Mesenchymal Stem Cells Reverse Diabetic Nephropathy Disease via Lipoxin A4 by Targeting Transforming Growth Factor β (TGF-β)/smad Pathway and Pro-Inflammatory Cytokines

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Background: Diabetic nephropathy (DN) is the leading cause of end-stage renal disease. Mesenchymal stem cells (MSCs) treatment has been proved to be effective in DN models by protecting renal function and preventing fibrosis. However, the underlying mechanism is unclear. Previous research indicated diabetes and associated complications may be attributed to failed resolution of inflammation, which is deliberately regulated by pro-resolving lipids, including lipoxins (LXs), resolvins (Rv) D and E series, protectins, and maresins. In this study, we monitored pro-resolving mediators in a DN model to explore the mechanism of MSCs treatment.

Material/Methods: The DN model was induced by STZ injection in SD rats. UPLC-MS/MS was performed to determine pro-resolving lipids in kidney tissue and serum of DN model before and after MSCs treatment, as well as in supernatants of HBZY-1-MSCs co-culture.

Results: LXA4 was highly accumulated in renal tissue of DN rats with MSCs treatment; *ex vivo*, LXA4 was significantly increased in the supernatants of HBZY-1 cells co-cultured with MSCs in a high-glucose (HG) medium. Western blot analysis indicated that ALX/FPR2, the receptor of LXA4, was markedly expressed in renal tissue of the DN-MSC group and HBZY-1 after incubating with MSCs in HG. Intraperitoneal injection of LXA4 inhibited renal fibrosis by targeting TGF-β/Smad signaling and downregulated serum TNF-α, IL-6, IL-8, and IFN-γ in DN rats. Notably, all the protective effects induced by MSCs or LXA4 were abolished by ALX/FRP2 blocking.

Conclusions: Our results demonstrate that MSCs intervention prevented DN procession via the LXA4-ALX/FPR2 axis, which inhibited glomerulosclerosis and pro-inflammatory cytokines, eventually contributing to kidney homeostasis.

MeSH Keywords: Diabetic Nephropathies • Inflammation • Lipoxins • Mesenchymal Stromal Cells • Receptors, Transforming Growth Factor beta

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Background

Diabetic nephropathy (DN), a serious complication of diabetes, is the principal cause of end-stage renal disease, with 30–40% mortality [1,2]. DN is characterized by progressive abnormality of renal function and renal fibrosis [2]. Considering that the current therapies, such as glycemic and blood pressure control, can only partly relieve progression of DN, it is imperative to develop a new therapeutic strategy for combatting DN.

With the advent of cell therapy, mesenchymal stem cells (MSCs) have provided a promising strategy to modify the development of DN. As a kind of multipotent stem cell, MSCs can be easily obtained from various tissues, including bone marrow, fat, skin, and liver [3]. They can proliferate rapidly *in vitro* and differentiate into bone, fat, and cartilage tissue [4], which suggests a potential application in regeneration medicine. Additionally, they are able to inhibit immune cell responses [5] and the lack of HLA-II [6]. Thus, it is possible that MSCs are immune-privileged and act as immune modulators. Because of these characteristics, MSCs have been applied in various diseases, including GvHD [7] and OI [8]. Notably, MSCs have been widely studied in DN treatment. In animal models, it has shown the attractive abilities to improve renal function and inhibit fibrosis, but the mechanisms remain unclear.

Previous studies demonstrated that diabetes and associated complications may be caused by inflammation resolution failure [9]. The development of renal fibrosis also indicates failure of resolution because the renal capacity for regeneration and repair is disorganized and normal functional parenchyma is filled with extracellular matrix (ECM) [10]. The importance of inflammation resolution was not recognized until the past decade, and it has since become clear that resolution is an active biochemical process orchestrated by endogenous lipid mediators, including LXs, RvD and E series, protectins, and maresins with both anti-inflammatory and pro-resolving abilities [11,12]. These facts inspired us to hypothesize that MSCs treatment protects renal function and inhibits fibrosis via secreting these pro-resolving lipids.

In the present study we investigated pro-resolving lipids in DN rats after MSCs transplantation and in HBZY-1-MSCs co-culture. We found that MSCs exhibited protective effects on DN via LXA4-ALX/FPR2 by targeting the TGF-β/Smad pathway and various pro-inflammatory cytokines. Our findings indicate that MSCs treatment or pro-resolution may be a promising strategy in DN treatment.

Material and Methods

Diabetic model establishment

Male SD rats (10 weeks old; weight, 280–300 g) were purchased from the Chinese Academy of Medical Sciences (Beijing, China).

After 1 week of adaptation, diabetes was induced by a single intraperitoneal (i.p.) injection of 60 mg/kg STZ (Sigma Aldrich, St. Louis, USA) dissolved into PBS after 1-night fasting, and 72 h later, the rats with a fasting random glucose level >16.7 mmol/L for 3 continuous days were confirmed as diabetic rats. The blood glucose levels were detected throughout the study with a glucometer ACCU-CHEK Advantage Meter (Andon Medical Electronics Co., Tianjin, China). All experimental protocols and studies were in line with the Animal Ethics Committee of the Kunming Medical University, which are consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

MSCs isolation and transplantation

Bone marrow-derived MSCs were isolated from rats by flushing the femurs and tibias of male SD rats (3-week, 100–120 g), and collected as previously described [13]. Cells were analyzed by promoting their differentiation into osteocytes, chondrocytes, and adipocytes with specific differentiation media and flow cytometry analysis to confirm stem cell characteristics, as previously described [13]. Marrow-derived MSCs, characterized by CD29+, CD90+CD44+CD34-CD45-CD11b-, were identified by flow cytometry (BD Biosciences, Franklin Lakes, NJ). At 12 weeks after the successful establishment of the diabetes model, the rats were randomly assigned to 6 groups as follows: (1) the normal control group (NC, n=12): healthy rats without STZ treatment injected of PBS instead of MSCs; (2) the DN group (n=12): diabetic rats received an injection of PBS instead of MSCs; (3) the DN-MSC group (n=12): diabetic rats received a tail vein injection of MSCs; (4) the DN-MSC plus WRW4 group: diabetic rats received a tail vein injection of MSCs plus WRW4 (1 mg/kg); (5) the DN-LXA4 group (n=12): diabetic rats received an injection of LXA4 (10 mg/kg, i.t, Santa Cruz Biotechnology); and (6) the DN-LXA4 plus WRW4 group: diabetic rats received an i.p. injection of LXA4 plus WRW4. MSCs were transplanted via tail vein at a concentration of 5×10^6 in 0.5 ml PBS once a week for 2 continuous weeks, as previously described [14].

Renal immunohistochemistry

Kidney tissues were fixed with formaldehyde. Paraffin sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Masson trichrome (MT) or subjected to immunohistochemistry for TGF-β (product code: ab64715, Abcam, USA).

Western blot

Samples were lysed in RIPA buffer containing PMSF. The protein quantification was determined by BCA protein assay (Cat No. P0068, Beyotime, China), and equal amounts of proteins (40 μg) were subjected to SDS/PAGE (12% gels). After electrophoresis, proteins were transferred onto PVDF membranes

(0.2 mm) in running buffer with 20% methanol. Non-specific sites were blocked with 5% (w/v) non-fat dried skimmed milk powder in TBST (2M Tris-HCl buffer, pH 7.6; 0.05 M NaCl; and 0.05% Tween-20) for 60 min at 37°C. The membranes were then incubated overnight at 4°C with the following antibodies, which were diluted in TBST: anti-TGF-β (product code: ab64715, Abcam, USA), anti-Smad2/3 (product code: ab236030, Abcam, USA), phosphorylated Smad2 (product code: ab53100, Abcam, USA), and phosphorylated Smad3 (product code: ab63403, Abcam, USA). After 4 washes in TBST, the membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies (Cat No. A0562, Beyotime) for 1 h in TBST (dilution of 1: 5000). Protein bands were visualized by using an Enhanced Chemiluminescence (ECL) Plus Western blotting detection kit (Cat. No. P0018-2, Beyotime).

Real-time PCR

Real-time PCR was performed as previously described [15]. Total RNA was extracted from cells with an RNA queousTM Micro kit (Cat. No. 00490515, Invitrogen). Real-time quantitative PCR was performed on a CFX384TM system (BIO-RAD). Primers used in this study: (Primer sequences 5'→3'): TGF-β-F: CATTGCTGTCCCGTGCAGA and TGF-β-R: AGG TAA CGC CAG GAA TTG TTG CTA; IFN-γ-F: TCATCGAATCGCACCTGAT and IFN-γ-R: GGATCTGTGGGTTGTTCACC; IL-8-F: CTTTCAGAGACAGCAGAG and IL-8-R: CTAAGTTCTTTAGCACTCC; IL-6-F: GTCAACTCCAT CTGCCCTTC and IL-6-R: TGTGGGTGGTATCCTCTGTG; TNF-α-F: GCTCC CTCTCATCAGTTCCA and TNF-α-R: GCTTGGTGGTTTGCTACGAC; β-actin-F: CACCCGCGAGTACAACCTTC and β-actin-R: CCCAT ACCCACCATCACACC; GAPDH-F: ACAAGATGGTGAAGGTCGGTG and GAPDH-R: AGAAGGCAGCCCTGGTAACC.

Serum cytokine expression

BD Cytometric Bead Array (CBA) was used to determine the cytokines in serum samples, including IL-6, IL-8, TNF-α, and IFN-γ, according to the manufacturer's protocol and as described previously [16]. The results were analyzed by FCAP Array V3 software (BD).

Pro-resolving lipids determination

Cell supernatants were collected for evaluating LXA4, RvD1, RvD2, Rvd3, RvE1, Protectin D1, maresin 1, and maresin 2 concentration with UPLC-MS/MS, as previously described [15]. Prior to sample extraction, d4-PGE2 (500 pg) was added to permit quantification. Extracted samples were separated by an Acquity UPLC I-Class system (Waters, MA) and mass spectrometry was performed on an AB Sciex 6500 QTRAP. PGE2 was analyzed using scheduled multiple reaction monitoring (MRM). Data acquisition was performed using Analyst 1.6.2 software (Applied Biosystems).

Cell co-culture assay

The rat glomerular mesangial cell line (HBZY-1) was purchased from the China Center for Type Culture Collection. Cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco). To investigate the effect of MSCs on HBZY-1 stimulated by high glucose (30 mmol/L glucose for 72 h) through paracrine effects, MSCs were then plated in the bottom compartment of the Transwell chamber at a density of 400 000 cells/well, with no direct contact with HBZY-1 cells.

Statistics

The number of animals used in the experiments was estimated to give sufficient power (>90%) on the basis of the effect sizes observed in our preliminary data. The statistical analysis was performed using Excel (Microsoft) or GraphPad Prism 7 (GraphPad Software). Statistical significance for binary comparisons was assessed by a 2-tailed t test. For comparison of more than 2 groups, ANOVA with Sidak's multiple comparisons test was used. For correlation analysis, Pearson's correlation coefficient was applied. Overall survival was calculated using the Kaplan-Meier method, and the differences in survival curves were analyzed using the log-rank test. All data are reported as n≥3 mean ±SEM. A P value of 0.05 or less was considered significant.

Results

MSCs ameliorate the renal function and extend survival in diabetic rats

At 12 weeks after STZ injection, diabetic rats showed significantly elevated fasting blood glucose levels (FBG>16.7 mM) and renal damage. We employed urine and serum analyses to assess renal injury among normal control rats, DN rats, and DN rats with MSCs injection. As we expected, DN rats showed a significant elevation in serum level of FBG, serum creatinine (Scr), and blood urea nitrogen (BUN), as well as glycosuria (GLU), microalbumin (MAU), and albumin-to-creatinine ratio (ACR) in urine; however, MSCs inhibited these above indices except FBG in DN rats compared with the DN group (Figure 1A, 1B). Consistently, the DN-MSC group showed slightly morphologic abnormalities (renal glomerular hypertrophy, fibrosis, and tubular dilatation) in renal tissue compared with the DN group (Figure 1C). At 12 weeks after diabetes onset, the survival of DN-MSC group rats (75.0%, 9/12) was markedly higher compared with 33.3% in the DN group (Figure 1D). These data indicate that treatment with MSCs inhibited renal injury and extended the lifespan of DN rats.
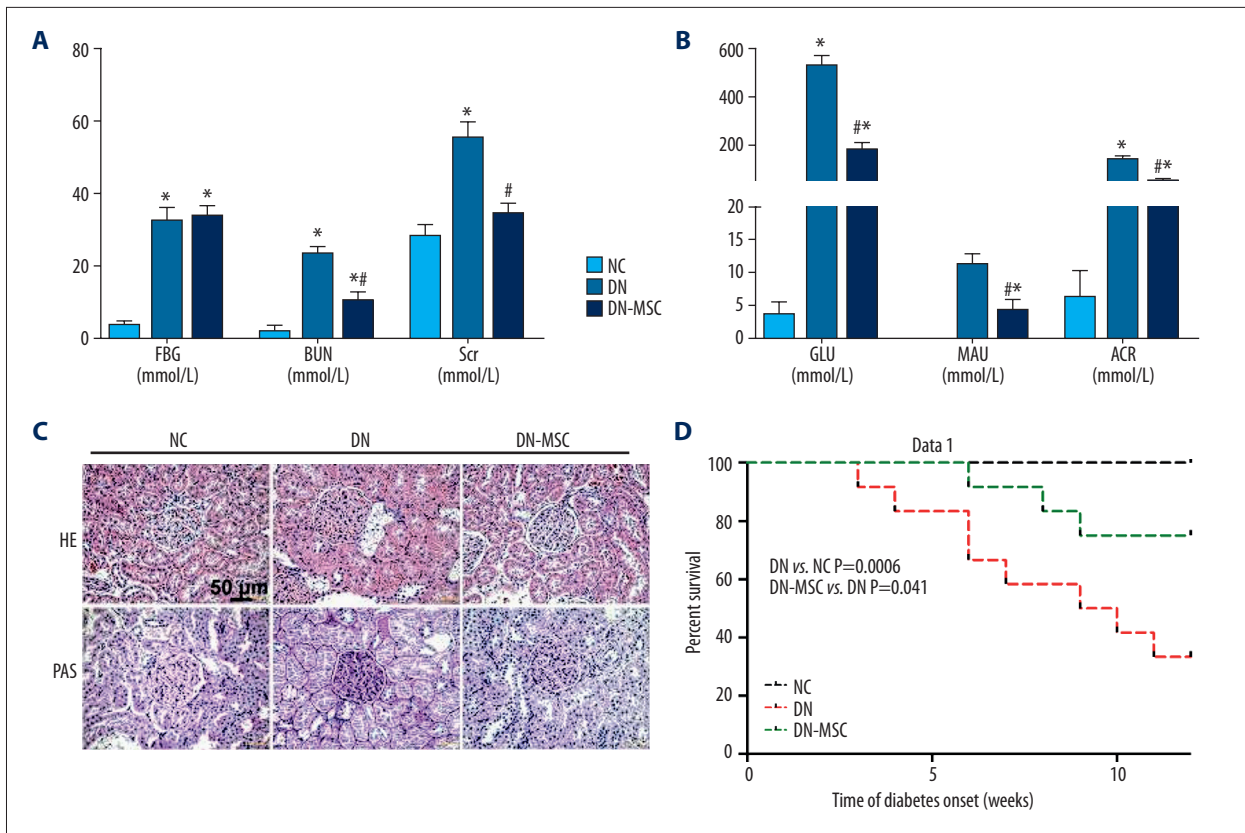
Figure 1. The effects of MSCs on renal function and survival rate in DN rats. Renal function indices, including FBG, Scr, and BUN (**A**), as well as GLU, MAU, ACR in urine (**B**), were measured in the DN model at 12 weeks after STZ injection. H&E- and PAS-stained sections of renal cortices at 12 weeks (**C**) after diabetes onset showed obvious morphologic lesions in DN kidneys (**D**). 12-week survival of 3 groups: NC group, 100%; DN group, 33.3%; DN-MSC group, 75%. * p<0.05 *vs.* normal group (NC), # p<0.05 *vs.* DN.

MSCs regulate the production of LXA4 and ALX/FPR2 in kidney tissue of DN rats and *in vitro*

Inflammation is considered to play a crucial role in the pathogenesis and progression of DN [17]. To investigate that whether MSCs treatment had an impact on pro-resolving lipid metabolism, UPLC-MS/MS was employed to analyze the pro-resolving lipids in the kidney of DN rats before and after MSCs transplantation. Intriguingly, only LXA4 was strongly increased in the DN-MSC group compared with the DN group, while the concentrations of other pro-resolving lipid mediators were not significantly changed (Figure 2A). When glomerular mesangial cells (HBZY-1) were incubated with MSCs in HG or normal glucose (NG) medium, we found that supernatants of the MSC-HG group, but not the NG, HG, or MSC-HG groups, produced a markedly higher level of LXA4 (Figure 2B). We further examined the LXA4 receptor ALX/FPR2 in MSCs and HBZY-1 cells, finding that ALX/FPR2 was commonly expressed in both MSCs and HBZY-1 cells, but it was significantly increased in HBZY-1 cells with MSCs by HG stimulation (Figure 2D). We also used Western blot analysis to assess ALX/FPR2 in kidney tissue of

DN rats with or without MSCs transplantation (Figure 2C). The level of ALX/FRP2 was strikingly increased in the MSCs group (Figure 2C), which was consistent with the result of HBZY-1-MSC co-culture with HG stimulation (Figure 2D). Together, these results demonstrate that MSCs treatment increased LXA4 and ALX/FPR2 in the kidney tissue of DN rats, as well as in HG-stimulated glomerular mesangial cells.

MSCs protect renal function and inhibit fibrosis in DN rats through LXA4-ALX/FPR2 axis

To find out whether MSCs protects renal function and extends the survival through LXA4 in DN rats, LXA4 was directedly administered in the DN model; intriguingly, the DN-LXA4 group showed a significant increase in survival and renal function compared with the DN group (Figure 3A, 3B). WRW4, an LXA4 receptor antagonist, was used to block ALX/FPR2. WRW4 significantly offset the protective effect of MSCs and LXA4 on both survival and renal function (Figure 3A, 3B). Since fibrosis is one of the leading causes of development and progression of DN [18], we used Masson's trichrome staining to estimate
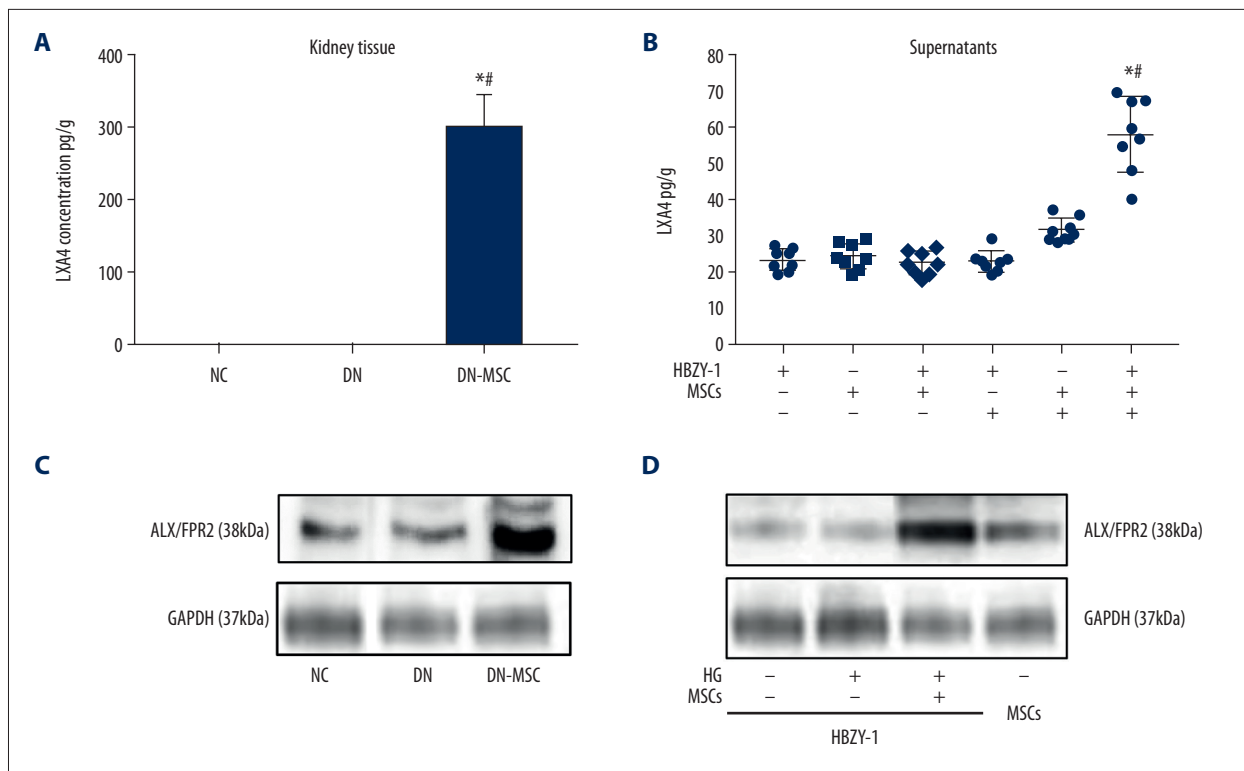
Figure 2. LXA4 and ALX/FPR2 were elevated in kidney tissue of DN-MSC and HBZY-1 cell exposed to HG stimulation and MSCs. The level of pro-resolving lipid mediators in NC, DN, and DM-MSC a 72 h after second MSCs transplantation (**A**) * p<0.05 *vs.* normal group (NC), # p<0.05 *vs.* DN. The level of LXA4 in supernatants of HBZY-1 with different incubation conditions: with or without MSCs; in or not in HG stimulation (**B**) * p<0.05 *vs.* MSCs alone or with HG, # p<0.05 *vs.* HBZY-1, HBZY-1 plus MSCs, or HBZY-1 plus HG. The protein level of ALX/FPR2 in renal tissue of the DN model with or without MSCs injection (**C**) and in HBZY-1 cells exposed to MSCs and HG (**D**).

fibrosis in kidney tissue in NC, DN, and DN-LXA4 groups. Significant reduction of fibrosis was observed in the DN-LXA4 group (Figure 3C), consistent with MSC treatment (Figure 1C). In summary, MSCs treatment decreased fibrosis and preserved renal function in DN rats via LXA4-ALX/FPR2.

MSCs-induced LXA4 inhibits fibrosis progression by targeting TGFβ/SMAD signaling

Diabetic nephropathy is characterized by progression of fibrosis in the kidney [19]. Transforming growth factor-β (TGF-β) is known to play fundamental roles in ECM stabilization, and dysfunction of TGF-β has been reported as a crucial mechanism for the pathogenesis of renal fibrosis [19]. Indeed, TGF-β staining showed TGF-β was normally expressed in renal tissue from the control group, while DN tissue expressed a strikingly high level of TGF-β, which was neutralized in DN-LXA4 groups (Figure 4A). Moreover, both mRNA and protein levels of TGF-β in the DN group were dramatically upregulated compared with the control group (Figure 4B, 4C), and phosphorylations of Smad2/Smad3 were also increased (Figure 4D). However, the level of TGF-β and Smad2/Smad3 phosphorylations were

strikingly downregulated in both DN-MSC and DN-LXA4 groups compared with the DN group (Figure 4B–4D). Notably, MSCs and LXA4-induced inhibitions of TGF-β and Smad2/Smad3 phosphorylations were abolished by administration of WRW4 (Figure 4B–4D). Together, these data indicate that MSCs suppressed pathogenesis progression of renal fibrosis via LXA4 by targeting TGF-β/Smad signaling in DN.

MSCs inhibited pro-inflammatory action in DN rats via LXA4

Inflammation plays a central role in DN [6]. Diabetic rats exhibited notably increased gene expression of pro-inflammatory cytokines, including TNF-α, IL-6, IL-8, and IFN-γ, compared with the non-DN rats. However, MSCs and LXA4 treatment resulted in notable decreases in gene expression of these factors at 12 weeks after diabetes onset (Figure 5A). We also assessed these 4 pro-inflammatory factors in the serum of DN rats; the DN group had increased serum levels of TNF-α, IL-6, IL-8, and IFN-γ in comparison with the control group and a significant decrease of IL-6, IL-8, and IFN-γ was observed in the DN-MSC and DM-LXA4 groups compared with the DN group. However, suppression of ALX/FPR2
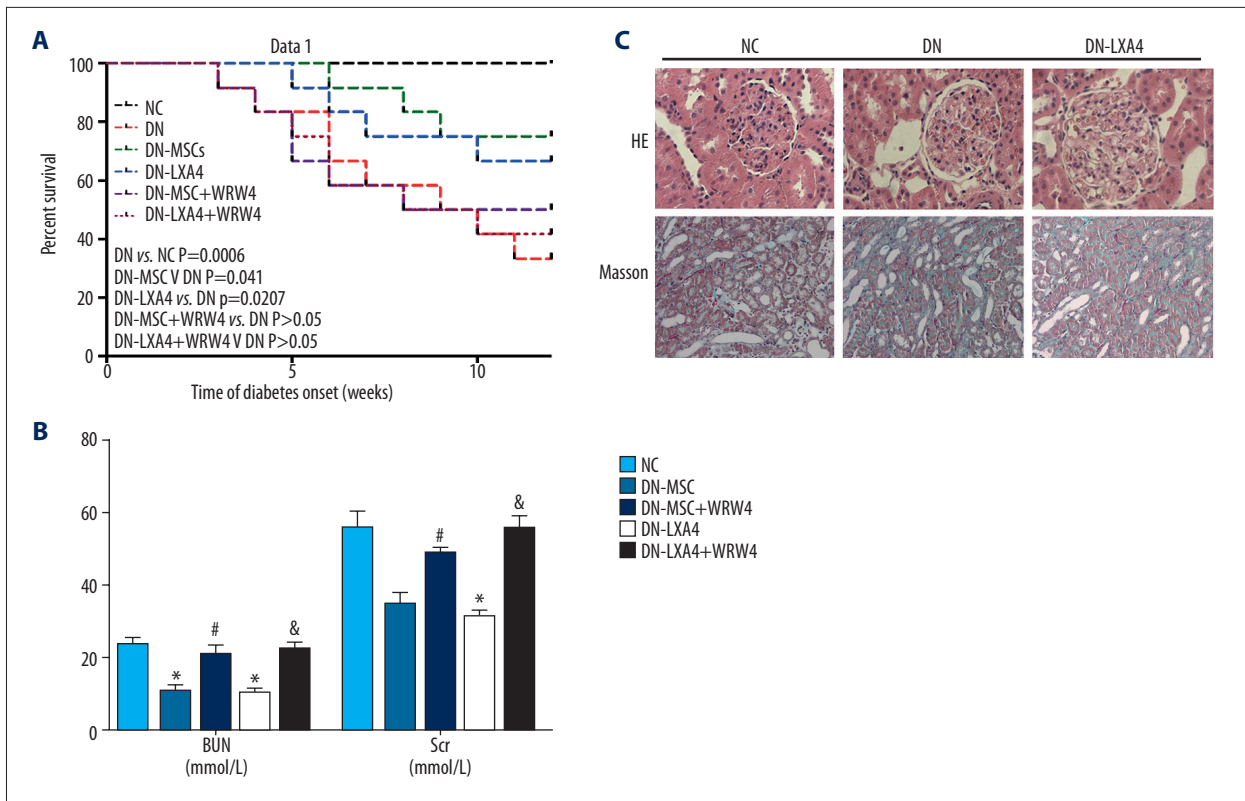
Figure 3. The effects of LXA4-ALX/FPR2 on renal function and survival in the DN model. Survival rates of DN-MSC and DN-LXA4 groups after ALX/FRP2 blocking (**A**). Renal function after ALX/FPR2 inhibition in DN-MSC and DN-LXA4 groups (**B**), * p<0.05 *vs.* DN, # p<0.05 *vs.* DN-MSC, & p<0.05 *vs.* DN-LXA4. Representative images of H&E- and Masson trichrome-stained sections of renal cortices from 3 groups of rats. Severe histologic changes were visible in the DN group, such as interstitial fibrosis. Recovery from most of the glomerular and tubular changes were observed in the DN-LXA4 group (**C**).

abrogated the anti-inflammatory effects induced by MSCs and LXA4 (Figure 5A, 5B). Intriguingly, the serum level of TNF-α was not significantly changed by MSCs or LXA4 treatment (Figure 5B).

Discussion

In DN animal models, MSCs treatment has been shown to improve renal function and inhibit renal fibrosis of DN animals, but the mechanisms remain unclear. Immune regulation has been thought to play a role in this process. In early studies, MSCs were shown to inhibit immune cell and suppress immune responses by inducing production of NO, PGE2, TGF-β, and HLA-G protein [20]. MSCs were also proved to modulate abnormal immune responses in many diseases [21]. In previous studies, MSCs were reported to modulate inflammation in the DN model [1]. However, how MSCs modulate the pathogenesis progression of DN is not fully understood.

In this study, we first demonstrated that MSCs exerted their protective effects by modulating pro-resolving lipid metabolism in the DN model. We believe that pro-lipid metabolism is

a critical process in the protective effects exerted by MSCs. First, we found that LXA4 was significantly increased the kidneys of the DN group rats after MSCs treatment. ALX/FPR2, the receptor of LXA4, was significantly increased in HBZY-1 cells by MSCs with HG stimulation. These data indicate that MSCs treatment promotes LXA4-ALX/FPR2 in the kidney microenvironment. To test the role of LXA4 in the protective effect of MSCs, we used WRW4, the LXA4 receptor antagonist, to block ALX/FPR2. The results showed that WRW4 abolished the protective effect of MSCs. In contrast, administration of LXA4 showed a similar protective effect on MSCs. These data indicate that LXA4 plays an important part in the protective mechanism of MSCs.

Then, we determined the levels of DN related fibrosis signaling pathway and pro-inflammatory cytokines. Both MSCs and LXA4 treatment inhibited TGF-β/Smad signaling and the expression of pro-inflammatory cytokines, including TNF-α, IL-6, IL-8, and IFN-γ. However, the administration of WRW4 abolished these effects. These data indicate that MSCs suppress progression of pathogenesis of DN via LXA4 by targeting TGF-β/Smad signaling and pro-inflammatory cytokines in DN.
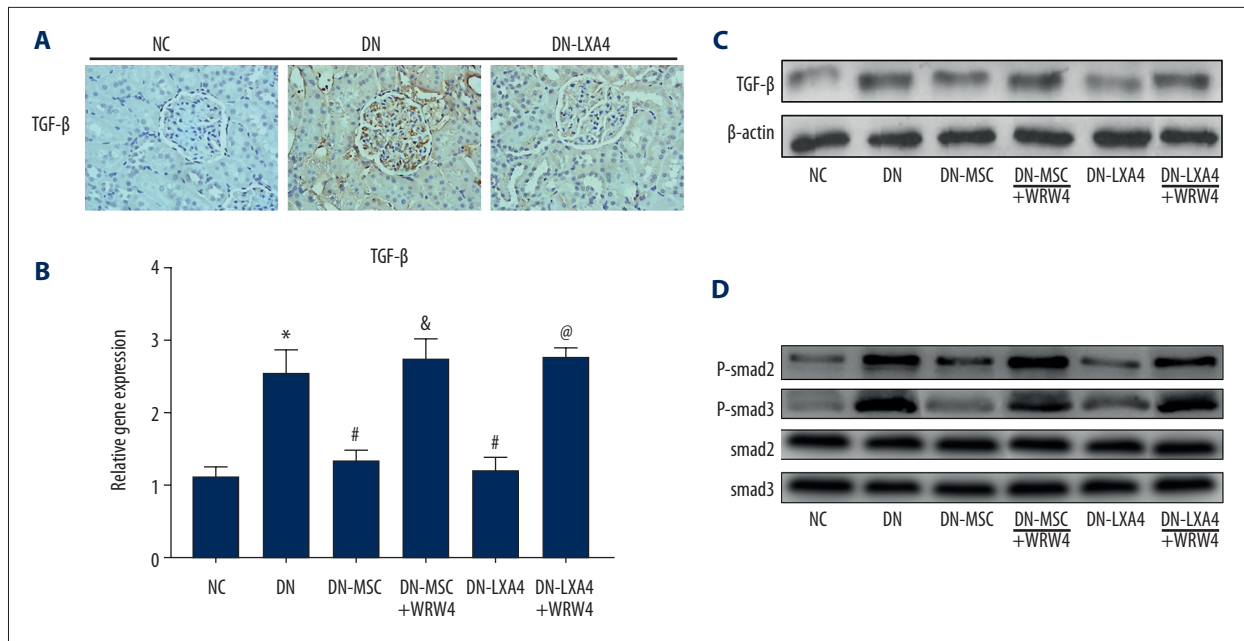
Figure 4. MSCs ameliorated renal fibrosis via LXA4-ALX/FPR2 by inhibiting TGF-β/Smad signaling. Representative images of Anti-TGF-β stained sections of renal cortices from 3 groups of rats. LXA4 markedly prevented renal fibrosis in DN rats (**A**). At 12 weeks after treatment, (**B**) the mRNA expression of TGF-β in kidney tissue was measured by real-time PCR (**B**), and the protein expressions of TGF-β (**C**), phosphorylated Smad2, and phosphorylated Smad3 (**D**) were measured by Western blot. * p<0.05 *vs.* NC, # p<0.05 *vs.* DN, & p<0.05 *vs.* DN-MSC, @ p<0.05 *vs.* DN-LXA4.
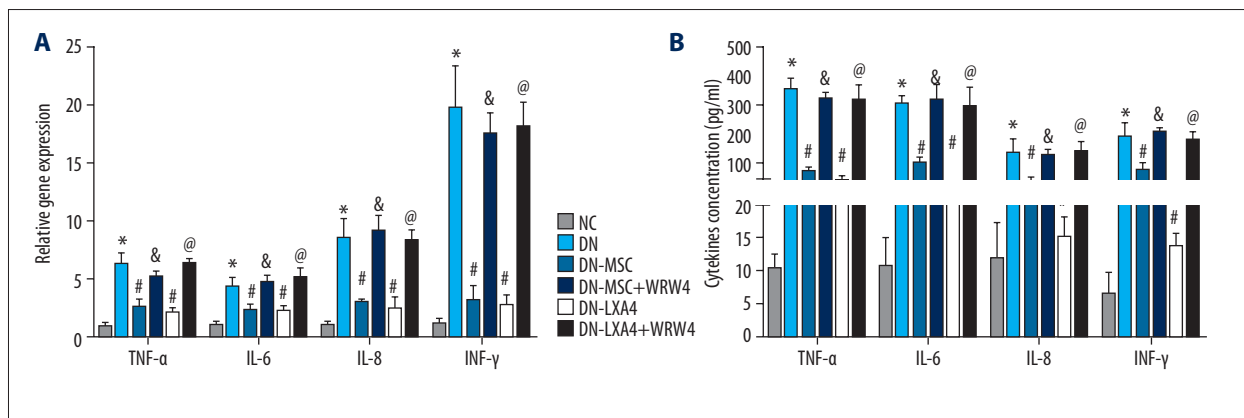

Figure 5. MSCs inhibited inflammation via LXA4-ALX/FPR2 by targeting pro-inflammatory cytokines. The mRNA expressions of several pro-inflammatory cytokines (TNF-α, IL-6, IL-8, and IFN-γ) were significantly suppressed in the DN-MSC and DN-LXA4 groups compared with the DN group at 12 weeks after diabetes onset (**A**). The levels of cytokines in the serum samples were determined at 12 weeks after diabetes onset. TNF-α, IL-6, IL-8, and IFN-γ levels were significantly suppressed in the DN-MSC and DN-LXA4 groups compared with the DN group (**B**). However, all protective effects of MSCs and LXA4 were ameliorated by WRW4. * p<0.05 *vs.* NC, # p<0.05 *vs.* DN, & p<0.05 *vs.* DN-MSC, @ p<0.05 *vs.* DN-LXA4.

Conclusions

Our study demonstrates that LXA4-ALX/FPR2 is an important mechanism in the protective effects of MSCs, which provides potential targets and promising strategies for DN treatment.

Conflict of interest

None.

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