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DOI: 10.1016/j.jdent.2014.07.007

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Document Version
Peer reviewed version

Citation for published version (Harvard):

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The effects of LPS on adhesion and migration of human dental pulp stem cells in vitro

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Keywords: LPS; hDPSCs; adhesion; migration; NF-κB; MAPK

Short title: The effects of LPS on adhesion and migration of hDPSC
The effects of LPS on adhesion and migration of human dental pulp stem cells in vitro

Abstract

Objectives: The aim of the present study was to investigate the effects of lipopolysaccharide (LPS) on the migration and adhesion of human dental pulp stem cells (hDPSCs) and the associated intracellular signaling pathways.

Methods: hDPSCs obtained from impacted third molars were exposed to LPS and in vitro cell adhesion and migration were evaluated. The effects of LPS on gene expression of adhesion molecules and chemotactic factors were investigated using quantitative real-time reverse-transcriptase polymerase chain (qRT-PCR). The potential involvement of Nuclear factor NF-kappa-B (NF-κB) or mitogen-activated protein kinase (MAPK) signaling pathways in the migration and adhesion of hDPSCs induced by LPS was assessed using a transwell cell migration assay and qRT-PCR.

Results: LPS promoted the adhesion of hDPSCs at 1 μg/mL and 10 μg/mL concentrations, 1 μg/mL LPS showing the greater effect. Transwell cell migration assay demonstrated that LPS increased migration of hDPSCs at 1 μg/mL concentration while decreasing it significantly at 10 μg/mL. The mRNA expressions of adhesion molecules and chemotactic factors were enhanced significantly after stimulation with 1 μg/mL LPS. Specific inhibitors for NF-κB and extracellular signal regulated kinases (ERK), c-Jun N-terminal kinase (JNK), and P38, markedly antagonized LPS-induced adhesion and migration of hDPSCs and also significantly abrogated LPS-induced
up-regulation of adhesion molecules and chemotactic factors. In addition, specific inhibitors of SDF-1/CXCR4, AMD3100 significantly diminished LPS-induced migration of hDPSCs.

**Conclusions:** LPS at specific concentrations can promote cell adhesion and migration in hDPSCs via the NF-κB and MAPK pathways by up-regulating the expression of adhesion molecules and chemotactic factors.

**Clinical significance:** LPS may influence pulp healing through enhancing the adhesion and migration of human dental pulp stem cells when it enters into pulp during pulp exposure or deep caries.

**Key words:** LPS; hDPSCs; adhesion; migration; NF-κB; MAPK
Introduction

Human dental pulp stem cells (hDPSCs) represent a post-natal mesenchymal stem cell population with the potential to differentiate into odontoblast-like cells in vitro and form dentin/pulp-like tissue in vivo.1-3 Regeneration of dentin-pulp after severe injury involves migration and adhesion of hDPSCs to sites of injury, followed by odontoblasts-like cell differentiation and tertiary dentin secretion giving rise to a dentin bridge.4 While carious bacteria and their products are important factors in tooth injury, their influence on repair mechanisms, such as hDPSC migration and adhesion and the underlying molecular mechanisms, remain unclear.

LPS, a gram-negative bacterial cell wall component, is commonly detected in infected pulp cavities and root canals.5 As well as being an important etiologic factor for human chronic periodontitis and apical periodontitis, LPS is also strongly implicated in pulpitis.6,7 LPS is postulated to have profound effects on alveolar bone resorption and pro-inflammatory cytokine production.8,9 Notably previous studies have demonstrated that LPS can decrease adhesion of endothelial progenitor cells (EPCs)10 and increase the migration of dental follicle progenitor cells (DFPCs)11 and bone marrow cells.12 However, the effects of LPS on migration of hDPSCs is unclear.

LPS specifically binds to TLR4 on eukaryotic cell walls leading to intracellular activation of the NF-κB or MAPK pathways downstream via myeloid differentiation factor 88 (MyD88)-dependent signaling.13 Notably MAPK has previously been reported to be involved in proliferation, migration and myofibroblastic (MF) differentiation of periodontal ligament (PDL)-derived endothelial progenitor cell (EPC)-like cells through MEK/ERK and JNK-mediated
signals. Recent studies have also demonstrated that NF-κB plays an important role in the migration and adhesion of several cell types when induced by LPS. Our previous research has recently demonstrated that LPS can activate MAPK and NF-κB signaling in hDPSCs, however the effects of these signaling pathways on the adhesion and migration of hDPSCs has not been assessed.

The aim of the present study was to investigate the influence of LPS on the adhesion and migration of hDPSCs, and the potential roles of MAPK and NF-κB signaling in mediating these effects.

Material and Methods

Reagents

Ultrapure Escherichia coli LPS was obtained from InvivoGen (San Diego, CA). Pyrrolidinedithiocarbamate (PDTC), a specific inhibitor of NF-κB, and AMD3100, a specific inhibitor of stromal cell-derived factor-1 (SDF-1)/CXC chemokine receptor type 4 (CXCR4) were obtained from Sigma-Aldrich (St Louis, MO, USA). U0126, a specific inhibitor of extracellular signal-regulated kinase (ERK); SB203580, a specific inhibitor of p38 kinase; and SP600125, a specific inhibitor of Jun N-terminal kinase (JNK), were purchased from InvivoGen (San Diego, CA).

E.Z.N.A. Total RNA Kit I was obtained from Omega (Omega, USA) and Prime Script RT-PCR Kit, Quantitect SYBR Green kits were obtained from Takara (Takara, Japan). All the primer sequences were synthesised and purchased from Sangon (Sangon Biotech (Shanghai) Co., Ltd.).
Isolation and culture of hDPSC

Healthy third molars extracted for orthodontic reasons were collected from patients (18-25 years) at the Stomatological Hospital affiliated with Fourth Military Medical University (FMMU). Informed consent was obtained from each patient and research protocols were approved by the University’s Ethics Committee. Dental pulps from extracted teeth were isolated and digested with 3 mg/mL type I collagenase and 4 mg/mL dispase (Sigma) for 45-60 min at 37 °C. Single-cell suspensions were seeded in 35-mm or 60-mm culture dishes and maintained in growth medium consisting of α-minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (Gibco-BRL, Grand Island, NY), 100 units/mL penicillin-G, and 100 mg/mL streptomycin (Invitrogen) in a humidified atmosphere of 5% CO₂ at 37 °C. Single-cell clones of DPSCs was isolated, passaged and characterized as previous described. Cultures of between the 2nd and 5th passages were used in subsequent experiments.

Adhesion assay

For the cell adhesion assay, 50 μl type I collagen diluted in PBS (40 mg/l) was added to each well of a 96-well plate and incubated overnight at 4 °C. After removal of unbound collagen, each well was washed with PBS and then blocked with 1% BSA for 1 h at 37 °C in a 5% CO₂ atmosphere. Cells were trypsinized, resuspended in serum-free medium at a cell density of 1 × 10⁵ cells/mL and 100 μl of the cell suspension were added to each well. Different concentrations of LPS with or without inhibitors of the MAPK and NF-κB pathways were combined with hDPSCs prior to seeding on the collagen-coated wells. Cells were incubated for 90 min at 37 °C and non-
adhered cells were removed by washing three times with PBS. The remaining cells were observed by inverted phase contrast microscopy (Olympus, Japan) and then fixed with 4% paraformaldehyde for 15 min and washed three times with PBS. Cells were stained with 0.5% toluidine blue for 10 min and washed three times with PBS prior to the addition of 100 μl of 33% acetic acid (v/v) to each well. Subsequently, the optical density (OD) was determined using a microplate reader (BIO-TEK, Winooski, VT, USA) at an absorbance of 595 nm.

**Transwell migration assay**

Cell migration was evaluated using a two chamber transwell system (8 mm pore size and 6.5 mm diameter) and the cell culture inserts are pre-coated with extracellular matrix proteins (Corning, N.Y, USA). Briefly, the cells were starved for 24 h with serum-free medium, then dissociated and resuspended in 100 μl serum-free medium, and re-seeded onto the top chamber of a transwell plate at a density of 3 × 10⁴ cells/mL. 600-800 μl of serum-free α-MEM containing various concentrations of LPS, inhibitors of SDF-1/CXCR4, or inhibitors of MAPK and NF-κB (Invitrogen) were added to the lower migration chamber. 100-150 μl cell suspension were added to the transwell upper chamber, then the chamber was placed into medium for 24h in an incubator at 37 °C and 5% CO₂. Cells migrating through the membrane were fixed in 4% paraformaldehyde for 15 min, while non-migratory cells were discarded. The transwell chamber was then immersed in 1 g/ml hematoxylin (Sigma-Aldrich, USA) for staining for 15 min. To quantify the migrated cells, ten random microscopic fields per filter at 200x magnification (Olympus, Japan) were selected for cell counting. Measurements were performed in triplicate and mean counts calculated for each experiment.

**Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)**

After exposure to LPS 1 μg/mL in the presence or absence of inhibitors, total RNA was isolated from cells using the E.Z.N.A. Total RNA Kit I (Omega, USA) according to the manufacturer’s protocol and digested with DNase I (RNase-free, RQ1; Promega). First-strand cDNA was synthesized from 1 μg of total RNA using Prime Script RT-PCR Kit (Takara, Japan). qRT-PCR
analyses were performed using an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the SYBR Green PCR master mix reagent (Takara, Otsu, Japan). Reaction mixtures were subjected to 35 cycles of PCR comprising denaturation for 10 seconds at 95 °C, annealing for 15 seconds at 60 °C, and extension for 10 seconds at 72 °C. The relative amount or fold change of the target gene was normalized relative to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and relative to a control (untreated cells). Primer sequences (Sangon Biotech (Shanghai) Co., Ltd.) for GAPDH, fibronectin (FN), intercellular adhesion molecule 1 (ICAM-1), beta1 integrin (Integrin β1), vascular endothelial growth factor (VEGF) and chemokines CXC chemokine receptor type 4 (CXCR4), fibroblast growth factor 2 (FGF2), monocyte chemoattractant protein 1 (MCP-1), stromal cell-derived factor-1 (SDF-1), macrophage inflammatory protein-1 alpha (MIP-1α), transforming growth factor β1 (TGF-β1) and laminin α5 (LAMA α5) are listed in Table 1.

Statistical Analysis

Data are represented as the mean ± standard deviation of 3 separate experiments performed in triplicate. The data were analyzed, where appropriate, by using the Student’s t test or one-way analysis of variance followed by the Student–Neumann–Keuls test using SPSS software (version 17.0; SPSS, Chicago, IL). A P value < 0.05 was considered statistically significant.

Results

3.1 Effects of LPS on hDPSCs adhesion

The effects of various concentrations of LPS on hDPSCs adhesion to extracellular matrix were
investigated. While there was no significant difference in adherent cell numbers between 0.1 μg/mL LPS and the control group (Fig. 1A and B), 1 μg/mL and 10 μg/mL LPS concentrations considerably increased adherent cell number compared with the control. Cell adhesion was more strongly promoted by 1 than 10 μg/mL LPS (Fig. 1A and B). LPS (0.1, 1 or 10 μg/mL) did not influence cell viability as assessed by the MTT assay (data not shown).

The mRNA expressions of key adhesion molecules were determined using real-time PCR after exposing the hDPSCs to various concentrations of LPS. The results showed that the expressions of FN, ICAM-1, Integrin-β1 and VEGF were significantly increased by LPS at concentrations of 1 μg/mL and 10 μg/mL, while no detectable differences were observed after exposure to LPS at 0.1 μg/mL (Fig. 1C). 1 μg/mL LPS strongly increased mRNA expression of FN, ICAM-1, Integrin-β1 and VEGF compared with 10 μg/mL LPS (Fig. 1C).

3.2 Involvement of NF-κB and MAPK Pathways in LPS-induced hDPSCs adherence

To investigate whether NF-κB or MAPK signaling was necessary for LPS-induced cell adhesion, cells were treated with 1 μg/mL LPS with or without pretreatment with specific inhibitors of NF-κB or MAPK signaling. Treatment with the NF-κB inhibitor (PDTC, 20 μmol/L), ERK1/2 MAPK inhibitor (U0126, 25 μmol/L), p38 MAPK inhibitor (SB203580, 20 μmol/L), or JNK MAPK inhibitor (SP600125, 25 μmol/L) significantly decreased adherent cell numbers compared with the LPS-treated control (Fig. 2A and B). Treatment of the cells with PDTC (20 μmol/L), U0126 (25 μmol/L), SB203580 (20 μmol/L) or SP600125 (25 μmol/L) did not influence cell viability as assessed by the MTT assay (data not shown).

The mRNA expression of adhesion molecules was examined using real-time PCR after
exposing the hDPSCs to 1 \mu g/mL LPS with or without pretreatment with the specific inhibitors of NF-\kappa B or MAPK signaling. The results demonstrated that PDTC, U0126, SB203580 and SP600125 significantly decreased LPS-induced mRNA expression of FN, ICAM-1, Integrin-\beta 1 and VEGF in hDPSCs (Fig. 1C).

### 3.3 Effects of LPS on the migration of hDPSCs

To explore the role of LPS on the migratory motility of hDPSCs, assays were performed using a two-chamber transwell system. The hDPSCs were treated with different concentrations of LPS for 24 h, and cells that had traversed the membrane to the bottom side were fixed and stained. The results showed that 1 \mu g/mL LPS significantly increased the migratory ability of hDPSCs, however, 10 \mu g/mL LPS significantly decreased migration of these cells (Fig. 3A and B).

The mRNA expression of chemotactic factors was determined using real-time PCR after exposing the hDPSCs to various concentrations of LPS. The expressions of SDF-1, CXCR4, MCP-1, LAMA-\alpha 5, FGF2, MIP-1\alpha and TGF-\beta 1 were significantly increased by LPS at 1 \mu g/mL and decreased at 10 \mu g/mL, while no detectable differences were observed after stimulation by LPS at 0.1 \mu g/mL (Fig. 3C).

### 3.4 Involvement of NF-\kappa B and MAPK signaling pathways in the LPS-induced migration of hDPSCs

To explore signaling pathways involved in the LPS-induced migration of hDPSCs, cells were treated with 1 \mu g/mL LPS with or without pretreatment with specific inhibitors of NF-\kappa B or MAPK signaling. The results showed that NF-\kappa B inhibitor (PDTC, 20 \mu mol/L) and ERK1/2
MAPK inhibitor (U0126, 25 μmol/L), p38 MAPK inhibitor (SB203580, 20 μmol/L), or JNK MAPK inhibitor (SP600125, 25 μmol/L) significantly decreased 1 μg/mL LPS-induced migration of hDPSCs, especially with PDTC and SP600125, which virtually abolished the effects of 1 μg/mL LPS (Fig. 4A and B).

The mRNA expression of chemotactic factors was determined using qRT-PCR after exposing the hDPSCs to 1 μg/mL LPS with or without pretreatment with specific inhibitors of NF-κB or MAPK signaling. The results showed that PDTC, SB203580, U0126 and SP600125 significantly antagonized LPS-induced mRNA expression of SDF-1, MCP-1, LAMA-α5, FGF2, MIP-1α and TGF-β1 (Fig. 4C).

3.5 Involvement of SDF-1/CXCR4 in LPS-induced migration of hDPSCs

To investigate the role of SDF-1/CXCR4 in LPS-induced migration of hDPSCs, cultures were treated with 1 μg/mL LPS with or without pretreatment with a specific inhibitor of SDF-1/CXCR4 (AMD3100, 1 μmol/L). The results showed that AMD3100 significantly diminished 1 μg/mL LPS-induced migration of hDPSCs (Fig. 4D and E). However, AMD3100 (1 μmol/L) did not influence cell viability as assessed by the MTT assay (data not shown).

Discussion

Adhesion is fundamental to cell behaviour in tissues. Previous studies have shown that in endothelial progenitor cells (EPCs), LPS at concentrations of 10 pg/ml, 100 pg/ml and 1 ng/ml has no effect on EPC adhesion, while 10 and 100 ng/ml LPS significantly decreases cell adhesion. However in breast cancer cells, LPS treatment increased cell adhesion. In addition, it was found
that the concentration of endotoxin in all root canal samplings ranged from 17–696 EU/mL (equate to 0.27-0.696 ug/mL). Endotoxin contents ranged from 17–228 EU/mL (equate to 0.17-0.228 ug/mL) in asymptomatic teeth. In contrast, higher levels of endotoxin were found in teeth which had clinical symptomatology, ranging from 270–696 EU/mL (equate to 0.27-0.696 ug/mL).

In the present study, 1 μg/mL and 10 μg/mL LPS significantly promoted adhesion of hDPSCs, while 0.1 μg/mL LPS had no effect. It indicated that maybe there was a threshold for the cells of dental pulp in response to bacterial endotoxin. These results also suggested that cell specific differences in adhesion may exist.

Cell adhesion is mediated by adhesion molecules via several signaling pathways. Previous studies have shown that molecules including FN, ICAM-1, Integrin-β1 and VEGF play important roles in cell adhesion. In the present study, our results showed that mRNA expression of FN, ICAM-1, Integrin-β1 and VEGF were significantly up-regulated by LPS at 1 μg/mL, which also enhanced adhesion of hDPSCs.

The effects of LPS on migration of DPSCs will impact on recruitment of DPSCs to sites of injury. The present data showed that 1 μg/mL LPS significantly increased migration of hDPSCs, however, 10 μg/mL LPS significantly decreased migration of these cells. Our unpublished data showed that 10 μg/mL LPS inhibited proliferation of hDPSCs although did not affect viability of these cells. Previous studies have shown that LPS promotes migration of dendritic cells (DCs) and Lewis lung cancer (LLC) cells as well as stem cell populations, including dental follicle progenitor cells (DFPCs) and bone marrow cells. Any differences in LPS effects on migration of these various cell populations likely reflects relative sensitivities of specific cell types to LPS.

Various chemotactic factors have been considered to be important in cell migration, including
SDF-1, CXCR4, MCP-1, FGF2, MIP-1α and TGF-β1. In the present study, we examined the expression of SDF-1, CXCR4, MCP-1, FGF2, MIP-1α and TGF-β1 to investigate their association with the migration of hDPSCs. Our result showed that all these chemotactic factors were significantly increased by LPS exposure at 1 µg/mL while being decreased at 10 µg/mL, however no detectable difference after exposure to LPS at 0.1 µg/mL, which paralleled the results for the migration assay. This suggests that SDF-1, CXCR4, MCP-1, FGF2, MIP-1α and TGF-β1 may be strongly associated with hDPSCs migration induced by 1 µg/mL LPS. In order to investigate the role of SDF-1/CXCR4 in LPS-induced migration of hDPSCs, a specific inhibitor of SDF-1/CXCR4, AMD3100 was used prior to LPS stimulation. The results showed that AMD3100 significantly diminished 1 µg/mL LPS-induced migration, which further indicated that SDF-1/CXCR4 was involved in LPS-induced migration.

NF-κB and MAPK pathways have been shown to be involved in cell adhesion and migration. In order to determine the role of NF-κB and MAPK pathways in 1 µg/mL LPS-induced cell adhesion and migration of hDPSCs, specific inhibitors for NF-κB and MAPK pathways were added prior to 1 µg/mL LPS treatment. Our results showed that specific inhibitors of NF-κB, JNK, P38, and ERK clearly repressed the migration and adhesion induced by 1 µg/mL LPS. Interestingly, the effects of PDTC and SP600125 were greatest and led to virtually complete inhibition of the LPS-induced migration of hDPSCs. These data suggests that NF-κB and JNK MAPK may play key roles in LPS-induced cell migration in hDPSCs. Such signaling may be cell-specific since inhibitor studies have implicated ERK signaling in mechano-growth factor (MGF)-induced mesenchymal stem cell migration. However, other studies have shown that P38MAPK pathway is important for the migration of ovarian cancer cells. Notably our previous research has
shown that LPS can activate NF-κB and MAPK signaling in hDPSCs, which may be associated with increased adhesion and migration of hDPSCs.

In conclusion, our results have shown that LPS at a concentration of 1 μg/mL can promote the adhesion and migration of hDPSCs and that NF-κB and MAPK pathways appear to be involved in up-regulating the expression of adhesion molecules and chemotactic factors.

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Figure legends

Fig. 1 - The effects of LPS on the adhesion of hDPSCs. Cells were incubated with LPS at the concentrations indicated for 90 min. Adherent cells were fixed and stained and examined by inverted microscopy (Fig. 1A). The stain was dissolved from the cells and quantified at 595 nm using a multiplate reader (Fig. 1B). In a separated experiment, total RNA was extracted, then mRNA expression of adhesion molecules including FN, ICAM-1, integrin-β1 and VEGF were measured by qRT-PCR (Fig. 1C). Results are shown as the mean ± S.D. deviation of three independent experiments performed in triplicate. CON represent the control group. *P < 0.05 represents a significant difference compared with the control. Scale bars indicate 100 μm.

Fig. 2 - Involvement of NF-κB and MAPK pathways in LPS-induced adhesion of hDPSCs.

hDPSCs were pretreated with NF-κB inhibitor (PDTC, 20 μmol/L), ERK1/2 MAPK inhibitor (U0126, 25 μmol/L), p38 MAPK inhibitor (SB203580, 20 μmol/L), or JNK MAPK inhibitor (SP600125, 25 μmol/L) for 30 min prior to stimulation with LPS (1 μg/mL) for 90 min. Adherent cells were fixed and stained and examined by inverted microscopy (Fig. 2A). The stain was dissolved from cells and quantified at 595 nm using a multiplate reader (Fig. 2B). In a separate experiment, total RNA was extracted and the mRNA expression of adhesion molecules including
FN, ICAM-1, integrin-β1 and VEGF were measured by qRT-PCR (Fig. 2C). Results are shown as the mean ± S.D. deviation of three independent experiments performed in triplicate. CON represent the control group. *P < 0.05 represents a significant difference compared with the control. Scale bars indicate 100 μm.

Fig. 3 - Effects of LPS on the migration of hDPSCs. Cell migration assays were evaluated using a two-chamber transwell system. Cells were treated with LPS concentration indicated for 24 h and then the migratory cells were fixed and stained (A and B). In a separated experiment, hDPSCs were treated with LPS at the concentrations indicated for 24 h, and then total RNA was extracted and the mRNA expression of chemotactic factors including SDF-1, CXCR4, MCP-1, LAMA-α5, FGF2, MIP-1α, TGF-β1 were assessed by qRT-PCR (Fig. 3C). Results are shown as the mean ± S.D. deviation of three independent experiments performed in triplicate. CON represent the control group. *P < 0.05 represents a significant difference compared with the control. Scale bars indicate 100 μm.

Fig. 4 - Involvement of NF-κB, MAPK or SDF-1/CXCR4 pathways in LPS-induced hDPSC migration. Cell migration assays were evaluated using a two-chamber transwell system. Cells were treated with LPS (1 µg/mL) for 24 h with or without pretreatment with PDTC (20 μmmol/L), SB203580 (20 μmmol/L), SP600125 (25 μmmol/L), U0126 (25 μmmol/L) or AMD3100 (1 μmol/L) for 1 h, and then the migratory cells were fixed, stained and counted (A - B and D - E). In a separated experiment, hDPSCs were treated with LPS (1 µg/mL) with or without indicated inhibitors, and then total RNA was extracted and the mRNA expressions of chemotactic factors
including SDF-1, CXCR4, MCP-1, LAMA-α5, FGF2, MIP-1α and TGF-β1 were assessed by qRT-PCR (Fig. 4C). CON represent the control group. * P< 0.05 when compared with the untreated control group. # P< 0.05 when compared with the LPS-treated group. Scale bars indicate 100 μm.
Acknowledgements

This work was supported by Grant 81271125 and 81070831 from the Chinese National Natural Science Foundation. The authors deny any conflicts of interest related to this study. We gratefully acknowledge the assistance of Dr. AJ Smith in the editing of this manuscript.
Highlights
Effects of LPS on hDPSCs adhesion
Effects of LPS on the migration of hDPSCs
Involvement of NF-κB and MAPK Pathways in LPS-induced hDPSCs adherence and migration
Involvement of NF-κB and MAPK signaling pathways in the LPS-induced of hDPSCs
Involvement of SDF-1/CXCR4 in LPS-induced migration of hDPSCs
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