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Human umbilical cord mesenchymal stem cells alleviate ongoing autoimmune dacryoadenitis in rabbits via polarizing macrophages into an anti-inflammatory phenotype

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Abstract

Mesenchymal stem cells (MSCs) exhibit beneficial effects on autoimmune dacryoadenitis. However, the underlying mechanisms are not fully understood. In this study, we investigated the therapeutic effect of human umbilical cord mesenchymal stem cells (hUC-MSCs) on rabbit autoimmune dacryoadenitis, an animal model of Sjögren's syndrome (SS) dry eye, and explored whether the effects of MSCs were related to their modulation on macrophage polarization. We have showed that systemic infusion of hUC-MSCs after disease onset efficiently diminished the chronic inflammation in diseased LGs and improved the clinical symptoms. Further analysis revealed that hUC-MSC treatment significantly inhibited the expression of pro-inflammatory M1 macrophage markers iNOS, TNF-α and IL-6, and promoted the expression of anti-inflammatory M2 macrophage markers Arg1, CD206, IL-10, IL-4 and TGF-β in LGs. Mechanistically, hUC-MSCs activated AKT pathway in macrophages, resulting in upregulation of M2-associated molecule Arg1, which was partly abolished by PI3K inhibitor, LY294002. Together, our data indicated that hUC-MSCs can skew macrophages into an M2 phenotype via affecting AKT pathway. These data may provide a new insight into the mechanisms of hUC-MSCs in the therapy of SS dry eye.

Keywords

Human umbilical cord mesenchymal stem cells; Autoimmune dacryoadenitis; Macrophages; Inflammatory mediators

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Conflicting interest statement

The authors declare that they have no competing financial interests.

1. Introduction

Sjögren's syndrome (SS) dry eye, a chronic and intractable autoimmune disorder, is characterized by immune cell infiltration affecting the lacrimal glands (LGs) and the ocular surface, ultimately resulting in impairment of vision (Foulks et al., 2015; Ogawa et al., 2018; Park et al., 2015). Although autoreactive T cells are key drivers in SS pathogenesis, monocytes/macrophages also play an important role in the development of SS (Ushio et al., 2018; Verstappen et al., 2019; Zhou and McNamara, 2014). Indeed, a critical pathological feature of SS is the accumulation of macrophages in LGs and salivary glands (Christodoulou et al., 2010; Schenke-Layland et al., 2008; Zhou et al., 2012).

Activated macrophages can be divided into classically activated M1 macrophages or alternative activated M2 subtypes with distinct functions. M1 macrophages exert pro-inflammatory effects through production of inflammatory factors such as TNF-α, IL-6, IL-1β and IL-12. Conversely, the alternative activated M2 macrophages contribute to inflammation resolution and tissue regeneration via secreting anti-inflammatory mediators such IL-10 and TGF-β (Shapouri-Moghaddam et al., 2018; Sica et al., 2015). iNOS and Arg1 are commonly used to characterize M1 and M2 phenotype, respectively (Kim et al., 2019). Accumulating data show that aberrant polarization of macrophages is closely associated with the progression of autoimmune diseases (Funes et al., 2018). M1-like polarization of islet macrophages has been proved to play critical roles in islet inflammation in type 2 diabetes (Eguchi and Nagai, 2017). You et al. found that macrophages largely exhibited an M1 phenotype in the conjunctiva in desiccation stress-induced dry eye mouse model (You et al., 2015). Moreover, the number of M2-like macrophages reduced as the lymphocytic infiltration grade increased in salivary glands from primary Sjögren's syndrome (pSS) patients (Aota et al., 2018).

Mesenchymal stem cells (MSCs) are a group of fibroblast-like multipotent stem cells with the ability to differentiate into multiple cell types (Uccelli et al., 2008). MSCs have been recently reported to drive the polarization of M2 macrophages both in vivo and in vitro (Chiossone et al., 2016; Kudlik et al., 2016; Zhang et al., 2018; Zheng et al., 2015). Compared to MSCs from other tissues, human umbilical cord mesenchymal stem cells (hUC-MSCs) are more easily harvested, proliferative, immunosuppressive (El Omar et al., 2014). Furthermore, hUC-MSCs do not raise ethical issues for clinical applications (Dalous et al., 2012). Thus, hUC-MSCs offer the best option for the cell-based therapy. For example, in a murine rheumatoid arthritis model, human umbilical cord blood derived MSCs have been found to ameliorate the severity of disease via promoting M2 macrophage polarization (Shin et al., 2016). We have previously demonstrated the protective role of adipose-derived mesenchymal stem cells (ADSCs) in autoimmune dacryoadenitis by inhibiting Th1/Th17 responses (Li et al., 2016). However, whether MSCs exert their therapeutic effects by modulating macrophage polarization in SS dry eye remains unknown.

In the current work, we investigated the therapeutic effects of hUC-MSCs on rabbit autoimmune dacryoadenitis and explored the underlying mechanisms. Our data demonstrated that intravenously infusion of hUC-MSCs after disease onset efficiently alleviated autoimmune dacryoadenitis in rabbits. Further mechanism study showed that

the therapeutic effects of hUC-MSCs may be partially attributed to their regulation on macrophage polarization.

2. Materials and methods

2.1. Animals

Adult New Zealand white rabbits (3.5–4 kg, female) were purchased from Vital River Laboratory Animal Technology (Beijing, China). All animal experimental procedures were performed in accordance with the guidelines of the Laboratory Animal Care and Use Committee of Tianjin Medical University and conformed to the ARVO Statement on Use of Animals in Ophthalmic and Vision Research. All rabbits were raised in the breeding room constant with the temperature at 25 °C ± 2 °C and relative humidity from 50% to 75% in a 12-h light-dark cycle (8 am–8 pm). A standardized ocular examination was undertaken to exclude any rabbits with pre-existing eye defects before the experimentation.

2.2. Induction of autoimmune dacryoadenitis

Rabbits were anesthetized and the left inferior LGs were surgically excised under aseptic conditions for isolating purified LG epithelial cells (pLGECs) according to the methods introduced by Guo et al. (2000). Peripheral blood lymphocytes (PBLs) from healthy rabbits and irradiated pLGECs were cultured separately at 37 °C for 2 days. Afterwards, the pLGECs were irradiated and co-cultured with autologous PBLs at the ratio of 1:1. After 5 days, the activated PBLs from mixed cell reactions were collected and adoptively injected (1.5×10^6 suspended in 100 μl sterile phosphate-buffered saline, [PBS]) back into rabbits via ear margin vein to induce autoimmune dacryoadenitis while the healthy group was injected with the same volume of PBS.

2.3. Cell culture

Fresh human umbilical cords were obtained and processed as soon as possible after delivery. All the subjects signed informed consent before delivery. The protocol was approved by the Ethics Committee of Tianjin Medical University Eye Hospital. The cords were washed twice, minced into 1 mm^2 pieces and then digested with 0.1% collagenase typeⅡ(Gibco, USA) for 1h at 37 °C. After that, all the liquids were centrifuged. Then the harvested pellets were resuspended and subsequently incubated at 37 °C. The medium was changed every 2–3 days. hUC-MSCs were characterized by MSC surface markers and their potential of differentiating into adipocytes and osteoblasts as previously described (Bai et al., 2017). Adipose-derived mesenchymal stem cells (ADSCs) from rabbits were prepared as previously described in our work (Li et al., 2016). Only hUC-MSCs and ADSCs in passages 3–5 were used for further experiments.

Human THP-1 monocytes (from Stem Cell Bank, Chinese Academy of Sciences) were cultured in RPMI 1640 medium with 10% FBS at a density of 1×10^6 cells/ml. To obtain monocyte-derived macrophages, THP-1 cells were treated with 160 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich, USA) for 24h. Then, the adherent cells were stimulated for further 24 h with 100 ng/ml lipopolysaccharide (LPS) and 50 ng/ml recombinant human interferon-γ (IFN-γ, R&D Systems, USA). After that, cells were

incubated with fresh medium alone or hUC-MSCs (macrophages: hUC-MSCs = 5:1) by trans-well for an additional 36 h.

2.4. Grouping and treatment procedures

In order to investigate the effects of MSCs on autoimmune dacryoadenitis, two administration schedules were arranged: 1) To compare the preventive effects of hUC-MSCs and ADSCs, rabbits were randomly assigned to four groups: ADSC-treated group (n = 9), hUC-MSC-treated group (n = 9), untreated group (n = 9) and healthy group (n = 9). Rabbits in the treated group were intravenously injected with 1×10^7 hUC-MSCs or ADSCs from day 1 to day 5 after adoptive transfer of activated PBLs. The MSC dose was at a final concentration of median 2.65 (range 2.5–2.8) $\times 10^6$ cells/kg (Hayes et al., 2015). As a control, rabbits in the untreated groups were injected with the same volume of PBS 2) To evaluated the therapeutic effects of hUC-MSCs, rabbits were randomly classified as follows: hUC-MSC-treated group (n = 9), untreated group (n = 9) and healthy group (n = 9). For the hUC-MSC-treated group, the injections of hUC-MSCs started at 2 w after adoptive transfer and continued for five consecutive days. The untreated group was treated the same as described above. Based on our preliminary experiments, this therapeutic protocol (intravenously injection of 1×10^7 MSCs per rabbit for five days) had been confirmed to be most effective for rabbit autoimmune dacryoadenitis(Li et al., 2016).

2.5. Clinical ocular surface assessments

Schirmer's test with anesthesia was performed for testing tear production. Schirmer strip was inserted into the lower fornix of the eye, the wetted length of the strip was measured after 1 min. Tear film stability was assessed by instilling 2% fluorescein in the middle of the lower eyelid and measuring the tear break-up time (BUT) after several blinks under cobalt blue slit-lamp illumination. Subsequently, the cornea punctate staining was recorded and scored as described previously (Xiao et al., 2013). The assessments were conducted every 2 weeks after the first hUC-MSC or ADSC administration.

2.6. Histologic analysis of lacrimal glands (LGs) and conjunctiva

Rabbits were sacrificed and then the right inferior LGs were surgically removed. Part of the glands was fixed in 10% buffered formalin. After dehydration, the samples were embedded in paraffin, transversely sectioned, and routinely stained with hematoxylin and eosin (H&E). H& E stained sections from four independent samples of each experiment group were scanned using light microscopy (BX51; Olympus Corporation, Tokyo, Japan). Two random fields of view were selected for each section and photographed using CellSen software (Olympus, Tokyo, Japan). The definition of foci was an aggregate of more than 50 lymphocytes (Vitali and Bombardieri, 1995). The total number of focus per 4 mm^2 of lacrimal tissue was recorded by a blinded pathologist (Shiboski et al., 2012). Samples of conjunctiva were also collected and treated as described above.

2.7. Quantitative real-time PCR (Q-PCR)

Total RNA was extracted from tissues or cells using the Trizol reagent (Invitrogen, USA) as previously described (Rio et al., 2010). The RNA sample was reversely transcribed into

the first-strand cDNA using a reverse transcription kit (Fermentas, Canada). Q-PCR analysis was performed using SYBR Green PCR master mix (Applied Biosystems, USA) on ABI 7900 HT Sequence Detection System. The gene-specific primer sequences are listed in Table 1 and Table 2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the endogenous healthyizing control. For each sample, the cycle threshold (Ct) values were obtained for both target and internal reference gene GAPDH, and the relative target mRNA expression was calculated by applying the $2^{[\Delta Ct(control)-\Delta Ct(target)]}$ method.

2.8. Western blot analysis

Frozen tissues or cells were thawed and homogenized in ice-cold lysis reagent. The extracted protein was quantified using the BCA Protein Assay Kit (Solarbio®, USA). Proteins of equal 50 μg were fractionated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane. After blocking in 5% fat-free milk, the membranes were incubated with primary antibodies against iNOS (1:500, Santa Cruz, sc-7271), Arg1 (1:500, Abcam, ab239731), Phospho-Akt (Ser473) (1:1000, Cell Signaling Technology, #12694), β-actin (1:2000, ZSGB-BIO, ZB-5301) overnight at 4 °C. An appropriate HRP-conjugated secondary antibody (1:5000, cell signaling, #7076s) was used to detect the binding of the specific antibody. The protein bands were visualized by Multispectral Imaging System (UVP, Upland, CA, USA). Quantitative analysis of target protein expression was performed using Quantity One software (Bio-Rad, Hercules, CA, USA).

2.9. Immunofluorescence staining

After fixation with 4% paraformaldehyde, THP-1 cells were blocked in 0.2% bovine serum albumin at room temperature for 1 h, and then incubated with primary antibody of Arg1(1:100, Abcam, ab239731) overnight at 4 °C. After being washed, the cells were incubated with anti-mouse IgG H&L (Alexa Fluor 488) secondary antibody (1: 500, Abcam, ab150113) at room temperature for 2 h, followed by counter-staining of the nuclei with 4,6-diamidino-2-phenylindole (DAPI) (Solarbio®, USA). Finally, samples were visualized and photographed by a blinded observer with an Olympus IX-71 fluorescence microscope (Tokyo, Japan). Quantification of $Arg1^+$ cells in at least five random fields of views was undertaken using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

2.10. Statistical analysis

Statistical analysis was performed using SPSS 25.0 software (IBM Corporation, Somers, NY, USA). All the data were presented as mean ± SD. The normality of the data was tested using the Shapiro-Wilk test. When normality was not rejected, comparisons were made using Student's *t*-test (comparisons of two groups) or one-way analysis of variance (ANOVA) (comparisons of three groups). Nonparametric data were compared using the Kruskal-Wallis test (comparison of three groups) or the Mann-Whitney *U* test (comparison of two groups). A P value less than 0.05 was considered as statistically significant.

3. Results

3.1. hUC-MSCs were as effective as ADSCs in preventing the development of rabbit autoimmune dacryoadenitis when administered before disease onset

We previously reported that ADSC treatment had a preventive effect on the development of rabbit autoimmune dacryoadenitis (Li et al., 2016). To examine whether hUC-MSCs were as effective as ADSCs in controlling autoimmune dacryoadenitis, ADSCs or hUC-MSCs were injected intravenously into rabbits for 5 successive days at the time of disease induction (transfer of activated PBLs) (Fig. 1A), and the disease severity was determined by evaluating tear production, tear break-up time (BUT), corneal epithelial integrity and histological scores. As shown in Fig. 1B–E, the untreated group developed a severe illness, characterized by decreased tear production, shortened tear BUT, stained corneal epithelium after disease induction. By contrast, recipients of hUC-MSCs or ADSCs showed remarkably attenuated clinical symptoms at all the time points observed, and the preventive effects of hUC-MSCs were similar to those of ADSCs.

To further validate the preventive effects of MSCs, a histological assessment was performed on day 42 post MSC administration. As shown in Fig. 1F, more severe acinar atrophy and more periductal or perivenous infiltration of inflammatory cells were observed in LGs of the untreated group than in those of the hUC-MSC or ADSC-treated group. Considering the future clinical application of MSCs, we next focused on the therapeutic effects and mechanisms of hUC-MSCs on the autoimmune dacryoadenitis.

3.2. hUC-MSCs administered after disease onset efficiently alleviated rabbit autoimmune dacryoadenitis

To investigate whether hUC-MSCs had therapeutic effects on rabbit autoimmune dacryoadenitis, hUC-MSCs were injected intravenously into rabbits after disease onset (2 weeks after transfer of activated PBLs) for 5 successive days (Fig. 2A). To evaluate the duration of the effect, we observed the clinical features till 42 days after hUC-MSC injection. As shown in Fig. 2B–D, the clinical scores were significantly reduced in the hUC-MSC-treated group compared with the untreated group at any observation time point. Histological examinations showed that the signs of inflammation and tissue damage in the LGs and conjunctiva were significantly attenuated in the hUC-MSC-treated group than those in the untreated group (Fig. 2E–G). All together, these data suggested that hUC-MSCs had a protective effect on inflamed LGs and conjunctivas in autoimmune dacryoadenitis.

3.3. hUC-MSC administration suppressed inflammation and promoted the expression of M2 macrophage-associated molecules in vivo

Macrophages are reported to be important regulators in inflammation of LGs (Li et al., 2010; Zhou et al., 2012). To investigate the modulatory effects of hUC-MSCs on macrophages in LGs, we collected LGs from healthy rabbits and disease-induced rabbits treated with or without hUC-MSCs, and analyzed the expression of M1/M2 macrophage markers and related cytokine genes by Q-PCR analysis. In comparison with the healthy group, the expression of iNOS (M1 macrophage marker) in LGs of the disease-induced rabbits without hUC-MSC treatment, was remarkably upregulated, together with a significant increase in

the expression of genes encoding pro-inflammatory cytokines, including tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 (Fig. 3A), indicating M1 macrophages may predominate in LGs of rabbit autoimmune dacryoadenitis. After hUC-MSC administration, iNOS showed a slightly non-significant downregulation, while a dramatically decreased expression of related pro-inflammatory cytokine was observed (Fig. 3A). In contrast, as revealed in Fig. 3B and 3D, the expression of M2 macrophage markers, Arg1 and CD206, and anti-inflammatory cytokines IL-10, IL-4 and transforming growth factor (TGF)-β was remarkably upregulated in the hUC-MSC-treated group. Besides, the increase of Arg1 expression in LGs of the hUC-MSC-treated group was further supported by the results from Western blot analysis (Fig. 3C). Given that monocyte chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony stimulating factor (GM-CSF) and matrix metalloproteinases (MMPs) have been shown to be associated with the infiltration and differentiation of macrophages (Ishikawa et al., 2017; Rajasekaran et al., 2019; Tavakoli et al., 2017), we therefore detected the mRNA expression of these inflammatory mediators in vivo. As shown in Fig. 3E, administration of hUC-MSCs significantly downregulated the expression of MCP-1, GM-CSF, MMP-9 and MMP-2 in inflamed LGs. Collectively, these findings suggested that hUC-MSCs could convert macrophages toward the anti-inflammatory M2 phenotype, and inhibit the chronic inflammation in LGs of rabbit autoimmune dacryoadenitis.

3.4. hUC-MSCs polarized inflammatory macrophages towards the anti-inflammatory M2 phenotype in vitro

We next investigated the modulatory effects of hUC-MSCs on macrophage polarization in vitro. To this end, PBLs isolated from model rabbits were co-cultured with irradiated pLGECs in the presence or absence of hUC-MSCs (PBLs: hUC-MSCs = 5:1) in a trans-well system. Five days later, the cells were collected and subjected to Q-PCR assay. The results showed that the expressions of M2-associated genes Arg1, CD206, IL-10 and IL-4 were significantly increased, whereas the mRNA levels of M1-associated genes iNOS, TNF-α, IL-6, MCP-1 and GM-CSF were reduced in the hUC-MSC group compared to the control group. No statistical difference was detected in IL-1β and TGF-β expression between two groups (Fig. 4A–B). Additionally, the upregulated protein level of Arg1 was detected after hUC-MSC co-culture (Fig. 4C).

To further confirm the influence of hUC-MSCs on macrophage phenotype, we performed an in vitro study using human monocytic cell line THP-1. THP-1 cells were stimulated with LPS + IFN-γ for 24h to induce M1 macrophage polarization (Yin et al., 2018), and then were co-cultured with or without hUC-MSCs in a trans-well system for 36h. Q-PCR analysis revealed higher expressions of M2 markers Arg1, CD206, IL-10 and IL-4 and lower expressions of M1 markers iNOS, TNF-α, IL-1β and IL-6 in the hUC-MSC group compared with the LPS + IFN-γ group (Fig. 5A–B). There was no statistical difference in the mRNA expression of TGF-β between two groups. Meanwhile, in comparison with the LPS + IFN-γ group, immunofluorescence staining showed an obvious increased percentage of $Arg1^+$ cells in the hUC-MSC group (Fig. 5C–D). Moreover, Western blot analyses showed the markedly downregulated protein level of iNOS and the significantly increased Arg1 protein expression after hUC-MSC incubation (Fig. 5E, 5G and 5H). Taken together, these results

suggested that hUC-MSCs effectively suppressed M1 activation and shifted macrophages into an anti-inflammatory M2 state in vitro.

3.5. hUC-MSCs modulated macrophage polarization via AKT pathway

We further explored the underlying molecular mechanism through which hUC-MSCs induced M2 macrophage polarization. Considering that Akt activation is critical for M2 macrophage conversion (Byles et al., 2013), we hypothesized that hUC-MSCs may regulate macrophage polarization via activation of AKT signaling. To test this, THP-1 cells were treated with LPS and IFN-γ, and co-cultured with or without hUC-MSCs in a trans-well system for 36h, and then the protein expression of phosphorylated Akt (p-AKT) in macrophages of each group was examined. Immunoblotting results demonstrated that the expression of p-AKT was significantly enhanced in the hUC-MSC group in comparison with the LPS + IFN-γ group, accompanied with remarkable elevation of Arg-1 (a hallmark of M2 macrophage) protein level (Fig. 5F–H), suggesting that activation of AKT signaling in macrophages may be linked with hUC-MSC induced M2 macrophage polarization. To further confirm whether maintaining AKT activation was critical for the modulatory effect of hUC-MSCs on macrophage phenotype, we pretreated LPS and IFN-γ-stimulated macrophages with LY294002, a specific pharmacological inhibitor of PI3K, or dimethyl sulfoxide (DMSO) for 30 min before hUC-MSC co-incubation. We found that after blockage of AKT, the upregulated protein level of Arg1 induced by hUC-MSCs was partly abolished (Fig. 5I–J). Collectively, these results indicated the importance of PI3K/AKT pathway in M2 macrophage polarization induced by hUC-MSCs.

4. Discussion

In recent years, MSCs have been proposed as a promising therapeutic alternative for inflammatory and autoimmune diseases (Guerrouahen et al., 2019; Weiss and Dahlke, 2019). In the present study, we demonstrated that hUC-MSCs administered after disease onset efficiently improved the clinical symptoms and reduced lacrimal and conjunctival impairment in rabbit autoimmune dacryoadenitis during an observation period of 42 days. hUC-MSC treatment induced the decreased M1 marker expression and upregulated M2 marker expression in LGs, accompanied with reduced pro-inflammatory cytokine production and enhanced anti-inflammatory cytokine expression. Importantly, we demonstrated that hUC-MSCs could drive the polarization of M2 macrophages via activating AKT signaling.

Macrophage polarization is closely associated with the pathogenesis of various inflammatory and autoimmune diseases. A prolonged activation of M1 macrophages can lead to inflammation and autoimmunity development (Sindrilaru et al., 2011; Van Raemdonck et al., 2019). In agree with these observations, our data showed that LGs from rabbit autoimmune dacryoadenitis expressed elevated pro-inflammatory M1 markers, implying the involvement of M1 macrophages in pathogenesis of rabbit autoimmune dacryoadenitis. Accumulated M1 macrophages in inflamed LGs might produce inflammatory mediators to enhance SS-like lesions. In contrast to the tissue-destructive functions of M1 macrophages, M2 macrophages have stimulated an emerging interest for their immunoregulatory roles in autoimmune disorders. Adoptive transfer of M2

macrophages has been found to prevent the progression of experimental colitis and type 1 diabetes (Haribhai et al., 2016; Parsa et al., 2012), illustrating the beneficial effects of M2 macrophages in autoimmune diseases. In the present study, we observed that LGs of the diseased group exhibited the lower expression of M2-related markers compared to the healthy group, suggesting a defective M2 activation in autoimmune dacryoadenitis. Given that M2 macrophages could secret anti-inflammatory cytokines and promote tissue repair (Bi et al., 2019), targeting M2 polarization may be a promising therapeutic strategy for treatment of SS dry eye.

The involvement of macrophages in mediating therapeutic efficacy of MSCs has been previously reported in several autoimmune disorders (Ko et al., 2016; Xie et al., 2016). Recently, using a murine model of lupus nephritis, Zhang et al. demonstrated that infused hUC-MSCs prevented podocyte injury and ameliorated lupus nephritis via polarizing macrophages into an anti-inflammatory phenotype (Zhang et al., 2019). Similarly, we found that hUC-MSC treatment promoted anti-inflammatory M2 macrophage polarization and diminished the inflammation in diseased LGs, and we further demonstrated that hUC-MSCs possessed the ability to shift pro-inflammatory M1 macrophages toward an M2 state in vitro. hUC-MSC-induced M2 macrophage polarization might suppress inflammatory responses and enhance subsequent reparative activities, thereby slow the progression of autoimmune dacryoadenitis.

Activation of the PI3K/AKT pathway is required for M2 activation (Vergadi et al., 2017). A crosstalk between STAT6 and PI3K activation is required for IL-4 induced M2 macrophage polarization in SHIP-deficient macrophages (Weisser et al., 2011). MSCs have been shown to increase the protein expression of p-STAT6 and Arg1 in peritoneal macrophages from type 2 diabetic rats (Xie et al., 2016). However, to date, there remains limited data to reveal the role of PI3K/Akt signaling in hUC-MSC modulated macrophage polarization. In this study, we found that hUC-MSCs could activate AKT signaling in macrophages simultaneous with the rescue of M2-related gene expression, and by using PI3K inhibitor LY294002, we further confirmed that AKT activation was necessary for hUC-MSC-induced M2 polarization. AKT activation may lead to phosphorylation of transcription factor STAT6, enabling nucler translocation and induction of target gene Arg1 transcription, resulting in subsequent polarization of M2 macrophages (Liu et al., 2019; Weisser et al., 2011).

Cytokines and chemokines in the inflammatory microenvironment are critical for the phenotype diversity of macrophages (Gordon and Martinez, 2010; Ruytinx et al., 2018). GM-CSF and the representative C–C motif chemokine MCP-1 play a key role in M1 macrophage polarization (Benmerzoug et al., 2018; Rajasekaran et al., 2019), while IL-4, IL-10, TGF-β, or a combination of these anti-inflammatory cytokines, are favorable for M2 polarization (Atri et al., 2018; Makita et al., 2015). In addition, it has been reported that matrix metalloproteinases (MMPs) also affect M1/M2 polarization. MMP-9 deletion was found to stimulate M2 macrophage polarization in ageing-associated cardiac inflammation (Ma et al., 2015). A recent study by Takahashi et al. demonstrated that MMP-2 promoted the differentiation of M1 macrophages in allergic bronchial asthma (Takahashi et al., 2019). In this study, we observed that hUC-MSC infusion significantly decreased the expression of MCP-1, GM-CSF, MMP-2 and MMP-9, while remarkably upregulated the expression of

IL-4, IL-10 and TGF-β in inflamed LGs, suggesting that hUC-MSC treatment may establish a milieu favoring the generation of M2 macrophages.

In summary, hUC-MSC treatment efficiently suppressed inflammation and restored LG function in rabbit autoimmune dacryoadenitis, and this effect may be partly ascribed to eliciting macrophages into an anti-inflammatory M2 sate via activating AKT pathway. Our study here provided a novel insight into the mechanisms of MSC-based therapy for autoimmune dry eye.

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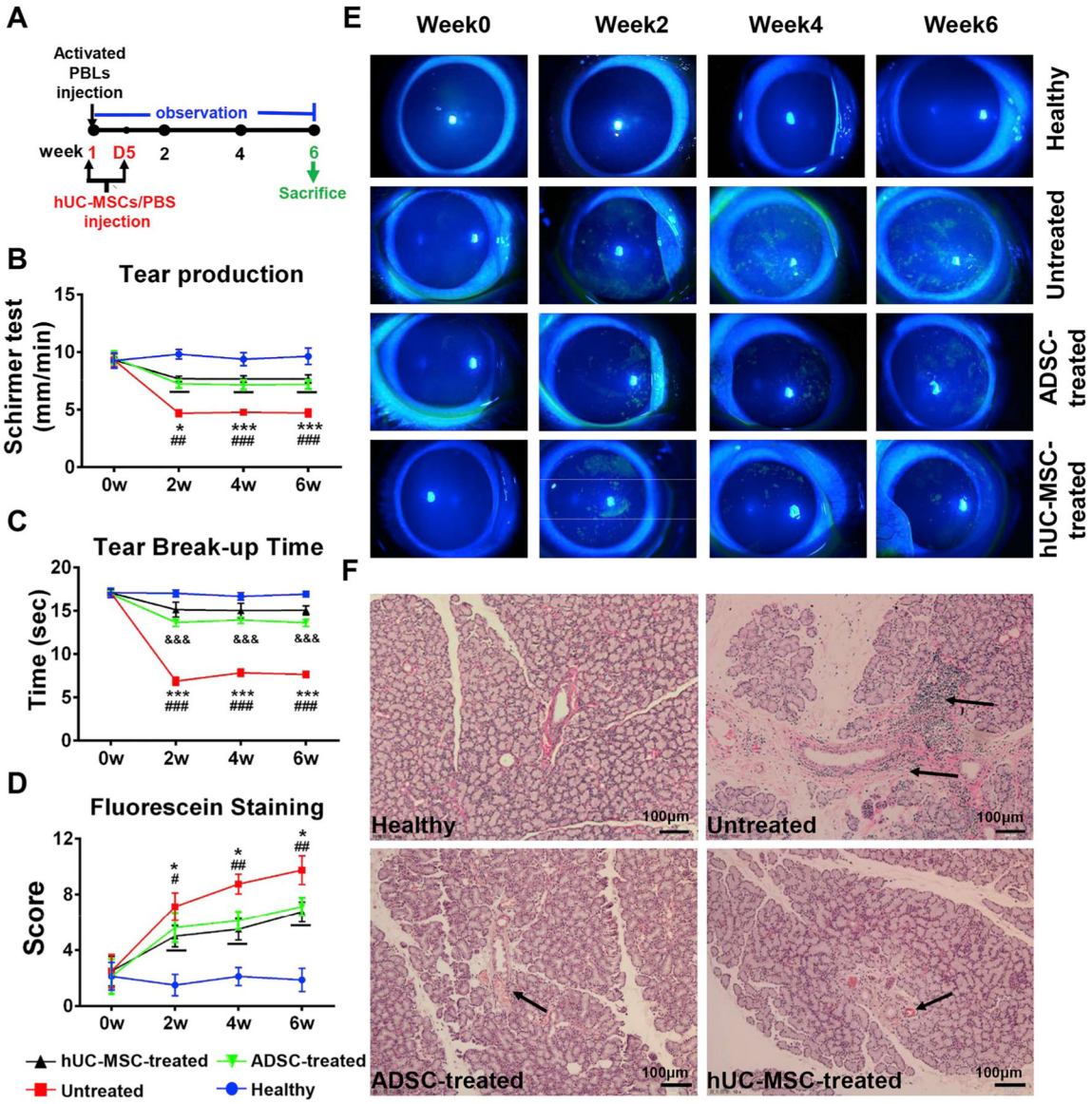

Fig. 1. The preventive effects of hUC-MSCs and ADSCs on rabbit autoimmune dacryoadenitis.

(A) Schema of hUC-MSC and ADSC Infusion in a rabbit model of autoimmune dacryoadenitis. (B) Tear production. Schirmer test was conducted before and after hUC-MSC or ADSC administration. (C) Tear break-up time. Slit-lamp examination was performed to evaluate the tear stability. (D and E) Scores and representative images of fluorescein staining. Detection of ocular surface detects with fluorescein staining was performed. (F) Representative images (H&E staining) of lacrimal gland sections from rabbits in the healthy, untreated, ADSC-treated and hUC-MSC-treated group. Arrows indicate infiltrating lymphocytes in the periductal or perivascular distribution. Data of (B–D) are presented as mean ± SD, n = 8 rabbits per group. * ADSC-treated group versus Untreated group, *P < 0.05,**P < 0.01, ***P < 0.001. # hUC-MSC-treated group versus Untreated group, #P < 0.05, ##P < 0.01, ###P < 0.001. & hUC-MSC-treated group versus

ADSC-treated group, &P < 0.05, &&P < 0.01, &&&P < 0.001. — represents no statistical difference between the hUC-MSC-treated group and ADSC-treated group.

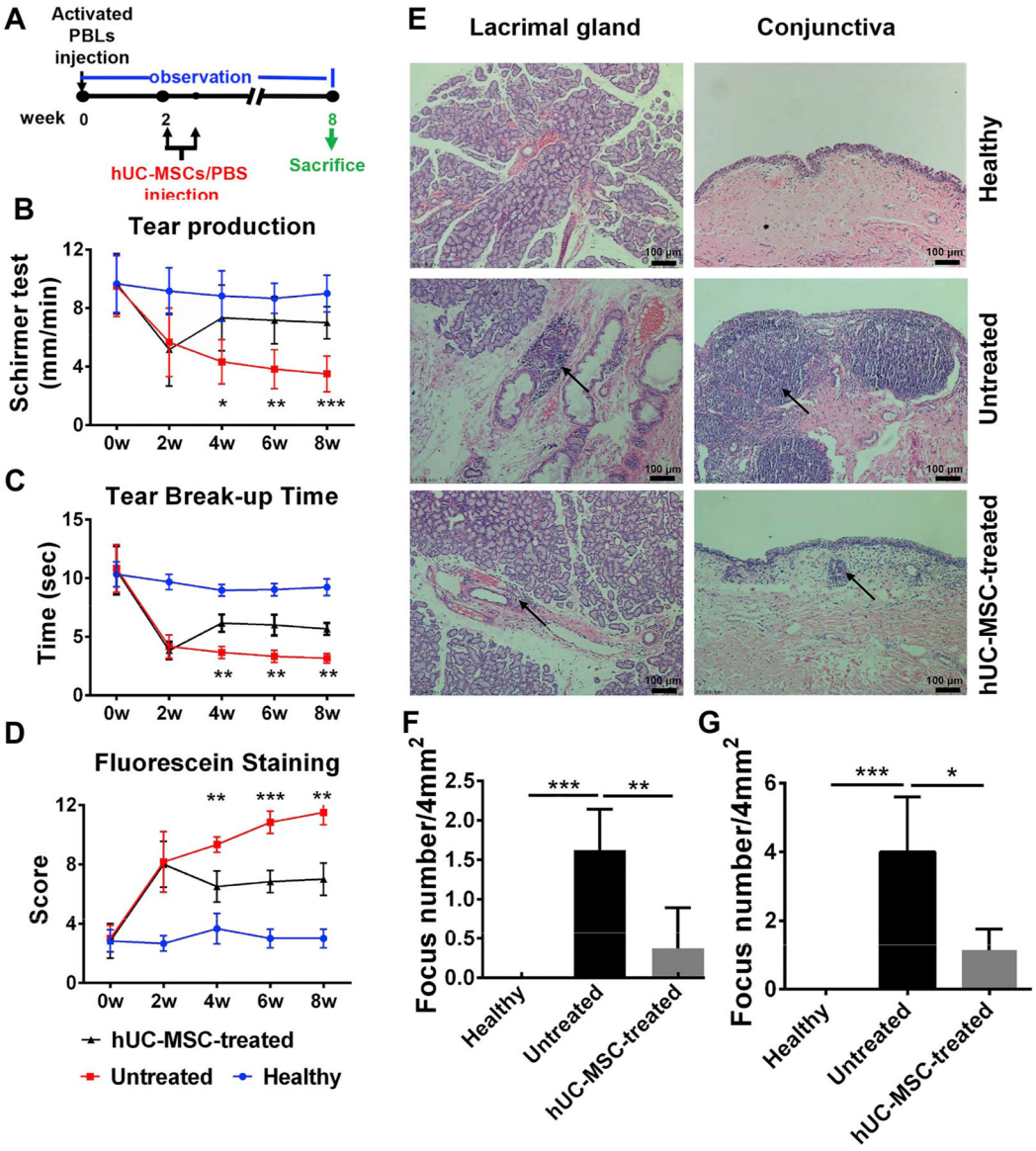

Fig. 2. hUC-MSCs administrated after disease onset efficiently attenuated rabbit autoimmune dacryoadenitis.

(A) Schematic representation of hUC-MSC administration in a rabbit model of autoimmune dacryoadenitis. (B) Tear production. (C) Tear break-up time. (D) Corneal fluorescein grading scores. n = 6 rabbits per group (B–D) (E) Representative H&E staining photographs of LGs and conjunctivas in the healthy, untreated and hUC-MSC-treated rabbits. Arrows indicate perivascular or periductal lymphocytic foci (> 50 infiltrating lymphocytes). (F) Quantification of the numbers of lymphocytic foci per 4 mm^2 in LGs (n = 8). (G) Numbers of lymphocytic foci per 4 mm^2 in conjunctivas were evaluated (n = 8). Data represent the mean ± SD (B–D, F, G). *P < 0.05, **P < 0.01.

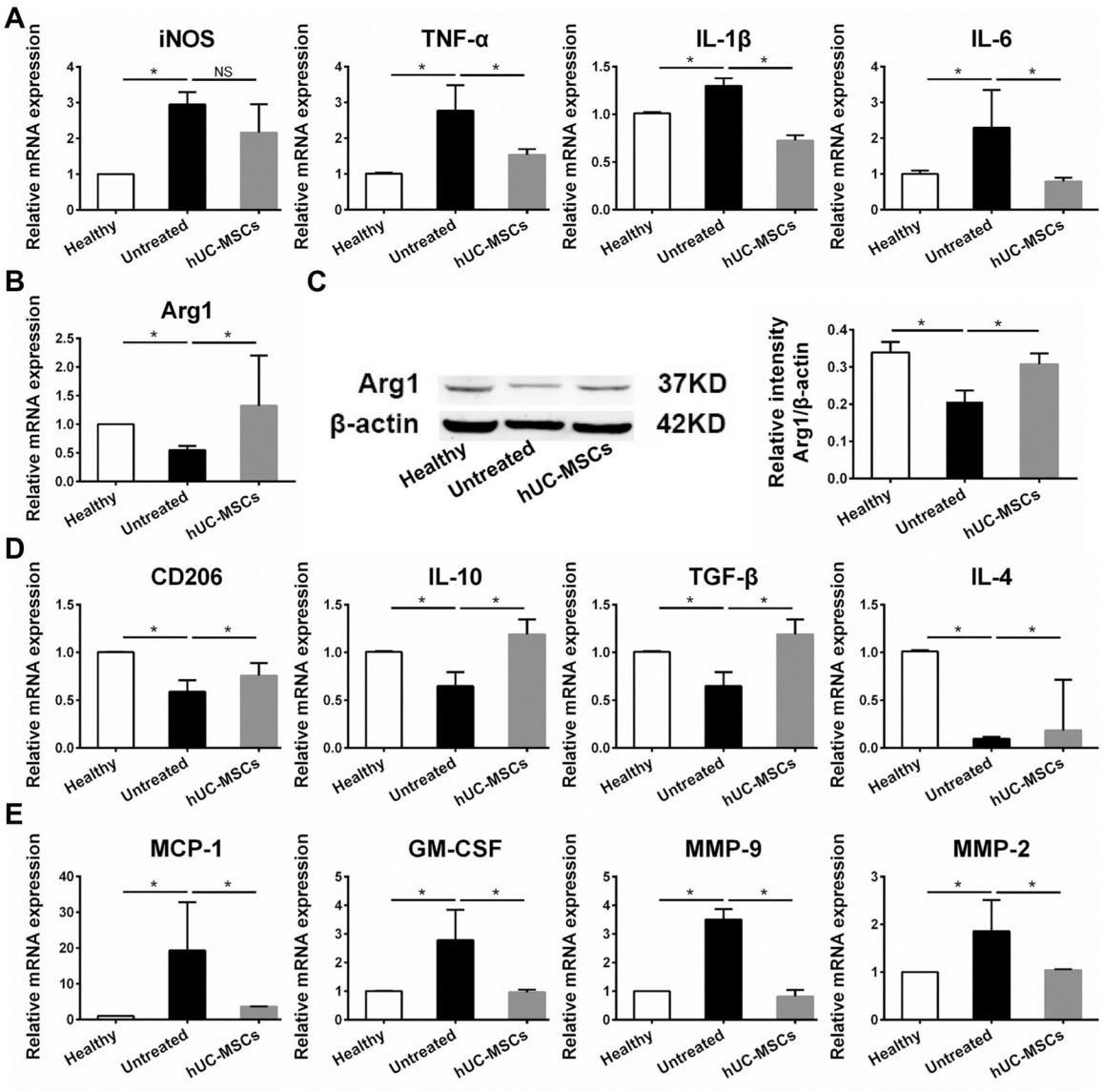

Fig. 3. hUC-MSC administration suppressed inflammation and promoted the expression of M2 macrophage-associated molecules in vivo.

LGs were collected from rabbits in the healthy, untreated and hUC-MSCs group at week 8, and then subjected to Q-PCR or Western blot analysis. (A)The relative expression of genes encoding M1 macrophage marker (iNOS) and inflammatory cytokines (TNF-α, IL-1β and IL-6). (B) Gene expression of Arg1 (a marker of M2 macrophage). (C) Western blot assay for Arg1 expression. Images shown are from one experiment representative of three independent experiments. Relative protein level is quantified by ratio of Arg1 to β-actin. (D) The mRNA expression of M2 macrophage marker (CD206) and anti-inflammatory molecules (IL-10, TGF-β and IL-4). (E) Gene expression profiles of inflammatory mediators (MCP-1, GM-CSF, MMP-9 and MMP-2). Data are representative of three independent experiments (n = 3 rabbits per group in each experiment) and bar graphs indicate mean ± SD. *P < 0.05. NS = not significant.

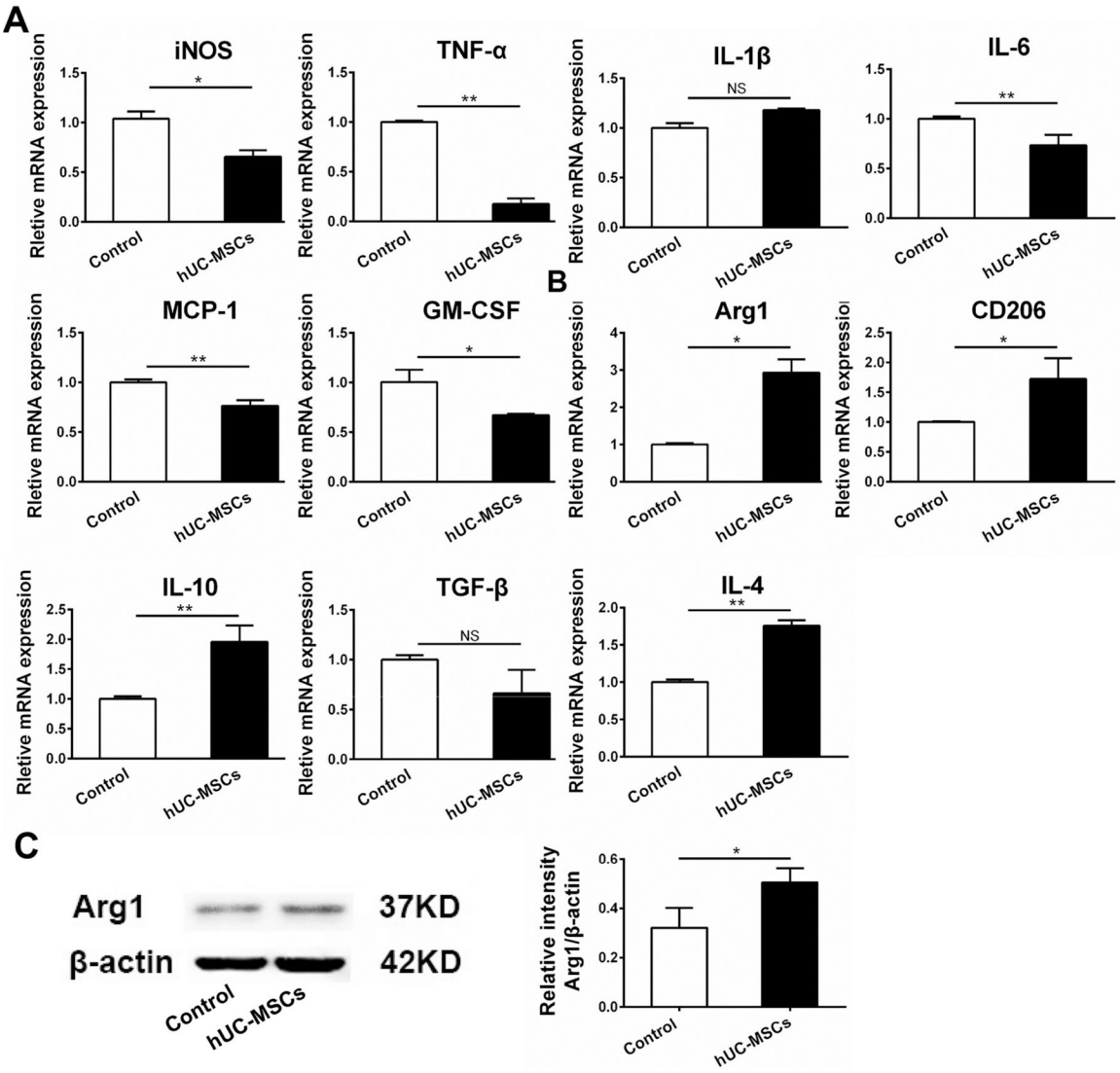

Fig. 4. hUC-MSCs polarized inflammatory macrophages towards the anti-inflammatory M2 phenotype in vitro.

PBLs isolated from diseased rabbits were co-cultured with or without hUC-MSCs in a trans-well system in the presence of irradiated pLGECs for 5 days. (A–B) Q-PCR analysis of iNOS, TNF-α, IL-1β, IL-6, MCP-1, GM-CSF, Arg1, CD206, IL-10, TGF-β and IL-4 mRNA expression in PBLs co-cultured with or without hUC-MSCs. (C) Western blot assay and quantitative analysis for Arg1 expression in PBLs after co-culture with hUC-MSCs. Representative images from three independent experiments are shown. Data are expressed as mean ± SD of three samples obtained from at least three independent experiments. *P < 0.05, **P < 0.01. NS = not significant.

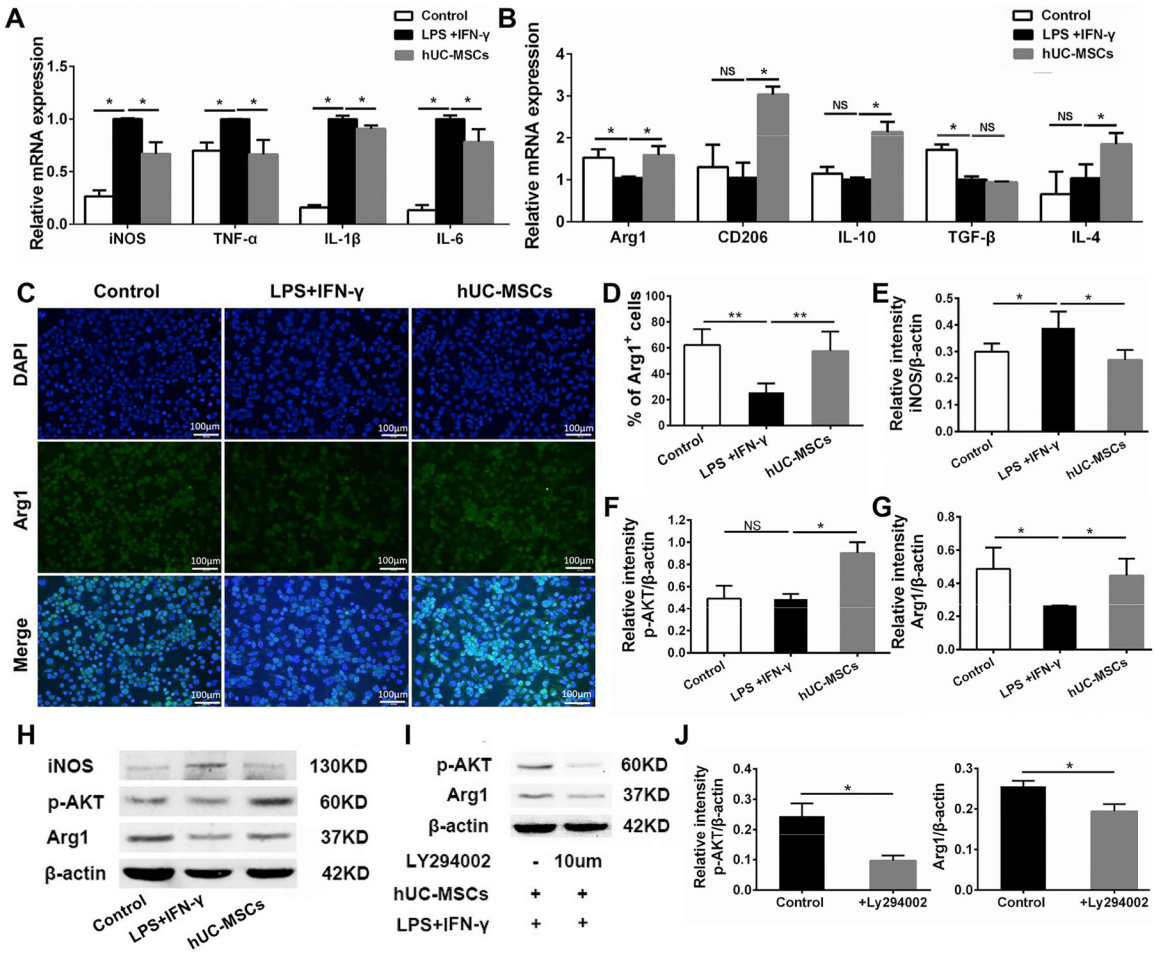

Fig. 5. hUC-MSCs facilitated M2 macrophage polarization in THP-1 cells stimulated by LPS and IFN-γ via activating AKT pathway.

Macrophage derived from THP-1 cells were cultured alone (control group) or stimulated by LPS and IFN-γ in the absence (LPS + IFN-γ group) or presence of hUC-MSCs (hUC-MSCs group). (A–B) Gene expressions of iNOS, TNF-α, IL-1β, IL-6, Arg1, CD206, IL-10, TGF-β and IL-4 in macrophages from the above three groups were detected by Q-PCR. (C) Representative images of immunofluorescence staining for $ARG1^+$-cells in the three groups. Scale bar, 100 μm. The percentage of $ARG1^+$-cells presented in (D) was quantified by evaluating at least five random fields of each section. (H) Western blot assay for iNOS, p-AKT and Arg1 expression in the three groups. (I) Macrophages in the hUC-MSCs group were treated with or without the PI3K inhibitor, LY294002. Protein expression of Arg1 and p-AKT was detected by Western blot. Images shown are from one representative of three independent experiments. β-actin was used as a loading control, and quantitative analysis of relative protein levels was presented in (E–G) and (J). Data shown are mean ± SD of three samples from three independent experiments. *P < 0.05, **P < 0.01. NS = not significant.

Table 1

Gene-specific primers used for Q-PCR (rabbit).

Gene	Forward Primer Seqence	Reverse Primer Sequence
GAPDH	5′-GGGTGGTGGACCTCATGGT-3′	5′-CGGTGGTTTGAGGGCTCTTA-3′
iNOS	5′-TCCACCAGGAGATGCTCAACT-3′	5′-TGGGTTTTCCACGCCTCTAC-3′
CD206	5′-CTGATAGATGGAGGGTGAGGTACA-3′	5′-CCAGATAGACGCATGCTGACTTC-3′
Arg1	5′-GAAGTAACTCGAACGGTGAACACA-3′	5′-TCCCGAGCAACTCCAAAAGA-3′
IL-10	5′-GGCTGAGGCTGCGACAAT-3′	5′-TGCCTTGCTCTTGTTTTCACA-3′
TGF-β	5′-CAAGGACCTGGGCTGGAA-3′	5′-AGGCAGAAGTTGGCGTGGTA-3′
IL-6	5′-GCAGAAAAACCAGTGGCTGAA-3′	5′-GGCCGCGCAGGATGA-3′
TNF-α	5′-AGCTTCTCGGGCCCTGAGT-3′	5′-CCACTTGCGGGTTTGCTACT-3′
Il-4	5′-GCACTCCGGCAGTTCTACCT-3′	5′-CTCCGTGCTCCTTGAAGCA-3′
IL-1β	5′-CTCCTGCCAACCCTACAACAA-3′	5′-TCCAGAGCCACAACGACTGA-3′
MMP-9	5′-CCAGTACCGAGAGAAAGCCTACTT-3′	5′-CCTCGTTCCGGGTACTCACA-3′
MMP-2	5′-GCGCGCCTTCCAAGTCT-3′	5′-CATCGTGGATTCGAGAAAACC-3′
MCP-1	5′-TGTAAAGGCAGGTGTGGTCTGT-3′	5′-ACGTTCCCGAGGCAAACA-3′
GM-CSF	5′-CCAGCCCTTGAAGCATGTG-3′	5′-TTACTGCGGCTCAGGATGATC-3′

Table 2

Gene-specific primers used for Q-PCR (human).

Gene	Forward Primer Seqence	Reverse Primer Sequence
GAPDH	5′-CTGGGCTACACTGAGCACC-3′	5′-AAGTGGTCGTTGAGGGCAATG-3′
iNOS	5′-CCCCTTCAATGGCTGGTACA-3′	5′-GCGCTGGACGTCACAGAA-3′
CD206	5′-CGCTACTAGGCAATGCCAATG-3′	5′-GCAATCTGCGTACCACTTGTTT-3′
Arg1	5′-GCGCCAAGTCCAGAACCA-3′	5′-CGTGGCTGTCCCTTTGAGAA-3′
IL-10	5′-TGAGAACAGCTGCACCCACTT-3′	5′-TCGGAGATCTCGAAGCATGTTA-3′
TGF-β	5′-CGCGCATCCTAGACCCTTT-3′	5′-CTGTGGCAGGTCGGAGAGA-3′
IL-6	5′-AGGGCTCTTCGGCAAATGTA-3′	5′GAAGGAATGCCCATTAACAACAA-3′
TNF-α	5′GCAGGTCTACTTTGGGATCATTG-3′	5′-GCGTTTGGGAAGGTTGGA-3′
IL-4	5′-TGGGTCTCACCTCCCAACTG-3′	5′-GCCGGCACATGCTAGCA-3′
IL-1β	5′-TCAGCCAATCTTCATTGCTCAA-3′	5′-TGGCGAGCTCAGGTACTTCTG-3′

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