mRNA of the pro-apoptotic gene *BBC3* serves as a molecular marker for TNF- α -induced islet damage in humans

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Abstract

Aims/hypothesis TNF- α plays important roles in the pathogenesis of type 1 and type 2 diabetes mellitus. In light of this, we examined the involvement of a pro-apoptotic gene, *BBC3* (also known as *PUMA*), in TNF- α -mediated beta cell dysfunction and destruction in human islets.

Methods Human islets were exposed in vitro to TNF- α alone or in combination with IFN- γ . Gene expression was assessed by RT-PCR using a set of single islets. Protein abundance and cellular localisation of *BBC3* were assessed by immunoblot and immunohistochemistry. A marginal number of islets were transplanted into diabetic NOD scid mice to correlate in vivo islet function with *BBC3* expression.

Results *BBC3* and *IL8* mRNA were upregulated in TNF- α -stimulated islets in a dose-dependent manner and enhanced through addition of IFN- γ , but not upregulated by IFN- γ alone. Immunohistochemistry revealed that TNF- α in combination with IFN- γ upregulated basal *BBC3* abundance in the cytoplasm of beta cells along with the perinuclear clustering of mitochondria partially co-localised with *BBC3*. TNF- α alone did not induce beta cell death, but did abrogate preproinsulin precursor mRNA

synthesis in response to high glucose stimulation, which was inversely associated with upregulation of *BBC3* mRNA expression by TNF- α . Higher *BBC3* mRNA expression in islets correlated with decreased graft function in vivo.

Conclusions/interpretation These results suggest that *BBC3* mRNA can serve as a molecular marker to detect early TNF- α -induced beta cell stress and may help identify islet-protective compounds for the treatment of diabetes.

Keywords *BBC3* · Human islets · Islet apoptosis · Preproinsulin · *PUMA* · TNF- α

Abbreviations

BAX	BCL-2-associated X protein
BBC3	BCL-2 binding component 3
BCL-2	B cell CLL/lymphoma 2
COX IV	Cytochrome <i>c</i> oxidase subunit IV
IEQ	Islet equivalent
NF- κ B	Nuclear factor- κ B
siRNA	Small interfering RNA
TMRE	Tetramethylrhodamine, ethyl ester, perchlorate
TNFRSF	TNF receptor superfamily

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Introduction

Proinflammatory cytokines are implicated in beta cell damage and apoptosis [1, 2]. Although the mechanisms remain elusive [3–5], TNF- α and IFN- γ work synergistically to induce beta cell apoptosis and are considered important factors in type 1 diabetes [6]. In type 2 diabetes, TNF- α is also an important mediator of insulin resistance associated with obesity [7–9]. TNF- α not only induces insulin resistance in insulin-sensitive tissues [10, 11], but also decreases glucose-stimulated insulin secretion [10], which

suggests that TNF- α mediates beta cell dysfunction and subsequent destruction.

TNF- α can induce two opposing signals, pro-apoptotic and anti-apoptotic, regulated by the activation of nuclear factor- κ B (NF- κ B) [12, 13]. TNF- α -mediated apoptosis occurs through the TNF receptor-associated death domain, to which the specific ligand receptor binds, leading to activation of caspase-8, followed by that of caspase-3 [14], which is known as an extrinsic pathway. Apoptosis through the intracellular (or intrinsic pathway) is initiated intracellularly by factors such as DNA damage, hypoxia, nutrient deprivation or reactive oxygen species, as well as via the mitochondrial pathway, and is tightly modulated by the B cell CLL/lymphoma 2 (BCL-2) proteins. Intracellular stress increases mitochondrial membrane permeability, which causes the release of cytochrome *c*, followed by activation of caspase-9, which activates caspase-3 [15]. Recent studies have shown that proinflammatory cytokines induce beta cell death through the intrinsic pathway by activating the BCL-2 homology 3 only subgroup of BCL-2 member proteins [16–18].

BCL-2 binding component 3 (BBC3), also known as p53 upregulated modulator of apoptosis (PUMA), is one of the most potent killers among these proteins [19, 20]. *BBC3* initiates apoptotic responses directly activated by p53-responsive elements in its promoter region [21] or acts independently of p53 through other transcription factors, including growth factors, cytokine deprivation [22] or endoplasmic reticulum stress [23]. *BBC3* can also be activated in response to TNF- α stimulation of the p65 component of NF- κ B through a κ B site in the *BBC3* promoter [24]. IL-1 β , together with IFN- γ , activates *BBC3* through NF- κ B activation, leading to mitochondrial BCL-2-associated X protein (BAX) translocation and induction of beta cell apoptosis [18]. It has not been determined whether *BBC3* induction by TNF- α plays a role in beta cell dysfunction and destruction.

The objective of the current study was to determine whether *BBC3* mRNA can serve as a biological marker that reflects beta cell damage/apoptosis mediated by TNF- α . We measured the expression of mRNA (or precursor mRNA) in human islets using a previously developed assay for determination of preproinsulin precursor mRNA levels and evaluated insulin biosynthesis using a set of single human islets [25]. *BBC3* expression induced in islets by TNF- α alone or with IFN- γ was compared with insulin synthesis and release, and beta cell apoptosis in vitro. *BBC3* mRNA levels were also compared with in vivo islet function in diabetic NOD*scid* mice.

Methods

Reagents and antibodies The following reagents and antibodies were used: for RT-PCR: M MLV reverse transcrip-

tase (Promega, San Luis Obispo, CA, USA), and SYBER green mix (Bio-Rad, Hercules, CA, USA); for tissue culture: recombinant human TNF- α , recombinant human IFN- γ , recombinant rat TNF- α and recombinant rat IFN- γ (R&D Systems, Minneapolis, MN, USA); BAY11-7082 (Calbiochem, San Diego, CA, USA); for western blot: BBC3, phospho-p65 (Ser536), caspase-9, cleaved caspase-8, cleaved caspase-3, β -actin, anti-rabbit IgG horseradish-peroxidase-linked antibody, LumiGLO chemiluminescent substrate (Cell Signaling Technology, Danvers, MA, USA); for immunohistochemistry: cytochrome *c* oxidase subunit IV (COX IV), BBC3 (Cell Signaling Technology), insulin (DAKO, Carpinteria, CA, USA), glucagon and DAPI (Sigma-Aldrich, St Louis, MO, USA), and corresponding secondary antibodies conjugated with AMCA, FITC, Texas Red, Cy5 (Jackson ImmunoResearch, West Grove, PA, USA); and for transfection and FACS: ON-TARGET^{plus} small interfering RNA (siRNA) reagents and siGLO transfection indicator (Dharmacon, Lafayette, CO, USA), tetramethylrhodamine, ethyl ester, perchlorate (TMRE) (Invitrogen, Carlsbad, CA, USA) and APC Annexin V (BD Biosciences, San Jose, CA, USA).

Human islet and acinar cell culture The use of human islets and acinar cells was approved by the Institutional Review Board of the City of Hope. Human islets and acinar cells isolated from pancreases approved for research use were obtained from the Southern California Islet Cell Resources Center, Beckman Research Institute of the City of Hope (Duarte, CA, USA) and used after 1 to 3 days in culture. Donors were of both sexes, with ages ranging from 18 to 67 (48 ± 14) years. Islet preparations with >70% purity and >90% viability were used. For mRNA assessment, islets between 150 and 300 μ m in diameter were hand-picked by a single experienced investigator under a dissection microscope without staining and a single islet per well was cultured in sextuplicate for each group in a non-tissue culture treated 96 well plate (Sarstedt, Newton, NC, USA) using CMRL1066-based (Mediatech, Holly Hill, FL, USA) serum-free medium. Islets were treated with recombinant human TNF- α (0, 1, 5, 50 ng/ml) alone or in combination with recombinant human IFN- γ (0, 10, 100, 1,000 U/ml) for up to 16 h. For other experiments, 500 to 1,000 islet equivalents (IEQ) were cultured at a concentration of 500 IEQ/ml in a Petri dish for up to 24 h in a specified condition. Acinar cells were kept in islet culture medium at 4°C immediately after isolation and used within 24 h. Five to ten clusters of acinar cells were placed in each well in triplicate and cultured in islet culture medium with or without TNF- α for up to 16 h. To assess islet-protective effects of various compounds, human islets were pre-incubated for 1 h with etanercept, FK506, ciclosporin, rapamycin, imatinib mesylate or SB203580 (p38 inhibitor),

followed by 4 h culture after addition of 5 ng/ml TNF- α . All compounds except etanercept (0.1 μ g/ml, dissolved in PBS) were dissolved in DMSO (0.1% wt/vol. at the final concentration) and used at a concentration of 10 μ mol/l.

Quantification of mRNA from a single islet After culture, handpicked single islets were transferred to a 96 well filter plate. Poly(A)⁺ RNA isolation was performed [Hem(A)⁺ System; Hitachi Chemical Research Center, Irvine, CA, USA] and cDNA was directly synthesised in each well as previously described [25]. The specific primer-primed cDNAs in the liquid phase were used for SYBR Green PCR (Bio-Rad). Differences in Cycle threshold (C_t) between the target and control mRNA (β actin) (ΔC_t) were used to quantify the relative amount of each target, calculated as $2^{-\Delta C_t}$. Primers used for gene expression assays have been previously described [25–28].

Measurement of INS precursor mRNA, mRNA synthesis and total insulin release Handpicked human islets were cultured for 16 h in 100 μ l RPMI 1640 medium containing 5% FBS and either low (3.3 mmol/l) or high glucose (17 mmol/l). To assess beta cell function, glucose-induced INS precursor mRNA [immature, pre-spliced poly(A)⁺ RNA] was measured by RT-PCR using the primers located in the intron. The results were normalized by the values of total INS mRNA measured using the primers located in the exon [25]. The culture supernatant fraction was collected from each well after 16 h culture to measure insulin release using a human insulin ELISA (Mercodia, Winston Salem, NC, USA).

Western blot Samples containing 500 IEQ human islets or siRNA-transfected INS-1 cells were collected before or 24 h after culture with TNF- α alone or together with IFN- γ , washed twice with ice-cold PBS and stored at -80°C until use. In other experiments, islets were pre-incubated for 1 h with 10 μ mol/l NF- κ B inhibitor, BAY11-7082, and subjected to cytokine stimulation. Cell lysis and western blot were performed as previously described [29].

Immunohistochemistry and beta cell apoptosis analysed by laser scanning cytometry Paraffin-embedded islet or pancreas sections were immunostained for BBC3, COX IV, insulin or glucagon as described previously [30]. Some sections were counterstained with DAPI for DNA. Images were obtained through the $\times 40$ objective of a fluorescent microscope (BX51; Olympus, Center Valley, PA, USA) equipped with a Pixera 600CL camera (Pixera, San Jose, CA, USA) or through the $\times 63$ objective of a laser scanning microscope (LSM510; Carl Zeiss, Thornwood, NY, USA). For apoptosis assessment, paraffin-embedded islet sections were stained using a fluorescein in situ apoptosis detection

kit (Apop Tag Plus; Chemicon, Temecula, CA, USA) followed by immunostaining for insulin and DAPI. Beta cell apoptosis was evaluated using a laser scanning cytometer (iCys; Compucyte, Westwood, MA, USA) as previously described [30].

siRNA transfection and flow cytometry analysis The rat *Bbc3* siRNA pool containing four individual siRNAs targeting *Bbc3* and a negative control siRNA (SMARTpool siRNA; Dharmacon) was transfected into a rat insulinoma cell line, INS-1, at 20 nmol/l concentration. Transfection was performed using siRNA reagents (ON-TARGETplus) according to the manufacturer's (Dharmacon) instructions. The INS-1 cells were cultured in RPMI 1640 medium containing 5% FBS and 15 mmol/l HEPES. Recombinant rat TNF- α (50 ng/ml) alone or with recombinant rat IFN- γ (1,000 U/ml) was added to the culture medium 24 h after transfection and further cultured for up to 48 h. Cultured cells were collected using TrypLE (Invitrogen) for FACS analysis performed on an analyser (CyAn ADP; Beckman Coulter, Fullerton, CA, USA). To assess mitochondrial membrane permeability, INS-1 cells were incubated for 30 min in culture medium containing 100 nmol/l TMRE and washed twice with PBS before FACS analysis. Cell death was analysed using FACS and staining with 1 μ g/ml of DAPI.

Assessment of in vivo islet function in diabetic NODscid mice Male NODscid mice, 10 to 12 weeks of age, were obtained from the Animal Resources Center of Beckman Research Institute of the City of Hope and used as islet recipients. Mice were rendered diabetic by intraperitoneal injections of 50 mg/kg streptozotocin (Sigma-Aldrich) for three consecutive days. Mice that exhibited hyperglycaemia (>19.4 mmol/l) for two consecutive days were used as recipients. Human islets (1,200 IEQ) were transplanted under the left kidney capsule of diabetic mice and blood glucose levels measured two to three times weekly. Recipient mice that maintained blood glucose levels <11.1 mmol/l were considered to have reversed diabetes. At the end of each experiment, a nephrectomy was performed to confirm graft dependence. In separate experiments, islets were isolated from male Lewis rats weighing 250 to 350 g (Charles River Laboratories, Wilmington, MA, USA) using our laboratory's standard procedure [31]. Handpicked islets ($n=250$) transfected with either *Bbc3* siRNA or control siRNA were transplanted into the liver of diabetic NODscid mice via the portal vein. All animal procedures followed protocols approved by the Institutional Animal Care and Use Committee of the City of Hope/Beckman Research Institute.

Statistical analysis Data are presented as a mean $\pm$ SEM. Paired two-tailed Student's *t* test was used to compare the

two groups. The correlation and analysis of variance procedures were applied to assess the strength of linear dependence between two variables (correlation coefficient: r). p values of $p < 0.05$ were considered significant.

Results

BBC3 mRNA is elevated in human islets following TNF- α stimulation Rat islets are known to produce one of the receptors for TNF- α , TNF- α receptor superfamily (TNFRSF)1A, but little or none of the other TNF- α receptor, TNFRSF1B [2]. We confirmed that isolated human islet and acinar cell expression of *TNFRSF1A*

mRNA was abundant, while *TNFRSF1B* mRNA was only detectable at a low level (electronic supplementary material [ESM] Fig. 1a).

To examine whether TNF- α treatment of human islets induces cell death through the intrinsic pathway, islets were treated with TNF- α (50 ng/ml, 16 h) and the expression of apoptosis-related BCL-2 family genes was assessed. TNF- α induced a 1.9- to 4.7-fold increase of *BBC3* mRNA over untreated controls, while other BCL-2 family mRNAs increased less than twofold ($n=4$) (Fig. 1a). After TNF- α exposure, *BBC3* mRNA in human islets was elevated within 1 h, peaked by 4 h and remained at near-peak levels for over 16 h (Fig. 1b). This *BBC3* mRNA expression was dependent on TNF- α dose (Fig. 1b). Along with *BBC3*, *IL8*

Fig. 1 TNF- α predominantly induces *BBC3* in human islets. **a** Induction of BCL-2 family mRNA by stimulation of human islets by TNF- α . Single islets, in sextuplicate, were stimulated with or without TNF- α (50 ng/ml) for 16 h. Expression of each gene was assessed by RT-PCR and normalised by *ACTB*. The fold increase was calculated by dividing TNF- α -treated islets by control islets; $n=4$; $p < 0.01$. *BAK*, also known as *BAK1*; *BIM*, also known as *BCL2L11*; *BCLXS*, also known as *BCL2L1*. **b** *BBC3*, **(c)** *IL8* and **(d)** *TNF* mRNA

expression kinetics normalised by *ACTB* measured in islets stimulated with 0 (white circles), 1 (black triangles), 5 (black squares) and 50 (black diamonds) ng/ml TNF- α for 0, 1, 2, 4 and 16 h; $n=3$, each in triplicate. **e** *BBC3* and **(f)** *IL8* mRNA expression in human islets stimulated for 4 h with TNF- α (1, 5, 50 ng/ml) and/or IFN- γ (10, 100, 1,000 U/ml). Results show representative data from three independent cases; * $p < 0.05$ and *** $p < 0.001$ vs control; † $p < 0.01$ vs TNF(50). All data (**a–f**) are presented as mean $\pm$ SEM

mRNA increased in the islets in a dose-dependent manner (Fig. 1c). Treatment with exogenous TNF- α induced a slight endogenous *TNF* mRNA expression in human islets (Fig. 1d). Acinar cells treated with TNF- α also showed increased *BBC3*, *IL8* and *TNF* mRNA expression; however, this expression peaked at 4 h and returned to basal levels by 16 h (ESM Fig. 1b–d).

To test whether passenger leucocytes residing in pancreatic tissue are responsible for *BBC3*, *IL8* and *TNF* mRNA expression, we treated human blood leucocytes with TNF- α . TNF- α treatment at doses up to 200 ng/ml did not induce *BBC3* mRNA, but did elevate *IL8* and endogenous *TNF* mRNA, confirming that passenger leucocytes were not involved in the *BBC3* mRNA measured in TNF- α -treated islets and acinar cells (data not shown).

Human islets were treated with recombinant human IFN- γ alone or in combination with TNF- α . IFN- γ alone induced neither *BBC3* nor *IL8* mRNA. However, when

combined with TNF- α , IFN- γ strongly augmented TNF- α -mediated *BBC3* mRNA expression (Fig. 1e, f).

BBC3 protein is upregulated in human islets through NF- κ B activation Translational changes of BBC3 over 24 h by TNF- α alone or in combination with IFN- γ (TNF- α + IFN- γ) were examined by western blot. BBC3 protein abundance was significantly increased by TNF- α alone (50 ng/ml, $p<0.05$), as well as by TNF- α + IFN- γ ($p<0.01$), but not by IFN- γ (1,000 U/ml) alone, as compared with control (Fig. 2a), these finding being consistent with mRNA expression. The involvement of NF- κ B in BBC3 upregulation was measured by the phosphorylation of p65 protein. Phosphorylation of p65 protein increased in islets treated with TNF- α , but not in those treated with IFN- γ . Phosphorylated p65 abundance induced by TNF- α further increased with the addition of IFN- γ (Fig. 2b). Pre-incubation of islets with an NF- κ B inhibitor, BAY11-7082,

Fig. 2 IFN- γ alone does not induce BBC3, but augments TNF- α -induced BBC3 production. IEQ (500) were cultured for 24 h with TNF- α (5 or 50 ng/ml) and/or IFN- γ (1,000 U/ml). Cell lysate was used for western blot to examine (a) BBC3 abundance and (b) phosphorylation of p65 component (Ser536) of NF- κ B in islets; $n=3$; * $p<0.05$, ** $p<0.01$. c, d BBC3 and phosphorylated p65 were examined by western blot in islets pre-incubated for 1 h with an NF- κ B inhibitor, BAY11-7082, prior to treatment with (c) TNF- α (50 ng/ml) alone and (d) TNF- α (50 ng/ml) + IFN- γ (1,000 U/ml) treatment; $n=4$; * $p<0.05$. Target protein abundance (a–d) is normalised by β -actin; all values are presented as mean $\pm$ SEM

significantly suppressed the upregulation of BBC3 protein mediated by TNF- α ($p<0.05$) (Fig. 2c), but not that mediated by TNF- α + IFN- γ (Fig. 2d).

Treatment of human islets with TNF- α and IFN- γ induced mitochondrial clustering and increased BBC3 protein in beta cell cytoplasm Confocal microscopy was used to examine BBC3 abundance in human islets treated with TNF- α + IFN- γ for 24 h. Paraffin sections of the islets were stained for BBC3, COX IV (a marker for mitochondria) and insulin. BBC3 abundance was upregulated in TNF- α + IFN- γ -treated islets (Fig. 3a, b). This treatment also increased mitochondrial condensation and perinuclear clustering as shown by COX IV staining in control cells vs TNF- α + IFN- γ -treated cells. Furthermore, higher BBC3 levels in the cytoplasm were associated with morphological changes and cellular redistribution of mitochondria. The merged image of BBC3, COX IV and insulin shows co-localisation of some BBC3 with mitochondria around the nucleus of beta cells (Fig. 3b, c). However, the majority of

cytoplasmic BBC3 was independent of COX IV. Figure 3c (merged panels, see legend) confirmed that insulin staining was independent of BBC3 and COX IV staining, respectively. To examine BBC3 protein abundance in minimally manipulated pancreatic cells, paraffin sections of pancreas tissue taken from cold preserved pancreases before islet isolation were stained for BBC3 and insulin or BBC3 and glucagon (ESM Fig. 1). BBC3 co-localised with beta cells, but not with glucagon-positive alpha cells. Acinar cells surrounding islets were also negative for BBC3.

Transfection of Bbc3 siRNA prevents TNF- α - and IFN- γ -induced beta cell death Western blot was used to detect activation of caspase-8, -9 and -3 to determine whether islet apoptosis induced by TNF- α or TNF- α + IFN- γ is regulated by the extrinsic or intrinsic pathway. Cleaved caspase-9 and cleaved caspase-3 were detected in islets treated with TNF- α and further increased by co-treatment with IFN- γ (Fig. 4a). In contrast, caspase-8 showed very low abundance in TNF- α - and TNF- α + IFN- γ -treated

Fig. 3 TNF- α + IFN- γ -induced upregulation of BBC3 and clustering of mitochondria in cytoplasm of human beta cells. Paraffin sections of human islets cultured for 24 h with or without TNF- α (50 ng/ml) + IFN- γ (1,000 IU/ml) were stained for BBC3 (red), COX IV (green) and insulin (blue). **a** Cultured with medium alone or **(b, c)** with TNF- α + IFN- γ . Arrows **(a, b)** indicate mitochondria clustered in the perinuclear region. **c** Enlargements of a representative beta cell marked

above **(b)**, which produces BBC3. Individual staining for BBC3 (red only), COX IV (green only) and insulin (blue only) is shown, along with merged double staining for BBC3 and insulin (red, blue), COX IV and insulin (green, blue), and BBC3 and COX IV (red, green). Triple staining for BBC3, COX IV and insulin is also shown. Arrows **(c)** indicate co-localisation of BBC3 and mitochondria. Scale bars, 20 μ m

Fig. 4 BBC3 is involved in TNF- α + IFN- γ -induced beta cell death. **a** Islets were examined by western blot to determine activation of caspase-8, caspase-9 and caspase-3 after culture for 24 h with TNF- α (5 and 50 ng/ml) and/or IFN- γ (1,000 U/ml). The blot shows one of three independent cases. **b** Paraffin sections of human islets cultured for 24 h with TNF- α (50 ng/ml) and **(c)** with combined TNF- α (50 ng/ml) and IFN- γ (1,000 U/ml) were stained for TUNEL and insulin. Apoptotic beta cells were then quantified using laser scanning cytometry and the percentage of apoptotic beta cells was calculated by dividing the TUNEL-insulin double-positive cell number by the total number of insulin-positive cells in each section; $n=3$, * $p<0.05$. **d**

INS-1 cells were transfected with *Bbc3* siRNA (black bars) or control siRNA (white bars) for 24 h before cytokine stimulation as indicated, and cultured for an additional 48 h before FACS analysis. Mitochondrial membrane permeability of INS-1 cells treated with cytokines was assessed by TMRE staining; $n=3$, ** $p<0.01$. **e** Western blot for cleaved caspase-3 in *Bbc3* siRNA or control siRNA transfected INS-1 cells treated with TNF- α or TNF- α +IFN- γ . The blot shows the results of one of four independent experiments. **f** Densitometric quantification of the bands shown above (**e**); $n=4$, * $p<0.05$. All values (**b**, **c**, **d**, **f**) are presented as mean $\pm$ SEM

islets. These results indicate that TNF- α -induced islet cell apoptosis occurs primarily through the intrinsic pathway. Induction of beta cell apoptosis by these cytokines was also examined by co-staining cytokine-treated human islets with the late stage apoptotic marker TUNEL and insulin. TNF- α (50 ng/ml) treatment did not increase percentages of apoptotic beta cells and thus TNF- α alone is assumed to not induce beta cell death (Fig. 4b), whereas TNF- α + IFN- γ did induce apoptosis in a significant number of beta cells (Fig. 4c).

To further determine the involvement of BBC3 in TNF + IFN- γ -induced beta cell death, INS-1 cells were transfected with *Bbc3* siRNA to silence the gene. *Bbc3* siRNA transfection (transfection rate >80%) suppressed *Bbc3* mRNA expression to 22.2 $\pm$ 8.6% of the control. Silencing *Bbc3* significantly reduced INS-1 cell death induced by TNF- α + IFN- γ in 48 h culture as assessed by FACS (27.1 $\pm$ 4.9% in the *Bbc3* siRNA group vs 57.7 $\pm$ 3.6% in the control siRNA group, $n=3$, $p<0.01$) (ESM Fig. 2a). The percentage of cells positive for TMRE was also higher in INS-1 cells transfected with *Bbc3* siRNA than in controls (65.7 $\pm$ 3.4% vs 37.5 $\pm$ 0.8%, $n=3$, $p<0.01$) (Fig. 4d, ESM Fig. 2b), indicating

that *Bbc3* siRNA protects the mitochondrial membrane potential from TNF- α + IFN- γ -induced damage. Furthermore, *Bbc3* is required for caspase-3 activation in response to TNF- α + IFN- γ treatment in INS-1 cells (Fig. 4e, f).

INS precursor mRNA synthesis, but not insulin release from human islets, is inhibited by TNF- α treatment Insulin release and synthesis by islets, as indicators of beta cell function, were assessed in single human islets treated with TNF- α using a previously developed method [25]. Addition of 50 ng/ml TNF- α to low or high glucose medium did not affect insulin release during the 16 h culture period (ESM Fig. 3a). However, glucose-induced *INS* precursor mRNA synthesis was totally abolished by TNF- α ($p<0.05$) (ESM Fig. 3b). We further examined the dose effect of TNF- α with or without IFN- γ on *INS* precursor mRNA synthesis. TNF- α , at a concentration as low as 5 ng/ml, abrogated *INS* precursor mRNA synthesis of human islets in response to high glucose stimulation. In contrast, IFN- γ alone did not impair glucose-induced *INS* precursor mRNA upregulation (ESM Fig. 3c). ESM Figure 3d, e show the

upregulation of *BBC3* mRNA and downregulation of *INS* precursor mRNA in human islets by TNF- α (50 ng/ml). *BBC3* mRNA levels inversely correlated with *INS* precursor mRNA levels in islets cultured in high-glucose medium with different doses of TNF- α or TNF- α + IFN- γ treatment (Fig. 5, ESM Fig. 3f).

BBC3 expression in pre-transplant islets closely correlates with post-transplant graft function in diabetic NODscid mice Since the above in vitro results indicated that *BBC3* expression correlates with the insulin gene transcription rate in response to glucose, we examined whether *BBC3* expression correlates with in vivo beta cell function by transplanting 1,200 IEQ human islets under the renal capsule of streptozotocin-induced diabetic NODscid mice. Levels of *BBC3* mRNA in human islets shortly after isolation varied between islet lots (ESM Fig. 4a). These variations may have been due to exposure to cytokines, including TNF- α , released during cold ischaemia and re-warming of the pancreas, islet isolation and culture [32, 33]. *BBC3* expression positively correlated with blood glucose levels 30 days after transplantation ($r=0.64$, $p<0.001$) (ESM Fig. 4a). Blood glucose levels of mice transplanted with islets expressing lower *BBC3* mRNA were consistently lower than those receiving islets with higher *BBC3* mRNA (ESM Fig. 4b). Islet lots with lower *BBC3* mRNA levels had lower beta cell apoptosis (%) (ESM Fig. 4c) and achieved euglycaemia by day 30, while those with higher *BBC3* mRNA tended to contain more apoptotic beta cells and failed to reverse hyperglycaemia. We further tested the role of *Bbc3* on in vivo islet function by transplanting a marginal number of rat islets in which *Bbc3* was suppressed by *Bbc3* siRNA transfection into the liver of diabetic NODscid mice. Although the transfection rate was low ($25.3\pm0.9\%$) (ESM Fig. 4d, e), *Bbc3* siRNA

transfection reduced early islet loss as indicated by blood glucose levels of 8.5 ± 1.4 mmol/l in the *Bbc3* siRNA group vs 24.8 ± 3.5 mmol/l in the control siRNA group on day 3 ($p<0.05$). Furthermore, diabetes was reversed in all mice receiving *BBC3* siRNA-transfected islets, while mice receiving control islets remained diabetic (ESM Fig. 4f, g). These results indicate an inverse correlation between *BBC3* (*Bbc3*) mRNA expression and islet function in vivo.

Potential of *BBC3* mRNA as a molecular marker to screen compounds that protect islets from TNF- α -induced damage

Results thus far indicate that *BBC3*, along with *IL8* and *TNF* mRNA, may be used as molecular markers for screening the effects of compounds on islets. We conducted a pilot study to test the effect of various compounds on human islets treated by TNF- α . Human islets were incubated for 1 h with several compounds (listed below), stimulated by TNF- α (5 ng/ml) for an additional 4 h, and expression of *BBC3*, *IL8* and *TNF* was measured. Compounds were selected on the basis of their specific traits: (1) etanercept, a TNF- α receptor blocker reported to improve glycaemic control in clinical trials in patients with type 1 diabetes [34]; (2) FK506, ciclosporin A and rapamycin, clinically used immunosuppressants that modulate inflammatory/immune reactions; (3) imatinib mesylate, a tyrosine kinase inhibitor that suppresses NF- κ B activation and has been shown to protect islets from combined cytokines in vitro and to prevent spontaneous onset of diabetes in NOD mice [35]; and (4) SB203580, a p38 mitogen-activated protein kinase inhibitor that has been shown to prevent beta cell death, in part, by regulating TNF- α abundance [32, 36, 37]. Pre-incubation of islets with etanercept prevented TNF- α -induced upregulation of *BBC3* (Fig. 6a), *IL8* (Fig. 6b) and *TNF* (Fig. 6c) mRNA expression ($p<0.05$ vs control). Pre-incubation of islets with imatinib mesylate also reduced the upregulation of all these mRNAs when compared with control ($p<0.05$).

Fig. 5 *BBC3* mRNA upregulation correlates with downregulation of glucose-mediated *INS* precursor mRNA in an islet. Correlation between *BBC3* mRNA and *INS* precursor mRNA levels in islets cultured in high-glucose medium and treated for 16 h with TNF- α (1, 5 and 50 ng/ml), IFN- γ (10, 100 and 1,000 U/ml) or a combination of both. Data points are representative data from three independent cases and represent 53 single islets; $r = -0.45$, $p<0.001$

Discussion

Inflammation contributes to islet cell dysfunction and destruction, impairs beta cell regeneration and even causes peripheral insulin resistance [38, 39]. We have shown dose-dependent induction of a pro-apoptotic gene, *BBC3*, in human islets, following exposure to TNF- α alone or together with IFN- γ .

In INS-1 cells, *Bbc3* activation by IL-1 β and IFN- γ leads to mitochondrial BAX translocation followed by cytochrome *c* release, and to caspase-3 cleavage [18]. However, little was known about the expression of *BBC3* in human beta cells. We have clearly shown that *BBC3* is upregulated by exposure of beta cells to TNF- α alone or in combination with IFN- γ . Interestingly, *BBC3* mRNA expres-

Fig. 6 *BBC3*, *IL8* and *TNF* mRNA were used as markers to screen compounds that protect islets from TNF- α -mediated damage. Single human islets, in sextuplicate, were pre-incubated for 1 h at 37°C with various drugs or control (DMSO), followed by stimulation with or without 5 ng/ml TNF- α for an additional 4 h. **a** *BBC3*, **(b)** *IL8* and **(c)**

TNF and *ACTB* mRNA were quantified and the results expressed as % *ACTB*. All data are representative data from three independent experiments and are presented as mean $\pm$ SEM; * p <0.05, ** p <0.01. CsA, ciclosporin; p38-IH, SB203580 (p38 inhibitor)

sion in control islets without TNF- α decreased over the 16 h culture period (Fig. 1b). The high expression of *BBC3* at time 0 may indicate that the islets had been exposed to cytokines released from passenger leucocytes, acinar and other contaminating cells during isolation and culture prior to the experiment. The decrease in *BBC3* mRNA was, in part, supported by the decrease in *TNF* mRNA expression as shown in Fig. 1d, as well as by previous findings presented by ourselves [37] and others [33].

Upregulation of *BBC3* is associated with translocation of mitochondria to the perinuclear area and partial co-localisation of *BBC3* with mitochondria (Fig. 3). Mitochondrial translocation near the nucleus is known to be specific to the TNF- α receptor-induced cytotoxic response linked to cytokine-mediated cell death, and is considered an early event of apoptosis [40]. Mitochondrial condensation and perinuclear clustering have also been reported to occur in several cell types following translocation of BAX from cytoplasm to mitochondria [41]. Co-localisation of *BBC3* and mitochondria has also been found in ischaemic neuronal cells [42]. The question of how *BBC3* interacts with other BCL-2 member proteins to induce apoptosis in human beta cells still needs to be elucidated. TNF- α alone mediates mitochondrial stress, but not cell death (Fig. 4b), as supported by western blot showing the presence of *BBC3* (Fig. 2a) and weakly positive caspase-9 and caspase-3 (Fig. 4a). Our evaluation of beta cell function indicates that TNF- α -mediated *BBC3* expression represents early-stage cell damage. Upregulation of *BBC3* had little effect on glucose-mediated insulin release, but markedly decreased glucose-stimulated *INS* precursor mRNA synthesis. Our results are consistent with previous results, in which TNF- α impaired beta cell function without affecting cell survival or proliferation of beta cell lines [43, 44]. It is important to recognise that abrogation of *INS* precursor mRNA synthesis in response to glucose was associated with upregulation of *BBC3* mRNA by TNF- α or TNF- α + IFN- γ (Fig. 5), although it is not known how these genes interact.

BAY11-7082, an NF- κ B inhibitor, suppressed *BBC3* protein production induced by TNF- α alone, but not induced by TNF- α + IFN- γ . Our results agree with those of Gurzov et al. [18], who showed that adenovirus-mediated inactivation of NF- κ B decreases *Bbc3* mRNA in INS-1 cells during 6 h, but not during 24 h treatment with IL-1 β +IFN- γ , suggesting the involvement of multiple pathways in *BBC3* regulation. *BBC3* mRNA expression or *BBC3* levels are different between isolated islets and acinar cells, and between isolated and intact acinar cells in pancreatic tissues. These differences may be due to different levels of NF- κ B activation and/or involvement of other pathways in *BBC3* upregulation. Further studies are required to elucidate mechanisms involved in human islet *BBC3* expression.

In summary, we have demonstrated that TNF- α alone or in combination with IFN- γ upregulates *BBC3* mRNA expression in human islets. *BBC3* upregulation is associated with mitochondrial stress and induction of apoptosis. TNF- α -induced upregulation of *BBC3* mRNA was inversely associated with glucose-induced *INS* precursor mRNA synthesis. These results suggest that *BBC3* mRNA may be an indicator for evaluating the effect of various compounds/drugs on TNF- α -mediated beta cell damage.

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References

- Eizirik DL, Mandrup-Poulsen T (2001) A choice of death—the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* 44:2115–2133
- Stephens LA, Thomas HE, Ming L et al (1999) Tumor necrosis factor- α -activated cell death pathways in NIT-1 insulinoma cells and primary pancreatic beta cells. *Endocrinology* 140:3219–3227
- Picarella DE, Kratz A, Li CB, Ruddle NH, Flavell RA (1993) Transgenic tumor necrosis factor (TNF)- α production in pancreatic islets leads to insulinitis, not diabetes. Distinct patterns of inflammation in TNF- α and TNF- β transgenic mice. *J Immunol* 150:4136–4150
- Satoh J, Seino H, Abo T et al (1989) Recombinant human tumor necrosis factor α suppresses autoimmune diabetes in nonobese diabetic mice. *J Clin Invest* 84:1345–1348
- Yang XD, Tisch R, Singer SM et al (1994) Effect of tumor necrosis factor α on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process. *J Exp Med* 180:995–1004
- Suk K, Kim S, Kim YH et al (2001) IFN- γ /TNF- α synergism as the final effector in autoimmune diabetes: a key role for STAT1/IFN regulatory factor-1 pathway in pancreatic beta cell death. *J Immunol* 166:4481–4489
- Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM (1994) Tumor necrosis factor α inhibits signaling from the insulin receptor. *Proc Natl Acad Sci USA* 91:4854–4858
- Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS (1997) Protection from obesity-induced insulin resistance in mice lacking TNF- α function. *Nature* 389:610–614
- Steinberg GR, Michell BJ, van Denderen BJ et al (2006) Tumor necrosis factor α -induced skeletal muscle insulin resistance involves suppression of AMP-kinase signaling. *Cell Metab* 4:465–474
- Bouzakri K, Zierath JR (2007) MAP4K4 gene silencing in human skeletal muscle prevents tumor necrosis factor- α -induced insulin resistance. *J Biol Chem* 282:7783–7789
- Tesz GJ, Guilherme A, Guntur KV et al (2007) Tumor necrosis factor α (TNF α) stimulates Map4k4 expression through TNF α receptor 1 signaling to c-Jun and activating transcription factor 2. *J Biol Chem* 282:19302–19312
- Aggarwal BB (2003) Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev* 3:745–756
- Wajant H, Pfizenmaier K, Scheurich P (2003) Tumor necrosis factor signaling. *Cell Death Differ* 10:45–65
- Hengartner MO (2000) The biochemistry of apoptosis. *Nature* 407:770–776
- Danial NN, Korsmeyer SJ (2004) Cell death: critical control points. *Cell* 116:205–219
- McKenzie MD, Carrington EM, Kaufmann T et al (2008) Proapoptotic BH3-only protein Bid is essential for death receptor-induced apoptosis of pancreatic beta-cells. *Diabetes* 57:1284–1292
- Grunnet LG, Aikin R, Tonnesen MF et al (2009) Proinflammatory cytokines activate the intrinsic apoptotic pathway in beta-cells. *Diabetes* 58:1807–1815
- Gurzov EN, Germano CM, Cunha DA et al (2010) p53 up-regulated modulator of apoptosis (PUMA) activation contributes to pancreatic beta cell apoptosis induced by pro-inflammatory cytokines and endoplasmic reticulum stress. *J Biol Chem* 285:19910–19920
- Sun Q, Ming L, Thomas SM et al (2009) PUMA mediates EGFR tyrosine kinase inhibitor-induced apoptosis in head and neck cancer cells. *Oncogene* 28:2348–2357
- Vousden KH (2005) Apoptosis. p53 and PUMA: a deadly duo. *Science* 309:1685–1686
- Yu J, Wang Z, Kinzler KW, Vogelstein B, Zhang L (2003) PUMA mediates the apoptotic response to p53 in colorectal cancer cells. *Proc Natl Acad Sci USA* 100:1931–1936
- You H, Pellegrini M, Tsuchihara K et al (2006) FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. *J Exp Med* 203:1657–1663
- Reimertz C, Kogel D, Rami A, Chittenden T, Prehn JH (2003) Gene expression during ER stress-induced apoptosis in neurons: induction of the BH3-only protein Bbc3/PUMA and activation of the mitochondrial apoptosis pathway. *J Cell Biol* 162:587–597
- Wang P, Qiu W, Dudgeon C et al (2009) PUMA is directly activated by NF- κ B and contributes to TNF- α -induced apoptosis. *Cell Death Differ* 16:1192–1202
- Omori K, Mitsuhashi M, Todorov I et al (2010) Microassay for glucose-induced preproinsulin mRNA expression to assess islet functional potency for islet transplantation. *Transplantation* 89:146–154
- Mitsuhashi M, Targan SR (2008) Ex vivo simulation of IgG Fc and T cell receptor functions: an application to inflammatory bowel disease. *Inflamm Bowel Dis* 14:1061–1067
- Mitsuhashi M, Tomozawa S, Endo K, Shinagawa A (2006) Quantification of mRNA in whole blood by assessing recovery of RNA and efficiency of cDNA synthesis. *Clin Chem* 52:634–642
- Mitsuhashi M, Endo K, Obara K, Izutsu H, Ishida T, Chikatsu N, Shinagawa A (2008) Quantification of drug-induced mRNA in human whole blood ex vivo. *Clinical Medicine: Blood Disorders* 1:1–11, Available from www.la-press.com/quantification-of-drug-induced-mrna-in-humanwhole-blood-ex-vivo-a491. Accessed 1 May 2011
- Omori K, Valiente L, Orr C et al (2007) Improvement of human islet cryopreservation by a p38 MAPK inhibitor. *Am J Transplant* 7:1224–1232
- Todorov I, Nair I, Avakian-Mansoorian A et al (2010) Quantitative assessment of beta-cell apoptosis and cell composition of isolated, undisturbed human islets by laser scanning cytometry. *Transplantation* 90:836–842
- Itakura S, Asari S, Rawson J et al (2007) Mesenchymal stem cells facilitate the induction of mixed hematopoietic chimerism and islet allograft tolerance without GVHD in the rat. *Am J Transplant* 7:336–346
- Ito T, Omori K, Rawson J et al (2008) Improvement of canine islet yield by donor pancreas infusion with a p38MAPK inhibitor. *Transplantation* 86:321–329
- Hanley S, Liu S, Lipsett M et al (2006) Tumor necrosis factor- α production by human islets leads to postisolation cell death. *Transplantation* 82:813–818
- Mastrandrea L, Yu J, Behrens T et al (2009) Etanercept treatment in children with new-onset type 1 diabetes: pilot randomized, placebo-controlled, double-blind study. *Diabetes Care* 32:1244–1249
- Hagerkvist R, Sandler S, Mokhtari D, Welsh N (2007) Amelioration of diabetes by imatinib mesylate (Gleevec): role of beta-cell NF- κ B activation and anti-apoptotic preconditioning. *FASEB J* 21:618–628
- Matsuda T, Omori K, Vuong T et al (2005) Inhibition of p38 pathway suppresses human islet production of pro-inflammatory cytokines and improves islet graft function. *Am J Transplant* 5:484–493
- Omori K, Todorov I, Shintaku J et al (2010) P38 α -selective mitogen-activated protein kinase inhibitor for improvement of cultured human islet recovery. *Pancreas* 39:436–443
- Eizirik DL, Colli ML, Ortis F (2009) The role of inflammation in insulinitis and beta-cell loss in type 1 diabetes. *Nat Rev* 5:219–226

39. Donath MY, Storling J, Maedler K, Mandrup-Poulsen T (2003) Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes. *J Mol Med (Berlin, Germany)* 81:455–470
40. De Vos K, Goossens V, Boone E et al (1998) The 55-kDa tumor necrosis factor receptor induces clustering of mitochondria through its membrane-proximal region. *J Biol Chem* 273:9673–9680
41. Desagher S, Martinou JC (2000) Mitochondria as the central control point of apoptosis. *Trends Cell Biol* 10:369–377
42. Niizuma K, Endo H, Nito C, Myer DJ, Chan PH (2009) Potential role of PUMA in delayed death of hippocampal CA1 neurons after transient global cerebral ischemia. *Stroke* 40:618–625
43. Kwon G, Xu G, Marshall CA, McDaniel ML (1999) Tumor necrosis factor alpha-induced pancreatic beta-cell insulin resistance is mediated by nitric oxide and prevented by 15-deoxy-Delta12,14-prostaglandin J2 and aminoguanidine. A role for peroxisome proliferator-activated receptor gamma activation and inos expression. *J Biol Chem* 274:18702–18708
44. Kim HE, Choi SE, Lee SJ et al (2008) Tumour necrosis factor-alpha-induced glucose-stimulated insulin secretion inhibition in INS-1 cells is ascribed to a reduction of the glucose-stimulated Ca^{2+} influx. *J Endocrinol* 198:549–560