Brief Communication

MOLECULAR METABOLISM

Inhibition of RIPK1 kinase does not affect diabetes development: β-Cells survive RIPK1 activation

Tatiana Takiishi [1,6], Peng Xiao [1,6], Marie Franchimont [1], Eduardo H. Gilglioni [2], Erick N. Arroba [1,2], Esteban N. Gurzov [2,3], Mathieu JM. Bertrand [4,5], Alessandra K. Cardozo [1,*]

ABSTRACT

Objectives: Type 1 diabetes (T1D) is caused by progressive immune-mediated loss of insulin-producing β-cells. Inflammation is detrimental to β-cell function and survival, moreover, both apoptosis and necrosis have been implicated as mechanisms of β-cell loss in T1D. The receptor interacting serine/threonine protein kinase 1 (RIPK1) promotes inflammation by serving as a scaffold for NF-κB and MAPK activation, or by acting as a kinase that triggers apoptosis or necroptosis. It is unclear whether RIPK1 kinase activity is involved in T1D pathology. In the present study, we investigated if absence of RIPK1 activation would affect the susceptibility to immune-mediated diabetes or diet induced obesity (DIO).
Methods: The RIPK1 knockin mouse line carrying a mutation mimicking serine 25 phosphorylation (*Ripk1*$^{S25D/S25D}$), which abrogates RIPK1 kinase activity, was utilized to assess the *in vivo* role of RIPK1 in immune-mediated diabetes or diet induced obesity (DIO). *In vitro*, β-cell death and RIPK1 kinase activity was analysed in conditions known to induce RIPK1-dependent apoptosis/necroptosis.
Results: We demonstrate that *Ripk1*$^{S25D/S25D}$ mice presented normal glucose metabolism and β-cell function. Furthermore, immune-mediated diabetes and DIO were not different between *Ripk1*$^{S25D/S25D}$ and *Ripk1*$^{+/+}$ mice. Despite strong activation of RIPK1 kinase and other necroptosis effectors (RIPK3 and MLKL) by TNF+BV6+zVAD, no cell death was observed in mouse islets nor human β-cells.
Conclusion: Our results contrast recent literature showing that most cell types undergo necroptosis following RIPK1 kinase activation. This peculiarity may reflect an adaptation to the inability of β-cells to proliferate and self-renewal.

Keywords RIPK1; Type 1 diabetes; Obesity; Apoptosis; Necroptosis

1. INTRODUCTION

Type 1 diabetes (T1D) is characterized by immune-mediated destruction of pancreatic β-cells. The exact mechanisms implicated in failure of immune regulation and β-cell demise in T1D are unclear. Receptor interacting protein kinase 1 (RIPK1) is activated downstream of immune receptors such as CD95, TNFR1 and TLR-4, all shown to be involved in β-cell death in T1D [1]. RIPK1 is an important modulator of inflammation and cell survival, having opposing roles depending on the cellular context [2]. Tumor necrosis factor (TNF) binding to TNFR1 will lead to complex I assembly, where RIPK1 is ubiquitinated by cIAP1/2, functioning as a scaffold protein to stabilize TRAF and cIAP1/2, regulating the NF-κB pathway and promoting cell survival; whereas deubiquitination of RIPK1 by use of cIAP inhibitors (e.g. BV6, a SMAC mimetic), will lead to complex II formation, and activation of RIPK1 kinase enzymatic function which can promote apoptosis and/or necroptosis [3,4]. Both apoptosis and necrosis are detected in pancreas from T1D and T2D patients and induced in islets treated with cytokines and death receptor ligands [5,6]. Interestingly, recent studies showed that hyperglycaemic conditions prime cells for RIPK1 kinase-dependent necroptosis [7,8]. Moreover, use of RIPK1 kinase inhibitor necrostatin-1 (Nec-1) in obese mice improved glucose tolerance, insulin resistance and enhanced insulin signaling [9]. Many studies have shown that β-cells are resistant to TNF-induced cell death, however a recent study showed that NIT-1 (a mouse β-cell line) were sensitive to TNF + BV6 cell death and protected by total

[1]Inflammation and Cell Death Signalling Group, Laboratoire de Gastroentérologie Expérimental et Endotools, Université libre de Bruxelles (ULB), Brussels, Belgium [2]Signal Transduction and Metabolism Laboratory, Laboratoire de Gastroentérologie Expérimental et Endotools, Université libre de Bruxelles (ULB), Brussels, Belgium [3]WELBIO, WEL Research Institute, Avenue Pasteur 6, Wavre, 1300, Belgium [4]VIB Center for inflammation Research, Ghent, Belgium [5]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

[6] Authors contributed equally.

*Corresponding author. Inflammation and Cell Death Signalling Group, Laboratoire de Gastroentérologie Expérimental et Endotools, Université libre de Bruxelles, Route de Lennik, 808, CP 697/02, 1070, Brussels, Belgium.

E-mails: tatiana.takiishi@ulb.be (T. Takiishi), peng.xiao@ulb.be (P. Xiao), marie.franchimont96@gmail.com (M. Franchimont), eduardo.hideo.gilglioni@ulb.be (E.H. Gilglioni), erick.arroba@ulb.be (E.N. Arroba), esteban.gurzov@ulb.be (E.N. Gurzov), mathieu.bertrand@irc.vib-ugent.be (M.JM. Bertrand), alessandra.kupper.cardozo@ulb.be (A.K. Cardozo).

Received October 17, 2022 • Revision received December 30, 2022 • Accepted January 19, 2023 • Available online 24 January 2023

https://doi.org/10.1016/j.molmet.2023.101681

RIPK1 deletion [10,11]. This data suggest that metabolic stress can regulate components of the RIPK1 pathway, however it is unclear if RIPK1 kinase activity and necroptosis play a role in diabetes. In this study, we investigated whether β-cell viability, glucose metabolism and diabetes presentation would be affected in mice lacking RIPK1 kinase-dependent cell death.

2. MATERIALS AND METHODS

2.1. Mice treatments and procedures

Ripk1$^{S25D/S25D}$ knock-in mice were generated by CRISPR/Cas9 and maintained in the C57BL/6 genetic background, as previously described [12]. The non-fasted-glycaemia and body weight were followed in male *Ripk1*$^{S25D/S25D}$ mice and wild-type (*Ripk1*$^{+/+}$) littermates. Hyperglycaemia was defined as non-fasting blood glucose levels >200 mg/dL in two sequential measurements. The multiple low-dose-streptozotocin (MLDSTZ) model was performed in 7–8-week-old male mice by injections of 42.5 mg/kg streptozotocin (STZ) (Sigma-Aldrich, Belgium) as previously described [13]. For the diet induced obesity (DIO) model, male mice aged 8 weeks were fed ad libitum high fat diet (D09100310i) or control diet (D09100304i) from Research Diets (New Brunswick, NJ, USA). Lean and fat mass were analyzed using EchoMRI™ 3-in-1 (NMR) body composition analyser (EchoMedical Systems, Houston, TX, USA). Intra-peritoneal glucose tolerance test (ipGTT), insulin tolerances tests (ITTs), pancreatic insulin content analysis were performed as previously described [13]. Mice were housed and handled according to the Belgian Regulations for Animal Care and animal protocols were approved by the ULB ethical committee (627N).

2.2. Cell treatments and analysis

EndoC-βH1 cells were purchased from UNIVERCELL-BIOSOLUTIONS (MTA BH1-201601171, Toulouse, France) and islets were isolated as previously described [13]. The EndoC-βH1 and isolated murine islets were cultured as previously described [13,14]. These cells were treated with a combination of cytokines (TNF, IL1-β, IFN-γ) or RIPK1 kinase activators (TNF, BV6, zVAD). WB of EndoC-βH1 and murine islets was performed as previously described [13]. Viability and glucose-stimulated insulin secretion assay (GSIS) of islets were performed as previously described [13]. Endoc-βH1 cell viability was measured by integration of viability dye (SYTOX™ Green, Invitrogen) [12]. For details of treatments, chemicals and antibodies see Supplementary data.

2.3. Statistical analysis

Data were presented as means ± S.E.M. Comparisons between two groups were performed by two-tailed unpaired Student's t-test. Multiple comparisons among three or more groups were performed by one-way ANOVA followed by Tukey post-test or two-way ANOVA followed by Šidák post-test. Repeated measurements analysis was performed by mix-model ANOVA post hoc Tukey test. A p-value of $p \leq 0.05$ was considered statistically significant.

3. RESULTS

3.1. RIPK1 enzymatic activity does not affect glycemic control nor immune-mediated diabetes susceptibility

Ripk1$^{S25D/S25D}$ mice that carry a mutation mimicking serine 25 phosphorylation of RIPK1, which prevents RIPK1 kinase-dependent cell death whilst not affecting its scaffold function, were used [12]. Follow-up of homozygous *Ripk1*$^{S25D/S25D}$ mice showed no abnormalities in weight, glycemia and body mass composition compared to wild-type littermates (*Ripk1*$^{+/+}$) until mature adult age (16 weeks) or old age (18 months) (Figure 1A–B). Furthermore, RIPK1 kinase inhibition did not affect glucose tolerance response *in vivo* (Figure 1C).

To investigate the role of RIPK1 in immune-mediated diabetes, mice received MLDSTZ injections. Diabetes presentation was similar between *Ripk1*$^{S25D/S25D}$ and *Ripk1*$^{+/+}$ mice; hyperglycaemia (>200 mg/dL) initiated around day 7 and progressed until 45 days post last STZ injection (Figure 1D). Glucose tolerance was also indistinguishable between *Ripk1*$^{S25D/S25D}$ and *Ripk1*$^{+/+}$ mice, at peak of insulitis and after inflammation was resolved (respectively, 14 and 45 days post-MLDSTZ exposure [15]) (Figure 1E–F). Loss of pancreatic insulin content in diabetic-MLDSTZ mice was unaffected by absence of RIPK1 kinase function (Figure 1G). Thus, immune-mediated diabetes was not influenced by RIPK1 kinase.

3.2. RIPK1 kinase activity does not affect glucose tolerance nor insulin resistance in diet induced obesity (DIO)

Next, we assessed if RIPK1 enzymatic activity had a role in obesity or impaired glucose metabolism associated with obesity. Both *Ripk1*$^{S25D/S25D}$ and *Ripk1*$^{+/+}$ littermates fed high fat (40 kcal%), fructose (20 kcal %), cholesterol (2% wt:wt) diet for 16 weeks presented normal blood glucose levels (Figure 2A). DIO mice showed a significant increase in bodyweight during the 16-weeks, while *Ripk1*$^{S25D/S25D}$ mice tended to gain more weight than *Ripk1*$^{+/+}$ mice, the difference was not significant (Figure 2B). DIO led to significant percentual increase in fat mass, however no differences in body composition were found between *Ripk1*$^{S25D/S25D}$ and *Ripk1*$^{+/+}$ (Figure 2C). Moreover, absence of RIPK1 kinase activation did not improve glucose tolerance nor insulin resistance in DIO (Figure 2D–E). *Ripk1*$^{S25D/S25D}$ mice presented similar insulin levels in plasma and pancreatic insulin content as *Ripk1*$^{+/+}$ littermates (Figure 2F–G). As expected, islets from DIO mice exhibited a suppression of insulin secretion relative to islets of control mice on high glucose challenge, however no difference was observed between genotypes (Figure 2H). These results demonstrate that inhibition of RIPK1 enzymatic activity had no effect on DIO-induced glucose metabolic dysfunction.

3.3. RIPK1 enzymatic activation does not lead to death of murine islets nor human β-cells

To potentially elucidate the absence of phenotype in these diabetes models, we investigated if RIPK1 could be activated in β-cells and if RIPK1 kinase activity influenced β-cell survival. Our data shows that exposure to STZ significantly increased murine islet cell death, however inhibition of RIPK1 kinase activity had no effect on the severity of mortality (Figure 3A). To verify whether activation of RIPK1 kinase, monitored by autophosphorylation of RIPK1 on serine 166 [12], was induced in β-cells under inflammatory conditions *in vivo*, we isolated the islets from MLDSTZ- or buffer-treated mice at 14 days after the last STZ injection [16]. Our results show that RIPK1 enzymatic function is not activated during insulitis (Figure 3B). Next, we evaluated whether conditions known to induce RIPK1 kinase activity and associated cytotoxicity in other cell types would lead to death in murine islets. RIPK1 enzymatic activation was obtained by stimulating the cells with a combination of TNF+BV6 and the pan caspase inhibitor zVAD.fmk, a potent trigger of RIPK1 kinase-dependent necroptosis in many cell types [12,17,18]. Our data showed that, as expected, TNF+BV6+zVAD led to the induction of pRIPK1 in *Ripk1*$^{+/+}$ islets, which was absent in *Ripk1*$^{S25D/S25D}$ or when Nec-1 was added (Figure 3B–C). Surprisingly, neither TNF+BV6+zVAD nor TNF+BV6 (for RIPK1 kinase-dependent apoptosis induction) translated to a

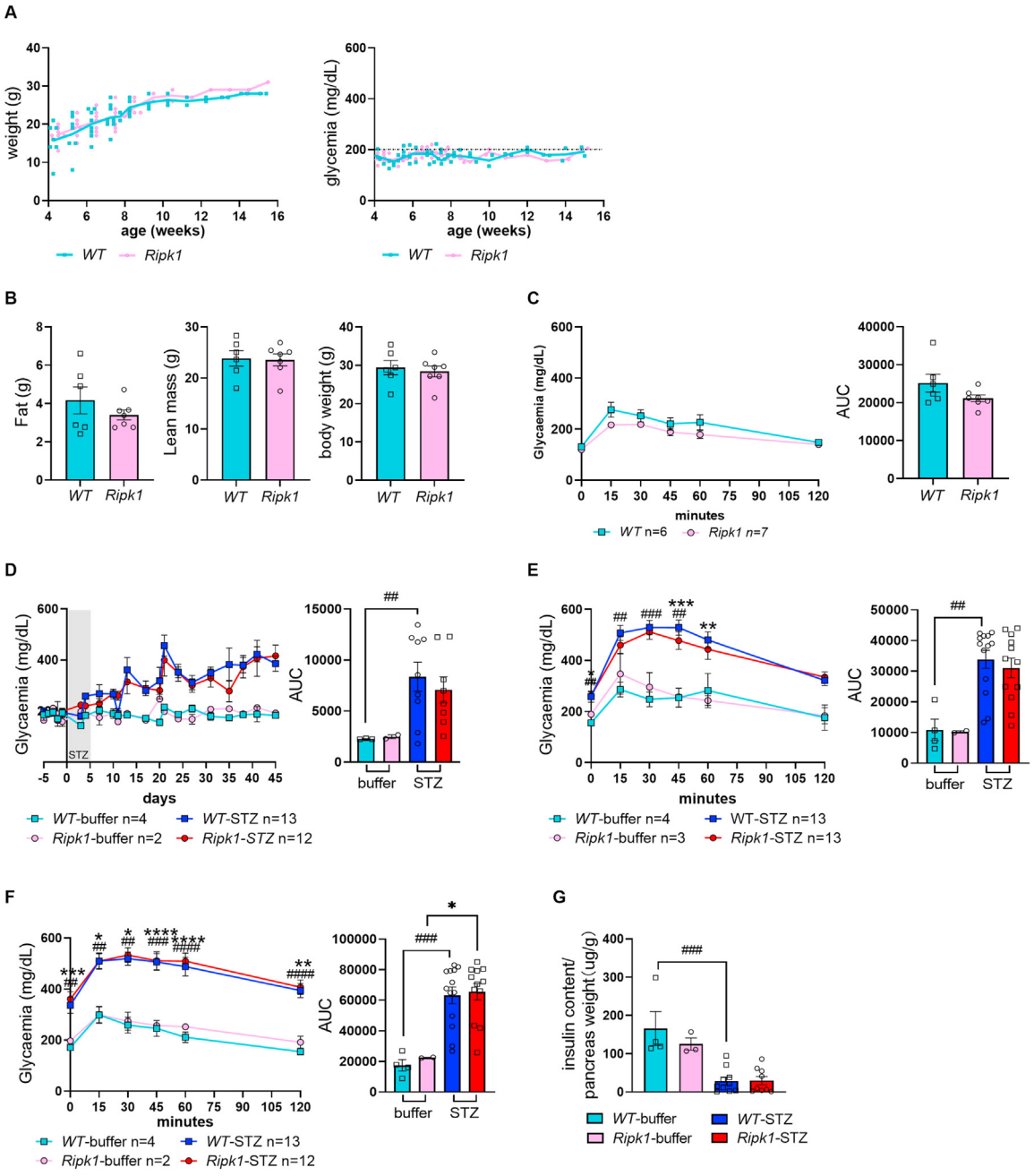

Figure 1: RIPK1$^{S25D/S25D}$ mice in homeostatic conditions (A–C) or in MLDSTZ-induced diabetes (D–G). (A) Male Ripk1$^{S25D/S25D}$ (Ripk1) and Ripk1$^{+/+}$ (WT) were followed for fed blood glucose and body weight from weaning until 16 weeks of age. (B) Fat, lean mass and body weight were measured in male Ripk1 and WT mice at 18 months of age. (C) IpGTT was performed on male Ripk1 and WT mice at 18 months of age. (D) 7–8-week-old male Ripk1 and WT were injected with streptozotocin (STZ) or vehicle (citrate buffer) and non-fasted glucose levels were measured prior to STZ injections (−3 day), during STZ treatment (1,2,3,4,5 days) and twice per week until 45 days after the last injection. (E–F) IpGTT was performed at (E) 14 days and (F) 45 days after last STZ injection. (G) Pancreatic insulin content was determined at the end of experiment; n = 3–10. (C–F) Left panels show raw data and right panels show quantitative analysis of the area under the curves (AUC). Data is presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001, Ripk1-STZ (red) vs Ripk1-buffer (pink). ##p < 0.01; ###p < 0.001, ####p < 0.0001, WT-STZ (dark blue) vs WT-buffer (light blue). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

significant increase in cell death in *Ripk1*$^{+/+}$ islets (Figure 3D), indicating that despite RIPK1 enzymatic activation, apoptosis and necroptosis were not triggered in murine islets.

We then analyzed if human β-cells were also resistant to RIPK1 kinase-dependent cell death. Interestingly, TNF+BV6 led to significant apoptotic cell death in the EndoC-βH1 cells (a human β-cell line [14]) (Figure 3E). TNF+BV6 induced caspase 3 and 8 cleavage and zVAD treatment significantly prevented caspase activation and cell death, whereas Nec-1 had no effect in cell survival (Figure 3E–F). These results indicate that TNF+BV6 induced RIPK1 kinase-independent apoptosis in these cells and that addition of zVAD did not switch the response to RIPK1 kinase-dependent necroptosis, in contrast to many other cell types.

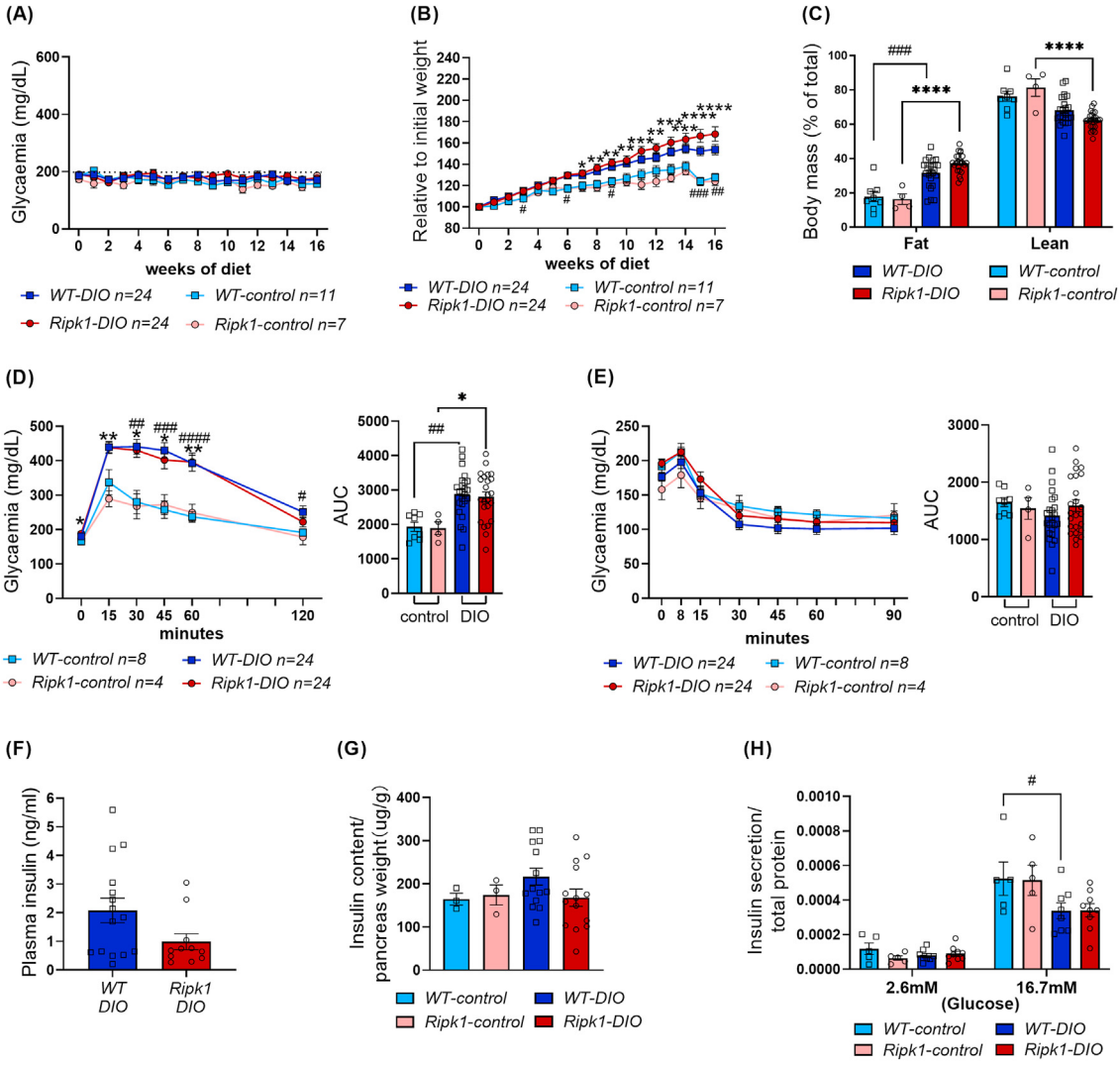

Figure 2: RIPK1 kinase has no effect on diet induced obesity. 8-Week-old male Ripk1$^{S25D/S25D}$ (Ripk1) and Ripk1$^{+/+}$ (WT) mice were fed a high fat (40 kcal%), fructose (20 kcal%), cholesterol (2% wt:wt) diet for 16 weeks. Weekly measures of (A) non-fasted blood glucose and (B) body weight were determined. (C) Body mass composition was analyzed after 16 weeks of DIO; n = 4–24. (D) IpGTTs and (E) iTTs were performed at 16 and 17 weeks of DIO, respectively. (F) Non-fasted plasma insulin levels and (G) pancreatic insulin content of DIO and control diet mice were determined at the end of the experiment. (F) n = 11–15. (G) n = 3–13. (H) Glucose-stimulated insulin secretion was performed in islets from Ripk1 and WT mice under DIO or control diet in low (2.8 mM) and high (16.7 mM); n = 5–9. glucose conditions. (D–E) Left panels show raw data and right panels show quantitative analysis of the area under the curves (AUC). Data is presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, Ripk1-DIO (red) vs Ripk1-control diet (pink). #p < 0.05; ##p < 0.01; ###p < 0.001; ####p < 0.0001, WT-DIO (dark blue) vs WT-control diet (light blue). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Nevertheless, TNF+BV6 induced pRIPK1 and the addition of zVAD greatly enhanced its activation (Figure 3F–H). We showed that phosphorylation of RIPK3 (pRIPK3) and MLKL (pMLKL), downstream and hallmark events of RIPK1 kinase-dependent necroptosis, were also induced in human β-cells by TNF+BV6+zVAD (Figure 3H). These results demonstrate that in murine islets and human β-cells, cell death was not triggered even with the RIPK1 enzymatic pathway highly activated. Overall, our data suggests that β-cells, contrary to other cell types, are resistant to RIPK1 kinase-dependent cell death.

4. DISCUSSION

For many years, apoptosis was accepted as the main form of T cell-mediated β-cell death in T1D, even though necrosis was also detected in human and rodent diabetic pancreas [19]. However, a recent study demonstrated necrosis to be the main mechanism of β-cell death in a murine model of diabetes using diabetogenic T-cells [20]. Interestingly, this study could not determine what led to necrosis in β-cells as neither loss of components of apoptotic signaling pathways (FAS, FAD, BCL-2) nor necroptotic executioner proteins (RIPK3 and MLKL) in β-cells prevented death by diabetogenic BDC2.5 T cells. Other studies demonstrated that high glucose concentrations (50 mM) together with TNF or FASL induced cell death that was inhibited by Nec-1 but not zVAD, accompanied by expression of pRIPK1, pRIPK3 and migration of MLKL to the cell membrane in different human cell lines or primary human T cells [7,8]. Despite the central role of RIPK1 in death and inflammation, its contribution to T1D pathology was never directly elucidated. To clarify the role of RIPK1 in autoimmune diabetes,

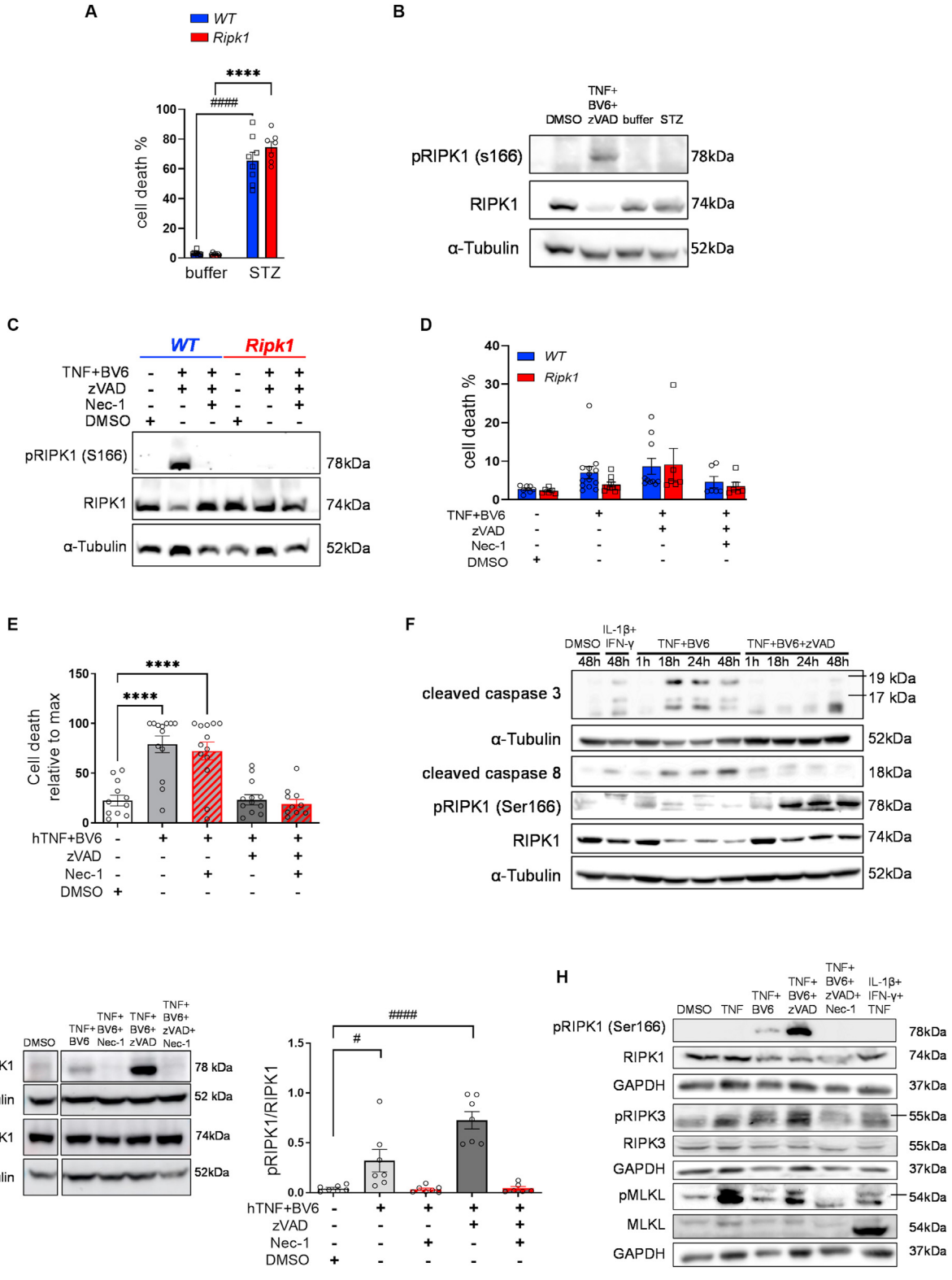

Figure 3: RIPK1 enzymatic activation does not lead to death of murine and human β-cells. (A) Viability of islets isolated from Ripk1$^{S25D/S25D}$ (Ripk1) and Ripk1$^{+/+}$ (WT) mice treated with STZ (1.1 mM) or vehicle (citrate buffer) was assessed; n = 6–9. (B–C) Expression of phosphoRIPK1 (ser166) (pRIPK1), RIPK1 and α-tubulin (loading control) on murine islets isolated from WT mice 2 weeks after last MLDSTZ or buffer injections or treated with TNF+BV6, zVAD, Nec-1 and/or DMSO for 18 h, as indicated, was assessed by WB. (D) Viability of murine islets treated with TNF+BV6, zVAD, Nec-1 and/or DMSO, for 24 h, as indicated was determined; n = 6–13. (E) Human β-cells (EndoC-βH1) were treated with TNF+BV6, zVAD, Nec-1 and/or DMSO, for 24 h, as indicated, and cell death was measured; n = 11–13. (F) Expression of cleaved caspase 8, cleaved caspase 3, pRIPK1, RIPK1and α-tubulin (loading control) was assessed by WB in EndoC-βH1 cells treated as indicated. (G) Expression of pRIPK1, RIPK1 and α-tubulin (loading control) was assessed by WB in EndoC-βH1 cells treated for 1 h, as indicated, quantification is shown on the right panel. (H) Expression of pRIPK1, pRIPK3, pMLKL, RIPK1, RIPK3, MLKL and GAPDH (loading control) were detected by western blot in EndoC-βH1 cells treated for 18 h, as indicated. (B, C, F, G, H) Representative image of WB is shown, n = 3–7. Data is presented as mean ± SEM. ****p < 0.0001; vs Ripk1$^{S25D/S25D}$ or treatments. #p < 0.05, ####p < 0.0001, vs WT or non-treated controls.

our group utilized MLDSTZ in *Ripk1*$^{S25D/S25D}$, which provokes immune-mediated diabetes in mice [16]. Our study showed no alteration in disease presentation in MLDSTZ-*Ripk1*$^{S25D/S25D}$ compared to MLDSTZ-*Ripk1*$^{+/+}$ and RIPK1 enzymatic activation was not detected in islets of hyperglycemic mice subjected to MLDSTZ, demonstrating that RIPK1 kinase had no relevance in the dynamics of immune-mediated diabetes development. This lack of phenotype may reflect other studies which showed that dysfunctional immune responses were related to aberrant RIPK1 (prosurvival) scaffold function, independent of kinase activity [21,22].

RIPK1 and downstream necroptotic signaling proteins such as MLKL have been associated with obesity and related comorbidities [9,23,24]. Overnutrition in zebrafish, in a model of muscle insulin resistance (zMIR), led to β-cell loss that was prevented by Nec-1 and GSK'872 (RIPK3 kinase inhibitor) but not zVAD, suggesting there could be a role for RIPK1 and RIPK3 in β-cell dysfunction and death [24]. They showed that at time of β-cell loss, punctuated RIPK3 expression (a sign of activated RIPK3 formation of amyloid fibers), but not RIPK1, was observed in β-cells and correlated with induction of IL1-β and NF-κB activation. In mice, inhibition of total RIPK1 with synthesized specific inhibitory antisense oligonucleotide (ASO) improved weight gain and insulin resistance in DIO [23]. Furthermore, knockdown of RIPK1 with ASO decreased liver and adipose tissue inflammation linked to recruitment of immune cells such as macrophages [23]. In this latter study, mice with a mutation of the RIPK1 catalytic lysine K45 (*Ripk1*^{K45A}) fed HFD, did not present any differences compared to WT-littermates [23]. The lack of improvement in glucose metabolism of obese *Ripk1*^{K45A} resembles our results in the *Ripk1*$^{S25D/S25D}$ mice. Both mouse models indicate that RIPK1 kinase is not involved in obesity contradicting the study which showed that RIPK1 kinase inhibition with Nec-1 improved insulin resistance in obese ob/ob mice [9]. However, Nec-1 also blocks indoleamine 2,3-dioxygnase (IDO), an immunoregulator that supresses effector immune responses, thus improvement of metabolic dysfunction in Nec-1-treated obese ob/ob mice may not be specific to inhibition of RIPK1 activity [25].

We report, for the first time, that both murine islets and the EndoC-βH1 human β-cell line are resistant to RIPK1-mediated cell death. The lack of cell death was in contrast with the activation of the main components of the necroptotic signaling pathway, namely phosphorylation of RIPK1, RIPK3 and MLKL. Of note, our results showed that the EndoC-βH1 human β-cell line was susceptible to TNF+BV6 mediated apoptosis, in contrast to murine islets, indicating there is a species-specific or cell-specific difference in the TNF signaling pathway [26,27]. Nevertheless, the TNF+BV6-induced cell death in EndoC-βH1 was prevented by zVAD but not Nec-1, indicating that it was independent of RIPK1 kinase-activity and therefore likely resulting from defective TNF-mediated NF-κB-dependent induction of prosurvival molecules such as cIAP1/2 [2–4].

A recent study by Contreras et al., reported that TNF+BV6 stimulation significantly activated caspases and elicited cell death in INS-1E, NIT-1 and in dispersed mouse islets, which was not prevented by zVAD [10]. Their data diverges from our current findings. In the study of Contreras et al., the islets cells were dispersed before treatment, thus it might be possible that the stress of dispersion may sensitize murine β-cells to TNF+BV6-induced cell death. It is known that β-cell function and viability in culture is better preserved within the islets of Langerhans when compared to dispersed cells [28,29]. To better understand the different results obtained between the cell lines, we reproduced some of the experiments of Contreras et al. in INS-1E cells (Supplementary Figure 1). Similar to what was observed, INS-1E cells were susceptible to TNF+BV6+zVAD-induced cell death. The divergence in caspase dependency of TNF+BV6-mediated cell death between INS-1E, NIT-1 cells and EndoC-βH1 cell death may be due to cell-line or species-specific differences [30]. Of note, NIT-1 cells express higher levels of TNFR1 compared to primary β-cells, making them more sensitive to TNF-induced death than primary β-cells [10,27]. In the case of the human β-cell line, EndoC-βH1 cells, previous studies have shown that they resemble human β cells in the expression of specific genetic and epigenetic markers [31]. Moreover, different studies showed that EndoC-βH1 also present similar responses to human islets after cytokine treatment [32,33]. Although, EndoC-βH1 are a well-established human β-cell line, further studies in primary human islets should be conducted to confirm our findings.

Regarding RIPK1, in the Contreras et al. study, total RIPK1 deletion was performed in NIT-1 cells and therefore, the specific role of RIPK1 enzymatic activity, could not be pinpointed as the causal effect in β-cell death [11,27]. Importantly, the lack of total RIPK1 protein prevents the activation of the downstream NF-κB and JNK signaling pathways, both pathways shown to contribute to β-cell death [34–37]. Overall, our *in vitro* and *in vivo* results indicates that RIPK1 activation (pRIPK1) is not involved in β-cell demise in diabetes.

It would be interesting to investigate why β-cells resist RIPK1 cytotoxicity even when strong RIPK1 activation is present. Necroptosis is an important mechanism for viral and bacterial clearance, thus cells with rapid turnover (e.g. macrophages, lymphocytes, epithelial cells and fibroblasts) can be killed by TNF exposure alone, which activates the necrosome complex [8,12,17,18]. Interestingly, necroptosis has also been linked to neuroinflammatory disorders and long-living neuronal cells are sensitive to necroptosis [38]. In contrast to neurons, virus and bacteria can directly encounter and infect β-cells, as gut microbiota translocate to pancreatic draining lymph nodes [39]. Thus, evading RIPK1 kinase-dependent cell death may be important to maintain β-cell mass over time, as β-cell renewal is a rare event.

Our study shows that RIPK1 enzymatic activity is not involved in the glucose-metabolic abnormalities caused by obesity nor affects disease outcome in immune-mediated diabetes. Interestingly, we found that β-cells are resistant to RIPK1-mediated cell death even when the necroptotic signaling pathway is activated.

AUTHOR CONTRIBUTIONS

T.T., P.X., M.F., E.N.G., M.B. and A.K.C. contributed to the study concept and design, analysis and interpretation of the data. T.T., P.X., M.F., E. H.G., E.N.A. contributed to the acquisition of the data. A.K.C., E.N.G., and M.B. contributed reagents/materials/analytical tools. T.T., P.X. and A.K.C. wrote/edited the manuscript. A.K.C. is responsible for its content. All authors revised the article and approved the final version.

DATA AVAILABILITY

Data will be made available on request.

ACKNOWLEDGMENTS

We thank M. Popa for technical assistance. Work in the A.K.C. group was supported by Fonds National de la Recherche Scientifique (FNRS) Excellence of Science grant (EOS 30826052) and research credit (CDR J.0109.220). P.X. is recipient of Fonds David et Alice Van Buuren, in partnership with the Jaumotte-Demoulin and Héger-Masson Foundations (Belgium). E.N.G. is supported by a FNRS-MIS grant (33650793), a European Research Council (ERC) Consolidator grant METAPTPs (GA817940), and a JDRF Career Development Award (CDA-2019-758-A-N). E.N.G. is

a Research Associate of the FNRS, Belgium. Research in the group of M.J.M.B is financially supported by the Vlaams Instituut voor Biotechnologie (VIB), by Ghent University (iBOF ATLANTIS), by grants from the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO) (G035320N, G044518N, EOS G0G6618N, EOS G0I5722N) and from the Flemish Government (Methusalem BOF09/01M00709 and BOF16/MET_V/007 — attributed to P. Vandenabeele).

DECLARATION OF COMPETING INTERESTS

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
Alessandra Kupper Cardozo reports financial support was provided by Fonds National de la Recherche Scientifique. Esteban N. Gurzov reports financial support was provided by Fonds National de la Recherche Scientifique. Esteban N. Gurzov reports financial support was provided by European Research Council. Esteban N. Gurzov reports financial support was provided by JDRF. Mathieu JM Bertrand reports financial support was provided by Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO). Mathieu JM Bertrand reports financial support was provided by Vlaams Instituut voor Biotechnologie (VIB), by Ghent University. Mathieu JM Bertrand reports financial support was provided by Vlaamse Overheid. Peng Xiao reports financial support was provided by Fonds David et Alice Van Buurenin partnership with the Jaumotte-Demoulin and Héger-Masson Foundations.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2023.101681.

REFERENCES

[1] Tai N, Wong FS, Wen L. The role of the innate immune system in destruction of pancreatic beta cells in NOD mice and humans with type I diabetes. J Autoimmun 2016;71:26—34.
[2] Weinlich R, Green DR. The two faces of receptor interacting protein kinase-1. Mol Cell 2014;56(4):469—80.
[3] He S, Wang X. RIP kinases as modulators of inflammation and immunity. Nat Immunol 2018;19(9):912—22.
[4] Vanden Berghe T, Kaiser WJ, Bertrand MJ, Vandenabeele P. Molecular crosstalk between apoptosis, necroptosis, and survival signaling. Mol Cell Oncol 2015;2(4):e975093.
[5] Allagnat F, Cunha D, Moore F, Vanderwinden JM, Eizirik DL, Cardozo AK. Mcl-1 downregulation by pro-inflammatory cytokines and palmitate is an early event contributing to beta-cell apoptosis. Cell Death Differ 2011;18(2):328—37.
[6] Steer SA, Scarim AL, Chambers KT, Corbett JA. Interleukin-1 stimulates beta-cell necrosis and release of the immunological adjuvant HMGB1. PLoS Med 2006;3(2):e17.
[7] LaRocca TJ, Sosunov SA, Shakerley NL, Ten VS, Ratner AJ. Hyperglycemic conditions prime cells for RIP1-dependent necroptosis. J Biol Chem 2016;291(26):13753—61.
[8] McCaig WD, Patel PS, Sosunov SA, Shakerley NL, Smiraglia TA, Craft MM, et al. Hyperglycemia potentiates a shift from apoptosis to RIP1-dependent necroptosis. Cell Death Discov 2018;4:55.
[9] Xu H, Du X, Liu G, Huang S, Du W, Zou S, et al. The pseudokinase MLKL regulates hepatic insulin sensitivity independently of inflammation. Mol Metab 2019;23:14—23.
[10] Contreras CJ, Mukherjee N, Branco RCS, Lin L, Hogan MF, Cai EP, et al. RIPK1 and RIPK3 regulate TNFalpha-induced beta-cell death in concert with caspase activity. Mol Metab 2022;65:101582.
[11] Kim HE, Choi SE, Lee SJ, Lee JH, Lee YJ, Kang SS, et al. Tumour necrosis factor-alpha-induced glucose-stimulated insulin secretion inhibition in INS-1 cells is ascribed to a reduction of the glucose-stimulated Ca^{2+} influx. J Endocrinol 2008;198(3):549—60.
[12] Dondelinger Y, Delanghe T, Priem D, Wynosky-Dolfi MA, Sorobetea D, Rojas-Rivera D, et al. Serine 25 phosphorylation inhibits RIPK1 kinase-dependent cell death in models of infection and inflammation. Nat Commun 2019;10(1):1729.
[13] Xiao P, Takiishi T, Violato NM, Licata G, Dotta F, Sebastiani G, et al. NF-kappaB-inducing kinase (NIK) is activated in pancreatic beta-cells but does not contribute to the development of diabetes. Cell Death Dis 2022;13(5):476.
[14] Ravassard P, Hazhouz Y, Pechberty S, Bricout-Neveu E, Armanet M, Czernichow P, et al. A genetically engineered human pancreatic beta cell line exhibiting glucose-inducible insulin secretion. J Clin Investig 2011;121(9):3589—97.
[15] Nackiewicz D, Dan M, Speck M, Chow SZ, Chen YC, Pospisilik JA, et al. Islet macrophages shift to a reparative state following pancreatic beta-cell death and are a major source of islet insulin-like growth factor-1. iScience 2020;23(1):100775.
[16] Luo Z, Solang C, Mejia-Cordova M, Thorvaldson L, Blix M, Sandler S, et al. Kinetics of immune cell responses in the multiple low-dose streptozotocin mouse model of type 1 diabetes. FASEB Bioadv 2019;1(9):538—49.
[17] Meng H, Liu Z, Li X, Wang H, Jin T, Wu G, et al. Death-domain dimerization-mediated activation of RIPK1 controls necroptosis and RIPK1-dependent apoptosis. Proc Natl Acad Sci U S A 2018;115(9):E2001—9.
[18] Moriwaki K, Bertin J, Gough PJ, Orlowski GM, Chan FK. Differential roles of RIPK1 and RIPK3 in TNF-induced necroptosis and chemotherapeutic agent-induced cell death. Cell Death Dis 2015;6:e1636.
[19] Thomas HE, Biden TJ. Bad news for beta-cell apoptosis. Diabetes 2009;58(8):1725—7.
[20] Zhao Y, Scott NA, Fynch S, Elkerbout L, Wong WW, Mason KD, et al. Autoreactive T cells induce necrosis and not BCL-2-regulated or death receptor-mediated apoptosis or RIPK3-dependent necroptosis of transplanted islets in a mouse model of type 1 diabetes. Diabetologia 2015;58(1):140—8.
[21] Moriwaki K, Park C, Koyama K, Balaji S, Kita K, Yagi R, et al. The scaffold-dependent function of RIPK1 in dendritic cells promotes injury-induced colitis. Mucosal Immunol 2022;15(1):84—95.
[22] Blanchett S, Boal-Carvalho I, Layzell S, Seddon B. NF-kappaB and extrinsic cell death pathways — entwined do-or-die decisions for T cells. Trends Immunol 2021;42(1):76—88.
[23] Karunakaran D, Turner AW, Duchez AC, Soubeyrand S, Rasheed A, Smyth D, et al. RIPK1 gene variants associate with obesity in humans and can be therapeutically silenced to reduce obesity in mice. Nat Metab 2020;2(10):1113—25.
[24] Yang B, Maddison LA, Zaborska KE, Dai C, Yin L, Tang Z, et al. RIPK3-mediated inflammation is a conserved beta cell response to ER stress. Sci Adv 2020;6(51).
[25] Vandenabeele P, Grootjans S, Callewaert N, Takahashi N. Necrostatin-1 blocks both RIPK1 and Ido: consequences for the study of cell death in experimental disease models. Cell Death Differ 2013;20(2):185—7.
[26] Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ 2003;10(1):45—65.
[27] Stephens LA, Thomas HE, Ming L, Grell M, Darwiche R, Volodin L, et al. Tumor necrosis factor-alpha-activated cell death pathways in NIT-1 insulinoma cells and primary pancreatic beta cells. Endocrinology 1999;140(7):3219—27.
[28] Chowdhury A, Dyachok O, Tengholm A, Sandler S, Bergsten P. Functional differences between aggregated and dispersed insulin-producing cells. Diabetologia 2013;56(7):1557—68.
[29] Kelly C, Guo H, McCluskey JT, Flatt PR, McClenaghan NH. Comparison of insulin release from MIN6 pseudoislets and pancreatic islets of Langerhans reveals importance of homotypic cell interactions. Pancreas 2010;39(7):1016—23.
[30] Gibbs RA, Weinstock GM, Metzker ML, Muzny DM, Sodergren EJ, Scherer S, et al. Genome sequence of the Brown Norway rat yields insights into mammalian evolution. Nature 2004;428(6982):493—521.

[31] Lawlor N, Marquez EJ, Orchard P, Narisu N, Shamim MS, Thibodeau A, et al. Multiomic profiling identifies cis-regulatory networks underlying human pancreatic beta cell identity and function. Cell Rep 2019;26(3):788–801 e786.
[32] Tsonkova VG, Sand FW, Wolf XA, Grunnet LG, Kirstine Ringgaard A, Ingvorsen C, et al. The EndoC-betaH1 cell line is a valid model of human beta cells and applicable for screenings to identify novel drug target candidates. Mol Metab 2018;8:144–57.
[33] Gurgul-Convey E, Mehmeti I, Plotz T, Jorns A, Lenzen S. Sensitivity profile of the human EndoC-betaH1 beta cell line to proinflammatory cytokines. Diabetologia 2016;59(10):2125–33.
[34] Patel S, Santani D. Role of NF-kappa B in the pathogenesis of diabetes and its associated complications. Pharmacol Rep 2009;61(4):595–603.
[35] Meyerovich K, Fukaya M, Terra LF, Ortis F, Eizirik DL, Cardozo AK. The non-canonical NF-kappaB pathway is induced by cytokines in pancreatic beta cells and contributes to cell death and proinflammatory responses in vitro. Diabetologia 2016;59(3):512–21.
[36] Brozzi F, Nardelli TR, Lopes M, Millard I, Barthson J, Igoillo-Esteve M, et al. Cytokines induce endoplasmic reticulum stress in human, rat and mouse beta cells via different mechanisms. Diabetologia 2015;58(10):2307–16.
[37] Ortis F, Cardozo AK, Crispim D, Storling J, Mandrup-Poulsen T, Eizirik DL. Cytokine-induced proapoptotic gene expression in insulin-producing cells is related to rapid, sustained, and nonoscillatory nuclear factor-kappaB activation. Mol Endocrinol 2006;20(8):1867–79.
[38] Yuan J, Amin P, Ofengeim D. Necroptosis and RIPK1-mediated neuroinflammation in CNS diseases. Nat Rev Neurosci 2019;20(1):19–33.
[39] Costa FR, Francozo MC, de Oliveira GG, Ignacio A, Castoldi A, Zamboni DS, et al. Gut microbiota translocation to the pancreatic lymph nodes triggers NOD2 activation and contributes to T1D onset. J Exp Med 2016;213(7):1223–39.

