TOLL-LIKE RECEPTOR 3 INFLUENCES GLUCOSE HOMEOSTASIS AND β-CELL INSULIN SECRETION

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ABSTRACT

Toll-like receptors (TLRs) have been implicated in the pathogenesis of type 2 diabetes. We examined the function of TLR3 in glucose metabolism and type 2 diabetes-related phenotypes in animals and humans. TLR3 is highly expressed in the pancreas, suggesting that it can influence metabolism. Using a diet-induced obesity model we show that TLR3-deficient mice had enhanced glycaemic control, facilitated by elevated insulin secretion. Despite having high insulin levels, Tlr3-/- mice did not experience disturbances in whole-body insulin sensitivity, suggesting that they have a robust metabolic system that manages increased insulin secretion. Increase in insulin secretion was associated with up regulation of islet glucose phosphorylation as well as exocytotic protein VAMP-2 in Tlr3-/- islets. TLR3 deficiency also modified the plasma lipid profile, decreasing very low-density lipoprotein levels due to decreased triglyceride biosynthesis. Moreover, meta-analysis of 2 healthy human populations showed that a missense single-nucleotide polymorphism (SNP) in TLR3 (encoding L412F) was linked to elevated insulin levels, consistent with our experimental findings. In conclusion, our results increase the understanding of the function of innate receptors in metabolic disorders and implicate TLR3 as a key control system in metabolic regulation.
INTRODUCTION

The prevalence of obesity and type 2 diabetes is radically rising worldwide, causing serious socioeconomic problems. Type 2 diabetes is a heterogeneous disorder with a complex pathogenesis, characterised by the progressive development of hyperglycaemia, dyslipidaemia and impaired insulin secretion from pancreatic β-cells (1). Experimental and clinical studies have demonstrated that inflammation mediates its pathophysiology, with Toll-like receptors (TLRs) playing critical functions (2).

In addition to the recognition of pathogen-derived structures, TLRs can activate innate immunity by recognition of endogenous molecules, such as lipids, fatty acids (FAs) and other mediators, that are elevated during tissue stress and cell death in chronic inflammatory diseases (3). Certain TLRs have been implicated in glucose metabolism and type 2 diabetes—TLR2- and TLR4-deficient mice are protected against diet-induced obesity and insulin resistance (4-9), and polymorphisms in human TLR4 have been linked to a lower risk of type 2 diabetes (10; 11).

TLRs are expressed predominantly in immune cells, but their expression in non-haematopoietic cells suggests that they have “non-immune” functions. Notably, TLR3, which is activated by viral double-stranded RNA (dsRNA) and messenger RNA (mRNA) from dying cells, is highly expressed in pancreatic β-cells (12; 13). Further, infectious agents, such as double-stranded RNA, that bind to TLR3 accelerate β-cell dysfunction and apoptosis, causing insulitis and autoimmunity (14). Although TLR3 has been examined in type 1 diabetes, its role in autoimmune diabetes remains unclear (15-18). Whether TLR3 has a role in obesity and the development of type 2 diabetes is unknown. In this study, we examined TLR3 function in glucose metabolism and type 2 diabetes-related phenotypes in animals and humans.
RESEARCH DESIGN AND METHODS

Animal studies

All mice were housed and used per the Stockholm North Committee for Experimental Animal Ethics and the Swedish National Board for Laboratory Animals. The hyperglycaemic clamp technique was performed at the National Mouse Metabolic Phenotyping Centre, University of Massachusetts Medical School, with approval by the local Institutional Animal Care and Use Committee (Worcester, MA). Male C57BL/6 (Charles River) and Tlr3<sup>-/-</sup> mice fully backcrossed (N14) onto C57BL/6 (19; 20) were weaned at 4 weeks and fed a high-fat diet (HFD) (34.9% fat, Altromin, Lage, Germany) or normal chow diet from age 5 weeks for 20 weeks unless otherwise stated.

Glucose, and insulin and pyruvate tolerance test

Intraperitoneal glucose tolerance test (ipGTT), intraperitoneal insulin tolerance test (ipITT) and intraperitoneal pyruvate tolerance test (ipPTT) were performed in overnight-starved mice (unless otherwise stated) by injecting glucose (1 g/kg body weight) insulin (0.8 U/kg body weight; Actrapid<sup>®</sup>) or pyruvate (1.5 g/kg body weight), respectively, and monitoring glucose concentrations (Abbott Scandinavia AB, Solna, Sweden) in tail vein blood. HOMA-IR was calculated as fasting glucose (mg/dL) x fasting insulin (mU/L)/405.

Hyperglycaemic clamp and insulin clearance

Following HFD for 26 weeks, a survival surgery was performed at 4–5 days before clamp experiments to establish an indwelling catheter in jugular vein. Mice were fasted overnight prior to the start of the experiment. A hyperglycaemic clamp was conducted in conscious
mice, starting with an infusion of 20% dextrose to quickly reach a target hyperglycaemia (~300 mg/dl glucose level) and maintain hyperglycaemia by adjusting glucose infusion rates. Plasma samples were collected before the start of infusion (baseline) and at indicated time points to measure glucose, insulin and C-peptide levels. Insulin clearance was estimated by the ratio of fasted C-peptide to insulin. At the end of the clamps, mice were sacrificed.

**Tissue processing**

Mice were euthanized with CO$_2$ after overnight-starvation, and blood was collected for plasma analysis. Organs were dissected after vascular perfusion with sterile RNase-free PBS, unless otherwise indicated. Tissues were snap-frozen for RNA and protein analysis or processed for immunohistochemistry.

**Blood and plasma analysis**

Whole blood was analysed by a Scil Vet abc hemocounter. Total plasma TG and cholesterol were measured using a colorimetric kit per the manufacturer (Randox, Crumlin, UK). Insulin (Crystal Chem INC., Illinois, USA), adiponectin (R&D Systems, Minneapolis, USA) leptin (Peprotech, Rocky Hill, USA), and Serum Amyloid A (SAA) (Life Technologies, Cergy Pontoise, France) were measured by ELISA per the manufacturers. Plasma TG and cholesterol lipoprotein profiles were examined as previously (21).

**Lipoprotein biosynthesis**

Mice were fed a HFD for 34 weeks and fasted 8 hours before the experiment. VLDL synthesis was assessed after irreversibly blocking VLDL catabolism using 10% (w/v) tyloxapol (500 mg/kg i.v.; Sigma-Aldrich). Mice were bled from the tail into EDTA-coated
tubes at the indicated times. Plasma TGs were measured as described above, normalized to baseline TGs and expressed as fold increase.

**Insulin signalling**

Insulin signalling in the liver was assessed by Western blot analysis of basal phosphorylated protein kinase B (PKB)/AKT in Tlr3-deficient and control mice. In a separate experiment, 10 minutes before freezing the tissue in liquid nitrogen, mice were injected with insulin (2U/kg body weight; Actrapid®). Number of mice and duration of HFD are indicated in the figure legend.

**Histopathology and immunohistochemistry**

Pancreatic tissue was dissected from the surrounding tissues, and specimens from the corpus/cauda regions were formalin-fixed and paraffin-embedded. Sections (4–10 µm) were stained with hematoxylin-eosin, insulin, glucagon and somatostatin by immunohistochemistry as described (22). Average islet area was calculated per total pancreatic area in 3 sections per sample. The detection of apoptotic cells was performed by TUNEL staining technique using an *in situ* cell death detection kit (Roche, Mannheim, Germany) per the manufacturer.

**Isolation, incubation and perifusion of pancreatic islets**

Islets of Langerhans were isolated from *Tlr3*−/− and control mice by collagenase digestion in HBSS, followed by sedimentation, and then cultured for 20-24 h as described (23). After culture, equal-sized islets were preincubated as in (23) followed by 60-min batch incubation (3 islets per tube in triplicate, 2.8 or 16.7 mmol/L glucose), perifusion, RNA
extraction or western blot. Islets from the batch incubations were treated with acid-ethanol to extract cellular insulin (23).

Secreted insulin and islet insulin content were analysed by RIA using $^{125}\text{I}$-labeled insulin and anti-porcine insulin (Department of Endocrinology, Karolinska Hospital (24)). Perifusion experiments were performed as described (23). Perifusate samples were collected every minute, and secreted insulin was analysed by RIA.

**PolyI:C treatment**

PolyI:C was administered three times per week simultaneously with the HFD in young C57/B6 mice (age 6 weeks). In total, mice received 12 i.p. injections of polyI:C (100 µg/mouse) or NaCl. Chow-fed C57/B6 mice (age 18 weeks) were treated 17 times with polyI:C (100 µg/mouse) or NaCl. Tlr3$^{-/-}$ mice received 11 i.p. injections polyI:C or NaCl during HFD. To assess insulin secretion, C57/B6 mice (age 9 weeks) received in total 17 i.p. injections of polyI:C or NaCl during HFD.

**Real-time PCR**

Total RNA was isolated using the RNaseasy Lipid Mini (Qiagen, Hilden, Germany) or RNaseasy Micro Kit (Qiagen, Hilden, Germany) and reverse-transcribed with Superscript-II (Invitrogen), random hexamers (pdN6), and RNasin (Life Technologies, Cergy Pontoise, France). RNA concentration was measured by spectrophotometry (Thermo Scientific), and RNA quality was assessed on a BioAnalyzer (Agilent Technologies, Waldbronn, Germany). cDNA was amplified on an ABI 7900HT (Applied Biosystems) by real-time PCR using primers and probes (Applied Biosystems, Foster City, CA) for the selected genes. Data were
calculated as \(2^{\Delta\Delta C_T}\), where \(\Delta\Delta C_T = \Delta C_T\) (sample) – \(\Delta C_T\) (calibrator = average \(C_T\) values of all samples in each group) and \(\Delta C_T\) is the target gene \(C_T\) minus the \(C_T\) of \(Hprt\).

**Western blot**

Pooled islets from 2–3 mice per sample were used for western blot as described (25). For insulin signalling analysis, a piece of liver was lysed and homogenized in ice-cold buffer (137mM NaCl, 2.7mM KCl, 1mM MgCl\(_2\), 1% Triton X-100, 10% Glycerol, 20mM Tris pH 7.8, 10mM NaF, 1mM EDTA, 5mM sodium pyrophosphate, 0.5 mM Na3VO4, 1µg/ml Leupeptin, 0.2 mM phenylmethyl sulfonyl fluoride, 1µg/ml Aprotinin, 1 µM microcystin). Homogenates rotated 30 minutes at 4°C before centrifugation (12,000 g for 15min at 4 °C). Primary antibodies were used at the following dilutions mouse anti-VAMP-2 1:10,000 (SYSY, Germany), rabbit anti-glucokinase (Gck) 1: 500 (Santa Cruz Biotechnology), mouse anti-beta-actin 1: 20,000 (Sigma Aldrich, USA), rabbit anti-caspase-3 and rabbit anti-cleaved caspase-3 (both 1:1000, Cell Signaling Technology, USA). After incubation with HRP-conjugated anti-mouse or -rabbit, bands were visualised by chemiluminescence (ECL, Pierce, USA).

**Human studies**

Male participants from the POLCA (n=625) and OLIVIA (n=306) cohorts were included. POLCA comprises healthy 50-year-old individuals who were free of coronary heart disease and recruited at random using a population registry (26), and OLIVIA includes healthy individuals aged 33–80 years, recruited for the PROCARDIS study (27). Subjects with type 2 diabetes (defined as diagnosis, use of anti-diabetic medication or fasting glucose \(\geq 7\) mmol/L) were excluded. All participants were genotyped using the Illumina Infinium 1M or 610K platforms at the SNP Technology Platform, Uppsala University, Sweden or Centre
National de Genotypage, Paris, France (see Supplementary Table 1 for the clinical characteristics and genotyping methods). SNPper (28) was used to identify SNPs in the TLR3, of which 24 were available in the POLCA and OLIVIA datasets. As only rs3775291 was the only SNP in the coding region, we focused on this. The association between rs3775291 and metabolic phenotypes was examined.

**Statistical analysis**

Results from the animal studies were expressed as mean ± SEM. Mann-Whitney U test was used for group comparisons, paired t-test of log-transformed values for pairwise observations. Associations between rs37795291 and metabolic phenotypes in the human cohorts were analysed by linear regression. Skewed variables were natural log-transformed before analysis. An additive genetic model was assumed, and adjustments were made for age and BMI. Fixed effects inverse variance meta-analyses were performed using METAL (29). P value <0.05 indicated a significant association.
RESULTS

*TLR3*−/− mice have enhanced glucose tolerance and increased circulating insulin upon glucose stimulation

After being fed a HFD or chow diet for 20 weeks, *TLR3*−/− and control mice experienced increased body weight, which did not differ between mice with either diet (Figure 1A). To examine the effects of TLR3 deletion on glucose homeostasis, we performed ipGTT. TLR3-deficient mice had better responses to glucose versus control mice, irrespective of diet (Figure 1B). Baseline glucose did not differ between control and *TLR3*−/− mice on chow or HFD (Figure 1C).

*TLR3*−/− mice on HFD had elevated circulating insulin under fasting conditions versus control mice (Figure 1D and E), which rose further after glucose challenge (Figure 1D). Similarly, incremental insulin was increased in HFD-fed *TLR3*−/− mice compared with control mice (Figure 1F). Insulin responses did not differ between chow-fed *TLR3*−/− and control mice (Figure 1D and E). HOMA-IR increased in HFD-fed *TLR3*−/− mice versus control mice (Figure 1G). Circulating levels of adiponectin and leptin (Supplemental Figures 1A and B, respectively) were similar between groups.

To determine the effects of TLR3 deficiency on glucose metabolism, the hyperglycaemic clamp technique was performed in HFD-fed mice (Figure 1H-J). Body weight was similar in HFD-fed control and *TLR3*−/− mice, and plasma glucose rose quickly and plateaued at ~300 mg/dl during infusion in both genotypes (Figure 1H). Glucose infusion rates were significantly higher in HFD-fed *TLR3*−/− versus control mice (Figure 1I). Insulin secretion in *TLR3*−/− mice climbed, as evidenced by elevated plasma insulin compared with control mice (Figure 1J). Further, plasma C-peptide levels increased slightly during clamping in *TLR3*−/− mice (Figure 1K), reflecting greater insulin production. The C-peptide to insulin
ration did not differ between the genotypes, indicating that insulin clearance was similar between the groups (Figure 1L).

*Tlr3*<sup>−/−</sup> and control mice have similar responses to insulin during ipITT and display no impairment of hepatic insulin sensitivity

By ipITT, insulin responses did not differ between *Tlr3*<sup>−/−</sup> and control mice with either chow (Figure 2A) or HFD (Figure 2B) for 20 weeks. To rule out the influence of starvation, ipITT was repeated in freely HFD-fed mice, yielding the same result (Figure 2C). Aged mice that were on a HFD for 12 months still responded better to glucose challenge (Figure 2D) and had increased insulin levels upon glucose stimulation (Figure 2E) without becoming insulin-resistant (Figure 2F). Despite the rise in the HOMA-IR index, the insulin responses did not differ between *Tlr3*<sup>−/−</sup> and control mice by *in vivo* ipITT, indicating that the increased insulin in *Tlr3*<sup>−/−</sup> mice did not affect whole body insulin resistance, irrespective of age or metabolic conditions. Liver specific insulin sensitivity was assessed by analysing insulin signalling (Figure 2G and H) and gluconeogenesis (Figure 2I and J). Basal phosphorylated AKT was reduced in Tlr3-deficient mice compared to control mice fed a HFD (Figure 2G). Insulin stimulated AKT activation was similar between the genotypes (Figure 2H). Reduced PEPCK mRNA in Tlr3-deficient mice (Figure 2I) was accompanied by a trend towards reduced glucose output from the liver compared to control mice (Figure 2J) demonstrating the ability of insulin to suppress glucose production in Tlr3-deficient mice.

**Systemic inflammation was increased in *Tlr3*<sup>−/−</sup> mice, but not locally in the pancreas**

Systemic inflammation was assessed by analysing peripheral blood cells and levels of SAA protein. Whereas total blood lymphocytes, granulocyte and monocyte number did not differ between the genotypes (Figure 3A), Tlr3-deficient mice fed a HFD displayed increased levels
of serum SAA (Figure 3B) indicating augmented systemic inflammation despite the lack of Tlr3.

Histopathological analysis of the pancreas showed no difference in the basic structure of the Langerhans cells between control and Tlr3⁻/⁻ mice (Figure 5C). Thus, size, number and shape of islets were in the normal range. No bleeding, necrosis or infiltration of granulocytes could not be detected.

**Tlr3⁻/⁻ mice have reduced circulating VLDL**

Dyslipidaemia is a common comorbidity in patients with obesity and type 2 diabetes, characterised by increased FA influx into the liver and higher plasma TG. Despite no difference in weight between control and Tlr3⁻/⁻ mice, the latter had decreased circulating TG (Figure 4A). This was attributed to reduced TG concentrations in the VLDL fraction (Figure 4B). Similarly, total cholesterol was lower overall and in the VLDL fraction in Tlr3⁻/⁻ mice (Figures 4C and D). Moreover, Tlr3⁻/⁻ mice had decreased circulating FFAs, the products of VLDL-TG hydrolysis, versus control mice (Figure 4E). No differences on LDL or HDL TG and cholesterol were observed (Figures 4B and D).

In examining the factors influencing lipoprotein levels, we found that mRNA expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) the rate-limiting enzyme in cholesterol biosynthesis, was significantly reduced in the liver of Tlr3⁻/⁻ mice (Figure 4F). Further, sortilin-1 that has been implicated in the regulation of VLDL catabolism rose in the liver of Tlr3⁻/⁻ mice (Supplemental Figure 2A). By inhibiting lipoprotein lipase-dependent (LPL-dependent) TG hydrolysis with tyloxapol, we show that Tlr3⁻/⁻ mice had significantly lower TG synthesis (Figure 4G). Interestingly, circulating TG raised significantly upon TLR3 stimulation with the TLR3 ligand polyI:C (Supplemental Figure 2B).
Increased insulin secretion in pancreatic islets from TLR3-deficient mice

After glucose challenge, islets of Langerhans from *Tlr3<sup>−/−</sup>* mice secreted significantly more insulin than control mice (Figure 5A), but islet insulin content was unchanged (Figure 5B). Pancreatic islet structure was similar between TLR3-deficient and control mice, wherein insulin-producing cells were central and glucagon- and somatostatin-expressing cells lay in the periphery (Figure 5C). Further, by islet morphometry, the islet area was similar between groups (Figure 5D).

Because TLR3 stimulation with synthetic dsRNA might increases cell death, we performed the TUNEL assay (Figure 5E) and analysed cleaved caspase-3 protein (Figure 5F) but could not detect increased islet apoptosis.

Glucose is the principal signal for insulin release from β-cells. To determine the mechanism of increased insulin secretion in *Tlr3<sup>−/−</sup>* mice, we measured Gck protein expression, the rate-limiting component of glucose metabolism in β-cells that phosphorylates glucose to glucose-6-phosphate (Figure 6A). Islets in *Tlr3<sup>−/−</sup>* mice expressed more Gck compared with control mice on a HFD, indicating improved glucose metabolism in the islets, which could affect greater insulin secretion. Furthermore, we measured mRNA levels of glucose transporters (GLUTs) in pancreatic islets and peripheral tissues (Figure 6B and Supplemental Figures 3A-C). GLUT2 did not differ significantly between *Tlr3<sup>−/−</sup>* and control mice in the islets or liver, and GLUT4 expression was similar in adipose tissue and muscle.

**TLR3 deficiency increases insulin secretion on glucose and K<sup>+</sup> stimulation, which is reversed by TLR3 stimulation**

Next, we examined glucose-stimulated insulin secretion from islets *in vitro* by perifusion of islets from *Tlr3<sup>−/−</sup>* and control mice. Stimulation with high glucose (16.7 mM)
induced biphasic insulin secretion from chow-fed, under physiological conditions, (Figure 7A) and HFD-fed (Figure 7E) control and TLR3-deficient mice. The enhancement in \( Tlr3^{-/-} \) mice involved the first and second phases of insulin secretion in response to high glucose in chow-fed (Figure 7A-C) and HFD-fed mice (Figure 7E-G). In addition, depolarisation with \( K^+ \) increased insulin secretion in \( Tlr3^{-/-} \) islets versus control mice (Figure 7A and D and 7E and H), indicating that the rise in insulin was not restricted to stimulation by glucose.

The fusion of insulin-containing vesicles with the \( \beta \)-cell membrane and subsequent release of insulin constitute the last crucial step of glucose-stimulated insulin secretion. By western blot, the exocytotic protein VAMP-2 was upregulated in the pancreatic islets of \( Tlr3^{-/-} \) mice (Figure 7I), which we speculate facilitates enhanced insulin secretion in \( Tlr3^{-/-} \) islets.

To examine TLR3 function in our model, we treated C57BL/6 chow (Figure 7J and K)- and HFD-fed mice (Figure 7L-N) with the TLR3 ligand polyI:C and analysed its effects on glucose control and VAMP-2 expression. PolyI:C reversed the glucose response in C57BL/6 mice under physiological conditions (Figure 7J) and on a HFD (Figure 7L). This impairment was accompanied by down regulation of VAMP-2 in pancreatic islets (Figure 7K and M) and a trend towards reduced insulin secretion in polyI:C treated C57BL/6 mice fed a HFD (Figure 7N). Treatment of Tlr3-deficient mice with polyI:C had no effect on glucose response (Supplemental figure 4A), baseline glucose (Supplemental figure 4B) or body weight (Supplemental figure 4C).

Next, we studied the function of lipotoxicity in TLR3-deficient mice, determining whether the improved \( \beta \)-cell function in \( Tlr3^{-/-} \) mice was due to enhanced insulin sensitivity and plasma lipids by reducing \( \beta \)-cell lipotoxicity. Upon stimulation of islets \( in vitro \) with palmitate, glucose-induced insulin secretion increased in \( Tlr3^{-/-} \) versus control mice (data not shown). Previous treatment with palmitate inhibited insulin secretion and decreased islet
insulin content (Supplemental Figures 5A–C) in the control and knockout mice. Insulin secretion was inhibited by 60% in Tlr3−/− animals versus 26% (as percent of no previous exposure to palmitate) in the control (Supplemental Figures 5A and B). Islet insulin content in the knockout fell to a similar extent as in the control on culture in palmitate (66% vs 62%), likely due to inhibition of pro-insulin biosynthesis.

**A TLR3 polymorphism is associated with metabolic risk factors in humans**

To determine whether TLR3 is linked to metabolic phenotypes in humans, we investigated the effect of genetic variants in TLR3 on insulin-related traits. We searched for all SNPs in TLR3 that could be identified by SNPper (28) and had been previously genotyped in the population studies (Figure 8). We found 24 SNPs (Figure 8), however only one, rs3775291, was found within the coding region. rs3775291 is predicted to damage TLR3 function (30) and has been studied primarily with regard to TLR3 function in viral infection (31-33). We measured the effects of rs3775291 on insulin-related traits.

A meta-analysis of rs3775291 in 2 healthy Swedish cohorts was performed and its minor allele (T) was associated with elevated fasting insulin levels (β=0.048, se=0.023, p=0.0340) but not blood glucose levels (β= -0.005, se=0.005, p=0.3365), consistent with our findings in Tlr3−/− mice.

**DISCUSSION**

Although TLRs have been implicated in type 2 diabetes, there is little evidence of their involvement, other than TLR2 and TLR4. In this study, we examined the function of TLR3 in the progression of diet-induced obesity in a mouse model and its effects on insulin and type 2 diabetes-related phenotypes in humans.
Our data show that the absence of functional TLR3 protects against metabolic disturbances due to fat intake. TLR3-deficient mice have enhanced glycaemic control on glucose challenge, which might be attributed to increased circulating insulin, caused by amplified insulin secretion from β-cells in Tlr3<sup>−/−</sup> mice, accelerating the response to glucose. Insulin content in β-cells and islet architecture and area were similar between Tlr3<sup>−/−</sup> and control mice.

Enhanced insulin secretion in Tlr3<sup>−/−</sup> mice was registered during the first and second phases of insulin secretion in response to high glucose by perifusion—a phenomenon that was glucose-dependent and induced by depolarisation with high K<sup>+</sup>.

VAMP-2 is a member of the v-SNARE complex of the exocytic apparatus and mediates insulin secretion from β-cells (34). Gck (proximal event) and VAMP-2 (distal event) were up regulated in the pancreatic islets of Tlr3<sup>−/−</sup> mice. Notably, VAMP-2 is down regulated in islets from diabetics with attenuated glucose responses (35), contrary to our murine model in which increased VAMP-2 protein expression parallels the ameliorated response to glucose. Further, stimulation of TLR3 signalling by polyI:C impairs this response and decreases VAMP-2 expression accompanied by decreased insulin secretion in mice on a HFD and under physiological, chow-fed, conditions. However, polyI:C treatment did not affect glucose response in Tlr3-deficient mice suggesting that TLR3 modulates the processes that effect increased insulin secretion in islets.

Our perifusion data demonstrate that insulin release is enhanced in the Tlr3<sup>−/−</sup> mice not only in response to elevated glucose but also as a response to elevated potassium levels. This kind of potassium increase functions as an artificial secretagogue that by-passes the regulation of secretion through K<sup>+</sup>-ATP channels, which is the normal mechanism for control of glucose signalling. Since TLR3 deficiency enhanced insulin secretion in response also to
potassium, TLR3 modulation of insulin secretion may operate on distal steps in the pathway, which are not directly dependent on glucose. However, a glucose-specific effect is not excluded, since glucose is known to influence insulin secretion also at steps distal to that of K+-ATP channels (36).

Dysfunctional lipid metabolism causes insulin resistance, reduced insulin secretion (lipotoxicity) and, in rare cases, enhanced insulin secretion, accompanied by hypoglycaemia (37), primed us to analyse the impact of Tlr3−/− deficiency on islet lipid metabolism. We tested the effects of palmitate on insulin secretion to determine whether β-cells in Tlr3−/− mice are resistant to lipotoxicity, thereby mitigating the damage of a HFD. Our results did not support this hypothesis—the inhibitory effects of palmitate were more extensive in Tlr3−/− mice versus control islets.

Tlr3−/− mice on a HFD had elevated basal fasting insulin levels and HOMA-IR values, which could indicate greater insulin resistance. However, the increased insulin response to intraperitoneally administered glucose was accompanied by better glucose tolerance versus controls—a typical primary effect on insulin secretion, not a secondary effect in response to insulin resistance. The primary effect of Tlr3-deficiency on insulin secretion was confirmed by hyperglycaemic clamp technique, which demonstrated enhanced insulin secretion at a fixed level of glycaemia—a conclusion that was supported by the islet experiments. Notably, the in vitro islet perifusion experiments were performed after identical culture times for control and Tlr3−/− islets, minimizing residual secondary effects of the in vivo environment. Collectively the ipITT results indicate that the Tlr3−/− mice do not become whole body insulin resistant. The only indication was the HOMA-IR value, a crude measure of resistance of which the validity has been debated (38). To rule out tissue specific insulin resistance, we assessed hepatic insulin sensitivity by analysing insulin signalling and gluconeogenesis.
Insulin stimulated AKT activation was similar between the genotypes, indicating that these effects are unlikely to be caused by impaired hepatic insulin sensitivity. Furthermore, we observed a trend towards reduced glucose output from the liver of Tlr3-deficient mice. This is most likely facilitated by the increased insulin, leading to suppression of PEPCK mRNA expression in the liver of Tlr3-deficient mice.

A further dissection of tissue specific insulin resistance, e.g. in muscle and adipose tissue, might be advisable in future studies to rule out a local effect of insulin in other peripheral tissues. Nevertheless, the systemic effects of increased insulin under several condition (physiological-chow, HFD, freely HFD-fed mice and aged HFD-fed mice), and the results obtained from the hyperglycaemic clamp in addition to our data on hepatic insulin sensitivity and gluconeogenesis make it unlikely that the phenotype of Tlr3<sup>-/-</sup> mice is caused by insulin resistance. Instead the data suggest that Tlr3<sup>-/-</sup> mice have a robust metabolic system with adaptive measures that enables them to cope with the increased insulin secretion over a prolonged time.

Lower circulating TG, cholesterol and FFA concentrations indicated that lipid metabolism in Tlr3<sup>-/-</sup> mice were affected. The decline in TG content occurred in the VLDL fraction and was due to reduced VLDL secretion, as revealed by inhibiting LPL-dependent lipoprotein catabolism. In line with this, polyI:C decreased TG levels. Tlr3<sup>-/-</sup> mice had higher mRNA levels for sortilin-1, which regulates VLDL secretion; this may account for at least some of the effects on VLDL (39; 40).

Consistent with this model, increased plasma insulin concentrations inhibit hepatic VLDL-TG production (39), and insulin-resistant obese patients experience greater basal VLDL secretion (41). The lower circulating TG levels in Tlr3<sup>-/-</sup> mice were accompanied by
decreased FFAs, which are associated with reduced insulin sensitivity and insulin-stimulated glucose uptake (42). In addition, prolonged elevation of FFAs impairs β-cell secretion.

TLR3 stimulation with a viral mimic accelerates the development of type 1 diabetes in rats (43; 44) and induces β-cell apoptosis through TLR3 (14) and Fas-Associated protein with Death Domain (FADD) recruitment, leading to activation of caspase-8. A dose-dependent effect governs the outcome of TLR3 stimulation—higher doses of exogenous TLR3 ligand induce insulitis and diabetes, and lower doses prevent autoimmune diabetes (15; 17; 45). Thus, TLR3 likely mediates viral recognition and protects against virally induced type 1 diabetes. The endogenous TLR3 ligand that mediates the effects in our study, in the absence of viral infection or exogenous stimulation, is unknown. TLR3 is activated by mRNA from damaged cells, but we could not detect islet cell death. Inflammation has been suggested by various studies to be a driving force in development of metabolic disturbances. The surprising finding that systemic inflammation was increased in the Tlr3-deficient mice is in line with findings from a study on atherosclerosis where infiltration of macrophages in atherosclerotic lesions of Tlr3−/− mice was observed (20). However, we could not detect any signs of local inflammation in the islets.

Notably, pancreatic tissue contains and secretes copious RNases; thus, a control system that involves TLR3, an innate RNA receptor, to detect and respond to abnormalities in this system is logical. Diabetics have increased concentrations of circulating nucleic acids, likely due to inhibition of plasma RNase activity (46). Circulating nucleases might prevent ligand-induced stimulation of inflammatory responses; thus, we speculate that this impairment in diabetics generates endogenous ligands of TLR3.
Our finding that TLR3 mediates glucose tolerance is supported by Wu et al (47) who demonstrated that TLR3 deficiency in mice improves glucose control. In our study, this improvement was mediated by elevated insulin secretion from \( Tlr3^{-/-} \) islets, at least partly due to increased Gck and VAMP-2 expression in pancreatic \( \beta \)-cells.

Last, we examined whether a well-characterised polymorphism in human \( TLR3 \) (rs3775291) was associated with glucometabolic alterations, supporting our findings in mice. In a meta-analysis of 2 healthy male populations, rs3775291 was associated with increased fasting insulin levels, but not fasting glucose. Rs3775291 is a non-synonymous SNP in the coding region of \( TLR3 \) that is predicted to impair protein function (30). How rs3775291 affects TLR3 function is unknown, but a recent report indicates that it reduces TLR3 ligand binding and impairs TLR3-mediated cell activation (48). Our results show that its relationship with fasting insulin levels is consistent with the predicted impairment in human TLR3 and the \( Tlr3^{-/-} \) mouse model findings. Unfortunately, the human studies do not allow assessment of insulin kinetics, thus we are not able to determine whether the increased insulin levels were due to increased production or reduced clearance.

Although TLRs have been shown to be involved in diabetes (17; 49), the role of innate immunity in glucose homeostasis and insulin secretion is largely unknown. Interestingly, Krus et al (50) demonstrate that the complement regulatory protein, CD59, modulates exocytosis machinery by directly interacting with syntaxin-1 and VAMP-2, thereby regulating insulin secretion. Interestingly, Tlr3-deficient mice displayed increased Gck and VAMP-2 protein expression. It remains to be determined whether TLR3 directly interacts with exocytosis proteins or if it is modulates glucose sensing via Gck. Tlr3 may also act distally, in other metabolic tissues and initiate processes that lead to changes in the islets. This notion is supported by the observation that Tlr3 increases hepatic production of VLDL. It is also in line
with published data indicating a role for Tlr3 in liver regeneration and hepatocyte proliferation (47; 51).

In summary, TLR3 deficiency affects glucose homeostasis, insulin secretion and lipid metabolism, likely by modulating proximal (improved glycolysis via Gck) and distal events (increased VAMP-2 expression). Further, the rs3775291 polymorphism in TLR3 influences glucose homeostasis in humans.

AUTHOR CONTRIBUTIONS

D.S and A.M.L wrote manuscript, performed experiments and analysed data; Z.M, T.W, D.F.J.K, D.E and S.F, performed experiments and analysed data, R.J.S and A.B analysed data, A.H, R.C, G.K.H and A.B contributed to discussion and critically edited the manuscript. All authors have reviewed and approved the manuscript. We declare no conflict of interest. D.S is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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FIGURE LEGENDS

Figure 1: Lack of TLR3 improves response to glucose and increases circulating insulin after glucose stimulation. (A) Body weight was measured in control and Tlr3−/− mice fed chow or HFD. (B) ipGTT was performed by injecting glucose (1g/kg; i.p.) and glucose concentration was analyzed in blood from the tail vein. (C) Baseline glucose. Levels of (D) circulating insulin and (E) baseline insulin were measured by ELISA. (F) Incremental insulin calculated by subtracting basal insulin (G) HOMA-IR. Hyperglycaemic clamps were performed on mice fed a HFD. (H) Plasma glucose (I) Glucose infusion rate (J) plasma insulin (K) C-peptide (L) Estimated insulin clearance calculated as fasted C-peptide to insulin. Number of animals included: BW and ipGTT N = 21-26 each genotype. Insulin release during ipGTT chow-fed mice N = 6+6, HFD-fed mice N = 18-19; HOMA-IR N = 6-8 each genotype; Hyperglycaemic clamps and insulin clearance N = 8-12 each genotype. Mice were fed a diet for 20w except for hyperglycaemic clamps (26w). AUC for glucose, insulin kinetic, and hyperglycaemic clamps were calculated. Open bars = control, black bars = Tlr3−/− mice. Means ± SEM. *P<0.05, **P<0.01, ***P<0.001.

Figure 2: Control and Tlr3−/− mice respond similarly to insulin and show no impairment of hepatic insulin sensitivity.

ipITT was performed on overnight starved mice (except (C) which were not starved) by injecting insulin (0,8 U/kg body weight; i.p) and glucose concentration was analysed in blood from the tail vein. Mice were fed (A) chow and (B and C) HFD for 20w. (D) ipGTT was performed on aged mice, fed a HFD for 12 month, by injecting glucose (1g/kg; i.p.) and glucose concentration was analyzed in blood from the tail vein. (E) Levels of circulating
insulin were measured by ELISA. (F) ipITT on aged mice (13 month old). (G) Basal insulin signaling as revealed by Western blot analysis of phosphorylated AKT in liver. Age at HFD-diet start = 4-5 weeks. HFD-diet duration = 20 weeks. (H) Insulin stimulated (2U/kg body weight) AKT phosphorylation. Age at HFD-diet start = 14-16 weeks. HFD-diet duration = 18 weeks. (G, H) Graphs are shown as AKT phosphorylation normalized to total AKT. (I, J) Gluconeogenesis was assessed by analyzing (I) PEPCK mRNA levels normalized to hprt and (J) by ipPTT in liver of mice fed a HFD for 20 and 15 weeks respectively. Diet and number of animals included in each experiment: (A) chow diet, N = 15-20; (B) HFD, N = 18; (C) freely fed, N = 11 each genotype; (D-F) aged HFD-fed, N = 4-5 each genotype; (G) HFD, N = 6 + 6; (H) HFD, N = 3 + 4 each genotype; (I) HFD, N = 18 per genotype; (J) HFD, N = 9 per genotype. The graphs for ipITT are shown as percentage reduce from baseline glucose. AUC for glucose kinetic and circulating insulin were calculated. Open bars = control, black bars = Tlr3−/− mice. Means ± SEM. *P<0.05, **P<0.01, ***P<0.001.

Figure 3: Systemic inflammation was increased in Tlr3−/− mice, but not locally in the pancreas

Blood analysis of A) total lymphocytes, granulocytes and monocytes per white blood cells (WBC) (B) SAA in mice fed a HFD for 20 weeks. N for total blood count = 8-18 per genotype, N for SAA = 15-17 per genotype.

Figure 4: Tlr3−/− mice have reduced TG in VLDL due to decreased TG-biosynthesis.

Control and Tlr3−/− mice fed HFD were analysed for plasma levels of TG (A) and cholesterol (C). Size analysis of lipoprotein profile was performed on plasma and the concentration of (B)
TG and (D) cholesterol were measured in each fraction and plotted against retention fraction number. 2-3 plasma samples were pooled per genotype and a total of 5 pools per group were analysed. Mean profiles are shown. AUC was calculated for each fraction of VLDL, LDL, and HDL. (E) FFA (N = 31-32 each genotype) were measured in plasma and (F) relative mRNA level of HMGCR (N = 18 each genotype) in liver was analysed and normalized to Hprt. (G) Newly synthesized VLDL-TG was assessed in HFD-fed (34w) mice by injecting Tyloxapol (i.v.). Graph is shown as fold increase ratio of newly synthesized VLDL-TG normalized to baseline TG. N = 6 each genotype. Open bars = control, black bars = Tlr3<sup>−/−</sup> mice. Means ± SEM. *P<0.05, **P<0.01, ***P<0.001.

**Figure 5: Tlr3<sup>−/−</sup> mice secrete more insulin from pancreatic islets.** (A) Insulin secretion and (B) insulin content were measured in islets from control and Tlr3<sup>−/−</sup> mice fed HFD. (C) Representative immunohistochemical staining for insulin, glucagon, and somatostatin in the islets of Langerhans of control and Tlr3<sup>−/−</sup> mice fed a HFD (D) Average islet area was calculated per total pancreatic area on three separate pancreatic sections per sample. (E) Detection of apoptosis by TUNEL staining was performed on pancreatic sections from control and Tlr3<sup>−/−</sup> mice fed HFD. Circle indicates islet area and duodenum villi were used as control for positive staining. N = 6-8 each genotype. (F) Cleaved caspase-3 was assessed by western blot and normalized to caspase-3 in mice fed a HFD for 18 weeks. N = 5-6 each genotype. Open bars = control, black bars = Tlr3<sup>−/−</sup> mice. Means ± SEM. ***P<0.001

**Figure 6: Lack of TLR3 increases Gck expression.** (A) Gck protein expression was analysed in islets from control or Tlr3<sup>−/−</sup> mice fed HFD with western blot and normalised to β-actin. N = 6 each genotype. (B) Relative Glut2 mRNA levels in islets was analysed in mice on
HFD and normalized to hprt (N = 7-9 each genotype). Open bars = control, black bars = Tlr3−/− mice. Means ± SEM. Means ± SEM. *P<0.05

**Figure 7: Insulin release in perifusion of islets from Tlr3−/− and control mice.** Islets control and Tlr3−/− fed a chow diet (A-D) or HFD (E-H) for 20w were perifused with 3.3 mmol/l glucose followed by 16.7 mM glucose and 20 mmol/l (K+). Samples of the perifused islets were collected every minute, and levels of secreted insulin was analysed by RIA. AUC was calculated for (B and F) first and (C and G) second phase of insulin secretion after glucose stimulation (indicated by dashed line in A and E), and (D and H) after K+ stimulation. Mean values from 5 mice per group are shown. (I) Protein expression levels of VAMP-2 was analysed in islets from control or Tlr3−/− mice fed HFD with western blot and normalised to β-actin. Samples were pooled and each band represents islets pooled from 2 mice. Total of 4 pools for each genotype. A representative western blot is shown. (J and K) Chow diet fed C57/B6 mice (age 18w) were treated with polyI:C (100µg / mouse) or NaCl respectively. (J) ipGTT was performed and AUC was calculated. N = 5-6 each genotype. (K) Protein expression of VAMP-2 was assessed by western blot and normalised to β-actin. Samples were pooled and each band represents islets pooled from 2-3 mice. (L and M) polyI:C and NaCl treatment respectively was started in young mice (age 6w) simultaneously with the HFD. (L) ipGTT was performed and AUC was calculated. N = 5-6 each genotype. (M) Protein expression of VAMP-2 was assessed by western blot and normalised to β-actin. Samples were pooled and each band represents islets pooled from 2-3 mice. (N) Insulin secretion displayed as percentage of insulin content in polyI:C treated HFD-fed C57/B6 mice. PolyI:C (100 µg/mouse) and NaCl treatment respectively was started in 9 weeks old mice simultaneously.
with the HFD. In total mice received 17 injections. N = 4-5 each genotype. Open bars = control, black bars = $Tlr3^{+/−}$. Means ± SEM. *$P<0.05$, **$P<0.01$

**Figure 8: Genetic structure of human TLR3.** A schematic diagram of the human TLR3 gene and the relative positions of the SNPs analysed in this study. Linkage disequilibrium (calculated from POLCA and OLIVIA) is given in $r^2$ (colour and numbers).
Figure 2

A

Chow diet

% Glucose from baseline

Time post insulin injection (min)

B

HFD

% Glucose from baseline

Time post insulin injection (min)

C

Freely HFD-fed

% Glucose from baseline

Time post insulin injection (min)

D

Aged HFD-fed

AUC HFD: * control vs. TLR3Δ

Time post glucose injection (min)

E

Aged HFD-fed

AUC HFD: * control vs. TLR3Δ

Time post glucose injection (min)

F

Aged HFD-fed

% Glucose from baseline

Time post insulin injection (min)

G

H

I

J

172x159mm (300 x 300 DPI)
Figure 3

A

Total blood cell count per WBC [x\(10^7\)]

control
TLR3\(^{\pm}\)

Lymphocytes
Granulocytes
Monocytes

B

Serum Amyloid A [\(\mu g/ml\)]

control
TLR3\(^{\pm}\)

*
Figure 4

A) Triglycerides (mg/dL)

B) Triglyceride profile

C) Cholesterol (mg/dL)

D) Cholesterol profile

E) FPA (mmol/L)

F) HMGCoA reductase

G) AUC HFD: * control vs. TLR3

167x144mm (300 x 300 DPI)
Figure 6

A

control  TLR3−/−

Gck
β-actin

300

200

100

0

control  TLR3−/−

B

Islets

1.5

1.0

0.5

0

control  TLR3−/−

Glut2/hprt

75x59mm (300 x 300 DPI)
Supplemental figure 1

A

Adiponectin [μg/ml]

control TLR3−/− control TLR3−/−

chow diet HFD

B

Leptin [ng/ml]

control TLR3−/− control TLR3−/−

chow diet HFD
Supplemental figure 3

A. Liver
B. Adipose tissue
C. Muscle

Glut2/hprt vs. Glut4/hprt
Supplemental figure 4

A. Glucose response to glucose injection over time in Tlr3\(^{+/−}\) and Tlr3\(^{+/−}\) mice injected with NaCl or polyI:C.

B. Baseline glucose levels in NaCl and polyI:C injected groups.

C. Body weight comparison between NaCl and polyI:C injected groups.
Supplemental figure 5

A

TLR3^-/-
glucose palmitate

B

Glucose Palmitate
Pre-treatment with palmitate - control TLR3^-/-

C

Insulin secretion [µU/h/islet]
Glucose Palmitate Glucose Palmitate
control TLR3^-/-
Online supplement

TOLL-LIKE RECEPTOR 3 INFLUENCES GLUCOSE HOMEOSTASIS AND \(\beta\)-CELL INSULIN SECRETION

Daniela Strodthoff\(^1,2\), Zuheng Ma\(^3\), Tina Wirström\(^3\), Rona J Strawbridge\(^4\), Daniel F. J. Ketelhuth\(^1\), David Engel\(^1\), Robert Clarke\(^3\), Sture Falkmer\(^6\), Anders Hamsten\(^4\), Göran K. Hansson\(^1\), Anneli Björklund\(^3\), Anna M. Lundberg\(^1\)

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5 Clinical Trial Service Unit and Epidemiological Studies Unit, University of Oxford, U.K.
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Online supplemental data

Palmitate experiment

FA solutions of palmitate were prepared as described previously (1). Isolated islets from chow diet treated control and \(Tlr3^{-/-}\) mice were cultured in 11 mmol/l glucose with and without 200 \(\mu\)mol/l palmitate for 48 h. After culture and 30 min of pre-incubation in low glucose (2.8mM glucose in KRB buffer) final batch incubations for 60 min were performed in 2.8 and 16.7 mmol/l glucose.
## Online supplemental table 1 Description of cohorts POLCA and OLIVIA

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Mean (standard deviation). *in PROCARDIS, fasting values only available for a subset of subjects (n=3243 nondiabetic subjects, n=478 diabetic subjects)
Supplemental figure legend

Supplemental Figure 1: No change in circulating adiponectin and leptin was observed.
Levels of circulating (A) adiponectin and (B) leptin were measured by ELISA. Chow diet N = 15-21, HFD N = 18 mice per genotype. Open bars = control, black bars = Tlr3−/− mice. Means ± SEM.

Supplemental Figure 2: TLR3 signaling leads to a changes of TG-level.
(A) Relative sortilin-1 mRNA levels in liver was analyzed in chow diet fed and normalized to hprt (N = 14-21 each genotype). (B) Plasma levels of TG in NaCl and polyI:C treated mice fed a chow diet were analyzed. N = 5-6 each genotype. Open bars = control, black bars = Tlr3−/−. Means ± SEM. *P<0.05

Supplemental Figure 3: Lack of TLR3 does not influence Glut2 and Glut4 expression.
Relative Glut2 mRNA levels in (A) liver, and Glut4 mRNA levels in (B) adipose tissue and (C) muscle was analysed in mice on HFD and normalized to hprt. Liver and muscle N = 17-18, adipose tissue N = 13-14. Open bars = control, black bars = Tlr3−/− mice. Means ± SEM.

Supplemental Figure 4: PolyI:C treatment does not affect glucose response in Tlr3-deficient mice. PolyI:C (100 µg/mouse) and NaCl treatment respectively was started in 9 weeks old mice simultaneously with the HFD. In total mice received 11 injections. (A) ipGTT was performed by injecting glucose (1g/kg; i.p.) and glucose concentration was analyzed in blood from the tail vein. Baseline glucose (B) and body weight (C) was measured in Tlr3−/− mice treated with NaCl or polyI:C. N = 4-5 each genotype. Open bars = NaCl treated Tlr3−/− mice, black bars = polyI:C treated Tlr3−/− mice.
Supplemental Figure 4 5: Assessment of lipotoxicity in Tlr3⁻/⁻ and control mice.

Lipotoxicity was assessed by long-term (48h) palmitate treatment of isolated islets from control and Tlr3⁻/⁻ chow-fed mice. (A and B) Insulin secretion and (C) insulin content were assessed (N = 4 each genotype). Open bars = control, grey bars = Tlr3⁻/⁻. Means ± SEM. *P<0.05

Reference