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ARTICLE

Baculoviral inhibitors of apoptosis repeat containing (BIRC) proteins fine-tune TNF-induced nuclear factor κB and c-Jun N-terminal kinase signalling in mouse pancreatic beta cells

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Abstract
Aims/hypothesis For beta cells, contact with TNF-α triggers signalling cascades that converge on pathways important for cell survival and inflammation, specifically nuclear factor κB (NF-κB), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase pathways. Here, we investigated the function of baculoviral inhibitors of apoptosis repeat containing (BIRC) proteins in regulating TNF signalling cascades.
Methods TNF regulation of Birc genes was studied by mRNA expression and promoter analysis. Birc gene control of cell signalling was studied in beta cell lines, and in islets from *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ mice, and from *Birc3*$^{-/-}$*Birc2*Δ beta cell mice that selectively lack *Birc2* and *Birc3* (double knockout [DKO]). Islet function was tested by intraperitoneal glucose tolerance test and transplantation.
Results TNF-α selectively induced *Birc3* in beta cells, which in turn was sufficient to drive and potentiate NF-κB reporter activity. Conversely, *Birc3*$^{-/-}$ islets exhibited delayed TNF-α-induced IκBα degradation with reduced expression of *Ccl2* and *Cxcl10*. DKO islets showed a further delay in IκBα degradation kinetics. Surprisingly, DKO islets exhibited stimulus-independent and TNF-dependent hyperexpression of TNF target genes *A20* (also known as *Tnfaip3*), *Icam1*, *Ccl2* and *Cxcl10*. DKO islets showed hyperphosphorylation of the JNK-substrate, c-Jun, while a JNK-antagonist prevented increases of *Icam1*, *Ccl2* and *Cxcl10* expression. Proteosome blockade of MIN6 cells phenocopied DKO islets. DKO islets showed more rapid loss of glucose homeostasis when challenged with the inflammatory insult of transplantation.
Conclusions/interpretation BIRC3 provides a feed-forward loop, which, with BIRC2, is required to moderate the normal speed of NF-κB activation. Paradoxically, BIRC2 and BIRC3 act as a molecular brake to rein in activation of the JNK signalling pathway. Thus BIRC2 and BIRC3 fine-tune NF-κB and JNK signalling to ensure transcriptional responses are appropriately matched to extracellular inputs. This control is critical for the beta cell's stress response.

Keywords Beta cell · BIRC · Diabetes · Gene · Inflammation · Islet · JNK · NF-κB · TNF

Abbreviations

ASK1	Apoptosis signal-regulating kinase 1
BIRC	Baculoviral inhibitors of apoptosis repeat containing proteins
DKO	*Birc3*$^{-/-}$*Birc2*Δ beta cell mice that selectively lack *Birc2* and *Birc3*
eGFP	Enhanced green fluorescent protein
eGFP-*Birc3*	pIRES2-eGFP-*Birc3*
IAP	Inhibitors of apoptosis
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
JNK	c-Jun N-terminal kinase
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B cells
NIK	NF-κB-inducing kinase
p38	p38 mitogen-activated protein kinases

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PDTC	Pyrrolidine dithiocarbamate
pIRES2	Plasmid containing an internal ribosome entry site two
RIPK1	Receptor-interacting protein kinase 1
TNFR	TNF receptor
TRAF	TNFR-associated factor
XIAP	X-linked inhibitor of apoptosis protein

Introduction

TNF-α is a pleiotropic cytokine that is involved in the pathogenesis of autoimmune type 1 diabetes and influences cell proliferation, inflammation and cell death. TNF-α mediates its actions through two distinct receptors, TNF receptor (TNFR) 1/p55 and TNFR2/p75, both of which are expressed on virtually all cell types [1]. TNFR1 is predominantly responsible for TNF signalling in most cell types [1], mediated by the sequential formation of two complexes upon TNFR1 ligation [2]. Complex I forms with the recruitment of TNFR1-associated death domain protein, which subsequently recruits TNFR-associated factor (TRAF) 2, TRAF5 and receptor-interacting protein kinase 1 (RIPK1) [2]. Within complex I, RIPK1 is essential for activating the nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) pathway [3], while TRAF2 is required for activation of the c-Jun N-terminal kinase (JNK) pathway [4]. These signalling cascades are imperative for the control of genes involved in immune response, inflammation and cell survival [1]. In addition, a secondary complex, spatially and temporally distinct from complex I, comprises Fas-associated death domain protein and caspase 8, which triggers a pro-apoptotic cascade [2]. Normally, TNF-α-stimulated cells are protected from the pro-apoptotic force of complex II by the expression of NF-κB-induced genes, including *A20* (also known as *Tnfaip3*) and *Cflip* (also known as *Cflar*), both of which are regulated via complex I. *A20* and *Cflip* prevent activation of caspase-8, possibly at the level of complex II [5, 6]. Thus, TNF-α triggers a co-ordinated and complex cellular program regulating cell proliferation, inflammation and cell death.

The contribution of TNF-α to type 1 diabetes pathogenesis is multifaceted. A characteristic feature of diabetes is the infiltration of the pancreatic islets with T cells, with TNFRI-null islets being protected from destruction by T lymphocytes [7]. TNF-α is an early cytokine detected within the immune infiltrate surrounding islets [8]. In the context of type 1 diabetes, TNF-α exerts detrimental effects, including heightened expression of inflammatory genes in pancreatic beta cells [9], the impairment of insulin secretion [10] and the triggering of beta cell apoptosis [5]. TNF-α's ability to induce and perpetuate diabetes has been clearly demonstrated in 3-week-old NOD mice, which upon administration of exogenous TNF-α had accelerated onset of diabetes and increased disease frequency [11]. In contrast, diabetes progression can be ameliorated by anti-TNF-α monoclonal antibody treatment [11]. Despite the importance of TNF-α in the pathogenesis of type 1 diabetes, the exact molecular machinery governing the TNF signalling cascade in islets and pancreatic beta cells remains poorly understood. An improved understanding of TNF signalling could lead to novel diagnostics or therapies for patients with type 1 diabetes.

Inhibitors of apoptosis (IAP) or baculoviral IAP repeat containing proteins (BIRC) are implicated in the regulation of downstream TNF signalling networks [12]. A total of eight BIRC proteins has been identified, namely: neuronal AIP (BIRC1), cellular IAP1 (BIRC2), cellular IAP2 (BIRC3), X-linked inhibitor of apoptosis protein (XIAP/BIRC4), survivin (TIAP/BIRC5), baculoviral IAP repeat-containing ubiquitin-conjugating enzyme (BRUCE/Apollon/BIRC6), Melanoma-IAP (ML-IAP/Livin/kidney-IAP/BIRC7) and ILP-2 (Testis Specific-IAP)/BIRC8). A key cellular function of BIRC proteins is the regulation of apoptosis [12]. Indeed, XIAP can prevent apoptosis in a number of cell types, including islets [13–16] by preventing direct IAP binding protein with low pI (DIABLO)-mediated cleavage of caspase 9 [17]. While BIRC proteins were thought to primarily act as regulators of apoptosis, this function has not been established for all its members; it is now recognised that BIRC proteins are also involved in the regulation of diverse cellular functions, including the regulation of signalling and inflammation [12]. Further to this, BIRC2 and BIRC3, together with TRAF2, have been identified as key components of the TNF signalling pathway that are necessary for TNF-mediated NF-κB activation [18]. With reference to beta cells, some studies have shown that beta cells express *Birc3*, and that *Birc3* expression is regulated by the TNF-α-mediated NF-κB pathway [5, 19]; however, its binding partner, *Birc2*, has not been examined. This suggested to us that in beta cells, BIRC3 may participate in a TNF signalling feedback loop, an idea that has not been tested to date. Here we examined the requirements for BIRC3 and BIRC2 in TNF signalling in beta cells.

Methods

Mouse strains BALB/c and C57BL/6 mice were from Australian BioResource (Mossvale, NSW, Australia). *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ mice were a kind gift of D. Vaux (Cell Signalling and Cell Death division, Walter and Eliza Hall Institute, Parkville, VIC, Australia). *Birc3*$^{-/-}$ mice containing *Birc2*$^{loxP/lox\text{-}P}$ were crossed with RIP-*Cre* mice (*Cre* driven by the rat insulin promoter) (Jax Mice, Bar Harbour, ME, USA) to generate beta cell-specific double knockout *Birc3*$^{-/-}$*Birc2*Δ beta cell mice that selectively lack *Birc2* and *Birc3* (double knockout [DKO]). Procedures complied with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes.

Cytokines Mouse islets were isolated as described [20]. Either 70–100 islets or 1×10^6 MIN6 beta cells [21] were stimulated with 200 U/ml of TNF-α for 4 h (RNA analysis) and 8 h (promoter analysis) (R&D Systems, Minneapolis, MN, USA). β-TC_3 cells were used for some transfection studies [22]. In some cases, cells were pretreated for 1 h with 1 μmol/l actinomycin D, 50 μmol/l SP600125, 1 μmol/l MG-132 and 50 μmol/l pyrrolidine dithiocarbamate (PDTC) (all from Sigma-Aldrich, St Louis, MO, USA), and with 20 μmol/l SB203580 (Cell Signalling Technology, Beverly, MA, USA), prior to cytokine stimulation. Western blotting was performed using standard protocols with IκBα antibody (9242L), phospho-c-Jun (Ser73, 9164S) (Cell Signalling Technology), β-actin (Clone AC-15; A5441; Sigma-Aldrich) and horseradish peroxidase-conjugated antibodies (Pierce, Rockford, IL, USA), and chemiluminescence (GE Healthcare, Uppsala, Sweden).

Quantitative RT-PCR Total RNA and cDNA were generated from beta cell lines and mouse islets using standard techniques previously described by this research group [5]. Relative gene expression is the ratio to the housekeeping gene, Cyclophilin A (*Ppia*); fold-changes were analysed using the $2^{-\Delta\Delta C_t}$ method. Primers used for PCR are provided in electronic supplementary material (ESM) Table 1.

Cloning of Birc3 and promoter The *Birc3* coding region was cloned from cytokine-stimulated mouse islets, using the primers shown in ESM Table 2, with a PCR system (Expand High Fidelity PCR; Roche, Indianapolis, IN, USA), and subcloned into a pIRES2-eGFP vector (Clontech Laboratories, Mountain View, CA, USA). Putative transcription factor binding sites in the *Birc3* proximal promoter were identified using PROMO3.0 [23]. The mouse *Birc3* 5′ untranslated region was amplified from genomic DNA using the primers shown in ESM Table 3 with KOD Hot Start DNA Polymerase (Merck, Darmstadt, Germany) and subcloned into a pGL3 basic vector (Promega, Sydney, NSW, Australia).

Transient transfections Transfection of MIN6 and β-TC_3 cells was performed using techniques and expression plasmids encoding NF-κB.Luc (Promega), *RelA*/*p65*, A20.Luc, and plasmid containing beta galactosidase under the control of the Rous Sarcoma virus (pRSV-β-galactosidase) as previously described by us [5]. Luciferase values (Luciferase Assay System; Promega) were normalised to β-galactosidase activity

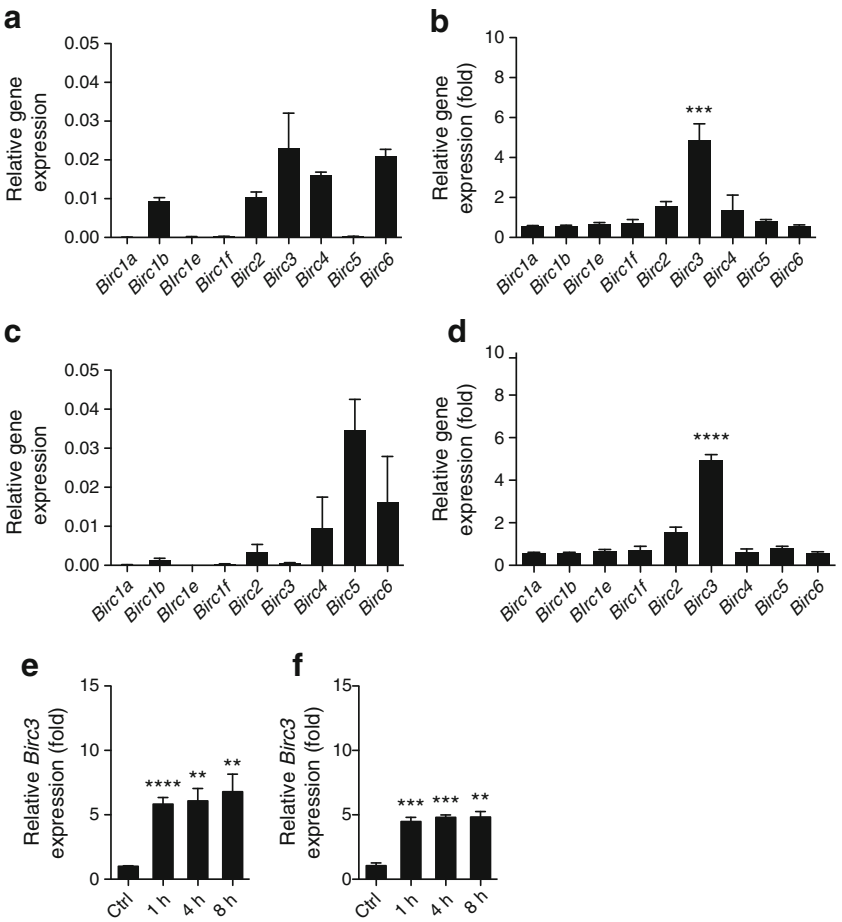

Fig. 1 *Birc3* is an early immediate-response gene. (**a**) Expression of Birc genes, as indicated, in primary mouse islets that were left untreated or (**b**) were stimulated with TNF-α for 4 h, and in (**c**) MIN6 cells left untreated or (**d**) stimulated with TNF-α for 4 h. (**e**) Expression of *Birc3* in primary islets and (**f**) in MIN6 cells stimulated with TNF-α for 1 to 8 h. Data represent mean ± SEM of gene expression relative to *Ppia* from at least three independent experiments. Statistical comparisons were by ANOVA with pair-wise multiple comparisons made using an unpaired *t* test or single unpaired *t* test between two groups. $^{**}p\leq0.01$, $^{***}p\leq0.001$ and $^{****}p\leq0.0001$. *Birc1a*, also known as *Naip1*; *Birc1b*, also known as *Naip2*; *Birc1e*, also known as *Naip5*; *Birc1f*, also known as *Naip6*; *Birc4*, also known as *Xiap*

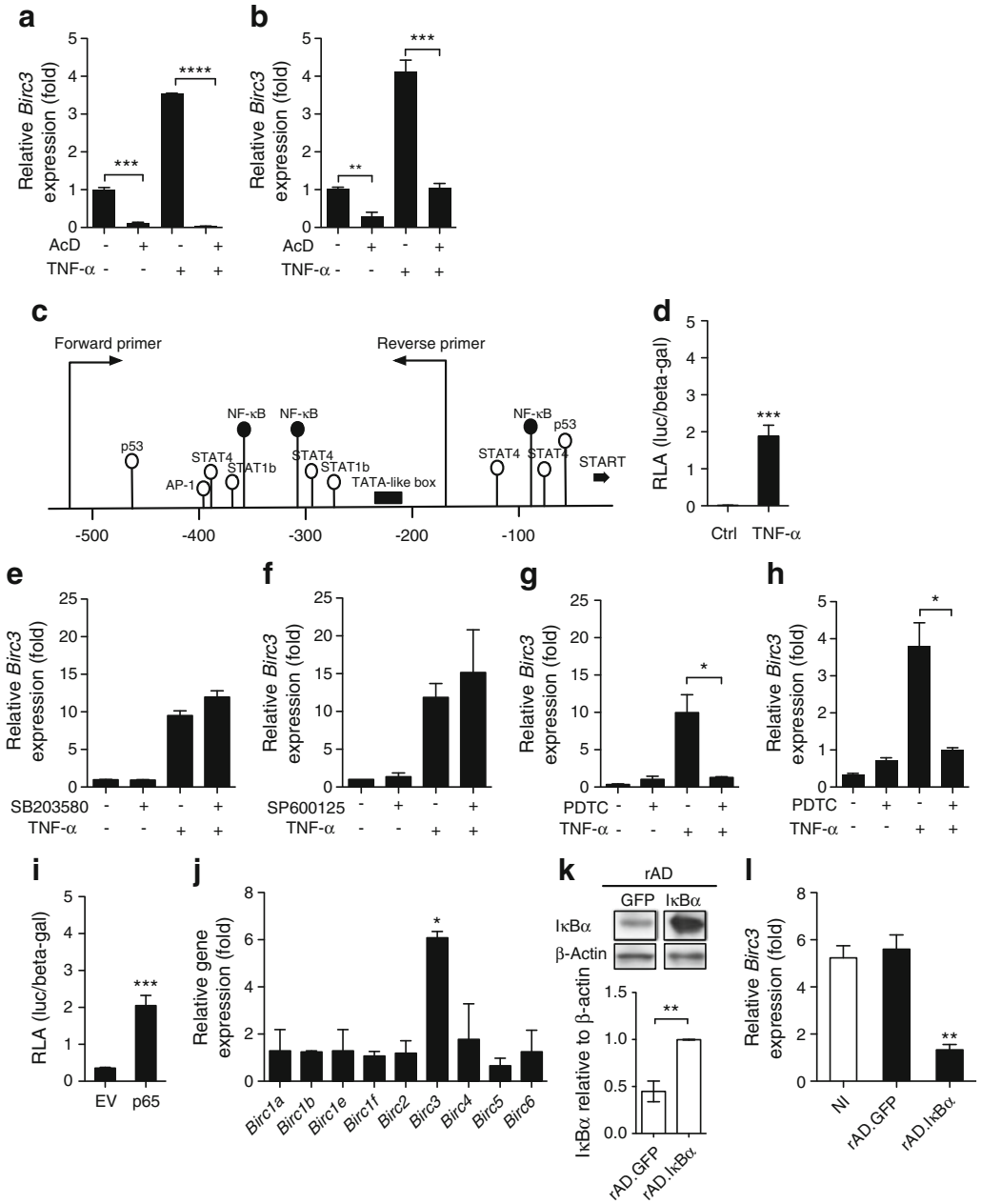

Fig. 2 *Birc3* is regulated by de novo transcription. *Birc3* expression in primary islets (**a**) or MIN6 cells (**b**) that were left untreated or were pretreated for 1 h with actinomycin D (AcD), with or without TNF-α stimulation for 4 h. (**c**) Diagram of putative transcription elements in the *Birc3* proximal promoter ~500 bp upstream of the transcription start site (START), identified using PROMO 3.0 software. AP-1, activator protein 1; STAT, signal transducer and activator of transcription. (**d**) Induction of the *Birc3* reporter in MIN6 cells by TNF-α exposure for 8 h. RLA, relative luciferase activity. (**e**) Expression of *Birc3* in MIN6 cells that were treated with or without TNF-α for 4 h, and were either left untreated or had been pretreated for 1 h with SB203580, (**f**) SP600125 or (**g**) PDTC. (**h**) Expression of *Birc3* in primary islets that were left untreated or were pretreated for 1 h with PDTC, with or without TNF-α stimulation for 4 h. (**i**) Induction of the *Birc3* reporter in MIN6 cells by *RelA*/*p65*. EV, control vector. (**j**) Induction by *RelA*/*p65* of endogenous Birc genes mRNA, as indicated, in β-TC_3 cells. *Birc1a*, also known as *Naip1*; *Birc1b*, also known as *Naip2*; *Birc1e*, also known as *Naip5*; *Birc1f*, also known as *Naip6*; *Birc4*, also known as *Xiap*. (**k**) Western blot analysis of IκBα levels in MIN6 cells transduced with rAD.GFP or rAD.IκBα. β-Actin was used as a loading control. Representative blots are shown. IκBα (39 kDa) was quantified by densitometry and expressed relative to β-actin. (**l**) Induction of *Birc3* by MIN6 cells left non-infected (NI), or transduced with rAD.GFP or rAD.IκBα prior to TNF-α stimulation for 4 h. (**a**, **b**, **e**–**h**, **j**, **l**) Data represent mean ± SEM of gene expression relative to *Ppia* from three independent experiments or (**d**, **k**) mean ± SEM from three independent experiments. Statistical comparisons were made by single unpaired *t* test between two groups. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$

(Galacto-Star; Applied Biosystems, Bedford, MA, USA) for relative luciferase activity.

Adenovirus transduction MIN6 cells (1×10^6) were infected for 1.5 h with recombinant adenovirus expressing GFP (rAd.GFP) or rAd.IκBα [24] at a multiplicity of infection of 100:1 in serum-free medium before adding FCS-enriched medium to achieve a 10% (vol./vol.) FCS concentration. Cells were incubated overnight and the medium was replaced prior to stimulation with TNF-α for 4 h.

In vivo studies Intraperitoneal glucose tolerance tests were performed on 12-week-old male mice administered 2 g/kg glucose following a 16 h fast. For islet transplantation, islets from donor (H-2^b) mice were transplanted into recipient mice (H-2^k) that had been rendered diabetic with 200 mg/kg streptozotocin (Sigma-Aldrich) as previously described [25].

Statistics Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA).

Results

Birc3 is an early immediate-response gene regulated by de novo transcription In the steady state, *Birc3* was highly expressed (Fig. 1a) and was selectively increased by TNF-α in primary islets ($p\leq0.0001$) (Fig. 1b) and MIN6 cells (Fig. 1c, d). Once induced, high levels of *Birc3* mRNA were maintained in primary islets ($p\leq0.01$ and $p\leq0.0001$) (Fig. 1e) and MIN6 cells ($p\leq0.01$ and $p\leq0.001$) (Fig. 1f).

To determine the mechanism of *Birc3* induction, primary islets and MIN6 cells were pretreated with the transcription inhibitor, actinomycin D, prior to TNF-α stimulation. Pretreatment with an optimal dose of actinomycin D (1 μmol/l) resulted in >70% suppression of TNF-α-induced *Birc3* expression in islets ($p\leq0.0001$) (Fig. 2a) and MIN6 cells ($p\leq0.001$) (Fig. 2b). Thus *Birc3* is an inflammation-regulated, early immediate-response gene regulated via de novo gene transcription.

NF-κB controls TNF-induced Birc3 expression The 5′ untranslated region of the *Birc3* proximal promoter contained three putative consensus NF-κB binding sites and a TATA-like box with the sequence TTTAAA' (Fig. 2c). Potential putative activator protein 1, signal transducers and activators of transcription 1 and protein 53 (p53) binding sites were also identified. This region was cloned to generate the *Birc3* reporter, which showed an approximately twofold increase upon TNF-α stimulation ($p\leq0.01$) (Fig. 2d). These results indicate that the *Birc3* proximal promoter contains cytokine responsive elements that direct *Birc3* gene expression.

To determine the molecular mechanisms driving *Birc3* transcription, MIN6 cells were left untreated, or were pretreated with SP600125, SB203580 or PDTC to selectively target the JNK, p38 mitogen-activated protein kinases (p38) or NF-κB pathways respectively, prior to TNF-α stimulation. *Birc3* mRNA levels were measured by quantitative RT-PCR. Neither SB203580 (Fig. 2e) nor SP600125 (Fig. 2f), but only PDTC pre-treatment inhibited TNF-α-induced *Birc3* expression, e.g. by ~90% for MIN6 cells ($p\leq0.05$) (Fig. 2g) and ~75% for islets ($p\leq0.05$) (Fig. 2h). These data show that NF-κB is necessary for TNF-α-induced *Birc3* expression.

NF-κB is sufficient to drive de novo Birc3 expression When MIN6 cells were co-transfected with *Birc3* reporter and a *RelA*/*p65* expression vector, *Birc3* reporter activity was increased by twofold ($p\leq0.001$) (Fig. 2i). To determine whether *RelA*/*p65* was sufficient to drive endogenous *Birc3* gene expression, β-TC_3 cells were transfected with the *RelA*/*p65* expression vector and Birc family gene expression determined by quantitative RT-PCR. In this case, *Birc3* was selectively induced (~sixfold,

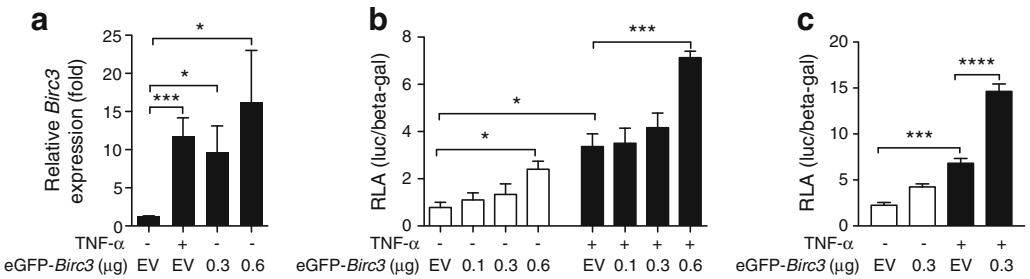

Fig. 3 Increased *Birc3* regulates NF-κB signalling. (**a**) Endogenous expression of *Birc3* in MIN6 cells transfected either with 0.6 μg control vector (EV) for 24 h, in the presence or absence of TNF-α for 4 h, or with 0.3 μg–0.6 μg eGFP-*Birc3*. Data represent mean ± SEM of gene expression relative to *Ppia* from six independent experiments. (**b**) Induction of the NF-κB reporter in MIN6 cells transfected with either 0.6 μg EV or 0.1 to 0.6 μg eGFP-*Birc3*, in the absence or presence of TNF-α for 8 h. Data represent mean ± SEM from three independent experiments. (**c**) Induction of *A20* reporter in β-TC_3 cells transfected with either 0.3 μg EV or 0.3 μg eGFP-*Birc3*, in the absence or presence of TNF-α for 8 h. Data represent mean ± SEM from four independent experiments. Statistical comparisons were made by single unpaired *t* test between two groups or by ANOVA with pairwise multiple comparisons made using an unpaired *t* test. *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$ and ****$p\leq0.0001$

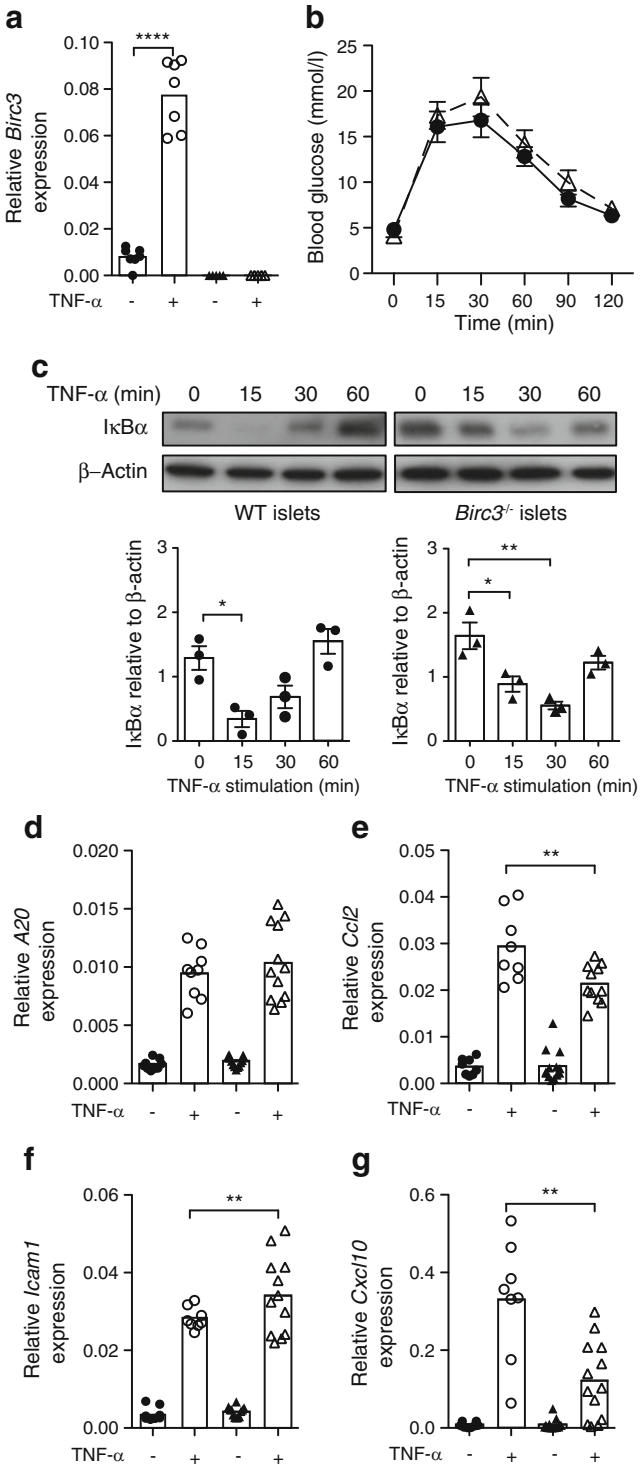

Fig. 4 A redundant role for *Birc3* in NF-κB signalling. (**a**) Expression of *Birc3* in wild type (WT) primary islets in the absence (black circles) or presence (white circles) of TNF-α for 4 h, and in *Birc3*$^{-/-}$ islets under the same conditions (absence, black triangles; presence, white triangles). Values from individual experiments are shown ($n \geq 5$ per group). Bars represent mean ± SEM of gene expression relative to *Ppia*. (**b**) Blood glucose concentrations during intraperitoneal glucose tolerance tests on 12-week-old male wild-type (black circles, solid line) and *Birc3*$^{-/-}$ (white triangles, dotted line) mice. Data represent mean ± SEM from four mice per group. (**c**) Western blot analysis of NF-κB activation measured by IκBα degradation in wild-type (WT) and *Birc3*$^{-/-}$ islets that were either left untreated or were stimulated with TNF-α at the indicated times. β-Actin was used as a loading control. Representative blots are shown. IκBα (39 kDa) was quantified by densitometry and expressed relative to β-actin. Data represent mean ± SEM from three independent experiments. (**d**) Expression of *A20*, (**e**) *Ccl2*, (**f**) *Icam1* and (**g**) *Cxcl10* in wild-type islets in the absence (black circles) or presence (white circles) of TNF-α for 4 h, and in *Birc3*$^{-/-}$ islets under the same conditions (absence, black triangles; presence, white triangles). Values from individual experiments are shown ($n \geq 11$ per group). Bars represent mean ± SEM of gene expression relative to *Ppia*. Statistical comparisons were made by single unpaired *t* test between two groups. *$p \leq 0.05$, **$p \leq 0.01$ and ****$p \leq 0.0001$

$p \leq 0.05$) (Fig. 2j). Moreover, overabundance of IκBα in MIN6 cells (Fig. 2k) resulted in a 77% suppression of TNF-α-induced *Birc3* expression ($p \leq 0.01$) (Fig. 2l). Thus, NF-κB is necessary and sufficient to drive de novo transcription of *Birc3* mRNA.

Increased Birc3 regulates NF-κB signalling Our results imply a model whereby TNF-α activates NF-κB, which subsequently induces increased *Birc3* expression. To elucidate the function of increased *Birc3* in pancreatic beta cells, MIN6 cells were transfected with the pIRES2-eGFP control vector or 0.3 to 0.6 μg pIRES2-eGFP-*Birc3* (eGFP-*Birc3*). eGFP-*Birc3*-transfected MIN6 cells expressed *Birc3* in a range similar to TNF-α-stimulated MIN6 cells (i.e. 12-fold, $p \leq 0.001$) (Fig. 3a). Subsequently, MIN6 cells were transfected with a luciferase reporter containing two tandem NF-κB responsive elements (NF-κB.Luc), plus either 0.1 to 0.6 μg eGFP-*Birc3*, or 0.6 μg control vector. In MIN6 cells, eGFP-*Birc3*-expressing cells showed a dose-dependent increase in NF-κB reporter activity (Fig. 3b), while a combination of TNF-α stimulation and 0.6 μg eGFP-*Birc3* showed a further increase of NF-κB reporter activity, from 3.4-fold ($p \leq 0.05$) with TNF-α alone to sevenfold with eGFP-*Birc3* ($p \leq 0.01$).

To elucidate how *Birc3* modulates a native NF-κB promoter, the effect of *Birc3* activity on the NF-κB-driven *A20* reporter [5] was examined. β-TC_3 cells were transfected with 2 μg *A20*.Luc and either 0.25 μg pIRES2-eGFP control vector or eGFP-*Birc3*. *A20* reporter activity increased from sevenfold ($p \leq 0.001$) for TNF-α alone to 9.5-fold for TNF-α and eGFP-*Birc3* (Fig. 3c). These data demonstrate that increased *Birc3* expression is sufficient to drive NF-κB activity and enhance TNF-α-induced NF-κB signalling.

A redundant role for Birc3 in NF-κB signalling The effect of *Birc3* loss-of-function on beta cell TNF-α-mediated NF-κB signalling was examined in *Birc3*$^{-/-}$ mice (Fig. 4a). *Birc3* is not necessary for beta cell function as indicated by the normal glucose tolerance of *Birc3*$^{-/-}$ mice (Fig. 4b). We analysed TNF-α-induced NF-κB signalling in wild-type or *Birc3*$^{-/-}$ islets via western blot analysis of IκBα levels [26]. Compared with wild-type islets, TNF-α-stimulated

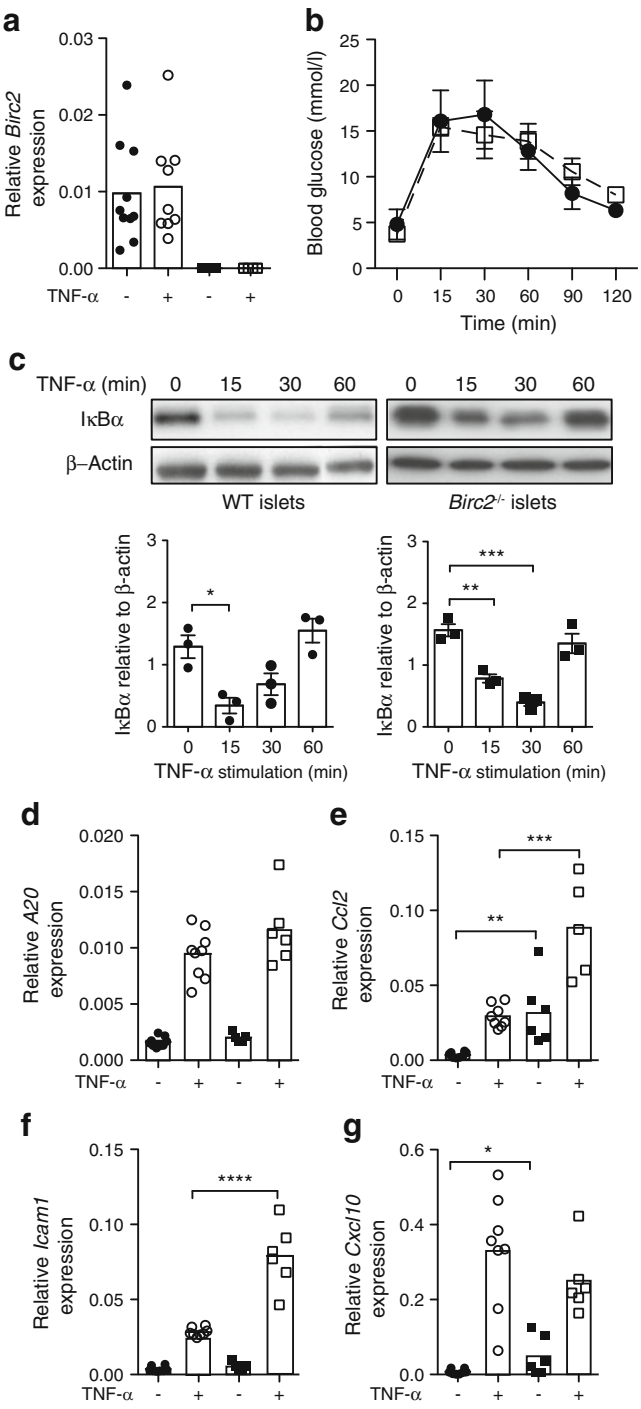

Fig. 5 *Birc2* provides negative control of TNF-pathways in beta cells. (**a**) Expression of *Birc2* in wild-type islets in the absence (black circles) or presence (white circles) of TNF-α for 4 h, and in *Birc2*$^{-/-}$ islets under the same conditions (absence, black squares; presence, white squares). Values from each experiment are shown ($n \geq 6$ per group). Bars represent mean ± SEM of gene expression relative to *Ppia*. (**b**) Blood glucose concentrations during intraperitoneal glucose tolerance tests on 12-week-old male wild-type (black circles, solid line) and *Birc2*$^{-/-}$ (white squares, dotted line) mice. Data represent mean ± SEM from four mice per group. (**c**) Western blot analysis of NF-κB activation measured by IκBα degradation in wild-type (WT) and *Birc2*$^{-/-}$ islets that were left untreated or were stimulated with TNF-α at the indicated times. β-Actin was used as a loading control. Representative blots are shown. IκBα (39 kDa) was quantified by densitometry and expressed relative to β-actin. Data represent mean ± SEM from three independent experiments. (**d**) Expression of *A20*, (**e**) *Ccl2*, (**f**) *Icam1* and (**g**) *Cxcl10* in wild-type islets in the absence (black circles) or presence (white circles) of TNF-α for 4 h, and in *Birc2*$^{-/-}$ islets under the same conditions (absence, black squares; presence, white squares). Values from individual experiments are shown ($n \geq 5$ per group). Bars represent mean ± SEM of gene expression relative to *Ppia*. Statistical comparisons were made by single unpaired *t* test between two groups. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$

Birc3$^{-/-}$ islets showed delayed IκBα degradation, but a normal return to baseline levels at 60 min (Fig. 4c). Analysis of NF-κB-regulated and TNF-α-induced genes in *Birc3*$^{-/-}$ islets revealed an abnormal expression pattern. While normal *A20* induction (Fig. 4d) was observed, expression of *Ccl2* ($p \leq 0.01$) (Fig. 4e) and *Cxcl10* ($p \leq 0.01$) (Fig. 4g) was reduced, with *Icam1* levels being modestly increased ($p \leq 0.01$) (Fig. 4f). These data indicate that BIRC3 is necessary to fine-tune cellular responses to TNF-α.

Birc2 provides negative control of TNF pathways in beta cells BIRC2 can compensate for BIRC3 [3, 27], therefore we examined NF-κB signalling in single *Birc2*$^{-/-}$ islets (Fig. 5a). *Birc2*$^{-/-}$ mice had normal glucose homeostasis (Fig. 5b). Regarding NF-κB signalling, *Birc2*$^{-/-}$ islets had delayed kinetics of TNF-α-stimulated IκBα degradation (Fig. 5c). Noticeably, in the absence of a pro-inflammatory stimulus, *Birc2*$^{-/-}$ islets expressed high levels of *Ccl2* (ninefold greater, $p \leq 0.01$) (Fig. 5e) and *Cxcl10* (sevenfold greater, $p \leq 0.05$) (Fig. 5g) compared with wild-type islets. Furthermore, while TNF-α-stimulated *Birc2*$^{-/-}$ islets expressed normal induced levels of *A20* (Fig. 5d), induction of *Ccl2* (threefold, $p \leq 0.001$) and *Icam1* (threefold, $p \leq 0.0001$) mRNA was significantly higher than for wild-type islets (Fig. 5e, f). These findings suggest that *Birc2* functions as a negative control factor, dampening expression of TNF target genes in islet cells.

Dysregulated gene expression in the absence of Birc2 and Birc3 As BIRC2 and BIRC3 can compensate for each other to allow NF-κB signalling [3, 27], we generated DKO mice. DKO islets exhibited loss of BIRC2 and BIRC3 (Fig. 6a, b), but normal glucose homeostasis, indicating that these two proteins are dispensable for physiological beta cell function (Fig. 6c). Analysis of TNF-α-induced IκBα degradation revealed that NF-κB signalling kinetics were significantly delayed in the absence of BIRC2 and BIRC3 (Fig. 6d). Moreover, DKO islets showed significant disturbances in the regulation of TNF target genes, expressing significantly higher stimulus-independent levels of *A20* (fourfold, $p \leq 0.0001$), *Ccl2* (eightfold, $p \leq 0.001$), *Icam1* (22-fold, $p \leq 0.0001$)

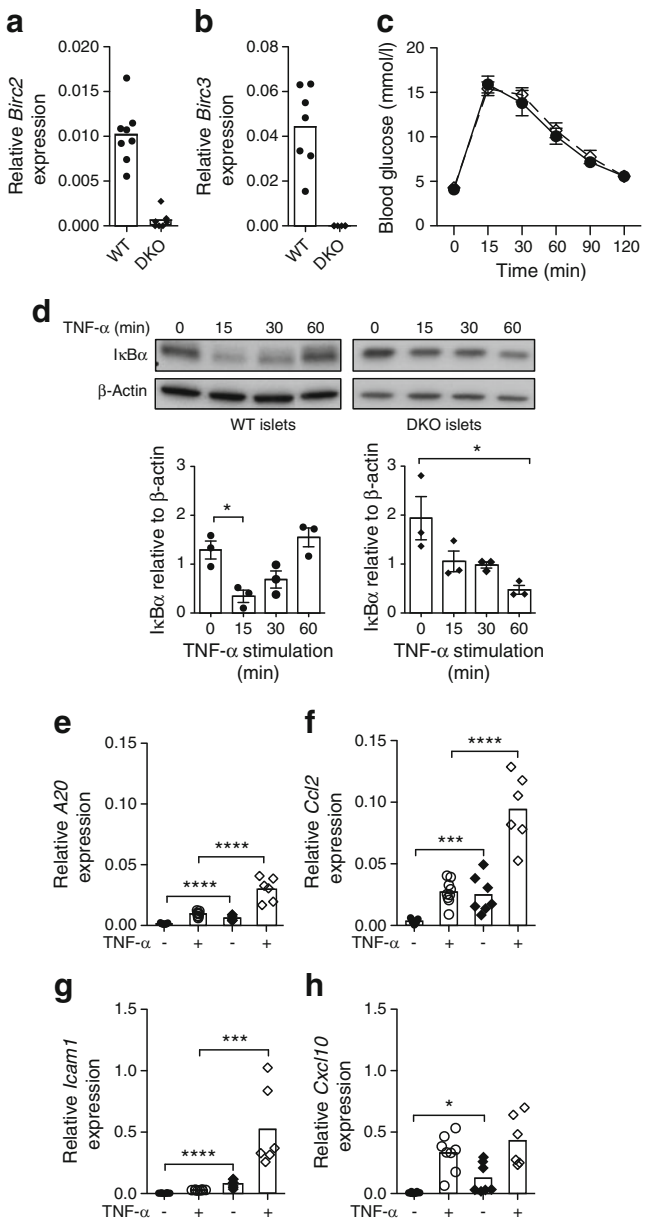

Fig. 6 Dysregulated gene expression in the absence of *Birc2* and *Birc3*. (**a**) Expression of *Birc2* and (**b**) *Birc3* in wild-type islets (black circles) and DKO islets (black diamonds). Values from individual experiment are shown ($n \geq 7$ per group). Bars represent mean ± SEM of gene expression relative to *Ppia*. (**c**) Blood glucose concentrations during intraperitoneal glucose tolerance tests on 12-week-old male wild-type (black circles, solid line) and DKO (white diamonds, dotted line) mice. Data represent mean ± SEM from at least seven mice per group. (**d**) Western blot analysis of NF-κB activation measured by IκBα degradation in wild-type (WT) and DKO islets that were left untreated or were stimulated with TNF-α at the indicated times. β-Actin was used as a loading control. Representative blots are shown. IκBα (39 kDa) was quantified by densitometry and expressed relative to β-actin. Data represent mean ± SEM from three independent experiments. (**e**) Expression of *A20*, (**f**) *Ccl2*, (**g**) *Icam1* and (**h**) *Cxcl10* in wild-type islets in the absence (black circles) or presence (white circles) of TNF-α for 4 h, and in DKO islets under the same conditions (absence, black diamonds; presence, white diamonds). Values from individual experiments are shown ($n \geq 6$ per group). Bars represent mean ± SEM of gene expression relative to *Ppia*. Statistical comparisons were made by single unpaired *t* test between two groups. *$p \leq 0.05$, ***$p \leq 0.001$ and ****$p \leq 0.0001$

and *Cxcl10* (17-fold, $p \leq 0.05$) compared with resting wild-type islets (Fig. 6e–h). Analysis of TNF-α-induced responses showed hyperinduction of *A20* (threefold, $p \leq 0.0001$), *Ccl2* (fourfold, $p \leq 0.0001$) and *Icam1* (18-fold, $p \leq 0.001$), but not of *Cxcl10* mRNA compared with wild-type islets (Fig. 6e–h). Therefore, *Birc2*$^{-/-}$- and *Birc3*$^{-/-}$-deficient beta cells show aberrant NF-kB signalling and dysregulated control of TNF target genes.

Birc2 and Birc3 fine-tune inflammatory gene expression in islet cells We examined the relative contribution of BIRC2 and BIRC3 to the phenotypes observed for DKO islets. We found that in the absence of a TNF-stimulus, *A20* ($p \leq 0.0001$) (Fig. 7a) and *Icam1* ($p \leq 0.0001$) (Fig. 7c) expression was increased in DKO islets, but not in *Birc2*$^{-/-}$ or *Birc3*$^{-/-}$ islets, indicating that neither BIRC2 nor BIRC3 alone can exert molecular control over *A20* and *Icam1* expression. In contrast, molecular control of *Ccl2* expression was strictly controlled by BIRC2, as the phenotype of *Birc2*$^{-/-}$ islets was identical to DKO islets ($p \leq 0.001$ and $p \leq 0.05$) (Fig. 7b). Like *Ccl2*, control of *Cxcl10* expression appears to be more strongly dependent on the presence of BIRC2 ($p \leq 0.05$ and $p \leq 0.05$) (Fig. 7d).

BIRC proteins regulate gene transcription through JNK When DKO islets were treated with actinomycin D, the stimulus-independent expression of *A20* (13-fold, $p \leq 0.0001$) and *Cxcl10* (tenfold, $p \leq 0.01$), but not of *Ccl2* or *Icam1*, was suppressed (Fig. 7e–h), indicating that basal *A20* and *Cxcl10* mRNA levels are regulated by active transcription in DKO islets.

We hypothesised that the increased stimulus-independent transcription rates of *A20* and *Cxcl10* in DKO islets might be due to loss of control of the normal regulatory paths that control TNF-α-induced gene expression. We first sought to identify the possible pathways involved in normal TNF-α-induced gene expression. To do this, MIN6 cells were left untreated, or were pretreated with PDTC, SP600125 or SB203580 to inhibit NF-κB, JNK or p38 respectively, prior to TNF-α stimulation for 4 h. Our findings show that, in MIN6 cells, TNF-α-stimulated *A20* was regulated by NF-κB, but not by the JNK or p38 pathways (Fig. 8a), whereas TNF-α-induced *Ccl2*, *Icam1* and *Cxcl10* expression was regulated by the NF-κB and the JNK pathways (Fig. 8b–d).

We next tested the idea that the increased stimulus-independent gene expression in DKO islets might be

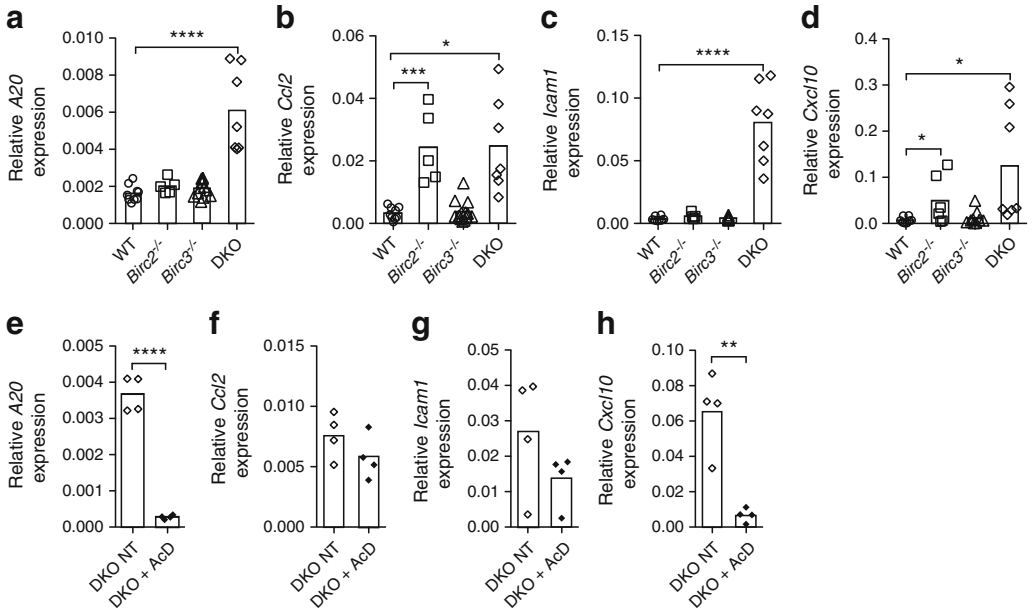

Fig. 7 *Birc2* and *Birc3* fine-tune inflammatory gene expression in islet cells. (**a**) Basal levels of *A20*, (**b**) *Ccl2*, (**c**) *Icam1* and (**d**) *Cxcl10* mRNA from wild-type (WT) (white circles), *Birc2*$^{-/-}$ (white squares), *Birc3*$^{-/-}$ (white triangles) or DKO (white diamonds) islets. Data sourced from Figs. 4, 5 and 6. Values from individual experiments are shown ($n \geq 5$ per group). (**e**) Basal levels of *A20*, (**f**) *Ccl2*, (**g**) *Icam1* and (**h**) *Cxcl10* from DKO islets either left untreated (NT) (white diamonds) or treated for 8 h with actinomycin D (AcD) (black diamonds). Values from individual experiments are shown ($n=4$ per group). Statistical comparisons were made by single unpaired *t* test between two groups. Bars represent mean ± SEM of gene expression relative to *Ppia*. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$

due to hyperactivation of those same pathways involved in the regulation of normal TNF-α-induced gene expression. To test this, DKO islets were treated with inhibitors and gene expression was analysed by quantitative RT-PCR. Notably, the stimulus-independent expression of *Ccl2*, *Icam1* and *Cxcl10* was sensitive to SP600125, but not to SB203580 or PDTC treatment (Fig. 8e–h). Moreover, examination of JNK activation by analysis of c-Jun phosphorylation showed hyperphosphorylation of c-Jun in DKO islets compared with wild-type islets (Fig. 8i). These findings indicate that loss of *Birc2* and *Birc3* dysregulates fine control over the JNK pathway, such that beta cells are hypersensitive to the triggering of JNK signalling, resulting in the loss of fine control over gene transcription.

Proteosome inhibition mimics BIRC2 and BIRC3 deficiency BIRC proteins function as ubiquitin modifying enzymes that target proteins for proteosomal degradation [12]. We tested the idea that, in the absence of BIRC proteins, the accumulation of signalling components normally targeted for degradation, perhaps regulatory kinases, could trigger activation of signalling pathways without appropriate external inputs. To do this, we treated MIN6 cells with the proteosome inhibitor, MG-132, to examine whether loss of proteosome activity could replicate the DKO islet phenotype. Compared with control MIN6 cells, MG-132-treated cells exhibited increased expression of *Ccl2* (50%, $p \leq 0.05$), *Icam1* (50%, $p \leq 0.001$) and *Cxcl10* (80%, $p \leq 0.05$), but not of *A20* (Fig. 9a–d). Moreover, based on analysis of c-Jun phosphorylation, MG-132-treated MIN6 cells also showed increased JNK pathway activation (Fig. 9e). Thus blockade of the proteosome resulted in dysregulated JNK pathway activation in a stimulus-independent manner, with hyperexpression of *Ccl2*, *Icam1* and *Cxcl10* inflammatory genes.

DKO islets have impaired function after transplantation JNK is acutely activated in islet cells during the process of islet isolation [28] and antagonisation of JNK signalling is beneficial for islet survival after isolation [29]. Therefore, we predicted that DKO islets would fair worse in vivo following transplantation. To test this idea, wild-type or DKO islets were transplanted into diabetic allogeneic recipients. Analysis of blood glucose levels showed that wild-type and DKO islets provided good metabolic control immediately after transplantation (Fig. 10). As early graft function can be affected by loss of islet mass due to cell death [30, 31], this suggests that DKO islets were not hypersensitive to death stimuli. However, by post-operative day 6, the function of DKO islets began to deteriorate, a process that worsened with time. In contrast, wild-type islets showed good metabolic control until at least post-operative day 18. The earlier

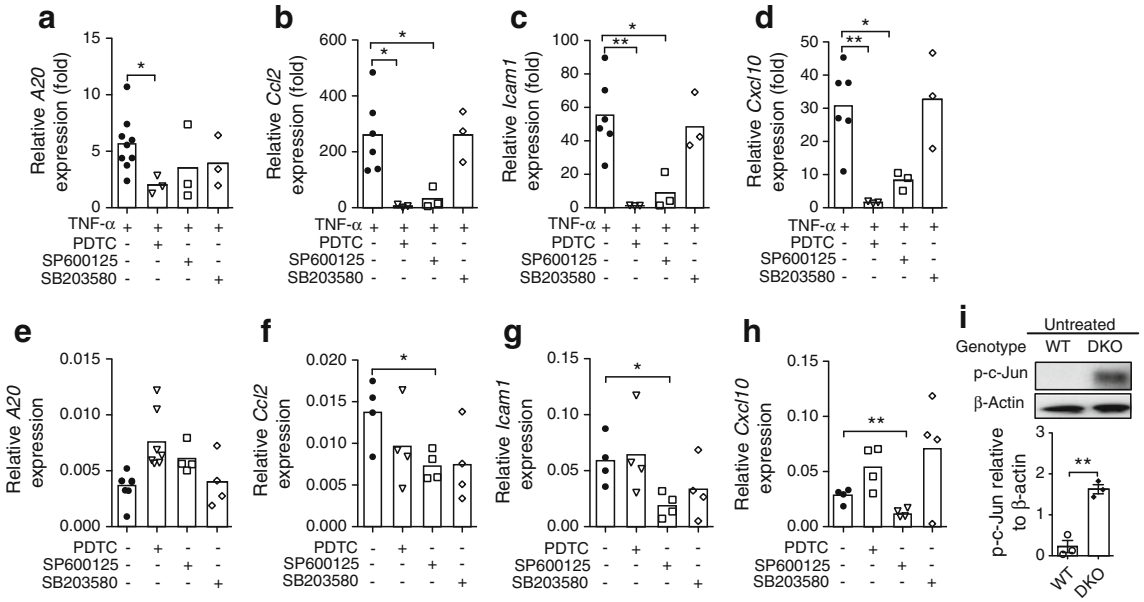

Fig. 8 *Birc2* and *Birc3* fine-tune TNF signalling in islet cells. (**a**) Expression of *A20*, (**b**) *Ccl2*, (**c**) *Icam1* and (**d**) *Cxcl10* in MIN6 cells left untreated (black circles) or pretreated for 1 h with PDTC (white inverted triangles), SP600125 (white squares) or SB203580 (white diamonds) prior to TNF-α stimulation for 4 h. Values from individual experiments are shown ($n \geq 3$ per group). (**e**) Expression of *A20*, (**f**) *Ccl2*, (**g**) *Icam1* and (**h**) *Cxcl10* in DKO islets left untreated (black circles) or treated for 8 h with PDTC (white inverted triangles), SP600125 (white squares) or SB203580 (white diamonds). Values from individual experiments are shown ($n=4$ per group). **a**–**h** Bars represent mean ± SEM of gene expression relative to *Ppia*. (**i**) Western blot analysis of JNK activation by c-Jun phosphorylation in resting wild-type (WT) and DKO islets. β-Actin was used as a loading control. Representative blots are shown. Phosphorylated c-Jun (48 kDa) was quantified by densitometry and expressed relative to β-actin. Data represent mean ± SEM from three independent experiments. Statistical comparisons were made by single unpaired *t* test between two groups. *$p \leq 0.05$ and **$p \leq 0.01$

loss of DKO islet function could reflect loss of control of inflammatory signalling.

Discussion

Our studies present novel insights into the molecular control of TNF signalling in primary pancreatic beta cells and the role of BIRC proteins in this process. Surprisingly, both single *Birc2*$^{-/-}$ and DKO islets, as well as MIN6 cells treated with proteosome inhibitors, exhibited dysregulated expression of select TNF target genes. The augmented stimulus-independent expression of *Ccl2*, *Cxcl10* and *Icam1* mRNA observed for DKO islets was substantially reduced by antagonising JNK signalling. Also, DKO islets showed stimulus-independent hyperphosphorylation of c-Jun. We were also able to demonstrate a role for the JNK pathway in the normal de novo regulation of these same genes in

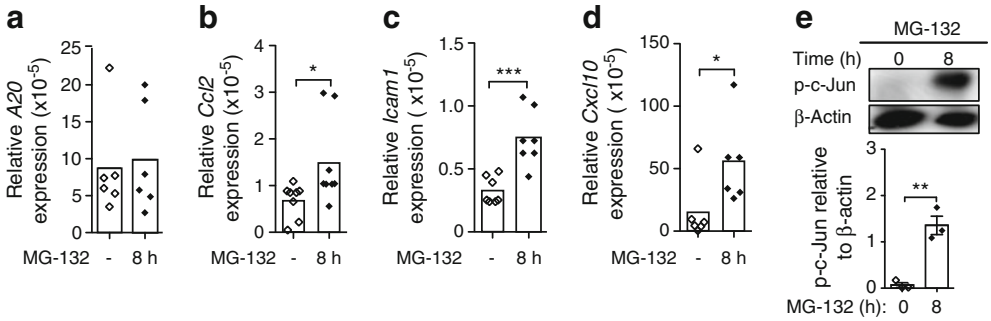

Fig. 9 Proteosome inhibition mimics BIRC2 and BIRC3 deficiency. Expression of *A20* (**a**), *Ccl2* (**b**), *Icam1* (**c**) and *Cxcl10* (**d**) in MIN6 cells left untreated (white diamonds) or treated for 8 h with MG-132 (black diamonds). Values from individual experiments are shown ($n \geq 5$ per group). Bars represent mean ± SEM of gene expression relative to *Ppia*. (**e**) Western blot analysis of JNK activation by c-Jun phosphorylation in MIN6 cells with or without MG-132 treatment for 8 h. β-Actin was used as a loading control. Representative blots are shown. Phosphorylated c-Jun (48 kDa) was quantified by densitometry and expressed relative to β-actin. Data represent mean ± SEM from three independent experiments. Statistical comparisons were made by single unpaired *t* test between two groups. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$

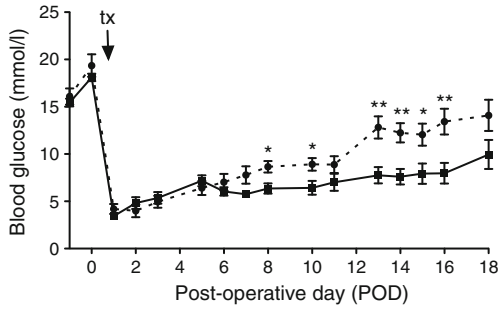

Fig. 10 DKO islets have impaired function after transplantation. Blood glucose values over time of diabetic CBA mice receiving allogeneic wild-type (solid line) or DKO islets (dotted line); tx, transplant day. Values are mean ± SEM ($n \geq 7$ per group). Statistical comparisons were made by single unpaired *t* test between two groups. *$p \leq 0.05$ and **$p \leq 0.01$

islets and beta cells. These data could indicate that BIRC2 and BIRC3 negatively regulate the JNK pathway and subsequent JNK/activator protein-1-regulated gene expression in islet cells.

Interestingly, for B lymphocytes, loss of BIRC2 also leads to prolonged TNF-mediated JNK signalling [32]. BIRC2 and BIRC3 are recruited to the TNFR1 complex via interaction with TRAF2 [18, 33]. TRAF2 is necessary for TNF-α-mediated JNK signalling, but is redundant in the NF-κB or p38 pathways [4, 34, 35]. BIRC2 modulates the duration of TNF-α-induced JNK signalling by targeting TRAF2 and the mitogen-activated protein kinase kinase kinase protein, apoptosis signal-regulating kinase 1 (ASK1), for proteosomal degradation [32, 36]. Our data indicate that this pathway may operate in beta cells. Indeed, treatment of MIN6 cells with the proteosome inhibitor, MG-132, phenocopied DKO islets, lending support to this concept. Hence, one possibility is that, in the absence of BIRC2 and/or BIRC3, TRAF2 and ASK1 accumulate, subsequently triggering hyperactivation of c-Jun, with dysregulated expression of downstream target genes. Hyperactivation of c-Jun might also affect the duration of signalling, contributing to the enhanced gene expression observed for DKO islets following TNF-ligation.

The stimulus-independent expression of *Cxcl10* and *A20* observed in DKO islets showed different sensitivities to JNK inhibition; hyperexpression of *A20* was not sensitive to JNK inhibition. In islets, *A20* is an NF-κB-regulated gene [5] that forms a feedback loop to modulate NF-κB activation [20, 37] and control inflammation [38]. Some data show that BIRC antagonists promote degradation of BIRC proteins, which can subsequently trigger stimulus-independent NF-κB signalling. This may be mediated by increased availability of RIPK1, allowing RIPK1–TNFR1 interactions to trigger NF-κB activation [39], subsequently driving expression of NF-κB target genes [40]. Another possibility is regulation of the non-canonical NF-κB component, v-rel reticuloendotheliosis viral oncogene homolog B (RelB). Indeed, $Relb^{-/-}$ fibroblasts exhibit increased NF-κB activity with stimulus-independent expression of NF-κB target genes [41]. Moreover, the non-canonical pathway is regulated at the level of NF-κB-inducing kinase (NIK) [42]. $Birc2^{-/-}$ mouse embryonic fibroblasts cells (MEFs) and $Birc2^{-/-}Birc3^{-/-}$ cancer cells accumulate high NIK levels, and NIK in turn phosphorylates IκB kinase (IKK)α, inducing activation of the non-canonical NF-κB pathway [39, 40]. Hence BIRC2 and BIRC3 provide negative regulatory control over non-canonical NF-κB signalling.

With regard to NF-κB, DKO islets, interestingly, exhibit altered kinetics of IκBα degradation, which presumably reflects a necessary role for BIRC proteins in controlling the speed of NF-κB signalling in beta cells. NF-κB activation is sensitive to proteosome inhibition [43, 44], and BIRC proteins, via their Really Interesting New Gene (RING) domains, can target substrates for proteosomal degradation through ubiquitin editing [3]. One BIRC substrate is RIPK1, which recruits the transforming growth factor (TGF)-beta activated kinase 1 (TAK1)–TGF-beta activated kinase 1/mitogen-activated protein kinase kinase kinase 7 (MAP3K7) binding protein 2 (TAB2)–TAB3 and IKKα–IKKβ–NF-κB essential modulator (NEMO) complexes, triggering TAK1-dependent phosphorylation of IKKβ [45]. The activated NF-κB signalosome [46] subsequently phosphorylates IκBα, initiating the necessary steps for NF-κB translocation [47]. Thus, by controlling the availability of RIPK1 through ubiquitin editing [39], BIRC proteins have the capacity to fine-tune the speed of NF-κB signalling.

Our data provide new insights into the mechanisms by which BIRC proteins control TNF signalling. TNF-α stimulation induces NF-κB activation, which drives early immediate expression of *Birc3* in beta cells. The increased levels of BIRC3 may be required to fine-tune TNF-induced inflammatory signalling for full expression of TNF-α-induced genes like *Ccl2* and *Cxcl10*, while suppressing others, including *Icam1*. Thus, increased BIRC3, in combination with BIRC2, acts simultaneously as a positive factor for NF-κB signalling, but also as a molecular brake that provides modulatory control over the JNK signalling axis. BIRC proteins prevent activation of this pathway in the absence of an overt extracellular signal normally provided by TNF. Thus under inflammatory conditions, pancreatic islets rapidly induce *Birc3* to fine-tune NF-κB and JNK signalling pathways to ensure beta cell transcriptional responses are appropriately matched to extracellular inputs. Maintaining a balanced response may be critical for beta cell function under conditions of cellular stress. Thus, for example, beta cell failure in the context of type 2 diabetes is associated with exacerbated *CXCL10* expression [48], while activation of the JNK pathway impairs islet survival and function [49, 50]. Moreover, as shown here in the context of islet transplantation, the absence of BIRC proteins impairs islet grafts, resulting in a more rapid loss of glucose homeostasis.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement BMT, NWZ, AOY, RS, SNW, EM and STG made substantial contributions to the study conception and design, the acquisition of data, and to the analysis and interpretation of data. They were all involved in drafting the article and or revising it critically for important intellectual content. All authors made final approval of the version to be published. STG directed the research and is the guarantor of the study.

References

1. Varfolomeev EE, Ashkenazi A (2004) Tumor necrosis factor: an apoptosis JuNKie? Cell 116:491–497
2. Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114:181–190
3. Mahoney DJ, Cheung HH, Mrad RL et al (2008) Both cIAP1 and cIAP2 regulate TNFalpha-mediated NF-kappaB activation. Proc Natl Acad Sci U S A 105:11778–11783
4. Habelhah H, Takahashi S, Cho SG, Kadoya T, Watanabe T, Ronai Z (2004) Ubiquitination and translocation of TRAF2 is required for activation of JNK but not of p38 or NF-kappaB. EMBO J 23:322–332
5. Liuwantara D, Elliot M, Smith MW et al (2006) Nuclear factor-kappaB regulates beta-cell death: a critical role for A20 in beta-cell protection. Diabetes 55:2491–2501
6. Cottet S, Dupraz P, Hamburger F, Dolci W, Jaquet M, Thorens B (2002) cFLIP protein prevents tumor necrosis factor-alpha-mediated induction of caspase-8-dependent apoptosis in insulin-secreting betaTc-Tet cells. Diabetes 51:1805–1814
7. Pakala SV, Chivetta M, Kelly CB, Katz JD (1999) In autoimmune diabetes the transition from benign to pernicious insulitis requires an islet cell response to tumor necrosis factor alpha. J Exp Med 189:1053–1062
8. Dahlen E, Dawe K, Ohlsson L, Hedlund G (1998) Dendritic cells and macrophages are the first and major producers of TNF-alpha in pancreatic islets in the nonobese diabetic mouse. J Immunol 160:3585–3593
9. Ortis F, Pirot P, Naamane N et al (2008) Induction of nuclear factor-kappaB and its downstream genes by TNF-alpha and IL-1beta has a pro-apoptotic role in pancreatic beta cells. Diabetologia 51:1213–1225
10. Zhang S, Kim KH (1995) TNF-alpha inhibits glucose-induced insulin secretion in a pancreatic beta-cell line (INS-1). FEBS Lett 377:237–239
11. Yang XD, Tisch R, Singer SM et al (1994) Effect of tumor necrosis factor alpha on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process. J Exp Med 180:995–1004
12. Gyrd-Hansen M, Meier P (2010) IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. Nat Rev Cancer 10:561–574
13. Emamaullee JA, Rajotte RV, Liston P et al (2005) XIAP overexpression in human islets prevents early posttransplant apoptosis and reduces the islet mass needed to treat diabetes. Diabetes 54:2541–2548
14. Plesner A, Liston P, Tan R, Korneluk RG, Verchere CB (2005) The X-linked inhibitor of apoptosis protein enhances survival of murine islet allografts. Diabetes 54:2533–2540
15. Ou D, Wang X, Metzger DL et al (2005) Synergistic inhibition of tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human pancreatic beta cells by Bcl-2 and X-linked inhibitor of apoptosis. Hum Immunol 66:274–284
16. Plesner A, Soukhatcheva G, Korneluk RG, Verchere CB (2010) XIAP inhibition of beta-cell apoptosis reduces the number of islets required to restore euglycemia in a syngeneic islet transplantation model. Islets 2:18–23
17. Shiozaki EN, Chai J, Rigotti DJ et al (2003) Mechanism of XIAP-mediated inhibition of caspase-9. Molecular Cell 11:519–527
18. Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV (1995) The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell 83:1243–1252
19. Sarkar SA, Kutlu B, Velmurugan K et al (2009) Cytokine-mediated induction of anti-apoptotic genes that are linked to nuclear factor kappa-B (NF-kappaB) signalling in human islets and in a mouse beta cell line. Diabetologia 52:1092–1101
20. Grey ST, Arvelo MB, Hasenkamp W, Bach FH, Ferran C (1999) A20 inhibits cytokine-induced apoptosis and nuclear factor kappaB-dependent gene activation in islets. J Exp Med 190:1135–1146
21. Miyazaki J, Araki K, Yamato E et al (1990) Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. Endocrinology 127:126–132
22. D'Ambra R, Surana M, Efrat S, Starr RG, Fleischer N (1990) Regulation of insulin secretion from beta-cell lines derived from transgenic mice insulinomas resembles that of normal beta-cells. Endocrinology 126:2815–2822
23. Farre D, Roset R, Huerta M et al (2003) Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. Nucleic Acids Res 31:3651–3653
24. Wrighton CJ, Hofer-Warbinek R, Moll T, Eytner R, Bach FH, de Martin R (1996) Inhibition of endothelial cell activation by adenovirus-mediated expression of I kappa B alpha, an inhibitor of the transcription factor NF-kappa B. J Exp Med 183:1013–1022
25. Walters S, Webster KE, Sutherland A et al (2009) Increased CD4+Foxp3+ T cells in BAFF-transgenic mice suppress T cell effector responses. J Immunol 182:793–801
26. Beg AA, Finco TS, Nantermet PV, Baldwin AS Jr (1993) Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. Mol Cell Biol 13:3301–3310
27. Varfolomeev E, Goncharov T, Fedorova AV et al (2008) c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation. J Biol Chem 283:24295–24299
28. Abdelli S, Abderrahmani A, Hering BJ, Beckmann JS, Bonny C (2007) The c-Jun N-terminal kinase JNK participates in cytokine- and isolation stress-induced rat pancreatic islet apoptosis. Diabetologia 50:1660–1669

29. Noguchi H, Nakai Y, Matsumoto S et al (2005) Cell permeable peptide of JNK inhibitor prevents islet apoptosis immediately after isolation and improves islet graft function. Am J Transplant 5:1848–1855
30. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E (2002) Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes 51:66–72
31. Grey ST, Longo C, Shukri T et al (2003) Genetic engineering of a suboptimal islet graft with A20 preserves beta cell mass and function. J Immunol 170:6250–6256
32. Zhao Y, Conze DB, Hanover JA, Ashwell JD (2007) Tumor necrosis factor receptor 2 signaling induces selective c-IAP1-dependent ASK1 ubiquitination and terminates mitogen-activated protein kinase signaling. J Biol Chem 282:7777–7782
33. Shu HB, Takeuchi M, Goeddel DV (1996) The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex. Proc Natl Acad Sci U S A 93:13973–13978
34. Yeh WC, Shahinian A, Speiser D et al (1997) Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. Immunity 7:715–725
35. Lee SY, Reichlin A, Santana A, Sokol KA, Nussenzweig MC, Choi Y (1997) TRAF2 is essential for JNK but not NF-kappa B activation and regulates lymphocyte proliferation and survival. Immunity 7:703–713
36. Samuel T, Welsh K, Lober T, Togo SH, Zapata JM, Reed JC (2006) Distinct BIR domains of cIAP1 mediate binding to and ubiquitination of tumor necrosis factor receptor-associated factor 2 and second mitochondrial activator of caspases. J Biol Chem 281:1080–1090
37. Wertz IE, O'Rourke KM, Zhou H et al (2004) De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature 430:694–699
38. Lee EG, Boone DL, Chai S et al (2000) Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. Science 289:2350–2354
39. Vince JE, Wong WW, Khan N et al (2007) IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 131:682–693
40. Varfolomeev E, Blankenship JW, Wayson SM et al (2007) IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell 131:669–681
41. Xia Y, Pauza ME, Feng L, Lo D (1997) RelB regulation of chemokine expression modulates local inflammation. Am J Pathol 151:375–387
42. Zarnegar BJ, Wang YY, Mahoney DJ et al (2008) Noncanonical NF-kappa B activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. Nat Immunol 9:1371–1378
43. Mathes E, O'Dea EL, Hoffmann A, Ghosh G (2008) NF-kappaB dictates the degradation pathway of IkappaBalpha. EMBO J 27:1357–1367
44. Chan JY, Biden TJ, Laybutt DR (2012) Cross-talk between the unfolded protein response and nuclear factor-kappaB signalling pathways regulates cytokine-mediated beta cell death in MIN6 cells and isolated mouse islets. Diabetologia 55:2999–3009
45. Bertrand MJM, Milutinovic S, Dickson KM et al (2008) cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Molecular Cell 30:689–700
46. Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P (1998) The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity 8:297–303
47. Henkel T, Machleidt T, Alkalay I, Kronke M, Ben-Neriah Y, Baeuerle PA (1993) Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. Nature 365:182–185
48. Schulthess FT, Paroni F, Sauter NS et al (2009) CXCL10 impairs beta cell function and viability in diabetes through TLR4 signaling. Cell Metab 9:125–139
49. Fornoni A, Pileggi A, Molano RD et al (2008) Inhibition of c-jun N terminal kinase (JNK) improves functional beta cell mass in human islets and leads to AKT and glycogen synthase kinase-3 (GSK-3) phosphorylation. Diabetologia 51:298–308
50. Varona-Santos JL, Pileggi A, Molano RD et al (2008) c-Jun N-terminal kinase 1 is deleterious to the function and survival of murine pancreatic islets. Diabetologia 51:2271–2280