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Mechanisms of toxicity by proinflammatory cytokines in a novel human pancreatic beta cell line, 1.1B4

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ABSTRACT

Background: Molecular mechanisms of toxicity and cell damage were investigated in the novel human beta cell line, 1.1B4, after exposure to proinflammatory cytokines — IL-1β, IFN-γ, TNF-α.
Methods: MTT assay, insulin radioimmunoassay, glucokinase assay, real time reverse transcription PCR, western blotting, nitrite assay, caspase assay and comet assay were used to investigate mechanisms of cytokine toxicity.
Results: Viability of 1.1B4 cells decreased after 18 h cytokine exposure. Cytokines significantly reduced cellular insulin content and impaired insulin secretion induced by glucose, alanine, KCl, elevated Ca^{2+}, GLP-1 or forskolin. Glucokinase enzyme activity, regulation of intracellular Ca^{2+} and PDX1 protein expression were significantly reduced by cytokines. mRNA expression of genes involved in secretory function — *INS*, *GCK*, *PCSK2* and *GJA1* was downregulated in cytokine treated 1.1B4 cells. Upregulation of transcription of genes involved in antioxidant defence — *SOD2* and *GPX1* was observed, suggesting involvement of oxidative stress. Cytokines also upregulated transcriptions of *NFKB1* and *STAT1*, which was accompanied by a significant increase in *NOS2* transcription and accumulation of nitrite in culture medium, implicating nitrosative stress. Oxidative and nitrosative stresses induced apoptosis was evident from increased % tail DNA, DNA fragmentation, caspase 3/7 activity, apoptotic cells and lower BCL2 protein expression.
Conclusions: This study delineates molecular mechanisms of cytokine toxicity in 1.1B4 cells, which agree with earlier observations using human islets and rodent beta cells.
General significance: This study emphasizes the potential usefulness of this cell line as a human beta cell model for research investigating autoimmune destruction of pancreatic beta cells.

1. Introduction

Type 1 diabetes is an autoimmune disease caused by destruction of pancreatic beta cells. Autoimmune invasion of endocrine pancreas and targeted attack leads to loss of beta cell mass due to induction of apoptosis by cytokines. Several factors have been shown to trigger autoimmunity leading to type 1 diabetes such as HLA (human leukocyte antigen) haplotypes — DQA1*0301–B1*0302, DQA1*0501–B1*0201 and certain high risk HLA DR haplotypes, viral infections, toxins and food components [1,2].

Enteroviral particles were detectable in islets of type 1 diabetic patients [3–5]. In INS-1E cells infected with Coxsackie viruses, expression of cytokines and chemokines was upregulated [4]. Viral infections, especially enteroviruses trigger release of cytokines and chemokines by beta cells which attract lymphocytes and macrophages resulting in immune invasion of islets. Activated lymphocytes and mononuclear cells release proinflammatory cytokines such as IL-1β (Interleukin-1β), IFN-γ (Interferon γ) and TNF-α (Tumour necrosis factor α) which participate in local inflammatory reactions — termed 'insulitis' [6–8]. Autoantibodies in the circulation that target autoantigens such as insulin and glutamic acid decarboxylase, cytokines released by infiltrating lymphocytes — IL-1β, TNF-α, IFN-γ and local inflammation cause progressive beta cell destruction [8,9]. A number of intracellular events take place following binding of cytokines to their cytokine receptors. Pro-inflammatory cytokines activate NFkB (Nuclear factor kappa B) which induces nitric oxide synthesis and production of reactive oxygen species (ROS) among other events [7].

Nitric oxide downregulates genes involved in beta cell specialised function. Notable among them are PDX1 (Pancreatic Duodenal Homeobox-1), GLUT-2 and SERCA2 (Sarco endoplasmic reticulum Ca^{2+} ATPase 2). PDX1 is essential for early pancreas formation, insulin secretion, mitochondrial metabolism, cell survival and regulation of insulin gene expression. PDX1 deficiency renders the beta cell susceptible to endoplasmic reticulum (ER) stress and stress-induced apoptosis [10]. Although several studies report involvement of ER stress in cytokine toxicity [7,11,12], a study by Akerfeldt et al. (2008) contradicts this, as they observed that ER stress-mediated cell death is not essential for the detrimental effects of cytokines [13]. Nitric oxide also triggers DNA strand breakage, leading to apoptosis. Apoptosis is programmed cell death, which occurs by one of the two pathways — intrinsic and extrinsic. Activation of JNK (c-Jun NH_2-terminal kinase), induction of nitric

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oxide synthesis, induction of expression of pro-apoptotic genes such as BID, BAX and BAK and activation of STAT1 (Signal transducer and activator of transcription) which induces expression of different caspases, nitric oxide-mediated ER stress and release of cytochrome C from mitochondria, are all proposed mechanisms of cytokine induced apoptosis [7,14–18].

Generation of pancreatic beta cell lines of human rather than animal origin has proven to be very difficult. Furthermore once created, there are substantial problems including instability in culture and loss of insulin production and secretion, as observed with previously generated human beta cell lines such as CM, βLOX5 and TRM-1 [19–23]. Recently, a genetically engineered human beta cell line, EndoC-βH1, derived by targeted oncogenesis of human foetal tissue was reported to possess intact secretory responses similar to normal beta cells [24]. However this cell line was not commercialized and hence is inaccessible to other researchers. Of late, McCluskey and colleagues reported establishment of four novel human pancreatic beta cell lines derived by electrofusion technology [25]. These cell lines demonstrated stability in culture, expression of beta cell enriched genes and glucose responsiveness. When one of these clonal human cell lines (1.1B4) was transplanted into streptozotocin diabetic SCID mice, it formed well vascularised cell masses and improved hyperglycemia [25]. In addition, configuration of human 1.1B4 cells as pseudoislets (functional islet like structures) enhanced expression of gap junction proteins and insulin secretion [26]. Investigations of the cellular responses of 1.1B4 cells to lipotoxicity also revealed key similarities between this novel cell line and primary human islets [27].

Although much research has been conducted on rodent beta cells, comparatively little information has been generated using human islets and more specifically isolated human beta cells. In the present paper, we have studied molecular mechanisms of cytokine toxicity in the recently described novel human pancreatic beta cell line, 1.1B4 [25]. We describe for the first time, effects of exposure to pro-inflammatory cytokines (IL-1β, IFN-γ and TNF-α) on 1.1B4 cell viability, secretory function, calcium handling, gene expression, antioxidant defence and apoptosis.

2. Materials and methods

2.1. Cell culture and viability

1.1B4 cells (passages 25–40) were routinely cultured in RPMI-1640 medium (Gibco®, Invitrogen, UK) containing 11.1 mM glucose, 10% (v/v) foetal bovine serum (FBS) (Gibco®, Invitrogen, UK) and 1% (v/v) antibiotics–penicillin (100 U/ml) and streptomycin (0.1 mg/l) (Gibco®, Invitrogen, UK), with 5% CO_2 and 95% air. For evaluation of effects of cytokines, cells were cultured for 18 h with IL-1β, IFN-γ, TNF-α or cytokine mixture as indicated in the Tables and Figures. Cytokine concentrations tested were similar to those used in many previous studies [28–32]. Viability of cells after exposure to recombinant human cytokines (Specific activity of IL-1βPromoKine, Heidelberg, Germany) was assessed by MTT assay [33]. The generation and basic characteristics of 1.1B4 cells, which are available from ECACC (Catalogue number: 10012801) are described elsewhere [25].

2.2. Insulin release, insulin content and glucokinase

1.1B4 cells were harvested and seeded at a density of 70,000 cells per well in 24 well plates and allowed to attach overnight. Following 18 h cytokine treatment, secretory function was assessed [25]. Cells were extracted using ice cold acid ethanol (75% v/v ethanol, 1.5% v/v concentrated HCl) for assessment of insulin content. Insulin was measured by radioimmunoassay using human insulin standards and expressed as ng per million cells per 20 min (Sigma, Poole, UK) [34]. Glucokinase activity was assessed using enzyme-coupled photometric assay [35].

2.3. Intracellular Ca^{2+} studies

FLIPR calcium 5 assay kit (Molecular Devices, Sunnyvale, CA, USA) instructions were followed to determine $[Ca^{2+}]_i$ responses. 1.1B4 cells were seeded at a density of 25,000 cells/well of 96 well black walled clear bottom plates and allowed to attach overnight. Following cytokine exposure, $[Ca^{2+}]_i$ responses to insulin secretagogues were monitored using FlexStation scanning fluorometer and area under curve was plotted [36].

2.4. Real time reverse transcription PCR

mRNA extraction and cDNA conversion were carried out using RNeasy mini kit (Qiagen, UK) and superscript II reverse transcriptase — RNase H kit (Invitrogen, UK) following manufacturer's instructions. Reaction mix of real time reverse transcription PCR consisted of 12.5 μl buffer (QuantiFast SYBR green PCR kit, Qiagen, UK), 1 μl primers (forward and reverse, Table 1, Invitrogen, UK), 1 μl respective cDNA and 9.5 μl RNase free water. Amplification conditions were initial denaturation at 95 °C for 5 min, final denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s for 34 cycles, followed by melting curve analysis at temperature range of 60 °C–90 °C. Real time data was acquired using MiniOpticon two-colour real time PCR detection system (BioRad, UK) and analysed using ΔΔCt method, with mRNA expression normalised to *ACTB* expression.

2.5. Protein extraction, quantification and western blot

Total protein from 1.1B4 cells was extracted using RIPA buffer containing 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl, pH 7.6 and protease inhibitor cocktail at 4 °C for 10 min. 30 μg protein of each sample was boiled at 95 °C with Laemmli buffer for 10 min, loaded on to pre-cast gels (NuPAGE 4–12% Bis-Tris gels, Invitrogen, UK) and subjected to SDS-PAGE (100 V, 45 min). After transfer, membranes were probed with rabbit polyclonal antibody to BCL2 (1:200)/rabbit polyclonal antibody to PDX-1 (1:5000)/mouse monoclonal antibody to ACTB (1:5000) (Abcam, UK). The membranes were then probed with ECL anti-rabbit IgG horseradish peroxidase-linked whole antibody (from donkey)/ECL anti-mouse IgG horseradish peroxidase-linked whole antibody (from sheep) (1:10000) (GE Healthcare, UK) and detected using Luminata Forte HRP substrate (Millipore, UK). Data were normalized to ACTB and expressed relative to untreated control.

2.6. Alkaline comet assay

For analysis of DNA damage after cytokine exposure, alkaline comet assay was carried out [37]. For positive control, cells were exposed to UV light for 20 min. After comet assay, the gels were stained with DAPI (4′,6-diamidino-2-phenylindole) (100 μg/ml) and viewed using epifluorescent microscope (Olympus system microscope, model BX51). DNA damage was assessed by CometScore software which analyses complete comets measuring densitometric and geometric parameters including % tail DNA and olive tail moment (product of tail length and fraction of total DNA in tail). 4 replicates per treatment and 100 cells per gel were analysed.

2.7. Caspase assay

To assess apoptosis, caspase 3/7 activity was determined using Caspase Glo 3/7 assay kit (Promega, UK) according to manufacturer's instructions. Briefly, 1.1B4 cells were lysed with RIPA buffer in the absence of protease inhibitor cocktail to protect caspase activity. Immediately before use, Caspase-Glo reagent was prepared by mixing Caspase-Glo and Caspase-Glo buffer. Equal amounts of samples and reagent were mixed and incubated for 1 h and luminescence was measured using

Table 1
List of human primers.

Gene symbol (accession number)	Alias/common name	Primer sequence (5′-nt-3′)	Product size (bp)	Annealing temperature (°C)	Cycle threshold (Ct)
Secretory function					
INS (NM_000207)	Insulin	F — TACCAGCATCTGCTCCCTCT R — TGCTGGTTCAAGGGCTTTAT	120	58	20
GCK (NM_000162)	Glucokinase	F — TGGACCAAGGGCTTCAAGGCC R — CATGTAGCAGGCATTGCAGCC	207	58	23
PCSK1 (NM_001177875)	PC1/3, proprotein convertase subtilisin/kexin type 1	F — TCGCGCCTCCTAGCTCTTCGCA R — GCAGACTCCAGGCTCTTCGCTC	173	58	25
PCSK2 (NM_001201528)	PC2, proprotein convertase subtilisin/kexin type 2	F — TCGACCAGGTGGTGCGGGAT R — AAAGGCGGATGTGCAGCGCT	137	58	26
GJA1 (NM_000165)	Connexin 43A, gap junction protein 1	F — GCTATTGTGAATGGGGTGCT R — CTGCCAAAATTGGGAACACT	493	58	23
Antioxidant defence					
SOD1 (NM_000454)	Superoxide dismutase 1 (Soluble)	F — ACGGGGTGCTGGTTTGCGTC R — TTCAGCACGCACACGGCCTT	121	58	18
SOD2 (NM_000636)	Superoxide dismutase 2, mitochondrial	F — TCCCAAGGGAAACACTCGGCTTT R — AAACCACTGGGTGACATCTACCAGA	95	55	21
CAT (NM_001752)	Catalase	F — CGTGCTGAATGAGGAACAGA R — AGTCAGGGTGGACCTCAGTG	118	58	20
GPX-1 (NM_000581)	Glutathione peroxidase 1	F — TCCCTGCGGGGCAAGGTACTAC R — TTCGTTCTTGGCGTTCTCCTGATG	171	55	23
Apoptosis					
BCL2 (NM_000633)	B cell CLL/lymphoma 2	F — TGTGGAGAGCGTCAACCGGGAG R — ATCAAACAGAGGCCGCATGCTG	160	58	21
BAX (NM_004324)	BCL2-associated X protein	F — TGGACTTCCTCCGGGAGCGG R — CTGGGGGCCTCAGCCCATCT	167	58	22
NFKB1 (NM_003998)	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1	F — CCTGGATGACTCTTGGGAAA R — TCAGCCAGCTGTTTCATGTC	173	58	20
STAT1 (NM_007315)	Signal transducer and activator of transcription 1	F — GCCAAAGGAAGCACCAGAGCCAAT R — AGGAGACATGGGGAGCAGGTTGT	136	58	19
NOS2 (NM_000625)	Inducible nitric oxide synthase	F — CCCACCAGACAGTGCGCCTG R — GGAGCAGCAGCTGGGTTGGG	141	58	29
ER stress response					
HSPA4 (NM_002154)	Hsp70, heat shock 70 kDa protein 4	F — AGCAGCGCTCTCGGTTGCAG R — AGACAGGACACGGACCCCCG	133	58	20
HSPA5 (NM_005347)	BiP, heat shock 70 kDa protein 5	F — TGCTGCTGCCCAACTGGCTG R — GAACACGCCGACGCAGGAGT	160	58	16
EIF2A (NM_032025)	Eukaryotic translation initiation factor 2A	F — ACGCCGCTCTTGACAGTCCG R — TTGCCCCAGGCAAACAAGGTCC	152	57	20
EIF2AK3 (NM_004836)	Eukaryotic translation initiation factor 2 alpha kinase 3	F — CCCCAACAAGGCCAGCCTGG R — GGACAGCCAGCCGTGTTCCC	168	57	19
Reference gene					
ACTB (NM_001101)	Actin, beta	F — AGAGCCTCGCCTTTGCCGATCC R — CACATGCCGGAGCCGTTGTCG	103	58	14
Cytokine receptors					
IL1R1 (NM_000877)	IL1 receptor type 1	F — CCTCCCAGGGGCTCCACCTG R — AGCTGGAGGACAGGGCAGGG	93	58	19
IFNGR1 (NM_000416)	IFN-γ receptor type 1	F — CCGTCGGTAGCAGCATGGCT R — AGATCCGCGGTGCCCATCTCA	85	58	22
TNFRSF1A (NM_001065)	TNF receptor superfamily, member 1A	F — GTCCTGCAGGGGCAAGCAGG R — CAGGCTCTTGAGCCCACGGC	143	58	22

Specific human primers were designed using Primer3 and BLAST. Primer sequences, annealing temperature, product size and approximate cycle threshold values are listed in the table. Primers with efficiencies between 90% and 110% were used.

FlexStation III (Molecular devices, Sunnyvale, CA, USA). Caspase 3/7 activity was expressed in terms of relative luminescence units.

2.8. Nitrite assay

Nitrite levels in culture supernatant were determined using Griess reagent kit (Invitrogen, UK). Briefly, Griess reagent was prepared by mixing equal amounts of sulfanilic acid and N-(1-naphthyl) ethylenediamine. Reaction mix for nitrite assay consisted of 150 μl sample, 20 μl Griess reagent and 130 μl deionised water. After 30 min incubation at room temperature, absorbance was read at 548 nm using microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.9. TUNEL assay

1.1B4 cells were exposed to cytokines for 18 h, harvested and allowed to attach on polysine slides for 15 min at 37 °C. The cells were then fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were then permeabilised using citrate buffer (pH 6.0) at 94 °C for 20 min and incubated with TUNEL reaction mixture (In Situ Cell Death Detection kit, Roche Diagnostics) at 37 °C for 1 h. The slides were then rinsed in PBS thrice and mounted using antifade mounting medium. The slides were viewed under FITC filter (488 nm) using fluorescent microscope (Olympus System microscope, BX51) and photographed using the DP70 camera adapter system.

2.10. Acridine orange/ethidium bromide assay

1.1B4 cells were exposed to cytokines for 18 h, harvested and then resuspended in Phosphate Buffered Saline (PBS). The cell suspension was then stained using acridine orange and ethidium bromide solution (20 μg/ml PBS each) for 5 min at room temperature. The cells were then mounted using antifade mounting medium and viewed under FITC filter (488 nm) and TRITC filter (594 nm) using fluorescent microscope (Olympus System microscope, BX51) and photographed using the DP70 camera adapter system. Apoptosis was analysed as follows — bright green nuclei — healthy cells; dense green nuclei (evident of chromatin condensation) — early apoptosis; bright yellow nuclei and yellow cytoplasm — late apoptosis; orange/red nuclei, orange cytoplasm — late apoptosis/necrosis. Membrane blebs and apoptotic bodies were also taken into account for analysis. Approximately 100 cells per replicate were analysed using ImageJ software.

2.11. Statistical analysis

Results were analysed in GraphPad PRISM (version 3.0) and presented as mean ± SEM. Statistical analyses were carried out by unpaired student's *t*-test (non-parametric) (with two-tailed P values and 95% confidence intervals) and one way ANOVA with Bonferroni post-hoc test wherever applicable. Results were considered significant if $p < 0.05$.

3. Results

3.1. Viability

IL-1β, IFN-γ and TNF-α caused 28%–54%, 42%–56% and 25%–46% reduction in viability respectively ($p < 0.05$ to $p < 0.001$, Fig. 1A, 1B, 1C). Lethal dose (LD_{50}) was found to be 893 U/ml for IL-1β, 477 U/ml for IFN-γ and 2742 U/ml for TNF-α. Synergistic action of these cytokines was evident from the observation that mixture containing approximately ten times lower concentrations of individual cytokines significantly reduced viability by 30%–40% ($p < 0.01$, $p < 0.001$, Fig. 1D). Based on these results, all subsequent experiments were performed using 800 U/ml IL-1β, 200 U/ml IFN-γ, 2250 U/ml TNF-α and a mixture comprising 100 U/ml IL-1β + 20 U/ml IFN-γ + 200 U/ml TNF-α. Exposure to cytokines at these concentrations also increased transcription of cytokine receptors — *IL1R1*, *IFNGR1* and *TNFRSF1* in 1.1B4 cells ($p < 0.05$, $p < 0.01$, $p < 0.001$, Table 2).

3.2. Secretory function

Cytokines did not affect insulin release at 5.6 mM glucose although a trend towards decreased output was observed, albeit not significant (Table 3). IL-1β and cytokine cocktail inhibited the secretory response to 16.7 mM glucose and all other secretagogues tested by 68%–80% and 48%–90% respectively ($p < 0.05$, $p < 0.001$, Table 3). IFN-γ also decreased glucose responsiveness but IFN-γ and TNF-α did not significantly affect the insulin responses to alanine and KCl, while the responses to elevated Ca^{2+}, GLP-1 and forskolin were inhibited by 40%–55% and 50%–52% respectively ($p < 0.001$,Table 3). Cytokines significantly reduced 1.1B4 cellular insulin content by 29%–72% ($p < 0.05$, $p < 0.001$, Fig. 2A). Glucokinase enzyme activity was also reduced by cytokines ($p < 0.001$, Fig. 2B) whereas PDX1 protein expression was downregulated markedly in IL-1β, IFN-γ and cytokine cocktail treated cells ($p < 0.01$, $p < 0.001$, Fig. 2C, D). mRNA expression of *INS* and *GCK* was markedly reduced by 29%–43% and 14%–34% in cytokine treated cells respectively ($p < 0.05$ to $p < 0.001$, Table 2). *PCSK2* transcription was downregulated significantly by IL-1β and cytokine cocktail while a downward trend was observed in IFN-γ and TNF-α treated cells ($p < 0.05$, Table 2). Cytokines upregulated *PCSK1* transcription in 1.1B4 cells ($p < 0.05$, $p < 0.01$, Table 2). IFN-γ significantly reduced *GJA1* transcription ($p < 0.05$, Table 2) while TNF-α and cytokine cocktail caused a trend towards downregulation in *GJA1* transcription.

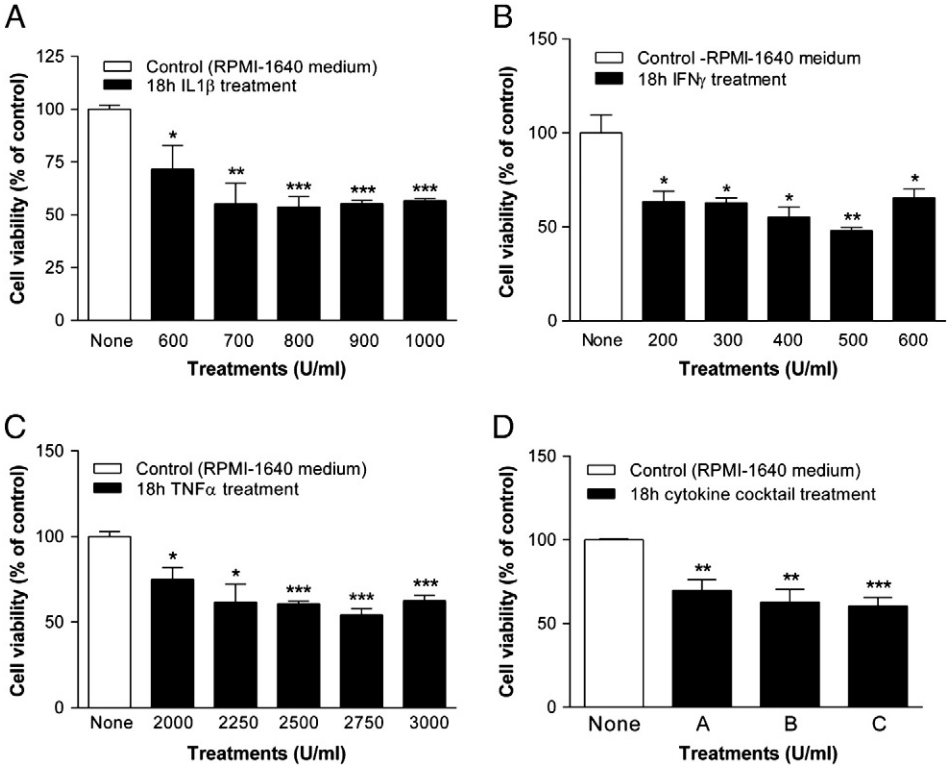

Fig. 1. Viability of 1.1B4 cells after 18 h exposure to cytokines — IL-1β (A), IFN-γ (B), TNF-α (C) and cytokine cocktail comprising A — 100 U/ml IL-1β + 20 U/ml IFN-γ + 200 U/ml TNF-α, B — 200 U/ml IL-1β + 30 U/ml IFN-γ + 250 U/ml TNF-α and C — 300 U/ml IL-1β + 40 U/ml IFN-γ + 300 U/ml TNF-α (D), as assessed by MTT assay. Values are mean ± SEM (n = 8). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to untreated control.

Table 2
1.1B4 gene expression after 18 h exposure to cytokines.

Gene	Control	IL-1β (800 U/ml)	IFN-γ (200 U/ml)	TNF-α (2250 U/ml)	Cytokine cocktail
Secretory function					
INS	100 ± 6	57 ± 5**	61 ± 8**	45 ± 2***	70 ± 3*
GCK	100 ± 7	73 ± 5	71 ± 5*	65 ± 5*	85 ± 3
PCSK1	100 ± 4	129 ± 7*	135 ± 6*	119 ± 5	148 ± 2**
PCSK2	100 ± 5	42 ± 11*	70 ± 7	71 ± 4	47 ± 13*
GJA1	100 ± 9	95 ± 15	37 ± 8*	73 ± 9	79 ± 8
Antioxidant defence					
SOD1	100 ± 7	117 ± 7	135 ± 3	111 ± 8	129 ± 9
SOD2	100 ± 18	255 ± 4**	165 ± 7	232 ± 16*	388 ± 16***
CAT	100 ± 4	115 ± 7	100 ± 4	95 ± 4	103 ± 6
GPX1	100 ± 7	117 ± 7	210 ± 9***	183 ± 8***	194 ± 9***
Apoptosis					
BCL2	100 ± 8	33 ± 8***	55 ± 6**	22 ± 5***	74 ± 3
BAX	100 ± 9	103 ± 9	101 ± 2	62 ± 4*	77 ± 2
NFKB1	100 ± 10	188 ± 8***	136 ± 4	159 ± 5**	298 ± 8***
NOS2	100 ± 8	171 ± 17*	179 ± 13*	191 ± 7**	206 ± 18**
STAT1	100 ± 7	126 ± 18	565 ± 11***	112 ± 24	545 ± 18***
ER stress					
HSPA4	100 ± 5	174 ± 9**	158 ± 12**	327 ± 5***	204 ± 9***
HSPA5	100 ± 7	60 ± 7	49 ± 9*	67 ± 11	55 ± 8*
EIF2A	100 ± 1	108 ± 8	72 ± 12	73 ± 3	79 ± 3
EIF2AK3	100 ± 15	165 ± 10	125 ± 14	108 ± 14	290 ± 15
Cytokine receptors					
IL1R1	100 ± 5	261 ± 18***	313 ± 10***	244 ± 13***	233 ± 15***
IFNGR1	100 ± 20	190 ± 13**	201 ± 13**	217 ± 4***	189 ± 5**
TNFRSF1A	100 ± 10	126 ± 4	199 ± 11***	150 ± 11*	158 ± 18**

Following cytokine exposure, mRNA was extracted and converted to cDNA. mRNA expression was normalized to *ACTB* expression. The cytokine cocktail comprised of 100 U/ml IL-1β, 20 U/ml IFN-γ and 200 U/ml TNF-α. Values are mean ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 compared to untreated control (100%).

3.3. Calcium handling

$[Ca^{2+}]_i$ responses in terms of relative fluorescence units are shown in Supplementary Fig. 1. IL-1β and TNF-α significantly reduced $[Ca^{2+}]_i$ responses to 16.7 mM glucose and 10 mM alanine ($p < 0.01$, $p < 0.001$, Fig. 3). IL-1β and TNF-α also caused a downward trend in response to 30 mM KCl and elevated Ca^{2+} (7.68 mM). IFN-γ significantly reduced $[Ca^{2+}]_i$ responses to elevated Ca^{2+} ($p < 0.05$, Fig. 3). Cytokine cocktail decreased $[Ca^{2+}]_i$ responses to alanine and KCl ($p < 0.05$, Fig. 3).

3.4. Antioxidant defence

Transcriptions of *SOD2* and *GPX1* were upregulated by all cytokines in 1.1B4 cells ($p < 0.05$, $p < 0.01$, $p < 0.001$, Table 2). Although *SOD1* transcription was not significantly affected by cytokines, a trend towards upregulation was observed. Although *CAT* transcription was unaffected in cytokine treated cells, catalase enzyme activity was significantly increased after 18 h exposure to cytokines ($p < 0.05$, $p < 0.01$, $p < 0.001$, data not included).

3.5. Nitrosative stress

Cytokines markedly upregulated *NFKB1* and *STAT1* transcriptions, which were accompanied by a significant increase in *NOS2* transcription ($p < 0.05$, $p < 0.01$, $p < 0.001$, Table 2). Nitrite levels in culture medium significantly increased by 2, 3, 5 and 7 folds in IL-1β, IFN-γ, TNF-α and cytokine cocktail treated cells respectively ($p < 0.01$, Fig. 4A).

3.6. ER stress

Cytokines increased transcription of *HSPA4* and decreased transcription of *HSPA5* ($p < 0.05$, $p < 0.01$, $p < 0.001$, Table 2). Upregulation of transcription of molecular chaperones is a marker for ER stress which could be mediated either by oxidative stress or nitric oxide in 1.1B4 cells.

3.7. Apoptosis

Oxidative and nitrosative stresses induced DNA damage was evident from significant increase in % tail DNA and olive tail moment in cytokine treated cells, as assessed by comet assay ($p < 0.001$, Fig. 4B, C). Execution of apoptosis was evident from 2 to 3.8 fold increase in caspase 3/7 activity in cytokine treated cells ($p < 0.01$, $p < 0.001$, Fig. 4D). DNA fragmentation and apoptosis activation were also confirmed by TUNEL assay and acridine orange/ethidium bromide assay. Representative images showing TUNEL positive or apoptotic cells are shown in Fig. 5A. Chronic 18 h cytokine exposure to cytokines increased DNA fragmentation by 3.7 to 4.7 fold and significantly increased number of apoptotic cells ($p < 0.01$, $p < 0.001$, Fig. 5B, C). Cytokines also caused a downregulation in *BCL2* transcription ($p < 0.01$, $p < 0.001$, Table 2). IFN-γ and cytokine cocktail significantly reduced BCL2 protein levels by 52% and 61% respectively while a trend towards downregulation was observed in IL-1β and TNF-α treated cells ($p < 0.05$, Fig. 6A, B).

4. Discussion

The cytokines IL-1β, IFN-γ and TNF-α each mediated cell death in 1.1B4 beta cells. Expression of cytokine receptors by these cells was confirmed by gene expression. Cytokine combination (IL-1β, IFN-γ and TNF-α) proved to be more lethal, confirming synergistic action of individual cytokines. Reduction in viability after exposure to IL-1β, IFN-γ, TNF-α and combinations of cytokines has been observed previously in rodent clonal beta cells as well as in FACS purified primary rat beta cells and islets from rat and humans [11,13,18,38–42]. However we did not observe dose dependent decrease in cell viability as observed

Table 3
Insulin release from 1.1B4 cells after 18 h exposure to cytokines.

Secretagogues	Insulin release (ng/million cells/20 min)				
	Control	IL-1β (800 U/ml)	IFN-γ (200 U/ml)	TNF-α (2250 U/ml)	Cytokine cocktail
Glucose (5.6 mM)	0.12 ± 0.01	0.06 ± 0.01	0.08 ± 0.02	0.09 ± 0.01	0.04 ± 0.01
Glucose (16.7 mM)	0.25 ± 0.04	0.06 ± 0.01***	0.09 ± 0.01	0.15 ± 0.02	0.03 ± 0.01***
Alanine (10 mM)	0.31 ± 0.01	0.09 ± 0.01***	0.17 ± 0.01	0.28 ± 0.03	0.16 ± 0.01*
KCl (30 mM)	0.46 ± 0.02	0.09 ± 0.01***	0.44 ± 0.07	0.33 ± 0.03	0.13 ± 0.02***
$CaCl_2 \cdot 2H_2O$ (7.68 mM)	0.96 ± 0.05	0.34 ± 0.02***	0.46 ± 0.07***	0.47 ± 0.01***	0.38 ± 0.01***
GLP-1 (20 nM) + glucose (16.7 mM)	0.67 ± 0.02	0.12 ± 0.03***	0.30 ± 0.07***	0.33 ± 0.04***	0.07 ± 0.02***
Forskolin (25 μM) + glucose (16.7 mM)	0.48 ± 0.03	0.09 ± 0.02***	0.29 ± 0.02	0.23 ± 0.03***	0.10 ± 0.02***

The cytokine cocktail comprised of 100 U/ml IL-1β, 20 U/ml IFN-γ and 200 U/ml TNF-α. Alanine, KCl and $CaCl_2 \cdot 2H_2O$ were tested at 5.6 mM glucose whereas other agents were tested at 16.7 mM glucose. Calcium concentration of buffer was 1.28 mM unless otherwise stated. Values are mean ± SEM (n = 8). *p < 0.05, ***p < 0.001 compared to respective secretagogues in untreated control cells.

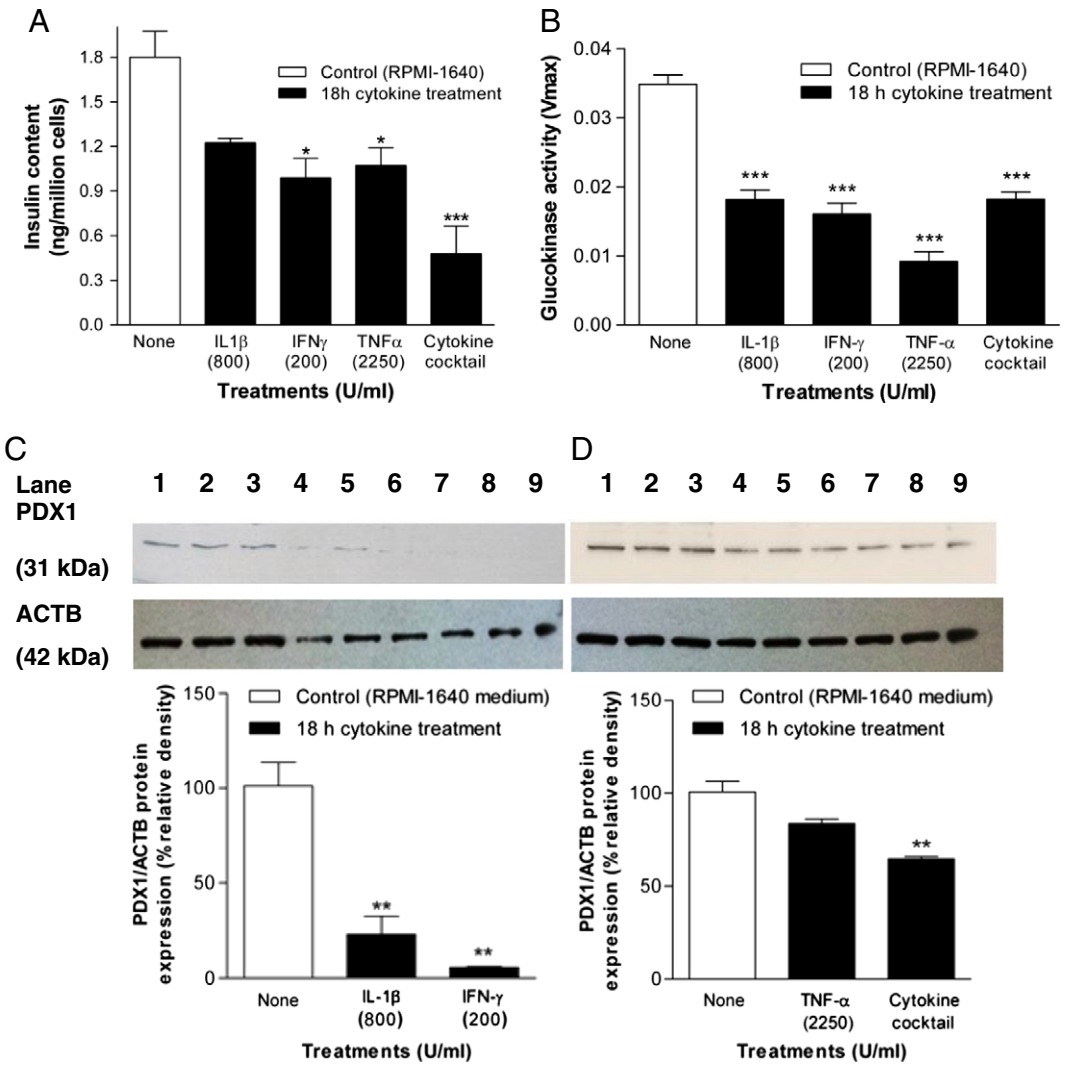

Fig. 2. A. Insulin content of 1.1B4 cells after 18 h cytokine exposure. Cytokine cocktail comprised of 100 U/ml IL-1β, 20 U/ml IFN-γ and 200 U/ml TNF-α. Values are mean ± SEM (n = 8). $^{*}p < 0.05$, $^{***}p < 0.001$ compared to untreated control. B. Glucokinase enzyme activity after 18 h cytokine exposure, expressed in terms of Vmax (U/mg), a Michaelis Menten constant. Values are mean ± SEM (n = 8). $^{***}p < 0.001$ compared to untreated control. C. PDX1 protein expression after 18 h exposure to IL1-β and IFN-γ. Lanes 1 to 3 — control, lanes 4 to 6 — IL-1β (800 U/ml) and lanes 7 to 9 — IFN-γ (200 U/ml). D. PDX1 protein expression after chronic 18 h exposure to TNF-α and cytokine cocktail. Lanes 1 to 3 — control, lanes 4 to 6 — TNF-α (2250 U/ml) and lanes 7 to 9 — Cytokine cocktail (100 U/ml IL-1β, 20 U/ml IFN-γ and 200 U/ml TNF-α). Protein expression was normalised to β-actin expression and plotted as % relative band density. Values are mean ± SEM (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$ compared to untreated control.

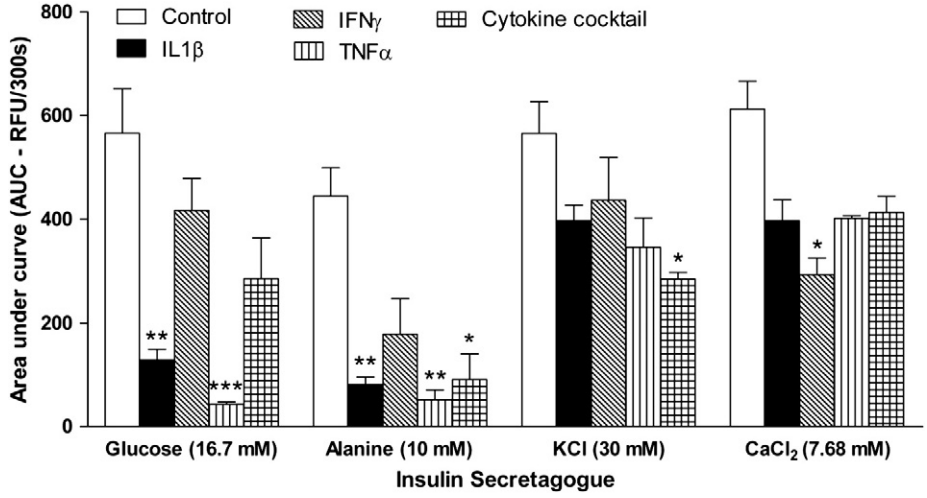

Fig. 3. $[Ca^{2+}]_i$ responses of 1.1B4 cells to 16.7 mM glucose, 10 mM alanine, 30 mM KCl, elevated 7.68 mM Ca^{2+} after 18 h exposure to cytokines — IL-1β, IFN-γ, TNF-α and cytokine cocktail comprising 100 U/ml IL-1β, 20 U/ml IFN-γ and 200 U/ml TNF-α. Data are expressed as area under curve. Temporal responses are given in Supplementary Fig. 1. Tests were performed at 5.6 mM unless otherwise stated. Values are mean ± SEM (n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control.

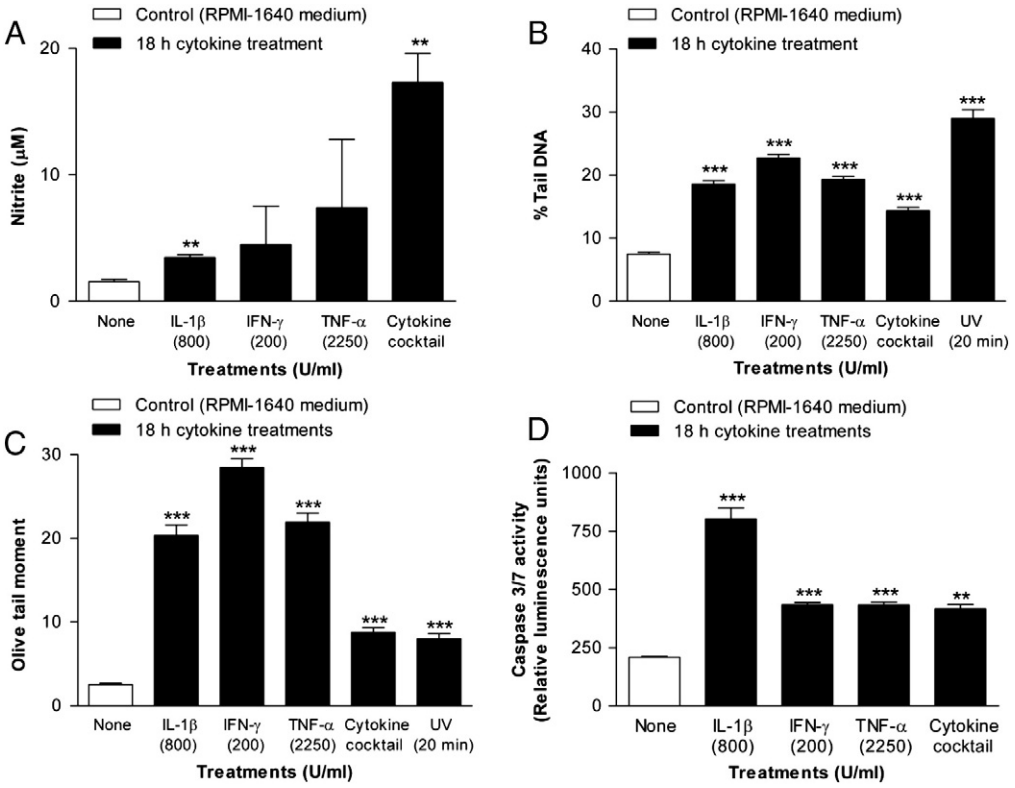

Fig. 4. A. Nitrite levels in the culture medium after 18 h cytokine exposure. Values are mean ± SEM (n = 3). $^{**}p < 0.01$ compared to untreated control. B. % tail DNA in comet tails and C. Olive tail moment of comets of 1.1B4 cells treated with cytokines. For positive control, cells were exposed to UV light for 20 min. Values are mean ± SEM (n = 4). 100 cells per gel were analysed. $^{***}p < 0.001$ compared to untreated control. D. Caspase 3/7 activity in 1.1B4 cells after chronic 18 h exposure to cytokines. Values are mean ± SEM (n = 4). $^{***}p < 0.001$ compared to untreated control.

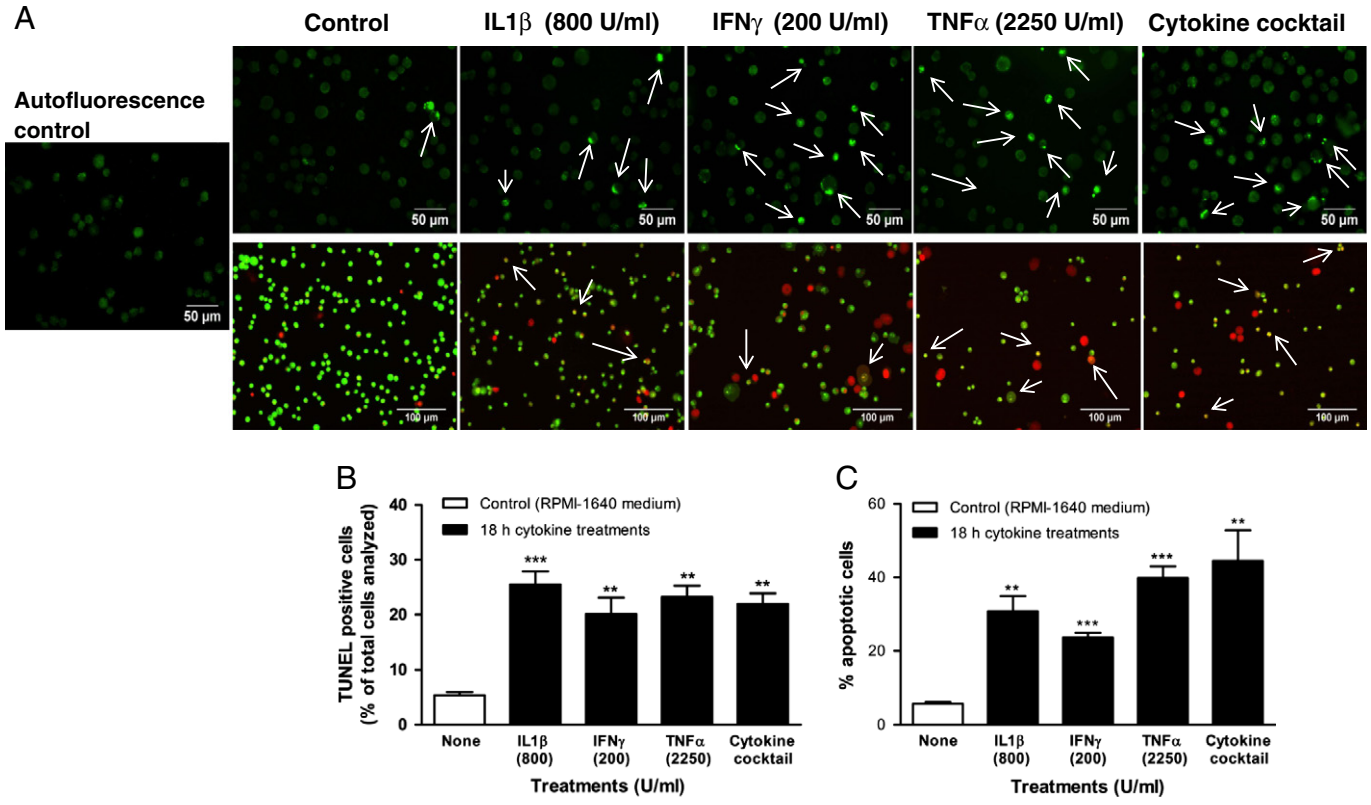

Fig. 5. A Representative images showing TUNEL positive cells and apoptotic cells after chronic 18 h exposure to cytokines — IL-1β, IFN-γ, TNF-α and cytokine cocktail comprising 100 U/ml IL-1β, 20 U/ml IFN-γ and 200 U/ml TNF-α. B. TUNEL positive cells, expressed as % of total cells analysed. C. Apoptotic cells, expressed as % of total cells analysed. Values are mean ± SEM (n = 4). 100 cells per gel were analysed. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to untreated control.

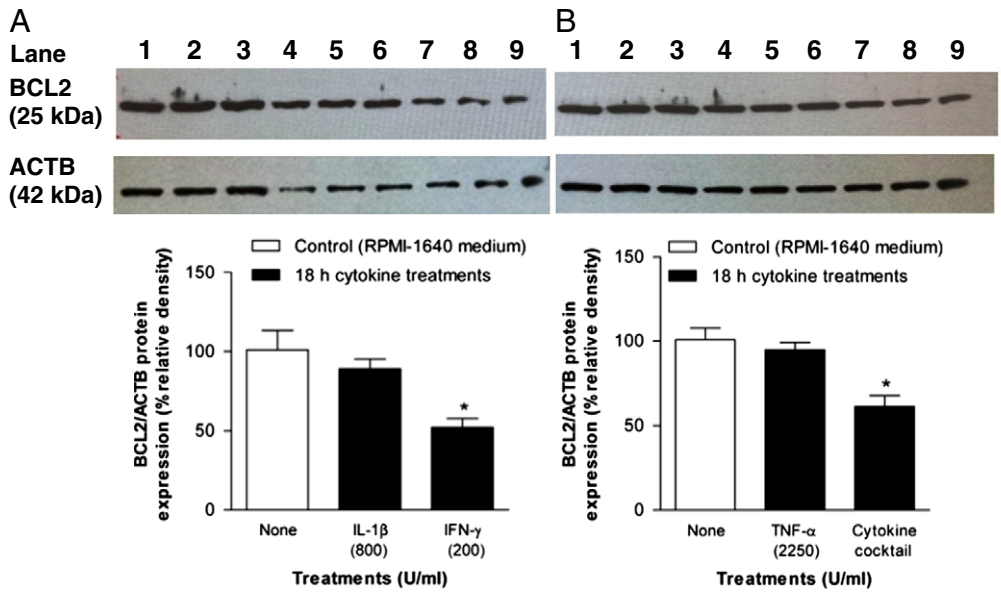

Fig. 6. A. BCL2 protein expression after chronic 18 h exposure to IL1-β and IFN-γ. Lanes 1–3 — control, lanes 4–6 — IL-1β (800 U/ml) and lanes 7–9 — IFN-γ (200 U/ml). B. BCL2 protein expression after chronic 18 h exposure to TNF-α and cytokine cocktail. Lanes 1–3 — control, lanes 4–6 — TNF-α (2250 U/ml) and lanes 7–9 — Cytokine cocktail (100 U/ml IL-1β, 20 U/ml IFN-γ and 200 U/ml TNF-α). Protein expression was normalised to β-actin expression and plotted as % relative band density. Values are mean ± SEM (n = 3). $^{*}p < 0.05$ compared to untreated control.

in other studies. Concentrations used in our study are similar to other studies. However the cytokine cocktail contained slightly higher levels than observed in the plasma of type 1 diabetic patients [43]. In vivo, autoimmune cells release other factors including nitric oxide, granzymes and perforins in addition to pro-inflammatory cytokines, which have potent synergistic effects, thus explaining the choice of concentrations in our study.

In 1.1B4 cells, the observed decrease in *GCK*, *INS* and *PCSK2* transcriptions and reduced cellular insulin content could be due to decreased PDX1 levels, as observed previously in INS-1E cells and isolated rodent islets [14,32,38,44–46]. Although glucokinase activity was reduced, measurements of ATP and ADP levels along with estimation of ATP/ADP ratio will confirm the effect of cytokines at the level of glucose phosphorylation. At the concentrations tested, glucose stimulated insulin secretion was impaired by IL-1β, IFN-γ and the cytokine cocktail, whereas basal insulin secretion was spared as observed in rodent and human islets, although a trend towards reduction was observed [14,38,28,47]. In cytokine treated cells, GLP-1 and to a lesser extent forskolin did not potentiate insulin secretion, which could signal disturbances in the cAMP messenger pathway. The responses to other secretagogues were also impaired and most notably alanine, depolarization with KCl or elevation of Ca^{2+} did not evoke insulin release following exposure to IL-1β and cytokine cocktail. This may be partly attributed to disturbances in Ca^{2+} handling following cytokine exposure. Indeed, glucose stimulated Ca^{2+} influx was significantly less in IL-1β and TNF-α treated cells, as previously observed in INS-1 cells and mouse islets [48,49]. Interestingly, there was not a direct match between the extent of disturbances of insulin secretion and Ca^{2+} influx in 1.1B4 cells. Thus the observed defects in secretion are partly due to other mechanisms as well as the apparent ability of cytokines to misdirect Ca^{2+} as observed for other cellular processes [49]. For example, inhibition of insulin secretion by cytokines could be partly mediated via nitric oxide, which inhibits aconitase and complexes of electron transport system, thereby reducing glucose oxidation [50]. In 1.1B4 cells, *NOS2* mRNA expression was induced by cytokines, which was accompanied by nitrite accumulation in the culture medium, as observed in INS-1E cells, RINm5F cells and isolated human islets [39,41,45,51,52]. Cytokine cocktail exerted the most potent effect on *NOS2* mRNA expression and nitrite production compared to individual cytokines, further confirming the synergistic actions of cytokines. Eizirik et al. (1994) reported similar effects on nitrite production in human islets after exposure to pro-inflammatory cytokines [28]. Nitric oxide and peroxynitrite react with tyrosine and cysteine residues on proteins, leading to ubiquitination and degradation and hence the observed reduction in glucokinase activity.

Nitric oxide affects Ca^{2+}-ATPase activity of SERCA pump, thus disturbing ER Ca^{2+} homeostasis and hence activating ER stress response [12,53,54]. In 1.1B4 cells, cytokines upregulated transcriptions of *HSPA4* and *EIF2AK3*, suggesting activation of ER stress response. EIF2AK3 phosphorylates EIF2A (eukaryotic translation initiation factor 2α), thereby inhibiting protein translation, further explaining the observed reduction in insulin and PDX1 levels. Further investigations should be carried out to assess ER stress response activation in cytokine exposed 1.1B4 cells by studying PERK, ATF6 and IRE1 pathways in detail.

In 1.1B4 cells, *CAT* and *SOD1* mRNA expressions were unaffected while *GPX1* expression was upregulated. Cytokines activate the transcription factor, NFkB, which controls transcription of over 100 genes including *NOS2*, *SOD2*, *CHOP* (involved in ER stress induced apoptosis), *HSPA4*, *IL-15* and *MCP-1* and downregulation of transcriptions of *INS*, *GCK*, and *SERCA2*. Activation of NFkB by oxidative stress results in upregulation of *SOD2*, an effect observed in 1.1B4 cells, INS-1E cells, RINm5F cells, primary rat beta cells and isolated human islets [42,45,46,55]. Cytokine exposure also results in STAT1 translocation to nucleus and hence transcription of genes involved in apoptosis [32,40,56]. A marked increase in mRNA levels of *NFkB* and *STAT1* was observed in 1.1B4 cells, as reported for INS-1E cells, RINm5F cells and FACS purified rat beta cells [32,40,56]. However, gene expression data does not reflect activity of these transcription factors and hence assessment of DNA binding by NFkB and STAT1 after cytokine exposure could help confirm involvement of NFkB and STAT1 in cytokine toxicity. It should also be noted that NFkB and STAT1 autoregulate their own expression [57,58] and hence upregulation of mRNA expression indirectly suggests increased DNA binding by NFkB and STAT1 in cytokine treated 1.1B4 cells. mRNA expression of *BCL2* and its protein expression was downregulated in cytokine exposed 1.1B4 cells while the expression of proapoptotic gene, *BAX* was unaffected. This effect has been observed earlier in rat beta cells exposed to cytokines [59]. Clearly a reduction

in expression of antiapoptotic protein and unaltered expression of proapoptotic protein favour apoptosis.

Nitric oxide, peroxynitrite and ROS induce DNA damage. Although we did not measure ROS levels in 1.1B4 cells, we measured DNA damage by comet assay and TUNEL assay to indirectly assess stress induced damage. Cytokines induced DNA damage in 1.1B4 cells, as previously demonstrated in isolated mouse, rat and human islets [14,17,50,60]. Excessive DNA damage or overactivation of ER stress response activates mitochondrial apoptotic pathway and activates caspases. Engagement of cytokine receptors that have intracellular death domains such as Fas and TNF-R1 also results in activation of caspase-8, which in turn activates other effector caspases. Indeed, cytokines significantly increased caspase 3/7 activity in 1.1B4 cells, as observed in various rodent cell lines, rat beta cells and islets [13,31,42,50,55,61]. The percentage of apoptotic 1.1B4 cells was higher after cytokine treatment, as demonstrated in INS-1E cells, MIN6 cells, primary rat beta cells, isolated mouse and human islets [11,12,18,32,59,61–63].

Overall, these data indicate that proinflammatory cytokines mediated 1.1B4 cell death via NFkB and STAT-1 which play central roles in multiple complex cellular pathways. Cytokines affected 1.1B4 cell integrity by inhibiting insulin secretion, impairing calcium handling, downregulating expression of beta cell enriched genes, generating nitric oxide and ROS, inducing DNA damage, activating ER stress response and inducing apoptosis. The present study also highlights disturbances in $[Ca^{2+}]_i$ responses in human beta cells after cytokine exposure. These observations using 1.1B4 cells agree with and extend earlier reports on cytokine toxicity in various primary and beta cell models. Most importantly, this novel human-derived pancreatic beta cell line appears to be resistant to cytokine toxicity compared to rodent beta cell lines, which may be a typical feature of human cells. Previously, research on beta cell dysfunction was carried out using rodent beta cell lines due to the limited availability of human islets. The present cellular and molecular profiles of 1.1B4 cells indicate that this cell line provides a useful and unlimited source of novel human beta cells for studying the mechanisms of diabetes induced beta cell death.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2013.08.022.

Declaration of interest

The authors declare no conflict of interest.

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