Coxsackievirus B Tailors the Unfolded Protein Response to Favour Viral Amplification in Pancreatic β Cells

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Abstract

Type 1 diabetes (T1D) is an autoimmune disease characterized by islet inflammation and progressive pancreatic β cell destruction. The disease is triggered by a combination of genetic and environmental factors, but the mechanisms leading to the triggering of early innate and late adaptive immunity and consequent progressive pancreatic β cell death remain unclear. The insulin-producing β cells are active secretory cells and are thus particularly sensitive to endoplasmic reticulum (ER) stress. ER stress plays an important role in the pathologic pathway leading to autoimmunity, islet inflammation, and β cell death. We show here that group B coxsackievirus (CVB) infection, a putative causative factor for T1D, induces a partial ER stress in rat and human β cells. The activation of the PERK/ATF4/CHOP branch is blunted while the IRE1α branch leads to increased spliced XBP1 expression and c-Jun N-terminal kinase (JNK) activation. Inter-estingly, JNK1 activation is essential for CVB amplification in both human and rat β cells. Furthermore, a chemically in-duced ER stress preceding viral infection increases viral rep-lication, in a process dependent on IRE1α activation. Our findings show that CVB tailors the unfolded protein response in β cells to support their replication, preferentially trigger-ing the pro-viral IRE1α/XBP1s/JNK1 pathway while blocking the pro-apoptotic PERK/ATF4/CHOP pathway.

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Introduction

Viruses are intracellular micro-organisms that depend on the host cell to accomplish their life cycle. Viruses have co-evolved with their host and adapted to their cellular responses. Specifically, they hijack cellular machinery for their own benefit, circumvent the anti-viral cellular re-sponses, and prevent early cell death to allow completion of the viral life cycle [1]. One of the cellular mechanisms affected by viral infection is the unfolded protein re-

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sponse (UPR). The UPR is activated in response to the endoplasmic reticulum (ER) stress caused by strong viral protein synthesis, alterations in the ER membranes, and imbalances in ER calcium concentrations [2]. The UPR aims to restore protein folding homeostasis by: (1) decreasing total protein translation and consequently the load of proteins entering the ER; (2) increasing the expression of chaperones to restore protein folding and maturation; (3) inducing the degradation of terminally misfolded proteins by the ER-associated degradation (ERAD) complex [3]. If these processes are severe and/or persistent, the UPR leads to the up-regulation of inflammation and innate immunity [3], and apoptosis of the stressed cell. The UPR is transduced via 3 main pathways triggered by transmembrane ER proteins: (1) inositol requiring ER-to-nucleus signal kinase 1α (IRE1α), that possesses both kinase and endonuclease activities; (2) activating transcription factor (ATF) 6; and (3) double-stranded RNA-activated kinase (PKR)-like ER kinase (PERK) [3] (Fig. 1a). The IRE1α endonuclease domain catalyses the splicing of the bZIP transcription factor, XBP1, generating its active form, XBP1-spliced (XBP1s). XBP1s regulates the expression of chaperones involved in protein-folding and also that of proteins regulating the ERAD [4]. The kinase domain of IRE1α can phosphorylate and activate c-Jun N-terminal kinase (JNK), contributing to late apoptosis [5]. Activated ATF6 translocates to the Golgi where it is cleaved, releasing a transcription factor involved in the regulation of genes encoding ER chaperones [6]. The kinase PERK phosphorylates eIF2α, promoting global protein synthesis inhibition, but, paradoxically, stimulating the translation of specific proteins such as ATF4 [7]. ATF4 regulates the expression of genes involved in the protection against oxidative stress, but it can also induce the expression of the pro-apoptotic genes CCAAT/enhancer-binding protein homologous protein (CHOP) and ATF3.

Type 1 diabetes (T1D) is an autoimmune disease characterized by the progressive destruction of insulin-producing pancreatic β cells by auto-reactive cytotoxic T cells and locally released pro-inflammatory cytokines [8]. Epidemiological and research findings indicate an association between enterovirus infection and the onset of autoimmunity against β cells in T1D [9]. *IFIH1* encodes melanoma differentiation-associated protein 5 (MDA5), a sensor of double-stranded RNA that initiates the innate immune response against infections by RNA viruses. Polymorphisms in *IFIH1* are associated with T1D. [10, 11]. Enterovirus, especially coxsackievirus B (CVB) [12], have a tropism for pancreatic β cells, and persistent CVB infection of β cells is detected in 60–70% of pancreases from T1D individuals compared to only 6% in non-diabetic individuals [13]. Coxsackievirus can persist in the murine pancreas by deletion of the 5′ genomic sequence [14], but whether these deleted forms of viral genomes are present in the pancreas of T1D patients has still to be demonstrated. The exact mechanisms leading to enterovirus-mediated β cell dysfunction and death and to autoimmunity are still to be clarified.

ER stress plays an important role in the pathologic pathway leading to autoimmunity, islet inflammation, and β cell death [15]. We report here that CVB infections induce ER stress in both rat and human β cells, activating both the IRE1α and PERK pathways. IRE1α-mediated activation of JNK1 is essential for CVB replication, while PERK pathway activation fails to induce the expected up-regulation of ATF4, ATF3, and CHOP, and the consequent death of β cells. These observations suggest that CVB tailors the UPR in the β cells to favour its own replication while preventing premature cell death.

Fig. 1. Coxsackievirus infection of pancreatic β cells triggers the unfolded protein response (UPR) and induces apoptosis. **a** Cellular pathways of the UPR. The accumulation of unfolded proteins in the ER leads to the activation of 3 transmembrane proteins: IRE1α, ATF6, and PERK. In response to ER stress, 1-ATF6 undergoes conformational change leading to its release from the ER and cleavage in the Golgi. The cytosolic domain of ATF6 is a transcription factor controlling the expression of genes encoding chaperones such as BiP; 2-PERK oligomerizes and autophosphorylates. Activation of PERK induces eIF2α phosphorylation, leading to a decrease in global protein synthesis and the accumulation of the transcription factor ATF4 which regulates the expression of ATF3 and CHOP. 3-IRE1α oligomerizes and autophosphorylates and its RNase domain becomes active and targets XBP1 mRNA, leading to the expression of the spliced form of XBP1 (XBP1s). XBP1s is a transcription factor controlling the expression of genes involved in protein folding and degradation. **b–k** INS-1E cells were infected or not with CVB5 at MOI 1 (**b**), MOI 5 (**c–k**), or MOI 20 (**g**), or treated with CPA (**g**) and followed for different time points. **b** CVB5-induced apoptosis was evaluated using nuclear dyes (*n* = 3–4). **c–k** The expression of the viral capsid protein (VP1), the ER stress pathway proteins (PERK/eIF2α/ATF4/ATF3/CHOP, BiP, and IRE1α/XBP1s) and JNK activation (p-JNK) were evaluated by Western blot at different time points and quantified by densitometry (*n* = 3–4). * $p < 0.05$, ** $p < 0.01$ vs. time 0 h (uninfected), ANOVA. Data are mean ± SEM.

(For figure see next page.)

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Material and Methods

Cells Culture

The rat pancreatic β cell line, INS-1E (a kind gift from Dr. C. Wollheim, Centre Médical Universitaire, Geneva, Switzerland) was cultured in medium containing RPMI 1640 GlutaMAX-I, 5% heat-inactivated FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 10 mM HEPES, 1 mM Na-pyruvate and 50 μM 2-mer-

captoethanol [16]. The experiments were performed in INS-1E cells (passages 60–72) after 48–72 h of plating.

The human pancreatic β cell line, EndoC-βH1 (kindly provided by Dr. R. Scharfmann, Centre de Recherche de l'Institut du Cerveau et de la Moelle Épinière, Paris, France), was cultured in plates pre-coated with Matrigel-fibronectin (100 and 2 mg/mL, respectively) in DMEM containing 5.6 mM glucose, 2% fatty acid-free BSA, 50 μM 2-mercaptoethanol, 10 mM nicotinamide, 5.5 μg/mL

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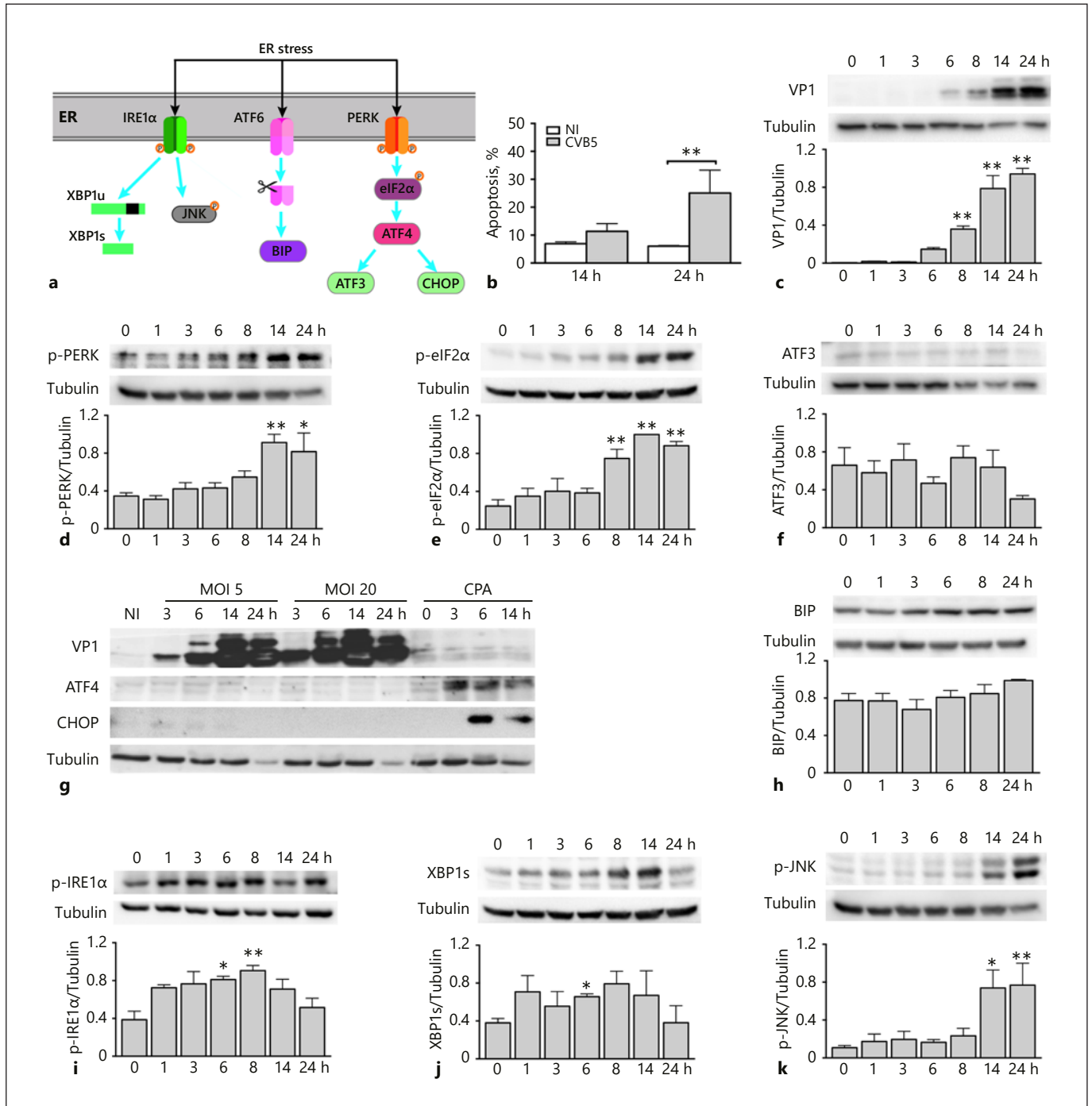
transferrin, 6.7 ng/mL selenite, 100 U/mL penicillin, and 100 μg/mL streptomycin [17]. EndoC-βH1 cells (passages 66–75) were used for the experiments after 72 h of plating as described [18].

Isolation of human islets was performed by collagenase digestion and density-gradient purification [19]. The isolated islets were cultured in M199 medium containing 5.5 mM glucose during shipment to Brussels. Upon arrival (1–5 days after isolation), the islets were left to recover overnight. The next day, they were dispersed and cultured in Ham's F-10 medium containing 6.1 mM glucose, 2 mM GlutaMAX, 50 mM 3-isobutyl-1-methylxanthine, 1% fatty acid-free BSA, 10% FBS, 50 mg/mL streptomycin, and 50 U/mL penicillin. The proportion of β cells in each preparation was evaluated by immunocytochemistry for insulin [20]. During the transfection of small interfering (si)RNAs, cells were cultured in the usual medium except that the antibiotics and BSA were removed.

Viral Infection

The prototype strains of enterovirus (CVB5/Faulkner; CBV4/ JVB, CVB1/ Conn-5) were obtained from ATCC. These viruses were passaged in GMK cells. The identity of the enterovirus was confirmed by a plaque neutralization assay with type-specific anti-sera.

Cells were infected with the indicated multiplicity of infection (MOI) of the virus preparation in medium without FBS. After adsorption for 2 h at 37°C, the inoculum virus was removed, and the cells were washed twice with medium. Culture medium containing FBS was then added to the plates and the virus was allowed to replicate for the indicated time periods. Viability assays were performed 24 h post-infection in CVB4- and CVB5-infected β cells at a MOI of 1 in INS-1E and a MOI of 5 in EndoC-βH1 cells, aiming to reach a cytopathogenic effect of around 30–50%. The viral strain CVB1 is particularly cytopathogenic to the human cell line EndoC-βH1 (100% mortality at MOI 1 24 h post-infection, data not shown), so the MOI 0.1 was used to reach 50% cell viability. CVB1 was not used in INS-1E because it was previously demonstrated that this serotype does not replicate in rat cells [21].

Viral Titration

Cells infected with coxsackievirus were frozen in their medium and then submitted to 3 cycles of thaw and freeze for viral release. Total infectivity was evaluated using end-point dilutions in microwell cultures of GMK cells. Cytopathic effects were read on day 6 by microscopy, and 50% tissue infection dose titers were calculated using the Kärber formula [22]. The described relative viral titers are the ratio between the viral titers obtained in the treated condition and those obtained under the control condition.

RNA Interference

The siRNAs used were: Allstars Negative Control siRNA (siCTL, Qiagen, Venlo, The Netherlands), and rat JNK1 (siJNK1) [23], rat JNK2 (siJNK2) [24], and rat IRE1α (siIRE1α), all from Invitrogen, Carlsbad, CA, USA [25]. The protocol for siRNA transfection was previously validated using a FITC-coupled siRNA (siGLO Green Transfection Indicator, Thermo Scientific, Chicago, IL, USA). Functional studies demonstrated that control siRNA does not affect gene expression or insulin release [26]. In brief, the transfection was performed overnight with 30 nM of siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions [26]. After a recovery period of 48–72 h, the cells were infected with different coxsackieviruses (CVB4 and CVB5), as described above.

Cell Treatment and JNK Inhibition

The JNK inhibitors SP600125 (Sigma-Aldrich) and JNK inhibitor V (Calbiochem, Darmstadt, Germany) were prepared in DMSO and used at concentrations of 10 and 5 μM, respectively. We have previously shown that this concentration induces specific JNK inhibition [27]. Since the JNK activation was not observed early during viral infection, SP600125, JNK inhibitor V, or DMSO alone (control) was added to the cell medium at the moment of the viral infection and maintained until the end of the experiment.

The chemical chaperone TUDCA (Calbiochem) was dissolved in water and used at a final concentration of 125 μM, based on previous dose-response experiments [18]. The cells were pre-treated for 24 h with TUDCA, and the drug was maintained during the entire treatment.

Tunicamycin (Sigma-Aldrich) was prepared in DMSO and used at the indicated concentrations. Cells were pre-treated with tunicamycin or DMSO alone (control) for 8 h before CVB infection, but the drug was not maintained after infection. In initial experiments, we performed dose-response studies to choose a concentration of tunicamycin that induces ER stress markers with minimal toxicity. Based on the dose-response assay presented in online supplementary S1 Figure (for all online suppl. material, see www.karger.com/doi/10.1159/000496034), 0.1 and 0.25 μg/mL tunicamycin induced the ER stress markers BiP and XBP1s, with no increase in β cell death. We thus selected 0.1 and 0.25 μg/mL tunicamycin for the subsequent experiments.

Cells were exposed to the pro-inflammatory cytokines IL-1β (10 U/mL) and IFN-γ (100 U/mL) (R&D Systems, Abingdon, UK) in order to evaluate JNK activation. Cyclopiazonic acid (12.5 μM) or thapsigargin (1 μM) (Sigma-Aldrich) was used as a positive control for UPR markers [18].

Assessment of Cell Viability

The cells were stained with the DNA-binding dyes propidium iodide (PI), 5 μg/mL, and Hoechst 33342 (HO), 5 μg/mL, (Sigma). The number of viable, apoptotic, and necrotic cells was determined by 2 independent observers, one of them unaware of sample identity. The agreement among observers counting was >90%. In each experimental condition, at least 600 cells were counted. Results are presented as percentage of apoptosis, calculated as number of apoptotic cells/total number of cells ×100, or as percentage of total cell death, calculated as number of apoptotic plus necrotic cells/total number of cells ×100. This method has been validated for use in β cells by systematic comparison with different techniques including electron microscopy, cleavage of the effector caspase 3, and DNA laddering [27, 28]. In selected experiments, apoptosis was confirmed by activation (cleavage) of caspase 3 by Western blot.

mRNA Extraction and Real-Time PCR

mRNA was extracted from cells with the Dynabeads mRNA DIRECT™ kit (Invitrogen Dynal, Oslo, Norway). The material obtained was then reverse-transcribed to generate its complementary (c)DNA [28]. cDNA was amplified by a real-time PCR reaction [28], using SYBR Green fluorescence on a LightCycler instrument (Roche, Mannheim, Germany) and correlated to a standard curve [29]. Gene expression was corrected for the housekeeping gene, GAPDH. The values are normalized by the highest value of each experiment considered as 1. GAPDH expression is not modified by viral infection of β cells [30]. The sequences of the primers for VP1 and GAPDH were described in [30].
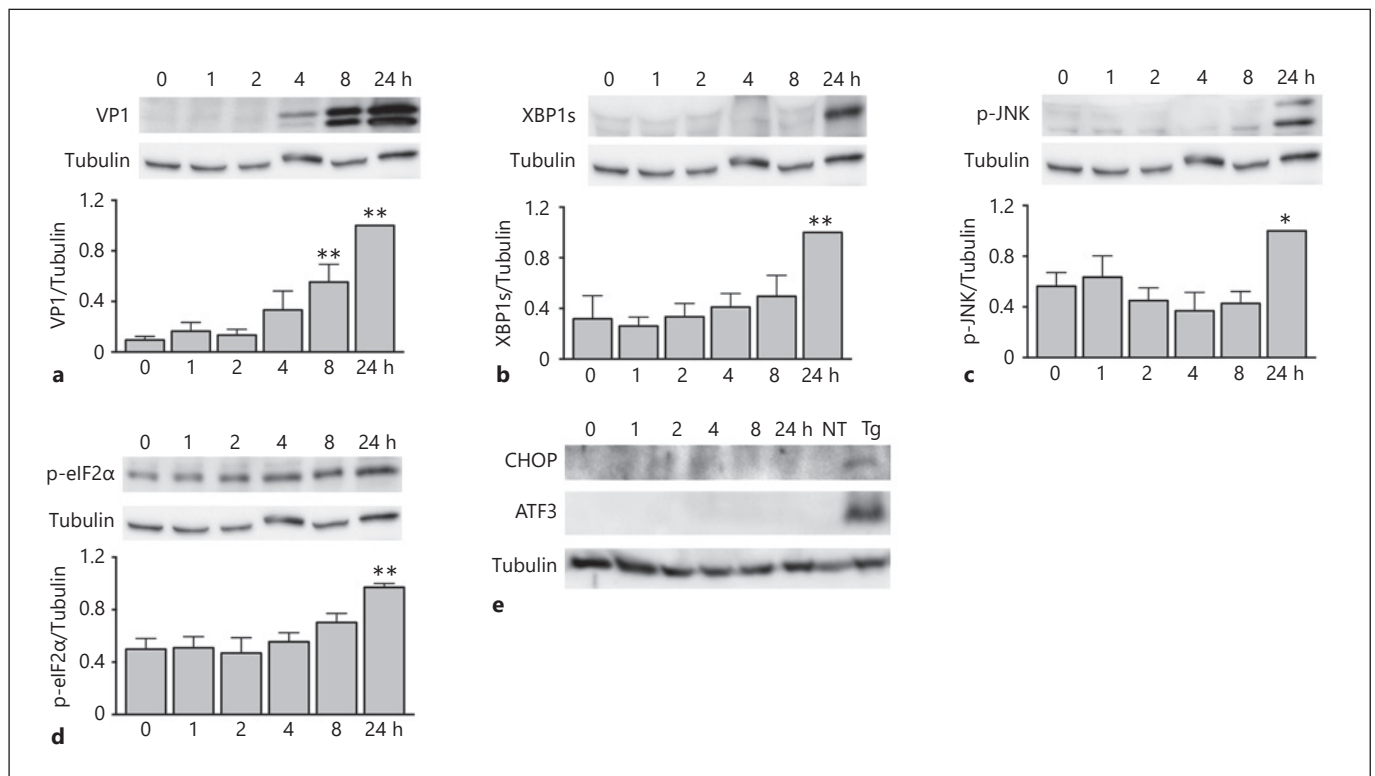
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Fig. 2. Infection of the human pancreatic β cell line EndoC-βH1 with CVB5 activates the IRE1α pathway. **a–e** EndoC-βH1 cells were infected with CVB5 at MOI 5 or treated with thapsigargin (**e**) and the protein expression of the viral capsid protein (VP1), ER stress markers (p-eIF2α, XBP1s, ATF3, and CHOP) and JNK phos-phorylation (p-JNK) were evaluated by Western blot at different time points and quantified by densitometry ($n = 3–4$). * $p < 0.05$, ** $p < 0.01$ vs. time 0 h (uninfected), ANOVA. Data are mean ± SEM.

Western Blot Analysis

For protein evaluation, the cells were washed once with cold PBS, and directly lysed with either Laemmli buffer (25 mM Tris-HCl pH 6.8, 10% glycerol, 1% SDS, 350 mM 2-mercaptoethanol, 175 mM dithiothreitol, and 0.005% bromophenol blue) or Phospho lysis buffer (1% NP40, 150 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$; 50 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM PMSF, and protease inhibitor cocktail [Roche]). Equal amounts of proteins were resolved by 6–12% SDS-PAGE gel and then transferred to a nitrocellulose membrane. Immunoblot analysis was performed with antibodies against ATF4, CHOP, XBP1 (Santa Cruz Biotechnology, CA, USA), p-IRE1α (Ser724) (Novus Biologicals, Minneapolis, MN, USA), BIP, phospho-JNK, total JNK, JNK1, JNK2, cleaved caspase 3, phospho-eIF2α, phospho-PERK (Cell Signaling, Danvers, MA, USA), enterovirus-specific polyclonal rabbit antiserum (KTL-510) [31], GAPDH (Trevigen, MD, USA) or α-tubulin (Sigma, Bornem, Belgium). Horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG were used as secondary antibodies (Lucron Bioproducts, De Pinte; Belgium). Immunoreactive bands were detected by a Bio-Rad chemi DocTM XRS+ (Bio-Rad Laboratories) using the SuperSignal West Femto chemiluminescent substrate (Thermo Scientific). Densitometric analysis of the bands was quantified using ImageLab software (Bio-Rad Laboratories). Protein expression was corrected by the housekeeping proteins α-tubulin or GAPDH and normalized by the highest value of each experiment considered as 1. *N* represents the number of independent experiments performed.

Statistical Analysis

Data are presented as mean ± SEM. Comparisons were performed by two-tailed paired Student's *t* test or ANOVA followed by Bonferroni correction as adequate using Prism software v6.0. A *p* value <0.05 was considered as statistically significant.

Results

Coxsackievirus Infection Induces Apoptosis and Activates a Partial UPR in Pancreatic β Cells

Infection of the rat insulin-producing INS-1E cells with CVB5 [32] promoted β cell apoptosis (Fig. 1b). In parallel, there was a progressive increase in the expression of the major capsid viral protein 1 (VP1) (Fig. 1c). Infection of INS-1E cells with CVB5 induced a >2-fold increase in PERK activation by 14 and 24 h (Fig. 1d), leading

to the phosphorylation of the PERK target, eIF2α (Fig. 1e). Unexpectedly, there was no induction of ATF4, ATF3, or CHOP, 3 classical ER stress proteins activated downstream of PERK/eIF2α (Fig. 1f, g). On the other hand, the chemical ER stressor cyclopiazonic acid (CPA), used as positive control, up-regulated the expression of both ATF4 and CHOP (Fig. 1g). In line with these findings, there were no significant changes in BiP (a downstream protein activated by ATF6) expression after the viral infection of β cells (Fig. 1h). On the other hand, IRE1α phosphorylation increased following viral infection, peaking at 8 h and decreasing after 14 h (Fig. 1i). Viral infection led also to a parallel increase of the spliced form of XBP1 (XBP1s), indicating activation of the endoribonuclease activity of IRE1α (Fig. 1j), and JNK phosphorylation, with a peak 6-fold activation at 14 h (Fig. 1k). The induction of c-jun mRNA, a transcription factor induced downstream of JNK activation [33], was detected by qRT-PCR, confirming the functional impact of JNK induction (online suppl. S2 Fig.). PKR is another virus-activated kinase that activates eIF2α. We next silenced PKR and PERK, using previously validated siRNAs (siPKR, [12] and siPERK, [34]), and evaluated the phosphorylation of eIF2α in CVB5-infected INS-1E cells. Transfection of INS-1E cells with siPERK decreased by >50% PERK protein expression when compared to a control siRNA (siCTL) (online suppl. S3 Fig. A), while siPKR inhibited (by 80%) the induction of PKR in CVB5-infected INS-1E (online suppl. S3 Fig. B). The silencing of PERK or PKR reduced CVB5-induced eIF2α phosphorylation by 30 and 50%, respectively (online suppl. S3 Fig. C), indicating that both kinases contribute to the activation of eIF2α during CVB5 infection. Similar to INS-1E cells, CVB5 infection of human insulin-producing EndoC-βH1 cells (Fig. 2a) induced splicing of XBP1 (Fig. 2b), JNK activation (Fig. 2c), and eIF2α phosphorylation (Fig. 2d), but no induction of the downstream proteins CHOP and ATF3 (Fig. 2e). The same unfolded protein response profile is observed in EndoC-βH1 infected by 2 other CVB serotypes associated with T1D development, namely CVB4 and CVB1 (online suppl. S4 Fig. A, B).

Taken as a whole, the findings described above in rat and human β cells indicate that CVB5 infection induces a partial ER stress response, selectively inducing IRE1α/ XBP1 signalling.

JNK Activation during Coxsackievirus Infection of Pancreatic β Cells Is IRE1α-Dependent

Different viruses, including enterovirus, activate JNK [35], but the mechanisms regulating this process are not well understood. To evaluate whether coxsackievirus-induced phosphorylation of JNK is secondary to IRE1α activation, we inhibited IRE1α expression using a previously validated siRNA against IRE1α (siIRE1α) [25]. Transfection of INS-1E cells with siIRE1α decreased IRE1α protein expression by >80% when compared to a control siRNA (siCTL) (Fig. 3a). Transfection of siCTL or siIRE1α does not affect INS-1E viability [25]. Silencing of IRE1α prevented JNK phosphorylation following viral infection of INS-1E cells (Fig. 3b). In line with these results, the chemical chaperone tauroursodeoxycholic acid (TUDCA), which alleviates ER stress [36], decreased CVB5-mediated JNK activation in β cells (Fig. 3c). Since viruses modulate their environment to favour amplification, we exposed cells to a low dose of the ER stressor tunicamycin (0.1 or 0.25 μg/mL) to test whether ER stress supports viral growth. Low doses of tunicamicyn induced BiP and XBP1s expression in the absence of augmented cell death (Fig. 3d, e). CVB5 infection of these mildly ER-stressed cells increased cell mortality, viral protein expression, and viral titer when compared to vehicle-treated cells (Fig. 3e–g). Silencing IRE1α abolished the viral-supporting effect of tunicamycin (Fig. 3h), confirming that IRE1α activation potentiates CVB5 amplification.

Inhibition of JNK Phosphorylation Reduces Pancreatic β Cells Apoptosis and Viral Replication

IRE1α promotes JNK activation via its serine/threonine kinase domain [5]. JNK activation contributes to β cell apoptosis induced by pro-inflammatory cytokines [18], free-fatty acids [27], and the mimetic viral double-stranded RNA [37]. Two independent chemical inhibitors of JNK (SP600125 and JNK inhibitor V) were used to study its role on virus-induced β cell apoptosis. Treatment of INS-1E cells with these inhibitors reduced SP600125 and JNK inhibitor V CVB-induced JNK phosphorylation by >60 and 80%, respectively (Fig. 4a). Inhibition of JNK activation promoted a significant decrease in CVB5-mediated β cell apoptosis as evaluated by nuclear dyes (Fig. 4b), and cleavage of the apoptosis effector caspase 3 (Fig. 4c). Similar results were obtained using CVB4, which has been previously identified in the pancreas of recent-onset T1D patients [38] (online suppl. S5 Fig.). JNK regulates viral replication in other cell types [39–41]. To investigate whether this is the case also in β cells, we infected INS-1E cells with CVB5 (Fig. 4) or CVB4 (online suppl. S5 Fig.) in the presence or absence of the JNK chemical inhibitors and then measured viral titers (Fig. 4d; online suppl. S5 Fig. D) and VP1 expression (Fig. 4e; online suppl. S5 Fig. E). Prevention of JNK acti-
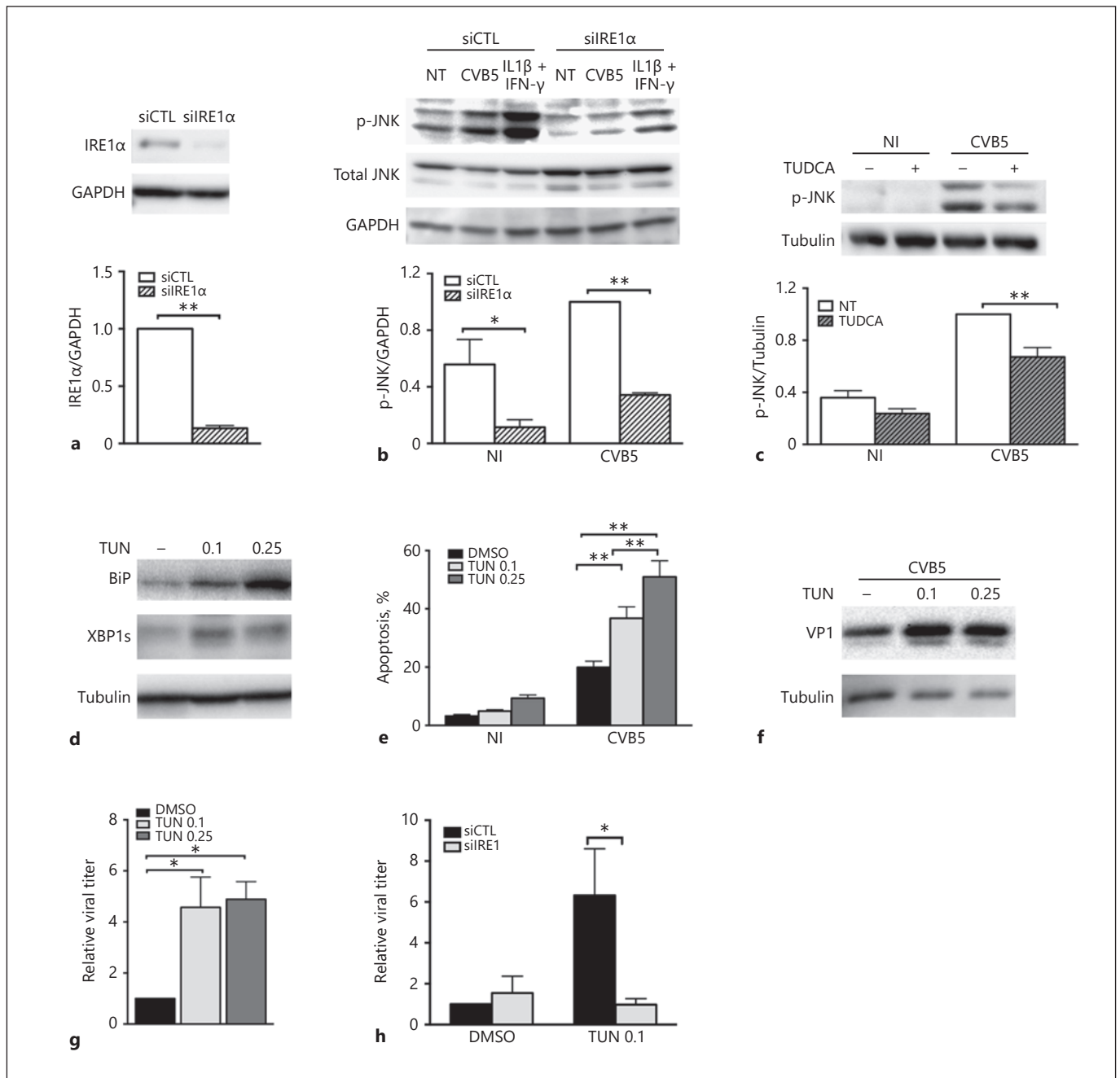
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Fig. 3. Activation of JNK during viral infection of pancreatic β cells is IRE1α-dependent and reduced by the chemical chaperone TUDCA. **a**, **b**, **h** INS-1E cells were transfected with a control siRNA (siCTL) or an siRNA against IRE1α (siIRE1α) for 72 h, and then infected or not with CVB5 at MOI 5 for 14 h. **a** The efficiency of IRE1α knockdown was determined at the protein level ($n = 3$). **b** CVB5-induced activation of JNK was evaluated in the presence or not of IRE1α silencing. The pro-inflammatory cytokines (IL-1β and IFN-γ) were used as positive control for IRE1α-dependent JNK activation ($n = 3$). **c** EndoC-βH1 cells were left untreated or pre-treated with TUDCA (125 μM) for 24 h. Cells were then infected or not with CVB5, alone or in combination with TUDCA. Phosphorylation of JNK was determined after 24 h and quantified by densitometry ($n = 4$). **d** Expression of the BiP and XBP1s proteins was evaluated by Western blot after 8 h of tunicamycin (TUN) treatment. Representative of 3 independent experiments. **e–h** INS-1E cells were exposed to different doses of tunicamycin for 8 h and then infected or not with CVB5 for 14 h. **e** Apoptosis was evaluated using DNA binding dyes ($n = 4$). **f** Viral protein expression (VP1) was evaluated by Western blot. Representative of 3 independent experiments. **g**, **h** Viral amplification is presented as the relative viral titer (described in Methods) ($n = 3–5$). * $p < 0.05$, ** $p < 0.01$ vs. the indicated conditions, t test (**a**), ANOVA (**b–h**). Data are mean ± SEM.
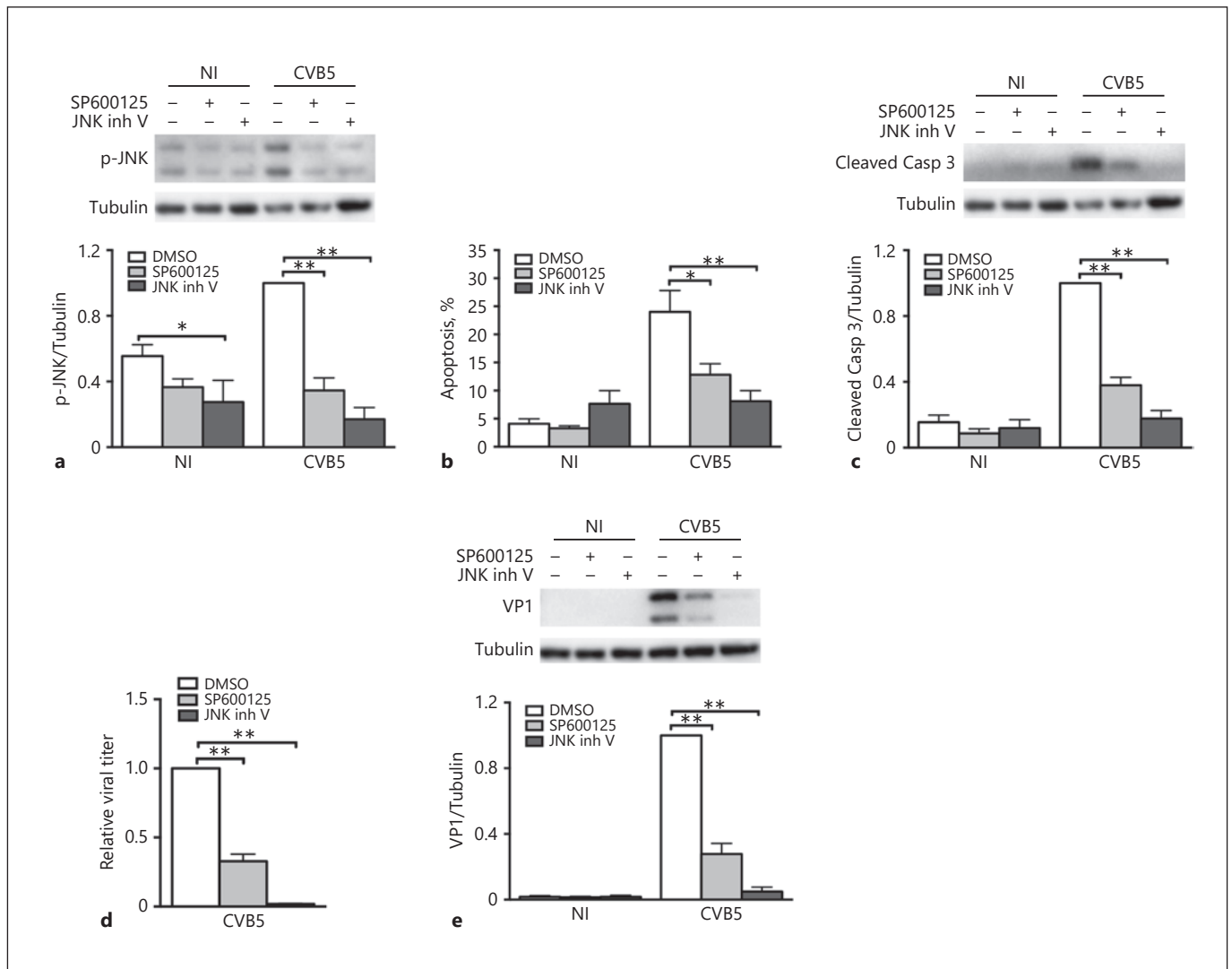
Fig. 4. JNK inhibition reduces CVB5-induced β cell apoptosis and viral replication in INS-1E cells. **a–e** INS-1E cells were infected with CVB5 at MOI 1 for 24 h in the presence or absence of the chemical JNK inhibitors SP600125 (10 μM) or JNK inhibitor V (JNK inh V; 5 μM). **a** Phosphorylation of JNK was determined by Western blot and quantified by densitometry (n = 3–4). **b, c** Apoptosis was evaluated using nuclear dyes and cleavage of caspase 3 (Casp 3), was evaluated by Western blot (n = 3–4). **d** Viral replication was quantified by viral titration (described in Methods) (n = 2–4). **e** VP1 expression was determined by Western blot and quantified by densitometry (n = 3). * $p < 0.05$, ** $p < 0.01$ versus the indicated conditions, ANOVA. Data are mean ± SEM.

vation by SP600125 or JNK inhibitor V decreased the viral titers of both viruses by >60 and >90%, respectively (Fig. 4d; online suppl. S5 Fig. D), which is in line with their potency as JNK inhibitors (Fig. 4a; online suppl. S5 Fig. A). JNK inhibition also reduced viral protein expression (Fig. 4e; online suppl. S5 Fig. E). A decrease in virus-induced apoptosis (Fig. 4b, c; online suppl. S5 Fig. B, C) may decrease viral spread and, consequently, the viral progeny. To evaluate if this explained the findings described above, we measured the VP1 expression in the presence or absence of JNK inhibition ahead of the start of apoptosis (at 8 h). JNK inhibitors reduced VP1 expression at 6 h and particularly at 8 h, suggesting that JNK affects viral replication independently of cell death (online suppl. S6 Fig.).

Enteroviruses are human pathogens and there are species-specific responses to viruses [42, 43]. We thus studied the role of JNK phosphorylation on the outcome of viral infection of the human β cell line EndoC-βH1 (Fig. 5; online suppl. S7 Fig.) and of human pancreatic islets
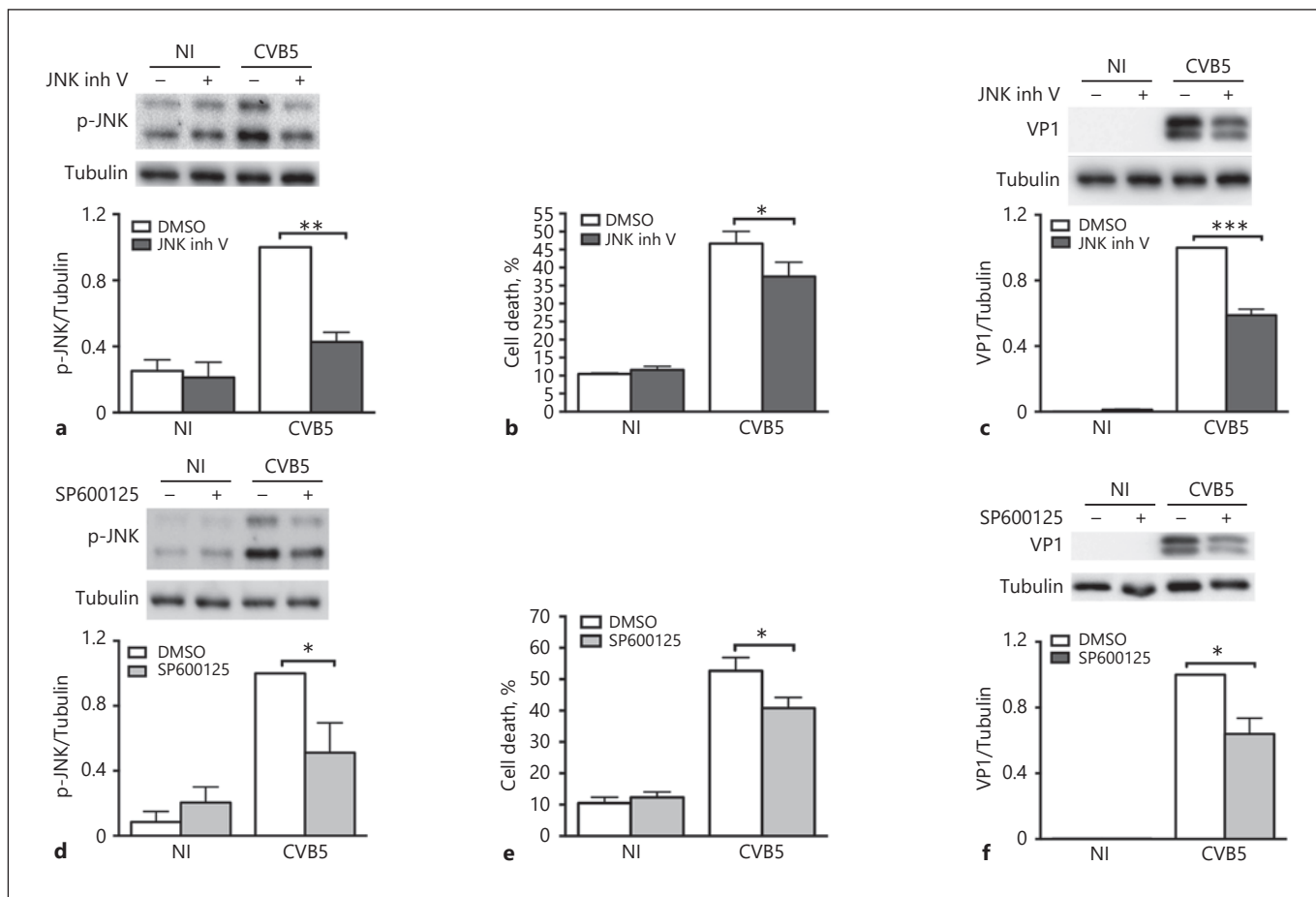
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Fig. 5. Prevention of JNK phosphorylation regulates virus-induced cell death and viral protein expression in the human β cell line, EndoC-βH1. **a–f** EndoC-βH1 cells were infected or not with CVB5 at MOI 5, alone or in combination with the JNK chemical inhibitors SP600125 (10 μM) or JNK inhibitor V (JNK inh V; 5 μM), for 24 h. **a**, **d** JNK activation was determined by Western blot ($n = 3$). **b**, **e** Cell viability was quantified using nuclear dyes ($n = 4$). **c**, **f** VP1 expression was evaluated by Western blot ($n = 3–4$). * $p < 0.05$, ** $p < 0.01$ versus the indicated conditions, ANOVA. Data are mean ± SEM.

(Fig. 6). In agreement with the findings observed in rat INS-1E cells, inhibition of JNK phosphorylation reduced CVB5- and CVB4-induced cell death in both human EndoC-βH1 cells (Fig. 5b, e; online suppl. S7 Fig. B, E) and primary human islets (Fig. 6a). Furthermore, the JNK inhibitor V decreased (by >60%) the viral titers in human islets infected with CVB5 or CVB4 (Fig. 6b). This was paralleled by a significant reduction in viral protein expression in CVB5 and CVB4-infected EndoC-βH1 cells (Fig. 5c, f; online suppl. S7 Fig. C, F) and human islets (Fig. 6c, d). Consistently, CVB1 expression is also decreased when EndoC-βH1 cells are infected in the presence of JNK inhibitors (online suppl. S8 Fig.). These results indicate that CVB5, CVB4, and CVB1 amplification require JNK activation.

JNK1, but Not JNK2, Regulates Coxsackievirus Replication and Pancreatic β Cell Apoptosis

JNK has 2 main isoforms, JNK1 and JNK2 [44]. JNK1 is the main contributor to cytokine-induced β cell apoptosis [45]. To clarify the role of these isoforms on viral replication, we used previously validated siRNAs targeting JNK1 or JNK2 [24, 45]. These siRNAs knocked down JNK1 and JNK2 protein expression by 55 and 65%, respectively (Fig. 7a, e). As observed using chemical JNK inhibitors (Fig. 4b, c; 5b, e; 6a), silencing JNK1 reduced β cell apoptosis after CVB5 infection (Fig. 7b). In addition, JNK1 reduced viral replication, as determined by the quantification of viral titers, and the expression of viral mRNA (data not shown) and protein capsid (Fig. 7c, d). On the other hand, inhibition of JNK2 sensitized pancreatic β cells to
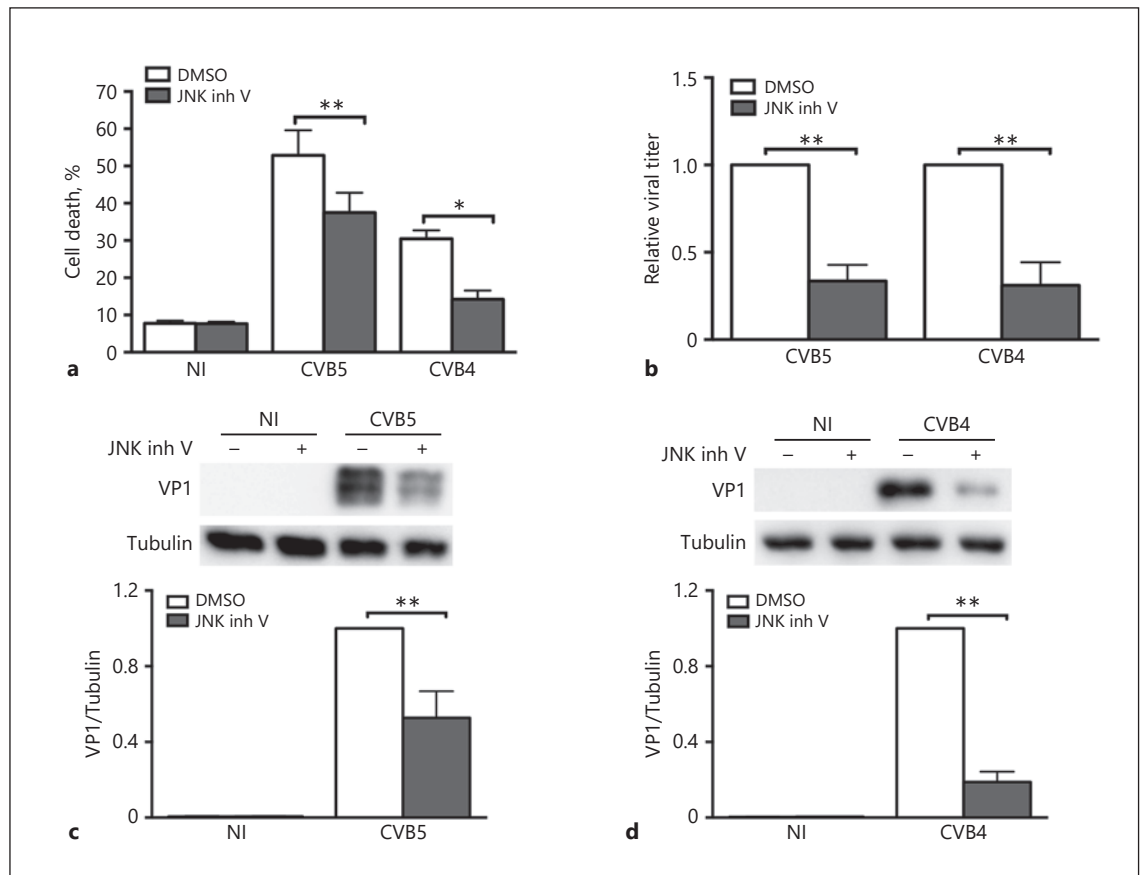
Fig. 6. JNK activation is required for CVB replication and cell death following infection of pancreatic human islets. **a–d** Dispersed pancreatic human islets were infected with CVB5 or CVB4 at MOI 5 for 24 h, in the presence or not of JNK inhibitor V (JNK inh V; 5 μM). **a** Cell viability was quantified using nuclear dyes ($n = 5$). **b** Viral titers were determined in the presence or absence of JNK inhibitor V ($n = 4$). **c, d** VP1 expression was evaluated by Western blot ($n = 3$–4). * $p < 0.05$, ** $p < 0.01$ versus the indicated conditions, ANOVA. Data are mean ± SEM.

virus-induced apoptosis (Fig. 7f) without modifying the viral protein expression or viral titer (Fig. 7g, h). These data indicate that JNK1 is the main isoform regulating the apoptosis and replication of CVB5 in β cells.

Discussion

We have shown that coxsackievirus infection of pancreatic β cells triggers a partial ER stress response. Thus, while the IRE1α/XBP1s branch of the UPR is fully activated, the PERK/eIF2α and the ATF6/BiP signalling pathways are prematurely arrested. The kinase domain of IRE1α induces the activation of JNK, which regulates β cell apoptosis and virus replication in both rat and human β cells. JNK1 is the main isoform regulating β cell death and viral replication, whereas JNK2 has predominantly

anti-apoptotic effects and does not modify the extent of viral replication.

Pancreatic β cells have developed a fine-tuned UPR to cope with the high demand of insulin synthesis, that may reach 50% of total protein synthesis during stimulation [46]. Many viral infections induce ER stress, but the profile of UPR activation depends on both virus and cell type [47, 48]. We show here that the infection of β cells by coxsackievirus promotes a partial ER stress characterized by an early phosphorylation of the kinase PERK and its downstream target eIF2α, leading to a decrease in protein translation. Picornaviruses, such as CVB, use internal ribosome entry sites (IRES) to initiate their translation. This translation requires canonical translation initiation factors, including eIF2α, for the viral replication [49]. Interestingly, poliovirus (PV), another member of the Picornaviridae family, switches translation from an eIF2α-dependent to an inde-
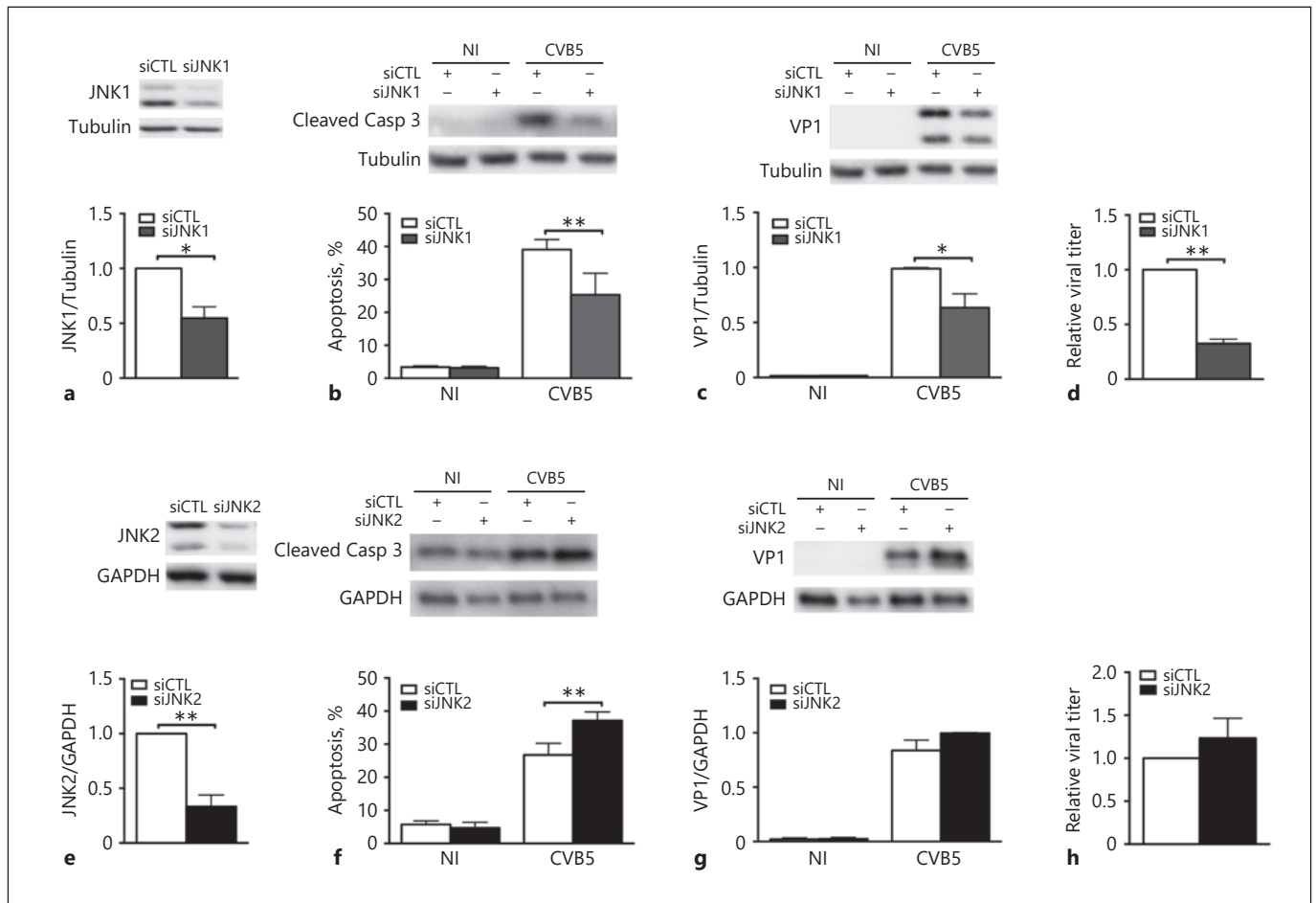
Fig. 7. JNK1 is the main isoform regulating CVB-induced apoptosis and viral replication in pancreatic β cells. **a–h** INS-1E cells were transfected with siCTL or specific siRNAs against JNK1 (**a–d**) or JNK2 (**e–h**), and, after 48 h of recovery, infected or not with CVB5 at MOI 1 for 24 h. **a, e** JNK1 and JNK2 knockdown was confirmed by Western blot ($n = 4–5$). **b, f** Cell viability was measured using nuclear dyes ($n = 4–8$) and confirmed by the activation of caspase 3 (Casp 3) in the presence or not of JNK1 or of JNK2 silencing (representative data). **c, g** VP1 protein expression was evaluated by Western blot ($n = 4–6$). **d, h** Viral titers were determined after silencing or not of JNK1 (**d**, $n = 5$) or JNK2 (**h**, $n = 3$). * $p < 0.05$, ** $p < 0.01$ vs. the indicated conditions, t test (**a, d, e, h**), ANOVA (**b, c, f, g**). Data are mean ± SEM.

pendent mode during the course of infection [50]. Some of the downstream components of the PERK/eIF2α signalling pathway have deleterious effects for propagation of the virus, as demonstrated during West Nile virus (WNV) infection, where silencing CHOP expression increases the viral titer and protein expression [51]. The pro-apoptotic protein CHOP is an important mediator of cytokine-induced β cell apoptosis [52]. Our findings suggest that CVB modulates the PERK/eIF2α branch of the UPR in β cells to prevent the early induction of CHOP-dependent early apoptosis, which would be detrimental for CVB replication. Similar UPR tailoring has been described during bacterial infection in other cell types, where LPS prevents

CHOP induction via the TLR/TRIF-dependent pathway [53]. To our knowledge, however, this has not been previously described in viral infections. The mechanisms responsible for this finding remain to be clarified.

Taken as a whole, these observations indicate that CVB5 activation of the UPR eventually leads to the activation of the IRE1α/XBP1s pathway while it inhibits the PERK/ATF4/ATF3/CHOP pathway. Other authors reported the induction of CHOP and BiP in CVB3-infected HL-1 (a mouse cardiac muscle cell line) and HeLa cells [47, 48]; however, these ER stress markers were not induced in pancreatic human and rat β cell lines infected with CVB1, CVB4, or CVB5 (present data). These observations suggest

Fig. 8. Proposed model for the role of ER stress in coxsackievirus infection of pancreatic β cells. During viral infection of β cells the IRE1α signaling pathway is activated, resulting in the generation of the active form of XBP1 (XBP1s) and phosphorylation of the 2 main JNK isoforms, JNK1 and JNK2. Activation of JNK1 contributes to viral replication and cell apoptosis, while JNK2 has anti-apoptotic effects that partly counteract the JNK1 pro-apoptotic effects. Activation of the PERK-eIF2α pathway is transitory, probably to delay β cell apoptosis and thus enable sufficient time for viral amplification.
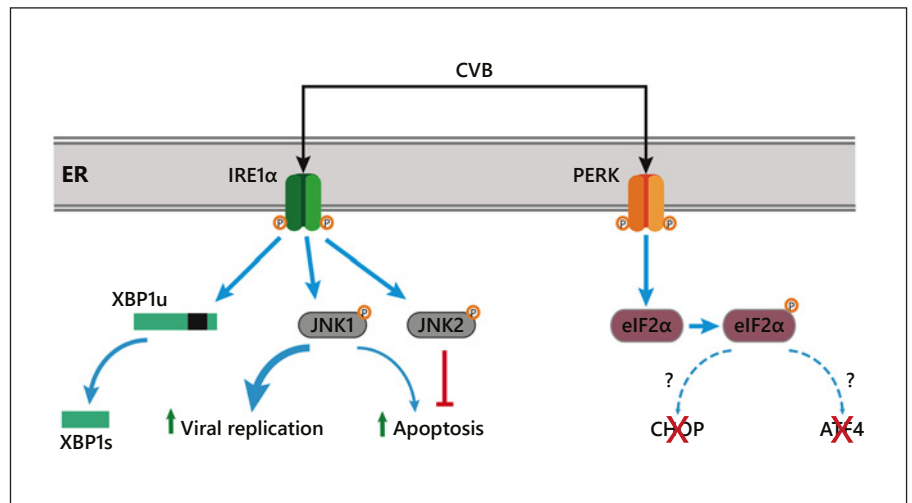
that the particular profile of UPR, i.e., the activation of the pro-viral IRE1α/PJNK pathway and the blocking of the pro-death PERK/PEIF2α/CHOP, is putatively specific to CVB-infected β cells. Interestingly, the activation of the IRE1α pathway plays a key role in the onset of diabetes in a mouse model of T1D, namely the non-obese diabetic (NOD) mouse. NOD mice develop spontaneous autoimmune diabetes, which shares many similarities to T1D [54]. ER stress is detected in the β cells of NOD mice before the onset of T1D [55]; the treatment of NOD mice with the chemical chaperone TUDCA [56] or imatinib, by blunting IRE1α RNAse hyperactivity, [57] decreases the incidence of diabetes in the T1D mouse model. Selectively activating the IRE1α pathway, CVB5 could thus trigger β cell-directed autoimmunity and demise.

The activation of JNK in enterovirus-infected cells has been previously described in other cell types [35, 58] but this was associated with an increase in viral amplification for enterovirus 71 only, by an unknown mechanism [41]. In β cells, CVB5 infection induces a significant increase in JNK activation that contributes to the early stages of the viral life cycle, preceding cell death. In line with this, a mild, non-lethal ER stress in β cells augments CVB5 proliferation, and this phenomenon is abolished by IRE1α silencing (our data). It was previously suggested that tunicamycin favours adenoviral entry and thus potentiates adenovirus infection via decreasing the glycosylation of the coxsackievirus and adenovirus receptor (CAR) [59]. Glycosylation of CAR, however, does not affect CVB entry [60]. Moreover, JNK inhibition did not induce differences in viral protein expression at the early time points of CVB5 infection (online suppl. S6 Fig.), and adding JNK inhibitors after CVB5 binding and cell entry had a similar

inhibitory effect on viral replication as adding them before viral infection (online suppl. S9 Fig.). Taken together, these findings suggest that JNK does not act via facilitation of the viral entrance, and that tunicamycin favours CVB5 replication in β cells by an IRE1α-JNK-dependent mechanism that remains to be identified.

In conclusion, we have shown that potentially diabetogenic viruses, namely CVB1, CVB4, and CVB5, trigger an incomplete UPR response in pancreatic β cells (Fig. 8). In this "partial UPR," the pro-apoptotic PERK pathway is arrested, while the IRE1α pathway is triggered, leading to the activation of kinase JNK1 that is then used by the virus to complete its life cycle. These finding suggest an interesting evolution of the host-virus interaction, where the virus actually takes advantage of a characteristic of pancreatic β cells, namely the active UPR response, to proliferate. This novel role for ER stress and the UPR in β cell permissiveness to infections by coxsackievirus raises two interesting topics for future research. (1) Childhood obesity accelerates the development of T1D. Since obesity (and the consequent insulin resistance and increased circulating free fatty acids) induces ER stress in β cells and contributes to triggering inflammation [3], it will be of great interest to determine whether this also amplifies particular CVB infections in the β cells. (2) Modulators of ER stress are being considered as an early therapeutic approach for T1D [3]. It will become relevant to evaluate whether these approaches will also render β cells more resistant to viral infections. Enterovirus infections are associated with several diseases. Today, no anti-viral drugs have yet been approved for the treatment of enteroviral infections. ER stress inhibitors and peptide JNK inhibitors are being tested in clinical trials in the context of

amyotrophic lateral sclerosis or hearing loss, respectively [61, 62]. Our data, showing the role played by ER stress and JNK activation in CVB amplification, opens the door for the development and testing of new families of molecules for the treatment of enterovirus-associated disease.

Acknowledgement

Statement of Ethics

Human islets were isolated from 6 non-diabetic brain-dead organ donors (online suppl. S1 Table) with approval from the local ethics committee in Pisa, Italy. According to Italian legislation, organ and tissue donation in Italy is regulated by Art. 23 of the National Law No. 91, issued on April 1st, 1999; in Tuscany the regional transplant organization (OTT, Organizzazione Toscana Trapianti: http://www.parlamento.it/parlam/leggi/99091l.htm) allows that organs not suitable for clinical transplantation are used for research purposes. Since the donors were brain-dead, provided informed consent was signed by the next-of-kin. Prof. Marchetti's group had access to donated pancreases for the preparation and study of isolated islets on the basis of approval by their local ethics committee, renewed in 2013. The use of the human islets for our research project was also been approved by the Ethics Committee of the Université Libre de Bruxelles and Erasmus Hospital, Belgium.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

M.L.C., D.L.E. and A.O.d.b. conceived, designed, and supervised the experiments. M.L.C., F.M.P. and A.O.d.b. acquired data. L.M., P.M., and M.R. contributed with material and reagents. M.L.C., D.L.E. and A.O.d.b. wrote the manuscript. All authors revised the manuscript. A.O.d.b. is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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