

Interferon- γ regulates the function of mesenchymal stem cells from oral lichen planus via indoleamine 2,3-dioxygenase activity

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BACKGROUND: Little is known about mesenchymal stem cells (MSCs) in normal or inflammatory oral mucosal tissues, such as in oral lichen planus (OLP). Our objectives were to identify, isolate, and characterize MSCs from normal human oral mucosa and OLP lesions, and to evaluate indoleamine 2,3 dioxygenase (IDO) activity in mediating immunomodulation of MSCs from these tissues.

METHODS: Expressions of MSCs-related markers were examined in isolated cells by flow cytometry. Self-renewal and multilineage differentiations were studied to characterize these MSCs. Interferon- γ (IFN- γ), IDO, and STRO-1 were assessed by immunofluorescence. MSCs from oral mucosa and OLP or IFN- γ -pretreated MSCs were co-cultured with allogeneic mixed lymphocyte reaction assays (MLR). Proliferation and apoptosis of MLR or MSCs were detected by CCK8 and the annexin V-FITC apoptosis detection kit, respectively. IDO expression and activity were measured by real-time PCR, Western blotting, and high-performance liquid chromatography.

RESULTS: Isolated cells from oral mucosa and OLP expressed MSC-related markers STRO-1, CD105, and CD90 but were absent for hematopoietic stem cell markers CD34. Besides, they all showed self-renewal and multilineage differentiation capacities. MSCs in OLP presented STRO-1/IDO+ phenotype by immunofluorescence. MSCs and IFN- γ -pretreated MSCs could inhibit lymphocyte proliferation via IDO activity, but not via cell

apoptosis. Long-term IFN- γ could also inhibit MSC proliferation via IDO activity.

CONCLUSIONS: Mesenchymal stem cells can be isolated from human oral mucosa and OLP tissues. Besides self-renewal and multilineage differentiation properties, these cells may participate in immunomodulation mediated by IFN- γ via IDO activity in human OLP.

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Introduction

Oral lichen planus (OLP) is a common chronic inflammatory condition with a variety of clinical presentations, including reticular, papular, plaque-like, atrophic, and ulcerative lesions (1). The main pathological features include band-like infiltration of lymphocytes into the subepithelial lamina propria, which is a structure of mesenchymal origin. The mechanism underlying the genesis of OLP remains unclear. Based on previous reports, the pathological characteristics of OLP are closely related to T cell-dominated infiltration, where these cells are stimulated to secrete pro-inflammatory cytokines, such as interferon- γ (IFN- γ) (1, 2). Local production of IFN- γ may maintain and contribute to the genesis and chronicity of this inflammatory disease (3–5).

A variety of progenitor/stem cells have been isolated from adult human organs (6–8), including dental and periodontal tissues (8, 9), and from inflamed tissues such as found in irreversible pulpitis and chronic periodontitis (10, 11). These mesenchymal stem cells (MSCs) possess the ability to differentiate into three germ layers, and they also demonstrate the potential for transdifferentiation (6, 12). To date, studies on human oral mucosal MSCs have been limited to normal masticatory or lining mucosa (13–17) and

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it is not known whether MSCs are present in OLP-affected mucosal tissues. MSCs in chronic inflammatory lesions such as OLP may provide important insights into their potential roles in immunomodulation.

It has been reported that MSCs can selectively migrate to corresponding parts of injured tissues and regulate T lymphocyte formation or inhibit the proliferation of allergenic T lymphocytes (18–20). This immunosuppressive mechanism has been investigated and found to act via indoleamine 2,3-dioxygenase (IDO) activity in MSCs from bone marrow, the placenta, or gingiva (19, 21, 22). IDO is often recognized as a therapeutic factor causing T cell inhibition (23). In previous research, IFN- γ -induced IDO activity was linked to antiproliferative effects on T cells that lead to the induction of immune tolerance (24, 25). However, it is not clear whether MSCs can migrate to the sites of infiltration of T lymphocytes in OLP and take part in regulating inflammation in oral mucosal tissue via IFN- γ -induced IDO activity.

Therefore, we aimed to isolate and characterize MSCs from OLP tissues, and investigate whether these cells have self-renewal capability and the capacity for multilineage differentiation. We further investigated the molecular interactions within its cellular mechanism(s) underlying the immunosuppressive function of these MSCs by focusing on IFN- γ -associated IDO expression or/and activity. Using flow cytometry, real-time PCR, immunofluorescence, mixed lymphocyte reaction assays, and Western blotting we found that MSCs are present in OLP-affected tissues and possess self-renewal and multilineage differentiation properties. MSCs treated with short time IFN- γ (48 h) showed inhibitive effects on T lymphocyte proliferation in an IDO-dependent manner, and thus, MSCs might participate in immunomodulation in OLP via IDO activity.

Materials and methods

Tissue sample collection

Oral lichen planus tissues were biopsied from the buccal mucosa of eight patients for definitive diagnosis after comprehensive inspections. Clinical and pathological diagnoses were made according to modified World Health Organization diagnostic criteria for OLP (26). A summary of individual patient information is shown in Table 1. Two pieces of normal oral mucosal tissues, 8 mm³ each in size, were obtained from buccal mucosa of patients who

Table 1 Summary of individual information

Control no.	Gender	Age	Patient no.	Gender	Age
Control 1	Female	40	OLP 1	Female	40
Control 2	Female	46	OLP 2	Female	55
Control 3	Female	45	OLP 3	Female	48
Control 4	Female	45	OLP 4	Female	46
Control 5	Male	43	OLP 5	Male	45
Control 6	Male	68	OLP 6	Male	61
Control 7	Male	30	OLP 7	Male	33
Control 8	Male	30	OLP 8	Male	30
Mean \pm SD		43.38 \pm 11.88			44.75 \pm 10.42

Ages in years were recorded at the time of biopsy. Diagnoses were made with clinical and histopathologic criteria.

underwent surgical removal of mucous retention cysts. Of which, one piece was fixed by 10% formalin and underwent hematoxylin–eosin staining, while the other was used for cell extraction. The study was approved by the Biomedical Institutional Review Board of the authors' institution (IRB00001052-12007), and every participant gave written informed consent.

Cell isolation and culture

The collected tissues were treated aseptically and incubated with dispase (2 mg/ml; Sigma–Aldrich, St. Louis, MO, USA) in sterile phosphate-buffered saline (PBS) to separate the epithelial and subepithelial lamina propria, which is made up of the mesenchymal structure. The mesenchymal tissues were minced into 1-mm³ fragments and digested with 3 mg/ml collagenase type I and 4 mg/ml dispase (1 h). The cell suspensions were filtered (Falcon, BD Lab-ware, Franklin Lakes, NJ, USA) and cultured in α -modified Eagle's minimum essential medium (α -MEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). The cells were used in subsequent experiments at passages 2–4. Bone marrow MSCs were selected as positive control cells (27).

Flow cytometry

Single-cell suspensions of MSCs from bone marrow, oral mucosa, and OLP ($>1 \times 10^6$ cells) were incubated with a STRO-1 antibody (R&D Systems, Minneapolis, MN, USA) or an isotype-matched immunoglobulin control IgM (eBioscience, San Diego, CA, USA). The cells were then treated with phycoerythrin (PE)-conjugated secondary antibody (R&D Systems). For direct immunostaining, cells were treated with 20 μ l of PE-conjugated or FITC-conjugated human CD90, CD105, and CD34 (BD Biosciences, San Jose, CA, USA), or isotype-matched control IgGs (Southern Biotechnology Associates). All groups were analyzed with a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Colony-forming unit fibroblast assay

To assess the colony-forming efficiency, single-cell suspensions were seeded in 10-cm Petri dishes at 1×10^3 cells/dish. These were cultured for 14 days and then fixed with 4% paraformaldehyde for subsequent staining with 1 g/l toluidine blue (Sigma, St. Louis, MO, USA). A colony-forming fibroblast unit was defined as a group of at least 50 cells. Colony-forming efficiency (%) = colony number/the number of cells in the initial seed (i.e., 1000) \times 100% (28).

Cell counting assay

For the cell proliferation assay, MSCs from bone marrow, oral mucosa, and OLP were seeded into 96-well tissue culture plates at a density of 1000 cells/well. The proliferation of cells was assessed using cell counting kit-8 (CCK8; Dojindo Laboratory, Kumamoto, Japan) according to the manufacturer's instructions. Optical density at 450 nm was measured using an absorbance microplate reader (ELx808; BioTek, Winooski, VT, USA), and the cell proliferation rates were calculated. In addition, CCK8 assays were also used for assessing proliferations of allogeneic T lymphocytes in

mixed lymphocyte reactions (MLR) assays by MSCs (29) and for assessing the long-term effects of IFN- γ on the proliferation of MSCs.

Multilineage differentiation

Mesenchymal stem cells from bone marrow, oral mucosa, and OLP were plated at 5×10^5 cells/well in 6-well plates for assessment of osteogenic and adipogenic differentiations. The osteogenic induction medium was α -MEM supplemented with 10 nM dexamethasone, 10 mM β -glycerophosphate, 0.1 mM L-ascorbic acid-2-phosphate, and 2 mM glutamine. At 28 days, alizarin red S (pH 4.2; Sigma) staining was performed to assess osteogenic mineralization (30). The adipogenic induction medium was α -MEM supplemented with 1 μ M dexamethasone, 0.5 mM 3-isobutylmethylxanthine, 10 mg/ml insulin, 60 mM indomethacin, and 2 mM glutamine. Samples were fixed with 4% paraformaldehyde after induction. At 21 days, oil red O (Sigma) staining was performed to assess the presence of adipogenic lipid droplets (31). For neurogenic differentiation, MSCs (5×10^4 cells) were plated in 24-well plates and induced with 100 μ M CoCl₂ (Sigma) for at least 3 days (32). Samples were fixed, and the expression of neuron-specific enolase (NSE) was detected by immunofluorescence. Negative controls were cultured by α -MEM containing 10% FBS without induction. RNA was harvested for real-time reverse-transcription polymerase chain reactions (real-time PCR) to determine expression levels of various genes after induction.

Immunofluorescence studies

For cellular immunofluorescence studies, rabbit antihuman vimentin or cytokeratin (1:200; Bioworld Technology, St. Louis Park, MN, USA; a mesenchymal or epithelial cell surface marker, respectively) and rabbit antihuman NSE (a neuronal cell marker) were incubated overnight at 4°C. After washing with PBS, samples were followed by FITC-conjugated goat anti-rabbit secondary antibodies.

For histological immunofluorescence studies, the collected tissues were embedded in paraffin. Then, 4 mm sections were prepared and incubated overnight at 4°C with primary antibodies: rabbit antihuman IFN- γ (1:200; Bioworld Technology), mouse antihuman STRO-1 (1:20; R&D Systems), and rabbit antihuman IDO (1:200; Proteintech lab, Chicago, IL, USA). Oral carcinoma tissues were selected as positive control groups. Negative control groups were incubated with PBS instead of primary antibodies at the same time. After rinsing with PBS, samples were incubated for 30 min at 37°C in the presence of goat anti-mouse or goat anti-rabbit secondary antibodies that were conjugated to FITC (1:500; Bioworld Technology), rhodamine (1:200; Santa Cruz Bio-

technology, Dallas, TX, USA) or mouse IgM secondary antibody (1:20; R&D Systems). At least three fields were randomly captured in each experiment for quantification. The percentage of positive staining was measured quantitatively with the Image J software.

Induction of IDO by IFN- γ pretreatment

To confirm MSCs from oral mucosa and OLP could generate activated IDO, the cells were treated with different concentrations of IFN- γ (0, 0.01, 1, 10, or 100 ng/ml; Proteintech) in 6-cm² plates for 48 h. Then, the cells were collected for the detection of IDO by real-time PCR and Western blot, and transferred into a 96-well plate for co-culture with allogeneic T lymphocytes in MLR assays. Supernatants were collected for tryptophan and kynurenine detection by high-performance liquid chromatography (HPLC) to determine IDO activity.

Reverse-transcription PCR and real-time PCR analysis

Total RNA was extracted with TRIzol[®] reagent (Invitrogen Life Technologies, Grand Island, NY, USA), and samples were quantified by spectrophotometry. Reverse-transcription reactions were performed using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Lithuania). Then, cDNA was used as template for each PCR using IQ SYBR Green. The primer sets used are listed in Table 2. To quantify relative gene expression, a relative quantitative analysis method was performed by comparison with the expression level of the housekeeping gene *glyceraldehyde phosphate dehydrogenase (GAPDH)*. PCR was performed using the 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA).

Western blot analysis

Cell protein samples were quantified by a BCA Kit and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Samples were incubated with the primary monoclonal antibodies including mouse anti-IDO (1:100; Proteintech) and rabbit antitubulin- β (1:1000; Sigma) overnight at 4°C. Then, secondary antibodies (1:20 000) conjugated to horseradish peroxidase were applied for 1 h at room temperature. Proteins were visualized and photographed with the ECL Detection and Analysis System and the Image J software.

HPLC analysis of IDO activity

High-performance liquid chromatography assays were performed to detect IDO activity in supernatants of MSCs pretreated with IFN- γ or MLR/MS co-cultures. First, supernatants were precipitated with 5% trichloroacetic acid

Table 2 Specific primers used for gene expression analysis

Gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
OCN	CACTCCTCGCCCTATTGGC	CCCTCCTGCTTGGACACAAAG
PPAR γ 2	GGGATCAGCTCCGTGGATCT	TGCACTTTGGTACTCTTGAAGTT
NSE	AGCTCTACGGGCATCTATGA	TTCTCAGTCCCATCCAACCTCC
IDO	GCCAGCTTCGAGAAAGAGTTG	ATCCCAGAAGTAGACGTGCAA
GAPDH	CGACAGTCAGCCGCATCTT	CCAATACGACCAAATCCGTTG

and centrifuged at 3000 *g* for 5 min. Then, the supernatants were used for HPLC analysis. Tryptophan and kynurenine were measured using UV absorption values at 278 and 363 nm, respectively. The samples were passed through a Waters Bondapak C18 column in a mobile phase (pH 5) consisting 80 mM sodium acetate/80 mM citric acid in 5% acetonitrile at a flow rate of 1 ml/min (33).

Effect of MSCs on MLR via IDO activity

To assess the potential role of MSCs in immunomodulation in OLP, their effects on proliferation of allogeneic T lymphocytes were assessed using a mixed lymphocyte reactions (MLR) assay (22, 28, 29). Human peripheral blood mononuclear cells (PBMC) were obtained and extracted via Ficoll gradient separation from eight healthy donors after informed consent. To obtain MLR, 1×10^5 irradiated allogeneic human PBMC (irradiated with 3000 Gray) from donor A were co-cultured with 1×10^5 responder PBMC from donor B for 72 h. Untreated MSCs from oral mucosa and OLP or MSCs pretreated with 100 ng/ml IFN- γ were seeded at various concentrations into wells of 96-well plates for 24 h before MLR were added. MSC/MLR co-cultures were set at ratios of 1:10, 1:50, and 1:100 MSCs to responder cells for 4 days. The supernatants were collected for assessment of IDO activity by HPLC or for assessing proliferation rates by CCK8. All experiments were performed at least thrice.

To further prove that MSCs inhibited allogeneic T lymphocytes proliferation in the MLR assay through IDO activity, the IDO competitive inhibitor 1-methyl-tryptophan (1-MT; Sigma) at 800 μ M or 100 ng/ml tryptophan (Sigma) was added before the MLR co-cultures were prepared (22).

Assessment of apoptosis

Untreated MSCs from oral mucosa and OLP or MSCs pretreated with 100 ng/ml IFN- γ was co-cultured with MLR at ratios of 1:10, 1:50, and 1:100 MSCs to responder cells for 4 days. The apoptotic effect of MSCs on MLR was assessed by the annexin V-FITC apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions. All groups were harvested and washed with PBS, and further incubated with 5 μ l annexin V-FITC for 3 min and then 10 μ l propidium iodide (PI) for 10 min at room temperature. Unstained and single-stained samples were used as controls. After resuspended with 300 μ l binding buff, samples were analyzed using a FACS Calibur flow cytometer.

Long-term IFN- γ effect on MSCs

To validate whether long-term IFN- γ exposure could influence the proliferation of MSCs, 100 ng/ml IFN- γ was added to MSCs for 12 days. Then, 1-MT or tryptophan inhibition studies were performed (as above) to determine the effects of these compounds on MSC proliferation via IDO activity. The apoptosis of MSCs treated with 100 ng/ml IFN- γ was also examined.

Statistical analysis

We performed three parallel experiments for each assay to test the reproducibility of the assays and the agreements among different study subjects. For each assay, data were

expressed as mean \pm SD from at least three independent experiments. Data analyses were performed using SPSS v.13.0 software. Differences among three or more groups were analyzed using one- or two-way analysis of variance (ANOVA) and the *post hoc* Bonferroni tests for pairwise comparisons. For comparison between two groups, independent two-tailed Student's *t*-tests were performed. $P < 0.05$ was considered to be statistically significant.

Results

OLP-affected tissues contain cells expressing MSC markers

Definitive diagnoses of OLP were made using widely accepted clinical and histopathologic criteria (26), and the most common type of OLP found exhibited a reticular pattern (Fig. 1A). A typical infiltration band-like zone was found in OLP patients (Fig. 1C), while no such change was observed in normal oral mucosa (Fig. 1B) when examined by hematoxylin-eosin staining. There was expression of vimentin, the mesenchymal cell surface marker (Fig. 1D,E), and no expression of cytokeratin, the epithelium cell surface marker (Fig. 1F,G), in cells from oral mucosa and OLP, thus demonstrating that the extracted stem cells originated from the mesenchymal tissue.

Mesenchymal stem cell-related markers were detected in isolated MSCs by flow cytometric analyses. Comparable to bone marrow MSCs, MSCs from oral mucosa and OLP showed characteristic MSC surface markers, including STRO-1, CD105, and CD90; however, the hematopoietic stem cell marker CD34 was not expressed (Fig. 1H). In addition, immunofluorescence assays demonstrated that STRO-1 was detected in both oral mucosa and OLP tissues (Fig. 3A–D). These results indicate that MSCs are found in the lamina propria of oral mucosa and these cells show features characteristic of MSCs *in vitro*.

MSCs from OLP possess self-renewal and multilineage differentiation capacity in vitro

In the colony-forming unit fibroblast assay, MSCs from oral mucosa and OLP showed similar colony growth capacity to bone marrow MSCs. The frequency of colony-forming cells formed by MSCs from oral mucosa was 72.83 ± 4.31 colonies/ 10^3 cells, which was greater than observed for MSCs from OLP (39.33 ± 3.67 colonies/ 10^3 cells) and bone marrow MSCs (52.33 ± 5.92 colonies/ 10^3 cells; $P < 0.01$; Fig. 2A,B). In the cell proliferation assay, the proliferation rate of MSCs from oral mucosa was greater than that of MSCs from bone marrow and OLP (Fig. 2C).

In the assessment of multilineage differentiation capacity, MSCs from oral mucosa and OLP maintained osteogenic, adipogenic, and neurogenic abilities in a similar way to bone marrow MSCs. Under osteogenic induction conditions, MSCs from oral mucosa and OLP formed mineralized nodules similar to that in bone marrow MSCs (Fig. 2D). There was no difference in mRNA expression of the mature osteoblastic gene osteocalcin (*OCN*) among these cell types when assessed by real-time PCR (Fig. 2G; $P > 0.05$). Under adipogenic induction, MSCs from oral mucosa and OLP also formed oil droplets (Fig. 2E). Furthermore, the expression of the adipogenic master gene *peroxisome*

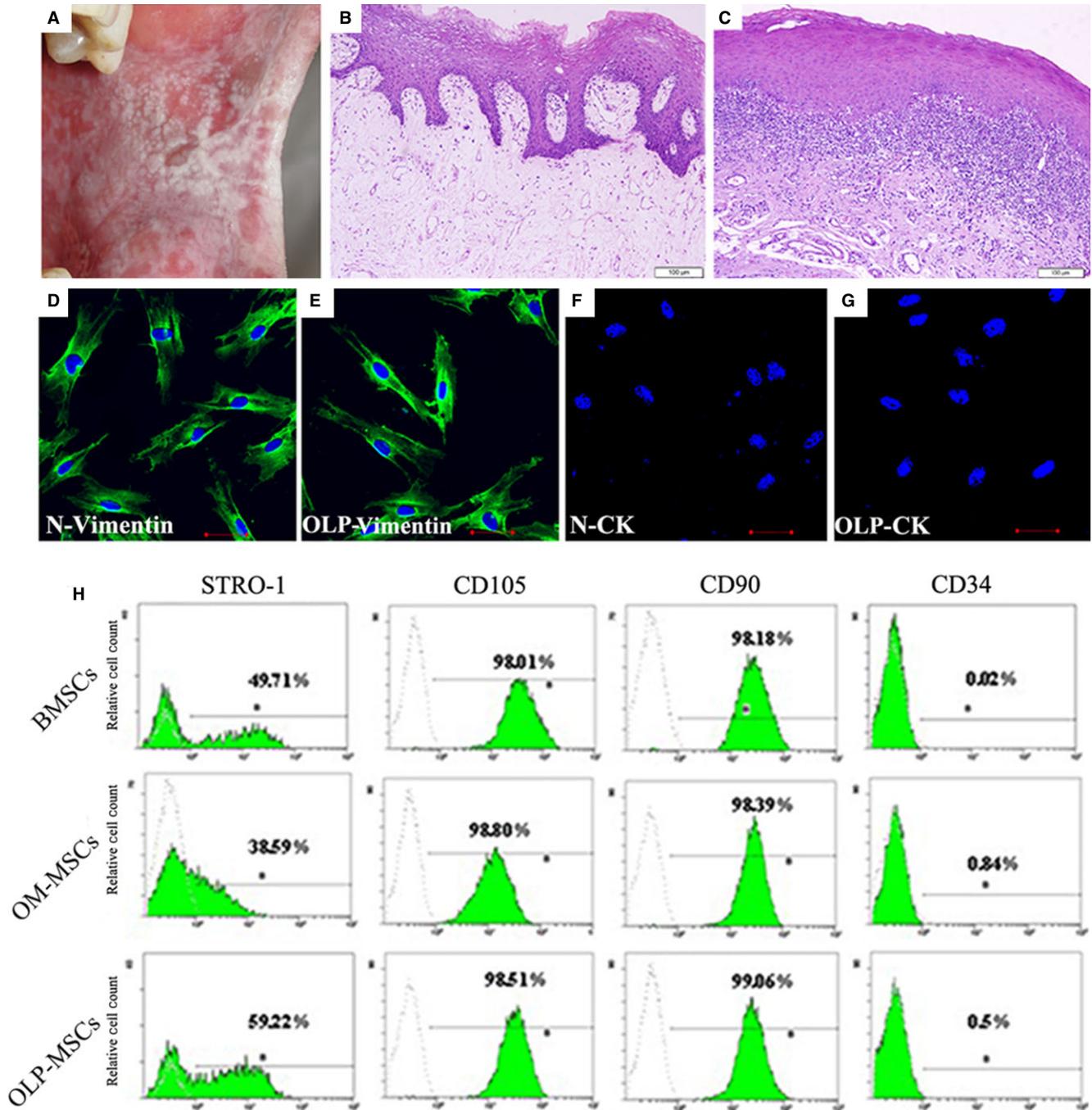


Figure 1 Clinical manifestation and histopathologic features of oral lichen planus, and identification of mesenchymal stem cells (MSCs). (A): Clinical manifestation of oral lichen planus. (B, C): Histopathologic features of normal oral mucosa (B) and oral lichen planus (C) by H&E staining. (D, E): Vimentin expression of cells from oral mucosa (D) and oral lichen planus (E) by immunofluorescent staining. (F, G): CK expression of cells from oral mucosa by immunofluorescent staining. (H): Flow cytometric analysis of the expression of cell surface markers related to MSCs (STRO1, CD90, and CD105) or hematopoietic stem cells (CD34) in all groups. Scale bar = 100 μ m in B and C; scale bar = 50 μ m in D–G. BMSCs, MSCs from bone marrow; OM-MSCs, MSCs from oral mucosa; OLP-MSCs, MSCs from oral lichen planus; H&E, hematoxylin and eosin.

proliferator-activated receptors (PPAR γ 2) mRNA was greater in MSCs from OLP than that in normal oral mucosa ($P < 0.01$; Fig. 2H). After neurogenic differentiation, the morphology of the MSCs presented neural changes (Fig. 2F). There was no difference in the expression of the NSE protein and its mRNA among the different cell types when examined by immunofluorescence assay and

real-time PCR, respectively (Fig. 2I,J; $P > 0.05$). There were no mineralized nodules, oil droplets, or neural changes in the negative control groups (Fig. 2D–F).

MSCs can generate IDO in OLP

STRO-1, IFN- γ , and IDO proteins were selected for conjunct staining in immunofluorescence assays. We found

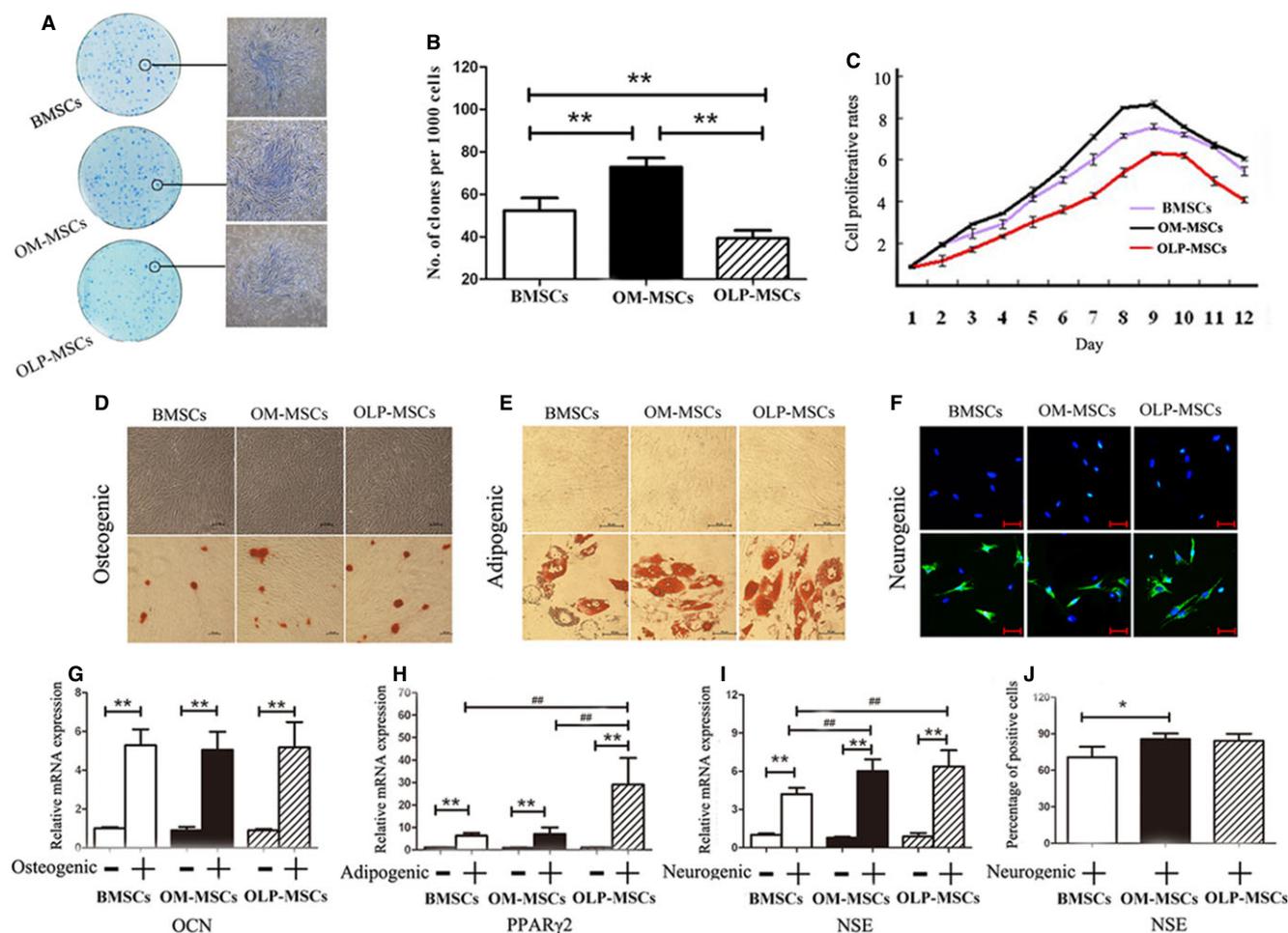


Figure 2 Characteristics of BMSCs, OM-MSCs, and OLP-MSCs. (A) Colony-forming units of BMSCs, OM-MSCs, and OLP-MSCs. (B) Statistical analysis of colony-forming efficiencies of BMSCs, OM-MSCs, and OLP-MSCs ($n = 6$; $*P < 0.05$, $**P < 0.01$). (C) Comparison of cell proliferation capacities. Proliferating capacity of OLP-MSCs was down-regulated. (D) Osteogenic differentiation potential of BMSCs, OM-MSCs, and OLP-MSCs. Mineralized nodules in induced groups as assessed by alizarin red staining. No mineralized nodules were formed in control groups. (E) Adipogenic differentiation potential of BMSCs, OM-MSCs, and OLP-MSCs. Oil red O staining of accumulated lipid droplets in induced cells. No lipid droplets were formed in control groups. (F) Neurogenic differentiation potential of BMSCs, OM-MSCs, and OLP-MSCs. The morphology of MSC neural changes immunofluorescently stained with neuron-specific enolase (NSE). No such changes were found in control groups. (G) Analysis of *OCN* mRNA expression during osteogenic differentiation as assessed by real-time PCR ($n = 8$; $**P < 0.01$). (H): Analysis of *PPARγ2* mRNA expression during adipogenic differentiation as assessed by real-time PCR ($n = 8$; $*P < 0.05$, $**P < 0.01$; $###P < 0.01$). (I): Analysis of *NSE* mRNA expression during neurogenic differentiation as assessed by real-time PCR ($n = 8$; $*P < 0.05$, $**P < 0.01$; $##P < 0.05$, $###P < 0.01$). (J): Semi-quantification of NSE positive cells after immunofluorescent staining ($n = 5$; $*P < 0.05$, $**P < 0.01$). Statistical analysis was performed by one-way ANOVA in B and J and by two-way ANOVA in G, H, and I. The results were representative of at least six independent experiments, and three parallel experiments were performed. Scale bar = 100 μm in D and F. Scale bar = 50 μm in E.

that STRO-1 expression in MSCs of OLP was greater than that in normal oral mucosa (Fig. 3A–E; $P < 0.01$). Meanwhile, IFN- γ and IDO were highly expressed in the lamina propria of OLP, but were almost completely absent in the normal oral mucosa (Fig. 3A–D,F,G; $P < 0.01$). There were no STRO-1, IFN- γ , and IDO expressions in the negative control groups. STRO-1 $^+$ cells did not express IFN- γ , while STRO-1 $^+$ /IDO $^+$ cells co-localized in OLP (Fig. 3D).

MSCs from OLP induce functionally active IDO after IFN- γ treatment

During exposure to IFN- γ , the induction of IDO by MSCs peaked at 100 ng/ml IFN- γ treatment for 48 h in MSCs from oral mucosa when assessed by real-time PCR and Western blot. In contrast, the level of IDO induction was the

highest when MSCs from OLP were stimulated with 10 ng/ml IFN- γ . As there were no statistical differences in IDO expression of MSCs from OLP stimulated by 10 ng/ml and 100 ng/ml IFN- γ (Fig. 4A,B), 100 ng/ml IFN- γ was selected for the subsequent experiments. IDO activity is assayed using HPLC by quantifying tryptophan catabolism and the generation of kynurenine (34, 35). Hence, we also determined IDO activity by quantifying conversion of tryptophan to kynurenine in the supernatants of MSCs after exposure to 100 ng/ml IFN- γ . Conversion of tryptophan to kynurenine by MSCs from oral mucosa was greater than that by MSCs from OLP, meaning that the IDO activity generated by MSCs from normal oral mucosa was stronger (Fig. 4C; $P < 0.05$). These results demonstrate that MSCs from oral mucosa and OLP could generate functionally

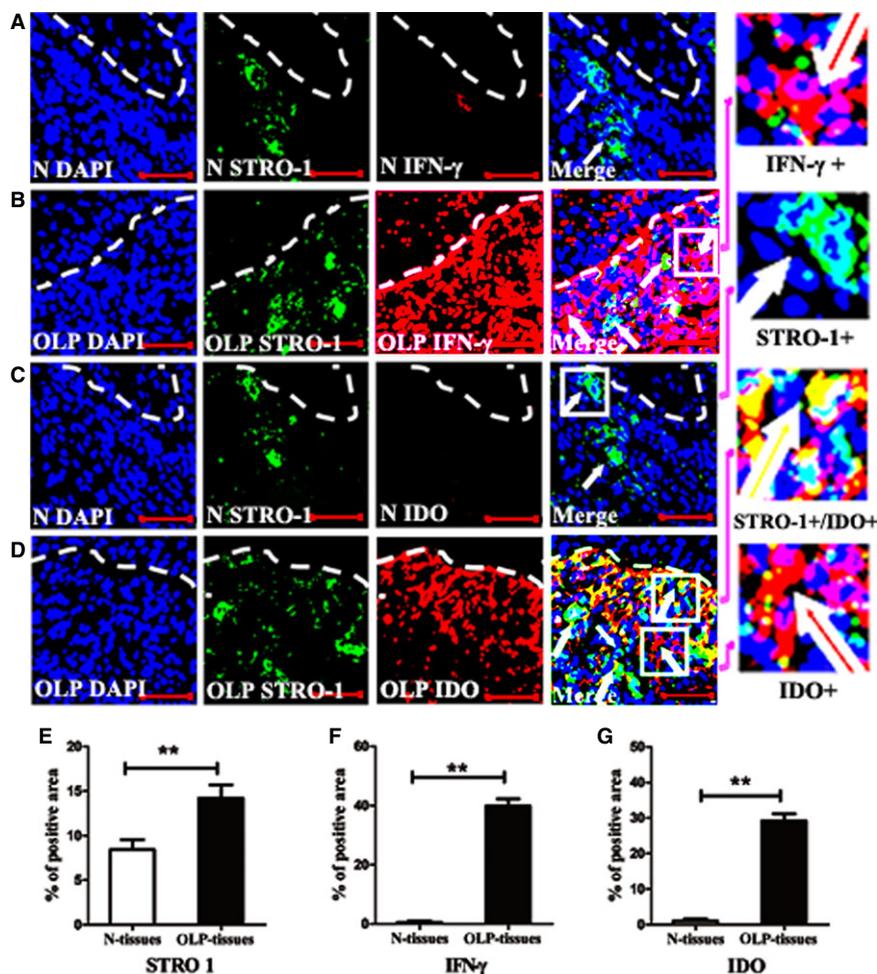


Figure 3 Expression of STRO-1, IFN- γ , and IDO in human normal and oral lichen planus tissues. (A, B): Macroscopic view of the cells against STRO-1 (green) and IFN- γ (red) in human normal oral mucosa (A) and oral lichen planus tissues (B) by immunofluorescent staining. (C, D): Macroscopic view of the cells against STRO-1 (green) and IDO (red) in human normal oral mucosa (C), and oral lichen planus tissues (D). STRO-1 and IFN- γ positive cells are shown by white and red arrows, respectively. Some MSCs in oral lichen planus lesions expressed IDO. STRO-1 and IDO dual-positive cells (yellow) are highlighted by yellow arrows. Nuclei were stained with DAPI (shown in blue). (E, F, G): Quantification of STRO-1 (E), IFN- γ (F), and IDO (G) by comparison with percentage of positive cells in normal oral mucosa and oral lichen planus tissues by independent two-tailed Student's *t*-tests ($n \geq 5$; $**P < 0.01$). The results were representative of at least five independent experiments, and three parallel experiments were performed. Scale bar = 50 μ m. IFN- γ , interferon- γ . IDO, indoleamine 2,3-dioxygenase.

active IDO in response to proportionate concentrations of IFN- γ .

MSCs pretreated with IFN- γ suppress allogeneic T cell proliferation via IDO activity

We found that MSCs from oral mucosa and OLP significantly suppressed allogeneic T cell proliferation and the most effective MSC/MLR co-cultures ratio was 1:50 after 4 days. This function was greater in MSCs from oral mucosa compared with MSCs from OLP ($P < 0.01$). MSCs pretreated with 100 ng/ml IFN- γ resulted in a significant suppressive effect as compared with untreated MSCs ($P < 0.01$; Fig. 5A).

Indoleamine 2,3 dioxygenase (IDO) activity, with kynurenine augmentation and tryptophan depletion, was increased in the supernatants from MSC/MLR co-cultures (ratio of 1:50). The IDO activity generated by MSCs from oral mucosa was greater than that by MSCs from OLP ($P < 0.01$). Moreover, this activity could be reinforced by

IFN- γ pretreatment of MSCs (Fig. 5B). By counteracting the tryptophan-depleting effects of IDO or blocking the enzymatic activity of IDO, tryptophan replenishment or addition of the specific inhibitor 1-MT was able to significantly attenuate this inhibition (Fig. 5C). These data provide further support that the inhibition of T lymphocytes by MSCs acts via IDO activity.

MSCs pretreated with IFN- γ do not inhibit allogeneic T cell proliferation by cell apoptosis

Untreated MSCs from oral mucosa and OLP or MSCs pretreated with 100 ng/ml IFN- γ did not inhibit T cell proliferation by inducing cell apoptosis. The apoptosis cell ratio was $<6\%$ (Fig. 6A,B). No significant difference was seen in the cell numbers of MLR among all treatments ($P > 0.05$), confirming that MSCs from oral mucosa and OLP or MSCs pretreated with IFN- γ do not induce T cell immunosuppression through induction of cell apoptosis.

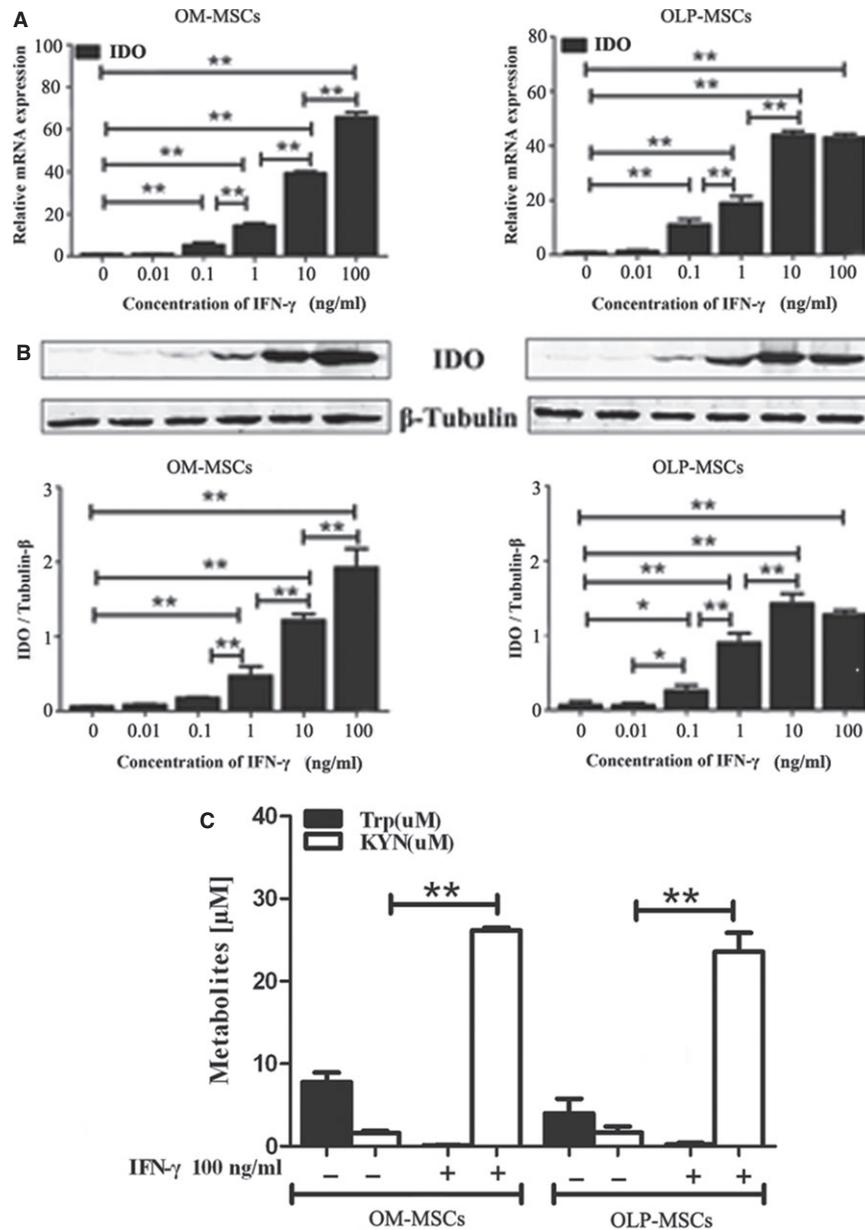


Figure 4 Incubation of OM-MSCs and OLP-MSCs with various concentrations of Interferon- γ (IFN- γ ; 0, 0.01, 0.1, 1, 10, and 100 ng/ml). (A) Analysis of IDO mRNA expression at 48 h in OM-MSCs and OLP-MSCs stimulated with IFN- γ as assessed by real-time PCR. (B) Expression and analysis of indoleamine 2,3 dioxygenase (IDO) protein at 48 h in OM-MSCs and OLP-MSCs by Western blot after stimulation with IFN- γ . (C) Concentration of kynurenine and tryptophan in culture supernatants determined by HPLC following treatment with 100 ng/ml IFN- γ . IDO activity was determined by assessing the conversion of tryptophan to kynurenine in the supernatants. Statistical analysis was performed by one-way ANOVA in A and B and by two-way ANOVA in C ($n \geq 3$; * $P < 0.05$, ** $P < 0.01$).

IFN- γ influences the proliferation of MSCs

After treatment with 100 ng/ml IFN- γ for 12 days, MSCs from oral mucosa grew rapidly after 5 days, but proliferation was inhibited significantly at 7 days (Fig. 7A). MSCs from OLP did not demonstrate accelerated proliferation during the first 5 days, but inhibition of MSC proliferation at 7 days was similar to that from oral mucosa (Fig. 7B). Inhibition of MSC proliferation at 7 days caused by IFN- γ was partly prevented by addition of tryptophan or 1-MT (Fig. 7C,D), which suggests that the antiproliferative effects of IFN- γ on these MSCs are at least partly linked to IDO activity. To determine whether the effect of 100 ng/ml

IFN- γ on MSC proliferation was related to cell apoptosis at 7 days, cell apoptosis of MSCs were also examined. The cell apoptosis ratio of MSCs from oral mucosa and OLP was <3% (Fig. 8A,B). No significant difference was seen in the numbers of MSCs ($P > 0.05$).

Discussion

MSCs can be isolated from OLP-affected tissues

In this study, stem cells from OLP were isolated and characterized. These cell populations presented several unique stem cell-like properties similar to those observed

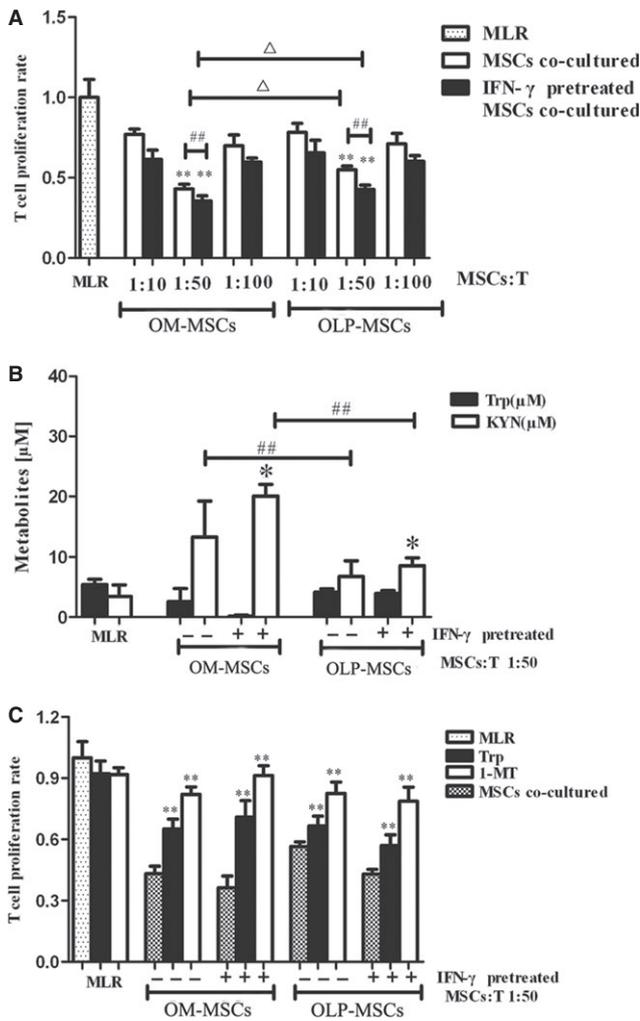


Figure 5 Effect of MSCs and IFN- γ -pretreated MSCs on proliferation of T lymphocytes in MLR. (A) Analysis of T cell proliferation rate after pretreating MSCs with 100 ng/ml IFN- γ when incubated with MLR at different ratios. OM-MSCs and OLP-MSCs suppressed the rate of allogeneic T cell proliferation, and the most effective ratio was 1:50. (B) Concentrations of kynurenine and tryptophan in culture supernatants from MSCs/MLR co-cultures (1:50) as assessed by HPLC. (C) Inhibition of IDO activity in MSCs restored MLR proliferation. Control MLR values were obtained in the absence of MSCs. Addition of tryptophan or 1-MT ameliorated the MSC immunosuppressive effect on MLR. Statistical analysis was performed by two-way ANOVA with Bonferroni's *post hoc* test ($n \geq 3$, * $P < 0.05$, ** $P < 0.01$; ### $P < 0.01$; $\Delta P < 0.05$). MLR, mixed lymphocyte reactions; KYN, kynurenine; Trp, tryptophan; and 1-MT, 1-methyl-tryptophan.

for bone marrow MSCs (6). First, MSCs commonly share similar expression of stem/progenitor cell surface molecules, such as STRO-1 (a putative stem cell surface marker used during the isolation of MSCs (36–39), SH2 (CD105), SH4 (CD73), CD90, CD146, and CD29 (6, 40–42); however, they typically lack hematopoietic stem cell markers, such as CD34 and CD45 (43, 44). The heterogeneity of STRO-1+/CD90+/CD105+ and CD34– MSCs from normal oral mucosa and OLP were also represented in the stem cell-enriched populations *in vitro*, in accordance with bone marrow MSCs. The presence of progenitors was also observed within the mesenchymal tissues of both

normal oral mucosa and OLP after staining for STRO-1, which offers definitive proof for the existence of stem cells *in situ*. In addition, these MSCs were identified as colony-forming unit fibroblast-like cells (45, 46). Further, we confirmed that the colony-forming ability and proliferation capacity of MSCs from normal oral mucosa was significantly greater than that observed for bone marrow MSCs. This higher colony-forming ability and proliferation capacity were also found in gingival MSCs compared with MSCs from bone marrow (BMSCs) (28). These findings suggest that MSCs found in buccal lining mucosa possess potent regenerative potentials similar to those found in attached gingiva (17). In a previous report, the healing of wounds in oral mucosa was found to be faster than observed for other tissues (47), which may be closely associated with the strong renewal ability of MSCs in oral mucosa. Third, MSCs from normal oral mucosa can differentiate into multiple cell lineages (6, 12), and the MSCs derived from OLP also showed osteogenic, adipogenic, and neurogenic capacities in a similar way to bone marrow MSCs. Therefore, our findings support that oral mucosa is an easily accessible source for MSCs for tissue regeneration and other cell therapies. MSCs in oral mucosa could possess a remarkable capacity to maintain local homeostasis and contribute to the rapid repair of oral mucosal tissues.

MSCs increase in number in OLP

Oral lichen planus is a long-term chronic inflammatory condition that involves the infiltration of abundant T lymphocytes (1). The number of MSCs around the infiltration of the T lymphocytes was increased in OLP when assessed by STRO-1 staining. This may be related to the tropic properties of MSCs that allow them to 'home' into inflammatory areas where they become activated (28, 48, 49). In addition, we found that the adipogenic capacity of MSCs from OLP was greater than that of oral mucosa MSCs. The tissues obtained adjacent to the buccal fat pad are rich in fat tissue, and some of the MSCs perhaps originated from adipose stem cells. When induced *in vitro*, the MSCs showed inherent adipogenic characteristics. Moreover, we found that the inflammatory cytokine IFN- γ promoted the proliferation of MSCs from oral mucosa for 5 days, which may be similar to the nonage genesis stage of OLP when the number of MSCs is stimulated to increase. Thus, the increase in number of MSCs in OLP lesions may partly be from the migration of circulating progenitor cells and partly be from existing progenitor cells in tissues adjacent to the lesions. MSCs from different tissue origins are likely to play different roles in modulating the disease process. Further studies are needed to clarify the activities of MSCs *in situ* in the OLP lesions as compared with those MSCs from circulating progenitor cells.

MSCs can generate IDO in OLP

The infiltration of T lymphocytes in OLP typically suggests that the progressive tissue destruction is due to local immune aggression (50). Thus, the immunosuppressive effects on T lymphocytes might be helpful for containing this inflammatory disease. IDO has been linked to the antiproliferative effects on T cells, and this can then lead to the induction of immune tolerance (24, 25). IDO expression

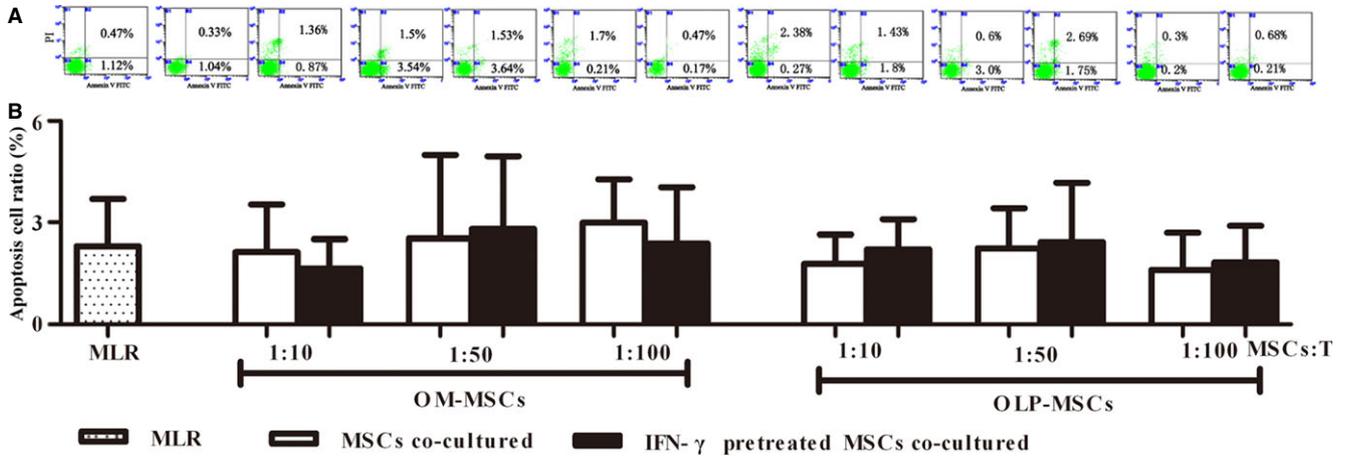


Figure 6 Effect of mesenchymal stem cells (MSCs) and Interferon- γ (IFN- γ)-pretreated MSCs on apoptosis of T lymphocytes in mixed lymphocyte reactions (MLR). (A): Analysis of T cell apoptosis ratio after pretreating MSCs with 100 ng/ml IFN- γ when incubated with MLR at different ratios using an annexin V-FITC apoptosis detection kit (B):OM-MSCs and OLP-MSCs do not immunosuppress through the induction of T cell apoptosis by two-way ANOVA ($n \geq 3$, $P > 0.05$).

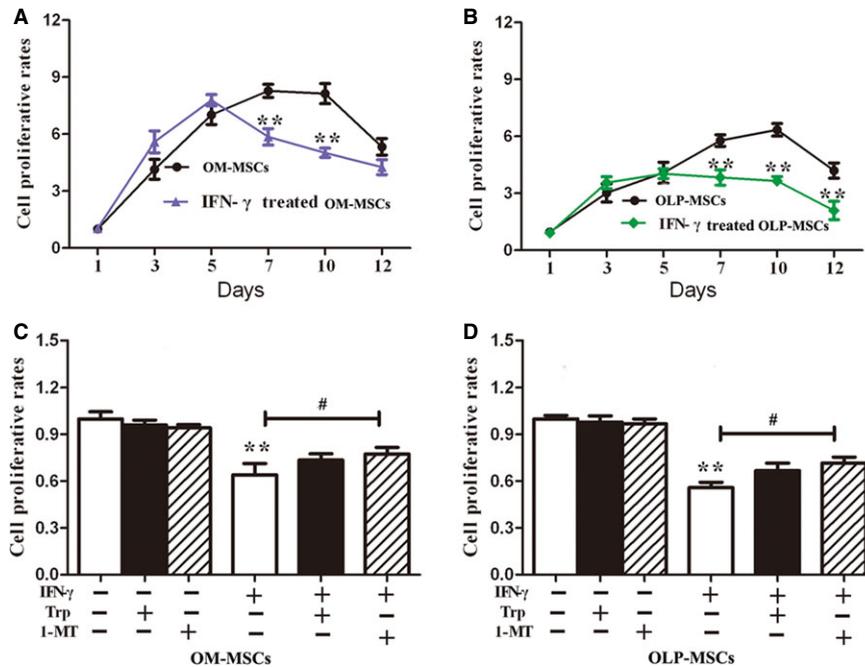


Figure 7 Long-term effect of Interferon- γ (IFN- γ) on the proliferation of OM-MSCs and OLP-MSCs. (A, B) Analysis of cell proliferation rates of OM-MSCs (A) and OLP-MSCs (B) after treatment with 100 ng/ml IFN- γ for 12 days. (C, D) Inhibition of IDO activity by tryptophan or 1-methyltryptophan (1-MT) in MSCs partly restored the proliferation of OM-MSCs (C) and OLP-MSCs (D) at 7 days. Statistical analysis was performed by two-way ANOVA ($n \geq 3$; $**P < 0.01$; $\#P < 0.05$).

increases within inflamed tissues after induction by infection and tissue injuries (51). At present, there are no reports concerning IDO expression in OLP, especially in MSCs isolated from OLP tissues. In our study, we found that the pro-inflammatory cytokine IFN- γ was not expressed by MSCs although MSCs could express IDO and that STRO-1+/IDO+ cells were co-localized in OLP. Thus, the expression of the anti-inflammatory cytokine IDO by MSCs might be proof that MSCs are activated and take part in regulating inflammation.

Effect of MSCs on the proliferation of T lymphocytes via IDO activity

Previous studies have shown that MSCs from some tissues, such as bone marrow, placental, and gingival tissues, immunosuppress the proliferative responses of T cells (ratio 1:4–1:100) (22, 28, 52). The mechanism of this immunosuppression is considered to be related to active IDO. The immune inhibitory effect of MSCs on T lymphocytes has been shown to be dependent on the depletion of tryptophan, coupled with the production of kynurenine (35). In our

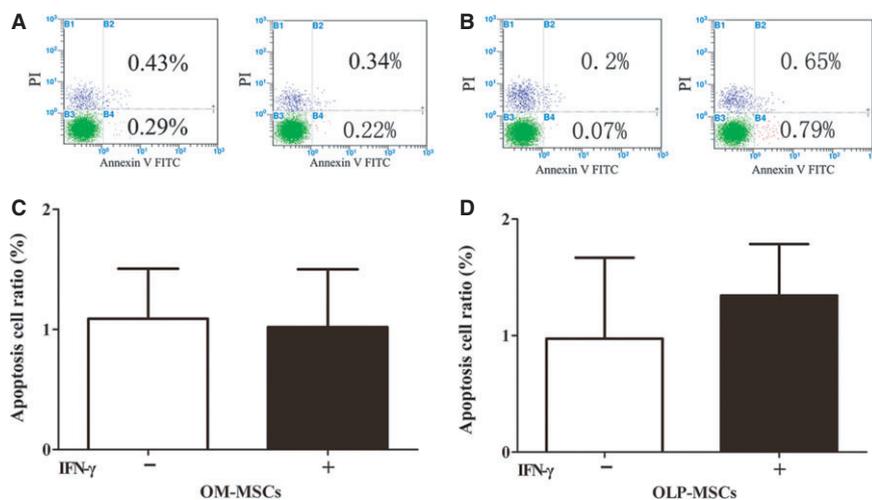


Figure 8 Long-term effect of Interferon- γ (IFN- γ) on the apoptosis of OM-MSCs, and OLP-MSCs. (A, B): Analysis for apoptosis ratio of OM-MSCs (A), and OLP-MSCs (B) after treatment with 100 ng/ml IFN- γ by an annexin V-FITC apoptosis detection kit at 7 days. (C, D): OM-MSCs (C) and OLP-MSCs (D) were not apoptotic by independent two-tailed Student's *t*-tests ($n \geq 3$, $P > 0.05$).

experiment, MSCs from oral mucosa and OLP could also regulate inflammation through antiproliferative effects on T lymphocytes in MLR, which was similar to the findings of previous research (22, 28, 52), and their immunosuppressive function was enhanced when pretreated with IFN- γ for 48 h. However, the antiproliferative effects of untreated and IFN- γ -pretreated MSCs on MLR were reversed by addition of tryptophan or 1-MT (which binds to active IDO). We have also shown that the antiproliferative effects of these MSCs were not associated with cell apoptosis of T lymphocytes in MLR. Therefore, the immunosuppression of T lymphocytes by MSCs was most likely via active IDO *in vitro*. However, this capacity was weaker in MSCs from OLP, which might demonstrate impairment of these MSCs in inflammatory regulation. MSCs show low immunogenicity and immunomodulatory functions in multiple inflammation-related diseases (20, 53). As such, our findings highlight the possible use of MSCs from oral mucosa or short time-pretreated MSCs as new clinical treatments for immune- and inflammatory-related disorders. As MSCs from normal mucosa show stronger immunomodulatory effects, future studies may focus on MSCs from normal tissues surrounding the OLP lesions. MSCs from tissues adjoining the OLP lesions may carry the same characteristics of MSCs from normal mucosa and be a useful sources of MSCs for cell-based therapies.

Effect of IFN- γ on the proliferation of MSCs

The pathological characteristics of OLP are closely related to T cells and their secretion of the inflammatory cytokine IFN- γ (1). Extended production of IFN- γ may maintain and contribute to the initiation and chronicity of this disease (3–5). Therefore, IFN- γ is the main pathogenic responsive cytokine in OLP, and reassuringly, IFN- γ also showed higher expression in OLP in this present study. However, IFN- γ works as a double-edged sword on the function of MSCs and immune regulation. IFN- γ is one of the most potent IDO inducers in the immune system such as at lymph nodes (33, 54, 55), and it can also mediate the immunosuppressive effects of MSCs on the proliferation of

T lymphocytes via IDO activity (21). However, against this, IFN- γ can inhibit the proliferation of mouse and human MSCs via activation of IDO (56).

Increasing attention has been paid to the immunosuppressive effects of MSCs through IFN- γ (57); however, there have been few studies on the impairment MSCs during immunoregulation. We found that, after treatment with IFN- γ for 7 days, proliferation of MSCs from oral mucosa and OLP was inhibited via IDO activity, which is in accordance with some aspects of previous research (56). In previous research, IFN- γ was capable of synergistically enhancing TNF- α -induced MSC apoptosis (58). Here, we demonstrate that treatment with 100 ng/ml IFN- γ did not induce MSC apoptosis *in vitro*. As MSCs from OLP tissues grew slower than MSCs from oral mucosa *in vitro*, we suggest that long-term IFN- γ might inhibit the proliferation of MSCs in OLP, which could contribute to the impairment of these cells and explain the protracted course of the disease.

In summary, first we provided cytological evidence supporting our hypothesis that MSCs exist in OLP-affected oral mucosa. These cells were obtained and identified to have clonogenic and multipotent differentiation properties for the first time. Second, MSCs from OLP may participate in immunomodulation by suppressing T lymphocytes proliferation via IDO activity. These findings further substantiate the potential roles of MSCs from oral mucosa in immunomodulation in inflammatory conditions (14, 59).

Further studies are warranted to confirm our observations *in vivo*. At present, we can confirm that the MSCs increased in number and IDO expression were greater in OLP, and a role for IDO-mediated immunosuppression was noted for the first time in MSCs from OLP. Accumulating evidence suggests that the anti-inflammatory and immunomodulatory functions of MSCs are flexible depending on the tissue origin, specific pathophysiological settings, and types of targeted immune cells (23). Our study contributes to the understanding of MSCs in regulating inflammation in OLP and provides insights into the therapeutic potential of MSCs in inflammatory diseases involving excessive T lymphocyte infiltration.

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Conflict of interest

The authors declare no conflicts of interest.