RESEARCH ARTICLE

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JNK1 ablation improves pancreatic β-cell mass and function in db/db diabetic mice without affecting insulin sensitivity and adipose tissue inflammation

Arianna Mazzoli[1] | Claudia Sardi[1] | Ludovic Breasson[1] | Franziska Theilig[2] |
Barbara Becattini[1] | Giovanni Solinas[1]

[1]The Wallenberg Laboratory and Sahlgrenska Center for Cardiovascular and Metabolic Research, Department of Molecular and Clinical Medicine, Institute of Medicine, University of Gothenburg, Gothenburg, Sweden

[2]Institute of Anatomy, Christian Albrechts-University Kiel, Kiel, Germany

Correspondence
Giovanni Solinas, The Wallenberg Laboratory and Sahlgrenska Center for Cardiovascular and Metabolic Research, Department of Molecular and Clinical Medicine, Institute of Medicine, University of Gothenburg, Gothenburg, Sweden.
Email: Giovanni.Solinas@wlab.gu.se

Abstract

The cJun N-terminal Kinases (JNK) emerged as a major link between obesity and insulin resistance, but their role in the loss of pancreatic β-cell mass and function driving the progression from insulin resistance to type-2 diabetes and in the complications of diabetes was not investigated to the same extent. Furthermore, it was shown that pan-JNK inhibition exacerbates kidney damage in the db/db model of obesity-driven diabetes. Here we investigate the role of JNK1 in the db/db model of obesity-driven type-2 diabetes. Mice with systemic ablation of JNK1 (JNK1$^{-/-}$) were backcrossed for more than 10 generations in db/+ C57BL/KS mice to generate db/db-JNK1$^{-/-}$ mice and db/db control mice. To define the role of JNK1 in the loss of β-cell mass and function occurring during obesity-driven diabetes we performed comprehensive metabolic phenotyping, evaluated steatosis and metabolic inflammation, performed morphometric and cellular composition analysis of pancreatic islets, and evaluated kidney function in db/db-JNK1$^{-/-}$ mice and db/db controls. db/db-JNK1$^{-/-}$ mice and db/db control mice developed insulin resistance, fatty liver, and metabolic inflammation to a similar extent. However, db/db-JNK1$^{-/-}$ mice displayed better glucose tolerance and improved insulin levels during glucose tolerance test, higher pancreatic insulin content, and larger pancreatic islets with more β-cells than db/db mice. Finally, albuminuria, kidney histopathology, kidney inflammation and oxidative stress in db/db-JNK1$^{-/-}$ mice and in db/db mice were similar. Our data indicate that selective JNK1 ablation improves glucose tolerance in db/db mice by reducing the loss of functional β-cells occurring in the db/db mouse model of obesity-driven

diabetes, without significantly affecting metabolic inflammation, steatosis, and insulin sensitivity. Furthermore, we have found that, differently from what previously reported for pan-JNK inhibitors, selective JNK1 ablation does not exacerbate kidney dysfunction in db/db mice. We conclude that selective JNK1 inactivation may have a superior therapeutic index than pan-JNK inhibition in obesity-driven diabetes.

KEYWORDS

hyperglycemia, insulin secretion, kidney, metabolic stress

1 | INTRODUCTION

Obesity and type-2 diabetes are a major public health issue of modern societies, but the molecular mechanisms linking excessive adiposity to loss of glucose homeostasis are only partially understood.[1] It was found that the cJun N-terminal Kinases (JNK) are chronically activated during obesity in different tissues and JNK activity was implicated in the pathogenesis of obesity and insulin resistance.[2-5] There are three JNK: JNK1 and JNK2 which are ubiquitously expressed; and JNK3 whose expression is restricted to few cell types including neurons and the pancreatic β-cell.[2-5] JNK1 and JNK2 activities were shown to promote diet-induced obesity and insulin resistance[6,7] whereas JNK3 activity reduces food intake and adiposity and thus protects mice from obesity and, as consequence, from obesity-driven insulin resistance.[8] JNK1 and JNK2 activities appear to be redundant in promoting adiposity, metabolic inflammation, and insulin resistance in obese mice, but with JNK1 being the most important isoform.[6,7,9,10] Hence, JNK1 and JNK2 specific inhibitors, which are highly selective against JNK3, may be effective therapeutics for the treatment of obesity-driven insulin resistance. However, to successfully exploit isoform-selective JNK inhibition for the treatment of obesity-driven diabetes, a better understanding of the role of the different JNK isoforms in the progression from insulin resistance to diabetes and in the complications of diabetes is necessary.

JNK activity is induced by most cellular stressors in virtually all cell types[2-5] and, depending on the specific context, JNK activation can result in dramatically different biological outcomes.[2] Indeed, JNK activity can either drive cell death or promote cell survival and proliferation, depending on the magnitude and duration of JNK activation and on the specific cell type.[2] Because of the importance of the context in determining the outcome of JNK signaling in stress response, it is necessary to evaluate the effects of JNK inactivation on different stages of the progression of obesity-driven diabetes. However, while the role of different JNK isoforms in obesity-driven insulin resistance has been extensively investigated,[6,7,9-20] the effects of JNK ablation on obesity-driven loss of functional pancreatic β-cells was not investigated to the same extent.

Several studies have consistently shown that JNK inhibition protects insulin producing cells from apoptosis and defective insulin expression and secretion in different cell culture models of β-cell stress.[17,21-30] However, it has also been shown that the role of JNK in β-cell stress response depends on the specific isoform and experimental conditions. Indeed, studies indicate that β-cell JNK3 signaling promotes β-cell mass and function[31-33]; and short term exposure of cultured β-cells to a low dose of IL-1β improves insulin secretion by a JNK dependent mechanism.[34] Finally, it has also been shown that JNK1 activity can protect the insulin producing cell-line INS1 from apoptosis induced by high levels of glucose and palmitate.[26] This dual role of JNK in cell culture models of β-cell stress response is overall consistent with the more general action of JNK activity in cell stress response.[2] Thereby, the role of JNK isoforms in β-cell dysfunction needs to be investigated in a specific in-vivo model of obesity-driven diabetes. However, the role of different JNK isoforms in β-cell failure was much less investigated in-vivo than in cell culture. In one study, sustained JNK activity was induced in pancreatic β-cells of mice by expression of a constitutively active upstream kinase (MKK7D), leading to loss of β-cell function and glucose intolerance.[35] Another study reported that mice lacking a functional JNK1 (JNK1⁻/⁻) are protected from streptozotocin (STZ)-induced islet injury[36]; and it was recently shown that mice treated with a pan-JNK inhibitor and mice lacking JNK1 (JNK1⁻/⁻) are protected from β-cell dysfunction induced by chronic glucose infusion.[37] These models indicate that JNK1 ablation protects islets from the stress induced by STZ and hyperglycemia in-vivo in lean mice, which is a different context than the loss of β-cell mass and function occurring in obesity-driven diabetes. db/db mice in the C57BL/KS genetic background develop obesity and diabetes due to insulin resistance and loss of β-cell mass and function and, to our understanding, is the animal model which, with high penetrance, more closely reproduces the loss of β-cell mass and function occurring in obesity-driven type-2 diabetes in humans. One study reported that pan-JNK inhibition improved glycaemia and insulin tolerance in db/db mice, but the effects of JNK inhibition on β-cell mass and function were not evaluated in this study.[38] Furthermore, the authors also observed that pan-JNK inhibition exacerbated albuminuria in db/db mice, which poses a significant concern for the safety of pan-JNK inhibitors in the

treatment of obesity-driven diabetes.[38] However, it is possible that the beneficial effects of JNK inhibition on obesity-driven diabetes may be dissociated from the deleterious effects on kidney function by targeting a specific JNK isoform.

Because JNK1 is the dominant JNK isoform in obesity and insulin resistance,[6] we have generated db/db-JNK1$^{-/-}$ mice and db/db control mice to define the specific role of the JNK1 isoforms in this in-vivo model of obesity-driven β-cell failure and albuminuria.

2 | MATERIAL AND METHODS

2.1 | Mice and in-vivo experiments

JNK1$^{-/-}$ mice in C57BL/6J background were backcrossed with db/+ mice in C57BL/KS genetic background (Jackson Laboratories) for 10 generations to generate db/+-JNK1$^{-/+}$ mice in pure C57BL/KS genetic background. db/+-JNK1$^{-/+}$ C57BL/KS were bred to generate littermate db/+-JNK1$^{-/-}$ mice and db/+ mice. Littermate db/+-JNK1$^{-/-}$ mice and db/+ mice were bred in parallel at the same time to generate age matched db/db-JNK1$^{-/-}$ mice (experimental) and db/db mice (controls) as well as db/+ lean non-diabetic mice. Only male mice were used for experiments. Mice were kept at the Experimental Biomedicine facility of the University of Gothenburg at 22°C under 12-hours light/dark cycles and were fed a chow diet. Body weight and fed glycaemia were recorded weekly. For the glucose tolerance test (GTT) mice were fasted for 4 h and then received 1 g D-glucose/kg body weight by intraperitoneal injection, blood was collected from the tail vein every 30 min for a total period of 120 min and glucose was measured using a glucometer. Insulin levels of serum samples collected during the GTT were measured by ELISA (Crystal Chem, #90080). Insulin tolerance test (ITT) was performed similarly to GTT, but instead of glucose mice received an intraperitoneal injection of 1 IU of insulin/kg body weight. Differences in the curves for GTT, ITT, and insulin levels during GTT of db/db and db/db-JNK1$^{-/-}$ mice were analyzed by two-way ANOVA for statistical significance. Mice were anesthetized with isoflurane (Baxter KDG 9623) and sacrificed by bleeding. All animal experiments were performed using protocols approved by the Research Animal Ethics Committee (Gothenburg, Sweden) and were performed according to local regulations and guidelines.

2.2 | Molecular and histopathological analysis of liver and adipose tissue

Liver and adipose tissue of db/db-JNK1$^{-/-}$ mice and db/db control mice were collected and a portion was fixed in 4% buffered formaldehyde and the rest was snap-frozen

into liquid nitrogen and grinded to powder in liquid nitrogen using mortar and pestle to extract RNA according to the guanidinium thiocyanate extraction method.[39] Total RNA quality and quantity was evaluated on a denaturing agarose gel. RNA was retro-transcribed using a commercial kit (Promega), and cDNA used to perform real-time quantitative PCR using a commercial SYBR-Green mix (Biorad) and specific primer sequences[11,40,41] listed in Table S1. For immunostaining, after fixation in 4% buffered formaldehyde, liver and fat tissues were embedded in paraffin, and cut into 5 μm thick sections. Hematoxylin and eosin staining of paraffin-embedded liver sections from the mice above was performed to evaluate hepatic steatosis, and three fields/section were scored blindly. Images were acquired at a 10× magnification. The number of adipose tissue crown-like structures (CLS) was quantified by immunostaining of paraffin embedded sections of adipose tissues from of db/db-JNK1$^{-/-}$ mice and db/db control mice using antibodies against Mac2 (Cederlane, #CL8942AP) as previously reported.[41,42] Images were acquired at a 20× magnification on a Zeiss AxioPlan microscope (AxioVision Software).

2.3 | Pancreatic insulin content, islet morphometry and composition, β-cell proliferation and islet inflammation

To evaluate the pancreatic insulin content, a portion of the pancreas of db/db-JNK1$^{-/-}$ mice and db/db control mice was collected, snap-frozen in liquid nitrogen, grinded to powder in liquid nitrogen using a mortar and pestle. 100 μl of Acid-Ethanol (7.5 ml EtOH 100%, 2.35 ml dd H$_2$O, 150 μl HCl 37%) was added to 100 μl of frozen pancreas powder in a 1.5 ml Eppendorf. The tissue was homogenized (Polytron) while in ice, centrifuged at 7000 rpm for 10 min at 4°C and transferred to a new Eppendorf.[41] The sample was diluted in PBS for protein quantitation and insulin content was measured by ELISA using a commercially available kit according to the manufacturer instructions (Crystal Chem, #90080).

For analysis of islet size and composition pancreas from db/+, db/db, and db/db-JNK1$^{-/-}$ mice were fixed in 4% buffered formaldehyde, embedded in paraffin, and cut into 5 μm thick sections. Paraffin embedded sections of pancreas from all three mice groups were stained using specific antibodies against insulin (Dako, #IR002), glucagon (Sigma-Aldrich, #G2654), and Nkx6.1 (DSHB, #F55A10) and slides were stained with DAPI as described.[40]

Images of pancreatic sections were acquired at a 5× and 20× magnification respectively to evaluate the number and size of pancreatic islets. Two random fields per section for each mouse were acquired and islets were counted and measured with the ImageJ software. Images were acquired at 40× magnification to evaluate islet cellular composition; three

randomly selected islets were analyzed for each mouse; (5 db/+, 6 db/db-JNK1$^{-/-}$ mice and 6 db/db control mice).

2.4 | β-cell proliferation, apoptosis, and islet inflammation

For analysis of β-cell proliferation, paraffin embedded sections from db/db, and db/db-JNK1$^{-/-}$ mice were co-stained with antibodies against insulin (Dako, #IR002), Nkx6.1 (DSHB, #F55A10), and the proliferation marker Ki-67 (Cell Signaling, #12202) followed by DAPI staining. Images were acquired at 40× magnification; three randomly selected islets for each mouse. To assess β-cell apoptosis on the mice above, TUNEL analysis was performed according to manufacturer's instructions (TMR Red-Roche) followed by insulin and DAPI co-staining. Images were acquired at 40× magnification; three randomly selected islets for each mouse.

Islet inflammation was evaluated by quantifying the number of islet-associated macrophages. Pancreatic sections from the mice above were co-stained using specific antibodies against the macrophage marker Mac2 and with anti-insulin antibodies to mark β-cells, and stained with DAPI. Images were acquired at 20× magnification and the number of islet-associated macrophages per islet surface was quantified.

All images were acquired on Zeiss AxioPlan microscope (AxioVision Software).

All antibodies used for immunohystochemistry and fluorescence staining are listed in Table S2.

2.5 | Functional, morphological, and molecular analysis of kidney function

To assess glomerular function, spot urines were collected from db/db-JNK1$^{-/-}$ mice and db/db control mice at the time of sacrifice and were used to measure the albumin creatinine ratio (μg/mg) with a commercial kit (Abcam). For kidney histopathological analysis, paraffin-embedded kidney tissue was cut into 5 μm sections and stained with Sirius red or Periodic Acid Schiff (PAS). Glomeruli were analyzed for features of diabetic nephropathy including thickening of the glomerular basement membrane, mesangial expansion, diffuse or nodular glomerulosclerosis, and arteriolar hyalinosis. The tubuloinsterstitium was judged for morphological appearance of the nephron segment, occurrence of tubular dilations or apoptotic tubules, tubular basement membrane thickening and fibrosis.

To assess oxidative stress and inflammation in kidney, a kidney from db/db-JNK1$^{-/-}$ mice and db/db control mice was collected snap frozen and grinded to powder using mortar and pestle in liquid nitrogen. The frozen kidney powder was used to measure the levels of oxidative damage marker malondialdehyde (MDA) using a commercial kit (ABCAM) and to extract total RNA to measure the mRNA levels of antioxidant genes and of markers for macrophages, macrophage activation, and inflammatory cytokines by real-time PCR as described above for liver and adipose tissue.

2.6 | Statistical analysis

Data are expressed as means ± standard errors. Statistical significance was defined by p-value less than 0.05. For statistical analysis of the differences in the curves from multiple data points of two groups, such as GTT, ITT, and insulin levels during GTT two-way ANOVA and Bonferroni post-test were used, whereas either Student t-test or Mann-Whitney were used for simple comparisons (e.g., area under the curve). Statistical analysis was performed using the GraphPad Software.

3 | RESULTS

3.1 | JNK1 ablation improves glucose homeostasis and insulin levels in db/db mice

To learn on the role of JNK1 in the loss of β-cell mass and function occurring in obese diabetic patients we ablated JNK1 in db/db mice (db/db-JNK1$^{-/-}$), a genetic model of obesity-driven diabetes. In this model, genetically obese mice fail to develop and sustain the insulin production capacity necessary to compensate for insulin resistance because of insufficient β-cell mass and function.

Compared to db/db control animals, db/db-JNK1$^{-/-}$ mice showed reduced body weight at weaning (4 weeks of age), a difference which tended to close over time, and that was minimal by the age of 10–12 weeks (Figure 1A), which is consistent with what previously reported in ob/ob C57Bl/6J mice lacking JNK1.[7]

At weaning, both db/db mice and db/db-JNK1$^{-/-}$ mice showed a mild fed hyperglycemia, which rapidly and dramatically worsened over time, and blood glucose levels were similar between genotypes at weaning and at the time of sacrifice (12 weeks-old mice) (Figure 1B). We observed an overall tendency for db/db-JNK1$^{-/-}$ mice to display lower glucose levels than control db/db mice, but this difference did not reach statistical significance (Figure 1B). A similar trend for lower glycemia in db/db-JNK1$^{-/-}$ mice was also observed during insulin tolerance test (ITT) for 11 weeks-old mice, but also these differences did not achieve statistical significance (Figure 1C-E). Furthermore, this trend was largely explained by a tendency toward an improved fasting glycemia in db/db-JNK1$^{-/-}$ mice as the trend disappeared when data on ITT were expressed as percentage of blood glucose from the baseline (Figure S1).
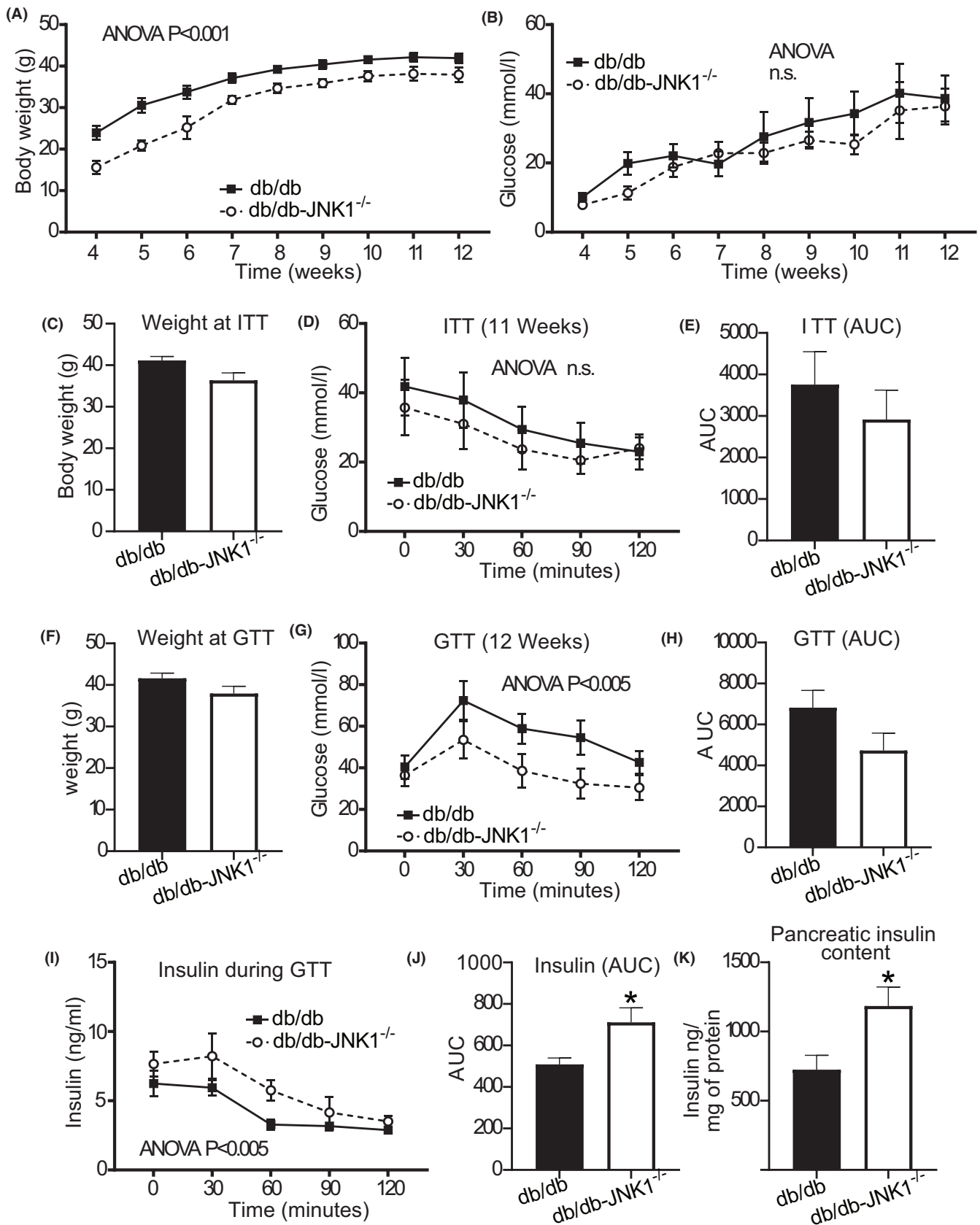
FIGURE 1 JNK1 ablation improves glucose tolerance and insulin levels in db/db diabetic mice. (A) Growth curves of db/db and db/db-JNK1$^{-/-}$ mice fed a chow diet. (B) Development of hyperglycemia in db/db and db/db-JNK1$^{-/-}$ mice. (C) Body weight of mice at the insulin tolerance test (ITT) time point. (D) ITT of mice at age 11 weeks. (E) Area under the curve (AUC) of the ITT in panel D. (F) Body weight of mice at the glucose tolerance test (GTT) time point. (G) GTT of mice at age 12 weeks. (H) AUC of the GTT in panel G. (I) Serum insulin levels during the GTT in panel G. (J) AUC of the insulin curve in panel I. (K) Pancreatic insulin content of mice at 12 weeks of age. Data are expressed as means ± standard errors, n = 9–8 mice/group, *$p < 0.05$
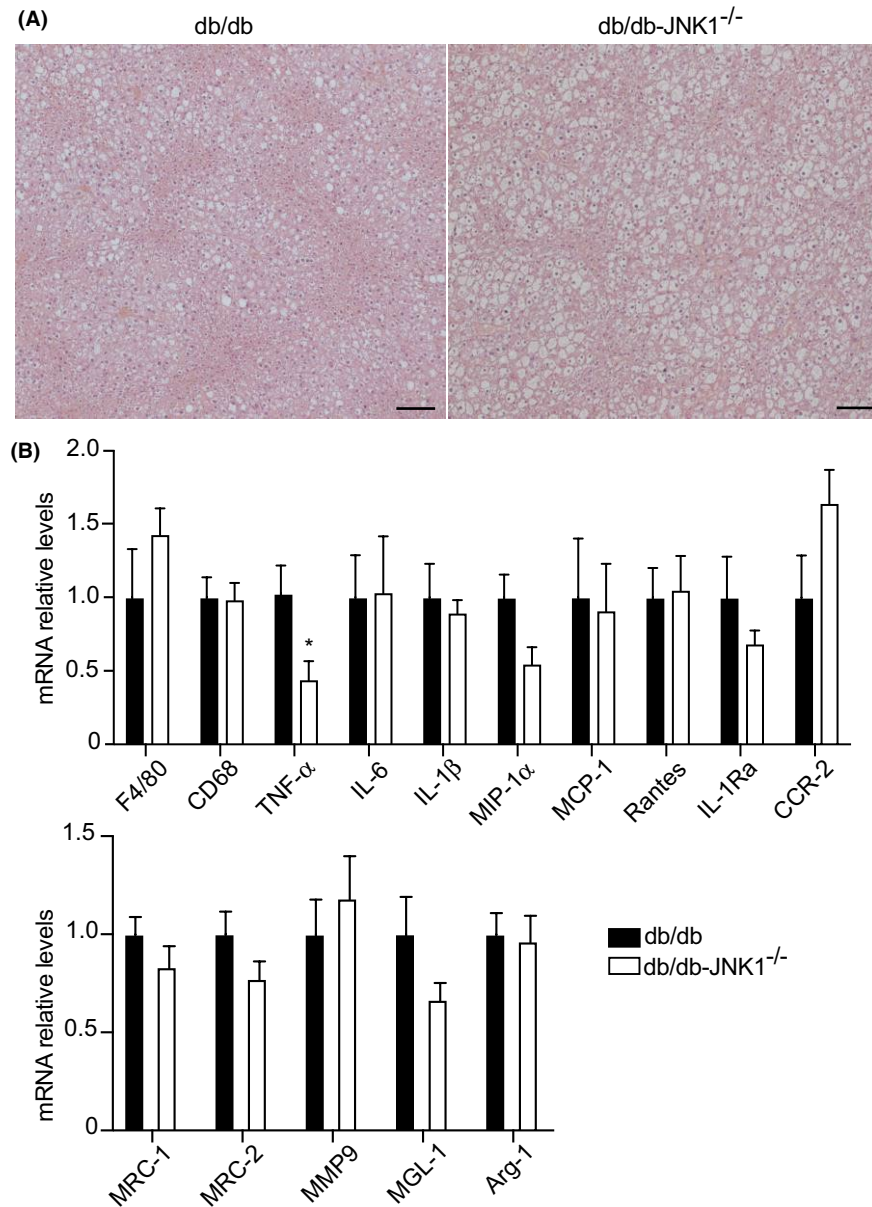
FIGURE 2 JNK1 ablation in db/db mice has marginal effects on the development of steatohepatitis. (A) Hematoxylin and eosin staining of liver sections of 12-weeks-old db/db and db/db-JNK1$^{-/-}$ mice (scale bar = 100 μm). (B) Real-time quantitative PCR analysis of mRNA abundance of macrophage marker genes and genes associated with classic and alternative macrophage activation in livers of the mice from above. Data are expressed as means $\pm$ standard errors, n = 9–7 mice/group, *$p < 0.05$

However, compared to db/db control mice, db/db-JNK1$^{-/-}$ mice displayed significantly improved glycaemia during the glucose tolerance test (GTT) (Figure 1F-H). Analysis of insulin levels during the GTT show that db/db-JNK1$^{-/-}$ mice displayed higher circulating levels of insulin than db/db control mice, which was significant by two-way ANOVA, and the area under the insulin curve was also significantly larger in db/db-JNK1$^{-/-}$ mice (Figure 1I,J). Overall, the data indicate that JNK1 ablation improves glucose tolerance in db/db mice because of improved circulating insulin levels. To further investigate the role of JNK1 in beta cell function we have measured the pancreatic insulin content in db/db-JNK1$^{-/-}$ mice and db/

db mice. The results show that db/db-JNK1$^{-/-}$ mice displayed a significantly and substantially higher insulin content in their pancreas compared to db/db mice (Figure 1K).

3.2 | JNK1 ablation in db/db mice has marginal effects on the development of steatohepatitis

Hematoxylin and eosin staining of paraffin embedded liver sections from db/db-JNK1$^{-/-}$ mice and db/db mice showed that JNK1 ablation did not protect db/db mice
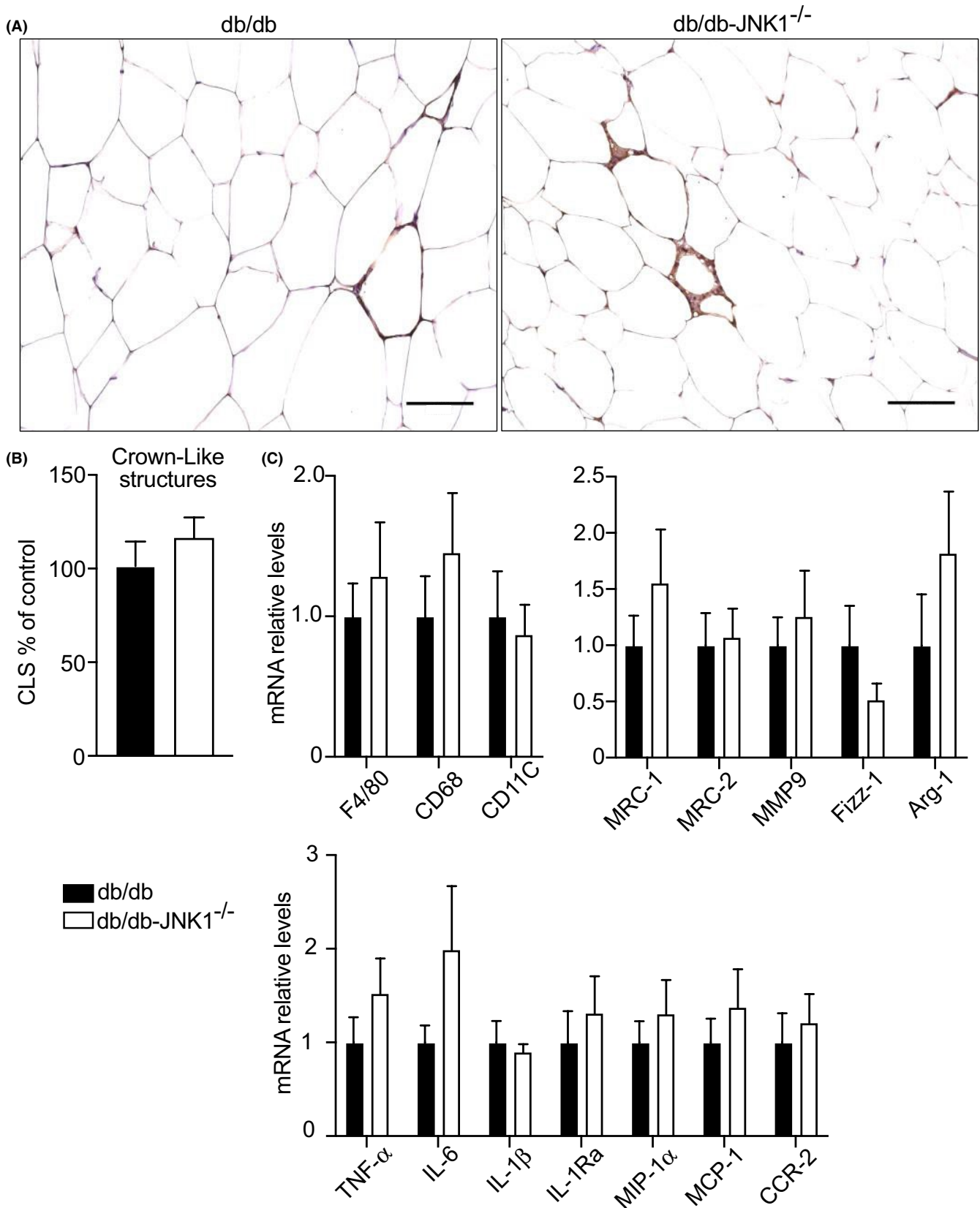
FIGURE 3 JNK1 ablation in db/db diabetic mice does not affect adipose tissue inflammation. (A) Mac2 staining of adipose tissue sections of 12-weeks-old db/db and db/db-JNK1$^{-/-}$ mice (scale bar = 100 µm). (B) Quantitation of crown like structures (CLS) from panel (A). (C) Real-time quantitative PCR gene expression analysis of macrophage marker genes and genes associated with classic and alternative macrophage activation in white adipose tissues of mice from above. Data are expressed as means ± standard errors, n = 6–7 mice/group, *$p < 0.05$

from developing fatty liver (Figure 2A). JNK1 was found to be involved in Kupffer cell activation,[10] thereby we have measured the abundance of macrophage markers, and of markers for classical (pro-inflammatory) "M1" and of alternative "M2" macrophage activation in livers from db/db-JNK1$^{-/-}$ mice and db/db mice. Our results indicate that JNK1 ablation in db/db mice did not affect the expression of general macrophage markers F4/80 and CD68 (Figure 2B). Furthermore, the impact of JNK1 ablation in db/db mice on the expression of markers of macrophage activation in livers was overall rather mild. We found that the expression of the pro-inflammatory cytokine TNFα was significantly reduced in livers from db/db-JNK1$^{-/-}$ mice, and we observed a similar trend for the inflammatory cytokine MIP-1α, but the latter was not statistically significant (Figure 2B). However, the abundance of all the other markers of macrophage activation was overall similar in db/db-JNK1$^{-/-}$ mice and db/db mice (Figure 2B).

Overall, we conclude that JNK1 ablation did not protect mice from fatty liver and, with the exception of reduced expression of TNFα, had no substantial effect on the overall inflammatory gene-expression profile.

3.3 | JNK1 is dispensable for the development of adipose tissue inflammation in db/db mice

To evaluate the effects of JNK1 ablation on adipose tissue inflammation of db/db diabetic mice we have quantified the number of crown-like structures (CLS) by Mac-2 immunostaining of macrophages in white adipose tissue from db/db-JNK1$^{-/-}$ mice and db/db mice. Our data indicate that loss of JNK1 does not affect the CLS number in the adipose tissue of db/db mice (Figure 3A,B). This observation is consistent with the fact that quantitative PCR analysis of gene-expression indicate that db/db-JNK1$^{-/-}$ mice and db/db mice showed similar mRNA levels of the macrophage markers F4/80; CD68; and CD11C in their white adipose tissue (Figure 3C). Finally, JNK1 ablation in db/db mice did not alter the mRNA abundance of several markers of classical M1 and alternative M2 macrophage activation (Figure 3C).

Altogether these results indicate that JNK1 is largely dispensable for the development of inflammation in the adipose tissue of diabetic db/db mice.

3.4 | db/db-JNK1$^{-/-}$ mice display larger islets and more β-cells per islet than db/db control mice

The improved glucose homeostasis observed in db/db-JNK1$^{-/-}$ mice, compared to db/db control mice, could be largely explained by higher levels of circulating insulin and increased pancreatic insulin content, which indicate an improved β-cell mass and/or function in these mice (Figure 1). Hence, we have stained pancreatic sections from db/db-JNK1$^{-/-}$ mice and db/db control mice with antibodies against insulin, the β-cell marker Nkx6.1, and glucagon to evaluate islet number, size and cellular composition. The results indicate that, compared to db/db mice, db/db-JNK1$^{-/-}$ mice showed in average larger islets and a trend toward an increased number of islets per pancreatic surface, although the latter did not achieve statistical significance (Figure 4A,B). Quantitation of the number of Nkx6.1$^+$ insulin$^+$ cells, Nkx6.1$^+$ insulin$^-$ cells, and glucagon$^+$ cells showed a general trend toward more cells in db/db-JNK1$^{-/-}$ mice compared to db/db control mice, but only the number of Nkx6.1$^+$ insulin$^+$ cells per islet was significantly elevated in db/db-JNK1$^{-/-}$ mice (Figure 4C,D).

Altogether, these results show that db/db-JNK1$^{-/-}$ mice have in average larger islets, and more insulin positive β-cells per islet than db/db control mice, which indicates that JNK1 ablation reduces the loss of functional β-cells occurring in db/db mice.

3.5 | Loss of JNK1 in db/db mice display a non-significant trend toward increased β-cell proliferation and no obvious effect on β-cell apoptosis

The larger islet size and higher number of β-cells per islet observed in db/db mice lacking JNK1 could be due either to a decreased β-cell death rate or to an increased β-cell regeneration rate. An increased β-cell regeneration rate should be reflected in an increased frequency of proliferating cells, and thus we have co-stained pancreatic sections from db/db-JNK1$^{-/-}$ mice and db/db control mice with antibodies against insulin, the β-cell marker Nkx6.1, and the proliferation marker Ki67. We observed a trend toward more proliferating β-cells in db/db-JNK1$^{-/-}$ mice (in average about 1.1% of total Nkx6.1$^+$ cells) compared to db/

FIGURE 4 db/db-JNK1$^{-/-}$ mice display larger islets and more β-cells per islet than db/db mice. (A) Immunostaining of pancreatic sections from 12-weeks-old db/+, db/db and db/db-JNK1$^{-/-}$ mice. Blue, DAPI; red, insulin (scale bar = 50 μm). (B) Average islet size relative to db/+ controls and islet number per pancreatic surface were quantified from the immunostaining in panel (A). (C) Images from the immunostaining of pancreatic sections from 12-weeks-old db/+, db/db and db/db-JNK1$^{-/-}$ mice were acquired at a higher magnification for analysis of islet cellular composition. (D) The number of Nkx6.1 positive cells, the percentage of Nkx6.1 positive-insulin negative cells (of total number of Nkx6.1 positive cells) and glucagon positive cells were quantified (scale bar = 50 μm). Data are expressed as means ± standard errors, (n = 5–6 mice/group)
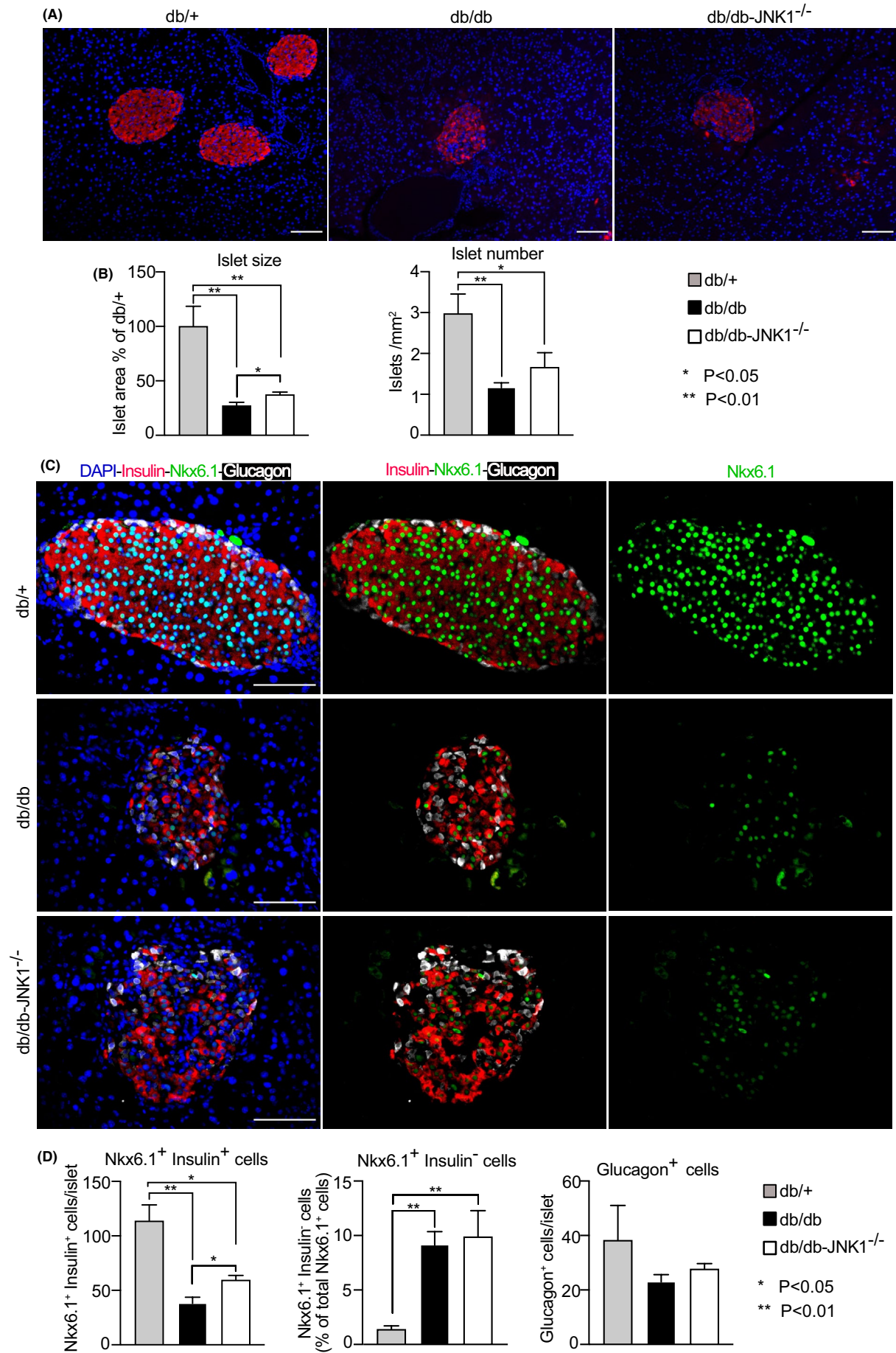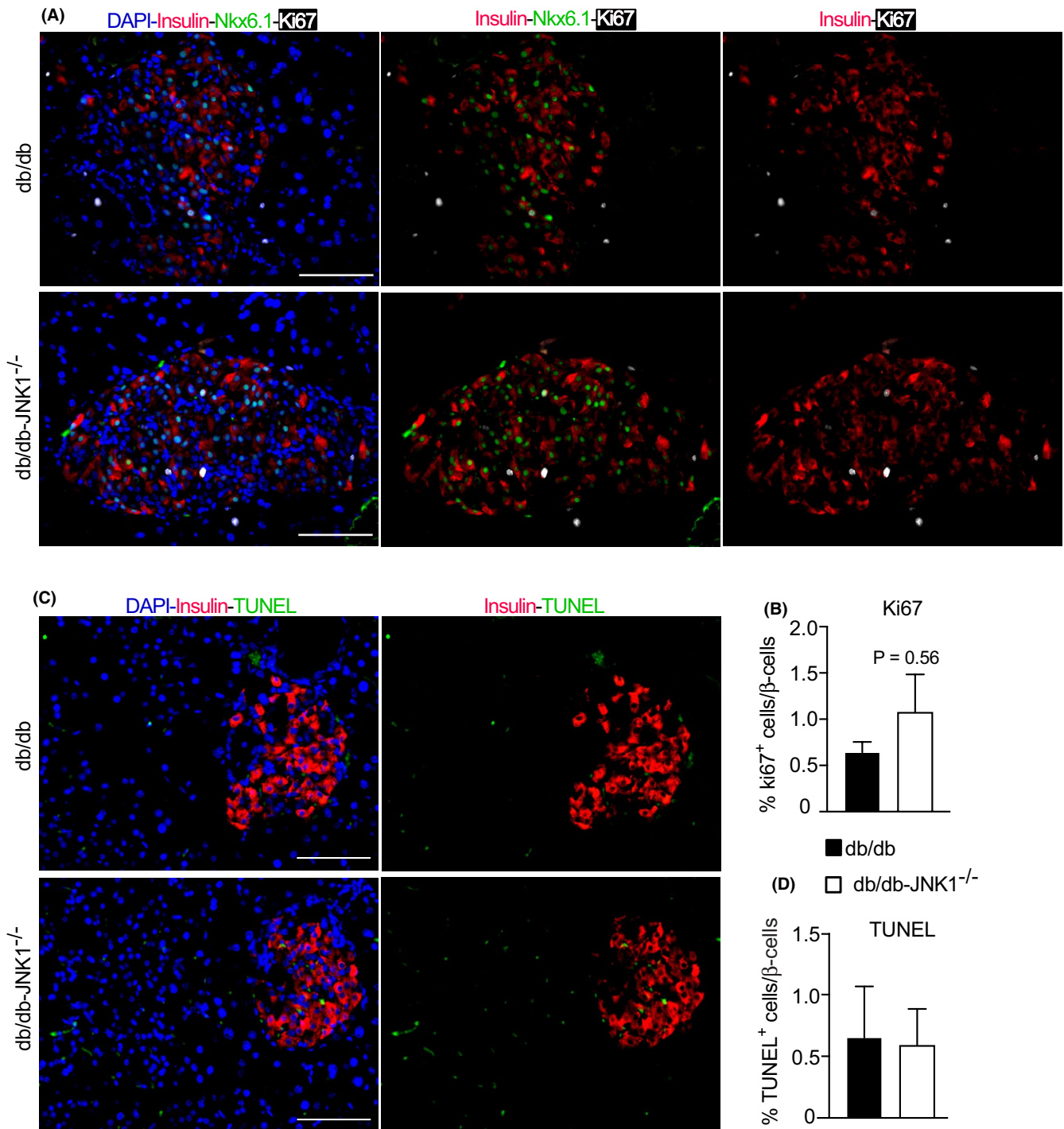
FIGURE 5 Loss of JNK1 in db/db mice display a non-significant trend toward increased β-cell proliferation and no obvious effect on β-cell apoptosis. (A) Ki-67, Nkx6.1, and insulin co-staining of pancreatic sections from 12-weeks-old db/db and db/db-JNK1$^{-/-}$ mice. Blue, DAPI; green, Nkx6.1; white, Ki67; red, insulin (scale bar = 50 μm). (B) Quantitation of the percentage of Ki-67 positive cells on total number of β-cells from the immunostaining in panel (A). (C) Mac-2 and insulin co-staining of pancreatic sections from 12-weeks-old db/db and db/db-JNK-1$^{-/-}$ mice. Blue, DAPI; green, TUNEL; red, insulin (scale bar = 50 μm). (D) Quantitation of the number of TUNEL positive cells on total number of β-cells, from the immunostaining in panel (C). Data are expressed as means ± standard errors, (n = 6 mice/group)

db control mice (in average 0.64% of total Nkx6.1$^+$ cells), but this difference did not achieve statistical significance (Figure 5A,B).

To evaluate the number of apoptotic cells we performed insulin and TUNEL co-staining of pancreatic sections from db/db-JNK1$^{-/-}$ mice and db/db control mice. TUNEL

positive β-cells were relatively infrequent in both db/db-JNK1$^{-/-}$ mice and db/db control mice and no difference was observed between genotypes (~0.65% for db/db and 0.59% for db/db-JNK1$^{-/-}$ mice) (Figure 5C,D).

Overall db/db-JNK1$^{-/-}$ mice and db/db control mice showed a relatively low number of proliferating and apoptotic β-cells, indicating a rather slow β-cell turnover. A trend toward increased β-cell proliferation was observed, but this difference did not achieve statistical significance.

3.6 | Loss of JNK1 in db/db mice does not affect the number of islet-associated macrophages

JNK1 plays an important role in innate immune responses within different pathological contexts, including the activation and accumulation of macrophages in the adipose tissue of obese insulin resistant mice.[2,9,10,12] Pancreatic islets of obese mice are infiltrated by macrophages, which were shown to play an important role in β-cell function and proliferation.[43]

Thereby, we have quantified the number of islet-associated macrophages by co-staining pancreatic sections from db/db-JNK1$^{-/-}$ mice and db/db control mice with antibodies against insulin and the macrophage marker Mac2. The results show that JNK1 ablation had no appreciable effect on the number of islet-associated macrophages (Figure 6A,B).

Overall our data indicate that JNK1 ablation in db/db mice did not affect macrophage infiltration into pancreatic islets.

3.7 | Selective JNK1 ablation does not exacerbate kidney dysfunction in db/db diabetic mice

It was shown that treatment with a highly specific peptidic pan-JNK inhibitor (targeting all JNK isoforms) exacerbates albuminuria in db/db mice despite improving glycaemia.[38] Hence, we have evaluated albumin/creatinine ratio in db/db-JNK1$^{-/-}$ mice and db/db control mice. The results indicate that selective JNK1 ablation did not exacerbate albuminuria in db/db diabetic mice, as we observed a non-significant trend toward reduced albumin/creatinine ratio in db/db-JNK1$^{-/-}$ mice (Figure S2A). db/db mice and db/db-JNK1$^{-/-}$ mice showed similar kidney weight (Figure S2B), and histopathological analysis of kidney sections from db/db-JNK1$^{-/-}$ mice and db/db mice by Sirius red and periodic acid-Schiff (PAS) staining did not reveal any obvious pathological difference between genotypes (Figure S2C,D).

We have also observed similar levels of the oxidative stress marker malondialdehyde (MDA) in kidneys from db/db-JNK1$^{-/-}$ mice and from db/db mice indicating that JNK1 ablation did not affect oxidative damage in kidneys of db/db diabetic mice (Figure S3A). Gene-expression profiling of antioxidant genes in kidneys from db/db-JNK1$^{-/-}$ mice and db/db mice by quantitative real-time PCR showed a small but significant increment of the mRNA levels of Sesn-2, Prdx-1, and Prdx-2 (Figure S3B). Furthermore, gene-expression analysis of the samples from above of macrophage markers and inflammatory genes by quantitative real-time PCR did not show any significant difference between genotypes (Figure S3C).
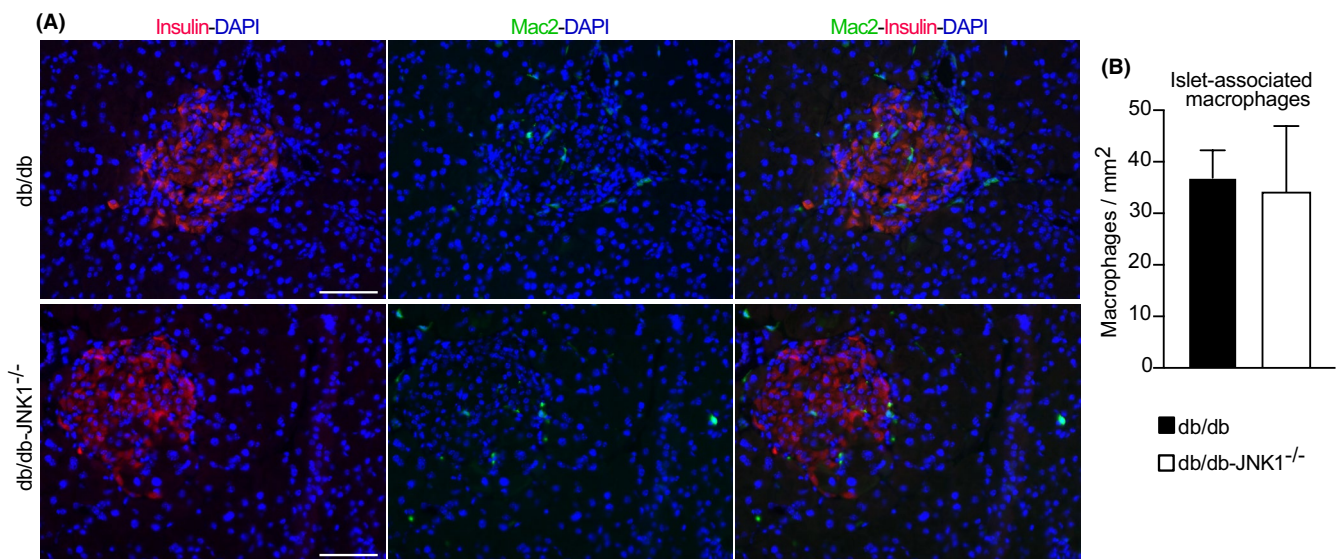

FIGURE 6 Loss of JNK1 in db/db mice does not affect the number of islet-associated macrophages. (A) Mac-2 and insulin co-staining of pancreatic sections from 12-weeks-old db/db and db/db-JNK-1$^{-/-}$ mice. Blue, DAPI; green, Mac2; red, insulin (scale bar = 100 μm). (B) Quantitation of the number of Mac-2 positive cells per surface of islet area, from the immunostaining in panel (A). Data are expressed as means ± standard errors, (n = 9–8 mice/group), *p < 0.05

Altogether, our data indicate that, differently from what previously reported on compound inhibition of all JNK isoforms,[38] systemic ablation of JNK1 does not exacerbate kidney damage in diabetic db/db mice.

4 | DISCUSSION

It is well established that JNK1 ablation protects mice from diet-induced obesity, metabolic inflammation, and insulin resistance,[6,7,9-11] but the role of JNK1 in the loss of β-cell mass and function driving the progression from insulin resistance to type-2 diabetes was not investigated to the same extent. Indeed, to our knowledge there is no data on the role of specific JNK isoforms in an in-vivo model of obesity-driven loss of pancreatic β-cell mass and function. Furthermore, because of the complex role of JNK in the cell response to stress, it is important to better understand the role of JNK isoforms in the complications of type-2 diabetes.[2]

In this study, we have found that JNK1 ablation improves glucose tolerance in the db/db model of obesity-driven type-2 diabetes, a phenotype which was largely explained by improved circulating levels of insulin and increased number of Nkx6.1$^+$ insulin$^+$ pancreatic β-cells. The positive action of JNK1 ablation on β-cells of db/db mice appears to be largely independent from the well-established role of JNK1 in obesity-driven metabolic inflammation and insulin resistance.[7,10,44] Indeed, insulin tolerance was only marginally improved in db/db-JNK1$^{-/-}$ mice compared to db/db control mice and mostly because of improved fasting glucose. Liver steatosis was similar between genotypes and, with the exception of reduced levels of TNFα in the liver, we did not observe other signs of reduced inflammation in db/db-JNK1$^{-/-}$ mice. The fact that JNK1 ablation did not significantly reduce metabolic inflammation in db/db mice can be explained by the more severe metabolic phenotype of db/db mice in the C57BL/KS genetic background compared to the one of ob/ob mice in C57BL/6J background or of WT C57BL/6J mice made obese by feeding an obesogenic diet. However, our data indicate that loss of JNK1 in db/db mice is partially compensated by another activity driving metabolic inflammation and insulin resistance. Although this activity remains to be identified, a logical candidate is JNK2, as it was shown that JNK1 and JNK2 activities are largely redundant in diet-induced obesity, metabolic inflammation, and insulin resistance.[6,44] This line of reasoning suggests that the ideal JNK inhibitor for the treatment of obesity-driven type-2 diabetes should specifically target JNK1 and JNK2 and be selective against JNK3, as JNK3 activity was shown to protect mice from diet-induced obesity[8] and to promote β-cell function in cell culture.[31,33] However, the complex role of specific JNK isoforms in the cell response to stress poses additional concerns. Indeed, pan-JNK inhibition exacerbates albuminuria in db/db mice and ablation of JNK2, but not JNK1, exacerbates the progression of kidney dysfunction and high albuminuria in mice made diabetic by STZ injection.[38] A possible interpretation of the results from this study is that the elevated albuminuria caused by pan-JNK inhibition in diabetic db/db mice could be mostly dependent on JNK2 activity. This interpretation is consistent with our data showing that, compared to control db/db mice, db/db-JNK1$^{-/-}$ mice did not show exacerbated albuminuria, or any obvious histopathological sign of exacerbated kidney pathology, or increased expression of inflammatory markers and levels of the oxidative damage marker MDA. Hence, we conclude that, differently from what observed in db/db mice treated with a pan JNK inhibitor or in mice lacking JNK2 made obese by STZ injection,[38] selective JNK1 ablation, in isolation, does not exacerbate albuminuria and kidney damage in db/db diabetic mice. It is, therefore, reasonable to deduce that a specific JNK1 inhibitor, selective against JNK2 and JNK3, may be safer than pan JNK inhibitors and of JNK1-JNK2 inhibitors, while largely retaining the protective effects of JNK inhibition in obesity-driven diabetes. Indeed, in mouse models of diet-induced obesity and insulin resistance loss of JNK1, in isolation, is sufficient to reduce adiposity, metabolic inflammation, and to improve insulin sensitivity[6,7]; and we now show in the db/db model of type-2 diabetes that selective JNK1 ablation is sufficient to improve circulating insulin levels, pancreatic insulin content, and β-cell number.

In our study, we could largely dissociate the protective effects of JNK1 ablation on improved β-cell mass from differences in insulin sensitivity or metabolic inflammation. However, whereas we have found that JNK1 activity is dispensable for macrophage accumulation in islets of db/db diabetic mice, our data do not exclude a possible role for JNK1 in the activation of islet-associated macrophages affecting β-cell function or proliferation. Differently from adipose tissue and liver macrophages, islet-associated macrophages from obese mice do not display an M2 to M1 polarization, and the gene expression signature associated with the effects of macrophages on islet mass and function is still largely unresolved.[43] Thus, a better understanding of the activation status and the functions of islet-associated macrophages in obesity, and the role of JNK1 in these processes is necessary.

The fact that, compared to db/db control mice, db/db-JNK1$^{-/-}$ mice showed reduced body weight at weaning, may suggest that the effects of JNK1 ablation on improved β-cell mass could be in part the indirect consequence of reduced adiposity. However, these differences in body weight were reduced over time; and an indirect protection consequent to reduced adiposity should have resulted in a more general improvement of the metabolic phenotype, including an improved fatty liver and insulin sensitivity, and reduced metabolic inflammation, which were not observed in db/db-JNK1$^{-/-}$ mice. Hence, the improved insulin levels and β-cell mass observed in db/db-JNK1$^{-/-}$ mice indicate a specific action for JNK1 in the loss of functional β-cells occurring in obesity-driven diabetes. Several studies indicate that JNK1 blockage protects β-cells from apoptosis in cell culture.[17,21-30] However, loss of JNK1 did not reduce the number

of TUNEL positive β-cells in db/db mice, indicating that the improved β-cell number observed in db/db-JNK1$^{-/-}$ mice may be independent from JNK1 action on apoptosis. Relatively to db/db mice, db/db-JNK1$^{-/-}$ mice showed a trend toward increased β-cell proliferation, which may explain the beneficial effects of JNK1 ablation on insulin levels and β-cell number in db/db mice, but this difference did not achieve statistical significance.

Furthermore, different laboratories have also shown that JNK activity reduces insulin expression in β-cells,[17,29,45-48] hence it is also possible that loss of JNK1 may sustain insulin gene expression, preventing the β-cell de-differentiation and insulin depletion occurring during the progression of type-2 diabetes.[49] However, we have found that the number of insulin-depleted β-cells (Nkx6.1$^+$ insulin$^-$) was not reduced in db/db mice lacking JNK1.

Thereby, whereas the improved insulin levels observed in db/db-JNK1$^{-/-}$ mice could be explained by an increased number of Nkx6.1$^+$ insulin$^+$ cells per islet, the specific mechanism by which JNK1 ablation preserves functional β-cells in db/db mice remains to be defined.

Overall, our study indicates that JNK1-selective inactivation may improve β-cell function in obesity-driven type-2 diabetes without exacerbating albuminuria, and therefore a specific JNK1 inhibition, selective against JNK2 and JNK3, may be the optimal therapeutic strategy to target JNK signaling in obesity-driven diabetes. Future clinical studies with isoform selective JNK1 inhibitors will be required to test this hypothesis.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHORS CONTRIBUTIONS

A.M. and B.B. performed most of experiments, analyzed data, and contributed to the manuscript preparation. C.S. generated the experimental mice performed some experiments, analyzed data, and contributed to the manuscript preparation. L.B. backcrossed the JNK$^{-/-}$ mice in db/+ C57BL/KS background. F.T. performed the histopathological analysis of kidney sections. G.S. conceived the project, directed the study, contributed to data interpretation, and wrote most of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.