Interleukin-25 primed mesenchymal stem cells achieve better therapeutic effects on dextran sulfate sodium-induced colitis via inhibiting Th17 immune response and inducing T regulatory cell phenotype

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Abstract: Aim: This study aimed to investigate the anti-inflammatory mechanism of IL-25 mediated mesenchymal stem cells (MSC) treatment for inflammatory bowel disease (IBD) in a DSS-induced rat colitis model. Methods: Rats with DSS-induced colitis were divided into control and treatment groups: normal control group (rats fed with water), DSS group (rats fed with DSS solution), MSC group (DSS-treated rats injected intravenously with GFP-MSCs), IL-25-MSC group (DSS-treated rats injected intravenously with IL-25 primed GFP-MSCs), and mesalazine group (DSS-treated rats fed with mesalazine). Results: In IL-25-MSC group, therapeutic efficacy (clinical symptoms) was better than in MSC group, but comparable to mesalazine group. In IL-25-MSC group and mesalazine group, fewer infiltrating inflammatory cells and lower pathological score were observed in the intestine. The FOXP3+ cells and IL-4+ cells decreased, but IL-17A+ cells and IFN-γ+ cells increased in the peripheral blood and colonic mucosa after DSS induced colitis, and these phenomena were reversed by MSC or mesalazine treatment. IL-17A+ cells reduced and FOXP3+ cells increased in IL-25-MSC group as compared with MSC group. The expressions of Ki67 and LGR5 were significantly elevated in MSC treatment groups as compared with normal control group, DSS group, and mesalazine group. Definite GFP positive cells were not observed in the intestine of MSC-treated rats. Conclusion: IL-25 primed MSCs exert improved therapeutic effects on the intestinal inflammation of IBD rats which may be related to the inhibition of Th17 immune response and induction of T Regulatory cell phenotype. Thus, IL-25 may be an attractive candidate for MSC-based therapy of IBD.

Keywords: Inflammatory bowel disease, mesenchymal stem cells, interleukin-25, colitis, therapeutic effect

Introduction

Inflammatory bowel diseases (IBD) include two types of chronic intestinal disorders including Crohn’s disease (CD) and ulcerative colitis (UC), which differ in disease distribution and histological features of the affected bowel segments. Although the etiology of IBD is still poorly understood, dysregulation of the mucosal immune response toward intestinal flora together with genetic and environmental factors has been found to play important roles in the pathogenesis of human IBD [1, 2]. Accumulating evidence has demonstrated that the imbalance between effector and regulatory T cells (Th1, Th2, Th17 and Treg) may induce pathological responses in the IBD intestinal mucosa, which is characterized by the elevated expression of adhesion molecules, chemokines, and other pro-inflammatory cytokines [3, 4]. Some drugs have been used for the treatment of human IBD including 5-aminosalicylic acid, glucocorticoids, immunosuppressants and monoclonal antibodies, but none is specific for IBD and may result in some adverse effects.

Mesenchymal stem cells (MSCs) are non-hematopoietic, multipotent, stromal precursor cells resident in most adult tissues. MSCs possess the capabilities of differentiation and regeneration, and can migrate to the injured site to repair the damaged tissues [5]. In addition, MSCs are
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also able to modulate immune response by regulating the recruitment, function, and fate of cells in the innate and adaptive immune system [6, 7]. Numerous animal and clinical studies have suggested that MSCs can suppress T cell proliferation and maintain them in a quiescent state by releasing related mediators and directing cell-cell contact [8-10]. Therefore, MSCs are emerging as a candidate for treatment of immune-mediated diseases, including IBD. In 1993 Drakos et al. first used autologous blood stem cell to treat a hematological malignancy patient with CD, and the symptoms of IBD were improved simultaneously [11]. Subsequent studies have also demonstrated the efficacy of MSC treatment in Crohn’s perianal fistula and refractory luminal CD [12, 13]. In recent years, investigators find that MSCs have better immunomodulatory potential after being primed with cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) [14, 15], and granulocyte colonystimulating factor (G-CSF) treated MSCs showed enhanced therapeutic effects on UC via an anti-inflammatory mechanism [16]. Available findings suggest the local microenvironment may affect immune related behaviors and therapeutic efficacy of MSCs.

Interleukin (IL)-25, a member of the cytokine IL-17 family, has been shown to stimulate the Th2 cell-mediated immune responses, resulting in epithelial cell hyperplasia and enhanced recruitment of inflammatory cells into injured tissues [17-19]. By decreasing the syntheses of several Th1 or Th17 related cytokines, IL-25 also attenuates the destructive inflammation in several autoimmune diseases [19-21]. Our previous study has proven that IL-25 was markedly decreased in the injured mucosa of IBD and could inhibit the activation and differentiation of IBD CD4+ T cells into Th1/Th17 cells in an IL-10-dependent manner [22]. In a mouse model of intestinal helminth infection, Saenz et al. found that IL-25 could promote the accumulation of a multipotent progenitor cell population in the gut-associated lymphoid tissue through inducing Th2 response [23]. In this study, a DSS-induced colitis model was established in rats and the therapeutic effects of IL-25 primed MSCs on the intestinal inflammation were further evaluated, in which the expression of Th-associated cytokines was measured and the underlying immunologic mechanism in MSC treatment of IBD was explored. Our study may provide evidence for the treatment of IBD and the development of new targets for IBD therapy.

Materials and methods

Animals

Sprague-Dawley rats (6-8 weeks, weighing 150-170 g) were purchased from Shanghai Laboratory Animal Research Center (Shanghai, China). Rats were maintained under specific pathogen-free conditions with controlled temperature (20-25°C) and 12-12 hour light-dark cycle and given ad libitum access to food and water. All animal experiments were approved by the Ethical Committee and Institutional Animal Care and Use Committee of Xiamen University.

MSC isolation, culture and characterization

Isolation and culture of MSCs were performed as described previously [24]. In brief, rats were sacrificed by cervical dislocation, and the tibiae was collected and flushed with Dulbecco's modified Eagle's medium (DMEM)-low glucose (Gibco Invitrogen, Carlsbad, USA). The bone marrow cells were harvested, then seeded into flasks and cultured at 37°C in an environment with 5% CO2. After 3-day culture, non-adherent cells were removed and the medium was refreshed every 2-3 days. Once the cell confluence reached approximately 80%, cells were treated with 0.25% trypsin-EDTA (HyClone, Utah, USA) and used in following experiments. Immunophenotyping of MSCs was done by flow cytometry using specific antibodies (CD44, CD90, CD73, CD34, CD29, CD45 and CD11b; BD Biosciences, San Diego, USA). After passaging twice, MSCs were transfected with lentiviral eGFP vector which uses a CMV promoter. The transfection efficiency and fluorescence intensity were determined by inverted fluorescent microscopy and flow cytometry.

Experimental colitis induction and MSC transplantation

Colitis was induced by drinking water containing 5% DSS (MW: 36,000-50,000 Da; MP Biochemicals, Shanghai, China) for 7 days. All rats were divided into five groups: control group (healthy rats drunk water alone), DSS plus PBS group (rats drunk DSS and were intravenously treated with PBS), DSS plus MSC group (rats
drunk DSS and were intravenously treated with MSCs), DSS plus IL-25-MSC group (rats drunk DSS and were intravenously treated with IL-25 primed MSCs), and DSS plus mesalazine group (rats drunk DSS and were intravenously treated with mesalazine). MSCs at a density of $5 \times 10^6$ cells/1 ml PBS or PBS were injected through the tail vein on days 1, 2 and 3. IL-25 primed MSCs was prepared by treatment of MSCs with 50 ng/ml IL-25 (ProSpec, NJ, USA) for 24 h. Mesalazine (SinoPharm, Shanghai, China) was administered intragastrically at 1000 mg/kg. Rats in each group were sacrificed on Day 8, and the peripheral blood and colon tissues were harvested for further detections.

Disease activity evaluation

During the study, the changes in the hair, mental status, and activity of these rats were observed daily. The weight loss and characteristics of the stool (including bloody stool) were recorded to determine the disease activity index (DAI) \[25\]. The scoring was performed by two investigators who were blind to the grouping.

Histological examination

The colon samples were fixed in 4% paraformaldehyde, embedded in paraffin, and sliced into 5-µm sections. Histological evaluation was performed after hematoxylin and eosin (H&E) staining based on the extent of edema, ulceration, crypt loss and infiltration of immune cells as previously described \[26\]. Total score was defined as the sum of each parameter. The investigators responsible for the histological examination were blind to the grouping. For immunohistochemistry, sections were incubated with Envision Flex Peroxidase-Blocking Reagent for 10 min, and then with rabbit anti-rat LGR-5 mAb (1:100, Novus, CO, USA), anti-rat Ki67 mAb (1:100, Abcam, MA, USA) or anti-rat GFP mAb (1:150, Abcam, MA, USA) overnight at 4°C. After washing in PBS, sections were treated for 30 min with brefeldin A (eBiosciences, CA, USA). Then, cells were stained with CD4-FITC (eBiosciences, CA, USA) and incubated for 20 min at 4°C in dark. After washing twice, intracellular staining was performed with IFN-γ-eFluor660, IL-17-PE, IL-4-eFluor660 or FoxP3-APC (Santa Cruz, TX, USA). Cytofix/Cytoperm (BD Biosciences, CA, USA) was used to wash and permeabilize the cells for intracellular staining. After incubation, washing buffer was added to each sample, followed by centrifugation at 500 g for 5 min at 4°C. Finally the samples were fixed in 0.5 ml of 3% paraformaldehyde buffer, and subjected to flow cytometry on a FACS Calibur instrument (BD Biosciences, CA, USA). Data were analyzed with CellQuest software according to our previously reported.
Statistical analysis

Statistical analysis was done with SPSS statistical software 17.0 (Chicago, IL, USA). Data were expressed as mean ± standard error (SEM). Differences between means were determined using the Student’s t test. A value of P < 0.05 was considered statistically significant.

Results

Clinical status and DAI scores in different groups

The immunophenotype of isolated MSCs was consistent with previously reported [13, 28]. Then, the therapeutic efficacy of MSCs was evaluated in rats with 5% DSS induced acute colitis. DSS resulted in severe colitis that was characterized by ruffled hair, loss of appetite, weight loss and bloody diarrhea, and the DAI score increased significantly (7.23±0.56). Compared with DSS group, rats in MSC group showed a slight improvement of symptoms and a small decrease in DAI score (5.26±0.33). However, in IL-25-MSC group, a better therapeutic efficacy (significant improvement of clinical symptoms) and a significant decrease in DAI score (3.45±0.22, *P < 0.05) were observed, and the DAI score was comparable to mesalazine treatment group (2.78±0.36). Moreover, macroscopic examination revealed that inflammation-related shortening of the colon was reduced after treatment with MSCs or mesalazine (3.45 cm±0.22 cm, 10.5 cm±0.22 cm vs 8.5 cm±0.85 cm, *P < 0.05), but IL-25-MSC treatment significantly attenuate the colon shortening (12.00 cm±0.62 cm). Data are shown in Figure 1.
Histological changes in different treatment groups

Then, the histopathology of the colon was examined in each group and the histological score was obtained. DSS-treated rats showed severe disruption of the crypt architecture and infiltration of inflammatory cells, including neutrophils, lymphocytes, and macrophages. The inflammatory lesions were located in the mucosa and sub-mucosa. Compared with DSS group, colitis rats treated with MSCs exhibited protective effects on DSS-induced histological damage, including fewer inflammatory infiltrates and milder crypt structure injury. The intestinal injury was also significantly improved in mesalazine treatment group. In addition, in IL-25-MSC group, a better therapeutic efficacy was observed, which displayed reduction of total histological score compared with DSS group or MSC group (4.67±0.48 vs 10.54±0.74, 7.28±0.61, P < 0.05). There was no significant difference in the histological score between IL-25-MSC group and mesalazine treatment group (4.67±0.48 vs 3.52±0.58, P>0.05) (Figure 2).

Transcription factor/cytokine expressions in peripheral blood and lamina propria CD4+ cells of rats

Increasing evidence shows that IL-25 plays an important role in the initiation of Th2-mediated immune response and the control of Th1/Th17-mediated inflammation [29, 30]. To investigate the potential role of IL-25 in the regulation of MSC-based IBD treatment, the IL-17A+ CD4+ cells, IFN-γ+ CD4+ cells, FOXP3+ CD4+ cells and IL-4+ CD4+ cells in the peripheral blood and intestinal mucosa were detected by flow cytometry and immunofluorescence staining, respectively. As shown in Figures 3 and 4, the FOXP3+ CD4+ cells and IL-4+ CD4+ cells decreased, but IL-17A+ CD4+ cells and IFN-γ+ CD4+ cells increased in rats with DSS induced colitis. This phenomenon was reversed by MSC treatment or mesalazine treatment. Interestingly, compared with MSC treatment, the IL-25 primed MSCs markedly lowered IL-17A+ CD4+ cells and elevated FOXP3+ CD4+ cells in the peripheral blood- and lamina propria-CD4+ cells of DSS-treated rats. The IL-4+ CD4+ cells and IFN-γ+ CD4+ cells were comparable between MSC group and IL-25-MSC group. These findings indicated that IL-25 could enhance the immunomodulatory ability of MSCs mainly via inhibiting Th17 immune response and promoting the immune regulation of Treg cells.

Transplanted MSCs in the colon of rats

IHC was performed to identify transplanted MSCs and their influence on the intestinal epithelial cells and intestinal stem cells in the colonic mucosa on day 8. The expressions of Ki67 and LGR-5 were determined by IHC. Results indicated that the expressions of Ki67...
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and LGR-5 were significantly elevated in MSC group compared with control group, DSS group, and mesalazine group ($P < 0.05$), but there was no marked difference between MSC group and IL-25-MSC group ($P < 0.05$) (Figure 5). Moreover, no definite GFP positive cells were observed in the colon of MSC-treated colitis rats. These findings suggest that IL-25 has no significant effect on the capability of MSCs to induce the differentiation of intestinal stem cells and the proliferation of intestinal epithelial cells.

Discussion

IL-25 is a member of the structurally related IL-17 cytokine family, possesses the lowest degree of homology to IL-17A, does not share biological functions with other members of the IL-17 cytokine family, but has been implicated in the promotion of Th2 immunity. Traditionally, IL-25 can induce the expression of IL-4, IL-5, and IL-13, thereby contributing to allergic diseases and host defense against helminthic parasites [31-33]. On the other hand, IL-25 has been found to suppress the development of Th1/Th17 immune response, and plays an anti-inflammatory role in autoimmune diseases, such as IBD, rheumatoid arthritis, and multiple sclerosis [34-36]. Recent report shows that IL-25 can induce a lineage negative multipotent progenitor (MPP) cell population at the inflammatory mucosa in a mouse model of Trichuris

Figure 3. Transcription factor/cytokine expression in peripheral blood CD4$^+$ cells of rats. Transcription factor/cytokine levels of peripheral blood cells were determined with flow cytometry. Results were obtained from three independent experiments. Data were presented as the mean ± SEM (*$P < 0.05$).
muris infection, and MPP cells have multipotent activity, giving rise to cells of monocytes/macrophages and granulocyte lineages both in vitro and in vivo [23]. Our previous study indicated that IL-25 was deficient in the sera and inflamed mucosa of IBD rats, and it inhibited the differentiation of IBD CD4+ T cells into Th1/Th17 cells but did not interfere with Th2-associated transcription factor expression [22]. In the present study, a well-established colitis model was established with DSS in rats, whether IL-25 could influence the therapeutic effects of MSCs on IBD was investigated, and then the potential mechanism was further explored. Our results showed that IL-25 primed MSC had a better protective effects on the intestinal symp-
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toms and histology of colitis rats compared with untreated colitis rats. Further analysis demonstrated that IL-25 acted via inhibiting Th17 and inducing Treg immune response. However, our study failed to confirm that IL-25 affected the migratory and regenerative capacities of MSCs in vivo. These finding indicate that IL-25 can augment the therapeutic efficacy of MSCs in the treatment of IBD.

MSCs can be easily separated from the bone marrow, where they provide a support for the growth and differentiation of hematopoietic progenitor cells in the bone marrow environment [37]. MSCs have multipotent differentiation potential, which allows them to differentiate into other cell types. In addition, they also have immunomodulatory capacity by controlling inflammation and modifying the proliferation and cytokine production of immune cells [38]. In 2002-2003, the observation that MSCs inhibited T-cell proliferation in vitro identified by three independent investigators opened a door for the use of MSCs in the treatment of autoimmune disorders [39-41]. The pathogenesis of IBD also has involvement of misregulation of T-cell subsets, which is accompanied by aberrant Th1- or Th17-mediated immune responses in CD, and a Th2-driven aberrant immune response in UC. Therefore, numerous reports have provided evidence that the effectiveness of MSC-based immunotherapy in IBD is associated with the decrease in inflammatory CD4+ T cells [42, 43]. The inhibitory effect of MSCs on pro-inflammatory T cells seems to depend on many parameters such as culture condition (an inflammatory environment). Thus, modification of culture condition for MSCs can enhance their immunomodulatory effects. Duijvestein et al. found that IFN-γ-stimulated MSCs, but not untreated MSCs, significantly attenuated DSS-induced colitis and TNBS-induced colitis via

Figure 5. Immunohistochemistry for Ki67 and LGR-5 in the colonic mucosa of rats (×200). Ki67 and LGR-5 positive cells were mainly located in the colonic villus and intestinal epithelium. The percentage of positive cells was calculated as follow: [(positive cells)/total cell number] × 100%. Results were obtained from three independent experiments. Data were presented as the mean ± SEM (*P < 0.05).
Regulatory T cells play a key role in the treatment of inflammatory diseases (such as IBD), mainly by inhibiting pro-inflammatory cytokine production, down-regulating costimulatory molecules on antigen presenting cells, and modulating T-cell proliferation and differentiation. Luz-Crawford et al. found that the suppressive effect of MSCs was associated with the increased functional CD4+CD25+Foxp3+ regulatory T cells and elevated IL-10 secretion [45]. In line with previous studies, the percentage of Tregs in peripheral blood- and lamina propria-CD4+ T cells increased significantly in rats after treatment with IL-25-primed MSCs as compared with MSC treated colitis rats and untreated colitis rats, suggesting that IL-25 helps MSCs ameliorate DSS-induced colitis by up-regulating Tregs. The MSCs mediated induction of regulatory T cells depends on local conditions. Under different inflammatory environments, MSC constitutively secrete transforming growth factor-β (TGF-β), indoleamine 2,3 deoxygenase (IDO) or IL-2, which acts as a growth factor for regulatory T cells [46]. Furthermore, MSC can induce the differentiation of type 2 macrophages, which produce IL-10, CCL-18 or PGE2 to induce regulatory T cells [47]. However, how IL-25 improves the ability of MSCs to induce regulatory T cells remains to be further identified.

In response to inflammation or tissue injury, MSCs are able to migrate to the injured sites, replace dysfunctional cells and differentiate into various tissues. The migration of MSCs to such sites may be dependent on the chemotactic signals derived from injured or inflamed tissues. Park et al. employed confocal imaging analysis to investigate the direct migration of MSCs in a DSS-induced colitis model, and they found that a larger amount of MSCs was detectable in either colon or regional mesenteric lymph nodes of the DSS-induced colitis group, most in the distal part of the colon, but few in the proximal part [13]. However, in our study, results failed to identify the migration of infused MSCs in the inflamed colonic mucosa in two MSC treatment groups on Day 8. We speculated that the absence of infused MSC was associated with the survival time of MSCs in the inflammatory tissues. Some studies have revealed that in vitro expanded MSCs have a short lifespan after in vivo administration [48]. Another study displays that the infused MSCs are detectable in the inflamed intestine until day 5, but not on day 7 in an experimental colitis model [49]. In mice suffering from TNBS-induced colitis, intravenous injection of MSCs leads to the increased expressions of Ki67 (a cell proliferation marker) and LGR5 (an intestinal stem cell markers) in damaged mucosa, indicating that MSCs induce the differentiation of intestinal stem cells and the proliferation of intestinal epithelial cells [50]. Our results are consistent with previous findings that MSCs up-regulate the expressions of Ki67 and LGR5, but those were comparable between MSC group and IL-25 primed MSC group in our study. Therefore, we postulate that IL-25 has no significant effect on the capability of MSCs to induce the differentiation of intestinal stem cells and the proliferation of intestinal epithelial cells.

In summary, our study demonstrates that IL-25 primed MSCs achieve better therapeutic effects on intestinal inflammation of IBD through inhibiting Th17 immune response and inducing T regulatory cell phenotype. Thus, IL-25 may become an attractive candidate for the MSC-based therapy of IBD.

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Disclosure of conflict of interest

None.

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