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A20 controls expression of beta cell regulatory genes and transcription factors

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Short title: A20 regulates islet gene expression

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19 **ABSTRACT**

20

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

41

42 **ABSTRACT:** 249 words

43 **INTRODUCTION**

44

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

57

58 More than 66 genes including *TNFAIP3/A20* are altered after challenge by cytokines released
59 in response to NF-kB activation (Cardozo *et al*., 2001; Cheng *et al*., 2016). The role of A20
60 has been extensively studied in various tissue types. Uncontrolled and multiorgan
61 inflammation has been observed in A20 deficient mice (Zheng *et al*., 2016). TNFAIP3fl/flCD19-
62 Cre mice have been shown to develop a disease resembling systemic lupus erythematosus
63 with increased production of autoantibodies (Umiker *et al*.,2014). The induction of A20
64 expression in cystic fibrosis airway epithelial cells is significantly delayed when compared with
65 healthy controls and is associated with an inability to form important signalling complexes
66 required to prevent NF-kB-driven inflammation (Kelly *et al*., 2013). B lymphoid cells devoid of
67 A20 are hyper-responsive to stimuli and show increased activation of NF-κB (Tavares *et al*.,
68 2010a). Dendritic cells lacking A20, mature at a faster rate, are more responsive to TLR
69 ligands, and produce more cytokines when compared to their wild type counterparts (Kool *et*

70 *al*., 2011). Loss of A20 in dendritic cells also has implications on populations of other cells of
71 the immune system (Kool *et al*., 2011; Hammer *et al*., 2012).
72
73 *TNFAIP3/A20* has been identified as the most highly regulated anti-apoptotic gene in the
74 pancreatic beta cell (Liuwantara *et al*., 2006) and protects against islet graft rejection in animal
75 models of transplantation (Grey *et al*., 2003; Zammit *et al*., 2019). Therapeutic administration
76 of A20 promotes immune tolerance and the survival of transplanted islets by increasing
77 inflammatory signaling thresholds (Zammit *et al.* 2019). In resting beta cells, basal expression
78 of A20 and activation of NF-κB are low and is increased upon challenge (Baker *et al*., 2011).
79 Knockdown of A20 in primary rat and mouse islet cells results in persistent cytokine-mediated
80 JNK activation (Fukaya *et al*., 2015). A20 was also shown to positively regulate Akt signalling
81 in beta cells contributing to the anti-apoptotic effect (Pepin *et al*., 2014). In the context of
82 human disease, mRNA and protein expression of A20 is reduced in blood mononuclear cells
83 from people with type 2 diabetes and adults with latent autoimmune diabetes (Cheng *et al.*
84 2014). Furthermore, A20 has been identified as a novel locus of interest within HLA class II
85 regions known to encompass a number of genetic risk factors for autoimmune pathologies
86 including type 1 diabetes (T1D) (Gough and Simmonds, 2007; Relle and Schwarting, 2012).
87
88 The protective effect of A20 in the pancreatic beta cell and the relationship with diabetes risk
89 is well established. Research has identified many of the signalling pathways through which
90 A20 acts in the beta cell including NF-kB, Akt and JNK. However, the direct impact of A20
91 deficiency on the regulatory machinery of the beta cell remains poorly defined. The current
92 study investigated the impact of A20 deficiency on beta cell survival, glucose-stimulated insulin
93 secretion and the expression of beta cell markers and transcription factors regulating beta cell
94 maturation and function.

95 **MATERIALS AND METHODS**

96

97 **Cell culture and treatment**

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

108

109 **Silencing of A20**

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

121 presence of transfection complexes for 72 hours without media change. Knockdown was
122 confirmed at the mRNA and protein level (Figure S2, ESM).
123
124 To confirm the specific role of A20, reversal of knockdown was achieved using 10 μm
125 recombinant A20 protein (Abcam) targeting the N terminal domain. Internalisation of
126 recombinant protein was achieved as previously described (Rust *et al.,* 2015; Zuris *et al.*,
127 2015) using 2.5 μL lipofectamine for 24h. Uptake of A20 into the cytoplasm of the cell was
128 confirmed by immunofluorescent staining of the cell as described below. Please see Figure
129 S3A-B, ESM for quantification of A20 uptake. The selected dose of recombinant A20 did not
130 affect apoptosis over 24h (Figure S3C).
131

132 **Quantitative real time PCR (qPCR)**

133 Total RNA was extracted from cells using the QIAGEN RNeasy Kit according to the
134 manufacturer's instructions. RNA concentration was measured using Nanodrop ND-1000
135 (Thermo Fisher; Loughborough, England) with 260/280 ratios of 1.8 - 2.1 accepted as
136 indicative of good quality RNA. RNA was sporadically run on 2% agarose gels to check RNA
137 integrity. A total of 500 ng RNA was reverse transcribed to cDNA using the Roche Transcriptor
138 cDNA synthesis kit (Welwyn Garden City, England) according to supplier instructions. qPCR
139 was performed on a Lightcycler 480 System (Roche) using custom designed probes (See
140 Table 1) and qPCR MasterMix (Roche) as previously described (Khan *et al*., 2019). Following
141 optimization, 18S was chosen as a reference gene for all experiments. Relative mRNA
142 expression was determined using $2^{\Delta Ct}$ method and normalised to 18S.
143

144 **Immunofluorescent staining of islet cells**

145 Cells were seeded into a 24 well plate containing 8 mm glass slides and allowed to attach until
146 90% confluency had been achieved. Media was aspirated, and the cells washed with cold
147 PBS. Cold methanol was added for 15 minutes to fix the cells, followed by a wash with cold
148 PBS. Permeabilization of the cells was performed using 0.1% Triton-X (Merck; Irvine, England)

149 for 10 minutes at room temperature followed by a wash with cold PBS. Donkey serum (5%,
150 diluted in PBS, Merck) was added and the cells incubated for 30 minutes at room temperature
151 followed by a wash with cold PBS. The cells were then incubated with a primary antibody
152 against A20 (Santa Cruz Biotech (sc-166692), 1:200. Santa Cruz, USA), washed with cold
153 PBS and incubated with an appropriate secondary antibody (Alexa Fluor® 594, Invitrogen
154 (A11032), 1:200. Invitrogen; Loughborough, England). The cells were then co-stained with
155 1:10000 DAPI (Merck) for five minutes at room temperature followed by a wash with cold PBS.
156 Slides were mounted in the dark with aqueous mounting media (Abcam; Cambridge, England)
157 and imaged under a fluorescent microscope (Zeiss Axio). Antigen positive cells were
158 expressed as a percentage of total cells (DAPI positive cells).
159

160 **Assessment of viability, cytotoxicity and apoptosis**

161 Apotox-Glo™ Triplex Assay by Promega (Southampton, England) allows for concurrent
162 measurements of cell viability, cytotoxicity and apoptosis. The assay implements fluorogenic
163 peptide substrate (bis-AAF-R110 Substrate) to measure dead-cell protease activity used to
164 assess viability and cytotoxicity of treated cells. Caspase-3/7 and Ultra-Glo™ Recombinant
165 Thermostable Luciferase luminesce measurements are used as direct measure of apoptosis.
166 The assay was used to assess the viability, cytotoxicity and activation of apoptosis in treated
167 BRIN-BD11 cells and human islets. All experiments were performed using the manufacturer's
168 protocol.
169

170 **Measurement of glucose-induced insulin secretion**

171 BRIN-BD11 cells and human islet cells were incubated for 40 minutes in Krebs solution (115
172 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
173 Bovine serum albumin) supplemented with 1.1 mM D-glucose at 37 ^{o}C. The media was then
174 removed, and cells supplemented with increasing concentrations of D-glucose (1.1 mM and
175 16.7 mM) for a further 20 minutes. The supernatant was collected and assessed for insulin
176 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 RT^2 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

205 readymade solution (Merck) and diluted to 30 μM in cell culture medium. Cells were first
206 allowed to reach 80% confluency and then exposed to 30 μM of JSH-23 or MG-132 for 1 hour
207 prior to experimentation.
208
209 **Data analysis**
210 Statistical analysis was performed using GraphPad PRISM (La Jolla, USA; version 7). Data
211 are presented as mean ± SEM for a given number of observations (n) as indicated in the
212 Figures. Differences between groups were compared using 2-tailed Student's *t* test as
213 appropriate. Statistical significance was accepted at $P<0.05$.
214
215
216 **RESULTS**
217
218 **Peak induction of A20 is observed 1 hour after TNFa stimulation**
219 Low level endogenous A20 expression is observed under basal conditions and expression is
220 rapidly induced following exposure to a range of cytokines, the best characterised of which, is
221 TNFa. BRIN-BD11 cells were therefore exposed to 10 ng/ml TNFa over a 24h period to
222 optimise time of exposure for subsequent experiments. Consistent with the first reports
223 describing A20 induction (Opipari *et al*., 1990), peak induction of *TNFAIP3/A20* mRNA was
224 observed 1h after TNFa stimulation (2.94 ± 0.36-fold increase, $P<0.001$; Fig. 1A). Expression,
225 thereafter, rapidly fell below basal levels. Induction at the protein level 1h after TNFa treatment
226 was also confirmed (3.87 ± 0.015-fold increase, $P<0.001$; Fig. 1B and 1C). Experiments in
227 human islet cells consistently observed significant increases following 1h TNFa treatment at
228 both the mRNA (2.86 ± 0.35-fold increase, $P<0.001$; Fig. 1D) and protein (1.795 ± 0.021-fold
229 increase, $P<0.001$; Fig. 1E and 1F) levels. TNFa exposure of 1h was used for all subsequent
230 experiments.
231
232 **Silencing of A20 increases apoptosis in islet cells.**

233 A20 was silenced in both BRIN-BD11 and human islets using siRNA against *TNFAIP3* and
234 expression compared with negative controls (scrambled siRNA). Knockdown was confirmed
235 at the mRNA (62.79 ± 0.085% knockdown in BRIN-BD11 cells, $P<0.001$, Fig. S2A; 92.38 ±
236 0.02% knockdown in human islet cells, $P<0.001$, Fig. S2D; and 52.42 ± 0.08% knockdown in
237 Jurkat cells, $P<0.001$, Fig. S2G (ESM)) and protein levels (61.18 ± 0.03% reduction in A20
238 positive cells in BRIN-BD11 cells, $P<0.001$, Fig. S2B and S2C; and 93.63 ± 0.01% reduction
239 in A20 positive cells in human islet cells, $P<0.001$, Fig. S2E and S2F (ESM)).
240
241 The impact of A20 silencing on cell viability, cytotoxicity and apoptosis was determined using
242 the AptoTox Glo^{TM} assay. Following transfection of *TNFAIP3* siRNA, BRIN-BD11 cells and
243 human islets were tested in the presence or absence of 10 ng/ml TNFa for 1h and differences
244 compared with negative controls. Significant changes in cell viability, cytotoxicity or apoptosis
245 were not observed in the absence of TNFa stimulation, consistent with the idea that A20 is an
246 inducible protein with limited activity under basal resting conditions. In the presence of TNFa,
247 differences in cell viability and cytotoxicity were not observed in either cell type examined (Fig.
248 2A and 2C). However, apoptosis was significantly increased in A20 silenced BRIN-BD11 cells
249 (2.85 ± 0.37-fold increase, $P<0.01$, Fig. 2B) and human islet cells (2.21 ± 0.15-fold increase,
250 $P<0.01$, Fig. 2D).
251
252 **Glucose-stimulated insulin secretion is reduced in A20 silenced islet cells**
253 The impact of A20 silencing on insulin secretion in response to acute exposure to basal (1.1
254 mM) and stimulatory (16.7 mM) concentrations of glucose was determined by ELISA. No
255 change in insulin release was observed in A20 silenced BRIN-BD11 cells (Fig. 3A) or human
256 islet cells (Fig. 3B) compared with negative controls in response to basal glucose
257 concentrations. However, significant reductions in stimulated insulin secretion were found in
258 both cell types (0.42 ± 0.0011-fold reduction in BRIN-BD11 cells, $P<0.05$, Fig. 3A; and 0.53 ±
259 0.0009-fold reduction in human islet cells, $P<0.01$, Fig. 3B).
260

261 **A20 regulates the expression of beta cell and inflammatory markers through release of**
262 **pro-inflammatory cytokines**
263 The mRNA expression of a range of beta cell markers, transcription factors regulating beta
264 cell maturation, and inflammatory markers (NF-kB subunits) was assessed by qPCR (Fig. 4).
265 Expression of all beta cell markers and all beta cell transcription factors tested was reduced
266 in A20 silenced BRIN-BD11 cells (P<0.05 – 0.001, Fig. 4A and 4B). Given the important role
267 of A20 in NF-kB activation, we also assessed the expression of *NFKB1* and *RelA*, which
268 encode the NF-kB subunits P50 and P65 respectively. We observed a reduction in the
269 expression of *NKFB1* (P<0.001), but an increase in *RelA* expression (P<0.001) following A20
270 silencing. This is consistent with data showing that A20 exerts its anti-inflammatory action in
271 a P65-dependent manner (Kelly *et al*., 2013). To determine if the changes in gene expression
272 were A20-dependent, we exposed BRIN-BD11 cells to recombinant A20 protein for 24h.
273 Recombinant A20 was readily internalised with the aid of lipofectamine. Cytoplasmic
274 expression of A20 in unstimulated BRIN-BD11 cells increased by 3.97 ± 0.03-fold (P<0.001;
275 Fig. S3, ESM) following 24-hour exposure to the recombinant protein. Consistently, BRIN-
276 BD11 cells exposed to *TNFAIP3* siRNA prior to exposure to recombinant A20 showed similar
277 3.91 ± 0.05-fold increase in expression (P<0.001; Fig. S3, ESM). With the exception of *RelA,*
278 administration of recombinant A20 independently enhanced the expression of all investigated
279 genes. In all instances, the pattern of gene expression following silencing of A20 was reversed
280 upon administration of the recombinant protein (Fig. 4).
281
282 Activation of the NF-kB pathway leads to the release of pro-inflammatory cytokines. TNFa,
283 IL1b and IFNg have been implicated in the pathogenesis of beta cell demise. We therefore
284 assessed the concentrations of these cytokines in supernatants taken from A20-silenced
285 BRIN-BD11 cells (Fig. 5A). TNFa was increased 3.64-fold (P<0.01), IL1b, 1.70-fold (P<0.01),
286 and IFNg, 2.16-fold (P<0.05). To assess whether this effect was specific to the beta cell,
287 experiments were repeated in the Jurkat cell line where consistent increases in the
288 concentrations of all three cytokines was observed (P<0.05 – 0.001, Fig. 5B).

289

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

298

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

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

313

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

317 *Kcnq1* and *Gck* expression in the presence of JSH23 persisted even when BRIN-BD11 cells
318 were exposed to siRNA against TNFAIP3. However, this was not true of *Abcc8, Kcnj11* and
319 *Scl2a2* expression where JSH23 failed to prevent A20-driven reductions in expression (Fig.
320 7A). In the case of beta cell transcription factors (Fig. 7B) and the expression of NF-kB
321 subunits (Fig. 7C), JSH23 administration was largely able to inhibit A20-driven changes in
322 gene expression. In almost all instances, the addition of MG132 was not able to fully restore
323 gene expression after A20 silencing (Fig. 7A – 7C).
324
325
326 **DISCUSSION**
327
328 *TNFAIP3* encodes a protein called A20 that is capable of acting as both a deubiquitinating
329 enzyme and an E3 ligase. A20 comprises two domains: (1) an OTU region capable of
330 deubiquitinating K63-linked polyubiquitin chains from target proteins to render them inactive
331 and (2) a c-terminal region that contain 7 zinc finger proteins, which act as E3 ligases by
332 adding K43 ubiquitin chains to target proteins triggering degradation by the 26S proteasome
333 (Makarova *et al*., 2000; Wertz *et al*., 2004). Together this dual function makes A20 a potent
334 negative regulator of several inflammatory and apoptotic pathways (Abassi *et al*., 2015). The
335 current study finds a role for A20 in regulating the expression of genes and transcription factors
336 that play a critical role in normal beta cell functioning and maturation.
337
338 Early work by Dixit and colleagues (1990) identified A20 as TNF-responsive gene with rapid
339 and transient induction. Peak A20 expression was observed 1 hour after treatment with TNFa
340 in human umbilical vein endothelial cells, which differed from other TNF-responsive genes
341 where significant expression persisted for at least 8 hours (Opipari *et al*., 1990). Consistently,
342 the present study found low level basal A20 expression in untreated beta cells with expression
343 significantly upregulated after 1-hour TNFa treatment. Although inducible in response to other
344 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

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

379

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

399

400 We treated BRIN-BD11 cells with TNFa, IL1b, IFNg, cytomix (all three cytokines in
401 combination) and conditioned media from Jurkat cells and found that the expression profile of
402 beta cell regulatory genes and transcription factors was remarkably similar to that of A20
403 silenced cells. It therefore seems likely that the inhibitory effect of A20 deficiency on beta cell
404 function stems from an increase in cytokine release, which in turn limits the expression of key
405 genes within the beta cell. Exposure of mouse islets to TNFa, IL1b or IL6 for 24 h decreased
406 mRNA expression of *Ins2, Slc2a2, Pdx1 and Nkx6-1* (Nordmann *et al*., 2017). Our findings
407 are consistent with these observations with all three cytokines resulting in major
408 downregulation of beta cell markers and transcription factors. The one notable exception was
409 *Hnf1a*, which was significantly downregulated in A20 silenced cells, but not in the presence of
410 recombinant cytokines. Recent work has shown that the beta cell response to pro-
411 inflammatory cytokines is dynamic and involves the transcription of several pathways involved
412 in T1D pathogenesis (Ramos-Rodríguez *et al*.,2019). To this end, pathway analysis on PCR
413 array data identified several candidate pathways for study in relation to A20 action within the
414 beta cell. Use of pharmacological inhibitors showed that changes in gene expression reported
415 in this study following silencing of A20 were largely, although not exclusively, NF-kB (P65)-
416 dependent. The finding that several key regulatory genes within the beta cell were not under
417 the control of NF-kB warrants further investigation to identify novel pathways through which
418 A20 may confer protection against beta cell demise.
419
420 In conclusion, the current study confirms A20 to be an important regulator of beta cell survival
421 and reveals a novel role in the regulation of gene expression within the beta cell. Control of
422 beta cell gene expression appears to be driven by heightened release of inflammatory
423 cytokines in A20 deficient cells. The observation of increased cytokine release in beta cell and
424 T cell lines indicates that A20 deficiency may lead to a double insult for the beta cell: firstly, by
425 enhancing cytokine release from the beta cell itself and secondly, by creating an inflammatory
426 environment that may ultimately prove detrimental to the beta cell.

427 **DECLARATION OF INTERESTS**

428 The authors declare no conflicts of interest

429

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435

436 **AUTHOR CONTRIBUTIONS**

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

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597 **TABLE AND FIGURE LEGENDS**

598

599 **Table 1. qPCR probes**

600

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

609

610 **Fig 2. Impact of A20 silencing on islet cell survival.** BRIN-BD11 cells **(A, B)** or human islet
611 cells **(C, D)** were treated with 100 ng siRNA against *TNFAIP3* or a negative control (scrambled
612 siRNA) and cell survival tested in the absence **(A, C)** or presence **(B, D)** of 10 ng/ml TNFa for
613 1h. The impact on cell viability, cytotoxicity and apoptosis was assessed using the ApoTox-
614 Glo^{TM} Triplex Assay (Promega). Data are presented as mean ± SEM (n = 5-6 for all
615 experiments). **P<0.01 compared with negative control (*t*-test).

616

617 **Fig 3. Impact of A20 silencing on glucose-induced insulin secretion.** BRIN-BD11 cells
618 (**A**) or human islet cells (**B**) were treated with 100 ng siRNA against *TNFAIP3* or a negative
619 control (scrambled siRNA) and subsequently exposed to basal (1.1 mM) or stimulatory
620 (16.7 mM) concentrations of D-glucose. Insulin secretion was assessed by ELISA. Data
621 are presented as mean ± SEM (n = 8 for BRIN-BD11 cells and n = 4 for human islet cells).
622 *P<0.05, **P<0.01, ***P<0.001 (*t*-test).

623

624 **Fig 4. A20 regulates expression of beta cell markers and inflammatory genes in BRIN-**
625 **BD11 cells.** BRIN-BD11 cells were treated with 100 ng siRNA against *TNFAIP3* or a negative
626 control (scrambled siRNA) ± 10 μM recombinant A20 protein. qPCR was used to assess the
627 expression of key beta cell regulatory genes (**A**), transcription factors (**B**) and NF-kB subunits
628 (**C**) which was standardized against the corresponding negative control using $2^{\wedge\Delta}$Ct. Data are
629 presented as mean ± SEM (n = 4 for all experiments). *P<0.05, **P<0.01, ***P<0.001,
630 ****P<0.0001 compared with corresponding negative control (*t*-test).
631
632 **Fig 5**. **A20 silencing increases basal cytokine concentrations.** BRIN-BD11 (**A**)
633 or Jurkat cells (**B**) were treated with 100 ng siRNA against *TNFAIP3* or a negative control
634 (scrambled siRNA). Following completion of the transfection process, fresh culture medium
635 was added to cells and collected 24 hours later. Cell free culture medium was assessed for
636 basal concentrations of IL1b, IFNg and TNFa by ELISA. Data are presented as mean ± SEM
637 (n = 4-6 for all experiments). *P<0.05, **P<0.01, ***P<0.001 compared with corresponding
638 control (*t*-test).
639
640 **Fig 6. Exposure to recombinant cytokine alters gene expression in BRIN-BD11**
641 **cells.** BRIN-BD11 cells were exposed to 10 ng/ml TNFa, IL1b, or IFNg, or a combination of
642 all three (cytomix), and conditioned media obtained from A20 silenced Jurkat T cells after 24
643 hours in culture (CM Jurkat). qPCR was used to assess the expression of key beta cell
644 regulatory genes (**A**), transcription factors (**B**) and NF-kB subunits (**C**), which was
645 standardized against the corresponding control using $2^{\wedge\Delta}$Ct. Data are presented as mean ±
646 SEM (n = 3-4 for all experiments). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared
647 with corresponding control (*t*-test).
648
649 **Fig 7. A20 regulation of beta cell gene expression is largely NF-kB dependent.** BRIN-
650 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 $2^{\Delta\Delta}$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).

656