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RESEARCH ARTICLE

Evaluation of immunomodulatory effects of co-culture or supernatant of dexamethasone or IFN-γ-treated adipose-derived mesenchymal stem cells on spleen mononuclear cells

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ABSTRACT. Although mesenchymal stem cells (MSCs) have exhibited promising immunomodulatory potential in preclinical studies, clinical studies have revealed variable results. These results often depend on environmental cues. Pre-conditioning MSCs with cytokines is one of the methods used to enhance their immunomodulatory effects. In this study, we harvested adipose-derived MSCs from mice and cultured them with different doses of the cytokine, IFN-γ, and the corticosteroid drug, dexamethasone, in order to investigate their effects on MSC immunosuppressive function. We found the co-culture or supernatant of MSCs, pre-conditioned with IFN-γ, together with spleen mononuclear cells resulted in a significant reduction of mononuclear cell proliferation. Although the supernatant of MSCs, pre-conditioned with dexamethasone, showed similar results, dexamethasone pre-conditioning of co-cultured MSCs increased mononuclear cell proliferation. The results further our understanding of immune-related effects of MSCs which may provide a basis for further *in vivo* studies to achieve better clinical results. We propose that pre-conditioning with cytokines might be an effective method to boost the immunomodulatory effects of MSCs.

Key words: immunomodulation, mesenchymal stem cells, IFN-γ, dexamethasone

Mesenchymal stem cells (MSCs) are multipotent cells that can be differentiated into several lineages including osteoblasts, chondrocytes, *etc*. There are various tissues including bone marrow, adipose tissue, and dental pulp, from which MSCs can be harvested. These cells are often identified by the expression of certain surface molecules such as CD105, CD90, and CD44. Although MSCs are traditionally known for their capacity to differentiate and use in regenerative medicine, their use is certainly not limited to this field. MSCs possess notable immunomodulatory properties that affect both innate and adaptive immunity. MSCs may therefore be potentially used as a method of treatment for diseases with chronic inflammation and autoimmune diseases [1-5]. Type 1 diabetes mellitus, rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease are some of the diseases for which MSC therapies are being developed. However, for MSCs to be translated into the clinic, they must first be cultured *in vitro* [6]. The effects of MSCs on the immune system can be either stimulatory or inhibitory; the result often depends on features of the cell culture microenvironment [7]. The immunomodulatory effects of MSCs largely depend on cytokines such as TNF-α, IL-1, and IFN-γ which are secreted from immune cells [8]. MSCs exert their effects on immune cells through the secretion of several immune mediators, including transforming growth factor (TGF)-β and prostaglandin E2 (PGE-2) [9].

MSCs and their supernatant are known to shift T-cells, B-cells, natural killer cells, and monocytes/macrophages towards anti-inflammatory phenotypes. Also, these cells are capable of inhibiting immune cell proliferation [2, 10-14]. One method of improving MSC immunoregulatory characteristics is to prime them with cytokines. IFN-γ is one of the cytokines which has previously been used to culture MSCs. Culturing with IFN-γ results in the upregulation of principal immunomodulatory agents, including PGE-2 and TGF- β [15]. Several MSC lines previously cultured with IFN-γ indicate that IFN-γ priming enhances MSC immunomodulatory effects both *in vitro* and *in vivo* [16]. However, immune-related effects from the supernatant of IFN-γ-treated MSCs have not been studied to the same extent.

IFN-γ is not the only cytokine that influences MSC immune-related effects. Since the immunoregulatory effects of MSCs highly depend on inflammatory cytokines present in the cell environment, their usage

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in patients who take immunosuppressive drugs is an area of concern. These drugs alter the inflammatory conditions in the body and can therefore affect MSC immunomodulatory effects when utilized in the clinic [17]. Corticosteroids are one of the major classes of immunosuppressive drugs which are used for many autoimmune diseases [18]. Dexamethasone (Dex) is a widely used and potent corticosteroid, which exerts its effects through alteration of gene expression. Dex is known to shift MSCs towards osteogenic lineages [19]. Also, Dex can regulate MSC effects of the immune system [20]. However, little is known about the effect of Dex on the immunoinhibitory function of MSCs.

In the current study, we obtained adipose tissue-derived MSCs (AD-MSCs) from C57BL/6 mice and seeded them with different dosages of IFN-γ or Dex. AD-MSCs or their corresponding supernatants were then used to culture mouse spleen mononuclear cells. Our results indicate that culturing mononuclear cells with MSCs or their supernatant, seeded with IFN-γ, particularly at high doses, significantly reduced the proliferation rate of mononuclear cells. Interestingly, although similar results were shown for the supernatant of MSCs pre-treated with Dex, MSC co-culture with Dex pre-treatment led to increased mononuclear cell proliferation.

MATERIALS AND METHODS

Isolation, cell count and viability analysis of MSCs

AD-MSCs were isolated from C57BL/6 mice as described previously [21]. Briefly, two six-week-old male C57BL/6 mice were purchased from Pasteur Institute, Tehran. The mice were killed by cervical dislocation and placed on a sterile surface. Abdominal fat tissue was harvested and washed 2-3 times with sterile phosphate-buffered saline (PBS) solution. The adipose tissue was cut into 1-2 mm^3 pieces and incubated with 0.1% type I collagenase solution for 30 minutes at 37°C. Condition medium, 5 cc, containing 10% foetal bovine serum (FBS) was added to stop the enzyme activity. The specimens were centrifuged at 1,500g for 15 minutes. After homogenizing in 6 mL Dulbecco's Modified Eagle Medium (DMEM), the cellular sediment was added to three flasks containing 3 mL of DMEM+10% FBS, and the supernatant was discarded. Cells were incubated in 37°C/5% CO_2 for 14 days and the medium was changed every other day. Cells were frozen and passaged three times using 0.25% trypsin-EDTA (Bioidea). After trypsinization, cells were centrifuged at 1,000g for five minutes. The sediment was added to 1 mL DMEM and mixed until homogenized. Cell count was performed using a light microscope and the following formula: $N = n * R * 10^4 * V$ (N=total number of cells, n=cells counted in 1 mm^3, R=dilution coefficient, V=volume of the cell containing medium [μL]). To assess the viability of cells, the trypan blue assay was performed.

Analysis and differentiation of MSCs

Analysis of AD-MSCs was performed by flowcytometry. FITC anti-CD44 antibodies and PerCP/Cy5.5 anti-CD105 antibodies were used as positive markers, and PE anti-CD34 antibodies and FITC anti-CD45 antibodies were used as negative markers. After incubation at 4°C for 45 minutes, the cells were centrifuged twice and washed with PBS. The supernatant was discarded and the cells were fixed using 4% paraformaldehyde suspension. Flowcytometry was performed using BD bioscience flow cytometers and the results were analysed using Flowjo software.

In order to assess the differentiation potential of stem cells, an osteogenic assay was performed using a previously described protocol [22]. Briefly, cells were cultured in osteogenic medium containing high-glucose DMEM, 10% FBS, 10 μM Dex, 10 mM b-Glycerol phosphate, and 50 μg ascorbic acid. Approximately, 1,000-10,000 cells were cultured per cm^2 and cells were incubated at 37°C for 21 days. Then, cells were washed with PBS, fixed with 4% paraformaldehyde and stained with Alizarin Red (AR) stain to assess tissue mineralization.

Isolation of mouse spleen mononuclear cells

After sterilization of the surgical site with 70% ethanol, the BALB/c mouse was placed in supine position. A V-shape cut was performed in the abdomen of the mouse and the spleen was excised and placed in a petri-dish. Isolation of mononuclear cells was performed as previously described [23]. Briefly, 10cc RPMI 1640 media was injected in the spleen and the ejected contents were transferred to a falcon tube. The tube was centrifuged for 5 minutes at 3,200 g. Lysis buffer, 4cc, was added for five minutes to lyse red blood cells and then 5cc of condition medium was added. The specimen was centrifuged again and the sediment was homogenized. Finally, spleen mononuclear cells were labelled with CSFE (Biolegend), using the manufacturer's protocol. Briefly, 10^7 cells were mixed with 1 mL PBS, and 5 μM CFSE solution was added to the mixture. After incubation at 37°C for 15 minutes, cells were washed with 5 mL RPMI medium containing 10% FBS. To ensure proper labelling, cells were compared with unlabelled cells using flowcytometry at Day 0.

AD-MSC culture with Dex or IFN-γ

In total, 10^4 MSCs were transferred into wells of a 96-well plate with different concentrations of Dex (Caspian Tamin) (0, 10, 100, 500 ng/mL) or IFN-γ (Peprotech) (5, 10, 20, 50 ng/mL). These concentrations were selected based on previous studies [24-26]. Since we intended to assess the effects of AD-MSC supernatant on mononuclear cell proliferation, we discarded the solution after 48 hours. We then washed the cells twice with PBS to clear the remaining Dex and IFN-γ in the plates. After 24 hours, cells and their supernatants were collected to be used for culture with mononuclear cells.

Co-culture of AD-MSCs and their supernatants with spleen mononuclear cells

In total, 10^5 CFSE-labelled spleen mononuclear cells were cultured with AD-MSCs or the corresponding supernatants separately in a 96-well plate. In order to stimulate mononuclear cells, phytohemagglutinin (PHA) (Gibco) was added to the plates to 1-2% of the final volume, which was approximately 200 μL. The

contents of each plate were gently mixed and the plates were incubated at 37°C for 72 hours.

Statistical analysis

Statistical analysis for this study was performed using IBM SPSS 20, Prism (GraphPad). ONE Way ANOVA was performed to compare the outcomes of study groups, and statistical significance was defined as $p<0.05$. Results were reported in the form of mean ± SD.

RESULTS

Isolation, culture and passage of AD-MSCs

AD-MSCs were isolated from C57BL/6 mice, as previously explained, and passaged three times. After the first passage, fibroblast-like cells and spindle-shaped cells were seen under the microscope. However, cells with other morphologies were also found. After the second and third passage, cell morphology more progressively moved towards that of spindle and fibroblast-like types and miscellaneous shapes were less commonly seen. *Figure 1* demonstrates cell morphology, as witnessed under the microscope with each passage.

Analysis of AD-MSC surface molecules to determine cell identity

To determine MSC identity, CD34, CD44, CD45, and CD105 surface molecules of AD-MSCs were examined by flowcytometry. Unlike CD34 and CD45 which were rarely observed on AD-MSCs (0.5% and 1.1%, respectively), CD44 and CD105 were abundantly expressed on AD-MSCs (99.7% and 24.5%, respectively). Flowcytometry data were analysed by Flowjo and the results may be visualized in *figure 2*.

Osteogenic differentiation of AD-MSCs confirms stemness characteristics

To further confirm MSC cell line identity and differentiation capacity, cells were cultured in an osteogenic medium for 21 days. After the osteogenic assay, cells were stained with Alizarin Red (AR) stain to assess calcium deposition in the extracellular matrix. MSCs which were cultured in the osteogenic medium exhibited strong AR staining, as presented in *figure 3*. On the other hand, the control group did not stain with AR. These results indicate that the isolated AD-MSC cell line has the appropriate potential to differentiate and further confirms the stemness of the isolated cells.

Immunomodulatory effects of AD-MSCs cultured with Dex or IFN-γ

In total, 10^5 spleen mononuclear cells were cultured with different doses of IFN-γ or Dex-treated AD-MSCs for three days. The mononuclear cells were pre-stained with CFSE and flowcytometry was performed to assess cell proliferation (*figure 4*). In the positive control group, CFSE-stained cells were treated with PHA to activate the cells. The negative control group was not treated with PHA. As seen in *figure 5*, adding PHA resulted in a significant increase in proliferation rate ($p<0.001$). Addition of untreated MSCs to PHA-treated splenocytes slightly increased the rate of splenocyte proliferation, however, this was not statistically significant. Treatment with 20 and 50 ng/mL IFN-γ resulted in a significant reduction of proliferation when compared with control (PHA+untreated MSC) ($p=0.02$ and $p<0.001$, respectively). Interestingly, culturing splenocytes with low-dose Dex-treated MSCs resulted in upregulation of proliferation; 10 and 100 ng/mL of Dex increased cell proliferation significantly ($p=0.009$ and $p=0.02$ respectively). On the other hand, higher doses of Dex (500 and 1,000 ng/mL) decreased proliferation, although this down-regulation was not statistically significant.

Immunomodulatory effects of MSC supernatant on mononuclear cell proliferation

In total, 10^5 spleen mononuclear cells were cultured with supernatants of AD-MSCs treated with different doses of IFN-γ or Dex for three days. Mononuclear cells were stained with CFSE to examine the proliferation rate by

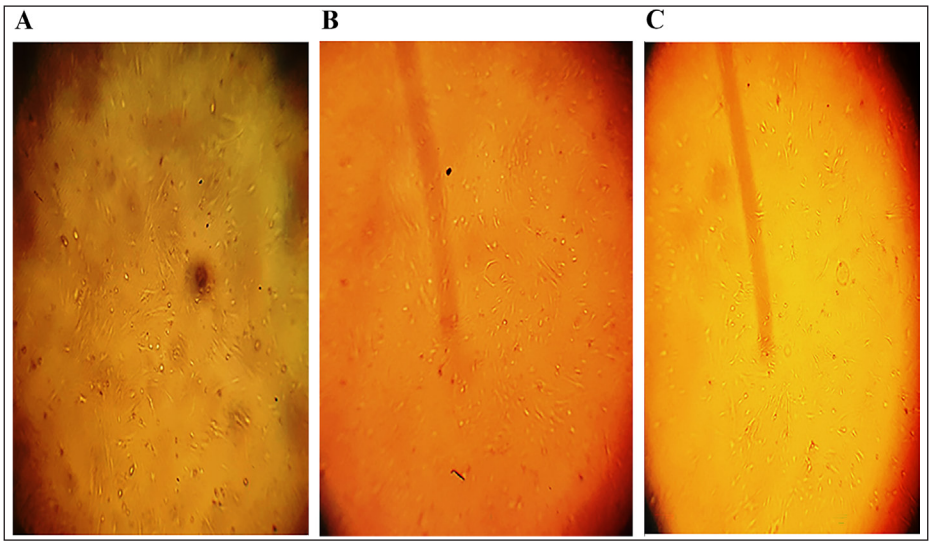

Figure 1
Morphology of AD-MSCs after Passages 1 to 3. **A)** Passage 1. **B)** Passage 2. **C)** Passage 3.

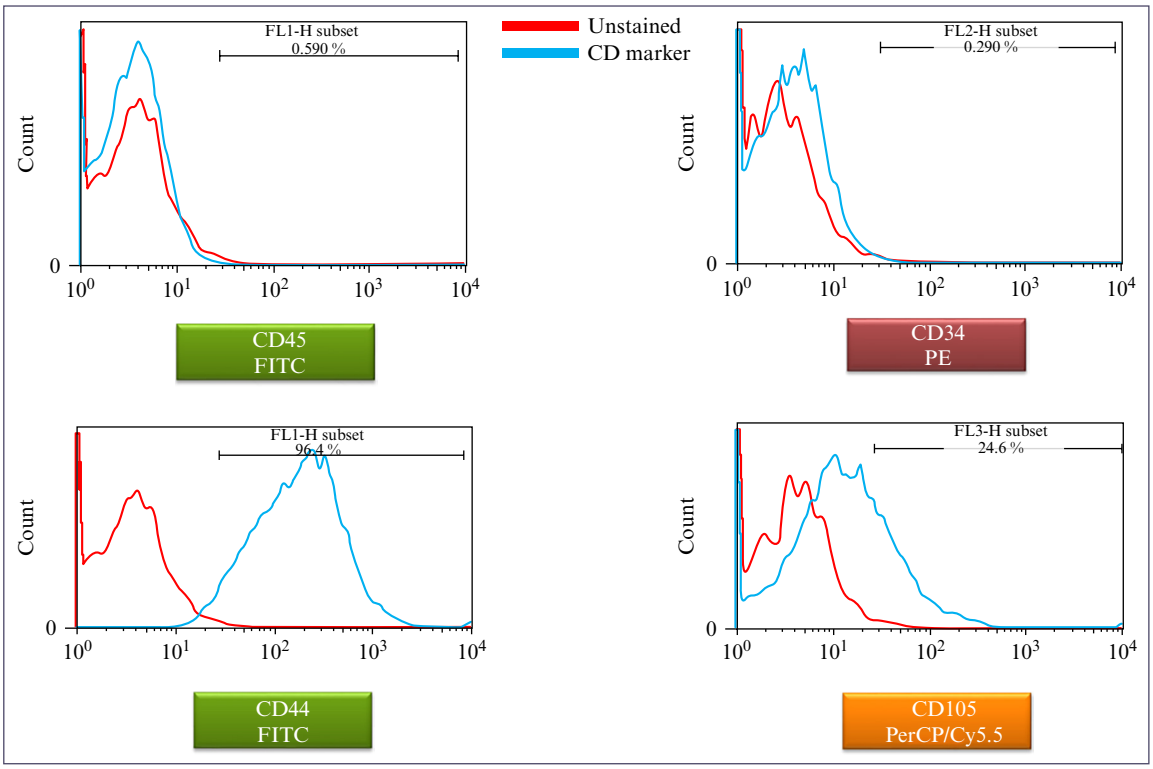

Figure 2
Immunophenotyping of AD-MSCs. Cells were examined for expression of CD34, CD44, CD45, and CD105 surface molecules. CD44 and CD105 were highly expressed on cells, whereas CD34 and CD 34 exhibited low expression levels.

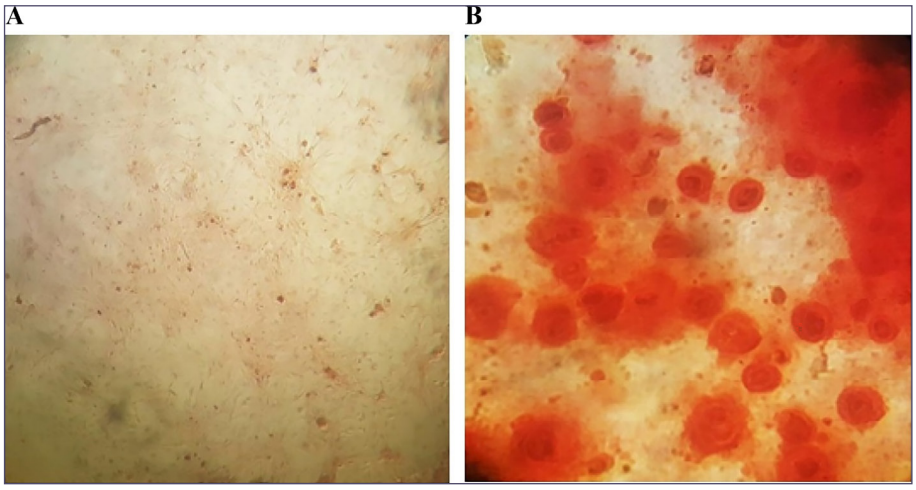

Figure 3
AR staining was performed to visualize tissue mineralization in order to assess osteogenesis. Unlike the control group **(A)**, cells cultured in the osteogenic medium **(B)** showed significant tissue mineralization.

flowcytometry. By comparing the mononuclear cell proliferation rate between the PHA group and PHA+untreated MSC supernatant group, the untreated MSC supernatant was shown to significantly increase mononuclear cell proliferation ($p<0.001$) (*figure 6*). The supernatant of IFN-γ-treated MSCs decreased mononuclear cell proliferation rate at all concentrations compared with the PHA+untreated MSC supernatant group. However, this decrease was only statistically significant with 5 and 50 ng/mL of IFN-γ ($p=0.038$ and $p=0.009$, respectively). All cell lines cultured with Dex-treated MSC supernatant exhibited lower proliferation levels compared with PHA+untreated MSC supernatant. This down-regulation was only significant with 500 ng/mL Dex ($p=0.004$).

DISCUSSION

One of the treatment methods for autoimmune diseases and sepsis, currently being studied, is to use MSCs. MSCs possess a wide range of immunomodulatory properties which affect several immune cell types. AD-MSCs are often harvested due to ease of access and the fact that an adequate number of cells may be obtained compared with other types [2]. MSCs are often characterized by their surface molecules. Several surface

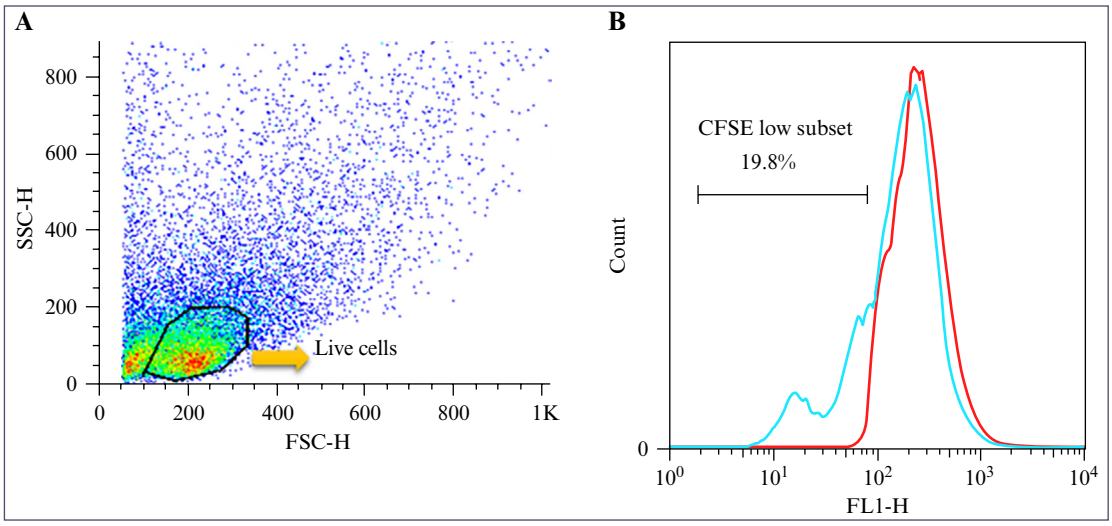

Figure 4
Flowcytometry analysis of the proliferation rate of splenocytes co-cultured with IFN-γ or Dex. **A**) Number of living cells based on forward scatter (FSC) and side scatter (SSC). **B**) Percentage of proliferation of PBMCs co-cultured with MSCs preconditioned with 10 ng/mL Dex compared to untreated cells.

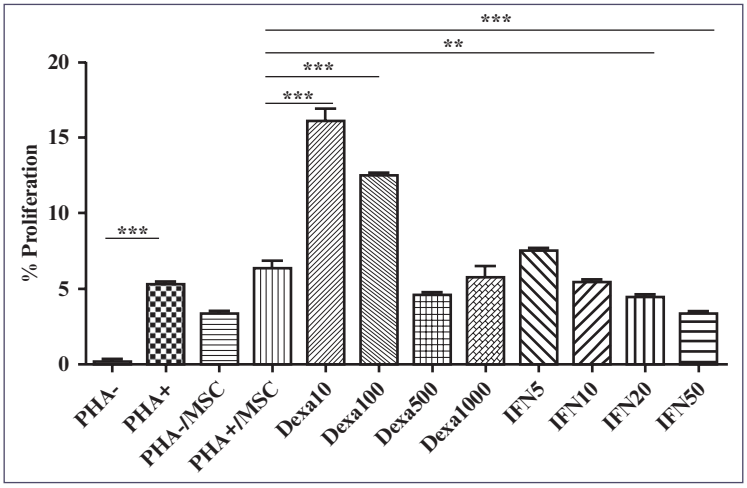

Figure 5
Analysis of the proliferation rate of CFSE-stained spleen mononuclear cells, co-cultured with MSCs, pre-treated with Dex or IFN-γ. After three days, flowcytometry analysis was performed to assess mononuclear cell proliferation. The ONE Way ANOVA test was used to compare the proliferation rate between study groups and the results are presented as mean ± SD. Statistically significant results are shown as *p<0.05*, p<0.01**, p<0.001***.

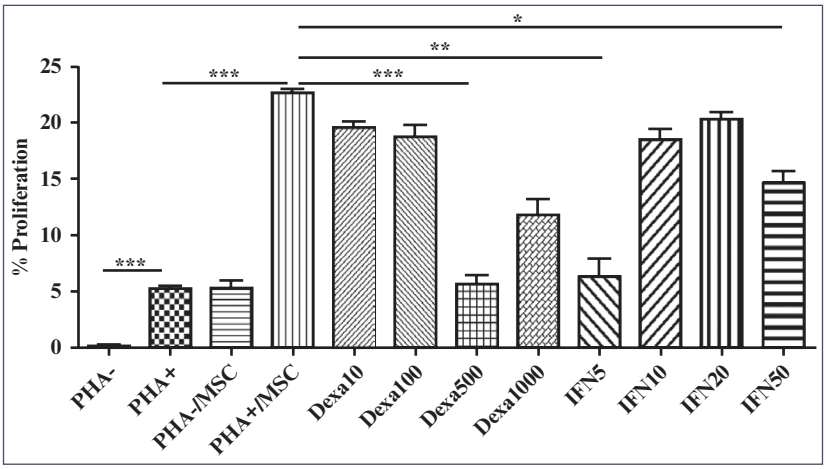

Figure 6
Analysis of the proliferation rate of CFSE-stained spleen mononuclear cells co-cultured with the supernatant of MSCs, pre-treated with Dex or IFN-γ. Flowcytometry was performed after three days of culture. Groups were compared using the ONE Way ANOVA test and results are reported as mean ± SD. Statistical significance is shown as p<0.05*, p<0.01**, p<0.001***.

molecules including CD44, CD90, and CD105 are associated with MSCs. On the other hand, CD34, CD45, and CD79 are rarely expressed on MSCs [27-29]. Based on a flowcytometry assay, we measured the level of a number of these surface molecules. The isolated cells were rich in CD44 and CD105 and poor in CD34 and CD45. In addition, another characteristic of MSCs is their inherent ability to differentiate into several cell lines [30]. We performed an osteogenic assay using a traditional osteogenic medium to examine osteogenic potential of the isolated cells. AR staining of cells after the osteogenic assay revealed a high level of matrix calcium deposition which is an indicator of osteogenesis. These results confirmed the stemness of the isolated AD-MSCs.

Comparison between splenocytes stimulated with PHA with and without MSC co-culture demonstrated that the presence of MSCs did not significantly affect the proliferation of splenocytes. Immunomodulatory effects of MSCs on immune cells can be stimulatory or inhibitory. Some studies show that the inhibitory or stimulating function of MSCs in the *in vitro* environment depends on the ratio between the number of MSCs and splenocytes and the amount of stimulating substance present in the *in vitro* environment, which may stimulate the proliferation of immune cells. The study by Herzig and colleagues, recently published in 2018 [31], shows that the ratio of the number of MSCs to immune cells is important regarding the type of MSC effect (inhibition or stimulation) on immune cells. MSCs are capable of inhibiting the proliferation of PBMCs *in vitro* if they are cultured at a high density (confluent MSCs) with these cells, and conversely, when co-cultured at a low density with PBMCs, the proliferation of PBMCs increases compared to the control group (PBMCs cultured with PHA). In our study, the ratio of the number of MSCs to splenocytes was 1 to 10, probably because MSCs were not confluent and were cultured with splenocytes in small numbers, thus our study is consistent with that of Herzig *et al*. Another variable which influences MSC immunomodulatory outcome is the concentration of the immune cell activator. Renner *et al*. found that, in contrast to low concentrations, high concentrations of concanavalin A and proinflammatory cytokines result in a more notable immunosuppression by MSCs [32]. In our study, we used PHA to induce mononuclear cell proliferation with a concentration of 1-2%, which has previously been reported to contribute to MSC immunosuppression [33]. We co-cultured AD-MSCs, primed with four concentrations of IFN-γ, with mouse spleen mononuclear cells, and immunosuppressive effects were observed with IFN-γ pre-treatment in a dose-dependent manner. Previous studies have reported similar results. In a study by De Witte *et al*. [34], IFN-γ-treated MSCs derived from human umbilical cord were co-cultured with PBMCs. The proliferation of PBMCs was significantly reduced when cultured with IFN-γ-primed MSCs, and the expression of IDO and PD-L1, immunoregulatory agents that block lymphocyte proliferation, was increased. It seems that IDO expression plays a key role in anti-inflammatory effects of MSCs. In a study by Valencic *et al*. [35], human AD-MSCs and Wharton's jelly MSCs were preconditioned with IFN-γ and co-cultured with PBMCs. Treatment with IFN-γ reduced PBMC proliferation and resulted in differential gene expression, affecting the expression of 512 genes, including *IDO*. Also, the JAK/STAT signalling pathway was shown to be responsible for IDO over-expression. Another consistent result from the study of Meisel and colleagues [36] was the finding that the expression rate of IDO inhibitory factor increased in a dose-dependent manner relative to an increasing concentration of IFN-γ (from 10 U/mL to 500 U/mL) in MSCs derived from bone marrow. François *et al*. [37] reported a similar result. In this study, after 24 hours of culture of MSCs with 10 ng/mL of the inflammatory factor, IFN-γ, the inhibitory effect of MSCs on PBMC proliferation increased significantly compared to the untreated group. As this effect is seen with a ratio of 1:3, but not 1:9 or 1:27, of MSC:PBMCs, it seems that the effect of a low ratio of MSCs:immune cells on the inhibitory function of MSCs may be improved by treatment with a high concentration of this inflammatory factor [37]. Other studies emphasize the necessity of other cytokines along with IFN-γ to increase the immunosuppressive activity of MSC [38, 39]. For example, Li and colleagues [38] considered the importance of the presence of other inflammatory cytokines along with IFN-γ and the addition of appropriate concentrations of these cytokines to trigger the immunosuppressive function of MSCs. In this study, the treatment of MSCs with different concentrations of IFN-γ and subsequent additional co-culture with splenocytes did not inhibit the proliferation of splenocytes, but by adding TNF-α together with IFN-γ to MSCs, a decrease in the proliferation of splenocytes was observed, indicating the importance of the presence of appropriate amounts of inflammatory cytokines in MSC culture medium and their synergistic effect to increase the immunosuppressive function of these cells.

Spleen mononuclear cells were also cultured with AD-MSCs preconditioned with four concentrations of Dex. At the concentrations of 10 and 100 ng/mL, Dex seemed to increase mononuclear cell proliferation. One of the most common immunosuppressive drugs is corticosteroid dexamethasone. Since most of the studies conducted so far on the effect of dexamethasone on MSCs have focussed on the proliferation and differentiation of these cells, very few studies have investigated the effect of this drug on the immunosuppressive function of MSCs. In our study, higher concentrations of Dex reduced proliferation, however, this reduction was not statistically significant. Collectively, Dex treatment did not reduce immune cell proliferation, and moreover, increased proliferation at low concentrations. Data from similar studies demonstrates the same results. Chen *et al*. [40] investigated the effects of Dex on MSC immunomodulation. The results from this study indicated that co-culturing anti-CD3 activated splenocytes with MSCs in the presence of a graded amount of Dex, at doses of up to 1 ng/mL, increased cell proliferation in a dose-dependent manner. However, at concentrations of 1-100 ng/mL of Dex, a downward trend in

splenocyte proliferation was observed. All in all, this corticosteroid seems to exhibit inhibitory effects on the immunosuppressive outcome of MSCs. The underlying reason for the small antiproliferative effect observed in the co-culture of MSCs and splenocytes could be due to the fact that, in the absence of inflammatory cytokines, MSCs may have gained a pro-inflammatory phenotype and caused an upregulation of proliferation. Moreover, in MSCs, Dex has been shown to exert its effects partly through the inhibition of mouse iNOS and human IDO transcription factors. In other words, Dex prevents inflammatory cytokine-induced immunosuppression by blocking iNOS or IDO expression which likely takes place via modulation of STAT1 phosphorylation in MSCs. Therefore, it seems that MSCs treated with Dex ought to exhibit greater inhibitory effects compared with the supernatant counterpart. The results from our study are in agreement with the study of Chen *et al*. [40]. Another study by Ankrum and colleagues [20] showed that the treatment of human MSCs with budesonide (a steroid drug similar to Dex) increased the expression of the inhibitory factor, IDO, in these cells. In this study, MSCs were initially cultured with budesonide (1 μM), and then, after 24 hours, IFN-γ was added. After 48 hours, the amount of IDO obtained from MSCs was measured by western blotting. The highest level of IDO expression from MSCs was obtained when budesonide was added along with IFN-γ; such an effect on the increase of IDO expression was not observed when added alone. The authors subsequently demonstrated that budesonide increased IDO expression mediated by glucocorticoid receptors which enhances MSC immunomodulatory function. Since glucocorticoid receptor may bind to other glucocorticoid steroids, repeating this step of the experiment with Dex (1 μM) produced a similar result. Therefore, it seems that the combination of this steroid drug with inflammatory factors can reduce its adverse effect and even increase the immunosuppressive function of MSCs [20].

The most recent study of the effect of Dex on the immunosuppressive function of MSCs, recently reported by Javorkova and colleagues [41], evaluated the effect of Dex on human MSCs. After treating MSCs with a concentration of 0.5 μg/mL of Dex, the expression of IDO did not change. Moreover, no increase in IDO expression was observed with the combination of IFN-γ and Dex, in contrast to the study of Ankrum and colleagues [20]. However, a combination of IFN-γ and Dex inhibited the expression of many other inhibitory factors, such as TSG-6 (tumour necrosis factor-inducible gene 6) and COX-2 enzyme (cyclooxygenase-2, which causes the production of PGE2 inhibitory factor) in MSCs compared to MSCs treated with IFN-γ; Dex alone did not increase the expression of these inhibitory factors compared to the control group (untreated MSCs) [41]. The study of Buron and colleagues [42] indicated that the concentrations of 0.01, 0.1 and 1 ng/mL of Dex in MSC and splenocyte coculture, with a ratio of 1:10 and 1:5, did not change the immunosuppressive performance of MSC, however, at a ratio of 1:20 with an increase in the concentration of Dex, the proliferation of splenocytes decreased and the immunosuppressive function of MSCs strengthened. The difference between the aforementioned studies and our study is the simultaneous presence of MSCs, splenocytes and Dex in this co-culture. Since MSCs were not pre-treated with Dex, we may thus eliminate a direct effect of dexamethasone in the culture medium.

Finally, we tested the effects of Dex or IFN-γ-treated MSC supernatants on spleen mononuclear cells. By comparing the proliferation of splenocytes stimulated with PHA, cultured with or without the supernatant from untreated MSCs, we demonstrate that the proliferation of splenocytes in the presence of MSC supernatant was significantly increased. In a similar study, Augello *et al*. [43] reported that although MSCs induce an inhibitory effect on T cell proliferation, culturing of T cells with MSC supernatants resulted in enhancement of proliferation. The results from microarray analysis indicate that several growth factors secreted by MSCs increase the proliferation of responding cells when cultured with the corresponding supernatants. Therefore, it is possible that the secreted growth factors from MSCs and disruption of direct contact between MSCs and spleen mononuclear cells cause a decrease in MSC inhibitory effects and increase in proliferation of mononuclear cells [44].

However, addition of Dex and IFN-γ resulted in down-regulation of proliferation. This down-regulation was significant at IFN-γ concentrations of 5 and 50 ng/mL and a Dex concentration of 500 ng/mL. Few studies have focused on the effects of activated MSC supernatants on immune cell proliferation. DelaRosa *et al.* [45] reported that supernatants of MSCs treated with IFN-γ, unlike untreated MSCs, significantly reduced human lymphocyte proliferation. Nevertheless, MSC supernatants are generally reported to reduce immune cell proliferation to a lesser extent compared to MSC co-culture [46, 47]. A recent study reported that although MSC co-culture suppresses proliferation to a greater extent relative to MSC supernatant, higher levels of FOXP3+/IL-17+ were found *in vivo* with MSC supernatant [48]. This suggests that MSC supernatant might exert immunosuppressive effects through other mechanisms than mere reduction of cell proliferation. Soluble factors, such as IDO, HGF, TGF-β1, and PGE-2, play important roles in supernatant-mediated effects [49-51]. Most of the studies in which MSC-derived soluble factors have effectively inhibited T cell proliferation have been conducted in transwell systems [46, 48, 52] or similar systems (such as encapsulated MSCs) where MSCs were also present in the culture medium at the same time, but without any direct cell contact with immune cells [53]. On the other hand, for most studies in which direct cell contact was considered necessary for the inhibitory effect of MSCs on T lymphocyte proliferation, the supernatant of these cells was used [44] or MSCs were previously removed from the culture medium following co-culture with T cells. Generally, in these studies, T cell-enriched cell populations have been investigated rather than mixed lymphocyte populations [54-56]. Differences and variations in the types of methods for investigating the effect of MSC soluble factors have clouded our understanding of the main mechanism involved in this type of MSC inhibitory effect [48]. Studies show that in

order to optimise investigation of the inhibitory effect of MSC supernatant relative to MSC co-culture with immune cells, the supernatant should be concentrated [54, 56, 57]. For the direct contact of cells during co-culture, a small dose of MSCs is sufficient to inhibit T cells. However, for the supernatant, a higher equivalent concentration of supernatant is necessary to observe an inhibitory effect [56-58]. Further studies are needed to fully grasp the effects of MSC supernatant on immunomodulation. Elucidating the mechanism governing this phenomenon may lead to development of therapeutic methods in the future.

CONCLUSION

In this study, we demonstrate that naïve MSCs do not possess immunomodulatory effects on spleen mononuclear cells. However, priming MSCs with IFN-γ or Dex can lead to upregulation or down-regulation of immune cell proliferation. Moreover, MSC supernatant exhibited similar results to MSC co-culture, however, collectively, stronger responses were achieved by MSC co-culture relative to supernatant, although supernatant effects could be enhanced by increasing the concentration or isolating/purifying certain cytokines. All in all, our results provide a better understanding of MSC-mediated immunomodulation and pave the way for *in vivo* studies with the goal of reaching the clinic as a novel treatment method for autoimmune diseases.

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