hUC-MSCs therapy for Crohn's disease: efficacy in TNBS-induced colitis in rats and pilot clinical study

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Summary

Background The use of mesenchymal stem cells (MSCs) has recently emerged as a promising new therapeutic strategy for many diseases including perianal fistulizing Crohn's disease (CD). Whether hUC-MSCs can promote the healing of luminal ulcer in CD has not been studied so far.

Methods The model of TNBS-induced colitis in rats was used to confirm the efficacy of hUC-MSCs in the treatment of CD. Then, seventeen CD patients refractory to or unsuitable for currently available therapies were enrolled and received once submucosal local injection through colonoscopy combined with once intravenous drip on the next day. All patients received a 24-week follow-up. Clinical and laboratory assessments were monitored at baseline, week 4, 8, 12, and 24. Endoscopic evaluations were conducted at baseline and week 12. Mucosal specimens were obtained at the margin of lesions by endoscopy biopsies and used for RNA sequencing. Two hUC-MSCs co-culture systems were established in vitro, one with the mucosa specimens and the other with M1 macrophages induced from THP1. The expressions of genes representing inflammation (TNFα, IL-6, and IL-1β) and intestinal barrier function (ZO1, CLAUDIN1, and CDH1) were tested by RT-PCR.

Findings hUC-MSCs treatment increased body weight and decreased disease activity index (DAI), colon macroscopic damage index (CMDI), and histopathological score (HPS) of rats with TNBS-induced colitis. The results of the clinical study also showed that this mode of hUC-MSCs application was associated with regression of intestinal ulceration. Eight patients (47%) got endoscopic responses (SES-CD improvement of ≥50% from baseline) and three patients (17.65%) got mucosal healing (SES-CD is zero), with a parallel improvement of clinical and laboratory parameters without serious adverse events. RNA sequencing showed hUC-MSCs therapy was associated with an upregulation of transcripts linked to intestinal epithelial barrier integrity and a downregulation of inflammatory signaling pathways in the intestinal mucosa, especially the TNF signaling pathway, IL-17 signaling pathway, and TLR signaling pathway. RNA expression of intestinal epithelial tight junction protein (ZO1, CLAUDIN1, and CDH1), and the RNA expression of major intestinal inflammatory factors in CD (IL-1β, IL-6, and TNFα, $p < 0.001$ for all) were improved significantly. Moreover, hUC-MSCs could attenuate the polarization of M1 macrophage induced from THP1, thereby decreasing the mRNA expression of IL-1β, IL-6, and TNFα significantly ($p < 0.05$ for all). TSG-6 expression was evaluated in hUC-MSCs culture supernatant after treatment with TNFα, IFNγ, and LPS for 48 h. And hUC-MSCs could inhibit the phosphorylation of JAK/STAT1 in the intestinal mucosa of CD patients.

Interpretation hUC-MSCs transplantation alleviated TNBS-induced colitis in rats. In this pilot clinical study, preliminary data suggested that this approach to administering hUC-MSCs might have potential for clinical efficacy and manageable safety in treating refractory CD, potentially providing hope for better outcomes. No serious adverse events were observed.

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Keywords: Human umbilical cord mesenchymal stem cells; Local submucosal injection; Intestinal ulceration; Crohn's disease; Macrophage; Intestinal barrier function

Research in context

Evidence before this study

The management of Crohn's disease (CD) represents a big challenge for clinicians. There are clear limitations with current treatment options, and healing rates remain far from what is expected. The use of mesenchymal stem cells (MSCs) has recently emerged as a promising new therapeutic strategy for many diseases including perianal fistulizing CD. Whether human umbilical cord derived MSCs (hUC-MSCs) can promote the healing of luminal ulcer in CD has not been studied so far. Our objective was to determine the safety and efficacy of a new mode of HUC-MSCs therapy in patients with refractory CD and explore the rationale and possible mechanism.

Added value of this study

In preclinical experiments, hUC-MSCs treatment increased body weight and decreased disease activity index (DAI), colon macroscopic damage index (CMDI), and histopathological score (HPS) of rats with TNBS-induced colitis. In the clinical study, hUC-MSCs also showed that this new mode of hUC-MSCs application induced regression of intestinal ulceration. Eight patients (47%) got an endoscopic response (SES-CD improvement of 50% or more) and three patients (17.65%) got mucosal healing (SES-CD is zero), with a parallel improvement of clinical and laboratory parameters in active CD patients without serious adverse events. In terms of mechanism, our results showed that hUC-MSCs therapy enhanced intestinal epithelial barrier integrity and downregulated inflammatory signaling pathways in intestinal mucosa by attenuating M1 macrophage polarization induced by THP1, and decreasing the expression of IL-1β, IL-6, and TNFα. TSG-6 secreted by hUC-MSCs could inhibit the phosphorylation of JAK/STAT1 in the intestinal mucosa of CD patients.

Implications of all the available evidence

This study highlights the efficacy and safety of this new mode of hUC-MSCs therapy in patients with refractory CD, indicating that this therapy could be a potential option for such patients. The therapeutic effect of hUC-MSCs on CD may be partially mediated by the secretion of TSG-6 to inhibit the phosphorylation of JAK/STAT1 to reduce M1 macrophage polarization, thereby reducing the secretion of proinflammatory cytokines and enhancing intestinal barrier function.

Introduction

Crohn's disease (CD) is one of the Inflammatory Bowel Disease (IBD) with increasing incidence and prevalence worldwide. Its clinical manifestations mainly include abdominal pain, chronic diarrhea, weight loss and fatigue, complications like intestinal stenosis and obstruction, abdominal abscess, fistula, and perianal lesions may occur in some patients due to the progressive nature of the disease. Intestinal ulcers are common endoscopic manifestation in active CD patients. The pathogenesis of CD may be related to the dysregulated immune response caused by complex factors such as genetic susceptibility, environmental and intestinal flora changes,[1] so the current mainstream therapeutic drugs are involving immune suppression such as steroids, immunosuppressants and biological agents with an associated risk of serious infections, malignant and autoimmune sequelae. Although drugs for CD have made a great leap in recent years, the overall efficacy rate is only 40–60% and approximately 25% of CD are refractory.[2] It is of great clinical significance to find a new treatment for CD.

Mesenchymal stem cells (MSCs) are a heterogeneous group of cells originating from the mesoderm during the early stage of embryonic development.[3] MSCs can be isolated from various tissues such as umbilical cord, adipose tissue, dermis, tendon, bone marrow, muscle, and dental pulp.[4] MSCs-based therapy has become a hot research topic owing to its regenerative, immunomodulatory, and anti-inflammatory functions. MSCs have been used to treat acute tissue injury syndromes, chronic degenerative disorders, cardiovascular diseases,[5] graft-versus-host disease (GVHD),[6] inflammatory disease, and autoimmune diseases such as rheumatoid arthritis, autoimmune hepatitis, systemic sclerosis and systemic lupus erythematosus. The mechanisms of

MSCs therapy for these diseases lie in its ability of differentiation, immune regulation, paracrine effects, anti-inflammatory, anti-fibrotic, and regulating non-coding RNA.[7] In recent years, a few studies have been done to treat CD and its complications such as anal fistula and intestinal stricture. The injection ways of MSCs were mostly intravenous infusion, and few of them are local administration. The former is simple and minimally invasive, but most of MSCs that entered the body were trapped in the lung capillary and only few reached the target site, all of these affected the effectiveness of treatment,[8,9] Therefore, multiple infusions were often required in the short term, resulting in increased treatment costs and possible adverse events. In comparison, local application of MSCs has certain advantages. Some studies on local injection of MSCs for treatment of anal fistula in CD have shown it is safe and effective, and a phase 3 clinical trial is under way.[10–12] Sophie Vieujean et also used local injection to treat CD patients with stenosis[13] and showed allogenic bone-marrow derived MSCs tropic injection in CD stricture was well tolerated and may offer a benefit. However, whether MSCs can alleviate or cure intestinal ulcers in CD has not been well studied.

Human umbilical cord MSCs (hUC-MSCs) have received increasing attention in recent years for the advantages of strong immunomodulatory ability, ease of collection, endless source of stem cells, less immune rejection, and no tumorigenic.[14] hUC-MSCs were effective and safe in immune and inflammatory diseases.[15] A phase I pilot study used hUC-MSCs to treat diabetic foot ulcers showed clinical benefits in patients.[16] Dilogo IH et al.[17] found hUC-MSCs significantly decreased pain measured by visual analogue scale in severe knee osteoarthritis (KOA) from initial to 6th-month follow-up and could be a potentially new regenerative treatment for KOA. However, there has been no study on local application of hUC-MSCs to treat intestinal ulcers in CD patients until now. In this study, we explored the effectiveness of hUC-MSCs in TNBS-induced colitis in rats firstly. Then we developed a new method of hUC-MSCs injection, that was local submucosal injection through colonoscopy combined with intravenous drip to treat intestinal ulcer of CD patient, and observed the safety and efficacy of this brand-new approach. Furthermore, we further explored the possible therapeutic mechanisms using in vitro co-culture system.

Methods

Animals and experimental design

All animal studies were reviewed and approved by the Ethics Committee of Shanghai East Hospital ([2020] Research Approval No. 122). Sixty male Sprague–Dawley rats (specific pathogen-free grade) aged 6–8 weeks were purchased from Shanghai Slake Experimental Animal Co., LTD. The rats were adaptively housed for one week before the experiment under the following conditions: temperature 22 ± 2 °C, humidity 55 ± 5%, and normal diet. On day 0, rats were randomly divided into three groups after 24 h of fasting: control group, 4,6-trinitrobenzene-sulfonic acid (TNBS) group, and TNBS + hUC-MSCs group (n = 20/group, the sample size was determined using a Resource Equation Approach).

The induction of colitis was performed by TNBS (Sigma–Aldrich). 5% TNBS and absolute ethanol were mixed at a volume ratio of 1:1 to make a 50% ethanol solution containing 2.5 mg/mL TNBS. On day 1, according to the standard of 100 mg/kg·body weight, the rats were enema at a distance of 8 cm from the anus, and the rats were kept inverted for about 30s to ensure that the mixture reached all the colons. For the TNBS + hUC-MSCs group, a 1 mL suspension of 1×10^6 hUC-MSCs was administered by tail vein injection 12 h after TNBS enema.[18] For the control group, an equal volume of 50% ethanol was administered by enema, followed by a tail vein injection of 1 mL of normal saline 12 h after the enema. Rats were anesthetized with ether before TNBS administration. After modeling, the rats were recorded daily for changes in body weight, stool shape, and presence and extent of rectal bleeding. Rats were sacrificed under anesthesia on day 8.

Disease activity index (DAI) and colon macroscopic damage index (CMDI)

DAI and CMDI were assessed in a blinded manner. The DAI[19] was determined by combining scores of body weight loss (0, no body weight loss; 1, 1–5%; 2, 6–10%; 3, 11–15%; and 4, >15%), stool consistency (0, normal; 2, loose; 4, diarrhea), and stool blood (0, normal; 2, hemoccult test positive; and 4, gross bleeding). The DAI was determined by averaging three separate scores.

CDMI includes the degree of colonic inflammation.[20] Colonic inflammation score:1 (focal hyperemia without ulcers), 2 (1 ulcer without hyperemia), 3 (1 ulcer with inflammation), 4 (>2 ulcers with inflammation), 5 (More than 2 ulcers and areas of inflammation greater than 1 cm in length), 6 (for ulcers and/or inflammation greater than 2 cm in length, the score is increased by 1 for each 1 cm increase in the extent of the lesion).

Histological evaluation

Colon tissue was sampled and colitis was assessed as described previously.[21] Briefly, the colons were removed, opened and washed with PBS. A portion of distal colon was fixed in formalin for histological examination. Fixed colon sections were stained with hematoxylin and eosin and assessed for severity of colitis using a semi-quantitative rubric. Colitis was assessed and scored by a board certified veterinary pathologist, experienced with assessing mouse intestinal pathology and blinded to the treatment groups.

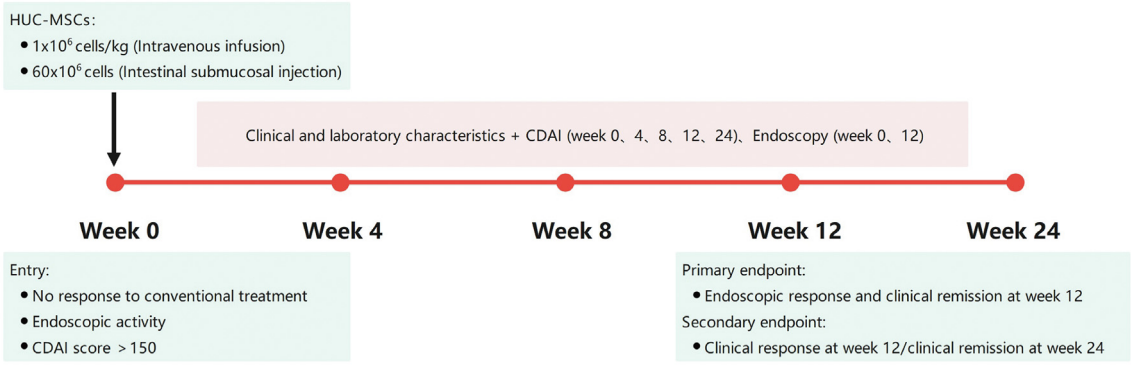


Fig. 1: The process of clinical trial.

Clinical study design and participants

We conducted an open-label, pilot, single-arm trial in Shanghai East Hospital (SEH), shanghai, China between November 2020 and October 2023. Patient inclusion criteria were as follows: (1) age: 18–75 years; (2)moderately to severely active CD for at least 3 months with a baseline Crohn's Disease Activity Index (CDAI) score of 220–450; (3) ulcers of any size on baseline endoscopic evaluation (SES-CD $\geq$ 3 on endoscopy); (4) no response or were intolerant to conventional therapy or advanced therapy (biologics such as anti-tumor necrosis factor-α monoclonal antibodies, vedolizumab, and ustekinumab); Key Exclusion criteria were as follows: (1) pregnancy or planning pregnancy within 1 year; (2) confounding Crohn's disease complications or other confounding comorbidity; (3) any intra-abdominal surgery or a hospitalization for bowel obstruction within 3 months of enrollment; (4) active and uncontrolled infection; (5) a history of malignancy within the past 5 years.

This trial was approved by the Ethics Committee of Shanghai East Hospital ([2020] Research Approval No. 081) and registered at https://www.chictr.org.cn/ (Identifier: ChiCTR2000040220). Written informed consents were obtained from all participants.

Sample size estimate

Traditional sample size determination is made to ensure specific power to detect targeted treatment effects. However, no clinical data exist on effects of hUC-MSCs or MSCs in humans or patients with intestinal ulcers Therefore, pilot trial sample size estimation (n = 17) was based on large standardized effect sizes (0.8) for a main trial designed with 90% power and two-sided 5% significance.[22]

Procedures

Eligible patients were admitted to the hospital and received baseline evaluation on day 0. The dosages of intravenous drip were referred to previous studies.[23] As for the dosage of hUC-MSCs used for submucosal injection, we determine it based on the dosage injected locally in intestinal strictures, knees, and perianal fistulas.[13,24,25] Finally, a total of 60×10^6 hUC-MSCs cells/10 mL were multiple-points submucosal injected around ulcer via colonoscopy on day 1 in all patients, and an intravenous weight-range-based dose of 1×10^6 cells/kg/100 mL was finished on day 2. The participants were hospitalized for 3 days and were observed for signs of acute adverse events. CDAI, clinical and laboratory examinations were determined at week 0, 4, 8, 12, and 24. Endoscopic evaluations were recorded at weeks 0 and 12. Laboratory and safety evaluations were done throughout the study. All patients maintained the original baseline medication therapy until the end of the study. The flow diagram of the study is shown in Fig. 1.

Outcomes

The primary outcomes were endoscopic response (SES-CD score decreased by ≥50% from baseline) and clinical remission (CDAI score < 150) at week 12. Major secondary endpoints included the following: evaluation of safety and tolerability; clinical response at week 12 and 24 (CDAI score decreased by ≥100 points from baseline) or clinical remission at week 24. Safety analyses included all safety data reported up to and including the week 24 visit for all patients.

Culture and preparation of hUC-MSCs

The hUC-MSCs used in this study were derived from the GMP laboratory of Stem Cell East Hospital affiliated with Tongji University, the National Stem Cell Translational Resource Center and were successfully produced on a large scale. hUC-MSCs produced in this base have passed the quality review inspection of the China Food and Drug Administration (report number: SH201903852/SH201903853) and met the requirements for clinical use. Human umbilical cords were obtained after receiving written informed consent from normal women in their full-term pregnancy at the Maternity Department of Shanghai East Hospital and

approved by the Hospital Ethics Committee. Methods for isolation and processing hUC-MSCs have been previously described in detail.[26] The fifth passage of hUC-MSCs was incubated with phycoerythrin or fluorescein isothiocyanate-conjugated mouse monoclonal antibodies against human CD11, CD19, CD31, CD34, CD45, human leukocyte antigen DR (HLA-DR), CD73, CD90, andCD105 (1:50) for 30 min, and flow cytometric analysis was performed (FACSCant II, BD Biosciences, San Jose, CA, USA). For differentiation identification, we replaced the culture medium with complete osteogenic (A10069 and A10066, Thermo Fisher), adipogenic (A10069 and A10066, Thermo Fisher), or chondrocyte differentiation medium (A10069 and A10064, Thermo Fisher) to induce osteogenesis, adipogenesis, and chondrogenesis, respectively. After stimulation, cells were stained with Alizarin Red S, Alcian Blue, or Oil Red O. The antibodies used were listed in Supplementary Table S1. The final products were delivered to the patient's physician on the day of injection.

Clinical and laboratory assessments

CDAI score and SES-CD score were used to assess the clinical and endoscopy improvement. Mucosal biopsies were obtained at the margin of lesion by endoscopy at week 0 and week 12. To measure the changes of laboratory biomarkers, including hemoglobin, concentrations of C-reactive protein (CRP), faecal calprotectin, pro-inflammatory cytokines (IL-6, IL-8) and CD4/CD8, blood samples were collected at baseline, week 4, 8, 12, 16, 20, and 24, then send to Laboratory Department in Shanghai East Hospital for testing.

RNA sequencing

Intestinal mucosal samples were collected via colonoscopy biopsy from three patients before and after hUC-MSCs transplantation. Total RNA of these samples was extracted using the mirVana miRNA Isolation kit (Ambion) according to the manufacturer's protocol. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and samples with RNA Integrity Number (RIN) $\geq$ 7 were subjected to the subsequent analysis. The libraries were constructed using TruSeq Stranded Total RNA with Ribo-Zero Gold according to the manufacturer's instructions. Then these libraries were sequenced on the Illumina sequencing platform (HiSeqTM 2500) and 150 bp paired-end reads were generated. Trimmomatic (v0.36) software was first used for adapter removal, and then low-quality bases and N-bases or low-quality reads were filtered out. Finally, we got high-quality clean reads. The filtered reads were mapped to human reference genome GRCh38 using Hisat2 (v2.2.1.0). The mapped reads were assembled using Stringtie (v1.3.3b). The expression levels were estimated with FPKM using Stringtie. Using the estimate Size Factors function of the DESeq2 (v1.18.0) (2012) R package to normalize the counts, and using the nbinom Test function to calculate q-value and fold change values for the difference comparison. Selecting differential transcripts with q-value < 0.05, |log2FoldChange|>1, and analyzing differential mRNA GO and KEGG enrichment by Hypergeometric Distribution Test. The data reported in this paper have been deposited in the OMIX, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (https://ngdc.cncb.ac.cn/omix: accession no. OMIX003527).

Co-culture system of human intestinal mucosa with hUC-MSCs

To clarify the mechanism of hUC-MSCs therapy for ulcer in CD, a co-culture system of human intestinal mucosa with hUC-MSCs was established and offered a relevant and practical experimental ex-vivo model. The details were as follows: fresh inflammatory mucosae were biopsy from the edge of ulcers, while the paired normal mucosae were obtained from the normal intestinal segment 5 cm away from the lesion of four CD patients via colonoscopy before hUC-MSCs transplantation. Samples washed nine times with PBS contained 2% Penicillin-Streptomycin Solution (PS), and co-cultured in RPMI-1640 (Contained 10% FBS and 2% PS) with or without hUC-MSCs in a six-well plate for 24 h.

Co-culture system of macrophage with hUC-MSCs

Macrophages play a crucial role in the pathogenesis of CD. To study whether hUC-MSCs affect macrophage polarization, human monocyte cell line THP-1 cells (purchased from the Cell Bank of the China Science Academy)were maintained in RPMI-1640 (Gibco) supplemented with 100 ng/mL PMA (Sigma) for 48 h and to generate M0 macrophages, subsequently cultured for another 48 h in RPMI-1640 medium supplemented with 100 ng/mL lipopolysaccharide (sigma) and 20 ng/mL IFN-γ (PeproTech) to induce M1-polarized macrophages, thereafter hUC-MSCs (2×10^5) were seeded into the top chamber of 0.4 μm pore transwell (Corning, NY) and co-cultured for further 48 h. All medium was supplemented with 100 units/mL of penicillin-streptomycin (Gibco) and 10% fetal bovine serum (Gibco). All the cells were cultured in 37 °C incubator with 5% CO2.

RNA extraction and quantitative real-time PCR

RNA of biopsy tissues or cells was extracted with TRIzol (Invitrogen, Carlsbad, CA, United States) and reverse-transcribed using the PrimeScript TM RT kit and gDNA Eraser (TaKaRa, Japan, RR047A). Quantitative Real-time PCR analysis was performed using the TB Green Premix Ex Taq (TaKaRa, Japan, RR420A) with the Applied Biosystems 7500 Fast Real-Time PCR System

(Thermo Fisher Scientific). The primers are listed in Supplementary Table S2.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed using the supernatant of hUC-MSCs treated with/without inflammatory cytokines. The supernatants were collected and protein content was determined using a Multiskan Spectrum (MutiskanTM GO, ThermoFisher). TNF-stimulated gene 6 protein (TSG-6) was measured using the ELISA kit according to the manufacturer's instructions.

Immunohistochemical staining

Immunohistochemical staining (IHC) was performed on 4 μm-thick paraffin sections of tissues fixed in buffered formalin. Sections were deparaffinized in xylene and rehydrated in graded alcohols. Endogenous peroxidase was blocked by 3% H2O2 followed by antigen retrieval. Slides were incubated with primary antibodies overnight at 4 °C and incubated with a secondary antibody at room temperature for 60 min. The following primary antibodies were used (Supplementary Table S1): pJAK1 (CY10423, Abways technology) and pSTAT1 (CY5702, Abways technology). The staining was developed using an EnVision Detection Rabbit Kit (GK500710, GeneTech).

Statistical analysis

This was a pilot trial of hUC-MSCs treatment for patients with CD. Analyses were done by intention to treat. Descriptive analyses of the variables were expressed as mean (SD), median (IQR), or number (%). To investigate the effects of hUC-MSCs treatment, we used the paired *t*-test on the statistical analysis of data. All variables of in-vitro experiments were expressed in mean ± SEM with at least 3 replicates in each group. One-way ANOVA analysis with Tukey's multiple comparisons test was used when comparing 3 or more groups. Statistical significance was defined as $p < 0.05$. All statistical analyses were done with GraphPad Prism 9.0 software (GraphPad Software, La Jolla, CA).

Role of funders

All of our funders played a role in providing the cost of basic experimental consumables, subsidizing clinical subjects, and providing the cost of paper processing. Study design, data collection, analysis, interpretation, and writing of the report, as well as the decision to submit the paper for publication, were done solely by the authors.

Results

Acquisition and culture of hUC-MSCs

hUC-MSCs were identified as previously described[27,28] by using flow cytometry, more than 95% were positive for CD73/CD90/CD105, and fewer than 2% were positive for CD11/CD19/CD31, CD34/CD45/HLA–DR (Supplementary Fig. S1A). Furthermore, the hUC-MSCs successfully differentiated into adipocytes, osteoblasts, and chondrocytes in vitro (Supplementary Fig. S1B). All these are the identification marks of MSCs.

hUC-MSCs treatment relieved TNBS-induced colitis in rats

To evaluate the efficacy of hUC-MSCs in the treatment of CD, we first established a TNBS-induced colitis model in rats and observed the effect of hUC-MSCs transplantation on colitis in rats (Fig. 2A). The results showed that the loss of body weight induced by TNBS in rats improved significantly by hUC-MSCs treatment (Fig. 2B). Meanwhile, the DAI of TNBS colitis rats also decreased after hUC-MSCs transplantation (Fig. 2C). Moreover, hUC-MSCs transplantation elevated survival probability of rats during the TNBS administration (Fig. 2D). Compared with TNBS group, TNBS + hUC-MSCs group showed a significant reduction in intestinal mucosal injury, hyperemia, edema and ulceration (Fig. 2E), and as shown in Fig. 2F, the CMDI of TNBS + hUC-MSCs group was mitigated obviously. Furthermore, hUC-MSCs therapy significantly reduced histological scores in TNBS-colitis rats (Fig. 2G–H). All these results hinted that hUC-MSCs transplantation maybe a promising way to relieve CD.

Patient demographics and disease characteristics

A total of 17 patients were enrolled with a mean age of 44 years (interquartile range [IQR]19–70 years), 47.06% (n = 8) of them were female, and 52.94% (n = 9) were male. Characteristics of all patients including age, sex, disease duration, disease location, and medical treatment were recorded (Table 1). At enrollment, the mean disease duration was 48 months (9 months–120 months). The mean SES-CD score was 7.94 (7.94 ± 3.02) and the mean CDAI score was 277.4 (277.4 ± 71) respectively. Only one patient was a current smoker.

All patients had received ≥1 prior conventional therapy or/and biologics at baseline and still ongoing the baseline medications or/and biologics at a stable dosage during the study. Nine patients were taking concomitant immunomodulators such as Azathioprine or Methotrexate, also ten patients were treated with biologics (five patients with TNF antagonist, two patients with Vedolizumab, and three patients with Ustekinumab). Four of the patients were on combination therapy (immunomodulators and biologics). All patients received hUC-MSCs treatments and were included in all outcome analyses.

Adverse events during hUC-MSCs treatment

The process of hUC-MSCs delivery by endoscopy was shown in Video 1. There was no serious adverse event associated with hUC-MSCs transplantation as showed

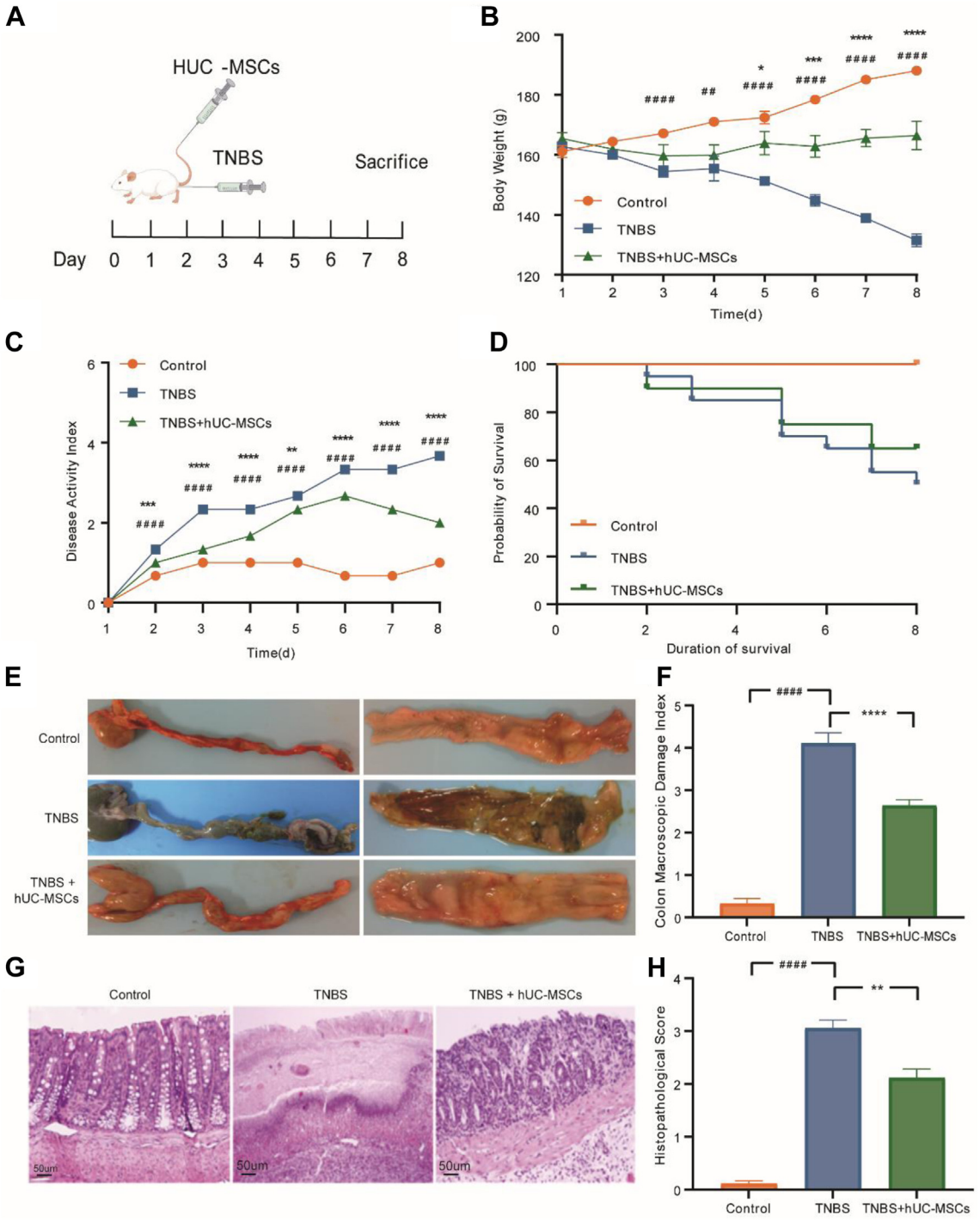


Fig. 2: hUC-MSCs transplantation relieved TNBS-induced colitis in rats. (A) Schematic diagram of the experiment. (B and C) The change of body weight and disease activity index (DAI) at different time points in rats of each group. (D) Survival probability during the TNBS administration. (E and F) The appearance of colons and colon macroscopic damage index (CDMI) in different groups. (G and H) Representative H&E staining of colonic sections in different groups and histopathological score of inflamed colonic tissue. Results are expressed as means ± SEM; n = 20 rats per group. Bars = 50 μm, TNBS group versus TNBS + hUC-MSCs group: *$p < 0.05$, **$p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$; Control group versus the TNBS group: #$p < 0.05$; ##$p < 0.01$, ###, $p < 0.001$, ####, $p < 0.0001$. (One-way ANOVA with Tukey's multiple comparisons test).

Parameter	n = 17 (%)
Male gender	8 [47.06]
Age at inclusion (years, median [IQR])	44 [30.5–62]
Disease duration (months, median [IQR])	48 [24–90]
Smoking habits	
Never	15 [88.2]
Past smoker	1 [5.9]
Active smoker	1 [5.9]
Medication during follow-up	
None	1 [5.9]
Salicylic acid preparations	5 [29.4]
Methylprednisolone	3 [17.6]
Immunomodulators	9 [52.9]
Anti-TNF therapy	5 [29.4]
Vedolizumab	2 [11.8]
Ustekinumab	3 [17.6]
Previous luminal surgery	
Yes	7 [41.2]
No	10 [58.8]

CDAI, Crohn's Disease Activity Index; IQR, interquartile range; SES-CD, Simple Endoscopic Score for Crohn's Disease; TNF, tumor necrosis factor.

Table 1: **Clinical characteristics of CD patients in baseline.**

in Supplementary Table S3. Only one patient had a low-grade fever (37.8 °C) after hUC-MSCs infusion and resolved spontaneously within 24 h. The above-mentioned findings indicated that hUC-MSCs treatment for CD patients was safe and tolerable.

hUC-MSCs treatment was associated with improvement of ulcers, clinical symptoms, and biomarkers in CD patients

To investigate the efficacy of hUC-MSCs transplantation in CD patients, the CDAI score, serum inflammatory markers, fecal calprotectin, hemoglobin, and SES-CD score were assessed. We first assessed the intestinal ulcers using endoscopy. Representative images of the intestinal ulcers before and after hUC-MSCs treatment in three patients were shown in Fig. 3A. It showed that 12 weeks after hUC-MSCs treatment, the intestinal ulcers showed improvement. SES-CD scores improved significantly at week 12 compared with week 0 (Fig. 3B). Eight patients (47%) got endoscopic response (SES-CD improvement of 50% or more) and three patients (17.65%) got mucosal healing (SES-CD is zero) (Fig. 3C). CDAI score at week 0, 4, 8, 12, and 24 was collected, as shown in Fig. 4A. Compared with baseline, the CDAI score decreased gradually over time after hUC-MSCs application and it reduced significantly at week 24, and all patients achieved clinical remission. Meanwhile, the hemoglobin concentrations showed small fluctuations over time within normal reference ranges in all patients. And it increased significantly at week 24 after hUC-MSCs treatment (Fig. 4B).

Inflammatory biomarkers such as CRP, CD4/CD8, IL-8, and fecal calprotectin (Cal) also decreased significantly at different timepoints after hUC-MSCs treatment, while IL-6 with significant changes at week 4 (Fig. 4C–F, Supplementary Fig. S2). These results indicated that hUC-MSCs treatment induced clinical remission, improved clinical indicators, and promoted ulcer healing in CD patients.

hUC-MSCs treatment enhanced intestinal epithelial barrier integrity and downregulated inflammatory signaling pathways in CD patients

To investigate the mechanism of hUC-MSCs improved intestinal ulcers in CD patients, mucosae were biopsied via colonoscopy at the margin of the lesions before and after hUC-MSCs treatment in three patients and collected for transcriptome sequencing. As shown in Fig. 5A, the transcriptome of intestinal mucosa changed significantly after hUC-MSCs treatment. Differentially expressed genes (DEGs) were shown by Volcano map (Fig. 5B). Then GO analysis suggested that hUC-MSCs treatment upregulated the expression of genes which maintained intestinal epithelial barrier integrity (PCDH20, SLC26A3, etc.) and downregulated the expression of genes related to inflammatory response (IL-1β, IL-6, etc.) (Fig. 5C and D). KEGG analysis also suggested that hUC-MSCs treatment down-regulated inflammation related signaling pathways such as TNF signaling pathway, IL17 signaling pathway, TLR signaling pathway, and so on, which are highly relevant to the pathogenesis of CD (Fig. 5E).

hUC-MSCs treatment restored intestinal tight junctions, attenuated macrophage M1 polarization and its related inflammatory factors

To further verify hUC-MSCs can promote intestinal epithelial barrier and alleviate inflammation, intestinal mucosa from CD patients and hUC-MSCs co-culture system was established. Normal intestinal mucosa and inflammatory mucosa were biopsied from CD patients by colonoscopy. Schematic diagram of co-culture method was shown in Fig. 6A (by Figdraw). After 24 h of co-culture, RT-PCR was used to assess the expression of genes related to tight junctions (ZO1, CLAUDIN1) and adherent junctions (CDH1, the gene encoding E-cadherin) of intestinal epithelial cells as well as inflammatory factors (IL-1β, IL-6, and TNFα, secreted mainly by M1 macrophage). As shown in Fig. 6B, co-culture with hUC-MSCs increased the expression of CDH1, ZO1, and CLAUDIN1, and inhibited the expression of IL-1β, IL-6, and TNFα in intestinal mucosa. These data suggested hUC-MSCs treatment enhanced tight junctions and decreased M1macrophage related inflammatory factors in the intestinal mucosa of CD patients in vitro.

Based on the above results and the key role of macrophages in the pathogenesis of CD, we speculated

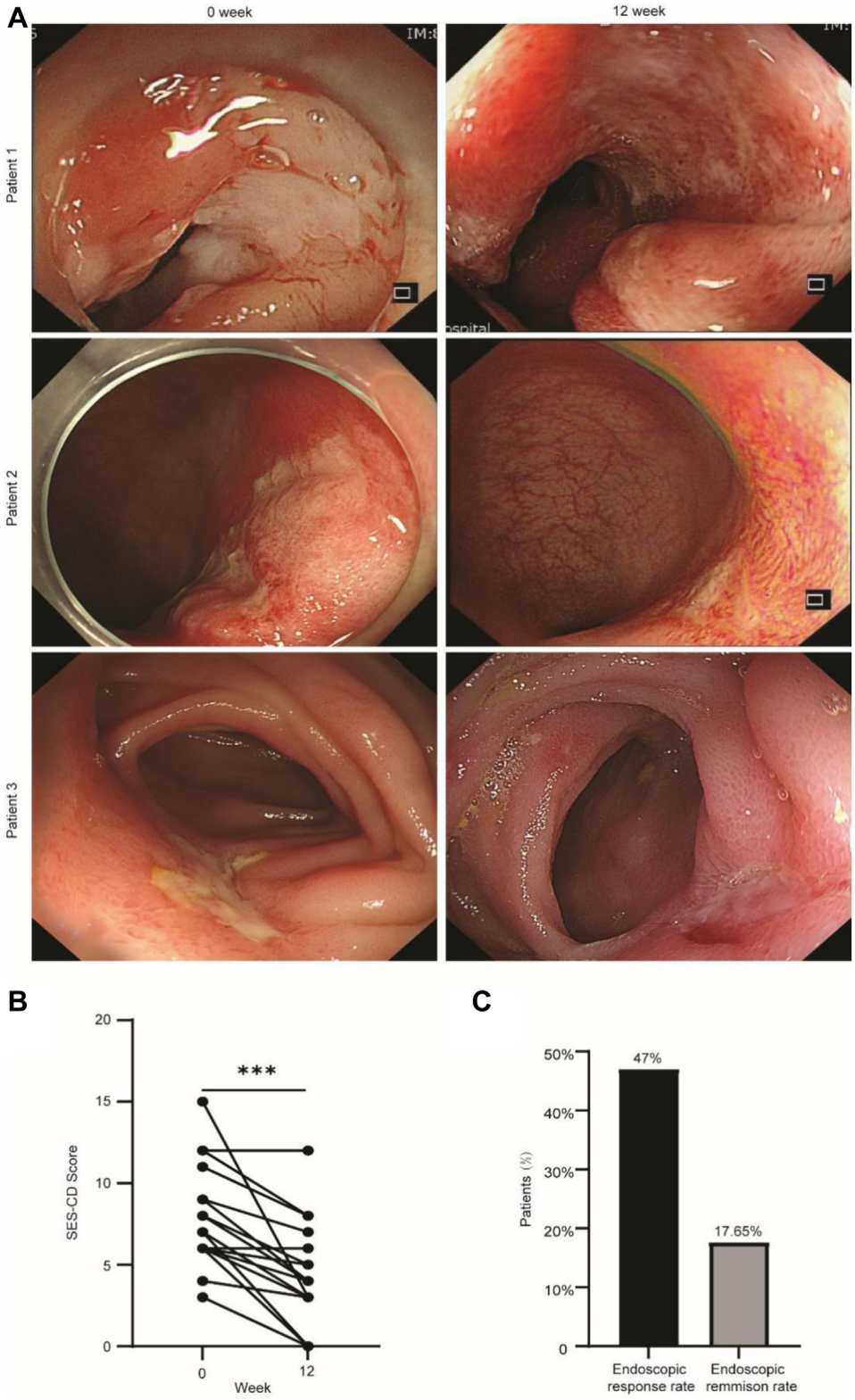


Fig. 3: hUC-MSCs therapy was associated with the healing of intestinal ulcers in patients with CD. (A) Representative endoscopic image of intestinal ulcers before and after hUC-MSCs treatment in three patients at Week 0 and week 12. (B) Evolution of Simple Endoscopy Score of Crohn's Disease (SES-CD) between week 0 and week 12 (n = 17). (C) Endoscopic response rate (SES-CD decreased 50% or more) and endoscopic remission rate (SES-CD reached 0) at week 12 after hUC-MSCs treatment. ***, $p < 0.001$ (paired t-test).

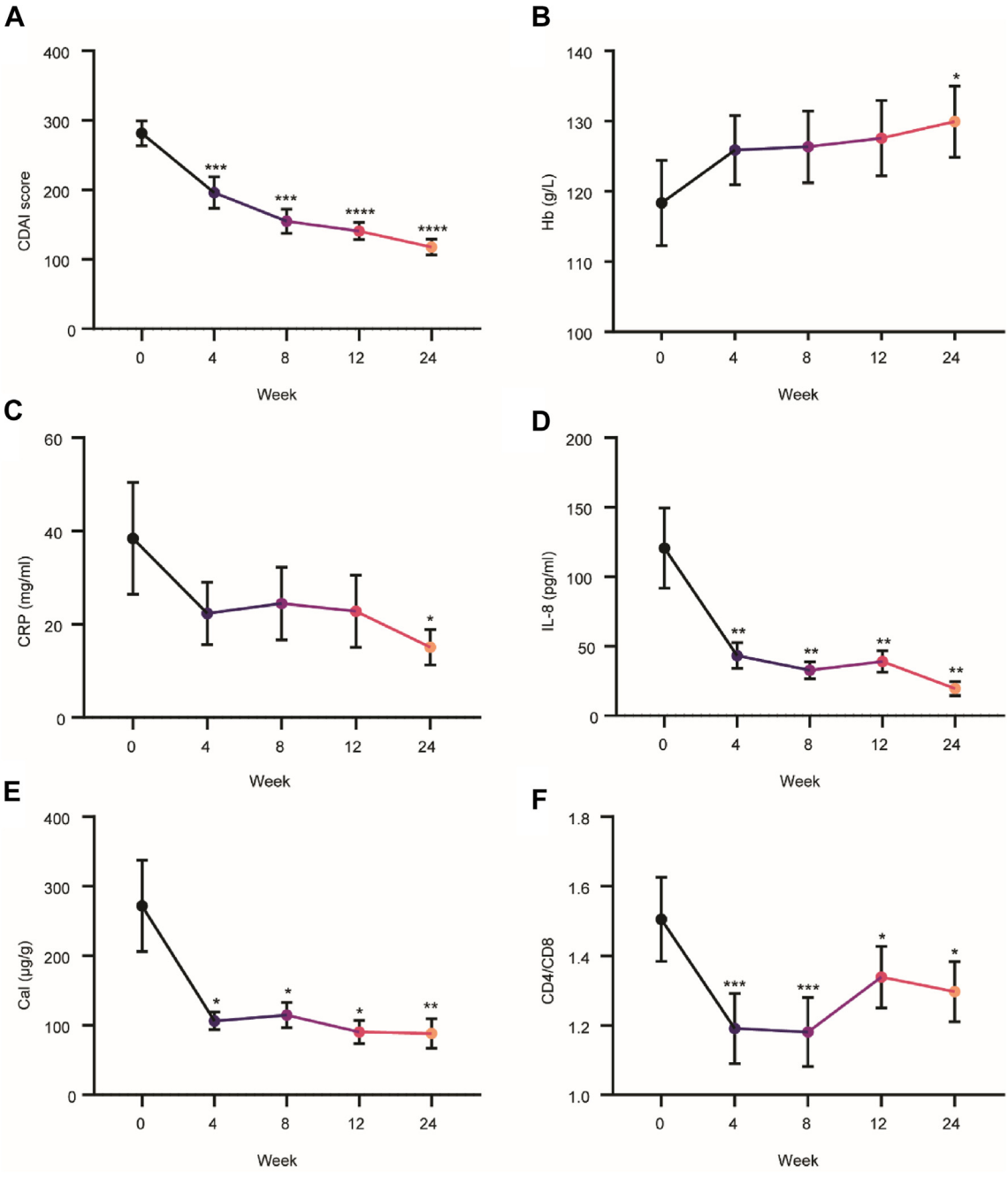
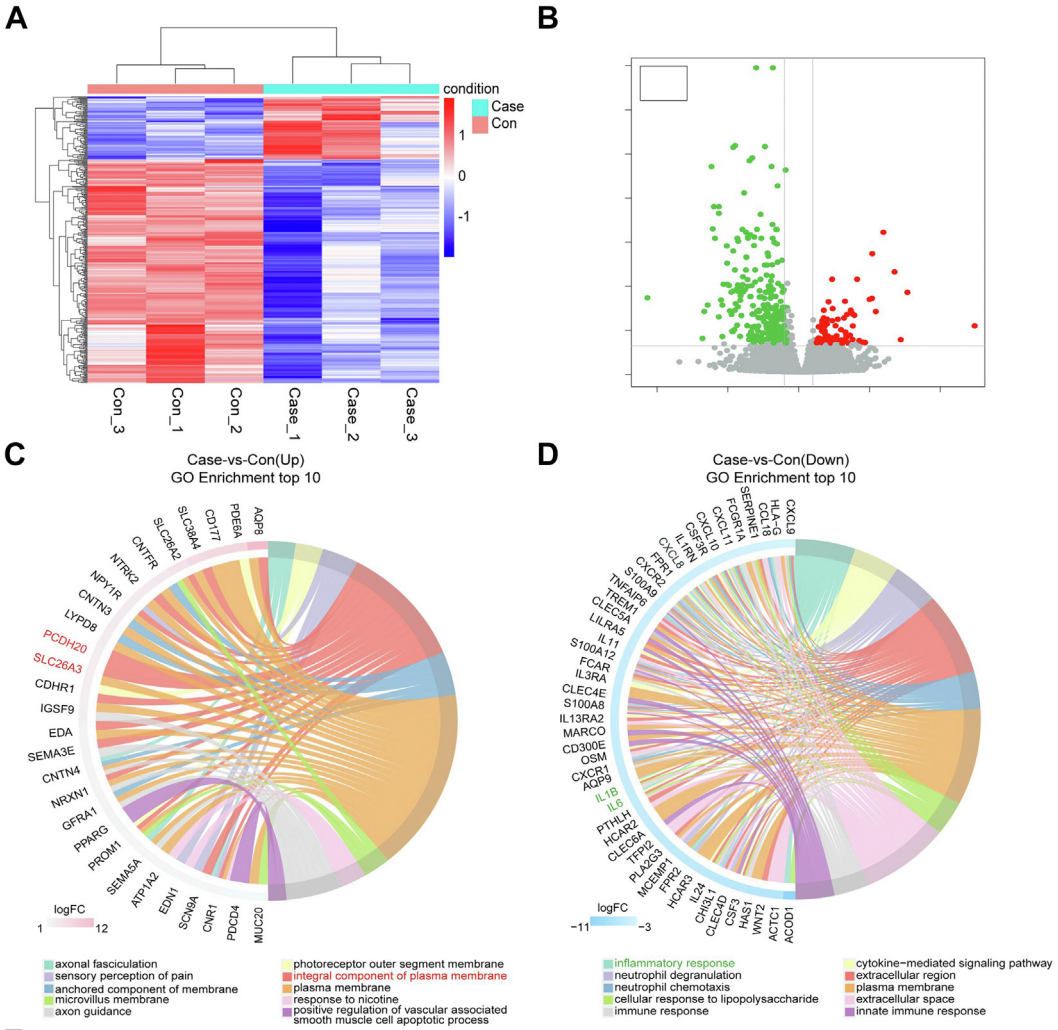


Fig. 4: hUC-MSCs therapy was associated with the improvement of patients' CDAI scores, anemia, and several inflammatory biomarkers. (A–F) Changes of the Crohn's Disease Activity Index (CDAI), hemoglobin (Hb), C-reactive protein (CRP), serum Interleukin 8 (IL-8), CD4/CD8 cells in the blood and fecal calprotectin (Cal) between Week 0, 8, 12, and 24. The data are expressed as the means ± SEM. n = 13–17. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ (paired t-test).

that the therapeutic target cells of hUC-MSCs might be macrophages. To confirm the hypothesis, a macrophage and hUC-MSCs co-culture system was set up. THP-1 cells were firstly induced to M0 by treated with 100 ng/mL PMA for 48 h and then cultured with 100 ng/mL lipopolysaccharide (LPS) and 20 ng/mL IFN-γ for another 48 h to induce M1polarization (Fig. 7A). As shown in Fig. 7B, the morphology of THP1, M0, and M1 macrophages were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PDE6A
CD177
SLC38A4
SLC26A2
CNTFR
NTRK2
NPY1R
CNTN3
LYPD8
PCDH20
SLC26A3
CDHR1
IGSF9
EDA
SEMA3E
CNTN4
NRXN1
GFRA1
PPARG
PROM1
SEMA5A
ATP1A2
EDN1
SCN9A
CNR1
PDCD4
MUC20
logFC
1
12
axonal fasciculation
sensory perception of pain
anchored component of membrane
microvillus membrane
axon guidance
photoreceptor outer segment membrane
integral component of plasma membrane
plasma membrane
response to nicotine
positive regulation of vascular associated smooth muscle cell apoptotic process
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Case-vs-Con(Down)
GO Enrichment top 10
CXCL9
HLA-J
SERPINE1
FCGR1A
CXCL11
CXCL10
CSF3R
IL1RN
CXCL8
FPR1
CXCR2
S100A9
TNFAIP6
TREM1
CLEC5A
LILRA5
IL11
S100A12
FCAR
IL3RA
CLEC4E
S100A8
IL13RA2
MARCO
CD300E
OSM
CXCR1
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HCAR3
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JAK-STAT signaling pathway
Malaria
Legionellosis
Leishmaniasis
Rheumatoid arthritis
Amoebiasis
Stapylococcus aureus infection
Tuberculosis
AGE-RAGE siganling pathway in diabetes complication
Osteoclast differentiation
IL−17 signaling pathway
Toll-like receptor siganling pathway
NOD-like receptor siganling pathway
Chemokine signaling pathway
CellP
EnvIP
HumD
OrgSy
4
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10
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5e−05

different. The mRNA expressions of CD86 and NOS2 were detected by RT-PCR to identify M1 polarization. As revealed in Fig. 7C, the expressions of CD86 and NOS2 mRNA were significantly up-regulated in M1 compared to M0 macrophages, and obviously down-regulated after co-cultured with hUC-MSCs. Meanwhile, similar patterns were seen in the expression of IL-1β, IL-6, and TNFα (Fig. 7C). These data suggested that hUC-MSCs improved CD by reducing the M1 polarization of macrophages and thereby reducing their secretion of pro-inflammatory cytokines.

As previously reported, hUC-MSCs secreted cytokines such as TSG-6, indoleamine 2,3-dioxygenase (IDO), and TGFβ,[15] while TSG-6 could suppress the expression of proinflammatory transcription factors pSTAT1 and might be a key factor for hUC-MSCs therapy to alleviate intestinal ulcers. To further explore the possible mechanism of hUC-MSCs on M1 macrophage polarization, we further explored our RNA-sequencing results. We found that the expression of STAT1 and STAT3 decreased significantly after treatment of hUC-MSCs (Supplementary Fig. S3A). Meanwhile, we observed a decreased expression of M1 specific makers (CD80 and CD86) and proinflammatory cytokines (Supplementary Fig. S3B). We then measured the levels of TSG-6 in the supernatant of hUC-MSCs culture by ELISA and the expression of pJAK1, and pSTAT1 in intestinal mucosa by IHC with/without hUC-MSCs co-culture. A higher level of TSG-6 was detected in hUC-MSCs culture supernatant after stimulated by TNFα, IFN,γ and LPS, which simulates an inflammatory environment (Fig. 8A), and the expressions of pJAK1 and pSTAT1 in intestinal mucosa of CD patients decreased significantly with hUC-MSCs co-culture (Fig. 8B and C). These results implied that TNFα-stimulated gene-6 (TSG6) secreted by hUC-MSCs may reduce M1 macrophage polarization by inhibiting the phosphorylation of JAK/STAT1, as shown in the graphical abstract of our study (Fig. 8D).

Discussion

CD is a chronic inflammatory bowel disease characterized by progressive transmural damage leading to complications. The effectiveness of existing medical treatments including biological agents does not exceed 60%, therefore, there is a need for new disease-modifying treatments with an alternative mechanism of action that are safe and well tolerated.

Three previous clinical studies investigated the safety and efficacy of local injection of MSCs, one for the treatment of short-segment stenosis and another two for the treatment of anal fistula in CD. The stem cells used in these studies were allogeneic bone marrow derived MSC and all proven to be safe and effective.[13,25,29] The efficacy of local injection of adipose-derived MSCs was also confirmed in several small series in fistulizing perianal CD.[30–33] Another study demonstrated safety of MSCs intravenous infusion for CD treatment, but efficacy required improvement.[34] Allogenic hBM-MSCs are generally obtained from bone marrow discarded from knee or hip surgery. The proliferative and differentiation capacities of these stem cells are significantly reduced, and their transplantation into allogeneic bodies may result in immune reactions, which limits their clinical applications. Compared to MSCs from alternative sources, the notable advantages of hUC-MSCs include fewer ethical concerns, painless acquisition from discarded umbilical cords, and absence of immune response.[35] So far, we have not found any previous report using hUC-MSCs submucosal injection combined with intravenous infusion to treat refractory ulcers in CD patients.

hUC-MSCs have been used in many research studies using different rat disease models, such as acute-on-chronic liver failure, subacute spinal cord injury, osteoarthritis, acute lung injury, and diabetes, which demonstrated that hUC-MSCs were beneficial for these diseases.[18,36–39] Consistent with previous studies, our results showed that hUC-MSCs ameliorated colitis in rats. Further clinical study demonstrated that the administration of hUC-MSCs by local injection combined with intravenous infusion was safe and well tolerated, with only one patient experiencing a self-limited fever. The CDAI and SES-CD score were improved after hUC-MSCs treatment, with few side effects. IL8 levels were reduced starting from week 4. CRP and fecal calprotectin, recognized as biomarkers for CD activity, also reached statistical significance in our study. For the primary endpoint of SES-CD, a reduction from baseline was reported at week 12. Of 12 patients, 8 (47%) had an endoscopic response, and three (17.65%) had endoscopic remission at week 12. It's reported that the cumulative rate of endoscopic remission with UST at week 16 was 17%,[40] which was similar with hUC-MSCs

Fig. 5: Results of transcriptome sequencing of intestinal tissues before and after hUC-MSCs treatment. (A) Hierarchical cluster analysis of all DEGs in the two groups, Con: before hUC-MSCs treatment, n = 3; Case: after hUC-MSCs treatment 12 weeks, n = 3. Q value < 0.05, |log2FC|>1. (B) Volcano map of mRNA expression before and after hUC-MSCs treatment. Q value < 0.05, |log2FC|>1. (C) Top10 of Gene Ontology (GO) analysis of up-regulated genes and their pathways after hUC-MSCs treatment. (D) Top10 of GO analysis of down-regulated genes and their pathways after hUC-MSCs treatment. (E) Top 20 pathways of KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment for the downregulated genes after hUC-MSCs treatment.

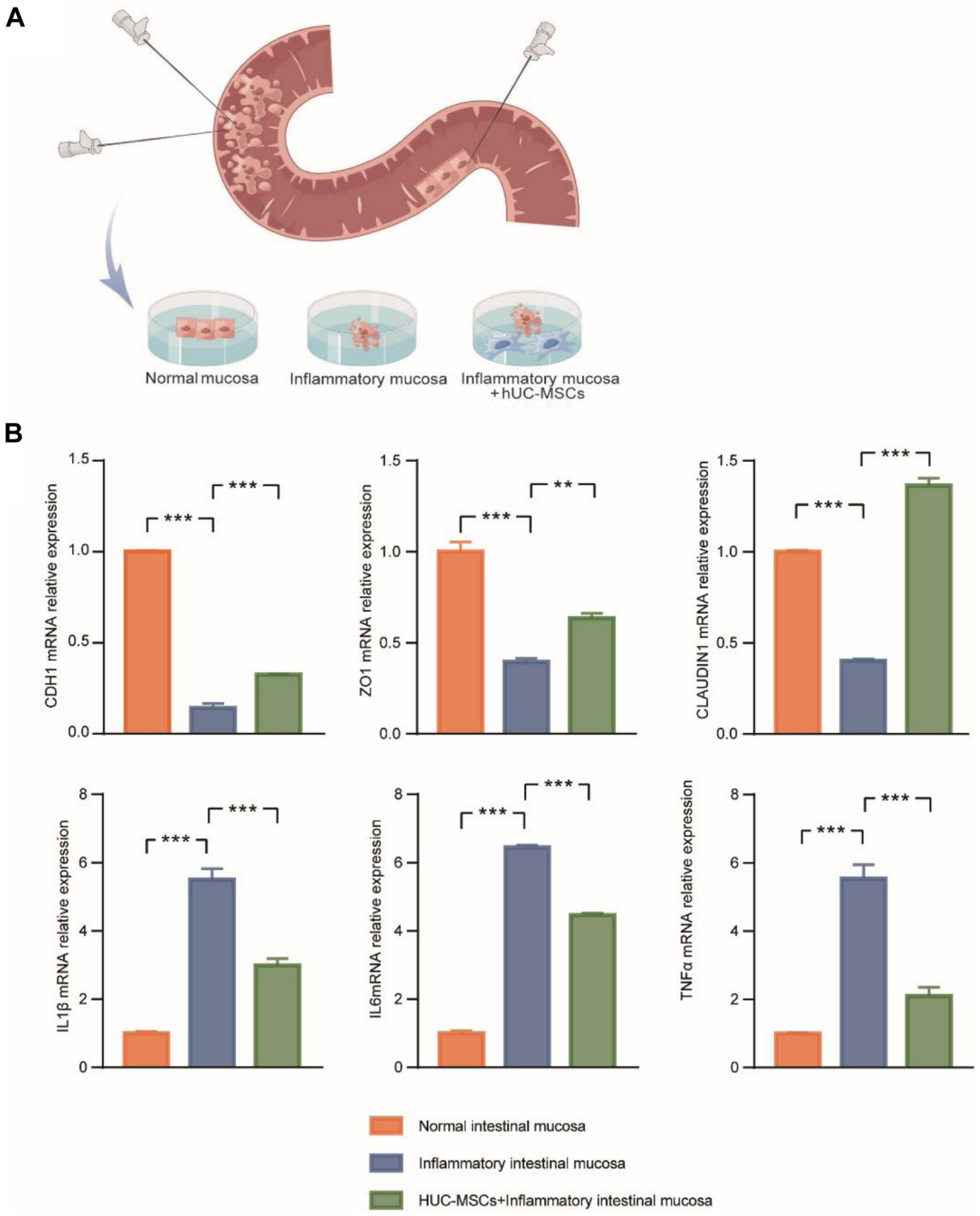

Fig. 6: Co-culture of hUC-MSCs and intestinal tissue in vitro. (A) Schematic diagram of co-culture. Normal intestinal mucosa, inflamed intestinal mucosa, and hUC-MSCs + inflamed intestinal mucosa was cultured in vitro for 24 h, separately. (B) Effect of hUC-MSCs on tight junctions and adherent junctions of intestinal mucosal epithelial cells, and expression of inflammatory factors (n = 4). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (One-way ANOVA with Tukey's multiple comparisons test).

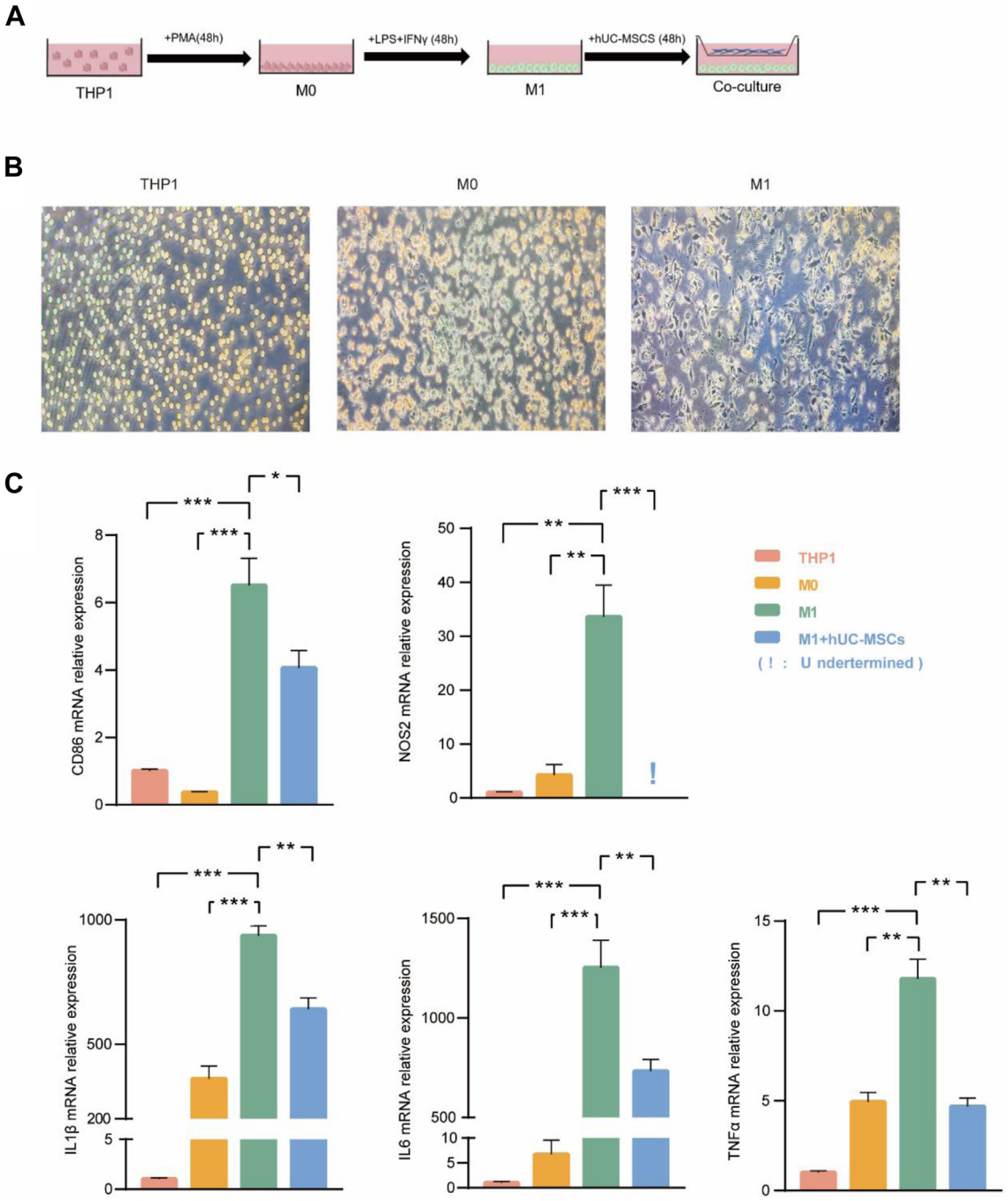

Fig. 7: Human THP-1 monocyte differentiation into M1 macrophages and hUC-MSCs attenuated macrophage M1 polarization. (A) Schematic diagram of the experiment. THP-1 cells were treated with 100 ng/mL PMA for 48 h and then cultured with 100 ng/mL lipopolysaccharide (LPS) and 20 ng/mL IFN-γ for a further 48 h. (B)The morphology of THP1, M0 (non-polarized THP-1 cells), and M1 macrophages. (C) The mRNA expression of M1 markers were measured by QT-PCR (n = 3). Data are presented as the mean ± SEM from three independent experiments. All results show the relative fold change compared to M1 macrophages. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$(One-way ANOVA with Tukey's multiple comparisons test). IL, interleukin; TNF-α, tumor necrosis factor-α; NOS2, nitric oxide synthase 2.

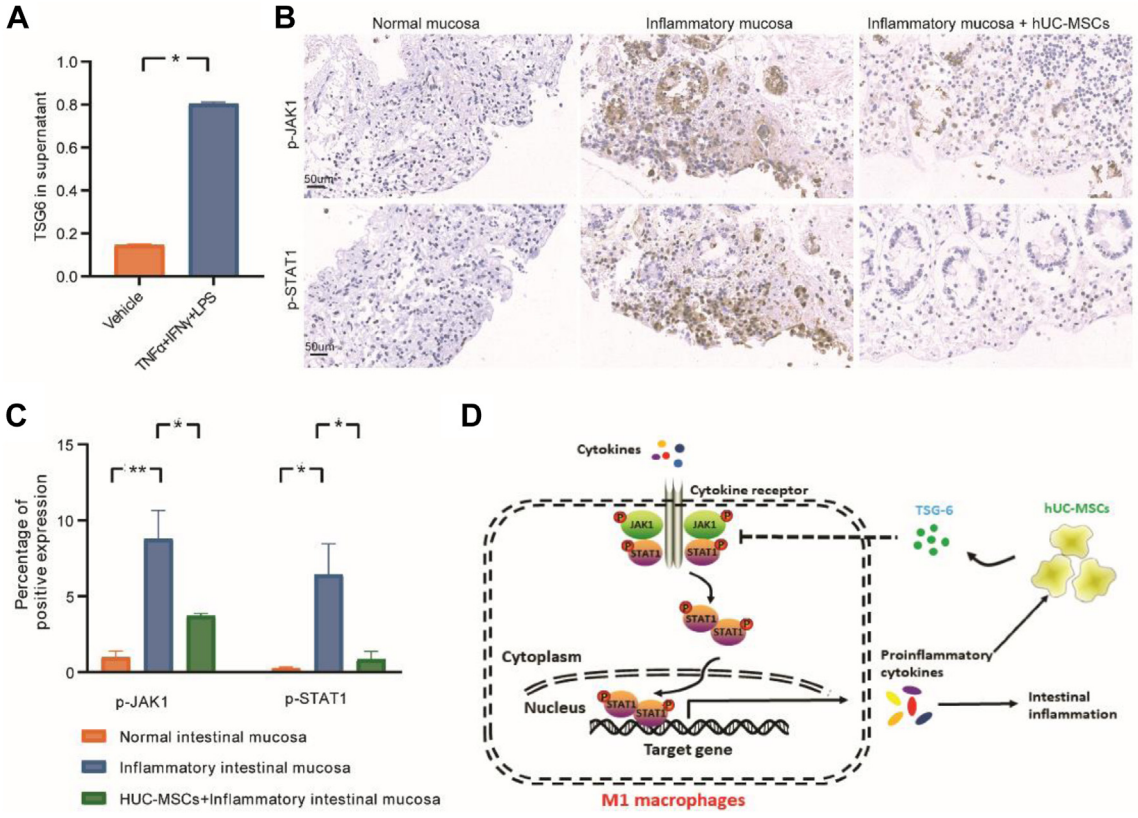

Fig. 8: TSG6 secreted by hUC-MSCs reduced M1 macrophage polarization by inhibiting the phosphorylation of JAK1 and STAT1. (A) TSG-6 expression in hUC-MSCs culture supernatant. HUC-MSCs were treated with TNFα (20 ng/mL), IFNγ (20 ng/mL) + LPS (10 pg/mL) for 48 h, and TSG-6 expression was detected in the culture supernatant using ELISA. (B), (C) The expression of p-JAK1/p-STAT1 was examined by IHC in intestinal mucosa with/without hUC-MSCs co-culture. (D) The graphical abstract of our study. *, $p < 0.05$; **, $p < 0.01$ (Two sample compared used unpaired t-test, and more than two samples used One-way ANOVA with Tukey's multiple comparisons test).

therapy in our study. In these patients with moderately or severely active CD, hUC-MSCs treatment was associated with clinical remission and healing of intestinal ulcers. Although the lack of a contemporaneous control group precludes definitive conclusions, the magnitude of the benefit observed on endoscopically defined disease activity is consistent with a meaningful treatment effect, and this-MSCs transplantation approach may show potential for further CD therapeutic development. Large-scale, randomized, double-blind, and placebo-controlled clinical studies are needed to reduce bias and confirm our findings in the future.

On the basis of the above-mentioned clinical study, we further explored the possible mechanism of hUC-MSCs in the treatment of intestinal ulcers in CD. We found that treatment with hUC-MSCs was associated with a reduction in the expression of genes involved in inflammatory response, such as IL-1β and IL-6, and the upregulation of genes related to maintenance of intestinal epithelial barrier integrity, such as SLC26A3[41] by GO enrichment analysis. According to KEGG enrichment analysis, we found that hUC-MSCs treatment was associated with the downregulation of TNF signaling pathway, IL17 signaling pathway, and TLR signaling pathway, all of which are thought to be major pro-inflammatory pathways in the pathogenesis of CD.[42] Further in vitro experiments with co-culture of inflammatory intestinal mucosa with hUC-MSCs also resulted in reduced expression of IL-1β, IL-6, and TNFα mRNA levels. In addition, the RNA expression of genes related to intestinal barrier integrity such as ZO1, CLAUDIN1, and CDH1 were increased by hUC-MSCs treatment. IL-1β, IL-6, and TNFα are mainly secreted by macrophages, which are known to recognize microbe-associated molecular patterns (MAMPs) via Toll-like receptors (TLRs).[43] Increased TNFα and IL-1β levels have been previously shown to impair the epithelial barrier function by injured tight junctions via NF-κB in IBD patients.[44,45] The level of IL-6 and its receptor have been reported to be increased in the serum and intestinal mucosa in CD patients,[46,47] while the anti-IL-6 receptor antibodies have been shown to improve intestinal inflammation in CD.[48] Therefore, we further used hUC-MSCs and M1 macrophages co-culture system to

investigate the influence of hUC-MSCs on macrophages polarization. As expected, hUC-MSCs inhibited macrophages M1 polarization and decreased the expression of IL-1β, IL-6, and TNFα, all of which impaired intestinal barrier integrity in CD. This was in line with other findings using experimental colitis models that hUC-MSCs could modulate immunity and restore intestinal epithelial barrier integrity.[49–51] The immunomodulatory activity of hUC-MSCs is mainly provided by the paracrine pathway.[52] The major paracrine factors included prostaglandin E2 (PGE2), IDO, TGFβ1, TSG-6, and so on. The secretion of IDO, PEG-2, and TGFβ inhibited the activation and proliferation of T cells; IL-10, PEG2, and TGFβ activated Treg cells; Activin A, PGE2, and HLA-G6 decreased the cytotoxicity of NK cells and its production of IFN-γ; TSG6 and MCP-1 induced M1 macrophages polarized to M2 macrophages.[15] Intestinal macrophages have a critical role in maintaining tissue homeostasis and inducing resolution after inflammation.[53] Studies showed that macrophage polarization disorder impaired the resolution of intestinal inflammation, and consequently attenuated bacterial clearance and stimulated excessive cytokine secretion in IBD patients.[54–56] Our results were consistent with previous studies mentioned above and suggested hUC-MSCs inhibited macrophages M1 polarization and decreased the expression of IL-1β, and IL-6, thereby restoring impaired intestinal barrier integrity in CD.

It has been reported that several pathways and key molecules were involved in M1 macrophages polarization, including STAT1, IRF5, NF-κB, and AP1.[57] In our study, the results of RNA-sequencing showed that the expression of STAT1, M1 macrophage-associated specific markers, and pro-inflammatory factors in intestinal mucosa decreased significantly in intestinal mucosa samples from CD patients after hUC-MSCs treatment. Moreover, it's reported that hUC-MSCs secreted lots of cytokines such as TSG-6, IDO, and TGFβ,[15] while TSG-6 could suppress the expression of proinflammatory transcription factors pSTAT1[58] and might be a key factor for hUC-MSCs therapy to alleviate intestinal ulcers. Our results found the level of TSG6 in the supernatant of hUC-MSCs culture elevated obviously by TNFα, IFN,γ and LPS stimulation and the expression of pJAK1/pSTAT1 in intestinal mucosa attenuated after co-culture with hUC-MSCs. And the mRNA expression of M1 related proinflammatory cytokines was also reduced both in intestinal mucosa and M1 macrophage after co-cultured with hUC-MSCs. All these elucidated that hUC-MSCs mitigated M1 macrophage polarization at least partly through TSG-6–JAK1/STAT1 axis.

Our study has some limitations: First, it is a small-scale, single-arm, non-double-blind, non-randomized, single-center, uncontrolled study; Second, further studies are needed to explore the in-depth mechanism of the treatment; Third, the follow-up time was 24 weeks, which may not be long enough compared with some other MSCs clinical studies. All these limitations will be addressed in our future research.

In summary, we established an approach of hUC-MSCs administration for CD treatment and provide data that suggest that hUC-MSCs may inhibit the inflammation response and improve intestinal barrier integrity by decreasing macrophages M1 polarization. Future follow-up research is needed to further confirm the efficacy of the treatment and to explore the underlying mechanisms of the therapy.

Contributors

Lan Zhong and Daxiang Cui contributed substantially to the study design. Qinjuan Sun and Shan Li wrote the manuscript. Qinjuan Sun, Shan Li, Guangxi Zhao, and Ritian Lin performed the basic experiments. Qinjuan Sun and Shan Li contributed to the statistical analysis and analysis and interpretation of data. Bin Liu provided material support. Yushuang Wei and Wenwen Jia prepared hUC-MSCs. Jinlai Lu, Miao Hu, Wei Wang, and Xiaoqing Yang completed the follow-up, recorded clinical and laboratory data after hUC-MSCs treatment. Yanni Hu, Wei Zhang, and Jiawen Zhu gave the intravenous of hUC-MSCs to patients. All authors contributed to data collection and interpretation, reviewed the manuscript, provided important intellectual contributions to its development, and approved the final version for publication. Qinjuan Sun and Shan Li have accessed and verified the data, and Lan Zhong was responsible for the decision to submit the manuscript.

Data sharing statement

The authors declare that all the analysis data can be found in the main text and the Supplementary files. The raw data generated in this study are not publicly available due to the privacy protection policy of personal medical data at our institution but are available for non-commercial purposes from the corresponding author on reasonable request.

Declaration of interests

The authors declare no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105128.

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