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Research paper

Mesenchymal stem cells overexpressing IL-35 effectively inhibit CD4$^+$ T cell function

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ABSTRACT

Mesenchymal stem cells (MSCs) have recently emerged as promising candidates for cell-based immune tolerance therapy. Interleukin 35 (IL-35) is a relatively newly identified cytokine required for the regulatory and suppressive functions of regulatory T cells (Treg), playing an important role in the prevention of autoimmune diseases. In this study, we isolated adipose tissue-derived MSCs, a good vehicle for cell therapy, which were transfected with a lentivirus vector for the overexpression of the therapeutic murine IL-35 gene. IL-35 levels in transfected MSCs (IL-35-MSCs) were quantified by ELISA. Co-culture of CD4$^+$ T cells and IL-35-MSCs resulted in the inhibition of CD4$^+$ T cell proliferation and IL-17A secretion. In addition, IL-35-MSCs induced IL-10 production by CD4$^+$ T cells, but did not affect IFN-γ. These findings suggested that MSCs over-expressing IL-35 had higher immunosuppressive capacity compared with non-transfected MSCs, and may provide a useful approach for basic research on gene therapy for autoimmune disorders.

1. Introduction

Interleukin (IL)-35 is a relatively newly identified heterodimeric cytokine containing the Epstein-Barr virus-induced gene 3 (EBI3) and p35 subunits. IL-35 is produced mainly by natural regulatory T cells (nTreg) and required for maximal regulatory activity of murine Tregs *in vitro* and *in vivo* [1]. IL-35 is involved in inflammatory diseases of the nervous, digestive, bone and joint, and respiratory systems. IL-35 also plays a certain role in allergic diseases and organ transplantation. Since its discovery, the predominant mechanism of suppression associated with IL-35 activity is its ability to suppress T-cell proliferation and effector functions.

Mesenchymal stem cells (MSCs) are undifferentiated adult cells and immune-privileged cells due to low expression of MHC-II and co-stimulatory molecules on their cell surface. MSCs can inhibit immune responses and influence all immune components as shown for T-, B-, natural killer-, monocytic and dendritic cells, both *in vitro* and *in vivo* [2,3]. Another unique feature of MSCs is accumulation at the site of damage or inflammation. These characteristics may be amplified by transforming them with genes that will improve their therapeutic ability [4,5]. Interestingly, MSCs have recently emerged as promising candidates for cell-based immunotherapy [6–8].

Although MSCs are currently most commonly obtained from the bone marrow, they are present in virtually all adult tissues assessed, including the adipose tissue. Several *in vitro* studies demonstrated that adipose tissue-derived MSCs (Ad-MSCs) are characterized by higher proliferation potential in comparison with bone marrow-derived MSCs (BM-MSCs). Therefore, Ad-MSCs represent a highly promising tool for stem cell-based therapy [9]. The current study demonstrated that Ad-MSCs overexpressing IL-35 can inhibit CD4$^+$ T cell function.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice (4–5 weeks old) were purchased from the Institute of Radiation Medicine Laboratory Animal Center (Tianjin, China), and maintained in the animal facility under standard conditions, according to institutional guidelines, all animal protocols

Abbreviations: Ad-MSCs, adipose derived mesenchymal stem cells; IL-35, Interleukin-35; Tregs, regulatory T cells; MOI, multiplicity of infection; GFP, green fluorescent protein.

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were reviewed and approved by the appropriate institutional review committee.

2.2. Isolation and culture of Ad-MSCs

Subcutaneous adipose tissues were obtained from C57/BL6J mice under aseptic conditions. After tissue mincing, typeⅠ collagenase was added for 1 h at 37 °C, with shaking. The digested adipose tissue was centrifuged at 1500 rpm for 10 min to obtain a cell pellet after addition of 3 volumes of PBS. The resulting pellet was washed twice with PBS, and resuspended in the DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic cocktail. Cells were plated at a density of 10^7 cells/cm^2 and incubated at 37 °C in a humidified environment containing 5% CO_2. Fresh culture medium was replaced every 48 h. Confluent third-passage cells were used for experiments.

2.3. Antibodies and flow cytometric analysis

A BD FACS CantoⅡ (BD Biosciences) instrument was used for flow cytometry, and data were analyzed with the BD FACSDiva software (BD Biosciences). Cells were collected, washed twice with PBS and stained according to the manufacturer's recommendations for flow cytometry. FITC conjugated anti-mouse Sca-1, FITC conjugated anti-mouse CD44, FITC conjugated anti-mouse CD34, PE conjugated anti-mouse CD29, PE conjugated anti-mouse CD45 were puchased from BioLegend (San Diego, CA, USA). FITC conjugated anti-mouse CD4, APC conjugated anti-mouse Foxp3, Percp-Cy5.5 conjugated anti-mouse IL-10, PE conjugated anti-mouse IL-17, APC conjugated anti-mouse IFN-γ were puchased from eBioscience (San Diego, CA, USA).

2.4. IL-35 gene cloning and lentivirus production

Murine IL-35 cDNA was amplified from the pSecTag2A-IL35 plasmid (a gift from the Glasgow Biomedical Research center), and subcloned into pCCS-Lv201 to generate the recombinant vector pCCS-IL35-Lv201. Lentivirus generation was performed by using GeneCopoeia 293Ta cells, according to the instructions of the lentivirus package kit (Lenti-Pac™ HIV, GeneCopoeia). Cell culture supernatants were collected and concentrated with the Lenti-Pac concentration kit (GeneCopoeia).

2.5. Ad-MSC transduction

Ad-MSCs were trypsinized and seeded at a density of 2×10^4 -cells in 1 ml DMEM-F12 supplemented with 10% FBS on 24 well plate. Then, 10 μL virus solution was added in presence of 5 μg/ml Polybrene (Sigma) followed by centrifugation for 1 min at 1500 rpm. Six hours later, the medium was refreshed. GFP positive transduced Ad-MSCs were observed by fluorescence microscopy, with transfection rates measured by flow cytometry 72 h after transduction. IL-35 protein levels in cell supernatants were measured using the mIL-35 ELISA kit (BioLegend) according to the manufacturer's instructions.

2.6. IL-35 gene expression assessment

Transfected or non-transfected Ad-MSCs were trypsinized and washed twice with PBS. Total RNA was extracted using TRIzol Reagent (Invitrogen) and reverse-transcribed by QuantScript RT Kit (Tiangen). IL-35 mRNA expression was quantified by PCR, with the following primers: forward, 5′-CCCCGGATCCCACTGAAA CAGCTCTCGTGGCTCT-3′; reverse, 5′- GGCGGCGGCCGCATAGCCCAT CACCCTGTTGA-3′.

2.7. Co-culture experiments

Transfected or non-transfected Ad-MSCs (2.5×10^4 cells/well) were seeded in 48-flat bottom plates and incubated for 24 h. Mouse $CD4^+$ T cells were isolated from splenocytes using mouse $CD4^+$ T cell isolation kit II (Miltenyi Biotec), following the manufacturer's instructions, with a purity exceeding 95%. $CD4^+$ T cells were added at different ratios, and optimal effects were found for a MSCs/T ratio of 1:5.

2.8. $CD4^+$ T cell proliferation

$CD4^+$ T cells were labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen), and added to cultured transfected or non-transfected Ad-MSCs the following day. Cells were cultured with soluble anti-CD3 (2 μg/ml), anti-CD28 (1 μg/ml) and IL-2 (2 ng/ml). After 72 h of incubation, cell proliferation was assessed by flow cytometry, data were analyzed by the Modifit software.

A 96-well Transwell plate with 0.4 μm pore size was also used (Corning) for the co-culture. Purified $CD4^+$ T cells mixed with soluble cytokines, as mentioned above, in the culture media, were placed in the lower chamber with IL-35-MSCs or MSCs in the upper chamber. Cells were cultured for 24 and 48 h, then removed the upper chamber and added 10 μL WST-8 dye (Boster) into the lower chamber. Cells were incubate at 37 °C for 2 h and the absorbance were finally determined at 450 nm using a microplate reader. The cell growth rates were calculated by the method that all the value of absorbance were divided by the mean absorbance of control group which contain $CD4^+$ T cells alone.

2.9. Cytokine secretion in $CD4^+$ T cells

Purified $CD4^+$ T cells were added to cultured transfected or non-transfected Ad-MSCs the following day with soluble cytokines. After 72 h of incubation, cell culture supernatants were collected and assessed by IL-17A (eBioscience), IL-10 (eBioscience) and IFN-γ (BioLegend) ELISA kits to determine the cytokine secretion levels.

A 24-well Transwell plate with 0.4 μm pore size was used for the co-culture. Purified $CD4^+$ T cells mixed with soluble anti-CD3, anti-CD-28 and IL-2 in the culture media, were placed in the lower chamber with IL-35-MSCs or MSCs in the upper chamber. Cells were cultured for 72 h, and $CD4^+$ T cells in the lower chambers were restimulated for 6 h by PMA and ionomycin (Sigma). Cells were collected and stained with anti-mCD4, anti-mIL-17, anti-mIL-10, anti-mIFN-γ or anti-mFoxp3. Then the IL-17, IL-10 and IFN-γ secrection level of $CD4^+$ T cells and the proportion of $CD4^+$-$Foxp3^+$Treg cells can be analyzed by flow cytometry.

2.10. Statistical analysis

GraphPad Prism (version: 5.04) was used to perform all statistical analyses. One-way analysis of variance (ANOVA) was used for group comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of Ad-MSCs

MSCs from mouse adipose tissues grew to sub-confluence after 7 days and showed a fibroblast-like morphology. Meanwhile Ad-MSCs grew faster and were passaged after about 3 days upon the initial passage. The phenotypes were analyzed by flow cytometry cells were positive for Sca-1 CD44 and CD29 and negative for