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Brief Report

Crosstalk Between Endoplasmic Reticulum Stress and The Unfolded Protein Response During ZIKA Virus Infection

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Abstract: Flaviviruses replicate in membranous factories associated with the endoplasmic reticulum (ER). The replication rate generates a high polyprotein integration that can contribute to ER stress. Therefore, the host cell can develop an Unfolded Protein Response (UPR) to this protein accumulation which will stimulate appropriate cellular responses in the infectious context (Adaptation, autophagy or cell death). These different stress responses can help to overcome the virus and usually support antiviral strategies initiated by infected cells. In the present study, we investigated the capacity of ZIKA virus (ZIKV) to induce ER stress in epithelial A549 cells with a special focus on the causal factors behind it. We observed that the cells respond to ZIKV infection by implementing an UPR through activation of the IRE1 and PERK pathway but surprisingly without activation of the ATF6 branch. When we examined the effects of modulating the ER stress response we found that UPR inducers significantly inhibit ZIKV replication. Interestingly, our findings provide evidence that ZIKV can altered the UPR in order to escape to the host cell defense system.

Keywords: ZIKA virus; ER stress; Unfolded Protein Response; ATF6

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1. Introduction

Endoplasmic reticulum (ER) is an essential cell compartment for several key mechanisms in cell physiology like membrane biosynthesis and calcium storage. These include also incorporation of newly synthesized proteins intended to be addressed or secreted and their post-translational modifications and folding. The correct folding of the proteins allowed to carry on their centrifugal transport in a cell depends on the N-linked protein glycosylation, the oxidative environment in the ER lumen which promotes the formation of disulfide bonds, and the presence of several Ca^{2+}-dependent molecular chaperones (calreticulin, GRP78 also named BiP and GRP94) which stabilize protein folding intermediates [1,2]. ER homeostasis can be perturbed in case of glucose starvation, hypoxia, calcium dysregulation or following a protein accumulation which will in turn provoke ER stress. ER homeostasis is then maintained through a tight association with the Unfolded Protein Response (UPR) that also mediates stress resolution. In cells infected by a virus, the huge and sudden amount of viral proteins that need to be processed usually leads to ER stress and UPR. This cell response was therefore shown to be involved in the implementation of several cell defenses, *i.e.* antiviral programs, immune responses [3] and commitment in autophagy or cell suicide [4,5]. During ER stress, the folding chaperone GRP78/BiP dissociates from three ER-resident transmembrane proteins *i.e.* PKR-like endoplasmic reticulum kinase (PERK), Inositol-Requiring Enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) [6]. Each will act as a stress transducer in three different pathways devoted to stress resolution. Thus, the activated kinase activity of PERK results in phosphorylation of eIF2α, followed by a transitory translational attenuation and consequently a reduced ER influx of newly synthesized proteins. For the activated IRE1, its endonuclease function produces a spliced form of the x-box binding protein 1(Xbp1) transcript. This spliced Xbp1 encodes a transcription factor in charge of genes coding for several factors aimed at resolving ER stress. Regarding ATF6, the soluble factor can egress from the ER to Golgi apparatus for maturation by S1P or S2P cleavage to generate ATF6f. Then ATF6f reaches the nucleus where it regulates UPR target gene expression. Transcriptional activity of ATF6 and Xbp1 leads to the increased expression of chaperones and proteins implicated in enhanced folding capacity or in ER-associated protein degradation (ERAD). Despite the implementation of an UPR with these adaptive mechanisms, an excessive, unresolved or prolonged ER stress will turn the cell's fate into autophagy or apoptosis. Crosstalks between ER stress, UPR, autophagy and apoptosis are of particular importance in the case of virally infected cells since each of these responses will condition the cell health and the success of the virus in replicating and spreading.

ZIKA virus (ZIKV) is a pathogenic single-stranded RNA virus belonging to the *Flaviviridae* family like Dengue virus (DENV), Yellow Fever virus (YFV) or West Nile virus (WNV). Among the pathogenic flaviviruses, ZIKV has gained notoriety in the last ten years, due to explosive

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outbreaks and serious clinical concerns. Neurological complications have been described, including Guillain-Barré syndrome (GBS) and congenital malformations, prompting specific vigilance for pregnant women in the event of a Zika epidemic [7,8]. Due to these atypical clinical manifestations, the ability of ZIKV to be sexually transmitted and not only by vectorization, and the evidence of its persistence in some tissues, studies have been conducted at molecular and cellular levels to highlight the singularity of ZIKV-host interactions among other flaviviruses. As for other flaviviruses, ZIKV replication occurs in ER invaginations of infected cells and leads to an increasingly important synthesis of viral proteins [9–11]. The genome translation is followed by an incorporation in the ER membrane of a polyprotein further cleaved proteolytically in three structural proteins (C, M, and E) and seven nonstructural proteins NS1, NS2A, NS2B, NS3 (helicase and protease), NS4A, NS4B, and NS5 (RNA-dependent RNA polymerase and methyltransferase). This important protein processing can potentially cause ER stress. It has previously been shown that ZIKV has the ability to instruct an UPR in response to ER stress [12–16] and that infection is accompanied by morphological modification of cell organelles [17].

However, deciphering the initiating modalities of the ER stress, which transducing branches are involved for the UPR implementation and how crosstalks are carried out between ZIKV, ER stress and the cell responses, deserve thorough investigation.

In this present work, using A549 cells as a model, we examined which causal component may trigger ER stress during ZIKV infection. Imaging of infected cells revealed typical ER morphological changes associated with high viral protein density. We then highlighted, for the first time, the production of significant proportions of oligomers with insoluble disulfide bonded viral E proteins (E-ZIKV), indicative of misfolded or unfolded proteins. When we looked at the stress transduction mechanisms, we discovered that the UPR response was initiated by the host cell but with no activation of the ATF6 branch. Considering that a partial or impaired UPR could be beneficial for the virus, we tested the effects of stress inducers on infection. We then confirmed that exogenously-induced UPR was detrimental to ZIKV. We finally conclude that ZIKV has the ability to modulate UPR to its benefits with a mechanism that remains to be discovered.

2. Materials and Methods

Virus, Cell culture, antibodies and reagents

The clinical isolate PF-25013-18 (PF13) of ZIKV has been previously described [18]. A549 cells (ATCC, CCL-185) were cultured at 37°C under a 5% CO_2 atmosphere in MEM medium

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supplemented with 10% heat-inactivated foetal bovine serum (FBS). For viral progeny quantification, plaque forming assay, were done as described before [19].

The mouse anti-pan flavivirus envelope E protein mAb 4G2 was produced by RD Biotech, the rat anti-EDIII ZIKV was described previously [20]. The rabbit anti-Calnexin antibody was purchased from Santa-Cruz Biotechnology (Clinisiences, Nanterre, France). Donkey anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 IgG antibodies were from Invitrogen (Thermofisher, Les Ulis, France). Horseradish peroxidase-conjugated anti-rabbit (ab97051) and anti-mouse (ab6789) antibodies were purchased from Abcam (Cambridge, UK). GRP78 (#3177) were from cell Signalling Technology (Ozyme, Saint-Cyr-l'École, France).

Thapsigargin (TG), an endoplasmic reticulum Ca^{2+}-ATPase inhibitor and Tunicamycin (Tm) an inhibitor of N-glycosylation were used to induce ER stress and UPR respectively at 1 µM and 2 µg.mL-1 for the indicated time in figure legends. These inhibitors were from Sigma-aldrich (Humeau, La Chapelle-Sur-Erdre, France)

Luciferase Reporter assay

Plasmids with GRP94 and GRP78 promoter upstream of F-Luc (pGRP78-Luc and pGRP94-Luc) were from Dr. Kazutoshi Mori [21]. $1x10^6$ cells were transfected with indicated plasmids using Lipofectamine 3000 according to manufacturer instructions (Invitrogen, Thermofisher, Les Ulis, France) and 12h after were separated into three pools, the first one was mock treated, the second one were infected with ZIKV PF13 at MOI of 5 for 24h and the third one was treated with thapsigargin (1µm) for 6 hours. Luciferase activities were measured using Luciferase Glo assay according to supplier's instructions (Promega, Madison, USA).

Cell fractionation and Western blot

Cells were washed with PBS and lysed at the concentration of $1x10^4$ cells.μl^{-1} in buffer A (0.2% Triton X-100, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5 mM EDTA) as before. The Triton X-100-insoluble fraction was separated by centrifugation at 3,400 g for 10 min. Pellets were enriched in non-folded proteins and supernatant in Triton X-100 soluble proteins. All fractions were proceeding for western blot. All western blot experiments were proceeded as before with only one modification: protein in Laemmli buffer were not reduced by DTT and not boiled at 95°C for 5 min [22].

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Total proteins cells extract was prepared by harvest of cells from plate in RIPA buffer after two washes with PBS. Cells were sonicated for 10" at 0.5 cycle with an amplitude of 50% using sonicator. Protein were treated in Laemmli buffer with DTT and boiled at 95°C for 5 min. western blot were perform as above.

Immunofluorescence

A549 cells were grown, infected or treated on glass coverslips and 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. Coverslips were incubated with primary antibodies (1:1000 dilution) in 1% PBS BSA for two hours and with Alexa Fluor-conjugated secondary antibodies (1:1000, Invitrogen) for one hour. 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).

RT-PCR

Semi quantitative RT-PCR experiments were performed as before [22]. Primers used for RT-PCR were CHOP: F 5'-GCACCTCCCAGAGCCCTCACTCTCC-3', R 5'-GTCTACTCCAAGCCTTCCCCCTGCG-3'; GRP78: F 5'-CATCACGCCGTCCTATGTCG-3', R 5'-CGTCAAAGACCGTGTTCTCG-3'; XBP1: F 5'-CCTTGTAGTTGAGAACCAGG-3', R 5'-GGGGCTTGGTATATATGTGG-3'; NS1: F 5'-AGAGGACCATCTCTGAGATC-3', R 5'-GGCCTTATCTCCATTCCATACC-3'; GAPDH: F 5'-GGGAGCCAAAAGGGTCATCA-3', R 5'-TGATGGCATGGACTGTGGTC-3'. PCR products were analyzed on a 1.5% agarose gel electrophoresis and stained as described before [19].

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. 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.

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Degrees of significance are indicated in the figure captions as follow: * p<0.05; ** p<0.01; *** p<0.001, **** p<0.0001, ns=not significant.

3. Results and Discussion

3.1. ZIKV infection induces ER morphological changes and oligomerization of viral proteins.

When stressed, ER exhibits morphological characteristics with luminal swelling and membrane expansion [23]. These morphological changes can be observed by fluorescence microscopy. When we compared Calnexin expression, an ER typical marker, between A549 cells infected with the epidemic strain of ZIKV (PF13) and control (mock infected cells), we noticed morphological shape changes of the ER with an obvious swelling aspect (Figure 1A). Imaging of the infected cells also showed a co-staining between the immunodetected viral envelope protein (E-ZIKV) and the calnexin-staining, confirming that viral proteins are present in an enlarged and globe-shaped compartment related to the ER (Figure 1 A).

Accumulation of partially processed or misfolded proteins is known to be responsible for ER stress. In the case of ZIKV infection, we looked for the E-ZIKV protein accumulation and signs of associated misfolding. Using cell fractionation, as described before for ERGIC-53 proteins, we separated soluble and insoluble proteins [22] and observed the repartition of E-ZIKV between these two fractions (Figure 1B). Detection of E-ZIKV with an antibody raised against the domain III of the E protein (EDIII) revealed an accumulation of insoluble forms of E-ZIKV with a high molecular weight instead of the 55 KDa predicted molecular weight of the monomer (M). This high molecular weight is consistent with oligomers of the protein (Oligomer, O). When the western blot membrane was incubated with 4G2, a pan-flavivirus antibody that binds a conformational epitope which overlaps the E protein fusion loop [24], we observed that the antibody recognized E-ZIKV mainly in a monomeric form in the soluble fraction (Figure 1B). These observations suggest that during ZIKV infection, E proteins are produced and accumulated in diverse forms corresponding to a soluble, native and folded form of the protein but also to oligomerized unfolded or misfolded proteins retained in an insoluble fraction.

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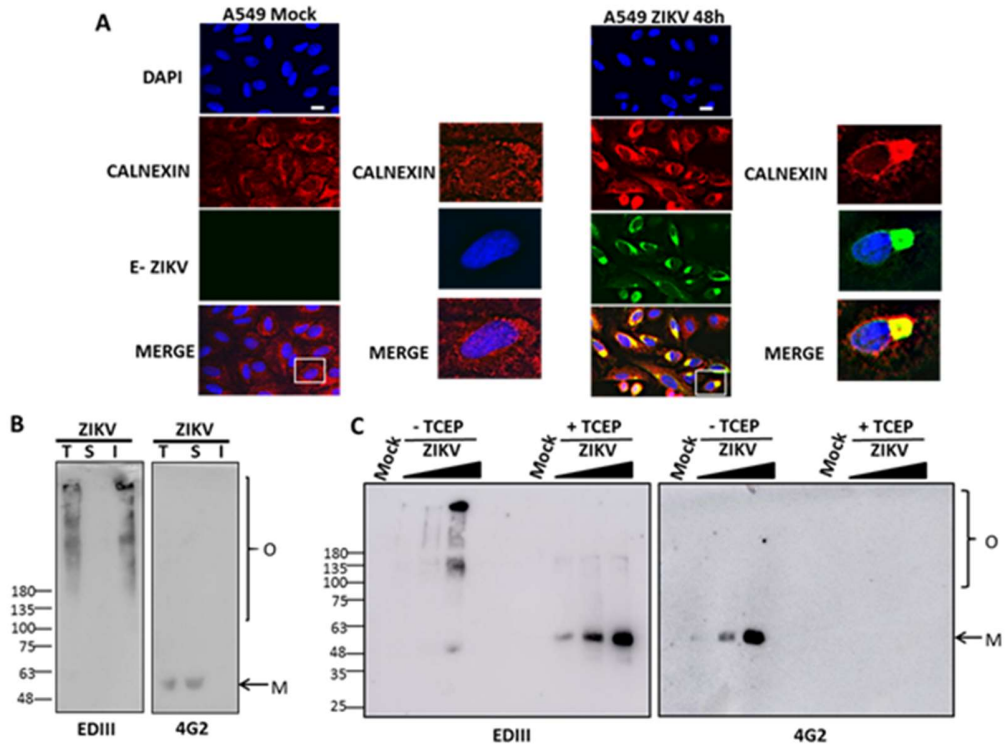

Figure 1. ZIKV infection is associated with morphological changes of ER and accumulation of insoluble disulfide bonded oligomers of E-ZIKV. **(A)** A549 cells were infected with ZIKV at MOI of 1 for 48h. Cells were immunostained for calnexin and E-ZIKV. Scale bar: 10 µm. Right panel series show magnified details of a selected cell from the x200 microscopic field (white square) **(B)** A549 cells were infected with ZIKV at MOI of 5. 48h post-infection cells were lysed in TX100 buffer A. An equal volume of total (T), soluble (S) and insoluble (I) fractions were separated under non-reducing conditions and immuno-blotted for E-ZIKV with rat anti-EDIII or 4G2 antibody. **(C)** A549 cells were infected with increased quantities of ZIKV (MOI 0.5, 1 and 5) and total fraction were treated or not with TCEP before western blotting as in (B). Molecular weight marker was indicated on the left. All experiments were representative of three independent experiments.

As non-native disulfides bonds can prevent correct folding and should be reduced or isomerized to form the correct, native disulfides bonds [25], we hypothesized that oligomers of E-ZIKV could result from non-native disulfide bonds formed between E-ZIKV monomers. To test this hypothesis, we treated cell protein extracts with Tris(2-carboxyethyl) phosphine (TCEP), a reducing agent, and performed immunodetection of E-ZIKV as above. With the antibody raised against the EDIII, we were able to detect mainly oligomers under non-reducing conditions and monomers upon reduction with TCEP (Figure 1C). With the 4G2 antibody, only the monomeric form of E-ZIKV could be detected and the signal was lost in the presence of TCEP (Figure 1C).

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This observation argues in favour of a native folded E-ZIKV form that depends on intra-disulfide bonds. Conversely, unfolded or misfolded E-ZIKV would have led to disulfide bonded aggregates. Accumulation of this oligomerized forms of E-ZIKV and the morphological deformation of the ER lumen observed during ZIKV infection are concrete elements that support an effective ER stress. To our knowledge this is the first observation reporting an accumulation of this type of aggregate during an infection by flaviviruses. ER stress being settled upon ZIKV infection, one could expect it to be resolved by an appropriate cell response and implementation of UPR pathways.

3.2. Unfolded Protein Response during ZIKV infection in A549 cells

Upon ER stress, cells initiate an UPR to prevent a damaging persistence of the stress and to restore ER homeostasis. In order to decipher the stress transduction mechanisms induced upon ZIKV infection in A549 cells, we analyzed each of the three main UPR pathways. For this purpose, we investigated the expression level of target genes specific of each activated branch.

3.2.1. Effect of ZIKV infection on PERK and IRE1 pathways of UPR.

Activation of the PERK pathway results in eIF2α phosphorylation which is followed by a reduced translation rate. It also leads to ATF4 upregulation. ATF4 is a transcription factor acting on several targets among which the C/EBP homologous protein (CHOP) encoding gene. This latter transcription factor plays a key role in stress resolution and in relationships between UPR, cell survival or cell death. We therefore followed activation of this PERK/ATF4 branch by a measure of the expression level of CHOP gene by RT-PCR (Figure 2A). Effective upregulation of CHOP at the transcriptional level suggests that the PERK branch of UPR was activated during ZIKV infection of A549 cells. This observation confirms previous findings in a model of ZIKV infected human neural stem cells [10].

The IRE1 pathway is characterized by the splicing of the Xbp1-transcript. The spliced Xbp1 (s-Xbp1) encodes a transcription factor able to transactivate genes mainly involved in ERAD. Splicing of Xbp1 was then followed by RT-PCR as an indicator of IRE1 branch activation. s-Xbp1 was detected upon ZIKV infection like in the thapsigargin (TG)-ER stress induced positive control

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(Figure 2A). This result on the IRE branch activation by ZIKV was also in accordance with previous findings [10].

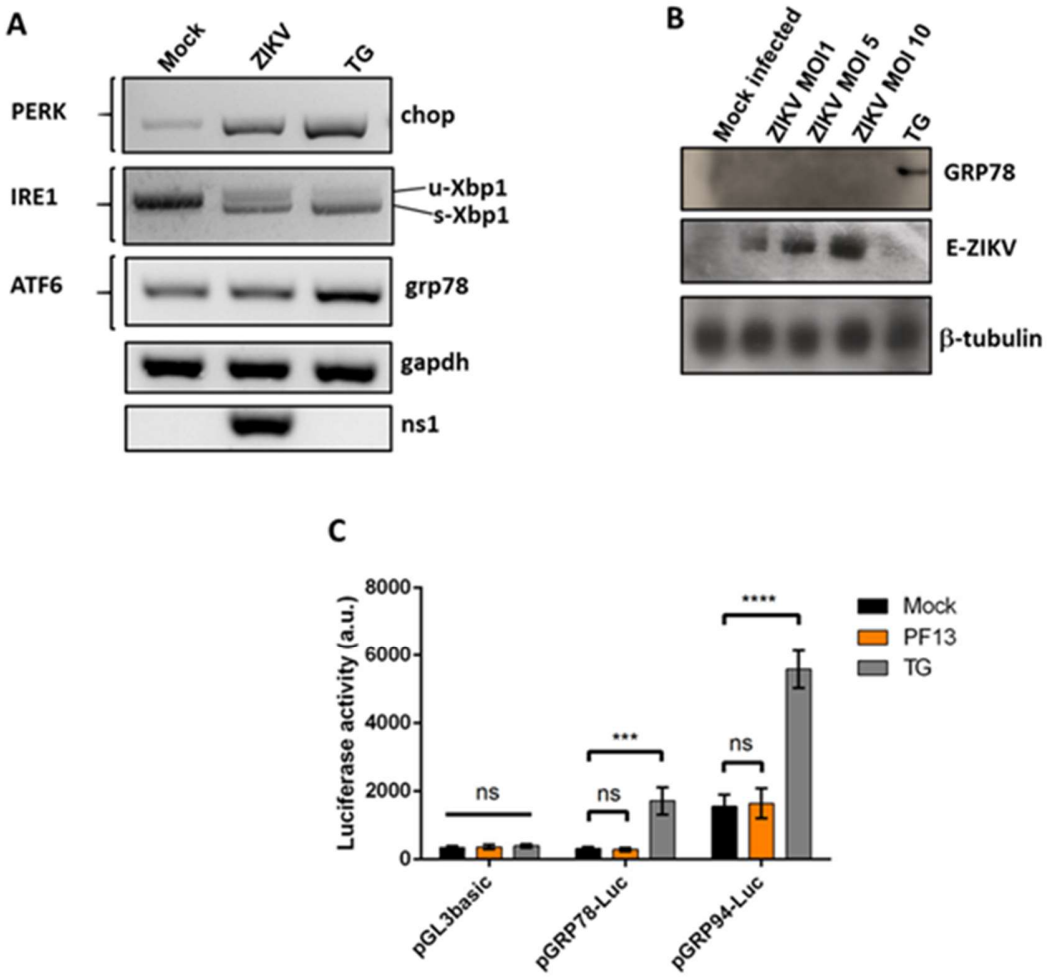

Figure 2. UPR branch activation during ZIKV infection. (A) A549 cells were infected with ZIKV at MOI of 1 for 24h. RT-PCR were performed to monitor transcriptional regulation of downstream gene targets of the UPR branch, chop (PERK pathway), Xbp1 splicing (IRE1 pathway) and grp78 (ATF6 pathway). u-Xbp1 correspond to unspliced Xbp1 and s-Xbp1 to spliced Xbp1. **(B)** A549 cells infected with ZIKV at indicated MOI for 48h were lysed in buffer A, total protein extracts, under reducing conditions were immuno-blotted for E-ZIKV with EDIII antibody, GRP78 and β-tubulin as a loading control. **(C)** A549 cells ($1x10^6$ cells) were transfected with the indicated reporter constructs. After 12h, transfected cells were plated at a density of $3x10^4$ cells per well and infected with ZIKV at MOI of 5. Luciferase activities were measured 24h post infection. For all experiments Thapsigargin-ER stress induced (TG) were used as positive control at 1 μM for 6h. RT-PCR and western blot experiments were representative of three independent experiments.

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3.2.2 Effect of ZIKV infection on ATF6 branch of UPR

The third branch of UPR is mediated by maturation of ATF6 upon S1P or S2P processing in Golgi apparatus. Once translocated to the nucleus, mature ATF6 upregulates the expression of chaperone encoding genes like GRP78 (Bip) and GRP94. Quantitative increase of these chaperones, involved in the refolding of proteins critically contributes to ER stress resolution. Unlike what was obtained upon thapsigargin treatment of A549 cells, RT-PCR results revealed that grp78 was not upregulated at the transcriptional level during ZIKV infection (Figure 2A). This observation is new and quite important as it could be a specificity being driven by ZIKV. Indeed, up to now, studies performed on the UPR branches implemented upon flaviviruses infections, like for DENV, do not report any inability of the infected cells to induce GRP78 in this A549 model [26].

We further investigated this lack of GRP78 upregulation by two approaches. We first checked the GRP78 protein levels by western blot in cells that were infected 48h by increased amount of ZIKV (MOI of 1, 5 and 10). Whatever the ZIKV-MOI condition and the related amount of viral protein produced, no expression of GRP78 could be detected (Figure 2B). We can notice that we have not been able to detect the protein GRP78 in the mock infected as in infected cells while a signal is obtained for grp78 mRNA in RT PCR. We assume that the sensitivity of the immuno-detection with the antibody used (which reveals the presence of GRP78 in TG treated cells) is not sufficient when low levels of protein expression are present.

In a second approach, we monitored ATF6 activity using luciferase reporter constructs. We transfected A549 cells with plasmids encoding a F-Luc reporter gene downstream of GRP78 or GRP94 promoter. These two promoters contain the ER stress response element (ERSE) which is transactivated by ATF6. The UPR inducer thapsigargin induced an expression of the reporter gene under control of ERSE. This was not the case after ZIKV infection (Figure 2C). All together these results suggest that ZIKV infection provides a partial transduction of the ER stress with activation of the PERK/ATF4 and IRE1 pathways of the UPR but without any involvement of the ATF6 branch. The absence of re-folding chaperone induction can have a main consequence, a failure of the adaptive response. This could lead to persistent ER stress or an interplay with programmed cell death by signaling pathway downstream of PERK activation with CHOP regulation of proteins involved in apoptosis like BIM, Bcl-2 and PUMA [27].

3.3 Crosstalk between UPR and ZIKV

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Given the implementation of UPR without ATF6 branch activation during ZIKV infection (Figure 2) associated with the accumulation of non-native disulfide bonds oligomers of E-ZIKV (Figure 1B, 1C) and considering that ZIKV can delay apoptosis [18,28,29], we hypothesized that ZIKV infection can lead to a persistent and unresolved ER stress. In light of this, we investigated the crosstalk between ZIKV and UPR by testing the effects of an exogenous induction of UPR on ZIKV infection.

3.3.1 UPR Affects ZIKV infection

ER stress can be induced by the action of tunicamicyn (Tm), an inhibitor of N-glycosylation. The resulting UPR can be qualified as 'standard' with activation of the PERK, IRE1 and ATF6 pathways [30]. In order to see the effect of exogenous activation of UPR on ZIKV infection, we added this inducer on infected cells, 2h after ZIKV inoculation. Cells were harvested and cell culture supernatant collected after 16h of treatment with Tm. When we looked at the viral protein expression in cells, as an indicator of viral replication efficiency, we observed a dose dependent reduction of E-ZIKV following Tm treatment (Figure 3A). When we looked at the viral progeny production in cell supernatants, we found that this production was completely abrogated even at low-dose of Tm (Figure 3B). Using thapsigargin (TG), an ER stress and UPR inducer by alteration of Ca^{2+} homeostasis, for a similar 16 h treatment, 2h after ZIKV inoculation, we observed the same inhibition of ZIKV infection (data not shown). These observations suggest that a 'standard' UPR has the ability to interfere with ZIKV replication efficiency, independently of the nature of the UPR inducer.

3.3.2 ZIKV Infection Modulates the UPR in A549 cells

As we found that ZIKV infection in A549 cells led to ER stress and UPR but with an impaired ATF6 branch with no GRP78 induction, we hypothesized that ZIKV could hijack this UPR branch and could take advantage of an unresolved ER stress. To test this hypothesis, we treated ZIKV infected cells with TG for 4h and analyzed GRP78 expression by WB (Figure 3C). As mentioned above, we observed a reduction in E-ZIKV expression, even with a shorter treatment with the UPR inducer (4h of TG instead of 16h of Tm treatment in figure 3A). We also confirmed that ZIKV infection was not able to upregulate GRP78 at the protein level. Furthermore, when we compared the TG treatment alone and in the presence of ZIKV, we found that the virus decreased the level of GRP78 protein induced by TG. All together these data favor our hypothesis that UPR affects

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ZIKV replication, but that the virus has acquired the ability to block partially this response. The interference with UPR can lead to unresolved and persistent ER stress.

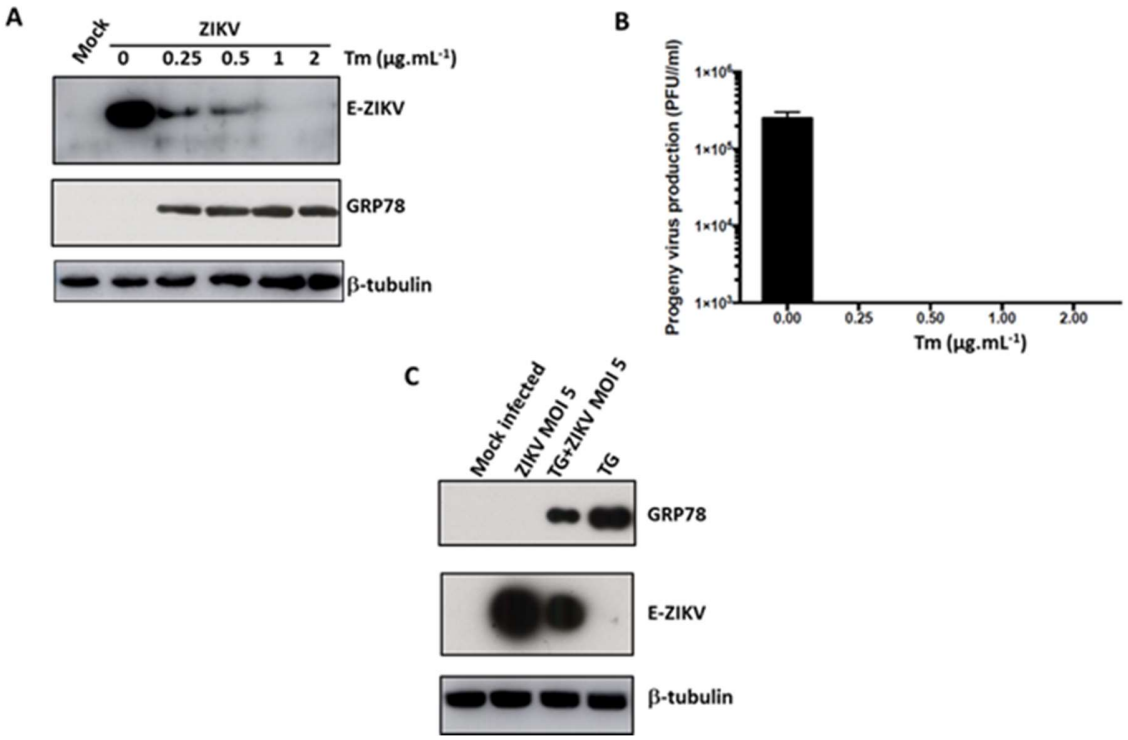

Figure 3. Crosstalk between UPR and ZIKV during infection. (A) A549 cells were infected with ZIKV at MOI of 5 for 18h. 2h post-infection tunicamycin (Tm) was added at the indicated concentration. Total protein extract under reducing conditions were immuno-blotted for E-ZIKV with EDIII antibody, GRP78 and β-tubulin as a loading control. **(B)** Viral progeny production in cell culture supernatants of cells infected and treated with Tm were determined by PFU assay on Vero cells. **(C)** A549 cells were infected or not with ZIKV for 16h at MOI of 5, followed by 4h treatment with TG at 1µM. Western blot were performed on total extract as in **(A)**. All western blot experiments were representative of three independent experiments.

4. Conclusion

Our study showed that ZIKV infection of epithelial A549 cells could trigger an ER stress through an accumulation of disulfide-bonded oligomers of viral proteins. We confirmed the activation of an UPR in this cell model, however, our observations suggest that ZIKV exhibits some

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unconventional specificities in terms of ER stress signaling, compared to other flaviviruses [31]. In addition, we found that an UPR can affect ZIKV efficiency during its infectious process, but that ZIKV has the ability to hijack the ATF6 branch of UPR. Therefore, we hypothesize that a mis-resolved and persistent ER stress is implemented during ZIKV infection and will favor the virus multiplication.

As ER dysfunctions combined with aggregated protein accumulation are responsible for neuronal degeneration in numerous human diseases, especially the ones related to conformational diseases [32,33], we guess that a virally orchestrated persistent ER stress with aggregated proteins accumulated in infected cells may contribute to the congenital complications observed during Zika epidemics, such as microcephaly. Indeed, maintaining a long lasting ER stress can have deleterious effects in the short and medium terms of pregnancy and *post-partum* period on neuronal cell sensitivity to aggregated proteins. This hypothesis could explain the occurrence of microcephaly in new-born babies several months after birth and mother's infection [34]. Further research in this area is needed to state this hypothesis.

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Conflicts of Interest: The authors declare no conflict of interest.

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