Human umbilical cord mesenchymal stem cells alleviate the imbalance of CD4+ T cells via protein tyrosine phosphatase non-receptor type 2/signal transducer and activator of transcription 3 signaling in ameliorating experimental autoimmune thyroiditis in rats

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Abstract. This study aimed to investigate the therapeutic effect of human umbilical cord mesenchymal stem cells (hUCMSCs) on experimental autoimmune thyroiditis (EAT) and the underlying mechanisms by utilizing a porcine thyroglobulin-induced EAT rat model. The rats received four tail vein injections of vehicle or hUCMSCs at an interval of 7 days and were sacrificed on day 28 after the first injection. Hematoxylin and eosin staining and enzyme-linked immunosorbent assays (ELISAs) were used to assess the therapeutic effects of hUCMSCs on EAT. Splenic lymphocytes were isolated from rats, and the proportions of CD4+ T cell subsets were analyzed by flow cytometry. Splenic CD4+ T cells from EAT rats were cocultured with hUCMSCs. A loss-of-function assay for protein tyrosine phosphatase non-receptor type 2 (PTPN2) was performed to explore the involvement of PTPN2/signal transducer and activator of transcription 3 (STAT3) signaling on the therapeutic benefit of hUCMSCs in EAT. hUCMSC treatment significantly alleviated inflammation, reduced serum thyroid antibody levels, and decreased the ratios of IL-17α+/CD25+FOXP3+ cells and serum IFN-γ/IL-4 in EAT rats. Furthermore, hUCMSC treatment upregulated PTPN2 protein expression in splenic lymphocytes of EAT rats as well as enhanced the PTPN2 protein level and attenuated phosphorylation of STAT3 in CD4+ T cells in vitro. Importantly, knockdown of Ptpn2 significantly reversed hUCMSC-mediated suppression of cell proliferation and hUCMSC-induced alterations in the expression of inflammatory cytokines in CD4+ T cells. Thus, hUCMSC treatment alleviates thyroid inflammation and the CD4+ T cell imbalance in EAT via PTPN2/STAT3 signaling, serving as a promising therapeutic approach for autoimmune thyroiditis.

Key words: Autoimmune thyroiditis, Human umbilical cord mesenchymal stem cells, Protein tyrosine phosphatase non-receptor type 2, Signal transducer and activator of transcription 3, CD4-positive lymphocytes

AUTOIMMUNE THYROIDITIS is an autoimmune disease characterized by inflammation of the thyroid gland and high titers of circulating antithyroid antibodies, representing the most common cause of hypothyroidism worldwide [1]. The annual incidence of autoimmune thyroiditis is approximately 0.8/1,000 in men and 3.5/1,000 in women [2]. It is a lifelong disorder with no effective cure, and the patients who develop hypothyroidism require long-term thyroid hormone replacement therapy [2]. Therefore, it is urgently needed to develop effective therapeutic methods for the early intervention of autoimmune thyroiditis.

Excessively stimulated CD4+ T lymphocytes play a critical role in the pathogenesis of autoimmune thyroiditis [3]. Activated CD4+ T cells differentiate into different subtypes, including the T-helper 1 (Th1), Th2, Th17, and regulatory T (Treg) cells, based on the secretion of specific cytokines [4]. Th1 cells produce tumor necrosis factor-beta (TNF-β), interferon-gamma (IFN-γ), and interleukin (IL) 2, and Th17 cells secrete IL-17α; both cell types promote tissue damage in autoimmune thyroiditis [5, 6]. On the other hand, Th2 and Treg cells produce anti-inflammatory cytokines, such as IL-4, IL-5, IL-10, and transforming growth factor-beta, thereby...
suppressing inflammatory responses in the thyroid gland in autoimmune thyroiditis [7, 8]. Studies have shown the presence of hyperactive Th1 and Th17 subsets along with insufficient Th2 and Treg subsets of peripheral lymphocytes in patients with autoimmune thyroiditis [9-11]. Therefore, targeting the imbalance of Th1/Th2 and Th17/Treg represents a promising therapeutic approach for the treatment of autoimmune thyroiditis [12, 13].

Protein tyrosine phosphatase non-receptor type 2 (PTPN2) is an intracellular phosphatase highly expressed in lymphoid cells [14]. PTPN2 regulates T cell proliferation and differentiation during inflammation [15, 16], playing a critical role in developing autoimmune diseases that exhibit a T cell subset imbalance, such as Crohn’s disease, rheumatoid arthritis, and type 1 diabetes [17-19]. Mechanistically, PTPN2 regulates the intracellular signaling pathway by dephosphorylating specific substrate proteins, and the signal transducer and activator of transcription 3 (STAT3) is an important target of PTPN2 [20]. PTPN2 negatively regulates STAT3 signaling by removing the phosphate from the tyrosine residue of phospho-STAT3 (p-STAT3) [21]. Studies have shown that overexpression of PTPN2 can suppress Th17 and Th1 differentiation while promoting Treg differentiation [15, 22, 23]. STAT3 activation is involved in the pathogenesis of autoimmune diseases by promoting and suppressing the differentiation of Th17 and Treg cells, respectively [24-26]. Blocking STAT3 signaling abrogates the differentiation of Th17 cells and enhances the development of Treg cells, leading to the amelioration of autoimmune diseases [26]. However, the involvement of PTPN2/STAT3 signaling in autoimmune thyroiditis remains unknown.

Mesenchymal stem cells (MSCs) are a type of mesoderm-derived stem cells that possess self-replication and multi-lineage differentiation potential. Studies have shown that MSCs regulate T cell proliferation and differentiation and that MSC-based treatment ameliorates autoimmune diseases [27-29]. MSCs are unable to activate T cells due to their deficiency of B7-1, B7-2, and FasL, as well as their weak expression of CD40, CD40L, and major histocompatibility complex (MHC) class I on the cell surface. The low immunogenicity of MSCs enables them to evade the immune system and allow their usage across MHC barriers [30, 31]. Compared with MSCs derived from other sources, the advantages of human umbilical cord mesenchymal stem cells (hUCMSCs) include easier isolation and expansion, lower immunogenicity, and higher differentiation potential [31], thus enhancing their clinical application in the treatment of autoimmune diseases. Our previous research has already shown that tail vein injection with hUCMSCs does not induce histopathological changes in the heart, liver, or kidney of rats, suggesting that hUCMSC transplantation does not induce immune rejection in rats [32]. hUCMSCs have displayed promising therapeutic effects in various autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes, and Crohn’s disease [33-36]. However, whether hUCMSCs can ameliorate autoimmune thyroiditis remains unexplored.

In the present study, we established a porcine thyroglobulin-induced experimental autoimmune thyroiditis (EAT) model in female rats because autoimmune thyroiditis is more prevalent in women than in men [37]. We explored the therapeutic effects of hUCMSCs on EAT. To reveal the underlying mechanism of hUCMSC-based treatment in EAT, we also investigated the alterations in CD4+ T cell proportions and the involvement of PTPN2/STAT3 signaling.

**Materials and Methods**

**Isolation and characterization of hUCMSCs**

hUCMSCs were provided by the Shandong Human Umbilical Cord Mesenchymal Stem Cell Bank (Shandong, China). The umbilical cord was obtained from a 25-year-old healthy mother who delivered a healthy full-term infant, with approval from the Ethics Committee of the local maternity hospital. Informed consent was obtained from the mother before delivery. hUCMSCs were isolated as previously described [38] and cultured in low-glucose Dulbecco’s modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 5% nonanimal-derived serum (Gibco) in a humified atmosphere of 5% CO₂ at 37°C. Cells at passage 4 were collected to determine the expression of surface markers and the differentiation capacity [39]. The cells were adjusted to 1 × 10^5/mL and incubated with anti-CD105-phycoerythrin (PE), anti-CD90-FITC, anti-CD34-PE, anti-CD45-FITC, anti-CD146-PE, and anti-HLA-DR-FITC antibodies (eBioscience, San Diego, CA, USA) at room temperature for 30 min in the dark. The marker expression was analyzed by using a FACS Calibur flow cytometer (BD Bioscience, San Jose, CA, USA).

To examine the differentiation capacity of hUCMSCs, cells at passage 4 were seeded in a 6-well plate at a density of 1 × 10^5/well. When the cells reached 80% confluence, osteogenic differentiation medium [DMEM/F12 medium, 10% fetal bovine serum (FBS), 10 mM sodium β-glycerophosphate, 0.1 mM dexamethasone, and 50 mg/L ascorbic acid] or adipogenic differentiation medium (DMEM/F12 medium, 10% FBS, 10 μM insulin, 1 μM dexamethasone, 200 μM/L indomethacin, and 0.5 mM/L 3-isobutyl-1-
methylxanthine) was added and refreshed every 3 days. At 21 days after induction, osteogenic differentiation and adipogenic differentiation were examined using Alizarin Red S staining and Oil Red O staining, respectively.

**Animals and treatment**

All animal care and experimental procedures were approved by the Ethics Committee of the local university (Shandong, China). All animal experiments were performed according to the Guidelines for Proper Conduct of Animal Experiments at the local university. Thirty-six female Wistar rats (4 weeks old, 100–110 g) were purchased from Jinan Pengyue Experimental Animal Center (animal qualification certificate #0140007; Shandong, China) and maintained under specific pathogen-free conditions.

After acclimatization for 1 week, the rats were randomly divided into the control, EAT, and hUCMSC-treated groups (n = 12/group). The EAT rat model was established as previously described [40]. On day 0, each rat in the EAT and hUCMSC-treated groups received a subcutaneous injection of 100 μg of porcine thyroglobulin (Sigma-Aldrich, St. Louis, MO, USA) emulsified in 100 μL of complete Freund’s adjuvant (Sigma-Aldrich) for the induction of EAT. To boost immunization, each rat was given the same dose of porcine thyroglobulin in 100 μL of incomplete Freund’s adjuvant (Sigma-Aldrich) on days 7, 14, and 21. hUCMSC treatment was conducted as previously described [41]. Briefly, hUCMSCs at passage 7 were resuspended in normal saline at a concentration of 1 × 10^7/mL. Each rat in the hUCMSC-treated group was given 0.5 mL of hUCMSCs in saline via tail vein injection on days 0, 7, 14, and 21. The rats in the control and EAT groups were administered 0.5 mL of saline. All rats were sacrificed on day 28. The serum, spleen, and thyroid were immediately collected from each rat for future studies.

**Hematoxylin and eosin staining**

Six rats were randomly selected from each group and sacrificed on day 28 after the first treatment. The thyroid glands were harvested and fixed with 4% paraformaldehyde for 48 h. The tissue sections (5-μm thick) were prepared and stained using a hematoxylin and eosin staining kit (Solarbio, Beijing, China), according to the manufacturer’s protocol. Histopathological changes, such as follicle destruction and inflammatory cell infiltration, were assessed. Images were captured at 200× magnification using an inverted microscope (Olympus, Japan). The infiltration index of inflammatory cells was scored as follows: 0, no infiltration; 1, interstitial accumulation of lymphocytes between two or more follicles; 2, the inflammatory foci reaching the size of one follicle; 3, the inflammatory foci occupying 10–40% of the thyroid tissue; and 4, the inflammatory foci occupying >40% of the thyroid tissue [42].

**Enzyme-linked immunosorbent assay (ELISA)**

Blood samples were collected from the orbit of each rat on day 28 post-treatment to measure the levels of serum antithyroglobulin antibody (TGAb), antithyroid peroxidase autoantibody (TPOAb), antithyroidmicrosome antibody (TMAb), total triiodothyronine (TT3), total tetraiodothyronine (TT4), thyroid-stimulating hormone (TSH), IL-4, and IFN-γ using the corresponding ELISA kit (MSKBio, Wuhan, Hubei, China), according to the manufacturer’s instructions.

**Isolation of splenic lymphocytes and flow cytometric analysis**

Six rats were randomly selected from each group, sacrificed, and soaked in 70% ethanol for 2 min. The spleen was harvested aseptically and placed on a cell strainer (70-μm pore size). The ground tissue was pressed through the mesh of the strainer using a syringe plunger. After washing with phosphate-buffered saline, the cell suspension was treated with rat peripheral blood lymphocyte separation solution (Solarbio) to isolate the lymphocytes. To analyze the proportions of CD4+ T cell subsets, the splenic lymphocytes were adjusted to 1 × 10^7/mL and labeled with CD8-FITC, CD25-PE, forkhead box P3 (FOXP3)-FITC, CD4-PE, and IL-17α-FITC antibodies (eBioscience), followed by flow cytometric analysis using a FACS Calibur flow cytometer (BD Biosciences).

**Construction of lentiviral expression vectors and transfection**

Short hairpin RNA against rat Ptpn2 (shPtpn2; 5'-GCA TTC TAC GGA AAC GTA TTC CGA AGA ATA CGT TTC CGT AGA ATGC-3') was cloned into a lentiviral vector (Genepharma, Shanghai, China) using the ViraPower™ packaging mix (Thermo Fisher Scientific, Waltham, MA, USA) [20]. Splenic CD4+ T cells from EAT rats were transfected with the expression vectors or the vectors expressing scrambled shRNA (shCon) using the transfection reagent in the ViraPower™ packaging mix (Thermo Fisher Scientific), according to the manufacturer’s protocol.

**Isolation of splenic CD4+ T cells and coculture with hUCMSCs**

Splenic T cells were isolated from splenic lymphocytes of EAT rats using a CD4+ T cell isolation kit (Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer’s instructions. hUCMSCs at passage 3 were seeded in a 25-cm² tissue culture flask at a density...
of $7.5 \times 10^5$/flask and incubated in low-glucose DMEM supplemented with 5% nonanimal-derived serum at 37°C for 24 h. After removal of the medium, $1.5 \times 10^7$ CD4+ T cells were resuspended in 15 mL of RPMI 1640 (Gibco) containing 10% FBS (Gibco) and 50 μg/mL phytohaemagglutinin [43] (Sigma-Aldrich). Furthermore, the cell resuspension was added into the flask and cocultured with hUCMSCs. CD4+ T cells transfected with shPtpn2 or shCon were cocultured with hUCMSCs at 3 days after transfection.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

Splenic CD4+ T cells from EAT rats were incubated with or without hUCMSCs for 3 days. The T cell suspension was transferred into a 96-well plate, and fresh RPMI 1640 medium was added to reach a final volume of 200 μL per well. Then, 20 μL of MTT (5 mg/L) was added to each well. After incubation at 37°C for 4 h, the cells were collected and centrifuged at 1,500 rpm for 10 min. After removing the MTT-containing supernatant, the cells were resuspended in 200 μL of dimethyl sulfoxide. The absorbance values were measured using a plate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 570 nm.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from CD4+ T cells at 3 days after incubation using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. One microgram of RNA was reverse-transcribed into cDNA using a reverse transcription kit (Tiangen Biotech, Beijing, China), according to the manufacturer’s protocol. Amplification was performed using a miScript SYBR Green PCR kit (Tiangen Biotech) and gene-specific primers (Table 1) on a qRT-PCR device (Eppendorf, Germany). Actb was used as an internal control.

Western blot analysis

CD4+ T cells were collected at 3 days after incubation and lysed with lysis buffer on ice. The protein concentrations were measured using a bicinchoninic acid protein assay kit (Elabscience, Wuhan, China). Proteins (40 μg) were separated on a 10% sodium dodecyl sulfate gel and transferred to a polyvinylidene fluoride membrane, followed by blocking with 5% skim milk for 1 h. The membrane was then incubated overnight at 4°C with primary antibody (Affinity Biosciences, Cincinnati, OH, USA) to rat GAPDH (1:1,000), PTPN2 (1:1,000), STAT3 (1:1,000), or p-STAT3 (1:1,000), followed by three washes with tris-buffered saline containing 0.1% Tween 20 (TBST). The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000; Elabscience) for 1 h at room temperature. After an additional three washes with TBST, the protein bands were visualized using an enhanced chemiluminescence reagent and analyzed using BandScan software (Glyko, Novato, CA, USA).

Statistical analysis

Statistical analyses were conducted using SPSS software (Version 13.0; SPSS, Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD). Comparisons among groups were conducted using one-way analysis of variance followed by post hoc analysis with Bonferroni correction for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

Results

hUCMSC treatment alleviates EAT in rats

hUCMSCs were isolated from the human umbilical cord. As shown in Fig. 1A, B, the spindle- or polygon-shaped hUCMSCs grew adherently, with high expression of CD105, CD90, and CD146 and low expression of CD34, CD45, and HLA-DR. Fig. 1C, D demonstrates the deposition of calcium and lipid droplets after osteogenic and adipogenic induction, respectively. These results indicate that the isolated cells were hUCMCs.

To explore the therapeutic effect of hUCMSCs on EAT, we established a rat EAT model and treated the rats with hUCMSCs. Examination of the hematoxylin-and-

| Table 1 | Primers for quantitative real-time PCR |
|---------|----------------------------------------|
| Gene    | Forward (5’–3’)                        | Reverse (5’–3’)                      |
| Ifng    | CGAATCCGACCTGATCAGCTATG                | GACTCCTTTTCCGGCGTTCCCTT              |
| Il4     | TGCACCCAGATGTTTTGTACCAG               | TGGCGAACCCCTTGAAG                   |
| Il17a   | GTGAAGGCACCGTGACTCA                    | TTCTGGAGCTGCTTTTGA                  |
| Fox3p   | TCACCTATGCCACCCCTCATCC                | CTTGCCAAACCTCAAATCTCATCAG           |
| Actb    | TGCTATGGTCGCTAGCTTCG                  | GTTGCCATAGGGCTTTTACGG               |

Ifng, interferon gamma; Il4, interleukin 4; Il17a, interleukin 17; Foxp3, forkhead box P3.
eosin-stained thyroid glands demonstrated that the EAT rats exhibited apparent follicle destruction and increased lymphocyte infiltration compared with the control rats, indicating that the EAT rat model was successfully established. Compared with vehicle treatment, hUCMSC treatment resulted in apparent alleviation in follicle destruction and lymphocyte infiltration (Fig. 2A) as well as a significant reduction in the infiltration index (Fig. 2B) in the thyroid tissue of the EAT rats. In addition, the levels of serum TPOAb, TGAb, and TMAb in the hUCMSC-treated group were significantly reduced compared with those in the vehicle-treated EAT group (Fig. 2C, D, E). Despite the nonsignificant change in the level of serum TT3 (Fig. 2F), we observed a significant elevation in the level of serum TT4 (Fig. 2G) and a significant reduction in the level of serum TSH (Fig. 2H). These results indicate that hUCMSC treatment alleviates EAT in rats.

**hUCMSC treatment alleviates the T cell subset imbalance in EAT rats**

We analyzed the splenic lymphocytes from rats using a flow cytometric assay to explore whether hUCMSCs could regulate the proportions of T cell subsets in EAT rats. The percentage of CD4+ cells in the total lymphocytes reached 94.3% (Fig. 3), which meets the requirement of the following experiments. As shown in Fig. 4A (upper panel), B, and D, the percentage of CD4+ T cells and the CD4+/CD8+ ratio were significantly increased in the EAT rats compared with the control rats. hUCMSC treatment only resulted in slight decreases in both the percentage of CD4+ cells and the CD4+/CD8+ ratio, compared with the vehicle treatment in the EAT group. No significant differences were observed in the percentage of CD8+ T cells among the groups.

Next, we sought to determine whether hUCMSC treatment could improve the imbalance of CD4+ T cell subsets in EAT by analyzing the expression of the Th17 marker IL-17α and the Treg markers CD25 and FOXP3 in the splenic CD4+ T cells and by measuring the levels of serum cytokines released by Th1 and Th2 cells. As shown in Fig. 4A (middle and lower panels), B, and D, in the EAT group, the percentage of IL-17α+ cells was significantly increased. In contrast, the percentage of CD25+FOXP3+ cells was significantly decreased compared with the control group. In addition, the ratio of IL-17α+/CD25+FOXP3+ cells in the EAT group was significantly higher than that in the control group. These
hUCMSC treatment alleviated histopathological changes in the thyroid gland and reduced the levels of serum thyroid antibodies in rats with experimental autoimmune thyroiditis (EAT). Female Wistar rats were randomly divided into the control, EAT, and hUCMSC-treated groups. The rats in the EAT and hUCMSC-treated groups received an injection of porcine thyroglobulin to induce EAT. The rats in the hUCMSC-treated group were given hUCMSCs via tail vein injection at passage 7 on days 0, 7, 14, and 21 after the porcine thyroglobulin injection. The rats in the control and EAT groups were administered normal saline. All of the rats were sacrificed on day 28. (A) Hematoxylin and eosin staining was performed to evaluate histopathological changes in the thyroid gland of the rats (n = 6/group). Representative images are shown. Scale bar = 100 μm. (B) Infiltration index of inflammatory cells. (C–E) Blood samples were collected from the orbit of each rat on day 28 after hUCMSC treatment to measure the levels of serum antithyroid peroxidase antibody, antithyroglobulin antibody, and antithyroid microsome antibody. (F–H) The levels of serum total triiodothyronine, total tetraiodothyronine, and thyroid-stimulating hormone. Data are expressed as the mean ± SD. *p < 0.05 vs. the control group, #p < 0.05 vs. the EAT group; n = 12. TPOAb, antithyroid peroxidase antibody; TGAb, antithyroglobulin antibody; TMAb, antithyroid microsome antibody; TT3, total triiodothyronine; TT4, total tetraiodothyronine; TSH, thyroid stimulating hormone.
data suggest a Th17/Treg imbalance in the EAT rats. Interestingly, hUCMSC treatment effectively reduced the percentage of IL-17α⁺ cells and elevated the percentage of CD25⁺FOXP3⁺ cells, compared with vehicle treatment in the EAT rats. Consistently, the IL-17α⁺/CD25⁺FOXP3⁺ cell ratio in hUCMSC-treated rats was significantly less than that in the vehicle-treated EAT rats. These results indicate that hUCMSC treatment alleviates the Th17/Treg imbalance in EAT.

In terms of cytokines, we did not observe a significant difference in the level of serum IL-4 produced by Th2 cells among the different groups (Fig. 4C). However, the level of serum INFγ produced by Th1 cells and the serum INF-γ/IL-4 ratio were significantly increased in the EAT group, compared with those in the control group (Fig. 4C, D). Importantly, hUCMSC treatment resulted in significant reductions in both the INF-γ level and the INF-γ/IL-4 ratio, compared with the vehicle treatment (Fig. 4C, D), suggesting that hUCMSC treatment might cause declines in the Th1 cell abundance and the Th1/Th2 ratio. Taken together, these results suggest that hUCMSC treatment improves the imbalance of T cell subsets when ameliorating EAT in rats.

**Coculture with hUCMSCs upregulates PTPN2 expression and downregulates the p-STAT3/STAT3 ratio in CD4⁺ T cells**

Considering the role of PTPN2 in CD4⁺ T cell differentiation during inflammation [13], we investigated whether hUCMSC treatment could regulate the proportions of CD4⁺ T cells in EAT rats by altering the PTPN2 expression. Western blotting revealed that hUCMSC treatment upregulated PTPN2 protein expression and downregulated the p-STAT3/STAT3 ratio in splenic lymphocytes from EAT rats, compared with vehicle treatment (Fig. 5A, B). Consistently, the in vitro coculture model showed that coculture with hUCMSCs significantly enhanced PTPN2 protein expression and attenuated STAT3 phosphorylation in CD4⁺ T cells, compared with CD4⁺ T cells cultured alone. Transfection with shPtpn2 displayed contrasting effects (Fig. 5C, D). These findings suggest that PTPN2 upregulation and STAT3 inactivation are involved in the therapeutic benefits of hUCMSCs on EAT.

**Knockdown of Ptpn2 reverses hUCMSC-mediated suppression of CD4⁺ T cell proliferation**

Next, we investigated whether PTPN2 is essential for the effect of hUCMSCs on CD4⁺ T cell growth by silencing Ptpn2 expression in T cells. Western blot analysis showed that transfection with shPtpn2 suppressed PTPN2 protein expression by 45% compared with shCon (Fig. 6). As shown in Fig. 7A, under a bright-field microscope, knockdown of Ptpn2 increased CD4⁺ T cell numbers and promoted cell agglomeration. In contrast, coculture with hUCMSCs reduced CD4⁺ cell numbers and inhibited cell agglomeration. Compared with the negative control, knockdown of Ptpn2 resulted in increases in the cell number and agglomeration of T cells cocultured with hUCMSCs, suggesting that knockdown of Ptpn2 abrogates the suppressive effect of hUCMSCs on T cell proliferation. The MTT assay further confirmed that knockdown of Ptpn2 partially but significantly reversed hUCMSC-mediated suppression of CD4⁺ T cell proliferation (Fig. 7B). Taken together, these results indicate that PTPN2 is required for the suppressive role of hUCMSCs in CD4⁺ T cell proliferation.

**Knockdown of Ptpn2 abrogates hUCMSC-induced alterations in CD4⁺ T cell-produced inflammatory cytokines**

Subsequently, we investigated whether Ptpn2 mediates hUCMSC-induced changes in the CD4⁺ T cell proportions by measuring the miRNA levels of inflammatory factors in CD4⁺ T cells. As shown in Fig. 7C, knockdown of Ptpn2 significantly upregulated the mRNA expression of Ifng and Iil17a while attenuating the mRNA expression of Foxp3 in CD4⁺ T cells. Coculture with hUCMSCs showed contrasting results. Compared with the negative control, knockdown of Ptpn2 partially but significantly abrogated the effects of hUCMSC coculture on Iil17a and Ifng expression in CD4⁺ T cells. Importantly, knockdown of Ptpn2 partially but significantly reversed the effects of hUCMSC coculture on the Ifng/Iil4 and Iil17a/Foxp3 mRNA ratios (Fig. 7D), suggesting that Ptpn2 at least partially mediates the regulation of hUCMSCs on the balance of CD4⁺ T cell subsets.
hUCMSC treatment improved the balance of CD4+ T cell subsets in EAT rats. (A) Representative flow cytometric analysis of rat splenic lymphocytes expressing CD4, CD8, IL-17a, CD25, or FOXP3. (B) Quantification of (A). (C) An enzyme-linked immunosorbent assay was performed to measure the levels of serum IL-4 and IFN-γ in the rats. (D) The ratios of CD4+ /CD8+ cells, IL17a /CD25+FOXP3+ cells, and serum IFN-γ/IL4 in the rats. Data are expressed as the mean ± SD. * p < 0.05 vs. the control group, # p < 0.05 vs. the EAT group; n = 6.
Although hUCMSCs have shown promising therapeutic benefits in various autoimmune diseases, the therapeutic effect of hUCMSCs on autoimmune thyroiditis remains unknown. In this study, we established a rat EAT model to explore the therapeutic effect of hUCMSCs on EAT. For the first time, we demonstrated that hUCMSC treatment alleviates EAT in rats, as evidenced by significantly reduced inflammation in the thyroid tissue and decreased levels of serum thyroid antibodies in hUCMSC-treated rats compared with vehicle-treated rats. We also found that hUCMSC treatment significantly reduced the ratios of IL-17a/CD25/FOXP3 cells and ameliorate EAT.

**Discussion**

**Fig. 5** Coculture with hUCMSCs upregulated PTPN2 expression and suppressed STAT3 phosphorylation in CD4+ T cells. (A, B) Western blotting was performed to determine the protein expression of PTPN2, phosphorylated-STAT3 (p-STAT3), and STAT3 in splenic lymphocytes from the rats. (C, D) CD4+ T cells were isolated from splenic lymphocytes of EAT rats. Untransfected splenic CD4+ T cells were cultured alone or cocultured with hUCMSCs. The cells transfected with small hairpin RNA of Ptpn2 (shPtpn2) or the control vector (shCon) were cocultured with hUCMSCs at 3 days after transfection. Western blotting was performed at 3 days after coculture to determine the protein expression of PTPN2, p-STAT3, and STAT3. GAPDH was used as the internal control. Data are expressed as the mean ± SD. *p < 0.05 vs. the control group, #p < 0.05 vs. the EAT group, ^p < 0.05 vs. the T group, $p < 0.05 vs. the T + hUCMSCs group, %p > 0.05 vs. the T + CD4+ T cells.
serum IFN-γ/IL-4 compared with vehicle treatment, suggesting that hUCMSC treatment alleviates Th17/Treg and Th1/Th2 imbalances in EAT rats. Thus, hUCMSC treatment is a promising therapeutic approach against autoimmune thyroiditis.

MSCs transplantation has emerged as a therapeutic strategy for autoimmune diseases owing to its immunosuppressive properties of MSCs. Although the mechanisms underlying the immunomodulatory activity of MSCs are incompletely understood, studies have shown that MSCs mediate immunosuppression by secreting soluble immune factors, adhering to the inflammatory sites, or directly interacting with various immune cells, including T cells [44]. The presence of MSCs induces up- and downregulation of multiple pathways in T cells [45]. It has been reported that T cell-specific Ptpn2-deficient mice have increased levels of IL-6, TNF, and IFN-γ and develop widespread inflammation and autoimmune disorders [46]. Loss of Ptpn2 in CD4+ T cells enhances induction of Th1 and Th17 cells but impairs the induction of Treg cells in Crohn’s disease [15]. Ptpn2-deficient T cells undergo rapid proliferation and differentiation [16]. Based on these reports, we wondered whether hUCMSC treatment could regulate CD4+ T cell activity in EAT rats by altering PTPN2 expression. As expected, our results confirmed that hUCMSC treatment induced upregulation of PTPN2 protein expression in splenic lymphocytes from EAT rats, suggesting the involvement of PTPN2 in the therapeutic benefits of hUCMSCs on EAT. Importantly, in the coculture model of hUCMSCs and CD4+ T cells from EAT rats, knockdown of Ptpn2 effectively reversed hUCMSC-mediated suppression on CD4+ T cell proliferation and regulation of the Ifng/Ii4 and Il17a/Foxp3 transcript ratios in CD4+ T cells, suggesting that hUCMSCs suppress CD4+ T cell proliferation and alleviate the imbalance of CD4+ T cell subsets in EAT by upregulating PTPN2 in CD4+ T cells. We also found that the shPtpn2 we chose from a published paper downregulated PTPN2 expression by 45%. Western blot analysis showed that the T + hUCMSC + shPtpn2 group and the T group had comparable PTPN2 protein levels. However, CD4+ T cell proliferation and the ratios of Ifng/Ii4 and Il17a/Foxp3 in the T + hUCMSC + shPtpn2 group were still less than those in the T group, suggesting that knockdown of Ptpn2 does not completely block the effect of hUCMSCs on cell proliferation, the Ifng/Ii4 ratio, and the Il17a/Foxp3 ratio in CD4+ T cells. These results suggest that in addition to PTPN2, hUCMSCs may regulate CD4+ T cell proliferation and polarization through other signaling pathways. To identify the signaling molecule downstream of PTPN2 in CD4+ T cells, we detected the phosphorylation status of STAT3 because STAT3 is an important substrate of PTPN2 and has been extensively implicated with autoimmune diseases [26]. Studies have shown that blocking STAT3 signaling regulates the differentiation of different subtypes of T cells, leading to amelioration of autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis [24-26]. However, it remains unknown whether STAT3 activation is involved in autoimmune thyroiditis and hUCMSC-based treatment. In this study, we found that coculture with hUCMSCs significantly enhanced PTPN2 protein expression in splenic lymphocytes from EAT rats, accompanied by a decrease in the phosphorylation of STAT3. This finding suggests that PTPN2-mediated STAT3 inactivation is associated with the role of hUCMSCs in EAT. Autoimmune diseases, including autoimmune thyroiditis, display defects in the number or function of Treg cells [11]. Activated STAT3 can bind to the FOXP3 promoter to suppress FOXP3 expression and inhibit Treg differentiation in inflammation [47]. STAT3 activation also suppresses the Th1 cell response in cancer [48]. Thus, inactivation or inhibition of STAT3 may enhance the development of Treg cells and, conversely, suppress the Th1 cell response, leading to amelioration of autoimmune diseases. Priceman et al. have reported that STAT3 promotes inflammation in the adipose tissue from mice with diet-induced obesity. In addition, STAT3 ablation promotes Treg polarization and reverses the high Th1/Treg ratio.

Fig. 6 Knockdown efficiency of Ptpn2. Western blotting was performed to determine the knockdown efficiency of Ptpn2 in CD4 T cells after shPtpn2 transfection. About half (45%) of the PTPN2 protein level was knocked down.
which in turn reduces local inflammation in the adipose tissue of obese mice [49]. Therefore, we speculate that hUCMSC treatment upregulates PTPN2 expression to inactivate STAT3 signaling in CD4\(^+\) T cells, thereby alleviating the imbalance of CD4\(^+\) T cell subsets and inflammation in the thyroid gland.

This study has some limitations. First, the impacts of time, dose, and frequency of hUCMSC administration require further investigation. Second, the bridge linking hUCMSCs and PTPN2 expression in CD4\(^+\) T cells remains unexplored. Third, Ptpn2 knockdown resulted in an incomplete reversal of hUCMSC-mediated regulation
in CD4+ T cells, suggesting that additional signaling pathways are also implicated in this process. Further studies are necessary to address these limitations. Fourth, the levels of PTPN2 did not change in the EAT group, and the role of PTPN2 in the etiology of EAT remains unknown. Additional studies are needed to reveal the role of PTPN2 in the pathogenesis of EAT.

In this study, we demonstrated that hUCMSC treatment ameliorated EAT in rats, as evidenced by significantly reduced follicle destruction and lymphocyte infiltration in the thyroid gland along with decreased levels of serum thyroid antibodies. hUCMSC treatment alleviated the imbalance of CD4+ T cell subsets via PTPN2/STAT3 signaling. Thus, hUCMSC implantation is a promising therapeutic approach for the treatment of autoimmune thyroiditis.

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Disclosure

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Competing interest
The authors declare no conflicts of interest.

References

1. Ramos-Levi AM, Marazuela M (2016) Pathogenesis of thyroid autoimmune disease: the role of cellular mechanisms. Endocrinal Nutr 63: 421–429.
2. Mincer DL, Jialal I (2021) Hashimoto Thyroiditis. In: StatPearls [Internet]. StatPearls Publishing, Treasure Island, Florida, USA.
3. Pyzik A, Grywalska E, Matyjaszek-Matuszek B, Rolinski J (2015) Immune disorders in Hashimoto’s thyroiditis: what do we know so far? J Immunol Res 2015: 979167.
4. Rydzewska M, Jaromin M, Pasierowska IE, Stozek K, Bossowski A (2018) Role of the T and B lymphocytes in pathogenesis of autoimmune thyroid diseases. Thyroid Res 11: 2.
5. Yasuda K, Takeuchi Y, Hirota K (2019) The pathogenicity of Th17 cells in autoimmune diseases. Semin Immunopathol 41: 283–297.
6. Drugar D, Negru S, Koreck A, Zosin I, Cristea C (2000) The pattern of a TH1 cytokine in autoimmune thyroiditis. Immunol Lett 71: 73–77.
7. Nepom GT, St Clair EW, Turka LA (2011) Challenges in the pursuit of immune tolerance. Immunol Rev 241: 49–62.
8. Goschl L, Scheinecker C, Bonelli M (2019) Treg cells in autoimmunity: from identification to Treg-based therapies. Semin Immunopathol 41: 301–314.
9. Jin X, Wang S, Zhao X, Jin Q, Fan C, et al. (2016) Coumestrol inhibits autoantibody production through modulating Th1 response in experimental autoimmune thyroiditis. Oncotarget 7: 52797–52809.
10. Vitales-Noyola M, Ramos-Levi AM, Martinez-Hernandez R, Serrano-Somavilla A, Sampedro-Nunez M, et al. (2017) Pathogenic Th17 and Th22 cells are increased in patients with autoimmune thyroid disorders. Endocrine 57: 409–417.
11. Xue H, Yu X, Ma L, Song S, Li Y, et al. (2015) The possible role of CD4+CD25hiFoxp3+/CD4+IL-17A+ cell imbalance in the autoimmunity of patients with Hashimoto thyroiditis. Endocrine 50: 665–673.
12. Zhang K, Wang Y, Ma W, Hu Z, Zhao P (2017) Genistein improves thyroid function in Hashimoto’s thyroiditis patients through regulating Th1 cytokines. Immunobiology 222: 183–187.
13. Shao S, Yu X, Shen L (2018) Autoimmune thyroid diseases and Th17/Treg lymphocytes. Life Sci 192: 160–165.
14. Doody KM, Bourdeau A, Tremblay ML (2009) T-cell protein tyrosine phosphatase is a key regulator in immune cell signaling: lessons from the knockout mouse model and implications in human disease. Immunol Rev 228: 325–341.
15. Spalinger MR, Kasper S, Chassard C, Raselli T, Frey-Wagner I, et al. (2015) PTPN2 controls differentiation of CD4+ T cells and limits intestinal inflammation and intestinal dysbiosis. Mucosal Immunol 8: 918–929.
16. Wiede F, La Gruta NL, Tiganis T (2014) PTPN2 attenuates T-cell lymphopenia-induced proliferation. Nat Commun 5: 3073.
17. Khor B, Gardet A, Xavier RJ (2011) Genetics and pathogenesis of inflammatory bowel disease. Nature 474: 307–317.
18. Pike KA, Tremblay ML (2018) Protein tyrosine phosphatases: regulators of CD4 T cells in inflammatory bowel disease. Front Immunol 9: 2504.
19. Wiede F, Brodnicki TC, Goh PK, Leong YA, Jones GW, et al. (2019) T-cell-specific PTPN2 deficiency in NOD mice accelerates the development of type 1 diabetes and autoimmune comorbidities. Diabetes 68: 1251–1266.
20. Zhu Y, Yao J, Duan Y, Xu H, Cheng Q, et al. (2020) Protein expression profile in rat silicosis model reveals upregulation of PTPN2 and its inhibitory effect on epithelial-mesenchymal transition by dephosphorylation
of STAT3. *Int J Mol Sci* 21: 1189.

21. Kim M, Morales LD, Jang IS, Cho YY, Kim DJ (2018) Protein tyrosine phosphatases as potential regulators of STAT3 signaling. *Int J Mol Sci* 19: 2708.

22. Li Y, Zhou H, Wang F, Han L, Liu M, et al. (2018) Overexpression of PTPN2 in visceral adipose tissue ameliorated atherosclerosis via T cells polarization shift in diabetic apoe−/− mice. *Cell Physiol Biochem* 46: 118–132.

23. Wu R, He Q, Chen H, Xu M, Zhao N, et al. (2017) MicroRNA-448 promotes multiple sclerosis development through induction of Th17 response through targeting protein tyrosine phosphatase non-receptor type 2 (PTPN2). *Biochem Biophys Res Commun* 486: 759–766.

24. Purvis HA, Anderson AE, Young DA, Isaacs JD, Hilkens CM (2014) A negative feedback loop mediated by STAT3 limits human Th17 responses. *J Immunol* 193: 1142–1150.

25. Durant L, Watford WT, Ramos HL, Laurence A, Vahedi G, et al. (2010) Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity* 32: 605–615.

26. Gharibi T, Babaloo Z, Hosseini A, Abdollahpour-Alitappeh M, Hashemi V, et al. (2020) Targeting STAT3 in cancer and autoimmune diseases. *Eur J Pharmacol* 878: 173107.

27. Uccelli A, de Rosbo NK (2015) The immunomodulatory function of mesenchymal stem cells: mode of action and pathways. *Ann N Y Acad Sci* 1351: 114–126.

28. Li H, Shen S, Fu H, Wang Z, Li X, et al. (2019) Immunomodulatory functions of mesenchymal stem cells in tissue engineering. *Stem Cells Int* 2019: 9671206.

29. von Bahr V, Millar JE, Malferttheiner MV, Ki KK, Passmore MR, et al. (2019) Mesenchymal stem cells may ameliorate inflammation in an *ex vivo* model of extracorporeal membrane oxygenation. *Perfusion* 34: 15–21.

30. Takikawa S, Yamamoto A, Sakai K, Shohara R, Iwase A, et al. (2013) Human umbilical cord-derived mesenchymal stromal cells promote sensory recovery in a spinal cord injury rat model. *Stem Cell Discovery* 3: 155–163.

31. Abbaspahab B, Momeni M, Ebrahimi M, Mousavi SH (2018) Advances in perinatal stem cells research: a precious cell source for clinical applications. *Regen Med* 13: 595–610.

32. Hu J, Wang F, Sun R, Wang Z, Yu X, et al. (2014) Effect of combined therapy of human Wharton’s jelly-derived mesenchymal stem cells from umbilical cord with sitagliptin in type 2 diabetic rats. *Endocrine* 45: 279–287.

33. Wang D, Niu L, Feng X, Yuan X, Zhao S, et al. (2017) Long-term safety of umbilical cord mesenchymal stem cells transplantation for systemic lupus erythematosus: a 6-year follow-up study. *Clin Exp Med* 17: 333–340.

34. Ma D, Xu K, Zhang G, Liu Y, Gao J, et al. (2019) Immunomodulatory effect of human umbilical cord mesenchymal stem cells on T lymphocytes in rheumatoid arthritis. *Int Immunopharmacol* 74: 105687.

35. Nagroho WS, Kusindarta DL, Susetya H, Fitriana I, Mulyani GT, et al. (2016) The structural and functional recovery of pancreatic beta-cells in type 1 diabetes mellitus induced mesenchymal stem cell-conditioned medium. *Vet World* 9: 535–539.

36. Zhang J, Lv S, Liu X, Song B, Shi L (2018) Umbilical cord mesenchymal stem cell treatment for Crohn’s disease: a randomized controlled clinical trial. *Gut Liver* 12: 73–78.

37. Calcatteria V, Nappi RE, Regalbuto C, De Silvestri A, Incardona A, et al. (2020) Gender differences at the onset of autoimmune thyroid diseases in children and adolescents. *Front Endocrinol (Lausanne)* 11: 229.

38. Hu J, Wang Y, Wang F, Wang L, Yu X, et al. (2015) Effect and mechanisms of human Wharton’s jelly-derived mesenchymal stem cells on type 1 diabetes in NOD model. *Endocrine* 48: 124–134.

39. Maleki M, Ghanbarvand F, Reza Behvarez M, Etemaei M, Ghadirkhomi E (2014) Comparison of mesenchymal stem cell markers in multiple human adult stem cells. *Int J Stem Cells* 7: 118–126.

40. Li H, Min J, Mao X, Wang X, Yang Y, et al. (2018) Edaravone ameliorates experimental autoimmune thyroiditis in rats through HO-1-dependent STAT3/Pi3K/Akt pathway. *Am J Transl Res* 10: 2037–2046.

41. Wang Z, Wei Q, Wang H, Han L, Dai H, et al. (2020) Mesenchymal stem cell therapy using human umbilical cord in a rat model of autoimmune-induced premature ovarian failure. *Stem Cells Int* 2020: 3249495.

42. Liu H, Tian Q, Ai X, Qin Y, Cui Z, et al. (2017) Dihydrotremcinisin attenuates autoimmune thyroiditis by inhibiting the CXCR3/Pi3K/AKT/NF-kappaB signaling pathway. *Oncotarget* 8: 115028–115040.

43. Beliaokova-Bethell N, Hezareh M, Wong JK, Strain MC, Lewinski MK, et al. (2017) Relative efficacy of T cell stimuli as inducers of productive HIV-1 replication in latently infected CD4 lymphocytes from patients on suppressive cART. *Virology* 508: 127–133.

44. Wang M, Yuan Q, Xie L (2018) Mesenchymal stem cell-based immunomodulation: properties and clinical application. *Stem Cells Int* 2018: 3057624.

45. van den Akker F, Vrijsen KR, Deddens JC, Buikema JW, Mokry M, et al. (2018) Suppression of T cells by mesenchymal and cardiac progenitor cells is partly mediated via extracellular vesicles. *Heliyon* 4: e00642.

46. Wiede F, Shields BJ, Chew SH, Kyparissoudis K, van Vliet C, et al. (2011) T cell protein tyrosine phosphatase attenuates T cell signaling to maintain tolerance in mice. *Clin Invest* 121: 4758–4774.

47. Egwuagu CE (2009) STAT3 in CD4+ T helper cell differentiation and inflammatory diseases. *Cytokine* 47: 149–156.

48. Lee H, Pal SK, Reckamp K, Figlin RA, Yu H (2011) STAT3: a target to enhance antitumor immune response. *Curr Top Microbiol Immunol* 344: 41–59.

49. Priceman SJ, Kujawski M, Shen S, Cherryholmes GA, Lee H, et al. (2013) Regulation of adipose tissue T cell subsets by Stat3 is crucial for diet-induced obesity and insulin resistance. *Proc Natl Acad Sci U S A* 110: 13079–13084.