Responses of GLP1-secreting L-cells to cytotoxicity resemble pancreatic β -cells but not α -cells

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Abstract

Little is known about responses of intestinal L-cells to chemical or cytokine-mediated attack and how these compare with pancreatic β - or α -cells. Administration of streptozotocin to mice induced severe diabetes, islet lymphocytic infiltration, increased α -cell proliferation and decreased numbers of β - and L-cells. *In vitro*, streptozotocin and cytokines reduced cell viability with higher lethal dose 50 values for α -TC1 cells. mRNA expression of *Glut2* was lower and *Cat* was greater in GLUTag and α -TC1 cells compared with MIN6 cells.

Cytotoxins affected the transcription of genes involved in secretion in GLUTag and MIN6 cells. They are also involved in upregulation of antioxidant defence enzymes, transcription of *NfκB* and *Nos2*, and production of nitrite in all cell types. Cytotoxin-induced DNA damage and apoptosis were apparent in all cells, but α -TC1 cells were less severely affected. Thus, responses of GLP1-secreting L-cells to cytotoxicity resemble β -cells, whereas α -cells are resistant due to differences in the expression of genes involved in cytotoxicity or antioxidant defence.

Key Words

- Cytokine
- L cells
- beta cells
- alpha cells
- streptozotocin

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Introduction

In type 1 diabetes, proinflammatory cytokines such as interleukin 1 β (IL1 β), interferon γ (IFN γ) and tumour necrosis factor α (TNF α), released by autoimmune cells, are important mediators of pancreatic β -cell death (Cnop *et al.* 2005). Cytokines activate master transcription factors – nuclear factor κ B (NF κ B) and STAT1 which transactivate the expression of genes involved in apoptosis and nitric oxide production (Gurzov *et al.* 2008, Moore *et al.* 2012). Nitric oxide participates in diffusion-controlled reaction with superoxide radical to form toxic peroxynitrite, which interacts with DNA, lipids and proteins via direct or indirect oxidative reactions (Pacher *et al.* 2007). Such reactions trigger necrosis or apoptosis due to limited expression of antioxidant enzymes in pancreatic β -cells

(Lenzen 2008, Mechlovich *et al.* 2010). The pathogenesis of cytokine-mediated β -cell death has been studied extensively using non-obese diabetic mice which demonstrate spontaneous autoimmunity and rodent β -cell lines.

Administration of streptozotocin in multiple low doses to mice destroys pancreatic β -cells in a fashion similar to cytokines (Shehata *et al.* 2011). Thus, unlike a single dose of streptozotocin which causes overt diabetes due to β -cell death caused by DNA damage-induced depletion of cellular NAD (Elsner *et al.* 2000, Lenzen 2008), administration of small doses causes islet lymphocyte infiltration and insulinitis similar to that observed in islets of patients with type 1 diabetes (Shehata *et al.* 2011). Interestingly, these events are associated with an increase in α -cell mass in diabetes

(Li *et al.* 2000, Yoon *et al.* 2003), accompanied by hyperglucagonaemia which contributes to metabolic disarray (Li *et al.* 2000, Meier *et al.* 2011). In streptozotocin-treated mice, α -cell mass expansion together with hyperglucagonaemia has been observed (Takeda *et al.* 2012, Vasu *et al.* 2014a). Thus, it is evident that α -cells survive conditions that are toxic to β -cells and hence appear to be more resistant to damage by cytotoxins. High expression of catalase, an antioxidant enzyme, was observed in pancreatic α -cells of diabetic mice (Bloch *et al.* 2007), suggesting that these cells employ defence mechanisms to evade cell death induced by cytotoxins.

In addition to α -cell expansion and hyperglucagonaemia, diabetes is associated with disturbances in the insulinotropic action and especially the secretion of GLP1 (Vilsboll *et al.* 2003, Holst 2007, Huml *et al.* 2011, Kielgast *et al.* 2011, Unger 2013). Recent investigations together with our unpublished observations have revealed that disturbances in the intestinal L-cell population may contribute to the loss-of-functional incretin response in diabetes (Kappe *et al.* 2014). In the context of type 1 diabetes pathogenesis, such disturbances may contribute to the observed metabolic abnormalities. Indeed, although pancreatic β -cell death is mediated by local inflammation, elevated systemic levels of IL1 β , IL6, IL17, IFN γ and TNF α were reported in type 1 diabetic patients (Espersen *et al.* 1993, Basu *et al.* 2005, Hamed *et al.* 2012, Sarikonda *et al.* 2014). Ho *et al.* (2014) have also reported elevated levels of TNF α with reduced levels of anti-inflammatory cytokine (IL10) after streptozotocin treatment. Thus, enteroendocrine cells are exposed to cytokines and also other cytotoxic agents just as β -cells, but whether these cells are susceptible to damage causing their malfunction is unknown. Multiple low dose of streptozotocin provides a good model of type 1 diabetes pathogenesis (Kolb 1987, Kolb-bachofen *et al.* 1988, Lukic *et al.* 1998, Shehata *et al.* 2011) and offers a straightforward method to assess the effects of local inflammation on pancreatic islets and low-grade systemic inflammation on intestinal L-cells.

The present paper seeks to answer two key questions. First, are intestinal L-cells susceptible to cytotoxic attack? Secondly, how do they compare with pancreatic α - and β -cells, in terms of antioxidant enzyme expression and ability to repair damage? To accomplish this, we investigated the effects of multiple low-dose streptozotocin-induced diabetes on intestinal L-cells and pancreatic α - and β -cells. Furthermore, we compared the gene expression and cellular responses of GLUtag, an intestinal L-cell model, α -TC1 clone 9, a pancreatic α -cell model, and

MIN6, a β -cell model after exposure to cytokines and streptozotocin.

Materials and methods

Animals

C57BL/6 mice, aged 8 weeks (Harlan UK Ltd, Shaw's farm, Blackthorn, UK) were housed individually in an air-conditioned room at $22 \pm 2^\circ\text{C}$ with 12 h light and 12 h darkness cycle. Standard rodent pellet diet (Trouw Nutrition, Cheshire, UK) and drinking water were supplied *ad libitum*. Streptozotocin (50 mg/kg body weight, freshly prepared in ice-cold 0.1 M sodium citrate buffer, pH 4.5) was injected (i.p.) for 5 consecutive days to induce diabetes. Blood glucose concentrations were determined using an Ascencia Contour glucose meter (Bayer Healthcare). The animals were culled, with collection of blood and tissues 5 days after the last dose of streptozotocin injection. All animal procedures were carried out according to UK Home Office regulations (UK Animal Scientific Procedures Act 1986) and 'Principles of laboratory animal care' (NIH Publication no. 86-23, revised 1985).

Cell culture and treatments

MIN6 cells (courtesy of Prof. Sigurd Lenzen, Institute of Clinical Biochemistry, Hannover, Germany) and α -TC1 clone 9 cells (courtesy of Prof. Kevin Doherty, University of Aberdeen) were cultured in 5% CO₂ and 95% air using DMEM (Gibco, Invitrogen) containing 25 mM glucose, 10% (v/v) foetal bovine serum and 1% (v/v) antibiotics. The GLUtag cells (courtesy of Prof. DJ Drucker, University of Toronto, Canada) were cultured in the same medium, but containing 5.5 mM glucose. All three cell lines were of murine origin. Recombinant human cytokines (Promokine, Heidelberg, Germany) or streptozotocin was added to the culture medium immediately before use. The viability of cells after cytotoxin treatment was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983).

Biochemical analyses

Terminal blood samples were collected into fluoride/heparin microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) and centrifuged for 30 s at 13 000 g. Plasma was collected and stored at -20°C . Insulin accumulated in culture media following exposure of MIN6 cells to cytotoxins was measured by RIA (Flatt & Bailey 1981). Glucagon and GLP1 in the culture media with α -TC1 cells or GLUtag cells

were measured by electrochemiluminescence assay using the Mouse/rat Glucagon Kit (total) or Total GLP1 Assay Kit (Multiarray system, Meso Scale Discovery, Rockville, MD, USA), as described previously (Irwin *et al.* 2012).

Immunohistochemistry

The resected pancreas and small intestine were fixed in 4% paraformaldehyde and processed using automated tissue processor (Leica TP1020, Leica Microsystems, Nussloch, Germany). The tissue sections (8 µm thickness, sectioned using microtome, Shandon Finesse 325, Thermo Scientific, UK) were deparaffinised, rehydrated and probed with primary antibodies: mouse anti-insulin antibody (1:500; Abcam), guinea-pig anti-glucagon antibody (PCA2/4, 1:200; raised in-house), rabbit anti-Ki67 antibody (ab15580, Abcam) or rabbit anti-GLP1 antibody (XJIC8, 1:200; raised in-house). The sections were then incubated with secondary antibody (Alexa Fluor 594 goat anti-guinea pig IgG (1:400) and Alexa Fluor 488 goat anti-rabbit IgG (1:400), Invitrogen) and mounted using anti-fade mounting medium. The slides were viewed under FITC filter (488 nm) or TRITC filter (594 nm) using a fluorescent microscope (Olympus System Microscope, model BX51) and photographed using the DP70 camera adapter system. For the analysis of infiltration of islets and small intestine by lymphocytes, pancreatic and intestinal tissue sections were stained using haematoxylin and eosin following established protocols. The images were analysed using Cell[^]F analysis software, and islet area together with related parameters were measured using the closed polygon tool. The percentage pancreatic β- or α-cell area (% of islet area or area of pancreas section) was calculated from insulin or glucagon-positive area/total islet area or total area of pancreatic section. Ki67/glucagon-positive cells were counted in a blinded fashion in ~1500 cells, and proliferation frequency was expressed as percentage of total cells analysed. For analysis of GLP1-positive cell distribution in small intestine, four tissue sections (8 µm thickness and sections were picked at an interval of ten sections) were stained for GLP1. GLP1-positive cells were counted in a blinded fashion and expressed as number per mucosal area (mm²). For scoring insulinitis, islets with lymphocyte infiltration (visible lymphocytes within islets) (Peterson *et al.* 1994) were counted in a blinded fashion and expressed as percent of total number of islets analysed.

Real-time RT PCR

mRNA was extracted using the RNeasy Mini Kit (Qiagen) following manufacturer's instructions and converted to

cDNA using superscript II reverse transcriptase – RNase H Kit (Invitrogen). Same amounts of mRNA were (under all conditions) for all three cell lines. The reaction mix of real-time RT PCR consisted of 12.5 µl (Quantifast SYBR green PCR kit, Qiagen), 1 µl primers (forward and reverse primers (between 90 and 110% efficiency), Table 1, Invitrogen), 1 µl 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. Each PCR experiment included negative control, positive control (*Actb*; forward – TGGATCGGTGGCTCCATCCTGG; reverse – GCAGCTCAGTAACAGTCCGCCTAGA; product length – 130 bp) and melting curve analysis at a temperature range of 60–90 °C. Real-time data were acquired using MiniOpticon two-colour real-time PCR detection system (BioRad). The results were analysed using ΔΔCt method and mRNA expression was normalised to *Actb*.

Alkaline comet assay

Cellular DNA damage after cytotoxin exposure was determined using alkaline comet assay (Lees Murdock *et al.* 2004). The gels were stained with 4',6-diamidino-2-phenylindole (DAPI) (100 µg/ml) and viewed using epifluorescent microscope (Olympus system microscope, model BX51). Densitometric and geometric parameters including % tail DNA and olive tail moment were measured by CometScore software. Approximately, 100 cells per gel were analysed (Meerza *et al.* 2012, Sirota *et al.* 2014, Vasu *et al.* 2014b).

Acridine orange/ethidium bromide assay for apoptosis

Apoptosis induction was determined using acridine orange/ethidium bromide staining (Vasu *et al.* 2014b). The cells were then mounted on glass slides and viewed using epifluorescent microscope (Olympus system microscope, model BX51) and photographed using digital camera adapter system (DP70). The cells with bright green nuclei were considered healthy, those with dense green nuclei (chromatin condensation) as early apoptotic, those with bright yellow nuclei and cytoplasm as late apoptotic and those with orange nuclei and cytoplasm as dead. Approximately 100 cells per replicate were analysed using cell counter plug-in of ImageJ software (Kempaiah & Kisiel 2008, Noor *et al.* 2014).

Nitrite assay

Griess Reagent Kit (Invitrogen) was used to determine nitrite levels in culture supernatant according to the

Table 1 The mouse primers used in this study. Primer efficiencies were between 90 and 110%. Mouse gene nomenclature guidelines were followed for gene symbols

Official gene symbol	Alias/common name	Primer sequence (5'-nt-3')	Product size (bp)	Annealing temperature T_m (°C)
Secretory function				
<i>Ins1</i>	Insulin	F – TAACCCCCAGCCCTTAGTGA R – ACACACCAGGTAGAGAGCCT	194	57
<i>Gcg</i>	Proglucagon	F – ACACCTGTTCGCAGCTCAGGC R – AAATCTTGGGCACGCGGGGAG	261	57
<i>Slc2a2</i>	Glucose transporter 2	F – ACCGGGATGATTGGCATGTT R – GGACCTGGCCCAATCTCAAA	140	56
<i>Pcsk1</i>	Pc1/3, proprotein convertase subtilisin/kexin type 1	F – GAATGGGCGGCGGAGATCCC R – CGGGACCTCCGAGGATGGCTT	140	58
<i>Pcsk2</i>	Pc2, proprotein convertase subtilisin/kexin type 2	F – CCGAGGAGGAGCTGAAAATGCAGAT R – CTGGCTGGCAGGGGTGCGAAT	104	55
Antioxidant defence				
<i>Cat</i>	Catalase	F – TCAGGGCCGCTTTTGCCT R – TCGAGCGCGGTAGGGACAGT	94	57
<i>Gpx1</i>	Glutathione peroxidase 1	F – CCCCCTGCGCTCATGACCG R – CGAAAGCGGCGGCTGTACCT	135	58
<i>Sod1</i>	Superoxide dismutase (soluble)	F – TTCCGTCCGTCGGCTTCTCGT R – CGCACACCGCTTTCATCGCC	96	58
<i>Sod2</i>	Superoxide dismutase 2, mitochondrial	F – TTCCAGGATGCCGCTCCGT R – GCGTCTGCTAGGCAGCGTCC	134	58
Apoptosis				
<i>Bax</i>	BCL2-associated X protein	F – TGAGCGAGTGTCTCCGGCGA R – CACGCGGCCCCAGTTGAAGT	155	57
<i>Bcl2</i>	B cell CLL/lymphoma 2	F – ATGGCCCCAGCATGCGACCTC R – CACTTGTGGCCCAGGTATGCACC	115	58
<i>Nfkb1</i>	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1	F – AGAAGTCTGGGCGCACAGCG R – TGGTGGACCTCTTCCGGCC	153	58
<i>Nos2</i>	Nitric oxide synthase 2, inducible	F – GCTCGCTTTGCCACGGACGA R – AAGGCAGCGGGCACATGCAA	146	58
ER stress				
<i>Hspa4</i>	Hsp70, Heat shock 70 kDa protein 4	F – GCACTGTGCACTGTACCAGGGGA R – TCTCCAGGGAAGTGGGCAGCTA	135	58
<i>Hspa5</i>	BiP, Heat shock 70 kDa protein 5	F – TGCCTGTGTGAGACCAGAAC R – CCCCAGTCGATGCCGACCAC	218	57
Cytokine receptors				
<i>Il1r1</i>	IL1 receptor 1	F – TAATGCTGTCCTGGGCTGACT R – CATCCAGCGACATTTCTCCC	100	58
<i>Ifngr1</i>	IFN γ receptor 1	F – GGAGTGGAGCTTTGACGAGC R – TCCCAGCATACGACAGGGTT	106	58
<i>Tnfrsf1a</i>	TNF receptor superfamily 1a	F – GTAGTGGGCTCTGTGCTTTC R – TGTGGGTGTGGCTTTATCGC	121	58

manufacturer's instructions (Vasu *et al.* 2014b). Absorbance was read at 548 nm using microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Results were analysed using GraphPad PRISM Software (version 3.0) and presented as mean $\pm$ S.E.M. Statistical analyses were carried out by one-way ANOVA with Bonferroni *post hoc* test or student's *t*-test (non-parametric) wherever applicable. For determination of lethal dose 50 (LD₅₀), XY graph (X – log (concentration) vs Y (values))

was plotted in GraphPad PRISM and concentration which caused 50% reduction in viability was computed using linear/non-linear regression curve. The results were considered significant if $P < 0.05$.

Results

Effect of multiple low-dose streptozotocin on islet and intestinal L-cells

Multiple low dose of streptozotocin induced severe diabetes (blood glucose >20 mmol/l), associated with

Figure 1

(A and B) Representative images showing insulin (red) and glucagon (green)-positive cells and (C and D) lymphocyte infiltration in islets of normoglycaemic (A and C) and streptozotocin-induced (B and D) diabetic mice. Arrows in islets indicate glucagon-positive cells (A and B) and lymphocyte infiltration (D). (E) β - and α -cell area, expressed as μm^2 . (F) Islet area, expressed as μm^2 . (G) Islet area, expressed as % of area of pancreatic section. (H) β -cell area, expressed as % of islet area. (I) β -cell area, expressed

as % of area of pancreatic section. (J) α -cell area, expressed as % of islet area. (K) α -cell area, expressed as % of area of pancreatic section. (L) Islet size distribution, expressed as % of all islets analysed. Values are mean $\pm$ s.e.m. ($n=6$ mice). ** $P<0.01$ and *** $P<0.001$ compared with normoglycaemic mice. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0214>.

lymphocytic infiltration of islets and β -cell destruction. Representative images showing insulin (red) and glucagon (green) staining and lymphocyte infiltration of control and streptozotocin-treated mice are shown in Fig. 1A, B, C, and D. Islet area and β -cell area were significantly reduced by streptozotocin treatment, while α -cell area increased markedly ($P<0.01$, $P<0.001$, Fig. 1E, F, G, H, and I). Percentage of islet area and total area of pancreas section occupied by α -cells significantly increased from 12 to 43% and 0.34 to 0.63% respectively ($P<0.001$, Fig. 1J and K). Islet size distribution was not affected by streptozotocin treatment (Fig. 1L). After streptozotocin treatment, lymphocyte infiltration was observed in $\sim 79\%$ of islets.

Representative images showing Ki67 (green) and glucagon (red) staining of control and streptozotocin-treated mice are shown in Fig. 2A. α -cell proliferation was undetectable in normal mice while proliferation

frequency was $\sim 6\%$ in streptozotocin-treated mice (Fig. 2B). Representative images showing haematoxylin/eosin staining and GLP1-positive cells in the intestine of control and streptozotocin-treated mice are shown in Fig. 3A. Streptozotocin treatment did not induce lymphocyte infiltration in intestine (Fig. 3A). Intestinal L-cell count was significantly reduced by 65% fold by streptozotocin administration ($P<0.01$, Fig. 3B).

Effects of streptozotocin and cytokines on clonal insulin, glucagon and GLP1-producing cells

Basal gene expression Basal levels of *Slc2a2* (*Glut2*) mRNA were significantly lower in α -TC1 and GLUTag cells ($P<0.05$, $P<0.01$, Table 2). mRNA expression of prohormone-processing enzymes coded by *Pcsk1* and *Pcsk2* was appreciably lower in α -TC1 and GLUTag cells compared

Figure 2

(A) Representative images showing Ki67 (green) and glucagon (red)-positive cells in islets of normoglycaemic and streptozotocin-induced diabetic mice. Arrows indicate Ki67-positive glucagon-positive cells. (B) α -cell proliferation frequency, expressed as % of total cells analysed. Values are mean $\pm$ S.E.M. ($n=6$ mice). Approximately 1500 cells were analysed. ND, none detected. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0214>.

with MIN6 cells ($P<0.05$, Table 2). *Gcg* mRNA levels were significantly lower in GLUTag cells when compared with α -TC1 cells ($P<0.01$, Table 2). mRNA expression of antioxidant enzymes coded by *Gpx1*, *Sod1*, and *Sod2* was similar in all three cell lines. However, mRNA expression of *Cat* was four- and 2.4-fold higher in α -TC1 and GLUTag cells respectively compared with MIN6 cells ($P<0.01$, $P<0.001$, Table 2). *Bcl2* transcription was significantly lower while *Bax* transcription was higher in GLUTag cells when compared with MIN6 cells ($P<0.05$, $P<0.001$, Table 2). *Nfkb1* mRNA levels were lower in α -TC1 and GLUTag cells ($P<0.05$, $P<0.01$, Table 2). *Nos2* was not expressed in healthy MIN6, α -TC1 and GLUTag cells. *Hspa5* expression was significantly lower in α -TC1 and GLUTag cells ($P<0.05$, Table 2). *Il1r1* expression was significantly higher in GLUTag cells when compared with MIN6 cells ($P<0.001$, Table 2). *Tnfrsf1a* expression was markedly lower in GLUTag cells when compared with MIN6 cells ($P<0.001$, Table 2).

Cellular viability Streptozotocin and cytokines caused a dose-dependent decrease in cell viability in all three cell lines. LD₅₀ for streptozotocin toxicity for MIN6,

α -TC1 and GLUTag cells was 3.5, 11.5 and 4.5 mM respectively, with LD₅₀ for α -TC1 cells significantly higher than MIN6 cells and GLUTag cells ($P<0.01$, $P<0.001$, Fig. 4A). LD₅₀ for IL1 β was markedly higher for α -TC1 cells compared with MIN6 and GLUTag cells ($P<0.001$, Fig. 4B). IFN γ caused similar levels of cell death in all three cell lines while LD₅₀ for TNF α was significantly higher for α -TC1 cells than MIN6 and GLUTag cells ($P<0.01$, Fig. 4C and D). Cytokine cocktail containing approximately one-tenth of effective concentrations of individual cytokines caused similar levels of cell death in all three cell lines (Fig. 4E). For α -TC1 cells, the cytokine cocktail contained higher concentrations of cytokines when compared with MIN6 and GLUTag cells, which could be attributed to enhanced protection from cytotoxicity.

Figure 3

(A) Representative images showing haematoxylin/eosin staining and GLP1 staining in intestine of normoglycaemic and streptozotocin-induced diabetic mice. Arrows indicate GLP1-positive cells in intestine. (B) GLP1-positive cell number, expressed as number per mucosal area (μm^2). GLP1-positive cells in small intestine were counted in a blinded fashion. Values are mean $\pm$ S.E.M. ($n=6$ mice, four tissue sections at ten sections apart). ** $P<0.01$ compared with normoglycaemic mice. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0214>.

Table 2 Comparison of gene expression profile between MIN6, α -TC1 clone 9 and GLUTag cells. Gene expression was normalised to *Actb* expression, with MIN6 cells or α -TC1 clone 9 cells as control (100%). Values are mean $\pm$ s.e.m. ($n=3$)

Gene	MIN6 (%)	α -TC1 clone 9 (%)	GLUTag (%)
Secretory function			
<i>Ins</i>	100 $\pm$ 2	NA	NA
<i>Gcg</i>	NA	100 $\pm$ 6	6 $\pm$ 12 [†]
<i>Slc2a2</i>	100 $\pm$ 10	3 $\pm$ 19*	21 $\pm$ 10 [†]
<i>Pcsk1</i>	100 $\pm$ 12	0.3 $\pm$ 10 [†]	12 $\pm$ 6 [†]
<i>Pcsk2</i>	100 $\pm$ 9	12 $\pm$ 10 [†]	7 $\pm$ 7 [†]
Antioxidant defence			
<i>Cat</i>	100 $\pm$ 20	400 $\pm$ 5 [†]	242 $\pm$ 5 [†]
<i>Gpx1</i>	100 $\pm$ 12	103 $\pm$ 5	80 $\pm$ 6
<i>Sod1</i>	100 $\pm$ 8	112 $\pm$ 6	72 $\pm$ 12
<i>Sod2</i>	100 $\pm$ 15	72 $\pm$ 10	93 $\pm$ 9
Apoptosis			
<i>Bax</i>	100 $\pm$ 15	144 $\pm$ 8	157 $\pm$ 7*
<i>Bcl2</i>	100 $\pm$ 9	74 $\pm$ 4	7 $\pm$ 2 [†]
<i>NfκB</i>	100 $\pm$ 7	43 $\pm$ 7 [†]	46 $\pm$ 11*
<i>Nos2</i>	Not expressed	Not expressed	Not expressed
ER stress			
<i>Hspa4</i>	100 $\pm$ 16	123 $\pm$ 9	84 $\pm$ 7
<i>Hspa5</i>	100 $\pm$ 18	11 $\pm$ 7*	12 $\pm$ 10*
Cytokine receptors			
<i>Il1r1</i>	100 $\pm$ 12	119 $\pm$ 14	203 $\pm$ 17 [†]
<i>Ifngr1</i>	100 $\pm$ 12	77 $\pm$ 16	93 $\pm$ 19
<i>Tnfrsf1a</i>	100 $\pm$ 14	53 $\pm$ 10	0.52 $\pm$ 20*

* $P<0.05$, [†] $P<0.01$, and [‡] $P<0.001$ compared with MIN6 or for proglucagon gene expression to α -TC1 clone 9 cells. NA, not assessed.

Hormone secretion and associated genes Streptozotocin and cytokine cocktail significantly decreased hormone secretion from MIN6 and GLUTag cells during 8-h exposure ($P<0.05$, $P<0.01$, Fig. 5A and C). In contrast, cytokine cocktail and streptozotocin significantly increased glucagon release from α -TC1 cells ($P<0.001$, Fig. 5B). These effects were associated with decreased *Ins* transcription in MIN6 cells ($P<0.05$, $P<0.001$, Table 3), and a downward trend in *Gcg* transcription in GLUTag

cells. In α -TC1 cells, cytokine cocktail significantly increased *Gcg* transcription while streptozotocin significantly decreased *Gcg* transcription ($P<0.05$, Table 4). Streptozotocin markedly reduced *Pcsk1* and *Pcsk2* transcription in GLUTag cells ($P<0.05$, $P<0.01$, Table 3). *Pcsk2* transcription was also reduced in MIN6 cells ($P<0.05$, Table 3). Both streptozotocin and cytokine cocktail decreased *Glut2* mRNA levels in MIN6 cells ($P<0.05$, Table 3). Cytokine cocktail significantly increased *Glut2* transcription in GLUTag cells ($P<0.05$, Table 3).

Genes involved in antioxidant defence mRNA expression of *Cat*, *Gpx1* and *Sod2* was upregulated in streptozotocin-treated MIN6 cells ($P<0.05$, $P<0.001$, Table 4). *Gpx1*, *Sod1* and *Sod2* transcription was downregulated in cytokine cocktail-treated MIN6 cells ($P<0.05$, $P<0.01$, Table 4). In α -TC1 cells, streptozotocin upregulated *Sod2* expression, whereas cytokine cocktail decreased *Cat*, *Gpx1* and *Sod1* transcription, with positive effects on *Sod2* ($P<0.001$, Table 4). In GLUTag cells, streptozotocin upregulated *Cat*, *Sod1* and *Sod2* transcription while cytokine cocktail upregulated *Cat* transcription ($P<0.05$, $P<0.01$, Table 4).

Cytokine receptors Cytokine cocktail exposure significantly increased *Il1r1* expression in MIN6 cells but not in α -TC1 nor GLUTag cells ($P<0.001$, Table 4). Cytokine exposure did not alter *Ifngr1* expression in any of the cell lines tested, but *Tnfrsf1a* expression was increased in GLUTag cells ($P<0.001$, Table 4).

DNA damage All three cell lines suffered DNA damage by streptozotocin and cytokine cocktail. Streptozotocin, a DNA alkylating agent, increased % tail DNA by four-, 2.9- and 2.7-fold and olive tail moment by 13-, 12- and eightfold in MIN6, α -TC1 and GLUTag cells respectively ($P<0.001$, Fig. 6A and B). Cytokine cocktail increased %

Figure 4

Lethal dose (LD₅₀) of streptozotocin (A), IL1 β (B), IFN γ (C) and TNF α (D). Values are mean $\pm$ s.e.m. ($n=6$). ** $P<0.01$ and *** $P<0.001$ compared with MIN6 cells. $\Delta\Delta P<0.01$ and $\Delta\Delta\Delta P<0.001$ compared with GLUTag cells. (E) Cell viability (% of control) of MIN6, α -TC1 and GLUTag cells after 8-h exposure

to cytokine cocktail, containing i) 60 U/ml IL1 β +20 U/ml IFN γ +200 U/ml TNF α ; ii) 100 U/ml IL1 β +25 U/ml IFN γ +250 U/ml TNF α ; and iii) 60 U/ml IL1 β +16 U/ml IFN γ +185 U/ml TNF α . Values are mean $\pm$ s.e.m. ($n=6$). *** $P<0.001$ compared with untreated control.

Figure 5

(A) Insulin release from MIN6 cells during 8-h exposure to streptozotocin and cytokine cocktail (60 U/ml IL1 β + 20 U/ml IFN γ + 200 U/ml TNF α). (B) Glucagon release from α -TC1 cells during 8-h exposure to streptozotocin and cytokine cocktail (100 U/ml IL1 β + 25 U/ml IFN γ + 250 U/ml TNF α). (C) GLP1 release from GLUTag cells during 8-h exposure to streptozotocin and cytokine cocktail (60 U/ml IL1 β + 16 U/ml IFN γ + 185 U/ml TNF α). Values are mean $\pm$ S.E.M. ($n=3$). * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ compared with untreated control.

tail DNA by two-, 1.2- and twofold and olive tail moment by 3.3-, three- and 3.7-fold in MIN6, α -TC1 and GLUTag cells respectively ($P<0.001$, Fig. 6A and B). Compared with MIN6 cells, DNA damage was significantly lesser in α -TC1 and GLUTag cells ($P<0.001$, Fig. 6A and B). Streptozotocin-induced DNA damage in GLUTag cells was significantly less than α -TC1 cells, while cytokine-induced DNA damage was significantly higher than α -TC1 cells ($P<0.05$, $P<0.01$, $P<0.001$, Fig. 6A and B). Toxic effects of streptozotocin and cytokines on DNA were comparable to DNA damage caused by exposure to ultraviolet light.

ER stress response, apoptosis and nitrite production

In GLUTag cells, cytokine cocktail increased *Hspa4* and decreased *Hspa5* transcription ($P<0.01$, $P<0.001$, Table 4). In contrast, streptozotocin decreased *Hspa5* transcription in all three cell lines ($P<0.05$, $P<0.01$, Table 4). Streptozotocin and cytokine cocktail significantly reduced *Bcl2* mRNA levels in MIN6 cells ($P<0.05$, Table 4). mRNA expression of *Bax* was unaffected in all three cell lines. Cytokines markedly increased *Nfkb1* transcription in all three cell lines, which was accompanied by a substantial increase in *Nos2* transcription ($P<0.01$, $P<0.001$, Table 4) and nitrite production (Fig. 6D). In our experiments, we considered untreated control cells with no expression of *Nos2* (Table 2) as 100% (cycle threshold value was >35 in PCR) and compared treated cells with this value. The percentage of apoptotic cells was significantly higher in all three cell lines after exposure to cytotoxins ($P<0.05$, $P<0.01$, $P<0.001$, Fig. 6C).

Discussion

Pancreatic β -cells are susceptible to streptozotocin and cytokine toxicity, whereas knowledge of the sensitivity of intestinal L-cells and pancreatic α -cells to these toxins is limited (Lenzen 2008). This paper evaluates the effects of multiple low-dose streptozotocin on these cell types in C57BL/6 mice, together with studies on cellular responses and related gene expression of intestinal L-cell and pancreatic β -, α -cell models under defined cytotoxic conditions *in vitro*.

Repeated injection of low sub-diabetogenic doses of streptozotocin over 5 days induced a severe form of diabetes (blood glucose >20 mmol/l) associated with lymphocytic infiltration of islets and β -cell destruction as is well known for this model (Shehata *et al.* 2011). Thus unlike a single large dose of streptozotocin that induces DNA damage and lethal depletion of NAD following DNA

Table 3 mRNA expression of genes involved in secretory function in MIN6, α -TC1 clone 9 and GLUTag cells after exposure to streptozotocin and cytokine cocktail. Gene expression was normalised to *Actb* expression, with respective untreated cells as control (100%). Values are mean $\pm$ S.E.M. ($n=3$)

Gene	Cell line	Control	Streptozotocin	Cytokine cocktail
<i>Ins</i>	MIN6	100 $\pm$ 2.4	67 $\pm$ 8*	40 $\pm$ 5*
<i>Gcg</i>	α -TC1 clone 9	100 $\pm$ 6.9	65 $\pm$ 10*	137 $\pm$ 5*
	GLUTag	100 $\pm$ 12	63 $\pm$ 11	82 $\pm$ 6
<i>Glut2</i>	MIN6	100 $\pm$ 10	38.7 $\pm$ 9*	45 $\pm$ 6*
	α -TC1 clone 9	100 $\pm$ 20	77 $\pm$ 20	114 $\pm$ 6
	GLUTag	100 $\pm$ 38	57 $\pm$ 16	226 $\pm$ 15*
<i>Pcsk1</i>	MIN6	100 $\pm$ 13	72 $\pm$ 12	113 $\pm$ 9
	α -TC1 clone 9	100 $\pm$ 11	73 $\pm$ 9	112 $\pm$ 9
	GLUTag	100 $\pm$ 6	45 $\pm$ 9 [†]	91 $\pm$ 8
<i>Pcsk2</i>	MIN6	100 $\pm$ 9	50 $\pm$ 5*	84 $\pm$ 6
	α -TC1 clone 9	100 $\pm$ 11	86 $\pm$ 8	123 $\pm$ 9
	GLUTag	100 $\pm$ 7	59 $\pm$ 9*	82 $\pm$ 9

* $P < 0.05$, [†] $P < 0.01$, and [‡] $P < 0.001$ compared with control.

repair in β -cells (Lenzen 2008), cytotoxic effects in this model are likely to involve both chemical and cell-mediated cytokine-induced attack (Sternesjo *et al.* 1997, Shehata *et al.* 2011). Lymphocyte infiltration was observed in $\sim 79\%$ of total islets analysed. Such effects were associated with reductions in both islet and β -cell areas, whereas α -cell area was increased, reflecting resistance of glucagon secreting cells to cytotoxicity as well as observed increase in α -cell proliferation. Destruction of β -cells with loss of normal paracrine inhibition by insulin may also contribute in this regard (Kelly *et al.* 2011).

Interestingly, multiple low-dose streptozotocin reduced the numbers of GLP1 secreting cells in the small intestine without any apparent induction of lymphocyte infiltration, suggesting induction of L-cell death, including possible deleterious effects on precursor stem cells that arise from within villus crypts (Gribble 2012). This apparent difference between α - and L-cells is notable given that both express the proglucagon gene and originate from similar cell lineage (Baggio & Drucker 2007). The turnover of intestinal L-cells is ~ 5 days and hence a reduction in L-cell number per mm² mucosal area, 5 days after the last streptozotocin administration might also relate to diabetic status of the animals and not to streptozotocin toxicity. In an independent study with similar experimental design, we observed that multiple low-dose streptozotocin treatment increased pancreatic and plasma glucagon, reduced pancreatic and plasma insulin while increasing plasma GLP1 (Vasu *et al.* 2014a). Such an increase in circulating GLP1 could be due to islet

α -cell-derived GLP1 in conditions of stress (Vasu *et al.* 2014a). Thus, many recent studies including our own have shown significant production of GLP1 by islet α -cells under conditions of stress. To investigate these issues in more detail, GLUTag and MIN6, α -TC1 cells, were used to examine cellular responses and expression of genes determining susceptibility to such cytotoxic insults.

Individual cytokines (IL1 β , IFN γ , TNF α) and streptozotocin decreased cell viability in all three cell lines in a dose-dependent fashion, an effect which has been documented previously in β -cells (Gao *et al.* 2000, Andersson *et al.* 2001, Eizirik *et al.* 2003, Kharroubi *et al.* 2004, Zhang *et al.* 2007). The LD₅₀ values for these agents were not significantly different for MIN6 and GLUTag cells, but they were 1.3- to 3.2-fold greater in α -TC1 cells exposed to streptozotocin, IL1 β or TNF α . The relative resistance of α -TC1 cells to IL1 β toxicity is consistent with previous observations (Hamaguchi & Leiter 1990). We also observed similar levels of cytokine receptor expression in MIN6 and α -TC1 cells. Thus resistance of α -cells to cytokine toxicity may be attributed to enhanced defence mechanisms and not due to differences in cytokine receptor expression. As streptozotocin mimics glucose in structure and enters cells via Glut2 transporter (Elsner *et al.* 2000, 2008, Lenzen 2008), lower *Glut2* mRNA levels in α -TC1 may contribute to the streptozotocin resistance.

Interestingly, GLUTag cells also expressed very low levels of *Glut2* mRNA compared with MIN6 cells, suggesting the involvement in L-cells of other glucose transporter isoforms or more likely the SGLT1 transporter (Gribble *et al.* 2003) in cellular uptake and action of streptozotocin. *In vivo*, the observed decrease in L-cell numbers could be due to direct toxic action of streptozotocin plus accompanying glucose toxicity as there was no evidence of intestinal lymphocyte infiltration. However, our *in vitro* observations indicate susceptibility of L-cells to cytokines and thus elevated concentrations of pro-inflammatory cytokines and glucose observed in circulation in type 1 diabetes (Lechleitner *et al.* 2000, Hanifi-Moghaddam *et al.* 2003, Chatzigeorgiou *et al.* 2010) could affect L-cell function and survival. Elevated level of TNF α after a single high-dose streptozotocin treatment has been reported recently (Ho *et al.* 2014), but further investigations are required to assess cytokine levels after multiple low-dose streptozotocin treatment. Concentrations used in our study were similar to other investigations on cytokine toxicity, while concentrations in the cytokine cocktail were slightly higher than those observed in the plasma of type 1 diabetic patients (Chatzigeorgiou *et al.* 2010). *In vivo* and *in vitro* conditions are different

Table 4 mRNA expression of genes involved in stress response in MIN6, α -TC1 and GLUTag cells after exposure to streptozotocin or cytokine cocktail. Gene expression was normalised to *Actb* expression, with respective untreated cells as control (100%). Values are mean $\pm$ S.E.M. ($n=3$)

Gene	Cell line	Control	Streptozotocin	Cytokine cocktail
Antioxidant defence				
<i>Cat</i>	MIN6	100 $\pm$ 12	284 $\pm$ 14 [‡]	86 $\pm$ 11
	α -TC1 clone 9	100 $\pm$ 6	111 $\pm$ 7	15 $\pm$ 7 [‡]
	GLUTag	100 $\pm$ 16	155 $\pm$ 10*	198 $\pm$ 13 [‡]
<i>Gpx1</i>	MIN6	100 $\pm$ 6	147 $\pm$ 12*	45 $\pm$ 4 [‡]
	α -TC1 clone 9	100 $\pm$ 5	100 $\pm$ 6	19 $\pm$ 8 [‡]
	GLUTag	100 $\pm$ 9	133 $\pm$ 10	98 $\pm$ 6
<i>Sod1</i>	MIN6	100 $\pm$ 9	91 $\pm$ 12	33 $\pm$ 4 [‡]
	α -TC1 clone 9	100 $\pm$ 7	85 $\pm$ 10	67 $\pm$ 8*
	GLUTag	100 $\pm$ 14	162 $\pm$ 11*	117 $\pm$ 10
<i>Sod2</i>	MIN6	100 $\pm$ 3	164 $\pm$ 3 [‡]	44 $\pm$ 3 [‡]
	α -TC1 clone 9	100 $\pm$ 11	134 $\pm$ 5 [‡]	157 $\pm$ 9 [‡]
	GLUTag	100 $\pm$ 10	146 $\pm$ 5*	83 $\pm$ 10
Apoptosis				
<i>Bax</i>	MIN6	100 $\pm$ 9	105 $\pm$ 7	100 $\pm$ 12
	α -TC1 clone 9	100 $\pm$ 9	79 $\pm$ 10	81 $\pm$ 9
	GLUTag	100 $\pm$ 7	96 $\pm$ 10	81 $\pm$ 8
<i>Bcl2</i>	MIN6	100 $\pm$ 9	55 $\pm$ 10*	57 $\pm$ 9*
	α -TC1 clone 9	100 $\pm$ 4	75 $\pm$ 8	76 $\pm$ 8
	GLUTag	100 $\pm$ 6	96 $\pm$ 9	97 $\pm$ 6
<i>Nfkb1</i>	MIN6	100 $\pm$ 10	102 $\pm$ 7	223 $\pm$ 9 [‡]
	α -TC1 clone 9	100 $\pm$ 7	140 $\pm$ 8*	185 $\pm$ 10 [‡]
	GLUTag	100 $\pm$ 6	90 $\pm$ 9	270 $\pm$ 6 [‡]
<i>Nos2</i>	MIN6	100 $\pm$ 6	92 $\pm$ 9	658 $\pm$ 22 [‡]
	α -TC1 clone 9	100 $\pm$ 9	58 $\pm$ 9*	7084 $\pm$ 12 [‡]
	GLUTag	100 $\pm$ 7	139 $\pm$ 11*	485 $\pm$ 10 [‡]
ER stress response				
<i>Hspa4</i>	MIN6	100 $\pm$ 9	76 $\pm$ 8	89 $\pm$ 8
	α -TC1 clone 9	100 $\pm$ 9	70 $\pm$ 8	102 $\pm$ 7
	GLUTag	100 $\pm$ 7	115 $\pm$ 9	197 $\pm$ 7 [‡]
<i>Hspa5</i>	MIN6	100 $\pm$ 12	31 $\pm$ 8 [‡]	62 $\pm$ 13
	α -TC1 clone 9	100 $\pm$ 7	73 $\pm$ 5*	14 $\pm$ 9 [‡]
	GLUTag	100 $\pm$ 10	50 $\pm$ 9*	118 $\pm$ 6
Cytokine receptors				
<i>Il1r1</i>	MIN6	100 $\pm$ 24	NA	960 $\pm$ 38 [‡]
	α -TC1 clone 9	100 $\pm$ 14	NA	173 $\pm$ 31
	GLUTag	100 $\pm$ 29	NA	172 $\pm$ 8
<i>Ifngr1</i>	MIN6	100 $\pm$ 12	NA	97 $\pm$ 35
	α -TC1 clone 9	100 $\pm$ 16	NA	161 $\pm$ 18
	GLUTag	100 $\pm$ 27	NA	91 $\pm$ 8
<i>Tnfrsf1a</i>	MIN6	100 $\pm$ 21	NA	133 $\pm$ 19
	α -TC1 clone 9	100 $\pm$ 10	NA	107 $\pm$ 19
	GLUTag	100 $\pm$ 20	NA	656 $\pm$ 29 [‡]

* $P<0.05$, [‡] $P<0.01$, and [‡] $P<0.001$ compared with control. NA, not assessed.

as autoimmune cells, in addition to pro-inflammatory cytokines, release other factors including nitric oxide, granzymes and perforins, which have synergistic effects, thus explaining the choice of concentrations in our study. Further investigations into the actions of cytokines were examined using cocktails containing approximately one-tenth of LD₅₀ dose of individual cytokines, thereby ensuring comparable reduction in viability of each cell line. As α -TC1 cells were relatively resistant to

streptozotocin, lower concentrations (which were lethal for MIN6 and GLUTag cells) may not induce any cellular responses. Hence we chose to test individual concentrations at which a significant reduction in viability of each cell line was observed.

The cellular actions of streptozotocin and cytokine cocktail were accompanied by decreased release of insulin and GLP1 during 8-h cultures of MIN6 and GLUTag cells, whereas glucagon release was enhanced from α -TC1 cells.

Figure 6

Effects of 8-h exposure of MIN6, α -TC1 clone 9 and GLUTag cells to streptozotocin and cytokine cocktail on (A) % tail DNA in comets, (B) olive tail moment of comets, (C) % apoptotic cells and (D) nitrite accumulation in culture medium. Cytokine cocktail consisted of i) 60 U/ml IL1 β + 20 U/ml IFN γ + 200 U/ml TNF α ; ii) 100 U/ml IL1 β + 25 U/ml IFN γ + 250 U/ml TNF α ; and iii) 60 U/ml IL1 β + 16 U/ml IFN γ + 185 U/ml TNF α . Values are mean $\pm$ S.E.M.

($n=4$). Approximately 100 cells per replicate were analysed for comet assay and acridine orange/ethidium bromide assay. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ compared with untreated control. $\Delta P<0.01$ and $\Delta\Delta P<0.001$ compared with respective treatment of MIN6 cells. $\phi P<0.05$, $\phi\phi P<0.01$, $\phi\phi\phi P<0.001$ compared with respective treatment of α -TC1 cells.

With exception of α -TC1 cells exposed to cytokines, cytotoxins decreased *Ins* and *Gcg* gene expression which undoubtedly contributed to decreased hormone output. Other contributing factors might be changes in proglucagon processing or damage to proteins such as those involved in granule transport and exocytosis. Indeed, streptozotocin markedly decreased *Pcsk2* transcription in MIN6 cells and *Pcsk1* and *Pcsk2* transcription in GLUTag cells but not in α -TC1 cells. Cytokines did not markedly affect transcription of prohormone convertases in any of the cell lines, but a trend towards downregulation of *Pcsk2* was observed in MIN6 and GLUTag cells. This difference clearly reflects different mechanisms of action which for cytokines involves activation of master transcription factors – NF κ B and STAT1, resulting in transactivation of a number of genes involved in function and cell survival (Gurzov *et al.* 2008, Moore *et al.* 2012).

The high susceptibility of insulin secreting cells to streptozotocin and other toxins, such as alloxan, is partly attributed to poor antioxidant defence mechanisms, including low levels of catalase, superoxide dismutase and glutathione peroxidase (Lenzen 2008). In this study, cytokines decreased transcription of *Gpx1*, *Sod1* and *Sod2* in MIN6 cells and *Gpx1*, *Cat* and *Sod1* in α -TC1 cells but upregulated *Cat* transcription in GLUTag cells. Streptozotocin upregulated transcription of *Gpx1*, *Cat* and *Sod1* in MIN6 cells; *Cat*, *Sod1* and *Sod2* in GLUTag cells and *Sod2* in α -TC1 cells, representing activation of defence mechanisms to counter cytotoxic attack. Oxidative stress activates NF κ B, a rapid acting transcription factor which activates either defence mechanisms via *Sod2* upregulation or apoptosis via *Bax* upregulation among other effects. We did not observe *Sod2* upregulation in

cytokine cocktail-treated MIN6 and GLUTag cells which could be due to the fact that NF κ B activation activates apoptosis pathways instead of cellular defence pathways in these cell types. Upregulation of *Sod2* in α -TC1 cells together with higher expression of *Cat* mRNA compared with MIN6 cells could offer protection from oxidative stress. In line with our observations, higher level of catalase enzyme expression was observed in pancreatic α -cells previously (Bloch *et al.* 2007).

Cytokine cocktail increased NF κ B1 transcription in all cell lines, which was accompanied by strong induction of *Nos2* expression and a significant increase in nitrite levels in the culture medium as observed previously in cytokine-exposed β -cell models, human islets and FACS-purified rat β -cells (Corbett *et al.* 1993, Lortz *et al.* 2000, Lakey *et al.* 2001, Sigfrid *et al.* 2003, Souza *et al.* 2004, Larsen *et al.* 2007, Zhang *et al.* 2007, Gurzov *et al.* 2008, Moore *et al.* 2011, 2012). Although gene expression data does not reflect activity of NF κ B, it should be noted that NF κ B autoregulates its own expression. Thus increase in NF κ B1 transcription indirectly suggests enhanced activity after cytokine exposure. Streptozotocin also upregulated NF κ B in α -TC1 cells but decreased *Nos2* expression without affecting nitrite production. *Nos2* was not expressed to any significant extent in healthy MIN6, α -TC1 or GLUTag cells. Nitrite interferes with ER Ca²⁺ homeostasis and activates the ER stress response which together with oxidative stress results in upregulation of expression of molecular chaperones. *Hspa4* transcription was noticeably increased in cytokine cocktail-treated GLUTag cells, suggesting the activation of ER stress response or existence of oxidative stress. Further, cytokine exposure increased transcription of cytokine

receptors in MIN6 and GLUTag cells (α -TC1 cells to some extent), suggesting the activation of master transcription factors STAT1 and NF κ B which activate transcription of cytokine receptors among other genes.

Consistent with different pattern of gene expression and susceptibility to cytotoxic attack, the level of DNA damage was modest in α -TC1 cells treated with toxins when compared with MIN6 or GLUTag cells. Excessive DNA damage activates mitochondrial apoptotic pathway and explains the observed downregulation of *Bcl2* transcription and increased numbers of apoptotic MIN6 cells. Although we used different concentrations of streptozotocin and cytokine cocktail for all three cell lines, lower degree of DNA damage even at higher concentration clearly suggests enhanced protection from cytotoxicity in α -TC1 cells. Induction of apoptosis was also of greater magnitude in MIN6 and GLUTag cells compared with α -TC1 cells. The cells in small intestine express lower levels of *Bcl2* and undergo spontaneous apoptosis due to lower threshold for induction of apoptosis, further confirming our observations (Potten 1992, Bach *et al.* 2000). Additional investigations using same concentrations of cytokines or streptozotocin would be useful to compare absolute differences in susceptibility of pancreatic α -, β - and intestinal L-cells to cytotoxicity.

In conclusion, streptozotocin and cytokines impaired secretory function, activated anti-oxidant defence mechanisms, induced DNA damage, reduced cell viability and triggered apoptosis in both GLUTag and MIN6 cells. In comparison, α -TC1 cells were substantially less susceptible to both streptozotocin and cytokines-evoked attack due to differences in the expression of genes involved in mediating their toxicity or anti-oxidant defence mechanisms. Additional studies evaluating gene expression at protein level would extend these data, but the present observations help to explain selective loss of β -cells in diabetes together with proliferation and enhanced function of glucagon secreting α -cells. Our data also suggest that GLP1-secreting L-cells are prone to cytotoxic attack and dysfunctional secretion, although this may be limited *in vivo* due to the rapid cell turnover of the intestinal mucosa and cellular replacement by differentiating stem cells that arise from the intestinal crypt (Roth *et al.* 1990, Bach *et al.* 2000, Schonhoff *et al.* 2004, Cani *et al.* 2007). Similar to β -cell compensation during early stages of diabetes and exhaustion in later stages, L-cell compensation may well precede L-cell exhaustion which in turn may further contribute to disease progression. Further investigations to assess the effects of chronic elevation of cytokines in circulation on

enteroendocrine cells could help to better understand the observed metabolic abnormalities in type 1 diabetes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S V and R C M contributed to acquisition of data, analysis, interpretation and drafted the manuscript. P R F and N H M conceived and designed the study and revised the manuscript critically for intellectual content. All authors approved the final version.

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