Autophagy Improves the Immunosuppression of CD4+ T Cells by Mesenchymal Stem Cells Through Transforming Growth Factor-β1

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Key Words. Autophagy • Mesenchymal stem cells • Transforming growth factor-β1 • Immunosuppression • CD4+ T cells

ABSTRACT

Mesenchymal stem cells (MSCs) have been extensively investigated as a promising approach to treat many autoimmune and inflammatory diseases. The stress condition would affect the therapeutic efficacy and induce autophagy of MSCs. However, whether autophagy would affect the immunosuppressive capacity of MSCs is largely unknown. The present study aimed to assess whether autophagy plays an important role in regulating the immunomodulation of MSCs and the underlying mechanisms. We successfully inhibited and induced autophagy of MSCs using 3-methyladenine (3-MA) and rapamycin, respectively. Our results demonstrated that rapamycin strengthened the capacity of MSCs to inhibit CD4+ T-cell proliferation, whereas 3-MA weakened the inhibitory ability of MSCs. Mechanistically, 3-MA-treated MSCs secreted less, whereas rapamycin-treated MSCs secreted more transforming growth factor-β1 (TGF-β1) compared with the control cells. Furthermore, exogenous TGF-β1 addition recovered the immunosuppressive capacity of 3-MA-pretreated MSCs, whereas exogenous anti-TGF-β1 antibody addition reduced the immunosuppressive capacity of rapamycin-pretreated MSCs. These results indicated that the autophagy level regulates the immunosuppression of CD4+ T cells by MSCs through affecting TGF-β1 secretion and provides a novel method for improving the therapeutic efficacy of MSCs by activating autophagy. Stem Cells Translational Medicine 2016;5:1496–1505

SIGNIFICANCE

Mesenchymal stem cell (MSC)-based therapy is a promising tool to treat many diseases. Autophagy occurred in MSCs during their application, especially in those exposed to stress conditions. However, whether autophagy will affect the therapeutic efficacy of MSCs is largely unknown. This study makes a significant contribution to demonstrate that autophagy could improve the immunosuppression of CD4+ T cells by mesenchymal stem cells through transforming growth factor-β1. Therefore, regulation of autophagy in MSCs would provide a promising strategy to improve the therapeutic efficacy of these cells.

INTRODUCTION

Autophagy is a lysosome-dependent degradation process that continuously recycles proteins and dysfunctional organelles and has a crucial role in different physiological and pathological conditions [1–3]. Furthermore, autophagy enables cells to respond to stresses such as starvation, inflammation, and hypoxia; thus, it is considered a protective mechanism [4–9]. A growing body of evidence suggests that autophagy has important roles in many different types of cells, such as lymphocytes, monocytes, and mesenchymal stem cells (MSCs) [10, 11]. Furthermore, some studies have shown that autophagy has a close relation with immunity, including innate immunity, the inflammatory response, and adaptive immunity. However, whether MSC autophagy affects its immunomodulation of peripheral blood mononuclear cells (PBMCs) is unknown.

MSCs are known as multipotential stem cells that are capable of self-renewal and differentiation into multiple lineages [12–14]. MSCs have been applied to treat many autoimmune and inflammatory diseases, such as graft-versus-host disease (GVHD) [15], Crohn’s disease [16], and ankylosing spondylitis [17]; these applications depended on the immunomodulatory effects and low host immune reactivity of these cells [18]. However, the individual treatment efficacy of these cells is currently varied and very limited, and the underlying mechanisms that affect the treatment efficacy are poorly understood. Stress
conditions such as inflammation, hypoxia, injury, or acid-base disturbances may affect the therapeutic efficacy of these cells; this possibility deserves further investigation. Moreover, stress conditions may induce autophagy in MSCs; some studies have demonstrated that downregulation of autophagy might protect MSCs against stressful conditions [19], whereas other studies take the opposite view. Thus, we hypothesize that autophagy may affect the therapeutic efficacy of MSCs.

The dominant view of autophagy is that paracrine factors of MSCs, in particular, as well as cell-cell contact, play important roles in the immunosuppressive capacity of MSCs. The cytokine transforming growth factor-β (TGF-β) plays an important role in regulating the immune response of MSCs. Three TGF-β members, TGF-β1, TGF-β2, and TGF-β3, have been described in mammalian species. Among these members, TGF-β1 is the predominant isoform of this family that is expressed in the immune system [20] and that is responsible for MSC-mediated inhibition of T cells [21]. Some studies demonstrated that TGF-β could activate the autophagy [22], whereas some other studies showed that autophagy could regulate the TGF-β expression of some cells [23, 24].

In our study, we demonstrated that autophagy could improve the immunosuppressive function of MSCs. Autophagy was inhibited using 3-methyladenine (3-MA) to weaken MSC immunosuppression of CD4+ T-cell proliferation, whereas autophagy was induced by rapamycin to improve MSC immunosuppression of CD4+ T-cell proliferation. We found that TGF-β1 secretion by MSCs was positively associated with autophagy of MSCs. Thus, activating autophagy may be a novel strategy for improving the therapeutic efficacy of MSCs.

Materials and Methods

Isolation and Culture of MSCs and PBMCs

This study was approved by the ethics committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University (Guangzhou, People’s Republic of China). All donors in our study were informed of the possible risks and the study objectives and signed the informed consent. Eighteen healthy donors (10 males, 8 females) between the ages of 20 and 30 years who had no history of any significant illness were selected for the study. Bone marrow was extracted from the posterior superior iliac spine under sterile conditions, and MSCs were isolated and purified according to a previously reported method. MSCs from passages 3 to 5 were used for the experiments. PBMCs were collected and purified from the peripheral blood of 18 healthy donors (10 males, 8 females) using Ficoll-Hypaque gradient centrifugation (GE Healthcare Life Sciences, Marlborough, MA, http://www.gelifesciences.com).

Flow Cytometry

MSCs were characterized by flow cytometry. MSCs were detached and incubated for 30 minutes at room temperature with the following specific antibodies: PE Mouse Anti-CD29 (IgG1, k; Clone: MAR4), FITC Rat Anti-Human CD44 (IgG2b, k; Clone: G44-26), FITC Mouse Anti-Human CD105 (IgG1, k; Clone: 266), FITC Rat Anti-Human CD45 (IgG2b, k; Clone: 30-F11), APC Mouse Anti-Human CD34 (IgG1, k; Clone: 563) and PE Mouse Anti-Human HLA-DR (IgG2a, k; Clone: G44-6) (all from BD Biosciences, San Jose, CA, http://www.bdbiosciences.com). Finally, the cells were assayed using a BD Influx Cell Sorter (BD Biosciences).

At the end of coculture, the PBMCs were harvested to analyze CD4+ T-cell proliferation. The carboxyfluorescein diacetate succinimidyl ester (CFDA-SE)-incubated PBMCs were incubated with PE Mouse Anti-Human CD4 (IgG1, k; Clone: RPA-T4; BD Biosciences) for 30 minutes and then washed with phosphate-buffered saline (PBS) before detection. We collected the pretreated MSCs to analyze MSC apoptosis using an Annexin V-PE Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer’s instructions.

Multipotent Differentiation Potential of MSCs

Osteogenic Differentiation

MSCs were seeded at a concentration of 1.5 × 10^5 cells per cm² in 12-well plates, and osteogenic differentiation was induced by the addition of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 IU/ml streptomycin, 0.1 µM dexamethasone, 10 mM β-glycerol phosphate, and 50 µM ascorbic acid (Sigma-Aldrich, St. Louis, MO, https://www.sigmaaldrich.com). The medium was replaced every 3 days for 21 days. Differenrtiated cells were stained with Alizarin Red S (ARS) to detect de novo formation of bone matrix.

Chondrogenic Differentiation

MSCs were grown as high-density pellets (5 × 10^5 cells) for 3 weeks in specific medium. Serum-free chondrogenic medium containing high-glucose DMEM supplemented with 1% ITS-Premix (Corning Life Sciences, Corning, NY, www.corning.com/lifesciences), 50 mg/L ascorbic acid (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 100 nM dexamethasone (Sigma-Aldrich), and 10 ng/ml recombinant human TGF-β3 (R&D Systems, Inc., Minneapolis, MN, https://www.rndsystems.com). On day 21, pellets were prepared for histology and stained with toluidine blue to detect the secretion of sulfated glycosaminoglycans.

Adipogenic Differentiation

For adipogenic induction, MSCs were induced in adipogenic medium consisting of DMEM supplemented with 1% ITS-Premix, 10% FBS, 1 µM dexamethasone (Sigma-Aldrich), 10 µg/ml insulin (Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), and 0.2 mM indomethacin (Sigma-Aldrich). The cells were stained with Oil Red O (ORO) on day 21.

Wortmannin, 3-MA, Rapamycin, and LiCl Preparation, and MSC Pretreatment

To establish the autophagy model of MSCs, wortmannin, 3-MA, rapamycin, and LiCl were added to the cultures. Wortmannin (100 nM), 3-MA (10 mM), and LiCl (20 mM) were dissolved in the culture medium, and rapamycin (3 µM) was dissolved in dimethyl sulfoxide (DMSO).

Cell Culture

MSCs were seeded and treated with 3-MA or rapamycin for 24 hours. Then, the medium was removed, and the cells were washed thoroughly with PBS three times. PBMCs were added to the coculture system with 2 ml medium at a ratio of 1:10 MSCs (0.5 × 10^5 cells):PBMCs (5 × 10^5 cells). For detecting CD4+ T-cell proliferation, PBMCs were incubated with 5 µM CFDA-SE (Thermo Fisher Scientific, Waltham, Massachusetts, USA).
USA, http://www.thermofisher.com) for 15 minutes and washed with PBS containing 10% FBS before coculture. Cells were cocultured in RPMI-1640 medium containing purified anti-CD3 (0.2 μg/ml, BD Biosciences) and anti-CD28 (1 μg/ml, BD Biosciences) antibodies to stimulate T cells to proliferate for 5 days.

Cell Proliferation Assay
MSCs were seeded in 96-well microplates and subjected to different treatments for the indicated times. A Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Rockville, MD, http://www.dojindo.com) was used to assess cell proliferation ability according to the manufacturer’s protocol.

Quantitative Real-Time Polymerase Chain Reaction
The quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using SYBR Premix Ex Taq (TaKaRa Bio, Inc., Kusatsu, Japan, http://www.takara-bio.com) and a LightCycler 480 Real-Time PCR system (Roche, Indianapolis, IN, https://www.lifescience.roche.com) to quantify mRNA levels of relative genes of our experiment. Briefly, total RNA was isolated from MSCs using TRIzol (Thermo Fisher Scientific,) according to the manufacturer’s instructions and was transcribed into cDNA using a PrimeScript RT reagent kit (TaKaRa). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene for quantification, and the relative expression levels of each gene were analyzed using the 2^-ΔΔCt method. The data were analyzed using real-time PCR analysis software (Fluidigm Corporation, South San Francisco, CA, https://www.fluidigm.com). The sequences for the primers used in this study are summarized in supplemental online Table 1.

Enzyme-Linked Immunosorbent Assay
The level of TGF-β1 secreted into the culture supernatants was measured using a Human TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich) according to the manufacturer’s instructions. Notably, unlike other ELISA kits, the TGF-β1 samples in this kit must be activated before use. Briefly, 1N HCl was used to activate latent TGF-β1 to its immunoreactive form, and then the protein was neutralized by adding 1.2 N NaOH/0.5 M HEPES (pH = 7.0–7.6). The results were normalized to the total protein content.

Western Blot Analysis
After the MSCs were subjected to different treatments, they were lysed, and protein contents were quantified as described. Equal amounts of protein lysates were loaded onto gels for 10% and 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis. After the proteins were separated, they were transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, http://www.emdmillipore.com), blocked with 5% nonfat milk, and incubated overnight at 4°C with primary antibodies directed against LC3 (Sigma-Aldrich; catalog no. L7543), p62 (Cell Signaling Technology, Danvers, MA, http://www.cellsignal.com; catalog no. 8025), TGF-β1 (Abcam, Cambridge, U.K., http://www.abcam.com; catalog no. ab27969), or GAPDH. After the membranes were washed three times, they were incubated with the specific secondary antibodies (1:3000; Santa Cruz Biotechnology, Dallas, TX, http://www.scbt.com) for 1 hour at room temperature. The membranes were washed again, and then the bands were detected using Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore). GAPDH protein served as an internal loading control. The intensity of each band was determined using ImageJ software, version 1.49e (National Institutes of Health, Bethesda, MD, https://imagej.nih.gov/ij).

Formation and Quantification of Exogenous GFP-LC3B
MSCs were seeded in 12-well plates and transfected with lentiviral vector containing GFP-LC3B (GenePharma, Shanghai, China, http://www.gene-pharma.com) for 24 hours, and the medium was replaced. Then, the cells were exposed to different treatments for 24 hours. The cells were washed three times with PBS, after which GFP-LC3 was detected and analyzed immediately under a fluorescence microscope.

Statistical Analysis
All quantitative data were reported as the means ± SDs, using one-way analysis of variance to analyze the differences among groups. SPSS version 13.0 (IBM, Armonk, NY, http://www.ibm.com) was used to carry out the statistical analysis. Statistical significance was set at p < .05.

RESULTS
Phenotypic Characterization and Multipotent Differentiation of MSCs
This study used plastic-adherent and spindle-shaped MSCs. All MSCs express CD29, CD44, and CD105 but lack CD34, CD45, and HLA-DR expression according to the classical antigens of MSCs (Fig. 1A). To explore the trilineage differentiation potential of these cells, MSCs were cultured in osteogenic, chondrogenic, or adipogenic medium for 21 days, and then ARS, toluidine blue, or ORO was used to detect differentiation (Fig. 1B). All results conformed to the standard criteria stated by the International Society for Cellular Therapy (Vancouver, British Columbia, Canada, http://www.celltherapysociety.org) [25].

Canonical Drug Can Regulate MSC Autophagy
Previous studies revealed that autophagy is involved in the molecular biology of MSCs. We investigated the autophagy of MSCs pretreated with wortmannin, 3-MA, rapamycin, or LiCl. Wortmannin and 3-MA are classical autophagy inhibitors, whereas rapamycin and LiCl are well-characterized autophagy inducers. Western blot analysis showed that the ratio of LC3 II/I decreased but that the ratio of p62/GAPDH increased after treatment with wortmannin or 3-MA for 24 hours, whereas treatment with rapamycin or LiCl displayed the reverse results (Fig. 2A). Furthermore, MSCs were transfected with lentiviral vector carrying GFP-LC3B and treated with different drugs. Puncta staining, which represents MSC autophagy, was then observed under a fluorescence microscope. The green puncta were obviously reduced in cells treated with wortmannin or 3-MA but increased dramatically in cells treated with rapamycin or LiCl (Fig. 2B). These results demonstrated that wortmannin or 3-MA could inhibit MSC autophagy, whereas rapamycin or LiCl could induce MSC autophagy. In particular, the cells treated with 3-MA and rapamycin presented dramatic changes compared with the control cells without treatment. Thus, we chose 3-MA and rapamycin in the following experiment.
Stimulation Times and Concentration Gradients of 3-MA and Rapamycin

MSCs were treated with 10 μM 3-MA for 8, 24, 48, and 72 hours. Autophagic response was detected by Western blot analysis using antibodies directed against LC3 II/I and p62/GAPDH. The ratio of LC3 II/I gradually decreased and reached the minimum value within 24 hours after the cells were treated with 3-MA; this response did not change, even when the stimulation time was prolonged to 48 hours and 72 hours. Because p62 is combined with LC3 and is degraded by the autophagy-lysosome pathway (30), the p62 protein level showed the crosscurrent compared with LC3 and autophagy (Fig. 3A). Then, MSCs were treated with 5, 10, 15, and 20 μM 3-MA for 24 hours, and the autophagy inhibitory capacity of 3-MA positively correlated with the concentration within 10 μM. The result suggested that the optimal stimulus concentration of 3-MA is 10 μM; this concentration results in the lowest LC3 II/I ratio and the highest p62/GAPDH ratio (Fig. 3B). Similarly, MSCs were exposed to 3 μM rapamycin for 8, 24, 48, and 72 hours. The ratio of LC3 II/I increased remarkably within 24 hours but had inconspicuous changes at 48 and 72 hours later, whereas p62/GAPDH reached the minimum level in 24 hours (Fig. 3C). Furthermore, we treated MSCs with 1, 3, 5, and 10 μM rapamycin for 24 hours. Autophagy was activated prominently, with an increase in the concentration of rapamycin within 3 μM, and reached a peak at 3 μM rapamycin (Fig. 3D). According to the above results, 10 μM 3-MA or 3 μM rapamycin treatment for 24 hours was chosen for the following experiments.

3-MA or Rapamycin Did Not Induce MSC Apoptosis or Affect MSC Proliferation

The immunomodulatory ability of MSCs depends on their state, proliferation, and culture conditions. Thus, we examined MSC apoptosis and proliferation after treatment with 3-MA or rapamycin. MSC apoptosis was analyzed by flow cytometry; the results suggested that 10 μM 3-MA or 3 μM rapamycin would not induce apoptosis (Fig. 4A). CCK-8 revealed that cell proliferation did not differ among control, 3-MA-, and rapamycin-treated cells (Fig. 4B). Altogether, the results indicate that the different immunosuppressive abilities of each MSC group do not depend on the MSC number or status.

Autophagy Regulates the Immunosuppressive Potential of MSCs

At the fifth day of coculture, the proliferation of CD4+ T cells was analyzed according to fluorescence intensities. The blank group (PBMCs: not activated by CD3/CD28, non-cocultured) was
defined as the nonproliferation group. The CD3/CD28<sup>+</sup> group of PBMCs were treated with purified CD3/CD28 antibody, and the proliferation rate of CD4<sup>+</sup> T cells elevated remarkably. When CD3/CD28-treated CD4<sup>+</sup> T cells were cocultured with MSCs, the proliferation of CD3/CD28-treated CD4<sup>+</sup> T cells could be inhibited effectively. The 3-MA and rapamycin groups showed opposite results compared with the control group. Our results demonstrated that the proliferation of CD3/CD28-treated CD4<sup>+</sup> T cells cocultured with MSCs pretreated with 3-MA was higher than the control but that a remarkably lower proliferation rate was observed in the rapamycin-pretreated group (Fig. 5). In summary, MSCs have certain immunosuppressive effects on CD4<sup>+</sup> T cells, and we can regulate this immunosuppressive potential by inhibiting or activating autophagy of MSCs.

Lower TGF-β1 Secretion Is Observed in 3-MA-Treated MSCs but More in Rapamycin-Treated MSCs Compared With the Control Group

As is shown in our results, autophagy might have an important role in MSC immunomodulation. Next, we attempted to analyze cytokines and factors such as TGF super family members (TGF-β1, cyclooxygenase-2 (COX2), indoleamine 2,3-dioxygenase (IDO), suppressor of cytokine signaling 1, isoform nitric oxide synthase, interleukin-6, interleukin-10, and interferon-γ (IFN-γ) that are involved in immunomodulation of MSCs. Our qRT-PCR results demonstrated a positive correlation between TGF-β1 and MSC autophagy. To be more precise, the TGF-β1 mRNA expression in the 3-MA-pretreated group decreased, whereas that in the rapamycin-pretreated group increased remarkably (Fig. 6A). In addition, we obtained a similar result when TGF-β1 was detected in the cell culture supernatant (Fig. 6B). Consistent with the ELISA and Western blot results, TGF-β1 secretion by 3-MA-pretreated MSCs decreased but increased in the rapamycin-pretreated group (Fig. 6C). Altogether, these results suggest that autophagy might regulate the secretion of TGF-β1 by MSCs, which would affect the immunosuppression of MSCs immediately.

Exogenous TGF-β1 Recovered the Immunosuppressive Capacity of 3-MA-Pretreated MSCs, Whereas Exogenous Anti-TGF-β1 Antibody Reduced the Immunosuppressive Capacity of Rapamycin-Pretreated MSCs

Exogenous TGF-β1 was added to the 3-MA group, and exogenous anti-TGF-β1 antibody was added to the rapamycin group to confirm the function of TGF-β1 in the immunosuppressive capacity of MSCs. Increasing concentrations of exogenous TGF-β1 gradually reduced the proliferation of CD4<sup>+</sup> T cells in the 3-MA-pretreated group. When exogenous TGF-β1 reached a concentration of 100 ng/ml, the result of CFDA-SE exposure was similar to that of the control group, but the proliferation rate of CD4<sup>+</sup> T cells in the 3-MA-pretreated group remained slightly higher than that of the control group. Moreover, exogenous anti-TGF-β1 antibody was added to the coculture of rapamycin group. The result demonstrated that exogenous anti-TGF-β1 antibody could decrease the immunosuppressive capability of MSCs in a concentration-dependent manner. When a concentration of 150 ng/ml anti-TGF-β1 antibody was used, the result was similar to the control group, but the proliferation rate of CD4<sup>+</sup> T cells was slightly below that of the control group. In summary, our results suggest that autophagy could regulate the immunosuppressive capacity of MSCs mediated by TGF-β1.

**DISCUSSION**

In this study, we successfully inhibited and induced autophagy of MSCs using 3-MA and rapamycin, respectively. Although 3-MA and rapamycin treatments did not display different effects on MSC proliferation and did not induce apoptosis compared with the control, rapamycin strengthened the capacity of MSCs to inhibit CD4<sup>+</sup> T-cell proliferation, whereas 3-MA weakened the inhibitory ability of MSCs compared with the control group. Our further results demonstrated that MSCs secreted less TGF-β1 after pretreatment with 3-MA but secreted more TGF-β1 after pretreatment with rapamycin, which could immediately affect T-cell
proliferation. The separate addition of exogenous TGF-β1 and anti-TGF-β1 antibody to 3-MA-pretreated and rapamycin-pretreated MSCs, respectively, could rectify changes in the inhibitory capability of MSCs. Taken together, these results infer that autophagy could regulate the immunosuppressive capacity of MSCs mediated by TGF-β1.

MSCs are stromal cells that can be harvested from bone marrow, adipose tissue, and umbilical cords [26–28]. These cells have been regarded as prospective therapeutic tools because of their characteristics, such as multipotent differentiation, self-renewal, immunomodulation, and immunoprivilege [29]. Among these characteristics, the immunomodulatory capacity of MSCs is currently the most important issue because this function of MSCs has been successfully applied in many inflammatory and autoimmune diseases. However, the underlying mechanism and influencing factors remain largely unknown. Previous studies demonstrated that inflammation, hypoxia, injury, and acid-base disturbances seem to critically influence the immunosuppressive effect of MSCs [30], which indicates the plasticity of immunomodulation by MSCs. Of the various effects, inflammation has been primarily studied. However, not all the above factors can explain the plasticity of MSCs in immunomodulation perfectly.

Some studies have demonstrated that inflammation, hypoxia, and injury also induce MSC autophagy. Autophagy has long been recognized as an important intracellular process used to degrade cellular contents through lysosomes [31]. Previous studies have demonstrated that autophagy has an intimate connection with immunity [32]. For example, stimulating TLR4 with lipopolysaccharide could induce autophagosomes [33]. Defective autophagy would decrease the number of naive T cells [34] and B cells [35] and facilitate CD4+ T-cell apoptosis after activation [36]. However, few studies have examined whether regulating MSC autophagy would change the immunosuppressive capacity of these cells. Fan suggested that autophagy is critical for the survival of MSCs under oxidative conditions [37]. Therefore, we speculated whether autophagy would affect the immunosuppressive capacity of MSCs.

![Figure 3. 3-MA reduces and rapamycin activates autophagy in MSCs. (A): MSCs were exposed to 10 μM 3-MA for different times, and LC3 II/I and p62/GAPDH were used to determine the autophagy level. (B): Based on the result of (A), MSCs were treated with different doses of 3-MA for 24 hours to determine the optimal stimulation dose. (C): MSCs were stimulated by 3 μM rapamycin for 8, 24, 48, and 72 hours, and then relative LC3 II and p62 expression levels were analyzed by Western blot. (D): MSCs were exposed to 1, 3, 5, and 10 μM rapamycin for 24 hours; the Western blot result demonstrated that 3 μM rapamycin was the optimal concentration. Abbreviations: 3-MA, 3-methyladenine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MSCs, mesenchymal stem cells; Rapa, rapamycin.](image-url)
Some studies have indicated that rapamycin, an immunosuppressive drug, could enhance the immunoregulatory potency of pretreated MSCs through subsequent diffusion into cocultures [38]. We showed a similar result in the rapamycin group; however, this result may have been caused by the elevated autophagy level of MSCs. To confirm our speculation, we used an autophagy inducer that was not immunosuppressive, LiCl, to treat MSCs. We observed a similar result for the LiCl group compared with the rapamycin group. In addition, 3-MA pretreatment decreased the inhibitory ability of MSCs. These results suggested the rapamycin could strengthen the inhibitory capability of MSCs on T cells by activating autophagy rather than the immediate effect of rapamycin.

Our results demonstrate that autophagy may regulate MSC immunoregulation. Briefly, we detected the proliferation rate of CD4^{+} T cells by MSCs. The nonactivated CD4^{+} T cells were defined as the blank group. The proliferation rate of CD4^{+} T cells was remarkably elevated when stimulated by CD3/CD28. When cocultured with MSCs, the activated CD4^{+} T cells could be inhibited, and we observed two opposite modulations between the 3-MA and rapamycin group compared with the control group. The values are presented as the means ± SD, using one-way analysis of variance to analyze the differences among groups. *, p < .05 between groups. Abbreviations: 3-MA, 3-methyladenine; CFDA-SE, carboxyfluorescein diacetate succinimidyl ester; MSC, mesenchymal stem cell; OD, optical density.

![Figure 4](image_url) Pretreatment with 3-MA and rapamycin did not induce MSC apoptosis or affect MSC proliferation. (A): Flow cytometry was used to analyze the apoptosis of MSCs exposed to 10 μM 3-MA or 3 μM rapamycin for 24 hours; no discrepancies were observed. (B): MSC proliferation after different treatments was analyzed by Cell Counting Kit-8. Abbreviations: 3-MA, 3-methyladenine; 7-AAD, 7-aminoactinomycin; MSC, mesenchymal stem cell; OD, optical density.

![Figure 5](image_url) Autophagy improves the immunosuppression of CD4^{+} T cells by MSCs. The nonactivated CD4^{+} T cells were defined as the blank group. The proliferation rate of CD4^{+} T cells was remarkably elevated when stimulated by CD3/CD28. When cocultured with MSCs, the activated CD4^{+} T cells could be inhibited, and we observed two opposite modulations between the 3-MA and rapamycin group compared with the control group. The values are presented as the means ± SD, using one-way analysis of variance to analyze the differences among groups. *, p < .05 between groups. Abbreviations: 3-MA, 3-methyladenine; CFDA-SE, carboxyfluorescein diacetate succinimidyl ester; MSC, mesenchymal stem cell; PE, phycoerythrin; SSC, side scatter.

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of CD4+ T cells at the end of coculture. We found that 3-MA pre-
treatment reduced the immunosuppressive capacity of MSCs,
whereas rapamycin pretreatment increased the inhibitory capac-
ity of MSCs compared with the control group. Similarly, animal
experiments have shown that infusion with rapamycin could pro-
mote the therapeutic efficacy of MSCs in transplantation [39, 40].
Conversely, some studies have shown that inhibition of auto-
phagy improved the immunosuppressive capacity of MSCs [41].
The different results raised a question regarding whether auto-
phagy would improve or decrease the immunomodulation of
MSCs. However, the previous studies and our current study
had many different factors that limit their comparison. First,
MSCs were harvested from humans and mice, respectively, and
cultured under different conditions. Second, the detected
T cells were harvested from human peripheral blood in our study,
whereas the cells were collected from mouse spinal cords and
spleen in their studies. Third, we treated MSCs with the classical
autophagy drugs 3-MA and rapamycin, but they used tumor ne-
crosis factor and IFN-γ. Thus, the above factors might have con-
tributed to this “discrepancy.” However, all cells in our study
were obtained from humans rather than mice; therefore, undoubtedly,
our study has greater correlation with the human body and is
more suitable to the study of MSC therapy.

We next attempted to determine the factors that affect the
observed differences among groups. Previous studies have
shown that autophagy limits some cells proliferation [42]. How-
ever, whether autophagy affects MSC proliferation remains con-
troversial. To determine whether cell viability and numbers
influence the differences observed in our study, we analyzed
the proliferation and apoptosis of MSCs after pretreatment with
3-MA or rapamycin. However, we were excited to see that the
proliferation rates of each group had no obvious difference and
that 3-MA or rapamycin pretreatment would not induce apopto-
sis. Thus, we hypothesize that the different inhibitory abilities
of each group of MSCs may depend on other factors, such as soluble
molecules derived from MSCs.

Previous studies showed that the MSC secretome and cell-cell
contact might play critical roles in the immunosuppressive capacity
of MSCs. The most accepted soluble factors, such as TGF-β, IDO, prostaglandin E2, nitric oxide, and COX2, have an immediate
function on PBMCs [43, 44]. Thus, we detected the gene levels of
these immunoregulatory molecules, and the results indicated

Figure 6. Less TGF-β1 secretion was observed in the 3-MA-pretreated MSCs but more secretion was observed in the rapamycin-pretreated
MSCs as compared with the control group. (A): Well-known soluble molecules associated with immunoregulation of MSCs were detected by
qRT-PCR; a positive correlation was found between TGF-β1 mRNA expression and MSC autophagy. (B): TGF-β1 protein levels were detected by
ELISA. The 3-MA-pretreated MSCs secreted less TGF-β1, whereas rapamycin-pretreated MSCs secreted more in contrast to the control. (C): The
Western blot result was consistent with the TGF-β1 ELISA result. The values are presented as the means ± SD, using one-way analysis of variance
to analyze the differences among groups. *, p < .05 between groups. Abbreviations: 3-MA, 3-methyladenine; COX2, cyclooxygenase-2; ELISA,
enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon-γ; IL-6, interleukin-6; IL-10, interleukin-10; iNOS, isoform nitric oxide synthase; MSC, mesenchymal stem cell; qRT-PCR, quantitative
real-time polymerase chain reaction; Rapa, rapamycin; SOCS1, suppressor of cytokine signaling 1; TGF-β1, transforming growth factor-β1.
that only TGF-β1 had a positive correlation with MSC autophagy. Notably, the changes in autophagy and TGF-β1 secretion were more obvious in the rapamycin group, which was consistent with the remarkable increase in the inhibitory ability of MSCs on CD4+ T cells. Therefore, we hypothesized that TGF-β1 may be the key molecule causing the differences among the groups. TGF-β1 is the most important prototype of the TGF-β family, which consists of three members (TGF-β1, TGF-β2, and TGF-β3) that are pleiotropic cytokines with important roles in immunoregulation [20]. Likewise, TGF-β1 was proven as a crucial factor in MSC-mediated immunoregulation [21]. Zou also demonstrated that TGF-β1 suppressed inflammation during MSC-based therapy for disc degeneration [45]. We found that exogenous TGF-β1 could enhance the inhibitory function of 3-MA-pretreated MSCs, whereas adding anti-TGF-β1 antibody to the rapamycin-pretreated group apparently increased the proliferation rate of CD4+ T cells. The values are presented as the means ± SD, using one-way analysis of variance to analyze the differences among groups. *p < .05 between groups. Abbreviations: 3-MA, 3-methyladenine; CFDA-SE, carboxyfluorescein diacetate succinimidyl ester; MSCs, mesenchymal stem cells; PBMC, peripheral blood mononuclear cell; Rapa, rapamycin; SSC, side scatter; TGF-β1, transforming growth factor-β1.

**CONCLUSION**

Our study demonstrates that the autophagy level regulates the immunosuppressive function of CD4+ T cells by MSCs through affecting TGF-β1 secretion. This finding provides a novel method for improving the therapeutic efficacy of MSCs by activating autophagy.

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**AUTHOR CONTRIBUTIONS**

L.G.: manuscript writing, data analysis and interpretation; S.C.: collection and/or assembly of data, manuscript writing; P.W.: data analysis and interpretation; Z.X. and J.L.: provision of study material or patients; Z.L., W.D., H. Su, X.W., S.W., and Y.O.: provision of test and analysis materials; Y.W.: conception and design, administrative support, final approval of manuscript; H. Shen: conception and design, financial support, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.
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