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Original Article

HucMSC-Ex alleviates DSS-induced colitis in mice by decreasing mast cell activation via the IL-33/ST2 axis

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Abstract: Background: Inflammatory bowel disease (IBD) is a chronic inflammatory disease that poses challenges in terms of treatment. The precise mechanism underlying the role of human umbilical cord mesenchymal stem cell-derived exosome (HucMSC-Ex) in the inflammatory repair process of IBD remains elusive. Mucosal mast cells accumulate within the intestinal tract and exert regulatory functions in IBD, thus presenting a novel target for addressing this intestinal disease. Methods: A mouse model of Dextran Sulfate Sodium (DSS)-induced colitis was established and hucMSC-Ex were administered to investigate their impact on the regulation of intestinal mast cells. An in vitro co-culture model using the human clonal colorectal adenocarcinoma cell line (Caco-2) and human mast cell line (LAD2) was also established for further exploration of the effect of hucMSC-Ex. Results: We observed the accumulation of mast cells in the intestines of patients with IBD as well as mice. In colitis mice, there was an upregulation of mast cell-related tryptase, interleukin-33 (IL-33), and suppression of tumorigenicity 2 receptor (ST2 or IL1RL1), and the function of the intestinal mucosal barrier related to intestinal tight junction protein was weakened. HucMSC-Ex treatment significantly reduced mast cell infiltration and intestinal damage. In the co-culture model, a substantial number of mast cells interact with the epithelial barrier, triggering activation of the IL-33/IL1RL1 (ST2) pathway and subsequent release of inflammatory factors and trypsin. This disruption leads to aberrant expression of tight junction proteins, which can be alleviated by supplementation with hucMSC-Ex. Conclusion: Our results suggest that hucMSC-Ex may reduce the release of mast cell mediators via the IL-33/IL1RL1 (ST2) axis, thereby mitigating its detrimental effects on intestinal barrier function.

Keywords: Mast cell, IBD, hucMSC-Ex, intestinal barrier

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are two fundamental types of inflammatory bowel disease (IBD), and their clinical manifestations usually include abdominal pain, diarrhea, rectal bleeding, and weight loss [1]. Recent epidemiological and clinical studies underscore the imperative of identifying novel therapeutic targets to effectively counteract the progression of IBD, given its chronic nature and profound impact on patients' quality of life [2]. Studies have shown that IBD arises from the combined influence of the environment, genes, intestinal microbes, and immunity. Human umbilical cord mesenchymal stem cells (hucMSCs), as a kind of stem cell with low immunogenicity and strong differentiation ability, have attracted attention due to their great potential in regenerative medicine. Exosomes derived from hucMSCs (hucMSC-Ex) are extracellular microvesicles that exhibit equivalent biological activities to their source cells. Exosomes derived from hucMSCs (hucMSC-Ex) are extracellular microvesicles and have the same biological activities as their source cells.

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The therapeutic efficacy of hucMSC-Ex in IBD has been substantiated primarily through its pivotal roles in inflammatory repair, immune regulation, and tissue regeneration [3, 4]. Therefore, elucidating the mechanism of action of hucMSC-Ex and developing new treatment programs may potentially yield a groundbreaking advancement in the treatment of patients with IBD.

The gut constitutes a complex environment wherein the intestinal mucosal barrier, nervous system, gut microbiota, and immune system coexist [5, 6]. The immune system comprises various cellular components, including mast cells (MCs), dendritic cells, neutrophils, macrophages, lamina propria, and intraepithelial lymphocytes, all of which interact with each other. Therefore, altering one element can affect gut homeostasis [7, 8]. Our previous study showed that hucMSC-Ex modulates pyroptosis of intestinal macrophage via the miR-378a-5p/NOD-like receptor protein 3 (NLRP3) axis for the restoration IBD [9]. HucMSC-Ex is also critical for neutrophil regulation, and our previous findings suggest that hucMSC-Ex is able to reduce extracellular regulated protein kinases (ERK) phosphorylation and reduce the polarization of neutrophils to a “proinflammatory” phenotype, thereby reducing inflammation levels [4]. Furthermore, hucMSCs were administered to mice with TNBS-induced colitis by other researchers, revealing the significant dose-dependent reduction in inflammation. This treatment increases the proportion of type-1 regulatory T (TR1) cells in spleen and mesenteric lymph nodes at different stages of colitis, and decreases the proportion of T helper (Th)-1 and Th17 cells [10]. Therefore, investigating the regulatory impact of hucMSC-Ex on immune cells represents a crucial area for exploration of IBD.

Mast cells are granular immune cells derived from hematopoietic stem cells. The intestinal mucosa serves as a site of mast cells in the human body. These cells can be detected in the lamina propria of the mucosa and in the muscularis adjacent to nerve fibers [11, 12]. Mast cells are activated in response to stimuli and secrete bioactive products with inflammatory or immunosuppressive properties, including tryptase, chymotrypsin, carboxypeptidase, cytokines, chemokines, and biogenic amines [11, 13]. According to the differential expression of surface receptors on mast cells, they exhibit distinct responses to external stimuli to regulate their microenvironment, mainly manifesting as surface receptor-ligands binding, inducing mast cell activation and the release of particles formed in the cytoplasm. Protease, histamine, and cytokines in the particles play a biological role in regulating intestinal balance [14, 15]. The role of mast cells in the intestinal barrier is intricate, encompassing the regulation of intestinal epithelium function and integrity, as well as the innate and adaptive immunity of the mucosa [16, 17]. In addition, hyperactivated mast cells have been shown to exert a detrimental impact on the integrity of the intestinal mucosal barrier [13, 18], as they exhibit a dualistic role in the pathogenesis of IBD. Inhibition of adenosine triphosphate (ATP)-mediated mast cell activation has been found to decelerate experimental colitis, implying that mast cell activation plays a pro-inflammatory function in this model [19]. Intestinal fibroblasts are activated by mast cell-derived tryptase via the protease-activated receptor 2/protein kinase B/the mammalian target of rapamycin (PAR-2/Akt/mTOR) pathway [12]. Conversely, mast cell-derived prostaglandin D2 (PGD2) has been shown to act as an inhibitor of colitis and colon cancer [20]. Mast cells appear to play an anti-inflammatory role in studies of spontaneous experimental colitis [21]. Furthermore, there is a widespread consensus that gastrointestinal mast cells play a crucial role in maintaining the integrity of the barrier function [13, 22]. Trypsin, which is released by mast cell activation, is thought to reduce junctional adhesion molecule-A (JAM-A) expression in intestinal epithelial cells, thereby compromising intestinal barrier function [23]. Therefore, elucidating the mechanism of mast cells in intestinal diseases may become a breakthrough point to curing intestinal inflammatory diseases.

IL-33 is abundantly expressed in intestinal epithelial cells and is released as an alarm signal when cells are damaged. It is recognized by the IL-33 receptor IL1RL1 (ST2) on the surface of mast cells, thus activating downstream signals to play a role. The literature suggests that IL-33 and its receptor IL1RL1 (ST2) pathway in mast cells exhibit a multifaceted mechanism

of action in inflammatory diseases, while also playing a crucial role in the reparative processes associated with experimental colitis [24]. Perhaps the target of IL-33 to intervene in mast cell activation in mice may offer novel therapeutic avenues for the treatment of various diseases [25]. Currently, the precise mechanisms of the IL-33/ST2 axis in mast cells in IBD remain elusive and necessitate further investigation. It has been reported that tonsil-derived MSC exosomes can inhibit mast cell activation under inflammatory conditions [26]. HucMSCs can reduce interstitial cystitis by inhibiting mast cells [27]. However, limited research has been conducted on the precise mechanism through which exosomes modulate the IL-33/ST2 axis of mast cells for alleviating IBD. Based on the role of mast cells in intestinal barrier destruction, we speculated that hucMSC-Ex might repair IBD by blocking the IL-33/ST2 pathway. We successfully isolated, purified, and identified hucMSC-Ex, and constructed an experimental mouse model of colitis induced by DSS treatment, as previously reported [9, 28, 29]. Based on this premise, we explored the role of IL-33/ST2 in IBD, and subsequently established a cell co-culture model to highlight the clinical application value of hucMSC-Ex as a mast cell stabilizer. This study provides a new experimental basis for the clinical application of hucMSC-Ex in the prevention and treatment of IBD and has far-reaching research significance and clinical application value in intestinal diseases.

Materials and methods

HucMSC isolation and culture

Freshly delivered human umbilical cord tissue was rinsed in PBS containing 1% Penicillin-Streptomycin-Amphotericin B (Solarbio, P7630, Beijing, China), and veins, arteries, and blood cells were removed. The cleaned tissue was sectioned into small pieces measuring approximately 1 mm^3 and pasted on the surface of a sterile petri dish. After the umbilical tissue was carefully positioned within the culture dish, the tissue blocks were covered with alpha minimal essential medium (α-MEM) (Hyclone, SH30265.01, Beijing, China) supplemented with 15% fetal bovine serum (FBS) (Excell Bio, FSP500, Uruguay) and 1% Penicillin-Streptomycin-Amphotericin B Solution medium and cultured at 5% CO_2, 37°C. The medium was replaced with a fresh one daily, and the spindle-shaped morphology of hucMSCs was observed. The hucMSCs were passed into new Petri dishes using α-MEM supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin-Amphotericin B Solution medium. In the following passage, after each generation of cells adhered to the wall, the medium was changed to the complete FBS medium with the absence of exosomes, and ultracentrifugation was carried out for more than 24 h, and the cell supernatant was collected.

Extraction and identification of hucMSC-Ex

The cell culture supernatant collected in the previous step was filtered using a 0.22 μm (SLGP033R, Millipore, Carrigtwohill Co. Cork, Ireland) filter for later use. Cell debris and organelles were eliminated through a series of centrifugation steps at 4°C with forces of 300 g, 2000 g, and 10000 g. The obtained supernatant was subjected to continuous centrifugation using a 100 kDa ultrafiltration tube (UFC9100, Millipore, Carrigtwohill Co. Cork, Ireland) at 2000 g for 30 minutes each time. The concentrated supernatant was further centrifuged at 4°C for 2 hours at 100,000 g, so that hucMSC-Ex could be sedimented at the bottom of the test tube in the form of a thin film. The supernatant was aspirated, followed by a PBS wash and subsequent centrifugation for 2 hours. Excess PBS was subsequently decanted. PBS solution was finally added according to the precipitation amount and stored in a refrigerator at -80°C for later use. Fresh unfrozen hucMSC-Ex was taken, and the particle number and size were detected by NanoSight nanoparticle tracking analysis (NTA) (ZetaView, Germany). Transmission electron microscopy (TEM) (Hitachi TEM system) was used to observe the structure of hucMSC-Ex. Western blot was used to detect the positive markers CD9 (1:500; AF5139, Affinity Biosciences LTD., Jiangsu, China), CD81 (1:500; sc-18877, Santa Cruz Biotechnology, USA), and the negative marker Calnexin (1:500; AF5362, Affinity Biosciences LTD., Jiangsu, China).

Cell culture and co-culture

Caco-2 (Procell, Wuhan, China) was cultured in the special medium of Caco-2 (CM-0050,

Procell, Wuhan, China) at 37°C in an incubator containing 5% CO_2. Human mast cell line LAD2 was purchased from Nanjing Saihongrui Biotechnology Co., Ltd. and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone SH30022.01, UT, USA) with 10% FBS and 1% Penicillin-Streptomycin-Amphotericin B Solution, and incubated at 37°C with 5% CO_2. For the co-culture, Caco-2 was incubated in the transwell insert plates (LABSELECT) for 3-5 days and 2×10^6 LAD2 cells were incubated in the 6-well (LABSELECT) plates for 24 h. When Caco-2 cells began to differentiate, transwell insert plates were placed in six-well plates containing LAD2. In the hucMSC-Ex group, 400 μg/mL hucMSC-Ex were applied to both the upper and lower sides. Caco-2 and LAD2 were cultured separately as controls for the coculture. After 24 h incubation, Caco-2 and LAD2 were collected respectively for analysis.

Establishment of IBD mouse model

Male BABL/C mice (6-8 weeks old, 20 ± 3 g) were purchased from the Animal Research Center of Jiangsu University (Jiangsu, China). The animals were kept in a room with a temperature of 21 ± 2°C, a relative humidity of 30-70%, and a light cycle of 12/12 hours. The study was carried out with the approval of the Ethics Committee and the Laboratory Animal Management and Use Committee of Jiangsu University (protocol code UJS-IACUC-2022120601 and date of approval 20230517). Fifteen mice were randomly divided into 3 groups (normal control-NC, DSS, and hucMSC-Ex). The mice in the NC group were provided with tap water for consumption, while the DSS and hucMSC-Ex groups received a 3% Dextran Sulfate Sodium Salt (DSS, 0216011080, MP Biomedicals, CA, USA) solution to induce acute enteritis. The mice in the hucMSC-Ex group were administered with hucMSC-Ex containing a total protein content of 1 mg through tail-vein injection three times (the third day, the sixth day, and the ninth day) [28, 29]. The mice were weighed daily and their survival conditions were meticulously documented. Upon manifestation of evident enteritis symptoms in the DSS mice, humane euthanasia was performed, followed by collection of their colons for subsequent analysis.

Determination of intestinal barrier function

The intestinal barrier function of mice was evaluated by fluorescein isothiocyanate (FITC)-Dextran (60842-46-8, Sigma-Aldrich, St. Louis, MO, USA) with a molecular weight of 4 kDa (FD4). Mice with an intact and functional intestinal barrier effectively prevent the entry of FD4 into the bloodstream, while a compromised intestinal barrier allows for leakage of FD4 into the bloodstream. On the day before the end of the IBD model, mice were starved for 8 hours and then fed with an FD4 concentration of 10 mg/mL (0.1 mg/g). Three hours later, the eyeball blood of mice was taken and centrifuged at 12,000 g for 3 minutes. The standard curve was made by FD4-fold dilution with the highest concentration of 0.02 mg/mL. The fluorescence intensity was quantified using a fluorescence microplate reader, with excitation at 490 nm and emission at 520 nm, followed by calculation of the FD4 concentration.

Western blot

The colon tissue of mice was washed with precooled PBS and the intestinal mucosa was scraped for analysis. The total protein of the mouse colon and the cultured cell was extracted with RIPA lysate (R0010, Solarbio, Beijing, China) supplemented with protease inhibitors PMSF (329-98-6, Solarbio, Beijing, China). The concentration of total protein was determined using a BCA kit (E112-01, Vazyme, Jiangsu, China). To denature the total protein, the loading buffer (B0007, Invitrogen, CA, USA) was added according to the manufacturer's instructions and immersed in a water bath at 100°C for 8-10 minutes. According to the molecular weight of the target molecule, electrophoresis was carried out with SDS gels with different concentrations. After electrophoresis, the protein was transferred to polyvinylidene fluoride PVDF membrane (IPFL00010, Millipore, Carrigtwohill Co. Cork, Ireland), blocked with 5% skimmed milk for 2 h, washed by TBST buffer (T1081, Solarbio, Beijing, China), and incubated with the corresponding primary antibodies at 4°C overnight. The excess antibody was washed with TBST buffer and incubated with secondary antibodies at 25°C for 1 hour. Protein bands were observed by a chemical gel imaging system (mageQuant LAS 4000mini, GE, USA). The antibodies used

Table 1. Primer sequence

Gene	Primer sequence
Mouse-Claudin-1-F	AGATACAGTGCAAAGTCTTCGA
Mouse-Claudin-1-R	CAGGATGCCAATTACCATCAAG
Mouse-Occludin-F	TGCTTCATCGCTTCCTTAGTAA
Mouse-Occludin-R	GGGTTCACTCCCATTATGTACA
Mouse-ZO-1-F	CTGGTGAAGTCTCGGAAAAATG
Mouse-ZO-1-R	CATCTCTTGCTGCCAAACTATC
Mouse-IL-33-F	CAGAAGACCAAAGAATTCTGCC
Mouse-IL-33-R	CATGCTTGGTACCCGATTTTAG
Mouse-IL1RL1-F	CGACAGTACGTGGATCAGATAA
Mouse-IL1RL1-R	GAGCTTTGCAGTTCTTAAACCA
Mouse-IL-6-F	GACATGAGGCTTCTGAGAGTAA
Mouse-IL-6-R	TCCGCAGAAATGTATCCAGTAG
Mouse-IL-10-F	CAGAAGACCAAAGAATTCTGCC
Mouse-IL-10-R	CATGCTTGGTACCCGATTTTAG
Mouse-TNF-A-F	ATGTCTCAGCCTCTTCTCATTC
Mouse-TNF-A-R	GCTTGTCACTCGAATTTTGAGA
Mouse-IL-1B-F	CACTACAGGCTCCGAGATGAACAAC
Mouse-IL-1B-R	TGTCGTTGCTTGGTTCTCCTTGTAC
Mouse-MCP-6-F	GACATGAGGCTTCTGAGAGTAA
Mouse-MCP-6-R	TCCGCAGAAATGTATCCAGTAG
Human-ZO-1-F	AAAGAGAAAGGTGAAACACTGC
Human-ZO-1-R	TTTTAGAGCAAAAGACCAACCG
Human-Claudin-1-F	TCTTGCAGGTCTGGCTATTTTA
Human-Claudin-1-R	TTGGGTAAGAGGTTGTTTTTCG
Human-Occludin-F	AACTTCGCCTGTGGATGACTTCAG
Human-Occludin-R	TTTGACCTTCCTGCTCTTCCCTTTG
Human-IL-6-F	CACTGGTCTTTTGGAGTTTGAG
Human-IL-6-R	GGACTTTTGTACTCATCTGCAC
Human-IL-33-F	GCTTTGCCTTTGGTATATCAGG
Human-IL-33-R	CTGATTCATTTGAGGGGTGTTG
Human-IL1RL1-F	GCTACCTGGAGAAGATGTAGTC
Human-IL1RL1-R	TCGTAGGCAAACTCCTTATTGT
Mouse-B-Actin-F	GTGCTATGTTGCTCTAGACTTCG
Mouse-B-Actin-R	ATGCCACAGGATTCCATACC
Human-B-Actin-F	CCTGGCACCCAGCACAAT
Human-B-Actin-R	GGGCCGGACTCGTCATAC

were: anti-CD9 (1:500; AF5139, Affinity Biosciences LTD., Jiangsu, China), anti-CD81 (1:500; sc-18877, Santa Cruz Biotechnology, USA), anti-Calnexin (1:500; AF5362, Affinity Biosciences LTD., Jiangsu, China), anti-Occludin (1:5000; 66378-1-Ig, Proteintech, Wuhan, China), anti-ZO-1 (1:1000; 21773-1-AP, Proteintech, Wuhan, China), anti-Claudin-1 (1:1000; 28674-1-AP, Proteintech, Wuhan, China), anti-β-actin (1:1000; sc-47778, Santa Cruz Biotechnology, USA), Goat anti-rabbit IgG (1:10000; RS0002, Immunoway, USA), and Goat anti-mouse IgG (1:10000; RS0001, Immunoway, USA).

QRT-PCR

Total RNA from cells and tissues were extracted by trizol (7E510A1, Vazyme, Jiangsu, China). The extracted mRNA was reverse transcribed by a reverse transcription kit (R312-01/02, Vazyme, Jiangsu, China). Real-time fluorescence quantitative PCR (qRT-PCR) was performed with Synergy Brands Green (SYBR) Detection (Q511-02, Vazyme, Jiangsu, China) to measure the transcript abundance of the gene. Using the mRNA expression of β-actin as a control, the relative expression level of the target gene was calculated. The sequences of the primers used are listed in **Table 1**.

Hematoxylin and eosin staining (H&E)

The colon tissue of mice was washed with PBS buffer, then immersed in 4% paraformaldehyde (P1110, Solarbio, Beijing, China) for fixation, processed, embedded in paraffin, and cut into 4 μm. After dewaxing and a series of ethanol clearance, hematoxylin and eosin was applied, followed by sealing and observation under light microscopy (10×). Images were obtained by pathological section scanner.

Toluidine blue staining

The intestinal tissue sections of mice were dewaxed, rehydrated, and immersed in toluidine blue staining solution according to the manufacturer's instruction (G3670, Solarbio, Beijing, China) for 15 min, washed with tap water for 2 min, decolorized with 95% ethanol, routinely dehydrated and sealed, and observed under light microscopy (Viyee Photoelectric Device, Tianjing, China). Images were obtained by pathological section scanner (Pannoramic MIDI, 3DHISTECN, Hungary).

Immunohistochemical analysis (IHC)

The intestinal tissue sections of mice were dewaxed, rehydrated, and washed with PBS, followed by the addition of 3% hydrogen peroxide to block endogenous peroxidase. The slices were immersed in citrate buffer, placed in a steamer, and steamed for 30 min to repair anti-

gens. After dropping 5% Bovine Serum Albumin (BSA) (SW3015, Sorlarbio, Beijing, China) to block the nonspecific binding site, the primary antibody was added and incubated at 4°C overnight with an anti-TPSAB1 antibody (1:200; 13343-1-AP, Proteintech, Wuhan, China). After washing the unbound primary antibody with PBS, a biotin-labeled secondary antibody was added according to the manufacturer's instructions (SA1020, BOSTER Biological Technology, Wuhan, China), and incubated at 37°C for 30 min. After washing with PBS, SABC in the kit was added dropwise, incubated at 37°C for 30 min, and color developed with DAB (AR1027, BOSTER Biological Technology, Wuhan, China). After double dyeing with hematoxylin, the excess dye was rinsed with clear water, dehydrated, and sealed. Pictures were obtained with a pathological section scanner.

Immunofluorescence analysis (IF)

The sections were dewaxed and blocked with 3% hydrogen peroxide to inhibit endogenous peroxidase. The slices were immersed in citrate buffer, placed in a steamer, and steamed for 30 min to repair antigens. After blocking with 5% BSA, PE-anti-mouse CD117 (c-Kit) antibody (105826, BioLegend, CA, USA) and FITC-anti-mouse FcεRIα antibody (134305, BioLegend, CA, USA) were added and incubated at 4°C for 2 h. The excess antibody was washed with PBS, and the sections were sealed with an anti-fade mounting medium containing DAPI (abs9235, Absin, Shanghai, China) and observed under a fluorescence microscope (Zeiss, Germany).

Statistical analysis

All data are presented as mean ± SEM. Student's *t*-test was used to compare the differences between groups. $P \leq 0.05$ was considered statistically significant. Data were processed with GraphPad Prism 5.01.

Results

Identification and characterization of hucMSC-Ex

HucMSCs were isolated and cultured from human umbilical cord tissue. The supernatant of the culture medium was prepared by adding exosome-depleted FBS, followed by ultracentrifugation to obtain hucMSC-Ex. Nanoparticle tracking analysis (NTA) results showed a hucMSC-Ex average diameter of 169.4 nm (**Figure 1A**) while the transmission electron microscopy (TEM) imaging image showed a typical concave double-layer disk (**Figure 1B**) as earlier reported [30]. Western blot confirmed that hucMSC-Ex expresses CD9 and CD81, but not Calnexin, while hucMSC expressed all three markers (**Figure 1C**).

Evaluation of mast cell infiltration in IBD patients and the reparative effect of hucMSC-Ex in IBD mice

Colon tissues of healthy individuals and IBD patients from Nanjing Jiangning Hospital were collected for H&E and immunohistochemical staining. Normal human colon tissue specimens exhibited well-defined gland structure and orderly cellular morphology, while IBD patients had colon glands with different sizes, disordered arrangement, and obvious infiltration of inflammatory cells (**Figure 2A**). The intracellular tryptase TPSAB1, found in mast cells, undergoes extracellular secretion upon degranulation following cellular activation and was quantified in this study. TPSAB1 is considered to be a specific gene marker for mast cell tryptase and in mast cell-related diseases, the detection of TPSAB1 can be used to judge the degree of mast cell activation [31]. We observed a decreased expression of TPSAB1 in normal colon tissue, whereas an increased expression of TPSAB1 was detected as numerous punctate positive spots in the colon mucosa of patients with IBD (**Figure 2B**). In order to investigate the relationship between IBD and mast cells, we established the mice model of IBD.

The repair effect of DSS-induced colitis was examined by administering hucMSC-Ex via tail vein injection in mice. While the weight of mice in the DSS group sharply decreased on the sixth day, hucMSC-Ex mitigated the weight loss compared to the DSS group (**Figure 2C**). The disease activity index (DAI) in the mice was evaluated by quantifying the degree of weight loss, diarrhea, and bloody stools. The findings revealed a progressive increase in DAI scores among mice in the DSS group over time, while those treated with hucMSC-Ex exhibited DAI scores intermediate between the NC and DSS

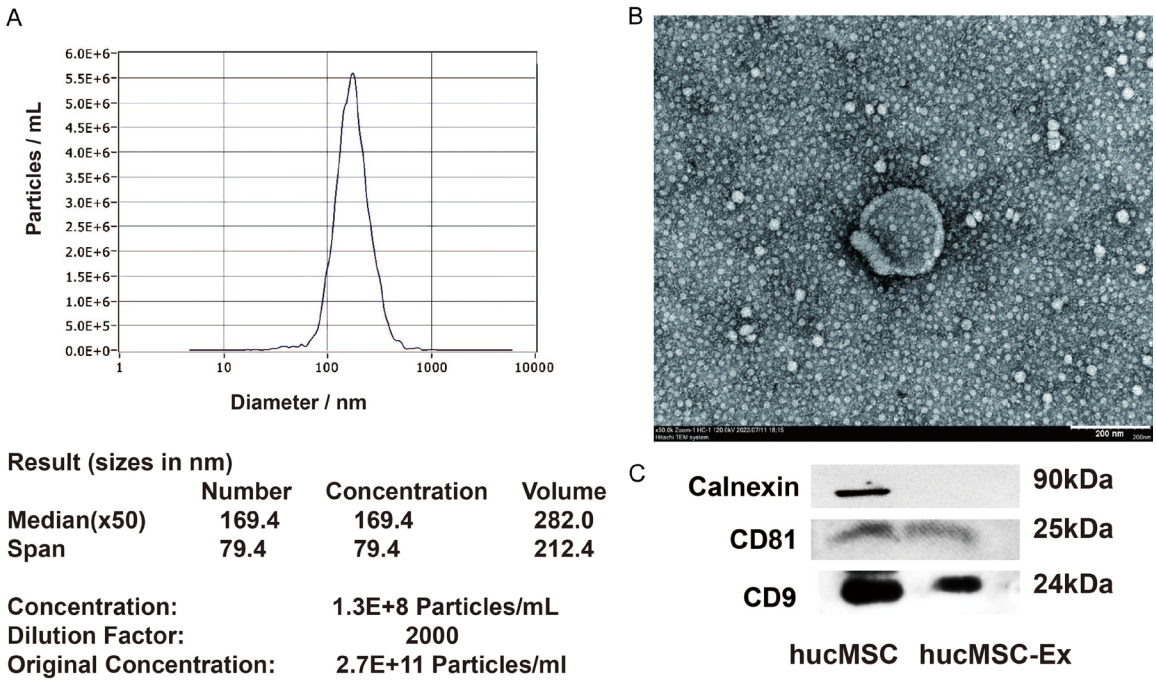
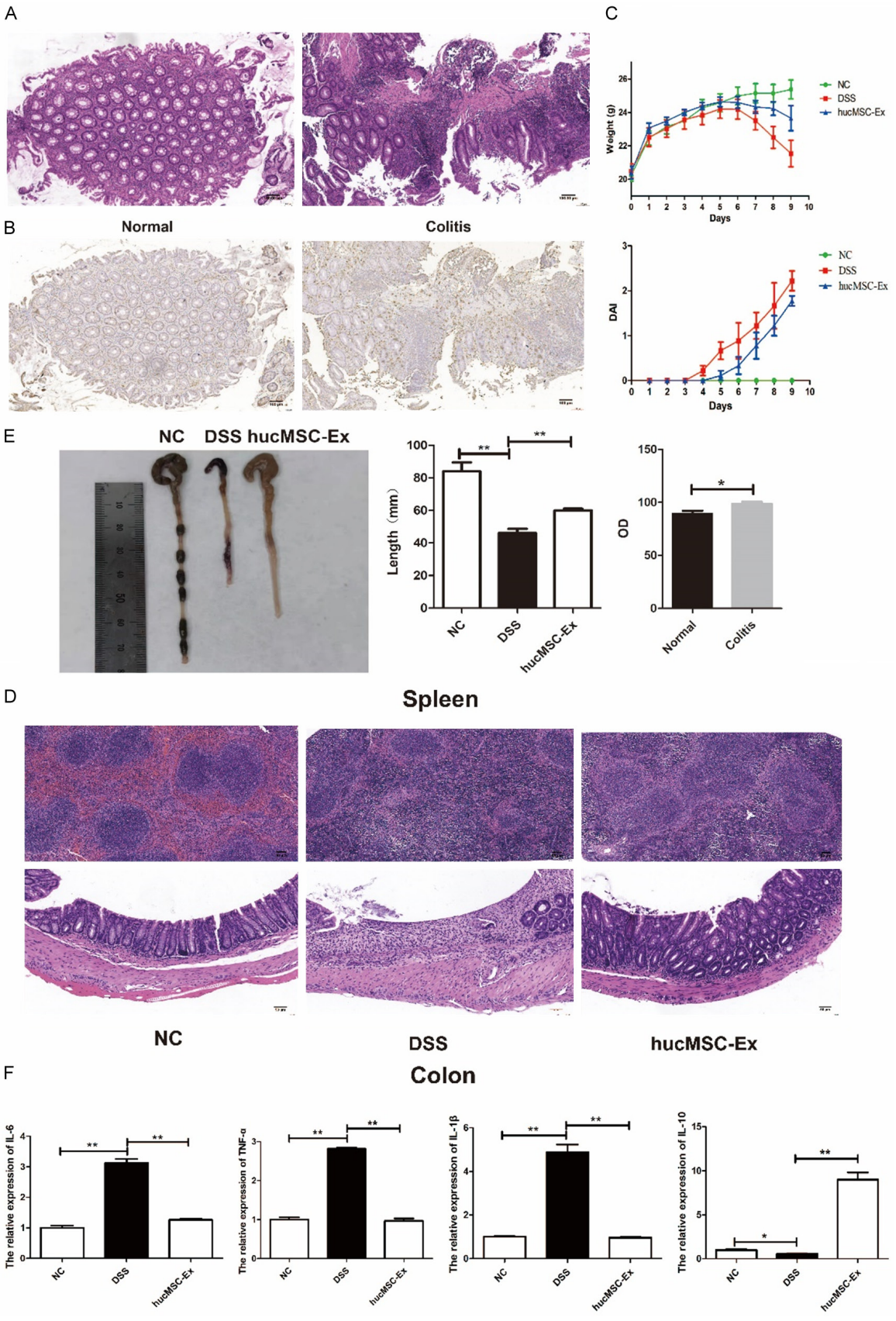


Figure 1. Identification of hucMSC-Ex. A. The size and concentration of hucMSC-Ex were determined using nanoparticle tracking analyzer (NanoSight); B. Identification of hucMSC-Ex was done through transmission electron microscopy (TEM); C. Western blot analysis was performed to identify the surface markers of hucMSC-Ex.

groups (**Figure 2C**). The H&E staining revealed aberrant intestinal mucosal structure and increased inflammatory cells in the DSS-induced mice colitis, while hucMSC-Ex significantly alleviated the mucosal destruction and inflammation. This observation was also reflected in the spleen, where the edges of the splenic nodules were blurred with unclear boundaries in the DSS group, while the structure of the splenic nodules in the hucMSC-Ex group was restored to a great extent (**Figure 2D**). Moreover, the colon length of mice in the DSS group was significantly reduced but largely restored in the hucMSC-Ex group (**Figure 2E**). The levels of proinflammatory factors IL-6, IL-1β, TNF-α in the colonic mucosa of mice in the DSS group were elevated, while the levels of anti-inflammatory factor IL-10 were slightly decreased. Administration of hucMSC-Ex resulted in decreased mRNA expression level of IL-6, IL-1β, TNF-α and increased level of IL-10 (**Figure 2F**).

HucMSC-Ex mitigates mast cell aggregation in the colonic mucosa of IBD mice

Our findings demonstrate that mast cells accumulate in human IBD colonic mucosa (**Figure 2A** and **2B**). Therefore, we investigated whether this phenomenon is also observed in the colonic tissue of mice with DSS-induced colitis. CD117 is highly expressed on the surface of mast cells, serving as a recognition marker or target of mast cells [32]. Moreover, the binding of immunoglobulin E (IgE) antibodies to antigens and subsequent interaction with the high-affinity IgE receptor, Fc epsilon RI (FcεRI), leads to the release of particulate matter following mast cell activation [33]. The aggregation of mast cells in the intestinal mucosa of mice was assessed by detecting CD117 and FcεRIα. The results demonstrated a homogeneous distribution of double positive fluorescent particles within the intestine of the NC group, while their abundance significantly increased in the DSS group (**Figure 3A**). The abundance of intestinal tryptase particles was significantly higher in the DSS group compared to the NC group, whereas treatment with hucMSC-Ex resulted in a reduction in the number of TPSAB1 positive particles (**Figure 3B**). This observation signifies the clustering of mast cells within the gastrointestinal tract of mice belonging to the DSS experimental group. The aggregation of mast cells in the DSS mice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Length (mm)
OD
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Colitis
D
Spleen
NC
DSS
hucMSC-Ex
F
Colon
The relative expression of IL-6
The relative expression of TNF-α
The relative expression of IL-1β
The relative expression of IL-10

Figure 2. HucMSC-Ex alleviates DSS-induced inflammatory injury in IBD mice. A. H&E staining of the colon in healthy and IBD patients; B. The expression of TPSAB1 was analyzed by immunohistochemical staining (scale bar = 100 μm) in the colon of healthy people and IBD patients; C. Changes in body weight and DAI score of mice; D. H&E staining was performed on sections of the colon and spleen in mice (scale bar = 100 μm in colon, scale bar = 50 μm in spleen); E. The length of the mice's colon; F. The mRNA level expression of IL-6, IL-1β, TNF-α, IL-10. $*P < 0.05$; $**P < 0.01$.

was also demonstrated by Toluidine blue staining (yellow arrows indicates the location of mast cells aggregation) (**Figure 3C**). The mouse homolog of human tryptase-β, known as mast cell protease-6 (MCP-6), has been demonstrated to enhance the presence of trypsin-expressing mast cells in the gastrointestinal tract of patients with IBD. In the presence of inflammation, mast cells expedite expression and release of tryptase into the microenvironment, resulting in the generation of pro-inflammatory factors and accumulation of other inflammatory cells, cascade leading to intestinal damage such as increased permeability [34]. MCP-6 mediates the expression of mouse mast cell protease in mice [35]. MCP-6 gene expression was up-regulated in the DSS group and decreased in the hucMSC-Ex group (**Figure 3D**).

HucMSC-Ex significantly ameliorates intestinal barrier function and reduces the expression of IL-33 and its receptor IL1RL1 in the intestinal mucosa of mice

The IL-33 derived from intestinal epithelial cells functions as an "alarm" in inflammatory disease and exhibits significantly elevated expression levels in patients with IBD [36]. Thus, we examined the effect of hucMSC-Ex on the expression of IL-33 and its receptor in the intestinal mucosa of mice. Immunofluorescence results showed that DSS mice had high levels of IL-33 and IL1RL1 (ST2), and hucMSC-Ex reduced the expression of both molecules (**Figure 4A**). This trend was also verified by QRT-PCR, indicating increased expression of IL-33 and IL1RL1 (ST2) in the DSS group, while hucMSC-Ex reduced both expressions (**Figure 4B** and **4C**).

Subsequently, we measured the restorative effect of hucMSC-Ex on the intestinal permeability of IBD mice. The concentration of FD4 in the bloodstream of mice in the DSS-induced colitis model was significantly up-regulated compared with that in the NC group, while the hucMSC-Ex group was significantly decreased (**Figure 4D**). The mRNA expression of intestinal tight junction proteins Occludin, Claudin-1, and ZO-1 in the DSS group decreased significantly, and hucMSC-Ex significantly restored their expression level (**Figure 4D**). The protein expression of mouse colonic tight-junction protein was matched with the mRNA level, where the fluorescence intensity of Claudin-1 and Occludin in the DSS group was significantly weaker than that in NC and hucMSC-Ex groups (**Figure 4E**). The same trend was observed in the recovery effect of hucMSC-Ex on ZO-1 protein (**Figure 4F**). Moreover, western blot results indicated that the expression of the three tight junction proteins in the DSS group was reduced compared to the NC group, while the hucMSCS-Ex group markedly recovered (**Figure 4G**).

HucMSC-Ex reduces mast cell activation by inhibiting the IL-33/IL1RL1 (ST2) axis

We constructed a cell co-culture model and analyzed the activation of mast cells (**Figure 5A**). The findings demonstrated that the co-cultivation of excessive mast cells with intestinal epithelial cells resulted in an upregulation of ST2 expression in mast cells. However, this trend was alleviated by hucMSC-Ex treatment (**Figure 5B**). IL-33 spills from epithelial damage, leading to mast cell inflammatory signal transduction [37]. We found that mast cell-derived IL-33 also increased in the co-culture group, and hucMSC-Ex treatment reduced its levels (**Figure 5C**). Consistent with the results from Taruselli et al (2022), IL-6 expression in mast cells was significantly up-regulated in the co-culture group, and hucMSC-Ex reduced this reaction [38] (**Figure 5D**). We also observed that coculture resulted in increased tryptase expression in mast cells, while hucMSC-Ex significantly reduced the expression levels (**Figure 5E**).

HucMSC-Ex relieves mast cell inflammation-induced reduction in barrier proteins of Caco-2 cells

We further examined the expression level of Caco-2 tight-junction protein in the co-culture system. The results demonstrated that exces-

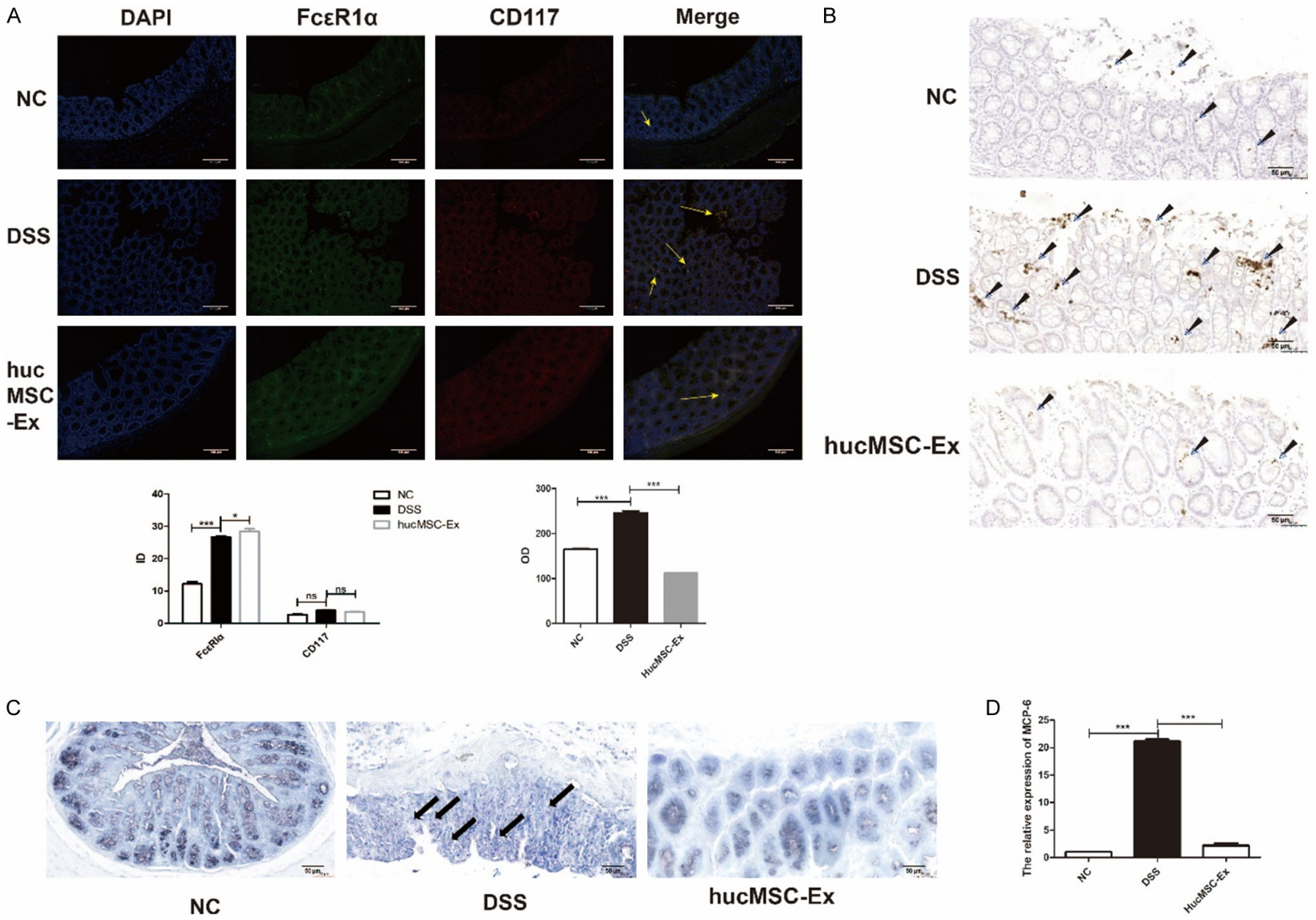
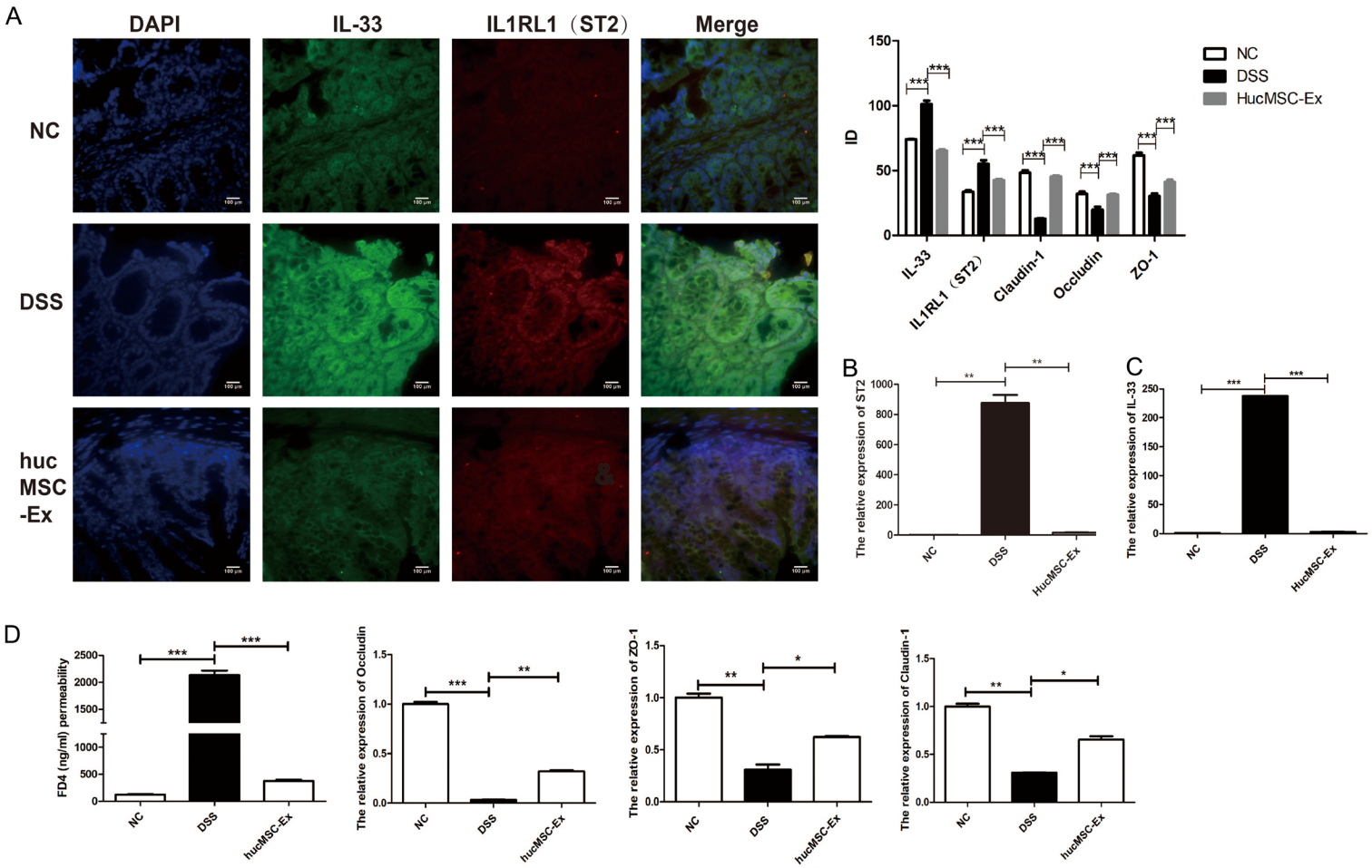


Figure 3. HucMSC-Ex reduces mast cell infiltration to relieve IBD. A. Representative images of immunofluorescence staining for FcεRIα and CD117 in colon tissues from mice (scale bar = 100 μm); B. The expression of TPSAB1 in the colon of mice by immunohistochemical staining (scale bar = 50 μm); C. Toluidine blue staining was performed on colon tissues of mice (scale bar = 50 μm); D. QRT-PCR analysis was conducted to measure the expression of MCP-6 mRNA in the colon tissue of mice. Data were presented as mean ± SD and analyzed by Student's *t*-test. **P* < 0.05; ****P* < 0.001.


A
DAPI
IL-33
IL1RL1（ST2）
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ID
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Claudin-1
Occludin
ZO-1
B
The relative expression of ST2
C
The relative expression of IL-33
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The relative expression of ZO-1
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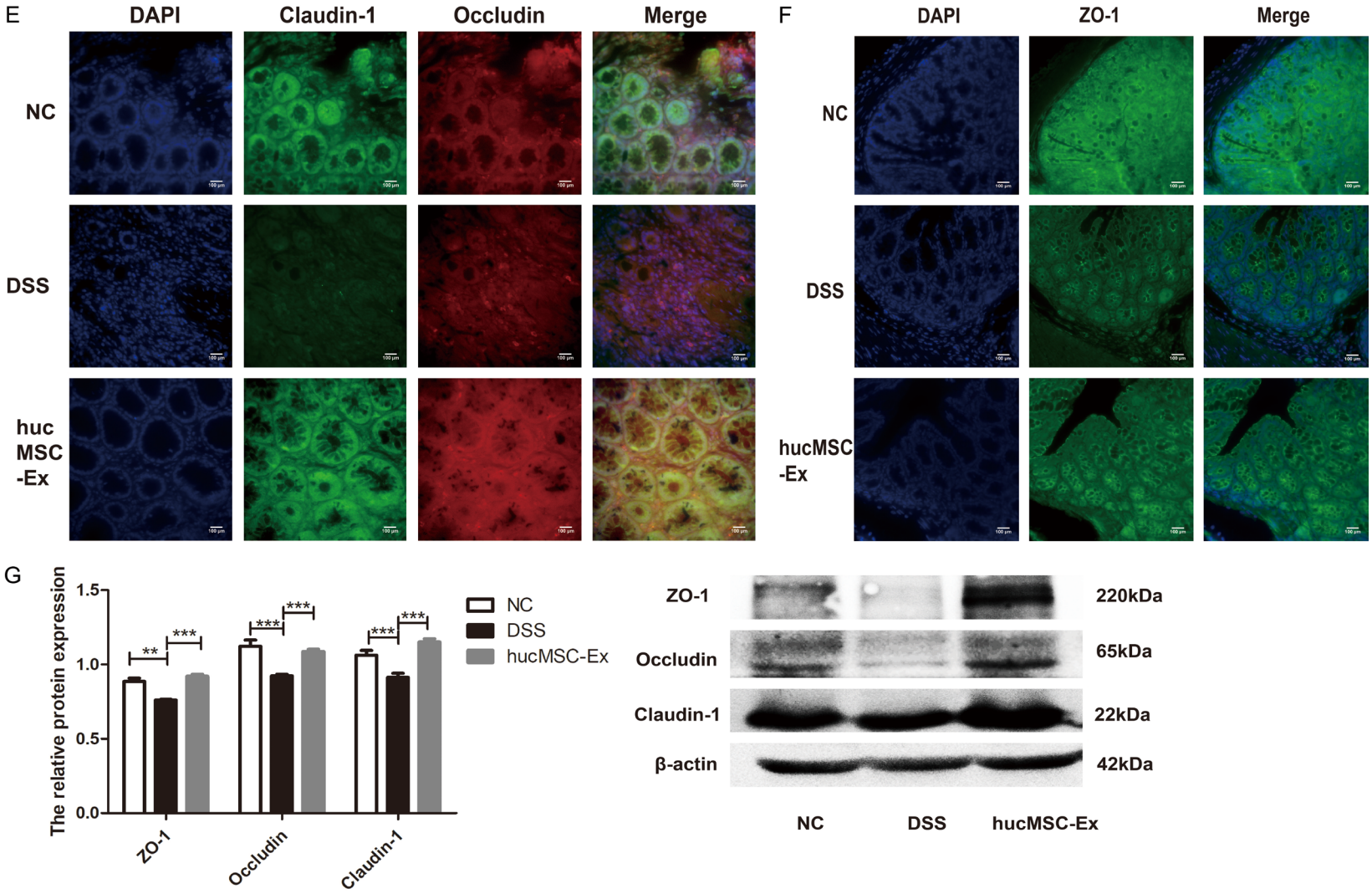

Figure 4. hucMSC-Ex effectively restores impaired intestinal barrier function and downregulates the expression of IL-33 and its receptor IL1RL1 in a murine model of IBD. (A) Representative images of immunofluorescence staining for IL-33 and IL1RL1 in colon tissues of mice (scale bar = 100 μm); (B) QRT-PCR analysis was performed to measure the expression of IL-1RL1 mRNA in mouse colon tissue; (C) QRT-PCR analysis was performed to measure the expression of IL-33 mRNA in mouse colon tissue; (D) FITC-carboxymethyl-dextran (FD4) determination of intestinal barrier permeability in mice and QRT-PCR analysis of tight-junction protein mRNA expression in mice colon tissue; (E) Representative images of immunofluorescence staining for Claudin-1 and Occludin in colon tissues of mice (scale bar = 100 μm); (F) Images of immunofluorescence staining for ZO-1 in colon tissues of mice (scale bar = 100 μm); (G) The western blot expression levels of tight junction protein; &, Fluorescence intensity (ID) of (A, E, F). Data were presented as mean ± SD and analyzed by Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

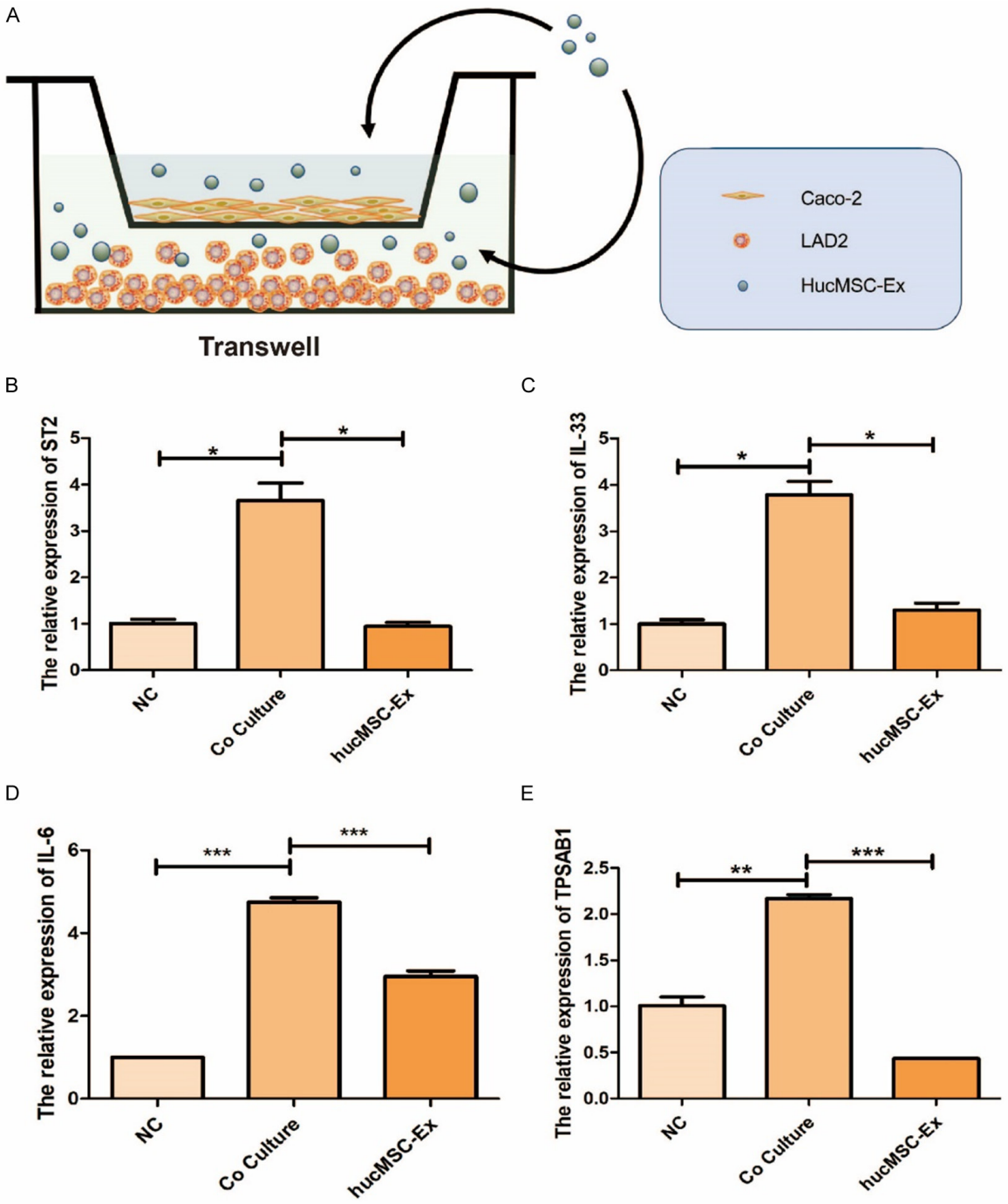

Figure 5. HucMSC-Ex inhibits mast cell activation induced by the IL-33/IL1RL1 (ST2) axis. A. A co-culture system of mast cells (LAD2) and Caco-2 cells was established. Caco-2 cells were inoculated in the upper layer of the chamber at a high density. After adhering to the wall, mast cells were inoculated in the lower layer. HucMSC-Ex with a total protein content of 400 μg/mL was added to both the upper and lower layers for incubation; B. QRT-PCR analysis of IL1RL1 (ST2) mRNA expression in LAD2; C. QRT-PCR analysis of IL-33 mRNA expression in LAD2; D. QRT-PCR analysis of IL-6 mRNA expression in LAD2; E. QRT-PCR analysis of TPSAB1 mRNA expression in LAD2. Data are presented as mean ± SD by student *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

sive mast cell aggregation led to a decrease in tight junction proteins. The expression levels of Claudin-1, Occludin, and ZO-1 genes were down-regulated in the co-culture group but

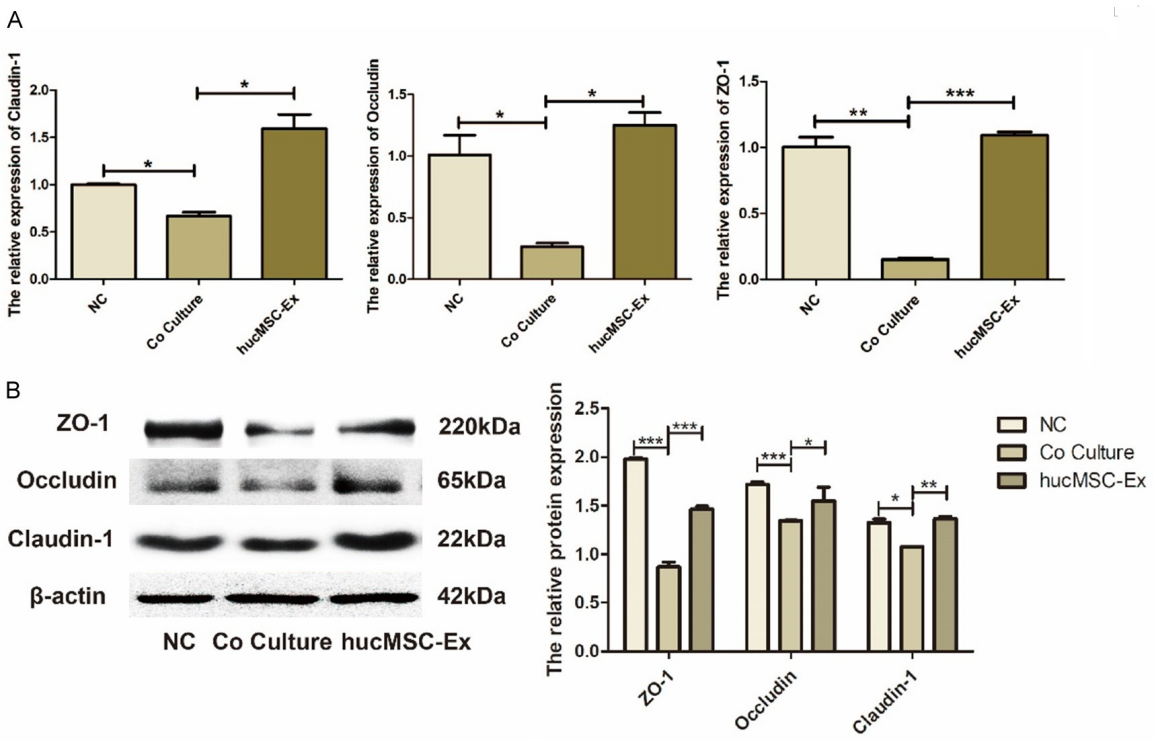

Figure 6. HucMSC-Ex relieves mast cell inflammation-induced reduction in barrier proteins in Caco-2 cells. A. QRT-PCR analysis of tight junction protein mRNA expression in Caco-2; B. Western blot analysis of tight junction protein expression levels. Data are presented as mean ± SD by student *t*-test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

increased in the hucMSC-Ex treated group (**Figure 6A**). Western blot results confirmed that the co-culture of mast cell and Caco-2 resulted in a reduced expression of Claudin-1, Occludin, and ZO-1, and the hucMSC-Ex reversed this trend (**Figure 6B**).

Discussion

The normal intestinal mucosal barrier consists of mucus, epithelial cells, normal intestinal flora, immune cells, and other components, which play an effective defensive role against external stimuli. Disturbance of intestinal homeostasis results in microbial invasion, inflammation, and immune system imbalance [39]. Mast cells reside in the gastrointestinal tract and have a profound significance in maintaining the function of the mucosal surface and regulating mucosal immunity [13]. However, abnormal aggregation and activation of mast cells due to inflammation, injury, or other immune responses can disrupt immune balance, resulting in allergic reactions or barrier damage [40, 41]. In this study, abnormal aggregation of mast cells was observed in the clinical samples of IBD, which is intricately linked to intestinal immune function. It is therefore reasonable to postulate that abnormal aggregation of mast cells was related to the pathological process of IBD, as corroborated by existing literature [19, 42-44]. Other researchers have proposed that mast cells play a dual role in the gut, contributing to both inflammatory repair and alleviation of gastrointestinal diseases [21]. However, in this study, we found an increase in tryptase-positive particles in IBD clinical samples, indicating elevated infiltration of mast cells in the inflamed area. One of the products of activation degranulation of intestinal mast cells is tryptase, which can disrupt intestinal barrier integrity [23]. Scholars have focused on targeting mast cell activation pathways to inhibit the degranulation and cytokine production of mast cells, aiming to alleviate barrier damage [45]. HucMSC-Ex has been extensively studied in the field of IBD mitigation due to its excellent repair properties [28]. We constructed a DSS-induced colitis model in mice and examined whether the repair effect of hucMSC-Ex is related to mast cells. Our results demonstrated that hucMSC-

Ex reduces the severity of intestinal inflammation in mice, highlighting its reparative potential. It was also found that DSS administration induces an increase in mast cell infiltration and tryptase release in the intestinal mucosa of IBD mice, while hucMSC-Ex not only reduced the number of mast cell infiltration but also the expression of tryptase, suggesting that hucMSC-Ex may reduce the activation of mast cells.

Mast cells express IL-33 receptor IL1RL1 (ST2) and the IL-33 released by mucosa stimulates the production of mast cell-derived chemokines and attracts macrophages to promote the development of gastric cancer [46]. Activation of mast cells through the IL-33/ST2 axis, leading to the production of pro-inflammatory cytokines, has been well established [38, 47]. Upon stimulation with IL-33, mast cells exhibit a cascade of signal transduction responses, leading to heightened cellular activation and the release of inflammatory mediators [48]. The production of IL-33 after epithelial injury may lead to the response of mast cells containing IL1RL1. We observed a significant upregulation of IL-33 and IL1RL1 in the damaged intestine of IBD mice, providing further evidence for the IL-33/IL1RL1 (ST2) axis may play a role in the activation of intestinal mast cells. Diets containing in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols are considered to stimulate mast cell activation and mucosal barrier destruction in a toll-like receptor (TLR)4-dependent manner. And that eventually leads to irritable bowel syndrome (IBS) [49]. Mast cell activation is also thought to be responsible for airway hyperresponsiveness and lung barrier dysfunction [50, 51]. Consequently, we posit that the activation of mast cells exhibits a strong correlation with dysfunction in the intestinal barrier. We found that DSS mice not only had an increase in the release of tryptase from mast cells but also exhibited impaired intestinal barrier function and increased permeability. The impairment of this barrier function may be attributed to the destruction and downregulating expression of tight junction proteins [23]. The application of hucMSC-Ex resulted in a reduction in mast cell infiltration, tryptase release, and IL-33 and ST2 expression in the intestinal mucosa, while also enhancing intestinal barrier function. It can be concluded that targeting the IL-33/IL1RL1 (ST2) pathway holds promise for treating IBD due to its association with mast cell activation and aggregation, tryptase release, and subsequent impairment of mucosal barrier function.

The administration of osthole reduced the inflammation of Caco-2 monolayer cells induced by lipopolysaccharide, it also alleviated permeability reduction caused by co-culture of Caco-2 with Tohoku Hospital Pediatric1 (THP-1) derived macrophages [52]. Thus, we also established a co-cultured cell model of mast cell lines LAD2 and Caco-2 to investigate the potential impact of an accumulation of mast cells in the intestinal mucosa on intestinal barrier integrity. We utilized the transwell chamber equipped with the smallest aperture to conduct our experiments. Caco-2 co-cultured with a large number of mast cells exhibited symptoms of the destruction of tight junction integrity owing to reduced junction proteins Claudin-1, Occludin, and ZO-1. It has been reported that neutralizing the IL-33/IL1RL1 (ST2) pathway significantly improves the signs of colitis, which may be related to the restoration of the cyto-protective factor connexin-43 expression [53]. Our results confirmed in vitro that when a large number of mast cells infiltrate the intestinal mucosa, the IL-33/IL1RL1 (ST2) pathway is activated, which may be the culprit that leads to the destruction of the intestinal mucosal barrier. Perhaps, the destruction of the intestinal epithelium triggers the release of IL-33, which activates the receptor on the surface of mast cells, as the mast cells themselves further produce IL-33, inducing a series of inflammatory reactions. HucMSC-Ex reduces the expression of IL1RL1 (ST2), resulting in reduced expression of inflammatory factors and tryptase in mast cells, repairing the intestinal barrier. However, our research currently lacks evidence demonstrating whether hucMSC-Ex directly modulates the expression of intestinal tight junction proteins and restores impaired intestinal barrier function. In addition, this study only explains this phenomenon and puts forward a hypothesis, but the specific mechanism of hucMSC-Ex needs further study.

Conclusion

The administration of hucMSC-Ex may ameliorate DSS-induced colitis in mice by inhibiting

the aggregation and activation of mast cells, as well as reducing tryptase production. These effects are potentially mediated through modulation of the IL-33/ST2 pathway.

Acknowledgements

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Disclosure of conflict of interest

None.

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