TOLL-LIKE RECEPTOR 4 EXPRESSION, INTERLEUKIN-6, -8 AND CCL-20 RELEASE, AND NF-KB TRANSLOCATION IN HUMAN PERIODONTAL LIGAMENT MESENCHYMAL STEM CELLS STIMULATED WITH LPS-*P. GINGIVALIS*

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Periodontal diseases, the major public health problem of the oral cavity, are clinically characterized by inflammation of the periodontal connective tissue that ultimately induces the destruction of periodontal tissue and the loss of alveolar bone. In chronic periodontitis, as well as aggressive periodontitis, the anaerobic gram-negative bacterium *Porphyromonas gingivalis (P. gingivalis)* is implicated. The pathogenicity of *P. gingivalis* is exerted by a wide variety of factors, including lipopolysaccharides (LPSs). LPSs activate the innate immune response during Gram-negative bacterial infections through the Toll-like receptor 4 (TLR-4)/myeloid differentiation protein 2 (MD-2) complex. In this study, the expression of TLR-4, the cell growth, the cytokine release, and the nuclear factor-KB (NF-kB) transcription factor expression in response to LPS-*P.Gingivalis* (LPS-G) were examined in Human Periodontal Ligament Mesenchymal Stem Cells (PDL-MSCs). The results obtained demonstrate that, in basal conditions, human PDL-MSCs express high levels of TLR-4. In inflammatory conditions mimicked by LPS-G challenge, the MTT assay carried out at different treatment times demonstrated the decrease of the cell growth. Moreover, the recognition of *P. gingivalis* components by TLR-4 culminated with the activation of secretion of inflammatory mediators such as: IL-6, IL-8 and CCL-20, and with the up-regulation of NF-kB, which was translocated into the nucleus. Our data intended to specify that TLR-4 expressed by PDL-MSCs is functional and plays a key role in inflammation.

Periodontal diseases, the major public health problem of the oral cavity, are clinically characterized by a bacterial biofilm-initiated inflammatory reaction which gives rise to gingival inflammation, bleeding, extracellular matrix (ECM) degradation, bone resorption, and tooth loss (1).

The development of periodontitis has been strongly associated with the presence of periodontogenic bacterial species, such as *Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans,* and *Tannerella forsythia*, in the subgingiva. The anaerobic gram-negative bacterium, *P. gingivalis*, is implicated not only in chronic periodontitis but also in aggressive periodontitis and its pathogenicity is exerted by a wide variety of factors,

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including, fimbriae, hemagglutinin, gingipains, and lipopolysaccharides (LPS) (2, 3). LPS, also known as endotoxin, is the pathogen-associated molecular pattern on Gram- bacteria that activates the innate immune response (4).

Several reports have demonstrated that Toll-like receptors (TLRs) are involved in the recognition of bacterial cell wall components (5). Toll-like receptor 4(TLR4)-deficient mice showed hyporesponsiveness to LPS, demonstrating that TLR4 is a critical receptor for LPS signaling (6). Recently, it was demonstrated that the binding of *P. gingivalis* LPS to TLR4 on Human gingival fibroblasts (HGFs) activates various second messenger systems (7), and in particular the common signaling feature among all TLRs is the activation of the transcription factor nuclear factor-kB (NF-kB) which has been implicated in controlling the expression of inflammatory cytokines and cell maturation molecules (8).

It was recently recognized that TLRs mediate stress responses of bone marrow progenitor cells and promote the migration and immune regulation of mesenchymal stem cells (9). In fact, it was demonstrated that cultured MSCs express TLR molecules 1-8 and activation of MSCs by TLR ligands induced IL-6 secretion and NF-kB translocation (10); in particular, Liotta demonstrated that LPS was able to induce NF-kB activation, and cytokine and chemokine production in MSCs (11).

Although studies suggest that activation of TLRs modulate the function of mesenchymal stem cells (MSCs) (12), little is known about the role of TLR4 on PDL-MSCs. Recently we isolated a heterogeneous population of MSCs from periodontal ligament (PDL) (13). Like bone marrow mesenchymal stem cells, PDL-MSCs express MSCs markers CD105, CD166, CD90 and CD73, differentiate into osteoblasts and adipocytes, and secrete IL-7 and SDFα (14). The PDL-MSCs contain a more immature mesenchymal stem cell CD349/FZD9 positive subpopulation, expressing the embryonic markers SSEA-1 and SSEA-4 and transcription factors nanog and Oct-4; furthermore, the frizzled-9/wnt pathway plays an important role in MSCs regulation in periodontal tissues (15).

Chang et al. (16) demonstrated that both TNF and LPS activated the I-kappa B kinase complex (IKK) in Dental Pulp Stem Cells (DPSC) to induce the phosphorylation and degradation of Ik-B, resulting in the nuclear translocation of NF-kB. Consistently, both TNF and LPS rapidly induced the expression of the NF-kB-dependent gene interleukin-8 (IL-8), suggesting that DPSCs may be involved in immune responses, and the activation of NF-kB during pulp infection.

In this study, to understand the role of and the response to LPS-G we examined cell growth, IL-6, IL-8 and CCL-20 release and the NF-kB signaling pathway in periodontal stem cells.

MATERIALS AND METHODS

Cell culture

Human PDL biopsies were carried out after informed consent on five volunteers aged 20–35 years. All subjects were free of systemic and oral diseases. Explants were obtained from alveolar crest and horizontal fibers of the PDL, by scraping the roots of non-carious third molars with a Gracey's curette (Carranza and Ubios, 2003). The PDL-MSCs were obtained and cultured in MSCM medium (Cambrex Company, Walkersville, MD, USA) according to the manufacturer's instructions. Cells migrated from the explants and on day 7 they were 80–90% confluent, as determined by phase contrast microscopy. Adherent cells were isolated using 0.1% trypsin solution and plated in tissue culture polystyrene flasks at $5x10^3$ cells/cm^2. PDL-MSCs at II passage were used in all experiments.

LPS challenge

PDL-MSCs were seeded at a density of 5×10^4 cells/well and incubated in the absence (controls) or presence of 5μg/mL of LPS from *P. gingivalis* (InvivoGen, San Diego, CA, USA). After 12, 24, 48 and 72 h of culture, the cells were analyzed by multiparametric analyses.

MTT assay

The cell viability was evaluated by MTT assay. Briefly, the cells were seeded into flat-bottom 96-well plates ($3x10^3$/well) in 100 μl medium. After 24 h incubation for attachment and starvation in 1% FBS-MSCBM , the medium was replaced with fresh medium for different time intervals (24, 48, and 72 h). At the designated time, 10 μl MTT (Promega, Milan, Italy) were added to each well and incubated for 3 h. Absorbance at 570 nm was measured with a reference wavelength of 630 nm. The cytotoxicity experiments were conducted independently in duplicate with six replicates for each per experiment.

Flow cytometer analysis

PDL-MSCs were characterized by flow cytometry using against TRL-4 PE-conjugated monoclonal antibody

(1:100, Becton-Dickinson, San Jose, CA). One million cells were incubated with antibodies for 30 min at 4°C. Excess antibody was removed by washing and the stained cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton-Dickinson). Cut-off markers were set individually for each measurement according to the negative control. Fluorescence intensity for a surface antigen was calculated as the geometric mean (MFI) of all cells above cut off.

Confocal laser scanning microscope analysis

Cells grown on glass coverslips were fixed for 10 min at RT with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS), pH 7.2. After washing in PBS, cultures were processed for immunofluorescence labeling. Briefly, PDL-MSCs were permeabilized with 0.5% Triton X-100 in PBS for 10 min, followed by blocking with 5% skimmed milk in PBS for 30 min. Primary monoclonal antibodies anti-human NF-kB(1:100) and TLR-4(1:100) (from Santa Cruz Biotechnology, Inc; Santa Cruz, CA, USA) were used, followed by Alexa Fluor 488 green fluorescence conjugated goat anti-mouse as secondary antibodies (1:200) (Molecular Probes, Invitrogen, Eugene, OR, USA). Subsequently, the PDL-MSCs were incubated with Alexa Fluor 594 phalloidin red fluorescence conjugate (1:400, Molecular Probes), marker of the actin cytoskeleton. Before mounting for microscope observation, samples were briefly washed with dH_2O and cell nuclei stained with TOPRO 1:200 (Molecular Probes) for 5 min. The Glass coverslips were placed face down on glass slides and mounted with Prolong antifade (Molecular Probes). Staining was visualized using a Zeiss LSM510META confocal system (Jena, Germany), connected to an inverted Zeiss Axiovert 200 microscope equipped with a Plan Neofluar oil-immersion objective (40x). Images were collected using an argon laser beam with excitation lines at 488 nm and a helium-neon source at 543 and 665 nm.

Western blot analysis

Thirty micrograms of proteins from treated and untreated PDL-MSCs were separated on SDS-PAGE and subsequently transferred to nitrocellulose sheets using a semidry blotting apparatus. Sheets were saturated for 60 min at 37°C in blocking buffer (1xTBS, 5% milk, 0.05% Tween-20), then incubated overnight at 4°C in blocking buffer containing primary antibodies to NF-kB(1:100) and β-actin (1:300) (from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After four washes in TBS containing 0.1% Tween-20, samples were incubated for 30 min at room temperature with peroxidase-conjugated secondary antibody diluted 1:1000 in 1x TBS, 5% milk, 0.05% Tween-20. Bands were visualized by the ECL method. The level of recovered protein was measured using the Bio-Rad (Hercules, CA) Protein Assay (detergent compatible) according to the manufacturer's instructions.

Cytokine assays

PDL-MSCs cells were cultured in 6-well plates in RPMI, without FCS for 24 h. The cells were then treated with LPS-G and after 24 h of culture the supernatants of treated and untreated PDL-MSCs were collected, and subsequently, to evaluate IL-6, IL-8, and CCL-20 (R&D System, Minneapolis, MN, USA) expression enzyme-linked immune-absorbent assay (ELISA) was performed. The test was carried out according to the manufacturer's instructions, and the supernatants were normalized according to the cell numbers.

Statistical analysis

The growth of treated and untreated PDL-MSCs was calculated as average and variance by two-way repeated measures Anova test. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Effects of LPS-G on cell morphology and cell proliferation

Light microscopy evaluation of *ex vivo*-expanded untreated PDL-MSCs at the second passage showed that the majority of cells were spindle shaped with extended cellular processes (filopodia and lamellipodia) and elliptical nuclei (Fig. 1A).

After 24 h of culture, morphological changes in treated samples were not evident. The cells had a morphologically homogeneous fibroblast-like appearance with a stellate shape and long cytoplasmic processes that made contact with neighbouring cells (Fig. 1B) .

Primary culture of PDL-MSCs at the II passage were used to examine the response of cell growth effects following treatment with 5μg/mL of LPS-G for 24, 48, and 72 h. The cell proliferation was assessed using the MTT assay, and the results obtained showed that when PDL cells were exposed to stimuli, cell viability was reduced in a time-dependent manner compared to controls (Fig. 1C). Fig. 1D shows the variance values recorded in the experimental groups.

TLR-4 receptor evaluation

The expression of TLR-4 receptor was evaluated

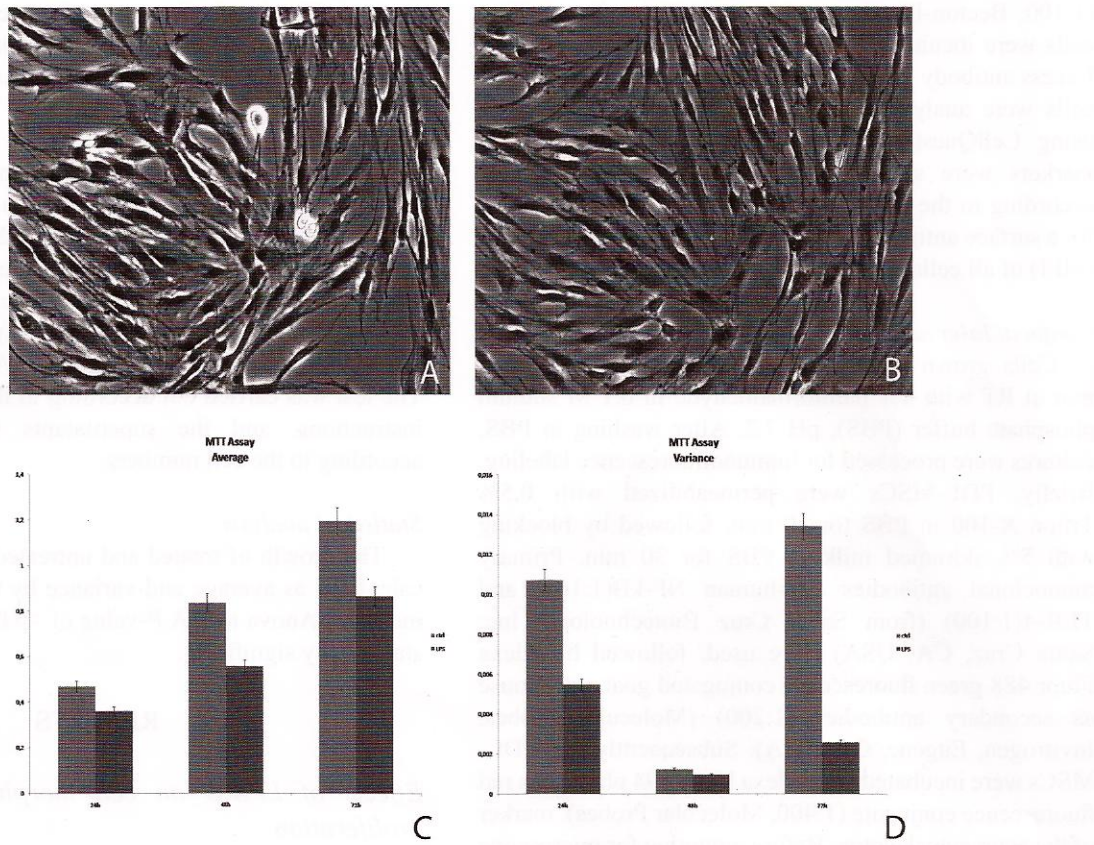

Fig. 1. *A) Micrograph of PDL-MSCs expanded ex vivo forming colonies observed under inverted light microscope. The cells show fibroblastoid-like morphology with stellate shape and long cytoplasmic processes.* ***B)*** *The cells do not show remarkable morphological changes after 24 h of LPS-G treatment. Original magnification 25x.* ***C)*** *MTT assay shows the cell viability of PDL-MSCs, at the II passages, exposed to LPS-G for 24, 48, and 72 h. During the treatment, there was a drastic reduction in cell viability starting at 24 h.* ***D)*** *Variance between the analyzed samples by Anova test.*

in PDL -MSCs by immunohistochemistry and cytofluorimetric analysis. The cytofluorimetric data indicated the presence of the TLR-4 receptor on the cell surfaces (Fig. 2A); this finding was supported by confocal fluorescence microscopy evaluation, in fact a marked positivity was detectable at cytoplasmic and membrane level (Fig. 2B).

NF-k B activation in PDL-MSCs after LPS-G treatment

The role of NF-kB pathways in PDL-MSCs was confirmed by western blotting and immunohistochemistry analysis. Western blotting analysis showed an up-regulation of the transcription factor starting at 12 h of treatment (line B), compared to basal sample (0 time, line A). The molecule expression increased in a time-dependent manner, as proved from results after 24 h of treatment (Fig. 2D, line C). Fig. 1 D shows the expression of β-actin in basal condition (line A), 12 h (line B) and 24 h of treatment (line C). The confocal microscopy analysis showed, in the control sample, the presence of the transcription factor localized only in the interchromatinic region (Fig. 2C).

Cytokine release

The IL-6 and IL-8 and CCL20 level, estimated using the immunoassay test, increased in the supernatant of treated cells in comparison with basal PDL-MSCs (Fig. 3). The samples were normalized

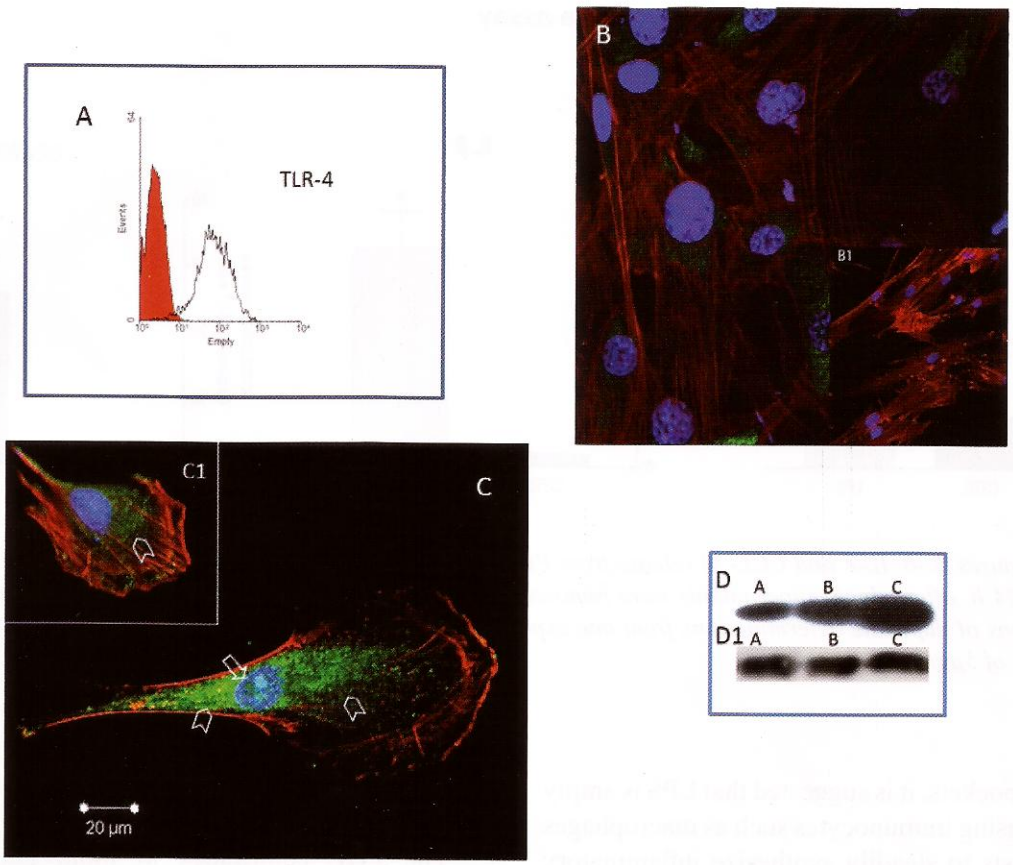

Fig. 2. *A) Cytofluorimetric profile of the surface expression of the TLR-4. These data are representative of five separate experiments. The red histogram represents the negative control.* ***B)*** *Triple labeling with TLR-4/phalloidin/TOPRO (green, red and blue fluorescence, respectively) of PDL-MSCs at the second passage. A punctuate TLR-4 labeling was observed on the cell membrane and on the cytoplasmic level. The inset (B1) shows a negative control following double labeling with phalloidin/TOPRO. Original magnification 40X.* ***C)*** *PDL-MSCs after 24 h of LPS-G treatment. The presence of NF-kB as specific immunoprecipitate was evident at cytoplasmic region. At nuclear level, in particular at the interchromatinic region, the translocated nuclear transcription was present. C1 shows the control sample. The immunoprecipitate was noticeable only to cytoplasmic compartment. Original magnification 40X.* ***D)*** *Western blotting analysis of PDL-MSCs treated for 0, 12, 24 h with LPS-G. An up-regulation of the NF-kB was evident starting at 12 h of treatment and in section D1 the expression of β-actin as housekeeping protein was analysed.*

according to the number of cells.

DISCUSSION

In humans, bone loss associated with periodontal disease is thought to occur from the persistence of inflammatory cells in gingival tissue that subject alveolar bone and PDL to injury and degradation (17). The host inflammatory response is thus an essential determinant in the progression and severity of periodontal disease (18).

Many basic and clinical studies support the concept that human periodontitis is a disease caused by several types of Gram-negative bacteria that are mainly present in dental plaque (19). In oral pathology, it is accepted that periodontal disease is the end result of the host immune response to LPS, rather than the result of a one-sided invasion of a periodontopathic bacterium.

When periodontopathic bacteria proliferate in

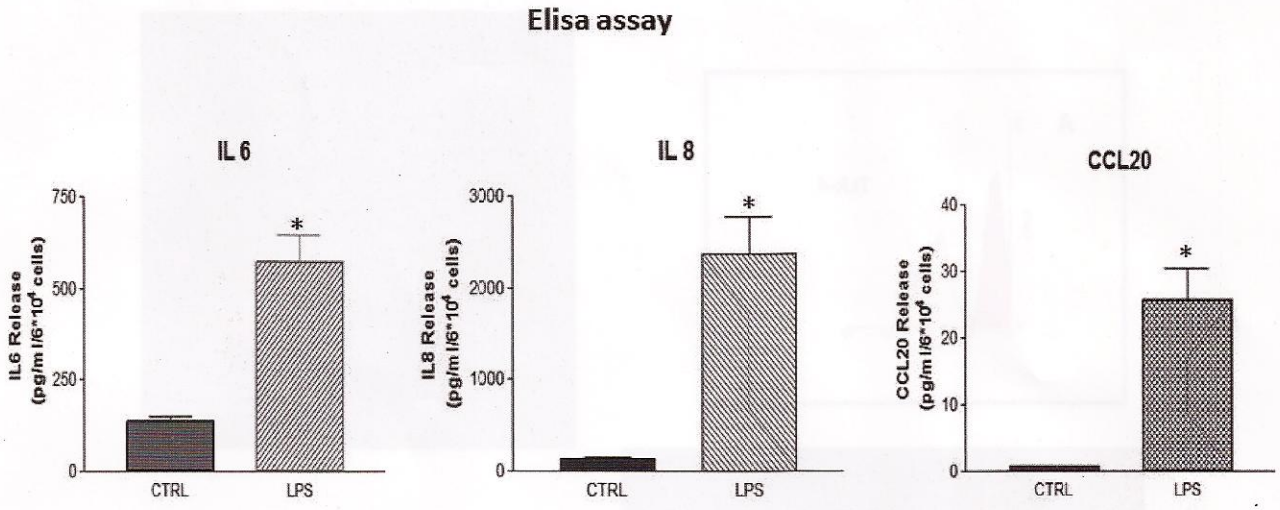

Fig. 3. *LPS causes IL-6, IL-8 and CCL-20 release from PDL-MSC. PDL-MSCs ($6*10^4$ /well) were plated in serum-free MSCBM for 24 h, after which, supernatants were removed and assayed for IL-6, IL-8 and CCL20 content by ELISA. Data are means of duplicate determinations from one experiment representative of three others. LPS-G was used at a concentration of 5μg/mL.*

periodontal pockets, it is suggested that LPS is amply released, causing immunocytes such as macrophages and fibroblasts to steadily synthesize inflammatory cytokines, including interleukin (IL)-1 and IL-6, that aggravate inflammation, destroying periodontal tissues, and inducing alveolar bone resorption (20).

Adults affected with periodontitis have a higher serum level of immunoglobulin G (IgG) antibodies against *P. gingivalis* than control individuals; many investigators have reported that patients with periodontitis have elevated levels of antibodies against sonicates of *P. gingivalis* in both the serum and gingival crevicular fluid (20). Moreover, it plays an important role in the pathogenic mechanism in systemic diseases (20). In fact, considerable evidence supports a plausible set of mechanisms by which periodontopathic bacteria may directly or indirectly contribute to cardiovascular diseases, such as blood platelet aggregation, enhanced low-density cholesterol and lipoprotein deposition in the arterial wall, invasion of cardiac and carotid endothelium, and a high level of inflammatory mediators in the circulation and tissues (21).

The periodontal ligament contains postnatal MSCs capable of regenerating several tissues of mesenchymal origin, such as bone, cartilage, and adipose tissue, as well as cementum/PDL-like structures which may take part in periodontal repair (13, 15).

The PDL contributes to tooth nutrition and homeostasis, helps to dampen mastication mechanical forces and plays an important role in adjacent bone remodeling. Periodontal ligament retains a regenerative capacity during adulthood as it contains progenitor cells, which maintain their proliferation and differentiation potential in various cell lineages including osteoblasts, fibroblasts, and cementoblasts (22).

Previously, our group characterized, phenotypically and functionally, human MSCs isolated from PDL and demonstrated similarities to bone marrow MSCs (13). In this work we have focused our attention on response of PDL-MSCs to LPS-G stimuli.

The data obtained demonstrated that periodontal stem cells express a functional TLR-4 receptor, and binding of the LPS receptor activates the NF-kB signaling pathways. In fact, an increased level of expression and a nuclear translocation of the transcription factor is evident in treated cells. Moreover, a substantial increase of pro-inflammatory cytokines IL-6 and IL-8 and CCL-20 chemokine production is visible after 24 h of stimulation with 5μg/ml LPS.

IL-6 belongs to an important family of mediators secreted from T cells, macrophages and osteoblasts. It is involved in the regulation of the acute-phase response to injury and infection (23). This cytokine plays a crucial role in haematopoiesis, liver and neuronal regeneration, and embryonic development and furthermore, it is related to inflammation and to immune response processes (23).

Deregulation of IL-6-type cytokine signaling contributes to the onset and maintenance of several diseases exerting its action through the activation of the JAK/ STAT (Janus kinase/signal transducer and activator of transcription) and MAPK (mitogen activated protein kinase) cascades (23).

IL-8 is produced by phagocytes and mesenchymal cells exposed to inflammatory stimuli, and it activates neutrophils inducing chemotaxis, exocytosis and the respiratory burst. *In vivo*, this chemokine is one of the major mediators of the inflammatory response; it elicits a massive neutrophil accumulation at the site of injection, functioning as a chemoattractant, and is also a potent angiogenic factor (24).

Recently, we demonstrated that Hema, a critical factor of inflammation, up-regulates IL-6 and IL-8 secretion in dental pulp stem cells (25).

CCL-20, is the only chemokine known to interact with CC chemokine receptor 6 (CCR6), a property shared with the antimicrobial β-defensins. The ligand-receptor pair CCL-20-CCR6 is responsible for the chemoattraction of immature dendritic cells (DC), effector/memory T-cells and B-cells and plays a role at skin and mucosal surfaces under homeostatic and inflammatory conditions, as well as in pathology, including cancer and rheumatoid arthritis (26).

A recent paper demonstrated the presence of CCL20/MIP-3a in mesenchymal/osteoprogenitor cells (27) and moreover, that CCL-20 induced the gene expression of IL-6, IL-lß, and COX-2 and upregulates the release of IL-6 and MMP-3 in human fibroblast-like synoviocytes (28). Additionally, strong evidence shows that CCL-20/CCR6 signaling through a PLCL1/PKC/MEK1/2/ERK1/2 pathway drives transcriptional and post-transcriptional control mechanisms (28).

In synthesis, our cell model seems to be a valuable tool for the evaluation of the response of stem cells to LPS-G, compounds that almost certainly are responsible for influencing periodontal diseases. The distinct ability of the periodontal ligament cells to secrete IL-6, IL-8 and CCL-20 emphasizes that these cells may contribute to the release of cytokines in LPS-challenged periodontium and offer the opportunity to better comprehend the degenerative processes in periodontal diseases.

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