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

Running title: The role of Mcl-1 in beta cell apoptosis

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bax translocation
intrinsic pathway

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)
eIF2 α , Eukaryotic translation initiation factor 2 subunit alpha
ER, Endoplasmic reticulum
FFA, Free fatty acid
GSIS, Glucose-stimulated insulin secretion
IL-1 β , 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
MOI, Multiplicity of infection
MOMP, Mitochondrial outer membrane permeabilization
PERK, PKR-like endoplasmic reticulum kinase
PTP, Permeability transition pore
TNF- α , Tumor necrosis factor alpha
siRNA, Small interference RNA

Abstract

Pancreatic beta cell apoptosis is a key feature of diabetes mellitus and the mitochondrial pathway of apoptosis is a major mediator of beta cell death. We presently evaluated the role of the myeloid cell leukemia sequence 1 (Mcl-1), an anti-apoptotic protein of the Bcl-2 family, in beta cells following exposure to well defined beta cell death effectors, e.g. pro-inflammatory cytokines, palmitate and chemical ER stressors. All cytotoxic stresses rapidly and preferentially decreased Mcl-1 protein expression as compared to the late effect observed on the other anti-apoptotic proteins Bcl-2 and Bcl-XL. This was due to ER stress-mediated inhibition of translation through eIF2 α phosphorylation for palmitate and ER-stressors and to the combined action of translation inhibition and JNK activation for cytokines. Knocking-down Mcl-1 using siRNAs increased apoptosis and caspase 3 cleavage induced by cytokines, palmitate or thapsigargin, while Mcl-1 overexpression partly prevented Bax translocation to the mitochondria, cytochrome c release, caspase 3 cleavage and apoptosis induced by the beta cell death effectors. Altogether, our data suggest that Mcl-1 downregulation is a crucial event leading to beta cell apoptosis and provide new insights in 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.

Introduction

Diabetes mellitus arises from the inability of the pancreatic beta cells to preserve metabolic homeostasis via 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 beta cell destruction and insulin deficiency. T2D is triggered by the combination of insulino-resistance and impaired beta cell function and survival, mostly secondary to metabolic factors through a process referred to as glucolipotoxicity (1). Both pathologies are characterized by decreased beta-cell mass secondary to apoptosis; beta-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 Bcl-2 protein family (2) plays a major role in pancreatic beta cell death both in T1D and T2D (3).

The Bcl-2 protein family can be divided into three groups, the anti-apoptotic proteins (Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1), the multi-domain pro-apoptotic proteins (Bax, Bak and Bok) and the pro-apoptotic BH3-only proteins (including Bim, Bid, DP5, 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, where 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 anti-apoptotic Bcl-2 proteins and pro-apoptotic BH3-only proteins. Among the anti-apoptotic proteins, overexpression of Bcl-2 and Bcl-XL have been shown to prevent beta cell

apoptosis in a variety of models (5-7), but they may hamper beta cell function (7). The molecular role of the myeloid cell leukemia sequence 1 (Mcl-1) in beta 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 via the JNK and 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 extra-cellular signals.

Pro-inflammatory cytokines (IL-1 β , TNF- α and 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 the present study we investigated the effect of cytokines, FFAs and chemical ER stressors on Mcl-1 expression and, using both siRNA strategy and adenoviral-mediated Mcl-1 overexpression, explored the role of Mcl-1 in beta cell function and survival. The data obtained indicate that Mcl-1 is a key anti-apoptotic protein in beta 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 beta cells. These novel observations place Mcl-1 as key determinant of beta-cell fate during severe stress.

Results

Pro-inflammatory cytokines and palmitate decrease Mcl-1 expression in pancreatic beta cells.

To evaluate the effect of pro-inflammatory cytokines on Mcl-1 expression, INS-1E cells were treated for 24 hours with IL-1 β , TNF- α and IFN- γ , alone or in combination, as indicated (Figure 1a). Western blot analyses revealed the presence of a doublet of Mcl-1 isoforms around 40/42kDA, as previously described (9). IL-1 β 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-1 β , leading to a 60% reduction of Mcl-1 expression (for quantification see supplemental data; Figure S1a). We have previously shown that cytokines induce ER stress in beta cells (10). To test whether ER stress contributes to Mcl-1 decrease, INS-1E cells were exposed to the ER stressors thapsigargin, CPA and tunicamycin for 15 hours, resulting in a similar 40 to 60% decrease in Mcl-1 expression (Figures 1b and S1b). Salubrinal, a selective inhibitor of the cellular phosphatase complex that dephosphorylates the eukaryotic translation initiation factor 2 subunit α (eIF2 α) (11) also significantly decreased Mcl-1 expression, although by 25% only (Figures 1b and S1b). INS-1E cells were also incubated for 15 hours in presence of the FFA oleate and the saturated FFA palmitate, the latter a known beta cell death effector (12, 13). Palmitate, but not oleate, decreased Mcl-1 expression by 50% (Figures 1c and S1b). Similar to what we observed in INS-1E cells, Mcl-1 expression was decreased in primary rat islets treated for 48 hours with TNF- α +IFN- γ or IL-1 β +IFN- γ or for 24 hours with thapsigargin, CPA or palmitate (Figure 1d). Time-course experiments of thapsigargin or IL-1 β +IFN- γ treatment

indicated that Mcl-1 protein expression is rapidly decreased (after 2 and 4 hours of thapsigargin or IL-1 β +IFN- γ treatment, respectively) as compared to the two other major anti-apoptotic proteins, Bcl-2 and Bcl-XL, whose expressions were decreased only after 15 and 24 hours of treatment with thapsigargin or IL-1 β +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 proteasomal inhibitor MG-132 displayed increased basal Mcl-1 expression and the inhibitor prevented IL-1 β +IFN- γ -, TNF- α +IFN- γ -, thapsigargin- and palmitate-induced Mcl-1 degradation (Figures 2b and S1c), indicating that proteasomal degradation is involved in Mcl-1 downregulation. Cytokine-induced nitric oxide (NO) production is instrumental in cytokine-mediated ER stress and beta cell apoptosis, while palmitate and thapsigargin do not induce NO production in these cells (10, 12). The NO synthase blocker LNMMA, which fully prevents cytokine-induced NO production in beta-cells (data not shown; (10)), had no effect on thapsigargin-mediated Mcl-1 decrease, whereas it completely prevented the cytokine effects on Mcl-1 expression (Figure 2c). On the other hand, forskolin, an adenylate cyclase activator which **partially** protects beta cells against cytokine- and palmitate-induced apoptosis (14, 15), failed to prevent Mcl-1 degradation (Figure 2c). INS-1E cells then were transfected with a siRNA targeting the PKR-like endoplasmic reticulum kinase (PERK), which is activated upon ER stress and phosphorylates the elongation factor eIF2 α , resulting in decreased protein synthesis (11). PERK knockdown **using two different siRNA (#1 and #2) 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 eIF2 α phosphorylation (Figures 2d-e and S1d). PERK knockdown using siPERK#1 prevented thapsigargin-, palmitate- and IL-1 β -induced Mcl-1 downregulation (Figures 2e and S1d). However, it only partially reversed the inhibitory effects of the cytokine combinations (IL-1 β +IFN- γ and TNF- α +IFN- γ) on Mcl-1 expression (Figures 2e and S1d). Similar observations were made using siPERK#2 (Figure S1e).

JNK has been reported to inactivate Mcl-1 (16) and cytokines induce a strong and rapid (1h) JNK phosphorylation in INS-1E cells (17, 18), leading to a peak of c-JUN phosphorylation between 6 and 8 hours. We thus exposed INS-1E cells to IL-1 β +IFN- γ for 8 hours in presence of the JNK inhibitor peptide (JNKi) (19) or the chemical JNK inhibitor SP600125 (20). 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 (Figure S1f). ER stress has been shown to induce a late JNK activation in beta cells (10), however the JNKi peptide did not prevent the effects of a 15 hours exposure to thapsigargin or palmitate on Mcl-1 downregulation. At this time point, JNK inhibition fully prevented the Mcl-1 downregulation induced by IL-1 β alone or TNF- α +IFN- γ , and partially prevented the effect of IL-1 β +IFN- γ (Figure 2g and S1c). These data indicate that palmitate and thapsigargin down-regulate Mcl-1 protein through ER stress-mediated inhibition of translation, while 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 extracellular signal-regulated kinase-1,2 (ERK1,2) and the glycogen synthase kinase 3 β (GSK-3 β) (8). IL-1 β +IFN- γ but not TNF- α +IFN- γ stimulate ERK phosphorylation in INS-1E cells (21, 22) The peak of IL-1 β +IFN- γ -induced ERK activation occurs after 4 to 8 hours of treatment, which is concomitant with Mcl-1 downregulation. However, blocking IL-1 β +IFN- γ -induced ERK activity using PD98059 had no effect of Mcl-1 down-regulation after 8 hours exposure to IL-1 β +IFN- γ (figure S2a). Time course experiments indicated that INS-1E cells have a high basal GSK-3 α,β phosphorylation, suggesting a low GSK-3 α,β activity since the phosphorylated form is inactive (Figure S2b). IL-1 β +IFN- γ further stimulated GSK-3 α,β phosphorylation after 8 hours, and the levels of phospho- and total GSK-3 β were decreased at later time points (16 and 24 hours). TNF- α +IFN- γ had no significant effect on P-GSK-3 α,β and total GSK-3 β (Figure S2c). We next analyzed Mcl-1 protein expression in the presence of two specific GSK-3 β inhibitors (SB216763 and BIO) (23, 24) in INS-1E cells treated or not for 8 hours with IL-1 β +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 (23). Both SB216763 and BIO increased beta-catenin expression in INS-1E cells, indicating that these compounds efficiently inhibit GSK-3 activity (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 beta cell apoptosis

To elucidate the role of Mcl-1 in beta 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 beta cell survival. Transfection with Mcl-1 siRNA#1 (Figures 3b and S3a) or #2 (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 FACS-purified primary beta cells using Mcl-1 siRNA#1 (Figures 3c and S3c).

Western blot analyses revealed that knocking-down Mcl-1 increased caspase 3 cleavage in control, cytokine-, thapsigargin- or palmitate-treated cells (Figure 4). Of note, 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 BiP, CHOP (Figure 4) and XBP-1s (data not shown).

Mcl-1 overexpression prevents beta cell apoptosis

We next evaluated whether Mcl-1 overexpression increases beta-cell viability following exposure to different stressful agents. For this purpose, an adenovirus overexpressing rat Mcl-1 (Ad-Mcl-1) was generated. As compared to 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 infection (MOI) 2, 5 and 10, displayed dose-dependently increased levels of Mcl-1 (Figure 5a). Western Blot analyses 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 co-localized with the mitochondrial marker CoxIV, whereas Bax was located in the cytosol (Figure 5b). Interestingly, Bcl-2 and Bcl-XL were present both in the cytosolic and mitochondrial fractions, in line with previous reports that Bcl-2 and Bcl-XL have a broader cellular distribution as compared to Mcl-1 (9). We next evaluated the effect of Mcl-1 overexpression on beta cell survival. As compared to non-infected (NI) or Ad-LUC-infected cells, Ad-Mcl-1-infected INS-1E cells displayed a 50% reduction in apoptosis following exposure to IL-1 β +IFN- γ , TNF- α +IFN- γ , thapsigargin, tunicamycin or palmitate (Figure 5c). Similar results were obtained in FACS-purified primary beta cells after treatment with IL-1 β +IFN- γ , thapsigargin or palmitate (Figure 5d). Time-course experiments revealed that the protection was continuous from 8 to 48 hours treatment with IL-1 β +IFN- γ (Figure S3a). Mcl-1 overexpression also reduced by 50% the caspase 3 cleavage induced by IL-1 β +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 (25). We studied Bax and cytochrome c localization by immunostaining in INS-1E cells overexpressing Mcl-1 and exposed to IL-1 β +IFN- γ . Upon apoptosis (determined by nucleus morphology using DNA labeling by Hoechst), INS-1E cells displayed a diffuse cytosolic cytochrome c staining whereas it was discrete and typically

mitochondrial in living cells, co-localizing 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 co-localized 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-1 β +IFN- γ (Figure 7c), as compared to Ad-LUC-infected cells. Similar data were obtained after a 15 hours exposure to thapsigargin (supplemental data, Figure S4), or palmitate (Figure S5), suggesting that Mcl-1 protects beta 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 don't affect beta cell function or proliferation

Bcl-XL overexpression has been shown to inhibit glucose-induced insulin secretion (7). In contrast, neither Mcl-1 knockdown (Figure S6a) nor overexpression (Figure S6b) altered the basal or glucose-stimulated insulin-secretion (GSIS). Mcl-1 overexpression did not prevent the GSIS inhibition induced by a 15 hours treatment with IL-1 β +IFN- γ (Fig S6c).

Mcl-1 transgenic mice have increased beta cell mass (26). Under the present experimental conditions, however, a 48-hour period of Mcl-1 overexpression did not modify INS-1E cells proliferation, as measured by BrDU incorporation (supplemental data, Figure S7).

Discussion

The aim of this study was to elucidate the role of the Bcl-2 protein family member Mcl-1 in beta cell function and survival. Knocking-down Mcl-1 sensitized beta cells to apoptosis while overexpressing it protected these cells against pathophysiologically relevant agents (pro-inflammatory cytokines or palmitate), as well as chemical ER stressors, demonstrating that Mcl-1 has an important anti-apoptotic role in beta-cells. Interestingly, Mcl-1 was rapidly degraded by cytokines, palmitate and chemical ER stressors, as compared to the other anti-apoptotic proteins Bcl-2 and Bcl-XL, setting Mcl-1 decrease as a precursor event in beta 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 beta 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 down regulation (8). In this study we confirm our previous findings that palmitate (13, 27) and cytokines (10) induce the PERK-eIF2 α pathway in beta cells, leading to decreased protein translation (11). We presently show using salubrinal that phosphorylation of eIF2 α is sufficient to significantly reduce Mcl-1 protein level. Knocking down PERK further demonstrate that eIF2 α phosphorylation is instrumental in palmitate- and thapsigargin-induced Mcl-1 downregulation. This is in line with previous reports of eIF2 α -dependent Mcl-1 downregulation in other cell-types (28, 29). The effect of cytokines is, however, more complex since blocking eIF2 α 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-3 β (8). Cytokines induce JNK in beta cells (20, 30) 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 hour) probably contributing to the early Mcl-1 degradation (4h - 8h). 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 down-regulation. ERK-mediated phosphorylation has been proposed to stabilize Mcl-1 and we previously showed that IL-1 β +IFN- γ induce ERK in beta-cells (21, 22). 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 beta cells. This is in accordance with previous studies showing that ERK plays a minor role in Mcl-1 regulation in other models (16, 31). GSK-3 has also been shown to phosphorylate Mcl-1, thereby facilitating its degradation (32), however our present data suggest that INS-1E cells have low GSK-3 β activity, and that the kinase is not involved in Mcl-1 regulation.

Altogether, our data demonstrate that palmitate and thapsigargin decrease Mcl-1 expression via ER stress-dependent inhibition of translation, while cytokines induce Mcl-1 downregulation via a combination of decreased translation and increased JNK activity (Figure 8).

Increasing evidence support the involvement of Bcl2 family members in beta cell apoptosis in response to pro-inflammatory cytokines, saturated FFA and ER stress (3, 5-

7, 13, 20, 33). Here, we demonstrate 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 demonstrating that Mcl-1, in spite of its preferential mitochondrial localization (34), is able to prevent Bax activation and translocation (25). The fact that Mcl-1 is unlikely to directly interact with Bax (34) suggest 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 conformational changes allowing oligomerization and translocation to the mitochondria (35) and tBid contributes to cytokine-induced beta cell apoptosis (36). Mcl-1 has been demonstrated to sequester tBid, thereby blocking Bax translocation and cytochrome c release (37). Further studies remain to be performed to 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 (38), suggesting that additional proteins involved in Bax translocation might be sequestered by Mcl-1. In line with this hypothesis, we recently demonstrated that the pro-apoptotic BH3-only proteins DP5/Hrk and PUMA, which are rapidly overexpressed in response to ER stress and cytokines in beta cells, are involved in beta cell apoptosis (20, 33). 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 beta cells; while Mcl-1 is degraded, BH3-only proteins (tBid, DP5, PUMA ...) are activated, thereby tipping the pro/anti-apoptotic balance toward apoptosis. Alternatively, other pro-apoptotic mechanisms involving additional

Bcl-2 family members (Bim, NOXA ...) and/or other pathways (XIAP, FLIPs ...) may contribute to beta cell apoptosis.

Many reports suggest that ER stress plays a major role in palmitate-induced beta cell apoptosis (12, 13, 27, 39). The present data support this hypothesis and provide new insights in the mechanism linking palmitate, ER stress and the intrinsic pathway of apoptosis through eIF2 α phosphorylation and Mcl-1 degradation. On the other hand, the role of ER stress in cytokine-induced beta cell apoptosis remains controversial (10, 39-41). We presently demonstrate 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 via ER stress-dependent and independent mechanisms. This may account for the contradictory findings in this field (10, 39-41). 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 beta 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 beta cell survival without impairing function, i.e. glucose-stimulated insulin secretion (GSIS). Bcl-XL overexpression improves beta cell survival but impairs glucose oxidation and GSIS (7) and the BH3-only protein BAD is also an important modulator of GSIS (42), suggesting that some members of the Bcl-2 family have “day” and “night” jobs in beta cells, affecting both cell survival and function (43). 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 beta 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 beta cell apoptosis in diabetes.

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RESEARCH DESIGN AND METHODS

Materials

The following chemicals were purchased from Sigma-Aldrich NV/SA (Bornem, Belgium) and used as indicated: MG-132 (1 μ M), tunicamycin (10 μ g/ml), cyclopiazonic acid (CPA 20 μ M), thapsigargin (100nM), salubrinal (75 μ M), forskolin (10 μ M), SP600125 (10 μ M), SB216763 (5 μ M), bromoindirubin-3'-oxime (BIO; 1 μ M), PD98059 (30 μ M) were dissolved in DMSO. NG-Methyl-L-arginine (L-NMMA, 1mM) was dissolved in distilled water. Oleate and palmitate (sodium salt; Sigma) 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, 27). The peptides JNK inhibitor L-TAT-JNKi and control peptide L-TAT (a kind gift of C. Bonny and M. Mathieu; XigenPharma, Lausanne, Switzerland) were dissolved in culture medium and used at 10 μ M, as previously described (19). The following cytokine concentrations were used: recombinant human IL-1 β (gift from C. W.Reinolds, National Cancer Institute, Bethesda, USA) at 10U/ml in INS-1E cells and 50U/ml in primary cells; recombinant rat IFN- γ (R&D systems, Abingdon, U.K.) at 100U/ml (0.0072 μ g/ml) in INS-1E cells and 500 U/ml (0.036 μ g/ml) in primary cells (10); recombinant human TNF- α (R&D systems, Abingdon, U.K.) at 1000U/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, 27)

Cell culture

The rat insulinoma cell line INS-1E (kindly provided by Prof. Claes Wollheim, CMU, University of Geneva) was maintained in the complete RPMI 1640 medium as previously described (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 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 beta cells were purified by autofluorescence-activated cell sorting (FACS; FACS Aria, BD Bioscience, San Jose, CA., U.S.A.) (10). The preparations used in the present study contained 90.1 ± 3.8 % beta cells ($n=11$). Dispersed islets or FACS-purified beta cells were pre-cultured in complete beta cell medium supplemented with 5% heat-inactivated fetal bovine serum (FBS) from 20 to 48 hours for recovery (13). Experiments were then conducted in medium without serum (10).

Western Blot Analyses

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 previously described (13, 20) 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., U.S.A.; **polyclonal anti-P-GSK-3 α , β** , **polyclonal anti-GSK-3 β** , **polyclonal anti-P-ERK1,2**, **polyclonal anti-ERK1,2**,

polyclonal anti-P-PERK, polyclonal anti-PERK, polyclonal anti-P-eIF2 α , polyclonal anti-CoxIV, polyclonal anti-BiP, polyclonal anti-cleaved caspase 3, polyclonal anti-Bcl-2 and polyclonal anti-Bcl-XL from Cell Signaling, Boston, MA., U.S.A.; polyclonal anti-Bak from BD Biosciences-Europe, Erembodegem, Belgium; 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#1: rat Mcl-1 ON-TARGETplus SMARTpool siRNA (Dharmacon RNAi technologies, Thermo Fisher Scientific, Aalst, Belgium). The SMARTpool is a mixture of

4 different siRNA: #1:GUAAGGACGAAGCGGGACU;
#2:GUAGAACAAAUCCGAGUUA; #3:GAAUUGUGGCUAACGAGAA;
#4:GAUCAGUUCUAGUGUAUUAU.

Mcl-1 siRNA#2: rat Mcl-1 *Silencer*[®] Select pre-designed siRNA: CGAGGACGAUGUAAAUCU from Ambion (Applied Biosystems, Lennik, Belgium).

PERK siRNA#1: rat Eif2ak3 ON-TARGETplus SMARTpool siRNA (Dharmacon);

#1:CAGGAUACGUGUCCCGAUA; #2:GAACAGGAGUCACGCGCGA;
#3:CCGUCAGGUCUCGGAAAA; #4: AUACAGUAAUGGUGCGCUU.

PERK siRNA#2: rat Eif2ak3 *Silencer*[®] Select pre-designed siRNA:

GUAUCCAUAUGACAACGGU from Ambion. Allstars Negative Control siRNA

(Qiagen, Venlo, Netherlands), which has little effect on beta cell gene expression and no effect on viability (21). siRNA transfection were conducted according to a protocol

developed in our laboratory (13, 21) using DharmaFECT 1 (Thermo Fisher Scientific, Aalst, Belgium) with a final concentration of 30 nM siRNA. The efficiency of transfection was >90% (13, 21). Cells were then cultured for a 48-hour recovery period before being collected or treated as indicated.

Generation of recombinant adenoviruses and cell infection.

Control adenovirus encoding luciferase (Ad-LUC) was purchased from Vector Biolabs, Philadelphia, PA, U.S.A. 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 beta cells were infected as previously described (44).

Assessment of cell viability.

The percentage of viable, apoptotic and necrotic cells was determined using the DNA-binding dyes Propidium Iodide (PI, 5 µg/ml) and Hoechst 33342 (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 sample identity. There was no difference in viability between non-treated cells (ctrl), cells exposed to the ER stressors solvent DMSO (0.005 to 0.1%) or cells incubated in the FFA medium (data not shown), thus the control (ctrl) data showed in viability figures are mean \pm SEM 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 beta-cells ($2.03\pm 0.34\%$: mean necrosis levels in all conditions). Apoptosis was confirmed by caspase 3 cleavage (described above in Western blot) 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, U.K.) and reverse transcribed as previously described (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, 27) The primers used in this study were: rat Mcl-1 Fw : 5'-CCTCCAGCCACCAACTACAT-3', rev : 5'-CCACTTTCTTTCTGCCGTGTTA-3' and rat GAPDH: 5'- AGTTCAACGGCACAGTCAAG-3', rev: 5'-TACTCAGCACCAGCATCACC-3'.

Subcellular fractionation

One million INS-1E cells plated in a 6 well plate were infected using Ad-LUC or Ad-Mcl-1. 48 hours later, cells were extracted using the PIERCE Mitochondria Isolation Kit

for Cultured Cells (Thermo Fisher Scientific, Aalst, Belgium) according to 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 hours in a 100 μ M BrdU solution prior to fixation. After PBS washing, DNA was denatured by incubating cells in HCl 2N for 30min. Acid solution was neutralized by washing twice in borate buffer 0.1M. 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 1h to appropriate Alexa fluor 488 or 555-conjugated antibodies (1/1000; N.V. Invitrogen SA, Merelbeke, Belgium). After washing, cells were stained with Hoechst, mounted and photographed using fluorescence microscopy (Axio Imager, Carl Zeiss, Zaventem, Belgium). A minimum of 600 cells was counted in each experimental condition by two independent observers, one of them unaware of sample identity.

Insulin secretion measurements

INS-1E cells were plated at a density of 150,000 cells/condition. 48 hours 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 preincubated for 30 min in glucose-free KRBH (Krebs–Ringer/bicarbonate–HEPES) buffer (20mM NaCl, 3.5mM KCl, 0.5mM NaH₂PO₄, 1.5mM CaCl₂, 5mM NaHCO₃, 10mM HEPES and 0.1% BSA). Cells were then incubated for 30 min in KRBH supplemented with 1.67 mM glucose, 16.7mM glucose or 16.7mM glucose + 10μM Forskolin. Insulin release was measured using the High Range Rat Insulin ELISA according to manufacturer's instruction (Merckodia AB, Uppsalla, Sweden).

Statistical Analyses

Data are presented as means ± SEM. 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 ≤0.05 was considered statistically significant.

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Figure 1 Mcl-1 protein expression is rapidly decreased upon beta cell exposure to pro-apoptotic agents.

Mcl-1 protein expression was measured by Western blot 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 (ctrl) for 24 h with different cytokines (IL-1 β , 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 cFFA (control medium for FFAs) oleate (ole) or palmitate (palm). (d) Rat islets treated for 24h with different cytokines (IL-1 β or TNF- α in combination with IFN- γ), palmitate, thapsigargin or CPA. (e-f) Time-course experiments of INS-1E cells treated with IL-1 β +IFN- γ (e) or thapsigargin (f). All data are representative of at least 4 independent Western blots.

Figure 2 Regulation of Mcl-1 expression

(a) Real-time PCR analyses of Mcl-1 and GAPDH mRNA expression in response to IL-1 β +IFN- γ , DMSO (solvent for the ER stressors), thapsigargin (thap), cFFA (control medium for FFAs, see Methods), oleate (ole) or palmitate (palm). *P<0.01, **P<0.001 vs. control (ctrl). (b) Mcl-1 protein expression was measured by Western blot in INS-1E cells exposed to cytokines (IL-1 β , 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 in INS-1E cells exposed to IL-1 β +IFN- γ or thapsigargin with or without L-NMMA or forskolin. (d) Western blot analysis of P-PERK, total PERK, P-eIF2 α and eIF2 α in INS-1E cells transfected with a control siRNA (siCtrl) or two PERK

siRNA (siPERK#1 or #2) and treated with thapsigargin (thap). (e) Western blot analysis of Mcl-1 and P-eIF2 α expression in INS-1E cells transfected with a control siRNA or the PERK siRNA#1 and treated with cytokines (IL-1 β , in combination or not with IFN- γ), thapsigargin or palmitate. (f) Western blot analysis of Mcl-1 in INS-1E cells treated or not (ctrl) for 8h with cytokines (IL-1 β +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 15h to cytokines (IL-1 β , in combination or not with IFN- γ), thapsigargin or palmitate, together or not with the L-Tat-JNKi (JNKi). (b-g) Representative blots of 4 independent experiments.

Figure 3 Mcl-1 knockdown increases beta cell apoptosis

(a) Representative Western blot of 5 independent experiments showing Mcl-1 expression in INS-1E transfected with a control siRNA (siCtrl) or two different Mcl-1 siRNAs (siMcl-1#1 and #2). (b) INS-1E cells were not transfected (grey bars) or transfected with siCtrl (black bars) or siMcl-1#1 (white bars) and treated; left panel: for 24h with cytokines (IL-1 β , TNF- α or IFN- γ , alone or in combination as indicated); right panel: for 15h with thapsigargin (thap) or FFAs (oleate or palmitate). (c) FACS-purified primary beta cells were transfected with the siCtrl (black bars) or siMcl-1#1 (white bars) and treated for 24h with cytokines, thapsigargin, oleate or palmitate, as indicated. (b-c) Prevalence of apoptosis was evaluated by HO-PI staining. Data are mean \pm SEM of at least 4 independent experiments expressed as the percentage of apoptotic cells over the

total number of cells counted. *P<0.01, **P<0.001 vs. respective non-treated condition. #P<0.01, ##P<0.001 vs. respective siCtrl-transfected condition.

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#1 (siMcl-1) and treated or not (ctrl) for 15h with IL-1 β +IFN- γ (cyt), DMSO (see Methods), thapsigargin (thap), cFFA (control for FFAs, see Methods), oleate (ole) or palmitate (palm). Data are representative of 4 independent Western blot experiments.

Figure 5 Mcl-1 overexpression prevents beta cell apoptosis

INS-1E (a-c) or FACS-purified primary beta cells (d) were infected or not (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 of 4 independent experiments showing expression of Mcl-1 and tubulin in total INS-1E protein extracts. (b) Representative Western blot of 4 independent experiments showing 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 not (NI) with Ad-LUC or Ad-Mcl-1, then treated or not (ctrl;black) for 15 hours with IL-1 β +IFN- γ (striped), TNF- α +IFN- γ (tiled), thapsigargin (white), tunicamycin (vertical stripes) or palmitate (horizontal stripes). (d) FACS-purified primary beta cells were infected or not (NI) with Ad-LUC or Ad-Mcl-1, then treated or not (black bars) for 24 hours with IL-1 β +IFN- γ (striped bars), thapsigargin (white bars) or palmitate (horizontal stripes). (c-d) Apoptosis was evaluated by HO-PI staining. Data are mean \pm SEM of at least 4 independent

experiments expressed as the percentage of apoptotic cells over the total number of cells counted. *P<0.01, **P<0.001 vs. respective non-treated condition. # P<0.01, ##P<0.001 vs. respective non-infected (NI) condition.

Figure 6 Mcl-1 overexpression prevents caspase 3 cleavage

INS-1E cells were infected or not (NI) with adenoviruses encoding luciferase (Ad-LUC) or Mcl-1 (Ad-Mcl-1) and treated or not for 15h with IL-1 β +IFN- γ (a), thapsigargin (b) or palmitate (c). Data are representative Western blot from 3 to 5 independent experiments.

Figure 7 Mcl-1 overexpression prevents Bax translocation to the mitochondria and cytochrome c release

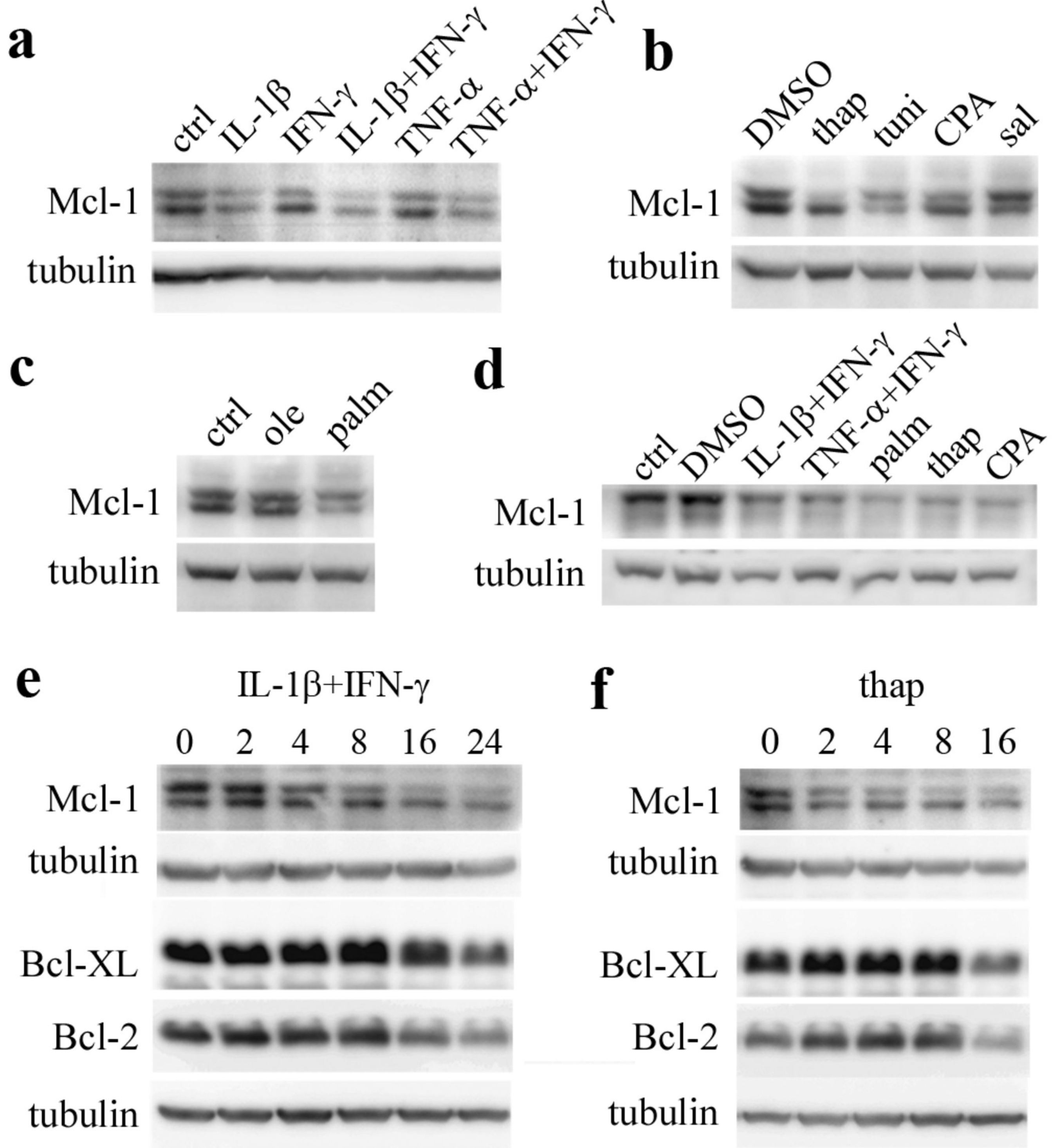
INS-1E cells were infected or not (NI) with adenoviruses encoding luciferase (Ad-LUC) or Mcl-1 (Ad-Mcl-1) at MOI 5 and treated with IL-1 β +IFN- γ for 24 hours. Five days after infection, cells were fixed and analyzed by immunofluorescence. (a) Representative field showing live (L) and apoptotic cells (A) stained using anti-cytochrome c, anti-AIF, the DNA labeling chemical Hoechst and a merge picture of all channels (red: cyt c; green: AIF; blue: Hoechst). (b) Representative field showing live (L) and apoptotic cells (A) stained using anti-ATPsynthase β , anti-Bax, Hoechst and a merged picture of all channels (red: ATPsynth; green: Bax; blue: Hoechst). (a-b) 100x magnification; the scale bar represents 10 μ m. (c) Data are mean \pm SEM of 4 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; vs. respective non-treated control. # P<0.01, ##P<0.001 vs. respective non-infected (NI) condition.

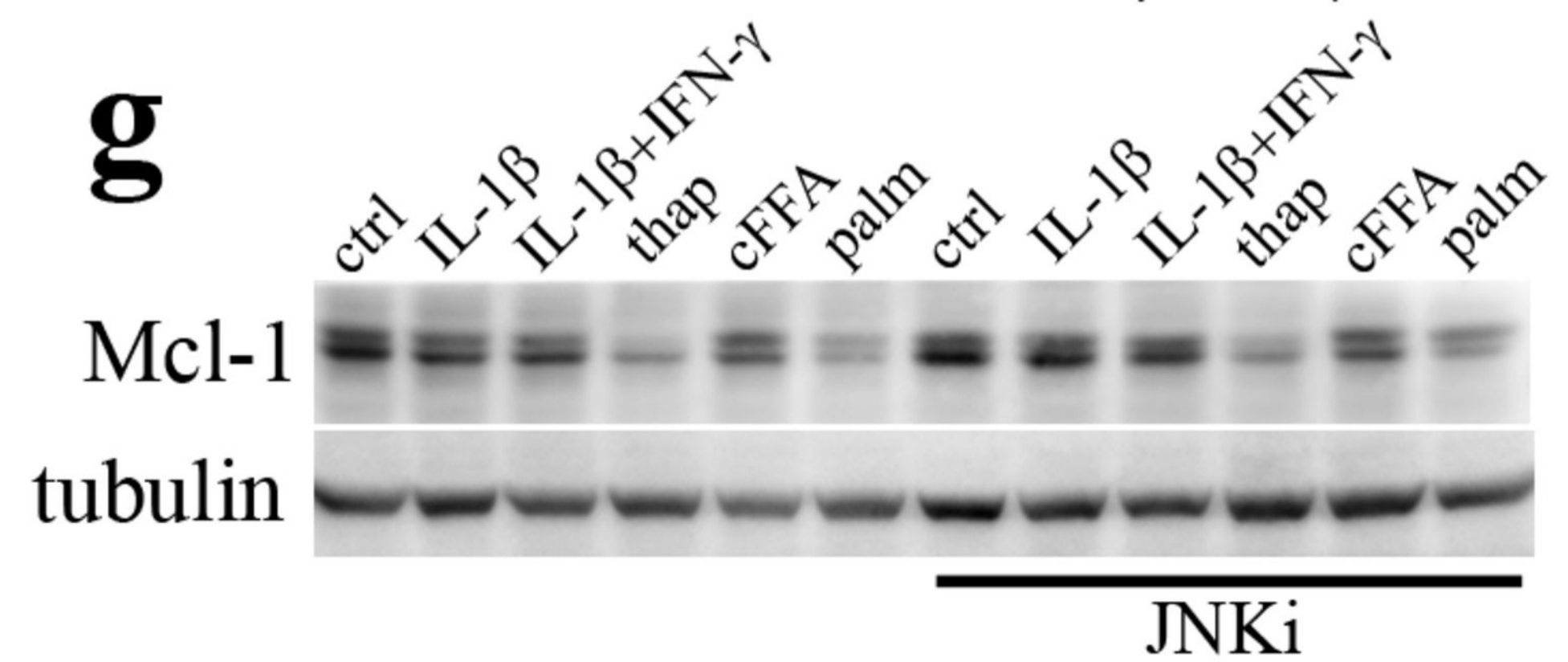
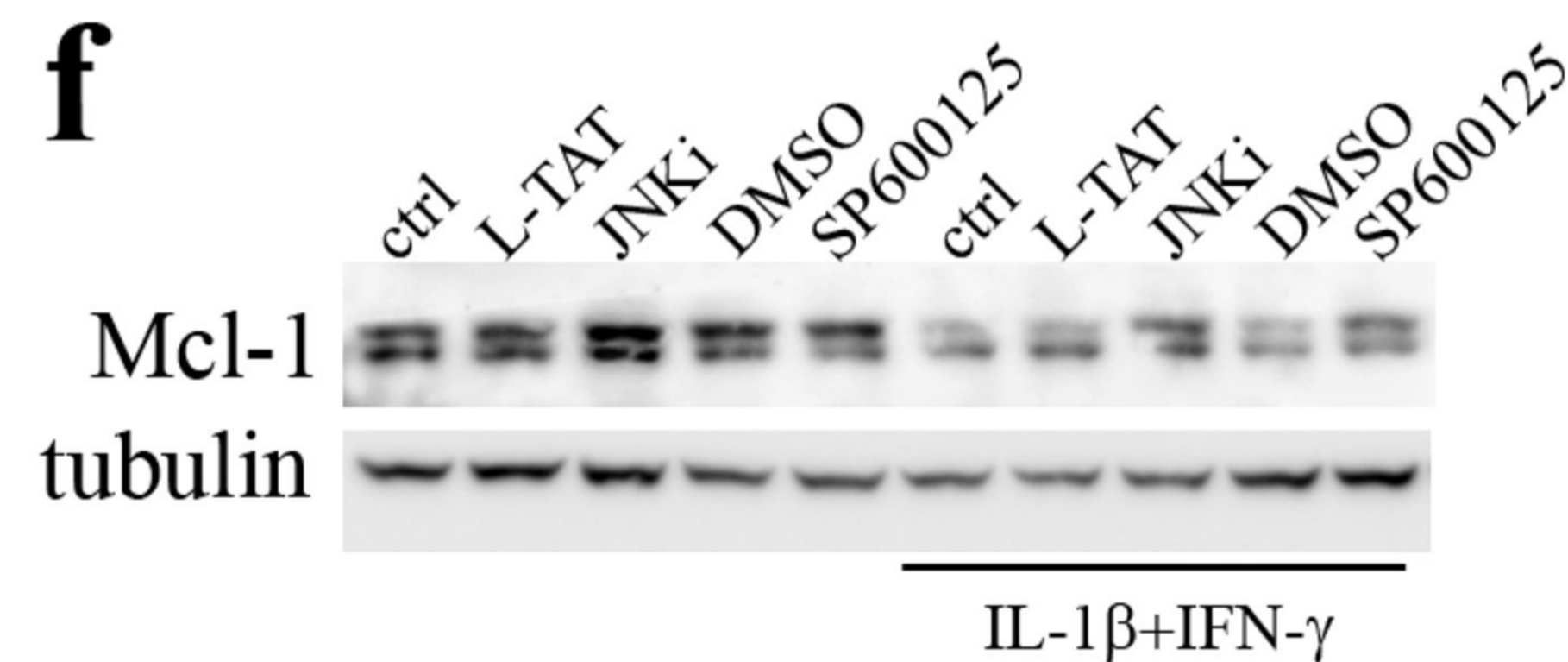
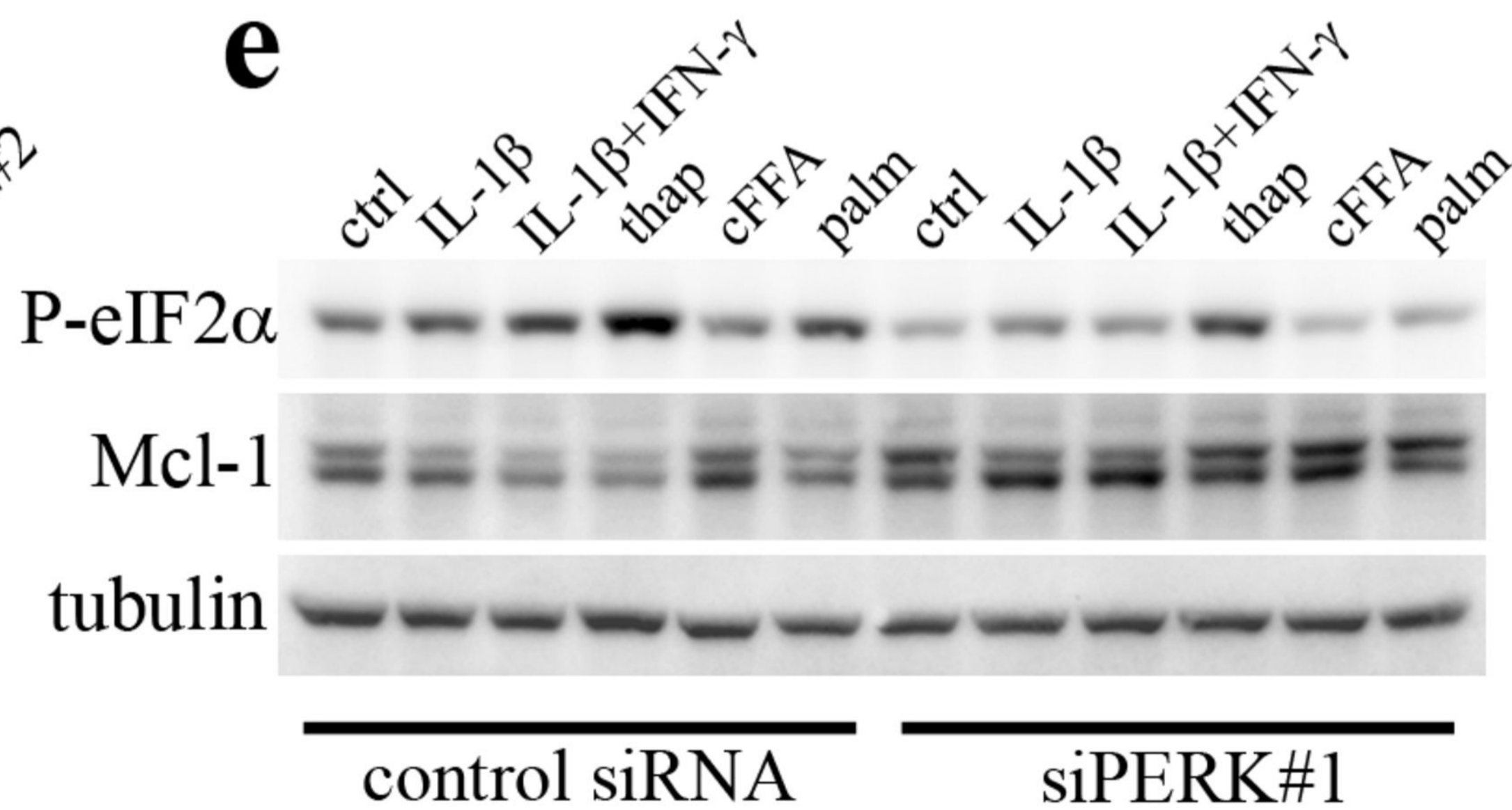
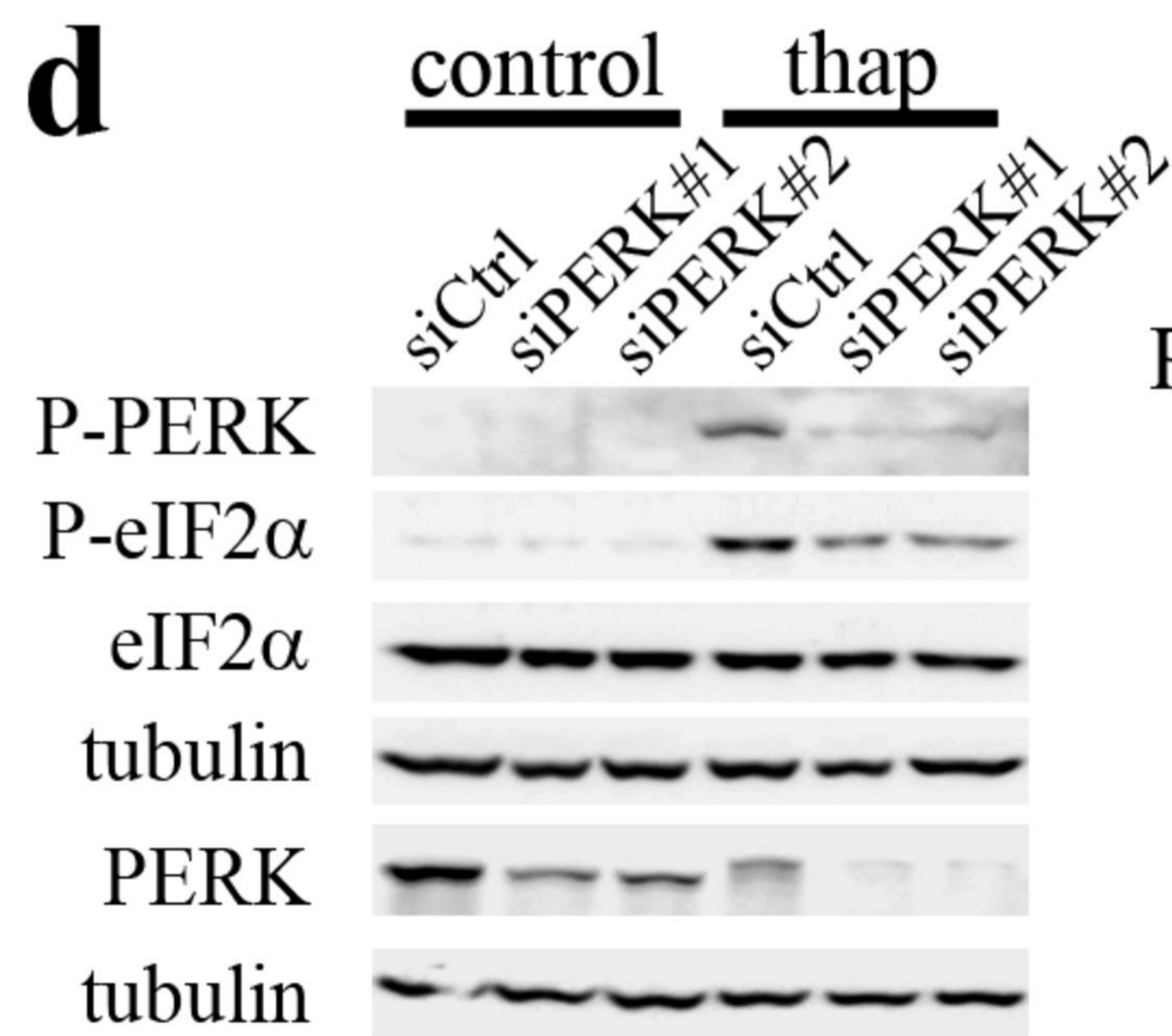
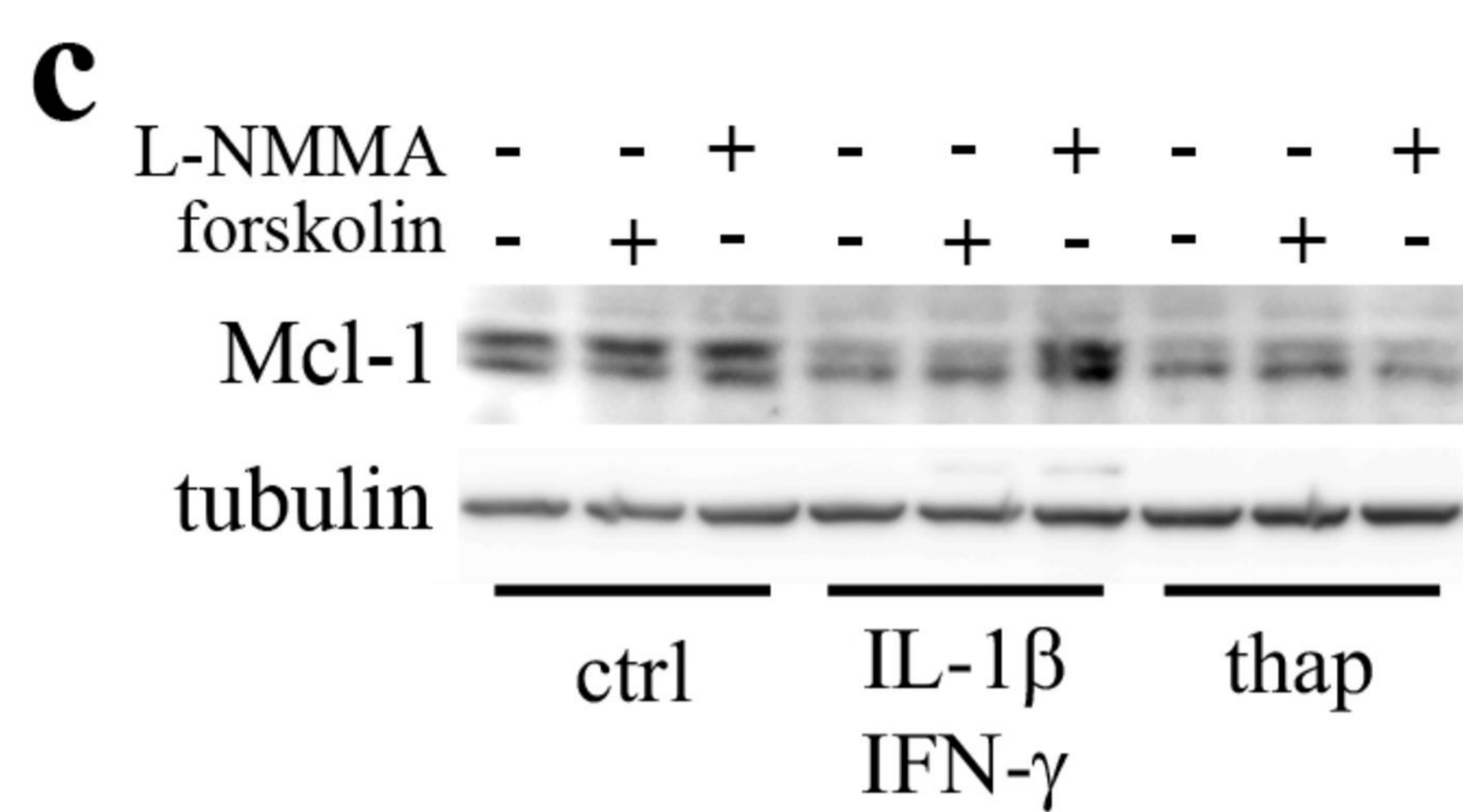
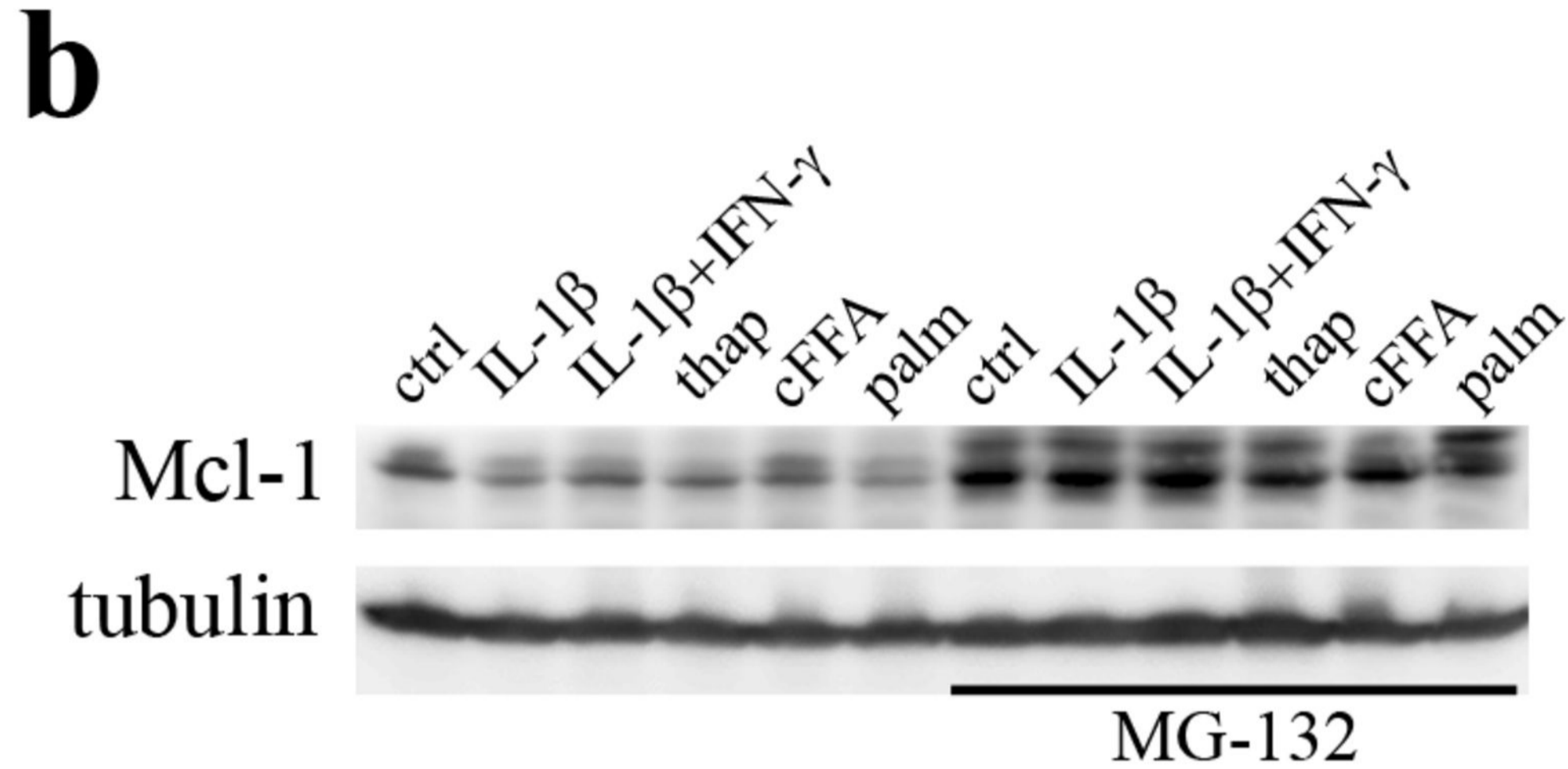
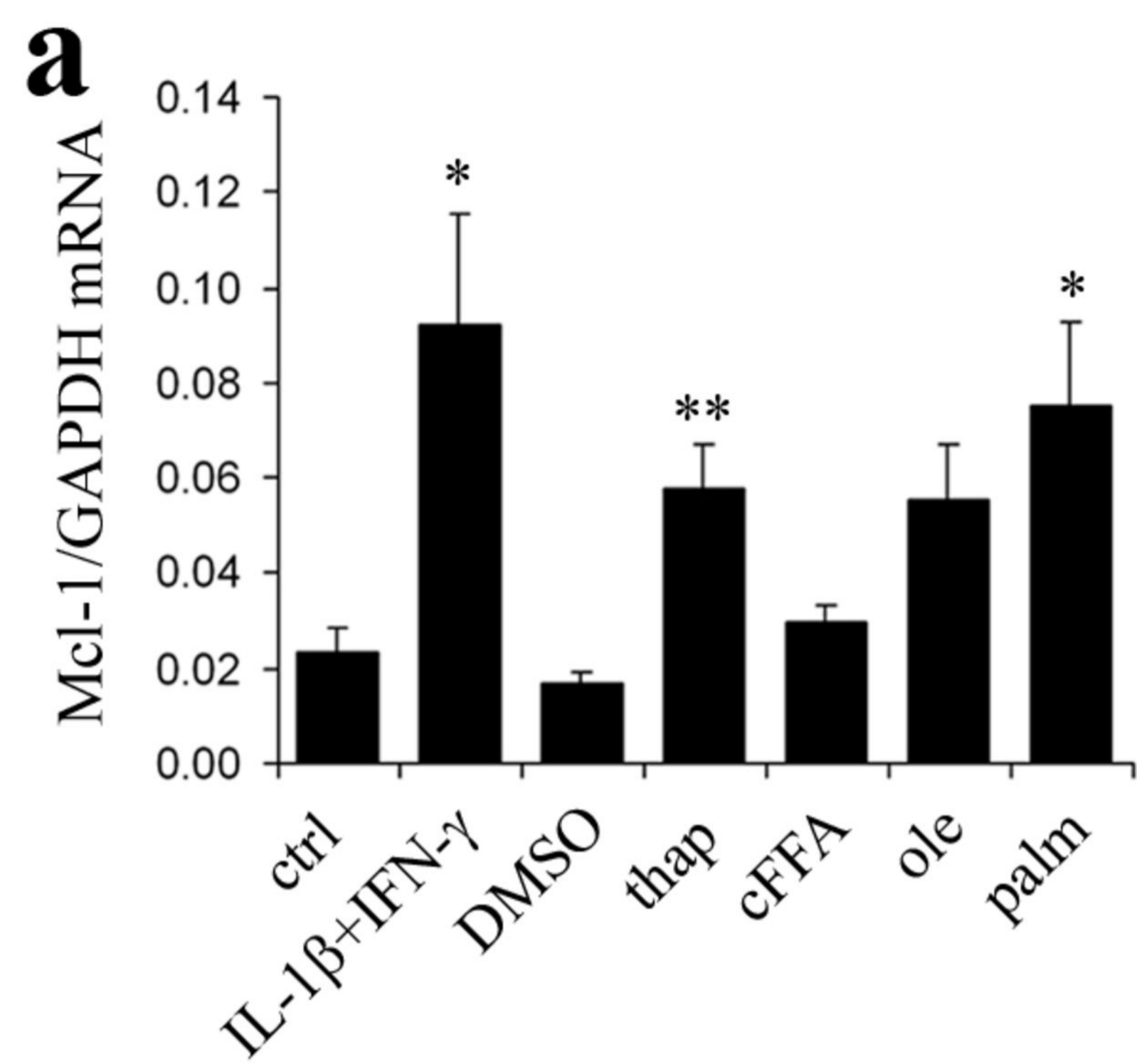
Figure 8 Scheme depicting the central role of Mcl-1 in apoptosis induced by cytokines, palmitate and ER stressors

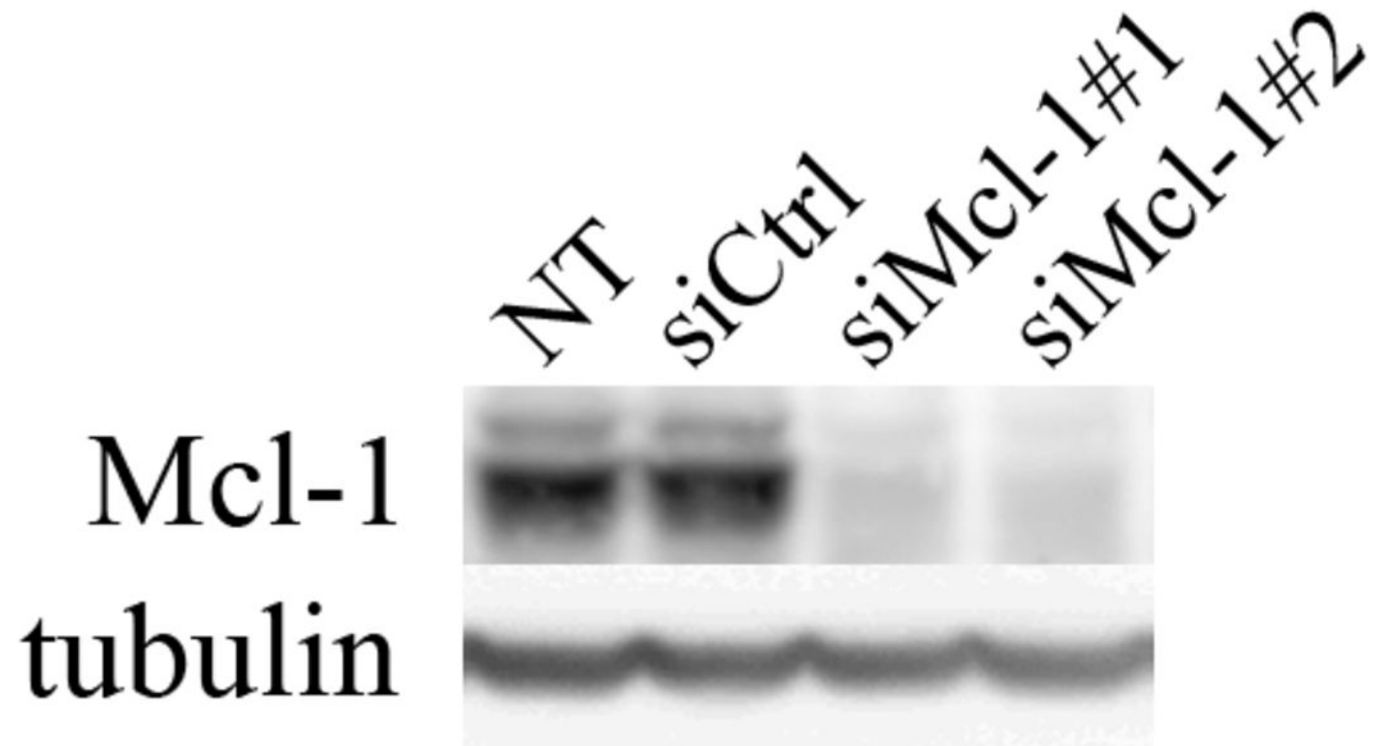
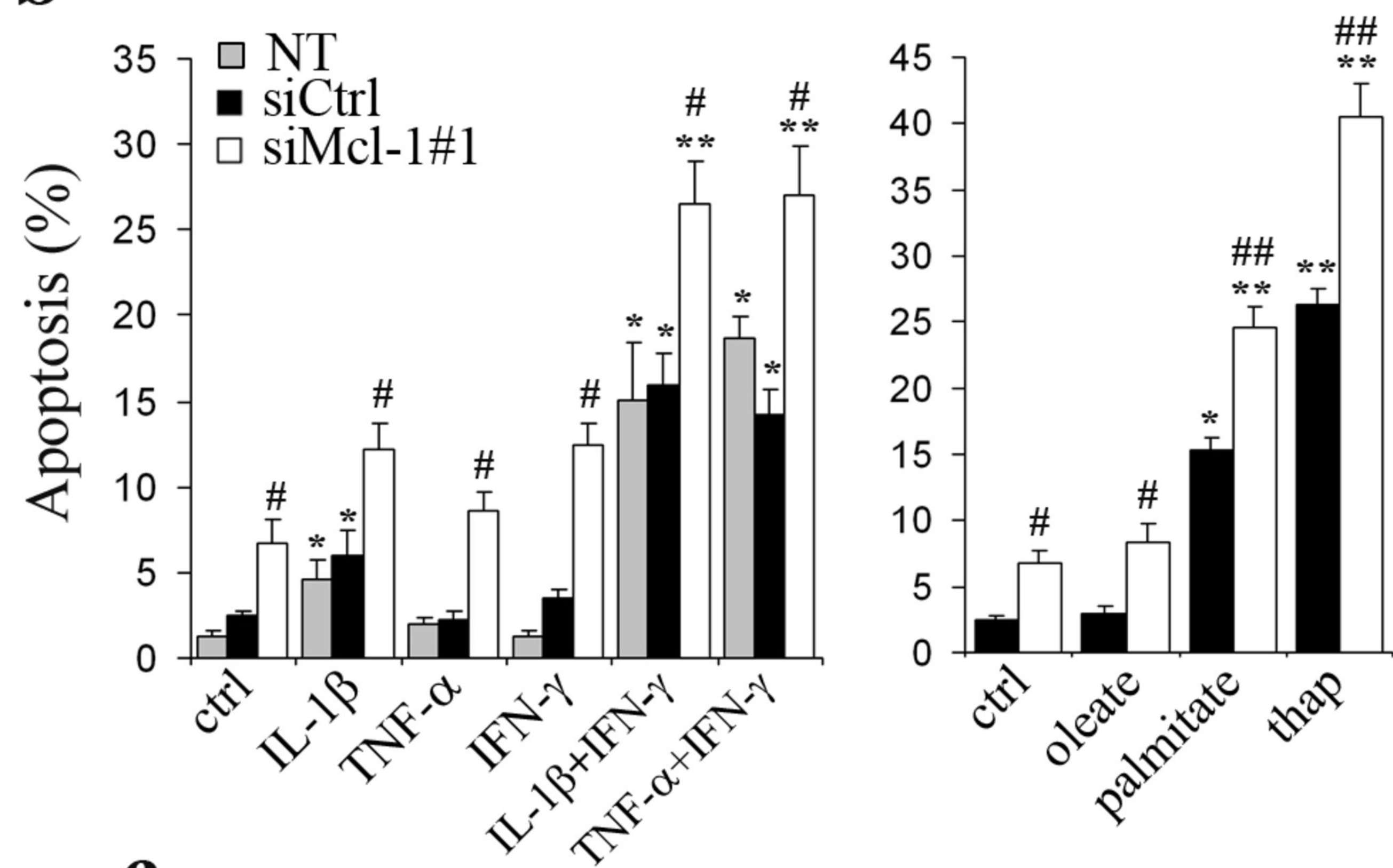
Palmitate and chemical ER-stressors (thapsigargin) induce PERK phosphorylation which phosphorylates eIF2 α , leading to decreased translation, hence reduced Mcl-1 synthesis.

Pro-inflammatory cytokines induce both phosphorylation of eIF2 α 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 where 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.





a**b****c**