Cell Death and Differentiation (2011) 18, 328–337

www.nature.com/cdd

Mcl-1 downregulation by pro-inflammatory cytokines and palmitate is an early event contributing to β-cell apoptosis

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Pancreatic β-cell apoptosis is a key feature of diabetes mellitus and the mitochondrial pathway of apoptosis is a major mediator of β-cell death. We presently evaluated the role of the myeloid cell leukemia sequence 1 (Mcl-1), an antiapoptotic protein of the Bcl-2 family, in β-cells following exposure to well-defined β-cell death effectors, for example, pro-inflammatory cytokines, palmitate and chemical endoplasmic reticulum (ER) stressors. All cytotoxic stresses rapidly and preferentially decreased Mcl-1 protein expression as compared with the late effect observed on the other antiapoptotic proteins, Bcl-2 and Bcl-xL. This was due to ER stress-mediated inhibition of translation through eIF2α phosphorylation for palmitate and ER stressors and through the combined action of translation inhibition and JNK activation for cytokines. Knocking down Mcl-1 using small interference RNAs increased apoptosis and caspase-3 cleavage induced by cytokines, palmitate or thapsigargin, whereas Mcl-1 overexpression partly prevented Bax translocation to the mitochondria, cytochrome *c* release, caspase-3 cleavage and apoptosis induced by the β-cell death effectors. Altogether, our data suggest that Mcl-1 downregulation is a crucial event leading to β-cell apoptosis and provide new insights into the mechanisms linking ER stress and the mitochondrial intrinsic pathway of apoptosis. Mcl-1 is therefore an attractive target for the design of new strategies in the treatment of diabetes.
Cell Death and Differentiation (2011) **18,** 328–337; doi:10.1038/cdd.2010.105; published online 27 August 2010

Diabetes mellitus arises from the inability of the pancreatic β-cells to preserve metabolic homeostasis by the regulated secretion of insulin. The two main forms of diabetes are type 1 (T1D) and type 2 (T2D).[1] T1D is primarily caused by an autoimmune attack leading to β-cell destruction and insulin deficiency. T2D is triggered by the combination of insulino-resistance and impaired β-cell function and survival, mostly secondary to metabolic factors through a process referred to as glucolipotoxicity.[1] Both pathologies are characterized by decreased β-cell mass secondary to apoptosis; β-cell loss is more marked in T1D and is a relative late event in T2D, probably contributing to secondary failure of oral therapies.[1] The mitochondrial intrinsic pathway of apoptosis controlled by the B-cell lymphoma 2 (Bcl-2) protein family[2] plays a major role in pancreatic β-cell death in both T1D and T2D.[3]

The Bcl-2 protein family can be divided into three groups, the antiapoptotic proteins (Bcl-2, B-cell lymphoma-extra large (Bcl-XL), Bcl-w, myeloid cell leukemia sequence 1 (Mcl-1) and A1), the multi-domain pro-apoptotic proteins (Bcl2-associated X protein (Bax), Bcl-2 homologous antagonist/killer (Bak) and Bok) and the pro-apoptotic BH3-only proteins (including Bim, Bid, neuronal death protein 5 (DP5, also known as Harakiri (Hrk)), Puma, Bad and Noxa). Upon death stimulus, Bax translocates from the cytosol to the mitochondria and oligomerizes with Bak, resulting in mitochondrial outer membrane permeabilization (MOMP). This allows proteins located in the mitochondrial intermembrane space, such as cytochrome *c*, to be released in the cytosol, in which they associate with cytosolic proteins to activate downstream executioner caspases resulting in apoptosis.[4] The Bax/Bak activation is regulated by a delicate equilibrium between antiapoptotic Bcl-2 proteins and pro-apoptotic BH3-only proteins. Among the antiapoptotic proteins, overexpression of Bcl-2 and Bcl-XL have been shown to prevent β-cell apoptosis in a variety of models,[5–7] but they may hamper β-cell function.[7] The molecular role of Mcl-1 in β-cells remains to be characterized. Mcl-1 is structurally unique among the Bcl-2 family; it contains two PEST sequences (polypeptide sequences enriched in proline (P), glutamic acid (E), serine (S) and threonine (T)), which favor Mcl-1 degradation, thereby reducing its half-life. Mcl-1 activity is also regulated by phosphorylation by the c-Jun NH2-terminal kinase (JNK)

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Keywords: Mcl-1; JNK; eIF2α; apoptosis; pancreatic β-cells; diabetes
Abbreviations: Ad-Mcl-1, adenoviruses expressing the rat Mcl-1; Ad-LUC, adenoviruses expressing the luciferase protein; Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl2-associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-XL, B-cell lymphoma-extra large; BiP, binding immunoglobulin protein, also known as glucose-regulated protein 78 (Grp78); CHOP, C/EBP homologous protein; CPA, cyclopiazonic acid; DP5, neuronal death protein 5, also known as Harakiri (Hrk); eIF2α, eukaryotic translation initiation factor 2 subunit alpha; ER, endoplasmic reticulum; FFA, free fatty acid; GSIS, glucose-stimulated insulin secretion; IL-1β, interleukin 1 beta; IFN-γ, interferon gamma; JNK, c-Jun NH2-terminal kinase, also known as mitogen-activated protein kinase 8 (Mapk8); Mcl-1, myeloid cell leukemia sequence 1; MOMP, mitochondrial outer membrane permeabilization; PERK, PKR-like endoplasmic reticulum kinase; TNF-α, tumor necrosis factor alpha; siRNA, small interference RNA
Received 04.3.10; revised 21.6.10; accepted 23.7.10; Edited by G Nunez; published online 27.8.10

and extracellular signal-regulated kinase (ERK) pathways in response to a variety of cell stimuli.[8] This highly regulated state suggests that Mcl-1, rather than other Bcl-2 pro-survival proteins, is an early pivotal protein in the regulation of cell survival in response to extracellular signals.

Pro-inflammatory cytokines (interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ)) and high concentrations of saturated free fatty acids (FFAs) such as palmitate are candidate mediators of beta cell apoptosis in T1D and T2D, respectively.[1] In this study, we investigated the effect of cytokines, FFAs and chemical endoplasmic reticulum (ER) stressors on Mcl-1 expression and, using both small interference RNA (siRNA) strategy and adenoviral-mediated Mcl-1 overexpression, explored the role of Mcl-1 in β-cell function and survival. The data obtained indicate that Mcl-1 is a key antiapoptotic protein in β-cells, which is rapidly degraded in response to pro-apoptotic stimuli, leading to increased Bax translocation to the mitochondria, cytochrome *c* release, cleavage of executioner caspase-3 and apoptosis in β-cells. These novel observations place Mcl-1 as key determinant of β-cell fate during severe stress.

Results

Pro-inflammatory cytokines and palmitate decrease Mcl-1 expression in pancreatic β-cells. To evaluate the effect of pro-inflammatory cytokines on Mcl-1 expression, INS-1E cells were treated for 24 h with IL-1β, TNF-α and IFN-γ, alone or in combination, as indicated (Figure 1a). Western blot analysis revealed the presence of a doublet of Mcl-1 isoforms around 40/42λkDa as described previously.[9] IL-1β alone decreased Mcl-1 expression by 30%. IFN-γ and TNF-α had no effect individually, but induced a 40% decrease in Mcl-1 expression when combined. IFN-γ also potentialized the effect of IL-1β, leading to a 60% reduction of Mcl-1 expression (for quantification see Supplementary data; Supplementary Figure S1a). We have previously shown that cytokines induce ER stress in β-cells.[10] To test whether ER stress contributes to Mcl-1 decrease, INS-1E cells were exposed to the ER stressors thapsigargin, cyclopiazonic acid (CPA) and tunicamycin for 15 h, resulting in a similar 40–60% decrease in Mcl-1 expression (Figure 1b and Supplementary Figure S1b). Salubrinal, a selective inhibitor of the cellular phosphatase complex that dephosphorylates the eukaryotic translation initiation factor 2 subunit alpha (eIF2α)[11] also significantly decreased Mcl-1 expression, although by 25% only (Figure 1b and Supplementary Figure S1b). INS-1E cells were also incubated for 15 h in the presence of the FFA oleate and the saturated FFA palmitate, the latter a known β-cell death effector.[12,13] Palmitate, but not oleate, decreased Mcl-1 expression by 50% (Figure 1c and Supplementary Figure S1b). Similar to what we observed in INS-1E cells, Mcl-1 expression was decreased in primary rat islets treated for 48 h with TNF-α + IFN-γ or IL-1β + IFN-γ, or for 24 h with thapsigargin, CPA or palmitate (Figure 1d). Time-course experiments of thapsigargin or IL-1β + IFN-γ treatment indicated that Mcl-1 protein expression is rapidly decreased (after 2 and 4 h of thapsigargin or IL-1β + IFN-γ treatment, respectively) as

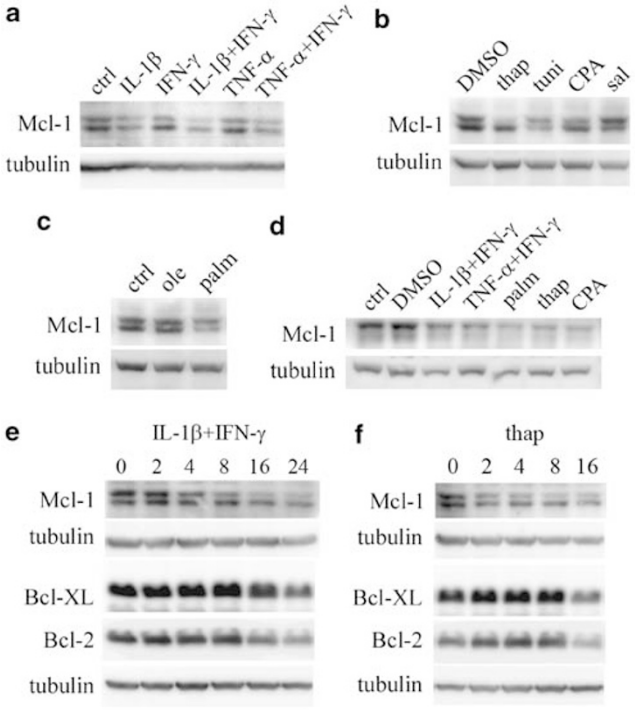

Figure 1 Mcl-1 protein expression is rapidly decreased upon β-cell exposure to pro-apoptotic agents. Mcl-1 protein expression was measured by western blot analyses in INS-1E cells and rat pancreatic islets treated with cytokines, chemical ER stressors or FFAs, as indicated. (**a**) INS-1E cells treated or not treated (ctrl) for 24 h with different cytokines (IL-1β, TNF-α or IFN-γ, alone or in combination as indicated). (**b**) INS-1E cells treated for 15 h with DMSO (solvent for the ER stressors), thapsigargin (thap), tunicamycin (tuni), cyclopiazonic acid (CPA) or salubrinal (sal). (**c**) INS-1E cells exposed for 15 h to ctrl (control medium for FFAs) oleate (ole) or palmitate (palm). (**d**) Rat islets treated for 24 h with different cytokines (IL-1β or TNF-α in combination with IFN-γ), palmitate, thapsigargin or CPA. (**e** and **f**) Time-course experiments of INS-1E cells treated with IL-1β + IFN-γ (**e**) or thapsigargin (**f**). All data are representative of at least four independent western blots

compared with the two other major antiapoptotic proteins, Bcl-2 and Bcl-XL, whose expressions were decreased only after 15 and 24 h of treatment with thapsigargin or IL-1β + IFN-γ, respectively (Figure 1e–f). On the other hand, *Mcl-1* mRNA expression was upregulated by cytokines, thapsigargin and palmitate, indicating that the Mcl-1 protein decrease is a post-transcriptional event (Figure 2a).

Mcl-1 is constitutively targeted for degradation by the proteasome.[8] INS-1E cells treated with the proteosomal inhibitor MG-132 displayed increased basal Mcl-1 expression and the inhibitor prevented IL-1β + IFN-γ-, TNF-α + IFN-γ-, thapsigargin- and palmitate-induced Mcl-1 degradation (Figure 2b and Supplementary Figure S1c), indicating that proteosomal degradation is involved in Mcl-1 down-regulation. Cytokine-induced nitric oxide (NO) production is instrumental in cytokine-mediated ER stress and β-cell apoptosis, whereas palmitate and thapsigargin do not induce NO production in these cells.[10,12] The NO synthase blocker *NG*-methyl-L-arginine (L-NMMA), which fully prevents cytokine-induced NO production in β-cells (data not shown; Cardozo *et al*.[10]), had no effect on thapsigargin-mediated Mcl-1 decrease, whereas it completely prevented the

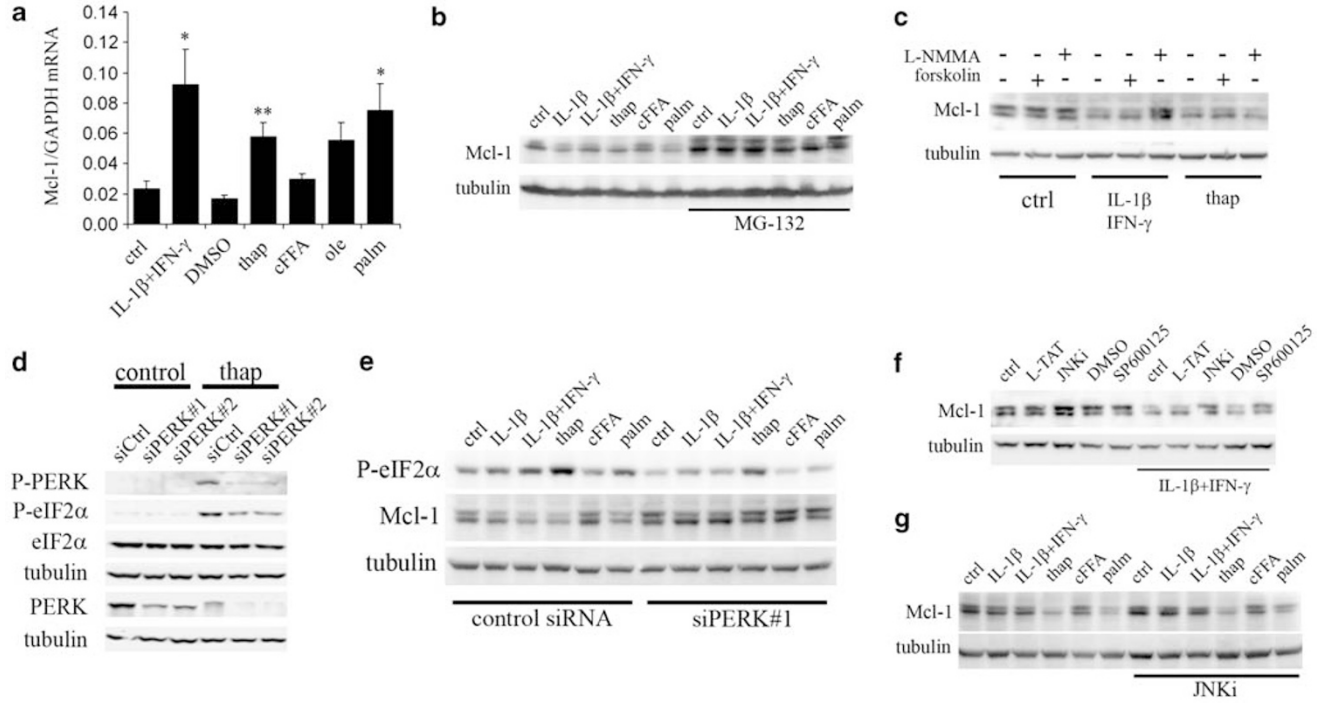

Figure 2 Regulation of Mcl-1 expression. (**a**) Real-time PCR analyses of *Mcl-1* and *GAPDH* mRNA expression in response to IL-1β + IFN-γ, DMSO (solvent for the ER stressors), thapsigargin (thap), cFFA (control medium for FFAs, see Materials and Methods), oleate (ole) or palmitate (palm). $^{*}P<0.05$, $^{**}P<0.01$ *versus* control (ctrl). (**b**) Mcl-1 protein expression was measured by western blot analysis in INS-1E cells exposed to cytokines (IL-1β, in combination or not with IFN-γ), thapsigargin or palmitate, with or without the proteasome inhibitor MG-132. (**c**) Mcl-1 protein was measured by western blot analysis in INS-1E cells exposed to IL-1β + IFN-γ or thapsigargin with or without L-NMMA or forskolin. (**d**) Western blot analysis of P-PERK, total PERK, P-eIF2α and eIF2α in INS-1E cells transfected with a control siRNA (siCtrl) or two PERK siRNA (siPERK nos. 1 or 2) and treated with thapsigargin. (**e**) Western blot analysis of Mcl-1 and P-eIF2α expression in INS-1E cells transfected with a control siRNA or the PERK siRNA no. 1 and treated with cytokines (IL-1β, in combination or not with IFN-γ), thapsigargin or palmitate. (**f**) Western blot analysis of Mcl-1 in INS-1E cells treated or not treated (ctrl) for 8 h with cytokines (IL-1β + IFN-γ), in combination with the L-TAT peptide alone, the L-TAT-JNK inhibitory peptide (JNKi) or the chemical JNK inhibitor SP600125. (**g**) Western blot analysis of Mcl-1 in INS-1E cells exposed for 15 h to cytokines (IL-1β, in combination or not with IFN-γ), thapsigargin or palmitate, together or not with the L-TAT-JNKi (JNKi). (**b**–**g**) Representative western blots of four independent experiments

cytokine effects on Mcl-1 expression (Figure 2c). On the other hand, forskolin, an adenylate cyclase activator, which partially protects β-cells against cytokine- and palmitate-induced apoptosis,[14,15] failed to prevent Mcl-1 degradation (Figure 2c). INS-1E cells then were transfected with an siRNA targeting the PKR-like endoplasmic reticulum kinase (PERK), which is activated upon ER stress and phosphorylates the elongation factor eIF2α, resulting in decreased protein synthesis.[11] PERK knockdown using two different siRNAs decreased total PERK expression by 50% and nearly completely abolished thapsigargin-induced PERK phosphorylation (Figure 2d). This resulted in a 50% decrease in basal and stress-induced eIF2α phosphorylation (Figure 2d–e and Supplementary Figure S1d). PERK knockdown using siPERK no. 1 prevented thapsigargin-, palmitate- and IL-1 β-induced Mcl-1 downregulation (Figure 2e and Supplementary Figure S1d). However, it only partially reversed the inhibitory effects of the cytokine combinations (IL-1β + IFN-γ and TNF-α + IFN-γ) on Mcl-1 expression (Figure 2e and Supplementary Figure S1d). Similar observations were made using siPERK no. 2 (Supplementary Figure S1e).

JNK has been reported to inactivate Mcl-1,[16] and cytokines induce a strong and rapid (1 h) JNK phosphorylation in INS-1E cells,[17,18] leading to a peak of c-Jun phosphorylation between 6 and 8 h. We thus exposed INS-1E cells to IL-1β + IFN-γ for 8 h in the presence of the JNK inhibitor peptide (JNKi)[19] or the chemical JNK inhibitor SP600125.[18] Both inhibitors partially prevented the effect of cytokines on Mcl-1 expression, suggesting that JNK is involved in the cytokine-induced Mcl-1 downregulation (Figure 2f). Western blot analysis of c-Jun phosphorylation confirmed the efficiency of the inhibitors in blocking JNK activity (Supplementary Figure S1f). ER stress has been shown to induce a late JNK activation in β-cells;[10] however, the JNKi peptide did not prevent the effects of a 15 h exposure to thapsigargin or palmitate on Mcl-1 downregulation. At this time point, JNK inhibition fully prevented the Mcl-1 downregulation induced by IL-1β alone or TNF-α + IFN-γ, and partially prevented the effect of IL-1β + IFN-γ (Figure 2g and Supplementary Figure S1c). These data indicate that palmitate and thapsigargin downregulate Mcl-1 protein through ER stress-mediated inhibition of translation, whereas cytokines decrease Mcl-1 through the combined action of translation inhibition and JNK activation.

In other cell types, Mcl-1 stability has been shown to be modulated through phosphorylation by the ERK1,2 and the glycogen synthase kinase-3β (GSK-3β).[8] IL-1β + IFN-γ but not TNF-α + IFN-γ stimulate ERK phosphorylation in INS-1E

cells.[20,21] The peak of IL-1β + IFN-γ-induced ERK activation occurs after 4–8 h of treatment, which is concomitant with Mcl-1 downregulation. However, blocking IL-1β + IFN-γ-induced ERK activity using PD98059 had no effect of Mcl-1 downregulation after 8 h exposure to IL-1β + IFN-γ (Supplementary Figure S2a). Time-course experiments indicated that INS-1E cells have a high basal GSK-3α,β phosphorylation, suggesting a low GSK-3α,β activity since the phosphorylated form is inactive (Supplementary Figure S2b). IL-1β + IFN-γ further stimulated GSK-3α,β phosphorylation after 8 h, and the levels of phospho (P)- and total GSK-3β were decreased at later time points (16 and 24 h). TNF-α + IFN-γ had no significant effect on P-GSK-3α,β and total GSK-3β (Supplementary Figure S2c). We next analyzed Mcl-1 protein expression in the presence of two specific GSK-3β inhibitors (SB216763 and bromoindirubin-3′-oxime (BIO))[22,23] in INS-1E cells treated or not treated for 8 h with IL-1β + IFN-γ. As a control for the effects of the compounds on GSK-3 activity, we evaluated β-catenin protein expression; β-catenin is targeted for proteosomal degradation upon phosphorylation by GSK-3.[22] Both SB216763 and BIO increased beta-catenin expression in INS-1E cells, indicating that these compounds efficiently inhibit GSK-3 activity (Supplementary Figure S2d). On the other hand, the two inhibitors had no effect on Mcl-1 expression, indicating that GSK-3-mediated Mcl-1 phosphorylation is not involved in Mcl-1 downregulation in INS-1E cells.

Mcl-1 knockdown increases β-cell apoptosis. To elucidate the role of Mcl-1 in β-cells, INS-1E cells were transfected with two siRNAs targeting the rat *Mcl-1*. Both siRNAs efficiently decreased Mcl-1 expression (Figure 3a). We then studied the effect of Mcl-1 knockdown on β-cell survival. Transfection with Mcl-1 siRNA no. 1 (Figure 3b and Supplementary Figure S3a) or no. 2 (Supplementary Figure S2b) significantly increased basal and induced apoptosis in all conditions and at all time points studied in INS-1E cells. Similar data were obtained in fluorescence-activated cell sorting (FACS)-purified primary β-cells using Mcl-1 siRNA no. 1 (Figure 3c and Supplementary Figure S3c).

Western blot analysis revealed that knocking down Mcl-1 increased caspase-3 cleavage in control, cytokine-, thapsigargin- or palmitate-treated cells (Figure 4).It is noteworthy that Mcl-1 knockdown did not affect Bcl-2 or Bcl-XL expression, and did not modify the effects of cytokines, thapsigargin or palmitate on the expression of the ER stress markers: binding immunoglobulin protein (BiP; also known as glucose regulated protein 78 (Grp78)), C/EBP homologous protein (CHOP) (Figure 4) and XBP-1s (data not shown).

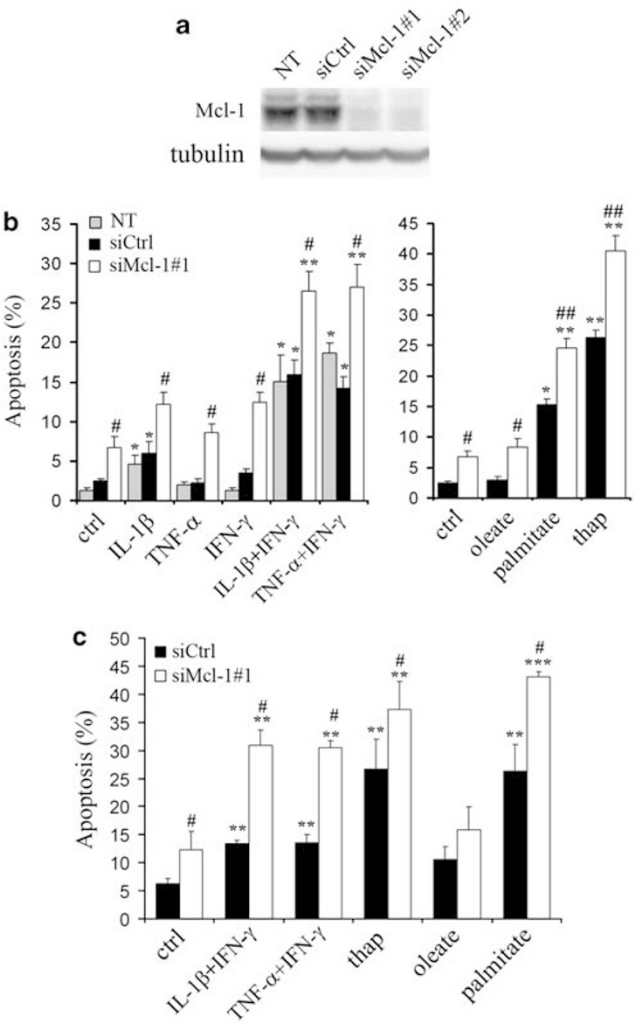

Figure 3 Mcl-1 knockdown increases β-cell apoptosis. (**a**) Representative western blot analysis of five independent experiments showing Mcl-1 expression in INS-1E transfected with a control siRNA (siCtrl) or two different Mcl-1 siRNAs (siMcl-1 nos. 1 and 2). (**b**) INS-1E cells were not transfected (gray bars) or transfected with siCtrl (black bars) or siMcl-1 no. 1 (white bars) and treated; left panel: for 24 h with cytokines (IL-1β, TNF-α or IFN-γ, alone or in combination as indicated); right panel: for 15 h with thapsigargin (thap) or FFAs (oleate or palmitate). (**c**) FACS-purified primary β-cells were transfected with the siCtrl (black bars) or siMcl-1 no. 1 (white bars) and treated for 24 h with cytokines, thapsigargin, oleate or palmitate, as indicated. (**b** and **c**) Prevalence of apoptosis was evaluated by HO-propidium iodide staining. Data are mean ± S.E.M. of at least four independent experiments, expressed as the percentage of apoptotic cells over the total number of cells counted. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *versus* respective non-treated condition. $^{\#}P<0.05$, $^{\#\#}P<0.01$ *versus* respective siCtrl-transfected condition

Mcl-1 overexpression prevents β-cell apoptosis. We next evaluated whether Mcl-1 overexpression increases β-cell viability following exposure to different stressful agents. For this purpose, an adenovirus overexpressing rat Mcl-1 (Ad-Mcl-1) was generated. As compared with non-infected (NI) cells or cells infected with the control virus encoding luciferase (Ad-LUC), INS-1E cells infected with the virus encoding Mcl-1 (Ad-Mcl-1) at multiplicity of infections 2, 5 and 10 displayed dose dependently increased levels of Mcl-1 (Figure 5a). Western blot analysis of mitochondrial and cytosolic fractions extracted from INS-1E cells infected with Ad-LUC or Ad-Mcl-1 revealed that the exogenous Mcl-1 is mostly localized in the mitochondrial fraction. The endogenous Mcl-1 protein was also located in the mitochondrial fraction and absent from the cytosolic fraction. As previously described in other cell types,[2] Bak colocalized with the mitochondrial marker CoxIV, whereas Bax was located in the cytosol (Figure 5b). Interestingly, Bcl-2 and Bcl-XL were present in both cytosolic and mitochondrial fractions, in line with previous reports that Bcl-2 and Bcl-XL have a broader

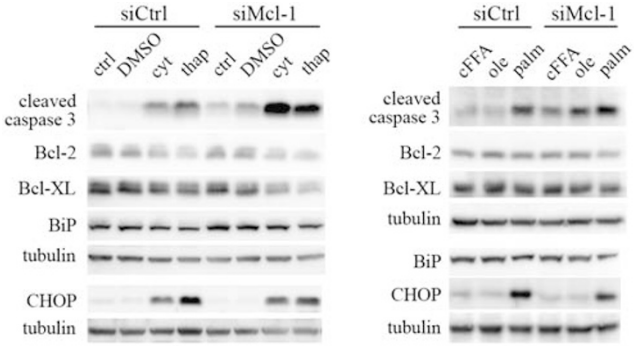

Figure 4 Mcl-1 knockdown increases caspase-3 cleavage. INS-1E cells were transfected with a control siRNA (siCtrl) or the rat Mcl-1 siRNA no. 1 (siMcl-1) and treated or not treated (ctrl) for 15 h with IL-1β + IFN-γ (cyt), DMSO (see Materials and methods), thapsigargin (thap), cFFA (control for FFAs, see Materials and methods), oleate (ole) or palmitate (palm). Data are representative of four independent western blot experiments

cellular distribution as compared with Mcl-1.[9] We next evaluated the effect of Mcl-1 overexpression on β-cell survival. As compared with NI or Ad-LUC-infected cells, Ad-Mcl-1-infected INS-1E cells displayed a 50% reduction in apoptosis following exposure to IL-1β + IFN-γ, TNF-α + IFN-γ, thapsigargin, tunicamycin or palmitate (Figure 5c). Similar results were obtained in FACS-purified primary β-cells after treatment with IL-1β + IFN-γ, thapsigargin or palmitate (Figure 5d). Time-course experiments revealed that the protection was continuous from 8 to 48 h treatment with IL-1β + IFN-γ (Supplementary Figure S3a). Mcl-1 overexpression also reduced by 50% the caspase-3 cleavage induced by IL-1β + IFN-γ (Figure 6a), thapsigargin (Figure 6b) or palmitate (Figure 6c), confirming the anti-apoptotic action of Mcl-1. Similar to Mcl-1 knockdown, Mcl-1 overexpression had no impact on Bax, Bak, Bcl-2, Bcl-XL, CHOP and BiP expression (Figure 6).

Mcl-1 overexpression prevents Bax translocation to the mitochondria. Mcl-1 has been shown to modulate Bax/Bak-mediated MOMP, release of cytochrome *c* and apoptosis.[24] We studied Bax and cytochrome *c* localization by immunostaining in INS-1E cells overexpressing Mcl-1 and exposed to IL-1β + IFN-γ. Upon apoptosis (determined by nucleus morphology using DNA labeling by Hoechst (HO)), INS-1E cells displayed a diffuse cytosolic cytochrome *c* staining, whereas it was discrete and typically mitochondrial in living cells, colocalizing with the mitochondrial marker apoptosis-inducible factor (AIF) (Figure 7a). The mitochondrial morphology, monitored using AIF (Figure 7a) or ATP synthase (Figure 7b), changed drastically between live and apoptotic cells, the latter showing disintegration of the tubular mitochondrial network and formation of punctiform, fragmented mitochondria. In contrast with the cytochrome *c* staining, the Bax labeling changed from a homogeneous cytosolic staining in live cells to a discrete punctate staining that colocalized with the mitochondrial marker ATP synthase in apoptotic cells (Figure 7b). Quantitative assessment revealed that Mcl-1 overexpression reduced by 70% Bax translocation and cytochrome *c* release induced by IL-1β + IFN-γ (Figure 7c),

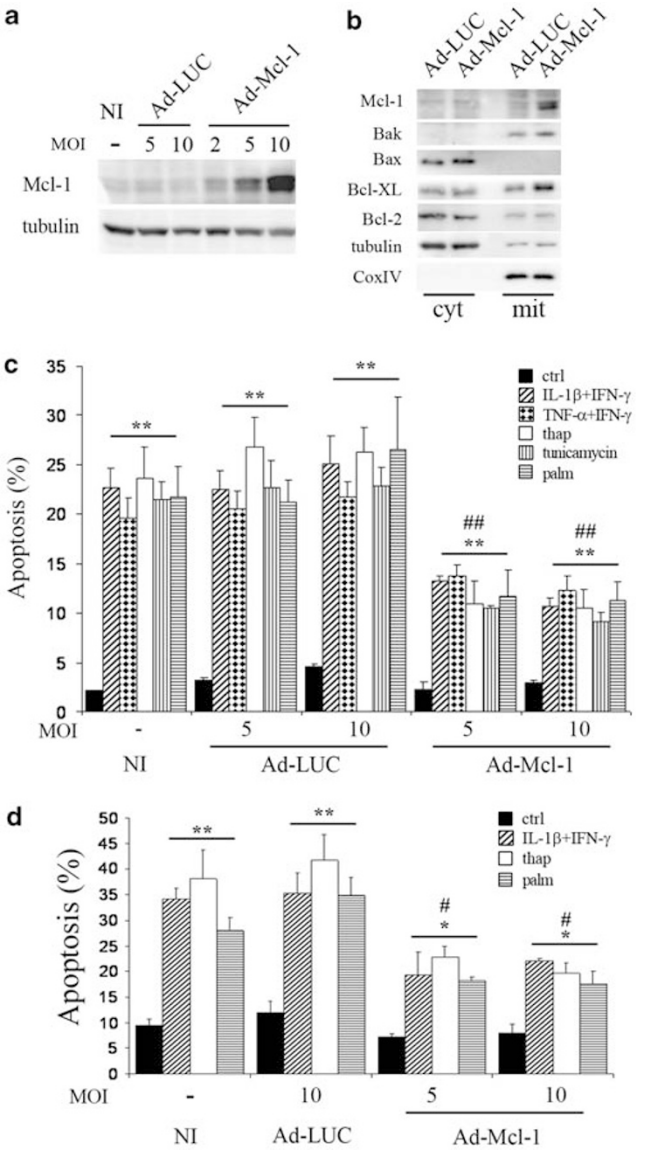

Figure 5 Mcl-1 overexpression prevents β-cell apoptosis. INS-1E- (**a**–**c**) or FACS-purified primary β-cells (**d**) were infected or not infected (NI) with adenoviruses encoding luciferase (Ad-LUC) or Mcl-1 (Ad-Mcl-1) at different multiplicity of infection (MOI), as indicated. (**a**) Representative western blot analysis of four independent experiments showing expression of Mcl-1 and tubulin in total INS-1E protein extracts. (**b**) Representative western blot analysis of four independent experiments showing the expression of Mcl-1, Bcl-2, Bcl-XL, Bax, Bak, tubulin and CoxIV in cytosolic and mitochondrial protein fractions. (**c**) INS-1E cells were infected or NI with Ad-LUC or Ad-Mcl-1, and then treated or not treated (ctrl; black) for 15 h with IL-1β + IFN-γ (striped), TNF-α + IFN-γ (tiled), thapsigargin (white), tunicamycin (vertical stripes) or palmitate (horizontal stripes). (**d**) FACS-purified primary β-cells were infected or NI with Ad-LUC or Ad-Mcl-1, and then treated or not treated (black bars) for 24 h with IL-1β + IFN-γ (striped bars), thapsigargin (white bars) or palmitate (horizontal stripes). (**c** and **d**) Apoptosis was evaluated by HO-propidium iodide staining. Data are mean ± S.E.M. of at least four independent experiments, expressed as the percentage of apoptotic cells over the total number of cells counted. $^{*}P<0.05$, $^{**}P<0.01$ *versus* respective non-treated condition. $^{\#}P<0.05$, $^{\#\#}P<0.01$ *versus* respective NI condition

as compared with Ad-LUC-infected cells. Similar data were obtained after a 15 h exposure to thapsigargin (Supplementary data, Supplementary Figure S4) or palmitate

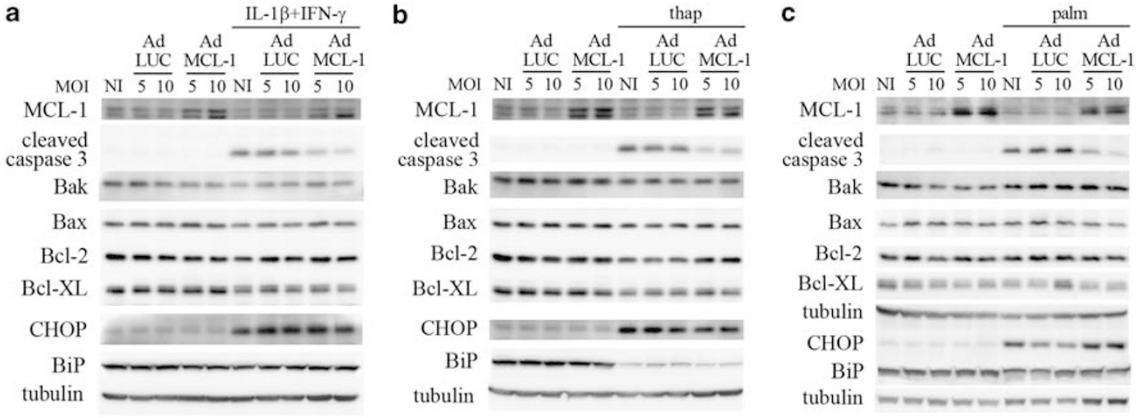

Figure 6 Mcl-1 overexpression prevents caspase-3 cleavage. INS-1E cells were infected or not infected (NI) with adenoviruses encoding luciferase (Ad-LUC) or Mcl-1 (Ad-Mcl-1) and treated or not treated for 15 h with IL-1β + IFN-γ (**a**), thapsigargin (**b**) or palmitate (**c**). Data are representative western blots of three to five independent experiments

(Supplementary Figure S5), suggesting that Mcl-1 protects β-cells from different pro-apoptotic stimuli by preventing Bax translocation and subsequent cytochrome *c* release (Figure 8).

Acute and short-term changes in Mcl-1 expression do not affect β-cell function or proliferation. Bcl-XL overexpression has been shown to inhibit glucose-induced insulin secretion.[7] In contrast, neither Mcl-1 knockdown (Supplementary Figure S6a) nor overexpression (Supplementary Figure S6b) altered the basal or glucose-stimulated insulin secretion (GSIS). Mcl-1 overexpression did not prevent the GSIS inhibition induced by a 15 h treatment with IL-1β + IFN-γ (Supplementary Figure S6c).

Mcl-1 transgenic mice have increased β-cell mass.[25] Under the present experimental conditions, however, a 48-h period of Mcl-1 overexpression did not modify INS-1E cells proliferation, as measured by BrDU incorporation (Supplementary data, Supplementary Figure S7).

Discussion

The aim of this study was to elucidate the role of the Bcl-2 protein family member Mcl-1 in β-cell function and survival. Knocking down Mcl-1 sensitized β-cells to apoptosis, whereas overexpressing it protected these cells against pathophysiologically relevant agents (pro-inflammatory cytokines or palmitate), as well as chemical ER stressors, showing that Mcl-1 has an important antiapoptotic role in β-cells. Interestingly, Mcl-1 was rapidly degraded by cytokines, palmitate and chemical ER stressors, as compared with the other antiapoptotic proteins Bcl-2 and Bcl-XL, setting Mcl-1 decrease as a precursor event in β-cell apoptosis. Mcl-1 downregulation was not a transcriptional event, but was secondary to ER stress-mediated inhibition of translation for palmitate and ER stressors, and to the combined action of translation inhibition and JNK activation for cytokines. Altogether, these novel observations strongly suggest that Mcl-1 degradation may be a decisive step leading to β-cell apoptosis in T1D and T2D.

Mcl-1 is a very labile protein with a 30–90 min half-life and inhibition of *de novo* Mcl-1 synthesis results in rapid Mcl-1 downregulation.[8] In this study, we confirm our previous findings that palmitate[13,26] and cytokines[10] induce the PERK-eIF2α pathway in β-cells, leading to decreased protein translation.[11] We presently show, using salubrinal, that phosphorylation of eIF2α is sufficient to significantly reduce Mcl-1 protein level. Knocking down PERK confirmed that eIF2α phosphorylation is instrumental in palmitate- and thapsigargin-induced Mcl-1 downregulation. This is in line with previous reports of eIF2α-dependent Mcl-1 downregulation in other cell types.[27,28] The effect of cytokines is, however, more complex as blocking eIF2α phosphorylation only partly prevents cytokine-induced Mcl-1 downregulation. Mcl-1 stability in other cell types is influenced through phosphorylation of various residues by multiple kinases, including JNK, ERK and GSK-3β.[8] Cytokines induce JNK in β-cells[18,29] and phosphorylation of Mcl-1 by JNK facilitates Mcl-1 degradation in HEK293 cells.[16] We presently observed that JNK contributes to cytokine-mediated Mcl-1 downregulation. Cytokine-induced JNK phosphorylation is a rapid and transitory event (peak at 1 h), probably contributing to the early Mcl-1 degradation (4–8 h). The role of JNK, however, is partial and restricted to cytokines (blocking JNK did not prevent palmitate- or thapsigargin-induced Mcl-1 degradation), indicating that translation arrest is the main event leading to Mcl-1 downregulation. ERK-mediated phosphorylation has been proposed to stabilize Mcl-1 and we previously showed that IL-1β + IFN-γ induce ERK in β-cells.[20,21] However, blocking ERK activity had no effect on Mcl-1 expression, suggesting that ERK activation is not a major player in cytokine-induced changes in Mcl-1 expression in β-cells. This is in accordance with previous studies showing that ERK plays a minor role in Mcl-1 regulation in other models.[16,30] GSK-3 has also been shown to phosphorylate Mcl-1, thereby facilitating its degradation;[31] however, our present data suggest that INS-1E cells have low GSK-3β activity, and that the kinase is not involved in Mcl-1 regulation.

Altogether, our data show that palmitate and thapsigargin decrease Mcl-1 expression by ER stress-dependent inhibition

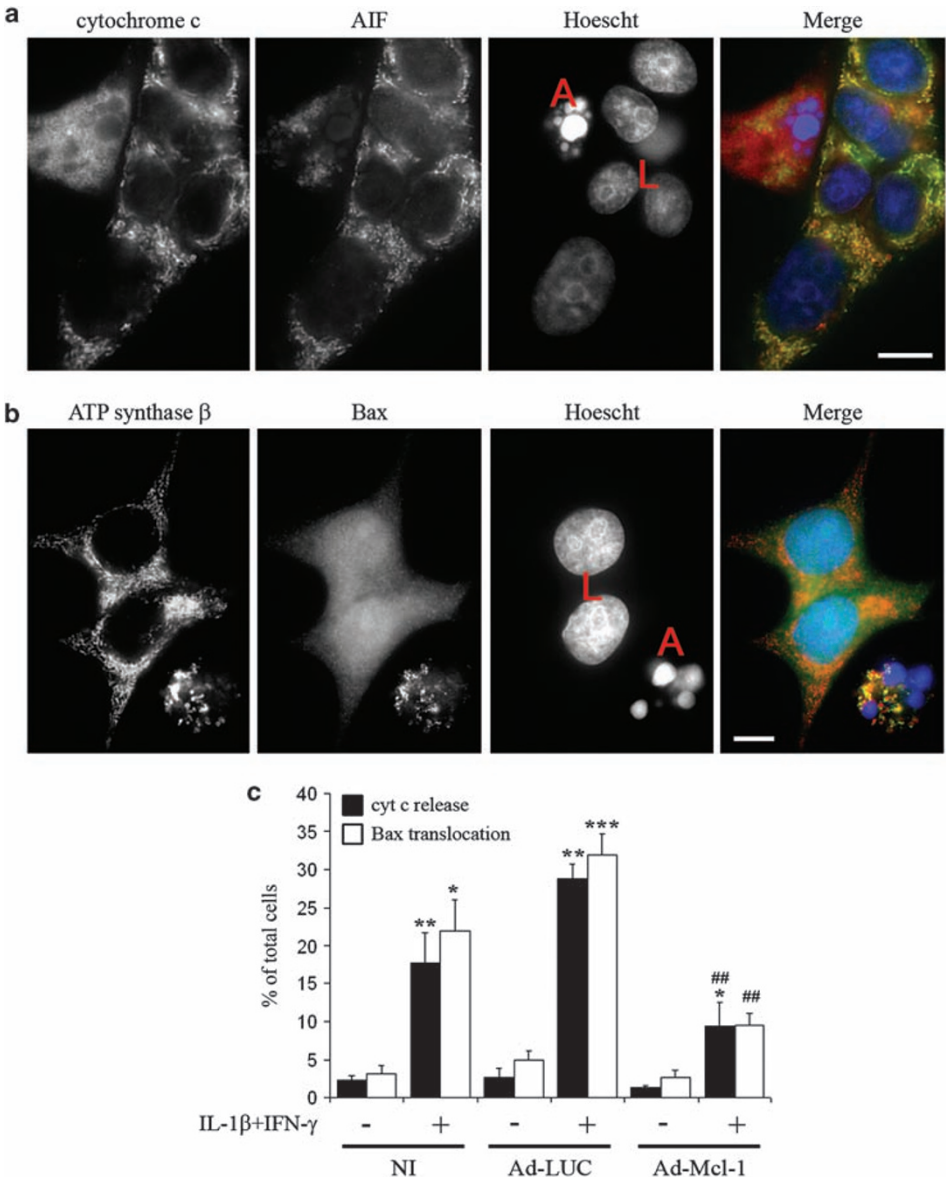

Figure 7 Mcl-1 overexpression prevents Bax translocation to the mitochondria and cytochrome *c* release. INS-1E cells were infected or not (NI) with adenoviruses encoding luciferase (Ad-LUC) or Mcl-1 (Ad-Mcl-1) at an MOI 5 and treated with IL-1β + IFN-γ for 24 h. At 5 days after infection, cells were fixed and analyzed by immunofluorescence. (**a**) Representative field showing live (L) and apoptotic cells (A) stained using anti-cytochrome *c*, anti-AIF, the DNA labeling chemical HO and a merge picture of all channels (red, cyt *c*; green, AIF; blue, HO). (**b**) Representative field showing L and A cells stained using anti-ATP synthase β, anti-Bax, HO and a merged picture of all channels (red, ATPsynth; green, Bax; blue, HO). (**a** and **b**) Magnification, $\times$ 100; the scale bar represents 10 μm. (**c**) Data are mean ± S.E.M. of four independent experiments, expressed as the percentage of cells showing Bax translocation and/or cytochrome *c* release over the total number of cells counted. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *versus* respective non-treated control. $^{\#\#}P<0.01$ *versus* respective NI condition

of translation, whereas cytokines induce Mcl-1 downregulation by a combination of decreased translation and increased JNK activity (Figure 8).

Increasing evidence support the involvement of Bcl2 family members in β-cell apoptosis in response to pro-inflammatory cytokines, saturated FFA and ER stress.[3,5–7,13,18,32] Here, we show that cytokines, palmitate and thapsigargin, induce Bax translocation, cytochrome *c* release and caspase-3 cleavage, independently of changes in Bcl-2, Bcl-XL, Bax and Bak expression levels, but partly as a consequence of Mcl-1 downregulation (Figure 8). Our data are in agreement with previous studies showing that Mcl-1, in spite of its preferential mitochondrial localization,[33] is able to prevent Bax activation and translocation.[24] The fact that Mcl-1 is unlikely to interact directly with Bax[33] suggests that additional factors are involved in the inhibitory action of Mcl-1 on Bax translocation. Truncated Bid (tBid) is one of the factors contributing to Bax translocation to the mitochondria,[34] and has been shown to be involved in cytokine-induced β-cell apoptosis.[35] Mcl-1 has been proposed to act by sequestering tBid, thereby blocking Bax translocation and cytochrome *c* release.[36] Further studies remain to be performed to

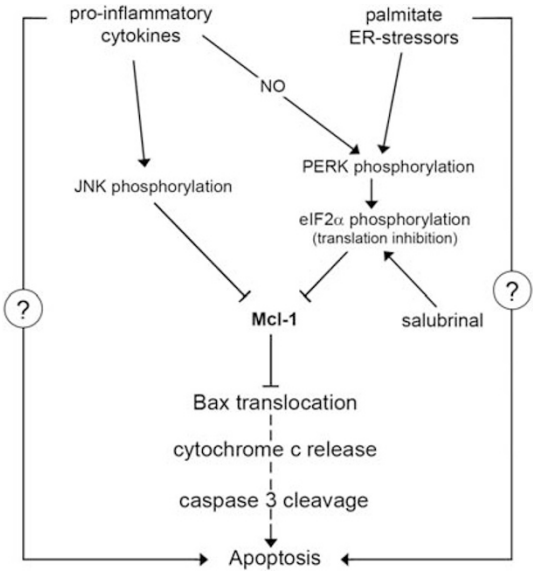

Figure 8 Scheme depicting the central role of Mcl-1 in apoptosis induced by cytokines, palmitate and ER stressors. Palmitate and chemical ER stressors (thapsigargin) induce PERK phosphorylation, which phosphorylates eIF2α, leading to decreased translation, and hence reduced Mcl-1 synthesis. Pro-inflammatory cytokines induce both phosphorylation of eIF2α and JNK activation, leading to decreased Mcl-1 translation and increased Mcl-1 degradation, respectively. The decrease in Mcl-1 protein level permits the translocation of Bax to the mitochondria, in which it oligomerizes with Bak to form a channel leading to mitochondrial outer membrane permeabilization (MOMP), release of cytochrome *c*, cleavage of effector caspases, including caspase-3, and apoptosis

determine whether Mcl-1 overexpression results in tBid sequestration, and whether this contributes to the blockade of Bax translocation. *In vitro* studies suggest that Mcl-1 may interact with other BH3-only proteins including Puma, Noxa, Bim and DP5,[37] suggesting that additional proteins involved in Bax translocation might be sequestered by Mcl-1. In line with this hypothesis, we recently showed that the pro-apoptotic BH3-only proteins DP5/Hrk and PUMA, which are rapidly overexpressed in response to ER stress and cytokines in β-cells, are involved in β-cell apoptosis.[18,32] Taken together, these data suggest that, at least in the case of cytokines, proteins of the Bcl-2 family are regulated at several levels in β-cells; while Mcl-1 is degraded, BH3-only proteins (tBid, DP5, PUMA, etc.) are activated, thereby tipping the pro-/antiapoptotic balance toward apoptosis. Alternatively, other pro-apoptotic mechanisms involving additional Bcl-2 family members (Bim, NOXA, etc.) and/or other pathways (XIAP, FLIPs, etc.) may contribute to β-cell apoptosis.

Many reports suggest that ER stress plays a major role in palmitate-induced β-cell apoptosis.[12,13,26,38] The present data support this hypothesis and provide new insights into the mechanism linking palmitate, ER stress and the intrinsic pathway of apoptosis through eIF2α phosphorylation and Mcl-1 degradation. On the other hand, the role of ER stress in cytokine-induced β-cell apoptosis remains controversial.[10,38–40] We presently show that the cytokine effects on Mcl-1 require ER stress-dependent and -independent mechanisms (JNK activation), suggesting that cytokines may, depending on the severity and duration of exposure, induce apoptosis by ER stress-dependent and -independent mechanisms. This may account for the contradictory findings in this field.[10,38–40] Altogether, our data point out Mcl-1 degradation, whether it is due to ER stress or JNK activation, as a novel and common mechanism for β-cell apoptosis triggered by potential death effectors involved in the onset of T1D and T2D (Figure 8).

New strategies to prevent the development of diabetes require improving β-cell survival without impairing function, for example, GSIS. Bcl-XL overexpression improves β-cell survival, but impairs glucose oxidation and GSIS,[7] and the BH3-only protein BAD is also an important modulator of GSIS,[41] suggesting that some members of the Bcl-2 family have 'day' and 'night' jobs in β-cells, affecting both cell survival and function.[42] This is apparently not the case for Mcl-1 as neither Mcl-1 knockdown nor overexpression altered GSIS. These observations strongly suggest that the role of Mcl-1 in β-cells is restricted to the control of apoptosis and underline Mcl-1 as a promising target for the development of new strategies aiming to prevent β-cell apoptosis in diabetes.

Materials and Methods

Materials. The following chemicals were purchased from Sigma-Aldrich NV/SA (Bornem, Belgium) and used as indicated: MG-132 (1 μM), tunicamycin (10 μg/ml), CPA (20 μM), thapsigargin (100 nM), salubrinal (75 μM), forskolin (10 μM), SP600125 (10 μM), SB216763 (5 μM), BIO (1 μM) and PD98059 (30 μM) were dissolved in DMSO. L-NMMA (1 mM) was dissolved in distilled water. Oleate and palmitate (sodium salt; Sigma-Aldrich) were dissolved in 90% ethanol, heated to 60°C and diluted (final concentration 0.5 mM) in RPMI 1640 with 1% BSA and 1% fetal calf serum (cFFA medium).[13,26] The peptides JNK inhibitor L-TAT-JNKi and control peptide L-TAT (a kind gift from C Bonny and M Mathieu; XigenPharma, Lausanne, Switzerland) were dissolved in culture medium and used at 10 μM as described previously.[19] The following cytokine concentrations were used: recombinant human IL-1β (gift from CW Reinolds, National Cancer Institute, Bethesda, MD, USA) at 10 U/ml in INS-1E cells and 50 U/ml in primary cells; recombinant rat IFN-γ (R&D Systems, Abingdon, UK) at 100 U/ml (0.0072 μg/ml) in INS-1E cells and 500 U/ml (0.036 μg/ml) in primary cells;[10] and recombinant human TNF-α (R&D Systems) at 1000 U/ml in INS-1E cells and primary cells. The selected concentrations of ER stressors, FFA and cytokines are based on previous dose–response studies from our group.[10,12,26]

Cell culture. The rat insulinoma cell line INS-1E (kindly provided by Prof. Claes Wollheim, CMU, University of Geneva, Geneva, Switzerland) was maintained in the complete RPMI 1640 medium as described previously.[10]

Male Wistar rats (Charles River Laboratories, Brussels, Belgium) were housed and manipulated according to the guidelines of the Belgian Regulations for Animal Care; all experiments performed were approved by the local ethics 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 FACS using the auto-fluorescent properties of β-cells (FACSAria, BD Bioscience, San Jose, CA, USA)[10] The preparations used in this study contained 90.1 ± 3.8% β-cells ($n = 11$). Dispersed islets or FACS-purified β-cells were pre-cultured in complete β-cell medium supplemented with 5% heat-inactivated fetal bovine serum from 20 to 48 h for recovery.[13] Experiments were then conducted in the medium without serum.[10]

Western blot analysis. Cells were washed once with cold PBS and directly lysed with Laemmli buffer. Lysates were then resolved by SDS-PAGE and transferred to a PVDF membrane. Immunoblot analyses were performed as described previously,[13,18] using the following antibodies: polyclonal anti-rat Mcl-1 from Biovision (Gentaur, Brussels, Belgium); polyclonal anti-CHOP/GADD153, monoclonal anti-β-catenin (D-10) and polyclonal anti-Bax (P-19) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); polyclonal anti-P-GSK-3α,β, polyclonal anti-GSK-3β, polyclonal anti-P-ERK1,2, polyclonal anti-ERK1,2, polyclonal anti-P-PERK, polyclonal anti-PERK, polyclonal anti-P-eIF2α, polyclonal anti-CoxIV,

polyclonal anti-BiP, polyclonal anticleaved caspase-3, polyclonal anti-Bcl-2 and polyclonal anti-Bcl-XL from Cell Signaling (Boston, MA, USA); polyclonal anti-Bak from BD Biosciences-Europe (Erembodegem, Belgium); and monoclonal anti-α-tubulin and horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG from Sigma-Aldrich.

RNA interference. The following siRNAs were used in this study:

Mcl-1 siRNA no. 1: rat Mcl-1 ON-TARGETplus SMARTpool siRNA (Dharmacon RNAi Technologies, Thermo Fisher Scientific, Aalst, Belgium). The SMARTpool is a mixture of four different siRNA: no. 1, GUAAGGACGAAGCGGGACU; no. 2, GUAGAACAAAUCCGAGUUA; no. 3, GAAUUGUGGCUAACGAGAA; and no. 4, GAUCAGUUCUAGUGUAUAU.

Mcl-1 siRNA no. 2: rat Mcl-1 *Silencer* Select pre-designed siRNA: CGAG GACGAUGUUAAAUCU from Ambion (Applied Biosystems, Lennik, Belgium).

PERK siRNA no. 1: rat Eif2ak3 ON-TARGETplus SMARTpool siRNA (Dharmacon) consisting of: no. 1, CAGGAUACGUGUCCCGAUA; no. 2, GAACAGG AGUCACGCGCGA; no. 3, CCGUCAGGUCUCGGAAAA; and no. 4, AUACAGUA AUGGUGCGCUU.

PERK siRNA no. 2: rat Eif2ak3 *Silencer* Select pre-designed siRNA: GUAUCCAUAUGACAACGGU from Ambion. Allstars Negative Control siRNA (Qiagen, Venlo, the Netherlands), which has little effect on β-cell gene expression and no effect on cell viability.[20] siRNA transfection were conducted according to a protocol developed in our laboratory[13,20] using DharmaFECT 1 (Thermo Fisher Scientific) with a final concentration of 30 nM siRNA. The efficiency of transfection was $>90\%$.[13,20] Cells were then cultured for a 48-h recovery period before being collected or treated as indicated.

Generation of recombinant adenoviruses and cell infection. Ad-LUC was purchased from Vector Biolabs (Philadelphia, PA, USA). The adenovirus encoding rat Mcl-1 was generated by Vector Biolabs using rat myeloid cell leukemia 1 complete cds (cDNA clone MGC: 93459; IMAGE: 7121699; GenBank: BC078835) inserted in the pExpress-1 vector backbone (purchased at ImaGenes GmbH, Berlin, Germany). INS-1E cells and FACS-purified β-cells were infected as described previously.[43]

Assessment of cell viability. The percentage of viable, apoptotic and necrotic cells was determined using the DNA-binding dyes propidium iodide (5 μg/ml) and HO (5 μg/ml; Sigma-Aldrich).[10] 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 the sample identity. There was no difference in viability between non-treated cells (control (ctrl)), cells exposed to the ER stressors solvent DMSO (0.005–0.1%) or cells incubated in the FFA medium (data not shown); thus, the ctrl data showed in viability figures are mean $\pm$ S.E.M. of the different controls used in the experiments. The necrosis levels were low throughout the experiments and were not significantly changed between different experimental conditions in INS-1E cells ($1.32 \pm 0.22\%$: mean necrosis levels in all conditions) and FACS-purified primary β-cells ($2.03 \pm 0.34\%$: mean necrosis levels in all conditions). Apoptosis was confirmed by caspase-3 cleavage (described above in 'Western blot analysis') and Bax translocation (see below in 'Immunofluorescence').

mRNA extraction and quantitative RT-PCR. Poly(A)$+$ mRNA was isolated from dispersed islet cells using the Dynabeads mRNA DIRECT kit (Invitrogen, Paisley, UK) and reverse transcribed as described previously.[10] 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*). Cytokines, FFAs or ER stressors treatments do not modify *GAPDH* expression in insulin-producing cells under the present experimental conditions.[10,12,26] The primers used in this study were: rat *Mcl-1*: Fw – 5′-CCTCCAG CCACCAACTACAT-3′; rev – 5′-CCACTTTCTTTCTGCCGTGTTA-3′; and rat *GAPDH*: Fw – 5′-AGTTCAACGGCACAGTCAAG-3′; rev – 5′-TACTCAGCACCAG CATCACC-3′.

Subcellular fractionation. One million INS-1E cells plated in a six-well plate were infected using Ad-LUC or Ad-Mcl-1. After 48 h, cells were extracted using the PIERCE Mitochondria Isolation Kit for Cultured Cells (Thermo Fisher Scientific) according to the manufacturer's instructions.

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°C in the presence of polyclonal rabbit anti-Bax (1/1000; Santa Cruz Biotechnology Inc.), monoclonal mouse anti-cytochrome *c* or anti-ATP synthase β (1/2000; BD Biosciences). For the proliferation assay using BrdU, INS-1E cells were incubated for 6 h in a 100 μM BrdU solution before fixation. After PBS washing, DNA was denaturated by incubating cells in HCl 2N for 30 min. Acid solution was neutralized by washing twice in borate buffer 0.1 M. Slides were then blocked using PBS-goat serum 5% and incubated overnight at 4°C in the presence of purified mouse anti-BrDU (1/500; BD Biosciences). Cells were then washed and further exposed for 1 h to appropriate Alexa Fluor 488- or 555-conjugated antibodies (1/1000; NV Invitrogen SA, Merelbeke, Belgium). After washing, cells were stained with HO, mounted and photographed using fluorescence microscopy (Axio Imager, Carl Zeiss). A minimum of 600 cells was counted in each experimental condition by two independent observers, one of them unaware of the sample identity.

Insulin secretion measurements. INS-1E cells were plated at a density of 150 000 cells per condition. At 48 h after infection or transfection, cells were incubated for 30 min in glucose-free INS-1E medium. Cells were washed in KRBH (Krebs–Ringer/bicarbonate–HEPES) buffer and pre-incubated for 30 min in glucose-free KRBH buffer (20 mM NaCl, 3.5 mM KCl, 0.5 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 10 mM HEPES and 0.1% BSA). Cells were then incubated for 30 min in KRBH supplemented with 1.67 mM glucose, 16.7 mM glucose or 16.7 mM glucose $+$ 10 μM forskolin. Insulin release was measured using the High Range Rat Insulin ELISA according to the manufacturer's instruction (Mercodia AB, Uppsalla, Sweden).

Statistical analyses. 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 comparison. A *P*-value $\leqslant 0.05$ was considered statistically significant.

Conflict of interest. The authors declare no conflict of interest.

Acknowledgements. This work was supported by grants from the Juvenile Diabetes Research Foundation (JDRF) International (1-2008-536), Fonds National de la Recherche Scientifique (FNRS – FRSM) Belgium, the Communauté Française de Belgique – Actions de Recherche Concertées (ARC), the European Union (Integrated Projects EuroDia in the Framework Programme 6 [FP6] and Naimit in the FP7 of the European Community) and the Belgium Program on Interuniversity Poles of Attraction initiated by the Belgium State (IUAP P6/40). AK Cardozo is a Research Associate of the FNRS; F Allagnat is supported by the JDRF; F Moore is the recipient of a Post-Doctoral Fellowship from FNRS, Belgium; and JM Vanderwinden is a Research Director of the FNRS. We thank MA Neef, G Vandenbroeck, M Urbain, R Leeman, AM Musuaya, R Makhnas and S Mertens from the Laboratory of Experimental Medicine, ULB, for excellent technical support.

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