Toll-like receptors TLR2 and TLR4 block the replication of pancreatic β cells in diet-induced obesity

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Consumption of a high-energy Western diet triggers mild adaptive β cell proliferation to compensate for peripheral insulin resistance; however, the underlying molecular mechanism remains unclear. In the present study we show that the toll-like receptors TLR2 and TLR4 inhibited the diet-induced replication of β cells in mice and humans. The combined, but not the individual, loss of TLR2 and TLR4 increased the replication of β cells, but not that of α cells, leading to enlarged β cell area and hyperinsulinemia in diet-induced obesity. Loss of TLR2 and TLR4 increased the nuclear abundance of the cell cycle regulators cyclin D2 and Cdk4 in a manner dependent on the signaling mediator Erk. These data reveal a regulatory mechanism controlling the proliferation of β cells in diet-induced obesity and suggest that selective targeting of the TLR2/TLR4 pathways may reverse β cell failure in patients with diabetes.

β cell replacement and regeneration therapies are promising approaches to the treatment of insulin-dependent type 1 diabetes (T1D) and type 2 diabetes (T2D). In rodents and humans, β cells in the pancreas are maintained by neogenesis of β cells from ductal precursor cells and the replication of pre-existing β cells1–5. Neogenesis takes place mostly during fetal and neonatal life, and β cell proliferation rates decline dramatically with age. Mitotic replication of β cells, also known as β cell self-renewal, compensates for increased metabolic demands in adults6–8. A Western diet or a high-fat diet triggers mild β cell proliferation and islet mass expansion9–12; however, the mechanism driving this adaptive process remains unclear. In diet-induced obesity, increased metabolic burden increases production of insulin by increasing secretion per β cell and/or by increasing β cell numbers. Insulin and glucose, as well as growth factors and nutrients, promote β cell replication, at least in part through the activation of pro-proliferative/pro-survival protein kinases such as Akt and extracellular signal-regulated kinase (Erk, or Mapk, mitogen-activated protein kinase)13. Activation of Akt and Erk/Mapk may induce β cell proliferation by activating cell cycle regulators such as the cyclin D2 and Cdk4 and/or anti-apoptotic factors14–16. This indicates potential negative regulatory mechanism(s) that limit diet-induced β cell replication, the precise identities of which remain largely unknown.

Toll-like receptor (TLR) proteins, a family of conserved cell surface and intracellular proteins, recognize a variety of pathogen-associated molecular patterns and induce innate immune responses. They link innate immunity to adaptive immune responses against all known pathogens such as viruses, fungi, bacteria and protozoa17–19. Among a dozen TLR proteins, TLR4 detects lipopolysaccharides (LPSs) found in most Gram-negative bacteria, whereas TLR2, together with TLR1 or TLR6, recognizes lipopeptides (for example, lipoteichoic acid, LTA) and other components of Gram-positive bacteria. It has been reported that TLR2 and TLR4 may mediate nutrient sensing and metabolic regulation in T2D by acting directly or indirectly as sensors for free fatty acids or other lipids in adipose tissue and macrophages20–22. In most reports, loss of either TLR2 or TLR4 in mice fed a high-fat diet (HFD) reduces systemic inflammation in the liver and adipose tissue, and improves insulin sensitivity20–22. However, Tlr2−/− and Tlr4−/− mice on an HFD exhibit mild metabolic phenotypes in terms of glucose and insulin sensitivity, which may be explained by redundancy in TLR2 and TLR4 function23. In the present study, we show that TLR2 and TLR4 regulated β cell proliferation. The combined action of the TLR2- and TLR4-mediated signaling pathways maintained β cell quiescence in an islet-intrinsic manner in mice and humans. Loss of TLR2 and TLR4, but not of each individually, increased β cell proliferation in an HFD-dependent manner.

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**Results**

Regulatory mechanisms counter HFD-induced β cell replication. We first performed immunofluorescent staining and confocal microscopy to quantify β cell proliferation, marked as Ki67+ (Ki67+Ins+) cells, as a function of age in C57BL/6j (B6) male mice on a low-fat diet (LFD) or an HFD with 13% and 60% of calories derived from fat, respectively. Mice were maintained on an LFD and, for some, were switched to an HFD at 6 weeks of age. The percentage of Ki67+Ins+ β cells decreased nearly 50-fold when LFD mice reached 20 weeks of age compared with 4-week-old LFD mice and declined by an additional 4-fold in 45-week-old LFD mice (Fig. 1a). HFD feeding for 39 weeks modestly, but not greatly, increased the percentage of Ki67+Ins+ β cells compared with those age-matched LFD mice aged 45 weeks (Fig. 1a and see Supplementary Fig. 1a). Treatment with the insulin receptor antagonist S961 (to induce systemic insulin resistance) for the last 2 weeks augmented the percentage of Ki67+Ins+ β cells by 3.5-fold in HFD mice compared with vehicle-treated HFD mice (Fig. 1a and see Supplementary Fig. 1a). By contrast, S961 increased the percentage of Ki67+Ins+ β cells by ~120 fold in 45-week-old LFD mice compared with vehicle-treated LFD mice, reaching a level that was threefold higher than in S981-treated HFD mice (Fig. 1a). These observations indicated the existence of a regulatory mechanism that restrained β cell replication in HFD mice.
TLR2 and TLR4 activation blocks β cell proliferation in mice and humans. Compared with those cultured in low (2.8 mM) glucose, treatment of mouse primary islets with high (22.8 mM) glucose for 3 days stimulated the incorporation of the nucleotide analog bromodeoxyuridine (BrdU) in replicating cells, as measured by flow cytometry (Fig. 1b). Treatment with the TLR2- and TLR4-specific agonists, lipoteichoic acid (LTA) and LPSs, for the last 2 days greatly reduced the percentage of BrdU+ β cells cultured with 22.8 mM glucose (Fig. 1b). The inhibitory effect of TLR2 and TLR4 agonists on β cell proliferation was blunted in Thr2−/−Thr4−/− mice (Fig. 1b). We next investigated the effect of TLR2/TLR4 activation in human islets isolated from three independent donors using the same BrdU incorporation assay (see Supplementary Fig. 1b). Activation of TLR2 and TLR4 by LPS + LTA treatment reduced high glucose-induced BrdU incorporation in islets from healthy controls and T2D patients, albeit with a large variability among the samples in terms of baseline proliferation (see Supplementary Fig. 1c,d). These data suggested that activation of TLR2 and TLR4 may attenuate hyperglycemia-induced, β cell proliferation.

TLR2/TLR4 deficiency plus HFD feeding leads to β cell accumulation. Six-week-old Thr2−/−Thr4−/− male mice on the B6 background fed an HFD gained body weight compared with age- and gender-matched, non-littermate, B6 HFD mice (bred separately), as shown previously (see Supplementary Fig. 2a). Serum insulin increased progressively in Thr2−/−Thr4−/− HFD mice compared with B6 HFD mice over a 21-week HFD feeding period (Fig. 1c). Histologic examination of the pancreas revealed progressively enlarged islet mass and increased abundance of Ins+ β cells in Thr2−/−Thr4−/− HFD mice compared with age-matched B6 HFD mice over a 51-week HFD feeding period (Fig. 1d and see Supplementary Fig. 2b,c). By contrast, the number of glucagon+ α cells per islet was unchanged.
in Tlr2−/− Tlr4−/− HFD mice compared with age-matched B6 HFD mice (see Supplementary Fig. 2d). After prolonged 51-week HFD feeding, islets from Tlr2−/− Tlr4−/− HFD mice were visible to the naked eye with a remarkable accumulation of β cells, but not islets from B6 HFD mice (Fig. 1e and see Supplementary Fig. 3a). HFD feeding was required for β cell accumulation in Tlr2−/− Tlr4−/− mice because the Ins+ β cell area was comparable between Tlr2−/− Tlr4−/− mice and control B6 mice on an LFD at 20 and 45 weeks of age (see Supplementary Fig. 3b–d).

To exclude possible genetic variability between non-littermate B6 mice and Tlr2−/− Tlr4−/− mice, we crossed the Tlr2−/− Tlr4−/− mice with B6 mice and then interbred the Tlr2−/− Tlr4−/− mice to generate Tlr2−/− Tlr4−/− and Tlr2+/+Tlr4+/+ as littermates. In keeping with the above findings, Tlr2−/− Tlr4−/− HFD mice had a lot more Ins+ β cells and elevated insulin in the serum compared with Tlr2+/+Tlr4+/+ HFD littermates after a 20-week HFD (Fig. 1f–h).

Islet hyperplasia and hyperinsulinemia were linked to insulin resistance in mice with a liver-specific deletion of the insulin receptor. However, 20-week-old Tlr2−/− Tlr4−/− HFD mice exhibited comparable insulin sensitivity to that of age-matched Tlr2+/+Tlr4+/+ HFD and B6 HFD mice after a 14-week HFD (Fig. 1i,j), suggesting that β cell accumulation in Tlr2−/− Tlr4−/− HFD mice was not due to systemic insulin resistance. Therefore, these data suggested that loss of TLR2 and TLR4 in mice fed an HFD leads to the accumulation of β cells, not triggered by systemic insulin resistance.

**TLR2 and TLR4 inhibit β cell proliferation in an HFD-dependent manner.** To assess the etiology of β cell accumulation, we next measured β cell proliferation by co-staining pancreatic sections with insulin and proliferation markers such as Ki67 and proliferating cell nuclear antigen (PCNA). Ki67+Ins+ β cells were barely detectable in 20- and 45-week-old Tlr2−/− Tlr4−/− and control B6 mice on an LFD (Fig. 2a). The percentage of Ki67+Ins+ β cells in 20- and 45-week-old HFD Tlr2−/− Tlr4−/− mice was increased by 14- and 7-fold, respectively, compared with HFD B6 mice (Fig. 2b,c and see Supplementary Fig. 4a). Indeed, Ki67+Ins+ β cells in 20-week-old Tlr2−/− Tlr4−/− mice after a 14-week HFD reached 9–10% of the total β cells (Fig. 2b,c). Consistently, expression of PCNA, another proliferation marker, in islets from 35-week-old Tlr2−/− Tlr4−/− HFD mice after 29 weeks of an HFD, was higher than in B6 HFD mice (Fig. 2d). Expression of the mature β cell marker PDX1 on β cells was unchanged in Tlr2−/− Tlr4−/− HFD mice compared with B6 HFD mice after 29 weeks of an HFD (Fig. 2e). The percentage of Ki67+Ins+ β cells was consistently sixfold higher in 26-week-old Tlr2−/− Tlr4−/− HFD mice than in Tlr2+/+Tlr4+/+ HFD littermates after a 20-week HFD (Fig. 2f), although few Ki67+Ins+ β cells were detected in 26-week-old Tlr2−/− Tlr4−/− and Tlr2+/+Tlr4+/+ littermates on an LFD (see Supplementary Fig. 4c). Ki67 immunostaining was virtually absent in Glucagon+ α cells in Tlr2−/− Tlr4−/− HFD, Tlr2+/+Tlr4+/+ HFD and B6 HFD mice (Fig. 2g and see Supplementary Fig. 4d). There was no detectable terminal deoxynucleotidyl transferase (TUNEL)+ β cells in Tlr2−/− Tlr4−/− HFD and B6 HFD mice (Fig. 2h).

Using flow cytometry, we noted that the expression of markers of cellular senescence, including p16ink4a (ref. 32) and β-galactosidase on β cells from 9-month-old Tlr2−/− Tlr4−/− HFD mice was decreased compared with age-matched B6 mice (see Supplementary Fig. 4e,f).
Intriguingly, when switched back to an LFD for 4 weeks, the percentage of Ki67+ β cells in Tlr2−/− Tlr4−/− HFD mice on 10-week (short-term) or 32-week (long-term) HFD dropped by more than 10-fold (Fig. 2i,j and see Supplementary Fig. 4g). These data excluded the possibility of oncogenic transformation of β cells or the development of insulinomas in Tlr2−/− Tlr4−/− HFD mice. Hence, loss of TLR2 and TLR4 enhanced the proliferation of β cells in mice on an HFD.

Tlr2 and Tlr4 block nuclear accumulation of the Ccnd2/Cdk4 complex. We next dissected the molecular events underlying the enhanced β cell proliferation in Tlr2−/− Tlr4−/− HFD mice. Ccnd2, a key regulator of the cell cycle, enters the nucleus to drive cell cycle progression in β cells. Using immunofluorescent staining, we noted that Ccnd2 was largely nuclear in β cells from Tlr2−/− Tlr4−/− HFD mice versus largely cytosolic in β cells from B6 HFD mice and Tlr2−/− Tlr4−/− HFD littermates (Fig. 3a,b). Ccnd2 was not detectable in α cells from HFD mice regardless of the genotypes (see Supplementary Fig. 5a, b). Nuclear accumulation of Ccnd2 in β cells from Tlr2−/− Tlr4−/− HFD mice was greatly reduced on switching to an LFD for 4 weeks (Fig. 3c) and, indeed, Ccnd2 was largely cytosolic in β cells from Tlr2−/− Tlr4−/− LFD mice (see Supplementary Fig. 5c). Consistently, Cdk4, which forms a complex with Ccnd2, was highly enriched in the nucleus of β, not α, cells from Tlr2−/− Tlr4−/− HFD mice compared with B6 HFD mice (Fig. 3d and see Supplementary Fig. 5d).

We next asked whether the production and secretion of insulin were affected in primary islets from 15-week-old Tlr2−/− Tlr4−/− and B6 mice on an HFD for 9 weeks. Cytosolic calcium flux and insulin secretion, in response to extracellular stimuli such as glucose and KCl, were either enhanced or comparable in islets from Tlr2−/− Tlr4−/− HFD mice compared with those from B6 HFD mice (Fig. 3e). Insulin granules in β cells from Tlr2−/− Tlr4−/− mice on an HFD for 51 weeks were similar in size and density relative to those from B6 HFD mice, as assessed by transmission electron microscopy (TEM) (Fig. 3f), indicating that the Tlr2−/− Tlr4−/− β cells retained a normal response to hyperglycemia. Together, these data indicated an increased abundance of nuclear Ccnd2/Cdk4 complex and normal secretory function of β cells from Tlr2−/− Tlr4−/− HFD mice.

Tlr2 and Tlr4 are redundant in controlling β cell replication. To address whether the effects of TLR2 and TLR4 on β cells are redundant or synergistic, we generated and characterized the islet phenotypes of Tlr2−/− Tlr4−/−, Tlr2−/−, Tlr4−/− and Tlr2−/− Tlr4−/− littermate mice on the B6 background after a 20-week HFD feeding starting at age 6 weeks. All mice gained weight comparably (Fig. 4a). The islet size and percentage of Ins+ β cell area per pancreas in the Tlr2−/− HFD or Tlr4−/− HFD mice were comparable to those in

**Fig. 4 | Redundant effect of TLR2 and TLR4 on β cell proliferation.** a–f. Assays using 6-week-old Tlr2−/− Tlr4−/−, Tlr2−/−, Tlr4−/− and Tlr2−/− Tlr4−/− littermates on a 20-week HFD showing: body weight (a), representative confocal images of Ins+ β cells with quantitation shown in c (scale bar: 2 mm; b,c), representative confocal images showing Ki67+Ins+ β cells with quantitation shown in e (scale bar: 100 μm (upper) and 10 μm (lower); d,e), representative confocal images showing Ccnd2 localization in Ins+ β cells (scale bars: 50 μm (upper) and 10 μm (lower); f), g. Flow cytometric analysis showing BrdU+ β cells of primary B6 mouse islets cultured in 2.8 mM or 22.8 mM glucose (Glc), treated with a combination of LPS and/or LTA for 72 h. a–e, n = 8, 9, 10, 4 mice for Tlr2−/− Tlr4−/−, Tlr2−/−, Tlr4−/− and Tlr2−/− Tlr4−/−, e, n = 74, 72, 83 and 42 islets for Tlr2−/− Tlr4−/−, Tlr2−/−, Tlr4−/− and Tlr2−/− Tlr4−/− mice. g, n = 9 mice. All the data were reproducible with at least two repeats. Values represent mean ± s.e.m. NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001, by one-way ANOVA with the Newman–Keuls post-test.
**TLR2 and TLR4 regulate islet expansion in an islet-intrinsic manner.** β cell proliferation can be controlled locally in a paracrine fashion or systemetically by hepatokines in an endocrine fashion. To distinguish between these possibilities we surgically joined CD45.1<sup>−/−</sup> Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> and CD45.1<sup>+</sup> B6 mice at age 8 weeks and, after a 3-week recovery period, we placed them on an HFD for 14 weeks. The B6-Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> parabiotic pairs exhibited similar weight gain on an HFD to control Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> and B6–B6 pairs on an HFD (Fig. 5a). Flow cytometry indicated the equilibration of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and CD19<sup>+</sup> B cells in the blood of the parabiotic pairs 3 weeks post-surgery (Fig. 5b). The Ins<sup>+</sup> β cell area did not change in the Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> or the B6 partners of the B6-Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> pairs compared with those in Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> and B6–B6 pairs, respectively (Fig. 5c), excluding a major effect of host-derived soluble factor(s) in the islet expansion of HFD Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> mice.

Next we addressed whether TLR2 and TLR4 were expressed in islets. As determined by PCR with reverse transcription (RT–PCR), Th2 and Tlr4 mRNA expressions were used in primary islets from age-matched B6 mice on an LFD or HFD for 7 months, starting at age 6 weeks (Fig. 5d). Although expression of Tlr4 mRNA decreased with age, Tlr2 expression remained unchanged in islets from 2- to 12-month-old B6 mice on an LFD (see Supplementary Fig. 6a). In humans, TLR2, but not TLR4, mRNA was greatly increased in islets isolated from T2D patients compared with the healthy controls (Fig. 5e).

To test whether the role of TLR2 and TLR4 in β cells was islet intrinsic we transplanted similar size primary islets isolated from LFD B6 and Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> mice (Fig. 6a) under the capsules of the left (B6) islets and right (Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> islets) kidneys of 10-week-old recipient B6 or Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> mice treated with a single high dose of the diabetogenic agent streptozotocin (STZ) to induce diabetes. After a 4-week recovery period, islet recipients were switched to an HFD diet (see Supplementary Fig. 6b) and steadily gained weight for a total of 14 weeks (Fig. 6b). Within a week post-transplantation, STZ-treated diabetic recipients exhibited improved glycemia (Fig. 6c), indicative of a successful islet transplantation. After 14 weeks on an HFD, Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> islet grafts grew much larger than B6 islet grafts in either B6 or Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> recipients (see Supplementary Fig. 6c). Histologic analyses indicated a much larger islet area and more Ins<sup>+</sup> cells in the Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> islet grafts compared with the B6 islet grafts (Fig. 6d). Confocal microscopy revealed elevated numbers of Ki67<sup>+</sup>Ins<sup>+</sup> β cells and more nuclear Cnd2<sup>+</sup> β cells in Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> islet grafts compared with B6 islet grafts (Fig. 6e,f). Blood vasculatization of the islet grafts was comparable between B6 and Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> islets (see Supplementary Fig. 6d), excluding a possible contribution of vasculatization in differential islet expansion. Together, these experiments argued against a role of circulating host-derived factors in islet expansion of Tlr2<sup>−/−</sup>Tlr4<sup>−/−</sup> HFD mice, and suggested that TLR2 and TLR4 deficiency affected HFD-induced β cell replication in an islet-intrinsic manner.
TLR2/TLR4 regulate HFD-induced β cell proliferation via Erk. We next explored the molecular link between TLR2/TLR4 deficiency and HFD-induced β cell proliferation. We performed unbiased global transcriptional profiling of islets from B6 and Tlr2−/−/Tlr4−/− mice after a 7-month HFD. Gene set enrichment analysis revealed a large increase in pathways linked to cell cycle progression and replication in islets from HFD Tlr2−/−/Tlr4−/− mice (see Supplementary Fig. 7a). We observed the differential regulation of ~101 genes, the levels of which were altered by more than 2.0-fold in islets from HFD Tlr2−/−/Tlr4−/− mice compared with those from HFD B6 mice (false discovery rate q < 0.05), including Reg2 (Fig. 7a and see Supplementary Fig. 7b). Reg2 is a gene highly induced in islet regeneration38 and required for compensatory islet proliferation and expansion in obesity and aging39. Similar results were obtained in islets after a short-term, 5-week HFD (Fig. 7a and see Supplementary Fig. 7c,d). The induction of Reg2 mRNA in Tlr2−/−/Tlr4−/− islets from HFD mice suggested a unique signature for the highly proliferative β cell population in the Tlr2−/−/Tlr4−/− HFD mice compared with B6 HFD mice.

We next analyzed the status of various signaling pathways known to be implicated in β cell proliferation. Phosphorylation of Erk1/2 proteins was increased by two- to threefold in islets from HFD Tlr2−/−/Tlr4−/− mice compared with HFD B6 mice (Fig. 7b), whereas phosphorylation of other kinases, including Akt, AMPK, Jnk, IκB and p-NFκB was unchanged (Fig. 7c). In line with elevated Erk activation, the upstream kinases MEK1/2 were hyperphosphorylated in islets from HFD Tlr2−/−/Tlr4−/− mice compared with HFD B6 mice (Fig. 7b), p16INK4a, a known downstream target of the Erk pathway in β cells30, was downregulated in islets from HFD Tlr2−/−/Tlr4−/− mice compared with HFD B6 mice (see Supplementary Fig. 4e). LPS + LTA treatment suppressed high glucose-induced Erk1/2 phosphorylation in islets from B6 LFD mice, which was blunted in islets from Tlr2−/−/Tlr4−/− LFD mice (Fig. 7e,f). Moreover, LPS + LTA treatment blocked the nuclear accumulation of Ccnd2 and Cdk4 proteins induced by high glucose in primary islets from B6 LFD mice in vitro (Fig. 7g and see Supplementary Fig. 8a,b). Treatment of B6 islets with either LPSs or LTA suppressed phosphorylation of Erk (see Supplementary Fig. 8c), suggesting the redundant function of TLR2 and TLR4 in blocking Erk phosphorylation. Treatment

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**Fig. 6** The effect of TLR2 and TLR4 on HFD-induced β cell proliferation is islet intrinsic. a, Representative light microscopic images showing the size of primary islets from age-matched Tlr2−/−/Tlr4−/− and B6 mice on an LFD used for transplantation under the kidney capsule: 100 B6 and 100 Tlr2−/−/Tlr4−/− primary islets were transplanted under the left and right kidney capsules of the same recipient, either B6 or Tlr2−/−/Tlr4−/−, respectively. b,c, Body weight (b) and blood glucose (c) of B6 recipients post-transplantation. The same trends were observed for Tlr2−/−/Tlr4−/− recipients (n = 4; not shown). d, Representative H&E images showing islets (arrows) from recipient kidneys received islet grafts from B6 (upper) or Tlr2−/−/Tlr4−/− (lower) mice. The dotted line outlines the boundary of the islet grafts. Scale bars: 1 mm (left) and 0.2 mm (right). e, Representative confocal images showing Ki67+ cells (e) or Ccnd2+ cells (f) of transplanted islets under kidney capsules. Scale bars: 100 μm (upper) and 20 μm (lower inset). The dotted line marks the boundary of the islets (note high autofluorescent signals of the surrounding kidney tubules); for Tlr2−/−/Tlr4−/− islets, the whole field is full of β cells (e). Representative image from four mice, each with two repeats. Values represent mean ± s.e.m. ***P < 0.001 by two-tailed, Student’s t-test (b,c), each compared with day 0.
with LPS + LTA in macrophages had the opposite effect on phosphorylation of Erk (see Supplementary Fig. 8d), pointing to a specific effect of TLR2/LTR4 activation in Erk activation in islets.

Lastly, oral administration of the small-molecule mitogen-activated protein kinase kinase (MEK) inhibitor MEK162, a Food and Drug Administration-approved drug known as binimetinib, blocked ERK activation (see Supplementary Fig. 8e), reduced the number of Ki67\^+Ins\(^+\) β cells (Fig. 7h and see Supplementary Fig. 8f) and the abundance of nuclear Ccnd2 in β cells (Fig. 7l) in 14-week-old Tlr2\(^{+/-}\)/Tlr4\(^{+/-}\) mice on an HFD for 8 weeks. Taken together, these data suggested that the TLR2/TLR4-signaling pathways in HFD mice attenuated β cell proliferation in a MEK/Erk-dependent manner.

**Discussion**

In the present study we describe a regulatory mechanism underlying the diet-induced β cell proliferation. Although activation of TLR2 and TLR4 in vitro blocks high glucose-induced β cell proliferation in primary mouse and human islets, the loss of TLR2 and TLR4, together but not individually, increases β cell proliferation and attenuates β cell senescence. This leads to massive β cell accumulation in a mouse model of diet-induced obesity. We further demonstrate that the effect of TLR2 and TLR4 on islet β cell accumulation in HFD mice is islet autonomous and depends on HFD feeding.

Our results suggest that β cell proliferation was regulated by both positive and negative signals in diet-induced obesity, with positive

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**Fig. 7 | TLR2 and TLR4 inhibit β cell proliferation via the Mek-Erk pathway.** a, Volcano plot analysis of the genome-wide cDNA profiling showing the gene expression change between Tlr2\(^{+/-}\)/Tlr4\(^{+/-}\) and B6 islets on a 7-month (upper) and 5-week (lower) HFD; n = 4 each for 7-month HFD cohorts, and 2, 4 mice for 5-week HFD Tlr2\(^{+/-}\)/Tlr4\(^{+/-}\) and B6 mice, respectively. The y-axis P value calculated by intensity-based moderated T-statistic (IBMT)-regularized t-test. b–d, Immunoblots showing the expression and phosphorylation of Mek1/2 and Erk1/2 (c, cells (Fig. 7i) and Ccnd2 in β cells (Fig. 7l) in 14-week-old Tlr2\(^{+/-}\)/Tlr4\(^{+/-}\) mice on an HFD for 8 weeks. Taken together, these data suggested that the TLR2/TLR4-signaling pathways in HFD mice attenuated β cell proliferation in a MEK/Erk-dependent manner.

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Our results suggest that β cell proliferation was regulated by both positive and negative signals in diet-induced obesity, with positive
molecule antagonists, genetic editing or bipotent TLR2/TLR4 antibodies, may enhance human β cell replication. Delineation of the immediate downstream pathways of TLR2/TLR4 in β cells may also help guide future efforts to target them as a promising strategy to expand β cells in diabetes.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41590-019-0396-z.

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Author contributions

Y.J. and S.S. designed and performed most of the experiments. N.S., L.B.D., H.K., D.A. and M.M. assisted in some of the experiments. I.S., R.N.K., B.A.C. and M.G. measured TLR expression in human and aged mouse islets. S.A.S. provided helpful discussions and suggestions. C.L. and A.N. provided human islets. Y.J.H., Y.W. and J.O. performed functional analysis of the mouse islets. S.K. performed microarray analyses. Y.J. and S.S. wrote the methods and legends. L.Q. designed the experiments, managed the project and wrote the manuscript. All authors edited and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to L.Q.

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Mice. Tlr2+/−Tlr4−/− mice were originally generated by S. Akira (Osaka University) and provided to us by L. Hjara (University of Washington) and David Russell (Cornell University). These Tlr2+/−Tlr4−/− mice have been backcrossed to the B6 background for six generations as previously described44,45. Non-littermate, wild-type mice (no. 000664) were purchased from the Jackson Laboratory and bred in our facility. To generate Tlr2+/−Tlr4+/− and Tlr2−/−Tlr4+/− as littermates, we crossed Tlr2−/−Tlr4−/− mice with B6 mice to generate Tlr2−/−Tlr4−/− mice, which then intercrossed to generate Tlr2−/−Tlr4−/−, Tlr2+−Tlr4−/− and Tlr2−/−Tlr4−/− wild-type littermates. All mice were reared, reared and housed in our specific pathogen-free facility at the University of Michigan Medical School. Age- and gender-matched mice were used as cohorts in the studies. Mice were fed an LFD composed of 13% fat, 67% carbohydrate and 20% protein from Harlan Teklad (no. 2914), as littermates, we used as cohorts in the studies. Mice were fed an LFD composed of 13% fat, 67% carbohydrate and 20% protein from Harlan Teklad (no. 2914), and continued once daily for the next 2–3 days. After a 4-week recovery, mice were given an HFD for 14 weeks, and the kidneys were collected for histologic analysis after euthanasia.

Parabiosis. Parabiosis was performed as previously described49. Female mice in the same cage were anesthetized using an isoflurane vaporizer and then shaved thoroughly, starting at 1 cm above the elbow to 1 cm below the knee, on the side to be connected during parabiosis. Then a longitudinal skin incision was performed at the shaved side and the skin was gently detached from the subcutaneous fascia to create about 0.5 cm of free skin. Mice were then joined by attaching the olecranon and the knee together, and the skin was sutured ventrally from the elbow toward the knee, followed by continuous dorsal stitches. Three weeks after the surgery, parabiotic pairs were fed on an HFD and monitored for body weight and blood glucose levels. After 14 weeks of HFD feeding, mice were sacrificed for histologic examination of the pancreas. To test blood chimerism, CD45.1−B6 mice (B6.SJL−129SvPep/Cd57, Jackson Laboratory) and CD45.1−Tlr2−/−Tlr4−/− mice were surgically joined, and the percentage of CD45.1+/CD45.1− hemopoietic cells in the blood of paired mice was determined by flow cytometry.

Drug treatment in vivo. For S961 studies, 45-week-old mice under an LFD or HFD were randomly and independently implanted with control (CET) or vehicle (S961) filled with insulin receptor antagonist S961 (ref. 46) or vehicle phosphate-buffered saline (PBS). Mice were infused with S961 at the dose of 10 nmol day−1 for 2 weeks, followed by euthanasia and histologic analysis of islets. For MEK162 studies, Tlr2−/−Tlr4−/− mice on an HFD for 8 weeks were orally gavaged with MEK162 (20 mg kg−1 body weight, LC Laboratories) or vehicle, twice daily for 2 days, followed by euthanization and histologic analysis.

Purification of primary mouse islets. The duodenal opening of the bile duct was clamped with a hemostat, and the bile duct was cannulated and perfused with 2 ml Liberase TL Research Grade (0.3 mg ml−1, Roche) in RPMI 1640 medium (Invitrogen). The perfused pancreas was removed and incubated at 37 °C for 20 min, after which the digestion was stopped by adding 20 ml of cold RPMI 1640 medium containing 10% serum (Fisher). Digested pancreas was then dissociated mechanically by vigorous shaking. After two washes in RPMI 1640 medium, the digested tissues were filtered through a 450-μm nylon mesh and resuspended in a Histopaque 1077 (Sigma)/RPMI 1640 medium and centrifuged at 950g for 20 min. The islets were collected from the interface between the medium and Histopaque, and resuspended in RPMI 1640 medium containing 10% serum. Islets were washed three times and hand-picked under a light microscope. The islets were cultured overnight in RPMI 1640 medium containing 10% serum for analyses.

Human islets. Human islets were received from the accredited Human Islet Research Center at the University of Pennsylvania. The pancreata were obtained from the donors through the local organ procurement organization. The islets were isolated using the protocols of the Clinical Islet Transplantation standard operating procedures for all islet isolations. The islets were distributed following the guidelines of the Clinical Islet Transplantation consortium protocol50. Briefly, the pancreas was digested after intraduodenal injection of Collagenase & Neutral Protease (SERVA) relatively uniformly at 1:50, PCNA (Santa Cruz, sc-56, 1:10) and Ki-67 (Abcam, ab15580, 1:50) were used. On arrival, islets were treated and monitored for body weight and blood glucose levels. After 14 weeks of HFD feeding, mice were sacrificed for histologic examination of the pancreas. To test blood chimerism, CD45.1−B6 mice (B6.SJL−129SvPep/Cd57, Jackson Laboratory) and CD45.1−Tlr2−/−Tlr4−/− mice were surgically joined, and the percentage of CD45.1+/CD45.1− hemopoietic cells in the blood of paired mice was determined by flow cytometry.
In vitro islet treatment. Islets were cultured in RPMI 1640 culture medium (10% FCS, 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), 33 mM glucose, 5 mM Fura-2/AM (Molecular Probes) and 2.5 μM rhodamine 123 (Sigma) for 30 min. The islets were then loaded into the temperature-equilibrated, microfluidic device mounted on an inverted epifluorescence microscope (Leica DMi800B). The Krebs’ Ringer buffer containing 14 mM glucose (20 min) or 30 mM KCl (15 min) was then administered to the islets. Dual-wavelength Fura-2/AM was excited at 340 and 380 nm and fluorescent emission was detected at 510 nm. These images were collected with a CCD (Retiga-SRV, Fast 1394, Qimaging). Simple PCI software (Hamamatsu Corp.) was used for image acquisition and analysis. Intracellular Ca++ was expressed as a ratio of fluorescent emission intensities F340/F380. All fluorescence signals were expressed as ‘change in percentage’ after being normalized against basal intensity levels established before stimulation. Perifusate samples were collected every 2 min at the outlet at a flow rate of 250 μl/min for insulin analysis using the Merckodia Rodent Insulin ELISA kit (Uppsala).

Transmission electron microscopy. Pancreatic tissues were collected from mice after a 51-week HFD feeding and immediately sliced into 1- to 2-mm² pieces and fixed, stained, dehydrated and embedded in Polybed 812 (Polysciences). Embedded samples were cut with a Leica Ultratric Ultramicrotome system and images were taken using a JEOL-1400 transmission electron microscope on a fee-for-service basis at the Electron Microscopy and Histology Core Facility of Weill Cornell Medical College.

Insulin and multiplex ELISA analyses. Serum insulin levels were measured after a 4-h fast using an ultrasensitive mouse/tat insulin ELISA kit (Crystal Chem) following the supplier’s instruction. For multiplex analysis, serum samples were collected from the indicated mouse cohorts after a 4-h fast and levels of various hormones were analyzed by Bio-Plex Pro Mouse Diabetes Panel 8-Plex (catalog no. 171-R7001M) following the manufacturer’s instruction.

RNA extraction, RT–PCR and qPCR. RNA extraction from cells and animal tissues, RT–PCR and qPCR analyses were performed as previously described5. The qPCR data were collected using Roche LightCycler 480 and gene expression was normalized to the ribosomal β2 gene for each sample. The qPCR primers used for house and human genes are as follows:

- **hTLR4** (TCATAGCTGCGGCTTGTTTCT, GAGGAGGAGAAAGGAGGG), hTLR9 (GGAGGAGGAGAAAGGAGGG), hCD45 (GGGAGGAGAAAGGAGGG), hCD19 (GGGAGGAGAAAGGAGGG)
- **mTlr2** (GTTCATCTGGTGGTCTTCC, ACACTCGGAATCTCTC), mTlr8 (GGAGGAGGAGAAAGGAGGG), mCD45 (GGGAGGAGAAAGGAGGG), mCD19 (GGGAGGAGAAAGGAGGG)
- **mTlr2**, mTlr8, mCD45, mCD19 (GCTTTCACCTCTGCCTTCAC, GAAACTGCCATGTTTGAGCA)

The qPCR conditions were: 94 °C for 5 min (94 °C for 15 s, 58 °C for 15 s and 72 °C for 30 s), 30–40 cycles according to individual template, followed by 70 °C for 10 min.

Microarray. Islets were collected from mice on an HFD for 5 weeks or 7 months and snap-frozen in liquid nitrogen. RNA was extracted as described above. RNA quality and concentration were determined using the RNA 6000 Nano kit on an Agilent 2100 bioanalyzer. The cDNA microarray of islet RNA was performed as previously described5. The Gene Expression Omnibus accession number for the microarray is GSE101392.

**Power analysis of the animal size.** Based on sample size formula of the power analysis, N = 8(β/2)1/2(1 – β/2)/(β/2), to reach the error = 0.05, power = 0.80, percentage change in means (PC) = 20%, coefficient of variation (CV) = 10 to ~15% (varies between the experiments), four to six mice per group are the minimal number of animals required using a one-tailed t-test assuming a Student’s t-test. Since we used a total of four to six mice per group, we routinely used a total of four to six mice in each study to ensure adequate power. The mice in each group were randomly chosen based on the age, genotype and sex.

**Statistical analysis.** Results were expressed as mean ± s.e.m. We performed the normality Kolmogorov–Smirnov test using the Prism software v6 or v8 first,
then parametric tests were performed on the experimental groups. Comparisons between groups were made using unpaired, two-tailed, Student’s t-test (two groups) and one-way analysis of variance (ANOVA) with the Newman–Keuls post-test (multiple groups). All experiments were repeated at least twice or performed with independent samples.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Microarray data supporting the findings of this study have been deposited into the Gene Expression Omnibus with accession number GSE101392. All other relevant data are available from the corresponding author upon request.

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
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- A description of all covariates tested
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- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
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- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
Confocal data of beta cell proliferation and beta cell mass were collected using NIS-elements C 4.60.00 (Nikon); H&E images of pancreatic sections were collected using Aperio Imagescope software v102.0.4.6; Flow data were collected using the FACSdiva v6.2 software (BD Biosciences); Western Blot data were collected by Image Lab software 4.1 (Bio-rad).

Data analysis
Prism software v6 and v8 was used for statistics analysis; Imaging data of beta cell proliferation and beta cell area were analyzed using the ImageJ, Fiji and Aperio Imagescope software v12.3.2.5030; Flow data were analyzed using the FACSDiva v6.2 software [BD Biosciences] and Flowjo (Flowjo.com); Western Blot data were analyzed by Image Lab software 4.1(Bio-rad).

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We state in our manuscript that Gene Expression Omnibus (GEO) accession number for the microarray study is GSE101392 [which is now public].
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Life sciences study design

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Sample size
Based on sample size formula of the power analysis, N=8/(CV^2)[1+(1-PC)/2][PC], to reach the error = 0.05, Power = 0.80, percentage change in means (PC) = 20%, coefficient of variation (CV) = 10—15% (varies between the experiments). 4-6 mice per group are the minimal number of mice to obtain statistical significance. Together with our prior experience, we routinely used a total of 4-6 mice or more in each study to ensure adequate power.

The sample size for each animal experiment was described in the figure legend. The way of predetermining sample size is described in the "Power analysis of the animal size".

Data exclusions
No animals or samples were excluded from the analysis.

Replication
All experiments were repeated at least twice or performed with independent samples. All experiments were successfully repeated.

This is described in text methods part, section "Statistical Analysis". The exact repeat times of experiments are indicated in the figure legends.

Randomization
Mice in each group were randomly chosen based on the age, genotype and gender.

Randomization of the mice is described in the methods.

Blinding
The investigators were not blinded to allocation during experiments and outcome assessment, as the differences described in this paper were very large.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
|     | Antibodies            |
|     | Eukaryotic cell lines |
|     | Palaeontology         |
|     | Animals and other organisms |
|     | Human research participants |
|     | Clinical data         |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
|     | ChiP-seq              |
|     | Flow cytometry        |
|     | MRI-based neuroimaging |

Antibodies

| Antibodies used |
|-----------------|
| Confocal immunofluorescent and immunohistochemistry staining: |
| Insulin (Abcam, #ab7842 or Linco # 4011, 1:200) |
| Glucagon (Sigma, #SAA4200685, clone K79b810, 1:1000) |
| Ki-67 (Abcam, #ab15580, 1:50) |
| PCNA (Santa Cruz, #sc-56, 1:100) |
| CD31 (Santa Cruz, #sc-1506, 1:100) |
| PDX1 (Cell Signaling, #5679, clone D59H3, 1:100) |
| Cdk4 (Santa Cruz, #sc-260, 1:100) |
| Ccnd2 (Santa Cruz, #sc-593, 1:100) |

Flow cytometric antibodies:
| BrdU-PE (# 556029, clone MOPC-21, 5ul/sample, BD Biosciences) |
| Insulin (Abcam, #ab7842, 1:100) |
| p16 (Santa Cruz, #sc-377412, 1:100) |
| CD4 (Biorad, #100408, clone GK1.5, 1:200) |
| CD8 (Biorad, #100712, clone Lyt2, 1:200) |
| CD45.1 (Biorad, #110726, clone Ly-5.1, 1:200) |
| CD19 (Biorad, #115912, clone 6D5, 1:200) |
Antibodies used in immuno blotting were:
HSP90 (Santa Cruz, #sc-7947, 1:6,000)
JNK1/2 (Santa Cruz, #sc-571, 1:2,000)
p-Thr202/Tyr204 ERK1/2 (Cell Signaling, #4370, 1:2,000)
ERK1/2 (Cell Signaling, #9102, 1:2,000)
p-Ser217/221 MEK1/2 (Cell Signaling, #9121, 1:1,000)
MEK1/2 (Cell Signaling, #4694, 1:1,000)
p-Thr183/Tyr185 JNK1/2 (Cell Signaling, #9255S, 1:2,000),
p-Ser473 AKT (Cell Signaling, #9271S, 1:2,000)
AKT (Cell Signaling, #9272, 1:2,000)
IκBα (Cell Signaling, #9242, 1:2,000)
p-Ser536 NFκB p65 (Cell Signaling, #3033, 1:1,000)
p-Thr172 AMPKα (Cell Signaling, #2535, 1:2,000)
AMPKα (Cell Signaling, #2532, 1:2,000)

Validation
The antibodies are from best available vendors and were validated by the vendors. Validation data are available on the manufacturer’s websites (checkable via the category # information above). We further verified specificity by the size of the band in Western blot, intracellular distribution in confocal image data, comparison with isotype controls as well as positive and negative control samples.

Eukaryotic cell lines
Policy information about cell lines
Cell line source(s) Ins-1 cell line were originally obtained from Thermo Fisher. The information is described in the Methods.
"Cell line."

Authentication The cells have been authenticated by morphology, insulin expression and insulin secretion.

Mycoplasma contamination No Mycoplasma contamination after testing.

Commonly misidentified lines (See IDTAC register) The cell line is not listed in that database.

Animals and other organisms
Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals Mice were all in the C57BL/6J background with different genetic modifications. This is described in methods part under the section of "Mice." Both males and females were used, and age are specifically indicated in the text and legends.

Wild animals No wild animals used in this study.

Field-collected samples No field-collected samples used in this study.

Ethics oversight All animal procedures were approved by and done in accordance with IACUC at Cornell University (#2007-0051) and the University of Michigan Medical School [APRO00006888].

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants
Policy information about studies involving human research participants

Population characteristics Human islet studies:
(a) Donor info for Q-PCR analysis of TLR expression in islets from the Joslin Diabetes Center (R.K.):
Gender: Ethnicity/Race Age BMI Diabetic donor status
Male: Hispanic/Latino 49 31.3 Non-DM
Female: White 52 31.4 Non-DM
Female: Hispanic/Latino 24 19.5 Non-DM
Male: Hispanic/Latino 58 31.2 Non-MD
Female: White 36 42.7 Non-DM
Male: Caucasian 49 28.2 T2D
Male: Unknown 53 31.0 T2D
Male: Caucasian 48 35.8 T2D
Male: Asian 49 23.9 T2D
Male: Asian 49 23.9 T2D

(b) Donor info for flow cytometric analysis of β cell proliferation in islets from the Integrated Islet Distribution Program (IIDP) at UPENN (C.L. and A.N.):
Gender: Ethnicity/Race Age BMI Diabetic donor status
Female (Donor 1): Hispanic 24 32.2 Non-DM
Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
The information is detailed on page 29-30 of the main text under the sections of ‘Flow cytometric analysis of β cell proliferation and senescence’ and ‘Flow cytometric analysis of parabiosis’.

Instrument
Samples were collected using BD LSR cell analyzer

Software
Sample are collected by using the FACSDiva v6.2 (BD Biosciences) and data are analyzed with using FACSDiva v6.2 and Flowjo (Flowjo.com).

Cell population abundance
N/A

Gating strategy
Gates and boundaries were clearly visible in the figures.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.