

PPAR γ activation reduces pancreatic beta cell death in type 1 diabetes by decreasing heparanase-dependent insulinitis

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ABSTRACT

While the majority of individuals with type 1 diabetes (T1D) receives lifelong exogenous insulin replacement therapy, a clinically significant subset remains refractory to achieving optimal glycemic targets, necessitating the exploration of novel adjunctive medications to enhance T1D treatment strategies. The aim of this study was to explore the efficacy of rosiglitazone (ROZ), a typical thiazolidinedione as selective agonists of the peroxisome proliferator-activated receptor gamma (PPAR γ), in the therapeutic management of T1D. The pharmacological effects of ROZ in different T1D mouse models induced by either multiple-low-dose (MLD) or single-high-dose (SHD) streptozotocin (STZ). Further morphological, bioinformatic, and in vitro experiments using cultured bone marrow-derived monocytes, were performed to explore the possible underlying mechanisms. In vivo findings revealed that ROZ primarily showed therapeutic effects in the MLD-STZ model, which is characterized by inflammatory damage to pancreatic beta cells, rather than SHD-STZ model. Mechanistically, PPAR γ activation, mediated by ROZ, downregulates the macrophage expression of heparanase, a specific endoglycosidase of the glycosaminoglycan heparan sulfate. This downregulation inhibits the degradation of intra-islet extracellular heparan sulfate, thereby enhancing the integrity of the physical barrier within the islets. Consequently, PPAR γ activation reduces the infiltration of inflammatory immune cells into the islets, thereby suppressing the damage to pancreatic beta cells associated with T1D. Our data emphasize the importance of sustained inflammation in the upregulation of heparanase in macrophages, while also underscoring the pivotal role played by the PPAR γ -heparanase axis. This study provides novel evidence for the potential targeting of PPAR γ -heparanase as an adjunctive treatment strategy for T1D.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by the

destruction of the insulin-producing pancreatic beta cells [1]. The interplay of genetic and environmental factors is incompletely understood; however, the recognition of autoantigens like insulin by

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autoreactive CD4- and CD8-positive T cells, along with the infiltration of macrophages and various other immune cells into the islets of Langerhans, ultimately leads to beta cell loss and absolute insulin deficiency, thereby precipitating severe hyperglycemia in patients with T1D [2] and a lifelong dependence on exogenous insulin injections [3]. However, during insulin therapy, over 70 % of patients with T1D fail to achieve optimal glycemic control [4] and frequently experience hypoglycemic events [5]. Given that T1D comprises 5–10 % of all diabetes cases and has a globally rising incidence [3], the exploration of novel adjunct medications is essential to enhance and refine the therapeutic strategies for T1D.

Some promising compounds that deserve further exploration are the thiazolidinediones, which were introduced in the 1990s as insulin-sensitizing glucose-lowering agents for the clinical treatment of type 2 diabetes (T2D) [6]. These drugs are insulin sensitizers that function as selective agonists of the peroxisome proliferator-activated receptor gamma (PPAR γ), a crucial regulator of metabolic pathways, thereby enhancing insulin action and glucose utilization [6]. However, whether thiazolidinediones have beneficial effects in patients with T1D remains controversial. In studies detailing the application of thiazolidinediones in patients with T1D, the outcomes are still contradictory, with some studies demonstrating efficacy while others failing to show any beneficial effect [7–9]. Therefore, elucidating the specific factors contributing to these differences is crucial when evaluating the potential application of thiazolidinediones for glycemic control in patients with T1D.

Significant inflammatory damage to pancreatic beta cells in T1D—a condition known as insulinitis [2], draws further attention to the anti-inflammatory properties exhibited by thiazolidinediones. By activating PPAR γ , thiazolidinediones effectively mitigate the pro-inflammatory activation of macrophages in several diseases, including chronic obstructive pulmonary disease (COPD), non-alcoholic fatty liver disease (NAFLD), and atherosclerosis [10–12]. This raises the possibility that activation of PPAR γ by thiazolidinediones could provide similar benefits in patients with T1D by reducing macrophage-related inflammatory damage to pancreatic beta cells. However, studies on the effects of thiazolidinediones on glycemic control in T1D animal models have produced inconsistent results [13–16], and clinical studies have reported contradictory findings. Some studies have indicated a protective role of thiazolidinediones on pancreatic beta cells in T1D mice models, but the intricate interplay between macrophages and other immune T cells in mediating inflammatory damage to islets continues to obscure the precise underlying mechanisms for this response.

The aim of the present study was to elucidate the potential role of thiazolidinedione-mediated PPAR γ activation in suppressing the inflammatory damage associated with pancreatic beta cells in T1D. We first compared the pharmacological effects of rosiglitazone (ROZ), a typical thiazolidinedione, after inducing T1D in two different mouse models by treatment with multiple-low-dose (MLD) or single-high-dose (SHD) streptozotocin. Further morphological, bioinformatic, and in vitro analyses suggested that PPAR γ activation, mediated by ROZ, exerted its protective effects on pancreatic beta cells in T1D by down-regulating the macrophage expression of heparanase, a specific endoglycosidase of the glycosaminoglycan heparan sulfate [17]. This downregulation inhibits the secretion of heparanase by infiltrating macrophages, thereby preventing the degradation of intra-islet extracellular heparan sulfate (HS), enhancing the integrity of the physical barrier within the islets, and preventing further infiltration of inflammatory immune cells into the islets. Thus, PPAR γ activation by thiazolidinediones contributes to the preservation of pancreatic beta cells in T1D.

2. Materials and methods

2.1. Animal models

All animal care and experimental procedures were approved by the

Animal Care and Ethics Committee of Nanjing Medical University. C57BL/6 J mice (male, 8 weeks old, 22 ± 2 g) were purchased from GemPharmatech Co., Ltd. (Nanjing, China). The mice were housed in a controlled environment with a 12 h light/12 h dark cycle and had free access to food and water. Streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO, USA) dissolved in sodium citrate buffer (pH 4.5) was used to induce the T1D phenotype. In the MLD-STZ group, mice received intraperitoneal injections of 50 mg/kg STZ for 5 consecutive days. In the SHD-STZ group, T1D was induced using a single intraperitoneal injection of STZ at a dose of 160 mg/kg. The control group mice were injected only with sodium citrate buffer. Mice were considered diabetic if two consecutive random blood glucose readings were 16.7 mmol/L or higher. After confirmation of the diabetic phenotype, the MLD-STZ and SHD-STZ groups were orally administered 10 mg/kg of rosiglitazone (ROZ) or vehicle daily for 4 weeks. The mice were monitored weekly to record body weights and blood glucose levels (Fig. 1A).

2.2. Glucose tolerance test (GTT) and glucose-stimulated insulin secretion

After the 4-week administration of ROZ, the mice in the five groups were fasted for 12 h and then received an intraperitoneal injection of glucose (2 g/kg body weight). A glucometer (Roche, Indianapolis, Indiana, USA) was used to record blood glucose levels in tail vein blood collected at 0, 30, 60, 90, and 120 min after glucose loading. Additional blood samples were collected at 0 and 30 min to measure serum insulin concentrations using a commercial insulin ELISA kit (EZassay, Shenzhen, China) following the manufacturer's protocol.

2.3. Morphological staining

After the 4-week administration period, all animals were euthanized, and the pancreas tissues were removed and fixed in 4 % formaldehyde. The tissues were embedded in paraffin, sectioned into 5 μ m slices, and subjected to immunohistochemistry and immunofluorescence analyses, as previously described [18]. In brief, paraffin sections were immersed in citrate buffer (0.01 M, pH 6.0) for heat-induced antigen retrieval. For immunohistochemical staining, after blocking with hydrogen peroxide and 3 % BSA, primary antibodies (Table S1) were applied, followed by visualization with an immunohistochemical DAB staining kit (Gene Tech, Shanghai, China); the cell nuclei were counterstained with hematoxylin. For immunofluorescence staining, fluorescent secondary antibodies were used, and the nuclei were stained with DAPI (Sigma). Apoptotic cells were stained using a TdT-mediated dUTP Nick-End Labeling (TUNEL) kit (Vazyme Biotech, Nanjing, China) following the manufacturer's protocol, and fluorescence images were captured using a confocal microscope (F1000, Olympus, Tokyo, Japan). The images were analyzed using Image J software (V1.8.0, NIH, Bethesda, MD, USA). The beta cell mass was calculated as the insulin-positive area divided by the total pancreas tissue area on the section and then multiplied by pancreas weight. For quantitative morphological analyses, all islets identified on pancreatic sections from each mouse (typically 5–15 islets per animal) were examined across the entire field of view. Data from multiple islets per animal were aggregated to generate a single biological replicate value. Group comparisons were performed using these individual animal data points ($n = 8$ mice per group).

2.4. RNA sequencing and single-cell RNA sequencing (scRNA-seq) data retrieval

RNA sequencing data were downloaded from the GEO database for pancreatic islet samples in healthy volunteers and patients with T1D (GSE181674) [19] and for pancreatic islet cells from autoimmune T1D NOD mice at different weeks of age (GSE141782) [20]. The expression of heparanase was analyzed using the R Limma package.

The subpopulation localization of heparanase was analyzed using a scRNA-seq expression matrix with non-endocrine cells isolated from

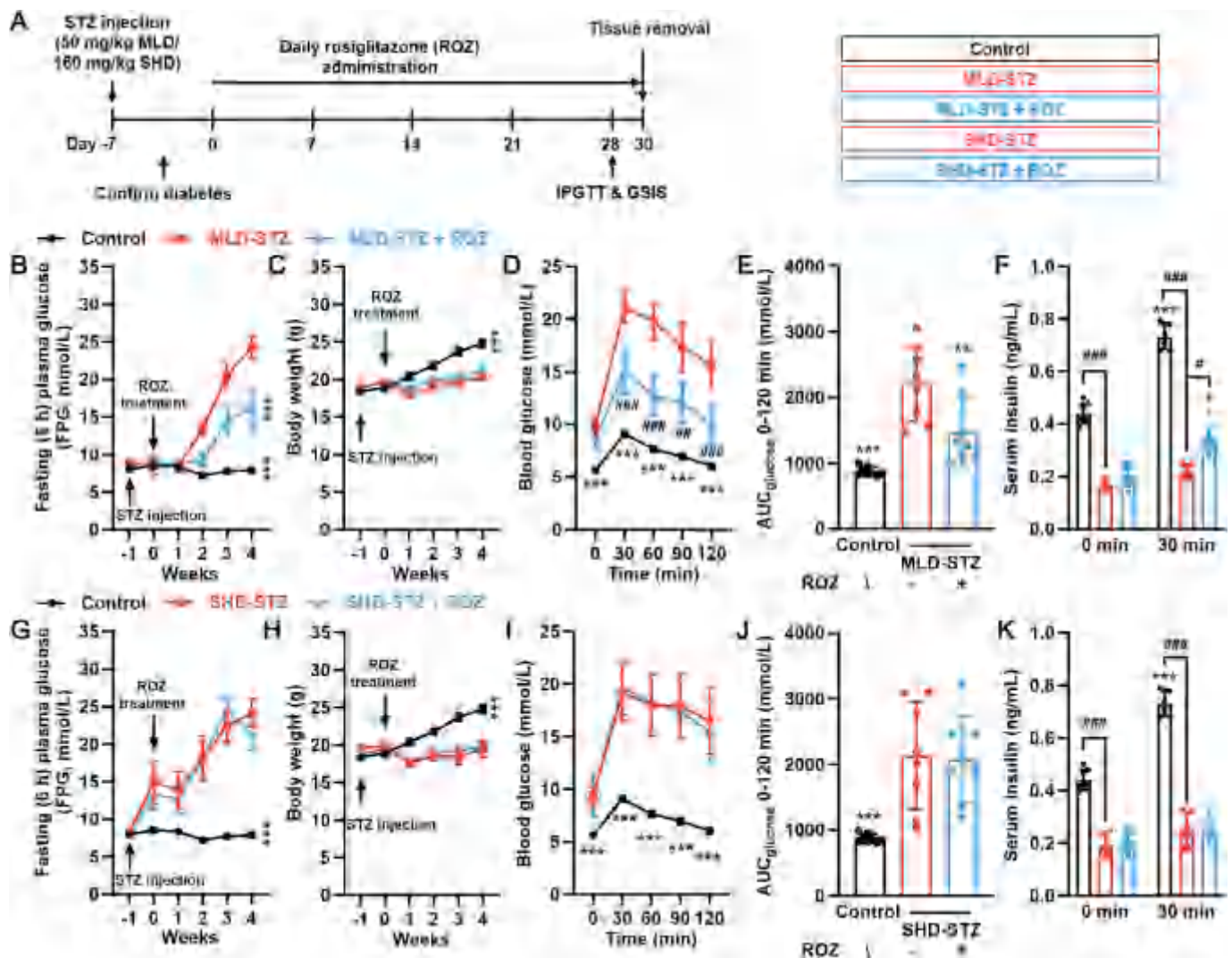


Fig. 1. The effect of rosiglitazone (ROZ) on glycemic control in T1D mouse models. (A) The experimental procedure schedule. (B) Fasting (6 h) plasma glucose (FPG) levels, (C) Body weight during administration in the MLD-STZ and control groups. (D) After a 4-week treatment with ROZ, glucose tolerance tests (GTTs) were performed in the MLD-STZ and control groups. (E) The area under curve (AUC) values for GTTs in (D). (F) Serum insulin levels were measured at 0 and 30 min during the GTTs in (D). (G) FPG levels, (H) Body weight during administration in the SHD-STZ and control groups. (I) After 4-week treatment with ROZ, GTTs were performed in the SHD-STZ and control groups. (J) The area under curve (AUC) for GTTs in (I). (K) Serum insulin levels were measured at 0 and 30 min during the GTTs in (I). ** $P < 0.01$, *** $P < 0.001$ in the ROZ-treated groups, # $P < 0.05$, ### $P < 0.001$ in the control group compared to the STZ groups, $n = 8$.

islet cells in NOD mice at different weeks of age (GSE141786) [20] and processed using the R package Seurat [21]. The gene expression data were normalized, and differentially expressed genes (DEGs) were identified. UMAP reduction was used for cluster visualization, and the R package ggplot2 was used to visualize gene expression. Monocle2 [22] was used to analyze the sequencing data based on the developmental trajectories of the cells.

2.5. Isolation and culture of mouse bone marrow-derived macrophages (BMDMs)

The mouse BMDMs were isolated as described in our previous study [23]. Briefly, bone marrow progenitors harvested from the femurs and tibias of pathogen-free C57BL/6 mice were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS and 1 % penicillin-streptomycin. To induce differentiation into macrophages, 20 ng/mL of Macrophage Colony-Stimulating Factor (M-CSF) (Sino Biological, P07141-1) was added on the day of seeding (Day 0) and the cells were maintained for 6 days, with a medium change on Day 3 and supplementation on Day 5. On Day 7, the BMDMs were stimulated either

with 100 ng/mL LPS and 50 ng/mL IFN γ to promote M1 polarization or with 100 ng/mL IL-4 to induce M2 polarization. The isolated mouse bone marrow cells were cultured overnight at 37 °C in a 5 % CO $_2$ incubator. Cells were preincubated with ROZ (50 μ M), pioglitazone (50 μ M), troglitazone (50 μ M) to investigate the effect of PPAR γ agonism on BMDMs. The effect of PPAR γ antagonism was investigated by co-treatment of GW9662 (10 μ M, MedChemExpress, Shanghai, China) An adenovirus vector containing mouse heparanase (AdHpse) was purchased from WZ Biosciences (Jinan, China) to conduct heparanase overexpression studies.

2.6. Real-time PCR and western blot analysis

After the different treatments, total RNA was extracted from BMDMs using TRIzol reagent (#12183555, Invitrogen, Carlsbad, CA, USA), followed by isopropanol and ethanol precipitation steps to remove DNA and protein impurities. For reverse transcription into complementary DNA (cDNA), 0.5 μ g of RNA was used with the reverse transcription and cDNA synthesis kit (#639506, Takara, Tokyo, Japan). Real-time quantitative PCR (qPCR) analysis was conducted using SYBR Green Premix

(Q111-02, Vazyme, Nanjing, China). Throughout this process, the fluorescence signal emitted by the bound fluorescent probes increases in proportion to the amplification of the target DNA, which is continuously monitored by the real-time PCR instrument. The primers used are listed in Table S2.

The western blot analysis was performed as described previously [24]. Briefly, after the different treatments, BMDMs were lysed in ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 7.4; 1 % NP-40; 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L phenylmethylsulfonyl fluoride). The western blotting was performed as previously described after protein content determination. The optical density of each band was analyzed by using the Image J software (National Institutes of Health, USA). The antibodies are listed in Table S1.

2.7. Data and statistical analysis

The data presented in this manuscript are expressed as mean \pm SD. One-way ANOVA or two-way ANOVA analysis followed by Dunnett's

post hoc tests were used to compare differences between different groups. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Rosiglitazone preferentially improved glycemic control in MLD-STZ-induced diabetic mice compared to SHD-STZ model mice

The T1D mouse models were established through either MLD-STZ or SHD-STZ injections, followed by daily oral administration of rosiglitazone (ROZ) or vehicle (Fig. 1A). As illustrated in Fig. 1, ROZ administration significantly mitigated MLD-STZ-induced hyperglycemia (Fig. 1B), with minimal changes in body weight compared to the vehicle-treated MLD-STZ group (Fig. 1C). After the 4-week intervention period, the ROZ-treated MLD-STZ mice exhibited significantly improved glucose tolerance (Fig. 1D, E) and their glucose-stimulated insulin secretion (GSIS) was enhanced compared to the vehicle-treated MLD-STZ mice (Fig. 1F). By contrast, ROZ administration had no effect on

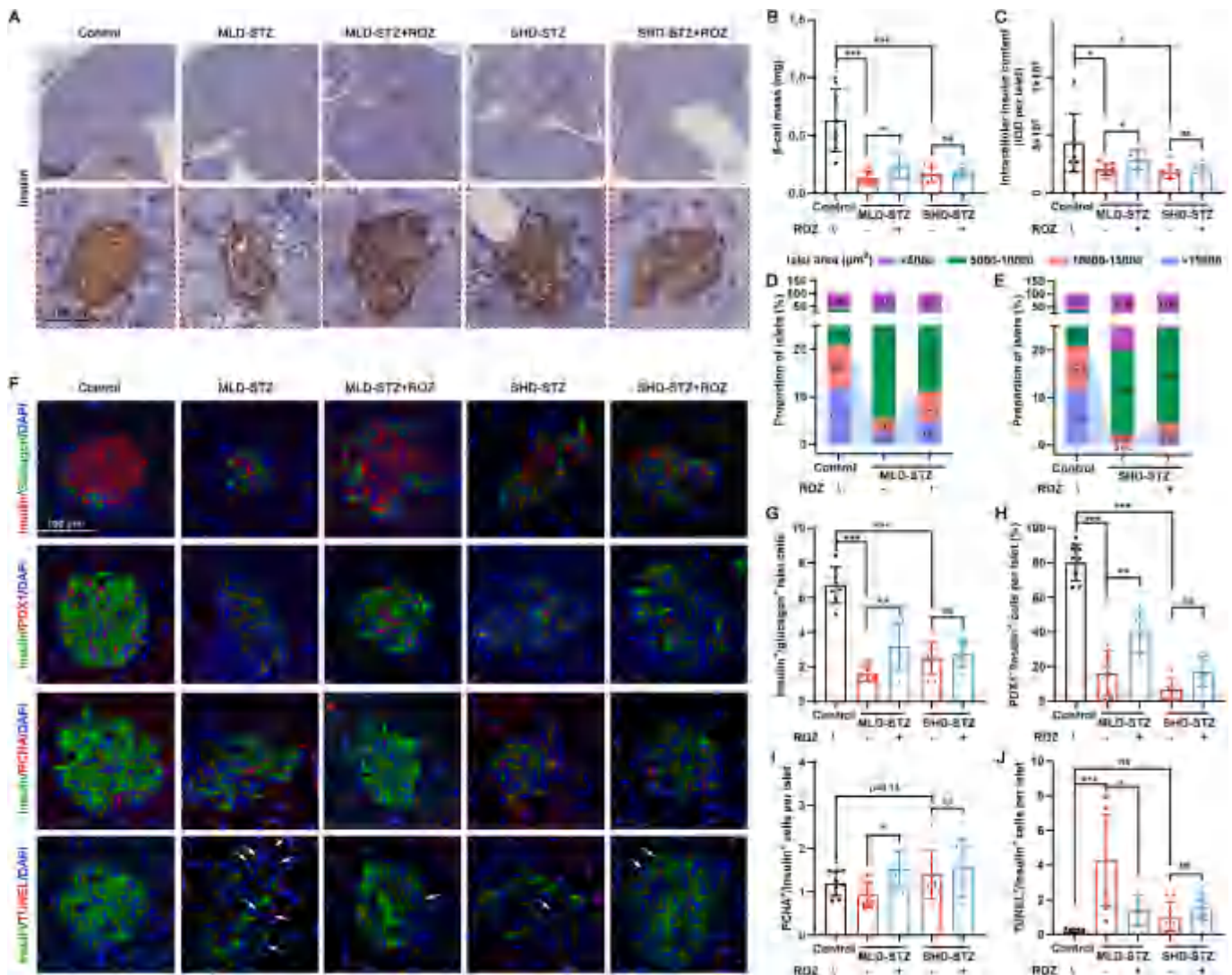


Fig. 2. The effect of ROZ administration on the morphology of pancreatic islets in T1D mouse models. (A) Representative images of insulin immunohistochemical staining in each group. Scale bar = 250 μ m for panels indicating 5 \times magnification; scale bar = 100 μ m for panels indicating 20 \times magnification. (B) The beta cell mass, (C) the integrated optical density (IOD) for insulin immunohistochemical staining, (D-E) the ratio of islets with different areas analyzed by insulin immunohistochemical staining. (F) Representative double immunofluorescence images for insulin (INS), glucagon, PDX1, PCNA, and TUNEL staining in pancreatic islets of each group; nuclei were dyed with DAPI. Scale bar = 100 μ m in all panels. (G) The ratio of insulin-positive (INS+) cells divided by glucagon-positive cells, (H) the ratio of PDX1+/INS+ cells, (I) the number of PCNA+/INS+ cells, (J) the number of TUNEL+/INS+ cells in the islets of the different groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant, as indicated; $n = 8$.

glycemic control, glucose tolerance, or GSIS in the SHD-STZ group (Fig. 1G–1K).

3.2. Rosiglitazone specifically reduced pancreatic beta cell death in MLD-STZ-treated mice

Immunohistochemical analysis of insulin antibodies revealed a significant reduction in beta cell mass and insulin content within the pancreatic islets of both the MLD-STZ and SHD-STZ mouse groups (Fig. 2A–2E). Notably, supplementation with 10 mg/kg ROZ partially mitigated this decrease in pancreatic beta cell mass within the islets of the MLD-STZ mice. Together with the increased medium and large islet ratio (Fig. 2D), the restoration of pancreatic islet morphology was particularly notable. No similar improvement was observed in the ROZ-

treated SHD-STZ mice (Fig. 2A–2E).

Morphologically, ROZ administration in the MLD-STZ group significantly increased the ratio of insulin/glucagon-positive (INS+/GCG+) cells (Fig. 2F, G), as well as the proportion of PDX1-positive (PDX1+) pancreatic beta cells within the islets, compared to the vehicle-treated MLD-STZ group (Fig. 2F, H). Although ROZ did not alter the PCNA-positive (PCNA+) pancreatic beta cell number (Fig. 2F, I), TUNEL staining demonstrated a significant decrease in the number of apoptotic pancreatic beta cells in MLD-STZ mice treated with ROZ (Fig. 2F, J). Consistent with the results from beta cell mass analysis, ROZ administration provided only a minimal benefit in mitigating beta cell death induced by SHD-STZ (Fig. 2F–2J).

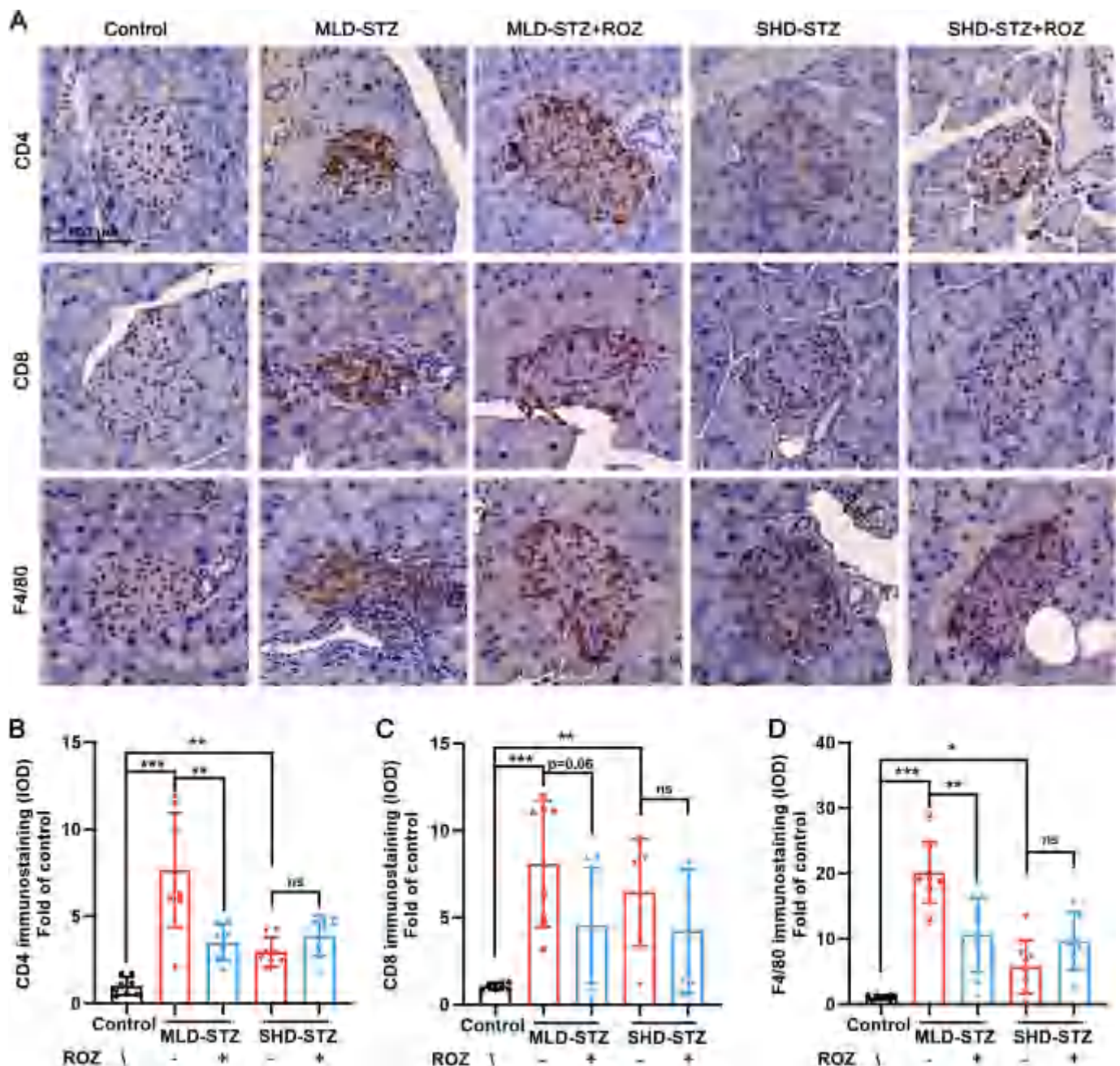


Fig. 3. The effect of ROZ administration on intra-islet immunocyte infiltration. (A) Representative images of CD4, CD8, and F4/80 immunohistochemical staining in the pancreatic islets of the different mouse groups. Scale bar = 100 μ m in all panels. The integrated optical density (IOD) of (B) CD4, (C) CD8, (D) F4/80 immunohistochemical staining in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant, as indicated, $n = 8$.

3.3. Rosiglitazone mitigated MLD-STZ-induced pancreatic beta cell death by reducing inflammatory immunocyte infiltration

The observed difference in the pharmacological efficacy of ROZ in MLD-STZ and SHD-STZ mice led us to delve further into the unique immunodynamic profiles of these two T1D mouse models. As depicted in Fig. 3, compared to the SHD-STZ group, the MLD-STZ group exhibited a marked increase in the intra-islet infiltration of F4/80-positive monocytes and CD4- or CD8-positive T cells. Quantitative analysis confirmed a decrease in the intra-islet infiltration of these immune cells in ROZ-treated MLD-STZ mice. No similar in vivo or morphological

differences were observed between the ROZ-treated and vehicle-treated SHD-STZ mice.

3.4. Reduced inflammatory polarization of macrophages contributes to the protective effect of rosiglitazone against MLD-STZ-induced pancreatic beta cell death

Considering the reported anti-inflammatory role of PPAR γ activation in macrophages, we examined the expression of markers associated with the inflammatory polarization of macrophages in the islets and pancreatic lymph nodes in the different mouse groups.

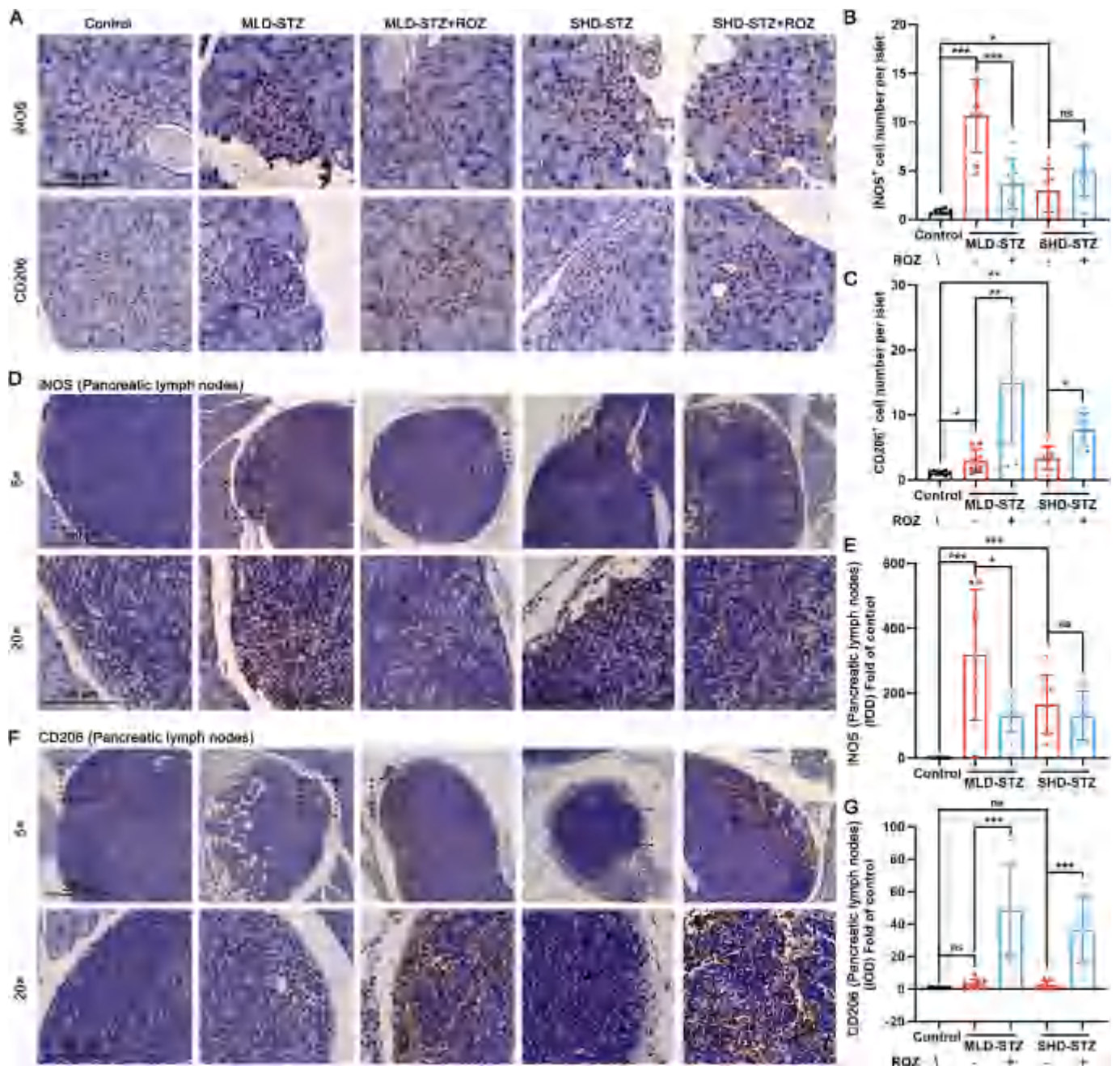


Fig. 4. The effect of ROZ administration on the expression of inflammatory markers in pancreatic macrophages. (A) Representative images of iNOS and CD206 immunohistochemical staining in the pancreatic islets of the different mouse groups. Scale bar = 100 μ m in all panels. (B) The number of iNOS-positive cells per islet, (C) CD206-positive cells per islet. (D) Representative images of iNOS immunohistochemical staining in the pancreatic lymph nodes of each mouse group. The magnifications and scale bar sizes are indicated. (E) The expression of iNOS immunohistochemical staining was calculated as the integrated optical density (IOD). (F) Representative images of CD206 immunohistochemical staining in the pancreatic lymph nodes of each group. The magnifications and scale bar sizes are indicated. (G) The expression of CD206 immunohistochemical staining was calculated as the IOD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant, as indicated, $n = 8$.

Immunohistochemical staining for iNOS, a well-established pro-inflammatory macrophage marker, demonstrated that MLD-STZ T1D induction, but not SHD-STZ T1D induction, significantly enhanced the pro-inflammatory polarization of monocytes within the pancreatic islets (Fig. 4A, B). Conversely, ROZ administration in the MLD-STZ mice decreased the numbers of pro-inflammatory macrophages and notably increased the numbers of CD206-positive anti-inflammatory macrophages within the islets (Fig. 4A–4C). Notably, although ROZ administration increased CD206 expression in both the MLD-STZ and SHD-STZ mouse pancreases, iNOS expression was reduced in the pancreatic lymph nodes (Fig. 4D–4G), indicating that ROZ administration had a systemic anti-inflammatory effect on macrophages.

3.5. Macrophage-specific heparanase expression is negatively correlated with PPAR γ during the development of type 1 diabetes

We further explored the underlying mechanisms underpinning the protective role of ROZ against islet beta cell damage in T1D by conducting bioinformatic analysis using a scRNA-seq expression matrix (GSE141786) of islet immune cells derived from autoimmune nonobese diabetic (NOD) mice with different durations of T1D. We identified the principal immune and non-endocrine cell populations using UMAP dimensionality reduction, as depicted in Fig. 5A. PPAR γ exhibits a predominant expression pattern within endothelial cells and macrophage clusters (Fig. 5B). Notably, a marked reduction in the expression of PPAR γ was observed in islet macrophages derived from NOD mice upon

development of T1D (Fig. 5C, D). The macrophages were further divided into the following five sub-populations, as previously described [20]: Mac-1 (Apoe), Mac-2 (Atf3), Mac-3 (Cxc19), Mac-4 (Prdx1), and Mac-5 (Stmn1) (Fig. 5E). The hallmark feature and the results of pseudotime alignment affirmed that the Mac-3 (Cxc19) subpopulation underwent a sequential and progressive pattern of pro-inflammatory activation during the course of autoimmune development (Fig. 5F). As expected, the expression of PPAR γ was clearly decreased along with this trajectory (Fig. 5G). We therefore conducted an analysis of genes that exhibited correlated expression with PPAR γ in macrophages within the GSE141786 scRNA-seq dataset (Fig. 5H), and we further validated whether these correlated genes were also notably upregulated in the pro-inflammatory Mac-3 (Cxc19) sub-cluster or in islets derived from human patients with T1D (GSE181674). The subsequent Venn diagram demonstrates that heparanase, which we have previously identified as a PPAR γ target gene [18], could potentially exhibit a strong correlation with the protective effects of PPAR γ agonism on pancreatic beta cells in T1D (Fig. 5I–5K).

3.6. Rosiglitazone inhibits the expression of macrophage-specific heparanase and reduces the intra-islet loss of heparan sulfate (HS) in MLD-STZ mice

We further elucidated the potential correlation between the protective efficacy of ROZ against T1D-induced pancreatic beta cell damage and the expression of macrophage-derived heparanase by investigating

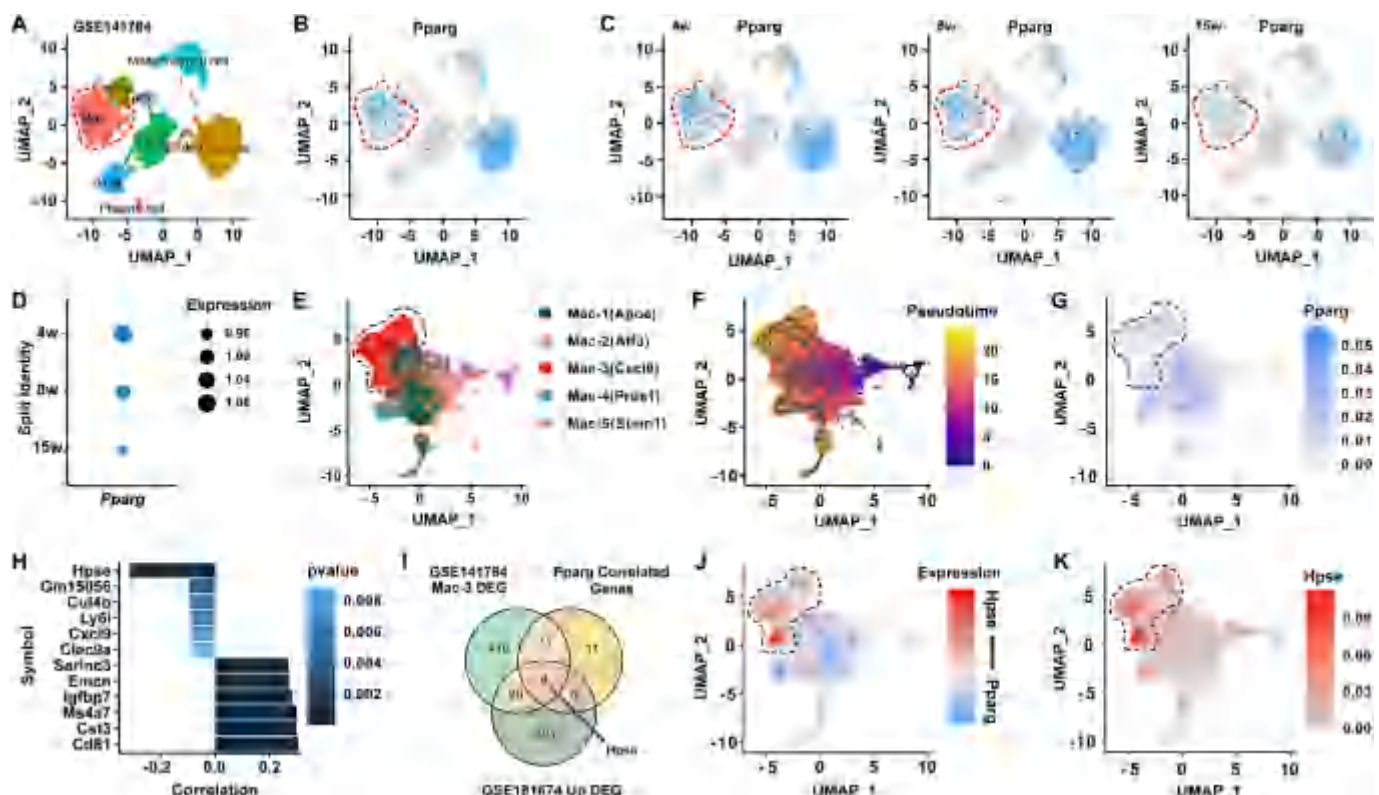


Fig. 5. Bioinformatic analysis reveals that heparanase is related to the protective effects of PPAR γ agonism on pancreatic beta cells in T1D. (A) UMAP dimensionality reduction and graph-based clustering of islet cells pooled together from 4-, 8-, and 15-week-old NOD mice in GSE141786, red dotted line indicates macrophage (Mac) cluster. (B) UMAP feature plots with cells colored according to expression of PPAR γ (Pparg). (C) The feature plots of Pparg expression in 4-, 8-, and 15-week-old NOD mice. (D) The bubble chart of Pparg expression in (C). (E) UMAP plot showing clusters of islet macrophages merged from 4-, 8-, and 15-week-old NOD mice samples in GSE141786, black dotted line indicates Mac-3 sub-population. (F) UMAP visualization of the cells arranged along trajectories by monocle2, colored by inferred pseudotime. (G) The feature plots of Pparg expression in the islet macrophages from NOD mice. (H) The correlate-expressed genes of Pparg in the macrophage cluster. (I) Venn diagram exhibits the intersection of the different expressed genes (DEGs) in Mac-3 compared to Mac-1, the DEGs in the islets from patients with T1D (GSE181674 dataset), and the genes in (H). (J) The feature plots of Pparg (blue) and heparanase (Hpse, red) expression in the islet macrophages. (K) The feature plots of Hpse expression in the islet macrophages. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the morphological expression patterns of heparanase and its physiological substrate, heparan sulfate (HS). In contrast to the control group, the expression of heparanase within the pancreatic islets was notably augmented in the MLD-STZ mice, whereas no similar increase was observed in the SHD-STZ mice. This augmentation was accompanied by a substantial depletion of intra-islet HS (Fig. 6A). Heparanase expression in the pancreatic lymph nodes was also specifically increased in the MLD-STZ group compared to the control group, whereas this expression was less altered in the SHD-STZ group (Fig. 6B). In the ROZ-treated MLD-STZ mice, the expressions of heparanase within the pancreatic islets and in the pancreatic lymph nodes were both decreased compared to the vehicle-treated MLD-STZ group, which exhibited higher HS levels within the islets (Fig. 6A–6E).

3.7. Additional stimulation of inflammatory cytokines increases the expression of heparanase in cultured bone marrow-derived macrophages (BMDMs)

The macrophages derived from 4-week-old NOD mice predominantly belonged to the Mac-1 (Apoe) and Mac-2 (Atf3) sub-clusters in the scRNA-seq dataset GSE141786. However, the proportion of macrophages in the pro-inflammatory Mac-3 (Cxcl9) sub-cluster increased significantly in 15-week-old NOD mice (Fig. 7A). As T1D progressed, macrophage-specific heparanase expression intensified, particularly within the Mac-3 (Cxcl9) sub-cluster (Fig. 7B). Notably, the DEGs between Hpse-positive (Hpse+) and Hpse-negative (Hpse-) macrophages were predominantly enriched in pathways associated with inflammation (Fig. 7C and D), suggesting strong relationship between heparanase expression and pro-inflammatory polarization of macrophages in T1D. Further, given the clear increase in heparanase expression in islets from both T1D patients (Fig. 7E) and aged diabetic NOD mice (Fig. 7F), we examined heparanase expression in cultured BMDMs following various stimuli (Fig. 7G). M1 polarization, induced by lipopolysaccharide (LPS) and IFN γ , elevated the expression of pro-inflammatory markers, such as iNOS (Nos2, Fig. 7H), whereas M2 polarization, simulated by interleukin 4 (IL4), significantly upregulated the expression of the anti-inflammatory marker Arg1 (Fig. 7I).

Surprisingly, neither M1 nor M2 polarization altered heparanase expression in cultured BMDMs (Fig. 7J). Therefore, we treated M1 BMDMs with additional inflammatory cytokines for 24 h (Fig. 7K). Additional stimulation with IFN γ , but not with tumor necrosis factor alpha (TNF- α) or interleukin 1beta (IL-1 β), resulted in dose-dependent and time-dependent increases in heparanase expression in M1 BMDMs (Fig. 7L–7P).

3.8. The inhibition of heparanase expression by rosiglitazone is not correlated with macrophage polarization

Given that the expression of heparanase was consistently negatively correlated with the expression of PPAR γ in macrophages during the progression of T1D (Fig. 8A), we confirmed that ROZ significantly inhibited the expression of heparanase after additional IFN γ stimulation (Fig. 8B and C), as well as other thiazolidinedione PPAR γ agonists pioglitazone or troglitazone (Fig. S1A). However, co-treatment with GW9662, an PPAR γ antagonist, and IFN γ resulted in minimal changes in heparanase expression levels compared to the IFN γ -only BMDMs (Fig. S1B). We ascertained whether the expression of heparanase can independently influence the polarization of macrophages by first infecting cultured BMDMs with AdHpse or AdVec to induce heparanase overexpression, followed by the application of different stimuli to induce M1/M2 polarization (Fig. 8D). Morphologically, the M1 BMDMs exhibited characteristic fried egg shapes, whereas M2 BMDMs displayed a mixed population that included spindle-shaped cells; however, overexpression of heparanase did not alter these typical shapes of the M1/M2 BMDMs (Fig. 8E). Furthermore, comparison of the expression of polarization markers revealed only a minimal difference between the

AdHpse- and AdVec-treated BMDMs (Fig. 8F and G).

4. Discussion

The administration of TZDs, an established therapeutic modality for T2D, for the treatment of T1D has remained a persistent area of exploration. Nevertheless, the efficacy of TZDs in T1D remains uncertain, based on results from studies on human patients with T1D or on T1D animal models. Rodent models of T1D are typically generated using diabetogenic chemicals, particularly streptozotocin (STZ) [25]. Typically, a multiple-low-dose streptozotocin (MLD-STZ) approach is used to simulate the autoimmune mechanisms underlying T1D because this treatment induces gradual pancreatic beta cell damage through an immune-mediated process that is characterized by immune cell infiltration and islet inflammation. Conversely, the single-high-dose streptozotocin (SHD-STZ) model typically results in a rapid and severe destruction of pancreatic beta cells, primarily through direct toxic effects, and it bypasses the immune-mediated pathway [26]. However, not all rodent models are comparable, as rats exhibit distinct tolerances to STZ [25], which limits the application of the MLD-STZ approach in T1D rat models. As a further complication, although previous studies aiming to investigate the efficacy of TZDs in resolving diabetic complications have frequently employed STZ to establish rodent T1D models [15], the assessment of the resulting T1D phenotypes, including blood glucose levels, has frequently been overlooked. These limitations in the previous animal studies have constrained comprehensive explorations of the potential efficacy of TZDs in T1D.

In the present study, we conducted a parallel examination of the efficacy of ROZ in both MLD-STZ and SHD-STZ mouse models. Our findings underscore that ROZ mitigation of hyperglycemia and alleviation of pancreatic beta cell damage are far superior in MLD-STZ model mice than in SHD-STZ mice. Our morphological analyses also revealed that ROZ administration to the MLD-STZ mice mitigated the infiltration of immune cells within the islets, while also reducing the pro-inflammatory activation of macrophages both within the islets and in the pancreatic lymph nodes. These observations align with some previous studies showing that ROZ could reduce the incidence of diabetes in the autoimmune T1D NOD mouse [27,28], thereby reinforcing the potential anti-inflammatory efficacy of ROZ in T1D. Notably, although some in vitro studies have reported a direct protective effect of TZDs and PPAR γ activation on cultured beta cell lines [29,30], the limited protective efficacy of ROZ in the SHD-STZ model implies that ROZ administration alone may not be sufficient to counteract STZ-mediated beta cell destruction. Phenotypic characterization revealed rapid β -cell destruction accompanied by marked hyperglycemia development within 7 days postmodeling in SHD-STZ mice. In contrast, MLD-STZ mice demonstrated progressive glycemic deterioration, with initial hyperglycemia emerging at 14–21 days postmodeling. Furthermore, ROZ intervention initiated during the prediabetic phase (day 7 postmodeling) significantly attenuated hyperglycemia progression in MLD-STZ mice. However, this therapeutic strategy showed limited efficacy in reversing established pancreatic β -cell depletion observed in SHD-STZ mice. These above distinct difference in efficacy between the two models indicates that the protective effect of ROZ may arise from its ability to mitigate the inflammatory infiltration of immunocytes, and this then has an indirect but beneficial impact on islet function and structural integrity in T1D mice, rather than directly preventing STZ-induced beta cell damage.

Given the complicated mechanisms that underlie beta cell damage in T1D, the present findings from mouse studies cannot definitively resolve the controversies pertaining to the efficacy of TZDs as clinical therapies for T1D. The underlying challenge stems from the significant clinical heterogeneity observed among patients with T1D. Although the pathogenesis of T1D is primarily characterized by autoimmune-mediated destruction of pancreatic beta cells, disease progression varies substantially across individuals. Notably, a subset of patients with milder manifestations of T1D are even misclassified and treated as T2D [31].

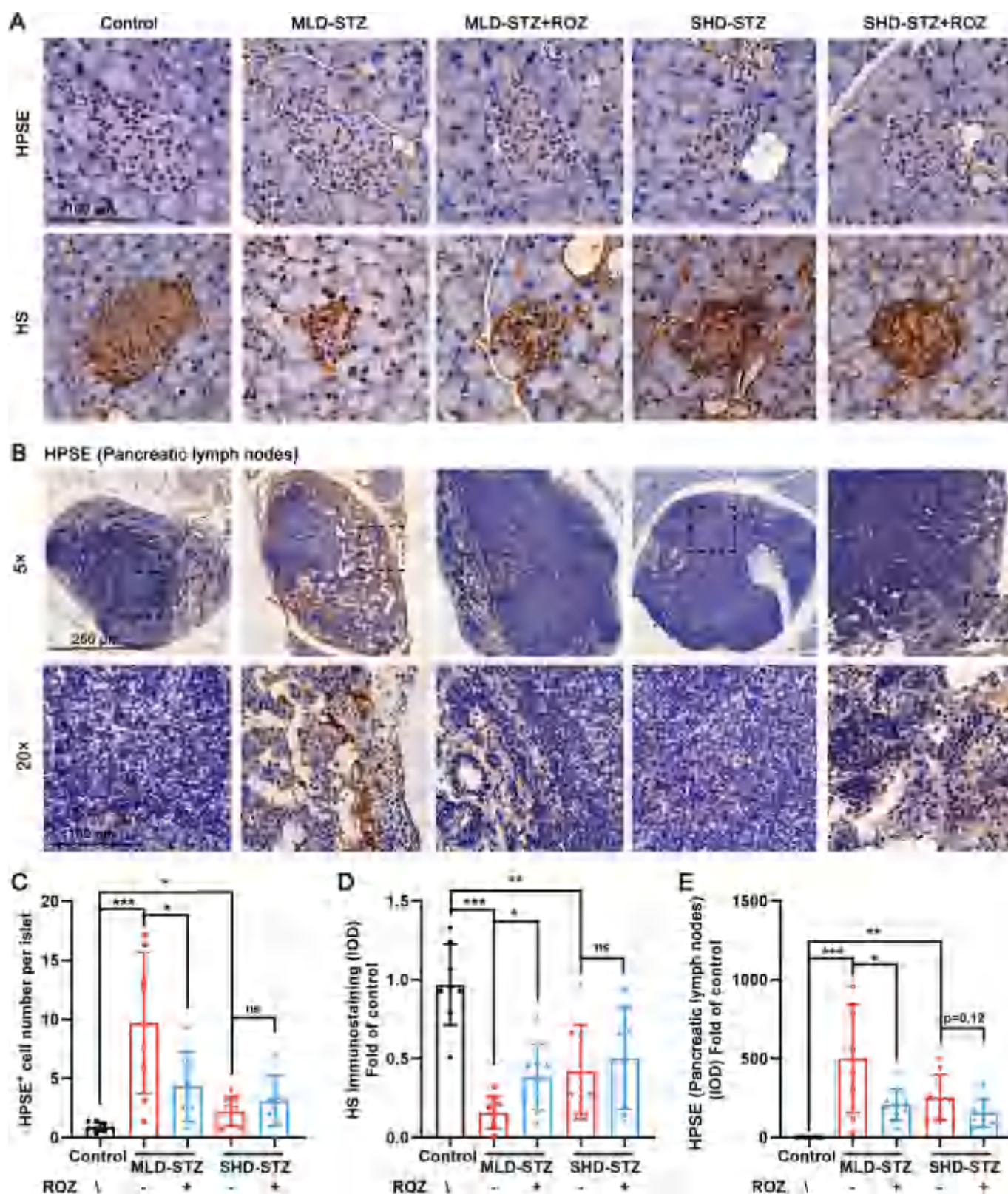


Fig. 6. The expression of heparanase (HPSE) and heparan sulfate (HS) in ROZ-treated STZ mouse models. (A) Representative images of HPSE and HS immunohistochemical staining in each group. Scale bar = 100 μ m in all panels. (B) Representative images of HPSE immunohistochemical staining in the pancreatic lymph nodes. Scale bar = 250 μ m in the panels with 5 \times magnification, scale bar = 100 μ m in panels with 20 \times magnification. (C) The number of HPSE-positive cells per islet. (D) The integrated optical density (IOD) of HS immunohistochemical staining in each group. (E) The IOD of HPSE immunohistochemical staining within pancreatic lymph nodes in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant, as indicated, $n = 8$.

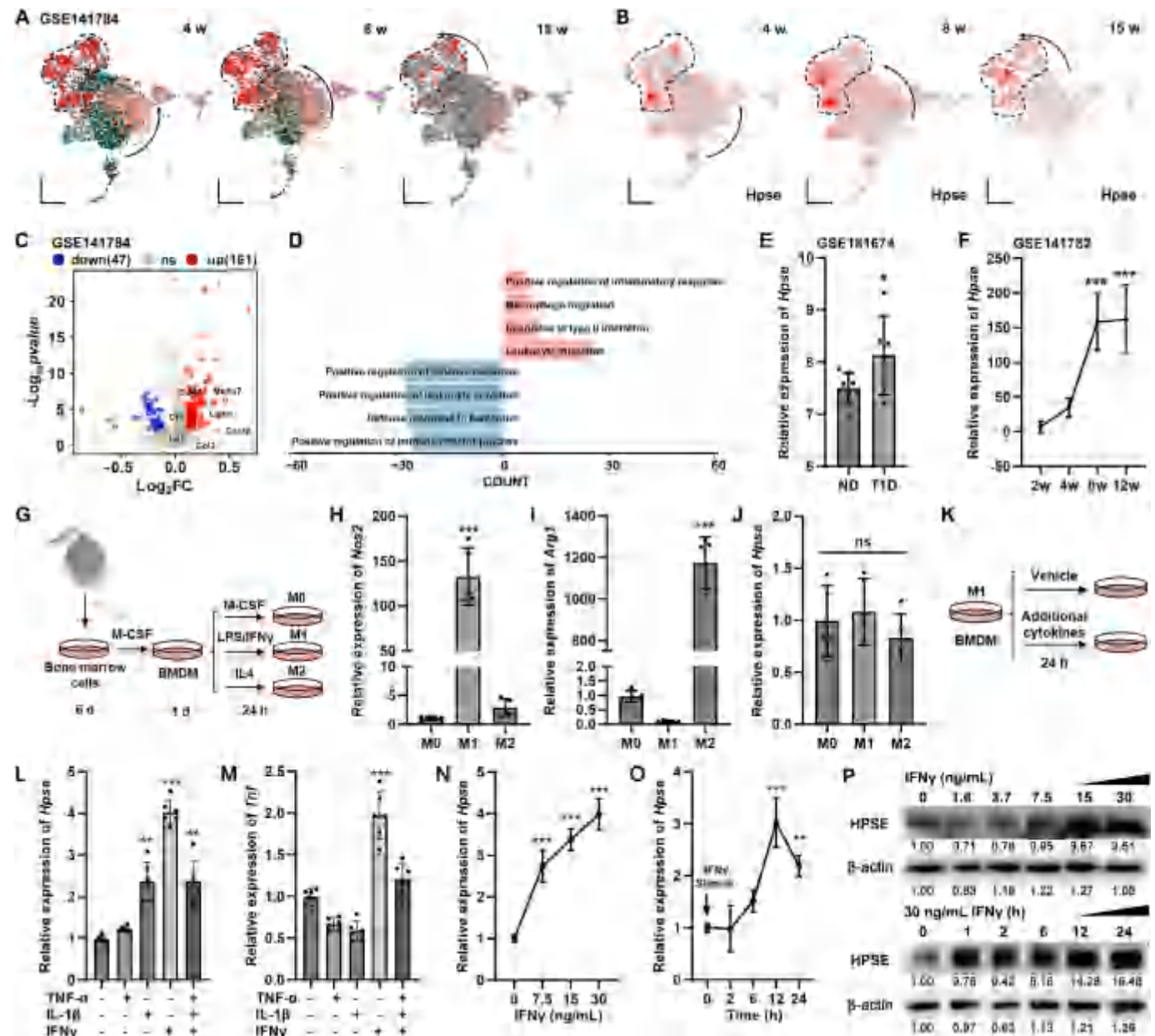


Fig. 7. The expression of heparanase (HPSE) by cultured bone marrow-derived macrophages (BMDMs) following different treatments. (A) Visualization of islet macrophage sub-clusters in 4-, 8-, and 15-week-old NOD mice in the GSE141786 dataset, colored according to the identified populations. (B) The feature plots of *Hpse* expression in the islet macrophages from 4-, 8-, and 15-week-old NOD mice. (C) Volcano plots indicating the representative differentially expressed genes (DEGs) between *Hpse*⁺ and *Hpse*⁻ macrophages in the GSE141786 dataset. (D) Pathway enrichment of DEGs in (C). (E) The expression of HPSE in the islets from non-diabetic (ND) volunteers and patients with T1D from the GSE181674 dataset, * $P < 0.05$. (F) The expression of *Hpse* in the islets from 2-, 4-, 8-, and 15-week-old NOD mice in the GSE141782 dataset, *** $P < 0.001$ compared to 2-week-old NOD mice. (G) The procedure for isolation and differentiation of BMDMs from C57BL/6 mice. (H) The expression of iNOS (*Nos2*), (I) *Arg1*, and (J) *Hpse* in M0, M1, and M2 BMDMs determined by qPCR assay. (K) The procedure for additional stimulation of M1 BMDMs. (L) The expression of *Hpse* and (M) *Tnf* in M1 BMDMs following different treatments. (N) The dose-dependent and (O) time-dependent expression of *Hpse* in M1 BMDMs following stimulation by additional interferon γ (IFN γ). ** $P < 0.01$, *** $P < 0.001$ compared to the vehicle-treated control, $n = 6$. (P) The dose-dependent and time-dependent expression of heparanase (HPSE) in M1 BMDMs following stimulation by additional IFN γ , determined by western blotting assay, β -actin was used as internal reference, the optical density of each band was analyzed.

Guclu, et al. reported that TZDs treatment exhibited better therapeutic efficacy in T1D patients with preserved residual β -cell function than others [32], provided clinical evidences that the pronounced patient heterogeneity poses significant challenges for accurately assessing the definitive therapeutic efficacy of TZDs in T1D clinical trials. In addition, previous clinical trials evaluating TZDs in T1D populations were limited in scale, featuring restricted sample sizes. Further larger-scale clinical trials targeting potential responder cohorts are warranted to elucidate the therapeutic utility of PPAR γ agonists. Nevertheless, building upon

the findings from the present study, ROZ treatment diminished immune cell infiltration within pancreatic islets—an early stage of T1D development. Recent work has identified teplizumab, an anti-CD3 monoclonal antibody, as a promising agent capable of delaying the onset of T1D and enhancing beta cell function as a viable early intervention strategy for T1D therapy [33]. Therefore, we hypothesize that the effectiveness of TZDs in various T1D patient cohorts may critically depend on the optimal therapeutic window for drug administration. Specifically, TZDs may be more effective in delaying the progression to

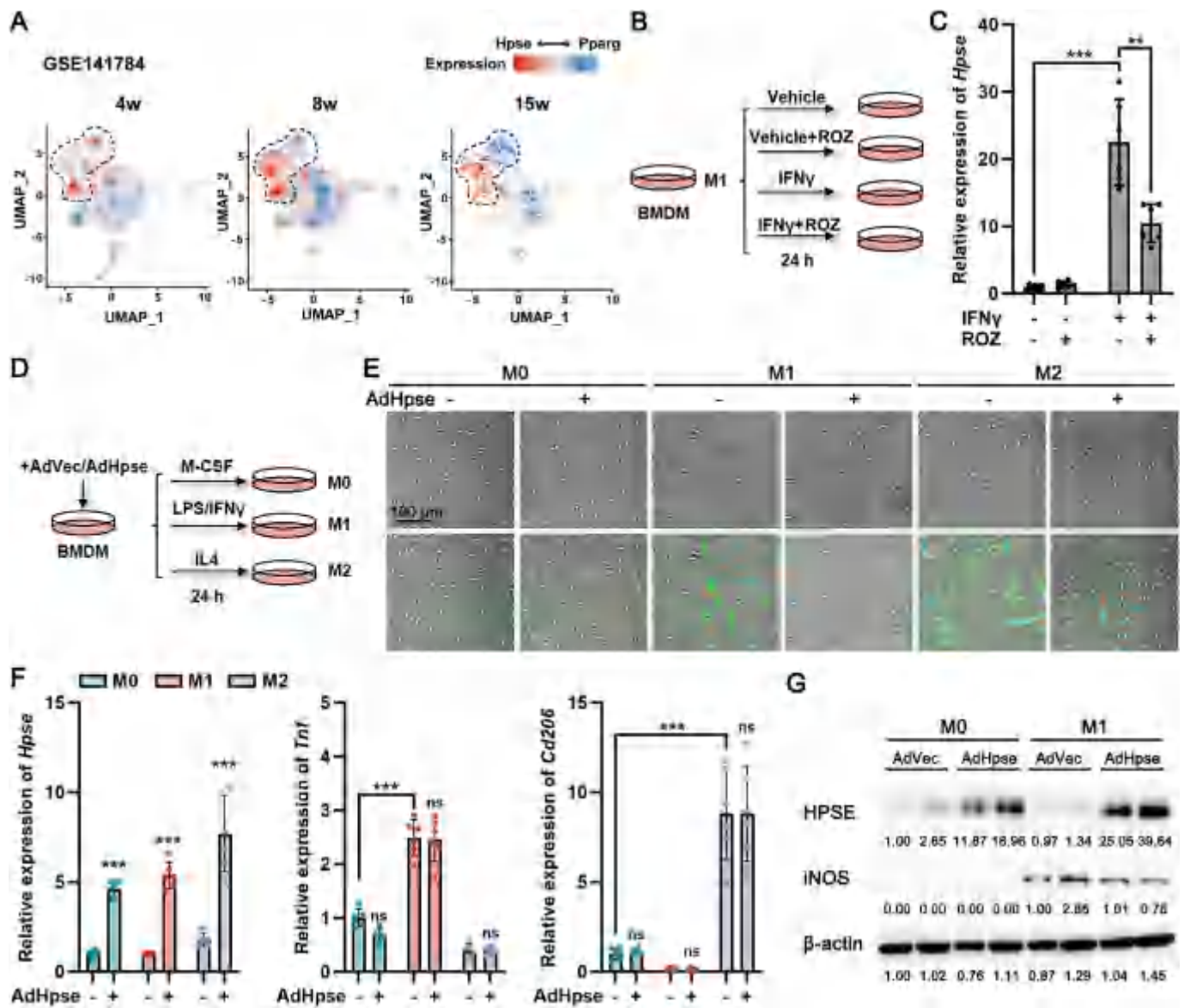


Fig. 8. The effect of heparanase expression on the polarization of BMDMs. (A) The feature plot of Pparg (blue) and Hpse (Red) expression in macrophages from 4-, 8-, and 15-week-old NOD mice in the GSE141786 dataset. (B) The procedure for ROZ-treated M1 BMDMs from C57BL/6 mice. (C) The expression of Hpse following additional IFN γ stimulation of M1 BMDMs in the presence of ROZ (50 μ M). (D) The procedure for Hpse overexpression before polarization stimuli of BMDMs from C57BL/6 mice. (E) The morphology of differently polarized BMDMs, green fluorescent protein (GFP) fluorescence indicates the infection efficacy. (F) After treatment, the mRNA expressions of Hpse, Tnf, and Cd206 in differently polarized BMDMs were detected by qPCR. (G) After treatment, the expressions of HPSE and iNOS were detected by western blotting, using β -actin as an internal reference, the optical density of each band was analyzed. ** $P < 0.01$, *** $P < 0.001$, ns = not significant, as indicated, $n = 6$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clinical T1D at an early stage, whereas their efficacy may diminish once clinical T1D has already manifested. The results presented here support an involvement of PPAR γ activation in this early response. Consequently, subsequent clinical trials should be designed to target early-stage T1D cohorts, like specifically antibody-positive adolescents exhibiting pre-hyperglycemic status, to evaluate the clinical potential of PPAR γ agonists as adjunctive therapy for T1D management.

Although the anti-inflammatory activity of PPAR γ agonism in macrophages has been well recognized [34,35], the detailed mechanisms underlying the protective effect of ROZ and its reduction of intra-islet immunocyte infiltration remain unclear. The pathogenic destruction of beta cells during T1D involves activities of pro-inflammatory monocytes and autoreactive CD4- and CD8-positive T cells [2], indicating the importance of determining whether the ROZ-induced anti-inflammatory effect in monocytes is sufficient to protect beta cells and halt the

progression of T1D. Our reanalysis of publicly available RNA-seq and scRNA-seq datasets utilizing islet samples derived from patients with T1D [19] and NOD mice at varying ages [20] aimed to identify potential genes and pathways that were correlated with PPAR γ signals in macrophage sub-populations from T1D islets. Heparanase, which we previously identified as a target gene of the PPAR γ transcription factor [18], warranted further investigation due to its significant expression.

Heparanase is the sole endoglycosidase currently known to catalyze the degradation of heparan sulfate (HS), a ubiquitous linear glycosaminoglycan that is widely distributed on the cell membrane surface and within the extracellular matrix and that possesses a diverse array of biological functions [17]. Physiologically, intra-islet HS may act as a physical barrier that maintains the integrity of the pancreatic islets—a possibility that is supported by the correlation between heparanase-mediated loss of intra-islet HS and the infiltration of immunocytes

during T1D [36]. We previously reported that hyperglycemia can increase the expression of heparanase in T1D; whereas inhibition of heparanase by a small molecule, OGT2115, reduces HS loss and prevents the infiltration of immunocytes within islets in the MLD-STZ mouse model [18]. In the present study, the expression of heparanase in ROZ-treated MLD-STZ mice and the levels of intra-islet HS were greatly reduced, yielding a comparable phenotype to that achieved with OGT2115. The subsequent bioinformatic analyses and *in vitro* experimentation utilizing cultured BMDMs collectively reinforced the negative correlation between heparanase activity and PPAR γ activation, suggesting that the protective effect exerted by ROZ on T1D islets is likely attributable to its ability to downregulate heparanase expression in macrophages, thereby preventing intra-islet HS loss, hindering immunocyte infiltration, and ultimately protecting pancreatic beta cells from inflammation damage.

We further clarified the role of this PPAR γ -heparanase axis in the pro-inflammation of monocytes by evaluating the role of heparanase in cultured BMDMs. Previous studies have revealed a biological role for heparanase in macrophages [37,38], and our study on the scRNA-seq data using islets of NOD mice revealed a notably increased heparanase expression of heparanase in the pro-inflammatory macrophage subset compared to other subpopulations. This finding suggested that the heparanase upregulation was presumably contingent upon the pro-inflammatory activation state of the macrophages. However, our *in vitro* results indicated that LPS/IFN γ , a commonly employed stimulus for inducing M1 polarization in cultured BMDMs, did not elicit any statistically significant increase in heparanase expression within this standard M1 polarization paradigm.

Given the robust correlation between heparanase expression and the progression of pro-inflammatory activation in macrophages, we found that the application of an additional stimulation using IFN γ significantly elevated heparanase expression in the M1 BMDMs, underscoring the potential importance of sustained inflammation in modulating heparanase-mediated processes in macrophages. The upregulation of heparanase expression induced by IFN γ can be significantly inhibited by PPAR γ agonists such as rosiglitazone and pioglitazone, confirming the negative regulatory role of PPAR γ in heparanase transcription. Interestingly, co-incubation with the PPAR γ inhibitor GW9662 did not enhance the upregulation of heparanase expression induced by IFN γ . We hypothesized that the possible explanation could be that PPAR γ acts as a transcriptional repressor of heparanase—where IFN γ functions as the “accelerator” driving transcription, PPAR γ agonism serves as a “braking system”. Thus, genetic knockdown or pharmacological inhibition of PPAR γ (like “releasing the brake”) does not enhance IFN γ -stimulated heparanase upregulation. This data provides additional support for the evidence that PPAR γ functions as a repressor of heparanase transcription. In addition, the overexpression of heparanase in BMDMs had only a minimal direct influence on macrophage polarization toward either the M1 or M2 phenotype. These findings strengthen the hypothesis that the downregulation of heparanase expression subsequent to PPAR γ activation is potentially independent of its anti-inflammatory activities. Rather, the attenuation of the heparanase activity, which in turn safeguards the intra-islet HS from degradation, ultimately contributes to the preservation of islet integrity. This alternative explanation could at least partially explain the protective effect of ROZ-mediated PPAR γ activation in T1D pancreatic beta cells. Although TZD-class PPAR γ agonists such as rosiglitazone and pioglitazone have been subjected to varying degrees of clinical restrictions due to adverse effects including cardiovascular risks [39], the significant clinical importance of PPAR γ as an insulin sensitizer in diabetes treatment underscores the ongoing value of dual/triple PPAR agonists, as well as other investigational non-TZD PPAR γ agonists [40], in the development of drugs for metabolic diseases.

In summary, this study describes a novel mechanism whereby ROZ mitigates pancreatic beta cell damage in T1D through the activation of PPAR γ and subsequent downregulation of heparanase expression in macrophages. The end result is a prevention of heparanase-mediated intra-islet HS loss and consequent preservation of islet integrity and

reduced infiltration of immunocytes into the islets. Our data emphasize the importance of sustained inflammation in the upregulation of heparanase in macrophages, while also underscoring the pivotal role played by the PPAR γ -heparanase axis. Recently, novel targeted therapies against inflammatory pathways—such as the interleukin-1 β (IL-1 β) receptor antagonist (IL-1 β Ra) [41] and antigen-specific apoptotic DNA immunotherapy [42]—continue to emerge in the drug discovery for T1D, however, multiplex immune pathway activation during insulinitis implies that blocking a single cascade (e.g., IL-1 β) may be insufficient to fully halt β -cell autoimmune destruction. Compared to single-pathway inhibitors, targeting the PPAR γ -heparanase axis may confer broader applicability as an adjunctive T1D therapy by restoring the pericellular barrier to reduce inflammatory cell infiltration into islets.

Although certain heparanase inhibitors, such as OGT2115 reported in our previous study [18], can mitigate insulinitis in the progression of T1D in mice model, the drug discovery based on small molecule inhibitors of heparanase remains an ongoing and challenging endeavor. Meanwhile, as reported in another of our published work, Muparfostat, a HS mimetic that enrolled into Phase III clinical trials, can bind to lipoprotein lipase on the vascular endothelial cells, to aggravate hyperlipidemia and hepatic steatosis in obese individuals, limit its potential usage in the treatment of diabetes [24]. Therefore, based on the strategy of repurposing of existing drugs, our current study proposes the use of PPAR γ agonists as a novel approach to target heparanase. Overall, this study provides novel evidence for the potential targeting of PPAR γ activation as an adjunctive treatment strategy for T1D.

CRediT authorship contribution statement

Qinyao Zhou: Methodology, Data curation, Writing – original draft, Formal analysis, Investigation. **Meiwei Li:** Methodology. **Jia Zhang:** Formal analysis, Investigation, Methodology. **Xiaohang Zhou:** Investigation, Methodology. **Qi Zhu:** Methodology. **Hailing Ni:** Methodology. **Yourong Hu:** Methodology. **Lei Wang:** Methodology. **Yuting Ge:** Methodology. **Kunxin Xie:** Methodology. **Guanting Li:** Methodology. **Yizheng Zhang:** Methodology. **Xiaowei Zhu:** Supervision, Resources. **Xinyuan Cao:** Investigation, Writing – review & editing, Conceptualization, Funding acquisition. **Xiao Han:** Writing – review & editing, Project administration, Resources, Validation, Conceptualization. **Peng Sun:** Writing – original draft, Funding acquisition, Writing – review & editing, Project administration, Conceptualization, Supervision, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2025.115183>.

Data availability

Data will be made available on request.

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