2015[2,3]. ZIKV is an arbovirus, mainly transmitted to humans through the bite of a mosquito vector from *Aedes* species [4]. Due to an increasingly global distribution of *Aedes*, ZIKV emergence is a threat in many areas that are no longer necessarily located in intertropical areas [5]. The ZIKV particle is composed of a single strand RNA molecule of about 11kb, inside a nucleocapsid, surrounded by a host-derived membrane that contains two virus encoded proteins (E and M). Phylogenetic analysis of viral sequences has identified two main virus lineages, African and Asian [6], the latter being the main cause of large current epidemics with millions of cases of infection, in particular those that recently affected Brazil and the Americas [7]. Once ZIKV has entered the human body, it targets many types of cells such as epithelial cells, in order to replicate and produce a viral progeny. Like for other flaviviruses, the life cycle of ZIKV leads to the release of its single-strand positive sense genomic RNA in the cell cytoplasm where it is translated into a single polyprotein which is then cleaved by host and viral proteases into three structural proteins (C, prM/M and E), and seven nonstructural proteins (NS) (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [8]. The viral cycle continues with the replication process and the production and maturation of envelope proteins, encapsidation, budding and release of virions by exocytosis.

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Like for many other viruses, interactions between ZIKV and its host trigger a variety of host responses in the body's attempt to resolve the infection [9]. Among these responses, apoptosis plays an important role as a host defense mechanisms [10]. Programmed cell death can quickly remove intracellular niches of viral replication and thus bypass the virus as it multiplies and spreads. As a result, many viruses have developed strategies to evade, delay or divert the cell death responses, often to their advantage [11]. However, the ability to exploit apoptosis beneficially is rather rare among viruses. One example is the case of chikungunya virus (CHIKV), an arbovirus of the alphavirus family, which takes advantage of massive apoptosis to hide in disseminating blebs and thus optimizes its spread [12]. Typically, alphaviruses such as CHIKV (but also Sindbis virus, Ross River virus, Semliki Virus) replicate extremely quickly and the infected cells are characterized by rapid and concomitant apoptosis [13]. Unlike alphaviruses, flaviviruses replication is relatively slow. For several of them such as DENV, Japanese Encephalitis virus (JEV) and WNV, apoptosis has been shown to be inhibited during the early stages of the viral cycle [14]. The role of viral proteins in the control of apoptosis has been extensively studied, with many observations in support of both pro-apoptotic activity and antiapoptotic effects. For WNV, a nuclear localization of the capsid was shown to induce a caspase-9-dependent apoptosis [15]. Whereas the WNV capsid protein was shown to suppress the activation of caspases 3 and 8 via Akt through a phosphatidylinositol 3-kinase-dependent mechanism (PI3K) [16]. Concerning ZIKV, *in cellulo* models have shown that infection can lead to cytopathic effects that are typical of apoptosis, and in previous work we observed late-onset apoptosis 48 hours after infection of A549 cells with ZIKV isolate PF13 [17]. In some other cell types (human fetal astrocytes), moderate apoptosis can occur even later and possibly contribute to persistent infection [18]. In the particular case of Zika pathology, homeostasis disorder; involving a lack of apoptosis control, a persistent inflammatory response and even viral persistence in the brain has been reported to explain the microcephaly observed in infected newborns [19].

In this study we examined the time course of cellular death associated with ZIKV infection. We confirm that, in A549 cells infected with the epidemic strain from Asian lineage (BeH819015, BR15^{MC}), apoptosis is quantitatively moderate and occurs late, after the maximum production of viral progeny. We investigated whether this delay was due to a protective effect of the virus itself. When intrinsic or extrinsic apoptosis was induced within 2 hours after infection, we could observe a significant decrease in cell death. As this protection was also obtained in cells expressing ZIKV "replicons", we deduced that viral replication was efficient at inhibiting apoptosis. ABT-737, an inhibitor of the anti-apoptotic Bcl-2 family proteins, abrogates the protective effects provided by ZIKV. This implies that, with a subversion mechanism that remains to be elucidated, ZIKV is able to maintain an anti-apoptotic status in infected cells while it completes its viral cycle.

2. Materials and Methods

2.1. Viruses, cell lines and reagents

The clinical isolate PF-25013-18 (PF13) and the molecular clones of ZIKV (BR15^{MC} and MR766^{MC}) have been previously described [20]. Vero cells (ATCC, CCL-81) and HEK 293T (CRL-3216) were cultured at 37 °C under a 5% CO_2 atmosphere in MEM medium (PAN Biotech, Aidenbach, Germany), supplemented with 5% heat-inactivated foetal bovine serum (FBS) (PAN Biotech, Aidenbach, Germany), A549-Dual™ cells (InvivoGen, Toulouse, France, a549d-nfis) designated hereafter as A549 cells in MEM medium supplemented with 10% heat-inactivated FBS. A549 cells were maintained in growth medium supplemented with 10 µg.mL^{-1} blasticidin and 100 mg.mL^{-1} zeocin (InvivoGen, Toulouse, France).

All reagents where from Sigma Aldrich (Humeau, La Chapelle-Sur-Erdre, France) except when indicated. The mouse anti-*pan* flavivirus envelope E protein mAb 4G2 was produced by RD Biotech. The rabbit anti-BAX and anti-caspase 3 antibodies were purchased from Cell Signalling Technology (Ozyme, Saint-Cyr-l'École, France), donkey anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 IgG antibodies from Invitrogen (Thermofisher, Les Ulis, France). Horseradish peroxidase-

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conjugated anti-rabbit (ab97051) and anti-mouse (ab6789) antibodies were purchased from Abcam (Cambridge, UK). ABT-737 (ab141336) was purchased from Abcam (Cambridge, UK).

2.2. Plaque forming assay

Viral titers were determined by a standard plaque-forming assay as previously described with minor modifications [17]. Briefly, Vero cells grown in 48-well culture plates were infected with tenfold dilutions of virus samples for 2 h at 37 °C and then incubated with 0.8% carboxymethylcellulose (CMC) for 4 days. The cells were fixed with 3.7% FA in PBS and stained with 0.5% crystal violet in 20% ethanol. Viral titers were expressed as plaque-forming units per mL (PFU.mL^{-1}).

2.3. Western blotting (WB)

Cell lysates were performed in RIPA lysis buffer. All subsequent steps of immunoblotting followed previous descriptions [21,22]. Primary antibodies were used at 1:1000 dilutions. Anti-rabbit immunoglobulin-horseradish peroxidase and anti-mouse immunoglobulin-horseradish peroxidase conjugates were used as secondary antibody (dilution 1:2000). Blots were revealed with ECL detection reagents using an Amersham Imager 680 (GE, Buc, France).

2.4. Induction of apoptosis

Apoptosis inducers were added 2 hours post-infection (hpi) or 2 hours before infection (hbi) for ZIKV infected cells. For the replicons, cells were treated 24 hours after transfection or passage of stable cells.

For intrinsic apoptosis, etoposide at 10 µM or blasticidin at 25 µg.mL^{-1} (InvivoGen, Toulouse, France) were added 16 h to 18h as indicated in the legends. Alternatively, A549DUAL cells are treated with a dose of 400 mJ of UV (Uvitec, Cambridge, UK) and cells were collected for death measurements 16 hours after treatment.

For extrinsic apoptosis, cells were treated 2 hpi or 2 hbi with TNFa (10 ng.mL^{-1}) and cycloheximide (10 µg.mL-1) (TNFa/CHX). The addition of cycloheximide prevents the activation of NFkB and the inhibition of apoptosis [23]. In any case, the drugs were added for 6 hours before quantification of cell death. For inhibition of the anti-apoptotic Bcl-2 family proteins, ABT-737 (10 µM) was added at the same time as TNF/CHX and the cells were treated as above. Previous experiments were set-up to shown that ABT-737 alone under these conditions did not induce cell death (data not shown).

2.5. Immunofluorescence assay

A549 cells grown on glass coverslips were fixed with 3.7% formaldehyde at room temperature for 10 min. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 4 min. Cells were stained using the mouse anti-pan flavivirus envelope E protein mAb 4G2 (1:1000 dilution), rabbit anti-Caspase 3 mAb (1:1000 dilution) and rabbit anti-BAX mAb (1:1000 dilution). Antigen staining was visualized with Alexa Fluor-conjugated secondary antibodies (1:1000, Invitrogen). Nucleus morphology was revealed by DAPI staining. The coverslips were mounted with VECTASHIELD® (Clinisciences, Nanterre, France), and fluorescence was observed using a Nikon Eclipse E2000-U microscope. Images were captured and processed using a Hamamatsu ORCA2 ER camera and the imaging software NIS-Element AR (Nikon, Tokyo, Japan).

2.6. Cytotoxicity assay

Necrotic cell damage was evaluated measuring lactate dehydrogenase (LDH) release resulting from a plasma membrane rupture. The supernatant of infected cells was recovered and subjected to a cytotoxicity assay, performed using the CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, USA) according to manufacturer's instructions. Absorbance of converted dye was measured at 490 nm using a microplate reader (Tecan, Grödig, Austria).

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2.7. Cell viability assay (MTT)

MTT (3-[4,5-dimethylthiazol-2-yl]−2,5-diphenyltetrazolium bromide) at 5 mg.mL^{-1} was added on A549 cells cultured in 96-well plate at a density of 5000 cells per well. Following a 1h incubation, MTT medium was removed and the insoluble formazan was solubilized with 100 µL of DMSO. Absorbance of converted dye was measured at 570 nm with a background subtraction at 690 nm.

2.8. Caspase-3/7 activity

A549 cells were cultured in 96-well plate at a density of 5×10^3 cells per well. Caspase-3/7 activity in crude cell lysates was measured using the Caspase Glo® 3/7 Assay Kit (Promega, Madison, USA) according to the manufacturer's protocols. Caspase activity was quantified by luminescence using a FLUOstar Omega Microplate Reader (BMG LABTECH, Orthenberg, Germany).

2.9. Generation of ZIKV Replicon by the ISA Method

The production of HEK 293T expressing a stable ZIKV RNA replicon with GFP as a reporter protein, named Rep ZIKV-GFP in the study, was based on the sequence of ZIKV strain MR766 Uganda 47-NIID (Genbank access # LC002520) and the ISA (Infectious Subgenomic Amplicons) method as described previously [24]. As a negative control, HEK 293T cells were transfected with pSilencer-puro 2.6 (Ambion, Thermofisher, Les Ulis, France) and pEGFP-C1 (Clontech, Ozyme, Saint-Cyr-l'École, France) plasmids with a ratio of 1 to 10 using lipofectamine 3000 according to supplier's instructions (Thermofisher, Les Ulis, France) and selected for 5 days in puromycin at 1µg.mL^{-1}.

The production of A549 cells transiently expressing a ZIKV RNA replicon was based on the ISA method and four amplicons overlapping the sequences of BeH819015 isolated in Brazil in 2015 [24] (Figure 5A). The amplicons were electroporated using the Gene pulser II apparatus according to supplier's instructions (Biorad, Marnes-la-Coquette, France) and treated within 2 days with apoptotic inducers. Cell controls in these experiments were A549DUALtransfected with a plasmid encoding GFP (pEGFP-N1) and A549 transfected with the same amplicons as above but lacking the first segment (the Z1 amplicon) and named REP NEG (Figure 5A).

2.10. Statistical analysis

All values are expressed as mean±SD of at least three independent experiments, as indicated in figure legends. After normality tests, comparisons between different treatments were analyzed by a one-way ANOVA tests as appropriate. Values of $p<0.05$ were considered statistically significant for a post-hoc Tukey's test. All statistical tests were done using the software Graph-Pad Prism version 7.01.

3. Results

3.1. ZIKV does not trigger apoptosis until the release of most of its progeny.

Our research team had previously demonstrated that a South Pacific epidemic clinical isolate of ZIKV (PF13-25013-18) was able to infect A549 epithelial cells. These cells are particularly permissive to the virus and therefore constitute a suitable model for studying *in cellulo* host-virus interactions [17]. In order to characterize the cellular death profile that accompanies ZIKV infection more precisely, we conducted a study of the cytopathic effects induced with the viral molecular clone of the epidemic strain from Asian lineage, BeH819015 isolated in Brazil in 2015 (BR15^{MC}) [25]. We infected A549 cells with BR15^{MC} at a multiplicity of infection (MOI) of 1 and followed for 3 days, the characteristics of the viro-induced cell death. We monitored different parameters representative of the induction and execution phases of apoptosis in infected cells and put them in focus with the results of viral production (Figure 1).

The measurement of LDH activity in infected cell culture supernatants, which results from a loss of cell integrity mainly reflecting secondary necrosis, revealed that significant cell mortality did not occur until 72 hours post infection (hpi) (Figure 1A). 24 hpi, there was no detectable sign of cell death

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by apoptosis (Figure B and C). Relocalisation of the pro-apoptotic factor BAX to mitochondria, an early marker of apoptosis, was only observed 48 hpi (Figure 1B) and only occurred in approximately 15 % of the cells that were immunolabeled with an antibody directed against the viral envelope protein E (ZIKV-Env) (Figure 1B). This low proportion of ZIKV-infected cells engaged in apoptosis 48 hpi, when 30% of CHIKV-infected cells are already apoptotic as early as 8 hpi [12] suggests a ZIKV-infection specific feature of the viro-induced apoptosis, together with a regulatory mechanism implemented by the virus. It should be noted that only the cells immunolabelled for ZIKV-Env had apoptotic characteristics. The percentage of uninfected cells with signs of death by apoptosis was always equivalent to that observed in the controlled cell cultures over time (data not shown). Analysis of apoptosis execution, such as the immuno-detection in infected cells of activated caspase3 (Figure 1C) or by WB (Figure 2B) supported the delay in cell death with respect to the course of viral multiplication. Moreover, significant signs of engagement in apoptosis occurred when the released viral progeny have already reached their maximum (Figure 1D).

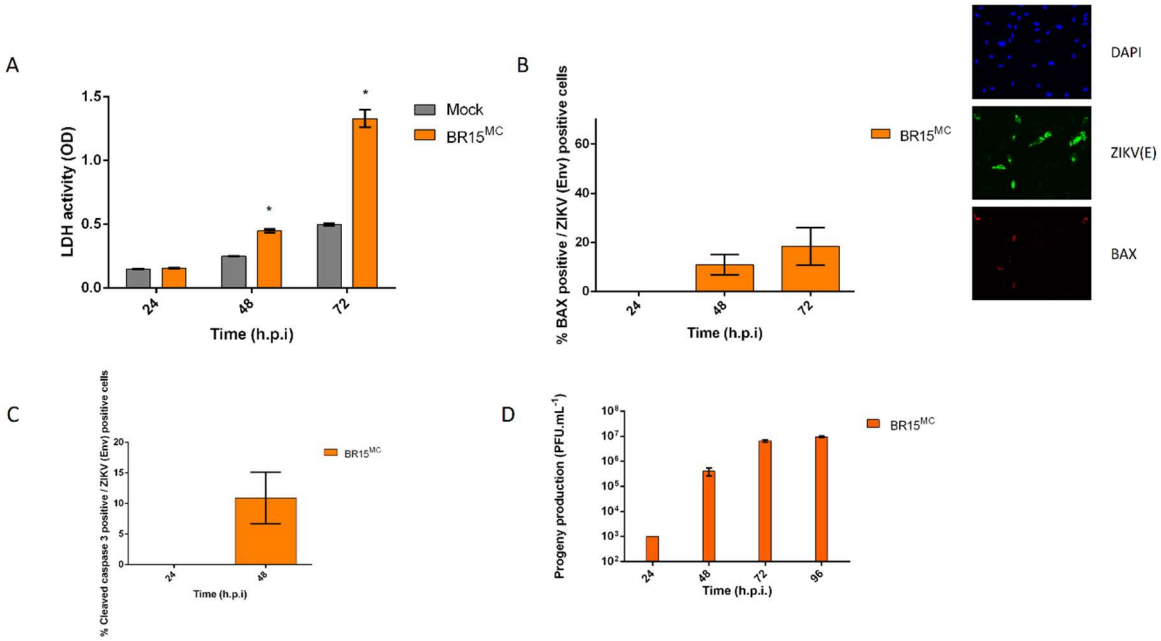

Figure 1. BR15^{MC} does not cause significant activation of apoptosis until late in infection. A549 cells were infected with BR15^{MC} at MOI of 1. (**A**) Released LDH activity was measured at 24, 48 and 72 hours post infection (hpi). (**B**) Percentage of A549 infected cells co-immunolabelled for ZIKV-Env (anti E) and for mitochondrial BAX (anti-BAX), among the ZIKV-Env positive cells were determined at 24, 48 and 72 hpi. (**C**) Percentage of A549 infected cells immunostained with anti-cleaved CASP3 antibody among the ZIKV-Env positive cells were followed at 24 and 48 hpi. (**D**) The infectious viral particles were collected from infected cell culture supernatants at 24, 48, 72 and 96 hours post infection (hpi) and titrated. Values represent the mean and standard deviation of three independent experiments (*p<0,05).

To rule out that delayed apoptosis in infected cells was not a feature of the epithelial cell line A549, we verified the respective courses of infection and cell death in other cell models. In the U251MG line of human brain glioblastoma-astrocytoma cells, infection kinetics was accompanied by a complete absence of apoptosis within the first 48 hours of infection before the maximum of progeny (Supplemental S1A and B). Thus we confirm that death by apoptosis induced by our BR15^{MC} viral molecular clone as for the Asian epidemic clinical isolate PF13 occurs late and is relatively moderate compared to the kinetics of induced viral death that can be observed in the case of infection by other viruses like alphaviruses [12].

3.2. ZIKV infected cells display resistance to apoptosis inducers.

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Apoptosis can be initiated by extrinsic or intrinsic pathways, the former being mediated by death receptors located on the cell surface, the latter being driven by various cellular stresses such as DNA damage. Activation of apoptosis-initiating caspases 8 or 9 results in outer membrane permeabilization via oligomerization and insertion of the proapoptotic factors BAX/BAK in the mitochondria of type II cells such as epithelial cells [26].

The late onset of apoptosis in infected cells led us to postulate that ZIKV may modulate the apoptotic response of the cell, delaying it through transient inhibition. To test this hypothesis, we investigated whether ZIKV could counteract the effect of death inducers added during the infection time course. We induced apoptosis through extrinsic and intrinsic pathways and tested the addition of the inducer at 2 hours prior-to and 2 hours post infection (Figure 2).

3.2.1. ZIKV provides a protection against death receptor mediated cell death

To drive an extrinsic apoptosis, we induced the TNF-Receptor using its ligand, TNF-alpha (TNFα), inhibiting the cytoprotective NFkb response by the addition of the translation inhibitor cycloheximide (CHX). Between 6 and 8 hours of treatment leads to an estimated 20% cell mortality in A549 cells, when counting BAX positive cells (Figure 2A). No variation in the percentage of dying cells (BAX+) 8 hours after the onset of treatment was observed when TNFα/CHX was added 2 hours prior to ZIKV infection.

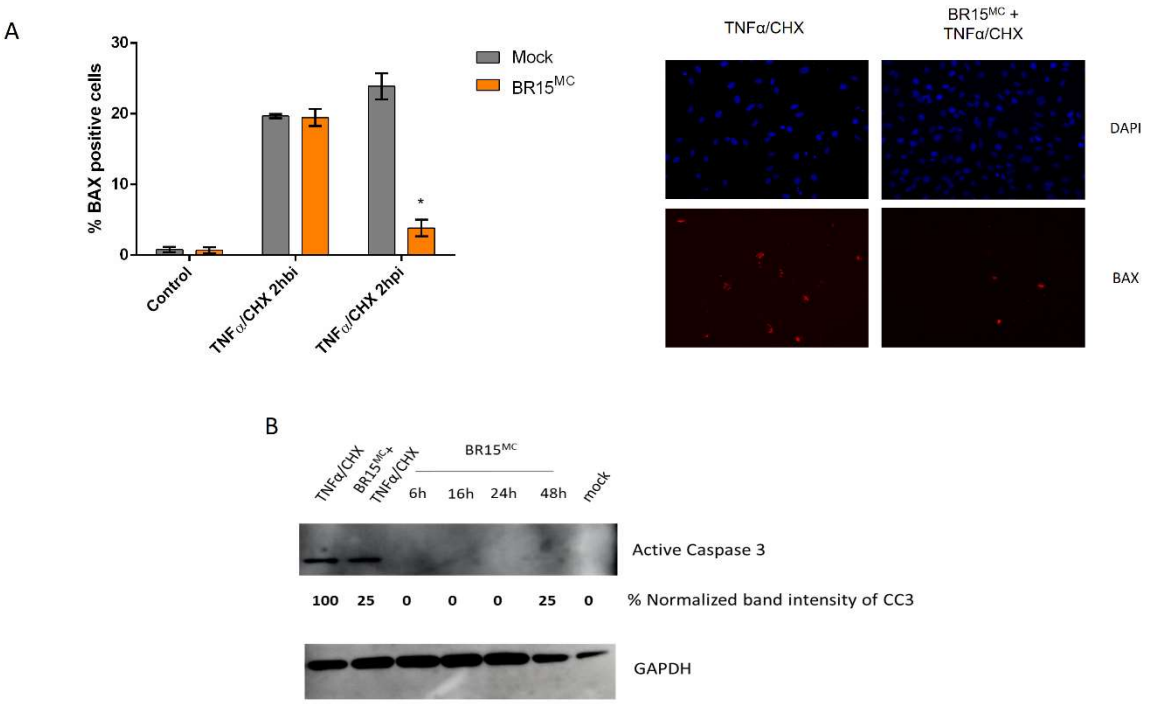

Figure 2. BR15^{MC} provides a protection against extrinsically induced cell death. A549 cells were infected with BR15^{MC} at MOI of 1 for 8 hours and treated with TNFα and cycloheximide (TNFα/CHX) 2 hpi or 2 hbi. The percentage of A549 cells immunolabelled with anti-BAX antibody was enumerated. Values represent the mean and standard deviation of three independent experiments (*p<0,05) (**A**). Immunoblot of active Caspase 3 (Cleaved caspase 3,CC3) during TNFα treatment and infection time course with BR15^{MC}. CC3 band intensity was normalized with GAPDH. WB is representative of three independent experiments (**B**).

Conversely, if TNFα/CHX was added 2 hours post infection, a drastic and significant drop in the % of cells engaged in apoptosis was observed. It is worth recalling that in this lapse of time, virally-induced apoptosis is undetectable and therefore is unlikely to interfere with the quantification of cell death induced by the action of TNFα/CHX. In WBs, detectable levels of the active form of

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caspase 3 in BR15^{MC}-infected cells were seen to reduce by around 75 % after treatment with TNFα/CHX for 6 hours (Figure 2B).

3.2.2. ZIKV provides a protection against intrinsically induced cell death

Intrinsically induced apoptosis was stimulated by chemical DNA damage. To do this, we used the genotoxic agent etoposide, a topoisomerase-II inhibitor which causes chromosome breaks during DNA replication. Overnight treatment with etoposide (18h) resulted in a percentage of cells with a mitochondrial BAX among the remaining adherent cells that was between 8 and 11%, depending on the experiment (Figure 3 and supplemental Figure S2-A). Similar to the induction of cell death by TNFα/CHX, although the effects are slightly more modest, apoptosis produced in A549 cells after 16 h of etoposide treatment was significantly reduced in the case where ZIKV was added 2h post-infection (Figure 3).

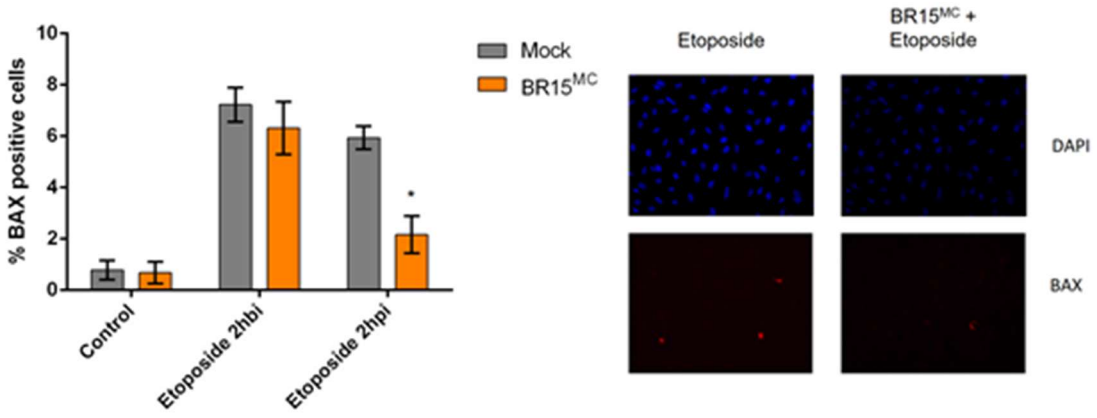

Figure 3. BR15^{MC} provides a protection against intrinsically induced cell death. A549 cells were infected with BR15^{MC} at MOI of 1 for 8 hours and treated with etoposide 2 hours before infection (2hbi) or 2 hours post infection (2hpi). Percentage of A549 cells immunostained with anti-BAX antibody were followed. Values represent the mean and standard deviation of three independent experiments (*p<0,05).

In order to ensure that the ZIKV mediated repression of apoptosis was conserved between ZIKV strains, we looked at the effect of apoptosis induction with etoposide after infection with the epidemic clinical isolate PF13 (ZIKV-PF13) but also with a molecular clone of historical strain from African lineage, MR766-NIID isolated in Uganda in 1947 (MR766^{MC}) (supplemental Figure S2 and S3). ZIKV infection resulted in reduced apoptosis for all tested strains. We also tested the effect of the cell line used for infection by repeating the procedure in Vero cells, in this instance using blasticidin to induce intrinsic apoptosis in response to the inhibition of translation. Dose-response time courses following several inducers of apoptosis again suggested that ZIKV infection represses apoptosis, regardless of cell type (Supplemental data, Figure S2). Convergent measurements of several parameters that establish the death rate and degree of protection (cell viability in Figure S2B and quantified activity of caspase 3/7 in Figure S2C) confirm that ZIKV interferes with the achievement of apoptosis in response to a death signal.

3.3. Apoptosis is repressed through ZIKV replication

Our data suggest that protection against an exogenous induced apoptosis is acquired once the virus has entered the cells and started its multiplication cycle. In order to determine the contribution of the replicative process in the protective mechanism, we exploited the “replicon” systems.

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3.3.1. Cells stably expressing a ZIKV replicon are protected from intrinsically and extrinsically mediated apoptosis.

Since both epidemic Asian (BR15^{MC}) and historical African (MR766^{MC}) strains of ZIKV, were found to be able to control apoptosis, we investigated the role of viral replication in the mechanism of apoptosis repression using a previously established MR766 replicon system in HEK-293 cells [24]. The Rep ZIKV-GFP cells have a self-replicating RNA encoding the viral NS proteins from MR766-NIID, a puromycin resistance gene to facilitate selection and GFP as a reporter. HEK-293 cells stably transfected with a plasmid encoding a GFP reporter gene and puromycin resistance without any viral material was used as a control (HEK CT) (Figure 4A). Apoptosis was induced either with TNFα/CHX, with etoposide or with blasticidin. We monitored apoptosis and in particular measured caspase 3/7 activity 6h post addition of TNFα/CHX or 16h post addition of etoposide or blasticidin. We compared the values related to caspase 3/7 activity in Rep ZIKV-GFP with those obtained with the control cells (HEK CT) (Figure 4A).

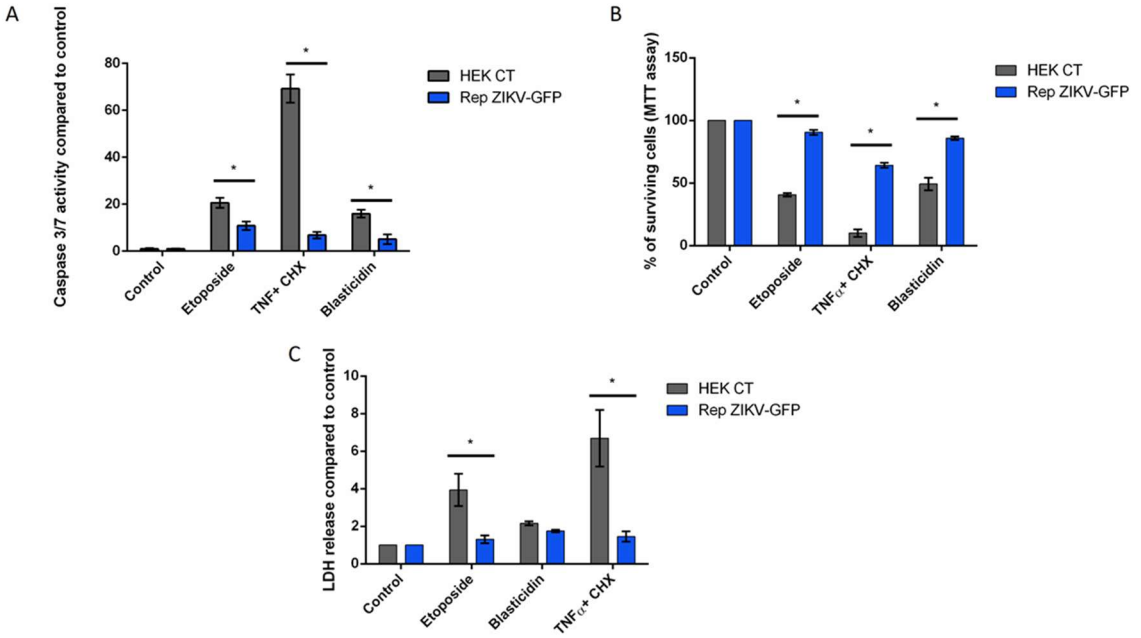

Figure 4. ZIKV replicon-expressing cells are protected from apoptosis. HEK-293 cells stably expressing a ZIKV replicon (Rep ZIKV-GFP) are protected from intrinsically and extrinsically mediated apoptosis. HEK-293 with Rep ZIKV-GFP were treated with TNFα and CHX for 6 hours, or treated with etoposide 10µM or blasticidin 25 µg.mL^{-1} for 16 hours and analyzed for : caspase 3/7 activity (**A**), cell viability (MTT assay) (**B**) and released LDH activity (**C**). Values represent the mean and standard deviation obtained with 3 different clones of Rep ZIKV-GFP (*p<0,05).

Monitoring apoptosis by caspase 3/7 activity (Figure 4A), together with the measure of cell viability (Figure 4B) and released LDH activity (Figure 4C) demonstrated that ZIKV replicon resulted in a significant reduction in the indicators of programmed cell death. An inhibition of apoptosis was not observed in the case of the cell control, selected for GFP and puromycin resistance (HEK CT) as well as in untransfected HEK-293 cells (data not shown). It can therefore be excluded that resistance to puromycin or the presence of a GFP encoding gene may be responsible for a protective effect.

It can therefore be stated that the presence of an autonomous replication of a viral RNA associated with the expression of ZIKV NS proteins, makes cells resistant to several extrinsic and intrinsic apoptotic inducers

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3.3.2. A549 cells transiently expressing a ZIKV replicon are protected from different intrinsically induced apoptosis.

To address a protective effect of the viral replication in a system consistent with the one in which the effect was revealed through infection, we adapted the ISA method to obtain A549 cells transiently expressing a ZIKV replicon with the non-structural sequences from BeH819015 (REP BR15). We used A549 cells transfected with an incomplete set of amplicons as a negative control (REP-NEG) (Figure 5A). Apoptosis was induced 48 hours after amplicon transfection by two parallel methods; etoposide (Figure 5B) and DNA damage through exposure to UV light (Figure 5C). Apoptosis was measured as the percentage of cells with mitochondrial BAX 16h after induction.

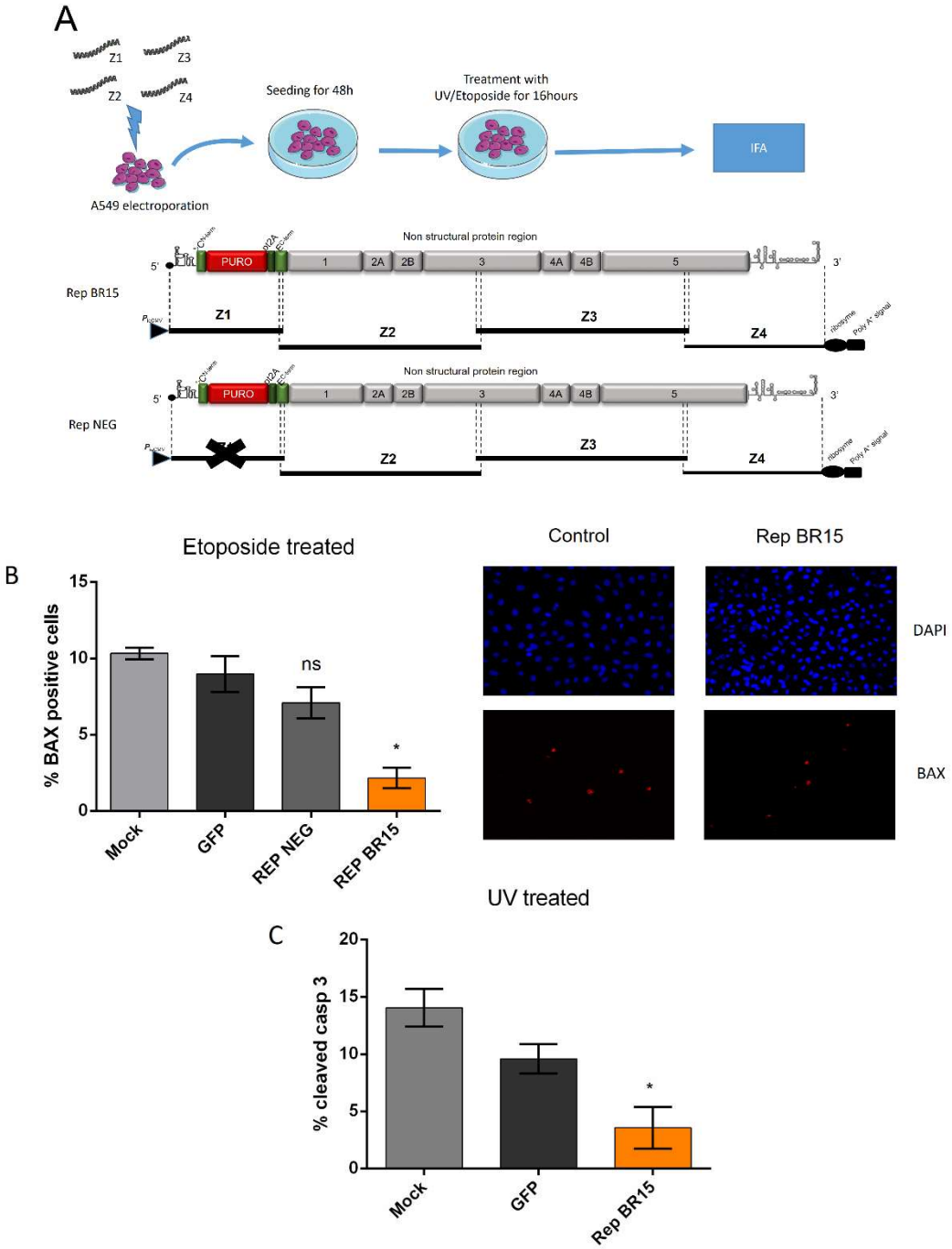

Figure 5. A549 cells transiently expressing a ZIKV replicon are protected against cell death by apoptosis. A549 cells were transfected with ZIKV amplicons (DNA fragments overlapping the viral genome) for production of REP BR15 and REP NEG or with pEGFP-N1 (**A**). 48h after transfection

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A549 cells were treated with Etoposide (**B**) or UV at 400 mJ (**C**) for 16 h. The percentage of A549 cells immunostained with anti-BAX antibody was monitored. Values represent the mean and standard deviation of three independent experiments (*p<0,05).

In cells treated with etoposide, REP BR15 expressing cells showed approximately half the number of apoptotic cells of the replicon control (REP NEG) (Figure 5B). The percentage of dying cells was even lower in cells expressing REP BR15 after UV treatment (Figure 5C). Thus, REP BR15 was able to confer resistance to apoptosis. All together these results suggest that replication of the ZIKV is capable of inhibiting apoptosis

3.4. ZIKV promotes an anti-apoptotic prevailing status in infected cells through the Bcl-2 family protein.

The long delay in the onset of viral apoptosis induced by ZIKV infection, particularly in the case of Asian viral strains responsible for current epidemics, and the demonstration that, when the viral RNA is present and replicating there is an inhibition of apoptotic induction, suggest that cells have acquired with the virus a status in which anti-apoptotic activity prevails. The protective effect acquired with ZIKV could be at the level of convergence of the intrinsic and extrinsic pathways. As we followed BAX relocalization we can argue in favor of protection around the mitochondria events and the control of the outer membrane permeabilization (OMP). A prominent anti-apoptotic factor involved in the regulation of early apoptosis, by operating mainly at the mitochondrial level for the control of its permeabilization is Bcl-2 and the related Bcl-XL protein [27]**.** To identify to which extent these anti-apoptotic factors play a role in the protection provided by ZIKV, we examined the effect of ABT-737 on cell death outcomes, with or without BR15^{MC} (Figure 4A and B). ABT-737 is a BH3 mimetic molecule that can bind to the hydrophobic groove of the members of the anti-apoptotic Bcl-2 protein family, Bcl-2 and Bcl-xL, and therefore inhibits their activity by shifting oligomerization mechanisms in favor of BAX/BAK dimerization [28].

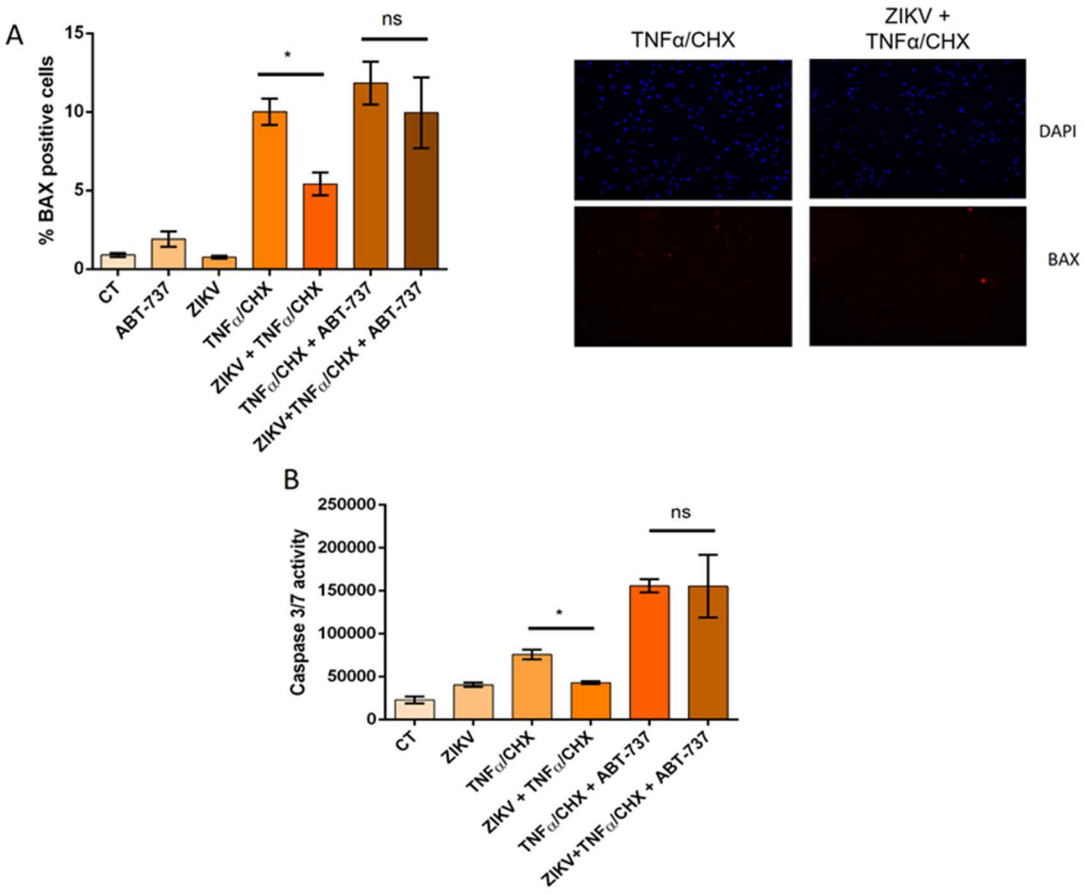

Figure 6. Inhibition of anti-apoptotic BCL-2 family proteins abrogates the protection mediated by ZIKV. A549 cells were infected with ZIKV at MOI of 1. TNFα and CHX were added 2 hours post

infection for 6 hours with or without ABT-737. (**A**) A549 cells were immunostained with an anti-BAX antibody (**A**) and Caspase 3/7 activity was followed after treatment (**B**). Values represent the mean and standard deviation of three independent experiments (*p<0,05).

When inducing apoptosis with TNFα/CHX, addition of ABT-737 restored the percentage of ZIKV-infected cells with mitochondrial BAX (Figure 6A) and caspase 3/7 activity (Figure 6B) to levels that were similar to cells that were not infected with ZIKV.

These observations suggest that ABT-737 has counteracted the protective effect acquired with the virus. It can legitimately be deduced that the viro-induced protective effect probably depends on the anti-apoptotic activity of Bcl-2 family proteins. When Bcl-2/Bcl-XL is inhibited, ZIKV no longer allows a quantitative reduction of apoptosis.

4. Discussion

ZIKV has recently earned its reputation in the field of medically important flaviviruses, which are responsible of world epidemics that are difficult to control [29]. To date, there is no effective treatment against this emerging pathogen despite significant efforts made to succeed in providing an effective vaccine. The characterization of the interaction modalities between the pathogen and host cells are important in order to better understand the strategies adopted by the virus to be effective in its replication and dispersal of its progeny. Among the responses of infected cells, a high priority must be given to virally induced programmed cell death since its completion can significantly interfere with the virus's multiplication cycle and hinder its spread [30]. Moreover, crosstalk between innate immune signaling and cell death pathways and how the viruses are able to manipulate each other are essential for viral clearance or persistence and for the global outcomes of the infection. Many studies support that ZIKV infection induces apoptosis *in vivo* as well *as in vitro* [17,31–33]. However, several of them also assert that ZIKV induced cell death could be delayed [17,18] We therefore wanted to define whether this capacity was specific to the strain of ZIKV responsible for the current epidemic and whether ZIKV was able to interfere with the induction of apoptosis.

Using of a molecular clone of the BeH819015 from the Brazilian 2015 outbreak (BR15^{MC}) and its comparison to the clinical isolate from the French Polynesian 2013 outbreak (PF13), our work has highlighted that ZIKV strains from the actual epidemic Asian lineage are particularly inclined to delay the onset of apoptosis in infected human epithelial cells (A549) as well as in human brain glioblastoma-astrocytoma cells (U251 MG) (Figure 1 and S1). These viruses are also characterized by a rather slow viral growth compared to the molecular clone of the historical strain of ZIKV MR766 from the African lineage [17,25]. Infection with MR766^{MC} is marked by a higher cytotoxicity over the duration of infection, with 10% of infected cells showing signs of entry into apoptosis as early as 24 hours, whereas epidemic strains have no signs of mortality before 48 hours. However, despite this behavior of MR766^{MC}, which seems more aggressive, it must be admitted that the mortality rate among infected cells remains limited until 48 hours post infection (Figure 2 and S3). We have proposed from these results that both viruses have the ability to interfere with the onset of apoptosis even though faster and more effective viral growth in the early hours of infection for MR766^{MC} than for BR15^{MC} has resulted in faster and more cytopathic effects. In both cases, the maximum mortality is only recorded when the virion production has reached its highest titer. This observation suggests a manipulation of apoptosis orchestrated by ZIKV in order to give it enough time to complete its entire production cycle. This phenomenon has already been described for other flaviviruses such as DENV, JEV and WNV as they can delay apoptosis through activation of Phosphatidylinositol 3-kinase (PI3K) and Akt pathway [16,34].

In a rather unexpected way, our work mainly shows that in the early stages of infection, ZIKV infection provides a solid protection against an exogenous induced cell death. We provide supporting evidence that a protection is acquired both with the Asian epidemic strains and with the African strain (Figure 2, 3, S2 and S3). This protection is effective against apoptosis mediated by an extrinsic death inducer (TNFα) as well as by an intrinsic signal (provided by the action of etoposide or blasticidin). Resistance to these induction modes has also been found to characterize cells expressing

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replicons, either HEK 293 cells stably expressing a MR766 replicon or A549 cells transiently expressing BR15 replicons or MR766 replicons (Figure 4, 5 and S4). The data obtained with the use of these replicons are in support of a greater protection granted by BR15. This is consistent with the data obtained with the whole virus that is responsible for the longest delay in apoptosis entry. Cell death inhibition ability acquired with the ZIKV replicons would imply that the single presence of a viral RNA leading to the production of the NS proteins and allowing its self-replication is the driving force behind the protection acquired against apoptosis.

This discovery is rather unusual when one considers the literature mainly in favor of pro apoptotic functions for the NS proteins [35]. However, studies have also shown that some NS may help ZIKV to evade antiviral immunity and cell death. NS2B in particular was proposed to be responsible for blocking RLR-triggered apoptotic cell death [36]. A thorough identification of the viral protein responsible for an inhibitory effect needs to be confirmed and further investigated. While our work suggests a possible role for NS proteins in the control of apoptosis, we cannot rule out the possibility that structural proteins may also act. Anti-apoptotic activities have been previously reported in relation to the capsid [16,18]. It should be noted that in the construct we used to generate the replicons, the polyprotein produced retains the first 33 amino acids of the capsid. It would be interesting to see if this part of structural protein, released after the cleavage of the GFP has a role in protection. We cannot also exclude a role of the viral RNA by itself. Recent work has also investigated the effects of viral RNAs, as flaviviruses are known to produce multiple small RNAs that may have interference activities in the cell physiology. It was recently described that recent epidemic Asian lineage display more negative-strand replicative intermediates than the historical African strain [37]. This important production could be a key element in the search for which viral determinants are crucial in the control of apoptosis. We also know how important are the viral genomic 3' UTR regions and the sfRNA produced, in the implementation of cellular responses to infection [38]. It remains to be discovered which factor associated with the replication process of ZIKV viral RNA is involved in the protection mechanism. What we already know from our study is that this mechanism requires the Bcl-2 family protein as ABT-737 abrogates the protection acquired against apoptosis by ZIKV infection (Figure 6). The anti-apoptotic capacity of the pro-survival Bcl-2 proteins is known to depend mainly on their ability to sequester pro-apoptotic proteins by binding their BH3 domains. A decrease in Bcl-2 leads to the disruption of associated pro-survival and pro-apoptotic Bcl-2 proteins and will promote apoptosis whereas overexpression of Bcl-2 will inhibit mitochondrial OMP [39,40]. A control of the stability and degradation of Bcl-2 is therefore a key in the subtle balance that takes place between pro and anti-apoptotic suits to determine the cell's fate [40]. Previous study showed the importance of Bcl-xL for cells survival, in deficient Bcl-2 cells during ZIKV infection [41], but here we cannot exclude a role for Bcl-2 and/or Bcl-xL protein as our model express these two anti-apoptotic proteins. As we used cycloheximide or blasticidin in our assays, we can therefore assume that the mechanism implemented by ZIKV does not involve a "*de novo*" synthesis of proteins. Based on these remarks, we formulate the hypothesis that ZIKV may allow a stabilization of the Bcl-2 protein over time (Figure 7). The mechanism by which the virus allows the Bcl-2 stabilization and blocks apoptosis needs further investigation.

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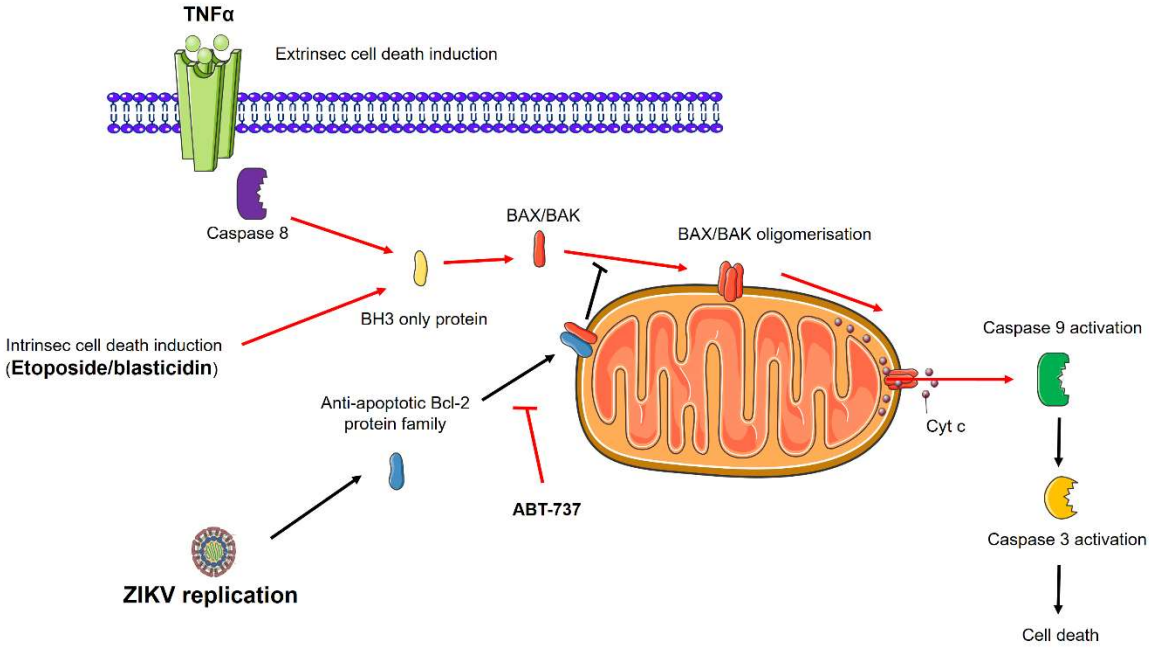

Figure 7. Model depicting the protective action of ZIKV infection against programmed cell death. Intrinsic or extrinsic activation of cell death occurs through the formation of mitochondrial outer membrane pore (MOMP) via the BAX-BAK complex. The anti-apoptotic family Bcl-2 members Bcl-2/Bcl-XL interfere with the complex formation by sequestering BAX via their BH3 domain. ZIKV replication interferes with BAX relocalization at the mitochondria. ABT-737 a BH3 mimetic, which inhibits Bcl-2/Bcl-XL, abrogates the inhibition induced by ZIKV. Indeed, the virus delays apoptosis during infection by modulating the homeostasis of Bcl-2/Bcl-XL.

Supplementary Materials: Figure S1: ZIKV-PF13 does not cause significant activation of apoptosis until late in infection in U251MG cells. U251MG cells were infected with ZIKV PF13 (clinical isolate) at MOI of 1 for 96h. (A) Percentage of U251MG cells infected immunostained with anti-BAX antibody were determined at 24, 48 and 96hpi. (B) The infectious viral particles were collected from infected cell culture supernatants during four days post infection (dpi) and titrated.Figure S2: ZIKV-PF13 provides a protection against induced cell death. A549 cells were infected with ZIKV-PF13 at MOI of 1 for 8 hours and treated with etoposide 2 h before infection (2hbi) or 2 h post infection (2hpi). The percentage of A549 cells immunostained with anti-BAX antibody was followed (A). Vero cells were infected with ZIKV-PF13 at MOI of 1 for 8 h and treated with blasticidin followed by MTT assay (B) or Caspase 3/7 activity (C). Values represent the mean and standard deviation of three independent experiments (*p<0,05). Figure S3: ZIKV-MR766 does not cause significant activation of apoptosis until late in infection and ZIKV-MR766 is able to control cell death. A549 cells were infected with $MR766^{MC}$ at MOI of 1 and during 96h. The percentage of A549 infected cells immunostained with anti-BAX antibody at 24, 48 and 72 h pi (A). The infectious virus was collected in supernatant of infected cells at 24, 48, 72 and 96 h pi for titration (PFU assay) (B). A549 cells were infected with $MR766^{MC}$ at MOI of 1 for 8 hours and treated with TNFα and CHX 2 h before infection (2hbi) or 2 h post infection (2hpi). Percentage of A549 cells immunostained with anti-BAX antibody (C). Values represent the mean and standard deviation of three independent experiments (*p<0,05). Figure S4: A549 cells transiently expressing a ZIKV-MR766 replicon are protected against cell death by apoptosis. A549 cells were transfected with amplicons of ISA methods to generate replicon for ZIKV-MR766 or with pEGFP-N1 (A). 48h after transfection A549 cells were treated with Etoposide for 16 h. Percentage of A549 cells immunostained with BAX antibody were monitored. Values represent the mean and standard deviation of three independent experiments (*p<0,05).

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