

C/EBP homologous protein contributes to cytokine-induced pro-inflammatory responses and apoptosis in β -cells

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Induction of the C/EBP homologous protein (CHOP) is considered a key event for endoplasmic reticulum (ER) stress-mediated apoptosis. Type 1 diabetes (T1D) is characterized by an autoimmune destruction of the pancreatic β -cells. Pro-inflammatory cytokines are early mediators of β -cell death in T1D. Cytokines induce ER stress and CHOP overexpression in β -cells, but the role for CHOP overexpression in cytokine-induced β -cell apoptosis remains controversial. We presently observed that CHOP knockdown (KD) prevents cytokine-mediated degradation of the anti-apoptotic proteins B-cell lymphoma 2 (Bcl-2) and myeloid cell leukemia sequence 1 (Mcl-1), thereby decreasing the cleavage of executioner caspases 9 and 3, and apoptosis. Nuclear factor- κ B (NF- κ B) is a crucial transcription factor regulating β -cell apoptosis and inflammation. CHOP KD resulted in reduced cytokine-induced NF- κ B activity and expression of key NF- κ B target genes involved in apoptosis and inflammation, including *iNOS*, *FAS*, *IRF-7*, *IL-15*, *CCL5* and *CXCL10*. This was due to decreased I κ B degradation and p65 translocation to the nucleus. The present data suggest that CHOP has a dual role in promoting β -cell death: (1) CHOP directly contributes to cytokine-induced β -cell apoptosis by promoting cytokine-induced mitochondrial pathways of apoptosis; and (2) by supporting the NF- κ B activation and subsequent cytokine/chemokine expression, CHOP may contribute to apoptosis and the chemo attraction of mononuclear cells to the islets during insulinitis.

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Type 1 diabetes (T1D) is a severe chronic disease resulting from an autoimmune destruction of the pancreatic β -cells. The incidence of T1D has been rising steadily in developed countries from the 1950s to the present day, with the recent, alarming prediction that it will double in children under the age of 5 years by 2020.¹ β -cell loss in T1DM occurs slowly over years and >80% of the β -cell mass is usually lost at the time of diagnosis. Because of the excessive mortality associated with complications of T1D and the increasing incidence of childhood diabetes,² there is an ongoing effort to develop novel strategies for a better treatment and hopefully, prevention of T1D.

In T1D, β -cells cooperate with the immune system to its own destruction by activating pro-apoptotic pathways and secreting chemokines/cytokines that contribute to islet inflammation.³ These responses are mostly triggered via the secretion of the pro-inflammatory cytokines interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) by the infiltrated immune cells. The mechanisms regulating

cytokine-mediated β -cell apoptosis and pro-inflammatory responses are intricate and include, but are not restricted to, the activation of the transcription factors nuclear factor- κ B (NF- κ B) and STAT-1, the c-Jun N-terminal kinases (JNK), endoplasmic reticulum (ER) stress pathways and the intrinsic mitochondrial apoptotic pathways.^{3–7}

NF- κ B activation is due to cytokine-dependent activation of the inhibitor of κ -light polypeptide gene enhancer in B-cells (I κ B) kinase (IKK) complex (IKK α , IKK β and IKK γ), which leads to I κ B phosphorylation, ubiquitination and proteasomal degradation, releasing NF- κ B for migration to the nucleus where it can drive gene expression.⁸ NF- κ B stimulates the expression of several chemokines and cytokines in β -cells, thereby contributing to islet inflammation in T1D.³ Although NF- κ B induces survival pathways in several cells types, it has mostly a pro-apoptotic role in β -cells. The pro-apoptotic effect of NF- κ B in β -cells is, at least partly, due to induction of TNF receptor superfamily member 6 (*FAS*) and the inducible nitric oxide (NO) synthase (*iNOS*). Excessive NO production

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Abbreviations: Bcl-2, B-cell lymphoma 2; BiP, binding immunoglobulin protein, also known as glucose regulated protein 78 (Grp78); CCL5, chemokine (C–C motif) ligand 5, also called RANTES; CHOP, C/EBP homologous protein, also known as GADD153 and DDIT3 (DNA damage inducible transcript 3); CPA, cyclopiazonic acid; CXCL10, chemokine (C–X–C motif) ligand 10, also called IFN-inducible protein 10 (IP-10); eIF2 α , eukaryotic translation initiation factor 2 subunit- α ; ER, endoplasmic reticulum; FAS, TNF receptor superfamily member 6; GADD34, growth arrest and DNA damage 34; GSIS, glucose-stimulated insulin secretion; I κ B, inhibitor of κ -light polypeptide gene enhancer in B cells; IKK, I κ B kinase; IL-1 β , interleukin 1 β ; IL15, interleukin 15; IFN- γ , interferon- γ ; iNOS/NOS2, inducible NO synthase; IRF7, IFN regulatory factor 7; Mcl-1, myeloid cell leukemia sequence 1; NO, nitric oxide; NF- κ B, nuclear factor- κ B; PERK, double-stranded RNA-dependent protein kinase-like ER kinase; TNF- α , tumor necrosis factor- α ; siRNA, small interference RNA

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depletes ER calcium, inducing ER stress and activating the unfolded protein response (UPR) in β -cells.⁹ The UPR involves translational attenuation, increase in the folding capacity of the ER by upregulation of ER chaperones, degradation of misfolded proteins and, in severe cases, apoptosis. The main UPR signaling cascades are initiated by three ER-localized protein sensors: IRE1 α (inositol-requiring 1 α), PERK (double-stranded RNA-dependent protein kinase-like ER kinase) and ATF6 (activating transcription factor 6). PERK phosphorylates the eukaryotic translation initiation factor subunit- α (eIF2 α), resulting in reduction of the general protein synthesis rate while favoring the translation of selective genes, including the transcription factor *ATF4*, which stimulates the expression of the C/EBP homologous protein (*CHOP*). Several studies point to a pro-apoptotic role for CHOP downstream of severe ER stress.^{4,10} Besides its pro-apoptotic role, recent studies also indicated a pro-inflammatory role for CHOP.^{11–13}

It is well established that cytokines induce ER stress in β -cells.⁴ However, the role of ER stress in cytokine-induced β -cell apoptosis remains controversial. We have recently shown that ER stress contributes to β -cell apoptosis via PERK-mediated downregulation of myeloid cell leukemia sequence 1 (Mcl-1), an important anti-apoptotic protein from the B-cell lymphoma 2 (Bcl-2) family that regulates the mitochondrial apoptotic pathway.¹⁴ Some studies suggest that CHOP and binding immunoglobulin protein (BiP) have, respectively, pro- and anti-apoptotic roles in this process, but others failed to find an association between CHOP and cytokine-induced β -cell death.^{15–17} Moreover, the molecular mechanisms underlying ER stress- and CHOP-induced apoptosis are still unclear.^{4,10}

Against this background, the goal of this study was to determine whether CHOP is involved in cytokine-induced apoptosis and inflammation in β -cells, and if so, what are the mechanisms involved. We observed that knocking down CHOP delayed cytokine-induced apoptosis in an insulinoma cell line (INS-1E cells), fluorescence-activated cell sorting (FACS)-purified rat β -cells and human islets. Moreover, CHOP knock-down (KD) resulted in decreased NF- κ B activity due to decreased I κ B degradation and subsequent p65 translocation to the nucleus. This correlated with decreased cytokine-driven induction of key NF- κ B target genes, including *iNOS*, *FAS* and several important chemokines. We further observed that knocking down CHOP prevented cytokine-mediated degradation of the anti-apoptotic proteins Bcl-2 and Mcl-1, thereby decreasing cytochrome *c* release from the mitochondria, subsequent cleavage of executioner caspases 9 and 3, and apoptosis. These observations suggest a role for CHOP in two key mechanisms of β -cell loss in T1D, namely apoptosis and local islet inflammation (insulinitis).

Results

To evaluate the effect of pro-inflammatory cytokines on CHOP expression, time-course experiments of TNF- α + IFN- γ or IL-1 β + IFN- γ treatment were conducted in INS-1E cells (Figure 1a, see Supplementary Figure S1A for quantification). Both TNF- α + IFN- γ or IL-1 β + IFN- γ induced CHOP protein after 8 h of treatment, with a peak around 16–24 h. Similar experiments were performed in isolated human islets, where

CHOP mRNA expression was induced after 24 h and maintained after 48 h treatment (Figure 1b). In contrast with INS-1E cells, TNF- α + IFN- γ induced higher levels of *CHOP* mRNA than IL-1 β + IFN- γ in human islet cells. The same pattern of expression was observed for *BiP* (Supplementary Figure S2A). Moreover, only a combination of TNF- α + IFN- γ induced XBP-1 splicing in human islets (Supplementary Figure S2B).

To elucidate the role of CHOP in cytokine-induced β -cells apoptosis, INS-1E cells were transfected with two small interference RNAs (siRNAs) targeting the rat CHOP. Both siRNAs decreased by about 80% cyclopiazonic acid (CPA)-induced CHOP protein overexpression (Figure 1c, see quantification in Supplementary Figure S1B). We confirmed at the mRNA level that transfection of the CHOPsiRNA#1 prevented both TNF- α + IFN- γ - or IL-1 β + IFN- γ -induced overexpression of *CHOP* (Supplementary Figure S1C) and its target gene *growth arrest and DNA damage 34 (GADD34)* (Supplementary Figure S1D) in INS-1E cells. We next evaluated the effect of CHOP KD on β -cell survival. As compared with non-transfected cells or cells transfected with a control siRNA (siCtrl), transfection with CHOP siRNA#1 or #2 had no effect on basal apoptosis, but it partly prevented apoptosis of INS-1E cells (50% reduction) induced by a 15-h treatment with cytokines or CPA (Figure 1d). This protection, however, was lost in INS-1E cells treated for 24 h with TNF- α + IFN- γ or IL-1 β + IFN- γ (Supplementary Figure S3A). Similar experiments were performed in FACS-purified primary β -cells. Primary cell transfection using CHOP siRNA#1 fully prevented TNF- α + IFN- γ - or IL-1 β + IFN- γ -induced CHOP mRNA expression (Figure 1e). FACS-purified primary β -cells are more resistant to cytokine-induced apoptosis than INS-1E cells.⁹ We tested the effect of CHOP KD on primary β -cells apoptosis after 24 and 48 h of cytokines exposure (IL-1 β + IFN- γ or TNF- α + IFN- γ). The CHOP siRNA#1 fully protected β -cells against apoptosis after 24 h (Figure 1f), but this protection was partial and not significant in β -cells treated for 48 h (Supplementary Figure S3B), indicating that both in INS-1E and primary β -cells, CHOP KD induces a transient protection against cytokine-induced apoptosis. CHOP was also knocked down in dispersed primary human islet cells using two different siRNAs. The percentage of CHOP KD was 68 \pm 12 and 63 \pm 14% for siCHOP#1 and siCHOP#2 (data not shown), respectively. The siCHOP#1 significantly protected human cells against apoptosis induced by a 48-h TNF- α + IFN- γ treatment and there was a tendency toward a protective effect of siCHOP#2 (Figure 1g).

Western blot analyses showed that knocking down CHOP partly prevents cleavage of caspase 3 and 9 induced by a 15-h treatment with IL-1 β + IFN- γ or TNF- α + IFN- γ (Figure 2), confirming the protection against cytokines. Cytokine-induced JNK activity is involved in β -cell apoptosis⁷ and cytokine-induced CHOP expression in β -cells is regulated at least in part by JNK.¹⁸ We performed a time-course analysis of IL-1 β + IFN- γ -induced CHOP in the presence or absence of the chemical JNK inhibitor SP600125.¹⁴ JNK inhibition partly, but significantly, hindered CHOP overexpression after 15 and 24 h of treatment, thus confirming that cytokine-induced CHOP overexpression is partly JNK-dependent (Supplementary Figure S4A). On the other hand, knocking

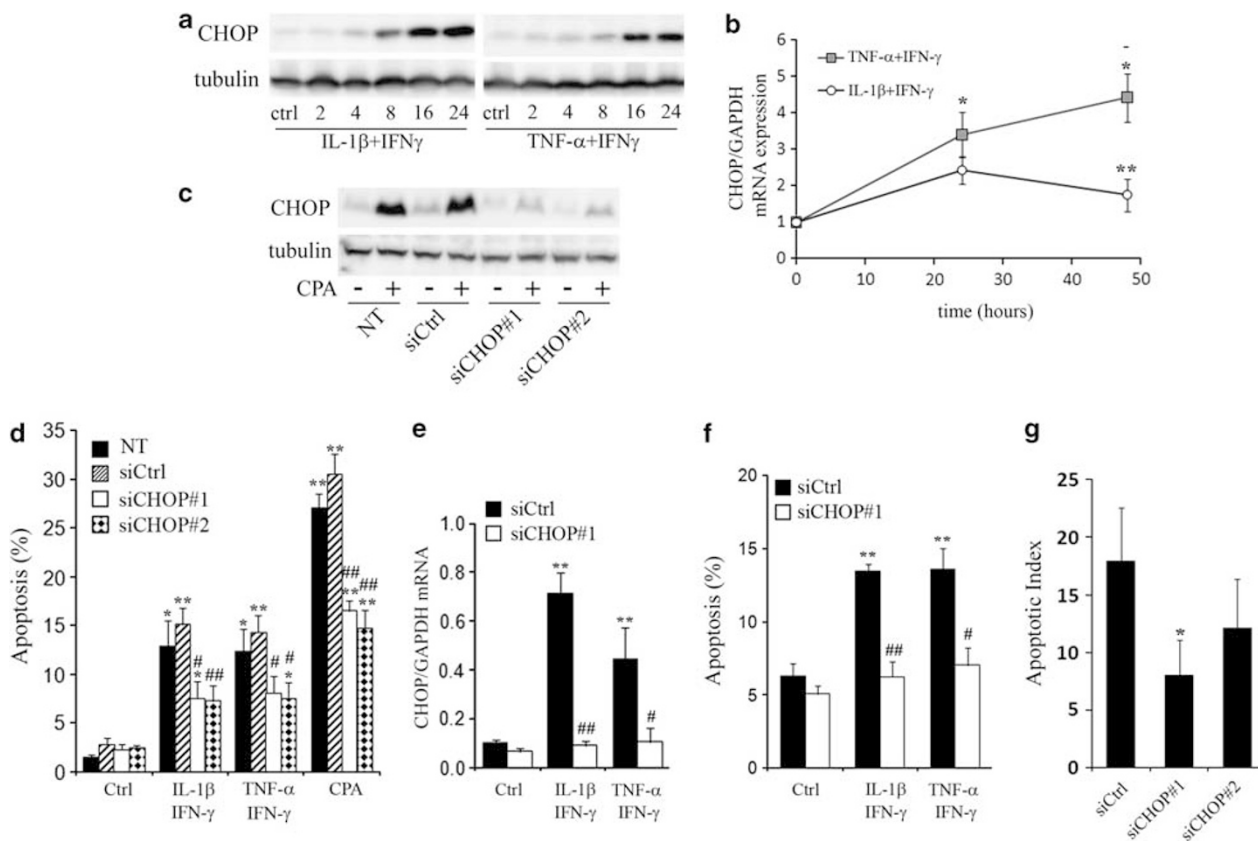


Figure 1 CHOP knockdown protects against cytokine-induced β -cell apoptosis. **(a)** Representative time-course experiment of CHOP protein expression in INS-1E cells treated with IL-1 β + IFN- γ (left panel) or TNF- α + IFN- γ (right panel). **(b)** Time-course analyses of CHOP mRNA expression in human islets exposed to TNF- α + IFN- γ (squares) or IL-1 β + IFN- γ (circles). Data are means \pm S.E.M. of three to five independent experiments. * P < 0.05, ** P < 0.01 versus non-treated condition. **(c)** Representative western blot of CHOP over tubulin expression in INS-1E untransfected (NT) or transfected with a control siRNA (siCtrl), or two different CHOP siRNA (siCHOP#1 and #2) and treated (+) or not (-) for 15 h with CPA. Data are means \pm S.E.M. of four independent experiments. **(d)** INS-1E cells were untransfected (NT, black bars), transfected with siCtrl (stripe bars), siCHOP#1 (white bars), or siCHOP#2 (dotted bars), and treated for 15 h with IL-1 β + IFN- γ , TNF- α + IFN- γ or CPA as indicated. **(e and f)** FACS-purified primary β -cells were transfected with the siCtrl (black bars) or siCHOP#1 (white bars), and treated for 24 h with IL-1 β + IFN- γ or TNF- α + IFN- γ . **(e)** Real-time PCR analyses of CHOP over GAPDH mRNA expression. **(d and f)** Prevalence of apoptosis was evaluated by HO-PI staining. **(d and f)** Data are mean \pm S.E.M. of at least four independent experiments. * P < 0.05, ** P < 0.01 versus respective non-treated condition. # P < 0.05, ## P < 0.01 versus respective untransfected and siCtrl-transfected condition. **(g)** Dispersed human islet cells were transfected with the siCtrl, the human siCHOP#1, or the human siCHOP#2 and treated for 48 h with TNF- α + IFN- γ . Prevalence of apoptosis was evaluated by HO-PI staining and expressed as the apoptotic index. Data are mean \pm S.E.M. of three independent experiments. * P < 0.05 versus respective siCtrl-transfected condition

down CHOP did not modify IL-1 β + IFN- γ -induced P-JNK and total JNK expression (Supplementary Figure S4B).

We next tested the impact of CHOP KD on β -cell function. Knocking down CHOP did not affect basal- (1.67 mM glucose) or glucose-stimulated insulin secretion (GSIS; 16.7 mM) under control conditions. In addition, CHOP KD did not prevent inhibition of the GSIS induced by a 15-h treatment with IL-1 β + IFN- γ (Supplementary Figure S5), indicating dissociation between the anti-apoptotic effects (Figures 1d, f, g) and functional effects of CHOP KD.

The Bcl-2 protein family controls the intrinsic pathway of apoptosis.⁵ The anti-apoptotic proteins Bcl-2 and B-cell lymphoma-extra large (Bcl-XL) have been shown to antagonize β -cell apoptosis^{19–21} and we recently demonstrated that degradation of the anti-apoptotic protein Mcl-1 contributes to cytokine-induced β -cell death.¹⁴ Mcl-1 and Bcl-2 expression were decreased in INS-1E cells after a 15- or 24-h exposure to IL-1 β + IFN- γ (Figure 3). Knocking down CHOP prevented the early (15 h) but not the late (24 h) decrease of

Bcl-2 (Figure 3a) and Mcl-1 (Figure 3b) expression. Similar results were obtained in INS-1E cells treated with TNF- α + IFN- γ (data not shown). Several pro-apoptotic BH3-only proteins, including PUMA,²² DP-5,²³ Bid²⁴ and BIM,²⁵ contribute to cytokine-induced β -cell death. Knocking down CHOP had no effect on the induction of these genes following an 8- or 15-h treatment with IL-1 β + IFN- γ or TNF- α + IFN- γ (Supplementary Figures S6A–F). Knocking down CHOP did not significantly affect the mRNA expression of Bcl-2 and Mcl-1 either (Supplementary Figures S6G and H), suggesting that the effects of CHOP on Bcl-2 and Mcl-1 are not transcriptional. Cytokines decrease Mcl-1 expression through the combined action of translation inhibition (through PERK-mediated eIF2 α phosphorylation) and increased degradation via JNK activation.¹⁴ On the other hand, CHOP exerts a retroactive effect on eIF2 α phosphorylation through induction of the phosphatase GADD34/PP1. We evaluated the impact of CHOP KD on eIF2 α phosphorylation. Surprisingly, knocking down CHOP did not exacerbate cytokine-induced eIF2 α phosphorylation

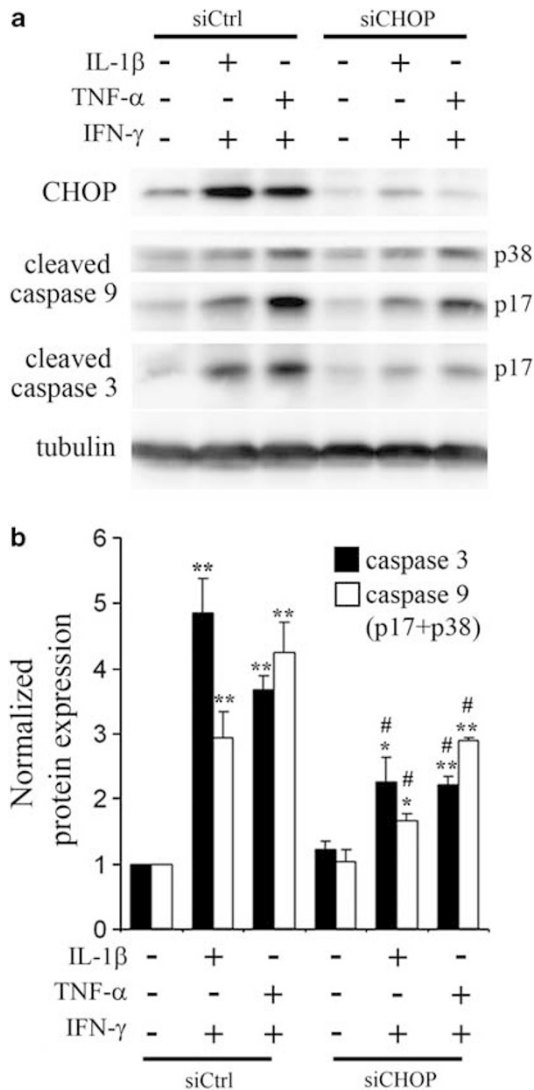


Figure 2 CHOP knockdown protects against cytokine-induced caspase 3 and 9 cleavage. INS-1E cells were transfected with a control siRNA (siCtrl) or siCHOP#1, and treated for 15 h with IL-1 β + IFN- γ or TNF- α + IFN- γ . (a) Representative western blot showing CHOP and the cleaved caspase 3 and 9 fragments normalized to the tubulin levels. (b) Quantitative assessment of four independent blots. * P < 0.05, ** P < 0.01 versus respective non-treated condition. # P < 0.05 versus respective siCtrl-transfected condition

(Supplementary Figure S7). Mcl-1 protein decrease can be prevented upon inhibition of the proteasome.¹⁴ This is also the case for Bcl-2, as Bcl-2 protein levels are restored to control values upon inhibition of proteasomal degradation using MG-132 (1 μ M) in INS-1E cells treated for 15 h with IL-1 β + IFN- γ , TNF- α + IFN- γ , or the chemical ER stressors CPA or thapsigargin (Figure 4a). To assess the contribution of CHOP to Bcl-2 and Mcl-1 protein degradation, time-course experiments of INS-1E cells treated with the translation inhibitor cycloheximide were performed under control condition or in cells treated with IL-1 β + IFN- γ . Bcl-2 is known to have a long half-life (12 h),²⁶ whereas Mcl-1 has a very short half-life (30 min).²⁷ To assess Bcl-2 and Mcl-1 stability, INS-1E cells were exposed to cycloheximide (30 μ M) for 4, 8 or 16 h, or for

20, 40 or 60 min, respectively. Under control condition, Bcl-2 has a half-life of about 12 h (Figure 4b) and Mcl-1 of 40 min (Figure 4c). Knocking down CHOP had no impact on the half-life of Bcl-2 and Mcl-1 in control conditions (data not shown). To assess the impact of cytokines on the stability of the proteins, INS-1E cells were treated for 16 and 6 h to decrease the levels of Bcl-2 and Mcl-1, respectively. Addition of cycloheximide to siCtrl-transfected cells led to a further decrease in the levels of both proteins. On the other hand, knocking down CHOP stabilized Bcl-2 (Figure 4b) and Mcl-1 (Figure 4c) proteins in INS-1E cells exposed to IL-1 β + IFN- γ . Bcl-2 and Mcl-1 are known to be degraded by the proteasome.²⁸ We thus studied the effect of CHOP KD on the activity of the 20S Proteasome, the catalytic core of the ATP-dependent proteolytic complex.²⁹ We observed that an 8- or 15-h cytokine treatment did not modify β -cells proteasomal activity. Knocking down CHOP also had no effect on the global 20S Proteasome activity (Supplementary Figure S8). Bcl-2 and Mcl-1 stability is regulated through poly-ubiquitination.²⁸ We next studied the effects of cytokines and CHOP knock-down on the expression of several E3-ligases involved in I κ B- α , Bcl-2 and Mcl-1 ubiquitination.²⁸ IL-1 β + IFN- γ induced the expression of *FBW7*, *FBW11*, *β -TRC* and *USP9X* at 16 h in INS-1E cells, whereas TNF- α + IFN- γ upregulated only *β -TRC*. On the other hand, none of those genes were regulated by CHOP (Supplementary Table 2).

Recent studies indicate a role for CHOP in inflammation,^{11–13} and we and others demonstrated that inflammation in β -cells largely depends on NF- κ B.³ We thus studied the impact of CHOP in cytokine-induced NF- κ B activity. Cytokines induce an early (30 min) NF- κ B activation that is sustained for 8–12 h and tends to decrease after at 24–48 h in INS-1E cells.³⁰ To study the potential role of CHOP in NF- κ B activity, we use 8 h of cytokine treatment, a time point when NF- κ B activity is still strong and CHOP is highly expressed. As expected, treating INS-1E cells with IL-1 β + IFN- γ or TNF- α + IFN- γ for 8 h strongly induced the activity of a NF- κ B-responsive promoter construct (Figure 5a). Knocking down CHOP had no effect on the basal promoter activity, but decreased by 50% the cytokine-stimulated activity (Figure 5a). Conversely, CHOP overexpression using a plasmid encoding the rat CHOP (Figure 5b) increased both the basal and cytokine-induced NF- κ B activity (Figure 5c). In line with these findings, knocking down CHOP in FACS-purified primary β -cells significantly decreased the 24 h cytokine-induced overexpression of *iNOS* (Figure 5d) and *FAS* (Figure 5e), two important NF- κ B target genes.^{31,32} *iNOS* (Supplementary Figure S9A) and *FAS* (Supplementary Figure S9C) were similarly regulated by CHOP in INS-1E cells after an 8-h treatment with IL-1 β + IFN- γ or TNF- α + IFN- γ . This resulted in a 20% diminution in NO production in those cells (Supplementary Figure S9B).

NF- κ B also controls several chemokine genes involved in inflammation.³ We studied the induction of the NF- κ B-dependent chemokine (C–C motif) ligand 5 (*CCL5*) in primary β -cells treated for 24 h with IL-1 β + IFN- γ or TNF- α + IFN- γ , and observed a 50% decrease in *CCL5* mRNA in CHOP knocked-down cells (Figure 6a). This was also observed in INS-1E cells after an 8-h treatment with cytokines (Supplementary Figure S9D) and resulted in a 50% decrease

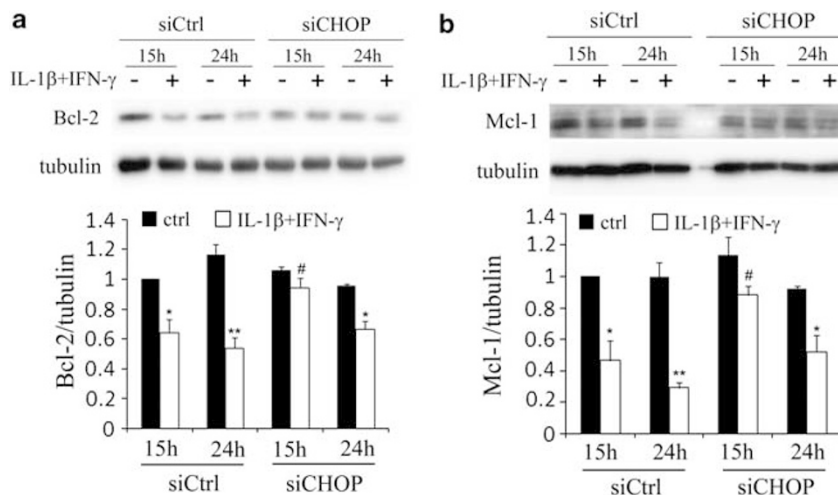


Figure 3 CHOP knockdown transiently protects against cytokine-induced Mcl-1 and Bcl-2 protein downregulation. (**a** and **b**) Western blot analyses of Bcl-2 (**a**) and Mcl-1 (**b**) over tubulin in INS-1E cells transfected with a control siRNA (siCtrl) or siCHOP#1, and treated for 15 or 24 h with IL-1 β + IFN- γ . Upper panels: representative western blot. Lower panels: quantitative assessment of four independent blots. * P < 0.05, ** P < 0.01 versus respective non-treated condition. # P < 0.05 versus respective siCtrl-transfected condition

in CCL5 release as measured by ELISA in response to IL-1 β + IFN- γ (Figure 6b) and TNF- α + IFN- γ (Figure 6c). Of note, the amount of CCL5 released by INS-1E cells treated with TNF- α + IFN- γ was 10-fold higher as compared with cells treated with IL-1 β + IFN- γ . Knocking down CHOP also partly blocked the induction of the chemokines (*C-X-C motif*) ligand 10 (*CXCL10*; Figure 6d) and the cytokine *IL15* (Figure 6e) in primary β -cells in response to TNF- α + IFN- γ , but had no effect on the minor induction obtained in response to IL-1 β + IFN- γ . These chemokines are co-regulated by IFN regulatory factor 7 (*IRF7*),³³ which is mostly a TNF- α -dependent gene. In line with this, knocking down CHOP decreased TNF- α + IFN- γ -induced *IRF-7* mRNA overexpression (Figure 6f).

Both CHOP and NF- κ B are transcription factors, and FAS has been shown to be co-regulated at the promoter level by the transcription factors C/EBP β and p65 (the active subunit of the NF- κ B complex).³¹ Co-immunoprecipitation experiments failed to show a direct CHOP/p65 interaction (Figure 7a). NF- κ B activity is controlled by the I κ B complex. In control condition, p65 is sequestered in the cytosol by the I κ B complex. Upon cytokine treatment, I κ B- α and β are degraded, allowing p65 translocation to the nucleus and induction of NF- κ B target genes. In an attempt to explain the CHOP effect on NF- κ B activity, we studied the impact of CHOP on p65 translocation and the stability of the I κ B complex. Knocking down CHOP attenuated the p65 nuclear translocation induced by an 8-h treatment with IL-1 β + IFN- γ or TNF- α + IFN- γ , and prevented I κ B- α degradation (Figures 7b and c). On the other hand, knocking down CHOP had no effect on IKK- β or I κ B- β degradation upon an 8-h cytokine treatment (Figure 7c).

Discussion

The purpose of this study was to elucidate whether CHOP contributes to cytokine-induced apoptosis, and if so, to determine the mechanism underlying this contribution. We presently demonstrate that CHOP expression is induced by cytokines, partly through JNK activation, in rodent β -cells and

human islets, and that it contributes to the early β -cell apoptosis induced by cytokines. CHOP is involved in cytokine-induced degradation of Bcl-2 and Mcl-1, two major anti-apoptotic proteins involved in the control of the intrinsic mitochondrial pathway of apoptosis. In addition, CHOP does not alter JNK activity, but promotes the activity of NF- κ B, a key transcription factor that controls the expression of several chemokines and cytokines, and has a major role in cytokine-mediated β -cell apoptosis.³ Altogether, these findings indicate that cytokines induce ER stress in rodent and human islets/ β -cells, and that cytokine-induced CHOP aggravates NF- κ B-driven mechanisms of inflammation and apoptosis, and favors the cytokine-induced mitochondrial apoptotic pathway.

The protective effects of knocking down CHOP on β -cell viability were transient, which may explain the conflicting findings in the literature concerning the involvement of CHOP in cytokine-induced apoptosis.^{15–17} The intrinsic mitochondrial pathway of apoptosis is controlled by a delicate equilibrium between anti-apoptotic and pro-apoptotic Bcl-2 proteins.^{5,34} Here we show that knocking down CHOP hinders NF- κ B activity and temporarily stabilize the anti-apoptotic Bcl-2 proteins Mcl-1 and Bcl-2. On the other hand, CHOP does not regulate the expression of several pro-apoptotic BH3-only proteins involved in cytokine-induced β -cell apoptosis, such as PUMA²² DP-5,²³ and BIM.²⁵ We propose that, by promoting Bcl-2 and Mcl-1 degradation, CHOP facilitates the stimulatory effects of BH3-only proteins on Bax translocation from the cytosol to the mitochondria, resulting in mitochondrial outer membrane permeabilization, release of mitochondrial intermembrane space proteins such as cytochrome *c*, recruitment of pro-caspase 9 to form the apoptosome, and finally cleavage of the executioner caspase 3, resulting in apoptosis. Together with studies in other tissues suggesting that CHOP stimulates Bax translocation to the mitochondria,¹⁰ the present data confirm a key role for CHOP in the regulation of the intrinsic pathway of apoptosis and provide new insights on the mechanism underlying CHOP pro-apoptotic action in β -cells (Figure 8).

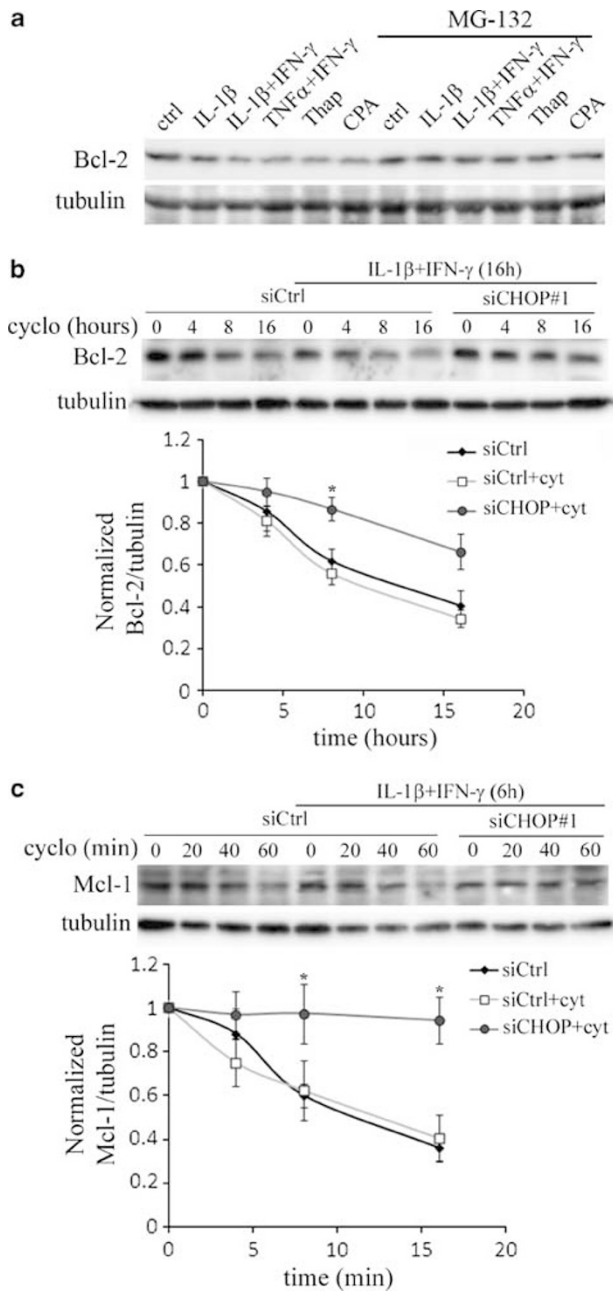


Figure 4 CHOP knockdown stabilizes Bcl-2 and Mcl-1 proteins in INS-1E cells treated with cytokines. (a) Representative western blot of Bcl-2 expression in INS-1E cells exposed for 15 h to cytokines (IL-1 β or TNF- α , \pm IFN- γ , as indicated), CPA or thapsigargin (thap), with or without the proteasome inhibitor MG-132. Figure is representative of four independent experiments. (b) INS-1E cells were transfected with siCtrl or siCHOP#1, and treated or not for 16 h with IL-1 β + IFN- γ . Cells were then further treated or not (time 0) for 4, 8 or 16 h with cycloheximide (30 μ M) to inhibit protein synthesis. Western blot analyses of Bcl-2 over tubulin were performed. Upper panel: representative blot. Lower panel: quantitative assessment of four independent blots. The results were normalized by setting the time zero of each condition as 1. * P < 0.05 versus respective siCtrl-transfected cells treated with cytokines. (c) INS-1E cells were transfected with siCtrl or siCHOP#1, and treated or not for 6 h with IL-1 β + IFN- γ . The cells were then further treated or not (0) for 20, 40 or 60 min with cycloheximide (30 μ M). Western blot analyses of Mcl-1 and tubulin were performed. Upper panel: representative blot. Lower panel: quantitative assessment of four independent blots. The results were normalized by setting the time zero of each condition as 1. * P < 0.05 versus respective siCtrl-transfected cells treated with cytokines

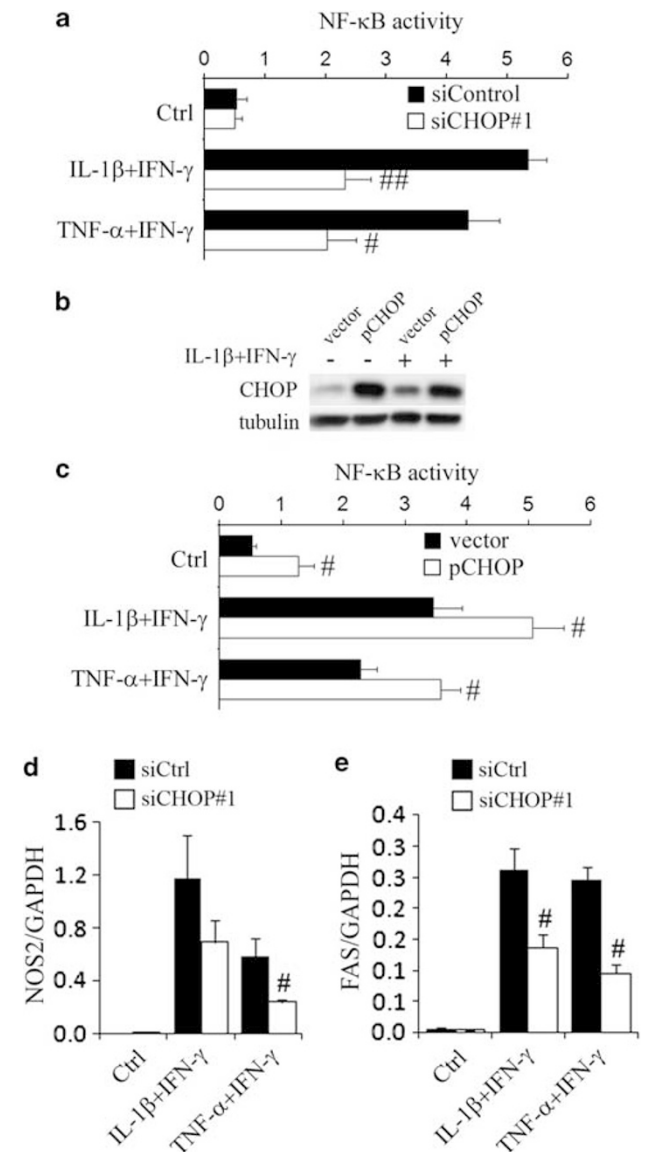


Figure 5 CHOP knockdown decreases cytokine-induced NF- κ B activity and expression of the NF- κ B target genes *iNOS* and *FAS*. (a) INS-1E cells were transfected with a NF- κ B responsive reporter construct and the control reporter plasmid pRLSV40renilla, together with a control siRNA (black bars) or the CHOP siRNA (white bars), and treated for 8 h with IL-1 β + IFN- γ or TNF- α + IFN- γ . Results are mean NF- κ B activities \pm S.E.M. of six independent experiments. (b and c) INS-1E cells were transfected with a NF- κ B responsive reporter construct and the control reporter plasmid pRLSV40renilla, together with an empty plasmid (vector) or a plasmid encoding the rat CHOP (pCHOP). (b) Representative western blot of CHOP over tubulin in cells treated or not for 15 h with IL-1 β + IFN- γ or TNF- α + IFN- γ , as indicated. (c) NF- κ B activity after 8 h of treatment with IL-1 β + IFN- γ or TNF- α + IFN- γ . (d) FACS-purified primary β -cells were transfected with the siCtrl (black bars) or siCHOP#1 (white bars), and treated for 24 h with IL-1 β + IFN- γ or TNF- α + IFN- γ . (e) Real-time PCR analyses of *NOS2* (d) and *FAS* (e) over *GAPDH* mRNA expression. Results are means \pm S.E.M. of four independent experiments. # P < 0.05, ## P < 0.01 versus respective siCtrl-transfected condition

We previously demonstrated that Mcl-1 is targeted for degradation upon ER stress and cytokine treatment,¹⁴ and we presently observed that cytokines also stimulate Bcl-2 degradation by the proteasome. This is consistent with

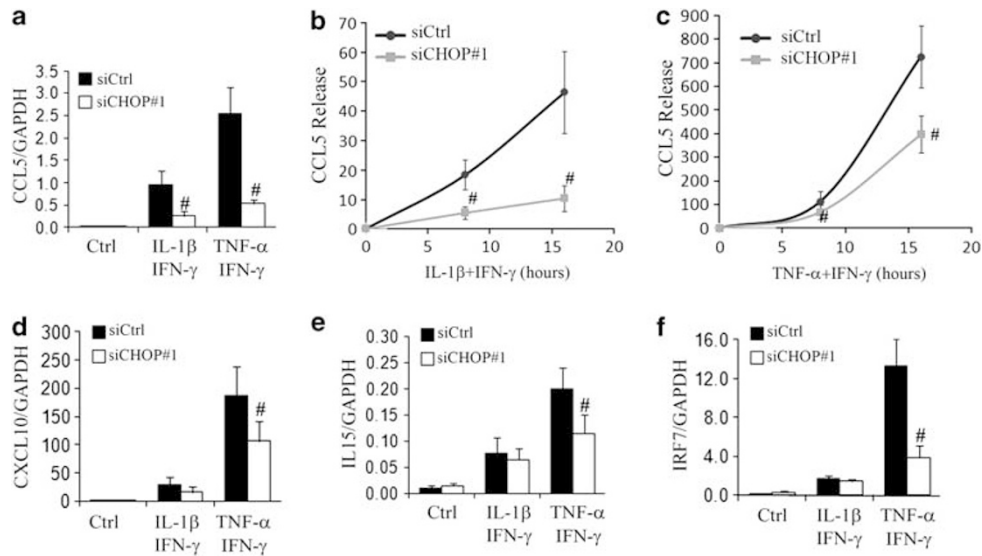


Figure 6 CHOP knockdown mitigates cytokine-induced expression of inflammation markers and release of CCL5. (a and d-f) FACS-purified primary β -cells were transfected with the siCtrl (black bars) or siCHOP#1 (white bars), and treated for 24 h with IL-1 β + IFN- γ or TNF- α + IFN- γ . Real-time PCR analyses of *CCL5* (a), *CXCL10* (d), *IL15* (e) and *IRF7* (f) over *GAPDH* mRNA expression. (b and c) INS-1E cells were transfected with siCtrl (black lines) or siCHOP#1 (gray lines), and treated for 8 or 15 h with IL-1 β + IFN- γ (b) or TNF- α + IFN- γ (c). CCL5 release was evaluated by ELISA of the cell supernatant. All data are means \pm S.E.M. of at least four independent experiments. # $P < 0.05$ versus respective siCtrl-transfected condition

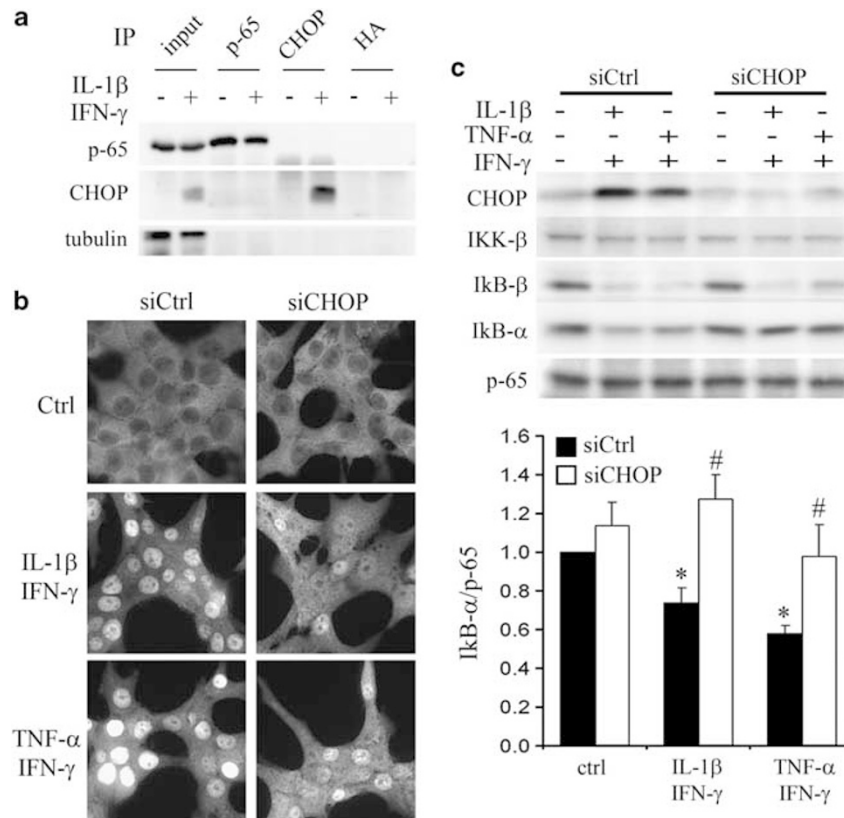


Figure 7 CHOP knockdown inhibits cytokine-induced p65 translocation and I κ B- α degradation. (a and c) INS-1E cells were transfected with siCtrl or siCHOP#1 and treated for 8 h with IL-1 β + IFN- γ or TNF- α + IFN- γ . (a) Representative western blot of p65 and CHOP in total extracts (input) or after co-immunoprecipitation using anti-p65, anti-CHOP or anti-HA antibodies. (b) p65 localization was analyzed by immunofluorescence in INS-1E cells. Data are representative of four independent experiments. (c) Upper panel: representative western blot showing CHOP, IKK- β , I κ B- β and I κ B- α , normalized to the p65 levels. Lower panel: quantitative assessment of I κ B- α over p65 expression of four independent blots. * $P < 0.05$ versus respective non-treated condition. # $P < 0.05$ versus respective siCtrl-transfected condition

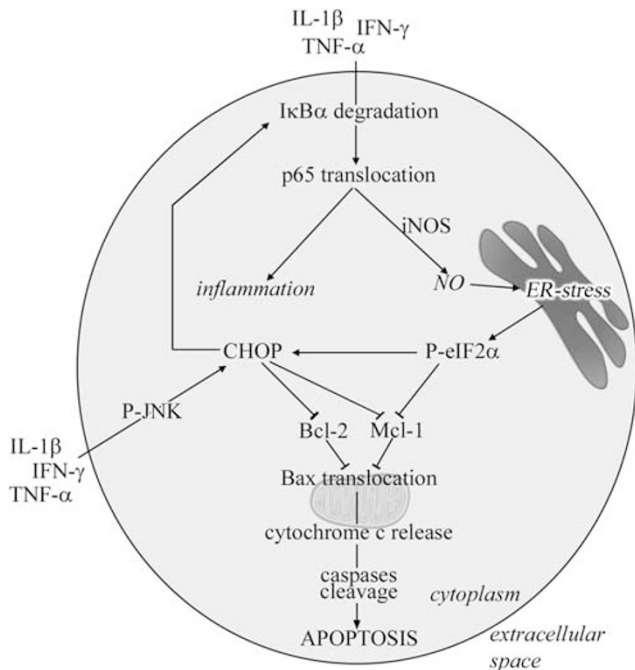


Figure 8 Scheme depicting the role of CHOP cytokine-induced NF- κ B activity and apoptosis. Pro-inflammatory cytokines stimulate NF- κ B activity and JNK phosphorylation, leading to NO production and ER stress, and resulting in eIF2 α phosphorylation and CHOP expression. In turn, CHOP facilitates the degradation of I κ B α , thereby amplifying NF- κ B activity. CHOP also contributes to the degradation of the anti-apoptotic proteins Bcl-2 and Mcl-1, leading to activation of the mitochondrial pathway of apoptosis. Furthermore, the impact of CHOP on NF- κ B activity may contribute to inflammatory processes triggered by cytokines in β -cells, putatively aggravating insulinitis

previous reports showing that Bcl-2 phosphorylation leads to proteosomal degradation, in particular in response to ER stress.^{35,36} We did not observe an effect of CHOP on the proteasome activity, indicating that that CHOP impairs the stability of a selected number of proteins, including Mcl-1 and Bcl-2, through a presently unknown mechanism. E3-ligases target proteins for degradation by the proteasome through ubiquitination.²⁸ Although cytokines modified the expression of some of the E3 ligases involved in Bcl-2 and/or Mcl-1 ubiquitination, CHOP did not significantly modulate these genes. We can't exclude however, that CHOP is regulating these ligases post-transcriptionally. Further studies need to be performed to evaluate the effect of CHOP on the ubiquitination of these proteins.

McCullough *et al.*³⁷ demonstrated that CHOP overexpression in rat fibroblasts decreases Bcl2 mRNA expression through an unknown mechanism. We presently observed that CHOP KD prevents cytokine-induced Bcl-2 protein degradation without a clear effect on Bcl-2 mRNA expression, suggesting that CHOP may control Bcl-2 expression at several levels. eIF2 α phosphorylation and reduced rate of protein translation are considered as protective mechanisms preventing oxidative stress and apoptosis during ER stress.³⁸ Thus, by inducing *GADD34*, CHOP may promote eIF2 α dephosphorylation and sustained protein synthesis, leading to apoptosis.³⁹ In this study, CHOP KD prevented *GADD34* overexpression, but did not decrease eIF2 α phosphorylation. Thus, the protective effect of CHOP KD was not mediated by increased translation

arrest. Besides, it is unlikely that increased translation arrest would be beneficial in our model as we recently showed that eIF2 α phosphorylation rapidly leads to decreased Mcl-1 protein levels, contributing to β -cell apoptosis.¹⁴

Our data suggest that CHOP contributes to β -cell apoptosis via increased cytokine-induced NF- κ B activity, which, by stimulating iNOS expression, mediates NO production and ER stress in β -cells. C/EBP family members may interact with NF- κ B subunits.⁴⁰ The major NF- κ B component induced by cytokines in β -cells is the p65/p65 dimer,³ and it was conceivable that CHOP stimulates NF- κ B activity by interacting with p65. Our data, however, indicate that there is no direct interaction between p65 and CHOP in response to cytokines. We observed that CHOP induces NF- κ B activity by promoting I κ B- α degradation and subsequent p65 translocation to the nucleus. This is in accordance with a recent study documenting that C/EBP β can stimulate p65 translocation and NF- κ B activity by inhibiting LPS-induced I κ B- α overexpression in HeLa cells.⁴¹ I κ B α degradation is mediated via ubiquitination by the E3 ligase β -TRC.²⁸ Here we show that IL-1 β + IFN- γ stimulate β -TRC expression, but that CHOP does not regulate this E3-ligase. On the other hand, our data demonstrate that CHOP contributes to cytokine-induced A20 (*TNFAIP3*) overexpression (data not shown), possibly indirectly as a result of its impact on NF- κ B activity. A20 is an ubiquitin-editing enzyme which has both ubiquitin and deubiquitinase activities.⁴² Further studies are required to verify whether A20 influences the stability of I κ B α , Mcl-1 and/or Bcl-2 proteins. Interestingly, a recent study indicates that IKK may directly phosphorylate and inactivate Bcl-2,⁴³ suggesting a cross talk between the NF- κ B pathway and the Bcl-2 family. Whether or not this cross talk exists in β -cells and is involved in the effects of CHOP on Bcl-2 and Mcl-1 stability also remains to be clarified.

As a consequence of its effect on NF- κ B activity, knocking down CHOP led to decreased induction of several NF- κ B target genes, including *iNOS*, *FAS* and *IRF-7*,^{31,32} and several cytokines/chemokines involved in the inflammatory response observed during insulinitis, such as IL-15, CXCL10 and CCL5.³ TNF- α + IFN- γ induces a higher expression of these pro-inflammatory genes in β -cells than IL-1 β + IFN- γ . This was previously shown to be mediated by TNF- α -induced IRF-7 expression.⁴⁴ As CHOP modulates *IRF-7* expression, the observed effects of CHOP KD on the expression of these chemokines probably occur through the combined decreased activity of NF- κ B and IRF-7. These data strongly suggest that CHOP contributes to inflammation. This is in agreement with several studies on the CHOP KO mice revealing a role for CHOP in *in vivo* models of inflammation.^{11–13} Moreover, CHOP KO mice are protected against diabetes induced by multiple low-dose streptozotocin (MLDSZT).⁴⁵ In contrast, backcrossing non-obese diabetic (NOD) mice with CHOP KO mice did not prevent diabetes, but delayed appearance of autoantibodies.⁴⁶ It is not surprising that deletion of a single pathway of apoptosis fails to protect NOD mice against a massive T-cell-specific attack. The observed protection against MLDSZT⁴⁵ is also in line with our previous report showing that inhibiting NF- κ B activity in β -cells protect mice against MLDSZT-induced diabetes.⁴⁷

In conclusion, our study suggests that cytokines induce ER stress in rodent and human islets/ β -cells, and that CHOP

induction has a dual role in promoting β -cell death in T1D. On one hand, CHOP directly contributes to cytokine-induced β -cell apoptosis by promoting activation of pro-apoptotic NF- κ B-dependent pathways (i.e., NO production) and regulating mitochondrial-mediated apoptosis (i.e., via the degradation of the anti-apoptotic proteins Bcl-2 and Mcl-1). On the other hand, the effect of CHOP on NF- κ B activation and subsequent cytokine/chemokine expression may amplify the chemoattraction of mononuclear cells to the islets during insulinitis aggravating local production of pro-inflammatory cytokines (Figure 8).

Materials and Methods

Materials. The following chemicals were purchased from Sigma-Aldrich NV/SA (Bornem, Belgium) and used as indicated: MG-132 (1 μ M), cyclopiiazonic acid (CPA 20 μ M), thapsigargin (100 nM) and SP600125 (10 μ M) were dissolved in DMSO; cycloheximide (30 μ M) was prepared in absolute ethanol. The following cytokine concentrations were used: recombinant human IL-1 β (R&D systems, Abingdon, UK) at 10 U/ml in INS-1E cells, and 50 U/ml in rat primary β -cells and human islets; recombinant rat IFN- γ (R&D systems) at 100 U/ml (0.0072 μ g/ml) in INS-1E cells and 1000 U/ml (0.036 μ g/ml) in rat primary β -cells;^{9,14} recombinant human IFN- γ (PreproTech, Rocky Hill, NJ, USA) at 1000 U/ml in human islets. Recombinant human TNF- α (R&D systems) at 1000 U/ml in INS-1E cells, rat primary cells and human islets. The selected concentrations of ER stressors and cytokines are based on our previous dose-response studies from our group.⁹

Cell culture. The rat insulinoma cell line INS-1E (kindly provided by Professor Claes Wollheim, CMU, University of Geneva) was maintained in the complete RPMI 1640 medium as previously described.⁹ Male Wistar rats (Charles River Laboratories, Brussels, Belgium) were housed and handled according to the guidelines of the Belgian Regulations for Animal Care; all performed experiments were approved by the local Ethical Committee. Rat islets were isolated by collagenase digestion followed by hand picking under a stereomicroscope. Islets were then dispersed, and primary β -cells were purified by auto-FACS (FACS Aria, BD Bioscience, San Jose, CA, USA).⁴⁸ The preparations used in the present study contained 90.1 \pm 3.8% β -cells ($n = 11$). FACS-purified β -cells were pre-cultured in complete β -cell medium supplemented with 5% heat-inactivated fetal bovine serum (FBS) from 20 to 48 h for recovery. Experiments were then conducted in medium without serum.⁹

Human islets were isolated from six (mRNA experiments) and four (viability experiments) non-diabetic organ donors (age 57 \pm 5 years; BMI 26 \pm 1 kg/m²) in Pisa, Italy, with the approval of the local ethics committee. Islets were isolated by enzymatic digestion and density-gradient purification, and cultured in M199 medium containing 5.5 mmol/l glucose. The human islets were shipped to Brussels within 1–5 days of isolation. After overnight recovery in Ham's F-10 containing 6.1 mmol/l glucose, 10% FBS, 2 mmol/l GlutaMAX, 50 mmol/l 3-isobutyl-1-methylxanthine, 1% BSA, 50 units/ml penicillin and 50 μ g/ml streptomycin, islets were exposed to cytokines in the same medium without FBS for 24–48 h. For viability experiments, islets were dispersed before siRNA transfection. The percentage of β -cells, examined in the dispersed islet preparations by staining with anti-insulin antibody (1 : 1000; Sigma-Aldrich) and donkey anti-mouse IgG rhodamine (1 : 200; Lucron Bioproducts, De Pinte, Belgium), was 53 \pm 6%.

In selected experiments, the cell culture supernatants were collected for determination of nitrite concentration (nitrite is a stable product of NO oxidation) using the Griess method.

Western blot analysis. Cells were washed once with cold PBS and directly lysed with Laemmli buffer. Preparation of nuclear extracts from INS-1E cells were performed as described previously.⁴⁹ Lysates were then resolved by SDS-PAGE and transferred to a PVDF membrane. Immunoblot analyses were performed as previously described^{14,49} using the following antibodies: polyclonal anti-rat Mcl-1 from Biovision (Gentaur, Brussels, Belgium); polyclonal anti-CHOP/GADD153, polyclonal anti-Bax; polyclonal anti-I κ B α or β ; polyclonal anti-p65; polyclonal anti-IKK α from Santa Cruz Biotechnology (Tebu-Bio nv, Boechout, Belgium); polyclonal anti-P-elf2 α , polyclonal anti-cleaved caspase 3 or 9, polyclonal anti-Bcl-2, polyclonal anti P-JNK and total JNK from Cell Signaling (Boston, MA, USA); monoclonal anti- α -tubulin and horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG from Sigma-Aldrich.

RNA interference. The siRNAs used in this study are described in Supplementary Table 1.

The Allstars Negative Control siRNA (Qiagen, Venlo, The Netherlands) has no effect on β -cell gene expression and viability.⁵⁰ The siRNA transfection was conducted according to a protocol developed in our laboratory,⁵⁰ using DharmaFECT 1 or lipofectamin 2000 (Invitrogen, Paisley, UK) with a final concentration of 30 nM siRNA. The efficiency of transfection is > 90%.⁵⁰ siRNA transfection under these conditions do not affect β -cell function.¹⁴ Cells were then cultured for a 48-h recovery period before being collected or treated as indicated.

Assessment of cell viability. The percentage of viable, apoptotic and necrotic cells was determined using the DNA-binding dyes propidium iodide (PI, 5 μ g/ml) and Hoechst 33342 (HO, 5 μ g/ml, Sigma-Aldrich).⁹ The cells were examined by inverted fluorescence microscopy (Axiovert 200, Carl Zeiss, Zaventem, Belgium). A minimum of 500 cells was counted in each experimental condition by two independent observers, one of them unaware of sample identity. In human islets experiments, cell viability was expressed as the apoptotic index calculated as [(% apoptotic cells in experimental conditions – % apoptotic cells in control)/(100–dead cells in control)] \times 100.³⁰

mRNA extraction and quantitative RT-PCR. Poly(A)⁺ mRNA was isolated from INS-1E cells or dispersed islet cells using the Dynabeads mRNA DIRECT kit (Invitrogen) and reverse transcribed as previously described.⁹ Quantitative PCR was performed using the IQ SYBR Green Supermix (BIO-RAD, Nazareth Eke, Belgium), in an IQ5 instrument (BIO-RAD). Expression values were corrected for the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). The presently utilized cytokines or ER stressors do not modify *GAPDH* expression in insulin-producing cells under the present experimental conditions.⁴⁴ The primers used in this study are described in Supplementary Table 1.

Immunofluorescence. INS-1E cells grown on glass culture slides (BD Biosciences Europe, Erembodegem, Belgium) were fixed for 15 min in fresh 4% paraformaldehyde, rinsed in PBS and permeabilized for 5 min in PBS-Triton X-100 0.1%. Slides were then blocked using PBS goat serum 5% and incubated overnight at 4 $^{\circ}$ C in the presence of polyclonal rabbit anti-p65 (Santa Cruz Biotechnology, Tebu-Bio nv). Cells were washed and further exposed for 1 h to Alexa fluor 488-conjugated antibodies (1/1000; NV Invitrogen SA). After washing, slides were mounted and photographed using fluorescence microscopy (Axio Imager, Carl Zeiss).

Insulin secretion measurements. Insulin secretion assay was performed in INS-1E cells as previously described.¹⁴ Briefly, transfected cells were incubated for 30 min in glucose-free INS-1E medium, then washed and pre-incubated for 30 min in glucose-free KRBH buffer. Cells were then incubated for 30 min in KRBH supplemented with 1.67 mM glucose or 16.7 mM glucose. Insulin release and content were measured using the High Range Rat Insulin ELISA according to manufacturer's instruction (Merckodia AB, Uppsala, Sweden).

Co-immunoprecipitation. INS-1E cells (2×10^6) were washed in PBS and resuspended in ice-cold IP cell lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, protease inhibitor cocktail complete (Roche Applied Science, Vilvoorde, Belgium) and 1 mM PMSF). Cell lysates were then sonicated on ice and microcentrifuge for 10 min at 14 000 \times g, 4 $^{\circ}$ C. Supernatants were pre-cleared using protein-agarose beads (Santa Cruz Biotechnology, Tebu-Bio nv) for 1 h at 4 $^{\circ}$ C. The protein concentration of the supernatants was determined by BCA protein assay (Thermo Fisher Scientific, Perbio Science, Erembodegem, Belgium) and adjusted to 1 mg/ml. Protein lysate (100 μ g) was incubated with 2 μ g of CHOP, p65 or HA antibody overnight at 4 $^{\circ}$ C. The protein-antibodies complexes were then immunoprecipitated using protein-agarose beads for 2 h at 4 $^{\circ}$ C. The beads were then washed five times with IP cell lysis buffer, resuspended in three times laemmli sample buffer, and loaded on a 12% SDS-PAGE.

Proteasome activity. Proteasome activity was determined using the 20S Proteasome Activity Assay Kit (Millipore (Chemicon), Brusselssesteenweg, Belgium) according to the manufacturer's instruction, using a Victor2 microplate reader (PerkinElmer, Zaventem, Belgium).

Promoter reporter assays. INS-1E cells were cotransfected using lipofectamine 2000 (Invitrogen) with the internal control pRL-CMV encoding Renilla luciferase (Promega, Madison, WI, USA) and the pNF- κ B-Luciferase (BD Biosciences, Palo Alto, CA, USA). After transfection (16 h), cells were exposed to cytokines for 8 h. Sample preparation, luciferase activities and values correction were performed as described previously.³³ Transient overexpression of rat CHOP was achieved using the full-length cDNA clone, cloned in the expression vector p-CMV-SPORT6 purchased from Open Biosystems (clone 7932840; Thermo Fisher Scientific, Perbio Science).

Chemokine measurements. Supernatants of INS-1E cells treated with IL-1 β + IFN- γ or TNF- α + IFN- γ for 8 and 16 h were collected and used to measurements of the chemokine CCL5 using an ELISA kit (R&D systems) as described by the manufacturer.

Statistical analysis. Data are presented as means \pm S.E.M. Comparisons were performed by two-tailed paired Student's *t*-test or by ANOVA, followed by *t*-tests with Bonferroni correction for multiple comparisons. A *P*-value ≤ 0.05 was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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Disclaimer

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)