Enhanced Immunomodulatory Effects of CXCR4/IL-35-Modified BMSCs on TNBS-Induced Colitis in Rats

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Abstract

Bone marrow-derived mesenchymal stem cells (BMSCs) hold great promise for the treatment of inflammatory bowel disease owing to their immunosuppressive property and tissue healing potential. The balance between regulatory T cells (Tregs) and T helper (Th)17 cells plays a crucial role in BMSC-mediated immunosuppression. Interleukin (IL)-35 is a newly identified anti-inflammatory cytokine required for the expansion of Tregs and suppression of Th17 cell differentiation. IL-35 can amplify the immunosuppressive property of BMSCs when overexpressed in these cells. However, the reparative capability of BMSCs in vivo is limited, partly due to the poor homing efficiency of BMSCs to inflamed colons. Up-regulation of CXC chemokine receptor 4 (CXCR4) expression in BMSCs may affect the directional homing of implanted BMSCs via stromal-derived factor-1. In this study, by lentivirus-mediated introduction of CXCR4 and IL-35 genes to modify rat BMSCs, we observed enhanced migration and strengthened immunomodulatory activities of the genetically engineering BMSCs. These results suggest that modification of BMSCs by dual expression of CXCR4 and IL-35 may provide an effective therapeutic strategy for inflammatory bowel disease.

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Keywords

Bone marrow-derived mesenchymal stem cellsCXCR4IL-35Inflammatory bowel diseaseTregs

1. Introduction

Inflammatory bowel disease (IBD), encompassing Crohn's disease and ulcerative colitis, is a chronic remittent or progressive inflammatory condition of the gastrointestinal tract that present with abdominal pain, bloody mucous diarrhea and tenesmus [1,2]. Affecting over 1 in every 300 people in Western countries, and with an accelerating incidence in Asian countries, IBD is thought to occur in genetically predisposed hosts by an aberrant cell-mediated immune response against enteric microflora [1,3]. Emerging evidence shows that the balance between regulatory T cells (Tregs) and T helper (Th)-17 cells plays a vital role in IBD by regulating immune homeostasis through the activation of forkhead box P3 (FOXP3) or related orphan receptor gamma t (RORγt), and the secretion of anti-inflammatory (i.e., interleukin (IL)-10) or pro-inflammatory (i.e., IL-17A) cytokines [4,5].

The self-renewal, hypoimmunogenic, and immunosuppressive potentials of bone marrow-derived mesenchymal stem cells (BMSCs) have made MSC-based therapy an attractive option in treating IBD [6]. One important mechanism of MSC-mediated immunomodulation is that they can regulate the balance between anti-inflammatory Tregs and inflammatory effector T cells [6,7]. Moreover, the immunosuppressive function of BMSCs can be propagated by collaborating with IL-35 [[8], [9], [10]]. IL-35, a heterodimeric cytokine comprised of the Epstein-Barr-virus-induced gene 3 (EBI3) and IL-12p35, is

produced mainly by Tregs and is required for maximum inhibitory activity [8]. The immunosuppressive function of IL-35 is characterized not only by the suppression of Th17 cells but also by stimulating Tregs to expand. These Tregs, in turn, secrete IL-35 to strengthen the immunosuppressive effect [9,11]. Studies have revealed that IL-35 has both regulatory and therapeutic effects on IBD [8,12].

Large numbers of systemically administered BMSCs are retained in the lung and liver. Consequently, only minimal therapeutic effects are observed [13,14]. Moreover, high cellular engraftment could increase the risk of respiratory and circulatory failure [14,15]. Therefore, migration of the implanted BMSCs to the injured intestinal mucosa successfully is crucial for MSC-based therapy of IBD. CXC chemokine receptor 4 (CXCR4), a seven-transmembrane G-protein–coupled receptor, plays an important role in directing the migration of BMSCs to injured sites through interaction with its ligand, stromal-derived factor-1 (SDF-1; also called CXCL12) [16,17]. SDF-1 levels are up-regulated in inflamed or injured tissues [17], including IBD mucosa [18,19]. In contrast, the expression of CXCR4 in BMSCs is significantly reduced during ex vivo expansion [20]. Up-regulation of CXCR4 expression may affect the directional homing of implanted BMSCs.

Based on these findings, we hypothesized that dual expression of CXCR4 and IL-35 could improve the therapeutic effects of BMSCs against IBD via regulating the Treg/Th17 balance. To test this hypothesis, in this study, we successfully performed dual transfection of lentivirus-mediated CXCR4 and IL-35 genes into rat BMSCs.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 160–180 g were provided by the experimental animal center of Huazhong University of Science and Technology (HUST; Wuhan, China), and housed under specific pathogen-free conditions. All animal procedures were conducted strictly according to the Animal Research Institute Committee guidelines of HUST, and approved by the Institutional Animal Care and Use Committee of HUST.

2.2. Lentiviral vectors expressing rat CXCR4 and IL-35 genes

The fusion fragments of CXCR4 and IL-35 genes were amplified by polymerase chain reaction (PCR) technology. Primer sequences for the fusion gene were 5′ -
GAGGATCCCCGGGTACCGGTCGCCACCATGGAAATATACACT

TCGGATAACTAC-3′ and 5′ - CACACATTCCACAGGCTAGTTAGGAGGAGCT

CAGATAGTTCATCACC-3′. The lentiviral vector, Ubi-MCS-SV40-EGFP-IRES-puromycin (named GV367; Genechem, Shanghai, China), was digested by the restriction enzyme AgeI/NheI. The fusion gene fragments were ligated into the lentiviral vector GV367. The primers (5′- GTCACTGTAGTGCACCCTGG-3′ and 5′-CGTCGCCGT CCAGCTCGACCAG-3′) located in the vector were used in PCR to identify positive transformants. Positive clones, as confirmed by PCR, were chosen for sequencing. Recombinant lentiviruses that co-expressed enhanced green fluorescent protein (EGFP), the anti-puromycin gene, and CXCR4 and IL-35 sequences were produced by 293T cells following co-transfection with GV367 and the packaging plasmids, pHelper 1.0 and 2.0 (Genechem). The virus titer was detected through a drug screening method.

2.3. Preparation of rat BMSCs, transfection with lentivirus, and culture expansion

BMSCs were obtained from healthy male rats 3 weeks of age as described previously [21]. Passage 2 (P2) BMSCs were identified by flow cytometric analysis and the phenotype was characterized with the use of anti-rat CD29, CD90, CD11b, and CD45 (BioLegend, San Diego, CA, USA). After identification, the adipogenic and osteogenic differentiation potentials of P2 BMSCs were investigated as described previously [22]. When P2 BMSCs reached 40–50% confluence, transfection was performed at a multiplicity of infection of 20 in the presence of 10 μg/mL polybrene (Genechem). CXCR4-IL-35-BMSCs were genetically engineered with recombinant lentivirus expressing the anti-puromycin gene, EGFP, CXCR4, and IL-35. Null-BMSCs were manipulated with lentivirus expressing the anti-puromycin gene and EGFP, and were used as a negative control. Successfully transfected cells were selected with puromycin at a final concentration of 2.5 μg/mL (Sigma-Aldrich, St. Louis, MO, USA) for 7 days. Afterwards, the culture medium was changed to low-Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Gibco, Scoresby, Australia).

2.4. Induction of experimental colitis and treatment

After an acclimation period of one week, male rats were assigned randomly to four groups: control, TNBS, null-BMSCs, and CXCR4-IL-35-BMSCs (n = 10 for each group). Colitis was induced by TNBS (Sigma-Aldrich) as described previously [21]. An equal volume of phosphate-buffered saline (PBS) was used instead of TNBS in the control group. On the third day after the enema, rats in the null-BMSC and CXCR4-IL-35-BMSC groups were given corresponding BMSCs (5 × 106 cells/mL) suspended in 1 mL PBS via tail vein injection[23]. The control and TNBS groups were injected with 1 mL PBS. On the seventh day after tail vein injection, animals were anesthetized, and colons were dissected and analyzed for various studies.

2.5. Assessment of inflammation

During the treatments, weight loss, stool viscosity, and hematochezia status were observed daily to score the disease activity index. Colons were dissected and processed for histological analyses as described previously [21].

2.6. Quantitative real-time PCR (qRT-PCR)

The expression of CXCR4, IL-35, IL-10, Foxp3, IL-17A, and RORγt mRNAs was quantified by qRT-PCR as described previously [21]. All mRNA primers are shown in Table 1.

Table 1. Primer sequences used for polymerase chain reaction.

Gene name	Primer sequences (5′ to 3′)
CXCR4	Forward
	ReverseTACACCGTCAACCTTTACAGCA
	GGGGATCCAGACACCCACAT
IL-35	Forward
	ReverseTGCACTGCTGGAGACATCG
	CAAGGCACAGGGTCATCATC
β-actin	Forward
	ReverseCGTTGACATCCGTAAAGACCTC
	TAGGAGCCAGGGCAGTAATCT
IL-10	Forward
	ReverseGCAGGACTTTAAGGGTTACTTGG
	ATCATTCTTCACCTGCTCCACT
FOXP3	Forward
	ReverseAAAGGAGAAGCTGGGAGCTATG
	GGATGAGGGTGGCATAGGTGA
IL-17A	Forward
	ReverseCTCAGACTACCTCAACCGTTCC
	CACTTCTCAGGCTCCCTCTTC
RORγt	Forward
	ReverseAGGCTTGTCATGTTTGATTTCC
	CCGATGTTTGTCTTTCTGGC

2.7. Western blotting and immunofluorescence

Western blotting and immunofluorescence analyses were performed as described previously [21]. Anti-CXCR4 (1:200, Abcam, Cambridge, UK), anti-EBI3 (1:500, Abcam), anti-p35 (1:500, GeneTex, Irvine, CA), anti-FOXP3 (1:2000, Abcam), and anti-RORγt (1:1000, Biorbyt, Wuhan, China) were used as primary antibodies for western blotting. Anti-EBI3 (1:100, Abcam) and anti-p35 (1:100, GeneTex) antibodies were used as primary antibodies for immunofluorescence.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-35, IL-10, and IL-17A in colon homogenate supernatants were detected with cytokine-specific kits using the quantitative sandwich enzyme immunoassay technique, according to the manufacturers' instructions [21]. The following ELISA kits were used: rat IL-35 (Cusabio, Wuhan, China), rat IL-10 (NeoBioscience, Shenzhen, China), and rat IL-17A(NeoBioscience).

2.9. Flow cytometry

Monocytes were isolated from mesenteric lymph nodes (MLNs) and spleen as described previously [24]. For Treg analysis, cells were stained with anti-CD4 and anti-CD25 antibodies (BD Biosciences, San Diego, USA) for 30 min at 4 °C in the dark, then fixed and permeabilized for 30 min in the dark, and finally stained with anti-FOXP3 antibodies (eBioscience, San Diego, CA). For Th17 cell analysis, cells were first treated with a leukocyte activation cocktail with BD GolgiPlug™ (BD Biosciences) in 5% CO2 at 37 °C for 8 h. Subsequently, cells were stained with anti-rat CD4 antibodies (BD Biosciences) for 15 min in the dark, then fixed and permeabilized for 20 min and finally stained with anti-IL-17A antibodies (eBioscience). After washing with permeabilization buffer (BD Biosciences), stained cells were analyzed by flow cytometry.

2.10. Statistical analyses

Data are presented as means ± standard deviation and were analyzed by SPSS 24.0 (IBM, Chicago, IL, USA) software. One-way analysis of variance followed by the least significant difference or Dunnett's T3 post hoc test (equal variances were not assumed) was used for multiple comparisons. The unpaired two-tailed Student t-test was used to compare two groups. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of BMSCs

To identify cultured cells, their immunophenotype was analyzed by flow cytometry and the pluripotent capacity was verified by exposing the cells to induction medium. As illustrated in Fig. 1A, cultured cells were positive for bone marrow progenitor cell surface markers, CD29 and CD90 (96.8%), and negative for hematopoietic cell surface markers, CD11b and CD45 (99.5%). After three weeks of osteogenic induction, calcium nodules were observed in P2 BMSCs and presented as red spots after alizarin red

staining (Fig. 1B). Four weeks after adipogenic induction, lipid droplet formation was obvious in P2 BMSCs and Oil Red O staining showed large amounts of lipid deposition (Fig. 1C). These results demonstrate the purity and differentiation potential of BMSCs.

Fig. 1

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Fig. 1. Expression of CXCR4 and IL-35 is up-regulated in CXCR4-and IL-35-transfected bone marrow-derived mesenchymal stem cells (BMSCs). (A) Flow cytometric characterization of BMSCs at passage 2. (B) Osteogenic differentiation of BMSCs stained with alizarin red. Original magnifcation, × 400. (C) Adipogenic differentiation of BMSCs stained with Oil Red O. Original magnifcation, × 400. (D) GFP-lentivirus transfected BMSCs under optical and fluorescence microscopes. Original magnifcation, × 100. (E)Levels of CXCR4 and IL-35 mRNA in lentivirus-transfected BMSCs. (F) Western blotting analysis of CXCR4, EBI3, and p35 proteins in lentivirus-transfected BMSCs. (G) Representative protein levels of CXCR4/β-actin, EBI3/β-actin, and p35/β-actin. Data are expressed as means ± SD (n = 3). *P < 0.05, **P < 0.01 vs. null-BMSCs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Expression of CXCR4 and IL-35 was up-regulated in CXCR4-and IL-35-transfected BMSCs

To validate whether lentivirus-mediated CXCR4 and IL-35 genes were transfected successfully into BMSCs, fluorescence labelling, qRT-PCR, and western blotting analyses were performed. Seventy-two hours after lentivirus transduction at a multiplicity of infection of 20, GFP expression in transfected BMSCs was observed under a fluorescence microscope (Fig. 1D). The expression of CXCR4 and IL-35 mRNAs increased 4.13- and 3.42-fold, respectively, in the CXCR4-IL-35-BMSCs (Fig. 1E). The protein levels of CXCR4, EBI3, and p35 increased 3.59-, 2.60-, and 3.31-fold in those cells, respectively (Fig. 1F, G). Taken together, the expression of CXCR4 and IL-35 was up-regulated significantly in CXCR4-IL-35-BMSCs compared with null-BMSCs.

3.3. Expression of CXCR4 and IL-35 promoted the migration of BMSCs and increased the levels of IL-35 in target tissues

To examine the migration efficiency of allogeneic BMSCs towards target tissues, GFP-labeled cells in colonic tissues were visualized by immunofluorescence microscopy. In addition, the distribution of IL-35 in colonic tissues was confirmed by co-localization of the EBI3 subunit and p35. We also detected the expression of IL-35 in colons with qRT-PCR and ELISA. The results showed that the GFP protein was expressed extensively in colons from rats treated with CXCR4-IL-35-BMSCs, but expressed sporadically in colons from rats treated with null-BMSCs(Fig. 2A). Similar results were observed in the distribution of IL-35 (Fig. 2B). Moreover, the IL-35 level in the CXCR4-IL-35-BMSC group was almost twice that in the null-BMSC group (Fig. 2C). Thus, our data suggest that more BMSCs migrate to the inflammatory sites, and the levels of IL-35 in colonic tissues are up-regulated, in CXCR4-IL-35-BMSC-treated rats.

Fig. 2

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Fig. 2. Expression of CXCR4 and IL-35 promots the migration of bone marrow-derived mesenchymal stem cells (BMSCs) and increases the levels of IL-35 in target tissues. (A) Representative micrographs of frozen colon sections were stained with green fluorescent protein for immunofluorescence. Original magnification, × 100. (B) Representative immunofluorescent detection of EBI3 and p35 expression in colon tissues. Colon tissue sections were labeled with antibodies to EBI3 (yellow) and p35 (red). Nuclei were stained with DAPI. Original magnification, × 400. (C) The expression of IL-35 in colonic tissues was analyzed by the quantitative real-time polymerase chain reaction (left) and an enzyme-linked immunosorbent assay (right). Data are expressed as means ± SD (n = 8).*P < 0.05, **P < 0.01 vs. 2,4,6-trinitrobenzene sulfonic acid (TNBS) group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Expression of CXCR4 and IL-35 enhanced the protective effects of BMSCs on TNBS-induced colitis

To evaluate the effects of CXCR4 and IL-35 in TNBS-induced colitis, we treated TNBS-induced rats with CXCR4-IL-35-BMSCs through tail vein injection. The disease activity index in the CXCR4-IL-35-BMSC group was decreased significantly compared to the TNBS group (Fig. 3A). Shortening of the colon is a reliable marker of colonic inflammation. Decreases of approximately 22.8, 4.05, and 18.3% in colon length were observed in the TNBS, CXCR4-IL-35-BMSC and null-BMSC groups, respectively when compared with the control group (Fig. 3B, C). Histological analyses showed the expression of CXCR4 and IL-35 mitigated colonic mucosal erosions and inflammatory cell infiltration, and a lower histological score was evident in the CXCR4-IL-35-BMSCgroup (Fig. 3D, E). Taken together, these results demonstrate that the expression of CXCR4 and IL-35 can enhance the ability of BMSCs to inhibit the intestinal mucosal inflammatory response in TNBS-treated rats.

Fig. 3

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Fig. 3. Expression of CXCR4 and IL-35 enhances the protective effects of bone marrow-derived mesenchymal stem cells (BMSCs) in rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. (A) The disease activity index (DAI) of rats was monitored daily. (B) Colons of rats from different treatments are shown. (C) Colonic length of rats. (D) Colonic histological scores. (E) Colon specimens stained with hematoxylin and eosin were analyzed. Original magnification, × 200. Data are expressed as means ± SD (n = 8).*P < 0.05, **P < 0.01 vs. TNBS group.

3.5. Expression of CXCR4 and IL-35 expanded Tregs and suppressed Th17 cells in TNBS-induced colitis rats

To confirm the anti-inflammatory effects of CXCR4-IL-35-BMSCs in vivo, we analyzed the balance between Tregs and Th17 cells in MLNs and spleen. We observed an increased percentage of Tregs and a decreased percentage of Th17 cells both in MLNs and spleen of different BMSC-treated rats. CXCR4-IL-35-BMSC-treated rats showed the most significant changes (Fig. 4A–D). Furthermore, we measured the levels of signature cytokines that are vital for the differentiation of Tregs and Th17 subsets in colonic tissues. Systemic infusion with CXCR4-IL-35-BMSCs significantly up-regulated the protein and mRNA levels of IL-10, and down-regulated the expression of IL-17A, compared to infusion with null-BMSCs (Fig. 4E, F). According to these results, the ability of CXCR4 and IL-35 to ameliorate TNBS-induced colitis is associated with an expansion of Tregs and a reduction of Th17 cells.

Fig. 4

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Fig. 4. Expression of CXCR4 and IL-35 expands Tregs and suppresses Th17 cells in rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. (A) The frequencies of Tregs in mesenteric lymph nodes (MLNs) and spleen were analyzed by flow cytometry. (B) Average frequencies of Tregs in MLNs and spleen. (C) Frequencies of Th17 cells in MLNs and the spleen. (D) Averagefrequencies of Th17 cells in MLNs and spleen. (E) Quantitative real-time polymerase chain reaction (qRT-PCR) analyses of IL-10 and IL-17A mRNA expression in colons. (F) Enzyme-linked immunosorbent assay analyses of IL-10 and IL-17A protein expression in colons. (G) FOXP3 and RORγt mRNA in colons were analyzed by qRT-PCR. (H) Western blotting analyses of FOXP3 and RORγt protein levels in colons. Each bar represents the mean ± SD(n = 8) from each group. *P < 0.05, **P < 0.01 vs. TNBS group.

3.6. Effects of CXCR4 and IL-35 on the expression of FOXP3 and RORγt in TNBS-induced colitis

To investigate the molecular mechanism by which CXCR4 and IL-35 affected Treg and Th17 subsets, we determined the expression of nuclear transcription factors of these subsets by qRT-PCR and western blotting. Treatment with CXCR4-IL-35-BMSCs enhanced the expression of FOXP3, but decreased the expression of RORγt, compared to treatment with null-BMSCs, at both the protein and mRNA levels (Fig. 4G, H). Taken together, thses results demonstrate that CXCR4-IL-35-BMSCs play a crucial role in preventing the development of intestinal inflammation and maintaining intestinal immune homeostasis.

4. Discussion

Extensive evidence has suggested that BMSCs hold great promise for the treatment of many autoimmune diseases [6]. Owning to their immunosuppressive property and tissue healing potential, BMSCs appear to be specifically promising in IBD where a dysregulated immune system leads to tissue damage [1,6]. The immunosuppressive properties of BMSCs could be amplified by transforming BMSCs with IL-35. Previous data had validated this effect in vivo where overexpression of IL-35 improved the immunosuppressive effects of MSCs in mice [10]. The majority of systemically infused BMSCs become emboli embedded in the lungs or are filtered out of the circulation by organs [14,15]. BMSCs migrate to injured sites in response to SDF-1. However, during ex vivo expansion, BMSCs reduce the surface

expression of the SDF-1 receptor, CXCR4 [17,20]. Our previous study demonstrated that up-regulating the expression of CXCR4 on BMSCs promoted the homing of these cells to inflamed colons via SDF-1 [25]. In the present study, with genetic modification of BMSCs by introducing CXCR4 and IL-35, significantly decreased disease severity and more GFP-labeled BMSCs were observed in the colons of rats with TNBS-induced colitis that were infused with CXCR4-IL-35-BMSCs.

Based on the decreased disease severity, we attempted to clarify the presumed mechanism by which overexpressing CXCR4-IL-35 could enhance the immunosuppressive activities of BMSCs. Inflammation is a tightly regulated defense process involving the release of inflammatory mediators and the migration of immune cells [26]. Of these immune cells, T cells and macrophages in the inflamed areas up-regulate the secretion of inflammatory mediators such as IL-17, which promote the development of colitis [1] and trigger T cell differentiation. Especially, Th17 cells secrete IL-17A and then recruit inflammatory cells such as monocytes and neutrophils to the inflamed sites [27], which would aggravate IBD. Tregs inhibit Th17 differentiation and IL-17A expression partially by releasing IL-10 [28]. Hence, the frequency of Th17 cells in TNBS-treated rats was higher than in the control group. The balance of Treg/Th17 plays a crucial role in BMSC-mediated immunosuppression [6]. Studies suggested that MSCs could up-regulate Tregs and down-regulate Th17 cells in systemic lupus erythematosus [29]. Here, our results revealed that CXCR4-IL-35-BMSCs potently increased the frequency of CD4+CD25+ FOXP3+ Tregs and significantly decreased the frequency of Th17 in MLNs and spleen. These findings were consistent with the reports mentioned above. CXCR4-IL-35-BMSCs also increased IL-10 expression and inhibited IL-17A expression. FOXP3, the Treg transcription factor, is required for the differentiation and suppressive function of these cells [30]. RORγt, the Th17 transcription factor, is necessary and sufficient for IL-17 expression and the differentiation of Th17 cells in mouse and human CD4+ T cells [31,32]. In the CXCR4-IL-35-BMSC-treated group, the expression of FOXP3 was significantly elevated while the expression of RORγt declined markedly. However, further studies will be required to clarify the individual contributions of CXCR4 and IL-35.

In conclusion, our study demonstrated that enhanced expression of CXCR4 and IL-35 by lentiviral-mediated gene transfer in BMSCs significantly increased the migration of these cells to inflamed colons in rats with TNBS-induced colitis. Furthermore, CXCR4-IL-35-BMSCs remarkably strengthened the immunomodulatory activity of BMSCs through the simultaneous expansion of Tregs and suppression of Th17 cells. Therefore, modification of BMSCs by lentivirus-mediated CXCR4 and IL-35 dual gene transfection may be a potential approach in the cell-based treatment of IBD.

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