Allogeneic adipose-derived stem cells suppress Th17 lymphocytes in patients with active lupus in vitro

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Interleukin-17 (IL-17)-producing CD4+ T cells (Th17 cells) have been proven to play a critical role in the pathogenesis of systemic lupus erythematosus (SLE). To shed light on the mechanism of immunoregulation of adipose-derived stem cells (ADSCs), we investigated the effects of allogeneic ADSCs on the Th17 lymphocytes of patients with active SLE by co-culturing ADSCs and peripheral blood mononuclear cells of these patients in vitro. The results indicated that ADSCs from passage 3 (P3) down-regulated the proportion of Th17 cells and their abilities to produce IL-17, whereas ADSCs from passage 8 (P8) had contrasting effect. The results also showed cell–cell contact played a role in P3 down-regulation. Blocking the functional pathway of IL-23 (both its ligand and its receptor) also contributed to this suppression. These results suggested that immunomodulation of ADSCs may be achieved by partially suppressing the number and capability of Th17 lymphocytes, indicating that ADSCs could be employed as therapeutic tools for the autoimmune diseases.

Keywords systemic lupus erythematosus; adipose-derived stem cells; Th17; cell therapy; IL-23

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Introduction

The potential of the stem cell to provide viable therapies for countless incurable diseases remains unrealized. Mesenchymal stem cells (MSC) have been used to treat graft versus host disease (GVHD) [1], because these cells exhibit low immunogenicity [2,3] and are capable of immune modulation [4,5] and injury repair [6,7]. Although the mechanisms underlying these activities remain unclear, favorable outcomes have been achieved, suggesting that MSC transplantation is a promising treatment for immune diseases. Th17 lymphocytes are a newly defined subset of CD4+ T cells, named after the signature cytokine that they produce, interleukin-17 (IL-17). By secreting this potent T cell-derived proinflammatory cytokine, Th17 cells have been found to play a major role in the pathogenesis of many autoimmune diseases, such as rheumatoid arthritis (RA), spondyloarthritis [8], and systemic lupus erythematosus (SLE). Down-regulating Th17 cells or neutralizing IL-17 may help to cure these autoimmune diseases [9]. Accumulating evidence suggests that Th17 plays an important role in SLE [10]. In this study, we analyzed the effect of allogeneic adipose-derived stem cells (ADSCs) on Th17 lymphocytes from patients with active SLE and attempted to investigate their mechanisms of action.

Materials and Methods

Adipose tissue and blood samples
Approximately 2 ml of raw subcutaneous adipose tissue was obtained from two patients (an 18-year-old female and a 20-year-old male) who underwent abdominal dermolipectomy at the plastic surgery department of Sun Yat-Sen Memorial hospital. Informed consent was obtained from the donor. All human tissue handling protocols were approved by the Research Ethics Committee of the hospital.

After informed consent was obtained, 10 patients (nine females and one male) with active SLE aged 24 ± 6.38 years old (mean ± SD) with an SLE disease activity index (SLEDAI) score ≥10 were enrolled in the study; the subjects were patients at the dermatology department of Sun Yat-Sen Memorial hospital. Ten milliliters of peripheral blood was obtained from each patient and mixed with the anticoagulant heparin. Peripheral blood mononuclear cells (PBMCs) were purified under sterile conditions within 1 h by density gradient centrifugation (Ficoll-Paque; Amersham Pharmacia, Piscataway, USA).
ADSCs preparation and culture

Human ADSCs were obtained as described previously [11]. The adipose tissue was immersed in sterile phosphate-buffered saline (PBS) supplemented with penicillin and streptomycin at concentrations of 600 IU/ml for 20 min at room temperature. Blood clots, vessels, invalid tissue, connective tissue, and dermas were removed. After being washed three times with PBS supplemented with antibiotics (100 IU/ml), the adipose tissue was homogenized and treated with 0.075% collagenase type I (Sigma–Aldrich, St. Louis, USA) in PBS for 30 min at 37°C with gentle agitation. The collagenase was inactivated with an equal volume of Dulbecco’s modified Eagle’s medium containing high glucose (DMEM-HG, Gibco, Grand Island, USA) and 10% (v/v) fetal bovine serum (FBS, Gibco). The digested tissue was centrifuged at 1000×g for 10 min. The cellular pellet was resuspended in DMEM-HG with 10% (v/v) FBS and filtered through a 100 μM filter to remove the debris. The filtrate was centrifuged at 1000×g for 5 min. Isolated stromal cells were resuspended with DMEM-HG containing 10% (v/v) FBS and seeded at a density of 4×10^6/cm² in 25 cm² culture flasks. The medium was changed every 3 days.

Surface antigens of ADSCs

ADSCs from passage 3 (P3) were harvested by 0.25% trypsin/0.02% EDTA (1:1; Bioind, Israel) digestion and stained with monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). All antibodies were from BD Biosciences (San Jose, USA), including CD13-PE, CD44-FITC, CD105-FITC, CD106-FITC, CD31-PE, CD49d-PE, CD29-PE, and CD34-FITC. Antibodies were from BD Biosciences (San Jose, USA), and stained with monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Isotype controls were used to ensure antibody specificity. At least 15,000 events were analyzed by flow cytometry on a FACScan with CellQuest software.

Confirmation of multilineage differentiation of the ADSCs

For adipogenic induction, the culture-expanded cells from passage 2 (P2) at 2×10⁴ cells/cm² were induced for 2 weeks in DMEM-HG supplemented with 10% (v/v) FBS, 1 μM dexamethasone, 10 μM insulin, 0.5 mM isobutylmethylxanthine, and 200 μM indomethacin (all from Sigma). After that, the cells were fixed in 10% formalin for 10 min and stained with fresh Oil Red-O solution (Sigma). For osteogenic induction, ADSCs from P2 at 2×10⁴ cells/cm² were induced for 3 weeks in DMEM-HG supplemented with 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbate-2-phosphate (Sigma). To reveal osteogenic differentiation, calcium phosphate precipitates were stained with von Kossa.

PBMCs collection and co-culture with ADSCs

After density gradient centrifugation, PBMCs were collected and suspended in DMEM-HG with 10% (v/v) FBS at a concentration of 2×10⁶ cells/ml. The ADSCs from P3 or passage 8 (P8) were collected by 0.25% trypsin/0.02% EDTA digestion, seeded into 24-well plates at a density of 4×10⁴ cells/well, and allowed to adhere for 24 h. After treatment with 25 mg/l mitomycin C (Sigma) for 1 h to arrest growth, the ADSCs were washed with PBS three times, followed by adding 0.2 ml of PBMC suspension (2×10⁶ cells/ml). The final volume was adjusted to 1.2 ml/well with culture medium. In the co-culture group with transwell, 0.2 ml PBMC suspension was added inside the transwell, and 0.8 ml DMEM/10% FBS was added outside. The ratio of PBMCs:ADSCs was 10:1 in all the co-culture groups.

The PBMCs from each SLE patient were divided into three co-culture groups and a control group. The co-culture groups included two groups (Groups I and II) from P3, and one group (Group III) from P8. In Group I, the ADSCs (P3) and the PBMCs were co-cultured directly (cell–cell contact). In Group II, the ADSCs (P3) and the PBMCs were co-cultured using a transwell chamber (Corning, New York, USA). In Group III, the ADSCs from P8 were co-cultured with the PBMCs directly. Double wells were set for each group. The cells were incubated at 37°C/5% CO₂ for 48 h. The viability of the PBMCs was evaluated by trypan blue staining at the beginning and at the end of the culture (>90%).

Th17 assay

The Th17 ratio of each group was analyzed by flow cytometry. In the wells for flow cytometry analysis, PBMCs were stimulated with 25 ng/ml phorbol-12-myristat-13-acetate (PMA, Sigma) and 1 μg/ml ionomycin in the presence of 10 μg/ml brefeldin A (BFA, Sigma) for 6 h before the analysis. Then the cells were incubated with anti-human CD3 conjugated to phycoerythrin-Cy5 (PE-Cy5) and FITC-conjugated CD8 antibodies (BD Biosciences) for 20 min, then fixed and permeabilized in a fixation/permeabilization solution according to the manufacturer’s protocol (eBioscience, San Diego, USA). Intracellular staining with PE-conjugated anti-human IL-17A (eBioscience) was also performed. Isotype controls were used to ensure antibody specificity.

Enzyme-linked immunosorbent assay

In the wells for enzyme-linked immunosorbent assay (ELISA), PBMCs of different groups were stimulated with 10 ng/ml PMA and 0.3 μg/ml ionomycin for 48 h. Then the cell-free culture supernatant was collected and stored at −80°C. The concentrations of IL-17A, IL-23, and IL-1β were measured by ELISA (BD Biosciences) according to...
the manufacturer’s instructions. All the samples were assessed in triplicate.

**Reverse transcription quantitative real-time polymerase chain reaction**

In the wells for quantitative polymerase chain reaction (qPCR), PBMCs of P3 co-culture Group I and the control group were stimulated with 10 ng/ml PMA and 0.3 μg/ml ionomycin for 48 h. Total RNA was extracted with Trizol according to the manufacturer’s instructions (Invitrogen, Carlsbad, USA). The synthesis of cDNA was performed by reverse transcription using an M-MLV first-strand kit (Invitrogen). The expression of the forkhead transcription factor protein 3 (**FOXP3**), IL-23 receptor (**IL-23R**), **IL-6R**, and **IL-1R** in the PBMCs (Group I vs. control group) was measured by qPCR on a LightCycler 480 (Roche, Basel, Switzerland). PCR primers (**Table 1**) were obtained from Takara Bio (Otsu, Japan). The qPCRs was performed with a platinum SYBR Green qPCR SuperMix-UDG mixture (Invitrogen). The PCR cycling conditions involved incubation at 50 °C for 2 min and 95 °C for 2 min, followed by 50 cycles of 15 s at 95 °C, 30 s at 60 °C, 15 s at 95 °C, 15 s at 65 °C, and 1 s at 40 °C. All the PCRs were conducted in triplicate. The results were analyzed with LightCycler 480 software 1.5. Human glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) was selected as the endogenous reference gene. The expression ratio of each gene was analyzed using the \( \Delta \Delta Ct \) method [12].

**Statistical analysis**

Statistical analysis was performed using SPSS version 16.0 for Windows (SPSS Inc., Chicago, USA). The results are expressed as mean ± standard error of mean (SEM). If the differences of two groups were derived from a normal population (Shapiro–Wilk test), a paired-samples \( t \)-test was conducted; otherwise, a Wilcoxon matched-samples rank sum test was carried out. The significance level was set at \( P < 0.05 \).

<table>
<thead>
<tr>
<th><strong>Table 1</strong> Primers used for qPCR</th>
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<td><strong>Genes</strong></td>
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| **FOXP3** | NM_001114377 | Forward: AAAGTGCGCCGGGATGTGAGA  
Reverse: GACATTGTGCCCTGCCCTTCT |
| **IL-23R** | NM_144701 | Forward: ACAGGGCACCTTACTTCTGACAA  
Reverse: AGCAAAGACGATCATTCCCAAT |
| **IL-6R** | NM_000565.2 | Forward: GAGGGCTTCTGCCATTTCTGAG  
Reverse: CCAGGTTCAGCTGACAACAAACA |
| **IL-1R** | NM_000877.2 | Forward: GGCCTAGCTTTCATTTGACACACA  
Reverse: TCAAGACGTGACATCCCTGCTC |

**FOXP3**, forkhead transcription factor protein 3; **IL**, interleukin; **R**, receptor.

**Results**

**ADSCs from primary culture**

The ADSCs adhered to the bottom of the culture flask within 4 h, exhibiting fibroblast-like morphology under a microscope [**Fig. 1(A)**]. When the cultures reached ~80% confluence, a passage was undertaken [**Fig. 1(B)**]. The ADSCs expressed CD29, CD44, CD105, CD13, and CD49d but not CD106, CD31, or CD34 (data not shown).

To confirm the multilineage differentiation, ADSCs were induced into two lineages. On completion of adipogenic induction, a significant fraction of the cells contained multiple, intracellular lipid-filled droplets that were stained by Oil Red-O [**Fig. 2(A)**]. They exhibited an expanded morphology, with the majority of the intracellular volume (90%–98%) occupied by lipid droplets, consistent with the phenotype of mature adipocytes. When induced in the osteogenic medium, the cells changed from spindle- to cuboid-shaped as they differentiated. Calcification was assessed using von Kossa stain, which appeared as black regions within the cell monolayer [**Fig. 2(B)**].

**ADSCs modulated Th17 ratio and the concentration of IL-17**

Two strains of ADSCs were used in this study. Each strain of ADSCs (P3 and P8) was used in co-culture with the PBMCs from five cases of active SLE. Between the two strains of ADSCs, the paired differences between two groups in relation to Th17 ratio and IL-17 concentration were analyzed by the Wilcoxon test. No significant difference was observed (all \( P > 0.05 \), data not shown). The differences between the four groups from all 10 SLE cases were analyzed by a paired-samples \( t \)-test.

Given that the expression of CD4 in human T cells was down-regulated during culture with the stimulants, we focused on CD3\(^+\), CD8\(^-\), and IL-17\(^+\) T cells [10,13]. When compared with the control group (1.96% ± 0.53%), the Th17 ratio of the P3 co-culture groups (0.39% ± 1.13% in Group I, 1.18% ± 0.41% in Group II) was decreased...
I vs. control $= 10.354$, $t_{II}$ vs. control $= 7.385$, $P$, 0.05), whereas the ratio was increased in the P8 co-culture group (Group III, $3.43\% + 1.16\%$, $t_{III}$ vs. control $= 6.983$, $P$, 0.05; Fig. 3(A–D)]. These data suggested that ADSCs from P3 can down-regulate the proportion of Th17 cells, but ADSCs from P8 have a contrasting effect. In relation to the two P3 co-culture groups, the Th17 ratio of Group I was lower than that of Group II ($t_I$ vs. $II = -6.337$, $P$, 0.05; Fig. 4(A)], which implied that cell–cell contact played a role in the P3 down-regulation. Consistent with the Th17 ratios, the concentration of IL-17 in the supernatant of the two P3 co-culture groups (293.14 $+ 49.40$ pg/ml in Group I, 701.19 $+ 65.46$ pg/ml in Group II) was lower ($t_I$ vs. control $= 16.191$, $t_{II}$ vs. control $= 6.997$, $P$, 0.05) compared with the control group (758.73 $+ 68.87$ pg/ml). While the concentration of IL-17 was increased in the P8 co-culture group (Group III, 921.53 $+ 119.66$ pg/ml, $t_{III}$ vs. control $= 6.834$, $P$, 0.05) compared with the control group (290.96 $+ 127.71$ pg/ml). No significant difference was observed between Group II and the control group ($P$. 0.05). These results suggest that ADSCs from P3 up-regulated the production of IL-17 by cell–cell contact.

Expression of FOXP3

To see whether Treg play a role in the Th17 suppression, we analyzed the expression of FOXP3 in PBMCs of P3 co-culture Group I and the control group after stimulation. Compared with the control group, mRNA transcription of FOXP3 in the P3 co-culture group was moderately increased but was not statistically significant ($P > 0.05$; Fig. 7), which implied that the ratio of Treg in PBMCs did not increase after the co-culture with P3 ADSCs.

P3 ADSCs suppressed the production of IL-23

To further study the mechanism of the Th17 suppression by P3 ADSCs, we investigated the production of IL-23 and IL-1β in the supernatant of the control group and the P3 co-culture groups (Figs. 5 and 6). Compared with Group II (111.40 $+ 30.44$ pg/ml) and the control group (117.68 $+ 25.72$ pg/ml), the IL-23 levels in the supernatant of Group I (73.21 $+ 20.49$ pg/ml) decreased ($t_I$ vs. control $= -3.83$, $t_{II}$ vs. control $= -2.77$, $P$, 0.05). The difference between Group II and the control group was not statistically significant ($P > 0.05$). The results suggested that P3 ADSCs reduce the production of IL-23 by cell–cell contact. The concentration of IL-1β in the supernatant of Group I (425.68 $+ 134.19$ pg/ml) was increased ($t_I$ vs. control $= 4.365$, $t_{II}$ vs. control $= 5.307$, $P$, 0.05) compared with that in Group II (306.19 $+ 135.52$ pg/ml) and the control group (290.96 $+ 127.71$ pg/ml). No significant difference was observed between Group II and the control group ($P$. 0.05). These results suggest that ADSCs from P3 up-regulated the production of IL-1β by cell–cell contact.

Adipose-derived stem cells suppress Th17 lymphocytes in vitro

Figure 1 ADSCs obtained by primary cultureADSCs exhibited fibroblast-like morphology ($\times 100$ magnification). (A) ADSCs on the third day of primary culture and (B) ADSCs on the eighth day of primary culture.

Figure 2 Adipogenic and osteogenic differentiation of ADSCs To confirm their ability to differentiate into multiple lineages, P2 ADSCs were induced by two differentiation pathways. (A) ADSCs after 2 weeks adipogenic induction (Oil Red-O staining, $\times 100$ magnification) and (B) ADSCs after 3 weeks of osteogenic induction (von Kossa staining, $\times 100$ magnification).
P3 ADSCs down-regulated IL-23R

The qPCR results showed that the mRNA expression level of IL-23R was significantly decreased in the P3 co-culture Group I compared with that in the control group (P < 0.05). For the expressions of IL-6R and IL-1R, no significant differences were observed (P > 0.05; Fig. 7). The results indicated that the expression of IL-23R in PBMCs was down-regulated by P3 ADSCs after the co-culture in vitro.

Discussion

The autoimmune disease SLE is currently incurable, with affected patients undergoing various therapies that have...
Adipose-derived stem cells suppress Th17 lymphocytes in vitro

Figure 5 Concentration of IL-23 in the supernatant of the Groups I and II, and the control group after stimulation by PMA and ionomycin. The concentration of IL-23 was measured by ELISA. The results are presented as means ± SEM from 10 cases, * P < 0.05.

Figure 6 Concentration of IL-1β in the supernatant of Groups I and II, and the control group after stimulation by PMA and ionomycin. The concentration of IL-1β was measured by ELISA. The results are presented as means ± SEM from 10 cases, * P < 0.05.

Figure 7 mRNA levels of FOXP3, IL-23R, IL-6R, and IL-1R in PBMCs of co-culture Group I and the control group after stimulation by PMA and ionomycin. Total RNA from the PBMCs of co-culture Group I and the control group was extracted with Trizol following 48 h stimulation by PMA and ionomycin. GAPDH was selected as the endogenous reference gene. The expression ratio of each gene was analyzed using the ΔΔCt method. The mRNA levels of FOXP3, IL-23R, IL-6R, and IL-1R in the co-culture group was normalized to the control group. The data are presented as means ± SEM, * P < 0.05.

Severe side effects and result in a low quality of life. The advent of stem cell research has significantly moved forward the development of stem cell therapies for autoimmune diseases, with applications in clinical practice yielding encouraging outcomes. At present, hematopoietic stem cell transplantation is the main stem cell therapy for SLE. However, the relatively high risk of mortality and the high recurrence rate combined with GVHD and the lack of histocompatibility leukocyte antigen compatibility restrict its application. The extensive proliferative potential, multilineage differentiation, injury repair abilities [6,7], immunomodulation, and low immunogenicity [2,3] of adult stromal stem cells show some promise for use in cellular therapy. ADSCs share many characteristics with bone marrow mesenchymal stem cells (BMSCs). The potential of ADSCs in cellular therapy of autoimmune disease [14] has been studied, and ADSC transplantation has been used to assist hematopoietic stem cell transplantation and attenuate or treat GVHD [1,15]. Although the mechanisms by which ADSCs impart their effects remain unclear, favorable outcomes imply their potential usefulness in the treatment of SLE. In this study, ADSCs were obtained by primary culture and appeared as a relatively homogenous population, exhibiting a fibroblast-like morphology. Flow cytometry analysis showed that the ADSCs were positive for CD13, CD44, CD49d, CD105, and CD29 but negative for CD31, CD106, and CD34, which was consistent with previous reports [16]. To confirm the multilineage differentiation capacity of these cells, ADSCs were successfully induced into adipogenic and osteogenic lineages in vitro.

Th17 lymphocytes are a newly defined CD4+ effector T cell subset distinct from Th1 and Th2 [17]. This subset is first identified in experimental autoimmune encephalomyelitis (EAE) [18]. This finding leads to increased understanding of the pathogenesis of many autoimmune diseases. Th17 is named after IL-17, a potent T cell derived pleiotropic inflammatory cytokine. A previous study has shown that Th17 can invade the target organ and promote the development of organ-specific autoimmune inflammation [19]. In addition to playing roles in RA [20], ankylosing spondylitis [8], and systemic scleroderma [21], Th17 also has a critical function in SLE [10,22]. Previous work has demonstrated that both the Th17 ratio and IL-17 concentration are increased in the peripheral blood of patients with active SLE [22]. IL-17 is an upstream mediator, which either directly or indirectly promotes the secretion of chemokines and other immune mediators to recruit inflammatory cells to target organs, such as the joints, kidney, and skin. It is proposed that targeting the Th17/IL-17 immune pathway may be beneficial in SLE therapy. Ko et al. [23] reported that BMSCs can inhibit the differentiation of CD4⁺ T cells into IL-17-secreting T cells in mice. In this study, we attempted to investigate the effect of PBMCs...
from patients with active SLE on the Th17 subset when they were co-cultured with allogeneic ADSCs in vitro. Our results demonstrated that ADSCs from an early passage (P3) down-regulated the Th17 ratio and IL-17 production, whereas those from a later passage (P8) had a contrasting effect. The immunomodulation property of ADSCs may be partly attributable to the suppression of the number and capability of the Th17 lymphocyte subset. ADSCs from a late passage (P8) not only lose the ability to suppress Th17 lymphocytes, but they may also result in the up-regulation of the number and capability of these lymphocytes. After a relatively long-term culture in vitro, ADSCs may have differentiated into stromal cells and lost their immunomodulation ability. Moreover, the differentiated ADSCs could be a source of cytokines (e.g. IL-6, IL-23, and IL-1β) [12,24] that can promote the differentiation of Th17 lymphocytes and the production of IL-17 upon stimulation by PMA and ionomycin. Further study is needed to test this hypothesis. Our results imply that ADSCs at an early passage may have potential utility in SLE treatment. When cultured in the transwell, P3 ADSCs were still able to down-regulate the number and IL-17-producing ability of the Th17 subset, but with slightly less efficiency, indicating that cell–cell contact is involved in the suppression mechanism.

To investigate the mechanism underlying the suppression of Th17 by allogeneic ADSCs (P3), we analyzed the production of cytokines and the mRNA transcription of the receptors. In the supernatant of the P3 co-culture group (Group I), the concentration of IL-23 was reduced, whereas IL-1β levels increased compared with the control group. Cell–cell contact was vital in these effects. Given that IL-23 is critical for the differentiation and functioning of the Th17 subset, P3 ADSCs may be able to suppress the Th17 subset in SLE patients by lowering the production of this cytokine. Although it has been reported [12] that IL-1β is vital in human Th17 induction, the elevation of IL-1β in the co-culture group implies that Th17 suppression is not dependent on the blocking of IL-1β expression. One possibility is that the P3 ADSCs are the extra source of IL-1β upon stimulation by PMA and ionomycin. Previous reports have shown that there is a balance between Treg and Th17, with Treg regulating the ratio and function of Th17 [10,25]. By analyzing the mRNA level of FOXP3 in PBMCs, we found that P3 ADSCs did not dramatically increase the number of Treg (P > 0.05) after ex vivo co-culture. Therefore, Th17 suppression may not be Treg dependent. In relation to the receptors that are important for Th17 lymphocytes, except for IL-23R, no statistical difference was observed in mRNA transcription between the P3 co-culture group (Group I) and the control group. The mRNA transcription level of IL-23R was down-regulated in Group I, but the mRNA levels corresponding to IL-6R and IL-1R did not change significantly (P > 0.05) under the co-culture. Combining the ELISA and qPCR results, the suppression of Th17 by ADSCs was achieved by blocking the functional pathway of the IL-23 ligand and its receptor. In EAE and collagen-induced arthritis animal models, in which Th17 plays a crucial role in pathogenesis, the disease development is blocked in mice deficient in IL-23 [26]. The present study suggests that allogeneic ADSCs of early passage have the potential to be employed in SLE therapy through regulating the pathogenic Th17 lymphocyte subset. Cell–cell contact and blockage of the IL-23 pathway may contribute to the effectiveness of the procedure.

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Adipose-derived stem cells suppress Th17 lymphocytes in vitro