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Lycopene ameliorates islet function and down-regulates TLR4/MyD88/NF-κB pathway in diabetic mice and Min6 cells

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Abstract

Inflammation of the pancreatic islets triggers β cells dysfunction and type 2 diabetes mellitus (T2DM) onset. While dietary lycopene consumption contributes to protect against T2DM in animal studies, the potential mechanisms of this compound in regulation of islet function in T2DM remain largely unclear. In the present study, by using anti-diabetic metformin as a positive control, we demonstrated that lycopene treatment suppressed islet inflammation and apoptosis in both high-fat diet (HFD)/streptozotocin (STZ)-induced diabetic mice and in Min6 cells exposed to high glucose/palmitic acid (HG/PA)-RAW264.7 conditioned medium. Lycopene intervention resulted in M1/M2 macrophages polarization homeostasis, which are associated with increased insulin secretion, decreased fasting blood glucose levels, and improved lipid profiles in diabetic mice. Furthermore, the protective actions of lycopene were associated with down-regulation of the TLR4/MyD88/NF-κB signaling pathway, which is positively related to inflammation in both diabetic mice and Min6 cells. Collectively, our findings indicated that lycopene ameliorates islet function and apoptosis and attenuates hyperglycemia and dyslipidemia, via the regulation of TLR4/MyD88/NF-κB signaling pathway. This study highlights dietary lycopene consumption as a novel strategy for the management of patients with diabetes.

Keywords

Lycopene; Diabetes; Islet function; β cells; Macrophages; TLR4/MyD88/NF-κB signaling pathway.

Abbreviations

AUC	Areas under the curve
Bcl-2	B-cell lymphoma-2
Bax	Bcl2-associated x
CRP	C-reactive protein
DAB	Diaminobenzidine
DAPI	4', 6-diamidino-2-phenylindole

FBG	Fasting blood glucose
FBS	Fetal bovine serum
H&E	Hematoxylin/Eosin
HFD	High-fat diet
HG/PA	High glucose and palmitic acid
IL-6	Interleukin-6
LDL	Low-density lipoprotein
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa-B
PDX1	Pancreatic duodenal homeobox-1
STZ	Streptozotocin
TLR4	Toll-like receptor 4
TC	Total cholesterol
TG	Triglycerides
TNFα	Tumor necrosis factor α
T2DM	Type 2 diabetes mellitus

1. Introduction

Diabetes mellitus is characterized by insufficient islets function to meet peripheral insulin requirement [1], and has become an epidemic in the world mainly due to unhealthy lifestyles [2, 3]. According to the International Diabetes Federation, 537 million people were suffering from diabetes around the world in 2021, and the number of affected patients is expected to reach 783 million by 2045. Of these, type 2 diabetes mellitus (T2DM) accounts for more than 90% of all diabetic cases, which is orchestrated by an elevated blood glucose level [4] and abnormal lipid profiles [5]. During the orchestra, chronic inflammation has attracted growing attention for its substantial contribution to islet β cells dysfunction and consequent the disorders of glucose and lipid metabolism [6-8].

The infiltration of macrophages into islets and its resultant overproduction of proinflammatory cytokines and chemokines, such as interleukin-6 (IL-6) and tumor necrosis factor α (TNFα), may lead to islet inflammation, β cell apoptosis and islet

dysfunction [9, 10]. There are two major phenotypes of macrophages involved in islets inflammation: classically activated M1 macrophages that mainly produce pro-inflammatory cytokines; and alternatively activated M2 macrophages that mainly exert anti-inflammation response [11]. Mounting evidence supports the concept that a polarity shift of macrophages from M1-like toward M2-like phenotype, contributes to insulin secretion and β cells function [7, 12-14].

Toll-like receptor 4 (TLR4), mainly derived from islet macrophages [15], signals through its downstream effectors myeloid differentiation factor 88 (MyD88) and nuclear factor kappa-B (NF-κB) to regulate islet inflammation during diabetes onset [6, 16]. Elevation of TLR4 expression activates islet macrophages to promote the secretion of inflammatory factors and subsequent β cells dysfunction [17]. By contrast, inhibition of TLR4 expression serves islets homeostasis through the TLR4/MyD88/NF-κB signaling pathway [18, 19]. Although islet β-cell does not express TLR4 [15], an upregulation of TLR immunoreactivity in islet macrophages is positively related to recruitment of macrophages to islets and their shift from M1-like to M2-like phenotype and consequent islet inflammation [20]. Clinically, TLR4 levels are elevated in the patients with diabetes [21, 22]. Therefore, limitation of the TLR4/MyD88/NF-κB signaling pathway may offer an effective therapeutic strategy to inhibit islet inflammation in diabetes.

Lycopene, a natural red pigment of fruits and vegetable, including tomatoes and watermelon, has been shown to alleviate hepatitis, diabetes, obesity, and other metabolic diseases due to its various biological activities, such as anti-inflammation, antioxidant and modulations of both glucose and lipid metabolism [23, 24]. We and others have demonstrated that lycopene improves glycolipid metabolism in diabetic and obese animals [25-29]. Moreover, lycopene may promote insulin secretion via preserving redox homeostasis by its anti-oxidant properties [25, 30-32]. However, whether and how lycopene regulates islet function in T2DM remain largely unknown.

In the present study, we investigated protective actions of lycopene on islet function in high-fat diet (HFD)/streptozotocin (STZ)-induced diabetic mice and in high glucose/palmitic acid (HG/PA)-RAW264.7 conditioned medium-stimulated Min6

cells. We also explored the potential underlying mechanisms involved in lycopene protection in both *in vivo* and *in vitro* models. We aimed to facilitate the development of new strategy to eliminate the islet dysfunction and provide new insights into the role of lycopene in protection against diabetes.

2. Materials and methods

2.1 Main materials

Lycopene (purity > 99.0%, F-003) was purchased from Chengdu Herbpurify CO., LTD (Chengdu, China). Metformin (purity>98.0%, ABF2453) was from Sino-American Shanghai Squibb Pharmaceuticals Ltd. Streptozotocin (STZ; S0130) was obtained from Sigma Chemicals.

2.2 Animal experiments

Eight-week-old male C57BL/6J mice [20 ± 2 g; Animal production license number: SCXK (Jing) 2016-0010] were purchased from Beijing SiBeiFu Animal Technology Co. Ltd. (Beijing, China), and maintained under standard conditions at 25 ± 2 °C and a relative humidity of 55 ± 5% with a 12 h light/dark cycle allowed free access to food and water during the experiments. All animal experiments were performed in compliance with the Animal Care Committee of Beijing University of Chinese Medicine (BUCM). Standard experimental protocols were approved by the ethics of animal experiments of BUCM [Animal licensee permit number: SCXK (Jing) 2016-0002; Approved No: BUCM-4-2016061701-3001].

After one week of acclimation, mice were randomly divided into normal control diet (NC) group and HFD (MD12033, Medicience Ltd., Jiangsu, China) group. Seven weeks after the first grouping, mice fed with HFD were injected intraperitoneally with 100 mg/kg STZ (Dissolved in pH 4.5 pre-chilled citrate buffer), while mice fed with the normal diet were subjected to the vehicle treatment only. Three days after STZ injection, mice that fasting blood glucose (FBG) levels ≥ 11.1 mM were considered to be a successful diabetic animal model. Subsequently, the diabetic mice were randomly divided into the following 3 groups: (a) Diabetic model (DM) group: The diabetic mice were orally administrated with sunflower oil; (b) Metformin (DM+Met) group: The

diabetic mice were orally administrated with 200 mg/kg of metformin in sunflower oil [33]; (c) Lycopene (DM+Lyc) group: The diabetic mice were administrated with 10 mg/kg of lycopene in sunflower oil by gavage [26]. The mice in the NC group were orally administered with same volume of sunflower oil. During the treatment, except for the NC group, mice in the other groups were kept on the HFD feeding until the end of the experiment. The body weight, water consumption, food intake, and FBG levels were measured during the treatment.

After 12 weeks of intervention, blood was harvested from the hearts of the anesthetized mice. And then serum was obtained by centrifugation (4000 rpm, 4 °C, 10 min) and stored at −80 °C until use. Meanwhile, pancreases were rapidly removed from the animal bodies and then weighed and stored at −80 °C or fixed in 4% neutral buffered formalin for histopathological observation.

2.3 Oral glucose tolerance test (OGTT)

After fasting 12 h, the mouse in each group was orally administered with 2 g/kg of glucose, and then the blood glucose levels were measured at 0, 30, 60, 90 and 120 min from the tail veins of mice using a blood glucose meter (Contour TS, Germany), respectively. The areas under the curve (AUC) of blood glucose levels were calculated to evaluate glucose tolerance.

2.4 Insulin tolerance test (ITT)

After fasting 4 h, the mouse in each group was injected with 0.5 U/mL of insulin, and then the blood glucose levels were measured at 0, 30, 60, 90 and 120 min from the tail veins of mice using a blood glucose meter (Contour TS, Germany), respectively. The areas under the curve (AUC) of blood glucose levels were calculated to evaluate glucose tolerance.

2.5 Body composition analysis

At the end of treatment followed by fasting overnight, all the mouse in each group was weighed, and then the body compositions (body fat mass and lean mass) were measured by EchoMRI-100H Body Composition Analyzer (Houston, USA) as described previously [34].

2.6 Hematoxylin/Eosin (H&E) staining

The formalin-fixed pancreases were embedded in paraffin and then were sectioned in a Lecia microtome. Then, four-μm sections were stained with H&E for histopathological examinations, according to the routine procedure as described before [35].

2.7 TUNEL staining

Cell apoptosis was assessed by a colorimetric TUNEL apoptosis assay kit (C1091; Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocol. Briefly, after deparaffinization and rehydration, pancreas sections were treated with protease K (20 mg/mL) for 20 min, and then were incubated with a TUNEL dye solution in dark at 37 °C for 60 min. Images were captured under an Olympus BX53 fluorescence microscope (Tokyo, Japan). Quantitative assessment of apoptotic cells per islet field was performed by Image-Pro Plus 6.0 software.

2.8 Serum biochemical analysis

Serum levels of triglycerides (TG), total cholesterol (TC) and low-density lipoprotein (LDL) were determined by the commercial kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Serum levels of IL-6 and TNFα were measured by ELISA kits according to the manufacturer's instructions (ABclonal Biotechnology Company, Wuhan, China). And serum insulin levels were determined using mouse insulin ELISA kits (American Laboratory Products Company, USA).

2.9 Immunohistochemical (IHC) and immunofluorescence (IF) staining analyses

For IHC staining analysis, pancreas sections were incubated at 4 °C overnight with the following primary antibodies: caspase 3 (Proteintech, Cat No. 66470-2-Ig; 1:500), TLR4 (Wanleibio, WL00196; 1:1000), MyD88 (Wanleibio, WL02494; 1:500), NF-κB (p65; Wanleibio, WL01980; 1:500), and p-NF-κB (S536; Wanleibio, WL02169; 1:1000), respectively, and then were incubated with the corresponding secondary antibody for 1 h at room temperature. Finally, the positive signals were visualized by staining with diaminobenzidine (DAB) solution and counterstaining with hematoxylin. Images were captured under an Olympus BX53 microscope (Tokyo, Japan) and

analyzed using Image-Pro Plus 6.0 software.

For IF staining analysis, the pancreas sections were incubated with the following primary antibodies: insulin (Proteintech, CL594-15848; 1:500), F4/80 (Proteintech, 28463-1-AP; 1:2000), CD86 (Bioss, bs-1035R; 1:1000) and CD206 (Proteintech, 18704-1-AP; 1:1000), respectively, at 4 °C overnight. Then on the 2nd day, the sections were incubated with the corresponding secondary antibody for 1 h at room temperature. Finally, the sections were re-stained by 4', 6-diamidino-2-phenylindole (DAPI). The immunofluorescence was captured under an Olympus BX53 fluorescence microscope (Tokyo, Japan) and the positive staining was analyzed using Image-Pro Plus 6.0 software.

2.10 Western blotting analysis

Total protein was extracted from pancreas tissues (about 30 mg) in a RIPA lysis buffer (about 450 μL) containing 1% PMSF, protease inhibitors and phosphatase inhibitors. And the protein concentration was determined by a bicinchoninic acid assay kit. Equal amounts of protein were loaded into 10% SDS-PAGE gel and then transferred onto a PVDF membrane. After blocking with 5% nonfat-dry milk, the membranes were incubated with pancreatic duodenal homeobox 1 (PDX1) antibody (Proteintech, 20989-1-AP; 1:1000) overnight at 4 ℃, followed by the corresponding secondary antibody for 1 h at room temperature. The bands were visualized using a ECL luminous liquid and the images were captured with Azure Bio-imaging systems (CA, USA). The gray values of the bands were quantified using an Image J software and normalized with the corresponding β-actin (Proteintech, 20536-1-AP; 1:20000) as the internal control.

2.11 Cell culture

Min6 cells (C582; Wuhan FineTest Biotechnology Co. Ltd; Wuhan, China) and RAW264.7 cells (1101MOU-PUMC000146; A gift from the laboratory of Professor Yu Hao of BUCM) were maintained in 1640 medium (Gibco; New York, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco; New York, USA) and 1% penicillin–streptomycin (Beijing Aoqing Biotechnology Co. Ltd, Beijing, China) in a humidified incubator at 37 ℃ with 5% CO_2.

2.12 Preparation of PA

The 20 mM solution of PA in 20 mM NaOH was incubated at 70 °C for 30 min. Then, 1 mL of 20 mM PA was mixed with 4 mL of 5% BSA and then filter-sterilized to obtain the stocked liquid of 4 mM PA. Finally, the stocked liquid of 4 mM PA was added to 1640 medium to achieve a final concentration of 0.3 mM PA.

2.13 Cell viability analysis

The cytotoxicity of lycopene was firstly measured using a cell counting kit-8 (CCK8; Beijing Aoqing Biotechnology Co. Ltd, Beijing, China). Briefly, Min6 and RAW264.7 cells (8-10×10^4/mL) were seeded into a 96-well plate overnight, respectively, and then incubated with various concentrations of lycopene (Dissolved in DMSO; 0, 2, 4, 6, 8 and 10 μM) for 24, 48 and 72 h in the presence or absence of HG/PA (20 mM/0.3 mM) for 6, 12 and 24 h. After that, CCK8 was added to each well and incubated for 1 h at 37 °C. Finally, the absorbance was measured at 450 nm using a FLUOstar Omega microplate reader (BMG Labtech, Germany).

2.14 Preparation of conditioned medium

To assess the effects of lycopene on inflammation responses in the islets, RAW264.7 cells (4×10^5/mL) were exposed to the following conditions in the three different groups: (1) Normal control group: RAW264.7 cells were incubated with 0.4% BSA; (2) HG/PA group: RAW264.7 cells were stimulated with HG/PA for 6 h; (3) HG/PA+lycopene group: RAW264.7 cells were pre-treated with lycopene (4 μM) for 24 h and then stimulated with HG/PA for 6 h. Subsequently, the conditioned medium was harvested from each group, and then separately added to Min6 cells for 6 h in the presence or absence of a TLR4 inhibitor [TAK242 (1 μM)].

2.15 RNA extraction and qRT-PCR analysis

The cells (2×10^5/mL) were seeded into a 12-well plate overnight. After intervention, total RNA was extracted from the cells using the Trizol reagent. First-strand cDNAs were synthesized by reverse transcription of 1 μg of total RNA using the Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit. qPCR assay was performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) using PowerUp™ SYBRTM Green Master Mix (Thermo Fisher

Scientific), 10 mM of each primer, and 300-500 ng of cDNA. Relative quantification for qRT-PCR was calculated using $2^{-\Delta\Delta Ct}$ method and β-actin was used as an internal control. Primer sequences were listed in Table 1.

Table 1. Primers for qRT-PCR amplification.

Gene	Primer	Sequences (5' to 3')	Species
Bax	F	GCTACAGGGTTTCATCCAGGATCG	Mus musculus
	R	TGCTGTCCAGTTCATCTCCAATTCG	
Bcl-2	F	CCAGCCTGAGAGCAACCCAATG	Mus musculus
	R	ACGACGGTAGCGACGAGAGAAG	
Caspase 3	F	TCTGACTGGAAAGCCGAAACTCTTC	Mus musculus
	R	GTCCCACTGTCTGTCTCAATGCC	
CD86	F	TCTGCCGTGCCCATTTACAAAGG	Mus musculus
	R	TGCCCAAATAGTGCTCGTACAGAAC	
CD206	F	GTCTGAGTGTACGCAGTGGTTGG	Mus musculus
	R	TCTGATGATGGACTTCCTGGTAGCC	
F4/80	F	TTCCTGCTGTGTCGTGCTGTTC	Mus musculus
	R	GCCGTCTGGTTGTCAGTCTTGTC	
Insulin	F	GGCTTCTTCTACACACCCAAGTCC	Mus musculus
	R	TGATCCACAATGCCACGCTTCTG	
MyD88	F	AGCAGAACCAGGAGTCCGAGAAG	Mus musculus
	R	GGGCAGTAGCAGATAAAGGCATCG	
NF-κB (p65)	F	AGACCCAGGAGTGTTCACAGACC	Mus musculus
	R	GTCACCAGGCGAGTTATAGCTTCAG	
PDX1	F	GTTCCAAAACCGTCGCATGAAGTG	Mus musculus
	R	CCGAGGTCACCGCACAATCTTG	
TLR4	F	GAGCCGGAAGGTTATTGTGGTAGTG	Mus musculus
	R	AGGACAATGAAGATGATGCCAGAGC	

2.16 Statistical analysis

All the results were analyzed using GraphPad prism 8.0 and expressed as the mean ± SEM. Data were analyzed by one-way ANOVA and Dunnett's T test. When the data met homogeneity of variances and the normal distribution, a one-way analysis of variance (ANOVA) was applied. When the data satisfied the normal distribution but did not achieve homogeneity of variance, or did not satisfy the normal distribution, Dunnett's T test or nonparametric test was used, respectively. A *p* value of less than 0.05 was considered to be statistically significant.

3. Results

3.1 Lycopene regulates glucose and lipid metabolism in diabetic mice

As shown in Fig. 1A, mice in the DM, DM+Met and DM+Lyc groups appeared hyperglycemic at the beginning of treatment. In addition, the results from OGTT (10^{th} week) showed that mice in the DM group were glucose-intolerant, and the AUC of OGTT was significantly greater than those in the NC group ($p<0.05$; Fig. 1B and C). Intriguingly, lycopene or metformin treatment significantly prevented these alterations in diabetic mice ($p<0.05$).

As shown in Fig. 1D-F, both body weight and body lean ratio were significantly decreased ($p<0.05$), and the body fat ratio was significantly increased in the mice of the DM group compared to those in the NC group ($p<0.05$). Similar to the action of metformin, lycopene significantly reversed the body weight, body lean ratio and body fat ratio in the diabetic mice compared to the vehicle-treated ones ($p<0.05$).

In addition, we assessed the alteration of calorie intake and water intake in each group of mice. As illustrated in Fig. 1G-H, both calorie and water intakes were markedly increased in the mice of the DM group in comparison with those in the NC group at the 4^{th}, 8^{th} and 12^{th} week ($p<0.05$). Notably, lycopene or metformin treatment significantly decreased the water intake ($p<0.05$), but did not affect the calorie intake in diabetic mice compared to the vehicle-treated ones at the different time points.

Moreover, serum levels of TC, TG and LDL were remarkably higher and serum HDL levels were significantly lower, respectively, in the mice of the DM group than those in the NC group (Fig. 1I; $p<0.05$). Intriguingly, either lycopene or metformin intervention significantly reversed the alterations of serum TC, TG, LDL and HDL levels in diabetic mice compared to the vehicle treatment ($p<0.05$). The results suggested that lycopene prevent dyslipidemia in diabetic mice, thusly contributes to correction of body composition. Collectively, these results indicated that lycopene could regulate glucose and lipid metabolism in diabetic mice.

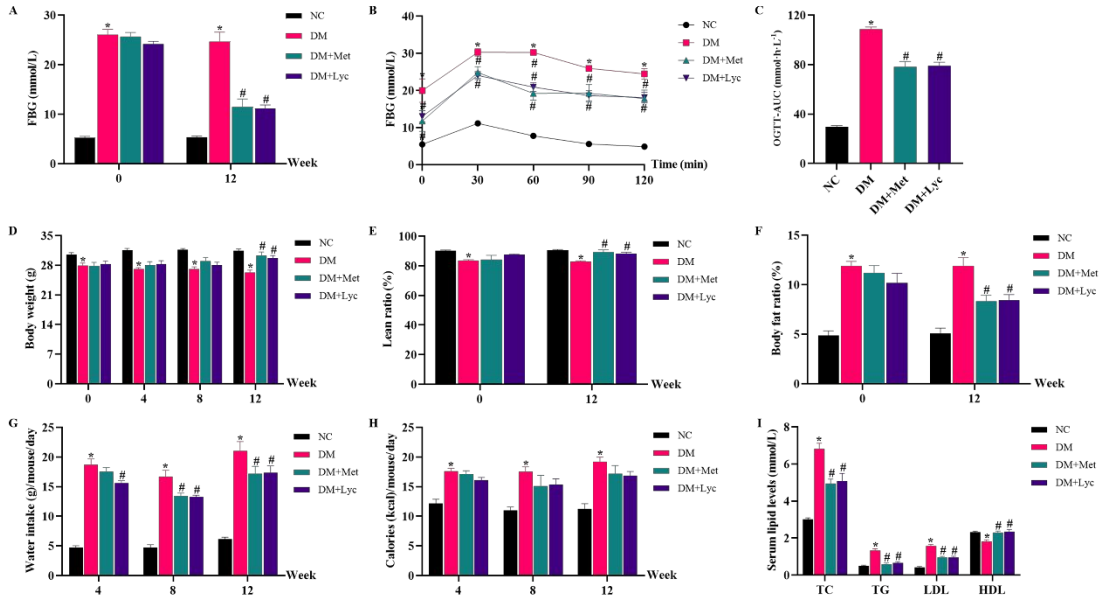

Figure 1. Lycopene regulates glucose and lipid metabolism in diabetic mice. (A) FBG levels at 0 and 12th week; (B) OGTT at 10th week, *compared with the NC group at the corresponding time point, #compared with the DM group at the corresponding time point; (C) AUC of OGTT at 10th week; (D) Body weight; (E) Lean ratio (Lean/body weight * 100%) at 0 and 12th week; (F) Body fat ratio (Body fat/body weight * 100%) at 0 and 12th week; (G) Water intake; (H) Calories consumption (kcal); (I) Serum levels of TC, TG, LDL and HDL. Data are presented as the mean ± SEM (n=6). *Compared with the NC group, #compared with the DM group. p<0.05 was considered a statistical difference. FBG: fasting blood glucose; OGTT: glucose tolerance test; AUC: area under the curve; TC: total cholesterol; TG: triglycerides; LDL: low-density lipoprotein; HDL: high-density lipoprotein.

3.2 Lycopene improves islet function in diabetic mice

We then examined the effects of lycopene on islet function in diabetic mice. The results from ITT (11th week) showed that insulin sensitivity was significantly lower, and the AUC of ITT was significantly greater in the mice of the DM group, respectively, than those in the NC group. Compared with the DM+Lyc group, the AUC of ITT in DM+Met group was significantly decreased (p<0.05; Fig. 2A-B). Moreover, as shown in Fig. 2C-D, serum insulin levels and HOMA-β were lower in the mice of the DM group than those in the NC group (p<0.05). As expected, lycopene or metformin treatment prevented these alterations in diabetic mice (p<0.05).

In addition, compared to the NC group, the islets in the DM group showed an abnormal micro-structure, including the disorganization of islet boundary, inhomogeneous islet cells, and cytoplasmic vacuolation (Fig. 2E). The expressions of islet insulin (Fig. 2F-G) and PDX1 (Fig. 2H), a transcriptional factor that regulated insulin expression and islet function [36], were remarkably decreased in the mice of the DM group determined by IF staining and western blots, respectively (p<0.05). As expected, lycopene or metformin treatment significantly prevented these alterations in diabetic mice (p<0.05).These results indicated that the impaired islet function in diabetic mice could be reversed by lycopene treatment.

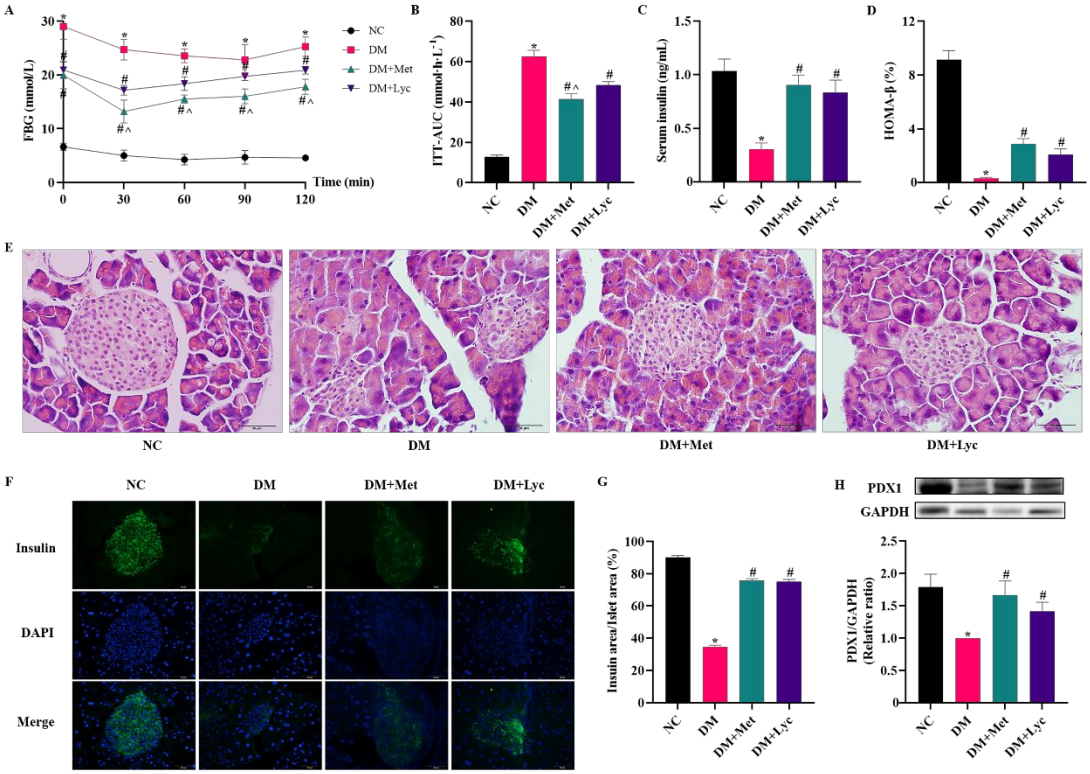

Figure 2. Lycopene improves islet function in diabetic mice. (A) ITT at 11th week (n=6), *compared with the NC group at the corresponding time point, #compared with the DM group at the corresponding time point, ^compared with the DM+Lyc group at the corresponding time point; (B) AUC of ITT at 11th week (n=6); (C) Serum insulin levels determined by ELISA (n=5); (D) HOMA-β (n=5); (E) Representative images of pancreatic histopathologic microstructure determined by H&E staining (400×; n=5); (F-G) The representative images of insulin expression in islets determined by immunofluorescence staining (400×; n=5); (H) The PDX1 expression determined by western blot (n=5). Data are presented as the mean ± SEM.

*Compared with the NC group, #compared with the DM group, ^compared with the DM+Lyc group. $p<0.05$ was considered a statistical difference. ITT: insulin tolerance test; AUC: area under the curve; PDX1: pancreatic duodenal homeobox 1.

3.3 Lycopene preserves M1/M2 macrophage homeostasis in the islets of diabetic mice and in HG/PA-stimulated RAW264.7 cells

Studies have shown that the sustained inflammation could induce islet dysfunction and consequently trigger the development of diabetes [37-39]. Accordingly, we investigated the effects of lycopene on altering inflammatory responses in diabetic mice, as compared with that of metformin treatment. As shown in Fig. 3A and B, serum levels of IL-6 and TNFα were significantly increased by 34.19% and 23.03%, respectively, in the DM group than in the NC group ($p<0.05$). Interestingly, lycopene or metformin treatment dramatically decreased serum IL-6 levels by 19.98% and 18.25%, and decreased serum TNFα levels by 16.77% and 16.67%, respectively, in diabetic mice in comparison with the vehicle-treated ones ($p<0.05$).

Since the disturbed homeostasis of M1/M2 macrophages triggers islet inflammation and thusly limit insulin secretion [40], we are interested to determine if lycopene could attenuate islet inflammation in HFD/STZ-induced diabetic mice. F4/80, a mouse macrophage marker, is closely related to the occurrence and development of inflammation [41]. As shown in Fig. 3C and D, the F4/80 expression in the islets of the DM group was significantly higher than that in the NC group ($p<0.05$). In contrast, lycopene or metformin treatment obviously prevented this alteration in the islets of diabetic mice ($p<0.05$).

In addition, the results from IF staining revealed that the expression levels of CD86 and CD86/CD206 were significantly upregulated in the islets of the DM group compared to those in the NC group (Fig. 3E-F; $p<0.05$). Interestingly, similar to metformin treatment, lycopene markedly downregulated the expression levels of CD86 and CD86/CD206 in the islets of diabetic mice compared to those with the vehicle-treated ones ($p<0.05$).

To further explore the effects of lycopene on the actions of macrophages and β

cells, Min6 cells and RAW 264.7 cells were exposed to HG/PA stimulation with or without lycopene treatment. We firstly determined the appropriate concentration for lycopene intervention. As shown in Fig. 3G-H, lycopene did not affect cell viability between 0-4 μM in Min6 and RAW264.7 cells, suggesting the non-toxic concentration of lycopene for both cell lines. Hence, 1, 2, and 4 μM of lycopene were selected for the further experiments. Next, we determined cell viability at different time points (6, 12 and 24 h) in Min6 cells and RAW264.7 cells in response to HG/PA stimulation. As shown in Fig. 3I, HG/PA significantly decreased the cell viability in a time-dependent manner in both cells (p<0.05). Lycopene (1, 2 and 4 μM) was used to pretreat HG/PA-stimulated Min6 cells and RAW264.7 cells, respectively. As displayed in Fig. 3J, cell viability was significantly lower in both cells under HG/PA stimulation, and this decrease was prevented by pretreating cells with 4 μM of lycopene (p<0.05). Therefore, lycopene at 4 μM was used to stimulate the cells for the following assays.

In order to assess the effects of lycopene on the shift of macrophage phenotypes in HG/PA-stimulated RAW264.7 cells, we examined the mRNA expressions of F4/80, CD86 and CD206 by qRT-PCR. As illustrated in Fig. 3K-L, HG/PA significantly increased the mRNA expressions of F4/80 and CD86/CD206 in RAW264.7 cells (p<0.05). We observed that lycopene treatment significantly decreased the relative mRNA expression of CD86 to CD206 (p<0.05), while lycopene did not affect F4/80 expression in HG/PA-stimulated RAW264.7 cells. These results suggested that lycopene may inhibit islet inflammation through a shift in polarization from a M1-like to M2-like macrophage phenotype in RAW264.7 cells induced by HG/PA.

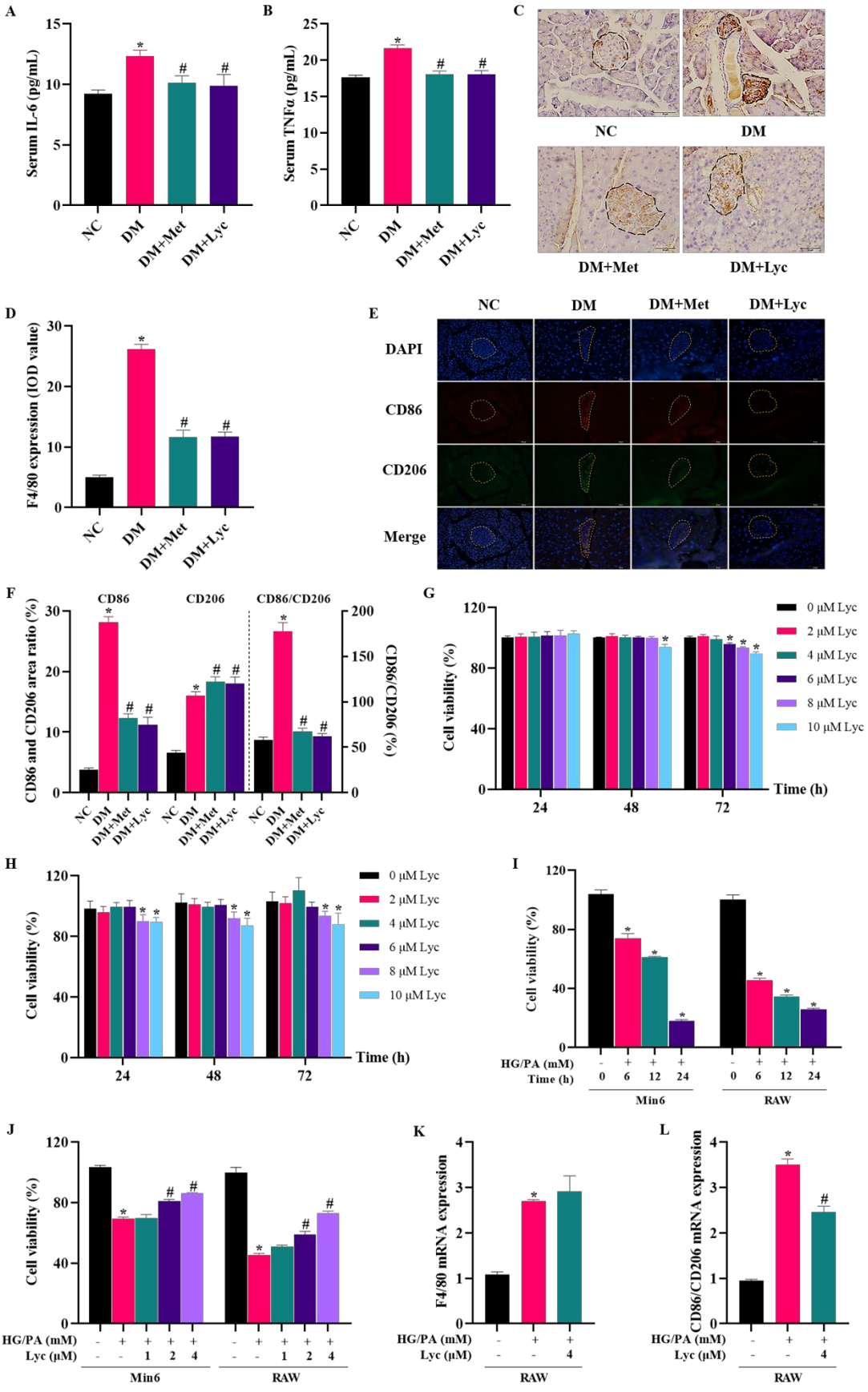

Figure 3. Lycopene preserves M1/M2 macrophage homeostasis in the islets of diabetic mice

and in HG/PA-stimulated RAW264.7 cells. (A) Serum levels of IL-6 in mice determined by ELISA (n=6); (B) Serum levels of TNFα in mice determined by ELISA (n=6); (C-D) The expression levels of F4/80 in the islets determined by immunohistochemical staining (400×; n=5); (E-F) The expression levels of CD86 (Left, F), CD206 (Middle, F) and CD86/CD206 (Right, F) determined by immunofluorescence analyses after 12-week treatment (400×; n=5); (G-H) The viability of Min6 and RAW264.7 cells at various concentrations of lycopene treatment (0-10 μM) (n=6); (I) The viability of Min6 and RAW264.7 cells in the presence of HG/PA stimulation for 6, 12, and 24 h (n=6); (J) The viability of Min6 and RAW264.7 cells after lycopene pre-treatment in the presence of HG/PA or HG/PA-RAW264.7 conditioned medium for 6 h (n=6); (K-L) The mRNA expressions of F4/80 and CD86/CD206 in HG/PA-stimulated (6 h) RAW264.7 cells with or without lycopene pre-treatment (n=3). Data are presented as the mean ± SEM. For mice, *compared with the NC group, #compared with the DM group. For cells, *compared with the vehicle treatment group, #compared with the HG/PA group. $p<0.05$ was considered a statistical difference. IL-6: interleukin-6; TNFα: tumor necrosis factor α.

3.4 Lycopene attenuates cell apoptosis in the islets of diabetic mice and in Min6 cells exposed to HG/PA-RAW264.7 conditioned medium

Inspired by the evidence that chronic inflammation and its consequent hyperglycemia could accelerate islet cells apoptosis [42, 43], we subsequently evaluated the effects of lycopene on β cells apoptosis in diabetic mice by a TUNEL assay and caspase 3 expression. As shown in Fig. 4A and B, the apoptosis rate of islet cells was significantly higher in the DM group than in the NC group ($p<0.05$). Moreover, the results from IHC staining (Fig. 4C and D) demonstrated that the expression levels of caspase 3 were also higher in the islets of the DM group than in the NC group ($p<0.05$). By contrast, lycopene or metformin intervention markedly attenuated islet cells apoptosis in the diabetic mice ($p<0.05$).

Moreover, we assessed the effects of lycopene on the alterations of apoptosis-related genes in Min6 cells exposed to HG/PA-RAW264.7 conditioned medium. HG/PA or HG/PA-RAW264.7 conditioned medium treatment (Fig. 4C-D) markedly

decreased the mRNA expression of B-cell lymphoma-2 (Bcl-2) and increased the mRNA expression of Bcl2-associated x (Bax) in Min6 cells (p<0.05). Notably, lycopene intervention only pronouncedly reversed the alterations in Min6 cells upon HG/PA-RAW264.7 conditioned medium stimulation (p<0.05). Moreover, as shown in Fig. 4E, the caspase 3 mRNA expression was only markedly increased in the HG/PA-RAW264.7 conditioned medium-stimulated Min6 cells (p<0.05), and lycopene treatment significantly prevented this alteration (p<0.05). These data suggested that lycopene may attenuate islet cells apoptosis through inhibition of inflammation in diabetic mice.

In light of the concept that islet cells apoptosis may diminish insulin secretion [44], we thusly investigated the actions of lycopene on Min6 cells in the presence of HG/PA-RAW264.7 conditioned medium. As shown in Fig. 4F and G, the mRNA expressions of PDX1 and insulin were obviously decreased in Min6 cells in HG/PA-RAW264.7 conditioned medium (p<0.05). However, lycopene treatment notably reversed the mRNA expressions of PDX1 and insulin in Min6 cells in the presence of HG/PA-RAW264.7 conditioned medium (p<0.05), but not in HG/PA stimulation. Collectively, these data indicated that lycopene may ameliorate islet function by limiting islet cell apoptosis via regulation of macrophage inflammatory response in diabetic mice.

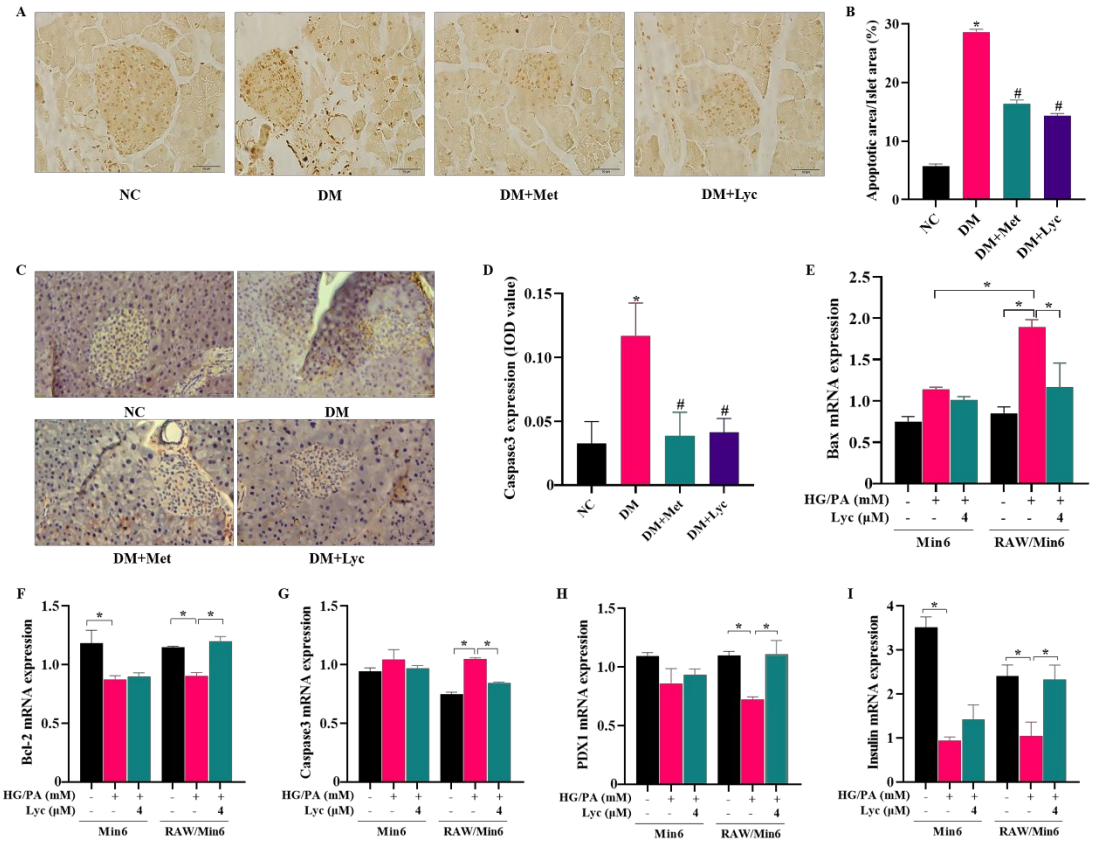

Figure 4. Lycopene attenuates cell apoptosis in the islets of diabetic mice and in Min6 cells exposed to HG/PA-RAW264.7 conditioned medium. The representative images (400×) of TUNEL staining and their analyses in the islets after 12-week treatment (A & B; n=5). The representative images (400×) of immunohistochemical staining and their analyses showed the expression levels of caspase 3 (C-D) in the islets after 12-week treatment (n=5). The mRNA expressions of Bax (E), Bcl-2 (F), caspase 3 (G), PDX1 (H) and insulin (I) were measured by qRT-PCR in Min6 cells (n=3) after lycopene pre-treatment for 24 h in the presence of HG/PA for 6 h. Data are presented as the mean ± SEM. For mice, *compared with the NC group, #compared with the DM group. $p<0.05$ was considered a statistical difference. Bax: bcl2-associated x; Bcl-2: b-cell lymphoma-2; PDX1: pancreatic duodenal homeobox 1.

3.5 Lycopene suppresses the TLR4/MyD88/NF-κB signaling pathway in the islets of diabetic mice and in Min6 cells exposed to HG/PA-RAW264.7 conditioned medium

The TLR4/MyD88/NF-κB signaling pathway is positively related to the enrichment of M1 macrophages in islets and resultant inflammation [45]. Accordingly,

we examined the actions of lycopene on the alterations of related factors involved in the TLR4/MyD88/NF-κB signaling pathway in diabetic mice. The results from IHC staining (Fig. 5A-H) demonstrated that the expression levels of TLR4, MyD88, NF-κB and p-NF-κB were higher in the islets of the DM group than in the NC group ($p<0.05$). While lycopene or metformin intervention significantly decreased the expression levels of TLR4, MyD88, NF-κB and p-NF-κB in diabetic mice compared to the vehicle treatment ($p<0.05$).

To further elucidate the underlying mechanisms, Min6 cells were exposed to HG/PA or HG/PA-RAW264.7 conditioned medium. As shown in Fig. 5I-K, the addition of HG/PA to Min6 cells significantly increased the mRNA expressions of TLR4, MyD88 and NF-κB ($p<0.05$). The potential was further augmented by HG/PA-RAW264.7 conditioned medium ($p<0.05$). However, lycopene treatment significantly decreased the mRNA expressions of TLR4, MyD88 and NF-κB in Min6 cells only in the presence of HG/PA-RAW264.7 conditioned medium ($p<0.05$). Additionally, the administration of TAK242, an inhibitor of TLR4, blocked the TLR4/MyD88/NF-κB signaling pathway in Min6 cells upon conditioned medium with HG/PA. These results demonstrated that lycopene may ameliorate islet function in diabetic mice via regulation of the TLR4/MyD88/NF-κB signaling pathway.

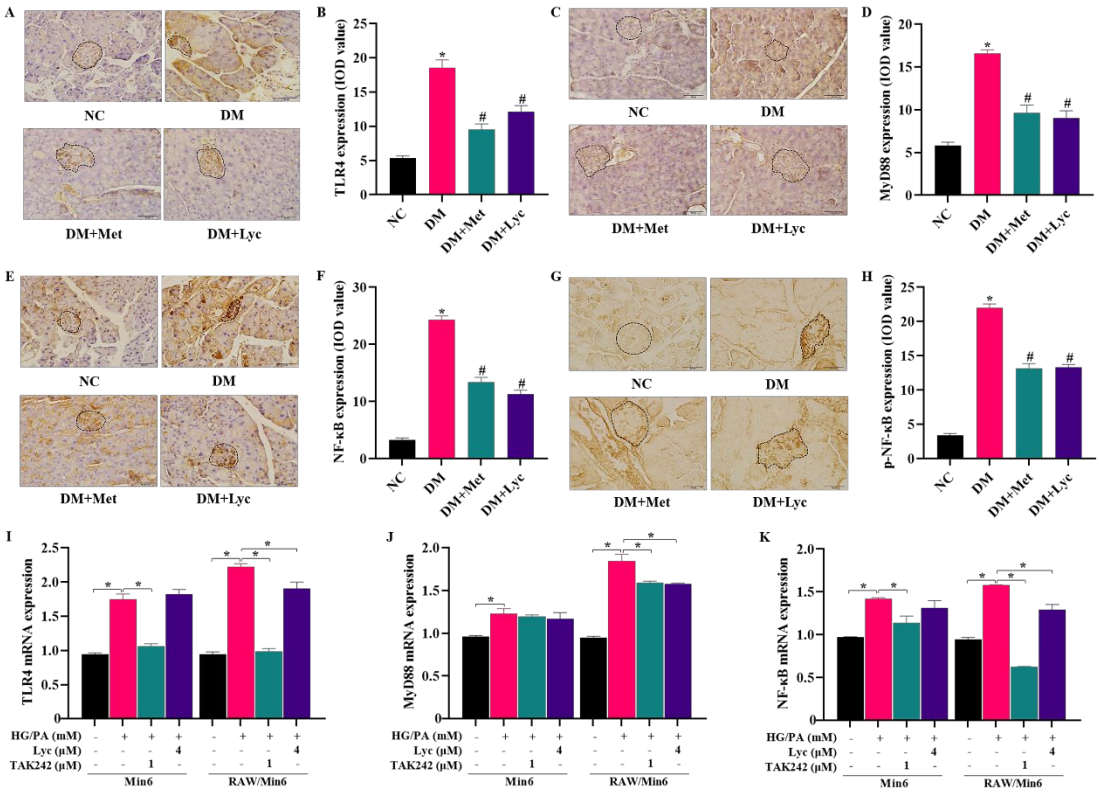

Figure 5. Lycopene suppresses the TLR4/MyD88/NF-κB signaling pathway in the islets of diabetic mice and in Min6 cells exposed to HG/PA-RAW264.7 conditioned medium. The representative images (400×) of immunohistochemical staining and their analyses show the expression levels of TLR4 (A-B), MyD88 (C-D), NF-κB (E-F) and p-NF-κB (G-H) in the islets (n=5). qRT-PCR results show the mRNA expression levels of TLR4 (I), MyD88 (J) and NF-κB (K) in Min6 cells with or without HG/PA or HG/PA-RAW264.7 conditioned medium (n=3). Data are presented as the mean ± SEM. For mice, *compared with the NC group, #compared with the DM group. $p<0.05$ was considered a statistical difference. TLR4: toll-like receptor 4; MyD88: myeloid differentiation factor 88; NF-κB: nuclear factor kappa-B.

4. Discussion

The chronic low degrade inflammation driven by the intra-islet accumulation of macrophages initiates β cell apoptosis and islet dysfunction [15, 46, 47]. Recently, natural products have attracted scientists' attention for their contribution in limitation of macrophage inflammatory response in the intra-islets and pancreatic β-cell death to deliver the benefits for diabetes management [45, 48, 49]. In the present study, we firstly demonstrated that lycopene alleviates the disorders of glucose and lipid metabolism in

HFD/STZ-induced diabetic mice. Secondly, lycopene treatment promotes the dynamic balance of M1/M2 macrophages in the islets of diabetic mice and in HG/PA-stimulated RAW264.7 cells. Thirdly, lycopene treatment results in attenuation of islet cells apoptosis and enhancement of insulin secretion. Finally, we provide a novel insight that lycopene may limit the activation of the TLR4/MyD88/NF-κB signaling pathway in HFD/STZ-induced diabetic mice and in Min6 cells exposed to HG/PA-RAW264.7 conditioned medium.

Exposure of mouse to HFD/STZ may cause hyperglycemia and dyslipidemia, and these alterations were prevented by lycopene intervention, including an improvement in glucose tolerance, FBG levels, calorie and water intake, and body composition, as well as blood lipid profiles (TC, TG, LDL and HDL), which was consistent with the previous reports that lycopene attenuates glucose and lipid disorders in HFD-induced obese rats [28, 29] and STZ-CCT (4% cholesterol, 1% cholic acid in diet and 0.5% thiouracil in drinking water) diet-induced hyperglycemia and hyperlipidemic rats [50]. Epidemiological investigations also indicate that lycopene consumption may deliver benefits for glucose and lipid metabolism [23]. However, in the present study, we found that mice in the NC group consumed fewer calories but were heavier than those in the DM group. One explanation for the phenomena is that the disorders of glucose and lipid metabolism may attenuate the protein synthesis and thusly lead to muscle atrophy and weight loss in the mice of the DM group [51, 52]. Collectively, these findings indicate that lycopene may prevent the development of diabetes by improving glucose and lipid metabolism.

Macrophage polarization and its consequent shift from M2-like to M1-like phenotype result in pro-inflammatory atmosphere in the islets [53-55]. Indeed, the present study demonstrated that the diabetic mice exhibit an increase in serum levels of proinflammatory cytokines (IL-6 and TNFα) and islet F4/80 expression, and a shift from M2-like to M1-like phenotype exhibited by an elevation in CD86 and CD86/CD206 expressions in the islets. This is in line with the observation that the distinct alterations of macrophages cause β cells dysfunctions during diabetes onset [56]. As is known that an inhibition of islet inflammation may offer benefits for diabetes

management [57]. Accordingly, we found that lycopene intervention could reverse these alterations in diabetic mice. Furthermore, our *in vitro* experiments showed that lycopene could decrease the relative ratio of CD86 to CD206 in HG/PA-stimulated RAW264.7 cells. Similarly, Albrahim et al. have shown that lycopene exerts an anti-inflammatory effect in HFD-induced obese rats [28]. Along with these findings, lycopene was demonstrated to attenuate hepatic inflammation and adipose tissue inflammation by reducing M1 polarization of macrophages in high cholesterol and fat-induced mice [58] and in HFD-induced mice [59]. Taken together, these findings suggest that the action of lycopene on inhibition of islet inflammation may be attributed to its ability of preventing macrophages polarization and preserving the M1/M2 macrophages homeostasis in diabetic mice.

The sustained macrophage inflammatory response in the intra-islets may result in β cell apoptosis [60], and further cause an inability of β cells to secrete sufficient insulin followed by the disorders of glucose and lipid metabolism [61]. Our findings herein demonstrated that HFD/STZ-induced diabetic mice appear abnormal cell apoptosis in the islets. And lycopene treatment restores islet cells apoptosis and promotes insulin secretion in HFD/STZ-induced diabetic mice. Our findings are in agreement with previous reports that lycopene promotes insulin secretion in diabetic rats [30], and inhibited cells apoptosis in neuronal cells [62] and bovine mammary epithelial cells [63]. Our *in vitro* experiments also revealed that lycopene augments insulin synthesis and inhibits apoptosis in Min6 cells only upon inflammation stimulation but not under direct HG/PA exposure. In consistence with our findings, the conditioned medium from PA-treated RAW264.7 cells may aggravate inflammation response on Min6 cells [6]. These findings suggested that lycopene may alleviate macrophages inflammation to attenuate cell apoptosis and promote insulin secretion in islets. It should to be noted that a study from Sandikci's group revealing that an increase in apoptotic cells was not observed in STZ-induced diabetic rats [64]. One potential explanation for the conflict findings may be attributed to that the different diabetes animal models between these two studies. While STZ was used to make hyperglycemia models in the previous investigation, HFD/STZ were used to establish diabetic animal models in our investigation.

It is known that an elevation of PDX1 is positively associated with an augment in insulin secretion and islet function [36, 65]. In the present study, we found that lycopene (10 mg/kg) treatment for 12 weeks promotes the PDX1 expression in HFD/STZ-induced diabetic mice. In addition, inhibition of M1 macrophages polarization contributes to islets insulin secretion and pancreas PDX1 expression [66]. Along with these observations, lycopene was demonstrated to inhibit M1 macrophages polarization, and increase PDX1 expression and insulin secretion in diabetic mice and in Min6 cells upon inflammation. Collectively, these findings in conjunction with the investigations from other groups suggested that lycopene could attenuate islet β cells dysfunction to prevent the development of diabetes through inhibition of islet cell apoptosis via blocking inflammation induced by M1 macrophages.

The present study revealed that lycopene may inhibit the TLR4/MyD88/NF-κB signaling pathway in the islets of diabetic mice, and in the HG/PA-RAW264.7 conditioned medium-stimulated Min6 cells. In addition, lycopene was not able to inhibit the TLR4/MyD88/NF-κB signaling pathway in Min6 cells upon direct HG/PA stimulation. Similarly, lycopene was reported to inhibit the TLR4/NF-κB signaling pathway in human endothelial cells [67]. Moreover, lycopene could reduce the risk of pancreatic cancer by regulating NF-κB signaling pathway [68], and inhibiting TLR4 expression and inflammatory cytokines secretion in respiratory epithelial cells [69]. Notably, inhibition of the TLR4/NF-κB signaling pathway could restore insulin secretion and protect islet β cells function in HFD-induced mice [70]. Therefore, these findings suggest that lycopene may improve islet function via regulation of the TLR4/MyD88/NF-κB signaling pathway.

In the present study, we used metformin as a positive control group, and indeed we demonstrated that metformin is able to improve glucose and lipid metabolism, inhibit inflammation response and islet cells apoptosis in HFD/STZ-induced diabetic mice. In addition, the present study demonstrated that lycopene is less effective than metformin by ITT. It is known that metformin is a first-line antidiabetic drug for clinical management of patients with diabetes. Further studies may be needed to fully observe the differences between metformin and lycopene. Moreover, we found that metformin

could inhibit the TLR4/MyD88/NF-κB signaling pathway in this diabetic animal model. Similarly, metformin has been reported to suppress the TLR4, MyD88 and NF-κB expressions in the femurs [71] and hippocampus [72] of diabetic mice. Thusly, these findings suggest that the anti-diabetic action of metformin may be associated with an inhibition of the TLR4/MyD88/NF-κB signaling pathway in the islets. However, further evidence is still needed to demonstrate the association.

In the present study, the dose of lycopene in the mouse was 10 mg/kg. According to the conversion of animal doses to human equivalent doses based on body surface area, the translational dose of lycopene into human was 1.1 mg/kg (66 mg/day). Indeed, a study from Veda Diwadkar-Navsariwala et al. [73] demonstrated that lycopene (10, 30, 60, 90, and 120 mg/day) had no adverse effects in healthy men. These doses should be considered safe because up to 120 mg/day of lycopene have been used in prostate cancer clinical trials with no detectable adverse effects [74, 75]. It should be noted that only 10-30% of lycopene could be absorbed in humans [23]. Since the bioavailability of lycopene in a mouse is less than that of a human, in the study conducted by Ying Guo et al. [76], 40 and 80 mg/kg of lycopene (in male mice) were used in order to see the beneficial effects of lycopene against diabetic nephropathy through diminishing inflammatory response and oxidative stress. Therefore, 10 mg/kg of lycopene in our study was safety when this dose was translated into human clinical trials.

There are several limitations in the present study when interpretation of the data. First, we used indirect co-culture condition (RAW264.7 conditioned medium) to investigate the effects of macrophages polarization on β cells and the action of lycopene on it. It is anticipated that direct co-culture system may facilitate to truly reflect the interactions between macrophages and β cells and lycopene. However, an investigation conducted by Kosei et al. have also demonstrated that the PA stimulated-macrophages conditioned medium limits β cells function [6]. Secondly, we did not employ TLR4-gene knockout in animals to examine the underlying mechanism of lycopene on islets inflammation. However, several studies reported that TLR4-knockout could attenuate PA-induced inflammation in HFD islets [70] and improve β cells function in pancreas of obese rats [18]. In addition, in the future, we will use different doses of lycopene and

TAK242 to further investigate the action of lycopene on islet function in vivo and in vitro because the potential interactions and complications of the treatment of two compounds together remain unknown. Thirdly, we did not employ an approach to deplete macrophages in islets, which may contribute to illustrate the direct action of lycopene on β cells function. Nevertheless, Böni-Schnetzler reported that inhibition of proinflammatory cytokine and chemokine expressions and macrophage infiltration improves insulin secretion and attenuates cell apoptosis [12].

5. Conclusions

In summary, our study for the first time revealed that lycopene could regulate glucose and lipid metabolism and attenuate cell apoptosis by improvement of islet function via regulation of the TLR4/MyD88/NF-κB signaling pathway in diabetic mice and Min6 cells (Fig. 6). This finding may open a new avenue for application of lycopene to the management of diabetes in translation value of this natural compound. Further investigations are needed to illustrate the anti-inflammation action of lycopene in the islets of patients with diabetes.

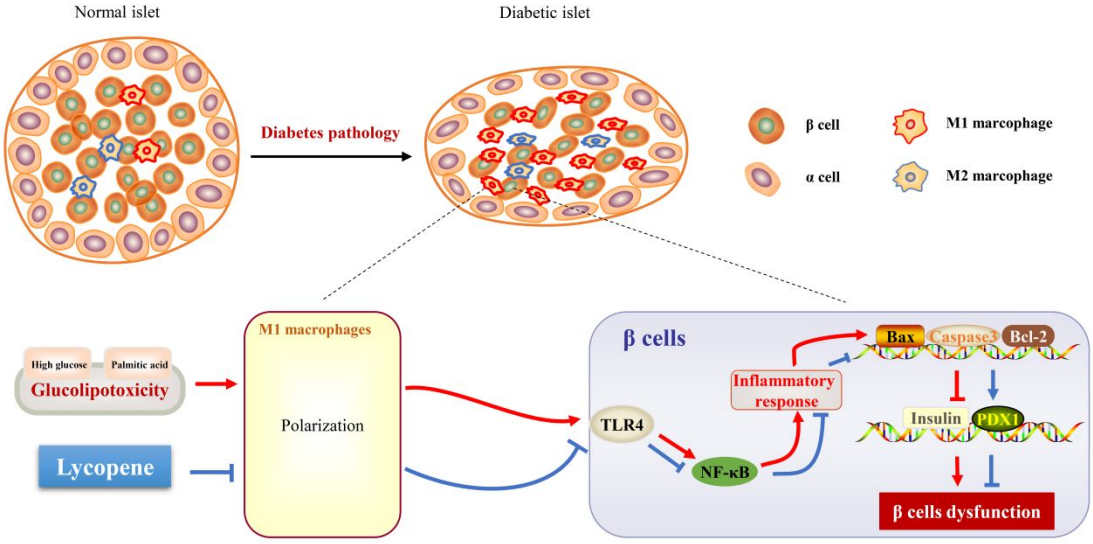

Figure 6. A proposed mechanism of the action of lycopene on inhibiting islet inflammation and promoting insulin secretion in HFD/STZ-induced diabetic mice. In diabetic islets, the infiltration of macrophages into the intra-islets triggers chronic inflammation and β cells apoptosis and consequent insufficiency of insulin secretion through activation of the

TLR4/MyD88/NF-κB signaling pathway. Lycopene may preserve M1/M2 macrophage homeostasis to attenuate islet inflammation, contributing to diabetes management.

Author contributions

YL, YT, LW and DwZ: **Conceptualization, Methodology, Software**. YL, YT, XD, TL, YZ and SW: **Data curation, Writing-Original draft preparation**. YL, YT, HS, JY, TX and RZ: **Visualization, Investigation**. YZ, DdZ, SG, LW and DwZ: Supervision. YL, YT: **Software, Validation**. YL, YT, YZ, XW, LW and DwZ : **Writing-Reviewing and Editing**.

Conflicts of Interest

The authors declared that they have no conflicts of interest to this work.

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