

Mesenchymal Stem Cells Promote Diabetic Corneal Epithelial Wound Healing Through TSG-6–Dependent Stem Cell Activation and Macrophage Switch

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PURPOSE. To explore the role and mechanism of bone marrow–derived mesenchymal stem cells (BM-MSCs) in corneal epithelial wound healing in type 1 diabetic mice.

METHODS. Diabetic mice were treated with subconjunctival injections of BM-MSCs or recombinant tumor necrosis factor- α –stimulated gene/protein-6 (TSG-6). The corneal epithelial wound healing rate was examined by fluorescein staining. The mRNA and protein expression levels of TSG-6 were measured by quantitative RT-PCR and Western blot. The infiltrations of leukocytes and macrophages were analyzed by flow cytometry and immunofluorescence staining. The effect of TSG-6 was further evaluated in cultured limbal epithelial stem/progenitor cells, macrophages, and diabetic mice by short hairpin RNA (shRNA) knockdown.

RESULTS. Local MSC transplantation significantly promoted diabetic corneal epithelial wound healing, accompanied by elevated corneal TSG-6 expression, increased corneal epithelial cell proliferation, and attenuated inflammatory response. Moreover, in cultured human limbal epithelial stem/progenitor cells, TSG-6 enhanced the colony-forming efficiency, stimulated mitogenic proliferation, and upregulated the expression level of Δ Np63. Furthermore, in diabetic mouse cornea and in vitro macrophage culture, TSG-6 alleviated leukocyte infiltration and promoted the polarization of recruited macrophages to anti-inflammatory M2 phenotypes with increased phagocytotic capacity. In addition, the promotion of epithelial stem/progenitor cell activation and macrophage polarization by MSC transplantation was largely abrogated by shRNA knockdown of TSG-6.

CONCLUSIONS. This study provided the first evidence of TSG-6 secreted by MSCs promoting corneal epithelial wound healing in diabetic mice through activating corneal epithelial stem/progenitor cells and accelerating M2 macrophage polarization.

Keywords: mesenchymal stem cells, TSG-6, corneal epithelial stem/progenitor cells, wound healing, macrophage polarization, diabetes

Patients with diabetes mellitus (DM) are at increased risk for developing ocular complications such as diabetic retinopathy, cataract, and neuro-ophthalmic disorders.^{1–3} In addition to these complications, ocular surface disorders, including impaired corneal sensation, superficial punctate keratitis, and delayed epithelial wound closure, are relatively common in DM patients. The nonhealing corneal epithelium defects increase the susceptibility to corneal ulcer, stromal opacification, and microbial keratitis, which may result in a decreased or even permanent loss of vision.⁴ Although ongoing efforts to develop pharmaceutical agents to treat delayed wound healing, such as autologous serum and neurotrophic factors, demonstrate promising results, there is no curative treatment for these complex disorders.^{5–7}

Recent studies revealed that the pathogenesis of diabetic keratopathy is closely related to impairment of corneal innervation, deposition of advanced glycation end products, and dysregulated intracellular signaling network of corneal epithelium.^{8–10} Moreover, chronic inflammation, a characteristic feature of diabetic wounds, is also linked to delayed wound

healing. The dysfunctional macrophages at the diabetic wound site are associated with higher burden of apoptotic cells and excessive expression of proinflammatory cytokines such as TNF- α and IL-1 β .^{11,12} Chronic low-grade inflammation, as well as unbalanced production of matrix metalloproteinases (MMPs), creates a deleterious microenvironment that is detrimental to the activation, proliferation, or even survival of limbal stem cells, thereby contributing to the diabetes-impaired epithelium wound healing.^{13,14}

Bone marrow–derived mesenchymal stem cells (BM-MSCs) are multipotent, self-renewing cells that have the capacity to differentiate into multiple lineages of the mesenchyme.¹⁵ Besides their multilineage differentiation capacity, MSCs can facilitate wound repair by modulating cellular responses in the cells of surrounding tissue and immune cells, creating a proregenerative and anti-inflammatory environment. Thus, these cells hold great therapeutic promise and have been investigated in animal models and in clinical trials for a wide variety of diseases, including diabetes and ocular disorders.^{16,17} The mechanisms by which MSCs exert these effects



are largely attributed to their unique anti-inflammatory and immune-modulatory properties via paracrine effects.¹⁸ Among the myriad trophic factors, tumor necrosis factor (TNF)- α -stimulated gene/protein-6 (TSG-6), a multifunctional anti-inflammatory protein produced in response to signals from injured tissue, has displayed remarkable therapeutic effects in a number of disease models in the eye and other tissues.^{19–24} However, the therapeutic potential of MSCs on diabetic corneal wound repair remains unclear. In the present study, we investigated whether a new treatment for facilitating diabetic corneal wound healing could be developed via TSG-6. Our data demonstrated that local transplantation of MSCs suppresses excessive inflammatory response, activates endogenous corneal progenitor cells, promotes alternative polarization of infiltrated macrophages, and subsequently enhances diabetic corneal wound repair in part by producing TSG-6.

MATERIALS AND METHODS

Animals

C57BL/6 mice (6–8 weeks old, male) were purchased from Beijing Pharmacology Institute, Chinese Academy of Medical Sciences (Beijing, China). All animal experiments were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Type 1 diabetic mice were induced as previously described.^{13,24–27}

Cell Culture

Bone marrow cells from femurs and tibias of 3- to 4-week-old C57BL/6 mice were flushed with 10 mL ice-cold α -Minimum Essential Medium (α -MEM; Life Technologies, Carlsbad, CA, USA) supplemented with 2% fetal calf serum (FCS) and separated by density gradient centrifugation with Ficoll-Paque PLUS (Sigma-Aldrich, Corp., St. Louis, MO, USA). The cells from the interface layer were resuspended with MesenCult Proliferation Kit with MesenPure (Mouse) (Stem Cell Technologies, Vancouver, Canada). A final concentration of 4×10^6 cells was seeded in 25-cm² tissue culture flasks (Sigma-Aldrich, Corp.) and grown at 37°C in a humidified atmosphere of 5% CO₂. Medium was changed twice per week until the cells reached around 80% confluence. The cells were then passaged with 0.05% trypsin/0.1% EDTA. MSCs of passage 3 were tested for purity by flow cytometry to detect cells that expressed the typical markers CD29 and Sca-1 and that were negative for CD11b, CD45, CD31, and CD34 (Supplementary Fig. S1A). Also, the cells were tested for their ability to differentiate into osteogenic, chondrogenic, and adipogenic lineages by a manufacturer of differentiation kits (Cyagen Biosciences, Inc., Guangzhou, China) (Supplementary Fig. S1B). For shRNA experiments, MSCs of passage 2 were transfected with shRNA for TSG-6 (sc-39820-V; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or scrambled short hairpin RNA (scr-shRNA) (sc-108080, Santa Cruz Biotechnology) with a commercial kit (Santa Cruz Biotechnology). TSG-6-silenced MSCs and MSCs transfected with scrambled shRNA were passaged and cultured with 2.5 μ g/mL puromycin. Passages 3 to 5 were used for all experiments.

Corneal Epithelial Wound Healing

Normal and diabetic mice were anesthetized by an intraperitoneal injection of xylazine (7 mg/kg) and ketamine (70 mg/kg) followed by topical application of 2% xylocaine. The corneal epithelium was removed up to the corneal/limbal border with a corneal rust ring remover (Algerbrush II; Alger

Equipment, Lago Vista, TX, USA) as previously described.^{24,28,29} The defects of corneal epithelium were visualized at 0, 24, 48, and 72 hours by instilling 0.25% fluorescein sodium and photographed under a BQ900 slit lamp (Haag-Streit, Bern, Switzerland). The staining area was analyzed by using ImageJ software (National Institutes of Health, Bethesda, MD, USA; in the public domain) and calculated as the percentage of residual epithelial defect. For MSC treatment, diabetic mice were injected subconjunctivally with 5×10^4 MSCs or shTSG-6-MSCs or scr-MSCs (5 μ L/eye) just after the scrape of corneal epithelium, while control mice were injected with PBS vehicle. For TSG-6 treatment, diabetic mice were injected subconjunctivally with 2 μ g recombinant mouse TSG-6 (rmTSG-6, 5 μ L/eye; R&D, San Diego, CA, USA) immediately after the removal of corneal epithelium, while control mice were injected with PBS vehicle ($n = 6$ per group).

Determination of MSCs Homing to Wounded Cornea

In vivo tracing of MSCs was determined by labeling with the fluorescent dye CM-DiI (Life Technologies) before in vivo administration. The dye was reconstituted at a concentration of 1 μ g/ μ L in dimethyl sulfoxide. Cultured MSCs were harvested and resuspended at a concentration of 10^6 cells in 1 mL PBS with 2 μ g CM-DiI dye. Cells were labeled by incubation at 37°C for 5 minutes followed by 4°C for 15 minutes. Cells were washed twice with ice-cold PBS and then resuspended in PBS at a concentration of 10^7 cell/mL. After corneal debridement, 5×10^4 MSCs were injected subconjunctivally. Forty-eight hours later, mice were killed, and 7- μ m-thick frozen sections of corneas were made. Visualization of labeled MSCs was based on the fluorescence of CM-DiI dye.

Colony Formation Assay

For the evaluation of colony-forming efficiency, primary human limbal epithelial cells (HLECs) were isolated and cultured as previously described.²⁵ One thousand passage 1 HLECs were seeded in wells of a 35-mm cell culture dish containing 0, 50, 100, and 200 ng/mL recombinant human TSG-6 (rhTSG-6; R&D) for 8 to 10 days. The colonies were fixed by 4% paraformaldehyde for crystal violet staining or immunofluorescence staining. The colony-forming efficiency (CFE) was calculated as the percent of the colony number/plated cell number.

Flow Cytometry Assay

Mouse corneas (three pooled corneas each group) were harvested 72 hours after surgery, digested in 50 μ L Liberase TL (2.5 mg/mL; Sigma-Aldrich, Corp.) for 30 minutes at 37°C with the undigested tissue removed by screen filters. Cell suspensions were collected and incubated for 30 minutes at 4°C with fluorescein-conjugated anti-mouse antibodies. The primary antibodies used were as follows: CD45-PE-cy5, F4/80-FITC, CD86-PE, and CD206-PE (Biolegend, San Diego, CA, USA). Flow cytometry analyses were performed using a FACS Calibur (BD Biosciences, San Jose, CA, USA). The gate was set on the CD45⁺ population, and further analysis of surface markers was done with in this gate. Data were analyzed using Flowjo program (Tree Star, Ashland, OR, USA).

Immunofluorescence Staining

Eyeballs were snap frozen in Tissue-Tek optimum cutting temperature compound (Sakura Finetechnical, Tokyo, Japan).

For immunofluorescence staining, frozen sections 7 μm thick were fixed by 4% paraformaldehyde for 15 minutes, permeabilized with 0.1% Triton X-100 for 5 minutes, and blocked with normal serum for 1 hour. The samples were stained with primary antibodies overnight at 4°C and subsequently with fluorescein-conjugated secondary antibodies at room temperature for 1 hour. All staining was examined under a confocal laser scanning microscope or an Eclipse TE2000-U microscope (Nikon, Tokyo, Japan) after counterstaining with 4',6-diamidino-2-phenylindole (DAPI).

Reverse Transcription Quantitative Polymerase Chain Reaction

Total RNA was extracted from mouse corneas (four pooled corneas each group) or cultured cells using Nucleospin RNA Kits (Macherey-Nagel, Düren, Germany). cDNAs were synthesized using the Primescript First-Strand cDNA Synthesis kit (TaKaRa, Dalian, China). Real-time PCR was carried out using SYBR Green reagents and the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The cycling conditions were 10 seconds at 95°C followed by 45 two-step cycles (15 seconds at 95°C and 1 minute at 60°C). The quantification data were analyzed with the Sequence Detection System software (Applied Biosystems) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

Western Blot Analysis

Mouse corneas were harvested 48 hours after surgery and total protein was extracted from the lysed samples in radio immunoprecipitation assay (RIPA) buffer (four pooled corneas each group). All samples were run on 10% SDS-PAGE gels for 2 hours at 110 V and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The blots were blocked in 5% nonfat dry milk dissolved in tris-buffered saline tween (TBST) for at least 1 hour and incubated with primary antibodies as follows: TSG-6 (1:1000; Abcam, Cambridge, MA, USA), indoleamine 2,3-dioxygenase (IDO, 1:1000; Abcam), cyclooxygenase-2 (COX-2, 1:1000; Abcam), interleukin-1 receptor antagonist (IL-1ra, 1:1000; Abcam), and TGF- β (1:1000; Abcam). The blots were washed three times, incubated with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ, USA), and finally visualized via enzyme-linked chemiluminescence using an ECL kit (Pierce Biotechnology, Rockford, IL, USA).

ELISA

Mouse corneas were harvested 48 hours after surgery and total protein was extracted from four corneas of each group by ice-cold tissue extract (Life Technologies). The supernatants were centrifuged and subjected to quantitative sandwich immunoassay with ELISA detection kits for mouse IL-1 β (Ebioscience, San Diego, CA, USA), MPO (USCN Life Science, Inc., Wuhan, China), and TNF- α (R&D) according to the manufacturer's instructions. Absorbance was read at 450 nm with a reference wave length of 570 nm by a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis

Data in this study were representative of at least three different experiments and presented as the means \pm SD. Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA) 1-way analysis of variance. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Local MSC Transplantation Promotes Diabetic Corneal Epithelial Healing

Diabetic keratopathy always exhibits impaired corneal epithelial wound healing.^{26,30,31} To assess the effect of MSCs on corneal epithelial wound healing, corneal epithelium was removed in diabetic mice and age-matched normal mice with or without MSC transplantation. To investigate MSC homing in vivo, MSCs were labeled with the fluorescent dye CM-Dil before transplantation. Cryosections were made 48 hours later. As shown in Supplementary Figure S2, MSCs were able to migrate to the wound edge of cornea. The corneal epithelial healing rate exhibited a significant difference from 48 hours after corneal epithelial debridement. The defect area of corneal epithelium in MSC-treated diabetic mice (48 hours: $23.8 \pm 7.8\%$; 72 hours: $2.6 \pm 2.3\%$) was significantly improved from that in untreated diabetic mice (48 hours: $49.5 \pm 11.5\%$; 72 hours: $22.7 \pm 5.9\%$) and reached a level equal to that in normal mice (48 hours: $19.6 \pm 5.7\%$; 72 hours: $3.7 \pm 3.1\%$) (Figs. 1A, 1B; $n = 6$ per group). Furthermore, at 72 hours post wound, a stronger staining intensity of proliferation marker Ki67 was found in the migrating area of corneal epithelium as compared with that in untreated diabetic corneal epithelium (Figs. 1C, 1D).

MSC Transplantation Alleviated Excessive Inflammatory Responses in Diabetic Cornea After Epithelium Debridement

We next evaluated the effects of MSCs on inflammatory response in diabetic cornea after epithelium debridement. The histologic analysis of the corneal sections showed more extensive infiltration of inflammatory cells in diabetic corneas than in control 72 hours post wound. In contrast, inflammatory infiltrates were markedly decreased in the diabetic corneas treated with MSCs (Fig. 2A). In addition to the histology, anti-inflammatory effects were further evaluated with regard to inflammatory cytokine production. ELISA analysis showed that the protein levels of MPO, a biomarker of activated neutrophils, and inflammatory cytokines TNF- α and IL-1 β , at day 3, were significantly lower in the corneas of MSC-treated eyes compared to those treated with PBS (Figs. 2B-D).

MSC Transplantation Upregulates TSG-6 Expression in Diabetic Cornea

To investigate the possible molecules responsible for immunosuppressive effects of MSCs on diabetic corneal wound healing, we analyzed gene expression changes in diabetic cornea treated with MSCs. Because MSCs have been shown to exert immunomodulatory effects through TSG-6, IL-1ra, COX-2, and IDO,^{19,32-34} we analyzed the mRNA expression changes of these molecules in diabetic corneas 48 hours after MSC transplantation. Four corneas were pooled for each sample. The results showed that no significant changes were observed for the expression of IL-1ra, COX-2 and IDO, whereas the expression of TSG-6 was significantly upregulated (Fig. 3A). Consistently, the results of Western blots also confirmed that MSC transplantation induced a 6.5 ± 1.9 -fold increase of TSG-6 compared with PBS treatment (Fig. 3B), while the expression of IL-1ra, COX-2, or IDO showed no significant change (Figs. 3C-E).

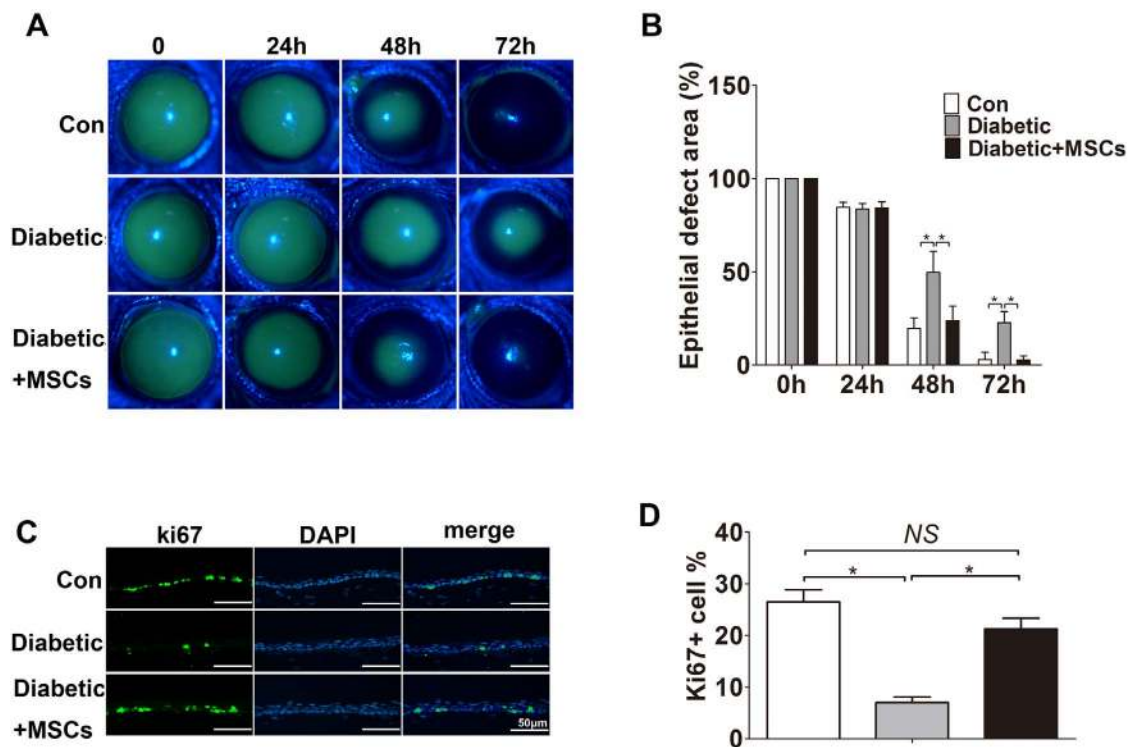


FIGURE 1. Local MSC transplantation promotes diabetic corneal epithelial healing. (A) Diabetic mice and normal control were injected subconjunctivally with 1×10^5 BM-MSCs just after the scrape of corneal epithelium, with PBS vehicle injection for control mice. The corneal epithelial wound was stained with fluorescein sodium at 24, 48, and 72 hours after the scrape of corneal epithelium ($n = 6$ per group). (B) Histogram of residual epithelial defect is presented as the percentage of the original wound size. (C) Frozen sections of cornea 7 μ m thick were made 72 hours after the scrape of corneal epithelium. The regenerating corneal epithelium was stained with Ki67 to show the activation of corneal epithelial stem/progenitor cells. (D) Staining was quantified by calculating the percentage of Ki67-positive cells in each image. Results are expressed as mean \pm SD; * $P < 0.05$.

MSCs and TSG-6 Promote Alternative Macrophage Polarization in Diabetic Cornea

Macrophages infiltrating to the injury site are known as a major source of inflammatory cytokines and growth factors, and therefore are key regulators of repair.³⁵ Aberrant macrophage polarization and impaired macrophage functions imposed by diabetic complications contribute to delayed wound healing in diabetes.^{36,37} To investigate whether MSCs or TSG-6 treatment might affect the infiltration and function of macrophages in diabetic corneas during wound repair, we analyzed the CD45-, F4/80-, CD86-, and CD206-expressing cells at 72 hours after surgery. Three corneas were pooled for each sample. Flow cytometric analysis revealed that the number of CD45⁺ cells, indicating infiltrated blood-borne immune cells, was markedly increased in diabetic corneas, and significantly reduced by subconjunctival injection of MSCs or TSG-6 (Supplementary Fig. S3). Further analysis after gating on CD45 showed that the number of CD45⁺F4/80⁺ macrophage cells was significantly increased in diabetic corneas compared to control, and was not altered by MSC or TSG-6 treatment (Figs. 4A, 4B). However, the ratio of classical activated macrophage (M1 phenotype), which was characterized by CD45⁺F4/80⁺CD86⁺, was dramatically decreased by MSC or TSG-6 administration (Figs. 4C, 4D), while the ratio of alternatively activated macrophage (M2 phenotype), which was characterized by CD45⁺F4/80⁺CD206⁺, was significantly increased in diabetic corneas when treated with MSCs or TSG-6 (Figs. 4E, 4F). Consistently, real-time PCR analysis revealed lower expression of genes encoding TNF- α , CD86, and MCP-1 (M1 macrophage) and higher expression of

genes encoding CD206, IL-10, and Arg-1 (M2 macrophage) in diabetic corneas treated with MSCs or TSG-6 (Fig. 4G). Furthermore, in vitro analysis confirmed that conditioned medium from MSCs or TSG-6 converted inflammatory monocytes to M2 macrophages (Supplementary Figs. S4A–D). Also, MSCs or TSG-6 could rescue diabetic defects in macrophage phagocytosis (Supplementary Fig. S4E). These results indicated that MSC or TSG-6 treatment promotes M2 polarization and limits the expression of genes encoding proinflammatory molecules in diabetic wounded cornea.

TSG-6 Accelerates Wound Healing in Diabetic Cornea and Stimulates the Activation of Corneal Epithelial Stem/Progenitor Cells

To examine whether exogenous TSG-6 improves wound response in the diabetic cornea, we administered 2 μ g rmTSG-6 in 5 μ L PBS to each cornea subconjunctivally and observed significantly accelerated wound closure in the diabetic corneas (Figs. 5A, 5B; $n = 6$ per group). To evaluate epithelial cell proliferation, Ki67 immunostaining was performed. In the TSG-6-treated cornea, the ratio of Ki67-positive cells was elevated compared to that in diabetic untreated corneas (Figs. 5C, 5D). Notably, CD44, the primary receptor that binds with TSG-6, was predominantly expressed in the basal layer of the human corneoscleral zone (Supplementary Fig. S5), where limbal stem cells reside, indicating that TSG-6 might selectively activate corneal epithelial stem/progenitor cells. To determine the influence of TSG-6 on the proliferative capacity of corneal epithelial stem/progenitor cells, human primary limbal cells were isolated and cultured as previously

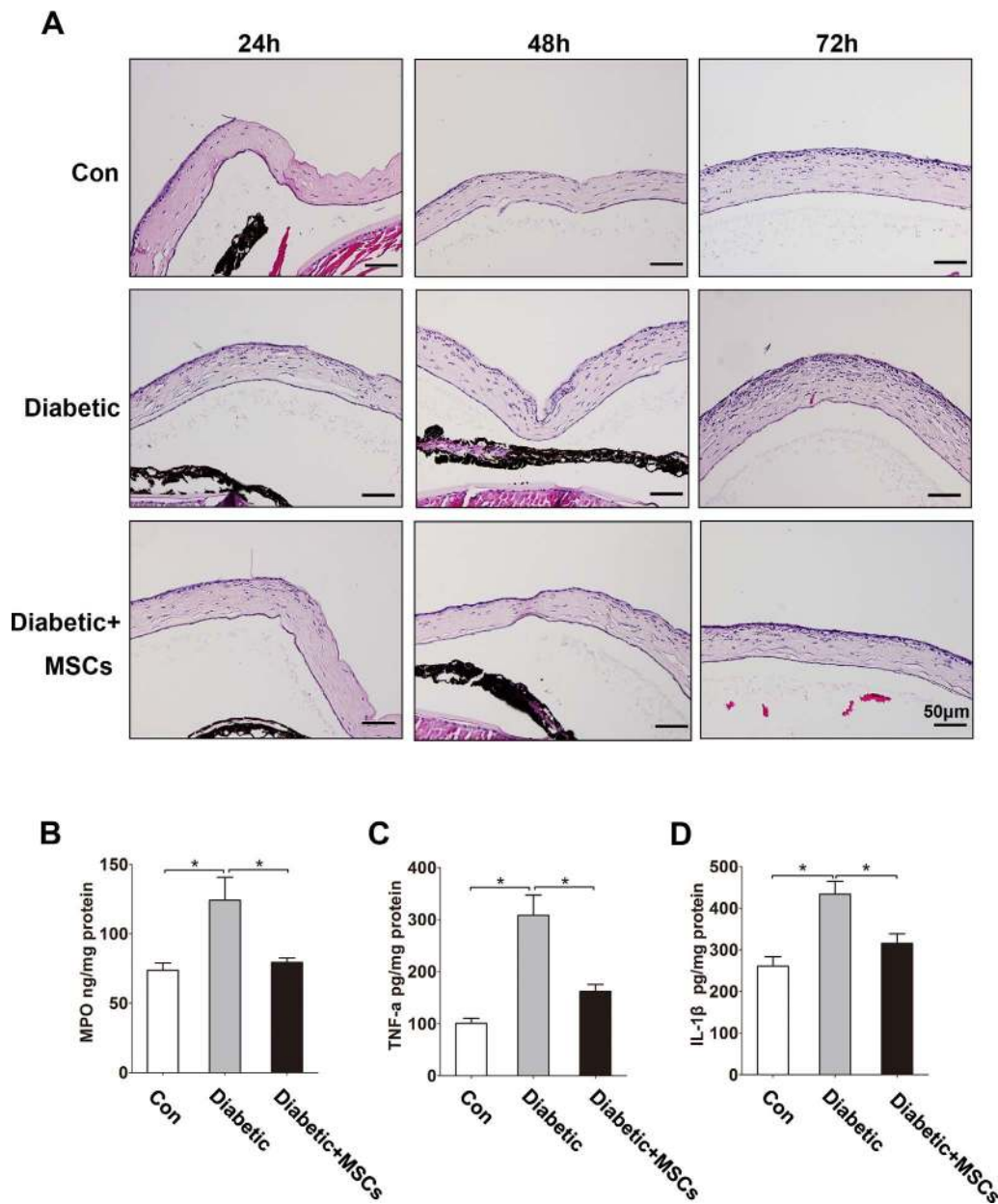


FIGURE 2. MSC transplantation alleviated excessive inflammatory responses in diabetic cornea after epithelium debridement. (A) Representative histologic appearance of healing corneas at the indicated time in control, diabetic, and MSC-treated mice. Seventy-two hours after injury, corneas were harvested, homogenized, and assayed for levels of MPO activity (B), TNF- α (C), and IL-1 β (D) by ELISA. Four corneas were pooled for each sample. Results are expressed as mean \pm SD; * $P < 0.05$.

described with 0 to 200 ng/mL rhTSG-6 for 9 to 12 days. The results showed that TSG-6 promoted the CFE when compared with vehicle control (Fig. 5E). The CFE increased by approximately 2.2-fold when treated with 100 or 200 ng/mL TSG-6 (Fig. 5F). Moreover, representative immunostaining of Δ Np63 and Ki67 showed a significantly increased fluorescence density in TSG-6-treated cells compared to that of control cells, while the expression of CK12 was reduced in the TSG-6-treated group (Figs. 5G–J).

Knockdown of TSG-6 Impairs the Enhancement of MSCs on Diabetic Corneal Epithelial Healing

To investigate whether or not TSG-6 is involved in the effects of MSCs on diabetic corneal epithelial healing, MSCs were

transfected with shRNA for TSG-6. The expression of TSG-6 was reduced by shRNA for TSG-6 (Fig. 6A). The MSCs transfected with shTSG-6 had little effect on the re-epithelialization of corneal abrasions in diabetic mice, whereas MSCs with the scrambled shRNA significantly accelerated the healing rate (Fig. 6B; $n = 6$ per group). In addition, immunofluorescence staining of cornea showed that knockdown of TSG-6 markedly attenuated the MSC-stimulated M2 macrophage polarization and increase of Ki67-positive cells within the wounded diabetic cornea (Figs. 6C, 6D). These results indicated that the secretion of TSG-6 from MSCs is largely responsible for macrophage polarization and epithelial cell proliferation associated with MSC-stimulated diabetic corneal epithelial healing.

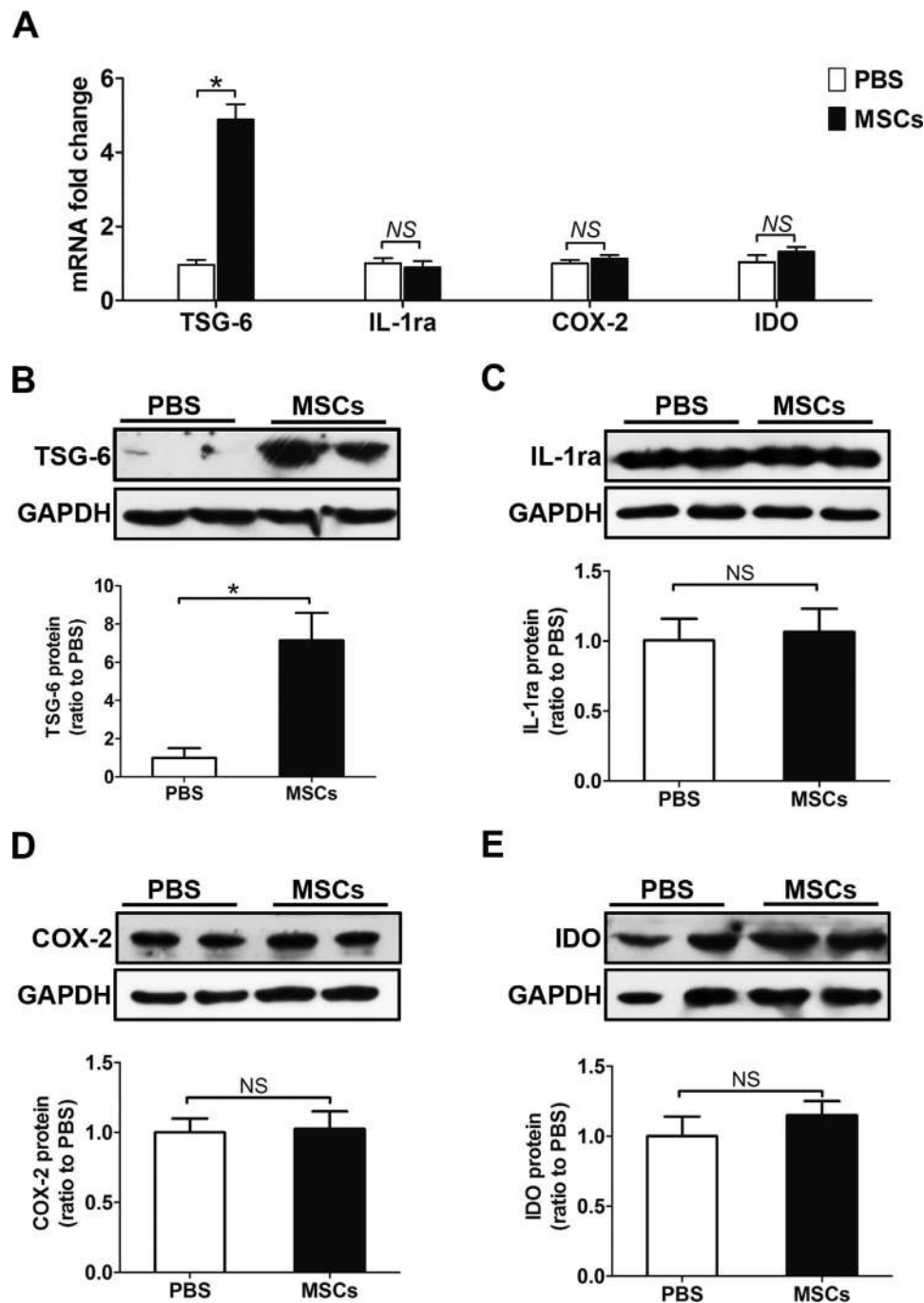


FIGURE 3. MSC transplantation upregulates TSG-6 expression in diabetic cornea. (A) Expression levels of mRNA of TSG-6, IL-1ra, COX-2, and IDO in corneas were determined by real-time RT-PCR at 48 hours after injury. (B–E) Corneas were collected 48 hours after injury, and the expression of TSG-6, IL-1ra, COX-2, and IDO was analyzed by Western blot. Four corneas were pooled for each sample. Results are expressed as mean \pm SD; * P < 0.05.

DISCUSSION

With the soaring prevalence of DM in the world, there is an unmet need for effective treatments of refractory ulceration in the diabetic cornea. In the present study, our data showed that local administration of BM-MSCs promoted diabetic corneal wound healing, both in vivo and in vitro, through modulating the immune response, inducing alternative activation of infiltrating macrophages and stimulating the mitogenic activation of corneal stem/progenitor cells. Notably, we identified TSG-6 as both necessary and sufficient to explain the paracrine

effect of BM-MSCs. Exogenous TSG-6 treatment could replicate the therapeutic potential of BM-MSCs, while MSCs transfected with a shRNA targeting TSG-6 failed to reverse the delayed wound repair in diabetic cornea.

TSG-6 is expressed by a variety of cells including MSCs in response to inflammatory stimuli. It has multiple functions that are linked to immunosuppressive modulation and extracellular matrix remodeling.³⁸ Previous studies have shown that MSCs promote mouse models for myocardial infarction, corneal injury, dry eye disease, and peritonitis via secreting TSG-6.^{19,22,39,40} Of note, TSG-6 can be used as a biomarker to

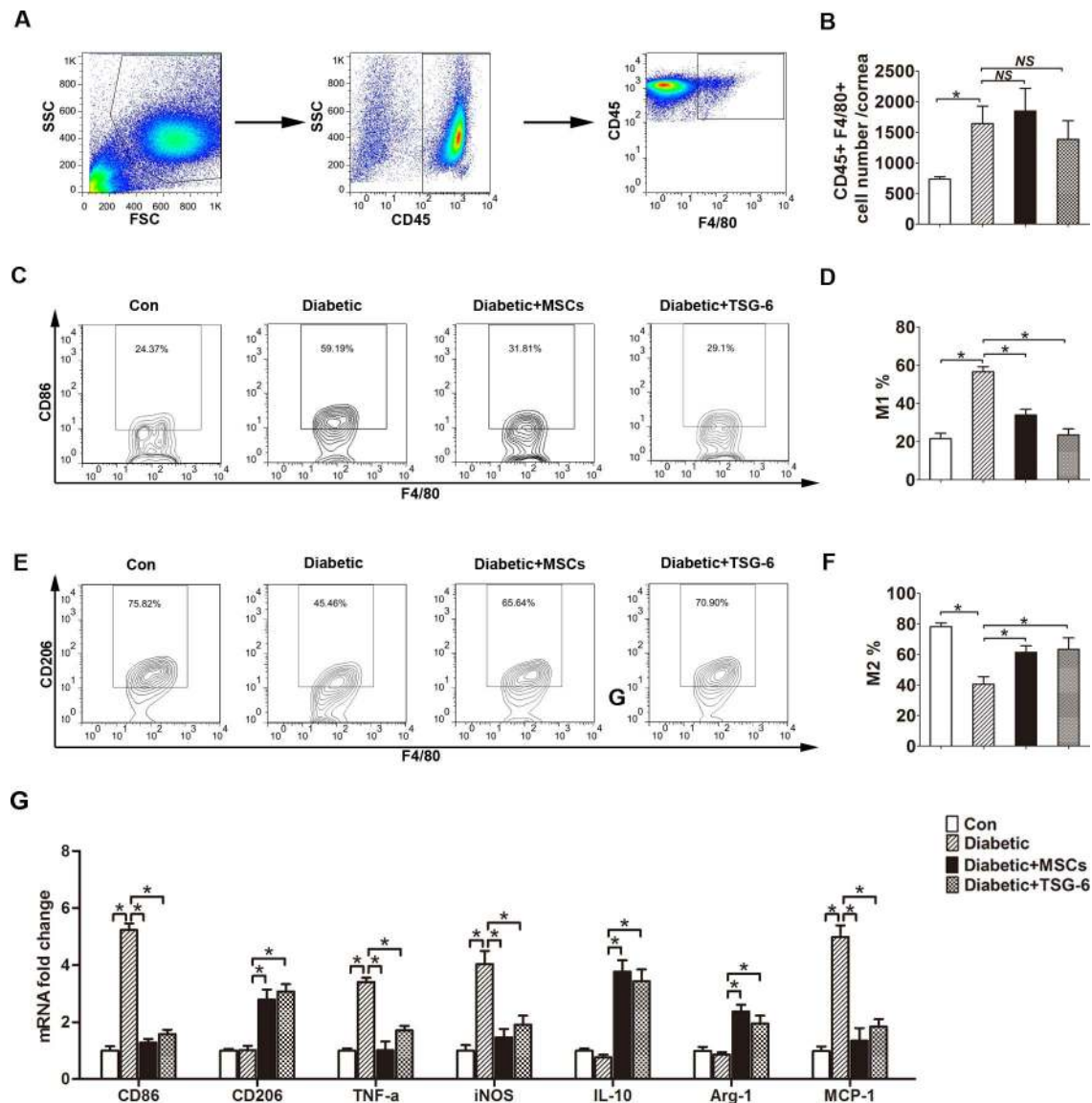


FIGURE 4. MSCs and TSG-6 promote alternative macrophage polarization in diabetic cornea. (A) Representative flow cytometric dot plots showing frequencies of CD45⁺F4/80⁺ macrophage infiltrated into the cornea 72 hours after injury (three corneas were pooled for each sample). (B) Cell number of CD45⁺F4/80⁺ macrophage infiltrated into cornea. (C) Representative flow cytometric contour plots showing frequencies of CD45⁺F4/80⁺CD86⁺ (M1) macrophages infiltrated into the cornea 72 hours after injury. (D) Ratio of CD45⁺F4/80⁺CD86⁺ macrophages infiltrated into cornea. (E) Representative flow cytometric contour plots showing frequencies of CD45⁺F4/80⁺CD206⁺ (M2) macrophages infiltrated into the cornea 72 hours after injury. (F) Ratio of CD45⁺F4/80⁺CD206⁺ macrophages infiltrated into cornea. (G) Real-time RT-PCR analysis of gene expression in corneas 72 hours after injury; results are presented relative to those of normal mice. Results are expressed as mean \pm SD; * P < 0.05.

predict the efficacy of MSCs in such sterile inflammatory models.²⁰ Consistently, in the present study, we found an increased level of TSG-6 in diabetic cornea treated with MSCs and correlated in vivo with amelioration of excessive inflammation. However, the expression of IL-1 α , COX-2, and IDO, all of which were reported to mediate the immunosuppressive effects of MSCs, remained unchanged when compared to the vehicle group. In addition, BM-MSCs were observed to lose their effectiveness in diabetic corneal wound healing after shRNA were used to knock down expression of TSG-6. Also, administration of recombinant TSG-6 reproduced most of the beneficial effects of the BM-MSCs, suggesting that TSG-6 is the key regulator in MSC-mediated promotion on diabetic corneal wound healing.

Recent observations from clinical and animal models of diabetes indicated that chronic inflammation underlies, in large

part, the failure of diabetic wounds to heal.^{41,42} Consistent with studies on other animal models, our data demonstrated an excessive inflammatory response predominantly by the accumulation of polymorphonuclear (PMN) cells in diabetic cornea. As a result of excessive inflammatory response, we found that wound healing was delayed in diabetic cornea and was accompanied by elevated levels of proinflammatory cytokines. Corneal wound healing is a complex process of well-defined overlapping events, wherein wound-site macrophages represent a key player in the removal of cellular debris and infiltrated leukocytes.⁴³ However, during impaired healing in diabetic mice, corneal wounds exhibit prolonged accumulation of macrophages that exhibit a sustained proinflammatory phenotype with an impaired upregulation of healing-associated factors. Growing evidence has suggested that MSCs show a unique immunomodulatory function by educating normal

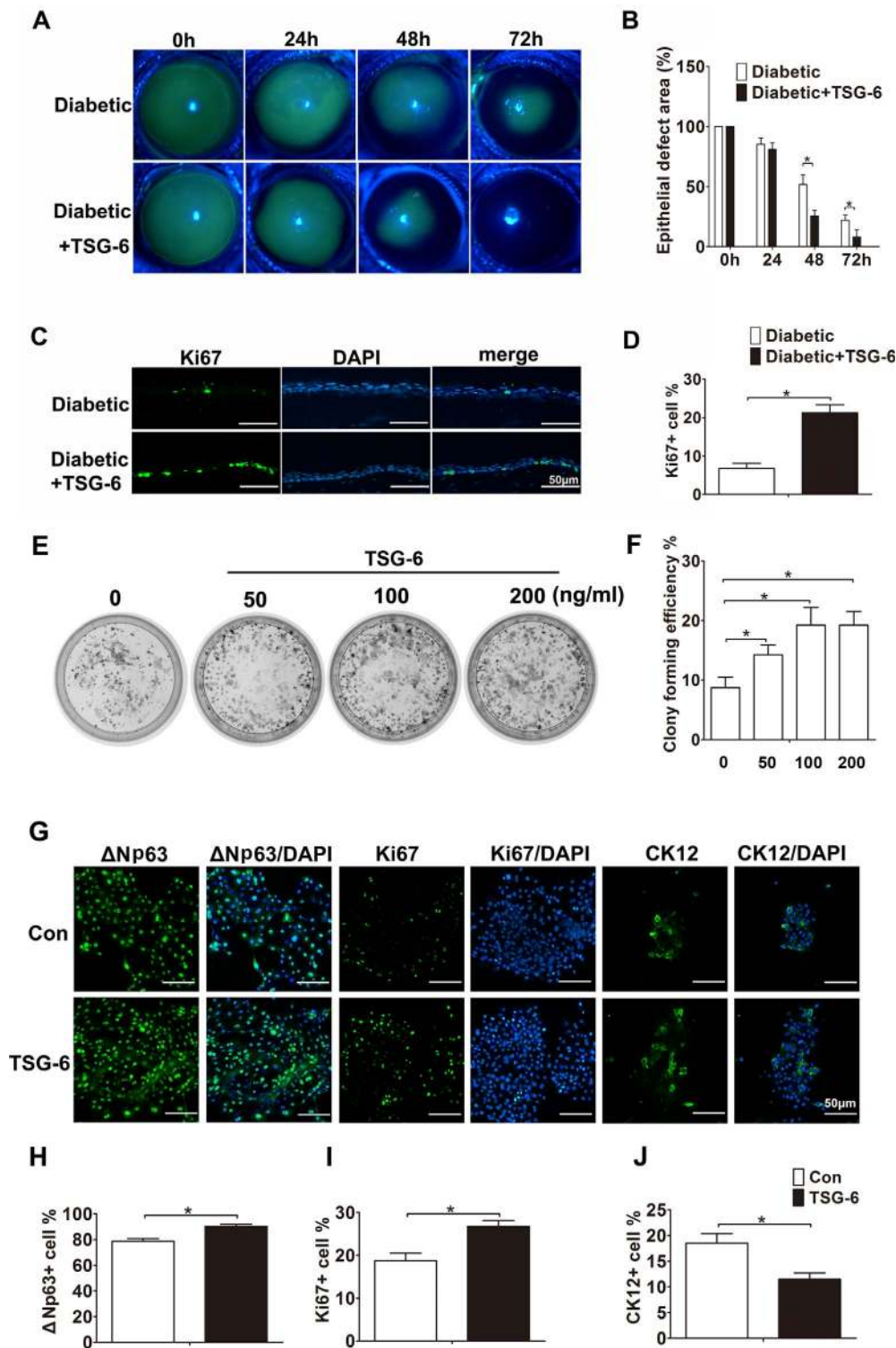


FIGURE 5. TSG-6 accelerates wound healing in diabetic cornea and stimulates the activation of corneal epithelial stem/progenitor cells. (A) The corneal epithelium of diabetic mice was removed with or without subconjunctival injection of 50 ng rmTSG-6. The corneal epithelial wound defect was stained with fluorescein sodium at 24, 48, and 72 hours after the scrape of corneal epithelium ($n = 6$ per group). (B) Histogram of residual epithelial defect is presented as the percentage of the original wound size. (C) The regenerating corneal epithelium was stained with Ki67. (D) Staining was quantified by calculating the percentage of Ki67-positive cells in each image. (E) Human primary limbal cells were incubated with 50, 100, and 200 ng/mL rhTSG-6 for 9 to 12 days. The corneal epithelial stem/progenitor cell colonies were stained with crystal violet. (F) The colony-forming efficiency was calculated as the percent of the colony number/plated cell number. (G) Cultured human corneal epithelial stem/progenitor cells were stained with Δ Np63, Ki67, and CK12. (H–J) Staining was quantified by calculating the percentage of positive cells in each image. Results are expressed as mean \pm SD; * $P < 0.05$.

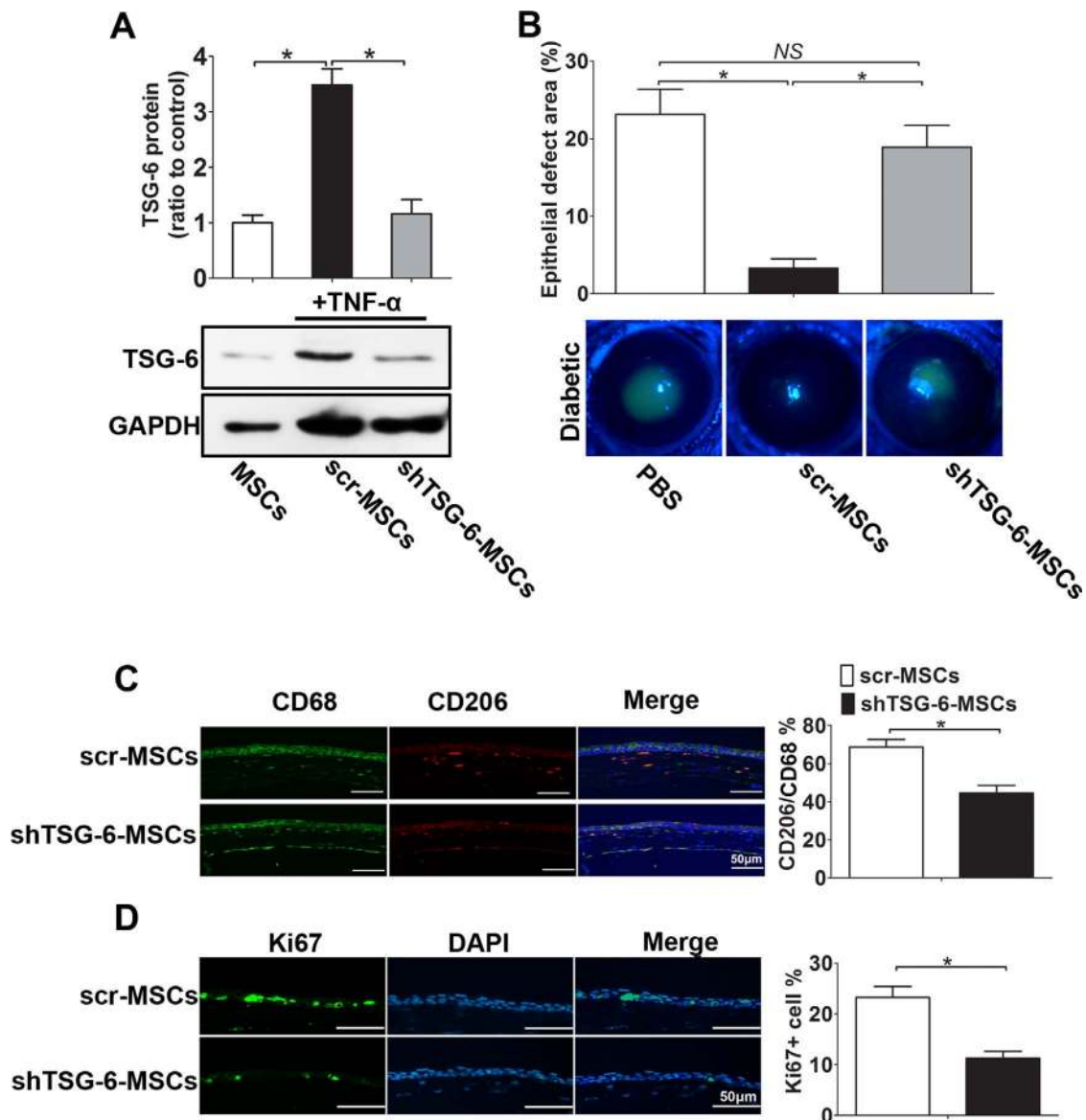


FIGURE 6. Knockdown of TSG-6 impairs the enhancement of MSCs on diabetic corneal epithelial healing. **(A)** BM-MSCs were transfected with shRNA targeted for TSG-6 (as the shTSG-6-MSCs); a scrambled shRNA was used as control (as the scr-MSCs); the gene silencing effect was tested by Western blot. **(B)** 1×10^5 shTSG-6-MSCs or scr-MSCs were injected subconjunctivally after the scrape of corneal epithelium in diabetic mice, with the vehicle (PBS) as control. The corneal epithelial wound defect was stained with fluorescein sodium at 72 hours after scrape of corneal epithelium. Histogram of residual epithelial defect is shown as the percentage of the original wound size ($n = 6$ per group). **(C)** Representative images of frozen corneal sections were stained for CD68 (green) and CD206 (red). Nuclei were counterstained with DAPI. **(D)** The regenerating corneal epithelium was stained with Ki-67. Results are expressed as mean \pm SD; * $P < 0.05$.

macrophages into M2 phenotype.^{44–46} However, whether and how MSCs regulate diabetic macrophages remains largely unknown, especially in the setting of chronic injury. We revealed here that, rather than altering the number of infiltrated macrophages, local transplantation of MSCs or TSG-6 significantly elevated the ratio of CD45⁺F4/80⁺CD206⁺M2 phenotype. Moreover, when treated with MSCs or TSG-6, the M1 markers in diabetic cornea, such as TNF- α , IL-1b, monocyte chemoattractant protein-1 (MCP-1), and inducible nitric oxide synthase (iNOS) were significantly reduced, while the M2 markers such as IL-10, CD206, and Arg1 were markedly increased. Importantly, macrophages from diabetic mice also showed reduced phagocytic activity, which was corrected by coculturing with BM-MSCs or TSG-6. Conversely, the effects of MSCs on macrophages were largely abrogated by

shRNA knockdown of TSG-6. Taken together, MSCs, when administered to diabetic wounded cornea, are able to secrete TSG-6, which then stimulates the reprogramming of infiltrated macrophages to M2 phenotype and their phagocytic activity.

The homeostasis of corneal epithelium is maintained by the stem/progenitor cells residing in the basal layer of the corneal epithelium.⁴⁷ Recent studies have revealed that corneal stem/progenitor cells are also altered in diabetes.⁴⁸ The expression of stem cell markers was significantly reduced in corneas of diabetic patients and can be restored c-Met-mediated gene therapy.^{14,49} Also, our previous studies demonstrated that the activation of corneal stem/progenitor cells is suppressed in STZ-induced type 1 diabetic mice, which can be rescued by neurotrophic factor CNTF or neuropeptide substance P.^{24,25} In the present study, our data showed a di-

rect effect of MSCs on stimulating the mitogenic activity of endogenous corneal stem/progenitor cells in diabetic cornea, via secreting TSG-6. In fact, MSCs have been recently identified in the superficial limbal stroma of the human cornea and as supporting the homeostasis and expansion of limbal stem cells.⁵⁰⁻⁵³ Moreover, our *in vitro* data showed that TSG-6 promotes the CFE of human limbal stem cells. Also, the immunostaining of CD44, the main receptor that binds with TSG-6, was predominantly expressed at the basal layer of human limbus, which further supports our hypothesis. However, future work will need to address the underlying mechanisms.

In summary, our data provide a novel therapy for delayed wound healing in the diabetic cornea by local administration of mesenchymal stem cells. In addition, the anti-inflammatory and proregenerative activities of MSCs are mediated through the release of TSG-6, which can be pursued as a novel and efficient therapeutic approach to such disease as an alternative to MSC-based therapy.

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