Toll-like receptor 9 negatively regulates pancreatic islet beta cell growth and function in a mouse model of type 1 diabetes

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Abstract
Aims/hypothesis Innate immune effectors interact with the environment to contribute to the pathogenesis of the autoimmune disease, type 1 diabetes. Although recent studies have suggested that innate immune Toll-like receptors (TLRs) are involved in tissue development, little is known about the role of TLRs in tissue development, compared with autoimmunity. We aimed to fill the knowledge gap by investigating the role of TLR9 in the development and function of islet beta cells in type 1 diabetes, using NOD mice.

Methods We generated Tlr9−/− NOD mice and examined them for type 1 diabetes development and beta cell function, including insulin secretion and glucose tolerance. We assessed islet and beta cell number and characterised CD140a expression on beta cells by flow cytometry. We also tested beta cell function in Tlr9−/− C57BL/6 mice. Finally, we used TLR9 antagonists to block TLR9 signalling in wild-type NOD mice to verify the role of TLR9 in beta cell development and function.

Results TLR9 deficiency promoted pancreatic islet development and beta cell differentiation, leading to enhanced glucose tolerance, improved insulin sensitivity and enhanced first-phase insulin secretory response. This was, in part, mediated by upregulation of CD140a (also known as platelet-derived growth factor receptor-α [PDGFRα]). In the absence of TLR9, induced by either genetic targeting or treatment with TLR9 antagonists, which had similar effects on ontogenesis and function of beta cells, NOD mice were protected from diabetes.

Conclusions/interpretation Our study links TLR9 and the CD140a pathway in regulating islet beta cell development and function and indicates a potential therapeutic target for diabetes prevention and/or treatment.

Keywords CD140a · Diabetes · Islet beta cell · PDGFRα · TLR9

Abbreviations
AM Acetoxymethyl
IPGTT Intra-peritoneal glucose tolerance test
ITT Insulin tolerance test
NGN3 Neurogenin 3
ODN Oligodeoxynucleotides
PDGF Platelet-derived growth factor
PDGFRα Platelet-derived growth factor receptor-α
PDX-1 Pancreatic and duodenal homeobox-1
qPCR Quantitative PCR
STZ Streptozotocin
TLR Toll-like receptor
WT Wild-type

Introduction
The innate immune system generates early inflammatory responses to a variety of environmental insults. A large number
of innate immune receptors, including the Toll-like receptors (TLRs), are important for immediate immune responses to infection, leading to later, more specific, adaptive immunity. On binding the appropriate ligand, the TLRs activate signalling pathways that lead to production of proinflammatory cytokines and upregulation of costimulatory molecules. TLRs were initially thought to be expressed mainly on immune cells, in particular antigen-presenting cells, but it is increasingly recognised that they are also expressed on many other cell types and have functions that range beyond activation of the immune system. We, and others, have shown that pancreatic beta cells express many TLRs in both mice and humans [1, 2]. Activation of TLR3, a receptor for double-stranded RNA, has been shown to induce beta cell apoptosis [1, 3, 4]. TLR4, the receptor for endotoxin, is involved in regulation of metabolism in a variety of tissues including beta cells [5–7]. TLR9 can also be detected easily in both mouse and human islets [1, 2].

Type 1 diabetes is a slowly progressing autoimmune disease. We, and others, have independently shown that TLR9-deficient (Tlr9−/−) NOD mice are protected from type 1 diabetes development [8–10]. This protection is mediated partly by impaired IFNα production from Th9−/− mouse dendritic cells [9] and by enhanced expression and regulatory function of CD73+ T cells [10]. However, increasing evidence suggests that TLRs recognise not only exogenous ligands from microbes but also endogenous ligands from both normal and damaged cells. Recent studies suggest that DNA released from both physiological and pathological dying cells can be a key stimulus to innate immune activation of TLR9 [11–14]. There is also evidence that TLRs regulate neurogenesis during development [15]. Considering that islet beta cells undergo significant growth and remodelling, early in life [16–19], it is likely that TLR9 plays an important role in the development of type 1 diabetes, beyond any direct immune function. However, to date, there have been no reports about the role of TLR9 in islet beta cell development. Therefore, we aimed to assess the role of TLR9 in the development and function of islet beta cells in both NOD and C57BL/6 mice.

**Methods**

**Mice** All the mice used in the study were housed in specific pathogen-free conditions with a 12 h dark–light cycle and were housed in individually ventilated filter cages with autoclaved food and bedding at the Yale University animal facility. The Tlr9−/− NOD mice were generated by backcrossing Th9−/− C57BL/6 mice [20] with our NOD mice, for over 11 generations. The purity of the NOD genetic background was confirmed by mouse genome SNP scan with Illumina Infinium panel (DartMouse, Lebanon, NH, USA). Th9−/− NOD.Scid mice were generated by breeding Th9−/− NOD mice with NOD.Scid mice, which were originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained at Yale University for ~25 years. Wild-type (WT) C57BL/6 (Th9+/+ C57BL/6) mice were also purchased from the Jackson Laboratory and maintained at Yale University for ~10 years. The use of the animals in this study was approved by the IACUC of Yale University. All mice used in different experiments were randomly selected from
different breeding cages and different litters. Experimenters were not blinded in this study.

**Natural history of diabetes development** Th9−/− NOD mice and Th9+/+ NOD littersmates were screened for glycosuria weekly for spontaneous diabetes development, up to 32 weeks of age. Diabetes was confirmed by blood glucose of ≥13.9 mmol/l with a FreeStyle glucose meter (Abbott, Chicago, IL, USA).

**Streptozotocin-induced diabetes development** Female Th9−/− NOD mice and Th9+/+ NOD littersmates (5–6 weeks old) were treated with either high-dose streptozotocin (STZ) (100 mg/kg, administered by two consecutive i.p. injections, 24 h apart) or low-dose STZ (40 mg/kg, administered by i.p. injection, once daily, for 5 days). Mice were screened for glycosuria daily for diabetes development and confirmed as above.

**Intra-peritoneal glucose tolerance test** Intra-peritoneal glucose tolerance tests (IPGTTs) were performed in 5–6-week-old Th9−/− NOD, Th9+/+ NOD, Th9−/− C57BL/6, Th9+/+ C57BL/6, Th9−/− NOD.Scid and Th9+/− NOD.Scid mice. The mice were fasted overnight with free access to water and the blood glucose was measured before (time zero) and after i.p. injection of glucose (1 g/kg) at different time points from blood samples. Blood glucose was measured by a FreeStyle glucose meter (Abbott). Data are shown from one out of three experiments, each confirming the significant difference.

**Insulin tolerance test** Insulin tolerance tests (ITTs) were performed in 5–6-week-old male Th9−/− C57BL/6 mice and Th9+/+ C57BL/6 mice. The mice were fasted for 6 h with free access to water and the blood glucose was measured before and after i.p. injection of insulin (Humulin-R, 0.75 U/kg; Eli Lilly, Indianapolis, IN, USA) at different time points, as described for IPGTT.

**Islet and beta cell isolation** Pancreatic islets were isolated as previously described [21]. Mice were euthanised by cervical dislocation. The pancreas was inflated with 3 ml cold collagenase (Sigma; St Louis, MO, USA) solution (0.3 mg/ml) through the bile duct with a 20G needle starting at the gall bladder. The pancreas was then removed into a siliconised glass tube containing 2 ml of 1 mg/ml collagenase solution and digested at 37°C in a water bath for 12–15 min. After three washes of the digested pancreas, islets were hand-picked and counted under a dissecting microscope for further experiments. For single-cell isolation, the islets were treated with Cell Dissociation Solution (Sigma) and the single-cell suspension was harvested. Beta cells from the dissociated islets were stained with fluorochrome-conjugated monoclonal antibodies to CD45 (BioLegend; San Diego, CA, USA), CD140a (BioLegend) and FluoZin-3-acetoxymethyl (AM) (CD45-FluoZin-3-AM*; ThermoFishier, Waltham, ME, USA) [22] before being analysed by flow cytometry (LSRII; BD Bioscience, San Diego, CA, USA).

**Quantitative PCR** Pancreatic islets were isolated as described above. RNA from islets of 3–4-week-old female Th9−/− NOD mice and Th9−/− NOD mice was extracted with an RNAeasy kit (Qiagen, Hilden, Germany) and quantified by NanoDrop (ThermoFisher). Equal amounts of RNA were reverse transcribed using SuperScript III First-strand synthesis kit with random hexamers (Invitrogen, Carlsbad, CA, USA). Quantitative PCR (qPCR) was performed using the Bio-Rad iQ5 qPCR detection system (Hercules, CA, USA) with the specific primers for Pdx-1 (also known as Pdx1) (5′-CAGC AGAACCGAGGAGAAT-3′ and 5′-CGACGGTTTGGAAA CCAGAT-3′) and Ngn3 (also known as Neurog3) (5′-CCCG CAGCTCTCTGTCTTT-3′ and 5′-GGGTCTCTTGAGGAC ACTTGG-3′) (Sigma). The relative expression of mRNA levels was determined with the 2−ΔΔCt method by normalisation with the housekeeping gene Gapdh (5′-AGGT CCGTGTTGAACGGATTTG-3′ and 5′-TG TGA GACCATGTAGTGGAGGTCA-3′).

**Cell staining for flow cytometry**

For direct staining, single-cell suspensions (~5 × 10⁴ to 2 × 10⁵ cells) of immune cells or islet cells were incubated with a 2.4G2 Fc-blocking antibody (10 mins, room temperature) prior to staining with pre-titrated amounts of monoclonal antibodies conjugated with different fluorochromes to combinations of CD3 (17A2), CD4 (GK1.5), CD44 (IM7), CD45 (30-F11) CD62L (MEL-14), CD140a (AP5) and a viability dye (all from BioLegend) in staining buffer (PBS containing 1% FCS) and kept on ice and in the dark for 30 min. The cells were washed twice with 2 ml staining buffer and fixed with 200 μl fixation buffer (eBioScience; San Diego, CA, USA) before analysis by flow cytometry. All antibodies were titrated using mouse splenocytes at different dilutions with the final dilution applied found to be most appropriate for the particular batch of antibody used and our flow cytometer set up.

**Intracellular staining** For intracellular staining, the single-cell suspension was treated with Perm/Fix buffer (eBioScience) followed by pre-titrated monoclonal antibodies conjugated with different fluorochromes to FoxP3 (FJK-16S, eBioscience) or FluoZin-3-AM (ThermoFisher). After 30 min incubation on ice or at room temperature, the cells were washed twice with 2 ml staining buffer and analysed by flow cytometry. FoxP3 was titrated using mouse splenocytes at different dilutions with the final dilution applied found to be appropriate for the batch used and our flow
cytometer set up. For Fluozin-3-AM, mouse islets were used to titrate the antibody, with 1:2000 dilution used found to be appropriate for the particular batch of antibody used and our flow cytometer set up. Dilutions were determined where they gave the clearest separation from the negative background or isotype control.

**Insulin release assay** An insulin release assay was performed as previously described [23] with modification. Hand-picked pancreatic islets from randomly selected \( Tlr9^{+/+} \) and \( Tlr9^{-/-} \) NOD or C57BL/6 mice (5–6 weeks old) were equally distributed to 30 islets/tube after stabilising with low-glucose KRB buffer. The islets were then stimulated with KRB containing high glucose (25 mmol/l) and the supernatant fractions were harvested every 5 min after glucose stimulation. Secreted insulin in the supernatant fractions was measured using the insulin RIA kit (EMD-Millipore, Burlington, ME, USA).

**Evaluation of islet mass** Ex vivo pancreases from randomly selected 5–6-week-old female \( Tlr9^{+/+} \) NOD and \( Tlr9^{-/-} \) NOD mice were fixed in peridate–lysine–paraformaldehyde, sucrose infused and then frozen in Tissue-Tek OCT (Bayer, Elkhart, IN, USA). The pancreas was cut in its entirety into hundreds of 10 μm thick sections and every tenth section was stained with haematoxylin alone (to better visualise the islets) and photographed under the microscope. Islet mass was measured using Image J software (NIH, Bethesda, MD, USA). H&E staining of sections was conducted purely for improving the contrast of the images for the photographs presented in Fig. 4b.

**In vitro TLR9 antagonist treatment** Freshly isolated islets from \( Tlr9^{-/-} \) NOD mice (5-week-old females) were cultured overnight with the TLR9 antagonist CpG-oligodeoxynucleotides (ODN) (2088; Invivogen, San Diego, CA, USA) or control CpG-ODN (Invivogen), both at 10 μg/ml. After extensive washing, a single-cell suspension was prepared as described earlier and stained with fluorochrome-conjugated monoclonal antibodies to CD45, CD140a and FluoZin-3-AM before analysis by flow cytometry. Another set of freshly isolated islets from \( Tlr9^{+/+} \) NOD mice was used for insulin release assay, after overnight culture in the presence of the TLR9 antagonist CpG-ODN or control CpG-ODN.

**In vivo treatment with TLR9 antagonist or chloroquine and diabetes development** Randomly chosen \( Tlr9^{+/+} \) female NOD mice were treated with TLR9 antagonist CpG-ODN (2088) or control ODN, 10 μg/mouse, administered as two i.p. injections, 3 days apart, 1 week after mating. Another set of randomly chosen \( Tlr9^{-/-} \) pregnant female NOD mice were treated with chloroquine (20 μg/g body weight), administered as two i.p. injections, 3 days apart. The female offspring from the treated mothers were investigated for CD140a-expressing islet beta cells, the number of islet beta cells and insulin-secreting function at ~5 weeks old. A third group of randomly chosen pregnant female \( Tlr9^{+/+} \) NOD mice were also treated with antagonist CpG-ODN or control ODN and the natural history of diabetes development was observed in the female progeny of the treated pregnant mice.

**Statistical analysis** No data were excluded and all viable mice within the different genotypes were included, with the exception of any obvious runts or under-developed mice. No outcomes or conditions were measured or used that are not reported in the results section. Statistical analyses were performed using GraphPad Prism software (San Diego, CA, USA). Diabetes incidence was compared using logrank test. The in vivo and in vitro assays were analysed with Student’s unpaired t test or ANOVA for statistical significance.

**Results**

**TLR9 deficiency suppressed type 1 diabetes development and enhanced islet beta cell function** Although the environment influences type 1 diabetes development [24], particularly in NOD mice, which are very sensitive to environmental changes [25], the protection from diabetes development seen in \( Tlr9^{-/-} \) NOD mouse has been consistent in our mouse colony over many years (Fig. 1a, b). This implies that environmental variation, including housing status (data not shown), does not play a major role in this protection. To test the hypothesis that, beyond its function in innate immunity, TLR9 may impact on pancreatic beta cells and investigate the role of TLR9 in islet beta cell function, we performed glucose tolerance tests (GTTs). \( Tlr9^{-/-} \) NOD mice had significantly better glucose tolerance, on glucose stimulation in vivo, than their \( Tlr9^{+/+} \) NOD (WT) littermates (Fig. 1c, d), at 5–6 weeks of age, when there is little beta cell destruction in the \( Tlr9^{-/-} \) NOD mice. To confirm that the improved glucose tolerance in \( Tlr9^{-/-} \) NOD mice was not due to the reduced insulitis in these mice [10], we generated \( Tlr9^{-/-} \) NOD.Scid, mice which are completely free of lymphocytic infiltration. We tested glucose tolerance in \( Tlr9^{-/-} \) NOD.Scid mice and \( Tlr9^{+/+} \) NOD.Scid mice, neither of which develop insulitis nor diabetes. The \( Tlr9^{-/-} \) NOD.Scid mice demonstrated significantly better glucose tolerance than their \( Tlr9^{+/+} \) NOD.Scid counterparts (Fig. 1e, f), similar to immune-sufficient \( Tlr9^{-/-} \) NOD mice.

To test whether the improved beta cell function in the absence of TLR9 was related to the NOD genetic background, we studied \( Tlr9^{-/-} \) C57BL/6 mice and \( Tlr9^{-/-} \) C57BL/6 mice. Interestingly, we consistently found enhanced glucose tolerance in \( Tlr9^{-/-} \) mice, regardless of their genetic background (Fig. 2a, b). Next, we investigated in vitro insulin secretion of islet beta cells, in response to glucose stimulation. Pancreatic islets isolated from young \( Tlr9^{+/+} \) NOD mice and \( Tlr9^{-/-} \)
NOD mice were cultured in high glucose concentrations and insulin release into the culture supernatant fractions was measured every 5 min. Consistent with the in vivo GTT results, more insulin was secreted from the islets isolated from both Tlr9+/− NOD and Tlr9+/− C57BL/6 mice vs Tlr9+/+ NOD and C57BL/6 mice (Fig. 2c, d). To further assess the insulin sensitivity, we also conducted an ITT and found improved glucose control in Tlr9+/− C57BL/6 mice vs Tlr9+/+ C57BL/6 mice (Fig. 2e).

Increased islet and beta cell number in the absence of TLR9 in NOD mice Based on our findings of enhanced beta cell function, we hypothesised that, in the absence of TLR9, beta cells were either more resistant to cell death or had increased cell growth. To investigate beta cell death, we treated Tlr9+/− NOD and Tlr9+/− NOD mice with STZ, a chemical causing beta cell death leading to clinical diabetes. However, there was no particular resistance to beta cell death in Tlr9+/− NOD mice, as diabetes onset was similar to the onset in their Tlr9+/− NOD counterparts after STZ treatment, both at high dose and multiple low doses (Fig. 3a, b). We also investigated islet apoptosis in response to STZ, directly ex vivo. Freshly isolated islet cells from Tlr9+/− NOD and Tlr9+/− NOD mice were cultured with STZ (40 ng/ml) for 3 h followed by staining for apoptosis using Annexin V and 7-AAD. In line with the results from the in vivo experiments, islet cells from Tlr9+/− NOD mice were as susceptible to apoptosis as the islet cells from Tlr9+/+ NOD mice (Fig. 3c).

Next, we investigated the alternative possibility of increased cell growth by analysing the islet mass. OCT-embedded frozen pancreatic tissue blocks from young Tlr9+/− NOD and Tlr9+/− NOD mice were completely sectioned at 10 μm/section and stained with haematoxylin alone to better visualise the islets. We examined every tenth section.
Increased islet beta cells expressing CD140a in the absence of TLR9 in NOD mice

We next investigated the molecular pathway(s) by which TLR9 could influence pancreatic islet development. We focused on CD140a (also known as platelet-derived-growth-factor receptor-α [PDGFRα]), as it has been reported to control proliferation of pancreatic beta cells [29]. We examined CD140a expression on dispersed beta cells from Tlr9+/− NOD and Tlr9−/− NOD mice, using anti-mouse-CD140a and the beta cell marker FluoZin-AM and its control CpG-ODN overnight and examined CD140a expression. It is interesting that the TLR9 antagonist was indeed able to induce CD140a expression in Tlr9+/− islets (Fig. 5c, d). Next, we tested the function of beta cells from TLR9 antagonist-treated WT islets by measuring insulin secretion in response to glucose stimulation. Pancreatic islets isolated from young Tlr9+/− NOD mice were cultured with TLR9 antagonist CpG-ODN or control CpG-ODN overnight. After extensive washing, the islets were further cultured in high glucose concentrations and insulin release into the culture supernatant fractions was measured every 5 min. Figure 5e shows that early-phase insulin secretion in response to glucose stimulation was significantly enhanced in Tlr9+/− NOD islets treated with TLR9 antagonist. To test whether inhibition of CD140a could reverse the effect of TLR9 antagonist on beta cells, we cultured islets from young Tlr9+/− NOD and Tlr9−/− NOD mice with a CD140a inhibitor (PDGFR inhibitor, Enzo Life Science, Oyster Bay, NY, USA), as we had done with the TLR9 antagonist. However, due to the cell toxicity of the inhibitor, most of the islet cells were not viable after overnight culture (data not shown).

**Treatment with TLR9 antagonist resulted in increased CD140a-expressing islet beta cells and number of beta cells, improved beta cell function and protected Tlr9+/− NOD mice from diabetes development**

A study by Chen and colleagues suggested that the expression of CD140a on islet beta cells was age-dependent [29]. We hypothesised that the enhanced development of pancreatic islets and the increased number of CD140a-expressing beta cells in Tlr9+/− NOD mice occur early in life. To test this in vivo, we treated Tlr9+/− NOD mice with TLR9 antagonist CpG-ODN and its control CpG-ODN
islets of 4-week-old female Tlr9+/+ mice (black circles) (p < 0.001). (b) Representative pancreas sections after staining with H&E are shown. Scale bar, 100 μm. (c) Beta cells from pancreatic islets of 4-week-old female Tlr9+/+ NOD and Tlr9−/− NOD mice (n = 4 mice for both groups) were harvested after treatment with Cell Dissociation Solution (Sigma). After staining with anti-CD45 and FluoZin-AM, beta cells (CD45− FluoZin+ cells) were enumerated by flow cytometry. Beta cell number was increased in the pancreas of Tlr9−/− mice when compared with Tlr9+/+ NOD mice (black diamonds) compared with Tlr9+/+ NOD mice (black circles) (p < 0.001). (d) Representative pancreas sections after staining with haematoxylin alone to visualise the islets better. Islet area was evaluated with ImageJ software. More islets were present in the pancreases of Tlr9−/− NOD mice (black diamonds) compared with Tlr9+/+ NOD mice (black circles) (p < 0.001). (e) Number of islets and beta cells per mouse, in the mice that received TLR9 antagonist treatment, we examined.

Discussion

In this study, we have identified a novel function of TLR9, quite distinct from its role in innate immunity. We showed that...
TLR9-deficient mice have more pancreatic islets and, correspondingly, more islet beta cells, with increased glucose-stimulated insulin secretion in vitro and improved glucose tolerance in vivo. This was not due to increased resistance to beta cell death. Rather, we found increased expression of genes encoding PDX-1 and NGN3, transcription factors associated with beta cell development, suggesting that the increase in beta cell mass was related to promotion of beta cell growth. Although many growth factors regulate islet beta cell development [28, 32], in linking TLR9 deficiency and islet beta cell development, we found that the proportion of islet beta cells expressing CD140a was increased in TLR9-deficient mice. Confirming that this effect was associated with TLR9 deficiency, we showed, using TLR9 antagonism, that inhibition of the TLR9 signalling pathway in islets from TLR9-sufficient mice led to an increased number of CD140a-expressing beta cells and enhanced insulin secretion in response to glucose stimulation. Our results thus demonstrate a novel link between TLR9 and CD140a, a growth factor that has been reported to regulate islet beta cell proliferation [29].

Islet beta cells display distinct phases of significant growth in the fetal and neonatal periods, whereas there is little increase in islet beta cell numbers in adulthood in either mice or humans [33, 34]. Proliferation and survival are among the functions promoted by platelet-derived growth factor (PDGF) signalling through the PDGF receptors, of which CD140a is one of two main receptor isoforms for PDGF. This is a
receptor tyrosine kinase and it is expressed in cells of mesenchymal origin, including the pancreas [35]. It has been suggested that the human CD140a promoter has a binding site for c-Rel [36], which is a subunit of the NFκB protein complex and plays an important role in development, immunity and diseases, including type 1 diabetes [37–39]. Expression of CD140a is normally age-dependent in mouse pancreatic islet beta cells, reaching a peak at around the age of 2 weeks and declining once mice reach adulthood [29]. If this receptor is lost prematurely, as shown by gene mutation experiments, it could play a role in tissue remodelling. Although the gut microbiota differs in composition when comparing Tlr9+/− NOD and Tlr9+/+ NOD mice, protection from diabetes and enhanced beta cell development and function were not associated with this difference (data not shown). It is particularly interesting that TLR9 is linked to pathological dying cells [40], it could play a role in tissue remodelling.

Fig. 7 Effect of TLR9 antagonist treatment on immune cells in Tlr9+/− NOD mice. (a) Regulatory T cells (Foxp3+ Tregs) in pancreatic islets. Immune progeny were extracted from pancreatic islets isolated from the female progeny (5–6 weeks old) of Tlr9+/− NOD mice treated with TLR9 antagonist ODN or control ODN during pregnancy (n = 3 pregnant dams/group). Cells were stained with monoclonal antibodies to CD3, CD4 and Foxp3. The percentage of Foxp3+CD4+ T cells is shown after gating for CD3+ cells (p = 0.235; n = 6 mice for both groups). (b) Reduced naive (CD44−CD62L+) CD4+ T cells in the spleen of the female progeny (5–6 weeks of age) from TLR9 antagonist-treated pregnant NOD mice. Splenocytes from these mice were isolated and stained with monoclonal antibodies to CD3, CD4, CD44 and CD62L. The percentage of memory CD4+ T cells is shown after gating for CD3+CD4+ T cells (p = 0.0106; n = 7 mice for both groups). Two independent experiments were carried out. Data are expressed as means (SD). Data were analysed by two-tailed unpaired Student’s t test. *p < 0.05, **p < 0.01

Fig. 8 The TLR9 inhibitor chloroquine promotes islet beta cell development. Pregnant Tlr9+/− NOD mice were treated with chloroquine (20 μg/g body weight, i.p. injection, twice, 3 or 4 days apart) or PBS (control). (a) Chloroquine treatment increased CD140a+ beta cell per cent. Islet beta cells from 4-week-old female progeny of chloroquine-treated (black circles) or PBS-treated (white circles) mothers (p = 0.0017; n = 4 mice for both) were isolated by flow cytometry for CD140a expression (p = 0.0011; n = 7 mice for both groups). (e) Chloroquine treatment increased the number of beta cells. Islet beta cells were isolated and stained with monoclonal antibodies to CD3 and Fluozin-AM. The number of beta cells (CD45−Foxp3−) was counted by flow cytometry (p = 0.0188; n = 5 mice for both). Two independent experiments were performed, with n = 4–6 mice per group per experiment. Data are expressed as means (horizontal line). Data were analysed by two-tailed unpaired Student’s t test. *p < 0.05, **p < 0.01
during prenatal development [41] but the capacity for neogenesis and regeneration of beta cells is lost later in life. Marked beta cell hyperplasia occurs during neonatal development and the role of CD140a in beta cell proliferation is age-dependent [29]. In our experiments, brief treatment of pregnant Tlr9+/+ NOD mice with a TLR9 antagonist oligonucleotide, as well as with chloroquine (which also antagonises TLR9), significantly enhanced beta cell growth. This was accompanied by enhanced insulin secretion in response to glucose stimulation, as the mice developed into adulthood, and also coincided with an increased percentage of CD140a-expressing beta cells, suggesting the association of the two processes. We did not examine the effect of chloroquine on islet beta cells directly in vitro, as we think it is important to study the drug effect in vivo; although chloroquine is not specific for islet beta cells, we did not observe any noticeable systemic adverse effects.

TLR signalling in mammals has been mainly linked to innate immunity. Our results suggest a novel effect of TLR9 on growth and development in addition to its role in innate immunity. The fact that this brief inhibition of TLR9 signalling, early in life, led to protection from autoimmune diabetes development in Tlr9+/+ NOD mice, similar to the phenotype seen in Tlr9−/− NOD mice, suggests a possible means of improving islet reserve. The inhibition of the development of diabetes is likely to be a combination of increased beta cell capacity (referring to increase in number of beta cells, improved sensitivity to glucose and increased insulin production), together with the immunological changes that we, and others, have previously reported [9, 10]. These changes include increased expression of CD73 and reduced production of proinflammatory cytokines [10], together with a reduction in activation of autoreactive diabeticogenic CD8 T cells, all of which occur as a result of TLR9 inhibition [9]. Inhibition of TLR9 has not been explored in human type 1 diabetes; however, pre-clinical tests for identifying an effective and safe dose, route and timing of any potential agent would need to be conducted first.

We conclude that TLR9 negatively regulates the development of pancreatic islets and insulin-secreting beta cells, mediated, at least in part, by CD140a. Our findings provide novel insight into the function of TLR9 beyond the immune cells and also suggest a new direction for the design of preventive and/or therapeutic strategies for diabetes.

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Data availability Data are available on request from the authors.

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

Contribution statement ML, JP, NT, CH and JG performed experiments. ML, JP, JAP, FSW and LW analysed the data and LH and HZ carried out bioinformatic analysis. LW and FSW wrote the manuscript and JAP edited the manuscript. All authors reviewed and approved the manuscript. LW initiated and designed the study and is the guarantor of the work.

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