Porphyromonas gingivalis lipopolysaccharide stimulation in human periodontal ligament stem cells: Role of epigenetic modifications to the inflammation

Francesca Diomede,[1] Soundara Rajan Thangavelu,[2] Ilaria Merciaro,[1] Monica D'Orazio,[3] Placido Bramanti,[2] Emanuela Mazzon,[2] Oriana Trubiani[1]

[1]Stem Cells and Regenerative Medicine Laboratory, Department of Medical, Oral and Biotechnological Sciences, University "G. d'Annunzio" Chieti-Pescara, Chieti, Italy
[2]IRCCS Centro Neurolesi "Bonino Pulejo", Messina, Italy
[3]Department of Rheumatology and Immunology, Medical University of Graz, Austria

Abstract

Periodontitis is a chronic oral inflammatory disease produced by bacteria. Gingival retraction and bone and connective tissues resorption are the hallmarks of this disease. Chronic periodontitis may contribute to the risk of onset or progression of neuroinflammatory pathological conditions, such as Alzheimer's disease. The main goal of the present study was to investigate if the role of epigenetic modulations is involved in periodontitis using human periodontal ligament stem cells (hPDLSCs) as an *in vitro* model system. hPDLSCs were treated with lipopolysaccharide of *Porphyromonas gingivalis* and the expression of proteins associated with DNA methylation and histone acetylation, such as DNMT1 and p300, respectively, and inflammatory transcription factor NF-kB, were examined. Immunofluorescence, Western blot and next generation sequencing results demonstrated that *P. gingivalis* lipopolysaccharide significantly reduced DNA methylase DNMT1, while it markedly upregulated the level of histone acetyltransferase p300 and NF-kB in hPDLSCs. Our results showed that *P. gingivalis* lipopolysaccharide markedly regulate the genes involved in epigenetic mechanism, which may result in inflammation induction. We propose that *P. gingivalis* lipopolysaccharide-treated hPDLSCs could be a potential *in vitro* model system to study epigenetics modulations associated with periodontitis, which might be helpful to identify novel biomarkers linked to this oral inflammatory disease.

Introduction

Periodontal disease is a chronic inflammatory disease caused by sub gingival bacterial biofilm.[1] This biofilm formation causes gingival retraction and bone and connective tissue resorption.[2] Moreover, it has been demonstrated that in chronic condition inflammatory modulators associated with periodontitis may cause systemic inflammation, participate in the onset or in the progression of neuroinflammatory conditions, including Alzheimer's disease (AD) and produce cognitive decline.[3-5] Although the real mechanism underlying the destruction of tooth supporting structures is not completely understood, bacterial interaction with the cells present in the oral cavity could be considered as a key pathogenetic step followed by biofilm synthesis. Moreover, mesenchymal stem cells (MSCs) present in the oral cavity are affected by bacteria and bacterial derivatives.[6] Different types of dental MSCs have been reported: dental pulp stem cells,[7] exfoliated deciduous teeth stem cells,[8] periodontal ligament stem cells,[9] apical papilla stem cells,[10] dental follicle stem cells,[11] and gingiva stem cells.[12,13] Human periodontal ligament stem cells (hPDLSCs) displayed substantial proliferation capacity *ex vivo*[14,15] and mesengenic differentiation capacity.[16]

Various bacteria and their pathogen associated molecules including lipopolysaccharides (LPS) activate a cascade of chronic inflammatory events and modulate the host tissue response. Many types of gram-negative anaerobic bacteria are involved in periodontitis. *Porphyromonas gingivalis* is the major periodontitis pathogen, that triggers initiation and progression of periodontal diseases.[17,18] In addition to bacteria, genetics and environmental factors also play a crucial role in the etiology of periodontitis *via* regulating epigenetic modifications.[19] Bacteria and their products can produce alterations in DNA methylation, which modifies the regulation of inflammatory genes followed by disease progression.[20-22] DNA methylation and histone acetylation are the major epigenetic modifications induced by diseases and environmental factors.[23,24] DNA (cytosine-5) methyltransferase 1 (DNMT1) and histone deacetylases (HDACs) are the key controllers, which regulate DNA methylation and histone acetylation, respectively.[25]

Correspondence: Oriana Trubiani, Department of Medical, Oral and Biotechnological Sciences, University "G. d'Annunzio" Chieti-Pescara, Via dei Vestini 31, 66100 Chieti, Italy.
Fax: +39.0871.3554033.
E-mail: trubiani@unich.it

Key words: Periodontitis; inflammation; human periodontal mesenchymal stem cells; epigenetic regulation; DNMT1; p300; Alzheimer's disease.

Acknowledgments: this work has been supported with OT ex 60% funds, University "G. d'Annunzio".

Contributions: FD, SRT, IM, experiments performing; MDO, samples collection; FD, SRT, MDO, data analysis, manuscript revision; EM, OT manuscript drafting; PB, EM, OT, study design. All authors have edited, read and approved the final manuscript.

Conflict of interest: the authors declare no conflict of interest.

Received for publication: 9 June 2017.
Accepted for publication: 2 August 2017.

European Journal of Histochemistry 2017; 61:2826
doi:10.4081/ejh.2017.2826

In periodontal disease condition, histone acetylation promotes the transcription of inflammatory genes such as p300/CBP histone acetyltransferase, NF-kB and other proinflammatory cytokines.[26] However, the impact of histone modifications during the progression of periodontitis remains unclear. NF-kB signaling pathway could be involved in sustained histone modifications which further augments the disease progression.[27] Nuclear transcription factor NF-kB has a key role to activate innate immunity which causes osteoclast differentiation and to induce bone resorption.[28] DNA methylation is regulated by two different types of DNA methyltransferases (DNMTs): *de novo* methyltransferases (DNMT3a and DNMT3b), which are active during early development[29] and maintenance methyltransferase (DNMT1), which regulates methylated and unmethylated CpG sites in the cells.[30-32]

In the present study, we have investigated the epigenetic modifications elicited by *Porphyromonas gingivalis* LPS (LPS-G) using hPDLSCs as a model system to study novel

biomarkers linked to this oral inflammatory disease. To this end, we have examined the expression of DNMT1, p300 and NF-kB followed by LPS-G treatment in hPDLSCs.

Materials and Methods

Ethic statement

The present study was approved by the Medical Ethics Committee at the Medical School, "G. d'Annunzio" University, Chieti, Italy (n. 266/17.04.14). All healthy volunteers enrolled in this study have signed the informative consent form. The Department of Medical, Oral and Biotechnological Sciences and the Laboratory of Stem Cells and Regenerative Medicine are certified according to the quality standard ISO 9001:2008 (certificate n. 32031/15/S).

Cell culture

Periodontal ligament biopsies were collected from premolar teeth of healthy volunteers. All patients provided written informed consent to participate in the study. Before the biopsy collection, each patient was pre-treated for one week with professional dental hygiene and chlorhexidine. Explants were obtained from alveolar crest and horizontal fibers of the periodontal ligament by scraping the roots using a Gracey's curette.[33] Periodontal tissue fragments were cut, washed with PBS (Lonza, Basel, Switzerland) and placed in a TheraPEAK™MSCGM-CD™ Bullet Kit serum free, chemically defined (MSCGM-CD) medium (Lonza) at 37°C for the growth of human MSCs. Cells spontaneously migrated from the explants after reaching about 80% of confluence were trypsinized (LiStar Fish, Milan, Italy), and subsequently subcultured until passage 2 (P2). Cells utilized for the experimental assays were at P2.

LPS-G treatment

hPDLSCs were divided in two groups: group 1, untreated control (hPDLSCs); and group 2, cells treated with 5 µg/mL LPS-G (InvivoGen, San Diego, CA, USA) (hPDLSCs/LPS-G) for 24 h.

Morphological evaluation

After 24 h, hPDLSCs and hPDLSCs treated with LPS-G were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 for 2 h, stained with toluidine blue solution and observed by inverted optical microscope Leica DMIL (Leica Microsystems, Milan, Italy).

MTT assay

Cell viability was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) test. 1.5×10^4 cells of each group were plated in 96-well plates and were incubated with 200 µl culture medium. After incubation, 20 µL MTT solution was added to each well and incubated for 3 h.[34] The absorbance was measured on an automated microplate reader (Sinergy HT, Biotek Instruments, Bad Friedrichshall, Germany) at 570 nm.

Immunofluorescence analysis

hPDLSCs and hPDLSCs/LPS-G were processed as previously reported by Trubiani *et al.*[35] The following primary monoclonal antibodies were used: anti-human NF-kB (1:250, rabbit) (OriGene Technologies, Inc., Rockville, MD, USA), anti-DNMT1 (1:250, rabbit) (OriGene), anti-p300 (1:250, rabbit) (OriGene). Then, cells were incubated for 1 h at 37°C with Alexa Fluor 568 red fluorescence conjugated goat anti-rabbit secondary antibodies (1:200) (Molecular Probes). Subsequently, cells were incubated with Alexa Fluor 488 phalloidin green fluorescence conjugate (1:200, Molecular Probes) to mark cytoskeleton actin. Cell nuclei were stained with TOPRO (1:200, Molecular Probes) for 1 h at 37°C. Glass coverslips were placed upside down on glass slides and mounted with Prolong antifade (Molecular Probes).[36] Samples were observed with Zeiss LSM510META confocal system (Zeiss, Jena, Germany) connected to an inverted Zeiss Axiovert 200 microscope equipped with a Plan Neofluar oil-immersion objective (63x). Images were collected using an argon laser beam with excitation lines at 488 nm and a helium-neon source at 543 and 665 nm.

Nuclei isolation

Treated and untreated hPDLSCs (5×10^6) were washed with PBS and resuspended in 500 µL of a hypotonic/detergent buffer [10 mM Tris-Cl pH 7.8, 0.125% Nonidet P-40, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 µL/mL cocktail inhibitor (Protease Inhibitor Cocktail, Sigma-Aldrich, St. Louis, MO, USA), 0.1 µg/mL soybean trypsin inhibitor]. Cells were sheared by 5 passages through a 22-gauge syringe needle.[37] Nuclei were recovered by low speed centrifugation (800×g for 10 min) and washed two times in 10 mM Tris-Cl pH 7.4, 2 mM MgCl2 plus protease inhibitors as mentioned above. The pellet, representing the intact nuclei fraction, was used for biochemical experiments.

Western blot analysis

Thirty micrograms of proteins from hPDLSCs and hPDLSCs/LPS-G and from extracted nuclei were separated on SDS-PAGE and subsequently transferred to nitrocellulose sheets using a semidry blotting apparatus. Sheets were saturated for 120 min at room temperature in blocking buffer (1xTBS, 5% milk, 0.1% Tween-20), then incubated overnight at 4°C in blocking buffer containing primary antibodies to NF-kB (1:2000, rabbit) (OriGene), DNMT1 (1:1000, rabbit) (OriGene), p300 (1:750, rabbit) (OriGene) and β-actin (1:750, mouse) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After four washes in TBS containing 0.1% Tween-20, samples were incubated for 60 min at room temperature with peroxidase-conjugated secondary antibody diluted 1:1000 in 1x TBS with 2.5% dry milk and 0.1% Tween-20.[38] Bands were visualized by ECL method and quantified by Alliance 2.7 (UVItec Ltd., Cambridge, UK).

Next generation sequencing (NGS)

Total RNA was isolated using a Reliaprep RNA Cell Miniprep System (Promega, USA). TruSeq RNA Access library kit (Illumina, Inc., San Diego, CA, USA) was applied to make RNA sequencing libraries. Fifty ng of total RNA was fragmented at 94°C for 8 min on a thermal cycler. First strand cDNA synthesis was produced with random hexameres and SuperScript II Reverse Transcriptase (Invitrogen) using the following temperature profile: 25°C for 10 min, 42°C for 15 min and 70°C for 15 min. Then, RNA templates were removed and double stranded cDNA was produced using dUTP. Subsequently, the 3`ends of the cDNA were adenylated and ligated with indexing adaptor. The following PCR temperature profile was used for the selective enrichment of adaptor-ligated DNA fragments: 98°C for 10 s, 60°C for 30 s and 72°C for 30 s (15 cycles). Then, the libraries were validated and were hybridized with the selective probes to capture exome. Probes with enriched libraries were captured using streptavidin coated magnetic beads, eluted from the beads, cleaned up with AMPure XP beads and processed for the second PCR amplification using following temperature profile: 98°C for 10 s, 60°C for 30 s and 72°C for 30 s (10 cycles). Later, the libraries were quantified with KAPA Library Quantification Kit-Illumina/ABI Prism® (Kapa Biosystems, Inc., Wilmington, MA, USA) and validated using Agilent High Sensitivity DNA Kit on a Bioanalyzer. DNA fragments were meas-

ured in the range of 200-650 bp. Peak was observed around 250 bp. After normalization to 12 pM, DNA libraries were processed for cluster and single read sequencing (150 cycles) using MiSeq tool (Illumina, Inc.). The libraries were loaded on a MiSeq Flow Cell v3 to make clustering and were sequenced. Later, the reads were grouped with specific indexes using demultiplexing software tool CASAVA (ver. 1.8.2, Illumina, Inc.) and were mapped against "Homo sapiens UCSC hg19" reference sequences using RNA-Seq Alignment ver. 1.0.0. TopHat 2 (Bowtie 1) was applied for the Read mapping. Fragments per kilobase of gene model per million mapped reads (FPKM) values were calculated as described by Rajan *et al*.[12]

Statistical analysis

All experiments were performed in triplicate. The data are presented as means ± standard error and were analyzed using SIGMASTAT (SPSS Inc., Chicago, Ill., USA). One-way repeated-measurement analysis of variance (ANOVA), followed by the *post-hoc* Holm-Sidak test (when appropriate) and the Student's paired *t*-test were used for statistical analysis. A P-value less than 0.05 was considered statistically significant. In NGS experiment, the statistical analysis on the read counts to measure the proportion of differentially expressed genes between two samples was performed by The Cufflinks Assembly & DE package ver. 2.0.0. False discovery rate (FDR; Q) value less than 0.05 was considered statistically significant.

Results

Morphological and viability evaluation

First, we have examined the morphological changes and viability in hPDLSCs treated with LPS-G. hPDLSCs (Figure 1A) and LPS-G treated hPDLSCs (Figure 1B) showed a fibroblastic-like shape. However, LPS-G treated cells showed long cytoplasmic processes and a low cell density when compared to the control cells. The proliferation rate of hPDLSCs was assessed by means of MTT assay. Obtained data showed a significant exponential cell growth during all endpoint in hPDLSCs, while LPS-G induces a significant reduction in cell viability (P<0.01) (Figure 1C).

LPS-G induces epigenetic modulations in hPDLSCs

Then, we have investigated the modulations in epigenetic regulation caused by LPS-G treatment. Expression and subcellular localization of NF-kB, DNMT1 and p300 were observed by immunofluorescence with confocal microscopy. Increased nuclear translocation of NF-kB was observed in LPS-G-treated hPDLSCs (Figure 2 A2), while its predominant cytoplasmic expression of NF-kB was noticed in control untreated cells (Figure 2 A1). Nuclear histone acetyl transferase p300 was enhanced in hPDLSCs treated with LPS-G (Figure 2 B2), while basal expression was observed in untreated cells (Figure 2 B1). On the contrary, DNMT1 expression was significantly suppressed in LPS-G- treated cells (Figure 2 C2), when compared to untreated cells (Figure 2 C1). These results were further confirmed with western blot analysis. Total expression of NF-kB was unaltered (Figure 3A), while nuclear expression of NF-kB was markedly increased in LPS-G-treated hPDLSCs, but not in the untreated cells (Figure 3B). Similarly, p300 level was increased in the cells treated with LPS-G (Figure 3D). As expected, DNMT1 was markedly reduced followed by LPS-G treatment (Figure 3C). Densitonetric analysis of specific band provided a protein quantification (Figure 3F). These results revealed that LPS-G significantly suppressed DNMT1, while upregulated the expression of p300 and NF-kB, which may trigger the transcription of inflammatory genes in hPDLSCs. NGS data supported these results. DNMT1 was downregulated and p300 was upregulated in LPS-G-treated hPDLSCs (Figure 4) (Q<0.05). Histone deacetylase HDAC1 was reduced while HDAC2 was increased after LPS-G treatment (Figure 4) (Q<0.05). Moreover, proinflammatory cytokine transcripts, tumor necrosis factor alpha induced protein 1 (TNFAIP1) and interleukin 6 signal transducer (IL6ST) were significantly enhanced in LPS-G-treated hPDLSCs (Figure 3) (Q<0.05).

LPS-G modulates genes associated with AD in hPDLSCs

Considering the connection between periodontitis and AD, we have examined the expression of genes linked with AD in LPS-G treated hPDLSCs. We have noticed that AD-linked genes such as amyloid beta precursor protein (APP), amyloid beta precursor protein binding protein 2 (APPBP2), interferon gamma receptor 1 (IFNGR1), matrix metallopeptidase 1 (MMP1), MMP2

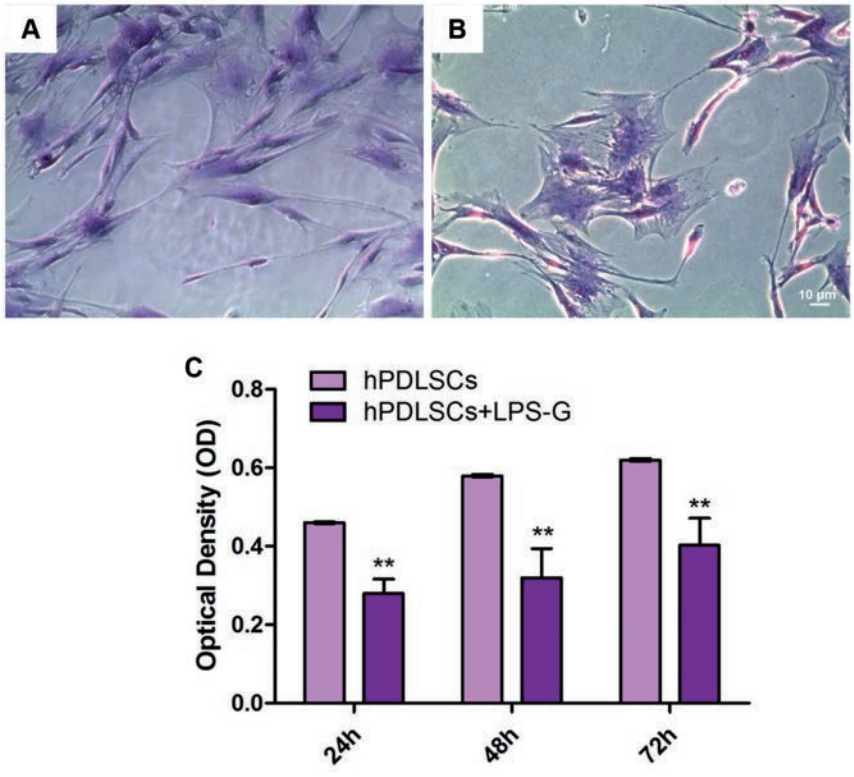

Figure 1. Morphological evaluation. A) Isolation and culture of hPDLSCs from periodontal ligament tissues; cells showed a fibroblastic morphology. B) After 24 h of LPS-G treatment, cells showed a low density and morphological changes, such as long and numerous cytoplasmic processes. C) MTT showed a reduction in cell proliferation rate in all examined endpoint in LPS-G treated cells. Magnification: 10x; **P<0.01.

and MMP16 were markedly increased in LPS-G-treated hPDLSCs than in control untreated hPDLSCs (Table 1; Q<0.0001). Relative log2 fold change expression of these genes in hPDLSCs and LPS-G-treated hPDLSCs are shown in Table 1. These results suggest that LPS-G-induced inflammation in hPDLSCs may activate the genes associated with AD.

Discussion

Periodontitis is a prevalent inflammatory disease arising from the gum plaques produced by oral pathogens including *Porphyromonas gingivalis*. Advanced form of periodontitis results in the retraction of gingiva, loss of periodontal ligament, and degeneration of surrounding alveolar bone. Chronic periodontitis may increase the risk of neuroinflammatory diseases such as AD.[39,40] Complex molecular mechanisms are involved in the stimulation and maintenance of inflammation, among which epigenetic pathways received special attention because of their upstream regulations. Epigenetic modifications include chemical alterations of DNA and associated proteins, leading to remodeling of the chromatin and activation or inactivation of gene transcription. These changes can contribute to the development and maintenance of cancer, autoimmune and inflammatory diseases, including periodontitis. Interestingly, some epigenetic modifications are reversible and can be stimulated by environmental factors.[41,42] Knowledge on the modification of epigenetic mechanisms may provide substantial inputs regarding the mechanism of the key regulatory pathways of the genes involved in the maintenance of chronic inflammation. Indeed, the crucial role of DNA and histone modifications, the two major epigenetic regulations, have been described in periodontitis.[26] Several studies have demonstrated that in periodontal disease, gene expression can be influenced by DNA methylation;[26] however, only few studies have reported the histone modifications induced by LPS-G stimulus.[43] Earlier studies have demonstrated that in B cells, LPS-G induced methylation and acetylation in H3 and H4 histone proteins.[26] Recently, it has been demonstrated that periodontitis is characterized by chronic inflammation that might be linked with aberrant DNA methylation in the gingival tissues.[44,45] The increased methylation in the gene promoter region is associated with a reduction in gene

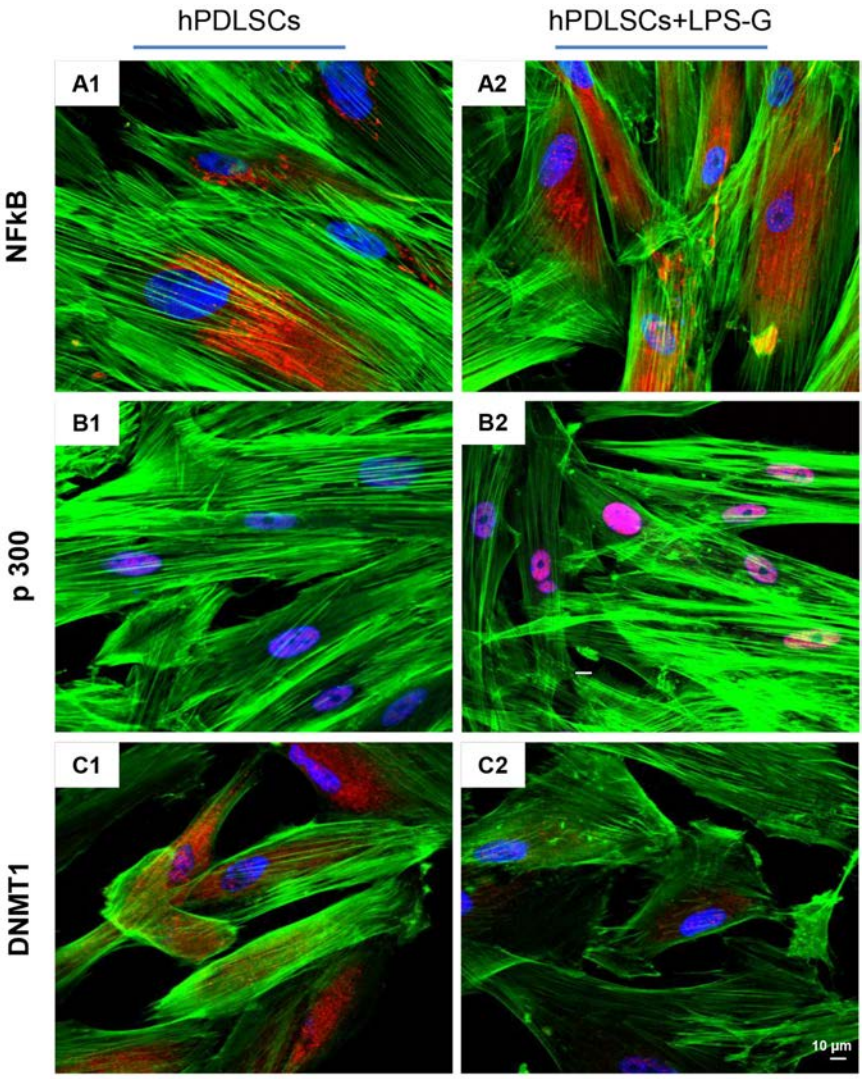

Figure 2. Immunofluorescence. Cells grown on coverslips were fixed and stained with the indicated primary and secondary antibodies and DAPI, and subjected to confocal microscopy. NF-kB expression in hPDLSCs (A1) and in hPDLSCs treated with LPS-G (A2). p300 expression in hPDLSCs (B1) and in hPDLSCs treated with LPS-G (B2). DNMT1 expression in hPDLSCs (C1) and in hPDLSCs treated with LPS-G (C2). Data are representative of three independent experiments. Magnification: 63x.

Table 1. LPS-G modulates genes associated with Alzheimer's disease. NGS data revealed that LPS-G treatment significantly increased the expression of AD-linked genes such as *APP, APPBP2, IFNGR1, MMP1, MMP2* and *MMP16* in hPDLSCs (Q<0.0001).

Gene	hPDLSCs	hPDLSCs+LPS-G	log2 fold change	Q-value
APP	405.63	1057.55	1.38	0.0001
APPBP2	4.48	6.94	0.63	0.0001
IFNGR1	14.82	34.78	1.23	0.0001
MMP1	13.84	161.31	3.54	0.0001
MMP2	975.87	1359.89	0.48	0.0001
MMP16	2.30	8.99	1.97	0.0001

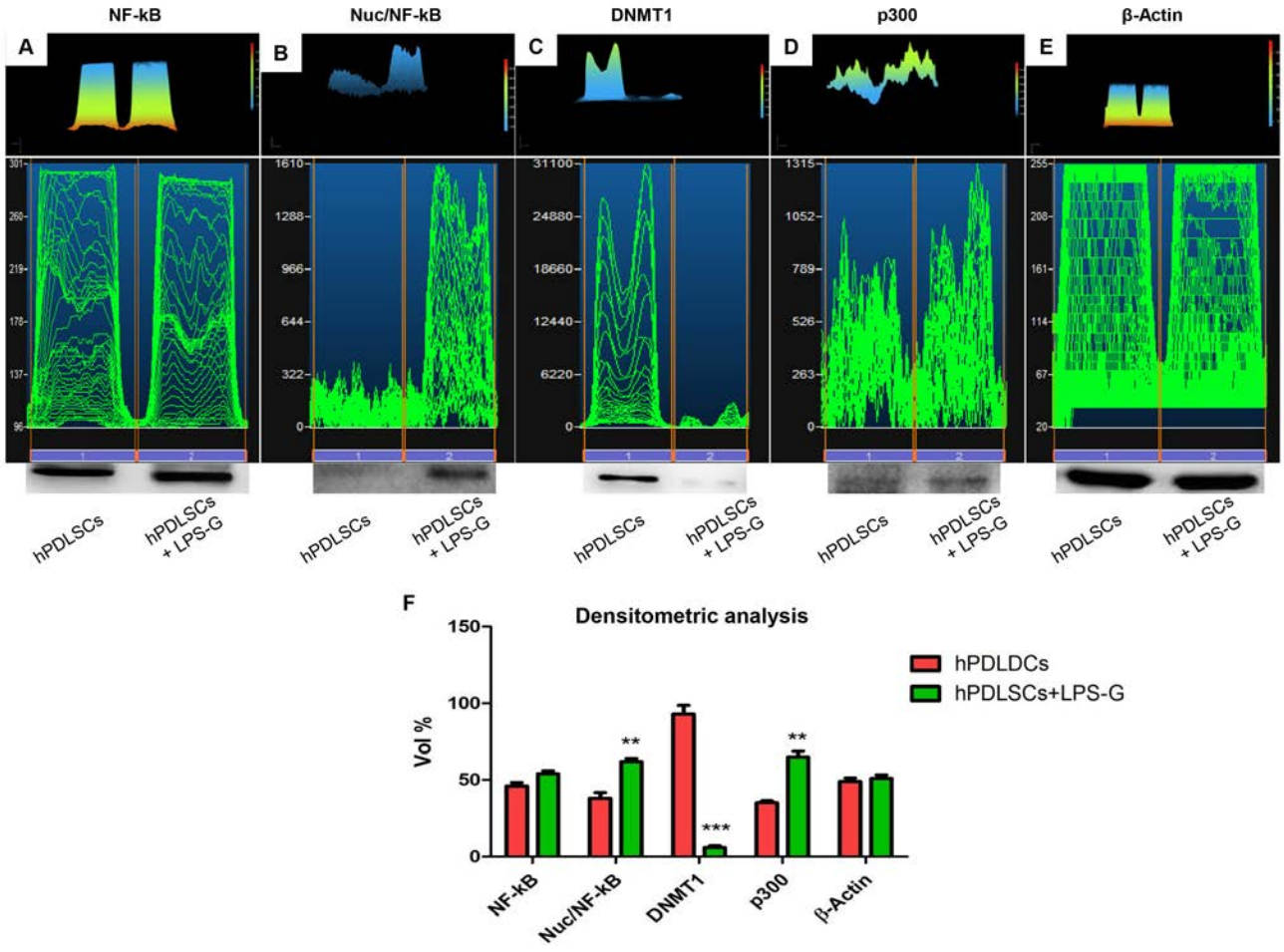

Figure 3. Western blot analysis of NF-κB (A), NF-κB in nuclear extract (B), DNMT1 (C) and p300 (D) expression in hPDLSCs and in hPDLSCs treated with LPS-G for 24 h. β-actin was used as a housekeeping protein (E). Densitometric analysis of protein specific bands (F). **P<0.01; ***P<0.001.

expression, while hypomethylation pattern is closely associated with transcriptional activation.[46] Moreover, it has been demonstrated that LPS-G downregulated the expression of DNA methylases DNMT3a and DNMT1 in keratinocytes.[44]

In our recent study we reported that in hPDLSCs, LPS-G treatment activates the TLR4/MyD88 complex, induces NF-kB nuclear translocation and triggers the secretion of proinflammatory cytokines including TNF-α.[47] Based on these findings, we assume that hPDLSCs may represent an appropriate stem cell modelling through which we may understand the biological machinery of epigenetic system and inflammation in response to LPS-G in periodontal tissues. To this end, we have investigated the expression of DNMT1, p300 and NF-kB in hPDLSCs administered with LPS-G. Immunofluorescence and Western blot results demonstrated that LPS-G treatment significantly suppressed the expression of DNMT1 in hPDLSCs. This result is corrob-

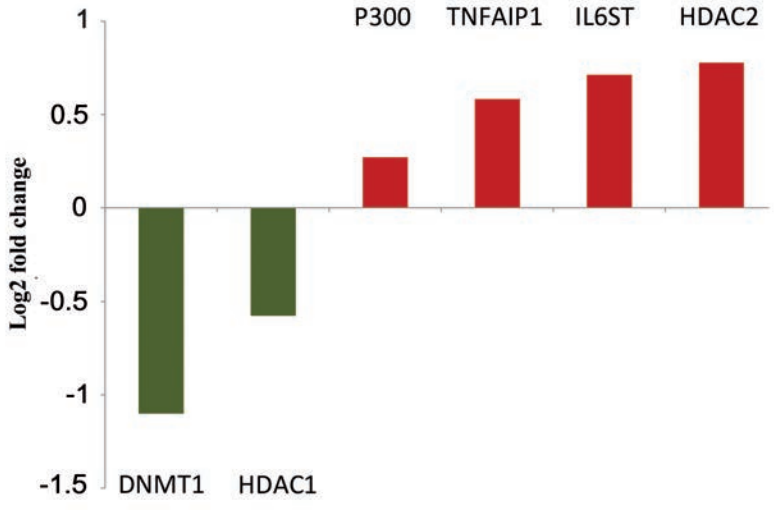

Figure 4. Gene expression. Next generation sequencing demonstrated the modulation of genes expressed in untreated and LPS-G treated hPDLSC. P300, TNFAIP1, IL6ST and HDAC2 were expressed greater than 2-fold (Log2 fold change; Q<0.05). DNMT1 and HDAC1 genes were significantly downregulated in LPS-G stimulated hPDLSCs when compared with untreated cells (Q<0.05). Up-regulated transcripts are highlighted in red color; downregulated transcripts are highlighted in green color.

orated with the findings from Yin and Chung,[48] which showed that P. *gingivalis* (whole bacteria) could cause a decrease in DNMT1 gene expression in oral epithelial cell and that *P. gingivalis* may modulate DNA methylation status of the genes involved in the pathogenesis of periodontitis. Moreover, LPS stimulation down regulated DNMT1 expression in human HaCaT keratinocytes and in oral ephitelial cells.[44]

LPS triggers many intracellular signaling cascades among which NF-kB pathway is a crucial one.[49,50] NF-kB represents a family of transcription factors essential for the induction of the most important classes of inflammatory genes, including genes involved in periodontitis progression. Both recruitment of NF-kB to target promoters and NF-kB-induced transcriptional genes could be modulated through chromatin modification.[50] CBP/p300 are general transcriptional co-activators that help NF-kB to bridge with the basal transcription machinery.[51] CBP/p300 interact with a large array of transcription factors to integrate multiple cellular signaling pathways.[52,53] In addition, CBP/p300 possess chromatin-remodeling capabilities owing to their histone- and transcription factor-acetylating properties. p300 is not only a transcriptional adaptor but also a histone acetyltransferase.[54] In our hPDLSCS-based stem cell model, we found that LPS-G increased the expression of NF-kB and its coactivator p300, indicating the critical regulatory effect of these molecules during the inflammation process triggered by LPS-G. Moreover, we noticed that histone deacetylase HDAC1 was decreased while HDAC2 was increased followed by LPS-G treatment. These results suggested the differential regulatory effect of these deacetylases over epigenetics regulation induced by LPS-G. In addition, NGS data revealed the upregulation of proinflammatory cytokine transcripts TNFAIP1 and IL6ST in LPS-G-treated hPDLSCs, which suggested the activation of inflammatory cytokines followed by LPS-G.

Emerging evidence suggests that poor oral health influences the initiation and/or progression of diseases such as atherosclerosis (with sequelae including myocardial infarction and stoke), diabetes mellitus and neurodegenerative diseases.[55] In particular, chronic periodontitis is associated with an increase in cognitive decline in Alzheimer's disease.[5] Considering the association between periodontitis and AD, in the present study we have examined if LPS-G treatment may modulate the genes linked with AD in hPDLSCs. NGS data showed that genes involved in AD pathogenesis such as APP, APPBP2, IFNGR1, MMP1, MMP2 and MMP16 were significantly upregulated in LPS-G-treated hPDLSCs. These data support the notion that periodontitis may increase the risk of AD. We assume that LPS-G-treated hPDLSCs might be a potential *in vitro* model system to explore the link between periodontitis and AD. The importance of epigenetics in periodontal disease has been studied with growing interest in the last few years due to its strict correlation to the systemic diseases Recent advances in epigenomic approaches allow mapping of the methylation state in the genome, which may help to identify new biomarkers as well as to develop novel therapeutic strategies for preventing periodontal disease.

In conclusion, we demonstrated the activation of epigenetic modifications in LPS-G-treated hPDLSCs. Reduced DNMT1 and increased NF-kB and p300 were noticed in hPDLSCs treated with LPS-G. We proposed that hPDLSCs-based stem cell model could be a potential *in vitro* tool to evaluate epigenetic changes associated with periodontitis.

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