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Preclinical assessment of IL-1β primed human umbilical cord mesenchymal stem cells for tendon functional repair through TGF-β/IL-10 signaling

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A B S T R A C T

Background: Inadequate repair capacity and disturbed immune compartments are the main pathological causes of tendinopathy. Transplantation of mesenchymal stem cells (MSCs) become an effective clinic option to alleviate tendinopathy. Interleukin-1β (IL-1β) could confer on MSCs enhanced immunoregulatory capability to remodel the repair microenvironment favoring tissue repair. Therefore, IL-1β activated UC-MSCs (1βUC-MSCs) may exert favorable efficacy in promoting tendon repair in a preclinical tendinopathy rat model.

Methods: Tendon-derived stem cells (TDSCs) were isolated and characterized. In vitro, the levels of immunoregulatory-related cytokines such as IL-1β, IL-6, IL-10, and TGF-β secreted by 1βUC-MSCs and unprimed UC-MSCs was measured. And tendon-specific markers expressed by TDSCs cultured with primed cultured medium (CM) or unprimed CM were detected. In vivo, Achilles tendinopathy was induced by 30 μL collagenase I injection in Sprague Dawley rats. One week later, the rats were randomly injected with UC-MSCs primed with IL-1β (10^6 cells per tendon), UC-MSCs, or PBS. After rats were sacrificed, histological evaluation, electron microscopy, biomechanical tests, gait performance were conducted to evaluate the structural and functional recovery of Achilles tendons. The inflammation and metabolic state of the extracellular matrix, and the potential mechanism were assessed by immunohistochemical staining and Western blot.

Results: UC-MSCs were activated by IL-1β to secrete higher levels of IL-10 and TGF-β while the secretion levels of IL-6 and IL-1β were not changed significantly, promoting a higher expression level of COL I and TNMD in TDSCs under proinflammatory environment. In vivo, the transplanted 1βUC-MSCs could survive up to 5 weeks after injection with tenogenic differentiation and improved tendon healing histologically semi-quantified by modified Bonar scores. This structural regeneration was further confirmed by observation of ultrastructural morphology, and led to good functional recovery including improved biomechanical properties and gait performance. During this process, the inflammatory response and metabolism of the extracellular matrix was improved through TGF-β/IL-10 pathway.

Conclusion: This study demonstrated that the transplantation of UC-MSCs activated by IL-1β exhibited satisfactory ability for promoting tendon functional repair in a tendinopathy rat model. During this process, the balance of inflammatory response and extracellular matrix metabolism was remodeled, and the TGF-β/Smad2/3 and IL-10 signaling pathways were activated simultaneously. We cautiously conclude that the IL-1β primed UC-MSCs could be a promising strategy for enhancing the ability of MSCs to treat tendinopathy.

Key Terms: umbilical cord-derived mesenchymal stem cells, tendinopathy; interleukin-1β, transforming growth factor-β.

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1. Introduction

Tendinopathy is a highly prevalent and refractory pathological condition characterized by chronic pain, reduced exercise tolerance, and poor movement function [1–3]. The incidence of lower-extremity tendinopathy is reported to be 1–2% per year, even surpassing the incidence of osteoarthritis [4]. Inflammation plays a crucial role among multiple theories about the relationship between pathophysiology and clinical manifestation of tendinopathy [5–7]. Overexpression of inflammatory cytokines IL-1β and IL-6 could exacerbate inflammatory response, inhibit tenogenic differentiation of histiocytes, interfere with the synthesis and decomposition of collagen fibers, destroying tendon structure and loss of biomechanical properties [5,6]. Current anti-inflammatory treatments, such as pharmaceutical and injection therapy can relieve their symptoms while having limited effects on the progression of pathological conditions, and may not eventually improve the quality of tendon healing [8]. Therefore, it is needed to develop an effective treatment to alleviate inflammation and promote tendon regeneration.

MSCs can promote tendon repair by secreting different kinds of cytokines and differentiating into tendon cells [9–12]. Lee et al. reported MSCs that were transplanted into the rat tendon could survive for at least four weeks, differentiate into the tenogenic lineage, and secrete type I collagen to enhance tendon repair [12]. Bone marrow-derived MSCs (BM-MSCs) and adipose tissue-derived MSCs (AT-MSCs), which were widely studied at present, have several disadvantages, including the invasive procedure of donors [13], low efficiencies of cell extraction [14]. and the risk of virus transmission [15]. Human umbilical cord-derived mesenchymal stem cells (UC-MSCs) have a more broad therapeutic potential because they are noninvasive, easier to obtain, and their superior characteristics of self-renewal, multi-lineage potential, hypo-immunogenicity, and immunomodulation [14]. However, few relevant studies reported on the efficacy of UC-MSCs in treating tendinopathy.

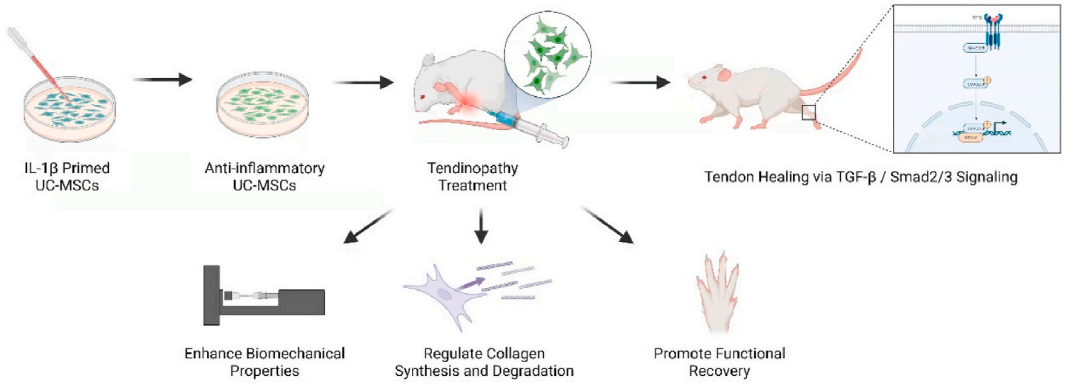
Recent researchers reported pro-inflammatory microenvironment could activate UC-MSCs in vitro, thus enhancing their anti-inflammatory and immunoregulatory properties. Castro et al. reported MSCs primed with IL-1β secreted more anti-inflammatory IL-10, leading to decreased expression of apoptotic markers in LPS-activated microglial cells [16]. Subsequent studies found that MSCs with enhanced immunomodulatory ability could improve the therapeutic outcomes of various inflammation-related diseases such as stroke, skin wounds, sepsis, and colitis [17–20]. Besides, UC-MSCs have a strong pro-trophic ability, which could secrete TGF-β (referring to TGF-β1 isoforms) much higher than that of BM-MSCs and AT-MSCs [21]. TGF-β is an essential inflammatory regulator and a well-documented tenogenic signal, which plays a crucial role in the development, differentiation, and regeneration of tendons [22, 23]. However, excessive levels of TGF-β may instead increase the risk of scar formation and tenocyte apoptosis, thereby impairing the effect of UC-MSCs in ameliorating tendinopathy [22,24].

Therefore, the present study aimed to investigate whether IL-1β activated UC-MSCs have an enhanced immunomodulatory ability to alleviate inflammation and accelerate tendon repair (Scheme 1). Our study may provide a novel strategy for enhancing the ability of MSCs to treat tendinopathy.

2. Materials and methods

2.1. Ethics statement

Institutional Animal Care and Use Committee (IACUC) of the Shanghai Jiaotong University affiliated Shanghai Sixth People's Hospital (DWLL2021-0625) approved all the procedures regarding animal maintenance and experiments. All efforts were made to minimize the suffering of experimental animals. All the male Sprague-Dawley rats weighing about 250g were housed under specific pathogen-free (SPF) conditions.



Scheme 1. Illustration of the IL-1β primed UC-MSCs with enhanced immunoregulatory ability for promoting tendon functional repair in a rat tendinopathy model.

2.2. Cell isolation, culture and identification

The UC-MSCs were acquired from the Cell Bank of the Chinese Academy of Sciences and passaged for expansion. UC-MSCs were cultured in α-MEM medium supplemented with 10 % FBS and 1 % penicillin/streptomycin (Gibco, Carlsbad, CA) at a set temperature of 37 °C and an atmosphere containing 5 % CO_2. Passages 4–6 were used in this study and seeded in a 12-well plate at 5000 cells/cm^2. And 24 h before treatment, the α-MEM medium containing 1 % FBS was replaced to synchronize the cells.

Primary tendon-derived stem cells (TDSCs) were isolated from Sprague-Dawley rats according to the previously established procedure [25]. Briefly, the Achilles tendons of Sprague Dawley rats were dissected after euthanasia. After carefully removing the tendinous sheath, the tendons were minced in PBS and digested in 3 mg/mL of collagenase I and 2 mg/ml of dispase (Sigma-Aldrich, St. Louis, MO) for 2 h at a constant temperature shaker. After that, the released cells were washed with PBS three times and centrifuged at 250 g for 5 min, then resuspended in α-MEM complete medium. When the degree of cell confluence reached 80 %, TDSCs were detached with 0.5 % trypsin-EDTA (Sigma-Aldrich). After counted, the cells were cultured as passage 0 cells. Cells from passages 3–4 were used in the subsequent experiments.

The surface markers were detected by flow cytometry to identify the stemness of TDSCs. The cells were trypsinized and resuspended in PBS at the desired concentration of 1×10^6 cells/mL, followed by incubating with antibodies against the following fluorescent-conjugated proteins: CD31-fluorescein isothiocyanate (FITC), CD34-FITC, CD44-FITC, CD45-FITC, CD90-FITC, and SSEA-4-FITC (dilution: 1:100; all from Biolegend, San Diego, CA). Flow cytometry was performed using a flow cytometer (Accuri C6 Plus, BD Biosciences, San Jose, CA), and the data were analyzed using Flowjo software (10.1 version).

The multi-lineage potential of TDSCs was assessed by tenogenic, adipogenic, chondrogenic, and osteogenic induction [25]. Briefly, TDSCs were seeded in 12-well plates at a density of 5000 cells/cm^2. After culturing for 24 h, the medium was replaced to α-MEM complete medium supplemented with 50 μg/ml ascorbic acid for induction of tenogenesis. And the specialized induction medium (Cyagen, Suzhou) was used for induction of osteogenic and adipogenic differentiation. For chondrogenic induction, the cell suspension with 10^5 cells was centrifuged, the supernatant was discarded, and the chondrogenic induction medium (Cyagen, Suzhou) was added. The centrifuged TDSCs can spontaneously form spheroids after 48 h of induction. The specialized induction medium was refreshed every two days during a 2-week induction period, then the cells were stained by Sirius red, Oil Red O, Alkaline Phosphatase, and Safranin O separately.

2.3. Enzyme-linked immunosorbent assay

UC-MSCs were respectively treated with recombinant human IL-1β (Sigma-Aldrich) for 0.5 h, 2 h, and 6 h at a concentration of 5 ng/ml to find a more appropriate stimulating concentration as previously reported [17,18,26]. Then, the medium was discarded. After the cells were washed with PBS, the fresh complete medium was added. After 48 h of incubation, the supernatant was collected. The concentrations of IL-1β, IL-6, IL-10, and TGF-β were measured by ELISA kit (R&D Systems). The manufacturer's instructions were followed carefully.

2.4. Immunofluorescence staining

After seeded in a 12-well plate at a density of 5000 cells/cm^2 for 12 h, TDSCs were simultaneously treated with 1 ng/ml IL-1β and cultured medium derived from UC-MSCs primed with IL-1β (Primed CM), cultured medium from unprimed UC-MSCs (Unprimed CM) and fresh medium (Control). IL-1β was used to simulate pro-inflammatory environment as previously reported [27]. After incubating for 48 h, the TDSCs were washed with PBS and immersed in 4 % paraformaldehyde for fixation. After permeabilized and blocked with 1 % BSA for 1 h, the cells were incubated with anti-COL I (dilution: 1: 200; Abcam, Boston, MA) and anti-TNMD (dilution: 1:150; Abcam) first antibodies overnight at 4 °C. Then the cells were washed with PBS three times, incubated with Cy3-conjugated or Alexa Fluor 488-conjugated secondary antibodies (dilution: 1:200; Biolegend) for an hour at ambient temperature. DAPI was used to counterstain the nuclei. And the images were captured with a fluorescence microscope (Olympus, Tokyo). Semi-quantitative analysis was conducted by using ImageJ software. Briefly, the images were initially prepared with the "Color-Split" function in ImageJ. For the measurement of COL I, Keep the green channel. For TNMD, Keep the red channel. Then, make sure "mean gray value" and "limit to threshold" are checked. And the mean gray value was "measured" as the mean fluorescence intensity.

2.5. Establishment of rat tendinopathy model

After a week of acclimatization, a total of thirty-six SD rats were anesthetized using pentobarbital sodium (40 mg/kg). After skin preparation and sterilization, the Achilles tendon was incised and well exposed. Under direct vision, 300 units of collagenase I (Sigma-Aldrich) dissolved in 30 μL PBS were injected into Achilles tendon using a 30-gauge needle [6]. After skin closure, each rat was kept separately, and free cage activity was allowed. One week after injection, the rats were randomly assigned to 3 groups: (1) 1βUC-MSCs group, transplantation with UC-MSCs primed with IL-1β (10^6 cells in 50 μL PBS); (2) UC-MSCs group, transplantation with UC-MSCs and the same volume of PBS; (3) PBS group, only 50 μL PBS injection without any treatment. The quantity of cells injected in a single session is based on previous studies [6,12]. And twelve SD rats were assigned to (4) Normal group, free movement in the cage without any injection. At five weeks after treatment, rats were sacrificed to analyze histology, ultrastructural morphology, functional recovery, inflammation response, and ECM-related proteins. Achilles tendons for Western blot analysis were snap-frozen and stored in liquid nitrogen.

2.6 Labeling of UC-MSCs for fluorescent trackingThe UC-MSCs were labeled with CFDA-SE fluorescent probes (Beyotime, Shanghai) for cell tracking in vivo. The manufacturer's protocol was followed strictly. Briefly, the UC-MSCs were trypsinized and resuspended in 1 mL of the CFDA-SE solution and incubated for 15 min at 37 °C. After centrifugation and washing with PBS three times, a cell concentration of 2×10^6 cells/100 μL was achieved [28]. Then, the suspended CFDA-SE labeled UC-MSCs were injected into the Achilles tendon of rats. The Achilles tendons were harvested at 3 days, 2 weeks, 5 weeks, then embedded in Optimum Cutting Temperature (O.C.T.) compound (Tissue-Tek). Cryostat sections were inspected and recorded under fluorescent microscopy (DMi8, Leica).

2.6. Histological staining and evaluation

Sections of Achilles tendons were made as previously described [29]. Briefly, the Achilles tendon was fixed with 4 % paraformaldehyde, dehydrated by standard procedures, and embedded in paraffin. Then the sections were cut with a thickness of 5 μm and stained with hematoxylin-eosin (H&E), Sirius Red, and Alcian blue. The specimens were observed using standard light microscopy (Leica, Germany) and polarized microscopy (Nikon, Tokyo). Modified Bonar score was used for semiquantitative histological evaluation [30]. Briefly, the scoring system consists of five features, including cell morphology, collagen arrangement, cellularity, vascularity, and ground substance deposition, each of which is graded as 0, 1, 2, 3. Additional 2.5 points will be calculated for each of calcification or adipocytes in each field of view. Accordingly, the total score with the most pathology will be 20, and a normal tendon will score 0. All observations and scoring processes were conducted by two examiners independently. The mean score was calculated for the analysis.

2.6.1. Scanning electron microscopy

The fresh tendons were cut into 1 mm^3 samples, fixed with 2.0 % glutaraldehyde for one day and 2 % osmium tetroxide at 4 °C for 2 h and rinsed well with PBS. After dehydrating with graded ethanol for three times, the samples were performed with critical point drying and sputter-coated with gold for 0.5 min at 20 mA. The characteristics of the collagen fibers were observed and captured by SEM (SU8020, Hitachi). [7]

2.7. Transmission electron microscopy

Excised tendon samples were double-fixed with glutaraldehyde and osmium tetroxide respectively, stained with lead citrate and uranyl acetate and embedded in epoxy resin, and the transverse and longitudinal sections were prepared with an ultramicrotome. The ultrastructure of the collagen fibers was recorded by TEM (HT7700, Hitachi). The distribution of collagen fibrils' diameters from TEM images were measured by randomly marking the total of fifty fibrils individually in Image J software.

2.8. Biomechanical tests

The mechanical experiment was carried out on the universal testing systems (Instron5969, United States) to examine the functional healing behavior of the Achilles tendons. In brief, the cross-sectional area of tendon samples was calculated by Vernier caliper. The proximal and distal ends of Achilles tendons were securely clamped and constantly moistened with saline during the test. The tendons were preloaded to 0.5 N and stretched at a displacement rate of 10 mm/min until failure. The criterion for the experiment's success was that the Achilles tendon broke at the middle point but not at the fixing clamp position.

2.9. Achilles functional index

The gait performance of healing tendons was assessed by Achilles functional index (AFI), using a modified method as previously reported [31,32]. Briefly, a defined roadway was lined with blank paper. After their hind paws were drenched with ink, the rats could move freely along the roadway. The related gait parameter, including print length (PL), toe spreading length (TS), and intermediary toe spreading length (IT) was measured by Image J software. Then, according to the difference between the experimental (E) the normal (N) values, three related parameters, including print length factor (PLF), toe spreading length factor (TSF) and intermediary toe spreading length factor (ITF) were calculated by the equations below: $PLF = (NPL - EPL)/EPL, TSF = (ETS - NTS)/\ NTS, ITF = (EIT - NIT)/NIT$. Therefore, we could calculate the AFI according to the established equation: $AFI = 74(PLF) + 161(TSF) + 48(ITF) - 5$ [32].

2.10. Immunohistochemistry staining

Slides of injured tendon were dewaxed, hydrated and recovered in citrate buffer solution at 95 °C. After blocked with 0.3 % hydrogen peroxide and goat serum (dilution:1:100; Beyotime, Shanghai, China), the slides were incubated with primary antibodies anti-CD68 (dilution:1:200; Abcam) at 4 °C overnight. Then the sections were washed with PBS and incubated with secondary antibodies for 30min at ambient temperature. DAPI was applied to counterstain the nuclei. The images were captured with a fluorescence microscope (Olympus, Tokyo). Semi-quantitative analysis was conducted by Image J software. Briefly, the images were prepared with the "color threshold" and "find maxima" functions in Image J. Next, the image is subtracted by the "image calculator" function, which can use the boundaries between cells to split cells, and finally use "analyze particles" function for cell counting and measurement.

2.11. Western blotting analysis

The experiment was conducted as described previously [11]. Summarily, total proteins from tendon tissues were harvested by RIPA lysis buffer and proteinase/phosphatase inhibitors (Beyotime). BCA assay was performed to calculate the concentrations of the proteins. Next, equal amounts of proteins were separated by SDS-PAGE, electro-transferred onto PVDF membranes (Millipore, MA), and blocked with 5 % non-fat milk for 1 h. Then the membranes were incubated with primary antibodies including anti-COL I (dilution: 1:1000; Abcam), anti-COL III (dilution: 1:1000; Abcam), anti-TNMD (dilution: 1:1000; Abcam), anti-MMP1 (dilution: 1:1000; Abcam), anti-MMP13 (dilution: 1:1000; Abcam), anti-TGF-β (dilution: 1:500; Abcam), anti-Smad2/3 (dilution: 1:500; Abcam), anti-*p*-Smad2/3 (dilution: 1:500; Abcam) and anti-GAPDH (dilution: 1:2000; Abcam) overnight at 4 °C. GAPDH was used as internal reference. After incubating membranes with horseradish peroxidase (HRP)-conjugated secondary antibodies (dilution: 1:3000; Abcam) for 30 min at room temperature, washing with TBST three times, Omni-ECL™ Light Chemiluminescence Kit (Epizyme, Shanghai, China) was used to visualize the proteins. The images were recorded by the ChemiDoc imaging system (Bio-Rad, Hercules, CA). And semi-quantification was conducted using ImageJ software. Briefly, the images were prepared with the "subtract background" and "invert" in ImageJ. Next, each protein band of interest was chosen and integrated optical density (IntDen) could be measured. The relative gray level was defined as the normalized value of the IntDen of interest protein to the IntDen of the internal reference GAPDH.

2.12. Statistical analysis

GraphPad Prism 8 was used to perform statistical analysis. Data are shown as the means ± standard deviation (SD). And multiple comparisons were conducted using one-way analysis of variance (ANOVA) with Tukey's post hoc test, and comparison between two groups was accomplished using Student's t-test. Statistical significance was set at P value < 0.05. All experiments were independently conducted at least three times.

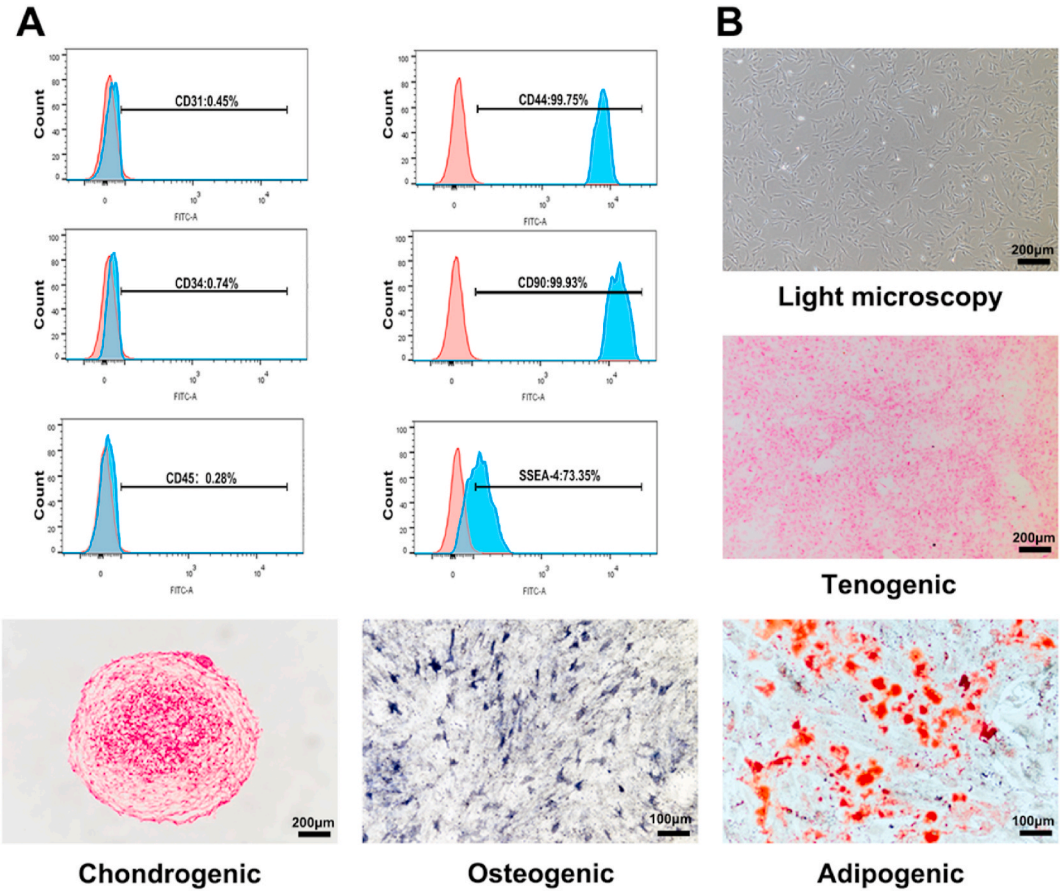


Fig. 1. Identification and characterization of TDSCs. A Flow cytometry analysis of the surface markers of TDSCs. The left part illustrated the expression of negative surface markers CD31, CD34, and CD45 and the right part showed the expression of positive stem markers CD44, CD90, and SSEA-4 of TDSCs. B Multi-directional differentiation capacity of TDSCs. TDSCs exhibited fibroblast-like morphology and could differentiate into tenocytes, adipocytes, chondrocytes and osteoblasts, which were stained by Sirius red, Oil Red O, Safranin O and Alkaline Phosphatase respectively.

3. Results

3.1. Identification of TSDCs

To determine the immuno-phenotypical profile of TDSCs, a series of stem cell surface markers was examined. Flow cytometry analysis demonstrated that the TDSCs positively expressed stem markers CD44, CD90 and SSEA-4, and negatively expressed CD31 (endothelial cell marker), CD34 (hematopoietic stem cell antigen), and CD45 (leukocyte common antigen) (Fig. 1A) [33]. Then, we examined the multi-differentiation potential of TDSCs. After tenogenic induction, the cells were stained with Sirius red, indicating collagen deposition. Upon adipogenic induction, lipid droplets within the cytoplasm were stained by oil red O. And the ability to differentiate into chondrocytes was demonstrated by Safranin O. Osteogenic differentiation showed most cells highly expressed alkaline phosphatase (ALP), an early marker of osteogenic differentiation (Fig. 1B).

3.2. UC-MSCs primed with IL-1β secreted higher levels of IL-10 and TGF-β

The concentration of inflammatory cytokines in the cultured medium derived from UC-MSCs primed with IL-1β (Primed CM) for 0.5h, 2h, and 6h, and cultured medium from unprimed UC-MSCs (Unprimed CM) was measured by ELISA kit. Although mean levels of inflammatory cytokines increased after IL-1β stimulation, there was no statistical difference between groups (Fig. 2A and B). However, the level of anti-inflammatory cytokine IL-10 increased significantly after half an hour of pretreatment (17.693 pg/ml, $P < 0.01$) (Fig. 2C). However, the protracted IL-1β priming didn't lead to a further increased ability of UC-MSCs to secrete IL-10 (Fig. 2C), but might instead resulted in reduced anti-inflammatory capability, which was consistent with previous reports [26]. Our results showed that IL-1β preconditioning could further increase the secretion of TGF-β, although it only has statistical significance at 0.5 h (2698.440 pg/ml, $P < 0.05$) (Fig. 2D). Therefore, primed CM of 0.5h priming exhibited better inflammation-modulating activity and was used for the following experiment.

3.3. Primed CM promoted higher expression of tendon markers of TDSCs in vitro

Based on previous studies, IL-1β could simulate pro-inflammatory environment and suppress proliferation and tenogenic differentiation of TDSCs [27,34]. Here, we used primed CM, and unprimed CM with different anti-inflammatory and pro-trophic capabilities to reduce the inhibitory effects of IL-1β on TDSCs. The fresh medium culture was used as a control. Cellular immunofluorescence showed that the unprimed CM pretreatment could enhance the expression levels of Type I collagen (COL I) and Tenomodulin (TNMD) in TDSCs (COL I: $P < 0.001$; TNMD: $P < 0.01$), compared with the control group (Fig. 3A–D). Furthermore, the CM derived from UC-MSCs primed with IL-1β (primed CM) further enhanced the expression of COL I and TNMD than the unprimed CM (COL I: $P < 0.01$; TNMD: $P < 0.05$), which may help accelerating the repair of injured tendons (Fig. 3).

3.4. Fluorescent tracking of injected UC-MSCs

The frozen sections demonstrated fluorescent signals of CFDA-SE-labeled UC-MSCs were abundant in the tendons at 3 days after the injection (Fig. 4A). Although the fluorescence intensity gradually declined at 2 weeks (Fig. 4B), we could still observe the fluorescence emitted from UC-MSCs at 5 weeks after transplantation (Fig. 4C). The morphology of the cells scattered between the collagen fibers of

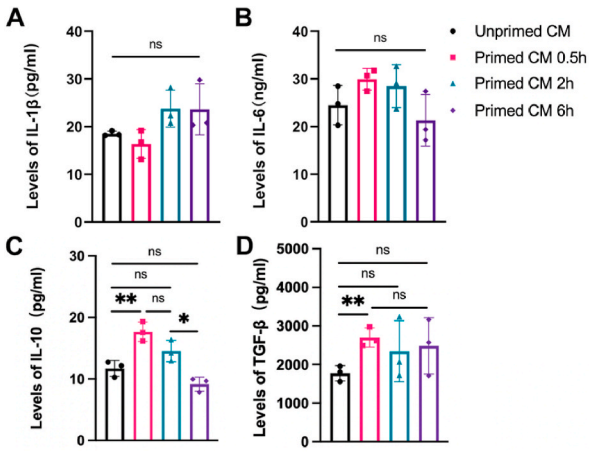


Fig. 2. UC-MSCs primed with IL-1β secreted higher levels of IL-10 and TGF-β in vitro. The levels of IL-1β (A), IL-6 (B), IL-10 (C), and TGF-β (D) in the cultured medium derived from UC-MSCs primed with IL-1β (Primed CM) for 0.5h, 2h and 6h were measured by ELISA kit. Cultured medium from unprimed UC-MSCs (Unprimed CM) were used as control (n = 3 per group). Data are presented as mean ± SD. **P < 0.01; ns, no statistical significance.

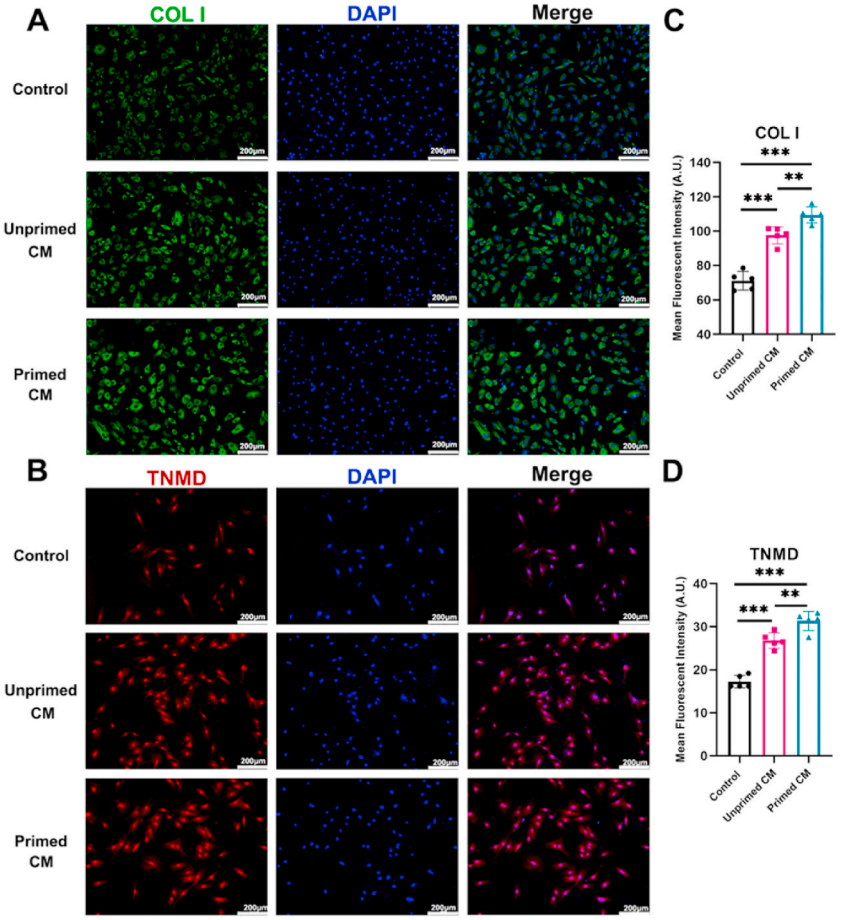


Fig. 3. Primed CM from UC-MSCs promoted higher expression of tendon markers in vitro. A-B Representative images of cellular immunofluorescence staining of the COL I (green) and TNMD (red) (n = 5 per group). C-D Semi-quantitative analysis of the mean fluorescent intensity. TDSCs of the primed CM group expressed higher levels of COL I and TNMD than the unprimed CM group and control group. The nuclei were stained with DAPI (blue). Scale Bar = 200 μm. Data are presented as mean ± SD. **P < 0.01; ***P < 0.001.

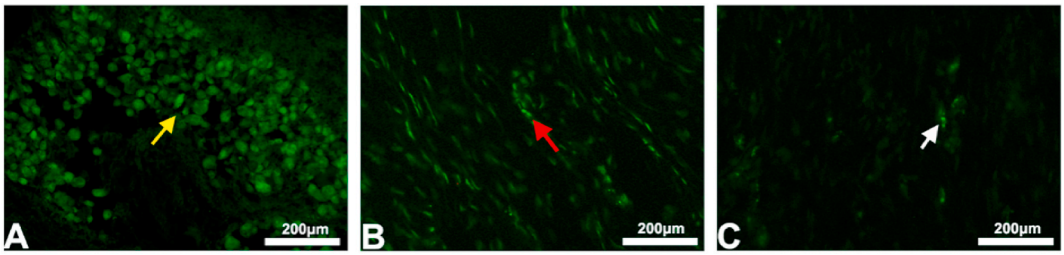


Fig. 4. Fluorescent microscopy of UC-MSCs labeled with CFDA-SE in Achilles tendon. Representative images of 3 days (A), 2 weeks (B), 5 weeks (C) after UC-MSCs injection. Yellow arrow: UC-MSCs with relatively high fluorescence intensity; Red arrow: elongated and oriented aligned UC-MSCs; White arrow: residual UC-MSCs with low fluorescence intensity. Scale Bar = 200 μm.

the Achilles tendon became elongated, which seemed to align along mechanical stress of the tendon.

3.5. Histopathological scale analysis reveals the degree of Achilles tendon degeneration

The rat collagenase-induced tendinopathy model could present several common pathological features of tendinopathy [35,36]. The semiquantitative Bonar histological scale was typically employed to assess the degree of tendon degeneration [30,37,38]. Here, we used the revisited histological score, which highlighted the major influence of the morphological changes of tendon cells. The fourth

line showed the morphology of the normal tendon. H&E staining showed the PBS group had the worst cell morphology in the four groups. Specifically, most of the cell nuclei in the field of view are rounded, prominent with abundant cytoplasm and even lacuna formation (chondroid change) (Fig. 5A–D). In the 1βUC-MSCs group, resident cells in the field of view were similar to the morphology of normal tendon cells and could be rated as Grade 0. Although the two transplantation groups seemed to undergo less inflammation, there was no statistical difference in the cell number in the visual field of the three groups (Fig. 5M – P). Polarized light images showed the separation of collagen fiber bundles and the loss of structure in the PBS group, reflecting the insufficiency of tendon repairability. The tendons in the 1βUC-MSCs group showed significant improvement in collagen organization (Fig. 5E–H). The tendons in the PBS group had new blood vessels and fatty tissues, which may signal the formation of ossification of the tendons, reflecting the severe degeneration of the tendons and the formation of fibrocartilage tissue. Although 1βUC-MSCs had stainable mucin between bundles, bundles were still discrete compared with abundant mucin throughout the section in the PBS group, reflecting the formation of

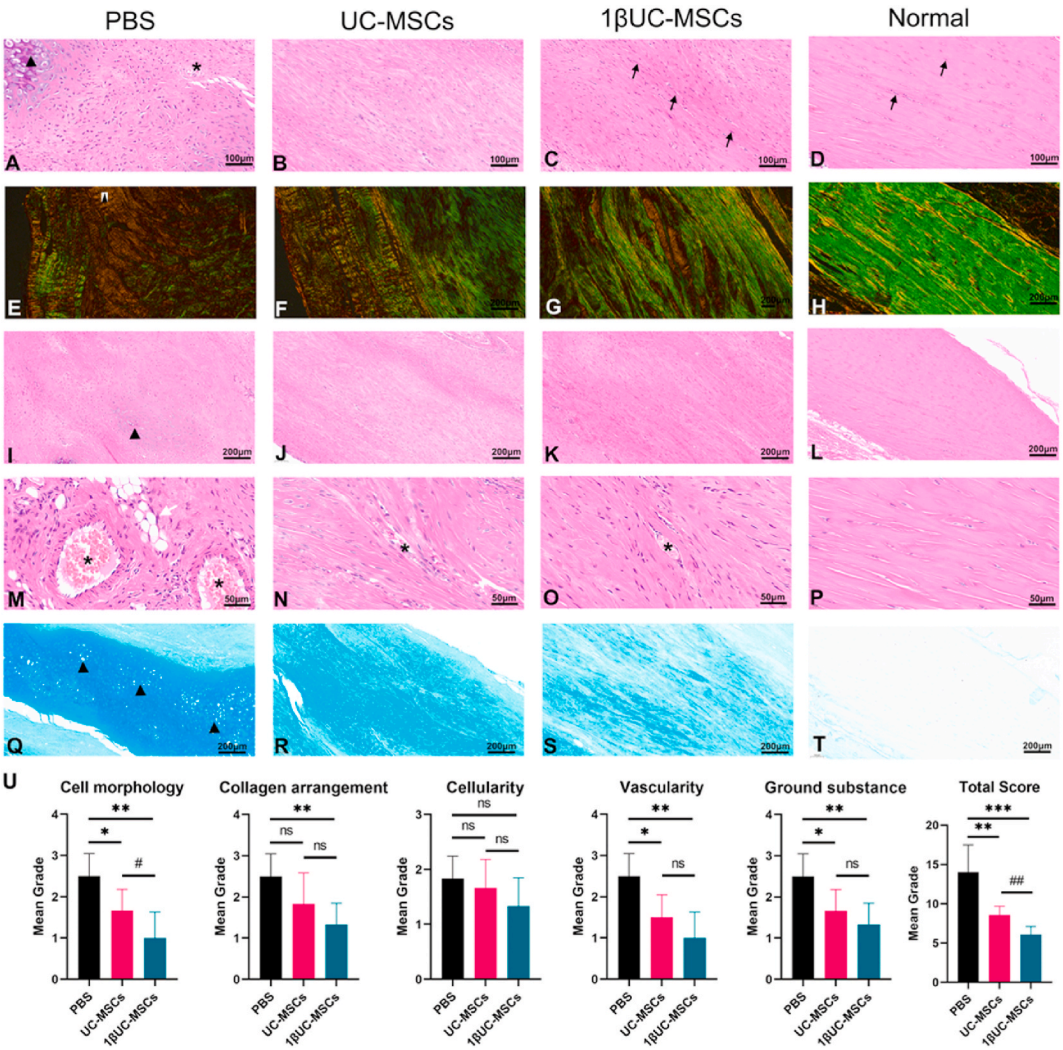


Fig. 5. Histological evaluation after transplantation of UC-MSCs at injured tendons. A-D Representative images of H&E staining to evaluate cell morphology. Tenocytes of 1βUC-MSCs group showed better morphology than that of the PBS group and UC-MSCs group (n = 6 per group). Black arrow: spindle-like resident cells; Asterisk: neovascularization; Triangle: chondroid cells with abundant cytoplasm. E-H Representative images of polarized light microscopy (stained with Sirius Red) to evaluate collagen arrangement. Collagen fibrils in the 1βUC-MSCs group were more orderly aligned and compact compared with PBS group and UC-MSCs group (n = 6 per group). I–P Representative H&E staining images to evaluate cellularity and vascularity of injured tendons. 1βUC-MSCs group and UC-MSCs group showed reduced cell infiltration and less vascularity in comparison with the PBS group (n = 6 per group). Asterisk: neovascularization; White arrow: fatty degeneration Q-T Representative Alcian Blue staining images to evaluate ground substance of injured tendons. Tendons from PBS group exhibited stronger positive staining, indicating more fibrocartilaginous tissue in this group (n = 6 per group). U Semi-quantitative analysis of histological performance of the three groups at 5 weeks after transplantation of UC-MSCs included cell morphology, collagen arrangement, cellularity, vascularity and ground substance, and the total scores were listed. Each feature was graded in six fields of view and the total score was calculated. Data are presented as mean ± SD. *: compared with PBS group; *P < 0.05; **P < 0.01; ***P < 0.001; #: compared with UC-MSCs group; #P < 0.05; ##P < 0.01; ns, no statistical significance.

fibrocartilage tissue in Alcian blue staining (Fig. 5Q-T) [31]. Overall, 1βUC-MSCs had the lowest total score (6.08 ± 1.02), followed by the UC-MSCs group (8.58 ± 1.11), and the PBS group exhibited the most severe tendon degeneration with the highest Bonar score (14.00 ± 3.49) (Fig. 5U).

3.6. IL-1β primed UC-MSCs improved ultrastructural morphology of injured tendons

The alignment, density, and diameters of collagen fibrils were crucial for the functional properties of regenerated tendons. SEM and TEM images intuitively revealed that the collagen fibers of the PBS group were in loose and chaotic arrangements and had reduced diameter of 40.29 ± 6.99 nm (Fig. 6A, E, I, M). And the diameter of UC-MSCs (Fig. 6B, F, J, N) was 49.19 ± 7.16 nm. The 1βUC-MSCs group (Fig. 6C, G, K, O) had more orderly aligned collagen fibrils with greater density and a larger diameter of 57.66 ± 8.37 nm than the UC-MSCs and PBS groups. But their collagen fibers were still smaller in diameter (160.38 ± 11.60 nm) than normal tendons (Fig. 6D, H, L, P), which may reflect that their biomechanical properties have not recovered to the level of native tendons.

3.7. IL-1β primed UC-MSCs promoted functional recovery of injured tendons

In addition to the morphological characteristics, the Achilles Functional Index (AFI) was analyzed to assess the functional recovery of healing tendon. According to Fig. 7A, the AFI value (−45.60 ± 3.23) of the control group was most negative, representing most serious hypomotility in all groups ($P < 0.001$). Notably, the 1βUC-MSCs group exhibited distinctly higher AFI values (−20.23 ± 1.14) than the UC-MSCs ($P < 0.05$), indicating a more satisfactory repair performance (Fig. 7B). The biomechanical properties of the degenerated tendon are also critical indicators of motor function. Therefore, the biomechanical properties of tendons was analyzed based on force-displacement curves (Fig. 7C). The failure force of Achilles tendons from PBS group, UC-MSCs group, 1βUC-MSCs group, and the normal group was 43.67 ± 9.03 N, 64.00 ± 9.27 N, 71.83 ± 8.8 N and 86.17 ± 12.02 N, respectively (Fig. 7D). The stiffness of the 1βUC-MSCs group (30.53 ± 2.77 N/mm) and UC-MSCs group (26.70 ± 2.95 N/mm) was higher than that of the PBS

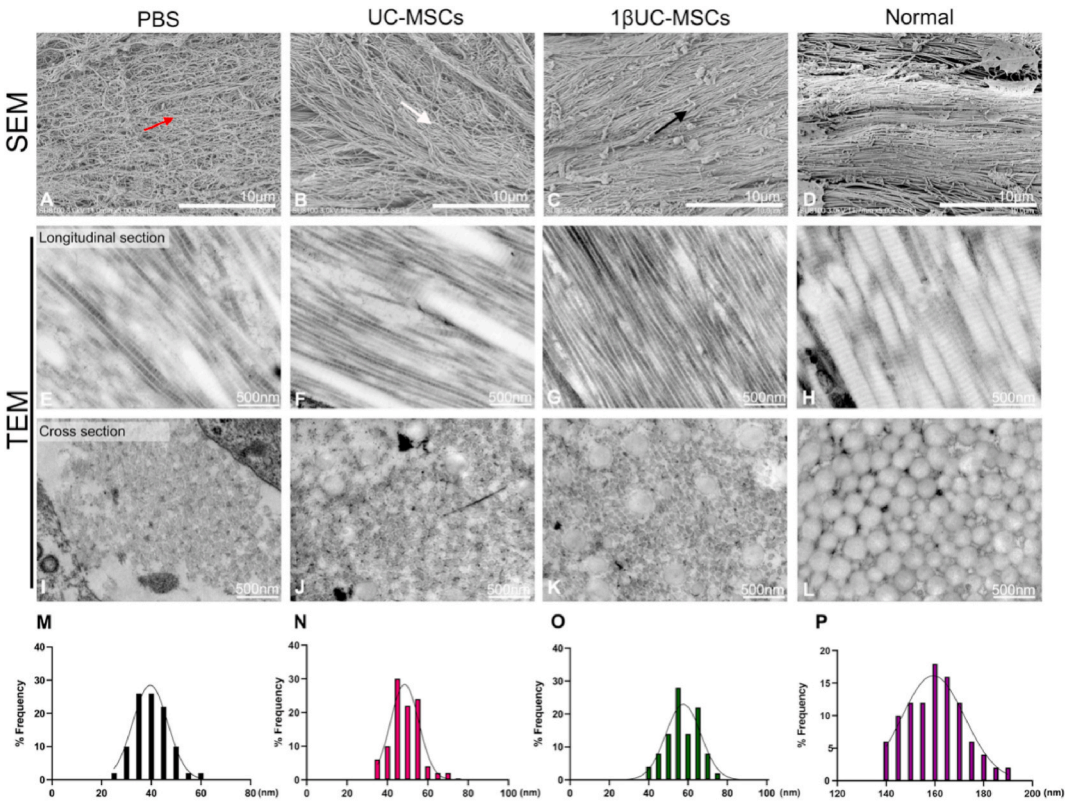


Fig. 6. IL-1β primed UC-MSCs improved ultrastructural morphology of injured tendons. A-D Representative SEM images of injured tendons from the PBS, UC-MSCs and 1βUC-MSCs group and native tendon at 5 weeks after UC-MSCs transplantation (n = 3 per group). Red arrow: collagen fibers with the disorganized and loose arrangement; White arrow: regenerated fibers with the more aligned arrangement than PBS group. Black arrow: parallel aligned and dense collagen fibers. Scale Bar = 10 μm. E-L Representative TEM (longitudinal and transverse) images of injured tendons (n = 3 per group). Scale Bar = 500 nm. M − P Representative images of the distribution of collagen fibril diameters (nm) at 5 weeks after UC-MSCs transplantation. The diameters of microfibrils were larger in the 1βUC-MSCs group than that of UC-MSCs and PBS groups, but still lower than the native tendons.

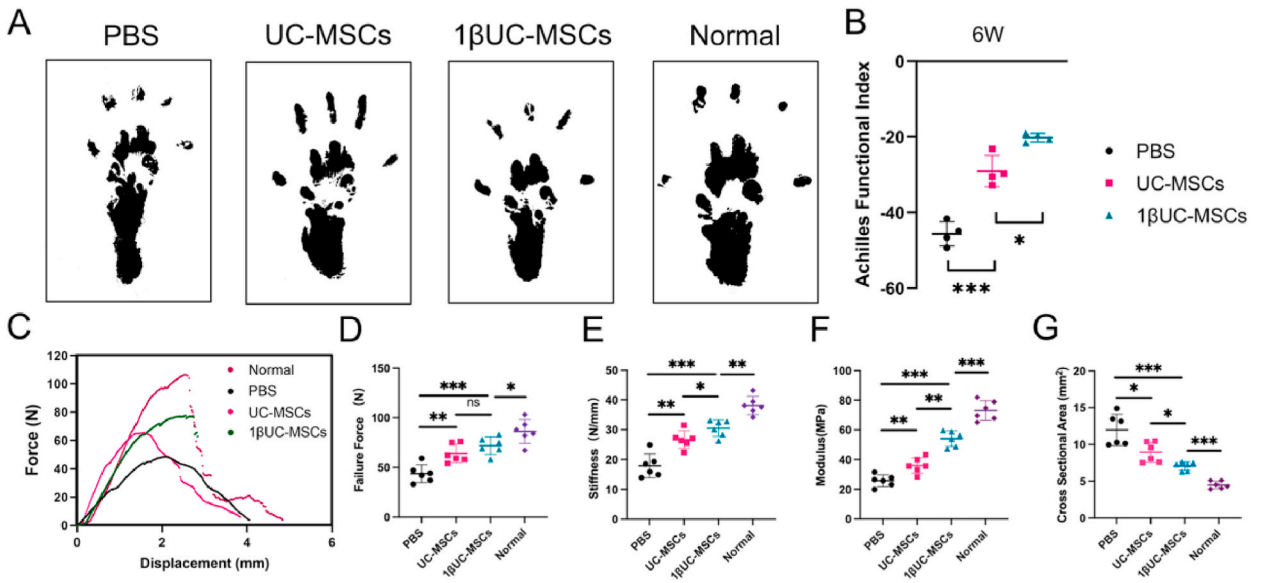


Fig. 7. IL-1β primed UC-MSCs promoted functional recovery of injured tendons. A Footprints of normal and experimental rats from the PBS, UC-MSCs and 1βUC-MSCs group (n = 4 per group). B Semi-quantification of Achilles Functional Index (AFI) was calculated. C Representative force-displacement curves from each group to calculate biomechanical properties. D-F The biomechanical properties (failure force, stiffness, and modulus) of healing tendons at 5 weeks after transplantation (n = 6 per group). G Cross sectional area (CSA) of injured tendons (n = 6 per group). Data are presented as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ns, no statistical significance.

group (17.92 ± 4.01 N/mm, P < 0.01), but still lower than normal group (38.15 ± 3.03 N/mm, P < 0.01) (Fig. 7E). The tensile modulus of the PBS group (25.75 ± 3.98 MPa, P < 0.01) was significantly weaker than the 1βUC-MSCs group (54.14 ± 5.22 MPa) and UC-MSCs group (36.02 ± 5.15 MPa). Although the 1βUC-MSCs group maintained the highest tensile modulus among the three groups, it was still lower than that of the normal group (73.02 ± 6.59 MPa, P < 0.001) (Fig. 7F). We also analyzed differences in the cross-sectional area (CSA) among the four groups. In general, native tendons tend to have smaller CSA due to their dense collagen fiber structure, and degenerative tendons have greater CSA due to their inflammatory response and fibrotic healing. PBS group has the largest CSA among the groups (11.98 ± 2.12 mm^2), and the CSA of UC-MSCs group, 1βUC-MSCs group, and the normal group was 8.94 ± 1.37 mm^2, 7.06 ± 0.59 mm^2 and 4.50 ± 0.52 mm^2 respectively (Fig. 7G).

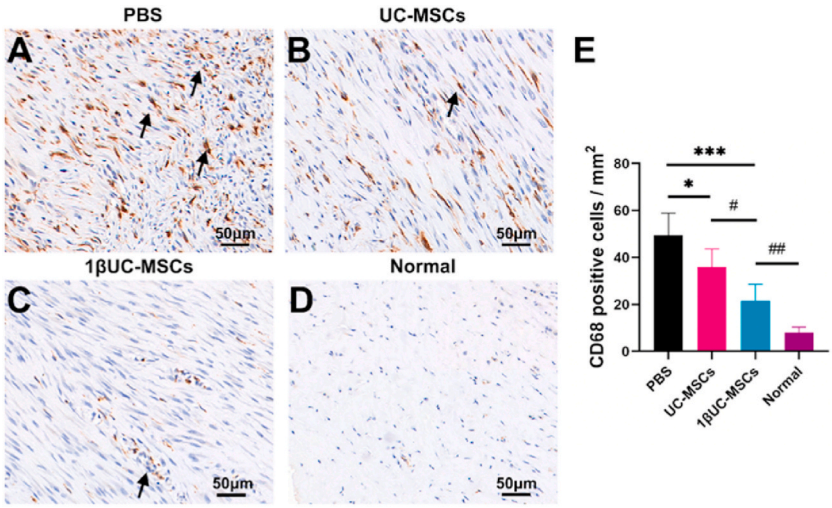


Fig. 8. IL-1β primed UC-MSCs alleviated infiltration of $CD68^+$ macrophages in injured tendons. A-D Representative images of immunohistochemical staining for CD68 expression of injured tendons from the PBS, UC-MSCs, 1βUC-MSCs and normal group at 5 weeks after transplantation (n = 5 per group). E Quantitation of CD68 positive cells was normalized by tissue area (mm^2). The number of macrophages in the 1βUC-MSCs group was lower than the PBS and UC-MSCs group. Black arrow: CD68 positive macrophages. Scale Bar = 50 μm. Data are presented as mean ± SD. *: compared with PBS group; *P < 0.05; **P < 0.01; ***P < 0.001; #: compared with 1βUC-MSCs group; #P < 0.05; ##P < 0.01.

3.8. IL-1β primed UC-MSCs alleviated infiltration of $CD68^{+}$ macrophages of injured tendons

The extensive infiltration of macrophages is a symbol of chronic inflammation for repairing tissue [39]. The immunomodulatory factors IL-10 and TGF-β can regulate the activation of macrophages and promote their polarization to the anti-inflammatory M2 phenotype, which is conducive to the early structural repair of tendons and collagen deposition, reducing the ongoing inflammation response [16]. According to the results of immunohistochemical staining for CD68 expression, the number of CD68 positive macrophages in the 1βUC-MSCs group (Fig. 8C) was significantly lower than the PBS (Fig. 8A) and UC-MSCs (Fig. 8B) group, which indicated transplantation of 1βUC-MSCs could alleviate inflammation effectively at six weeks after tendon injury (Fig. 8D and E).

3.9. UC-MSCs impacted the collagen synthesis and degradation of injured tendons

Large amounts of type III collagen are usually secreted in the early stages of injury and lead to scar formation, which leads to the loss of mechanical properties of the tendon [31]. However, type I collagen could gradually replace type III collagen in the remodeling phase. Our WB results showed that the 1βUC-MSCs and UC-MSCs groups had a significantly higher COL I/COL III ratio than the PBS group (fold change: 4.42 vs. 0.73, 4.30 vs. 0.73, respectively. $P < 0.05$), and there was no significant difference between the two cell transplantation groups (Fig. 9). As a specific marker of tendon maturation, TNMD has the highest expression in the 1βUC-MSCs group (fold change: 0.80). Higher levels of MMP-1 and MMP-13, which can decompose COL I and COL III, have been reported in tendinopathy and acute tendon ruptures and may be related to the early healing of tendon injuries [40,41]. The expression level of MMP-1and MMP-13 was significantly higher in the PBS group than the 1βUC-MSCs group (fold change: 0.83 vs. 0.42, 0.77 vs. 0.35, $P < 0.01$, respectively). It has been reported that the TGF-β/SMAD2/3 signaling pathway could stimulate migration, proliferation, and expression of tendon-specific genes of tendon cells and promote deposition of tendon-related proteins [42]. The level of TGF-β in the 1βUC-MSCs group was relatively higher than the PBS group and UC-MSCs group (fold change: 0.71 vs.0.52, 0.70 vs. 0.26, $P < 0.05$, respectively). Meanwhile, the phosphorylation level of SMAD2/3 was significantly increased in the 1βUC-MSCs group and UC-MSCs group (fold change: 0.54 vs. 0.29, 0.46 vs. 0.29, $P < 0.05$, respectively).

4. Discussion

This study demonstrated that IL-1β preconditioning could enhance the immunoregulatory and paracrine-mediated protrophic capability of UC-MSCs to promote structural and functional repair of tendon. During this process, the TGF-β/Smad2/3 and IL-10

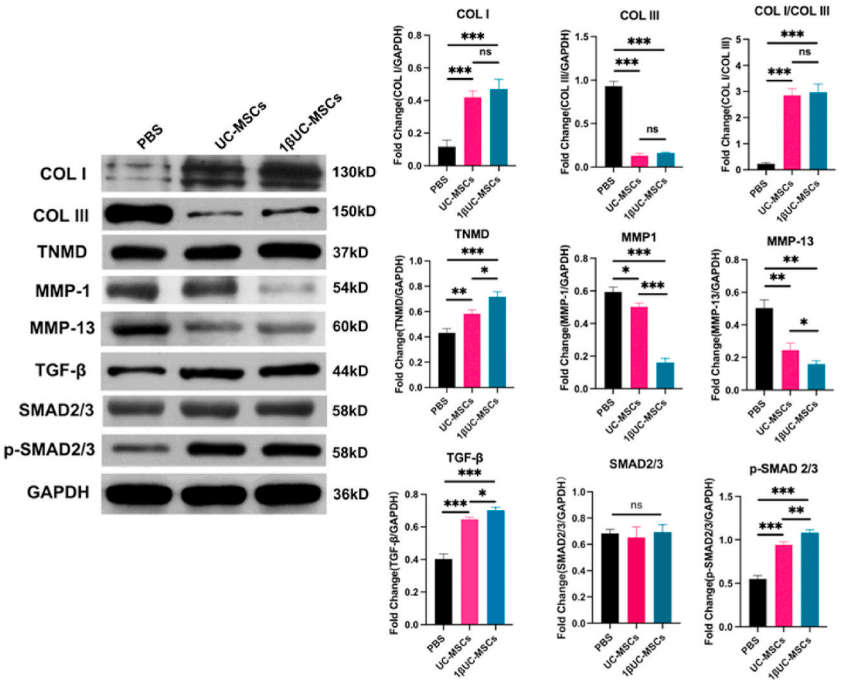


Fig. 9. Transplantation of UC-MSCs impacted the collagen synthesis and degradation of injured tendons through activating TGF-β/SMAD2/3 pathway. Represented images of the protein expression level and semi-quantification of COL I, COL III, the ratio of COL I/COL III, TNMD, MMP-1, MMP-13, TGF-β, SMAD2/3 and *p*-SMAD2/3 at 5 weeks after transplantation. Transplantation of UC-MSCs and 1βUC-MSCs improved the ratio of COL I to COL III expression level, promoted the expression of TNMD, and reduced the expression level of MMP-1 and MMP-13 (n = 3 per group). The level of TGF-β and phosphorylation of SMAD2/3 was significantly elevated in the 1βUC-MSCs group. GAPDH was used as the internal reference. Data are presented as mean ± SD. *: compared with PBS group; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

signaling pathways were activated simultaneously (Scheme 1). In vitro, IL-1β primed UC-MSCs could secrete more immunoregulatory cytokines IL-10 and TGF-β. TDSCs incubated with primed CM in an inflammatory environment showed enhanced expression of COL I and TNMD, demonstrating that 1βUC-MSCs could enhance collagen synthesis and tenogenic differentiation of TDSCs in vitro. In vivo, the transplantation of 1βUC-MSCs improved the quality of tendon healing histologically by semiquantitative modified Bonar scores. The rat collagenase-induced tendinopathy model could present several common pathological features of tendinopathy, including collagen disorganization, inflammation infiltration, neovascularization, lipogenesis and heterotopic ossification, which has been widely used to study the condition of chronic tendon diseases in human [35,36]. The semiquantitative boner histological scale was typically employed to assess the degree of tendon degeneration [30,37,38]. Alex et al. found that the worst cell morphology accompanied the most advanced degenerative change area [30]. And histological results were further confirmed by observation of ultrastructural morphology of the collagen fibers. The structural repair resulted in functional recovery of the Achilles tendon, including biomechanical properties and gait performance. Finally, we discovered that the therapeutic effect may derive from regulating the inflammatory response, and the balance of synthesis and decomposition of the extracellular matrix.

Human UC-MSCs are characterized by low immunogenicity and superior secretion of immunomodulatory factors. Its biological safety and immunomodulatory ability have been confirmed in a large number of experiments using SD rat models [16,43–45]. UC-MSCs have also been demonstrated in previous studies to repair rotator cuff injuries without significant immunosuppression successfully [10,46]. Likewise, a marked reduction in tissue inflammation in the cell transplant group in our histological sections and immunohistochemical staining of macrophages (Figs. 5 and 8) was observed. Further, tissue inflammation in the UC-MSCs group pretreated with IL-1β was alleviated to a greater extent. Several studies reported the safety and effectiveness of UC-MSCs for treating tendon defects. Ji et al. recently applied UC-MSCs to repair rotator cuff injury in a rat model, and they found that UC-MSCs could form the specific extracellular matrix and improve histology and biomechanical properties of the rotator cuff [10]. Yea et al. reported that the freshly thawed UC-MSCs were comparable to cultured UC-MSCs in treating a full-thickness tendon defect (FTD) of rotator cuff tendon in a rat model [46]. And the transplanted cells tended to differentiate into tendon cells at 4 weeks after surgery, which could increase the synthesis of collagen fibers and reduce fibrocartilage formation. Moreover, there was no significant immune response. Our study also demonstrated that the 1βUC-MSCs could still be detected after 5 weeks of transplantation. Multiple priming conditions, including hypoxia, mechanical stimuli, 3D environment, cytokines, and hormones, have been developed to mimic microenvironmental stimuli and make MSCs gain relevant phenotypes for specific therapeutic purposes [44,47–50]. It has been reported that primed MSCs could adapt to the microenvironments of injured tissues more rapidly and repair damaged tissues with more powerful efficacy [14]. Kim et al. found pioglitazone could stimulate the proliferation and enhance the secretion of VEGF (vascular endothelial growth factor) of MSCs. And the pioglitazone-primed MSCs could promote the proliferation, migration, and increase the expression of tendon-related markers such as COLI, scleraxis, and tenascin-C of the co-cultured tenocytes [51]. Besides, several studies have preliminarily demonstrated that the inflammatory factors primed MSCs therapy or MSCs-based modulators of inflammation could regulate the levels of inflammation and the polarization of macrophages after tendon injury [52–54]. Aktas et al. found that the transplantation of TNF-α primed MSCs could significantly increase IL-10 levels and decrease IL-1α levels in vivo. And the ratio of M1/M2 macrophage was also regulated considerably. This inflammatory-modulating capacity increased the concentration of type I procollagen and improved the failure stress of the injured tendon [54].

IL-1β pretreatment has been studied in a variety of inflammation-related diseases. Schnabel et al. found that IL-1β-licensed bone marrow-derived MSCs could improve inflamed cytokine microenvironment better than the naïve MSCs in the surgically induced tendon injury model [55]. Liu et al. reported IL-1β primed MSCs could alleviate chronic prostatitis/chronic pelvic pain syndrome by restoring systemic immunologic homeostasis [56]. Several studies also confirmed that IL-1β primed MSCs could improve the therapeutic effects of various inflammation-related diseases such as stroke, skin wounds, sepsis, and colitis [17–20]. However, there are no relevant studies about IL-1β primed MSCs for treating tendinopathy. Specifically, this study first found that UC-MSCs primed with IL-1β could further enhance the expression of tendon markers COL I and TNMD and achieve structural and functional regeneration of Achilles tendon in vivo. COL I is the main component of the extracellular matrix synthesized by tendon cells in normal tendons. TNMD is a tendon-specific marker, which could support the self-renewal of TDSCs and prevent their senescence. Besides, TNMD is essential for collagen maturation. It has been reported that loss of TNMD leads to inferior early tendon repair characterized by fibrovascular scaring, and persistently cause deficient repair during later repair stages [57]. In other musculoskeletal diseases, inflammatory cytokines-primed MSCs treatment have also been developed. Cassano et al. found that IFN-γ-primed MSCs had a superior ability to protect chondrocytes from catabolic stimulation when exposed to pro-inflammatory macrophages, which may help decrease the progression of osteoarthritis in vivo [58].

With the encouraging effect of IL-1β primed UC-MSC in the treatment of tendinopathy, we have carried out further research on how the specific mechanism works. IL-1β primed MSCs could suppress the activation of M1-type macrophages and induce the M2-type polarization, which is of great significance in inflammatory regulation and tissue repair [59]. This study confirmed that IL-1β primed MSCs could effectively reduce chronic inflammation characterized by the continuous infiltration of macrophages. It has been reported that elevated levels of immunoregulatory cytokines IL-10 could enhance the proliferation and migration of TDSCs in vitro [60]. Furthermore, IL-10 could stimulate the synthesis of extracellular matrix in vivo. During the late inflammatory phase, elevated level of IL-10 is helpful to reduce scar formation and improve biomechanical strength [61,62]. TGF-β is a well-documented cytokine that plays an essential role in tendon formation, development, and healing after injury [63]. They are necessary for the recruitment and differentiation of tendon cells, synthesis, and remodeling of the extracellular matrix [64–66]. TGF-β/SMAD2/3 is one of the most crucial signal pathways in the process of tendon differentiation [63,67]. A previous study demonstrated that the activation of the TGF-β/SMAD2/3 signaling cascade could promote the expression of the downstream molecules COL1A1, thereby accelerating tendon healing [67]. In this study, we confirmed the IL-10 and TGF-β/SMAD2/3 signaling pathways were activated in the 1βUC-MSCs group.

However, previous studies have also confirmed that excessive levels of TGF-β1 may increase the risk of tenocyte apoptosis and fibrosis formation, which may be detrimental to the improvement of tendinopathy [22,24]. In our view, rational temporal and spatial regulation of TGF-β1 levels may be the key to causing tendon functional regeneration rather than fibrosis. In terms of pro-inflammatory cytokines IL-1β and IL-6, it has been found that IL-1α–miR-146a/b and IL-6–C/EBP-β pathway could result in the senescence-associated secretory phenotype (SASP) and increased activity of β-galactosidase [68]. The aging cells may be eliminated by the immune system, thus reducing the treatment efficacy. We optimized the pretreatment time to ensure the secretion of inflammatory cytokines was not significantly upregulated. Above all, our results first demonstrated IL-1β could alleviate inflammation response, regulate the dynamic synthesis and degradation of extracellular matrix, eventually enhance the therapeutic efficacy of UC-MSCs for treating tendinopathy through TGF-β/IL-10 signaling pathway.

There are several limitations in this study. Firstly, we only investigated one testing point after transplantation of UC-MSCs. More testing time points should be investigated to comprehensively assess the efficacy of 1βUC-MSCs for promoting tendon regeneration in future studies. Secondly, future work should assess the durability of the primed phenotype of MSCs and the necessity of multiple injections in vivo. Thirdly, the optimal IL-1β primed condition of MSCs lacks a comprehensive study. In future studies, optimizing the combination of duration and concentration of IL-1β priming to obtain the most beneficial preclinical outcome is the most worthwhile in-depth work. Taken together, 1βUC-MSCs administration could be a promising strategy for treating tendinopathy but needs more comprehensive evaluation to achieve optimal preclinical results.

5. Conclusion

Our results demonstrated that the transplantation of UC-MSCs activated by IL-1β exhibited satisfactory ability 0for promoting tendon functional repair in a tendinopathy rat model. During this process, the balance of inflammatory response and extracellular matrix metabolism was remodeled, and the TGF-β/Smad2/3 and IL-10 signaling pathways were activated simultaneously. We cautiously conclude that the IL-1β primed UC-MSCs could be a promising strategy for enhancing the ability of MSCs to treat tendinopathy.

Ethics declarations

This study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of the Shanghai Jiaotong University affiliated Shanghai Sixth People's Hospital, with the approval number: DWLL2021-0625.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Shikun Wang: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Zhixiao Yao:** Data curation, Investigation, Methodology. **Lei Chen:** Data curation, Investigation, Methodology, Resources, Writing – original draft. **Juehong Li:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Shuai Chen:** Methodology, Supervision, Visualization, Writing – original draft. **Cunyi Fan:** Conceptualization.

Declaration of competing interest

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

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Abbreviations

UC-MSCs Human umbilical cord-derived mesenchymal stem cells.
TDSCs Tendon-derived stem cells.
BM-MSCs Bone marrow-derived MSCs.
AT-MSCs Adipose tissue-derived MSCs.
MSCs Mesenchymal stromal cells.
NSAIDs Nonsteroid anti-inflammatory drugs.
SSEA-4 Stage-specific embryonic antigen-4.
TNMD Tenomodulin.

MMP1 Matrix metalloprotease 1.
PBS Phosphate-buffered saline.
DAPI 4′,6-Diamidino-2-phenylindole.
CFDA-SE Carboxyfluorescein diacetate succinimidyl ester.
TEM Transmission electron microscope.
SEM Scanning electron microscope.
WB Western blotting.
HE Hematoxylin-eosin.
ELISA Enzyme-linked immunosorbent assay.
AFI Achilles functional index.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21411.

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