

Human periodontal ligament stem cells secretome from multiple sclerosis patients suppresses NALP3 inflammasome activation in experimental autoimmune encephalomyelitis

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Abstract

Research in recent years has largely explored the immunomodulatory effects of mesenchymal stem cells (MSCs) and their secretory products, called “secretome,” in the treatment of neuroinflammatory diseases. Here, we examined whether such immunosuppressive effects might be elicited due to inflammasome inactivation. To this end, we treated experimental autoimmune encephalomyelitis (EAE) mice model of multiple sclerosis (MS) with the conditioned medium or purified exosomes/microvesicles (EMVs) obtained from relapsing-remitting-MS patients human periodontal ligament stem cells (hPDLSCs) and investigated the regulation of NALP3 inflammasome. We noticed enhanced expression of NALP3, Cleaved Caspase 1, interleukin (IL)-1 β , and IL-18 in EAE mouse spinal cord. Conversely, hPDLSCs-conditioned medium and EMVs significantly blocked NALP3 inflammasome activation and provided protection from EAE. Reduction in NALP3, Cleaved Caspase 1, IL-1 β , and IL-18 level was noticed in conditioned medium and EMVs-treated EAE mice. Pro-inflammatory Toll-like receptor (TLR)-4 and nuclear factor (NF)- κ B were elevated in EAE, while hPDLSCs-conditioned medium and EMVs treatment reduced their expression and increased I κ B- α expression. Characterization of hPDLSCs-conditioned medium showed substantial level of anti-inflammatory IL-10, transforming growth factor (TGF)- β , and stromal cell-derived factor 1 α (SDF-1 α). We propose that the immunosuppressive role of hPDLSCs-derived conditioned medium and EMVs in EAE mice may partly attribute to the presence of soluble immunomodulatory factors, NALP3 inflammasome inactivation, and NF- κ B reduction.

Keywords

inflammasome, mesenchymal stem cells, multiple sclerosis, NALP3, regenerative medicine, secretome

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Introduction

Multiple sclerosis (MS) is a long-lasting progressive autoimmune neurological disorder of the central nervous system.¹ Immune cells invasion, axonal injury, and myelin sheath deformation are the common hallmarks of MS, which eventually produce neurological disability.² Based on heterogeneous

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clinical indices, MS has been classified into four subtypes: relapsing-remitting (RR), primary progressive, secondary progressive, and progressive relapsing.³ About 2.5 million people are affected by MS worldwide.⁴ It is predominant in women than in men and that RR type is the most common form of MS with 85% occurrence.^{5,6} However, few immunomodulatory drugs are available only for RR form, and no medicine is available yet for progressive form.⁷

In recent years, mesenchymal stem cells (MSCs), owing to their role in regenerative medicine, has drawn a center of attraction toward the treatment for MS.⁸ MSCs are pluripotent stromal cells, greatly emphasized for their self-renewal capacity and the ability to differentiate into various kinds of cell types such as neural cells, chondrocytes, and adipocytes.⁹ Human MSCs are largely present in adult tissues including bone marrow, adipose, dental, and placenta.¹⁰ In particular, neural crest MSCs from human dental tissues such as periodontal ligament, dental pulp, and gingiva attained greater attention owing to the minimal invasive procedure involved in surgical dental tissue explants removal, remarkable differentiation ability toward neurogenic and other cell lineages, its cost effectiveness, and free from ethical concerns.¹¹ Indeed, our research group and others have demonstrated the immunosuppressive and regenerative effects of dental MSCs in *in vivo* models including MS, muscular dystrophy, myocardial infarction, dental tissue-associated and connective tissue damages, and neurotrauma.^{12,13}

At molecular level, evidence shows that the therapeutic effects of MSCs, particularly immunomodulatory effects, might be promoted by the presence of small membranous exosomes/microvesicles (EMVs).¹⁴ EMVs contain a pool of soluble cytokines, proteins, nucleic acids, and lipids. Upon receiving pathological signals, such as inflammatory cues, these bioactive molecules are homed into the damaged site, where they suppress pro-inflammatory responses, inhibit apoptosis, and induce tissue-specific precursor cells differentiation via paracrine signaling.¹⁵ Indeed, regenerative potential of EMVs and other secretions, also called as “secretome,” has been investigated in preclinical and clinical studies associated with miscellaneous diseases including neurodegenerative diseases.^{16–18} Recently, we demonstrated the immunosuppressive role of human periodontal ligament stem cells

(hPDLSCs)-derived conditioned medium (CM) and purified EMVs in mice experimental autoimmune encephalomyelitis (EAE) models.¹⁹ Given the substantial immunoregulatory role of stem cells-derived EMVs in MS, one might presume that EMVs may have a modulatory role against inflammasomes activation in MS development. Inflammasomes are a multicomplex of scaffold proteins localized in cytoplasm, activated during sterile inflammatory diseases including neuroinflammatory disorders, metabolic diseases, and atherosclerosis.²⁰ Under appropriate stimuli, the nucleotide-binding oligomerization domain-like receptors (NLRs) assembled in the inflammasomes form a complex with apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) and regulate inactive procaspase-1 processing into active cleaved caspase-1, which in turn modifies the synthesis of active pro-inflammatory cytokines interleukin (IL)-1 β and IL-18 from their respective precursor forms.²¹ Among other NLRs, the NACHT, LRR, and PYD domains-containing protein 3 (NALP3; also known as NLRP3) is a key regulator and that NALP3 inflammasome pathway has been widely studied in various autoimmune diseases including MS.²² Nevertheless, modulation of NALP3 pathway with respect to the mechanistic actions of EMVs has not been well documented in MS.

In this study, we investigated the regulation of NALP3 inflammasome pathway in EAE mice administered with hPDLSCs-derived CM and purified EMVs obtained from RR-MS patients. Expression of NALP3 inflammasome pathway proteins NALP3, inactive and active caspase-1, IL-1 β , and IL-18 was analyzed in the spinal cord. In addition, we examined transcription factor NF- κ B signaling modulation in terms of NALP3 inflammasome activation.

Materials and methods

Ethics statement for human sampling

The procedure and informed agreement from human periodontal ligament biopsies were performed according to the approved guidelines of Medical Ethics Committee at the Medical School, “G. d’Annunzio” University, Chieti, Italy (no. 266/17.04.14). The formal consent form was signed by all subjects before samples collection

was carried out. The Department of Medical, Oral and Biotechnological Sciences and the Laboratory of Stem Cells and Regenerative Medicine are certified in accordance with the quality standard ISO 9001:2008 (certificate no. 32031/15/S).

hPDLSCs culture establishment

Human periodontal ligament biopsies were performed in healthy donors and RR-MS patients ($n=5$ for each). Before sampling, each subject was pretreated with 0.2% chlorhexidine solution (1 min). Sample tissues were collected from horizontal fibers present in the periodontal ligament of premolar teeth by root scaling. Periodontal tissue samples were cut into small pieces and washed with phosphate-buffered saline (PBS; LiStarFish, Milan, Italy). Then, the specimens were plated with human mesenchymal stem cells growth medium—chemically defined (MSCGM-CD) (Lonza, Basel, Switzerland). The medium was replaced with the fresh medium twice a week. Once 80% confluence was attained, hPDLSCs spontaneously migrated from the tissue explants were trypsinized and subcultured. hPDLSCs at second passage were used for the experiments.²³ Morphological assessment, stem cell surface markers expression, and mesengenic differentiation ability were carried out as reported previously.¹²

Preparation of hPDLSCs-CM

CM of hPDLSCs (15×10^3 cells/cm²) grown in MSCGM-CD was harvested after 72 h of incubation. hPDLSCs-CM was centrifuged at 1200 r/min for 5 min (4°C), and the resulting supernatant was further centrifuged at 3000 r/min for 3 min (4°C) to obtain the secondary supernatant. Then, 1 mL of secondary supernatant was mixed with 3 mL of ice-cold acetone, followed by overnight incubation (4°C). Afterward, hPDLSCs-CM:acetone mix was centrifuged at 16,000 r/min for 12 min (4°C) (Centrifuge 5804 R; Eppendorf, Milan, Italy). The resulting suspension was lysated in radioimmuno-precipitation assay buffer and protein estimation was quantified by Bradford assay.

Preparation of hPDLSCs-EMVs

EMVs were purified from hPDLSCs-CM using a commercial agglutinating agent Exoquick TC (System Biosciences, Mountain View, California,

USA) as per the manufacturer's instructions. In brief, 10 mL of hPDLSCs-CM was added with 2 mL of Exoquick TC and the mix was incubated for overnight (4°C). Then, the hPDLSCs-CM:Exoquick TC mix was centrifuged at 1500g for 30 min (4°C) and the resulting EMVs pellet was resuspended in 200 μ L PBS. Protein quantification was performed in EMVs to confirm their release from hPDLSCs.

Enzyme-linked immunosorbent assay

Supernatants from hPDLSCs and MS-hPDLSCs-CM, plated at a density of 10^6 cells/mL, were collected for cytokines/chemokines detection of IL-10, IL-15, stromal cell-derived factor 1 α (SDF-1 α), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1 α), and transforming growth factor (TGF)- β after 24 h of culture. Cytokines/chemokines release was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (eBioscience, San Diego, California, USA).

Animals

A 12-week-old, male C57BL/6 mice weighing 20–25 g (Harlan, Milan, Italy) were kept in separate ventilated cages. Food and water were supplied ad libitum. The housing room was maintained under constant temperature and humidity conditions. All animals were subjected into 12-h/12-h light/dark cycle.

Ethics statement for animal use

Mice were maintained appropriately in accordance with the European Organization Guidelines for Animal Welfare. All the experimental procedures were performed under the guidelines approved by the Ministry of Health "General Direction of animal health and veterinary drug" (Authorization 621/2015- D.lgs 26/2014). The experiments were designed in such a way to reduce the total number of mice required for the study.

EAE induction

Mice were anesthetized with a mixture of tiletamine and xylazine (10 mL/kg, intraperitoneal (i.p.)). Subsequently, EAE was induced in mice using Myelin Oligodendrocyte Glycoprotein peptide (MOG) 35–55 (MEVGWYRSPFSRVVHLYRNGK;

% peak area by high-performance liquid chromatography (HPLC) ≥ 95 , AnaSpec, EGT Corporate Headquarters, Fremont, CA, USA) as reported by Paschalidis et al.²⁴ In brief, mice were immunized subcutaneously in the flank with 300 μ L of emulsion (300 μ g of (MOG) 35–55 in Complete Freund's Adjuvant (CFA) with 300 μ g of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories Sparks, MD, USA)). An i.p. injection of *Bordetella pertussis* toxin (500 ng in 100 μ L; Sigma-Aldrich, Milan, Italy) was administered immediately after (MOG) 35–55 injection and after 48 h. After 14 days of EAE induction, active encephalitogenic responses in EAE-induced mice were identified with the visible pathological signs such as tail flaccidity and loss of hind legs movement.

Experimental design

Mice were arbitrarily divided into following groups (N=30 total animals): (a) naïve (N=5): normal mice with no (MOG) 35–55 or other immunization, (b) EAE (N=10): mice subjected to EAE with no other treatment, (c) EAE+hPDLSCs-CM (N=5): EAE mice intravenously (i.v.) injected (tail) with RR-MS patients-derived hPDLSCs-CM ($\approx 1600 \mu$ G of hPDLSCs-CM/mouse) after 14 days of EAE induction, (d) EAE+hPDLSCs-EMVs (N=5): EAE mice i.v. injected (tail) with RR-MS patients-derived hPDLSC-EMVs ($\approx 24 \mu$ G of hPDLSCs-EMVs/mouse) after 14 days of EAE induction, (e) naïve+hPDLSCs-CM (N=5): normal mice i.v. injected with RR-MS patients-derived hPDLSCs-CM ($\approx 1600 \mu$ G of hPDLSCs-CM/mouse) after 14 days of EAE induction, and (e) naïve+hPDLSCs-EMVs (N=5): normal mice IV injected with RR-MS patients-derived hPDLSCs-EMVs ($\approx 24 \mu$ G of hPDLSCs-EMVs/mouse) after 14 days of EAE induction.

On 28th day after EAE induction, mice were sacrificed with Tanax (5 mL/kg body weight; i.p.). Spinal cord tissues were collected and processed for biochemical analyses.

Clinical disease score evaluation. From day 14 after EAE immunization, MS pathological symptoms such as hind limb paralysis and tail tonus reduction were noticed in EAE mice. The severity of encephalitogenic responses was assessed using a 0–10 scoring system as reported by Campbell et al.,²⁵ where score 0 represents no disease condition and score 10 represents EAE-associated mortality. To obtain

more disease parameters and to perform statistical analysis,²⁶ we used a 0–10 scoring system in our study. Gait disturbances, righting reflexes impairment, tail tonicity, and limb tonicity were calculated to obtain disease scores, daily. The scoring system used in the present is as follows. (a) Gait was calculated as normal (0), marginally abnormal (+1), moderately abnormal (+2), or severe (+3). (b) Righting reflex analyses were accomplished by placing the animal on its back and recording the capacity to return rapidly to all four limbs. Righting reflex was counted as normal (0), slow (+0.5), or absent (+1). (c) Loss of tail tonicity was scored as normal (0), distal loss of tone (+0.5), or completely loss of tone (+1). (d) Limb weakness was measured by placing the animal in upturned position on a grid for 20 s and recording the ability to hold the grid. Limb tonicity was scored as normal (0), weak (+0.5), near paralysis where limb movement was limited with inability to hold the limbs under the body (+1), or paralyzed where limb movement was completely stopped (+1.5). Animal with both forelimb and hind limb paralysis was given the score 9 and animal died due to EAE was given the score 10.

Initial clinical disease score was measured on the day of EAE induction (day 0), and all the succeeding measurements were documented every 24 h until sacrifice. In addition, the daily variation of the clinical score of the disease has been expressed in comparison with a day of EAE induction (day 0). The value day has been reported as mean \pm standard deviation (SD) of all animals for each experimental group.

Light microscopy. Spinal cord tissues (fixed in 10% (w/v) PBS-buffered formaldehyde) were embedded in paraffin and sectioned into 7 μ m thin slices. After processing into xylene deparaffinization and subsequent rehydration steps, sections were stained with eosin and hematoxylin. Sections were visualized under optical microscope (Leica microscope ICC50HD).

Immunohistochemistry. Paraffin-embedded tissue slices were deparaffinized with xylene, rehydrated with alcohol series, and incubated in 0.01 M citrate buffer (pH 6) for 4 min to retrieve antigen. Then, the slices were incubated with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min to quench endogenous peroxidase and blocked with normal goat serum in PBS (2% v/v) for 20 min. Afterward, slices were incubated with

selective primary antibodies for overnight at 4°C. The primary antibodies applied for immunohistochemical analysis are as follows: anti-IL-18 antibody (1:250 in PBS v/v; Abcam, Cambridge, UK), anti-TLR4 antibody (1:100 in PBS v/v; Abcam, Cambridge, UK), anti-I κ B- α antibody (1:250 in PBS v/v; Cell Signaling Technology, Leiden, The Netherlands), and anti-NF- κ B antibody (1:250 in PBS v/v; Cell Signaling Technology, Leiden, The Netherlands). Then, the slices were washed with PBS and incubated with avidin/biotin blocking reagent (DBA, Milan, Italy) to block endogenous avidin and biotin binding sites. Afterward, slices were incubated with universal biotinylated secondary antibody followed by avidin-horseradish peroxidase (HRP)-conjugated solution (Vectastain ABC kit, Vector Laboratories, Burlingame, California, USA) according to the manufacturer's instructions. Slices were then incubated with hydrogen peroxide/DAB kit (Vectastain DAB kit, Vector Laboratories, Burlingame, California, USA) according to the manufacturer's instructions. Counterstaining was performed with nuclear fast red. To verify nonspecific background immunostaining, slices were incubated with either primary or secondary antibody alone. No staining was noticed in these controls. Slices were visualized using LEICA DM 2000 combined with LEICA ICC50 HD camera. Images were acquired using Leica Application Suite V4.2.0 software.

Western blot analysis. Spinal cord tissues were homogenized using ice-cold lysis buffer with following ingredients: 10 mM Tris-HCl pH 7.4, 0.32 M sucrose, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-*bis*(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 50 mM NaF, 5 mM NaN₃, 10 mM 2-mercaptoethanol, and protease inhibitor tablets (Roche Applied Science, Monza, Italy). Homogenates were clarified by centrifugation at 1000g for 10 min at 4°C, and the resulting supernatant was served as cytoplasmic fraction. The pellets were further lysed using ice-cold extraction buffer consisting of 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and protease inhibitors. Homogenates were clarified by centrifugation at 15,000g for 30 min at 4°C. The resulting supernatant was served as nuclear fraction. Protein concentration was assayed by using Bio-Rad Protein Assay (Bio-Rad, Segrate, Milan, Italy). Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis,

followed by blotting with polyvinylidene difluoride (PVDF) membranes (Immobilon-P Transfer membrane, Millipore, Vimedrone, Milan, Italy). Then, membranes were incubated in blocking solution (5% skimmed milk in 1 \times PBS) for 45 min at room temperature. Subsequently, membranes were incubated with selective primary antibodies for overnight at 4°C. The primary antibodies used were NALP3 (1:500; R&D Systems, Minneapolis, USA), Pro/cleaved caspase 1 (1:500; Abcam, Cambridge, UK) and IL-1 β (1:500; Cell Signaling Technology, Leiden, The Netherlands). Later, membranes were incubated with appropriate secondary antibodies conjugated with HRP (1:2000; Santacruz Biotechnology Inc., Dallas, Texas, USA) for 1 h at room temperature. The relative protein expression was observed using enhanced chemiluminescence kit (Luminata Western HRP Substrates, Millipore, Vimedrone, Milan, Italy). To assess equal loading of proteins, membranes were stripped and reprobed with HRP-conjugated GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody (1:1000; Cell Signaling Technology, Leiden, The Netherlands). Images of protein bands were captured by ChemiDoc™ MP System (Bio-Rad, Segrate, Milan, Italy), and relative densitometric level of the protein bands was evaluated with a software program (ImageJ Bethesda, Maryland, USA). Experiments were repeated for three separate times, and statistical analysis was performed on blots acquired from three separate experiments.

Statistical analysis. Data were analyzed statistically using GraphPad Prism version 6.0 program (GraphPad Software, La Jolla, CA). One-way analysis of variance (ANOVA) and post hoc Bonferroni multiple comparison test were performed. A *P* value of 0.05 or less between the groups was considered with statistical significance. Study data are reported as means \pm SD.

Results

RR-MS patients-derived hPDLSCs-CM and purified EMVs reduce the EAE progression

EAE-induced mice showed severe dysfunction in neurological functions. Clinical disease score assessment revealed the active encephalitogenic challenges occurred in EAE mice. We found that EAE induction caused severe phenotypic impairments. EAE-associated clinical disease parameters such as severe problem in gait, righting reflex

deficiency, and loss of tail and limb tonicity were noticed in EAE mice. On the contrary, EAE mice administered with hPDLSCs-CM and EMVs showed significant improvement. Progression of the disease has been remarkably reduced in the treated EAE mice (Figure 1(a)). Clinical parameter scores evaluated in naïve, EAE, EAE+hPDLSCs-CM, and EAE+hPDLSCs-EMVs groups have been reported in Table 1. E/H staining revealed marked inflammatory cells invasion in EAE mice spinal cord (Figure 1(e)). Conversely, treatment with hPDLSCs-CM and EMVs significantly terminated the invasion of inflammatory cells (Figure 1(f) and (g), respectively). No inflammatory cells infiltration was observed in naïve (Figure 1(b)), naïve+hPDLSCs-CM (Figure 1(c)), and naïve+hPDLSCs-EMVs (Figure 1(d)). These findings suggest that hPDLSCs-CM and EMVs may possess effective anti-inflammatory capacity, which in turn protect the EAE mice from inflammatory cells infiltration.

EAE-induced NALP3 inflammasome activation was repressed by RR-MS patients-derived hPDLSCs-CM and purified EMVs

Then, we investigated whether hPDLSCs-CM and purified EMVs obtained from RR-MS patients could modulate NALP3 inflammasome activation. We noticed that in EAE mice spinal cord, NALP3 inflammasome-associated proteins were prominently increased. Western blot results showed that NALP3 expression was enhanced in EAE mice (Figure 2(a)). Consequently, active cleaved caspase 1 was increased only in EAE mice (Figure 2(b)), while its precursor protein procaspase 1 showed no changes in their expression in all mice groups. Cleaved caspase 1 activation resulted in the synthesis of active pro-inflammatory IL-1 β (Figure 2(c)). In addition, Immunohistochemistry data showed increased expression of active pro-inflammatory IL-18 (Figure 2(g)) mediated by active cleaved caspase 1. Interestingly, hPDLSCs-CM and EMVs treatment inhibited NALP3 inflammasome activation in EAE mice. Significant reduction in NALP3 and cleaved caspase 1 expression was noticed in EAE mice administered with hPDLSCs-CM and EMVs ($P < 0.0001$ vs EAE). As expected, decreased cleaved caspase 1 resulted in substantial reduction of active IL-1 β and IL-18 (Figure 2(h) and (i), respectively; $P < 0.0001$ vs EAE) level. NALP3 expression in naïve,

naïve+hPDLSCs-CM, and naïve+hPDLSCs-EMVs group mice were unaltered, while cleaved caspase-1 and IL-1 β were undetectable in these groups. Similarly, IL-18 was undetectable in naïve (Figure 2(d)), naïve+hPDLSCs-CM (Figure 2(e)) and naïve+hPDLSCs-EMVs (Figure 2(f)) group mice. These findings suggested that RR-MS patients-derived hPDLSCs-CM and purified EMVs might elicit anti-inflammatory response in EAE mice via blocking NALP3 inflammasome activation.

RR-MS patients-derived hPDLSCs-CM and purified EMVs prevents TLR4-mediated pro-inflammatory response of NF- κ B in EAE mice

Then, we evaluated the expression of pro-inflammatory transcription factor NF- κ B signaling with reference to NALP3 stimulation. Immunohistochemistry results revealed that the expression of NF- κ B positive regulator TLR4 and NF- κ B was markedly elevated in EAE mice spinal cord (Figures 3(d) and 5(d), respectively), while NF- κ B inhibitor protein I κ B- α was absent in EAE mice (Figure 4(d)) similar to that of naïve mice (Figure 4(a)). On the contrary, EAE mice treated with hPDLSCs-CM and EMVs showed negative staining of TLR4 (Figure 3(e) and (f), respectively; $P < 0.0001$ vs EAE) and NF- κ B (Figure 5(e) and (f), respectively; $P < 0.0001$ vs EAE) similar to that of naïve mice (Figures 3(a) and 5(a), respectively). Interestingly, we noticed that I κ B- α was significantly enhanced after treatment with hPDLSCs-CM and EMVs (Figure 4(e) and (f), respectively; $P < 0.0001$ vs EAE). Negative staining of TLR4 and NF- κ B was noticed in naïve (Figures 3(a) and 5(a), respectively), naïve+hPDLSCs-CM (Figures 3(b) and 5(b), respectively), and naïve+hPDLSCs-EMVs (Figures 3(c) and 5(c), respectively). Similar negative staining was observed for I κ B- α in naïve+hPDLSCs-CM (Figure 4(b)) and naïve+hPDLSCs-EMVs (Figure 4(c)). These results showed that RR-MS patients-derived hPDLSCs-CM and EMVs suppress EAE-induced NF- κ B activation.

Immunoregulatory cytokines and chemokines expression in healthy subjects and RR-MS patients-derived hPDLSCs-CM

Finally, we investigated the expression of immunoregulatory molecules present in RR-MS hPDLSCs-CM. ELISA analysis showed marked presence

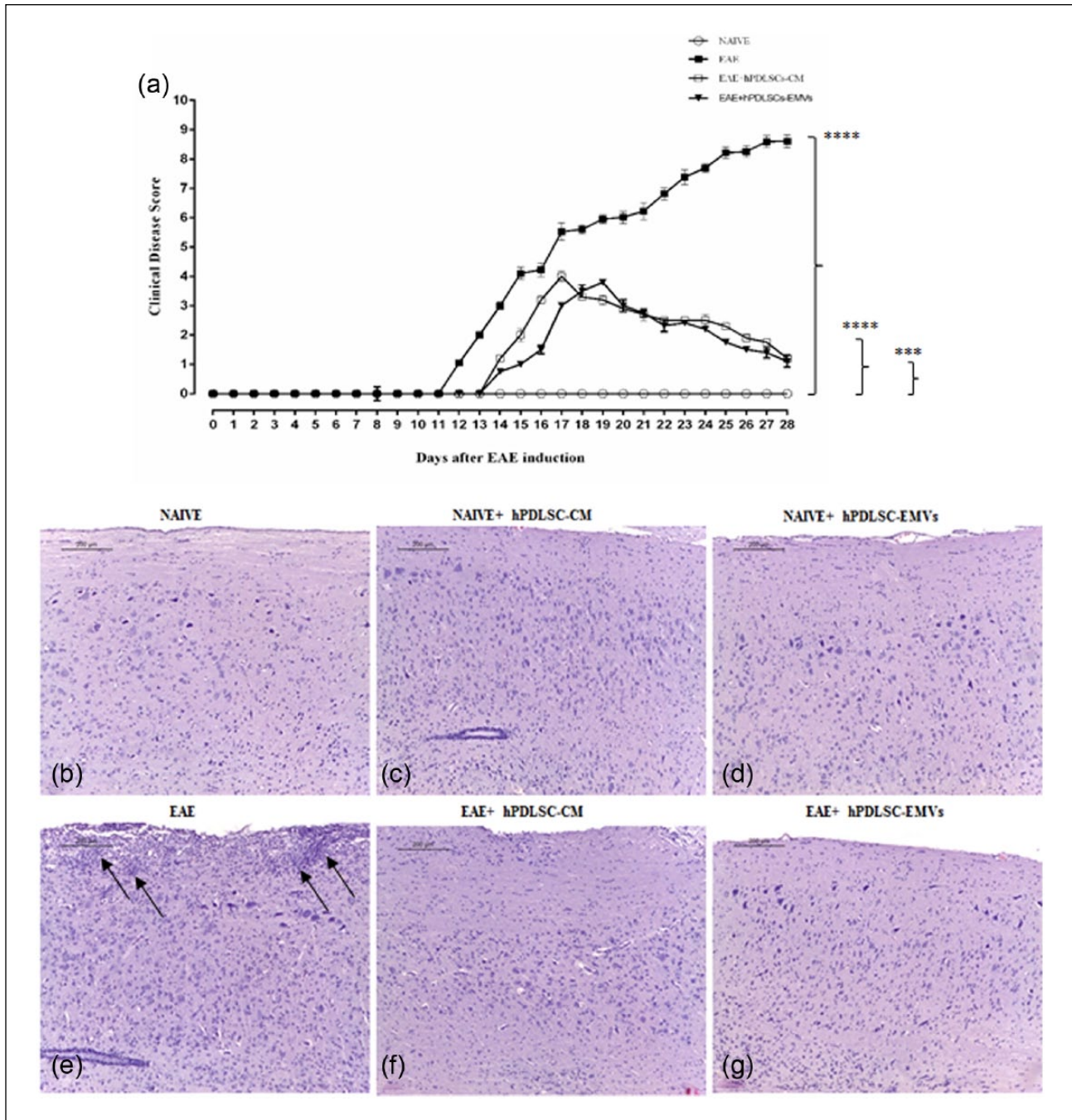


Figure 1. RR-MS patients–derived hPDLSCs-CM and purified EMVs reduce EAE progression. Clinical disease score evaluation showed severe impairment in EAE mice, while (a) treatment with hPDLSCs-CM and EMVs significantly suppressed EAE progression. $****P < 0.0001$ for naïve versus EAE; $****P < 0.0001$ for naïve versus EAE+hPDLSCs-CM; $***P = 0.0002$ for naïve versus EAE+hPDLSCs-EMVs. *P* values were calculated using one-way ANOVA followed by Bonferroni multiple comparison test. Data are represented as mean \pm SD. E/H staining displayed severe infiltration of inflammatory cells (black arrows) in (e) EAE spinal cord white matter, while treatment with (f) hPDLSCs-CM and (g) EMVs attenuated the invasion of inflammatory cells. Infiltration of inflammatory cells were unnoticed in (b) naïve mice and naïve mice treated with (c) hPDLSCs-CM and (d) EMVs.

of anti-inflammatory cytokines IL-10 and TGF- β (Figure 6). Substantial amount of immunoregulatory chemokine SDF-1 α was noticed. In addition, immunomodulatory cytokine IL-15 and chemokines monocyte chemoattractant protein-1 (MCP-1) and macrophage

inflammatory protein-1 α (MIP-1 α) were also present. Interestingly, expression of these immunomodulatory molecules present in RR-MS patients–derived hPDLSCs-CM was similar with healthy subjects–derived hPDLSCs-CM.

Table 1. Clinical parameters of EAE.

Group	Incidence (%)	Day of disease onset (avg) ± SD	Peak disease (avg) ± SD	Cumulative disease score (CDS) ± SD	Mortality
Naïve	0	0	0	0	0/5
EAE	100	13.1 ± 0.22	8.60 ± 0.50	11.97 ± 1.01	1/10
EAE + hPDLSCs-CM	100	15.3 ± 0.25	4.00 ± 0.74	3.71 ± 0.87	0/10
EAE + hPDLSCs-EMVs	100	14.9 ± 0.35	3.80 ± 0.60	3.20 ± 0.30	0/10
P value	<0.0001	0.04	0.002	<0.0001	<0.0001

SD: standard deviation; EAE: experimental autoimmune encephalomyelitis; hPDLSCs-CM: human periodontal ligament stem cells–derived conditioned medium; EMVs: exosomes/microvesicles; ANOVA: analysis of variance.

Incidence: percentage of mice that displayed any clinical signs of disease. Day of onset: the first day mice showed clinical signs. Peak of disease: the maximum value score observed between days 0 and 28. Cumulative disease score: mean cumulative disease scores were calculated by summing the daily clinical scores of living mice divided by the number of animals in the groups. One-way ANOVA followed by Bonferroni multiple comparison test was used to determine the statistical significance of differences.

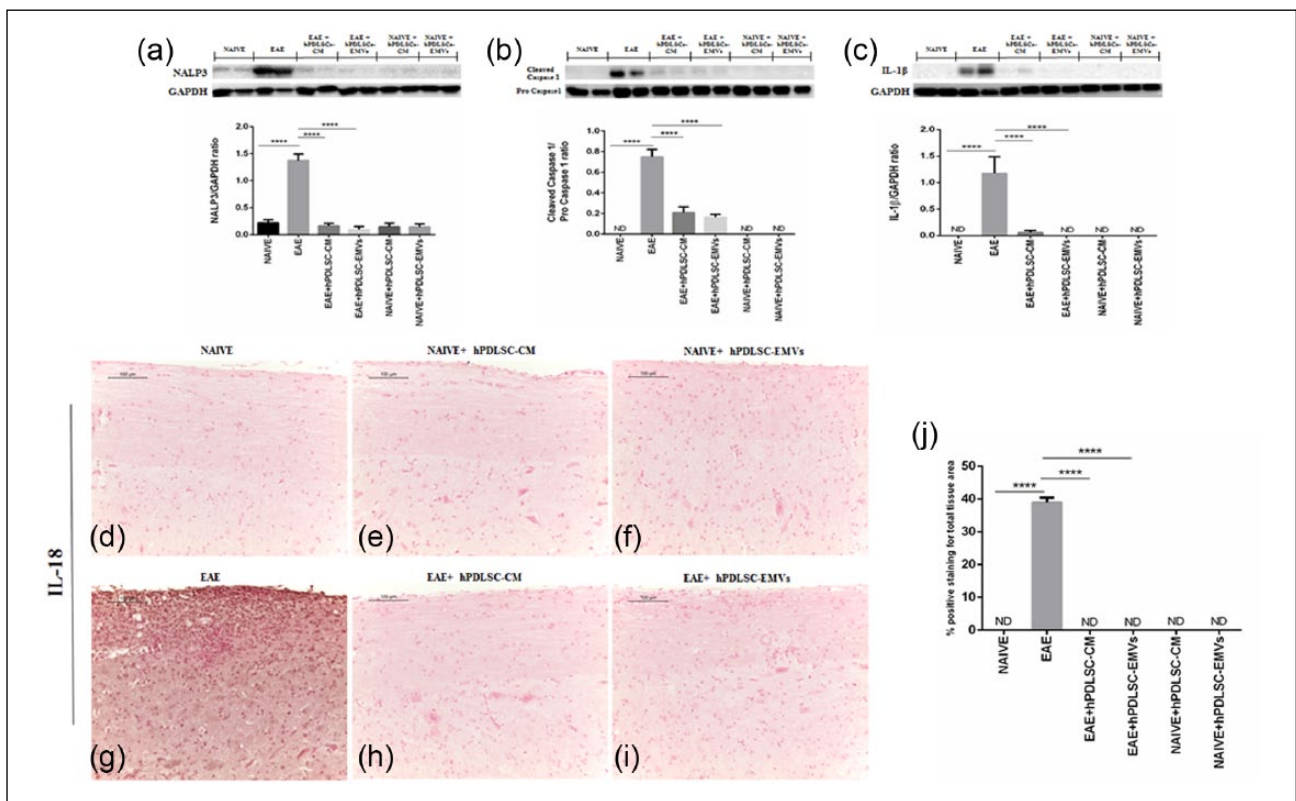


Figure 2. RR-MS patients–derived hPDLSCs-CM and purified EMVs inhibits EAE-induced NALP3 inflammasome activation. EAE induction stimulated NALP3 inflammasomes in the spinal cord of EAE mice. Western blot results showed elevated level of (a) NALP3, (b) active cleaved caspase 1, and (c) IL-1 β in EAE mice. On the contrary, significant reduction was observed in EAE mice administered with hPDLSCs-CM and EMVs. **** $P < 0.0001$ versus EAE. Data expressed as mean \pm SD. Representative bands of three separate experiments are shown. (g) Immunohistochemistry results showed significant positive staining for IL-18 in EAE mice, while in EAE mice treated with (h) hPDLSCs-CM and (i) EMVs, negative staining was noticed as that of (d) naïve mice. Naïve mice treated with (e) hPDLSCs-CM and (f) EMVs showed negative staining for IL-18. Magnification: 10 \times . (j) Densitometric analysis of IL-18 expression (**** $P < 0.0001$ vs EAE). P values were calculated using one-way ANOVA followed by Bonferroni multiple comparison test. Data are represented as mean \pm SD. ND: not detectable.

Discussion

Exploring the immunoregulatory effects of adult MSCs and their secretome has become a significant research area in the therapeutic arena of MS,

a most prevalent and debilitating autoimmune disease of the central nervous system, and other degenerative diseases.^{27,28} Dental tissues have emerged as a simple and potential alternative autologous MSCs resource due to the minimal

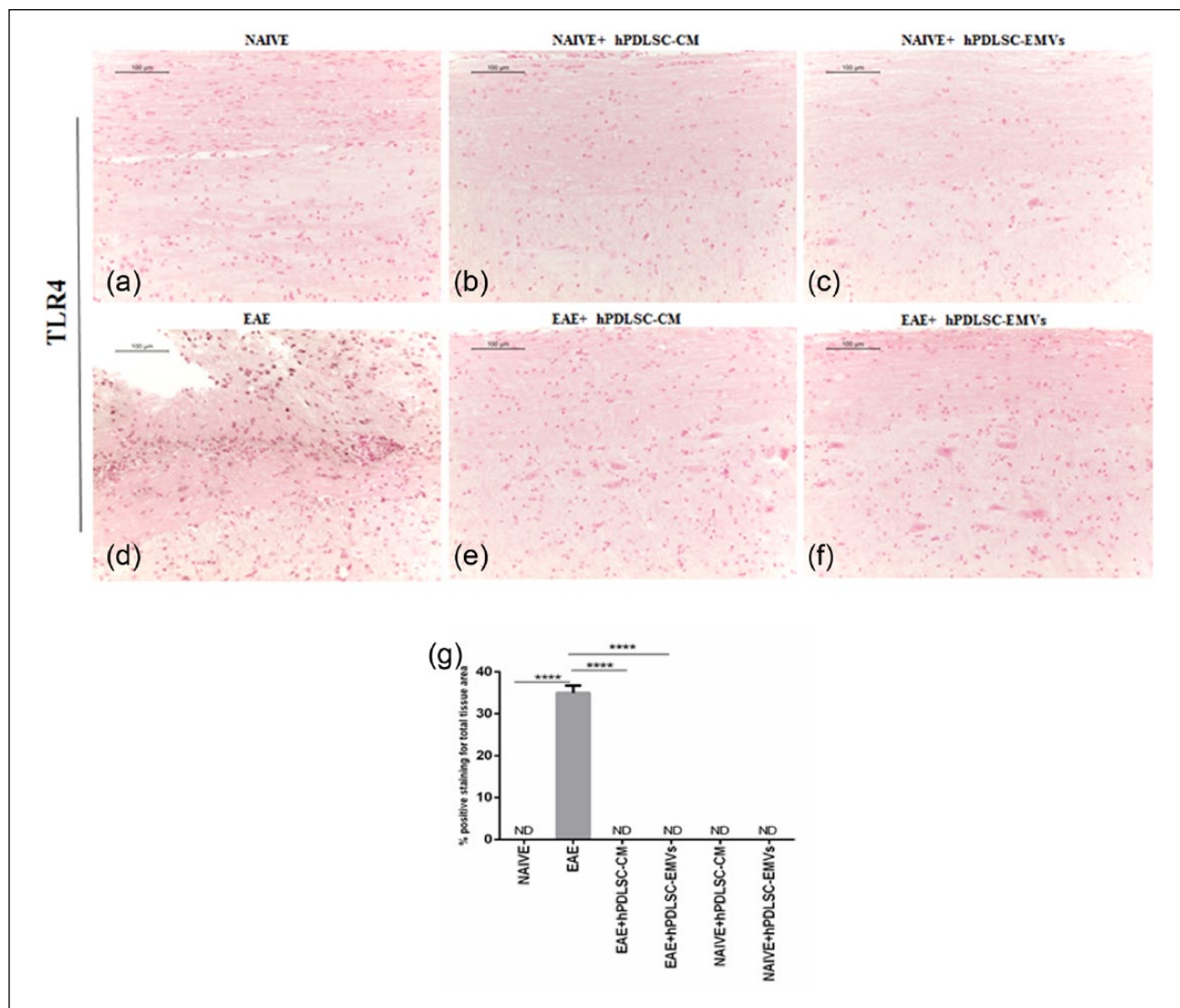


Figure 3. RR-MS patients–derived hPDLSCs-CM and purified EMVs prevents TLR4 expression in EAE mice. Immunohistochemistry data revealed that (d) EAE induction markedly activated TLR4 expression in the spinal cord with strong positive staining. On the contrary, hPDLSCs-CM and EMVs treatment significantly inhibited TLR4 activation. Negative staining was observed in (e) hPDLSCs-CM and (f) EMVs-treated EAE mice as that of (a) naïve. Similar negative staining was noticed in naïve mice treated with (b) hPDLSCs-CM and (c) EMVs. Magnification: 10×. (g) Densitometric analysis of TLR4 expression (**** $P < 0.0001$ vs EAE). P values were calculated using one-way ANOVA followed by Bonferroni multiple comparison test. Data are represented as mean \pm SD. ND: not detectable.

invasive procedure. In addition, dental MSCs possess remarkable self-renewal and differentiation capacities toward neurogenic, osteogenic, and adipogenic lineages.²⁹ Recently, we demonstrated the neuroprotective and immunosuppressive role of hPDLSCs and their CM and EMVs in EAE mice.^{12,19} Given the considerable role of inflammasome activation in MS and in *in vivo* EAE model,³⁰ in this study, we investigated the regulatory role of RR-MS patients hPDLSCs–derived CM and purified EMVs on NALP3 inflammasomes activation in EAE mice.

NALP3 inflammasome activation and its subsequent positive regulatory role in cleaved caspase 1, IL-1 β , and IL-18 in EAE mice have been already demonstrated in the previous studies.^{31,32} Likewise, in our study, we noticed that NALP3 inflammasome-linked proteins were significantly modulated. NALP3 expression was elevated in EAE mice spinal cord, which in turn proteolytically cleaved the inactive procaspase 1 into active cleaved caspase 1. Increased level of cleaved caspase 1 processed the synthesis of active IL-1 β and IL-18 from their inactive

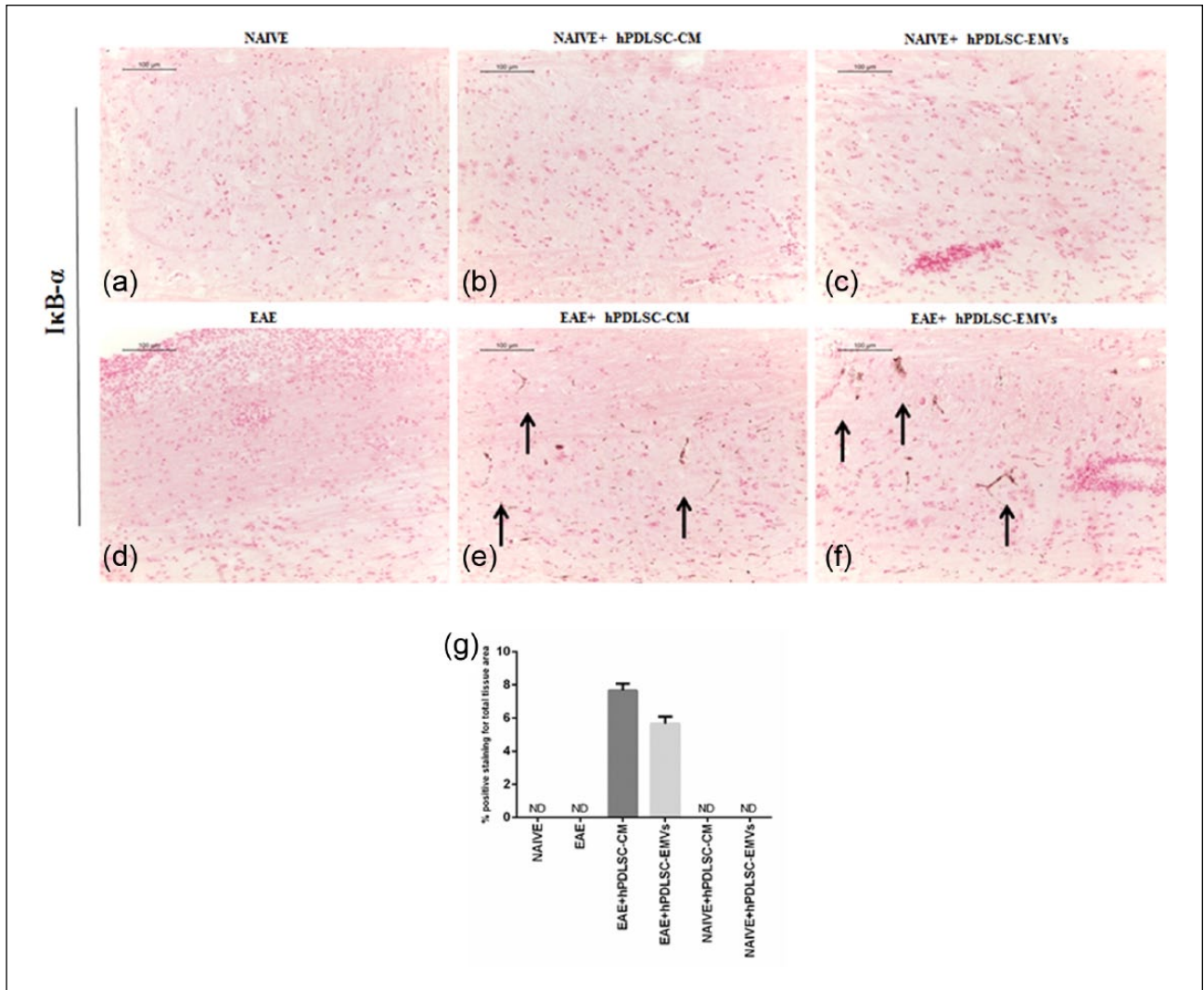


Figure 4. RR-MS patients–derived hPDLSCs-CM and purified EMVs induces IκB-α expression in EAE mice. Immunohistochemistry data revealed negative staining for IκB-α expression in (a) naïve and (d) EAE mice spinal cord. Similarly, naïve mice administered with (b) hPDLSCs-CM and (c) EMVs displayed negative staining. Conversely, significant positive staining for IκB-α was noticed in (e) hPDLSCs-CM and (f) EMVs-treated EAE mice. Magnification: 10×. (g) Densitometric analysis of IκB-α expression (*** $P < 0.0001$ vs EAE). P values were calculated using one-way ANOVA followed by Bonferroni multiple comparison test. Data are represented as mean ± SD.

ND: not detectable.

precursor forms. Interestingly, we found significant inhibition of NALP3 inflammasome activation in EAE mice treated with CM and EMVs. NALP3 expression was unaltered in CM and EMVs-administered EAE mice. These data were corroborated with the significant reduction of active cleaved caspase 1, IL-1 β , and IL-18. It is interesting to mention here that treatment with interferon (IFN)- β or steroids in MS patients resulted in the reduction of IL-1 β and the upregulation of endogenous IL-1 receptor antagonists.^{33–35} From our in vivo findings, we may

assume that IL-1 β downregulation followed by IFN- β or steroids administration in MS patients may partially depend on NALP3 suppression.

Moreover, it has been reported that NALP3 priming is a prerequisite for inflammasome formation. NF- κ B signaling is a most common pathway involved in NALP3 priming, which in turn enhanced the cytosolic NALP3 level.³⁶ Consequently, we assessed NF- κ B pathway in EAE mice. As reported earlier,^{37–40} augmented expression of TLR4, an upstream inducer of NF- κ B, and NF- κ B was found in EAE mice. NF- κ B inhibitor protein IκB-α was

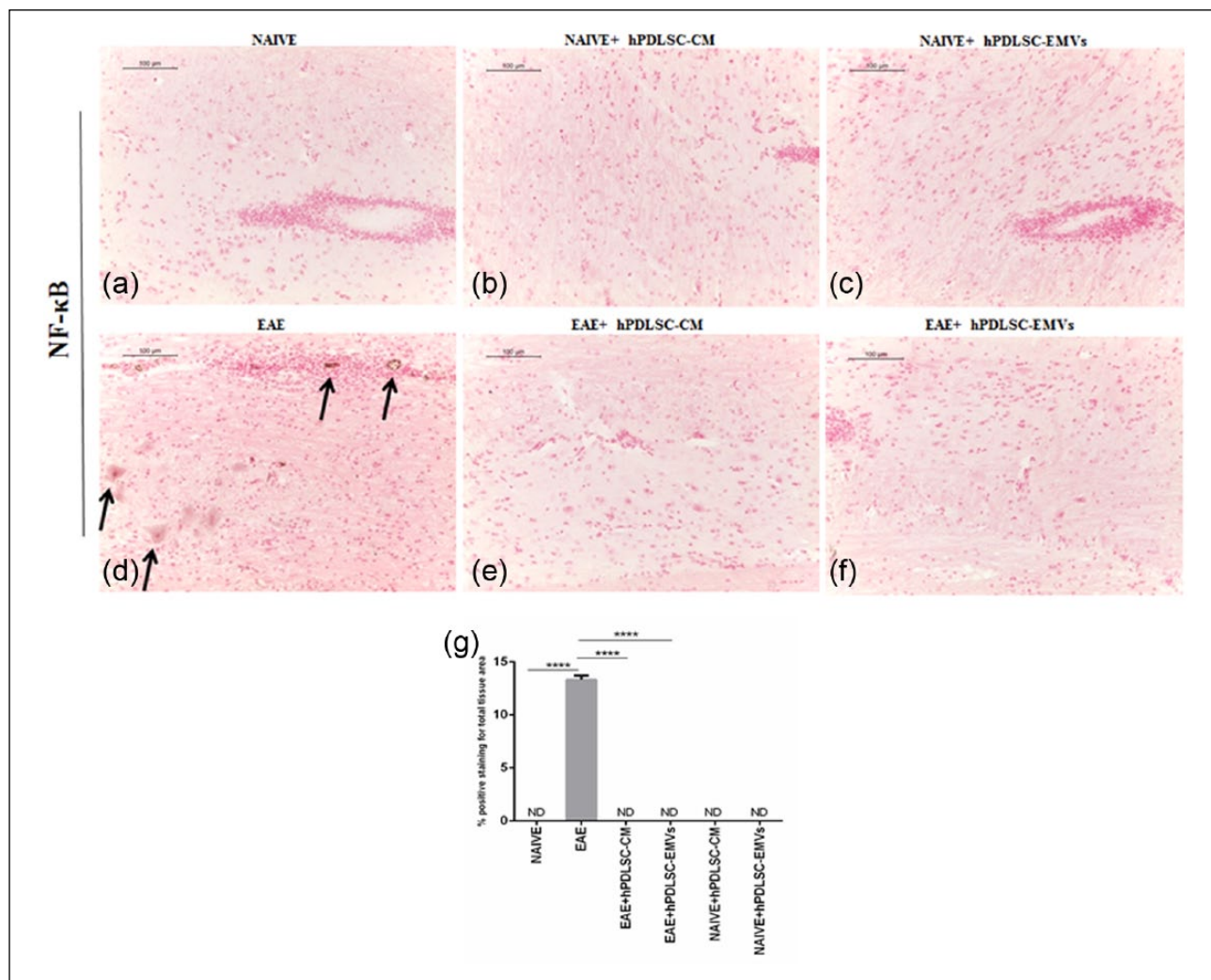


Figure 5. RR-MS patients–derived hPDLSCs-CM and purified EMVs prevents NF- κ B expression in EAE mice. (d) Immunohistochemistry data displayed positive staining for the expression of inflammatory transcription factor NF- κ B in EAE mice spinal cord. On the contrary, NF- κ B activation was completely terminated in EAE mice treated with (e) hPDLSCs-CM and (f) EMVs. Negative staining was noticed in (a) naïve mice and naïve mice treated with (b) hPDLSCs-CM and (c) EMVs. Magnification: 10 \times . (g) Densitometric analysis of NF- κ B expression (**** $P < 0.0001$ vs EAE). P values were calculated using one-way ANOVA followed by Bonferroni multiple comparison test. Data are represented as mean \pm SD. ND: not detectable.

decreased. However, CM and EMVs significantly inhibited TLR4 and NF- κ B activation, while increased the level of I κ B- α in EAE mice. Our results suggest that the anti-inflammatory role of hPDLSCs-CM and -EMVs in EAE mice may be due to the suppression of NF- κ B stimulation, which in turn inhibited NALP3 inflammasome activation.

To understand the molecular mechanisms underlying the immunosuppressive effects of hPDLSCs-derived secretome, we studied the expression of immunomodulatory cytokines and chemokines secreted from hPDLSCs. Characterization of hPDLSCs-CM derived from

RR-MS patients showed increased level of IL-10 and TGF- β . Anti-inflammatory effects of IL-10 and TGF- β have been well documented in EAE.^{41–44} Indeed, recently we demonstrated the presence of IL-10 and TGF- β in hPDLSCs-EMVs as well.¹⁹ Moreover, it has been demonstrated that IFN- β treatment in MS patients increased the synthesis of IL-10 and TGF- β , suggesting the beneficial role of these anti-inflammatory cytokines in the treatment of MS.^{45,46} Interestingly, negative regulatory role of IL-10 on NALP3 inflammasome activation has been reported earlier in lipopolysaccharide-activated macrophages.⁴⁷ Thus, we believe that

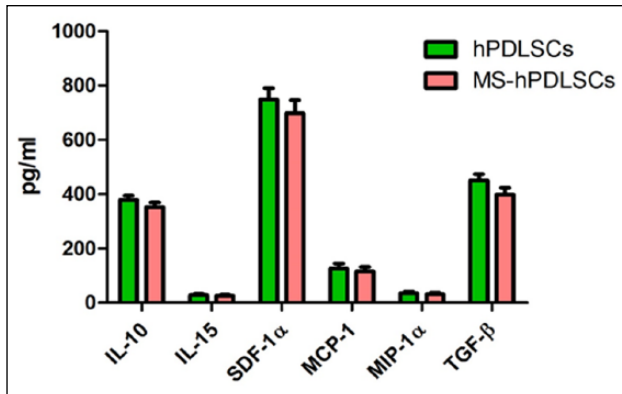


Figure 6. Immunomodulatory cytokines and chemokines expression in healthy subjects and RR-MS patients–derived hPDLSCs-CM. ELISA data revealed the presence of substantial amount of anti-inflammatory IL-10 and TGF- β cytokines and chemokine SDF-1 α in hPDLSCs-CM obtained from normal healthy subjects and RR-MS patients. Immunomodulatory cytokine IL-15 and chemokines MCP-1 and MIP-1 α were also expressed in hPDLSCs-CM.

IL-10 mediated NALP3 inflammasome reduction might be a crucial mechanism underlying hPDLSCs-derived CM and EMVs-mediated anti-inflammatory responses. In addition, hPDLSCs-CM showed a lesser presence of IL-15, a pro-inflammatory cytokine, which activates and promotes the survival of natural killer cells and memory CD8⁺ T-cells during inflammation.⁴⁸ Unlike the distinguished effector role of CD4⁺ cells (Th1 and Th17) in EAE, the role of CD8⁺ T-cells in EAE is unclear as studies have demonstrated both stimulative and suppressive effects during EAE development.⁴⁹ Recent studies demonstrated that IL-15 inhibits EAE progression by reducing CD4⁺ cells proliferation.^{49,50} Our results support this finding that the presence of IL-15 in hPDLSCs-CM, although present in low amount, may produce immunosuppressive effects by CD4⁺ cells inhibition in EAE. As reported in our previous work, reduced expression of CD4 in the spinal cord and spleen of EAE mice treated with hPDLSCs-CM¹⁹ further support our notion on negative regulation of CD4⁺ cells by IL-15.

In our study, we noticed substantial amount of immunomodulatory chemokine SDF-1 α in hPDLSCs-CM. CXC type SDF-1 α (also called as CXCL12) is a chemokine, which acts as a potent chemoattractant for B and T cells⁵¹ and promotes neural precursor cells migration in pathological conditions such as ischemic stroke.⁵² In EAE, SDF-1 α was addressed to be a suppressive factor

of the disease progression by redirecting the polarization of Th1 cells into IL-10 secreting Treg cells.⁵³ Interestingly, it has been reported that intrathecal application of SDF-1 α reduced the expression of NALP3 inflammasome activation in rat spinal cord injury model.⁵⁴ SDF-1 α expression in hPDLSCs-CM from our study corroborated these previous studies, suggesting a potential negative regulatory during EAE progression, which may depend on NALP3 inflammasome inactivation and Treg cells activation. In addition, we observed a lesser amount of MCP-1 and MIP-1 α chemokines in hPDLSCs-CM. Pro-inflammatory role of MCP-1 and MIP-1 α has been reported in EAE development and progression.⁵⁵ Conversely, these chemokines are also involved in neural stem cells migration followed by neuroinflammatory conditions such as striatal lesion and cerebral ischemia.⁵⁶ Given their low amount of secretion in hPDLSCs-CM, we assume that MCP-1 and MIP-1 α may not exert considerable immunoregulatory or neuroprotective effects in EAE mice due to the presence of substantial level of IL-10, TGF- β , and SDF-1 α .

Taken together, we assume that the immunosuppressive effects stimulated by RR-MS patients hPDLSCs-CM and -EMVs in EAE mice might occur due to the existence of these inflammation regulatory molecules and that NALP3 inflammasome inactivation might be partly involved in the overall anti-inflammatory response. Interestingly, expression of these cytokines and chemokines in CM and EMVs derived from hPDLSCs of RR-MS patients was similar to that of hPDLSCs derived from healthy subjects, which suggest that MS pathology, per se, has no marked role in regulating the expression of cytokines and chemokines. Thus, our results propose hPDLSCs as a simple autologous MSCs resource in stem cell therapy for MS patients. Furthermore, we observed that the anti-inflammatory effects triggered from CM and purified EMVs were analogous with respect to EAE suppression. Considering the requirement of enormous amount of time and money for EMVs purification, results from our study suggest that hPDLSCs-CM alone could be adequate for therapeutic intervention in MS patients. However, comprehensive high-throughput analysis of the proteome present in both CM and purified EMVs is necessary before proceeding to clinical trials.

Conclusion

In summary, our research findings suggest that the secretory molecules present in CM and purified EMVs obtained from hPDLSCs of RR-MS patients modulate NF- κ B level, inhibit NALP3 inflammasome activation, and exert protection in EAE mice. We conclude that hPDLSCs might be a simple and potential autologous stem cell resource in the treatment of MS patients. Both hPDLSCs-derived CM and purified EMVs exert comparable immunosuppressive effects. We propose that CM alone obtained from hPDLSCs may serve as effective and economical therapeutic tool in MS treatment.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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