A20 controls expression of beta cell regulatory genes and transcription factors

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ABSTRACT

TNFAIP3 encodes a zinc finger protein called A20, which has potent anti-inflammatory and anti-apoptotic properties. A20 promotes beta cell survival and protects against islet graft rejection in experimental models. The current study sought to investigate the mechanisms underlying the protective role of A20 in the pancreatic beta cell. Two islet cell types were used for experiments: The insulin-secreting BRIN-BD11 cell line and human islet cells. A20 was silenced using siRNA against *TNFAIP3* and knockdown confirmed by qPCR and immunostaining of cells. Cell viability, cytotoxicity and apoptosis was assessed using the ApotoxGlo assay. Glucose-stimulated insulin secretion and production of inflammatory cytokines (TNFa, IL1b and IFNg) were measured by ELISA. Expression of beta cell regulatory genes (*Abcc8, Kcnj11, Kcnq1, Gck, Scl2a2*) and transcription factors (*Hnf1a, Pdx1, Nkx6.1, Ngn3*) was determined by qPCR. A20 deficiency increased apoptosis, impaired glucose-induced insulin secretion, and reduced expression of beta cell regulatory genes and transcription factors. Addition of recombinant A20 normalized gene expression profiles. TNFa, IL1b and IFNg were elevated in A20 deficient cells and found to independently elicit changes in gene expression. Analysis of PCR array data suggests that A20 action in the beta cell is largely, although not exclusively, driven by the P65 subunit of NF-kB. The current report demonstrates a role for A20 in controlling beta cell integrity and survival, which likely results from regulation of inflammatory signaling. Of particular note is the impact that A20 deficiency has on the expression of transcription factors regulating the maturation and normal function of beta cells.

ABSTRACT: 249 words

INTRODUCTION

TNFAIP3 (Tumor necrosis factor, alpha-induced protein 3) encodes a zinc finger protein called A20 that exerts dual ubiquitin editing properties (Wertz *et al*., 2004). A20 is an endogenous negative regulator of inflammatory and apoptotic pathways including NF-kB (Nuclear Factor kappa B) and JNK (c-Jun N-terminal kinase) that is rapidly and transiently inducible in response to a range of inflammatory stimuli of which TNFa, IL1b and bacterial LPS are best characterised (Opipari *et al*., 1990; Lin *et al*., 2008). A20 interrupts the activation of these pathways early in the biological process, after recruitment of signaling molecules to the receptor-ligand complex by firstly deubiquitinating its target molecule to render it inactive (Shembade *et al*., 2010). Subsequently, A20 ubiquitinates the target molecule triggering proteasomal degradation and preventing further downstream activation of the pathway (Kerscher *et al*., 2006). These properties make A20 a potent anti-inflammatory and anti-apoptotic protein.

More than 66 genes including *TNFAIP3/A20* are altered after challenge by cytokines released in response to NF-kB activation (Cardozo *et al*., 2001; Cheng *et al*., 2016). The role of A20 has been extensively studied in various tissue types. Uncontrolled and multiorgan inflammation has been observed in A20 deficient mice (Zheng *et al*., 2016). TNFAIP3fl/flCD19-Cre mice have been shown to develop a disease resembling systemic lupus erythematosus with increased production of autoantibodies (Umiker *et al*.,2014). The induction of A20 expression in cystic fibrosis airway epithelial cells is significantly delayed when compared with healthy controls and is associated with an inability to form important signalling complexes required to prevent NF-kB-driven inflammation (Kelly *et al*., 2013). B lymphoid cells devoid of A20 are hyper-responsive to stimuli and show increased activation of NF-κB (Tavares *et al*., 2010a). Dendritic cells lacking A20, mature at a faster rate, are more responsive to TLR ligands, and produce more cytokines when compared to their wild type counterparts (Kool *et*

al., 2011). Loss of A20 in dendritic cells also has implications on populations of other cells of the immune system (Kool *et al*., 2011; Hammer *et al*., 2012).

TNFAIP3/A20 has been identified as the most highly regulated anti-apoptotic gene in the pancreatic beta cell (Liuwantara *et al*., 2006) and protects against islet graft rejection in animal models of transplantation (Grey *et al*., 2003; Zammit *et al*., 2019). Therapeutic administration of A20 promotes immune tolerance and the survival of transplanted islets by increasing inflammatory signaling thresholds (Zammit *et al.* 2019). In resting beta cells, basal expression of A20 and activation of NF-κB are low and is increased upon challenge (Baker *et al*., 2011). Knockdown of A20 in primary rat and mouse islet cells results in persistent cytokine-mediated JNK activation (Fukaya *et al*., 2015). A20 was also shown to positively regulate Akt signalling in beta cells contributing to the anti-apoptotic effect (Pepin *et al*., 2014). In the context of human disease, mRNA and protein expression of A20 is reduced in blood mononuclear cells from people with type 2 diabetes and adults with latent autoimmune diabetes (Cheng *et al.* 2014). Furthermore, A20 has been identified as a novel locus of interest within HLA class II regions known to encompass a number of genetic risk factors for autoimmune pathologies including type 1 diabetes (T1D) (Gough and Simmonds, 2007; Relle and Schwarting, 2012).

The protective effect of A20 in the pancreatic beta cell and the relationship with diabetes risk is well established. Research has identified many of the signalling pathways through which A20 acts in the beta cell including NF-kB, Akt and JNK. However, the direct impact of A20 deficiency on the regulatory machinery of the beta cell remains poorly defined. The current study investigated the impact of A20 deficiency on beta cell survival, glucose-stimulated insulin secretion and the expression of beta cell markers and transcription factors regulating beta cell maturation and function.

MATERIALS AND METHODS

Cell culture and treatment

All initial experiments and optimization protocols were performed in the insulin-secreting BRIN-BD11 cell line (passages 11-30), which was purchased from ECACC (Salisbury, England). Experiments were confirmed in human islet cells (passages 3-7), purchased from CelProgen (cat no. 35002-04; California, USA) and the Jurkat T-cell line (passages 12-20), also purchased from ECACC (Salisbury, England). All cells were cultured and maintained according to the suppliers' instructions. Further information on the isolation and characterisation of CelProgen human islets can be found in the Electronic Supplementary Material (ESM, Figure S1). Cells were treated with either 10 ng/mL TNFa, 10 ng/mL IL1b, 10 ng/mL IFNg, or cytomix (TNFa, IL1b, IFNg combined), (all PeproTech; London, England) as indicated in the Figures.

Silencing of A20

BRIN-BD11, human islet cells, and Jurkat cells were transfected using siRNA against *TNFAIP3* (Flexitube siRNA, Qiagen; Manchester, England). A total of 100 ng siRNA and 2.5 µL lipofectamine 2000 (Qiagen) were added to 100 µL serum free RPMI media (Gibco; Loughborough, England). A negative control consisting of 100 ng scrambled siRNA (AllStars Negative Control siRNA, Qiagen), 2.5 µL lipofectamine, and 100 µL serum free RPMI media and mock control containing 2.5 µL lipofectamine and 100 µL serum free media were also employed. Tubes were allowed to incubate for 15 minutes at room temperature to allow the formation of transfection complexes and thereafter, added dropwise to the cells. Islet cells were incubated in the presence of the transfection complexes for 48 hours. After this time, transfection complexes were removed, and cells were incubated with fresh complexes for a further 24 (BRIN-BD11) or 48 (human islet cells) hours. Jurkat cells were maintained in the

presence of transfection complexes for 72 hours without media change. Knockdown was confirmed at the mRNA and protein level (Figure S2, ESM).

To confirm the specific role of A20, reversal of knockdown was achieved using 10 μm recombinant A20 protein (Abcam) targeting the N terminal domain. Internalisation of recombinant protein was achieved as previously described (Rust *et al.,* 2015; Zuris *et al.*, 2015) using 2.5 μL lipofectamine for 24h. Uptake of A20 into the cytoplasm of the cell was confirmed by immunofluorescent staining of the cell as described below. Please see Figure S3A-B, ESM for quantification of A20 uptake. The selected dose of recombinant A20 did not affect apoptosis over 24h (Figure S3C).

Quantitative real time PCR (qPCR)

Total RNA was extracted from cells using the QIAGEN RNeasy Kit according to the manufacturer's instructions. RNA concentration was measured using Nanodrop ND-1000 (Thermo Fisher; Loughborough, England) with 260/280 ratios of 1.8 - 2.1 accepted as indicative of good quality RNA. RNA was sporadically run on 2% agarose gels to check RNA integrity. A total of 500 ng RNA was reverse transcribed to cDNA using the Roche Transcriptor cDNA synthesis kit (Welwyn Garden City, England) according to supplier instructions. qPCR was performed on a Lightcycler 480 System (Roche) using custom designed probes (See Table 1) and qPCR MasterMix (Roche) as previously described (Khan *et al*., 2019). Following optimization, 18S was chosen as a reference gene for all experiments. Relative mRNA expression was determined using 2^ΔCt method and normalised to 18S.

Immunofluorescent staining of islet cells

Cells were seeded into a 24 well plate containing 8 mm glass slides and allowed to attach until 90% confluency had been achieved. Media was aspirated, and the cells washed with cold PBS. Cold methanol was added for 15 minutes to fix the cells, followed by a wash with cold PBS. Permeabilization of the cells was performed using 0.1% Triton-X (Merck; Irvine, England)

for 10 minutes at room temperature followed by a wash with cold PBS. Donkey serum (5%, diluted in PBS, Merck) was added and the cells incubated for 30 minutes at room temperature followed by a wash with cold PBS. The cells were then incubated with a primary antibody against A20 (Santa Cruz Biotech (sc-166692), 1:200. Santa Cruz, USA), washed with cold PBS and incubated with an appropriate secondary antibody (Alexa Fluor® 594, Invitrogen (A11032), 1:200. Invitrogen; Loughborough, England). The cells were then co-stained with 1:10000 DAPI (Merck) for five minutes at room temperature followed by a wash with cold PBS. Slides were mounted in the dark with aqueous mounting media (Abcam; Cambridge, England) and imaged under a fluorescent microscope (Zeiss Axio). Antigen positive cells were expressed as a percentage of total cells (DAPI positive cells).

Assessment of viability, cytotoxicity and apoptosis

Apotox-Glo™ Triplex Assay by Promega (Southampton, England) allows for concurrent measurements of cell viability, cytotoxicity and apoptosis. The assay implements fluorogenic peptide substrate (bis-AAF-R110 Substrate) to measure dead-cell protease activity used to assess viability and cytotoxicity of treated cells. Caspase-3/7 and Ultra-Glo™ Recombinant Thermostable Luciferase luminesce measurements are used as direct measure of apoptosis. The assay was used to assess the viability, cytotoxicity and activation of apoptosis in treated BRIN-BD11 cells and human islets. All experiments were performed using the manufacturer's protocol.

Measurement of glucose-induced insulin secretion

BRIN-BD11 cells and human islet cells were incubated for 40 minutes in Krebs solution (115 mM NaCl; 4.7 mM KCl; 1.28 mM $CaCL_6H_20$; 1.2 mM KH_2PO_4; 1.2 mM MgSO4.7H20; 1 mg/mL Bovine serum albumin) supplemented with 1.1 mM D-glucose at 37 ºC. The media was then removed, and cells supplemented with increasing concentrations of D-glucose (1.1 mM and 16.7 mM) for a further 20 minutes. The supernatant was collected and assessed for insulin release using the Mercodia (Uppsala, Sweden) Ultrasensitive Rat insulin ELISA for BRIN-BD11

177 cells and Mercodia Ultrasensitive human insulin ELISA for human islet cells. All ELISAs were
178 conducted according to the manufacturer's instructions.
179
180 **Determination of cytokine concentration**
181 A20 was silenced in BRIN-BD11 cells and Jurkat cells as described above. Following
182 completion of the transfection process, fresh culture medium was added to cells and collected
183 24 hours later. The concentration of TNFa, IL1b and IFNg was assessed using commercially
184 available ELISA kits (PeproTech) according to the manufacturer's instructions.
185
186 **Investigating the mechanism of A20 action in the beta cell**
187 To investigate the mechanisms by which A20 regulates beta cell survival and function, two
188 RT² Profiler™ PCR arrays (Qiagen; Manchester, England) were used to assess differences in
189 the expression of genes involved in apoptotic and inflammatory pathways in BRIN-BD11 cells
190 treated with siRNA against *TNFAIP3* or a scrambled control. The arrays were preformed
191 according to manufacturer's instructions. The generated results were analysed by the
192 QIAGEN RT^2 PCR array data analysis software with a Ct threshold of 35 cycles. Significant
193 differences in gene expression were accepted for genes with fold changes >2 and $P<0.05$.
194 Differentially expressed genes were taken forward for pathway analysis. Obtained P values
195 and fold changes for each gene of interest were imputed into Ingenuity Pathway Analysis
196 software (Qiagen; Manchester, England). Core analysis was performed to identify canonical
197 pathways predicted to be affected by *TNFAIP3* deficiency. Based on pathway analysis, NF-
198 kB signalling and the protein ubiquitination pathway, were targeted for further investigation.
199
200 **Inhibition of NF-kB signalling and ubiquitination**
201 JSH-23 (5 mg, ≥98% (HPLC)), a P65 inhibitor was acquired from Merck (Darmstadt, Germany)
202 in solid form. The substance was resuspended in warm DMSO at a concentration of 1.10 g/mL
203 and diluted to 30 μM in cell culture medium. MG-132 (≥90% (HPLC)), a proteasome inhibitor
204 that reduces the degradation of ubiquitin-conjugated proteins was obtained in 10mM DMSO

readymade solution (Merck) and diluted to 30 μM in cell culture medium. Cells were first allowed to reach 80% confluency and then exposed to 30 μM of JSH-23 or MG-132 for 1 hour prior to experimentation.

Data analysis

Statistical analysis was performed using GraphPad PRISM (La Jolla, USA; version 7). Data are presented as mean ± SEM for a given number of observations (n) as indicated in the Figures. Differences between groups were compared using 2-tailed Student's *t* test as appropriate. Statistical significance was accepted at $P<0.05$.

RESULTS

Peak induction of A20 is observed 1 hour after TNFa stimulation

Low level endogenous A20 expression is observed under basal conditions and expression is rapidly induced following exposure to a range of cytokines, the best characterised of which, is TNFa. BRIN-BD11 cells were therefore exposed to 10 ng/ml TNFa over a 24h period to optimise time of exposure for subsequent experiments. Consistent with the first reports describing A20 induction (Opipari *et al*., 1990), peak induction of *TNFAIP3/A20* mRNA was observed 1h after TNFa stimulation (2.94 ± 0.36-fold increase, $P<0.001$; Fig. 1A). Expression, thereafter, rapidly fell below basal levels. Induction at the protein level 1h after TNFa treatment was also confirmed (3.87 ± 0.015-fold increase, $P<0.001$; Fig. 1B and 1C). Experiments in human islet cells consistently observed significant increases following 1h TNFa treatment at both the mRNA (2.86 ± 0.35-fold increase, $P<0.001$; Fig. 1D) and protein (1.795 ± 0.021-fold increase, $P<0.001$; Fig. 1E and 1F) levels. TNFa exposure of 1h was used for all subsequent experiments.

Silencing of A20 increases apoptosis in islet cells.

A20 was silenced in both BRIN-BD11 and human islets using siRNA against *TNFAIP3* and expression compared with negative controls (scrambled siRNA). Knockdown was confirmed at the mRNA (62.79 ± 0.085% knockdown in BRIN-BD11 cells, $P<0.001$, Fig. S2A; 92.38 ± 0.02% knockdown in human islet cells, $P<0.001$, Fig. S2D; and 52.42 ± 0.08% knockdown in Jurkat cells, $P<0.001$, Fig. S2G (ESM)) and protein levels (61.18 ± 0.03% reduction in A20 positive cells in BRIN-BD11 cells, $P<0.001$, Fig. S2B and S2C; and 93.63 ± 0.01% reduction in A20 positive cells in human islet cells, $P<0.001$, Fig. S2E and S2F (ESM)).

The impact of A20 silencing on cell viability, cytotoxicity and apoptosis was determined using the AptoTox Glo™ assay. Following transfection of *TNFAIP3* siRNA, BRIN-BD11 cells and human islets were tested in the presence or absence of 10 ng/ml TNFa for 1h and differences compared with negative controls. Significant changes in cell viability, cytotoxicity or apoptosis were not observed in the absence of TNFa stimulation, consistent with the idea that A20 is an inducible protein with limited activity under basal resting conditions. In the presence of TNFa, differences in cell viability and cytotoxicity were not observed in either cell type examined (Fig. 2A and 2C). However, apoptosis was significantly increased in A20 silenced BRIN-BD11 cells (2.85 ± 0.37-fold increase, $P<0.01$, Fig. 2B) and human islet cells (2.21 ± 0.15-fold increase, $P<0.01$, Fig. 2D).

Glucose-stimulated insulin secretion is reduced in A20 silenced islet cells

The impact of A20 silencing on insulin secretion in response to acute exposure to basal (1.1 mM) and stimulatory (16.7 mM) concentrations of glucose was determined by ELISA. No change in insulin release was observed in A20 silenced BRIN-BD11 cells (Fig. 3A) or human islet cells (Fig. 3B) compared with negative controls in response to basal glucose concentrations. However, significant reductions in stimulated insulin secretion were found in both cell types (0.42 ± 0.0011-fold reduction in BRIN-BD11 cells, $P<0.05$, Fig. 3A; and 0.53 ± 0.0009-fold reduction in human islet cells, $P<0.01$, Fig. 3B).

A20 regulates the expression of beta cell and inflammatory markers through release of pro-inflammatory cytokines

The mRNA expression of a range of beta cell markers, transcription factors regulating beta cell maturation, and inflammatory markers (NF-kB subunits) was assessed by qPCR (Fig. 4). Expression of all beta cell markers and all beta cell transcription factors tested was reduced in A20 silenced BRIN-BD11 cells (P<0.05 – 0.001, Fig. 4A and 4B). Given the important role of A20 in NF-kB activation, we also assessed the expression of *NFKB1* and *RelA*, which encode the NF-kB subunits P50 and P65 respectively. We observed a reduction in the expression of *NKFB1* (P<0.001), but an increase in *RelA* expression (P<0.001) following A20 silencing. This is consistent with data showing that A20 exerts its anti-inflammatory action in a P65-dependent manner (Kelly *et al*., 2013). To determine if the changes in gene expression were A20-dependent, we exposed BRIN-BD11 cells to recombinant A20 protein for 24h. Recombinant A20 was readily internalised with the aid of lipofectamine. Cytoplasmic expression of A20 in unstimulated BRIN-BD11 cells increased by 3.97 ± 0.03-fold (P<0.001; Fig. S3, ESM) following 24-hour exposure to the recombinant protein. Consistently, BRIN-BD11 cells exposed to *TNFAIP3* siRNA prior to exposure to recombinant A20 showed similar 3.91 ± 0.05-fold increase in expression (P<0.001; Fig. S3, ESM). With the exception of *RelA,* administration of recombinant A20 independently enhanced the expression of all investigated genes. In all instances, the pattern of gene expression following silencing of A20 was reversed upon administration of the recombinant protein (Fig. 4).

Activation of the NF-kB pathway leads to the release of pro-inflammatory cytokines. TNFa, IL1b and IFNg have been implicated in the pathogenesis of beta cell demise. We therefore assessed the concentrations of these cytokines in supernatants taken from A20-silenced BRIN-BD11 cells (Fig. 5A). TNFa was increased 3.64-fold (P<0.01), IL1b, 1.70-fold (P<0.01), and IFNg, 2.16-fold (P<0.05). To assess whether this effect was specific to the beta cell, experiments were repeated in the Jurkat cell line where consistent increases in the concentrations of all three cytokines was observed (P<0.05 – 0.001, Fig. 5B).

BRIN-BD11 cells were subsequently exposed to recombinant versions of these cytokines and to Jurkat conditioned media. The expression of beta cell markers, transcription factors and NF-kB subunits were assessed by qPCR (Fig. 6). The pattern of expression in this instance was remarkably similar to that observed in Fig. 4. Most genes were significantly downregulated (Fig 6A-6C, $P<0.001$) with only *RelA* showing a significant increase in expression ($P<0.001$, Fig. 6C). However, there was one notable exception that did not follow the pattern of expression observed after A20 silencing. The expression of *Hnf1a* was not significantly altered in response to any cytokine tested or Jurkat conditioned media (Fig. 6B).

Silencing of A20 activates the NF-kB and protein ubiquitination pathways

To better understand the mechanisms by which A20 confers protection in the beta cell, we performed PCR arrays on two separate panels of genes related to inflammatory and apoptotic pathways. Of the 164 genes tested, 15 were significantly differentially expressed (>2-fold change, $P<0.05$) between *TNFAIP3* deficient BRIN-BD11 cells and negative controls (scrambled siRNA) (Fig. S4A, ESM). Obtained *P* values and fold changes for each gene of interest were imputed into Ingenuity Pathway Analysis software, which predicted several candidate pathways affected by *TNFAIP3* deficiency (Fig. S4B, ESM). Based on relevance to the beta cell, ability to target, and overall significance, we selected the NF-kB and protein ubiquitination pathways for further study. Since our data showed that P65 and not P50 was significantly upregulated in A20 deficient cells, we used the selective P65 inhibitor, JSH23. The 26S proteasome inhibitor MG132, which prevents degradation of ubiquitin-conjugated proteins was also employed. Both Inhibitors were used at a concentration of 30 μM (Kelly *et al.,* 2013) and cells were found to tolerate this dose well over a 24h period (Fig. S5, ESM).

In all instances, the expression of beta cell regulatory genes (Fig. 7A) was downregulated in A20-silenced cells. Addition of JSH23 to negative controls (BRIN-BD11 cells exposed to scrambled siRNA) partially or fully reversed these reductions in expression. Restoration of

Kcnq1 and *Gck* expression in the presence of JSH23 persisted even when BRIN-BD11 cells were exposed to siRNA against TNFAIP3. However, this was not true of *Abcc8, Kcnj11* and *Scl2a2* expression where JSH23 failed to prevent A20-driven reductions in expression (Fig. 7A). In the case of beta cell transcription factors (Fig. 7B) and the expression of NF-kB subunits (Fig. 7C), JSH23 administration was largely able to inhibit A20-driven changes in gene expression. In almost all instances, the addition of MG132 was not able to fully restore gene expression after A20 silencing (Fig. 7A – 7C).

DISCUSSION

TNFAIP3 encodes a protein called A20 that is capable of acting as both a deubiquitinating enzyme and an E3 ligase. A20 comprises two domains: (1) an OTU region capable of deubiquitinating K63-linked polyubiquitin chains from target proteins to render them inactive and (2) a c-terminal region that contain 7 zinc finger proteins, which act as E3 ligases by adding K43 ubiquitin chains to target proteins triggering degradation by the 26S proteasome (Makarova *et al*., 2000; Wertz *et al*., 2004). Together this dual function makes A20 a potent negative regulator of several inflammatory and apoptotic pathways (Abassi *et al*., 2015). The current study finds a role for A20 in regulating the expression of genes and transcription factors that play a critical role in normal beta cell functioning and maturation.

Early work by Dixit and colleagues (1990) identified A20 as TNF-responsive gene with rapid and transient induction. Peak A20 expression was observed 1 hour after treatment with TNFa in human umbilical vein endothelial cells, which differed from other TNF-responsive genes where significant expression persisted for at least 8 hours (Opipari *et al*., 1990). Consistently, the present study found low level basal A20 expression in untreated beta cells with expression significantly upregulated after 1-hour TNFa treatment. Although inducible in response to other inflammatory stimuli including IL1b and bacterial LPS, the downstream effects of TNFa

345 stimulation on A20 action is best studied, and therefore, was selected as the stimulant for
346 experiments in this study.
347
348 Knockdown of A20 predictably resulted in an increase in apoptosis in beta cells irrespective if
349 treated with TNFa. Prior work has shown that deletion of A20 sensitises cells to TNF-induced
350 apoptosis through ubiquitin-dependent and independent mechanisms following a single TNF
351 stimulation (Priem *et al*., 2019). Within minutes of TNF sensing, A20 binds to the TNFR1
352 signalling complex via the zinc finger domain and stabilises the ubiquitin network associated
353 with the signalling complex independently of its E3 ligase activity (Priem *et al*., 2019).
354 Additionally, A20 was also shown to exert deubiquitinase activity to protect against TNF-driven
355 apoptosis (Priem *et al*., 2019). Work from the INS-1E pancreatic beta cell line, primary rat
356 islets and islets from beta cell-specific A20 knockout mice reveal that the anti-apoptotic
357 potential of A20 extends beyond negative regulation of NF-kB (Fukaya *et al*., 2016). In these
358 experiments, A20 was found to promote beta cell survival via actions on Akt signalling and
359 inhibition of the intrinsic apoptotic pathway in response to IL1b stimulation (Fukaya *et al*.,
360 2016).
361
362 The protective role of A20 in maintaining beta cell mass and protecting beta cell function is
363 well established (Grey *et al*., 2003; Liuwantara *et al*., 2006). Islet grafts overexpressing A20
364 are found to resist apoptosis and sustain beta cell function in animal models (Grey *et al*.,
365 2003). We found significant reductions in insulin secretion following A20 silencing. However,
366 this was not solely attributable to the increase in apoptosis. When data was corrected for
367 protein content, only a modest restoration of glucose-induced insulin secretion was observed.
368 Upon further examination, it was found that A20 deficiency had a significant negative impact
369 on the expression of many genes related to the beta cell secretory machinery, including those
370 encoding the K_{ATP} channel, GLUT2 and glucokinase. Furthermore, the expression of several
371 transcription factors involved in the development and normal function of mature beta cells was
372 also significantly impaired. This was associated with upregulation of *RelA*, which encodes the

P65 subunit of NF-kB. To our knowledge, there are no other reports of alterations in the expression of these genes in response to A20 deficiency and a logical mechanism by which A20 would directly control the transcriptional regulation of the beta cell machinery was not apparent. It seemed likely that soluble secreted factors released from cells in the absence of adequate expression of A20 may influence the expression of genes essential to the normal functioning of beta cells.

We examined the concentrations of TNFa, IL1b and IFNg, known to increase beta cell apoptosis by inducing the formation of oxygen free radicals and nitric oxide (Rabinovitch *et al*., 1998). All three cytokines were elevated in A20 silenced beta cells. To determine if this effect was beta cell specific, we also investigated the concentrations of these cytokines in A20 silenced Jurkat cells where the concentrations were also significantly increased. NF-kB activation (including increased expression of the P65 subunit) regulates the expression of several cytokines including TNFa through a positive feedback loop (Kagoya *et al*., 2014). Prior work has shown that exposure of human islets to TNFa, IL1b and IFNg upregulates both proapoptotic and anti-apoptotic genes including *TNFAIP3* through activation of NF-kB (Sakar *et al*., 2009). Cytokines activated as part of the NF-kB pathway are thought to be integral to balancing the pro-apoptotic and anti-apoptotic response and are critical to maintain islet integrity and viability (Sakar *et al*., 2009). Overexpression of A20 is associated with protection against IL1b and IFNg-induced apoptosis. Silencing of A20 in INS-1E cells and subsequent exposure to TNFa, IL1b or IFNg resulted in universal apoptosis (Fukaya *et al*., 2016). Furthermore, short-time pre-treatment of pancreatic beta cells with TNFa, IL1b or IFNg alone or in cytomix combination led to significant inhibition of insulin secretion (Wang *et al*., 2010). Consistently, we observe a reduction in glucose-stimulated insulin secretion and elevated apoptosis in association with enhanced cytokine release. Of particular interest, however, was the impact these cytokines may be having on gene expression within the beta cell.

We treated BRIN-BD11 cells with TNFa, IL1b, IFNg, cytomix (all three cytokines in combination) and conditioned media from Jurkat cells and found that the expression profile of beta cell regulatory genes and transcription factors was remarkably similar to that of A20 silenced cells. It therefore seems likely that the inhibitory effect of A20 deficiency on beta cell function stems from an increase in cytokine release, which in turn limits the expression of key genes within the beta cell. Exposure of mouse islets to TNFa, IL1b or IL6 for 24 h decreased mRNA expression of *Ins2, Slc2a2, Pdx1 and Nkx6-1* (Nordmann *et al*., 2017). Our findings are consistent with these observations with all three cytokines resulting in major downregulation of beta cell markers and transcription factors. The one notable exception was *Hnf1a*, which was significantly downregulated in A20 silenced cells, but not in the presence of recombinant cytokines. Recent work has shown that the beta cell response to pro-inflammatory cytokines is dynamic and involves the transcription of several pathways involved in T1D pathogenesis (Ramos-Rodríguez *et al*.,2019). To this end, pathway analysis on PCR array data identified several candidate pathways for study in relation to A20 action within the beta cell. Use of pharmacological inhibitors showed that changes in gene expression reported in this study following silencing of A20 were largely, although not exclusively, NF-kB (P65)-dependent. The finding that several key regulatory genes within the beta cell were not under the control of NF-kB warrants further investigation to identify novel pathways through which A20 may confer protection against beta cell demise.

In conclusion, the current study confirms A20 to be an important regulator of beta cell survival and reveals a novel role in the regulation of gene expression within the beta cell. Control of beta cell gene expression appears to be driven by heightened release of inflammatory cytokines in A20 deficient cells. The observation of increased cytokine release in beta cell and T cell lines indicates that A20 deficiency may lead to a double insult for the beta cell: firstly, by enhancing cytokine release from the beta cell itself and secondly, by creating an inflammatory environment that may ultimately prove detrimental to the beta cell.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest

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AUTHOR CONTRIBUTIONS

WR, SA and CK designed the study. WR conducted all experiments. WR and CK wrote the initial draft of the manuscript. All authors revised and approved the final submission.

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TABLE AND FIGURE LEGENDS

Table 1. qPCR probes

Fig 1. Induction of A20 in BRIN-BD11 cells. BRIN-BD11 cells or human islet cells were exposed to 10 ng/ml TNFa for 0-24h and the expression of *TNFAIP3* mRNA measured by qPCR (**A, D** respectively). Relative expression against 18S was calculated using 2^ΔCt. The induction of A20 expression was confirmed at the protein level with representative images shown in **(B, E).** A20 positive cells shown in red and the nuclear stain DAPI shown in blue. A20 positive cells were quantified as a percentage of total cells (DAPI staining, **C, F**) Data are presented as mean ± SEM (n = 4). ***P<0.001 compared with expression at 0h and $^{\Delta\Delta\Delta}P$<0.001 compared with expression at 4h (*t*-test).

Fig 2. Impact of A20 silencing on islet cell survival. BRIN-BD11 cells **(A, B)** or human islet cells **(C, D)** were treated with 100 ng siRNA against *TNFAIP3* or a negative control (scrambled siRNA) and cell survival tested in the absence **(A, C)** or presence **(B, D)** of 10 ng/ml TNFa for 1h. The impact on cell viability, cytotoxicity and apoptosis was assessed using the ApoTox-Glo^{TM} Triplex Assay (Promega). Data are presented as mean ± SEM (n = 5-6 for all experiments). **P<0.01 compared with negative control (*t*-test).

Fig 3. Impact of A20 silencing on glucose-induced insulin secretion. BRIN-BD11 cells (**A**) or human islet cells (**B**) were treated with 100 ng siRNA against *TNFAIP3* or a negative control (scrambled siRNA) and subsequently exposed to basal (1.1 mM) or stimulatory (16.7 mM) concentrations of D-glucose. Insulin secretion was assessed by ELISA. Data are presented as mean ± SEM (n = 8 for BRIN-BD11 cells and n = 4 for human islet cells). *P<0.05, **P<0.01, ***P<0.001 (*t*-test).

Fig 4. A20 regulates expression of beta cell markers and inflammatory genes in BRIN-BD11 cells. BRIN-BD11 cells were treated with 100 ng siRNA against *TNFAIP3* or a negative control (scrambled siRNA) ± 10 μM recombinant A20 protein. qPCR was used to assess the expression of key beta cell regulatory genes (**A**), transcription factors (**B**) and NF-kB subunits (**C**) which was standardized against the corresponding negative control using $2^{\wedge\Delta}$Ct. Data are presented as mean ± SEM (n = 4 for all experiments). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 compared with corresponding negative control (*t*-test).

Fig 5. **A20 silencing increases basal cytokine concentrations.** BRIN-BD11 (**A**) or Jurkat cells (**B**) were treated with 100 ng siRNA against *TNFAIP3* or a negative control (scrambled siRNA). Following completion of the transfection process, fresh culture medium was added to cells and collected 24 hours later. Cell free culture medium was assessed for basal concentrations of IL1b, IFNg and TNFa by ELISA. Data are presented as mean ± SEM (n = 4-6 for all experiments). **P*<0.05, ***P*<0.01, ****P*<0.001 compared with corresponding control (*t*-test).

Fig 6. Exposure to recombinant cytokine alters gene expression in BRIN-BD11 cells. BRIN-BD11 cells were exposed to 10 ng/ml TNFa, IL1b, or IFNg, or a combination of all three (cytomix), and conditioned media obtained from A20 silenced Jurkat T cells after 24 hours in culture (CM Jurkat). qPCR was used to assess the expression of key beta cell regulatory genes (**A**), transcription factors (**B**) and NF-kB subunits (**C**), which was standardized against the corresponding control using $2^{\wedge\Delta}$Ct. Data are presented as mean ± SEM (n = 3-4 for all experiments). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 compared with corresponding control (*t*-test).

Fig 7. A20 regulation of beta cell gene expression is largely NF-kB dependent. BRIN-BD11 cells were treated with 100 ng siRNA against *TNFAIP3* or a negative control

651 (scrambled siRNA) ± 30 μM JSH23 or MG132. qPCR was used to assess the expression of
652 key beta cell regulatory genes (**A**), transcription factors (**B**) and NF-kB subunits (**C**) which was
653 standardized against the corresponding negative control using $2^{\wedge\Delta}Ct$. Data are presented
654 as mean ± SEM (n = 4-5 for all experiments). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001
655 compared with corresponding negative control (*t*-test).
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