

Evidence against a role for NLRP3-driven islet inflammation in *db/db* mice



H.L. Kammoun^{1,*}, T.L. Allen¹, D.C. Henstridge¹, S. Barre¹, R.C. Coll², G.I. Lancaster¹, L. Cron³, S. Reibe³, J.Y. Chan³, M. Bensellam³, D.R. Laybutt^{3,4}, M.S. Butler², A.A.B. Robertson², L.A. O'Neill⁵, M.A. Cooper², M.A. Febbraio^{1,3,4,**}

ABSTRACT

Objectives: Type 2 diabetes (T2D) is associated with chronic, low grade inflammation. Activation of the NLRP3 inflammasome and secretion of its target interleukin-1 β (IL-1 β) have been implicated in pancreatic β cell failure in T2D. Specific targeting of the NLRP3 inflammasome to prevent pancreatic β cell death could allow for selective T2D treatment without compromising all IL-1 β -associated immune responses. We hypothesized that treating a mouse model of T2D with MCC950, a compound that specifically inhibits NLRP3, would prevent pancreatic β cell death, thereby preventing the onset of T2D.

Methods: Diabetic *db/db* mice were treated with MCC950 *via* drinking water for 8 weeks from 6 to 14 weeks of age, a period over which they developed pancreatic β cell failure. We assessed metabolic parameters such as body composition, glucose tolerance, or insulin secretion over the course of the intervention.

Results: MCC950 was a potent inhibitor of NLRP3-induced IL-1 β *in vitro* and was detected at high levels in the plasma of treated *db/db* mice. Treatment of pre-diabetic *db/db* mice with MCC950, however, did not prevent pancreatic dysfunction and full onset of the T2D pathology. When examining the NLRP3 pathway in the pancreas of *db/db* mice, we could not detect an activation of this pathway *nor* increased levels of its target IL-1 β .

Conclusions: NLRP3 driven-pancreatic IL-1 β inflammation does not play a key role in the pathogenesis of the *db/db* murine model of T2D.

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Keywords Type 2 diabetes; Inflammasome; Interleukin-1 β ; MCC950; *db/db* mice

1. INTRODUCTION

Diabetes mellitus is growing world-wide and is expected to affect almost 600 million people by 2035 [1]. Type 2 Diabetes (T2D) is recognized as a chronic inflammatory disease, and it is now established that inflammation and immune cell dysfunction are strongly associated with both insulin resistance and insulin secretion dysfunction [2]. The ability of immune cells to affect pancreatic function and, more specifically, β cell insulin-secretory activity in islets, is well established in the context of type 1 diabetes [3,4]. In contrast, the role of inflammation and immune cells in the pathology of T2D is a relatively recent phenomenon (for review see [5]). Specifically, macrophages have been shown to infiltrate pancreatic islets both in T2D patients as well as in rodent models of insulin resistance and diabetes [6,7]. This infiltration, in addition to the nutrient-stressed β

cells, contributes to elevated cytokine levels in the diabetic islet [8,9]. In particular, the pro-inflammatory cytokine interleukin-1 β (IL-1 β) has been highlighted as a potent driver of beta cell dysfunction [9,10]. IL-1 β secretion is triggered in the islet in response to high level of glucose, initiating a pro-inflammatory milieu that contributes to the recruitment and activation of macrophages, which, in turn, sustain islet inflammation [9]. Indeed, suppressing macrophage recruitment in *db/db* mice (that harbor a mutation of the leptin receptor leading to hyperphagia, obesity and eventually β cell failure) using clodronate liposomes improved insulin secretion [11]. Mature IL-1 β is mainly produced through the multi-protein inflammasome complexes such as NOD-like receptor pyrin domain containing protein 3 (NLRP3) inflammasome. Pro-inflammatory danger-associated molecular patterns (DAMPs), endogenous molecules such as extracellular ATP, or uric acid crystals are detected by the NLR scaffolding protein leading to the

¹Cellular and Molecular Metabolism Laboratory, Baker Heart & Diabetes Institute, Melbourne, Australia ²Institute for Molecular Bioscience, The University of Queensland, St Lucia, Australia ³Division of Diabetes & Metabolism, Garvan Institute of Medical Research, Sydney, Australia ⁴St Vincent's Clinical School, University of New South Wales, Sydney, New South Wales, Australia ⁵Inflammation research, Trinity Biomedical Sciences Institute, Dublin, Ireland

*Corresponding author. Cellular & Molecular Metabolism Laboratory, Baker Heart & Diabetes Institute, PO Box 6429, St Kilda Road Central, Melbourne 8008, VIC, Australia. E-mail: helene.kammoun@baker.edu.au (H.L. Kammoun).

**Corresponding author. Cellular & Molecular Metabolism Laboratory, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, 2010, NSW, Australia. E-mail: m.febrario@garvan.org.au (M.A. Febbraio).

Abbreviations: T2D, Type 2 Diabetes; IL-1 β , interleukin-1 β ; NLRP3, Nod-like receptor family pyrin domain-containing protein 3; DAMPs, danger-associated molecular patterns

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recruitment of the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1. The assembly of the NLRP3 inflammasome results in the cleavage and activation of caspase-1 which in turn activates the precursor form of IL-1 β through proteolytic cleavage [9]. In the context of T2D, several different stimuli have been proposed to trigger pancreatic NLRP3 activation and increased IL-1 β . We have shown that accumulation of islet amyloid polypeptide (IAPP), a protein known to accumulate into amyloid deposits in the pancreas of T2D patients, could activate the NLRP3 inflammasome and promote IL-1 β production [12]. In addition to IAPP, saturated fatty acids, ATP from apoptotic cells, endocannabinoids, ER stress, and oxidative stress have also been reported to potentiate NLRP3-induced IL-1 β production and contribute to islet inflammation [9]. Targeting IL-1 β signaling using Anakinra (IL-1 receptor antagonist) and Canakinumab (anti-IL-1 β antibody) to treat T2D patients yielded modest but promising results [13–15]. Specific targeting of the NLRP3 inflammasome to prevent pancreatic cell death, however, could be a viable treatment strategy for T2D, because it would not compromise IL-1 β -associated immune function, initiated by pathways other than NLRP3. We have shown that the orally available small molecule MCC950 potently and specifically inhibits NLRP3 activation and that it is efficacious in several pre-clinical models of inflammatory diseases including NLRP3-driven auto-inflammatory conditions [16–21]. We hypothesized that treating a diabetic mouse with MCC950 would prevent the pancreatic islets death and delay, if not completely block, the onset of T2D.

2. MATERIAL AND METHODS

2.1. Mouse models and drug treatment

db/db and *db/+* littermate control mice (BKS.Cg-Dock7$m^{+/-}$ Lepr$^{db}/J$) were purchased from Jackson laboratories (USA) at 4 weeks of age and left to acclimatize for 2 weeks. All mice were housed at the Alfred Medical Research and Education Precinct Animal Centre in a pathogen free facility under controlled environmental conditions and exposed to a 12:12 h light:dark cycle. Mice were fed a normal chow diet (Specialty Feeds, Australia) and provided with food and water *ad libitum*. From 6 weeks of age, mice received MCC950 (40 mg/kg) *via* drinking water for a period of 8 weeks. Water intake was recorded daily for each cage. The dose of MCC950 was adjusted every 3 days according to mice weight gain/loss and averaged water intake per cage. Animal experiments were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee and conducted in accordance with the National Health and Medical Research Council of Australia Guidelines for Animal Experimentation.

2.2. Metabolic measurements

2.2.1. Plasma insulin measurements

Insulin concentrations were measured using a Mouse Ultrasensitive Insulin ELISA kit (ALPCO, Salem, NH, USA) according to manufacturer's instructions.

2.2.2. Body composition

Mouse body composition (fat mass (FM) and lean body mass (LBM)) were measured weekly with a 4-in-1 EchoMRI body composition analyzer (Columbus Instruments, USA) and standard laboratory scales.

2.2.3. oral Glucose Tolerance Test (oGTT)

oGTT (2 g/kg LBM) were performed in 5 h (for week 2 oGTT) or 12 h (for week 7 oGTT) fasted mice as previously described [22].

2.3. RNA extraction and real time quantitative PCR

Pancreatic islets from 16 weeks old *db/+* and *db/db* mice were isolated as previously described [23]. Total RNA was isolated from tissues with Tri Reagent® (Sigma Aldrich) and reverse transcribed to cDNA with the use of random hexamers. Gene expression analysis was performed by Real-time PCR on a 7500 fast sequence detector (Applied Biosystems) using TaqMan gene expression assays (Applied Biosystems), including an 18S probe and primers for housekeeping measurements (Taqman references are included in Supplementary Fig. 3).

2.4. Blood parameters

For hematological assessment, a small volume (20 μ L) of whole blood was diluted 1:7 in Sysmex CELLPACK™ (Sysmex, Japan) diluent and assessed using an automated hematology analyzer (Sysmex XS-1000i, Japan).

2.5. MCC950 quantification

MCC950 was measured in the plasma by Liquid chromatography-tandem mass spectrometry as previously described [16] for plasma. For tissue analysis, fat, liver and kidney were weighed and homogenised in saline using a Qiagen tissue lyser. The quantification was performed as described in the Supplementary Material.

2.6. Western blotting

Renal cortex was isolated from frozen kidney samples and western blotting was performed on tissue homogenates as previously described [24] and quantified relative to relevant loading controls.

2.7. *In vitro* experiment

Bone marrow derived macrophages were obtained and cultured as previously described [25]. Cells were treated with LPS, ATP and MCC950 as described in the figure legend before the supernatant was harvested and used to measure IL-1 β using the R&D Duoset Mouse ELISA.

2.8. Statistical analysis

Data are presented as mean \pm standard error of the mean (SEM). Two-way analysis of variance (ANOVA) was used to detect main effects for genotype (*db/+* vs. *db/db*) and treatment (vehicle vs. MCC950). Post-hoc analyses (Holm-Sidak) were performed when a significant interaction was detected. Analyses were performed using a statistical computer program (Sigma Stat Version 3.5). $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. MCC950 inhibits IL-1 β secretion in BMDM stimulated with NLRP3 inflammasome activators

We first aimed to verify the potency of MCC950 to inhibit the NLRP3 inflammasome in our experimental conditions. We pre-treated bone marrow derived macrophages with LPS in order to induce IL1b gene expression and pro-IL-1 β production. We next treated these cells with MCC950 before inducing the NLRP3 inflammasome activation with ATP. We observed a near complete prevention of ATP-induced NLRP3-mediated IL-1 β release in the cells treated with the two doses of MCC950 confirming its strong inhibitory potency (Supplementary Fig. 1)

3.2. MCC950 does not impact body weight or fat and lean mass in *db/db* mice

We next proceeded to test the *in vivo* effect of MCC950 in a mouse model of T2D. *db/db* and *db/+* (lean littermate) mice were fed a chow

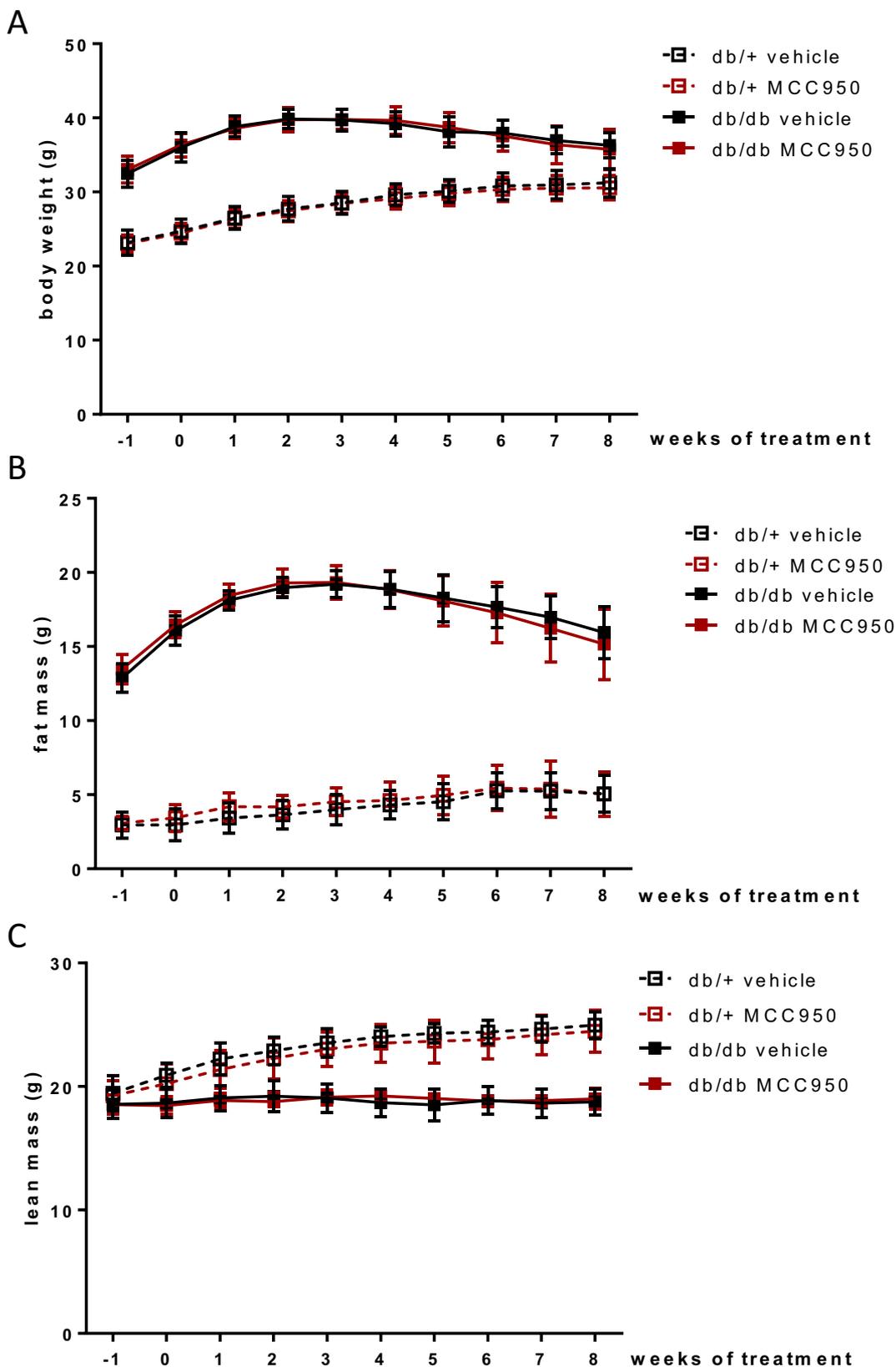


Figure 1: MCC950 does not impact body weight or fat and lean mass in *db/db* mice. 6 weeks old *db/db* mice were given 40 mg/kg MCC950 through their drinking water for an 8 weeks period. (A) Body weight (B) fat mass and (C) lean mass were assessed by EchoMRI a week before and weekly throughout the treatment period. Data presented as Mean \pm SEM, n = 10.

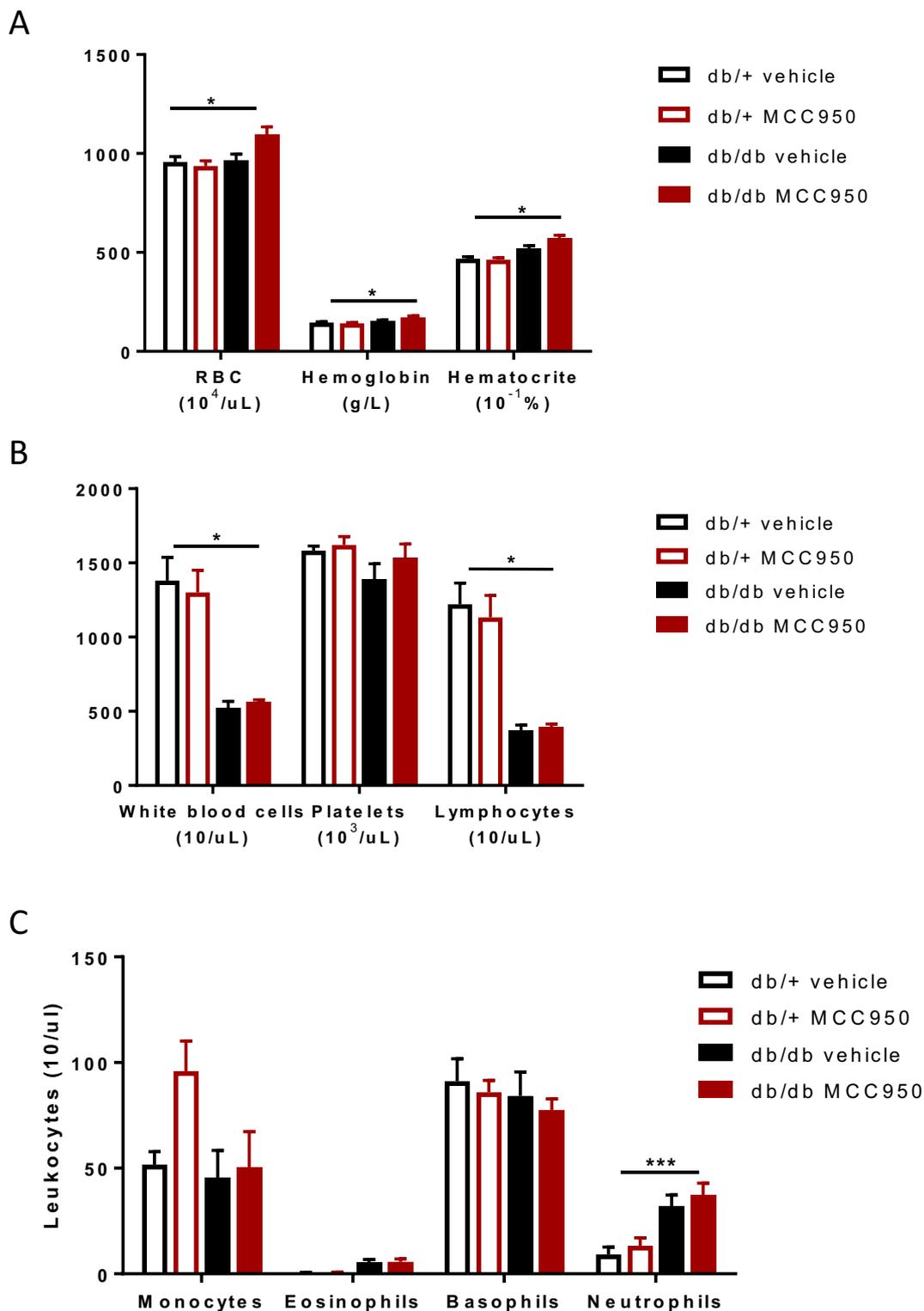


Figure 2: MCC950 is bioavailable in plasma but does not affect immune cell profile in *db/db* mice. 6 weeks old *db/db* mice were given 40 mg/kg MCC950 through their drinking water for an 8 weeks period. (A) Red blood cell parameters (B) White blood cell, platelets, lymphocyte counts, and (C) leukocyte counts were measured in the blood at the 8 weeks endpoint. Data presented as Mean \pm SEM, n = 10. Statistical analysis by 2 way ANOVA, *p < 0.05; ***p < 0.001 Main effect for *db/db* vs *db/+*.

diet for 8 weeks while receiving MCC950 (40 mg/kg) or plain water through their drinking water. As expected, the *db/db* mice became severely obese with a markedly increased fat mass, and decreased lean mass, compared with *db/+* mice (Figure 1). Treatment of both groups of mice with MCC950 did not, however, affect these parameters (Figure 1).

3.3. MCC950 is bioavailable in plasma and tissues but does not affect immune cell profile in *db/db* mice

After observing no impact on the animals' body composition in response to MCC950 treatment, we aimed to verify the bioavailability of MCC950. We analyzed plasma from all mice at termination of the intervention and assessed circulating levels of MCC950 using liquid chromatography-tandem mass spectrometry. As expected, we detected high circulating levels of our compound in both *db/+* and *db/db* treated groups as expected (Supplementary Fig. 2) consistent with previous pharmacokinetic studies that have shown efficacy of the compound at this dose [16]. Interestingly, we observed a ~5 fold higher ($P < 0.05$) plasma MCC950 concentration in *db/+* compared with *db/db* mice (Supplementary Fig. 2), suggesting that some of the compound might get trapped in the large fat stores in the obese *db/db* mice. Hence, we set to investigate the amount of compound in several tissues as well as in urine (Supplementary Fig. 2). Surprisingly, the compound was found in negligible quantities in the visceral fat depot of both *db/+* and *db/db* and the liver and kidney presented with the same pattern as the plasma with up to 10 times more compound being located in the liver of the *db/+* compared to the *db/db* mice. However, a major result arise from the urine analysis. *db/db* mice excreted MCC950 metabolite at a higher rate (1.5 fold versus *db/+*) and given that their urine output was almost 10 times higher (data not shown), it reveals that the discrepancy between the groups in relation to

circulating levels was due to the compound being excreted in *db/db* mice at a higher rate. Importantly, the circulating concentration in the *db/db* mice was nonetheless >1000 ng/mL, which was proven sufficient to inhibit NLRP3 *in vivo* [16]. We next determined whether MCC950 administration could result in any side effects by measuring several blood markers. MCC950 did not affect either hemoglobin, or hematocrit concentrations, nor did it affect red blood cells number (Figure 2A). White blood cells, including platelets and lymphocytes (Figure 2B) or circulating monocytes, neutrophils, eosinophils, and basophils (Figure 2C) were also unaffected by the treatment. Of note, the *db/db* mice displayed lymphocytopenia (Figure 2B) and mild neutrophilia, consistent with previous reports [26,27].

3.4. MCC950 does not improve glucose metabolism nor prevent pancreatic islet failure in *db/db* mice

We next determined whether MCC950 could better maintain glucose homeostasis in *db/db* mice. Six week old *db/db* mice, displayed both frank hyperglycemia (Figure 3A) and markedly increased water consumption (Figure 3C) throughout the intervention period compared with *db/+* mice. Importantly, MCC950 treatment did not affect these parameters (Figure 3A,C). The failure of MCC950 to affect hyperglycemia was associated with a lack of effect on insulin secretion (Figure 3B). Indeed, we measured fasting insulin levels along the course of the intervention and MCC950 treatment was unable to prevent the progressive decrease of insulin secretion observed in the *db/db* mice, an indication of the transition from β cell compensation to β cell failure (Figure 3B).

We next measured glucose tolerance by performing oral glucose tolerance test (OGTT; 2 g/kg LBM) at both week 2 and week 7 of the treatment period. Consistent with the failing pancreatic function of both *db/db* groups and the fasting glycemia observations, MCC950

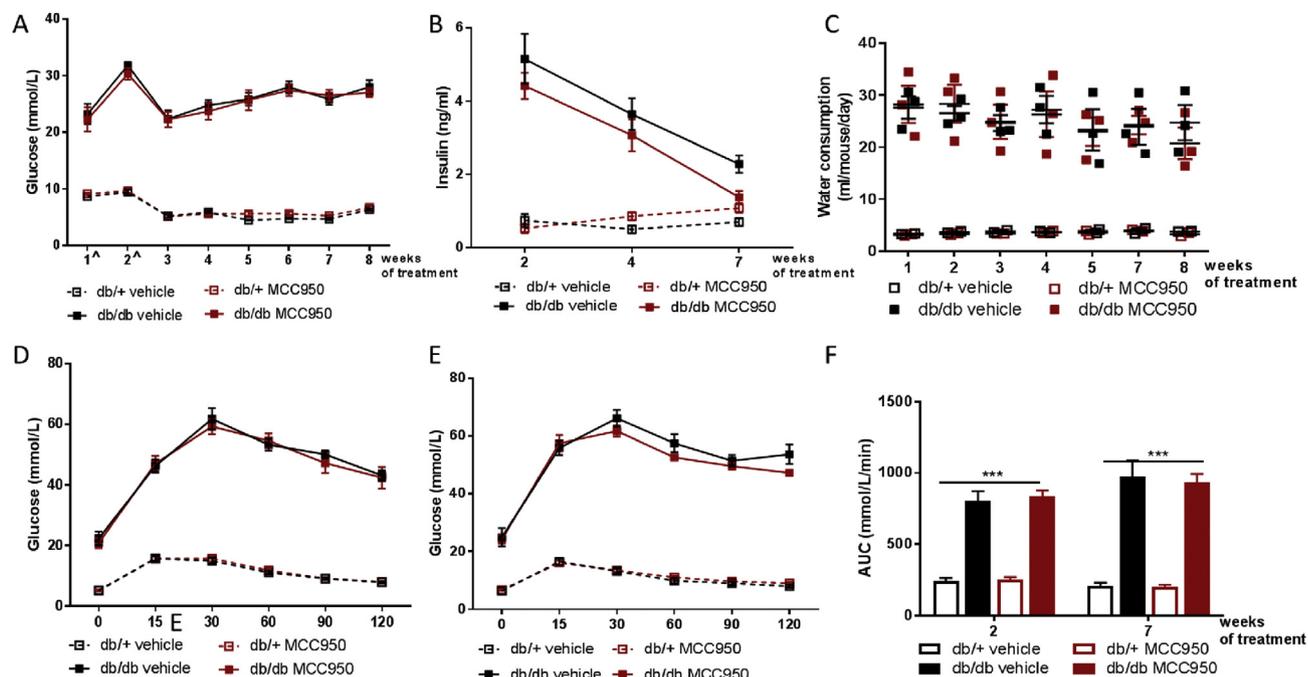


Figure 3: MCC950 does not improve glucose metabolism nor prevent pancreatic islet failure in *db/db* mice. 6 weeks old *db/db* mice were given 40 mg/kg MCC950 through their drinking water for an 8 week period. Glycemia (A) and circulating insulin levels (B) were measured after a 5 h (time points marked \cdot) or 12 h (all other time points) fasting period. (C) Water consumption per mouse per day was measured in the 3 different cages of each experimental group. (D–F) Glucose tolerance was assessed through an oGTT following a 5 h fast at 2 weeks of treatment (D) and a 12 h fast at 7 weeks of treatment (E) and was used to generate area under the curve (F). Data presented as Mean \pm SEM, $n = 10$. Statistical analysis by 2 way ANOVA, *** $p < 0.001$ Main effect for *db/db* vs *db/+*.

had no effect on the severe glucose intolerance displayed by *db/db* relative to *db/+* mice during the OGTT at both intervention periods (Figure 3D–F).

3.5. *db/db* mice do not display inflammasome activation in pancreatic islets

Since MCC950 inhibited NLRP3 activation *in vitro* (Supplementary Fig. 1) and was clearly bioavailable (Supplementary Fig. 2), our data raised the possibility that, in this particular model, NLRP3 was not activated in the pancreas of *db/db* mice. Indeed, previous data from our laboratory found no difference in IL-1 β expression in pancreatic islets of 6 weeks old *db/db* compared to *db/+* islets [28]. In addition, we isolated pancreatic islets isolated from *db/+* and *db/db* mice at both 6 and 16 weeks and analyzed the mRNA expression of *NLRP3* as well as *IL-1 β* in the islets of the 16 weeks old animals (Figure 4A,B). Given the current literature and work from our own group [12], we were surprised to observe no increase in the mRNA expression of either *IL-1 β* or *NLRP3* in the cDNA extracted from *db/db* islets compared with that obtained the *db/+* islets (Figure 4A,B). Of note, *IL-1 β* expression at 16 weeks was quite heterogeneous in the *db/db* group, probably reflecting different stages of the diabetes progression in these mice. Interestingly, and in line with previously published data [29], we were able to detect increased inflammasome components in the renal cortex of *db/db* mice compared with *db/+* mice (Figure 4C). We could not detect a significant effect of MCC950 administration on

the NLRP3 component by western blotting despite the accumulation of the drug in the kidney (Figure 4C and Supplementary Fig. 2).

4. DISCUSSION

Activation of the NLRP3 inflammasome has been implicated in the pathology of obesity and insulin resistance [30–32]. These previous studies demonstrated a role for the NLRP3 inflammasome in the adipose tissue and liver and mostly attributed its activation and the associated secretion of IL-1 β to infiltrating macrophages in these tissues.

In addition, the NLRP3 inflammasome has been increasingly studied for its role in pancreatic dysfunction in the context of T2D [5]. The activation of this inflammasome complex leading to the production of the pro-inflammatory cytokine IL-1 β has been described in the pancreas in response to several different stimuli. In the pancreatic islet, it appears that both glucose stressed- β cells as well as resident pro-inflammatory macrophages contribute to increased levels of IL-1 β and the subsequent insulin secretion impairment [9]. Reports are still unclear whether the myeloid-derived or β cell-produced IL-1 β , if not both, is the culprit in β -cell dysfunction. Interestingly, a recent report studying diabetic nephropathy in *db/db* mice also suggested a strong role for the NLRP3 inflammasome and the subsequent IL-1 β secretion in non-myeloid cells. Indeed, mice lacking NLRP3 are protected from diabetic nephropathy, even when transplanted with

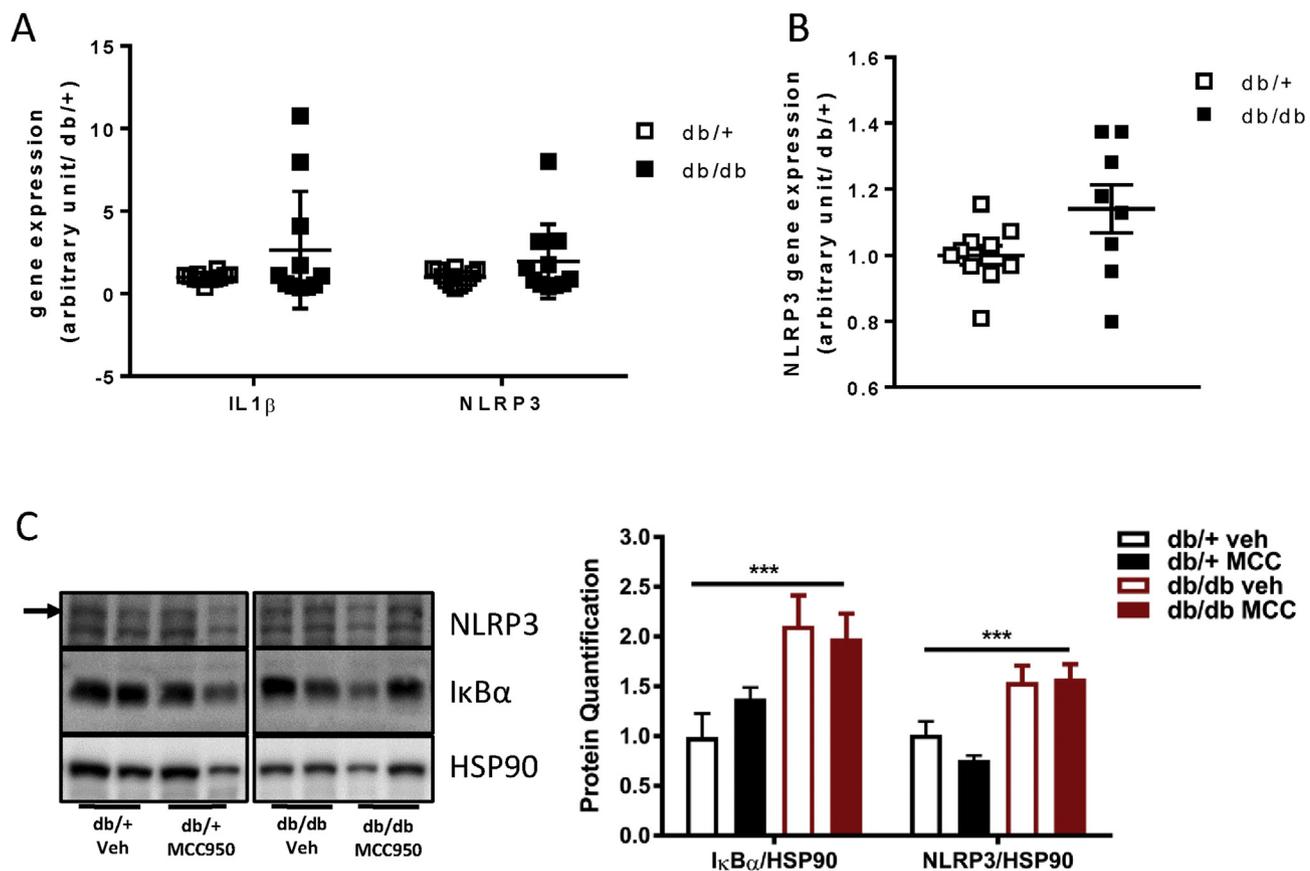


Figure 4: MCC950 treatment in *db/db* mice does not impact pancreatic inflammasome components. (A) Gene expression for IL-1 β and NLRP3 was measured in isolated pancreatic islet of 16 weeks old *db/+* and *db/db* mice. (B) Gene expression for NLRP3 was measured in isolated pancreatic islet of 6 weeks old *db/+* and *db/db* mice. (C) 16 weeks old *db/db* mice were given 40 mg/kg MCC950 through their drinking water for an 8 weeks period. I κ B α and NLRP3 were measured on renal cortex by western blotting at the end of the treatment period. Data presented as Mean \pm SEM, A–B; n = 11–12; C; n = 5–8.

wild-type bone marrow but conversely, transfer of NLRP3 deficient bone marrow into wild-type animals fails to prevent diabetic nephropathy [29]. This report emphasized a role for IL-1 β in the diabetic pathology of *db/db* mice as they presented a six-fold increase in the cytokine circulating concentration between 4 and 12 weeks of age in these animals [29]. These previous publications prompted us to examine the efficacy of our specific NLRP3 inhibitor in the *db/db* mice in order to arrest the transition from β -cell compensation to failure. We were, therefore surprised that, in our hands neither NLRP3 nor IL-1 β mRNA expression were elevated in the islets from *db/db* mice. In retrospect, it was not surprising that MCC950 had no effect in this experimental model of T2D.

Our data contrast a number of reports indicative of an NLRP3-dependent mechanism to explain IL-1 β activation and its role in pancreatic dysfunction. Youm et al. demonstrated that aged mice fed a high fat diet until reaching one year of age displayed ASC dependent glucose intolerance, but, more importantly, they observed a conserved insulin secretory function in aged mice on a high fat diet lacking the adaptor ASC or NLRP3 itself [33]. It is critically important, however, to note that in this previous study the authors did not use littermate controls for the ASC^{-/-} and NLRP3^{-/-} mice. This could represent an artifact, as it was shown that basal and challenged insulin levels and glucose metabolism are strongly influenced by the strains but also more importantly by the sub-strain of C57Bl/6 mouse used [34,35]. Indeed, data from our laboratory have shown that metabolic phenotype differences when comparing genetic mouse models are abolished when appropriate littermate control studies are performed [25]. Moreover, the work of others [21] has demonstrated that glucose tolerance and insulin secretion capacity could be significantly different in two sub-strains studied side by side on a high fat diet [35].

Interestingly, Kim et al. also proposed a role for the NLRP3 inflammasome in the same model used herein as they treated *db/db* mice with the modified form of Vitamin E γ -tocotrienol (γ T3) [36]. They report an improved glucose tolerance accompanied by an enhanced glucose stimulated insulin secretion *in vivo*. Importantly, however, they did not demonstrate the activation of NLRP3 in the *db/db* pancreatic islet and their γ T3 compound appears to have several targets in addition to NLRP3, making it difficult to ascertain the role of this inflammasome in the observed metabolic effects. Indeed, they also report a significantly decreased food intake that could likely mediate the decreased fasting glycemia, given the fasting insulin levels were not different between treated and control *db/db* animals. In addition, they also observed increased AMPK activation in the γ T3-treated mice, a known metabolic activator associated with improved glucose metabolism [37,38].

Of note, MCC950 targets the NLRP3 inflammasome activation meaning that not only the IL-1 β inflammatory pathway but also IL-18 driven inflammation or pyroptosis would be inhibited, ruling out a role for these pathways as well in the *db/db* model [39].

Finally, recent reports by Donath and colleagues indicate that the role of IL-1 β might not be just detrimental in the pancreas as they report that IL-1 β is secreted physiologically after a meal. Indeed, mice deficient for IL-1 β exhibited decreased insulin levels in response to refeeding or to a high fat diet [40]. This might add some complexity to the pharmaceutical field considering IL-1 β as a therapeutic target for diabetes.

In conclusion, it appears that the NLRP3 inflammasome is not responsible for the β -cell insulin secretory failure observed in the *db/db* mouse model of T2D. The use of a genetic model lacking the leptin receptor such as the *db/db* mouse might explain part of the

discrepancy as leptin signaling and the downstream STAT3 pathway have been implicated in inflammation modulation [41,42]. However, given the increasing number of human studies targeting IL-1 β and other inflammation components for the treatment of T2D [13,43–46], our data caution the use of *db/db* mice as an appropriate pre-clinical model of T2D, particularly when testing the efficacy of drugs that target insulin secretory pathways and islet inflammation.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.molmet.2018.02.001>.

CONFLICT OF INTEREST

MAC currently holds a fractional Professorial Research Fellow appointment at the University of Queensland with his remaining time as CEO of Inflazome Ltd. a company headquartered in Dublin, Ireland that is developing drugs to address clinical unmet needs in inflammatory disease by targeting the inflammasome.

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