BIOCELL
2024 48(7): 1115-1126
ARTICLE

Tech Science Press

Mesenchymal stromal cells modulate unfolded protein response and preserve β-cell mass in type 1 diabetes

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Key words: Type 1 diabetes, Mesenchymal stromal cells, Endoplasmic reticulum stress, Unfolded protein response, Non-obese diabetic mice

Abstract: Introduction: Transplantation of mesenchymal stromal cells (MSCs) is a promising therapy for type 1 diabetes (T1D). However, whether the infused MSCs affect the endoplasmic reticulum stress or subsequent unfolded protein response in β cells remains unclear. **Methods:** To investigate this, we induced early-onset T1D in non-obese diabetic mice using streptozotocin. Subsequently, T1D mice were randomly assigned to receive either MSCs or phosphate-buffered saline. We observed the *in vivo* homing of MSCs and assessed their effectiveness by analyzing blood glucose levels, body weight, histopathology, pancreatic protein expression, and serum levels of cytokines, proinsulin, and C-peptide. **Results:** Infused MSCs were found in the lungs, liver, spleen, and pancreas of T1D mice. They exhibited various effects, including reducing blood glucose levels, regulating immunity, inhibiting inflammation, increasing β-cell areas, and reducing the expression of key proteins in the unfolded protein response pathway. Fasting serum proinsulin and C-peptide levels were significantly higher in the MSCs treatment group than in the T1D model group. However, there was no significant difference in the biomarker of β-cell endoplasmic reticulum stress, the ratio of fasting serum proinsulin to C-peptide, between the two groups. **Conclusion:** Our findings reveal that MSCs infusion does not alleviate endoplasmic reticulum stress in β cells directly but modulates the unfolded protein response pathway to preserve β-cell mass and function in T1D mice.

Abbreviations

ATF-6	Activating transcription factor-6
BiP	Immunoglobulin heavy-chain binding protein
CHOP	C/EBP-homologous protein
ER	Endoplasmic reticulum
IPGTT	Intraperitoneal glucose tolerance tests
MSC(WJ)	Mesenchymal stromal cells derived from Wharton's jelly of the umbilical cord
NBG	Non-fasting blood glucose
NOD	Non-obese diabetic
PI	Proinsulin
STZ	Streptozotocin
UPR	Unfolded protein responsess

Introduction

Type 1 diabetes (T1D) is the primary form of diabetes affecting children [1]. Over the past three decades, T1D incidence has increased at an average annual rate of 3%–4% worldwide [2]. A cohort study conducted in Sweden based on national registers revealed that children and adolescents with T1D have a reduced average life expectancy of 10–16 years [3]. The necessity for lifelong exogenous insulin administration and the high risk of serious complications highlight the urgent need for innovative therapies to address this condition.

Contrary to the long-held belief that over 90% of the β cells are lost at T1D onset, recent studies have shown that a notable proportion of β cells, ranging from 14% to 60%, can be found in patients after diagnosis, though their function is diminished [4,5]. When hyperglycemia is alleviated through insulin administration, the remaining β cells can recover their function, leading to a so-called honeymoon period with low or no need for exogenous insulin. However, most cells are destroyed within a few months of T1D progression. These findings underline the importance of preserving the residual β cells as a key strategy for preventing or reversing T1D [5].

Owing to their high secretory activity, pancreatic β cells are susceptible to an increased demand for insulin or inflammation [6], resulting in the production of excessive

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Received: 08 February 2024; Accepted: 19 April 2024;
Published: 03 July 2024

Doi: 10.32604/biocell.2024.050493

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proinsulin (PI) that exceeds the processing capacity of the endoplasmic reticulum (ER). This leads to an accumulation of the unfolded and misfolded proteins in the ER lumen, known as ER stress [7]. In response to physiological ER stress, the unfolded protein response (UPR) is activated to restore ER homeostasis. This is achieved by reducing the synthesis of new proteins, increasing the folding ability of the ER, and degrading the misfolded proteins [8]. However, if ER stress persists, it over-activates the UPR pathway, ultimately leading to β-cell death [9,10]. Therefore, a combined therapy that modulates immunity and alleviates ER stress or UPR in β cells may be preferable to reverse T1D [11,12].

Mesenchymal stromal cells (MSCs) transplantation is a promising therapy for T1D because of its ability to modulate immunity and repair injured tissues [13,14]. Many experimental and clinical studies have shown that cultured MSCs are safe and effective in the treatment of T1D [15–18]. However, the mechanism of action remains incompletely understood. A recent study indicated that MSCs could enhance β-cell function by down-regulating the mRNA expression of key pancreatic UPR pathway proteins, such as immunoglobulin heavy-chain binding protein (BiP), activating transcription factor-6 (ATF-6), and C/EBP-homologous protein (CHOP), in T1D rats [19]. However, it did not analyze the expression level of these proteins or investigate β-cell ER stress.

Therefore, we aimed to explore this aspect by assessing a novel indicator of ER stress in β cells, the ratio of fasting serum PI to C-peptide [20], and the protein level of BiP, ATF-6, and CHOP in the pancreas. After inducing early-onset T1D in non-obese diabetic (NOD) mice, we administered a single dose of MSCs derived from Wharton's jelly of the umbilical cord (MSC(WJ)) [21] immediately following diagnosis. Our findings suggest that MSC(WJ) infusion has the potential to modulate the UPR pathway and preserve β-cell mass and function.

Materials and Methods

Isolation, culture, and characterization of MSC(WJ)

A segment of the umbilical cord was obtained from a healthy woman who gave birth at the Chinese PLA General Hospital in February 2023. Written informed consent was obtained from the donor before delivery. The protocol was approved by the Ethics Committee of the Chinese PLA General Hospital (No. S2022-107-19; Date: June 15th, 2022). MSC(WJ) was harvested from Wharton's jelly of the umbilical cord, as previously described [22], and characterized by phenotype analysis and induced differentiation *in vitro* [23]. The identification process is as described previously [14]. Briefly, the MSC(WJ) phenotype was determined by labeling with antibodies against CD14 (Biolegend, 325603, San Diego, USA), CD19 (Biolegend, 302205, San Diego, USA), CD34 (Biolegend, 343503, San Diego, USA), CD45 (Biolegend, 384405, San Diego, USA), CD73 (Biolegend, 344015, San Diego, USA), CD90 (Biolegend, 328107, San Diego, USA), CD105 (Biolegend, 323203, San Diego, USA), and HLA-DR (Biolegend, 327005, San Diego, USA), then detected using full-spectrum flow cytometry (Cytek, NL-CLC, San Diego, USA). MSC(WJ) cultures were induced to differentiate into adipocytes, osteocytes, and chondrocytes using an adipogenesis differentiation kit (Applied Cell, AC-1001029, Shanghai, China), osteogenesis differentiation kit (Applied Cell, AC-1001027, Shanghai, China), and chondrogenesis differentiation kit (Applied Cell, AC-1001028, Shanghai, China), respectively. After approximately 3 weeks, adipocytes were identified using Oil Red O (Solarbio, G1262, Beijing, China) staining, osteocytes with Alizarin Red S (Solarbio, G1450, Beijing, China) staining, and chondrocytes with Alcian Blue (Solarbio, G1565, Beijing, China) staining. Primary cells were expanded for 1–2 passages and cryopreserved following the Instructions of Serum-Free Medium for MSCs (version 2019). In this experiment, MSC (WJ) was cultured for approximately 72 h after thawing and used at the third passage. To demonstrate the homing of MSC(WJ) *in vivo*, the cells were labeled with CellTracker CM-DiI (Invitrogen, C7000, Carlsbad, USA) 2 h before transplantation, according to the manufacturer's protocol.

Animals and treatment

All procedures involving animals were performed under the Guide for the Care and Use of Laboratory Animals (8th Edition, USA) and were approved by the Ethics Review Committee for Animal Experiments of the Chinese PLA General Hospital (No. 2022-X18-77; Date: July 20th, 2022). Female NOD and Institute of Cancer Research (ICR) mice, aged 3–4 weeks, were purchased from HFK Biotechnology Co. Ltd. (Beijing, China) and housed in a specific pathogen-free facility at the Medical Laboratory Animal Center of the Chinese PLA General Hospital. Before sampling, the mice were anesthetized with 1% pentobarbital sodium (JHBio Co., Ltd., 57330, Beijing, China) to minimize their suffering.

After a 1-week acclimation period, 4-week-old NOD mice were intraperitoneally injected with streptozotocin (STZ, Sigma-Aldrich, S0130, St. Louis, USA) for 4 consecutive days (45 mg/kg/day) to accelerate T1D onset, which may be similar to childhood-onset T1D. ICR mice served as the control group (n = 6) and were administered the diluent. Non-fasting blood glucose (NBG) levels were monitored every 5 days via the tail vein using a glucometer (Johnson & Johnson, 06940202A, New Brunswick, USA). NOD mice with blood glucose levels >13.9 mmol/L for 2 consecutive days were diagnosed as overt T1D (n = 24) [24,25]. The mice were then randomly assigned to either the MSC(WJ) treatment group (n = 18) or the T1D model group (n = 6). Mice in the MSC(WJ) treatment group received an infusion of 1×10^6 MSC(WJ) suspended in 0.1 mL of phosphate-buffered saline (PBS, Gibco, 20012027, Carlsbad, USA) via tail vein, with a viability of 87.4%, on the same day they were diagnosed with T1D. The T1D model and control groups received 0.1 mL of PBS only. The cell suspension and PBS were infused slowly for more than 10 s.

MSC(WJ) homing and pancreatic histology

To evaluate the homing of MSC(WJ), we randomly selected and sacrificed two mice from the MSC(WJ) treatment group

at 12 h and 1, 2, 3, 5, and 7 days after MSC(WJ) infusion. The pancreases were collected and preserved in Tissue-Tek O.C.T. Compound (Sakura Finetek, 4583, Torrance, USA), flash-frozen in liquid nitrogen, and sliced into 5 μm sections with a 300 μm interval. Sections were stained with Hoechst 33342 (Beyotime, G1127, Shanghai, China) following an established protocol and examined using an inverted fluorescence microscope (Olympus, IX-51, Tokyo, Japan).

The remaining mice (six in each group) were sacrificed on day 16 after infusion, and three mice in each group were used for histological analysis. Paraffin-embedded pancreatic tissues were sectioned at 4 μm and stained with hematoxylin (Servicebio, G1004, Wuhan, China) and eosin (Servicebio, G1001, Wuhan, China). Three sections at a 300-μm interval were prepared for each mouse to ensure observation of different islets, with 20 islets per mouse evaluated. Insulitis was scored on a 0–4 scale. Additionally, the insulin and PI contents in the islets were detected by immunohistochemistry, as previously reported [26]. The insulin- and PI-positive areas, islet areas, and average optical density of these areas were measured using ImageJ software (NIH, Bethesda, USA). The sizes of the insulin- or PI-positive areas in each image were quantified by calculating the percentage of the islet area.

Intraperitoneal glucose tolerance tests (IPGTT) and enzyme-linked immunosorbent assay (ELISA)

Before administering STZ and after diagnosing T1D, IPGTT was performed to assess β-cell function in ICR and NOD mice. Six mice in each group were fasted for 7 h and injected intraperitoneally with glucose (1.5 g/kg). Blood glucose levels were measured in the tail vein at 0, 30, 60, 90, and 120 min post injection. The data were used to determine the area under the curve, which was visualized as a bar graph for each group.

Blood samples were obtained from the right ventricle of the mice under anesthesia, and plasma was collected after blood coagulation. The levels of PI, C-peptide, interleukin (IL)-10, and interferon (IFN)-γ were measured with ELISA kits (Mercodia, 10-1232-01, Uppsala, Sweden; ALPCO, 80-CPTMS-E01, Salem, USA; Proteintech, KE10008, Rosemont, USA; Proteintech, KE10094, Rosemont, USA), following the manufacturer's instructions.

Flow cytometric analysis

To obtain single-cell suspensions, the spleens were passed through 70-μm cell strainers (Corning Falcon, 352350, Corning, USA), and the remaining red blood cells were lysed with red blood cell lysis buffer (Biolegend, 420301, San Diego, USA). The resulting cells were counted using a Countess Cell Counter (Invitrogen, AMQAX2000, Carlsbad, USA) and resuspended in Roswell Park Memorial Institute 1640 medium (Gibco, 11875093, Carlsbad, USA) at a concentration of 1×10^7 cells/mL for flow cytometry analysis. The cells were then stained with a Zombie NIR Fixable Viability Kit (Biolegend, 423105, San Diego, USA) and resuspended in a cell-staining buffer (Biolegend, 420201, San Diego, USA). Subsequently, the cells were stained with monoclonal antibodies against Brilliant Violet 510-CD3 (Biolegend, 100234, San Diego, USA), PE-CD4 (Biolegend, 100407, San Diego, USA), PE/Cyanine7-CD8 (Biolegend, 100722, San Diego, USA), Brilliant Violet 421-CD25 (Biolegend, 100923, San Diego, USA), FITC-CD45 (Biolegend, 157214, San Diego, USA), and Foxp3 (Thermo Fisher Scientific, 17-5773-82, Waltham, USA). Data were acquired using full-spectrum flow cytometry (Cytek, NL-CLC, San Diego, USA) and analyzed with the FlowJo software (FlowJo, Ashland, USA).

Western blot analysis

Proteins were extracted from the pancreatic tissues of the remaining three mice in each group. The tail of the pancreas (20 mg) per mouse was added to RIPA lysis buffer (0.2 mL, Baiqiandu, Ba1004, Wuhan, China) and vibrated at 30 Hz for 5 min using TissueLyser (Qiagen, 85300, Hilden, Germany). The homogenate was then placed on ice for 30 min and centrifuged at 4°C and 13,000 × *g* for 5 min. The supernatant was carefully collected, and the protein content was measured with a bicinchoninic acid protein quantification kit (Baiqiandu, Ba1086, Wuhan, China). Protein samples (200 μg) from each mouse were resuspended in sodium dodecyl sulfate loading buffer (50 μL, Baiqiandu, Ba1011, Wuhan, China) and heated at 100°C for 5 min. After cooling to room temperature (20°C–24°C), the protein solutions were loaded onto five pieces of PAGE gels (Aqlabtech, AQ120-01, Beijing, China) and subjected to electrophoresis. The proteins were then transferred to polyvinylidene difluoride membranes (Millipore, IPVH00010, Burlington, USA). The membranes were blocked in 5% skim milk (Baiqiandu, Ba1033, Wuhan, China) for 1 h at room temperature and incubated overnight at 4°C with one of the following primary antibodies: BiP (Cell Signaling Technology, 3177S, Danvers, USA, 1:1,000), ATF-6 (Cell Signaling Technology, 65880S, Danvers, USA, 1:1,000), CHOP (Cell Signaling Technology, 5554S, Danvers, USA, 1:1,000), and GAPDH (Cell Signaling Technology, 5174S, Danvers, USA, 1:1,000). After three 5-min washes with tris-buffered saline containing 0.1% Tween-20 (Baiqiandu, Ba1100, Wuhan, China), the membranes were incubated with the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3,000, Servicebio, GB23303, Wuhan, China), at room temperature for 1 h. After another four washes, the protein bands were visualized in an enhanced chemiluminescence system (Peiqing Technology, JS-1070P, Shanghai, China), and the optical density of the bands was measured using ImageJ software (NIH, Bethesda, USA).

Statistical analysis

The mean ± SD represents all data presented. Statistical analyses were performed with SPSS 21.0 software (IBM, Armonk, USA). Two sets of data were compared using Student's *t*-test, while three sets were analyzed using one-way ANOVA followed by the *post hoc* Tukey test. If the assumption of homogeneity of variances was violated for three sets of data, they were then subjected to the Kruskal-Wallis test. Statistical significance was set at $p < 0.05$.

Results

Identification of MSC(WJ) characteristics

To determine the properties of the cultured MSC(WJ), we examined its immunophenotypic features and multi-lineage differentiation potential. Over 98% of the MSC(WJ) showed a significant presence of CD73, CD90, and CD105, with little presence of CD14, CD19, CD34, CD45, and HLA-DR (Fig. A1(A)). Adherent MSC(WJ) exhibited shuttle-shaped and fibroblast-like morphology (Fig. A1(B)) and the capacity to differentiate into osteoblasts, adipocytes, and chondroblasts under appropriate conditions (Figs. A1(C)–A1(E)). The results demonstrate that cultured MSC(WJ) possesses characteristics of MSCs.

Schedule, IPGTT, NBG, and body weight of the mice

In our preliminary experiments, NOD mice were administered 40 mg/kg/day STZ for 4 or 5 days, as described previously [26,27]. However, the blood glucose levels of the mice were either below the diagnostic criteria or excessively high, leading to death and severe weight loss after 2–3 weeks. To address this aspect, we adjusted the dosage to 45 mg/kg/day for 4 days (Fig. 1A), resulting in moderate hyperglycemia, and the mice survived for more than 5 weeks without exogenous insulin. Using this protocol, we administered STZ to 40 NOD mice to accelerate the onset of T1D, of which 24 developed diabetes before 6 weeks of age.

To assess the success of the induced models, we conducted the IPGTT both before and after T1D diagnosis (Fig. 1B). Before STZ administration, NOD mice displayed a significantly lower area under the curve of blood glucose compared to ICR mice; however, after one week, when diagnosed with T1D, the mice in the T1D model group exhibited a markedly higher area under the curve of blood glucose than those in the control group (Fig. 1C). These results indicate that the basal blood glucose levels of NOD mice are lower than those of ICR mice, but when diagnosed with T1D, the glucose metabolism function of NOD mice is significantly impaired.

NBG levels and body weight were monitored for 25 days. The MSC(WJ) treatment group had significantly lower NBG levels than the T1D model group at 10 and 15 days post-transplantation (Fig. 1D). Although the body weight of the MSC(WJ) treatment group was slightly higher than that of the T1D model group 15 days post-transplantation, no

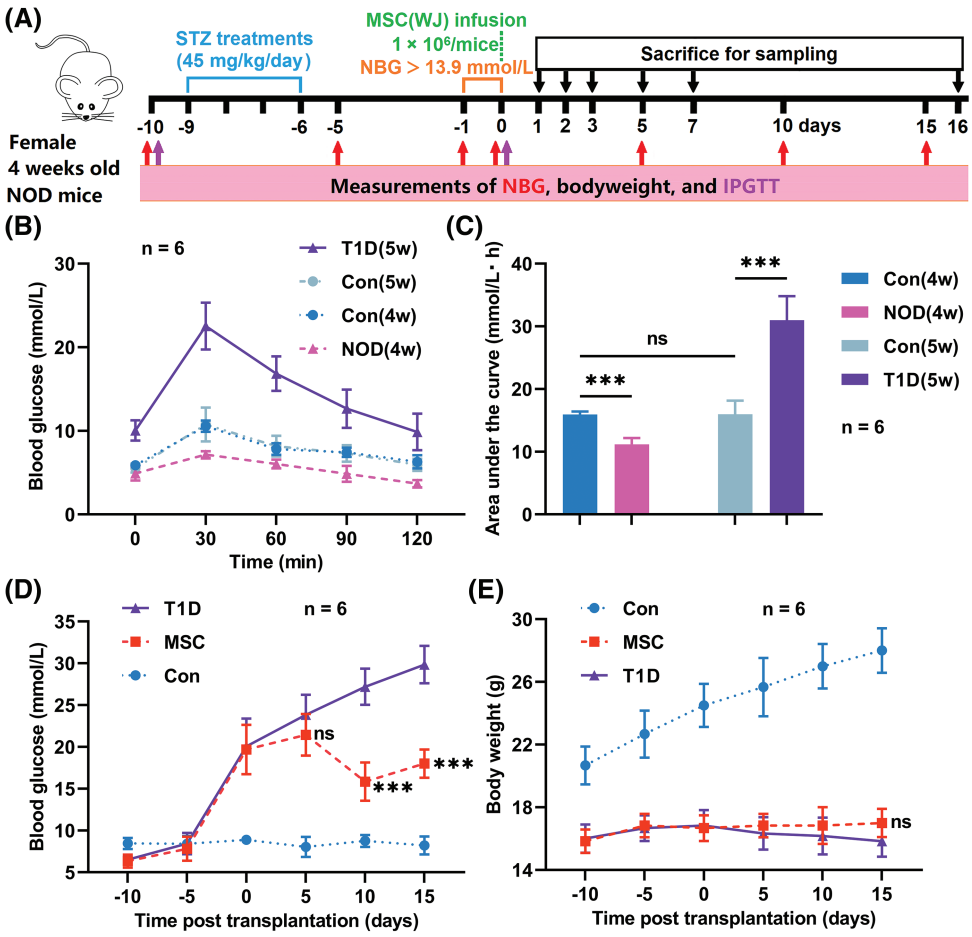

FIGURE 1. Experimental processes and monitoring indicators. (A) Schedule of the experiment. (B and C) Results of intraperitoneal glucose tolerance tests (IPGTT) and the area under the curve of IPGTT, before and after streptozotocin (STZ) injections. (D and E) Non-fasting blood glucose (NBG) levels and body weight of the mice. MSC(WJ), Mesenchymal stromal cells derived from Wharton's jelly of the umbilical cord; NOD, non-obese diabetic. Con(4w) and Con(5w), the control groups of 4-week-old and 5-week-old ICR mice; NOD(4w), the group of 4-week-old NOD mice before STZ injections; T1D(5w), the type 1 diabetes model group of 5-week-old NOD mice; MSC, the MSC(WJ) treatment group. Data are presented as mean ± SD; n = 6. ***$p < 0.001$ *vs.* T1D; ns, no significant differences.

significant disparity was found between the two groups (Fig. 1E). Hence, these findings suggest that infused MSC (WJ) can reduce blood glucose levels in newly diagnosed T1D mice; however, its effect on body weight is not remarkable in the short term.

Homing of MSC(WJ)

To track MSC(WJ) *in vivo*, we systemically infused CM-DiI-labeled cells into T1D mice. After 12 h, a significant number of MSC(WJ) was identified in the lungs, liver, and spleen, whereas a small quantity was observed in the pancreatic lymph nodes and pancreas. These cells remained present for at least 7 days (Figs. 2 and A2). These findings demonstrate the homing of MSC(WJ) to the lungs, liver, spleen, and pancreas in T1D mice.

Immunomodulatory effects of MSC(WJ) on T1D mice

To investigate the effects of MSC(WJ) on the immune system of mice, we measured insulitis, serum IL-10 and IFN-γ levels, and spleen T-lymphocyte subsets on day 16 post-infusion. In the T1D model group, only 1.67% of the pancreatic islets showed no insulitis, whereas approximately 50.0% displayed severe insulitis (>50% infiltration) or complete monocyte infiltration. In contrast, the MSC(WJ) treatment group showed no monocyte infiltration in 20.0% of the pancreatic islets, whereas severe insulitis was observed in 23.3% of the islets (Figs. 3A, 3B). The T1D model group showed a significantly higher mean insulitis score than that of the MSC(WJ) treatment group (Fig. 3C). Moreover, the MSC (WJ) treatment group exhibited significantly higher levels of IL-10 (Fig. 3D) and lower levels of IFN-γ (Fig. 3E) in peripheral blood, compared with the T1D model group. Although the percentages of CD4+ and CD8+ T cells in the spleen were not significantly different between the two groups (Figs. 3F, 3G), the ratio of CD4+ T cells to CD8+ T cells was significantly lower in the MSC(WJ) treatment group than in the T1D model group (Fig. 3H). In contrast, the frequency of CD4+ CD25+ Foxp3+ regulatory T cells was significantly higher (Fig. 3I). These results indicate that MSC(WJ) treatment modulates the immunity of T1D mice.

MSC(WJ) preserving β cells in T1D mice

To evaluate the impact of infused MSC(WJ) on β cells in T1D mice, we analyzed the immunohistochemical staining sections of pancreatic islets and measured the levels of fasting serum PI and C-peptide. Both insulin and PI immunohistochemical staining showed that MSC(WJ) infusion preserved β-cell mass in islets on day 16 post-transplantation (Fig. 4A). The MSC(WJ) treatment group showed significantly higher percentages of insulin- and PI-positive areas than did the T1D model group (Figs. 4B, 4C), both indicating an improvement in residual β-cell areas. However, compared with the T1D model group, the average optical density of insulin-positive areas in the MSC(WJ) treatment group was slightly decreased (Fig. 4D), while the average optical density of PI-positive areas was significantly decreased (Fig. 4E), indicating that the decrease of insulin concentration in islets was not significant in the MSC(WJ) treatment group. Additionally, the MSC(WJ) treatment group exhibited significantly higher levels of fasting serum PI and C-peptide than did the T1D model group (Figs. 5A, 5B). These results suggest that infused MSC(WJ) helps to preserve the residual β-cell mass and function.

Effect of MSC(WJ) on ER stress and UPR pathway in β cells

To investigate the impact of infused MSC(WJ) on ER stress and UPR in β cells, we assessed the ratio of the serum PI to C-peptide and examined the expression of key proteins in the UPR pathway. Although the ratio of fasting serum PI to C-peptide was slightly higher in MSC(WJ)-treated mice than in the T1D model mice, there was no significant difference between the two groups (Fig. 5C). However, we observed a significant decrease in the expression of UPR sensors—BiP, ATF-6, and CHOP—in the MSC(WJ) treatment group compared with those in the T1D model group (Figs. 5D, 5E). Consequently, although infused

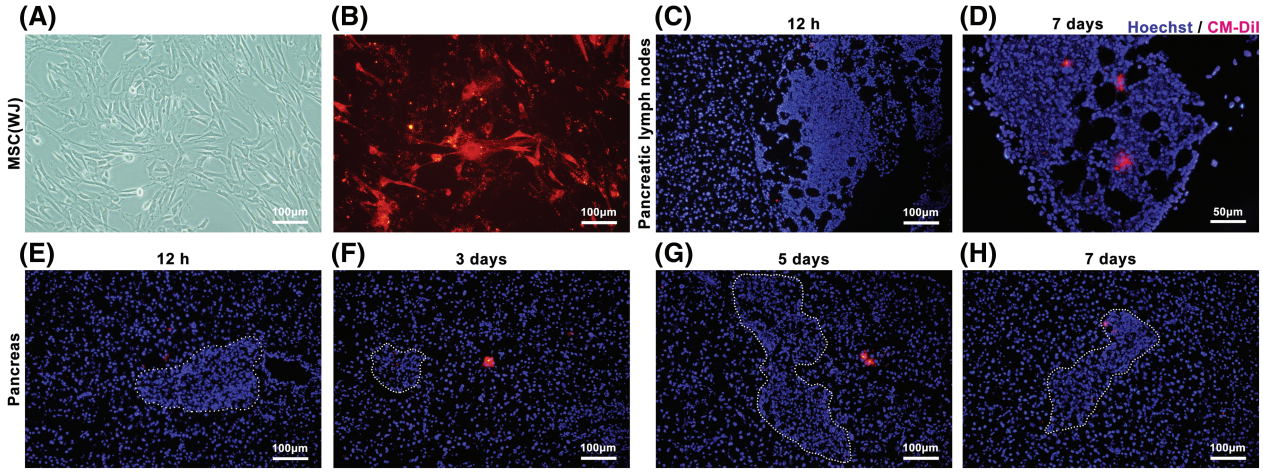

FIGURE 2. Mesenchymal stromal cells derived from Wharton's jelly of umbilical cord (MSC(WJ)) homing *in vivo*. (A and B) The morphology of the third-passage MSC(WJ) cultured *in vitro* and labeled with CM-DiI. Scale bar = 100 μm. (C and D) Representative images of pancreatic lymph nodes of mice with type 1 diabetes at 12 h (Scale bar = 100 μm) and 7 days (Scale bar = 50 μm) after MSC(WJ) infusion. (E–H) Representative micrographs of the pancreas of mice with type 1 diabetes at different time points after MSC(WJ) infusion. The areas marked by the dotted line indicate the presence of pancreatic islets. Scale bar = 100 μm.

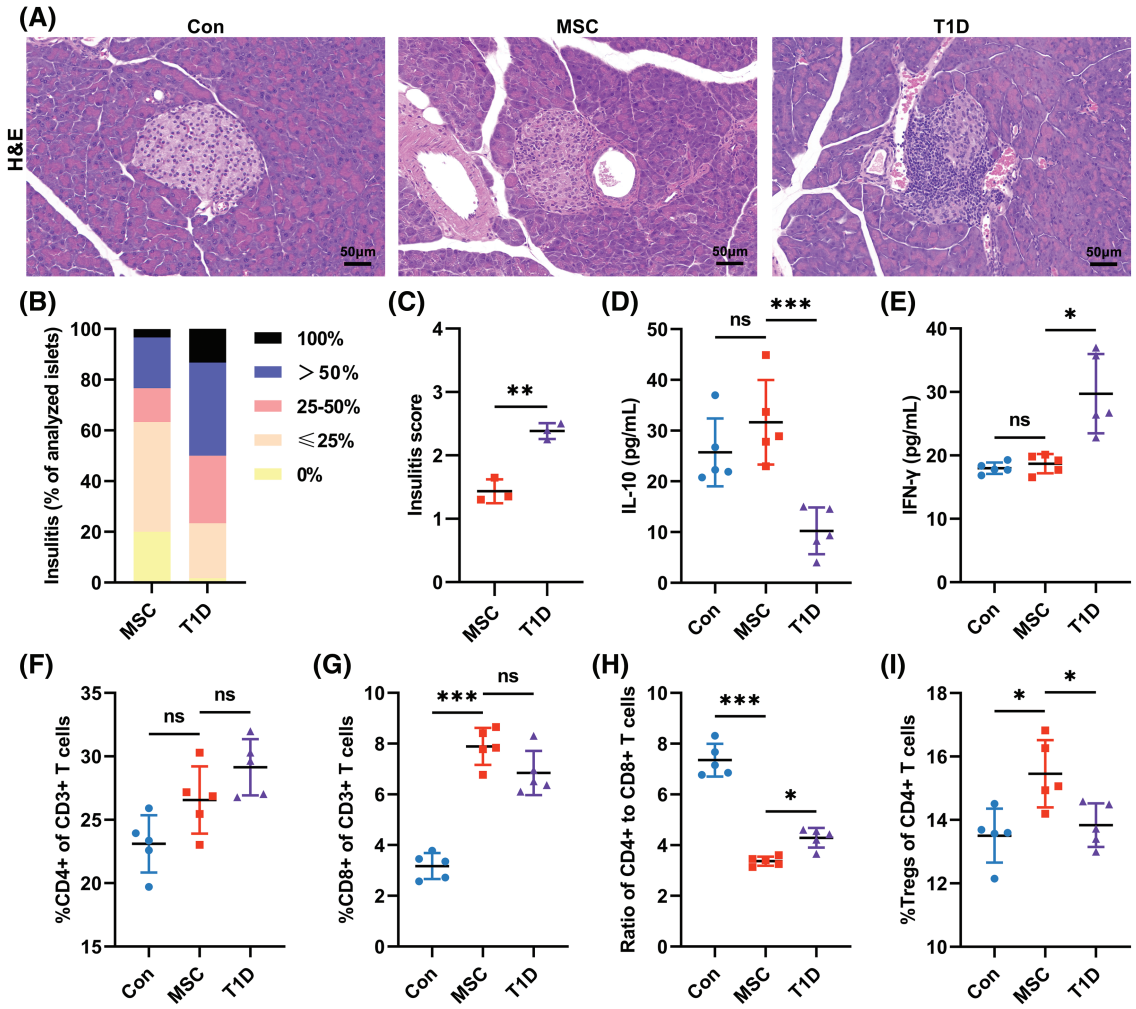

FIGURE 3. Effects of infused Mesenchymal stromal cells derived from Wharton's jelly of the umbilical cord (MSC(WJ)) on the immune system of mice. (A) representative micrograph of islets stained with hematoxylin and eosin (H&E) on day 16 post-transplantation. Scale bar = 50 μm. (B) Percentage of islets in each infiltration category (grade 0, no insulitis; grade 1, monocyte infiltration ≤25%; grade 2, monocyte infiltration >25%, but ≤50%; grade 3, monocyte infiltration >50%, but <100%; and grade 4, complete monocyte infiltration). Three mice in each group were analyzed, and 20 islets of each mouse were evaluated. (C) Insulitis scores of mice. n = 3. (D and E) Fasting serum IL-10 and IFN-γ levels. (F and G) Percentages of CD4+ T cells and CD8+ T cells in the CD3+ T cell population. (H) The ratio of CD4+ T cells to CD8+ T cells. (I) Percentage of regulatory T cells (Tregs) in the CD4+ T cell subset. Con, the control group; T1D, the type 1 diabetes model group; MSC, the MSC(WJ) treatment group. Data are presented as mean ± SD. n = 5. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns, no significant differences.

MSC(WJ) may not mitigate ER stress in β cells, it inhibited the UPR pathway.

Discussion

T1D is a chronic autoimmune disease characterized by the destruction of β cells, which is partly caused by persistent ER stress and over-activated UPR. In this preclinical study, we used a robust early-onset T1D model in NOD mice and observed that MSC(WJ) infusion effectively decreased blood glucose levels, regulated immunity, reduced the expression of sensors in the UPR pathway, and increased residual β-cell mass and insulin production. However, it did not alleviate the stress status of β cells. Our study provides novel evidence for understanding the potential mechanism of MSC(WJ) in T1D treatment. These findings suggest that the UPR pathway may be an important target for MSC(WJ) to preserve β cells in treating T1D and point out a direction for further improving the therapeutic effect of MSC(WJ) on T1D in the future.

Cellular senescence is a critical issue in cytotherapy. Despite researchers having a deep understanding of the mechanisms and signs of MSCs senescence, they are still unable to reverse or prevent it [28]. As the number of passages increases, MSCs cultured *in vitro* age gradually. The secretome of senescent MSCs loses its beneficial functions and can even negatively impact tissue homeostasis and repair [29,30]. Therefore, this study used young third-passage MSC(WJ) for transplantation.

There are many animal models available for T1D research, but only a few are appropriate for studying childhood-onset T1D [31]. NOD mice injected with multiple low doses of STZ have both a hereditary autoimmune basis and partial β-cell destruction, as well as self-antigen exposure caused by STZ. This modeling method accelerates the original autoimmune response and leads to

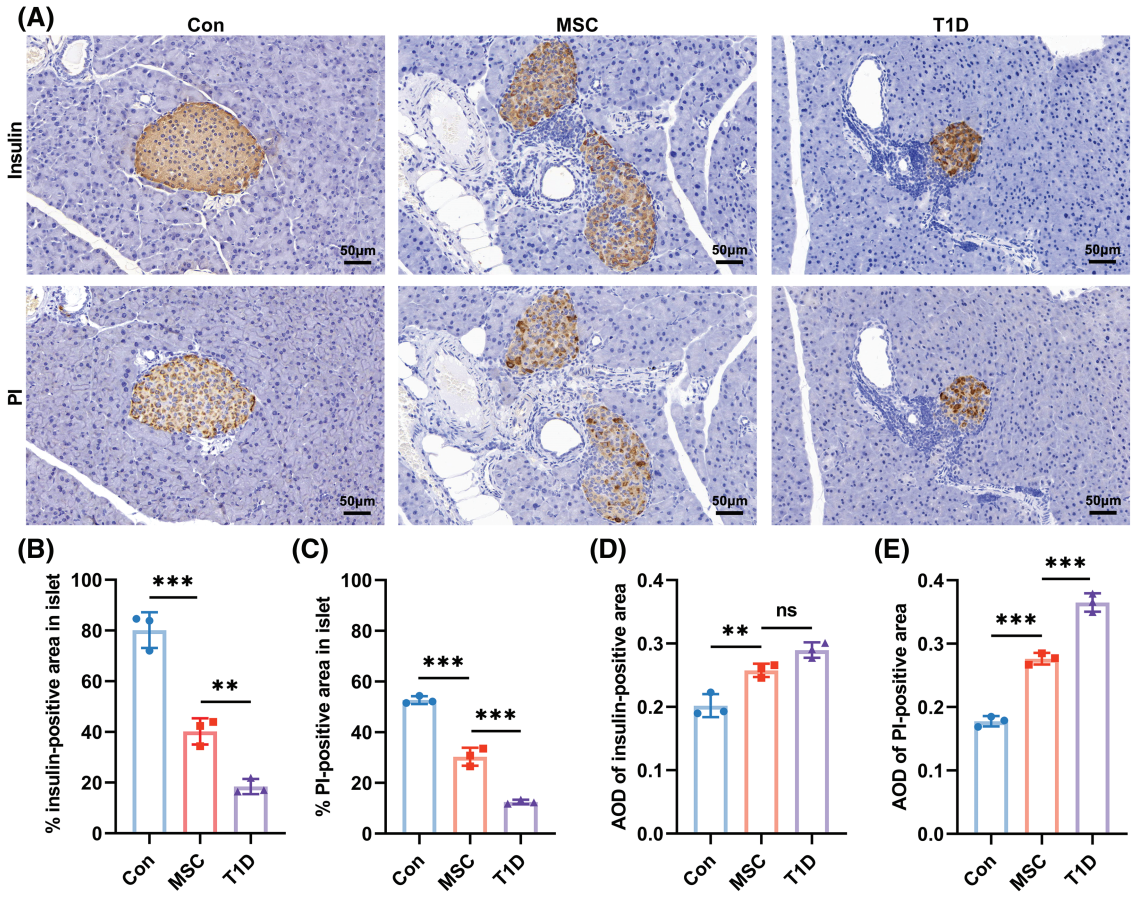

FIGURE 4. Effects of Mesenchymal stromal cells derived from Wharton's jelly of umbilical cord (MSC(WJ)) infusion on β-cell mass. (A) Representative micrographs of islets stained by immunohistochemistry on day 16 post-transplantation. Scale bar = 50 μm. (B and C) Percentage of insulin- and proinsulin (PI)-positive areas in islets. (D and E) Average optical density (AOD) of insulin- and PI-positive areas in islets. The areas and AOD of these areas were measured using ImageJ software. Three mice in each group were analyzed, and 10 islets of each mouse were evaluated. Con, the control group; T1D, the type 1 diabetes model group; MSC, the MSC(WJ) treatment group. Data are presented as mean ± SD. n = 3. $^{**}p < 0.01$, $^{***}p < 0.001$; ns, no significant differences.

the early development of T1D [32,33]. Although there are few reports on this modeling method [26,27], it may be suitable for studying childhood-onset T1D. In this experiment, we assessed the practicality of this modeling method by monitoring blood glucose levels, evaluating glucose metabolism ability, and examining pancreatic pathological changes in NOD mice.

Our study demonstrated that the infused MSC(WJ) effectively migrated to the pancreas and spleen in T1D mice, where they remained for at least 7 days. This infusion significantly reduced insulitis, increased serum IL-10 levels, decreased serum IFN-γ levels, lowered the ratio of CD4+ T cells to CD8+ T cells, and enhanced the frequency of regulatory T cells in T1D mice. These findings align with studies that also reported infused MSC(WJ) homing to the pancreas and spleen in diabetic mice [34,35]. This homing mechanism of MSC(WJ) enables it to exert immune-modulating effects through direct inter-cellular contact, paracrine secretion, and the induction of M2 polarization of macrophages [36]. Other studies have reported similar results, where intravenously infused MSC(WJ) has attenuated insulitis, reduced T helper 17 cells, and increased regulatory T cells in T1D mice [26,37].

Equal amounts of insulin and C-peptide are produced through PI cleavage. To accurately assess basal insulin production, we measured fasting serum C-peptide levels, as they remain more stable in peripheral blood. Our findings indicate that the infusion of MSC(WJ) effectively preserved β-cell mass and increased insulin production. These results align with previous findings and support the notion that systemic administration of MSC(WJ) can maintain secretory function by preserving the remaining β-cell mass [26,38].

As an immunomodulator [39], MSC(WJ) has shown potential in preserving β-cell function in clinical trials [38,40]. However, its effect on ER stress or UPR pathway in β cells has not been examined. Immunomodulatory therapies for T1D are being transitioned from the bench to the clinic [41], making it crucial to assess their impact on β-cell stress. Our results demonstrated that treatment with MSC(WJ) significantly reduced the expression of BiP, ATF-6, and CHOP in the pancreas of T1D mice. This is consistent with a recent report on pancreatic mRNA expression, which suggests that MSCs enhance β-cell function by modulating inflammatory, UPR, and apoptosis signaling pathways in T1D rats [19]. Another study on mice with type 2 diabetes (T2D) showed that MSCs can preserve pancreatic islets and their function by modulating the UPR pathway [42]. Inflammatory cytokines can induce the translocation of BiP to the plasma membrane in β cells, thereby triggering apoptosis [43]. Over-activated UPR pathway leads to the

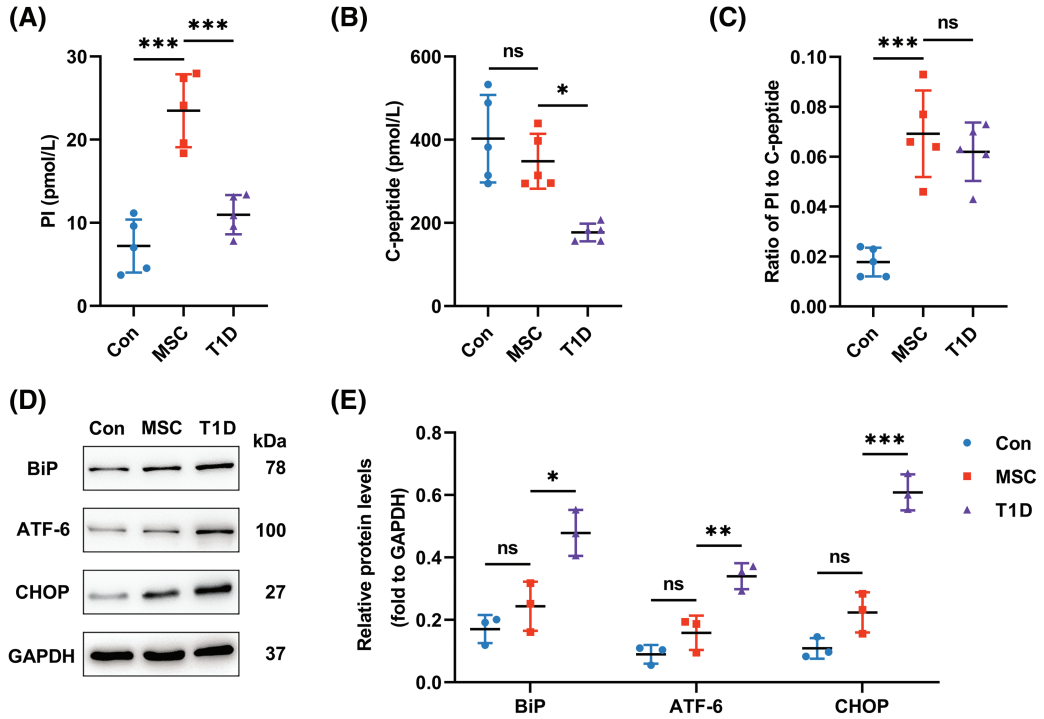

FIGURE 5. Impacts of infused Mesenchymal stromal cells derived from Wharton's jelly of the umbilical cord (MSC(WJ)) on β-cell function and pancreatic protein expression. (A and B) Fasting serum proinsulin (PI) and C-peptide levels determined by ELISA. n = 5. (C) Ratio of fasting serum PI to C-peptide. (D) Representative western blots of key proteins in the unfolded protein response pathway. n = 3. (E) Expression level of the key proteins. The optical density of the proteins was normalized against GAPDH. Con, the control group; T1D, the type 1 diabetes model group; MSC, the MSC(WJ) treatment group. Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, no significant differences.

upregulation of the transcription factor CHOP, leading to apoptosis of β cells in T2D [44]. However, modulating the UPR sensors, such as BiP, ATF-6, and CHOP, can protect β cells against immune assault in T1D [10]. MSCs can protect endothelial cells from hypoxic injury by suppressing the expression of BiP, ATF-6, and CHOP [45]. Therefore, this result explains why infused MSC(WJ) improved the survival of residual β cells in T1D mice.

Our results suggest that MSC(WJ) alone may not alleviate ER stress in β cells. However, some studies have reported that MSCs could relieve β-cell ER stress in the context of diabetes [19,46]. The controversy may be attributed to differences in biomarkers. Our study used a newly established biomarker of β-cell ER stress, the ratio of fasting serum PI to C-peptide [20], whereas the previous studies used the expression of sensors in UPR that cannot directly reflect the stress status of β cells. This new biomarker of β-cell stress has been applied in high-quality clinical trials to evaluate β-cell function in patients with T1D. For example, in a multicenter, double-blind, randomized controlled trial involving 84 youth with newly diagnosed T1D, after 52 weeks of treatment and evaluation, golimumab was found to stabilize β-cell stress in patients compared with controls [47]. Another trial conducted on 76 high-risk individuals in stage 2 of T1D [48] found that one course of teplizumab treatment did not affect the ER stress of β cells after 72 months of follow-up [49].

Inflammation and increased insulin demand can lead to ER stress in β cells [10]. Although infused MSCs regulate inflammation, they cannot differentiate into insulin-producing cells *in vivo* [50]. Consequently, the burden of insulin secretion remains heavy on the residual β cells. Therefore, our findings provide MSC(WJ) infusion alone may not alleviate ER stress in β cells.

The UPR pathway and inflammatory signaling share common regulators in cells. For example, the expression of BiP increases in coxsackievirus B3-induced myocarditis, possibly due to elevated levels of inflammatory cytokines such as IL-6 and tumor necrosis factor-α [51]. Additionally, IL-22 administration has been shown to suppress inflammation and ER stress in pancreatic β cells, leading to the reversal of hyperglycemia in T2D mice [52]. The anti-inflammatory cytokine IL-10 aids in protein folding and ER-associated degradation, thereby preventing protein misfolding [53]. Conversely, the inflammatory cytokine IFN-γ induces CHOP expression and downstream pro-apoptotic signaling in spinal cord neurons of patients with sporadic amyotrophic lateral sclerosis through the induction of inducible nitric oxide synthase [54]. Our findings indicate that MSC(WJ) regulates the BiP/ATF-6/CHOP pathway by upregulating IL-10 and downregulating IFN-γ, thereby preserving residual β-cell mass.

Recent clinical trials have shown promising early results in using verapamil and imatinib to treat patients with recent-onset T1D [55,56]. However, it is important to consider the potential harm to other cells when using these UPR inhibitors, as they primarily target β cells. Combining immunotherapies with β-cell stress therapies in T1D, specifically targeting β cells, could potentially provide an opportunity for prevention or treatment of the disease.

In clinical trials, exogenous insulin has been used as a standard treatment to improve glucose metabolism in patients with T1D, however, this approach may interfere with the evaluation of β-cell stress. Our experiment focused on the direct impact of infused MSC(WJ) on ER stress and UPR in β cells, providing valuable insights into potential therapies for childhood-onset T1D.

Our study has several limitations. First, the small sample size and short observation period may have led to subtle variations within the experiments, potentially yielding false-negative outcomes. Second, although repeated dosing with MSC(WJ) may offer additional benefits or prolong therapeutic efficacy [57], we did not test this strategy. Third, due to the limited volume of blood obtained from mice, our study only assessed fasting serum PI and C-peptide levels at the end of the experiment; this may restrict the comprehensive evaluation of β-cell function and ER stress. Fourth, to accurately reflect changes in the UPR pathways of β cells, protein expression in islets or β cells should be assessed. However, isolating islets or β cells can significantly alter their microenvironment [58], thereby interfering with ER stress and UPR in β cells. Therefore, we directly analyzed the islet-rich pancreatic tail. Finally, the body weight and the ratio of fasting serum PI to C-peptide in mice showed no significant difference between the MSC (WJ) treatment group and the T1D model group. This lack of difference could be attributed to the limited number of cells transfused in our study, as existing literature suggests that MSCs exhibit a dose-dependent effect within a specific range [59,60]. In future experiments, we intend to increase the quantity of transplanted cells and the frequency of infusion to assess their impact. Additionally, we plan to conduct further validation experiments using spontaneous T1D models such as BB rats or NOD mice.

In conclusion, by assessing the novel marker of β-cell ER stress and the expression level of key proteins in the UPR pathway, we demonstrate that MSC(WJ) infusion cannot alleviate ER stress in β cells. However, it does modulate the UPR pathway, leading to the preservation of remaining β-cell mass and function in newly-onset T1D mice. These findings indicate that MSC(WJ) transplantation is a cytotherapy with multiple targets (modulating immunity and UPR pathway) in treating T1D. Further research is necessary to validate our findings and clarify the molecular mechanisms by which MSC(WJ) affects the UPR pathway, ultimately improving its efficacy. Clinical trials will be essential to determine the impact of MSC(WJ) infusion on the stress status of β cells in patients with T1D. In the future, the incorporation of this cytotherapy as a supplementary treatment for childhood-onset T1D is anticipated to enhance the long-term prognosis of pediatric patients.

Acknowledgement: None.

Funding Statement: The authors received no specific funding for this study.

Author Contributions: The authors confirm their contribution to the paper as follows: study conception and design: ZL, SYL; data collection: YZ, YY; analysis and interpretation of results: DY, QW, ZYW; draft manuscript preparation: ZL, SYL. All authors reviewed the results and approved the final version of the manuscript.

Availability of Data and Materials: The datasets generated during the current study are available in the (Mendeley) repository, https://data.mendeley.com/preview/svyjsn9b69?a=ca03a699-4ef7-4293-8ab8-955a41a50dff.

Ethics Approval: A segment of the umbilical cord was obtained from a healthy woman who gave birth at the Chinese PLA General Hospital in February 2023. Written informed consent was obtained from the donor before delivery. The protocol was approved by the Ethics Committee of the Chinese PLA General Hospital (No. S2022-107-19; Date: June 15th, 2022). All procedures involving animals were performed under the Guide for the Care and Use of Laboratory Animals (8th Edition, USA) and were approved by the Ethics Review Committee for Animal Experiments of the Chinese PLA General Hospital (No. 2022-X18-77; Date: July 20th, 2022).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Appendix

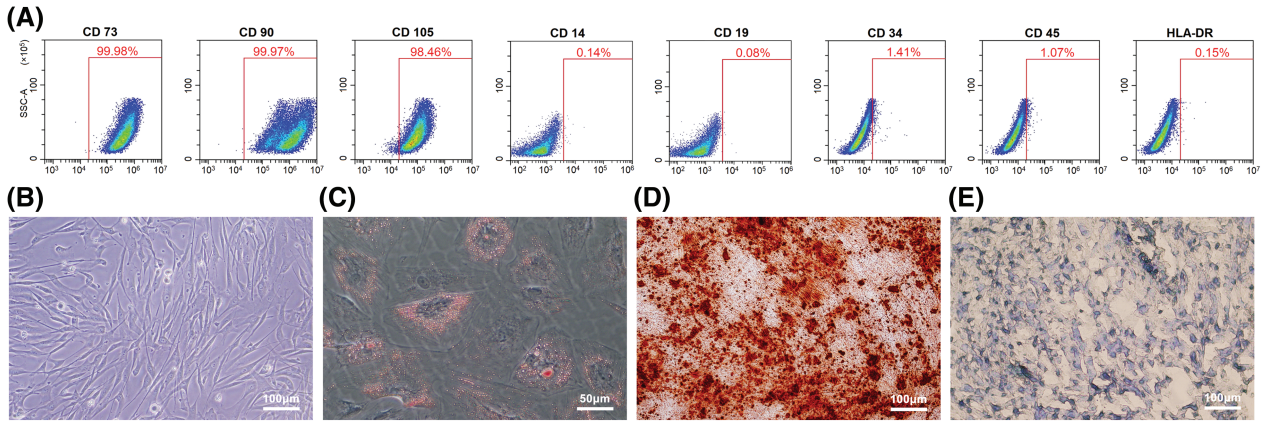

FIGURE A1. Characterizations of Mesenchymal stromal cells derived from Wharton's jelly of umbilical cord (MSC(WJ)). (A) Expression levels of CD73, CD90, CD105, CD14, CD19, CD34, CD45, and HLA-DR on the surface of MSC(WJ) analyzed using flow cytometry. (B) Representative picture of cultured MSC(WJ) observed under a microscope. Scale bar = 100 μm. (C) Micrograph of adipogenic differentiation identified by Oil Red O staining. Scale bar = 50 μm. (D and E) Representative pictures of osteogenic differentiation identified by Alizarin Red S staining, and chondrogenic differentiation identified by Alcian Blue staining. Scale bar = 100 μm.

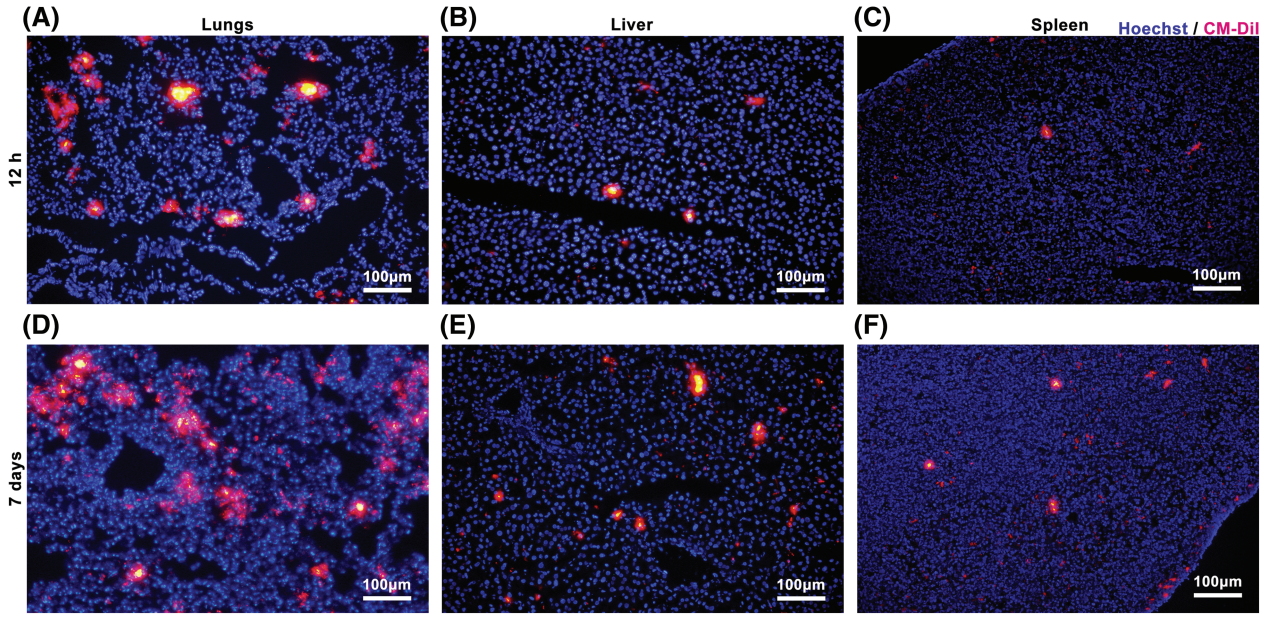

FIGURE A2. Mesenchymal stromal cells derived from Wharton's jelly of umbilical cord (MSC(WJ)) homing *in vivo*. Representative micrographs of the lungs, liver, and spleen of mice with type 1 diabetes at 12 h (A–C) and 7 days (D–F) after MSC(WJ) infusion. Scale bar = 100 μm.