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Endoplasmic reticulum stress sensitizes pancreatic beta cells to interleukin-1β-induced apoptosis via Bim/A1 imbalance

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We have recently shown that the crosstalk between mild endoplasmic reticulum (ER) stress and low concentrations of the pro-inflammatory cytokine interleukin (IL)-1β exacerbates beta cell inflammatory responses via the IRE1α/XBP1 pathway. We presently investigated whether mild ER stress also sensitizes beta cells to cytokine-induced apoptosis. Cyclopiazonic acid (CPA)-induced ER stress enhanced the IL-1β apoptosis in INS-1E and primary rat beta cells. This was not prevented by XBP1 knockdown (KD), indicating the dissociation between the pathways leading to inflammation and cell death. Analysis of the role of pro- and anti-apoptotic proteins in cytokine-induced apoptosis indicated a central role for the pro-apoptotic BH3 (Bcl-2 homology 3)-only protein Bim (Bcl-2-interacting mediator of cell death), which was counteracted by four anti-apoptotic Bcl-2 (B-cell lymphoma-2) proteins, namely Bcl-2, Bcl-XL, Mcl-1 and A1. CPA + IL-1β-induced beta cell apoptosis was accompanied by increased expression of Bim, particularly the most pro-apoptotic variant, small isoform of Bim (Bim$_S$), and decreased expression of A1. Bim silencing protected against CPA + IL-1β-induced apoptosis, whereas A1 KD aggravated cell death. Bim inhibition protected against cell death caused by A1 silencing under all conditions studied. In conclusion, mild ER stress predisposes beta cells to the pro-apoptotic effects of IL-1β by disrupting the balance between pro- and anti-apoptotic Bcl-2 proteins. These findings link ER stress to exacerbated apoptosis during islet inflammation and provide potential mechanistic targets for beta cell protection, namely downregulation of Bim and upregulation of A1.

Type 1 diabetes (T1D) is a chronic autoimmune disease, characterized by islet inflammation (insulitis) and progressive immune-mediated apoptosis of pancreatic beta cells via local production of pro-inflammatory cytokines, activation of FasL-Fas and other mechanisms.[1,2] The incidence of T1D is rising worldwide, especially among the youngest generation.[3,4] This rapid augmentation in T1D incidence suggests an increased environmental pressure upon individuals with genetic predisposition to the disease.[5,6] We hypothesized that the induction of beta cell endoplasmic reticulum (ER) stress by obesity-induced insulin resistance and/or by other mechanisms such as viral infections or other environmental insults that remain to be elucidated may contribute to the pathogenesis of T1D.[7,8]

When faced with compromised ER homeostasis and ER stress, cells trigger the unfolded protein response (UPR) through activation of three ER transmembrane proteins: activating transcription factor-6 (ATF-6), inositol-requiring enzyme 1α (IRE1α) and RNA-activated protein kinase-like eukaryotic initiation factor 2α kinase (PERK).[7,9–11] The UPR attenuates the protein load on the ER by decreasing global protein synthesis in parallel to increasing translation of ER chaperones and foldases involved in correct protein maturation; terminally misfolded proteins are degraded. If the stress cannot be resolved, the UPR triggers apoptosis.[9,10,12,13]

The UPR crosstalks with other pathways, particularly inflammation,[8] while on the other hand inflammation may elicit ER stress. The pro-inflammatory cytokines interleukin-1-β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) induce ER stress in vitro in rodent and human beta cells.[14–16] Islets from prediabetic, nonobese diabetic mice have increased expression of ER-stress markers and activation of the pro-inflammatory transcription factor nuclear factor κ-B (NF-κB),[17] whereas islets from T1D patients show increased expression of the UPR markers C/EBP homologous protein (CHOP) and BiP, but not X-box-binding protein-1 (XBP1).[18] We have recently shown that mild ER

stress sensitizes pancreatic beta cells to the pro-inflammatory effects of the cytokine IL-1β via enhanced NF-κB activation,[19] leading to augmented chemokine and Fas expression levels. A detailed study of the three UPR branches identified the IRE1α/XBP1s (XBP1-spliced) pathway as being responsible for this amplified pro-inflammatory response.[19]

Protracted or excessive ER stress and pro-inflammatory cytokines trigger beta cell death through the 'mitochondrial' or intrinsic pathway of apoptosis. This pathway is regulated by the Bcl-2 (B-cell lymphoma-2) protein family, which includes pro- (such as the Bcl-2 homology 3 (BH3)-only proteins) and anti-apoptotic proteins.[12,20,21] Recent studies suggest that death protein 5 (DP5, also known as Harakiri),[22] p53-upregulated modulator of apoptosis (PUMA)[23] and Bcl-2-interacting mediator of cell death (Bim)[21,24–26] are the key pro-apoptotic BH3-only proteins in cytokine-treated beta cells, whereas relevant anti-apoptotic proteins include Bcl-2, Bcl-XL, myeloid cell leukemia sequence-1 (Mcl-1) and A1 (also known as Bcl-2A1).[23,25,27,28] It remains to be defined, however, how the balance between these proteins regulates the activation of Bcl-2-associated X protein (Bax) and Bcl-2 antagonist/killer (Bak), the consequent activation of caspases 9 and 3, and the execution of beta cell apoptosis.[12]

Triggering of beta cell apoptosis is the ultimate mechanism regulating the transition from insulitis to clinical diabetes. Here, we studied the mechanisms implicated in the crosstalk between the UPR and inflammatory signals that lead to beta cell apoptosis. For this purpose, beta cells were exposed to a mild ER stress induced by the chemical stressor cyclopiazonic acid (CPA; 6.25 μM) and then cultured in the presence of a low dose of the cytokine IL-1β (0.5 U/ml; this concentration was selected based on dose–response studies as the lowest concentration that induces NF-κB activation[19]). At the selected concentration, CPA induces a 2–5-fold increase in the expression of CHOP, XBP1s and BiP, with only mild (<10%) apoptosis[29] or insulin mRNA degradation (see below). After determining that CPA + IL-1β treatment exacerbates apoptosis, we performed a detailed mechanistic analysis of the ER stress pathways and Bcl-2 proteins involved in cytokine- and/or ER stress + cytokine-induced beta cell apoptosis. This allowed us to reach three main conclusions: (1) Bim is the central pro-apoptotic BH3-only protein in the context of inflammation-induced beta cell apoptosis; (2) ER stress + inflammation-induced beta cell apoptosis is triggered by an imbalance between the pro-apoptotic protein Bim and the anti-apoptotic protein A1; (3) there are different molecular mechanisms underlying the potentiation by ER stress of cytokine-induced inflammation[19] and beta cell death (present data).

Results

ER stress enhances IL-1β-induced beta cell apoptosis in a XBP1-independent manner.
A dose–response analysis of ER stress in CPA-treated INS-1E cells indicated that 6.25 μM clearly induces the expression of XBP1s and CHOP, but not degradation of Ins-2 mRNA, which is an indicator of severe ER stress[30] (Supplementary Figure 1); similar observations were obtained at later time points[19,29] and

data not shown). Based on these results, 6.25 μM CPA was selected for subsequent experiments aiming to induce a mild ER stress. A time-course analysis of beta cell apoptosis in INS-1E cells after a 6 h CPA treatment followed by exposure to IL-1β (Figure 1a) indicated that IL-1β alone did not induce beta cell apoptosis at any time point analyzed, whereas CPA increased cell death in a time-dependent manner. The CPA pretreatment sensitized cells to IL-1β, aggravating cell death both in INS-1E (Figure 1a) and primary rat beta cells (Figure 1b). These findings were confirmed by protein analysis of cleaved caspases 9 and 3 (Figures 1c–e), indicating a clear increase in caspase activation with CPA + IL-1β as compared with either agent alone, and confirming that apoptosis takes place via the intrinsic mitochondrial pathway.[12]

Previous results from our group[19] indicated that ER stress crosstalks with the NF-κB pathway via XBP1s, leading to an enhanced pro-inflammatory response. To examine whether the same pathway was responsible for the observed increase in cell death, we silenced XBP1 and treated cells with CPA for 6 h, followed by IL-1β for 24 h. XBP1 knockdown (KD, Figure 2a) augmented apoptosis and did not protect against cell death under the ER stress ± cytokine conditions (Figure 2b), suggesting different mechanisms for UPR-induced inflammation and apoptosis.

A1 and Mcl-1 inhibition sensitizes to cytokine-induced Bim-mediated apoptosis.
We next examined which components of the intrinsic pathway are relevant for cytokine-induced beta cell apoptosis. In a well-described model of apoptosis induced by two cytokines, namely IL-1β and IFN-γ,[12,31] we assessed the role of the anti-apoptotic proteins A1, Bcl-2, Bcl-XL and Mcl-1. An early time point (8 h) was selected for these experiments, as KD of some of the anti-apoptotic Bcl-2 proteins led to massive cell death after 16–24 h, rendering data interpretation difficult (data not shown). The KD of each single Bcl-2 anti-apoptotic protein (confirmed in Supplementary Figure 2) increased apoptosis under basal conditions (Figure 3). KD of A1 and Mcl-1, but not of Bcl-2 and Bcl-XL, sensitized beta cells to cytokine-induced apoptosis. Concomitant KD of the pro-apoptotic protein Bim protected against apoptosis when A1 (Figure 3a), Bcl-2 (Figure 3b) or Mcl-1 (Figure 3d) were inhibited. In the case of Bcl-XL, this protection was only partial (Figure 3c). In additional experiments, we extended the observations with A1 and/or Bim KD to 16 h (Supplementary Figure 3), observing again the sensitization to basal and cytokine-induced cell death by A1 KD, and full protection by Bim KD.

A1 degradation and Bim$_S$ (small isoform of Bim) induction are central to the enhancement of IL-1β-induced beta cell apoptosis by ER stress.
In addition to the central role of Bim in cytokine-induced apoptosis (Figure 3 and Supplementary Figure 3), inhibition of Bim (Figures 4a and c) abrogated the sensitization of both INS-1E cells (Figure 4b) and primary rat beta cells (Figure 4d) by ER stress to IL-1β-induced cell death. Under the same experimental conditions, there was no significant increase in DP5 expression and a nonsignificant trend for higher PUMA expression (data not shown); PUMA KD, however, failed to protect against CPA + IL-1β-induced cell death (data not
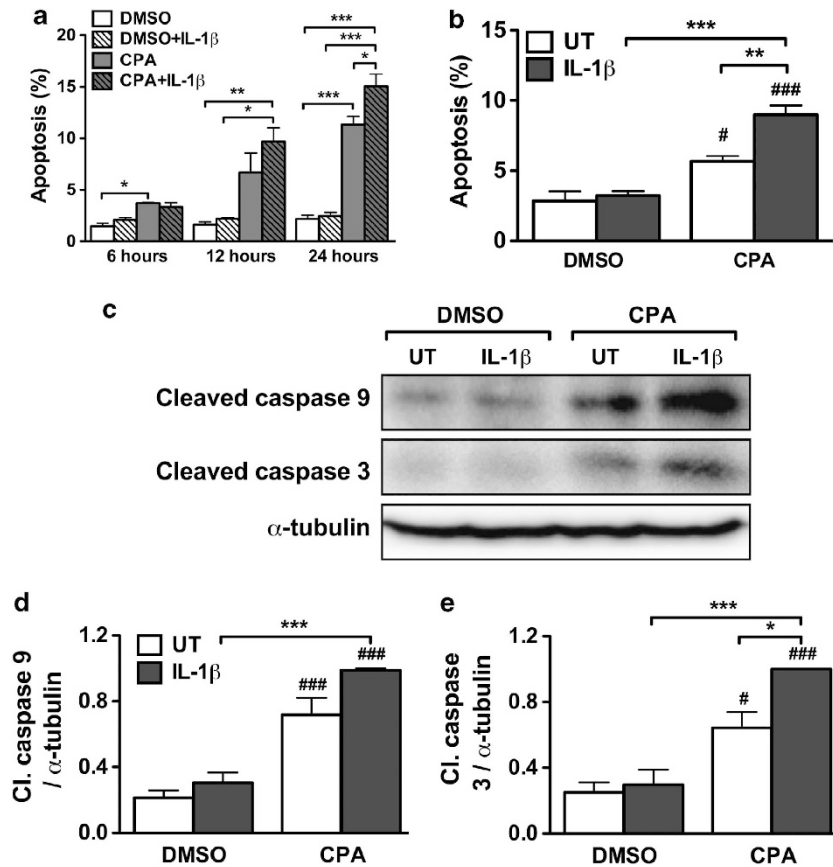
Figure 1 CPA pretreatment sensitizes beta cells to IL-1β-induced beta cell apoptosis. (a) INS-1E cells were exposed to CPA (6.25 μM) or DMSO for 6 h and then treated or not with IL-1β (0.5 U/ml) for the indicated time points. Apoptosis was evaluated by Hoechst 33342/PI staining. Results are the mean of three to six experiments, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus indicated bars, ANOVA. (b) Primary rat beta cells were treated as in (a) (6 h of CPA followed or not by 24 h with IL-1β). Apoptosis was evaluated by Hoechst 33342/PI staining. Results are the mean of four independent experiments. #$P < 0.05$ and ###$P < 0.001$ versus DMSO (UT), *$P < 0.05$ versus indicated bars, ANOVA. (c) INS-1E cells treated as in (a) were harvested and analyzed by western blot. A representative picture of cleaved caspases 9 and 3 is shown. (d and e) western blots of caspases 9 and 3, respectively, were quantified by densitometry. Results are the mean ± S.E.M. of four independent experiments. UT denotes untreated. #$P < 0.05$ and ###$P < 0.001$ versus DMSO, *$P < 0.05$ and ***$P < 0.001$ versus indicated bars, ANOVA

shown). To further study the role of Bim in CPA + IL-1β-induced apoptosis, we analyzed by western blot its expression after 6 h of pretreatment with CPA, followed by a 24 h IL-1β exposure (Figure 5a). The quantification of Bim isoforms revealed that the most pro-apoptotic variant Bim$_S$[32] is 2.9-fold upregulated (Figure 5d), compared with a small increase (40%) in the isoforms Bim$_{EL}$ (Figure 5b) and Bim$_L$ (Figure 5c).

Beta cell apoptosis depends on the balance between pro- and anti-apoptotic Bcl-2 proteins.[12] Our previous findings indicated that cytokines and ER stress inhibit the anti-apoptotic proteins Bcl-XL[21,23] and Mcl-1.[27] A1 was recently described by our group to be present in human and rodent beta cells.[28]

Beta cell apoptosis was evaluated after A1 (Figure 6a) and/or Bim (Figure 6b) KD. As observed for cytokine treatment (Figure 3a), A1 KD increased apoptosis in both untreated and treated (CPA and CPA + IL-1β) conditions (Figure 6c). Inhibition of Bim protected beta cells from apoptosis induced by CPA + IL-1β and A1 KD.

It was previously shown that cytokines cause Mcl-1 degradation,[27] contributing to beta cell apoptosis. We thus performed a time-course study to determine whether A1 protein expression is similarly affected after IL-1β + IFN-γ exposure or CPA (6 h) + IL-1β (12 or 24 h) treatment. This analysis indicated that A1 is induced by IL-1β + IFN-γ at 2 h, but returns to the basal level after a 24 h treatment (Figure 7a). CPA ± IL-1β exposure progressively decreased A1 expression, reaching a nadir at 24 h (Figures 7b and c). Collectively, these results demonstrate that ER stress induces beta cell sensitivity to IL-1β by degrading the anti-apoptotic Bcl-2 protein A1 and by increasing the expression of the highly pro-apoptotic Bim$_S$ isoform.

Discussion

We presently describe that mild ER stress sensitizes pancreatic beta cells to the pro-apoptotic effects of the cytokine IL-1β by altering the balance between the anti-apoptotic Bcl-2 protein A1 and the pro-apoptotic BH3-only protein Bim.

The clinical manifestations of T1D represent the end stage of a chronic autoimmune process characterized by insulitis and progressive beta cell apoptosis.[1,12] The preclinical stage
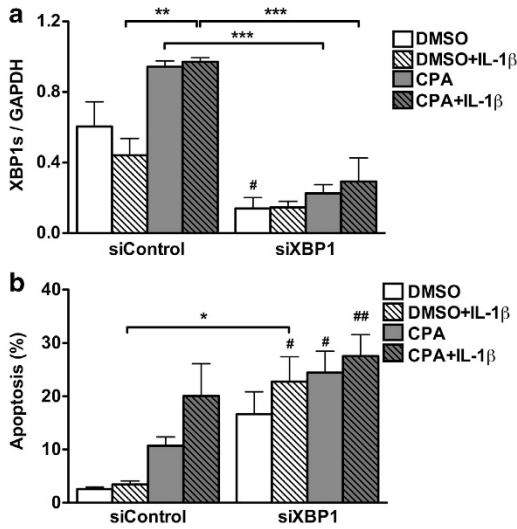
Figure 2 KD of XBP1 does not prevent $CPA + IL-1\beta$-induced beta cell apoptosis. INS-1E cells were transfected with a siRNA against XBP1 (siXBP1) or with a negative control siRNA (siControl). After 48 h, cells were exposed to CPA (6.25 μM) or DMSO for 6 h and then treated with IL-1β (0.5 U/ml) or left untreated for 24 h. (**a**) Cells were harvested to determine the mRNA expression of XBP1s and the housekeeping gene *GAPDH*. (**b**) Beta cell apoptosis was evaluated by Hoechst 33342/PI staining. Results are the mean $\pm$ S.E.M. of four independent experiments. [#]$P < 0.05$ and [##]$P < 0.01$ *versus* siControl + DMSO, *$P < 0.05$, **$P < 0.01$ ***$P < 0.001$ *versus* indicated bars, ANOVA

is silent and may last for years.[2] It is conceivable that during this period nonimmunologic factors, such as ER stress in beta cells, accelerate local inflammation and beta cell loss.[8] ER stress may be induced by obesity and insulin resistance (through the augmented demand placed on the pancreatic beta cells and/or via increased levels of circulating fatty acids), by viral infections or by other environmental insults that remain to be clarified.[7] In the diabetes-prone BioBreeding rat, an animal model of T1D, increasing the plasma-free fatty acid levels by two-fold significantly impaired beta cell function and augmented islet cytokine levels.[33]

We have recently shown that CPA-induced ER stress and IL-1β potentiate the pro-inflammatory response of beta cells, characterized by increased expression of chemokines and Fas, via activation of the transcription factor NF-κB by the IRE1α/XBP1 branch of the UPR.[19] XBP1 silencing, however, did not protect beta cells against the increased apoptosis induced by CPA + IL-1β (present data), indicating that the pro-inflammatory and pro-apoptotic effects of ER stress are mediated by different pathways. Recent findings indicate that thioredoxin-interacting protein (TXNIP) is induced by ER stress and contributes to sterile inflammation and UPR-dependent beta cell apoptosis.[34,35] In our experimental model, TXNIP was induced by CPA treatment, but its expression was not enhanced by the addition of IL-1β (data not shown), suggesting that other mechanisms explain beta cell apoptosis caused by mild ER stress plus IL-1β.

Our previous findings,[21,24–26] together with the present detailed analysis of the role of pro- and anti-apoptotic Bcl-2 proteins in basal or cytokine-induced apoptosis, indicate that Bim is a central mediator of beta cell death (Figure 3). Importantly, the presence of four anti-apoptotic proteins,
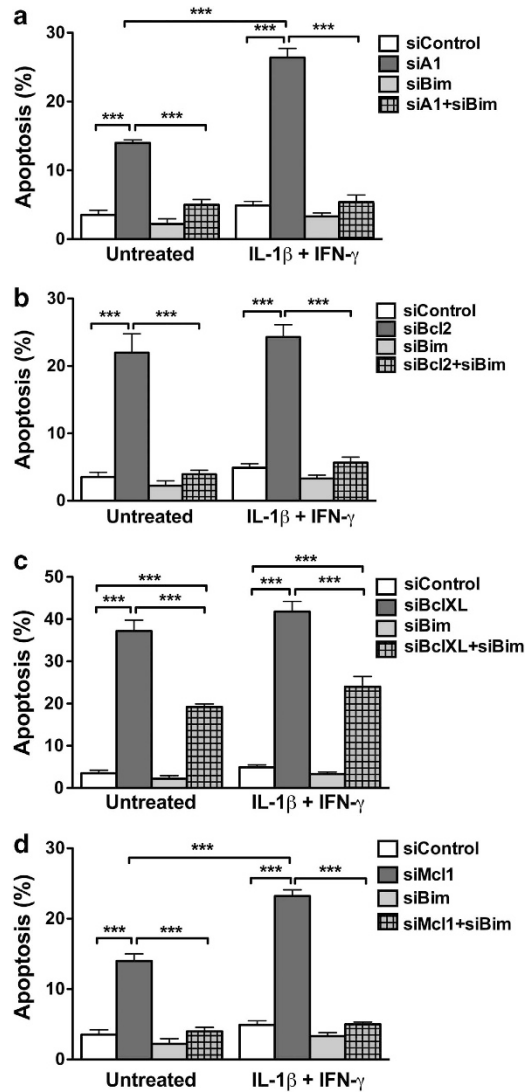

Figure 3 Bim KD protects against beta cell apoptosis induced by inactivation of Bcl-2 proteins and/or cytokine treatment. INS-1E cells were transfected with siRNAs against A1 (siA1, panel **a**), Bcl-2 (siBcl2, panel **b**), Bcl-XL (siBclXL, panel **c**), Mcl-1 (siMcl1, panel **d**) alone or together with siRNA against Bim (siBim) or with a negative control siRNA (siControl). After 48 h, cells were treated with IL-1β + IFN-γ (10 and 100 U/ml, respectively) for 8 h or left untreated. Beta cell apoptosis was evaluated by Hoechst 33342/PI staining. Results are the mean $\pm$ S.E.M. of five independent experiments. ***$P < 0.001$ *versus* indicated bars, ANOVA

namely A1, Bcl-2, Bcl-XL and Mcl-1, is necessary to counteract the pro-apoptotic effects of Bim. Although Bim KD abrogated the increase in cell death observed after KD of A1, Bcl-2 or Mcl-1, the protective effect observed following Bcl-XL inhibition was only partial. Bcl-XL counteracts the effects of both Bim and PUMA in beta cells,[21,23] and it is conceivable that the residual cell death observed following Bim and Bcl-XL KD is PUMA-dependent. Furthermore, Bcl-XL induces the retrotranslocation of Bax from the mitochondrial outer membrane to the cytoplasm, acting very early in the multistep Bax activation.[36] Thus, Bcl-XL inhibition may predispose cells to death in a broad way that cannot be prevented by the simultaneous KD of a single BH3-only protein.
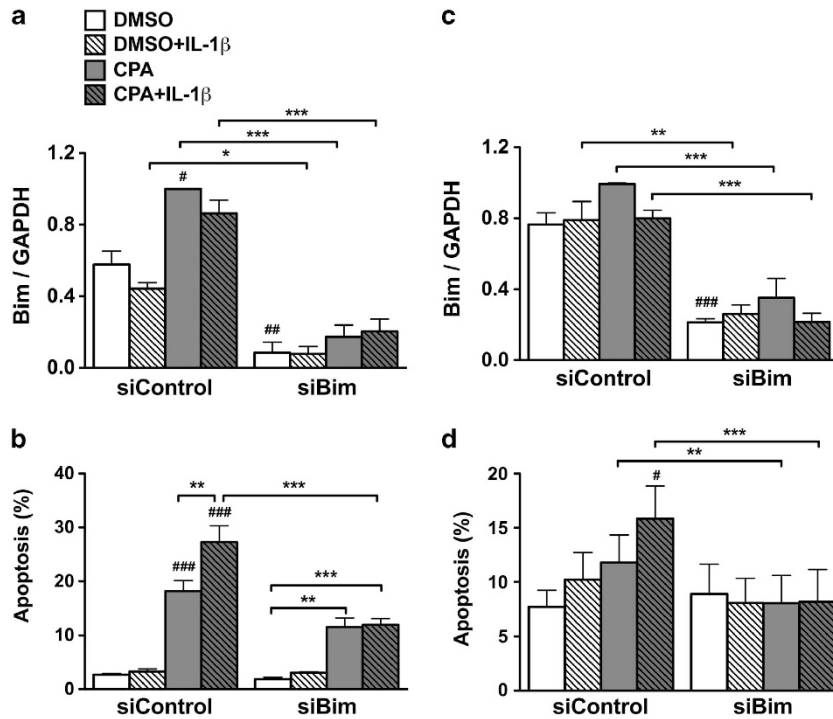
Figure 4 Bim KD protects against CPA + IL-1β-induced beta cell apoptosis in INS-1E and primary beta cells. INS-1E cells and primary rat beta cells were transfected with siRNA against Bim (siBim) or with negative control siRNA (siControl). After 48 h, cells were exposed to CPA (6.25 μM) or DMSO for 6 h and then treated or not with IL-1β (0.5 U/ml) for 24 h. INS-1E and primary beta cells (**a** and **c**, respectively) were harvested for determination of mRNA expression of Bim and the housekeeping gene *GAPDH*. Beta cell apoptosis was evaluated in INS-1E and primary beta cells (**b** and **d**, respectively) by Hoechst 33342/PI staining. Results are the mean ± S.E.M. of four independent experiments. #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ *versus* siControl + DMSO, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ *versus* indicated bars; ANOVA

Bim has a key role for ER stress plus IL-1β-induced apoptosis in INS-1E cells and primary beta cells (Figure 4), and it is also a central mediator of apoptosis following ER stress in other cell types.[37] CPA + IL-1β exposure preferentially induces the expression of the Bim$_S$. Bim$_S$ is the most pro-apoptotic variant of Bim, as it lacks the dynein domain that sequesters it to the cytoskeleton;[32] this allows Bim$_S$ to directly bind and activate Bax and Bak. In line with this, the preferential induction of Bim$_S$ is a key mechanism for apoptosis induced by B-RAF inhibitors in melanoma cells.[38] Bim activation is not the sole mechanism by which mild ER stress predisposes beta cells to apoptosis. Thus, we have previously described that ER stress leads to Mcl-1 degradation[27] and have presently observed that even a mild ER stress triggers a progressive inhibition of A1 expression. This contrasts with the observed increase in A1 mRNA expression induced by CPA. A similar phenomenon was observed with Mcl-1, that is, an increase in mRNA accompanied by a decrease in protein expression.[27] This suggests that both A1 and Mcl-1 are among the many proteins whose translation is inhibited by ER stress. A1 is regulated at the post-translational level by phosphorylation, cleavage and proteasome-mediated degradation,[39] which may further contribute to its progressive depletion during the UPR. As the four main anti-apoptotic proteins, namely Bcl-2, Bcl-XL, Mcl-1 and A1, are required to counteract the pro-apoptotic effects of Bim, a situation causing loss of any of these anti-apoptotic proteins will favor cell death. Here we demonstrate that ER stress in combination with an inflammatory cytokine leads to increased Bim and decreased A1 expression, culminating in beta cell death.

In conclusion, we presently demonstrated that the crosstalk between ER stress and the pro-inflammatory cytokine IL-1β sensitizes pancreatic beta cells to apoptosis mainly via an imbalance in the ratio of the anti-apoptotic A1 and the pro-apoptotic BH3-only protein Bim. This combination of factors may lead beta cells to overreact to a mild inflammatory insult, leading to increased beta cell demise, augmented insulitis and, consequently, increased risk of clinical T1D.

Materials and Methods

Culture of INS-1E cells and primary rat beta cells. The rat insulin-producing INS-1E cell line (passages 60–70, a kind gift from Dr. C Wollheim, Centre Medical Universitaire, Geneva, Switzerland) was cultured in RPMI 1640 GlutaMAX-I (Invitrogen, Carlsbad, CA, USA) with 5% heat-inactivated fetal bovine serum (FBS) and supplemented as described.[40]

Male Wistar rats (Charles River Laboratories, Brussels, Belgium) were housed and treated according to the Belgian Regulations for Animal Care guidelines. Rat pancreatic islets were isolated and pancreatic beta cells purified by fluorescence-activated cell sorting (FACSAria, BD Bioscience, San Jose, CA, USA).[41,42] The preparations used in this study contained 96 ± 1% of beta cells ($n = 7$). Purified beta cells were cultured in Ham's F-10 medium containing 10 mM glucose, 2 mM GlutaMAX, 50 μM 3-isobutyl-L-methylxanthine, 0.5% fatty acid-free BSA (Roche, Indianapolis, IN, USA), 5% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin. The same medium without FBS was used during the treatments described below.

Cell treatment. INS-1E cells and primary rat beta cells were exposed to 6.25 μM CPA (Sigma-Aldrich, Steinheim, Germany), a concentration that induces
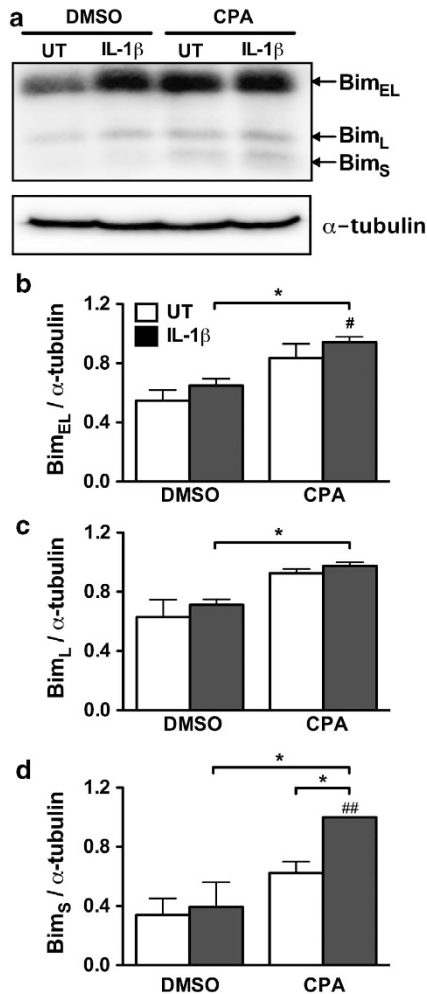
Figure 5 Augmented Bim expression correlates with increased beta cell apoptosis. INS-1E cells were exposed to CPA (6.25 μM) or DMSO for 6 h and then treated with IL-1β (0.5 U/ml) or left untreated (UT) for 24 h. Cells were harvested for Bim western blot (**a**). Expression of (**b**) Bim$_{EL}$, (**c**) Bim$_L$ and (**d**) Bim$_S$ was quantified by densitometry and corrected by α-tubulin. Results are the mean $\pm$ S.E.M. of four independent experiments. $^\#P < 0.05$ and $^{\#\#}P < 0.01$ versus DMSO, $^*P < 0.05$ versus indicated bars; ANOVA

mild ER stress in these cells[19,29] (Supplementary Figure 1). CPA was dissolved in dimethylsulfoxide (DMSO), which was used as control condition at the concentration of 0.03%. Cells were incubated for 6 h with CPA or DMSO and then treated with IL-1β (0.5 U/ml, R&D Systems, Abingdon, UK) or left untreated. This combined treatment was previously shown to exacerbate the pro-inflammatory response.[19] Alternatively, INS-1E cells were treated with IL-1β + IFN-γ (10 and 100 U/ml, respectively, R&D Systems) or left untreated. These cytokine concentrations were selected based on previous time-course and dose–response analyses.[31,43]

mRNA extraction and real-time RT-PCR. INS-1E cells and rat primary beta cells were lysed and harvested for mRNA extraction and reverse transcribed as described.[42] Gene expression was evaluated by real-time RT-PCR using SYBR Green.[42] Standard primers were used to construct a curve of cDNA with known copy numbers, to which unknown samples were compared.[44] Expression values were corrected by the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and normalized by the highest value of each experiment (considered as 1). GAPDH expression is not altered by the treatments used[45–47] (data not shown). The primers used for this study are described in Supplementary Table 1.
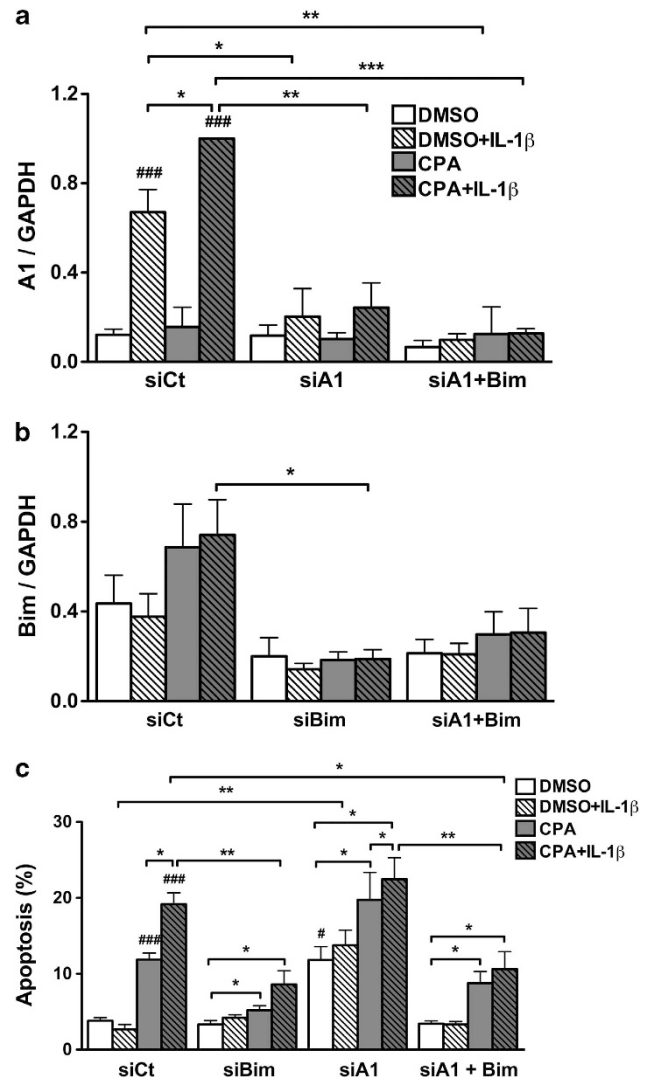

Figure 6 Bim KD protects against beta cell apoptosis induced by inactivation of A1 and CPA + IL-1β treatment. INS-1E cells were transfected with siRNA against A1 (siA1) alone or together with siRNA against Bim (siBim), or with negative control siRNA (siCt). After 48 h, cells were incubated with CPA (6.25 μM) or DMSO for 6 h and then treated or not with IL-1β (0.5 U/ml) for 24 h. Cells were harvested to determine A1 (**a**) and Bim (**b**) mRNA expression, which was corrected by the housekeeping gene GAPDH. (**c**) Beta cell apoptosis was evaluated by Hoechst 33342/PI staining. Results are the mean $\pm$ S.E.M. of five independent experiments. $^\#P < 0.05$ and $^{\#\#\#}P < 0.001$ versus siCt + DMSO, $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus indicated bars; paired t-test

Small interfering RNA (siRNA) transfection. Specific siRNAs were used to silence XBP1,[48] Bim,[21,24] Bcl-2, Bcl-XL,[23] A1 (Invitrogen)[28] and Mcl-1 (Thermo Scientific, Chicago, IL, USA).[25,27] These siRNAs have been previously tested and validated by our group, including confirmation of findings with a second siRNA.[21,23–25,27] An overnight transfection was performed with 30 nM siRNA mixed with Lipofectamine RNAiMAX (Invitrogen) as described.[26,49] Allstars Negative Control siRNA (Qiagen, Venlo, The Netherlands) was used as a negative control. We have previously shown that transfection with this control siRNA does not affect beta cell function, gene expression or viability as compared with nontransfected cells.[47,48,50]

Apoptosis assays. INS-1E cells and rat primary beta cells were incubated with the DNA dyes Hoechst 33342 (10 and 20 μl/ml, respectively) and propidium iodide (PI, 10 μl/ml) for 15 min. Beta cell viability was evaluated in at least 500
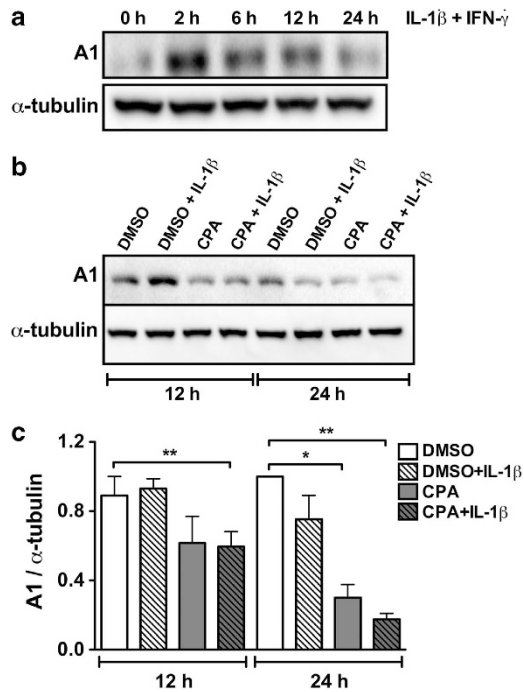
Figure 7 CPA and IL-1β progressively decrease A1 expression. (**a**) INS-1E cells were treated with IL-1β + IFN-γ (10 and 100 U/ml, respectively) for the indicated time points. (**b**) INS-1E were pretreated with CPA (6.25 μM) or DMSO for 6 h and then exposed or not to IL-1β (0.5 U/ml) for the indicated time points. Cells were harvested and A1 and α-tubulin protein expression was determined by western blot. (**c**) A1 expression was quantified by densitometry and corrected by α-tubulin. Results are the mean ± S.E.M. of three independent experiments. *P < 0.05 and **P < 0.01 *versus* indicated bars, paired t-test

cells per condition by two observers, one of them unaware of the sample identity.[42,51] In some experiments, presence of apoptosis was confirmed by examining expression of cleaved caspases 9 and 3 (see below).

Western blot. INS-1E cells were lysed and collected in Laemmli buffer. Whole-cell lysates were resolved by 10–14% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with antibodies targeting cleaved caspase 9, cleaved caspase 3, Bim, Bcl-XL, Bcl-2 (Cell Signaling, Danvers, MA, USA), Mcl-1 (Biovision, Milpitas, CA, USA) and A1 (Abcam, Cambridge, UK); α-tubulin was used as a control for protein loading (Sigma-Aldrich). Anti-rabbit or anti-mouse horseradish peroxidase-labeled anti-IgG (Lucron Bioproducts, De Pinte, Belgium) was used as secondary antibody. Immunoreactive protein bands were revealed using a chemiluminescent substrate (SuperSignal West Femto, Thermo Scientific), detected by ChemiDoc XRS$^+$ and quantified with ImageLab software (Bio-Rad, Hercules, CA, USA).

Statistical analysis. Data are presented as mean ± S.E.M. Comparisons were performed by two-tailed paired Student's t-test or by ANOVA, followed by Student's t-test with Bonferroni correction as indicated. A P-value < 0.05 was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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