

# Role of the TLR signaling molecule TRIF in $\beta$ -cell function and glucose homeostasis

Meredith J.H. Hutton,<sup>1</sup> Galina Soukhatcheva,<sup>1</sup> James D. Johnson<sup>2,3</sup> and C. Bruce Verchere<sup>1,3,\*</sup>

Departments of <sup>1</sup>Pathology and Laboratory Medicine; <sup>2</sup>Cellular and Physiological Sciences; and <sup>3</sup>Surgery; Faculty of Medicine; University of British Columbia; Child and Family Research Institute; Vancouver, BC Canada

**Key words:** islet, toll-like receptors, type 2 diabetes, insulin secretion

**Abbreviations:** AUC, area under the curve; IPGTT, intra-peritoneal glucose tolerance test; ITT, insulin tolerance test; MyD88, myeloid differentiation primary response gene (88); TLR, toll-like receptor; TRIF, TIR-domain-containing adaptor-inducing interferon- $\beta$

Type 2 diabetes is a metabolic and inflammatory disease characterized by deteriorating islet function and increased levels of inflammatory cytokines. The inflammatory milieu induced in type 2 diabetes exacerbates islet dysfunction and insulin resistance, and therapies that target inflammation can improve glycemic control in patients with type 2 diabetes. Inflammation in type 2 diabetes may be the result of the stimulation of Toll-like receptors (TLRs), one of the many mediators of inflammation.

TLRs can be activated by both exogenous and endogenous ligands, and are responsible for activating NF $\kappa$ B and interferon-inducible inflammatory gene expression. We examined the role of the TIR-domain containing adaptor-inducing interferon- $\beta$  (TRIF or TICAM-1), a major signaling molecule for TLR3 and TLR4, in  $\beta$ -cell function and glucose homeostasis by examining mice lacking TRIF (*Trif*<sup>-/-</sup>), TLR3 (*Tlr3*<sup>-/-</sup>) or TLR4 (*Tlr4*<sup>-/-</sup>).

Male, 10-week old *Trif*<sup>-/-</sup> mice exhibit a moderate but significant increase in fasting blood glucose compared to C57BL/6 controls (12.0  $\pm$  0.9 vs. 9.7  $\pm$  0.4 mM; *p* < 0.05) as well as impaired glucose tolerance revealed by IPGTT (AUC: 2850  $\pm$  236 vs. 2050  $\pm$  108; *p* < 0.005) whereas *Tlr3*<sup>-/-</sup> and *Tlr4*<sup>-/-</sup> mice have normal glucose tolerance. Interestingly, *Trif*<sup>-/-</sup> mice have normal insulin sensitivity yet have increased plasma insulin levels (180  $\pm$  22 vs. 89  $\pm$  24 pM; *p* < 0.05). Islets isolated from *Trif*<sup>-/-</sup> mice have impaired glucose-stimulated insulin secretion, with a diminished first-phase insulin response to glucose. Immunohistological analysis revealed that age-matched *Trif*<sup>-/-</sup> and control mice have normal islet morphology, although *Trif*<sup>-/-</sup> mice have increased  $\beta$ -cell mass (3.5  $\pm$  0.9 vs. 1.7  $\pm$  0.2 mg; *p* < 0.05). In summary, mice lacking TRIF have hyperglycemia associated with  $\beta$ -cell dysfunction that may be partly compensated for by increased  $\beta$ -cell mass. These studies suggest a role for TLR signaling in glucose homeostasis, and raise the possibility that TRIF signaling is required for normal  $\beta$ -cell function.

## Introduction

Type 2 diabetes (T2D) affects millions of people worldwide and is characterized by a progressive loss of  $\beta$ -cell function and mass, impaired glucose tolerance, fasting hyperglycemia and insulin resistance.<sup>1-4</sup> T2D is also an inflammatory disease, characterized by high levels of circulating inflammatory cytokines, such as TNF $\alpha$  and IL-6.<sup>5</sup> Inhibitors of inflammation such as salicylates have been shown to reverse insulin resistance in diet-induced obesity as well as improve glycemic control.<sup>6,7</sup>

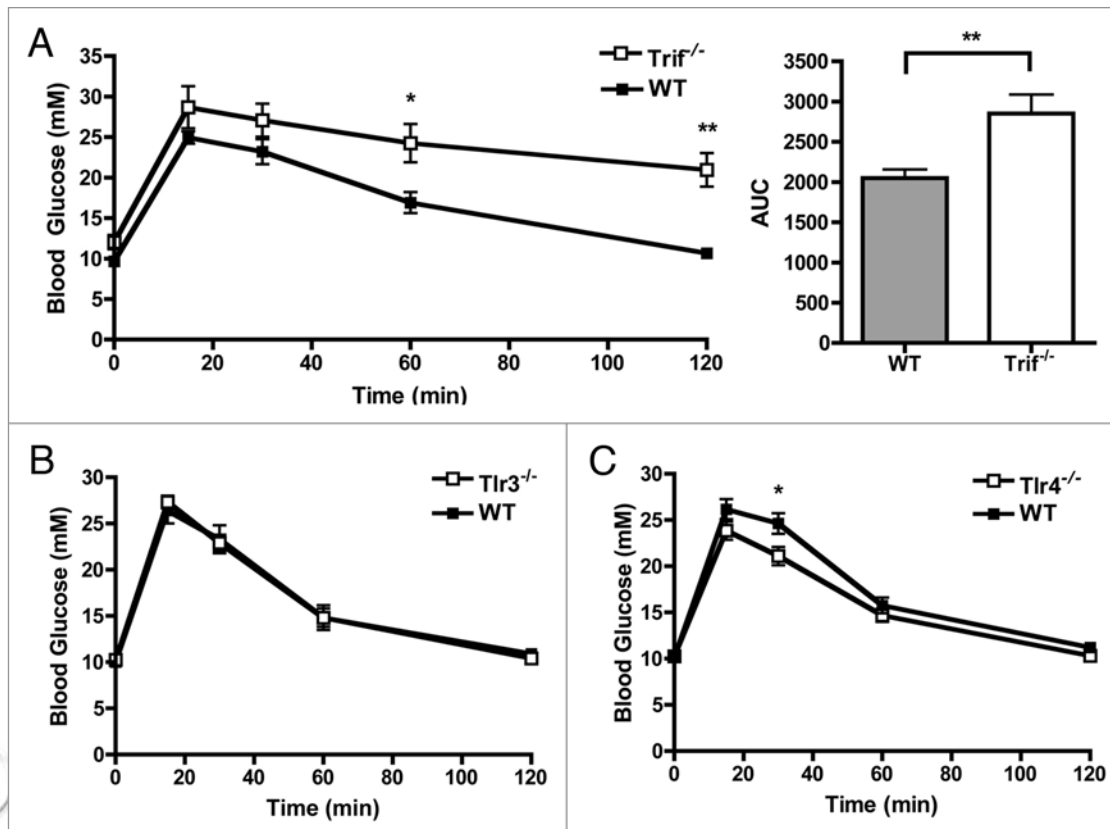
Toll-like receptors (TLRs) have been implicated in mediating chronic inflammatory disease, including obesity and diabetes.<sup>8-10</sup> Toll-like receptors are part of the innate immune system and recognize exogenous pathogen-associated molecular patterns. They also recognize endogenous ligands such as saturated fatty acids and necrotic cell products.<sup>9,11</sup> There are 11 TLRs in mammals, of which

TLRs 1-9 are conserved between humans and mice.<sup>12</sup> They are expressed on a range of cell types including macrophages, dendritic cells, endothelial and epithelial cells and pancreatic islets.<sup>13,14</sup> TLRs signal via two downstream molecules, MyD88 and TRIF; MyD88 is used by all TLRs with the exception of TLR3, which signals solely through TRIF. Upon TLR ligation, downstream signaling molecules upregulate NF $\kappa$ B or interferon (IFN)-inducible inflammatory gene expression, leading to an increase in inflammatory chemokines and cytokines.<sup>12</sup> Glucose and saturated fatty acids can contribute to TLR expression and activation in human monocytes, as cell surface expression of TLR4 is upregulated in a high glucose environment and saturated fatty acids can induce inflammatory cytokine production via TLR4.<sup>9,15</sup> Interestingly, lack of TLR4 protects mice from diet-induced obesity.<sup>9</sup> A recent study examined the role of MyD88 in glucose homeostasis and reported that while mice lacking MyD88 have normal glucose tolerance and fasting

\*Correspondence to: C. Bruce Verchere; Email: verchere@interchange.ubc.ca

Submitted: 07/31/09; Revised: 01/12/10; Accepted: 01/14/10

Previously published online: www.landesbioscience.com/journals/islets/article/11209



**Figure 1.** Mice lacking TRIF, but not TLR3 or TLR4, are glucose intolerant. (A) *Trif*<sup>-/-</sup> (n = 8; WT: n = 10) mice have significantly impaired glucose tolerance compared to wild-type controls, whereas (B) *Tlr3*<sup>-/-</sup> (n = 8; WT: n = 8) and (C) *Tlr4*<sup>-/-</sup> mice (n = 5; WT: n = 8) have normal glucose tolerance. Comparison of AUC values was used to determine significance. *Tlr4*<sup>-/-</sup> mice have slightly improved glucose clearance with significantly lower blood glucose levels 30 minutes post injection, compared to wild-type controls. Male, age-matched mice were fasted for four hours prior to the start of the IPGTT. Mice were injected with 50% dextrose and monitored over 120 minutes. (\*\*p < 0.005, \*p < 0.05).

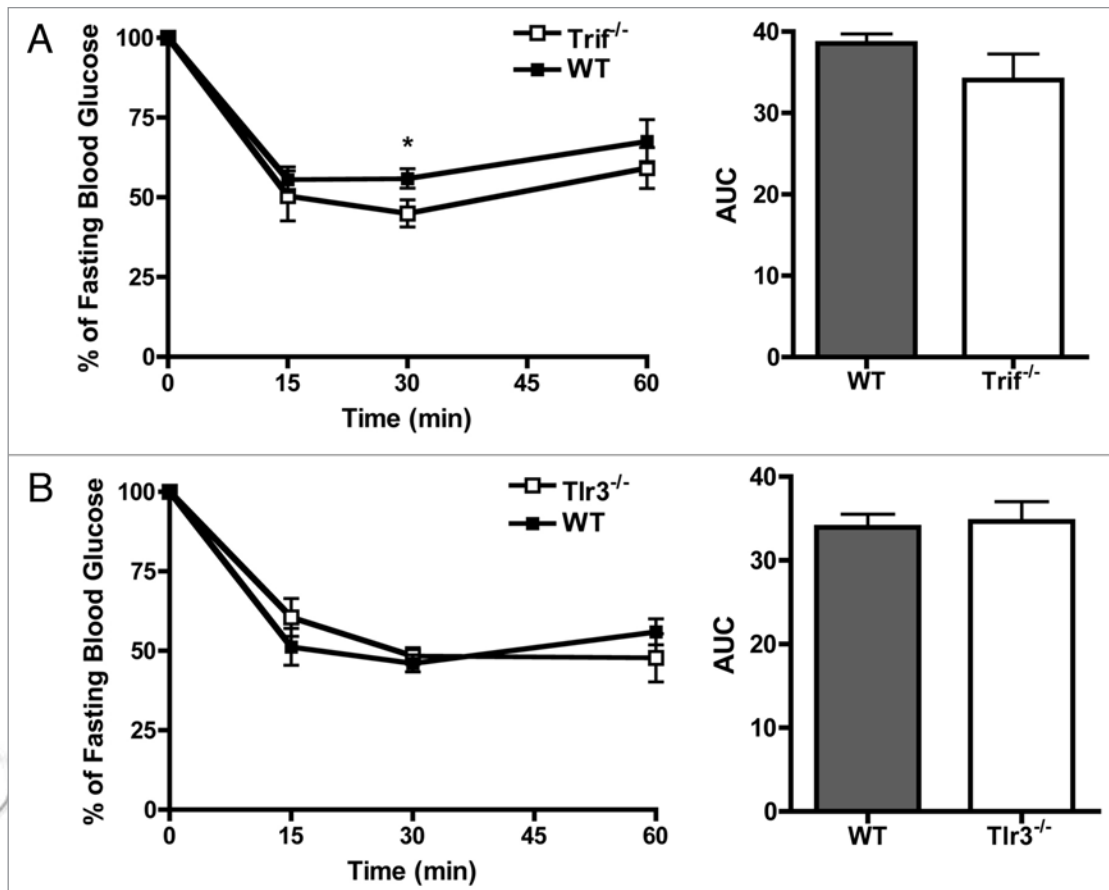
blood glucose levels, they have decreased  $\beta$ -cell mass compared to wild-type controls.<sup>16</sup> These findings provide evidence that TLR signaling may be important in the generation and/or replication of  $\beta$ -cells. Interestingly, this group also reported that when treated with a multiple low-dose streptozotocin (STZ) regimen to induce  $\beta$ -cell death, mice lacking MyD88 developed glucose intolerance. This finding points to a role for TLRs in preventing  $\beta$ -cell inflammation, dysfunction and apoptosis. As MyD88 is also the major signaling molecule for the IL-1 receptor, it remains to be determined whether these findings reflect loss of TLR signaling, or if they are a result of loss of action of other inflammatory receptors.<sup>17</sup> Given that TLRs appear to be important in mediating inflammation in diabetes and obesity, and may play a role in  $\beta$ -cell homeostasis, we sought to understand how TLR signaling via TRIF may impact  $\beta$ -cell function and glucose homeostasis in vivo. To this end, we examined  $\beta$ -cell function and glucose homeostasis in mice lacking TRIF, or either of the two TLRs that utilize TRIF for downstream signaling, TLR3 or TLR4.

## Results

***Trif*<sup>-/-</sup> mice are glucose intolerant.** To determine how lack of TRIF signaling may impact glucose tolerance, we performed

intra-peritoneal glucose tolerance tests (IPGTTs) on mice lacking TRIF, as well as mice lacking TLR3 or TLR4. After a four-hour fast, *Trif*<sup>-/-</sup> animals had significantly increased blood glucose levels compared to wild-type, age-matched controls ( $12.0 \pm 0.9$  vs.  $9.7 \pm 0.4$  mM respectively;  $p < 0.05$ ). In *Trif*<sup>-/-</sup> mice, blood glucose levels remained significantly higher than wild-type mice 60 ( $p < 0.05$ ) and 120 ( $p < 0.005$ ) minutes following glucose administration (Fig. 1A). However, glucose tolerance in both *Tlr3*<sup>-/-</sup> and *Tlr4*<sup>-/-</sup> mice was similar to wild-type controls, and interestingly, *Tlr4*<sup>-/-</sup> mice had significantly lower blood glucose levels 30 minutes after glucose administration, indicating moderately improved glucose clearance (Fig. 1B and C). Therefore, a significant impairment in glucose tolerance was only apparent in mice lacking TRIF.

***Trif*<sup>-/-</sup> and *Tlr3*<sup>-/-</sup> mice are insulin sensitive.** To understand the mechanism underlying the decreased glucose tolerance exhibited by *Trif*<sup>-/-</sup> animals, we assessed insulin sensitivity by insulin tolerance test (ITT). Despite significantly higher fasting blood glucose levels at the start of the test, *Trif*<sup>-/-</sup> mice had similar insulin sensitivity compared to controls, with significantly lower glucose levels 30 minutes after insulin administration. Similarly, *Tlr3*<sup>-/-</sup> (Fig. 2B) and *Tlr4*<sup>-/-</sup> (data not shown) mice were also insulin sensitive. Interestingly, *Trif*<sup>-/-</sup> mice (n = 10) had elevated



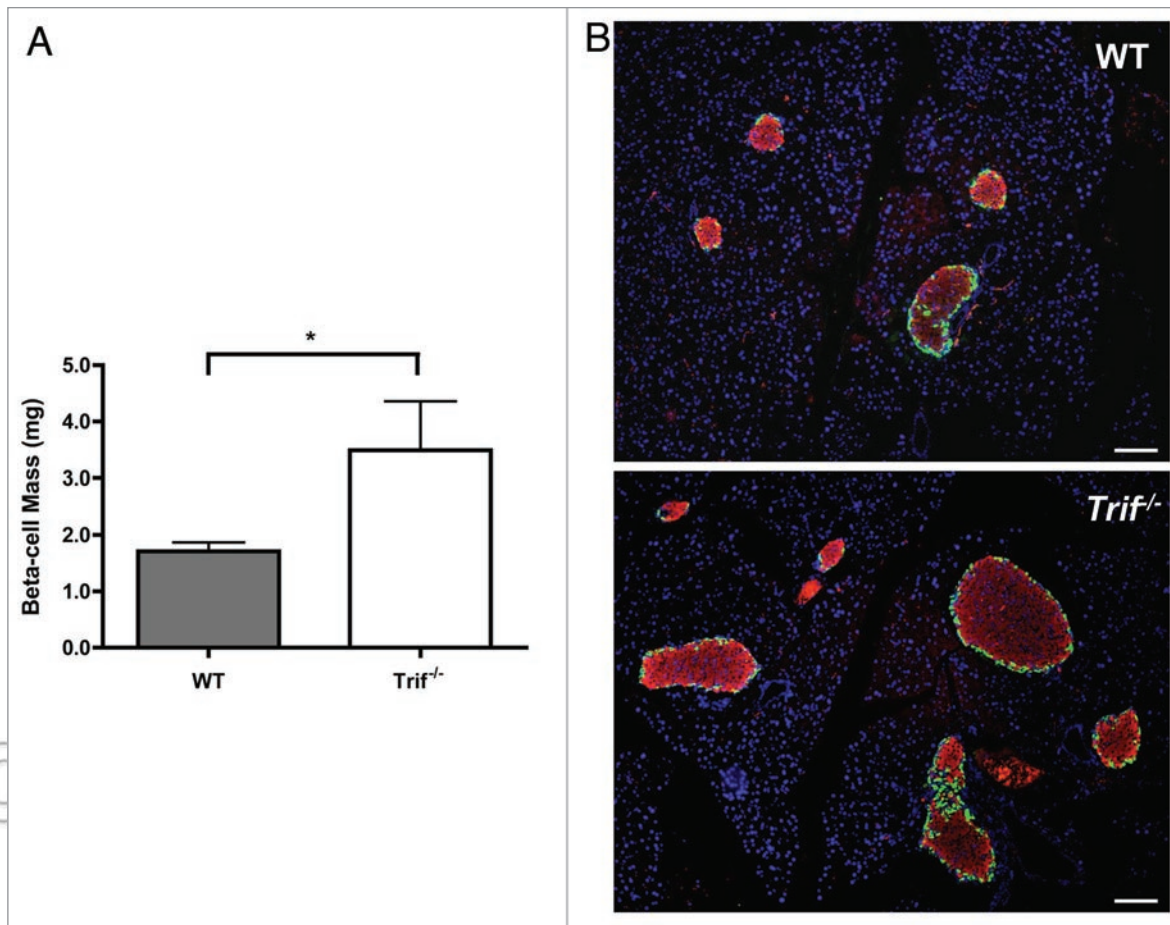
**Figure 2.** Lack of TRIF or TLR3 signaling in mice does not affect insulin sensitivity. (A) Male, *Trif*<sup>-/-</sup> mice (n = 9; WT n = 7) and (B) male, *Tlr3*<sup>-/-</sup> mice (n = 7; WT n = 7) have similar insulin sensitivity to wild-type control mice. *Trif*<sup>-/-</sup> mice have moderately albeit significantly improved blood glucose levels 30 minutes after insulin administration (\*p < 0.05). AUC values were used to determine significance (p = NS). All mice were fasted for four hours prior to the start of the insulin tolerance test and injected with 0.75 units of insulin per kilogram of body weight.

plasma insulin levels compared to wild-type controls (n = 6) ( $180 \pm 22$  vs.  $89 \pm 24$  pM;  $p < 0.05$ ), while *Tlr3*<sup>-/-</sup> mice (n = 5) had similar plasma insulin levels to wild-type controls ( $122 \pm 17$  vs.  $89 \pm 24$  pM;  $p = \text{NS}$ ). These findings suggest that despite high plasma insulin levels, *Trif*<sup>-/-</sup> mice are insulin sensitive and therefore glucose intolerance in *Trif*<sup>-/-</sup> mice is not likely to be due to reduced insulin sensitivity but rather changes in  $\beta$ -cell mass or function.

***Trif*<sup>-/-</sup> mice have increased  $\beta$ -cell mass.** To determine if the observed phenotype in mice lacking TRIF in vivo was associated with changes in  $\beta$ -cell mass and/or a defect in  $\beta$ -cell function, we next quantified  $\beta$ -cell mass in *Trif*<sup>-/-</sup> and wild-type animals (Fig. 3A). Surprisingly,  $\beta$ -cell mass was increased in *Trif*<sup>-/-</sup> animals (n = 6) compared to wild-type (n = 7) ( $3.5 \pm 0.9$  vs.  $1.7 \pm 0.2$  mg;  $p < 0.05$ ). Immunostaining with antibodies to insulin and glucagon showed that *Trif*<sup>-/-</sup> islets have normal islet architecture, with insulin-producing  $\beta$ -cells in the islet core and glucagon-producing alpha-cells in the periphery (Fig. 3B). Islet morphology in *Tlr3*<sup>-/-</sup> and *Tlr4*<sup>-/-</sup> mice also appeared normal (data not shown). These data suggest that defects in  $\beta$ -cell mass cannot explain the impairment in glucose tolerance in mice lacking TRIF and point to a defect in  $\beta$ -cell function.

***Trif*<sup>-/-</sup> islets have impaired first-phase insulin secretion.** We examined glucose-stimulated insulin secretion in static incubations of islets isolated from *Trif*<sup>-/-</sup> (n = 3) and wild-type (n = 3) mice (Fig. 4A). At basal glucose levels (1.67 mM), *Trif*<sup>-/-</sup> islets had significantly lower insulin secretion than wild-type controls. When stimulated with high glucose (16.67 mM), *Trif*<sup>-/-</sup> islets responded with a three-fold increase in insulin secretion, whereas wild-type islets secreted eight-fold more insulin. Insulin content was similar in both groups (data not shown). Thus when normalized to insulin content, glucose-stimulated insulin secretion was significantly higher in wild-type islets compared to *Trif*<sup>-/-</sup> islets. These data suggest that  $\beta$ -cells lacking TRIF have a functional impairment. In contrast, *Tlr3*<sup>-/-</sup> islets have normal function, as they have similar static glucose stimulated insulin secretion compared to wild-type islets (Fig. 4B).

To understand further the defect in glucose-stimulated insulin secretion, we performed perfusion studies on isolated islets to assess dynamics of insulin secretion. Islets from *Trif*<sup>-/-</sup> (n = 6) mice exhibited reduced first-phase insulin response to glucose compared to wild-type (n = 5) mice (Fig. 4C); AUC values for the first-phase insulin response (0 to 20 minutes after glucose stimulation) from wild-type islets were significantly



**Figure 3.** *Trif*<sup>-/-</sup> mice have increased  $\beta$ -cell mass. (A) Male, 10-week old *Trif*<sup>-/-</sup> mice (n = 6) have significantly increased  $\beta$ -cell mass compared to wild-type (n = 7) controls (\*p < 0.05). (B) *Trif*<sup>-/-</sup> islets have similar morphology to wild-type islets revealed by insulin (red) and glucagon (green) immunostaining (scale bar: 100  $\mu$ m).

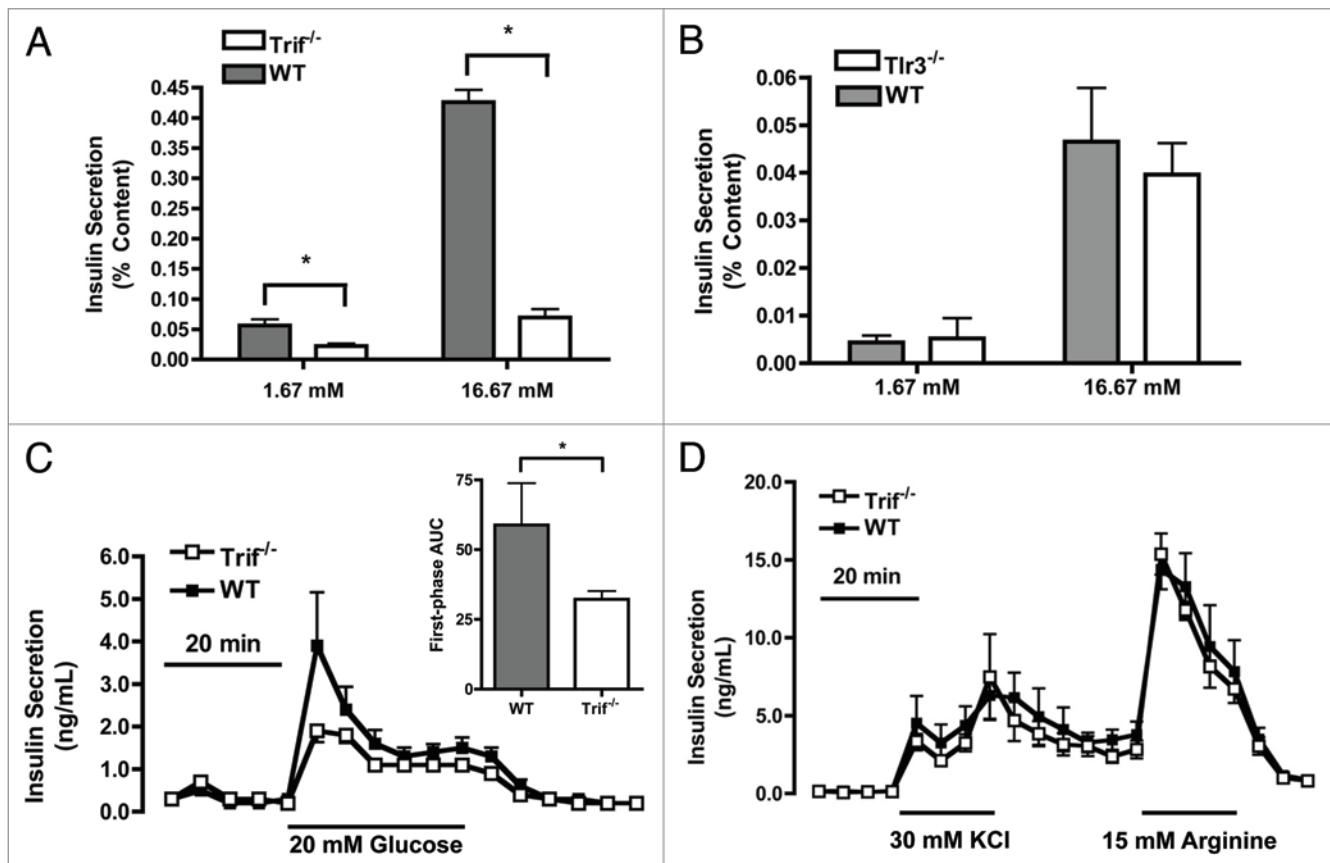
higher compared to *Trif*<sup>-/-</sup> islets (Fig. 4C inset). By contrast, second phase glucose-stimulated insulin secretion remained intact. Insulin secretion in response to the non-glucose  $\beta$ -cell secretagogues KCl and arginine was similar in wild-type and TRIF-deficient islets (Fig. 4D). These findings suggest that the  $\beta$ -cell exocytotic machinery required for insulin secretion is intact, and that the defect in glucose-stimulated insulin secretion in *Trif*<sup>-/-</sup> islets lies upstream of membrane depolarization, insulin granule docking and exocytosis. Together, these data raise the possibility that TRIF plays a previously unexpected role in glucose-sensing in the  $\beta$ -cell.

### Discussion

Type 2 diabetes is a metabolic and inflammatory syndrome, and the inflammatory state of diabetes may in part be mediated by TLR signaling. Inhibition of TLR activation has been previously shown to improve insulin resistance and prevent diet-induced obesity.<sup>9,10,18</sup> In this study, we examined glucose homeostasis and  $\beta$ -cell function in mice lacking the TLR adaptor molecule TRIF, a major signaling molecule for TLR3 and TLR4 and compared their phenotype to wild-type mice and

mice lacking TLR3 or TLR4. Interestingly, we observed that TRIF deficiency induces decreased glucose tolerance and  $\beta$ -cell dysfunction. Contrary to reports that TLR signaling is only detrimental to islet function, our findings suggest that TLR signaling via TRIF is required for normal  $\beta$ -cell function and glucose tolerance. This finding is seemingly counterintuitive as lack of TLR signaling and the subsequent prevention of inflammation might be predicted to have a positive effect on  $\beta$ -cell homeostasis. Although TLR pathways are known to be important sensors of invading pathogens or “danger signals”, our data indicate that an additional role for TLR signaling molecules exists in regulation of  $\beta$ -cell mass and function. Our findings share similarities to those in a recent study by Bollyky et al. who observed that lack of MyD88 increased susceptibility to STZ-induced apoptosis and caused a decrease in  $\beta$ -cell mass.<sup>16</sup> We report here that lack of the TLR signaling molecule TRIF is similarly detrimental to the  $\beta$ -cell. Taken together, these data point to an important role for TLR signaling in the maintenance of  $\beta$ -cell mass and function.

Because we studied mice with global TRIF deficiency and not mice with tissue specific deletion of TRIF, we cannot ascertain the critical tissues in which TLR-signaling deficiency leads



**Figure 4.** *Trif*<sup>-/-</sup> mice have impaired glucose-stimulated insulin secretion. (A) In a static incubation, *Trif*<sup>-/-</sup> islets secreted significantly less insulin compared to wild-type islets in a low and high glucose environment. Islets from *Trif*<sup>-/-</sup> (n = 3) and wild-type (n = 3) donors were incubated at low glucose (1.67 mM) for one hour prior to stimulation with high glucose (16.67 mM). *Trif*<sup>-/-</sup> and wild-type islets had similar insulin content. (B) Islets from *Tlr3*<sup>-/-</sup> (n = 3) and wild-type (n = 3) mice have similar glucose-stimulated insulin secretion in a static incubation. (C) Perfusion studies revealed that islets from *Trif*<sup>-/-</sup> mice (n = 6) have reduced first-phase insulin release but have similar second-phase insulin secretion compared to islets from wild-type mice (n = 5). (C: inset) AUC values comparing first-phase insulin release (0 to 20 minutes after glucose stimulation) show that wild-type islets secreted significantly more insulin than *Trif*<sup>-/-</sup> islets. (D) Insulin secretion in response to KCl and arginine was similar with *Trif*<sup>-/-</sup> and wild-type islets. Basal media contained 3 mM glucose. (\*p < 0.05).

to impaired glucose homeostasis. We observe  $\beta$ -cell dysfunction and glucose intolerance in the absence of insulin resistance; however, further studies will be needed to elucidate the effect of TRIF deficiency on other sites of glucose homeostasis such as the liver and adipose tissue. Static incubation of isolated islets from mice lacking TRIF revealed a marked defect in glucose-stimulated insulin secretion. Subsequent perfusion experiments using other secretagogues, and to examine the dynamics of insulin secretion, confirmed the presence of this defect and moreover showed that it is characterized by a specific loss of first-phase insulin secretion in response to glucose. In this regard, the  $\beta$ -cell defect in mice lacking TRIF resembles that seen in patients with type 2 diabetes, who typically also have impaired first phase glucose-stimulated insulin secretion.<sup>19</sup> The impaired insulin secretion could not be attributed to a loss of  $\beta$ -cell mass. In the absence of TRIF, islet morphology appeared normal and  $\beta$ -cell mass was increased, not decreased, perhaps as partial compensation for the insulin secretory defect.

Unlike TRIF-deficient animals, mice lacking TLR4 or TLR3 had normal glucose tolerance, even though these TLRs signal

via TRIF. Although TLR4 could still signal via MyD88 in the absence of TRIF, TLR3 signaling is thought to be completely TRIF-dependent, and as a result lack of TLR3 would be expected to induce the same effects as lack of TRIF. We found, however, that this is not the case, as lack of TRIF, but not TLR3 or TLR4, induces  $\beta$ -cell dysfunction and glucose intolerance. One possible interpretation of these data is that other TLRs can compensate for the deficiency in TLR3 or TLR4 signaling, and therefore events downstream of TRIF signaling can still occur even in the absence of TLR3 or TLR4. However, as our study and the recent report by Bollyky et al. demonstrate, when TLR signaling molecules are functionally impaired,  $\beta$ -cell function or survival can be affected.<sup>16</sup> Taken together these findings point to the importance of the TLR adaptor molecules TRIF and MyD88 as important regulators of  $\beta$ -cell mass and function.

Multiple molecules downstream of TRIF are known to play established roles in not only inflammatory signaling, but also in replication and cell survival, as well as cell function. TLR signaling has been implicated in regulating cell cycle entry by targeting cell cycle inhibitor p27.<sup>20,21</sup> TRIF interacts with TBK-1

(TANK binding kinase-1), a kinase essential in the activation of IRF-3 (IFN regulatory factor 3) and subsequent production of IFN $\beta$ , and TBK-1 has been reported to regulate p27 expression, an established regulator of  $\beta$ -cell proliferation.<sup>20,22,23</sup> IRF-3 is capable of activating NF $\kappa$ B, which in turn can play a pro- or anti-inflammatory role within the islet.<sup>24,25</sup> In addition, TLR3 activation of TRIF has been shown to require activation of the PI3K/Akt pathway to induce gene expression.<sup>26</sup> Since Akt has been shown to be an important regulator of  $\beta$ -cell function, one possible mechanism is that TRIF deficiency leads to  $\beta$ -cell dysfunction via decreased Akt signaling.<sup>27</sup> Recently, CXCL10 activation of TLR4 was shown to lead to  $\beta$ -cell death by switching Akt signals from proliferation to apoptosis.<sup>28</sup> Since  $\beta$ -cell mass was increased in TRIF-deficient animals, and Akt activation is associated with  $\beta$ -cell proliferation, it seems unlikely that loss of Akt signaling in TRIF-deficient animals could explain the changes in  $\beta$ -cell mass, but it is possible that this pathway underlies loss of glucose-sensing pathways, leading to  $\beta$ -cell dysfunction. Understanding the molecular mechanism underlying the impaired glucose-stimulated insulin secretion and increased  $\beta$ -cell mass in TRIF-deficient mice will require further study.

Despite glucose intolerance, *Trif*<sup>-/-</sup> mice are insulin sensitive. This seems somewhat surprising given that plasma insulin levels in *Trif*<sup>-/-</sup> mice are significantly higher than wild-type controls, and elevated fasting insulin levels are a marker of insulin resistance. It is possible that differences in insulin sensitivity exist but could not be detected due to the limited sensitivity of the insulin tolerance test. Euglycemic clamp studies might reveal differences in insulin sensitivity between genotypes. Interestingly, *Tlr4*<sup>-/-</sup> mice are protected from diet-induced insulin resistance.<sup>9</sup> This mechanism of protection potentially centers around the ability of saturated fatty-acids to activate TLR4 signaling and may not involve TRIF signaling, as TRIF only represents the latent, second phase of TLR4 signaling.<sup>12</sup>

Numerous reports indicate that TLR activation can lead to peripheral insulin resistance as well as  $\beta$ -cell dysfunction and death.<sup>28-32</sup> Our surprising observations indicate that although increased TLR signaling may be harmful to the  $\beta$ -cell during inflammation, signaling through TRIF is essential for maintaining glucose homeostasis under normal conditions. We therefore propose a previously unrecognized role for TRIF in glucose homeostasis and normal  $\beta$ -cell function. Further studies and cell-specific knockout models should help determine the mechanism behind the impaired glucose homeostasis and  $\beta$ -cell dysfunction in TRIF-deficient mice.

## Materials and Methods

**Animals.** C57Bl/6J-*Ticam1*<sup>1-p21</sup>/J (*Trif*<sup>-/-</sup>), B6;129S1-*Tlr3*<sup>tm1Flv</sup>/J (*Tlr3*<sup>-/-</sup>), C57Bl/10ScNJ (*Tlr4*<sup>-/-</sup>) and C57Bl/6J (wild-type; WT) breeder pairs and were purchased from The Jackson Laboratory (Bar Harbor, Maine). *Trif*<sup>-/-</sup>, *Tlr3*<sup>-/-</sup>, *Tlr4*<sup>-/-</sup> and wild-type mice were bred, and male offspring, aged 8–10 weeks were used in the study. *Tlr3*<sup>-/-</sup> mice were backcrossed with C57Bl/6 mice for eight generations prior to use in study and genotyping was performed according to the standard protocol and primers

given by The Jackson Laboratory. All animals were housed and maintained at the Child and Family Research Institute Animal Care Facility in compliance with Canadian Council on Animal Care guidelines.

**In vivo metabolic testing.** Age-matched *Trif*<sup>-/-</sup>, *Tlr3*<sup>-/-</sup>, *Tlr4*<sup>-/-</sup> and wild-type mice were fasted for four hours prior to the start of all metabolic tests [intra-peritoneal (i.p.) glucose and insulin tolerance tests] and measurements for blood glucose and plasma insulin. Basal blood glucose measurements were made using a glucometer (OneTouch, Burnaby, BC). Blood was collected from the saphenous vein in EDTA-coated microvette tubes (Sarstedt Inc., Montreal, QC) and plasma was extracted after centrifugation. Insulin was quantified from plasma using a rat insulin ELISA (Crystal Chem Inc., Downers Grove, IL). To measure glucose tolerance, mice were injected with 50% dextrose (Sigma Aldrich, St. Louis, MO) at a dose of 1.5 g dextrose/g of body weight. Blood glucose levels were measured at 15, 30, 60 and 120 minutes post-injection and a blood sample was taken for mice whose blood glucose rose above the sensitivity of the glucometer (>34 mM). Blood glucose concentration was then confirmed by assay (BioAssay Systems Inc., Hayward, CA). To test insulin tolerance, mice were injected with 0.33 U/mL insulin (Novolin<sup>®</sup> ge Toronto; Novo Nordisk Canada, Mississauga, ON) at a dose of 0.75 U/kg of body weight. Blood glucose levels were measured at 15, 30 and 60 minutes post injection. Blood glucose levels and area-under-the-curve (AUC) values were used to compare groups in both tests.

**Islet isolation and glucose-stimulated insulin secretion.** Pancreata from euthanized islet-donor mice (*Trif*<sup>-/-</sup>, *Tlr3*<sup>-/-</sup> or wild-type) were perfused retrogradely via the pancreatic duct with collagenase (1,000 U/mL type XI; Sigma Aldrich, St. Louis, MO) dissolved in HBSS (Cat. 14185, Gibco, Invitrogen Canada, Burlington, ON), followed by digestion in a 37°C water bath (14 minutes static plus 1 minute of shaking by hand) and purification with a 70  $\mu$ m nylon cell strainer (BD Biosciences, Mississauga, ON). Islets were then hand-picked from the filtrate and contaminating exocrine tissue was removed. Islets were then incubated overnight at 37°C in RPMI 1640 plus 10% fetal bovine serum (Cat. 11875077, Gibco). Islets were plated in triplicate for each condition [low (1.67 mM) and high (16.67 mM) glucose] with 20 islets/well in a 96 well plate. Islets were pre-incubated in Krebs-Ringer bicarbonate (KRB) buffer containing 10 mM HEPES (pH: 7.4), 0.25% BSA and 1.67 mM glucose for two hours at 37°C, followed by stimulation with KRB-1.67 mM or KRB-16.67 mM for one hour at 37°C. Media and islets were collected from each well to measure insulin secretion and content, respectively. Islets were lysed in 150  $\mu$ L 5% acetic acid plus 1% BSA (Sigma) by boiling samples for 10 minutes, followed by centrifugation for 10 minutes at 12,000 rpm at 4°C. Insulin levels in islet extracts and media were determined using a mouse insulin ELISA (ALPCO Diagnostics, Salem, NH). Three animals per genotype were pooled for each experiment; data shown are representative of four experiments with *Trif*<sup>-/-</sup> islets and two experiments with *Tlr3*<sup>-/-</sup> islets.

**Insulin secretion analysis.** The dynamics of insulin secretion were measured from isolated mouse pancreatic islets using

a standard perfusion protocol as described previously.<sup>33</sup> Briefly, islets were isolated from *Trif*<sup>-/-</sup> and wild-type mice as above, and incubated overnight in RPMI 1640 plus 10% FBS (Gibco). Groups of 100 size-matched islets were suspended with Cytodex microcarrier beads (Sigma) in 300  $\mu$ L plastic chambers of an Acusyst-S perfusion apparatus (Endotronics, Minneapolis, MN). Islets were perfused at 37°C and 5% CO<sub>2</sub> at 0.5 mL/min with a KRB buffer containing: 129 mM NaCl, 5 mM NaHCO<sub>3</sub>, 4.8 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM HEPES, 3 mM glucose and 5 g/L radioimmunoassay-grade BSA (Sigma). Prior to sample collection, islets were equilibrated in basal (3 mM glucose) conditions for 1 hour. Samples were frozen prior to analysis of insulin levels by specific radioimmunoassay (Millipore/Linco, Billerica, MA).

**Histology and  $\beta$ -cell mass calculation.** Pancreata from *Trif*<sup>-/-</sup> and wild-type mice were fixed in Z-fix (Anatech Ltd., Battle Creek MI) and processed for histology. Sections (5  $\mu$ m) were deparaffinized in xylene and hydrated through 95% and 70% ethanol to distilled water. Slides were immunostained with insulin, using a polyclonal antibody raised in guinea-pig (1:100, A0564, Dako Canada, Mississauga, ON), and glucagon, using a polyclonal antibody raised in rabbit (1:75, A0565, Dako). Slides were then incubated with Alexa 594 goat anti-guinea-pig and Alexa 488 goat anti-rabbit secondary antibodies (1:200; Molecular Probes, Invitrogen Canada). Sections were visualized using a Leica DM4000B microscope and images were obtained using a Qimaging Retiga 1300i FAST camera and OpenLab 4.0.2 software (Improvision, Waltham, MA).

To calculate  $\beta$ -cell mass, extracted pancreata were weighed prior to fixation. Serial sections were cut from five representative regions throughout the pancreas, separated by 100  $\mu$ m. Following deparaffinization, sections from each region were immunostained with insulin antibody (1:500, Dako) followed by incubation with a biotinylated anti-guinea pig secondary antibody raised in goat (1:200; Jackson Immuno Research, West Grove, PA). Insulin-positive tissue was visualized using liquid DAB (BioGenex, San Ramon, CA), followed by a counterstain with Zymed hematoxylin (Invitrogen Canada). Sections were then visualized using an Aperio ScanScope GL (Aperio Technologies Ltd., Vista, CA) and the insulin-positive ratio (insulin positive pixels/total pixels) was calculated using the Positive Pixel Algorithm with ImageScope software (Aperio Technologies). The insulin positive ratio was then combined with pancreas mass to determine  $\beta$ -cell mass.

**Data and statistical analysis.** Data presented are mean  $\pm$  SEM (standard error of the mean). Statistical significance was determined using the Student's t-test;  $p < 0.05$  was considered statistically significant.

#### Acknowledgements

The authors would like to thank Betty Hu and Mitsuo Komba for technical assistance with perfusion analysis and mouse genotyping, respectively. C.B.V. is a Michael Smith Foundation for Health Research (MSFHR) Senior Scholar. This work was supported by a Canadian Institutes of Health Research Operating Grant (MOP-64427) and the MSFHR.

#### References

1. WHO. Diabetes. World Health Organization, 2008:Diabetes, Fact Sheet Number 312.
2. Canadian Diabetes Association 2008 Clinical Practice Guidelines for the Prevention and Management of Diabetes in Canada. Canadian Journal of Diabetes 2008; 32:1.
3. Donath MY, Storling J, Maedler K, Mandrup-Poulsen T. Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes. J Mol Med 2003; 81:455-70.
4. Kahn SE. Clinical review 135: The importance of beta-cell failure in the development and progression of type 2 diabetes. J Clin Endocrinol Metab 2001; 86:4047-58.
5. Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. J Clin Invest 2006; 116:1793-801.
6. Fleischman A, Shoelson SE, Bernier R, Goldfine AB. Salsalate improves glycemia and inflammatory parameters in obese young adults. Diabetes Care 2008; 31:289-94.
7. Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, Shoelson SE. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science 2001; 293:1673-7.
8. Curtiss LK, Tobias PS. Emerging role of toll-like receptors in atherosclerosis. J Lipid Res 2008.
9. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest 2006; 116:3015-25.
10. Davis JE, Gabler NK, Walker-Daniels J, Spurlock ME. Tlr4 deficiency selectively protects against obesity induced by diets high in saturated fat. Obesity (Silver Spring) 2008; 16:1248-55.
11. Andrade CF, Waddell TK, Keshavjee S, Liu M. Innate immunity and organ transplantation: the potential role of toll-like receptors. Am J Transplant 2005; 5:969-75.
12. Takeda K, Akira S. TLR signaling pathways. Semin Immunol 2004; 16:3-9.
13. Rifkin IR, Leadbetter EA, Busconi L, Viglianti G, Marshak-Rothstein A. Toll-like receptors, endogenous ligands and systemic autoimmune disease. Immunol Rev 2005; 204:27-42.
14. Wen L, Peng J, Li Z, Wong FS. The effect of innate immunity on autoimmune diabetes and the expression of Toll-like receptors on pancreatic islets. J Immunol 2004; 172:3173-80.
15. Dasu MR, Devaraj S, Zhao L, Hwang DH, Jialal I. High glucose induces toll-like receptor expression in human monocytes: mechanism of activation. Diabetes 2008; 57:3090-8.
16. Bollyky PL, Bice JB, Sweet IR, Falk BA, Gebe JA, Clark AE, et al. The toll-like receptor signaling molecule Myd88 contributes to pancreatic beta-cell homeostasis in response to injury. PLoS One 2009; 4:5063.
17. O'Neill LA, Dinarello CA. The IL-1 receptor/toll-like receptor superfamily: crucial receptors for inflammation and host defense. Immunol Today 2000; 21:206-9.
18. Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, et al. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. Diabetes 2007; 56:1986-98.
19. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006; 444:840-6.
20. Hasan UA, Caux C, Perrot I, Doffin AC, Menetrier-Caux C, Trinchieri G, et al. Cell proliferation and survival induced by Toll-like receptors is antagonized by type I IFNs. Proc Natl Acad Sci USA 2007; 104:8047-52.
21. Hasan UA, Trinchieri G, Vlach J. Toll-like receptor signaling stimulates cell cycle entry and progression in fibroblasts. J Biol Chem 2005; 280:20620-7.
22. Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, et al. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 2003; 4:491-6.
23. Georgia S, Bhushan A. p27 Regulates the transition of beta-cells from quiescence to proliferation. Diabetes 2006; 55:2950-6.
24. Kim S, Millet I, Kim HS, Kim JY, Han MS, Lee MK, et al. NFkappaB prevents beta cell death and autoimmune diabetes in NOD mice. Proc Natl Acad Sci USA 2007; 104:1913-8.
25. Ortis F, Piro P, Naamane N, Kreins AY, Rasschaert J, Moore F, et al. Induction of nuclear factor-kappaB and its downstream genes by TNFalpha and IL-1beta has a pro-apoptotic role in pancreatic beta cells. Diabetologia 2008; 51:1213-25.
26. Sarkar SN, Peters KL, Elco CP, Sakamoto S, Pal S, Sen GC. Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. Nat Struct Mol Biol 2004; 11:1060-7.
27. Bernal-Mizrachi E, Fatrai S, Johnson JD, Ohsugi M, Otani K, Han Z, et al. Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced Akt activity in pancreatic islet beta cells. J Clin Invest 2004; 114:928-36.
28. Schulthess FT, Paroni F, Sauter NS, Shu L, Ribaux P, Haataja L, et al. CXCL10 impairs beta cell function and viability in diabetes through TLR4 signaling. Cell Metab 2009; 9:125-39.
29. Donath MY, Ehes JA, Maedler K, Schumann DM, Ellingsgaard H, Eppler E, Reinecke M. Mechanisms of beta-cell death in type 2 diabetes. Diabetes 2005; 54:108-13.
30. Schwarzna A, Hanson MS, Sperger JM, Schram BR, Danobeitia JS, Greenwood KK, et al. IL-1beta receptor blockade protects islets against pro-inflammatory cytokine induced necrosis and apoptosis. J Cell Physiol 2009; 220:341-7.

- 
31. Song MJ, Kim KH, Yoon JM, Kim JB. Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes. *Biochem Biophys Res Commun* 2006; 346:739-45.
  32. Rasschaert J, Ladrerie L, Urbain M, Dogusan Z, Karabua B, Sato S, et al. Toll-like receptor 3 and STAT-1 contribute to double-stranded RNA<sup>s</sup> interferon-gamma-induced apoptosis in primary pancreatic beta-cells. *J Biol Chem* 2005; 280:33984-91.
  33. Dror V, Nguyen V, Walia P, Kalynyak TB, Hill JA, Johnson JD. Notch signalling suppresses apoptosis in adult human and mouse pancreatic islet cells. *Diabetologia* 2007; 50:2504-15.

©2010 Landes Bioscience.  
Do not distribute.