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Dual expression of CXCR4 and IL-35 enhances the therapeutic effects of BMSCs on TNBS-induced colitis in rats through expansion of Tregs and suppression of Th17 cells

Zhen Nan [1], Heng Fan [1], Qing Tang*, Man Zhang, Meng Xu, Qianyun Chen, Yujin Liu, Yalan Dong, Hui Wu, Shuangjiao Deng

Department of Integrated Traditional Chinese and Western Medicine, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

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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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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 [8–10]. 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

* Corresponding author.
E-mail address: 256497630@qq.com (Q. Tang).
[1] These authors contributed equally to this study.

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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 (5×10^6 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.

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,

Table 1
Primer sequences used for polymerase chain reaction.

Gene name		Primer sequences (5′ to 3′)
CXCR4	Forward	TACACCGTCAACCTTTACAGCA
	Reverse	GGGGATCCAGACACCCACAT
IL-35	Forward	TGCACTGCTGGAGACATCG
	Reverse	CAAGGCACAGGGTCATCATC
β-actin	Forward	CGTTGACATCCGTAAAGACCTC
	Reverse	TAGGAGCCAGGGCAGTAATCT
IL-10	Forward	GCAGGACTTTAAGGGTTACTTGG
	Reverse	ATCATTCTTCACCTGCTCCACT
FOXP3	Forward	AAAGGAGAAGCTGGGAGCTATG
	Reverse	GGATGAGGGTGGCATAGGTGA
IL-17A	Forward	CTCAGACTACCTCAACCGTTCC
	Reverse	CACTTCTCAGGCTCCCTCTTC
RORγt	Forward	AGGCTTGTCATGTTTGATTTCC
	Reverse	CCGATGTTTGTCTTTCTGGC

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