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Insights from *in vitro* studies with probiotics and specialized pro-resolving mediators into new strategies in the control of periodontitis

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Advisor: Profa. Dra. Marinella Holzhausen Caldeira

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RESUMO

Souza EA. Perspectivas de estudos *in vitro* com probióticos e mediadores de resolução da inflamação sobre novas estratégias no controle da periodontite [tese]. São Paulo: Universidade de São Paulo, Faculdade de Odontologia; 2019. Versão Corrigida.

O controle das diversas formas de periodontite tem se mostrado um desafio, especialmente nos casos de maior severidade. Considerando a etiologia microbiana e imunológica dessa doença, diversas estratégias podem ser usadas com o intuito de alterar a microbiota disbiótica e controlar a resposta imune do hospedeiro. Nesse sentido, o uso de probióticos assim como de mediadores especializados na resolução da inflamação podem ser vistos como alternativas promissoras no controle desta doença. Entretanto, estudos *in vitro* devem ser realizados para que se entendam melhor os mecanismos pelos quais ambas alternativas podem alterar o perfil micro-imunológico de susceptibilidade associado à periodontite, de forma a modificar a composição do biofilme disbiótico e melhorar a atividade regenerativa dos tecidos perdidos no processo inflamatório. Esta tese tentou avaliar ambos os aspectos. Primeiro, estudamos através de uma triagem com diversas espécies de probióticos a interação desses microorganismos com células epiteliais gengivais (CEGs) desafiadas com cepas de *Porphyromonas gingivalis*, no intuito de selecionarmos aqueles probióticos com maior potencial no controle desse periodontopatógeno assim como da resposta imune inata mediada por esse microorganismo. Nesse sentido, os probióticos regularam a resposta imune mediada por CEGs ao prevenir a morte celular induzida por *P. gingivalis* e reduzir a adesão e a invasão desse patógeno às CEGs, ao mesmo tempo em que aumentaram sua própria adesão às CEGs. Este controle da interação de células desafiadas com *P. gingivalis* resultou em uma redução na síntese de IL-1β e TNF-α e em um aumento na concentração de CXCL8. Os probióticos também alteraram a transcrição de genes que codificam receptores de reconhecimento de padrões moleculares, peptídeos antimicrobianos e genes reguladores de apoptose, em geral de uma maneira cepa-específica. Segundo, avaliamos como mediadores lipídicos de resolução da inflamação (MaR1, RvE1) podem alterar as propriedades regenerativas de células-tronco do ligamento periodontal (CTLPs) em um ambiente inflamatório (IL-1β, TNF-α), em relação a biomarcadores relacionados a formação de ligamento periodontal, osso alveolar e cemento. A proporção de citocinas pró-inflamatórios e mediadores lipídicos de pro-resolução alterou as

atividades regenerativas periodontais de CTLPs. Um ambiente predominantemente inflamatório sob estímulo com IL-1β/TNF-α reduziu a *stemness* de CTLPs, diminuiu a expressão de seus biomarcadores de regeneração, dificultou a cicatrização de feridas *in vitro* e diminuiu suas propriedades cemento-osteogênicas. No entanto, a indução de um ambiente de resolução com a adição de MaR1/RvE1 reverteu este processo, resgatando parcialmente a expressão de biomarcadores de *stemness* e melhorou as propriedades regenerativas relacionadas às CTLPs. Portanto, um controle adequado do ambiente inflamatório parece ser fundamental para que haja uma melhora no processo de regeneração tecidual que deve suceder a destruição dos tecidos periodontais perdidos na periodontite. Terceiro, formulamos uma hipótese a respeito de como probióticos podem auxiliar no controle da inflamação através da regulação da síntese de mediadores lipídicos de resolução da inflamação.

Palavras-Chave: Periodontite, Probióticos, Inflamação, Células-epiteliais, Células-tronco.

ABSTRACT

Souza EA. Insights from *in vitro* studies with probiotics and specialized pro-resolving mediators into new strategies in the control of periodontitis [thesis]. São Paulo: Universidade de São Paulo, Faculdade de Odontologia; 2019. Corrected Version.

The control of the periodontitis has been a challenge, especially in cases where the severe form is present. Considering the microbial and immunological etiologies of this disease, several strategies can be used to alter the dysbiotic related-microbiota and control the host immune response. In this context, the use of probiotics as well as specialized pro-resolving lipid mediators (SPMs) can be seen as promising alternatives in the control of this disease. However, *in vitro* studies should be carried out to better understand the mechanisms by which both alternatives can alter the micro-immunological profile of susceptibility associated with periodontitis, in order to modify the composition of the dysbiotic biofilm and to improve the regenerative activity in tissues lost for the uncontrolled inflammatory process. This thesis tried to evaluate both aspects. Firstly, we studied the interaction of several species of probiotics with gingival epithelial cells (GECs) challenged by *Porphyromonas gingivalis* strains in order to select those probiotics with greater potential in the control of this periodontopathogen as well as in the innate immune response mediated by this microorganism. In this sense, probiotics regulated the response mediated by GECs preventing cell death induced by *P. gingivalis*, reducing pathogen adhesion and invasion at the same time as they increase their own adhesion to GECs. The control of the interaction of GECs with *P. gingivalis* resulted in a reduction in the synthesis of IL-1β and TNF-α with concomitant increase in the release of CXCL8. Probiotics also altered the transcription of genes encoding pattern recogntition receptors, antimicrobial peptides and apoptosis regulatory genes, overall in a strain-specific manner. Secondly, we evaluated how SPMs (MaR1, RvE1) can alter the regenerative properties of human periodontal ligament stem cells (hPDLSCs) in an inflammatory environment (IL-1β, TNF-α), regarding biomarkers related to the formation of periodontal ligament, alveolar bone and cementum. The ratio of pro-inflammatory mediators and pro-resolving lipid mediators altered periodontal regenerative activities. A predominantly inflammatory environment under IL-1β/TNF-α stimulus reduced the stemness of hPDLSCs, downregulated the expression of their regenerative biomarkers, impaired their *in vitro* wound

healing, and decreased their related cementum-osteogenic properties. However, the induction of an pro-resolving milue with the addition of MaR1/RvE1 reversed this process by partially rescuing biomarkers of stemness and ameliorate hPDLSCs regenerative-related activities. Therefore, an adequate control of the inflammatory environment was shown to be fundamental for an improvement in the process of tissue regeneration that must overcome the destruction of periodontal tissues. Thirdly, we formulate a hypothesis about how probiotics can help in the control of inflammation by regulating the synthesis of SPMs.

Keywords: Periodontitis; Probiotics; Inflammation; Epithelial Cells; Stem Cells.

LIST OF FIGURES

LIST OF ABBREVIATIONS, ACRONYMS, INITIALS, AND SYMBOLS

ARS	Alizarin Red Staining
ALP	Alkaline Phosphatase
APC	Allophycocyanin
ATCC	American Type Culture Collection
Bcl	B-Cell Lymphoma
BSA	Bovine Serum Albumin
CAP	Cementum Attachment Protein
CASP	Caspase
CEMP1	Cementum Protein 1
DAPI	4′,6-Diamidino-2-Phenylindole
HLA-ABC	Human Leukocyte Antigens A,B, And C
CFU	Colony-Forming Units
ChemR23	Chemerin Receptor 23
CXCL8	C-X-C Motif Chemokine Ligand 8
FITC	Fluorescein Isothiocyanate
FBS	Fetal Bovine Serum
FC	Flox Cytometry
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GECs	Gingival Epithelial Cells
hPDLSCs	human Periodontal Ligament Stem Cells
hBD	human Beta-Defensins
IL-1β	Interleukin-1β
IL-10R	Interleukin 10 Receptor Subunit Alpha

KSFM	Keratinocyte-Serum Free Medium
MaR1	Maresin-1
MFI	Mean Fluorescence Intensity
MOI	Multiplicity Of Infection
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyl Tetrazolium Bromide
NLRP3	Nlr Family, Pyrin Domain Containing 3
NOD	Nucleotide-Binding Oligomerization Domain
OBA-9	Immortalized Human Gingival Epithelial Cells
Oct-4	Octamer-binding transcription factor 4
OCN	Osteocalcin
PE	Phycoerythrin
PRSTN	Periostin
RT-Qpcr	Quantitative Real-Time Reverse Transcription PCR
Runx2	Runt-Related Transcription Factor 2
RvE1	Resolvin-E1
Sox-2	Sex Determining Region Y-Box2
SPMs	Specialized Pro-Resolving Mediators
TLR	Toll-Like Receptor
TNMD	Tenomodulin
TNF-α	Tumor Necrosis Factor-α
WB	Western Blotting
WH	Wound Healing
α-MEM	α-Minimum Essential Medium
α-SMA	α-Smooth Muscle Actin

CONTENTS

1 INTRODUCTION

Periodontitis is a biofilm-induced chronic inflammatory disease that affects the supporting periodontal tissues surrounding the teeth, i.e. cementum, alveolar bone, and periodontal ligament, besides exerting an impact on systemic health. Low-abundance pathogens colonizing subgingival biofilms, such as *Porphyromonas gingivalis*, can orchestrate the host inflammatory response by inducing dysbiosis [1]. This microorganism evolved sophisticated strategies to evade or subvert components of the innate immunity, such as the epithelial barrier. In this context, an uncontrolled host response activates the synthesis of an exaggerated amount of pro-inflammatory cytokines, such as IL-1β and TNF-α, leading to the destruction of periodontal tissues by activating mechanisms of osteoclastogenesis [2,3].

Over the past few years, the adjunctive antibiotic therapy to scaling and root planning associated with biofilm control has been used in the treatment of severe forms of periodontitis. However, the ever-increasing evidence of a "double-edged sword" nature of the periodontal host response turns the immunomodulation into an important emerging strategy for managing periodontitis. Importantly, it is becoming clear that keystone pathogens are 'inflammophilic', capitalizing the theory of controlling the inflammation to control the infection [4]. Therefore, strategies to prevent and control periodontitis should restore the homeostasis between the microbiome and the host response, by influencing the interaction of host cells with keystone pathogens and/or affecting the immune response triggered by periodontopathogens. Moreover, new treatment perspectives should also focus on a possible restoration of the inflammatory environment, so that tissue-specific stem cells involved in the local regeneration activity could play their role in the neo-formation of destroyed structures.

For many years the understanding of this disease, as well as other chronic inflammatory disorders, has been limited to the study of pro-inflammatory mediators and the resolution physiology has been neglected or understood as a passive process [5]. In this context, beyond the canonical pathways that activate inflammation, there is a cascade of events that determine the synthesis of specialized pro-resolving lipid mediators (SPMs) that act to counterbalance this pathological response [6-8]. Among them, lipid endogenous agonists derived from polyunsaturated fatty acids, including

resolvins and maresins, have presented a wide variety of functions ranging from inducing changes in biofilm composition, reorganizing the host response, enhancing bacterial phagocytosis and efferocytosis to stimulating pro-regenerative activities in order to reverse tissue destruction [9-11].

In fact, the understanding of the host immune response in periodontitis should be analyzed from a broader point of view. In addition to factors related to the interaction of proinflammatory cytokines and SPMs in the context of regenerative activity, another look should also be launched from a microbiological perspective. In this sense, the use of probiotics bacteria have emerged as a promising alternative to treat periodontal diseases, especially considering that such microorganisms appear to play a role as immunomodulators, besides their direct effects on pathogens [12,13]. Randomized controlled trials indicated that probiotics belonging to *Lactobacillus* and *Bifidobacterium* genera yield promising results in the treatment of periodontitis [14].

Therefore, in view of the multiple immunomodulation alternatives in the control of periodontitis forms, the studies from which this thesis emerged focused on two aspects. Firstly, we evaluated the effect of the interaction of probiotic species with *P. gingivalis* on the immune response performed by human gingival epithelial cells. Secondly, we studied the effect of the interaction of pro-inflammatory cytokines and SPMs on the regenerative activity of human periodontal ligament stem cells.

2 PROPOSITION

i. To evaluate immunoregulatory properties of several clinical isolates and commercially available *Lactobacillus* sp. and *Bifidobacterium* sp. on gingival epithelial cells challenged by *P. gingivalis* in order to point out those probiotics with greater potential for the control of periodontitis.

ii. To evaluate the effect of specialized pro-resolving mediators (MaR1 and RvE1) on periodontal-related regenerative activities and stemness of periodontal ligament stem cells under an inflammatory environment with IL-1β and TNF-α.

iii. To organize a hypothesis from the findings related to propositions i and ii, pointing out a new perspective in the relationship between probiotic bacteria and the synthesis of bioactive pro-resolving mediators.

3 CHAPTER I: Screening of probiotics candidates for the treatment of periodontitis*

ABSTRACT

Background: Recent studies have revealed the potential use of probiotics to treating periodontitis, although little is known about their interactions with gingival epithelial cells. Also, it is unclear which probiotic strain has immunomodulatory properties upon cells challenged by key pathogens in periodontitis such as *Porphyromonas gingivalis*.

Objective: To perform a screening study and to evaluate the regulatory effect of probiotic lactobacilli and bifidobacteria on OBA-9 cells challenged by *P. gingivalis* (capsulated, W83; acapsulated ATCC33277).

Methods: OBA-9 cells (~2x 10^5cells/well) were co-infected with each *P. gingivalis* strain and/or one of the 12 probiotics (bifidobacteria and lactobacilli) tested strains at a multiplicity of infection (MOI) of 1:1,000 for 2h. Bacterial adhesion and invasion were determined by antibiotic exclusion analysis. OBA-9 viability was measured by MTT assay. Inflammatory mediators (TNF-α, IL-1β, and CXCL8) were determined by ELISA, and expression of genes encoding transmembrane and intracellular receptors (TLR2, TLR4, NOD1, NOD2, NLRP3, and IL-10R), human beta-defensins [BD1 and BD3], chemokine [CXCL8] and apoptosis regulators [CASP3, CASP9, BCL2, and BCL6] were evaluated by RT-qPCR.

Results: Probiotics maintained cell viability despite *P. gingivalis* challenge, and reduced pathogen adhesion and invasion. Furthermore, probiotics reduced IL-1β and TNF-α synthesis in OBA-9 challenged by *P. gingivalis*. Also, *Lactobacillus acidophilus* LA-5 increased the secretion and transcription of CXCL8, downregulated the expression of TLR2, TLR4, NOD1, and NOD2, upregulated IL10R and BD1, and modulated apoptosis regulators genes.

Conclusion: Probiotics can modulate the innate immune response triggered by gingival epithelial cells challenged by *P. gingivalis,* and *L. acidophilus* emerges as a potential immunomodulator.

Key words: Probiotics; *Porphyromonas gingivalis*; Innate immunity.

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3.1 Introduction

According to the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), probiotics are defined as live microorganisms, mainly bacteria, safe for human consumption, that have beneficial effects on health [1]. Since 1994, WHO has encouraged the development of probiotic research, emphasizing it as one of the most important therapeutic alternatives when commonly prescribed antibiotics have no effect due to bacterial resistance [1,2].

Some of the most common probiotic strains belong to the genus Lactobacillus and Bifidobacterium, including *L. acidophilus*, *L. johnsonii*, *L. casei*, *L. rhamnosus*, *L. gasseri*, *L. reuteri*, *B. bifidum*, *B. longum* and *B. infantis* [2,3]. The use of such probiotics is one of several approaches that has been considered in clinical trials for the treatment of periodontal diseases [3-6]. Nevertheless, the literature is scarce in studies that identify the most effective probiotic strains to control the inflammatory response induced by keystone pathogens in the context of the periodontitis, such as *P. gingivalis* [7], as well as associated mechanisms triggered by this interaction.

In fact, despite the probiotics effects on the modulation of immunological parameters, on bacterial translocation or by providing bioactive or regulatory metabolites, [3] little is known on their effects on gingival epithelial cells (GECs). These bacteria can interact with several epithelial cell lines, preventing adhesion and invasion of pathogens, improving the integrity and controlling the permeability of the epithelial barrier [8,9], but their interactions with oral epithelial cells still need to be elucidated. These evaluations would bring stronger scientific evidences to guide the development of new products for the treatment and control of periodontitis and the conception of further clinical trials.

Therefore, the purpose of this *in vitro* screening study was to evaluate the effect of several clinical isolates and commercially available *Lactobacillus* sp. and *Bifidobacterium* sp. on GECs challenged by *P. gingivalis* strains with respect to essential functions in the microbiota-host interaction, such as control of bacterial adhesion and invasion, maintenance of cellular viability, reduction of pro-inflammatory cytokines, modulation of immune-related receptors and proteins.

3.2 Material and Methods

3.2.1 Cell culture

Immortalized human gingival epithelial cells OBA-9 [10] were cultured in Keratinocyte-Serum Free Medium (KSFM)(GIBCO™, Life Technologies, Carlsbad, CA,USA) supplied with human recombinant epidermal growth factor (EGF), PenStrep GIBCO™ (10,000 units.mL^{-1} of penicillin, 10,000 µg.mL^{-1} streptomycin), and 25 µg.mL^{-1} amphotericin B, at 37°C in 5% CO_2.

3.2.2 Bacterial strains and growth conditions

Twelve probiotic strains [*Lactobacillus reuteri* DSM 17938, *L. rhamnosus* Lr-32™, *L. rhamnosus* HN001™ (DuPont™ and Danisco®, Madison, WI, USA), *L. acidophilus* LA-5™ (CHR Hansen Holding A/S, Hørsholm, Denmark), *L. acidophilus* NCFM® (DuPont™ and Danisco®, Madison, WI, USA), *L. casei* 324m (clinical isolate, Institute of Biomedical Sciences, University of São Paulo, Brazil), *Bifidobacterium longum* subsp. *infantis* ATCC15697, *B. animalis* subsp. *lactis* BB-12™ (CHR Hansen Holding A/S), *B. breve* 1101A, *B. longum* 51A, *B. pseudolongum* 1191A, *B. bifidum* 1622A (clinical isolates from faeces of healthy children) [11] were evaluated. *P. gingivalis* W83 (capsulated) and *P. gingivalis* ATCC33277 (acapsualated) were used to challenge GECs. All strains were maintained in Brain Heart Infusion Broth with 20% glycerol, at -80^{o}C. Bifidobacteria were grown under anaerobic conditions (90% N_2, 5% CO_2 and 5% H_2, 37°C) in BSM broth and agar (Bifidus Selective Medium, Sigma-Aldrich, St. Louis, MO, USA) in an anaerobic chamber (PLas Labs Model 855, Lansing, MI, USA). Lactobacilli were cultivated under microaerophilic conditions (5% CO_2, 37°C) in Lactobacilli MRS broth and agar (Lactobacilli MRS, Difco Laboratories, Detroit, MI, USA). *P. gingivalis* were grown under anaerobic conditions (90% N_2, 5% CO_2 and 5% H_2, 37°C) in blood agar plates [Tryptic Soy agar (Difco Laboratories) enriched with 5% defibrinated sheep blood, 0.5 mg.mL^{-1} hemin (Sigma-Aldrich) and 1

mg.mL^{-1} menadione (Sigma-Aldrich)] and TS broth [Tryptic Soy broth (Difco Laboratories) with 0.5 mg.mL^{-1} hemin, and 1 mg.mL^{-1} menadione (Sigma-Aldrich)]. For the different assays, bacteria were inoculated in liquid media and grown until early-stationary phase was reached, and cell concentration adjusted to an optical density (OD_{495}) equivalent to 2.0x10^8 Colony-Forming Units (CFU)/ml.

3.2.3 Adhesion and invasion assays

OBA-9 cells were seeded in 24-well culture plates at a cell density of 2.0x10^5 cells per well, in KSFM without antibiotic. After 24h, cells were challenged with *P. gingivalis* strains at a multiplicity of infection (MOI) of 1:1,000. After 2h incubation, unattached bacteria were removed by washing, and fresh medium without antibiotic (adhesion assay) or containing 200 μg.mL^{-1} of metronidazole and 300 μg.mL^{-1} of gentamicin (invasion assay) was added. The plates were incubated for 1h, and OBA-9 cells were lysed using 1mL of sterile water for 20 min. Lysates were serially diluted, inoculated on the surface of agar plates (in duplicate) and incubated for 5 to 10 days for determination of the CFU/mL. Lactobaciili and Bifidobacteria counts were performed after cultivation in selective agar for 5 days. *P. gingivalis* counts were determined after 10 days of incubation in blood agar plates, and this specie was differentiated from the probiotics by its characteristic colony morphology (brown/black colonies). The number of adherent bacteria was obtained by subtracting the number of internalized bacteria from the total number of bacteria obtained in the absence of antibiotics [12].

3.2.4 MTT assay

OBA-9 viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) [13]. OBA-9 cells in 96-well plates (~2.0x10^{5}cells/well) were co-infected with probiotics and/or pathogens at a multiplicity of infection (MOI) of 1:1,000 for 2h diluted in KSFM without antibiotics, as described above. The OD at 570 nm was measured using the Bio-Rad™ Model 680 (Hercules, CA, USA) microplate reader. The percentage of

surviving cells was calculated as: (OD_{570nm} treated cells/OD_{570nm} control [untreated] cells)×100.

3.2.5 Quantification of inflammatory mediators

After co-infection with *P. gingivalis* and/or probiotics, supernatants were collected and stored at -80°C until further analysis. Quantification of IL-1β, TNF-α, and CXCL8 was performed using PeproTech® ELISA Development kits (Rocky Hill, NJ, USA), according to the manufacturer's recommendations. Data were acquired with the use of the Bio-Rad™ Model 680 microplate reader. Mediator concentrations of samples were estimated based on a standard curve ranging from 1-1000 $pg.ml^{-1}$ using a third-order polynomial equation and the GraphPad Prism 5 software and expressed as $pg.ml^{-1}$.

3.2.6 Gene expression (RT-qPCR)

OBA-9 cells were lysed and total RNA was extracted using RNeasy KIT (QIAGEN, Valencia, CA, United States). The quality and concentration of the extracted RNA were measured using a NanoDrop™ One Spectrophotometer (Thermo Scientific, Waltham, MA, United States). The ratio of absorbance at 260 and 280 nm was used to assess the purity of RNA. RNA from each sample was immediately reverse transcribed into cDNA using SuperScript VILO MasterMix (Invitrogen, Waltham, MA, United States). The conditions for reverse transcription were 10 min at 25°C, 60 min at 42°C, and 5min at 85°C. Quantitative real-time reverse transcription PCR (RT-qPCR) was performed using TaqMan® Gene Expression Master Mix (Applied Biosciences, Foster City, CA, United States), TaqMan primers and probes for Toll-Like Receptors [*TLR2* (*Hs00152932_m1*), *TLR4*(*Hs01060206_m1*)], NLR family, pyrin domain containing 3 [*NLRP3 (Hs00918082_m1)*], nucleotide-binding oligomerization domain [*NOD1 (Hs01036720_m1), NOD2 (Hs01550753_m1)*], interleukin 10 receptor subunit alpha [*IL10RA (Hs00155485_m1)*], human beta-defensins [*BD1(Hs00608345_m1), BD3 (Hs04194486_g1)*],

C-X-C motif chemokine ligand 8 [*CXCL8 (Hs00174103_m1)*], caspases [*CASP3(Hs00234387_m1), CASP9 (Hs00962278_m1)*], apoptosis regulator, B-cell lymphoma [*Bcl2 (Hs00608023_m1), Bcl6 (Hs00153386_m1)*], and *GAPDH* (*Hs02786624_g1*) and 40 ng of cDNA in each reaction. The RT-qPCR comprised an initial step of 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 s, and 50°C for 1 min, using StepOnePlus™ System (Applied Biosciences, Foster City, CA, United States). Relative expression analysis was performed by the ΔΔCT method [14], and GAPDH was used as endogenous control.

3.2.7 Statistical analysis

Data were expressed as mean ± standard deviation (SD) from three independent experiments. One-way ANOVA with post-hoc Tukey's test was used. Data analysis were based on differences between the positive control (*P. gingivalis* challenged OBA-9 cells) or negative control (non-challenged OBA-9 cells) and the probiotic + *P. gingivalis* challegend OBA-9 cells or probiotic challenged OBA-9 cells. Adhesion of probiotics strains were estimated based on differences between probiotic strain challenged OBA-9 cells (probiotic controls) and probiotic strain + *P. gingivalis* challenged OBA-9 cells. A significance level of 0.05 was established for all tests and the data analysis was performed using the GraphPad Software (GraphPad Prism™ Version 6.0c, La Jolla, CA, USA).

3.3 Results

The screening tests were performed in three stages. Firstly, it was observed that 5 probiotics (*B. breve* 1101A, *B. pseudolongum* 1191A, *B. bifidum* 1622A, *L. acidophilus* LA-5, *L. rhamnosus* Lr-32) were able to reduce pathogen adhesion or invasion, in addition to maintaining OBA-9 cell viability despite *P. gingivalis* challenge. Secondly, we carried out the quantification of inflammatory mediators, and TLR2 and TLR4 gene expression tests that led to the selection of two strains (*L. acidophilus LA-5*, *B. pseudolongum 1191A*) which presented distinct modulatory effects on TLRs while exhibited comparable profiles regarding the synthesis of chemo/cytokines. Thirdly, transcriptional analyzes of other receptors, beta-defensins and apoptosis regulatory genes were performed to assess different patterns that could be involved in the interaction of these probiotics with OBA-9 cells. The findings highlighted *L. acidophilus* LA-5 as an important immunomodulating probiotic of the interaction between gingival epithelial cells and *P. gingivalis* (Figure 3.1).

Figure 3.1 - Flowchart of the screening of probiotic candidates for the treatment of periodontitis

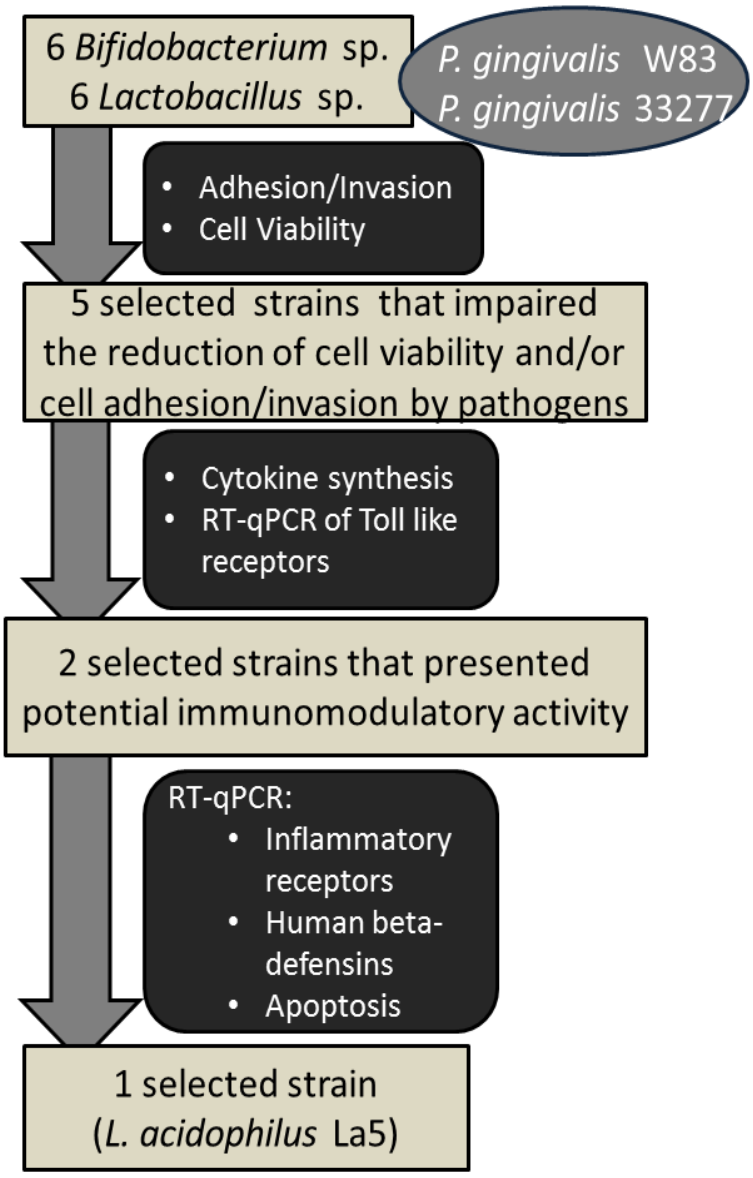
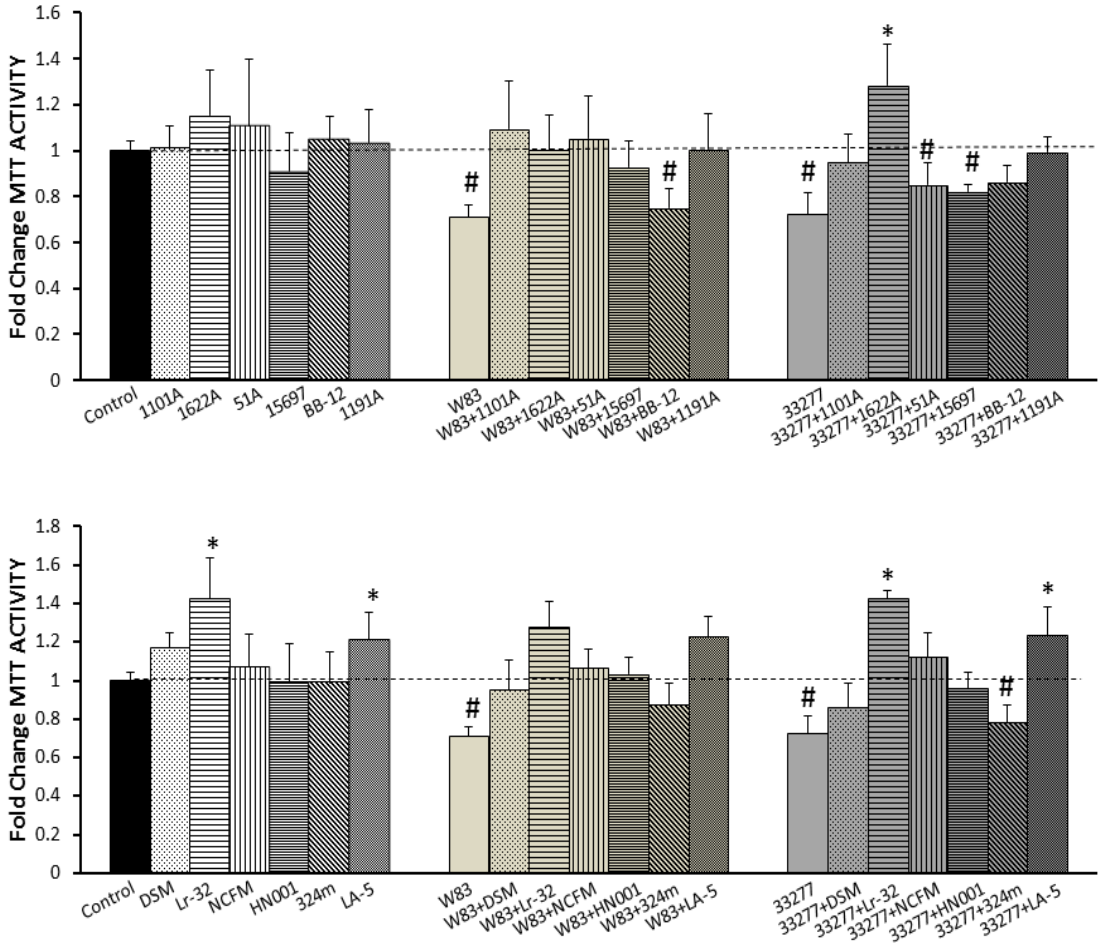


Figure 3.2 - MTT assay presenting the Fold Change of viable cells. The values from non-challenged OBA-9 cells (negative control) were normalized to 1 and values from the other groups established as proportion of it. (*) Significant increase in cell viability by infection with probiotics in relation to the negative control; (#) Infection with *P. gingivalis* (positive controls) reduced cell viability in relation to the negative control and co-infection with probiotics did not neutralize this effect. All tests were performed using a level of 5% at one-way ANOVA with post-hoc Tukey's test



3.3.1 Probiotic species prevent cell mortality caused by *P. gingivalis*

Both *P. gingivalis* strains significantly reduced cell viability, as determined by MTT, when compared to control (OBA-9 in KSFM without antibiotic). The challenge with *P. gingivalis* W83 reduced cell viability in 30.02% ± 5.12 ($p=0.0022$); whereas the reduction was 29.12% ± 9.90 ($p=0.0025$) with *P. gingivalis* 33277 challenge. Mono-infections with probiotics did not alter cell viability, although a slight increase in OBA-9 viability was observed in mono-infection with *L. rhamnosus* Lr-32 and *L. acidophilus* LA-5 ($p<0.01$) (Figure 3.2).

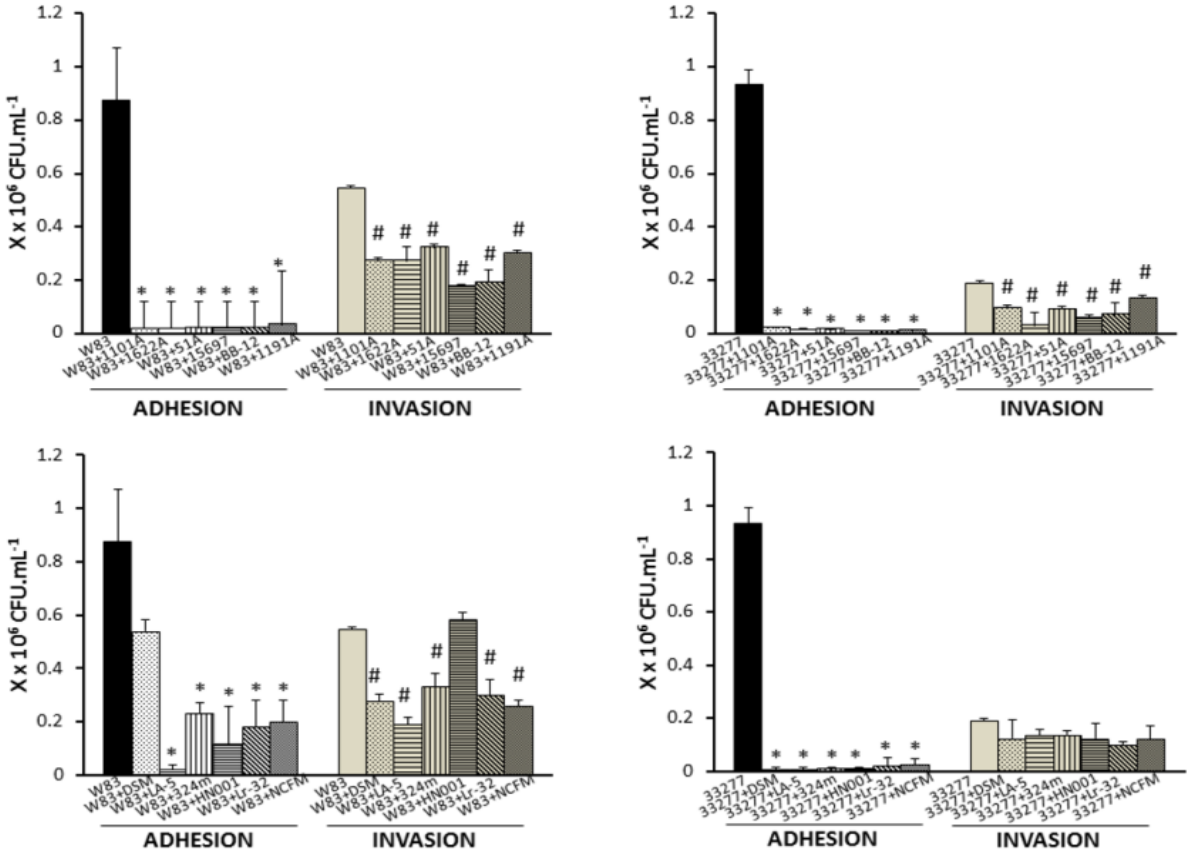
The deleterious effect of *P. gingivalis* on OBA-9 cells viability was abolished when the cells were exposed to a co-infection with most probiotics, except for the co-infections with *P. gingivalis* W83 and *B. animalis* BB-12, *P. gingivalis* 33277 and *B. longum* 51A, *P. gingivalis* 33277 and *B. longum* subsp. *infantis* 15697, and *P. gingivalis* 33277 and *L. casei* 324m. Moreover, the co-infection of OBA-9 cells challenged with *P. gingivalis* 33277 with the probiotics *B. bifidum* 1622A, *L. rhamnosus* Lr-32 and *L. acidophilus* LA-5 has not only prevented the loss in OBA-9 cell viability, but even increased the MTT values compared to control non-infected cells ($p<0.05$) (Fig. 3.2).

3.3.2 Probiotics reduced adhesion and invasion of *P. gingivalis* to gingival epithelial cells

The interaction of *P. gingivalis* W83 and ATCC 33277 with OBA-9 cells, resulted in similar adhesion efficiencies [0.87 (±0.2) x 10^6 CFU.mL^{-1}(~0.43%) and 0.9 (±0.05) x 10^6 CFU.mL^{-1} (~0.49%), respectively]. Both strains were able to invade GECs, as shown by the gentamicin and metronidazole exclusion method, although the percentage of internalized cells was significantly higher for strain W83 than for 33277 ($p<0.01$) [0.54 (±0.07) x 10^6 CFU.mL^{-1} (~0.27%) and 0.20 (±0.04) x 10^5 CFU.mL^{-1} (~0.12%), respectively] (Fig. 3.3). On the other hand, the adhesion of both *P. gingivalis* strains to OBA-9 cells was decreased when the cells were co-infected with the tested probiotics (except for *L. reuteri* 17938 in *P. gingivalis* W83 infected cells). Furthermore, all tested probiotics were able to inhibit invasion of *P. gingivalis* W83, except for the co-infection with *P. gingivalis* W83 and *L. rhamnosus* HN001.

Additionally, *Bifidobacterium* probiotics were able to inhibit invasion of *P. gingivalis* 33277 (Fig. 3.3).

Figure 3.3 - Adhesion and invasion of *P. gingivalis* (acapsuled ATCC33277; capsuled W83) to OBA-9 cells in mono-infection or co-infection with bifidobacteria and lactobacilli for 2 hours. All assays were performed at a MOI of 1: 1,000 (a bacterial suspension of $2.0x10^8$ $CFU.mL^{-1}$ in early-stationary phase). The total number of adhered/invaded pathogens was obtained using an antibiotic exclusion assay. (*), Significant difference in relation to the number of adhered pathogens in mono-infection; (#), Significant difference in relation to the number of invaded pathogens in mono-infection, at the 5% level using one-way ANOVA with post-hoc Tukey's test

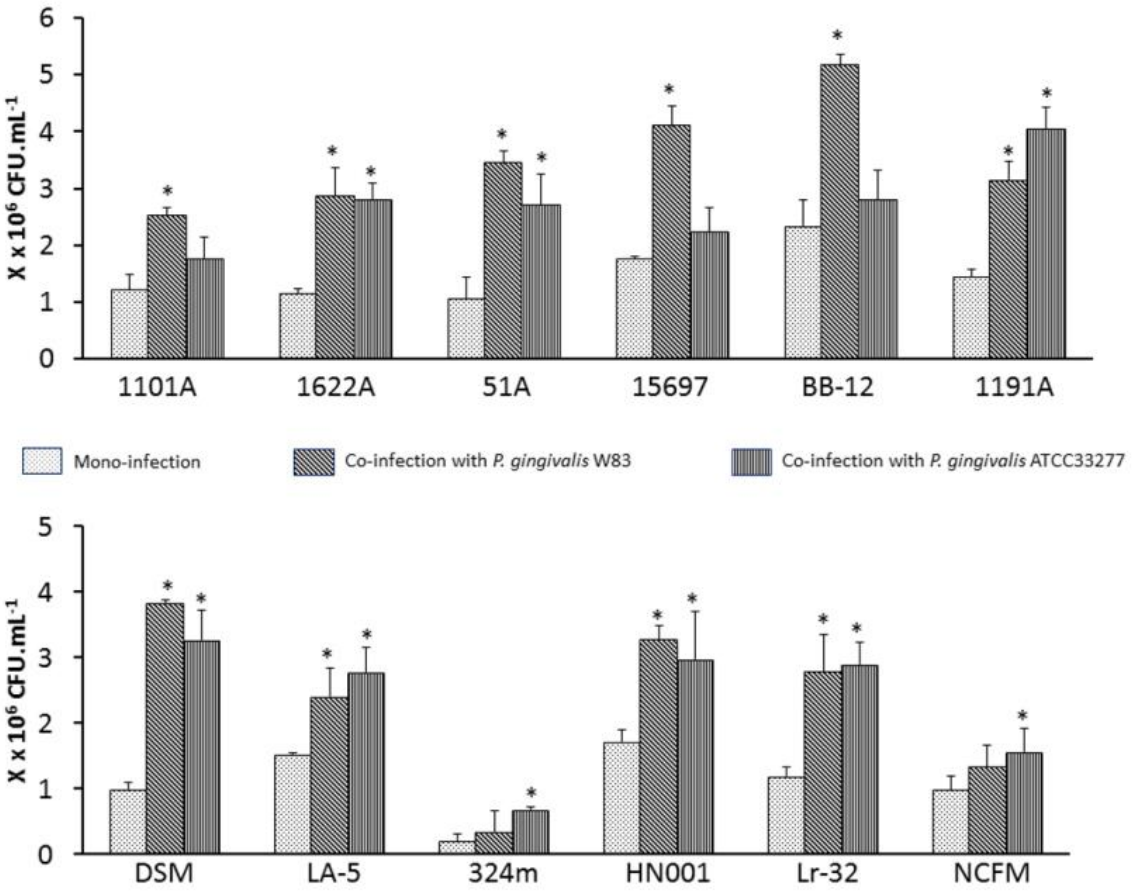


3.3.3 Increased adhesion of probiotics to gingival epithelial cells occurred in co-infection with *P. gingivalis*

Our data also indicated that the tested probiotics were able to adhere to OBA-9 cells. Furthermore, a significant increase in the adhesion of most probiotic strains to OBA-9 cells ($p<0.001$) occurred when cells were challenged with *P. gingivalis*, when compared to mono-infected cells (Fig 3.4).

Overall, the adhesion efficiency of all bifidobacteria increased in the co-infection with *P. gingivalis* W83. However, only three *Bifidobacterium* species showed increased adhesion in the presence of *P. gingivalis* 33277. On the contrary, all *Lactobacillus* species increased their adhesion efficiency in combination with *P. gingivalis* 33277 than in monoculture, but co-infection with *P. gingivalis* W83 increased adhesion efficiency of only four lactobacilli.

Figure 3.4 - Adhesion of bifidobacteria and lactobacilli to OBA-9 cells in mono-infection or co-infection with *P. gingivalis* strains for 2 hours. All assays were performed at a MOI of 1: 1,000 (a bacterial suspension of $2.0x10^8$ $CFU.mL^{-1}$ in early-stationary phase). The total number of adhered probiotics was obtained using an antibiotic exclusion assay. (*), Significant difference in relation to the number of adhered probiotics in mono-infection, at the 5% level using one-way ANOVA with post-hoc Tukey's test



Five probiotics (*B. breve* 1101A, *B. pseudolongum* 1191A, *B. bifidum* 1622A, *L. acidophilus* LA-5 and *L. rhamnosus* Lr-32) were selected for further analysis based on their effects on the adhesion/invasion/viability assays. These probiotics were evaluated by the production of inflammatory mediators and expression of *TLR2* and *TLR4* in *P. gingivalis* challenged GECs (Fig 3.6).

3.3.4 Probiotics altered the cytokine profile of gingival epithelial cells challenged by *P. gingivalis*

The effect of probiotics on the production of IL-1β, TNF-α and CXCL8 is shown in fig 3.5. Mono-infection of OBA-9 cells with both *P. gingivalis* strains increased the synthesis of IL-1β and TNF-α ($p<0.05$), but did not affect CXCL8 production. However, mono-infection of OBA-9 cells with most probiotics reduced the production of IL-1β and TNF-α below basal levels, but did not affect CXCL8 levels. Bifidobacteria attenuated the effect of *P. gingivalis* challenge on IL-1β and TNF-α produced by GECs, except for IL-1β in OBA-9 cells co-infected with *B. breve* 1101A and *P. gingivalis* W83. Co-infection with *L. acidophilus* LA-5 has also induced a pronounced decrease in IL-1β and TNF-α levels, but *L. rhamnosus* Lr-32 was not able to neutralize IL-1β production induced by both *P. gingivalis* strains. In contrast, *L. acidophilus* LA-5 and *L. rhamnosus* Lr-32 increased the production of CXCL8 in co-infection with *P. gingivalis*, when compared with the infection of *P. gingivalis* alone ($p<0.0001$).

3.3.5 Probiotics modulated the transcription of *TLR2* and *TLR4*

Both *P. gingivalis* strains reduced the expression of *TLR2* and increased the transcript levels of *TLR4*, when compared to non-infected control cells. Mono-infection of these cells with most tested probiotics downregulated the transcription of *TLR2* but did not alter *TLR4* mRNA levels. On the other hand, *L. acidophilus* LA-5 promoted a downregulation in*TLR4* transcription but did not alter *TLR2* transcripts levels (Fig 3.6).

Figure 3.5 – Fold Change of the concentration of cytokines (IL-1β, TNF-α) and chemokine (CXCL8) in the collected supernatant. The values from non-challenged OBA-9 cells (negative control) were normalized to 1 and values from the other groups established as proportion from it. (*), Significant difference in relation to the negative control; ($^{\Delta}$), Significant difference in relation to the mono-infection with *P. gingivalis* 33277; ($^{\#}$), Significant difference in relation to the mono-infection with *P. gingivalis* W83, at the 5% level using one-way ANOVA with post-hoc Tukey's test.

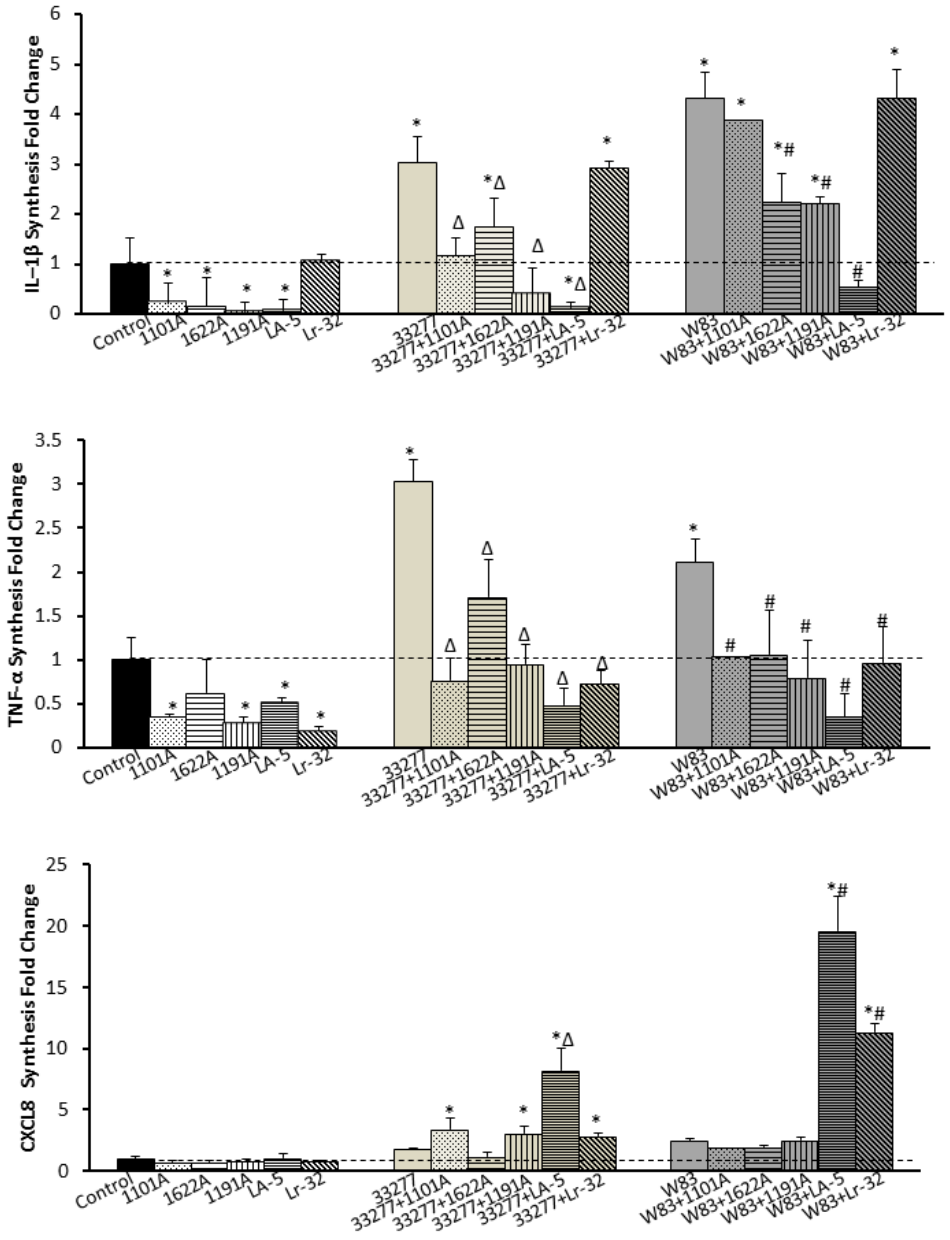
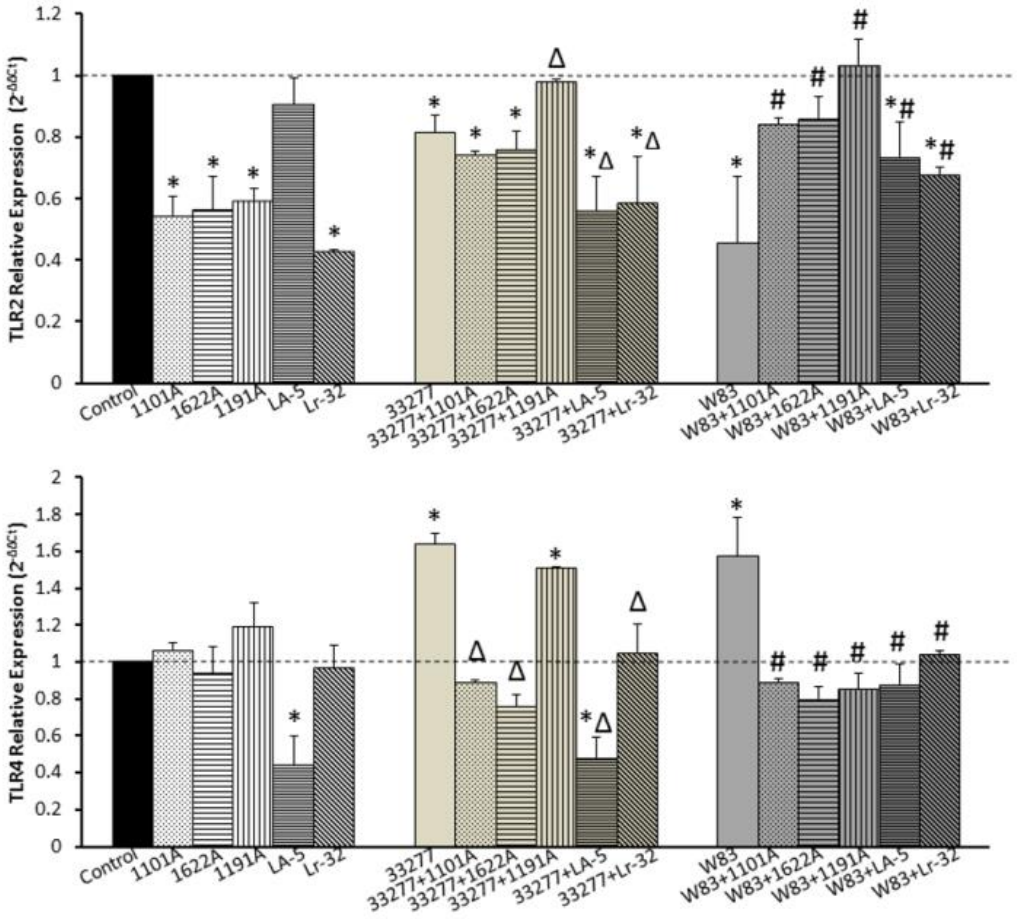


Figure 3.6 - Relative gene expression (average fold change ± SD) of Toll-like receptors (*TLR2*, *TLR4*) in *P. gingivalis* 33277 or W83 challenged OBA-9 cells (positive controls), and experimental groups in mono-infection or co-infection with probiotics in relation to non-challenged OBA-9 cells (negative control), for 2 hours, applying the ΔΔCT method and using GAPDH as endogenous control.(*), Significant difference in relation to the negative control; ($^{\Delta}$), Significant difference in relation to the mono-infection with *P. gingivalis* 33277; ($^{\#}$), Significant difference in relation to the mono-infection with *P. gingivalis* W83, at the 5% level using one-way ANOVA with post-hoc Tukey's test



The presence of probiotics species abolished the effect of both *P. gingivalis* strains on the transcription of *TLR4* (except for *B. pseudolongum* 1191A in *P. gingivalis* 33277 challenged cells). In contrast, both *P. gingivalis* strains decreased the *TLR2* expression, however, the addition of the probiotic species, especially *B. pseudolongum* 1191A, increased the expression of the *TLR2* in the co-infection with both *P. gingivalis* strains.

3.3.6 Probiotics altered the transcription of genes encoding other inflammatory-related receptors, antimicrobial peptides and apoptosis regulatory genes

In the analyzes of other receptors (IL-10R, NLRP3, NOD1, and NOD2) carried out with two strains (*B. pseudolongum* 1191A and *L. acidophilus* LA-5), both probiotics reduced the expression of NOD1, NOD2, and NLRP3, meanwhile only *B. pseudolongum* 1191A increased the transcription of IL10R. Infections with *P. gingivalis* strains also followed a distinct profile and only *P. gingivalis* 33277 up regulated NLRP3, NOD1, and IL-10R ($p<0.01$). The addition of probiotics modulated such networks and *L. acidophilus* LA-5, for example, downregulated NLRP3, NOD1 and NOD2 in OBA-9 challenged by *P. gingivalis* 33277 and enhanced expression of IL-10R in co-infection with *P. gingivalis* W83($p<0.01$) (Fig 3.7).

In addition, a modulatory activity on human β-defensins revealed that *L. acidophilus* LA-5, *B. pseudolongum* 1191A and both *P. gingivalis* strains (in co-infection or not) reduced the expression of BD3. In relation to BD1, while mono-infection with both pathogens did not alter its transcription, co-infection with *L. acidophilus* LA-5 upregulated the transcription of this antimicrobial peptide (Fig 3.7).

Pro-apoptotic genes (CASP3 and CASP9) were downregulated by infection with *P. gingivalis* W83 ($p<0.05$) and were not altered by the ATCC 33277 strain ($p>0.05$). However, co-infection of *P. gingivalis* W83 with *B. pseudolongum* 1191A or *L. acidophilus* LA-5 became the expression of CASP3 similar to controls and upregulated the transcription of CASP9. Still, while *P. gingivalis* W83 downregulated the anti-apoptotic gene Bcl-2, *P. gingivalis* 33277 increased the expression of Bcl-6. However, co-infection of OBA-9 with *L.*

acidophilus LA-5 and *P. gingivalis* W83 induced an increase in the transcription of both genes with proliferative activity ($p<0.01$) (Figure 3.7).

Figure 3.7 - Color code indicating relative gene expression (fold change) of transmembrane (TLR2, TLR4, IL-10R) and intracelular receptors (NOD1, NOD2, NLRP3), β-defensins (BD1, BD3), chemokine (CXCL8), and apoptosis regulators (CASP3, CASP9, Bcl-2, Bcl-6) in relation to the control (only OBA-9 cells), for 2 hours, appling the ΔΔCT method and using GAPDH as endogenous control. Green, significantly increased; Red, significantly reduced; Black, unchanged at the 5% level using one-way ANOVA with post-hoc Tukey's test

GENE	LA-5	1191A	W83	W83 + LA-5	W83 + 1191A	33277	33277+ LA-5	33277+ 1191A
TLR2		0.58	0.45	0.73		0.79	0.56	
TLR4	0.52		1.52			1.62	0.48	1.51
NLRP3	0.40	0.58		0.48		2.02	0.39	1.41
NOD1	0.36	0.51				1.31		
NOD2	0.37	0.52	1.38		1.81	1.44		
IL-10R		3.11		2.28	2.03	2.57	3.53	2.16
hBD1				4.74			5.10	
hBD3	0.19	0.56	0.19	0.18	0.13	0.30	0.14	0.20
CXCL8	6.41	104.07		84.49	10.67	4.53	17.59	44.27
CASP3	0.71		0.66				0.70	0.77
CASP9			0.70	1.34	1.26			
Bcl-2	0.77		0.73	1.34			1.57	1.54
Bcl-6		1.26		1.84	1.32	1.45	1.55	1.50

3.4 Discussion

Bacterial challenge to gingival epithelial cells constitutes the first step towards the innate-adaptive immune cell crosstalk in the early stages of chronic inflammatory periodontal disease, which eventually leads to a destruction of tissues supporting the teeth, including alveolar bone [3,15]. Our data indicate that the effect of a keystone pathogen triggered a response in the epithelial cells, which can be modulated by specific probiotic species. We also revealed that the mechanisms of action from these commercial probiotic species, which are mainly involved in the maintenance of cell viability by reducing the adherence and invasion of periodontal pathogens and by the modulation of the host response, focus on the alteration of recognition of pathogen-associated molecular patterns and on the reduction of the secretion of inflammatory mediators.

Both strains of *P. gingivalis* were able to reduce the viability of OBA-9 cells. In fact, this pathogen has been implicated in different mechanisms of cell death, including apoptosis and autophagy [15,16]. In this context, lysine gingipain from *P. gingivalis* can cleave active caspases and hydrolyze actin in a dose and time-dependent manner, and invasion of the pathogen in epithelial cells is linked to cell death and actin cleavage [17,18]. Moreover, this pathogen can activate cellular autophagy to provide a replicative niche intracellularly [16]. Interestingly, most tested probiotics reduced *P. gingivalis* adhesion/invasion. The decreased adhesion/invasion of *P. gingivalis* to GECs co-infected with probiotics may indicate competition for the same receptor on the epithelial cell surface and/or decreased fitness of the pathogen. Adhesins of lactobacilli and bifidobacteria may compete with pathogens for receptors on the gut [19]. Alternatively, probiotics could have induced a stressful environment by producing acids, bacteriocins or oxidative compounds, altering the transcription profile of the pathogen. This hypothesis is supported by data showing that the transcription of genes involved in biofilm formation, survival and virulence of *P. gingivalis* are regulated by environmental changes [19,20].

The inhibition of adhesion and invasion of *P. gingivalis* promoted by certain probiotics was concomitant to a reduction in the loss of OBA-9 viability, despite the pathogen challenge, suggesting that these effects are linked. Notwithstanding, co-infection with probiotics seems to protect against the pathogen either by a direct effect on *P. gingivalis*, by killing or reducing the expression of virulence factors; by competing for growth factors and

for adhesion/activation sites; [21] and by influencing pathogen-cell signaling, either by altering epithelial cell receptors or changing the microenvironment. Such events may occur simultaneously, enhancing the beneficial effect of probiotics [8].

Additionally, an increase in the adhesion of probiotic strains occurred when co-cultured with the pathogenic strains, resulting in a reduction in adhesion capacity of both *P. gingivalis* strains. This suggests that *P. gingivalis* could induce changes that favor the adhesion of the probiotic strains to epithelial cells. These aspects are relevant, since adhesive properties of probiotics may contribute to their beneficial effects [19,22]. Adhesion of probiotics to host cells is mediated by extracellular polysaccharides and surface layer proteins [19,23]. Expression of these surface components is regulated by environmental conditions, thus affecting their hydrophobic properties and adhesion efficiency [23-26]. Furthermore, certain adhesins of lactobacilli are proteases-sensitive whereas others are resistant [23], suggesting that adhesive properties of probiotics may be altered by *P. gingivalis* proteolytic activity. Other data pointed out that extracellular proteases from *P. gingivalis* may induce degradation of epithelial cells surface proteins [27], possibly altering receptors on the cell surface.

Lactobacillus and *Bifidobacterium* are saccharolytic bacteria, resident in the oral cavity, and both genera can be found in subgingival sites [28,29]. The ability of the studied strains to survive in the oxygen-poor, host-glycans-rich environment of the subgingival sites and alter the microbiome remains to be elucidated. However, our *in vitro* data suggested that *P. gingivalis* colonized sites may be more susceptible to colonization by these organisms. Therefore, further experiments are required to reveal how the interactions between probiotic strains, pathogens, and host cells can affect their phenotypes at the proteomic level [26] and the immunological response in complex biofilms [30].

Epithelial cells respond to pathogens challenge by producing and secreting chemokines and cytokines. *B. pseudolongum* 1191A, *B. breve* 1101A, *L. acidophilus* LA-5, and *L. rhamnosus* Lr-32 increased synthesis of CXCL8 by GECs co-infected with *P. gingivalis*, with emphasis on *L. acidophilus* La-5. As mentioned above, it is already known that the adhesion of probiotics to epithelial cell lines can be mediated by surface layers proteins, which have been also explored as immunomodulators [31]. These proteins have hydrophobic properties that facilitate the autoaggregation of such microorganisms and mediate their high adhesive capacity to epithelial cells, which favors the immunomodulatory

activity of probiotics [19]. For instance, BopA, a cysteine-anchored outer surface lipoprotein involved in the adhesion of bifidobacteria to intestinal epithelial cell lines, increases the release of CXCL8 by Caco-2 cells [32,33]. This chemokine is produced by components of the innate immune response, such as GECs, in order to stimulate migration of polymorphonuclear leukocytes [34], but is also involved in the induction of cell proliferation through the epidermal growth factor receptor [35,36].

Clinical studies indicated an increased expression of CXCL8 in biopsies of gingival tissues from periodontitis sites, although reduced levels were shown in the gingival crevicular fluid [34]. Moreover, deficiencies in the transmigration and attraction of Nφ were reported in sites undergoing severe forms of periodontitis [37,38]. In this sense, the current knowledge suggests that a reduction or no stimuli of the secretion of CXCL8 is a remarkable way of evasion handled by virulent bacteria such as *P. gingivalis* [15]. Indeed, cysteine proteases secreted by *P. gingivalis* strains may even increase the transcription of CXCL8, but arginine gingipains (RgpA/B) consecutively degrade this chemokine [39], preventing the recruitment of defense cells. Furthermore, *P. gingiva*lis inhibition of CXCL8 depends on the production of a serine phosphatase (serB) secreted after *P. gingivalis* epithelial cell invasion, which dephosphorylate NF-kB RelA/p65 [40].

Thus, prevention of *P. gingivalis* invasion in GECs promoted by probiotics may interrupt the inhibition of transcription of *CXCL8* gene, and alter cell signaling pathways and gene expression patterns. This activity of probiotics may not only affect *P. gingivalis* colonization levels, but may also prevent the mechanisms by which this keystone pathogen favors the growth of the entire microbial community and leads to dysbiosis by promoting a localized chemokine paralysis [41].A recent study revealed that co-culture of *L. paracasei* and human gastric epithelial cell lines infected with *H. pylori* resulted in decreased adhesion and reduced *CXCL8* expression, which is elevated in the gastric mucosa by *Helicobacter pylory* [42]. This difference clearly illustrates that probiotics modulatory properties should be explored according to the imbalance in innate response induced by specific pathogens on mucosa surfaces. Taking together the above findings, an increase in the synthesis of *CXCL8*, as observed in co-infection with some probiotics, could bring additional benefits in the control of periodontitis. This hypothesis needs to be evaluated in future studies, as well as the pathways by which probiotics regulate CXCL8 production.

TLR-signaling networks initially recognize and respond to microbial components and play an important role for the innate response mediated by GECs [43]. In this line, structures provide *P. gingivalis* with an unusual ability to modulate and interact with TLR2 and TLR4

[44,45]. However, a recent study indicated that *P. gingivalis* LPS pro-inflammatory activity is mediated exclusively through TLR4 in human blood cells [46]. Our data indicated that *P. gingivalis* strains upregulated the expression of TLR4 in OBA-9 human epithelial cells, however downregulated TLR2. Furthermore, *P. gingivalis* induced an increase in the synthesis of pro-inflammatory mediators (e.g. TNF-α and IL-1β) mainly via TLR4 activation, as indicated by previous studies [47,48]. These cytokines may help to perpetuate the local inflammatory response, induce degradation of the extracellular matrix in the connective tissue underlying the periodontal pockets, and osteoclastogenesis [7, 49].

The co-infection of *P. gingivalis* challenged GECs with probiotics triggered an opposite response, since *B. breve* 1101A, *B. pseudolongum* 1191A, *B. bifidum* 1622A, *L. acidophilus* LA-5, and *L. rhamnosus* Lr-32 were able to modulate the transcription of genes encoding TLR2 and TLR4. Furthermore, co-infection of *P. gingivalis* challenged GECs with probiotics resulted in decreased cytokine release into cells supernatants. Previous data indicated that probiotics lipoteichoic acid-TLR2 crosstalk leads to a downregulation of TLR4 and an increase in the expression of its negative regulators (A20, TOLLIP, IRAK-M) [50,51]. However, the influence of probiotics on TLRs expression and activation seems to be strain-specific (Fig. 6). In fact, previous studies have shown that *L. jensenii* attenuates the expression of pro-inflammatory cytokines caused by *E. coli* LPS challenge in intestinal epithelial cell lines by downregulating TLR4-dependent activation [52], whereas *B. longum* subsp. *infantis* can present similar anti-inflammatory effects by upregulating TLR4 in fetal human enterocytes [53]. Our observations are particularly relevant to periodontitis, since TLR4 expression is increased in gingival tissues of periodontitis sites compared to health [54].

However, this regulatory activity of probiotics does not appear to be restricted to TLRs, but also to receptors related to anti-inflammatory mediators (e.g. IL-10R) and a diversity of intracellular receptor proteins, such as NOD1 and NOD2, that respond to a diverse set of molecules involved in the metabolism of bacteria. On this basis, under challenge conditions with pathogens it seems that probiotics can also control IL-10R expression since while W83 strain infection did not alter its transcription, co-infection with *B. pseudolongum* 1191A and *L. acidophilus* LA-5 upregulated it. In humans, IL10/IL10R crosstalk plays critical roles in controlling the intestinal mucosal homeostasis, regulating the host cell function, and shaping the intestinal microbiome [55]. In addition, it also inhibits TLR4 signaling by inducing a downregulation of pro-inflammatory cytokines [56].

In this context, NOD1 and NOD2 respond to diaminopimelic acid and muramyl dipeptide from Gram-negative bacteria and sequentially activate pro-inflammatory canonical pathways [57,58]. At the same time, such components from Gram-positive microorganisms seem to interact with NOD2 and increasing the synthesis of defensins [58]. Our data showed that both *P. gingivalis* strains increased NOD2 expression, but only *P. gingivalis* 33277 upregulated NOD1 and NLRP3, which could help to distinguish it from the virulent characteristic of subverting the immune system via host inflammatory response impairment attributed to the capsuled strains, such as W83 and W50 [59]. However, *L. acidophilus* in both mono- and co-infection reduced the expression of all these pattern recognition receptors, which highlights it as an interesting immunomodulator for bacterial species that increase the transcription of such receptors.

Such regulation seems to be important in a future therapeutic perspective in the periodontology since it is well-accepted that a tight control of the immune system that resolves infection and tissue damage is fundamental to health. Our data are corroborated by proteomic analyzes which indicated that peptidoglycan or N-acetylmuramic acid on the cell wall of *L. acidophilus* presents anti-inflammatory properties [60,61]. Moreover, *L. acidophilus* S-layer associated proteins have recently gained attention due to their potential role in cell proliferation, adhesion, and immunomodulation. It should be also mentioned that factors such as growth phase may alter the S-layer composition [26], which can result in different properties for the same strain.

3.5 Conclusion

In summary, our data support the hypothesis that probiotics can modulate the inflammatory response mediated by *P. gingivalis* in GECs. This screening study pointed out that although probiotics may prevent cell death and reduce bacterial adhesion and invasion, their ability to interfere in the synthesis of chemo/cytokines as well as to dictate the expression of patter recognition receptors can be strain-specific. Among the twelve evaluated probiotic strains, *L. acidophilus* LA-5 emerges as an important therapeutic alternative for periodontitis due to its immunomodulatory potential (Fig. 1). However, since only living microorganisms have been analyzed, further studies are necessary to evaluate which structural

or secreted products of this oral probiotic candidate are involved in the cell signaling and pathways activated from specific sensor-receptor crosstalks.

3.6 References

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4 CHAPTER II: MaR1/RvE1 improve regerative-related activities of human periodontal ligament stem cells under IL-1β/TNF-α stimulus*

ABSTRACT

Background: Maresin-1 (MaR1) and Resolvin E1 (RvE1) are specialized pro-resolving mediators (SPMs) that regulate inflammatory processes and previous studies have demonstrated their regenerative potential *in vivo*.

Objective: We evaluated the impact of MaR1 and RvE1 in an inflammatory environment on regerative-related activities of human periodontal ligament stem cells (hPDLSCs).

Material and Metods: hPDLSCs were treated with MaR1 (10nM) and/or RvE1 (10nM) with or without IL-1β (10ng/mL) and/or TNF-α (10ng/mL) for 24h. Flow cytometry (FC) was used to evaluate stemness markers (CD45, CD11b, CD73, CD90, CD105, CDHLA-ABC, Oct-4, and Sox-2). Cell proliferation (MTT assay), apoptosis activity (AnnexinV staining), and *in vitro* wound healing were also measured. Biomarkers of periodontal ligament regeneration [tenomodulin, α-SMA, and periostin] were analyzed by RT-qPCR, Immunofluorescesce, and FC. After osteogenic differentiation treatment, alizarin red staining was performed, alkaline phosphatase was determined by ELISA, cemento-osteogenesis biomarkers (Runx2, Osteocalcin, CEMP1, CAP) were evaluated by RT-qPCR and WB, and ChemR23 was analysed by FC.

Results: Overall, IL-1β/TNF-α stimulus reduced the stemness of hPDLSCs, the expression of fibrogenesis regenerative biomarkers, impaired *in vitro* wound healing by inducing cell death, and decreased cementum-osteogenic activity. However, MaR1/RvE1 reverse this process partially rescuing biomarkers of stemness, ligament regeneration and accelerating wound healing by inducing cell proliferation and reducing apoptosis. MaR1/RvE1 also increased cementum-osteogenesis, which at least in relation to the MaR1-TNF-α stimulus may be related to a different regulation of the ChemR23 receptor.

Conclusion: MaR1 and RvE1 are important denominators for regenerative activity of periodontal tissues lost to inflammatory milieu.

Key words: Mesenchymal Stem Cells; Inflammation Mediators; Osteogenesis.

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4.1 Introduction

The understanding of the inflammatory response in the periodontal diseases has been limited to the study of pro-inflammatory mediators and resolution of inflammation has been understood as a passive process [1-3]. However, beyond the mediators that activate inflammation, there is a cascade of events that determine the synthesis of specialized pro-resolving mediators (SPMs) that act as endogenous agonists. These lipids, including resolvins and maresins, activate physiological pathways that determine the beginning of the resolution phase and the end of the acute inflammatory response. In this process, SPMs have presented a wide variety of functions ranging from inducing changes in biofilm composition, reorganizing the host response, enhancing bacterial phagocytosis and efferocytosis to stimulating pro-regenerative activities in order to reverse tissue destruction [4-7].

In the context of the regeneration, Maresin 1 [macrophage mediator in resolving inflammation (MaR1)] has presented potential to accelerate surgical wound in planaria, providing new insights that can potentially link organ regenerative response and tissue healing [8,9], as well as resolvins, such as RvE1, have promoted *in vivo* periodontal regeneration [10]. However, in spite of this promising perspective, little is known about the action of these mediators on regenerative-related activities, namely proliferation, migration and differentiation of human periodontal ligament stem cells (hPDLSCs) that are responsible for maintaining the tissue homeostasis and for the self-renewal processes that appear to be altered in the inflammatory microenvironment of the periodontitis [11,12].

In this perspective, trying to understand how this complex network of natural endogenous mediators such as inflammatory cytokines (e.g. IL-1β, TNF-α) and bioactive SPMs (e.g. MaR1, RvE1) may alter the stemness of cells, such as hPDLSCs, would be a key step towards to control their properties and in order to turn the stem cell-based therapy a successful approach. Moreover, in the context of the periodontitis must also be important the study of periodontal-ligament related regenerative biomarkers [e.g. periostin, tenomodulin and α-smooth muscle actin (α-SMA)] that are involved in the adhesion, migration and differentiation of hPDLSCs [13-16]. Still, an analysis of the cementum-osteogenesis phenomenon and correlated receptors (e.g. Chemerin Receptor 23 (ChemR23)] that may mediate the action of several mediators under inflammatory conditions [17-19].

Therefore, the objective of this study was to evaluate the impact of MaR1 and RvE1 on the stemness of hPDLSCs under an inflammatory milue with IL-1β and TNF-α as well as on their periodontal-related regenerative activities.

4.2 Material and Methods

4.2.1 Ethics Statement

The protocol and informed consent to use human periodontal ligament biopsy specimens were reviewed and approved by the Research Ethics Committee at Forsyth Institute (IRB #14-10) and conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000.

4.2.2 hPDLSC Isolation, Culture and Phenotyping

Two human third molars removed for impact reasons from two systemically healthy patients (ages 24-25 years) without periodontitis or predisponent associated factors, such as dental calculus and caries, were used. The isolation and culture of hPDLSCs were carried out as previously described in the literature, with minor modifications [20,21]. Briefly, the teeth were rinsed with α-minimum essential medium (α-MEM, Life Technologies, NY, USA) plus antimicrobial solution [0.1% Amphotericin B 250μg/mL (CellGro, VA, USA) + 1% PenStrep (Gibco, Life Technologies,NY, USA)] three times, and PDL tissues were separated from the surface of the middle third of the root. Then, the collected PDL tissues were placed in a centrifuge tube and digested with α-MEM supplemented with 3 mg/mL collagenase Type I and 4 mg/mL dispase (both from Sigma-Aldrich, MO, USA) for 1 hour at 37 C (at 20 minute intervals the sample had to be shaken with a Vortex during 30 seconds). Then, the tissues were transferred to 6-well plates containing α-MEM supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, GA, USA), 0.292 mg/mL glutamine, and 1% PenStrep. After 24h unattached cells and debris were washed out, and new medium added. Then, culture

medium were replaced every 48 hours. After reaching semiconfluence, subculture was performed using 0.25% Trypsin-EDTA (Gibco, Life Technologies, NY, USA), and then storage (Passage 0) or spread out on a T75 Primaria culture flask (Passage 1). Only passages from P3 to P5 were phenotyped and used in all experiments. Stemness biomarkers were evaluated as previously described [22]. The hPDLSC phenotype was determined by flow cytometry using the following surface antibodies: CD11b/FITC, CD44/APC/Cy7, CD45/FITC, CD73/Pacific Blue, CD90/AlexaFluor 700, CD105 APC, human leukocyte antigens (CDHLA-ABC)/PE (all from Biolegend, CA, US). Pluripotent embryonic markers, octamer-binding transcription factor 4 (OCT4)/BrilliantViolet421, and (sex determining region Y)-box2 (SOX2)/AlexaFluor647 were also evaluated. Compensation beads were used for each antibody and unstained cells were used as negative controls. Cells were analyzed with the Attune NxT (Invitrogen, ThermoFischer, USA) using the FlowJo software.

4.2.3 Experimental Design

hPDLSCs were seeded in 24-well or 6-well plates in α-MEM (1% FBS+1% PenStrep) at concentrations from $1x10^5$ cells/well to $1x10^6$ cells/well, and divided into 12 experimental groups, as follows: (1) control (unstimulated); (2) MaR1 (10nM); (3) RvE1 (10nM); (4) MaR1 (10nM) + RvE1 (10nM); (5) Il-1β (10ng/mL); (6) Il-1β (10ng/mL) + MaR1 (10nM); (7) Il-1β (10ng/mL) + RvE1 (10nM); (8) TNF-α (10ng/mL); (9) TNF-α (10ng/mL)+ MaR1 (10nM); (10) TNF-α (10ng/mL) + RvE1 (10nM); (11) Il-1β (10ng/mL) + TNF-α (10ng/mL); (12) Il-1β (10ng/mL) + TNF-α (10ng/mL)+ MaR1 (10nM) + RvE1 (10nM). After 24h incubation, stemness phenotyping was carried out using the biomarkers described above and regenerative properties of periodontal interest were carried out as follows.

4.2.4 Regenerative-Related Periodontal Ligament Biomarkers

4.2.4.1 Immunofluorescence

hPDLSCs were seeded in 24-well plates in semi-confluence (~$1x10^5$ cells/well) on coverslips and after 24h treatment they were fixed using 4% paraformaldehyde for 10 min at room temperature. Then, cells were permeabilized for 10 min with 0.1% Triton X-100, and incubated with 1% BSA for 30min to block unspecific binding of the antibodies. Periostin (PRSTN) rabbit anti-human (Invitrogen, IL, USA), α-Smooth muscle actin (α-SMA) mouse anti-human (R&D System, MN, USA), Tenomodulin rabbit anti-human (Abcam, MA, USA) diluted in 1%BSA/PBS to 1:100 ratio were used as primary antibodies and incubated for 60 min at RT. Alexa-Fluor 568 goat anti-rabbit IgG and Alexa-Fluor 488 goat anti-mouse IgG diluted in 1%BSA/PBS to 1:1000 ratio were used as secondary antibodies and incubated for 60 min at RT in the dark. For counter staining was used DAPI (0.1μg/mL) (Sigma-Aldrich, MO, USA) for 1 min. Then, coverslips were mounted with a drop of Fluoroshield mounting medium (Abcam, MA, USA). Images were taken using the Zeiss Axio Observer A1 and analysed using the Zen software.

4.2.4.2 RT-Qpcr

Reverse transcription followed by quantitative real-time PCR (RT-qPCR) was used to assess relative gene expression of hPDLSCs after 24h treatment with the experimental groups. After cell lysis, total RNA was extracted using RNeasy KIT (QIAGEN, CA, USA). The quality and concentration of the extracted RNA were determined by measurement of absorbance at 260 and 280 nm in a NanoDrop™ One Spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA was reverse transcribed into cDNA using SuperScript VILO Master Mix (Invitrogen, Waltham, MA, USA). The conditions for reverse transcription were 10 min at 25°C, 60 min at 42°C, and 5 min at 85°C. PCR was performed using TaqMan® Gene Expression Master Mix (Applied Biosciences, Foster City, CA, USA), TaqMan primers and probes (ThermoFisher, IL, USA) for α-SMA (*Hs004263835_g1*), TNMD (*Hs00943209_g1*), PRSTN (*Hs01566737_g1*), and GADH (*Hs02786624_g1*), and 40 ng of cDNA in each reaction. The qPCR comprised an initial step of 50°C for 2 min, 95°C for 10

min followed by 40 cycles at 95°C for 15 s, and 50°C for 1 min, using StepOne Plus™ System (Applied Biosciences, CA, USA). Relative expression analysis was performed by the ΔΔCT method [23], and *GAPDH* was used as endogenous control.

4.2.4.3 Flow Cytometry

hPDLSCs were seeded in 6-well plates (~$1x10^6$ cells/well) and after 24h-treatment monolayers were washed with PBS1x, dettached with 0.25% Trypsin-EDTA and centrifuged at 400 x g for 5 min/4°C. Fixation, permeabilization, blocking and immunostaining for primary and secondary antibodies were performed according to the steps described in the Immunofluorescence section. Unstained cells were used as negative controls and all experimental groups were analyzed with the Attune NxT (Invitrogen, ThermoFischer, USA) using the FlowJo software.

4.2.5 Wound Healing Assay

Scrath assay to study cell migration *in vitro* was design according to Liang et al. (2007) [24]. Briefly, scratches were created with a p200 pipet tip on confluent cell monolayers in 24-well plates (~2.5 x 10^5 cells/well). Scratches of approximately similar size (800-1000μm^2/field in 10x magnification at 0h) were carried out for all experimental groups in order to minimize any possible variation caused by the difference in the width of the scratches. Images of three fields with reference markings per well were taken at different time-points (0h, 8h, 16h, 24h) using a 10x magnification digital inverted-phase microscope (Olympus CK40, Spach Optics, NY, USA), and the wound area was calculated using the ImageJ software. The percentage of wound healing (%WH) was calculated at each time-point by the ratio: Wound surface area at Time-point X / Wound surface area at Time-point 0h X 100.

4.2.5.1 Cell Viability Assay

hPDLSC viability in all time-points of the wound healing assay was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) [25]. The OD at 570 nm was measured using the SpectraMax 340PC (Marshall Scientific, NH, USA) microplate reader. The number of surviving cells was calculated based on the number of viable cells immediately after the scratch was performed (N_0, T_0), as follows: Number of viable cells in T_x (N_x) = OD_{570nm} in T_x x N_0/OD_{570nm} in T_0. N_0 was standardized for $2x10^5$ cells in all experimental groups.

4.2.5.2 Apoptosis Assay

After 24h of the wound healing assay, hPDLSCs were detached by 0.25% Trypsin-EDTA as previously described (Hemming et al. 2014) and the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, MA, USA) was used to measure apoptotic cells following the manufacturer's recommendations.

4.2.6 Cementum-Osteogenic Differentiation

4.2.6.1 Alizarin Red S Staining

hPDLSCs seeded in 24-well plates in semi-confluence (~$1x10^5$ cells/well) were cultivated in osteogenic differentiation medium (Oricell, Cyagen, CA, USA) with the experimental groups dercribed above. Every three days the medium was changed and the experimental conditions renewed. After 7-days treatment Alizarin Red S Staining (ARS) quantification assay was performed in order to measure the amount of calcium deposits in cell culture following the manufacturer's recommendations (ARed-Q, ScienCell, CA, USA). The

concentration (mM) of ARS in the samples was calculated based on a calibrated OD_{405nm} standard curve.

4.2.6.2 Alkaline Phosphatase ELISA

After 7-days treatment, supernatants were also collected in order to measure the concentration of Alkaline phosphatase (ALP) produced by differentiated hPDLSCs under experimental conditions. The human ALPI/Alkaline Phosphatase ELISA kit (LSBio, LifeSpan BioSciences, WA, USA) was used according to manufacturer's recommendations. The concentration (ng/mL) of ALP in the supernatant of the samples was calculated based on a calibrated OD_{450nm} standard curve.

4.2.6.3 RT-qPCR

Gene expression of cementum-osteogenic markers of hPDLSCs treated in differentiation medium was carried out using Taqman probes (ThermoFisher, IL, USA) [Runx2 (*Hs01047973_m1*), Osteocalcin (OCN) (*Hs01587814_g1*), Cementum Protein 1 (CEMP1) (*Hs04185363_s1*), and Cemmentum Attachment Protein (CAP) (*Hs00171965_m1*)] by RT-qPCR following the protocol described above.

4.2.6.4 Western Blotting

Western blotting was performed as previously described (Siddiqui et al. 2019). Briefly, hPDLSCs were seeded in 6-well plates in semi-confluence (~$1x10^6$ cells/well), treated in differentiation medium with the experimental groups for 7 days, and then lysed using CelLytic M (Sigma-Aldrich, MO, USA) and 1:100 protease inhibitor cocktail (CellSignaling, MA, USA). After centrifugation of the cell lysate, protein concentration was determined with the Pierce BCA protein assay kit (ThermoFisher, IL, USA). Twenty

micrograms of each sample were prepared using 25% TruPAGE LDS Sample Buffer 4x and 2.5% 2-mercaptoethanol (Sigma-Aldrich, MO, USA), loaded in 12% TruPAGE Precast gels (Sigma-Aldrich, MO, USA), runned at 100V for ~1h, and transferred onto a PVDF membrane at 66mA, overnight. Then, membranes were incubated overnight at 4°C with primary antibodies [Runx2 (ZR002) (1:500) (ThermoFisher, IL, USA), OCN (1:1,000) (ThermoFisher, IL, USA), CEMP1 (1:1,000) (Abcam, MA, USA), CAP (1:250) (Abcam, MA, USA), GADPH (1:1,000) (CellSignaling, MA, USA)], and incubated for 2h at RT with secondary HRP-linked antibodies IgG (1:2,000) (CellSignaling, MA, USA). The membranes were developed with SuperSignal West Pico Chemiluminescent substrate (ThermoFisher, IL, USA), and bands were analysed in the GBox F3 (Syngene, NC, USA) using the GeneSnap software. Densitometric analysis was performed using ImageJ software, and protein expression was calculated using GAPDH as internal control.

4.2.7 ChemR23 Analysis

The role of the ChemR23 during the cementum-osteogenic differentiation was evaluated by Flow Cytometry. hPDLSCs seeded in 6-well plates in semi-confluence (~$1x10^6$ cells/well) were treated in both conditions, differentiation medium or non-differentiation medium [α-MEM (1% FBS+1% PenStrep)], with MaR1 and/or TNF-α for 7 days. Flow Cytometry was carried out using anti-ChemR23/APC (Miltenyi Biotec, MA, USA) following the manufacturer's recommendations.

4.2.8 Statistical Analysis

All experiments were carried out in triplicate using the hPDLSCs isolated from the two donors. The data were expressed as mean ±SEM. ANOVA with pos-hoc test Tukey was used to analyse statistical differences between experimental groups and controls (unstimulated) and IL-1β/TNF-α treated groups. Differences were considered significant when $p < 0.05$.

4.3 Results

4.3.1 IL-1β/TNF-α stimulus reduce the stemness of hPDLSCs but treatment with MaR1/RvE1 partially rescue their biomarkers

hPDLSCs may undergo changes as a result of stimuli present in the extracellular milieu, however it is still little unknown whether mediators involved in the inflammatory cascade may alter their stemness immunophenotype. Therefore, we evaluated whether pro-inflammatory or pro-resolution conditions could alter hPDLSCs phenotype characterized by typical stemness biomarkers within 24h treatment.

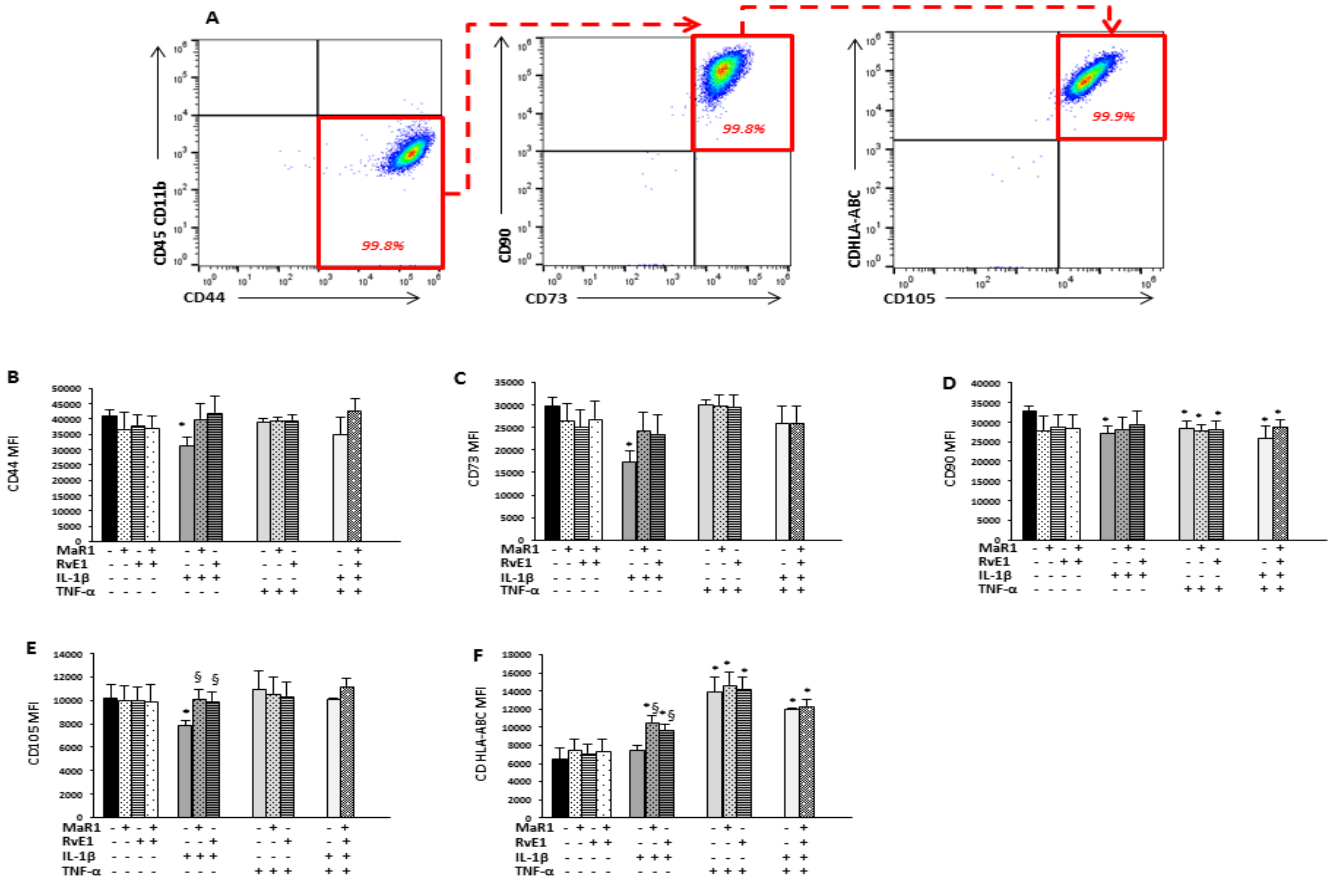
We observed that neither proinflammatory mediators (IL-1β/TNF-α) nor those involved in the pro-resolution cascade (MaR1/RvE1) altered the percentage of hPDLSCs immunophenotyped as $CD45^{-}11b^{-}CD44^{+}CD73^{+}CD90^{+}CD105^{+}CDHLA\text{-}ABC^{+}$. Following the flow gate strategy desplayed in the Fig. 8, ~100% of the cells in all experimental groups showed this typical phenotype of hPDLSC (Supporting information Fig. S1). However, MFI analysis pointed out that IL-1β treatment reduced the expression of CD44, CD73 and CD105, which are responsible for stimulating cell adhesion and migration, improve cell barrier under inflammatory hypoxia, and serve as receptor for TGF-β superfamily ligands, respectively [26-28]. Interestingly, co-treatment of this group with MaR1 or RvE1 rescued this deleterious effect rendering the expression of these surface markers similar to the untreated control (Fig. 4.1B, Fig. 4.1C, Fig. 4.1E). In addition, both pro-inflammatory conditions also decrease the expression of CD90 which is responsible for cell-extracellular matrix interactions [26]. However, co-treatment of IL-1β with MaR1 or RvE1 prevented the reduction in the expression of this biomarker (Fig. 4.1D).

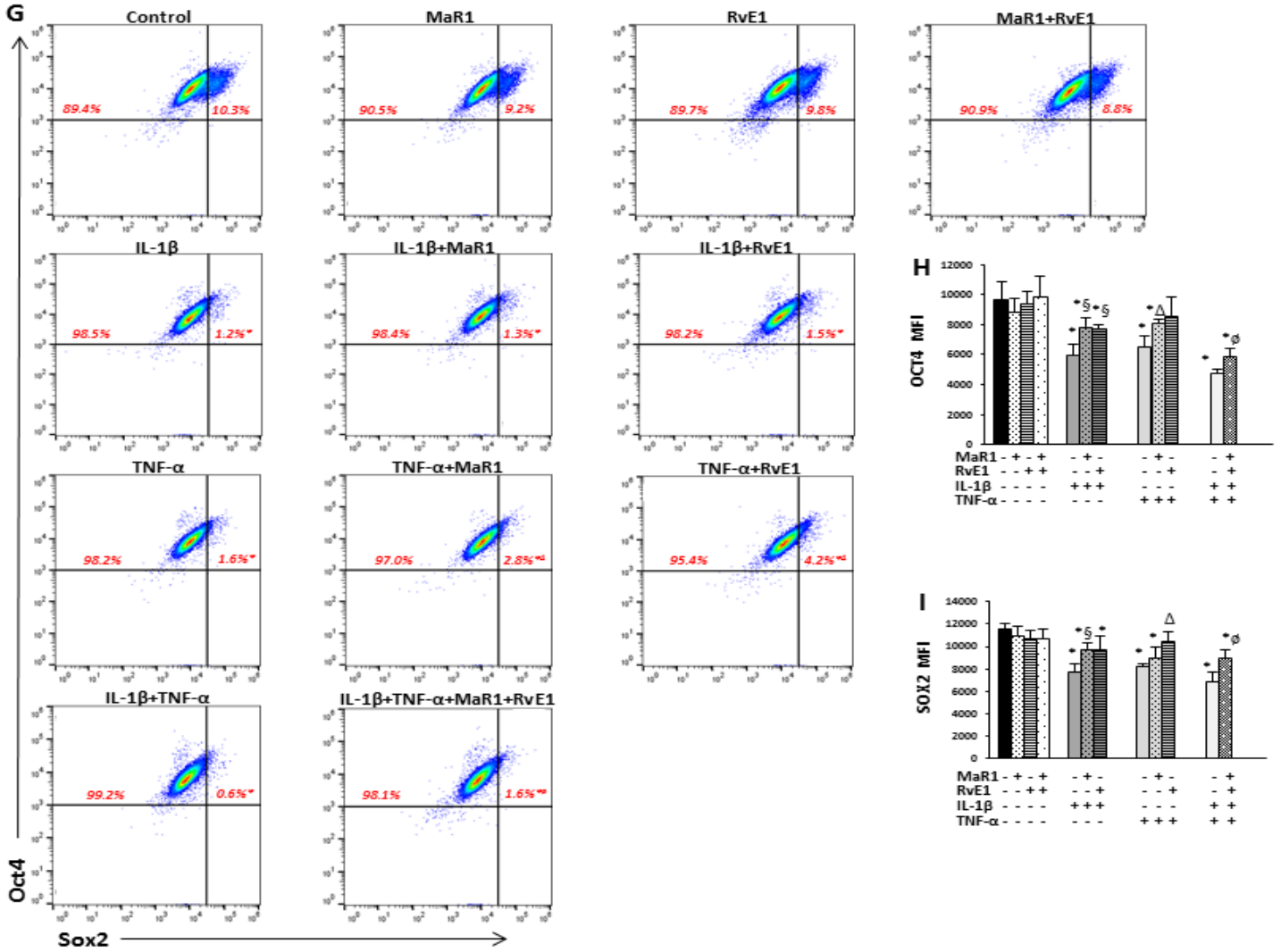
Furthermore, MHC Class I molecules are also expressed in hPDLSCs [22], which is corroborated with the present study (Fig. 8F, Supporting information Fig. S1). While HLA-A is constitutively highly expressed in human mesenchymal stem cells, HLA-B and -C have low expression [29]. On this subject, inflammatory stimuli such as INF-γ (25 ng/mL) for 24 h can reverse this ratio, and it is important to note that low constitutive expression of HLA-B and –C hamper complementary-dependent cytotoxicity [30]. Unfortunately, we could not measure how the MaR1/RvE1 - IL-1β/TNF-α stimuli change A/B/C ratio since HLA-ABC mutual

immunostaining was used. Still, although both miliues do not change the total percentage of positive cells for simultaneous labeling with all these HLA class I molecules (~ 100% HLA-ABC$^+$ cells, Supporting information Fig. S1) at least the intensity in this proportion can be altered. Co-treatment of IL-1β with MaR1/RvE1 as well as TNF-α alone or in combination with both pro-resolving mediators increased the overall expression of HLA-ABC (Fig. 4.1F). Considering that the development of immunocompatible pluripotent stem cells is important for a successful cell based therapy with respect to the transplantation process [31], and pro-resolving/pro-inflammatory mediators ratio seems to alter MHC Class I molecules, more detailed analysis will be required in order to evaluate further *in vivo* applications.

In addition to surface markers, we also analysed transcription factors that usually characterize the stemness pluripotency (e.g. Sox2 and Oct4), which appear to be mutually counter-regulated in stem cells [22]. In fact, Sox2 binds to DNA cooperatively with Oct4 at non-palindromic sequences to activate transcription of key pluripotency factors [32]. Acoordingly, while Oct4 could play its role by sustaining self-renewal capacity of adult somatic stem cells, Sox2 would be responsible for their proliferative activity [33,34]. Still, a double Sox2$^+$Oct4$^+$ immunostaining would determine an increase in their osteogenic potential [35]. In this context, although hPDLSCs were immunopositive for Oct4 independently of the experimental condition (Supporting information Fig. 1, Fig. 4.1G), only a small percentage of these cells had double Sox2$^+$Oct4$^+$ staining which underwent significant variations according to the treated groups. While the application of MaR1/RvE1 did not alter the percentage of Sox2$^+$Oct4$^+$ cells compared to the untreated control (~10%, $p>0.05$), in the groups under inflammatory stimuli this population was practically abolished (0.2-1.8%, $p<0.05$). Moreover, TNF-α reduced the percentage of this population to 1.6%, but co-treatment with Mar1 and RvE1 significantly ($p<0.05$) increased to 2.8% and 4.2%, respectively, although it still remained below the control (Fig. 4.1G). Similar situation was observed in the MFI analysis. Pro-inflammatory conditions Il-β/TNF-α reduced the fluorescence of Sox2$^+$ and Oct4$^+$ cells and although co-treatment with MaR1/RvE1 overall increases the MFI of these transcription factors, they still remain lower than controls (Fig. 4.18H, Fig. 4.1I). Thus, these findings point out that pro-inflammatory mediators reduce the stemness of hPDLSCs while bioactive lipid endogenous mediators produced in the pro-resolution phase restore their phenotype.

Figure 4.1 - hPDLSC phenotyping analysis with negative (CD45, CD11b) and positive (CD73, CD90, CD105, CDHLA-ABC) surface stemness biomarkers and pluripotent embryonic markers (Sox2, Oct4) after 24h of stimuli with mediators.**A**, Representative dot plots showing the flox gate strategy for surface immunophenotyping. **B-F**, Mean fluorescence intensity (MFI) analysis of positive surface markers. **G**, Dot plot showing the percentage of $Sox2^{+}$ and $Oct4^{+}$ hPDLSCs. **H-I,** MFI analysis of embryonic markers. (*), $p<0.05$ versus control (non-stimulated); (§) $p<0.05$ versus group treated with Il-1β; Results are given as the mean ± SEM




G
Control
89.4%
10.3%
MaR1
90.5%
9.2%
RvE1
89.7%
9.8%
MaR1+RvE1
90.9%
8.8%
IL-1β
98.5%
1.2%
IL-1β+MaR1
98.4%
1.3%
IL-1β+RvE1
98.2%
1.5%
TNF-α
98.2%
1.6%
TNF-α+MaR1
97.0%
2.8%
TNF-α+RvE1
95.4%
4.2%
IL-1β+TNF-α
99.2%
0.6%
IL-1β+TNF-α+MaR1+RvE1
98.1%
1.6%
Oct4
Sox2
H
OCT4 MFI
MaR1
RvE1
IL-1β
TNF-α
I
SOX2 MFI
MaR1
RvE1
IL-1β
TNF-α

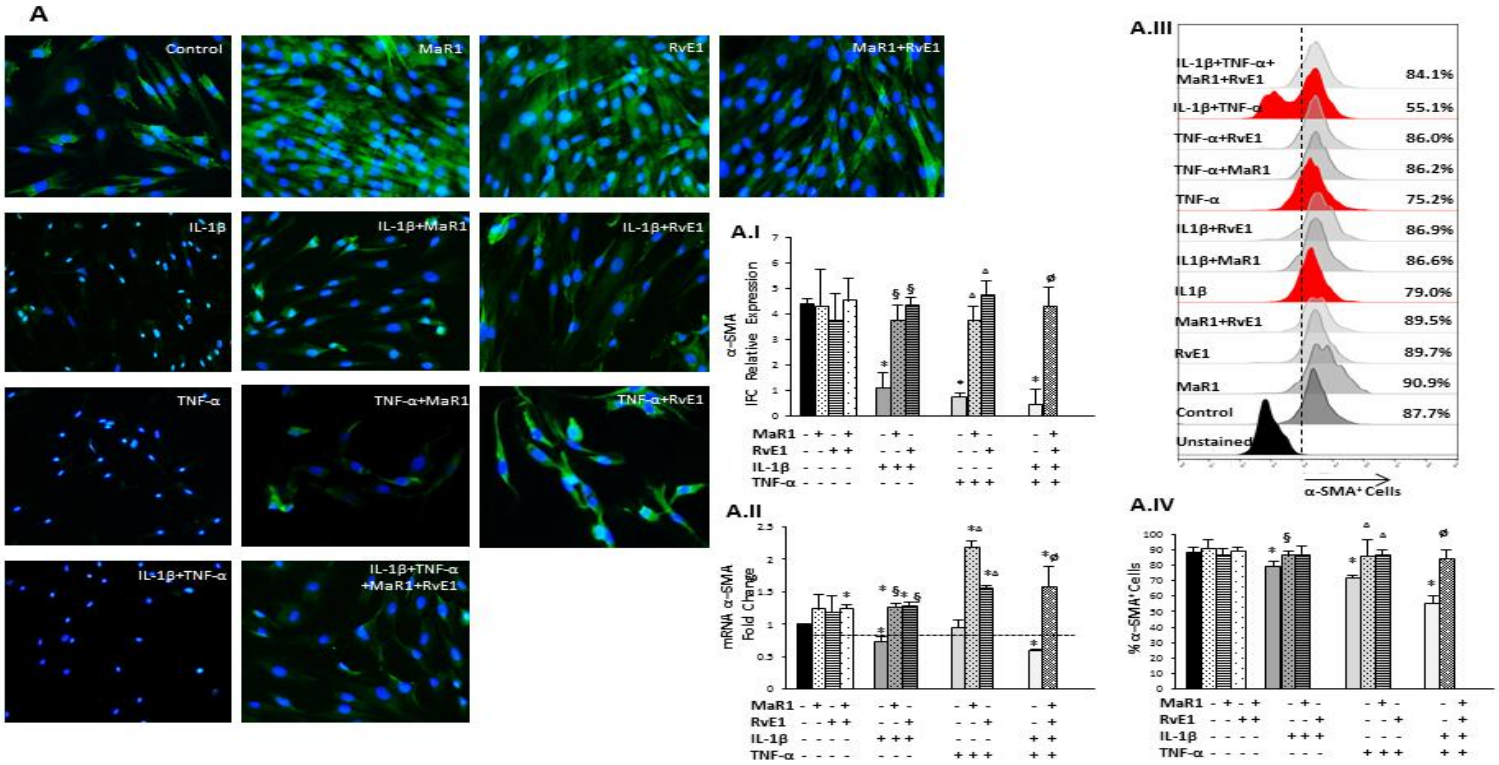
4.3.2 IL-1β/TNF-α reduce the expression of periodontal ligament regenerative biomarkers by hPDLSCs but Mar1/RvE1 reverse this deleterious effect

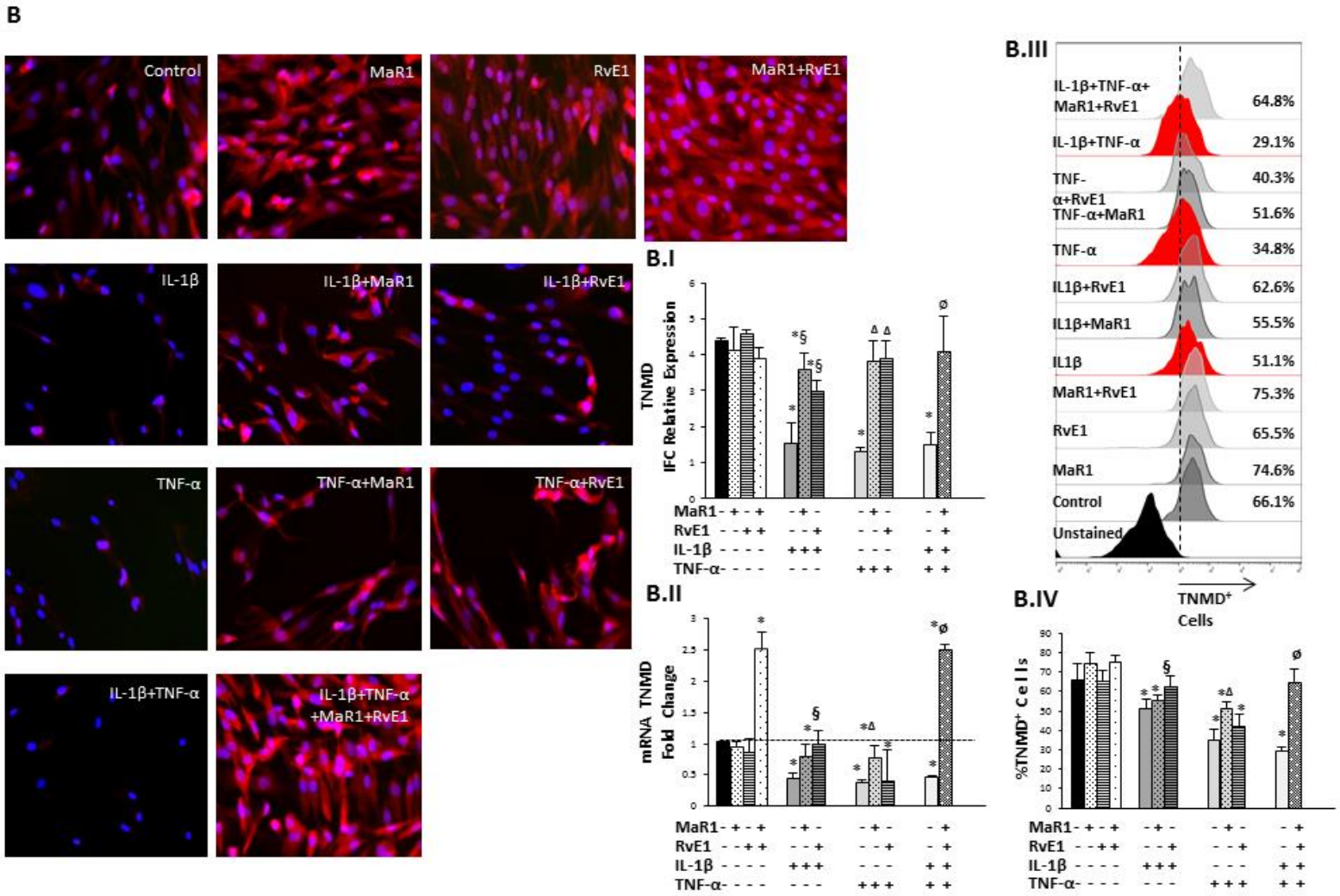
Structures responsible for supporting the teeth are destroyed by the inflammatory environment during the course of periodontitis and several biomarkers have been highlighted as crucial for the process of periodontal regeneration and stem cell activity. In this context, periostin (PRSTN) is an important regulator of periodontal tissue formation by promoting collagen fibrillogenesis and migration of fibroblasts, osteoblasts [14] and mesenchymal stem cells [36]. However, inflammatory mediators such as TNF-α reduce the expression of this biomarker in periodontal ligament cells such as PDLSCs [16] and fibroblasts [37]. In addition to PRSTN, tenomodulin (TNMD) is one of the best-known mature markers for ligament lineage cells [15] and it is expressed in the PDL of eruptive and post-eruptive teeth, where it promotes maturation or maintenance of the PDL by positively regulating cell adhesion [38]. Subpopulations of stem cells overexpressing TNMD have enhanced teno/ligamentogenesis [39] which seems to be important for the regeneration of tissues that are constantly under tensile-oclusive forces. Moreover, during the process of self-renewal another pivotal biomolecule is the α-SMA [40], which together with TNMD act to improve the contractile capacity of stem cells [38,41]. Actually, α-SMA can be used as a marker of undifferentiated cells and expressed along stress fibers [42]. Together with other biomarkers, e.g. PRSTN and TNMD, α-SMA can be also involved in ligamentogenesis [43] and they are useful to study periodontal development and regeneration [13]. Thus, we evaluated gene and protein expression of α-SMA, PRSTN and TNMD in hPDLSCs under pro-inflammatory and pro-resolving environments.

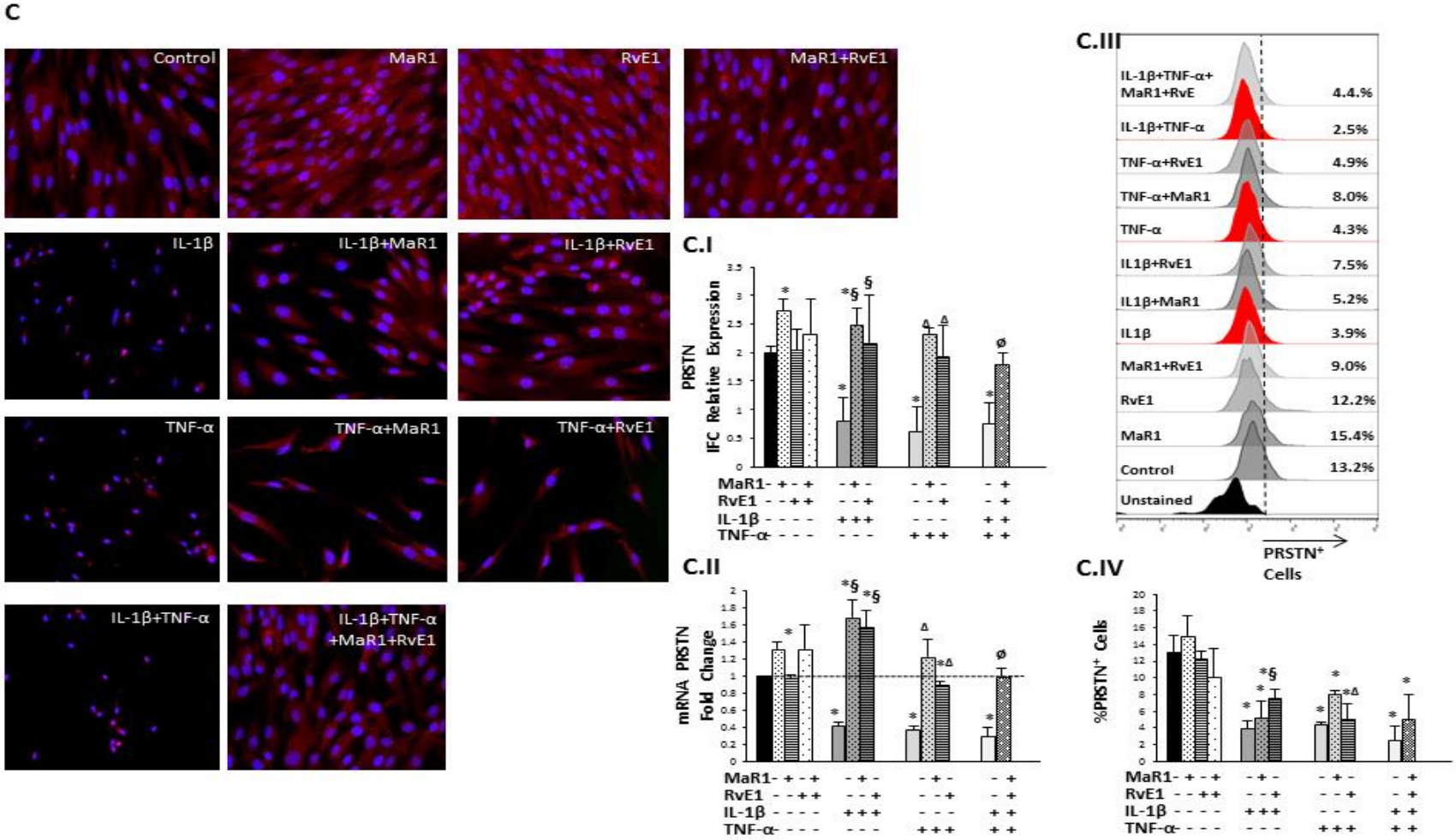
Treatment with MaR1 or RvE1 did not alter gene or protein expression of α-SMA (Fig. 4.2.A) or TNMD (Fig.4.2B), nor did they alter the percentage of hPDLSCs positive for these biomarkers ($p>0.05$) (Fig. 4.2A-IV, Fig. 4.2.B-IV), but the co-treatment with both MaR1 and RvE1 increased the expression of these molecules compared to untreated controls ($p<0.05$) at the mRNA level [1.25(±0.05) and 2.5(±0.28), respectively] (Fig. 4.2A-II, Fig. 4.2B-II). With respect to PRSTN, overall both SPMs also did not alter its expression, although MaR1 alone upregulated gene [1.32(±0.08), $p<0.05$] and protein [2.74(±0.2), $p<0.05$] expressions (Fig. 4.2.C). On the other hand, IL-1β/TNF-α reduced gene and protein expression of α-SMA, PRSTN and TNMD in hPDLSCs after 24h treatment. While controls

presented 87.7% (±4.2) α-SMA^{+}, 66.1%(±8.0) TNMD^{+}, and 13% (±2.5) PRSTN^{+} cells, the environment with IL-1β and/or TNF-α reduced the percentage of hPDLSCs positive for these biomarkers and the most deleterious effect was observed in the combination IL-1β/TNF-α that reduced ($p < 0.01$) these percentages to 55.1% (±5.5), 29.1%(±2.0), and 2.5%(±1.6), respectively. However, co-treatment of these groups with MaR1 and/or RvE1 partially rescued or even increased the expression of these biomarkers compared to untreated controls. For example, co-treatment of IL-1β with MaR1 or RvE1 determined a fold change of 1.28 (±0.05) and 1.3 (±0.04) for mRNA α-SMA, and 1.69(±0.2) and 1.57(±0.2) for mRNA PRSTN, respectively (Fig. 4.2A, Fig. 4.2C). Still, the co-treatment IL-1β/TNF-α/MaR1/RvE1 upregulated mRNA TNMD to 2.5(±0.28) ($p<0.01$) (Fig. 9B-II), and this same condition abolished the deleterious effect of the combination IL-1β/TNF, restoring the percentage of α-SMA^{+} cells to 89.5%(±2.3) and of TNMD^{+} cells to 75.3%(±3.0), which became similar to untreated controls ($p>0.05$) (Fig. 4.2A-IV, Fig. 4.2B-IV).

Figure 4.2 - Analysis of biomarkers related to periodontal ligament regeneration, α-SMA (**A**), tenomodulin [TNMD], (**B**), and periostin [PRSTN] (**C**) after 24h of stimuli with mediators. Immunofluorescence representative images in 40X magnification. **AI, BI, CI**, Protein expression analysed by immunofluorescence relative expression in relation to DAPI. **AII, BII, CII**, relative gene expression evaluated by RT-qPCR using GAPDH as endogenous control. **AIII-AIV, BIII-BIV, CIII-CIV**, Flow cytometry analysis showing the percentage of hPDLSCs for each biomarker. Unstained cells were used to set positive cell populations. (*), $p<0.05$ versus control (non-stimulated); (§) $p<0.05$ versus group treated with Il-1β; (Δ) $p<0.05$ versus group treated with TNF-α; (ø) $p<0.05$ versus group treated with Il-1β +TNF-α. Results are given as the mean ± SEM




B
Control
MaR1
RvE1
MaR1+RvE1
IL-1β
IL-1β+MaR1
IL-1β+RvE1
TNF-α
TNF-α+MaR1
TNF-α+RvE1
IL-1β+TNF-α
IL-1β+TNF-α +MaR1+RvE1
B.I
TNMD IFC Relative Expression
MaR1
RvE1
IL-1β
TNF-α
B.II
mRNA TNMD Fold Change
B.III
IL-1β+TNF-α+ MaR1+RvE1
64.8%
IL-1β+TNF-α
29.1%
TNF-α+RvE1
40.3%
TNF-α+MaR1
51.6%
TNF-α
34.8%
IL1β+RvE1
62.6%
IL1β+MaR1
55.5%
IL1β
51.1%
MaR1+RvE1
75.3%
RvE1
65.5%
MaR1
74.6%
Control
66.1%
Unstained
TNMD+ Cells
B.IV
%TNMD+ Cells


C
Control
MaR1
RvE1
MaR1+RvE1
IL-1β
IL-1β+MaR1
IL-1β+RvE1
TNF-α
TNF-α+MaR1
TNF-α+RvE1
IL-1β+TNF-α
IL-1β+TNF-α
+MaR1+RvE1
C.I
PRSTN
IFC Relative Expression
MaR1- + - + - + - + - +
RvE1 - - + + - - + - - +
IL-1β - - - - + + + - - -
TNF-α- - - - - - - + + +
C.II
mRNA PRSTN
Fold Change
MaR1- + - + - + - + - +
RvE1 - - + + - - + - - +
IL-1β - - - - + + + - - -
TNF-α- - - - - - - + + +
C.III
IL-1β+TNF-α+
MaR1+RvE
4.4%
IL-1β+TNF-α
2.5%
TNF-α+RvE1
4.9%
TNF-α+MaR1
8.0%
TNF-α
4.3%
IL1β+RvE1
7.5%
IL1β+MaR1
5.2%
IL1β
3.9%
MaR1+RvE1
9.0%
RvE1
12.2%
MaR1
15.4%
Control
13.2%
Unstained
PRSTN+
Cells
C.IV
%PRSTN+ Cells
MaR1- + - + - + - + - +
RvE1 - - + + - - + - - +
IL-1β - - - - + + + - - -
TNF-α- - - - - - - + + +

Therefore, while the inflammatory milue reduces the expression of key-biomarkers involved in the periodontal ligament regeneration (TNMD, PRSTN, and α-SMA), bioactive SPMs act by reversing this process in order to increase gene transcription, protein expression and the number of positive hPDLSCs for these biomarkers.

4.3.3 IL-1β/TNF-α reduce in vitro wound healing of hPDLSCs by inducing cell death but MaR1/RvE1 reverse this process by improving cell proliferation and reducing cell apoptosis

The migration and survival capacity of stem cells are inhibited by pro-inflammatory cytokines that can promote cell death by inducing apoptosis [44,45]. However, it is still unknownt whether MaR1/RvE1 can regulate this deleterious effect on hPDLSCs, although lipoxins have already proved to stimulate proliferation and migration of hPDLSCs [22].

On this basis, we observed that SPMs accelerated *in vitro* wound closure of scratches with hPDLSCs in monolayers ($p < 0.01$). Untreated controls had 22.2% (±12) wound healing (WH) after 24h while groups treated with MaR1 or RvE1 presented 83.6% (±4.8) and 82% (±6.9), respectively (Fig. 4.3E). At the same time, we observed a significant increase in the proliferative activity/cellular viability of hPDLSCs by performingMTT assay. While 2.9(±0.08) x 10^5 viable cells was found in the control group, treatment with MaR1 or RvE1 increased this number to 3.5(±0.28) and 3.8(±0.3) x10^5 cells, respectively (Fig. 4.3I). On the other hand, pro-inflammatory conditions had an opposite effect by preventing WH and reducing the number of viable cells (Fig. 4.3F-H, Fig.4.3J-L). The most deleterious response was observed with the combination IL-1β/TNF-α, which increased wound area by 112% (±15) and reduced cell viability to 0.3 x 10^5 cells ($p<0.01$) (Fig. 4.3F, Fig. 10L). However, co-treatment with SPMs reversed this response and IL-1β/TNF-α/MaR1/RvE1 treated group presented a WH [72%(±12)] and a number of viable cells [3.1 x 10^5] even higher than those found in untreated controls ($p<0.01$).

In addition to the WH and MTT analyzes, we also investigated cell death by apoptosis using AnnexinV (Fig. 4.3M, Fig. 4.3N). In line with the MTT assay, SPMs alone or in combination did not alter the apoptotic activity of hPDLSCs, but the inflammatory miliue increased the percentage of early apoptotic cells ($AnnexinV^{+}PI^{-}$), from 7.54% to 16.2% in the group treated with Il -1β/TNF-α ($p<0.01$) (Fig. 4.3N).

These data argue for two different patterns in the inflammatory response. Pro-inflammatory mediators lead to an increase in the apoptotic activity of hPDLSCs as well as in the wound area and concomitant reduction in cell migration. On the other hand, mediators synthesized in the latter stages of inflammation favor the events responsible for the repair, by increasing the percentage of WH/migration and proliferative activity of hPDLSCs besides reducing cell apoptosis.

4.3.4 IL-1β/TNF-α reduce the expression of cementum-osteogenic biomarkers by hPDLSCs but MaR1/RvE1 rescue this activity and accelerate it

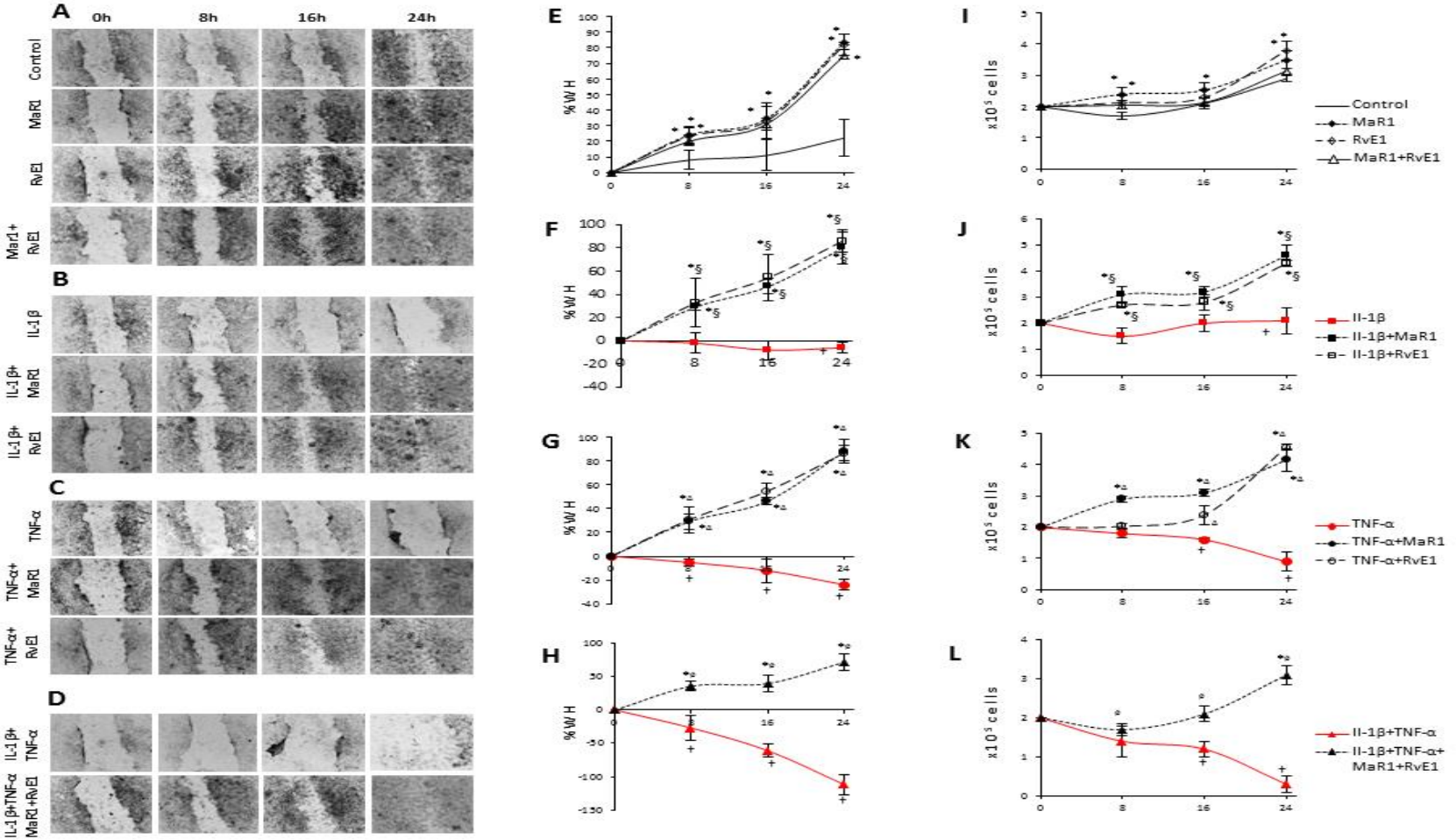
Previous studies have shown that mediators such as TNF-α may act by reducing the osteogenic activity of hPDLSCs [46] and IL-1β also exhibits the same function in a dose-dependent manner [47]. However, these activities still seem paradoxical bearing in mind that calcification processes may appear due to the inflammatory response. In order to clarify this puzzle we should evaluate the inflammatory process as a whole, and instead of focusing only on the analysis of pro-inflammatory mediators also evaluate the function of other agonist mediators, such as MaR1 and RvE1, that play a primordial role in the landscape of this response.

As shown in Fig. 4.4, MaR1 and/or RvE1 increased the calcification process of hPDLSCs treated in osteogenic medium (Fig. 4.4A, Fig. 4.4D), elevated ALP activity in the cell supernatant (Fig. 4.4G), and increased the expression of genes related to osteogenesis (OCN and Rux2) (Fig. 4.4B-C) and cementogenesis (CAP and CEMP1) (Fig. 4.4E-F). At the protein level, however, RvE1 reduced the expression of Runx2 and CEMP1, although an increase in CAP expression was also observed (Supporting information Fig.. S2, Fig. 11H). In general, both SPMs, MaR1 and RvE1, appear to exert distinct activities regulating the process

of cementum-osteogenesis in a mediator-specific manner. On the other hand, IL-1β and/or TNF-α overall reduced gene or protein expression of biomarkers related to cementum-osteogenesis (CAP, CEMP1, OCN and Rux2). The IL1-β/TNF-α combination also reduced the concentration of ALP in the supernatant of cells treated in osteogenic medium, besides being the most deleterious as observed at the protein level. Nevertheless, the addition of SPMs to the inflammatory miliue reverses this deleterious process. MaR1 or RvE1, in combination with IL-1β, increased ARS quantification, and when these SPMs were used combination with TNF-α increased ALP levels. Additionally, the addition of SPMs in the inflammatory environment also favored an increase in the gene and protein expression of biomarkers of cementum-osteogenesis (Fig. 4.4).

In summary, the proinflammatory environment may delay the process of periodontal regeneration by reducing the cementum-osteogenic potential of hPDLSCs but SPMs rescue such activity and they can act by stimulating boné and cementum neoformation.

Figure 4.3 - Analysis of hPDLSCs migration, proliferation and apoptosis in wound closure assay using scratches in monolayers with ~2x10^5 cells unstimulated or stimulated in different experimental conditions. **A-D**, representative images in 10X magnification at each time-point (baseline/0h, 8h, 16h, and 24h). **E-H**, percentage of wound healing at each time-point. Positive values correspond to reduction of the wound area and negative values correspond to an increase of this area. **I-L**, values relative to the total number of viable cells using MTT assay at each time-point. **M**, representative histograms of flow cytometry analysis using AnnexinV set in unstained cells to analyse positive populations. **N**, Percentage of AnnexinV$^+$ hPDLSCs (early apoptotic cells) after 24h treatment under different conditions. (*), p<0.05 versus control (non-stimulated); (§) p<0.05 versus group treated with Il-1β; (Δ) p<0.05 versus group treated with TNF-α; (ø) p<0.05 versus group treated with Il-1β +TNF-α. Results are given as the mean ± SEM



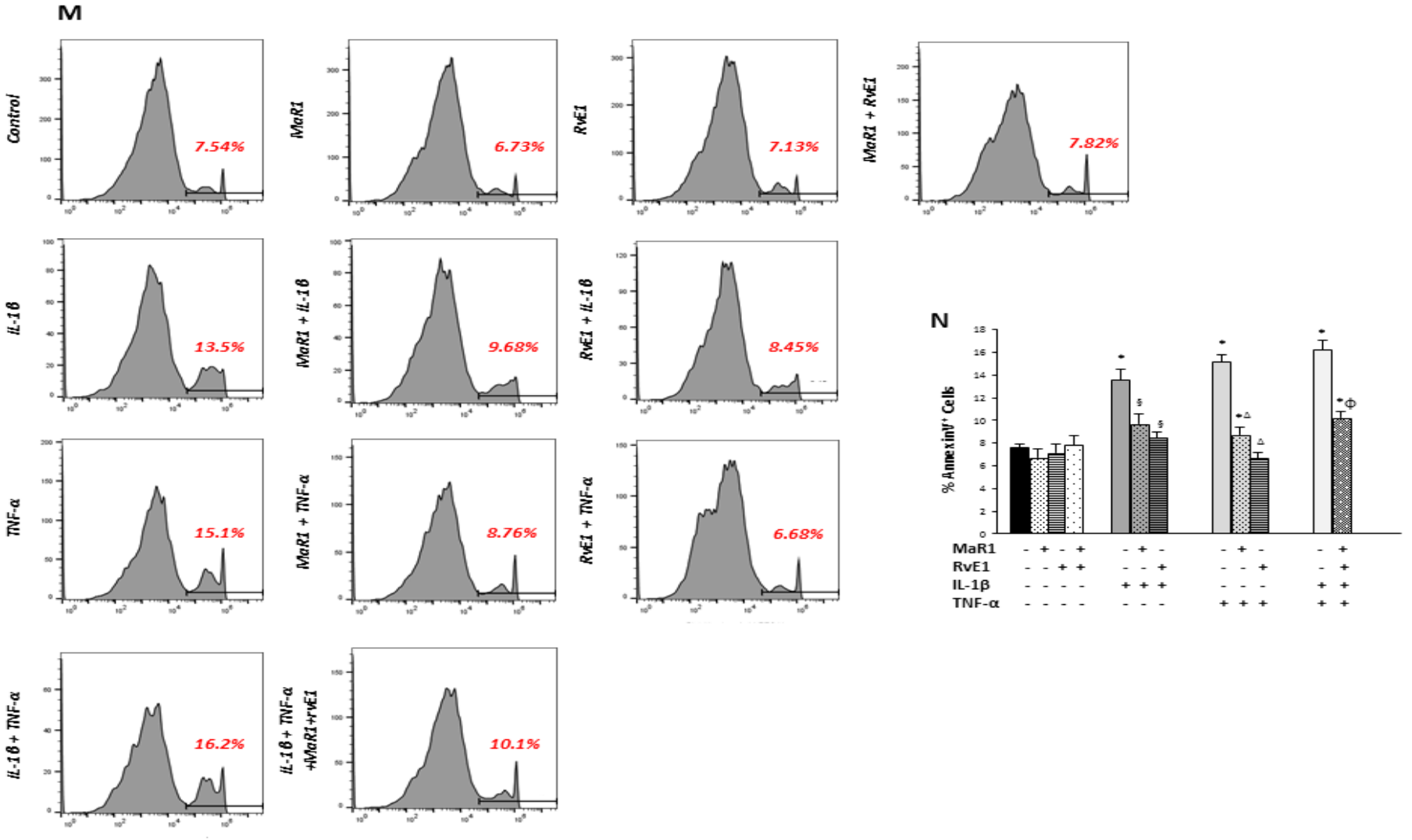
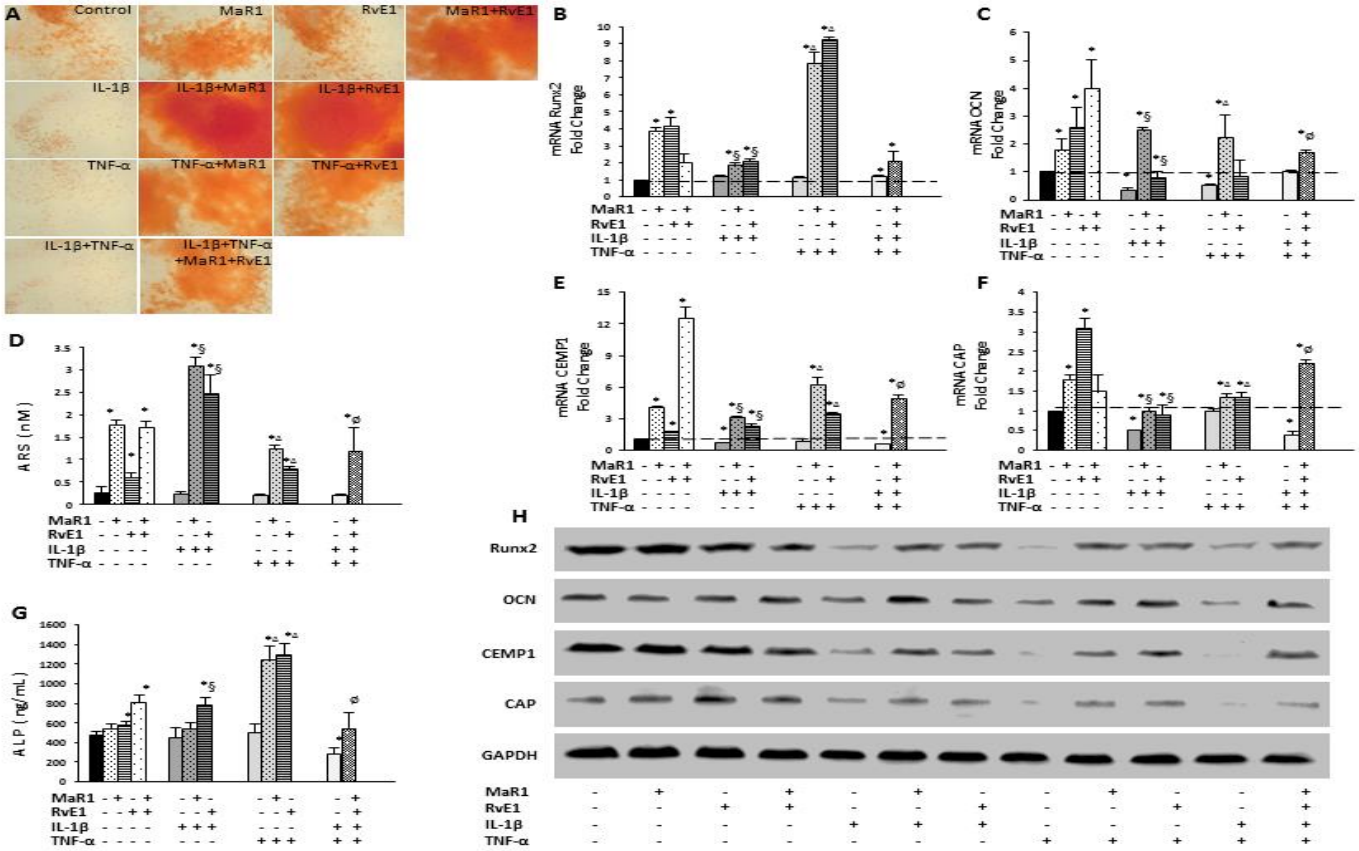
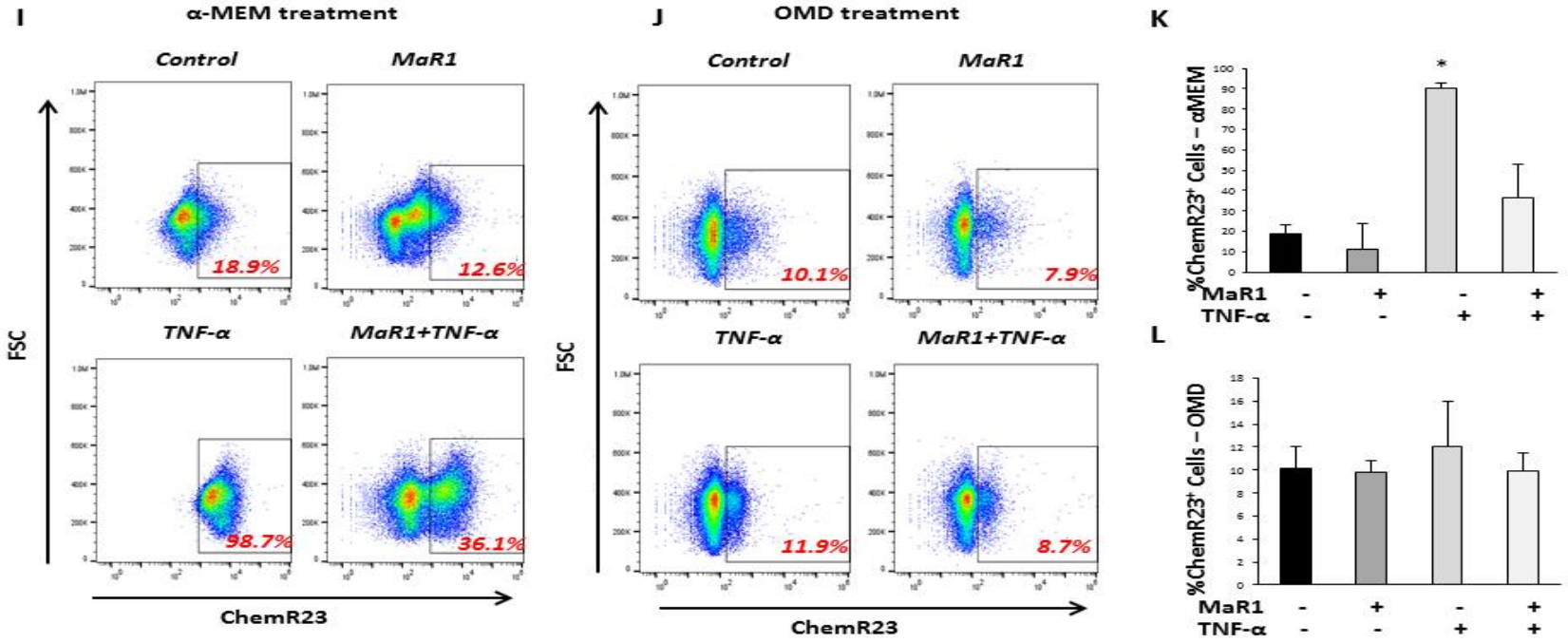

M
Control
7.54%
MaR1
6.73%
RvE1
7.13%
MaR1 + RvE1
7.82%
IL-1β
13.5%
MaR1 + IL-1β
9.68%
RvE1 + IL-1β
8.45%
TNF-α
15.1%
MaR1 + TNF-α
8.76%
RvE1 + TNF-α
6.68%
IL-1β + TNF-α
16.2%
IL-1β + TNF-α +MaR1+rvE1
10.1%
N
% AnnexinV+ Cells
MaR1
RvE1
IL-1β
TNF-α

Figure 4.4 - Analysis of hPDLSCs cementum-osteogenic differentiation after 7-days treatment in osteogenic medium (OMD) with different experimental conditions. **A**, qualitative ARS anaylis with representative figures for each condition showing calcified deposits. **B,C**, relative gene expression of osteogenic biomarkers (Runx2, Osteocalcin/OCN) evaluated by RT-qPCR using GAPDH as endogenous control. **D,** spectrophotometric quantification (in nM) of extracted ARS. **E,F,** relative gene expression of cementogenic biomarkers (CEMP1, CAP) evaluated by RT-qPCR using GAPDH as endogenous control. **G,** concentration of ALP (in ng/mL) measured at supernatant using ELISA kit. **H,** western blot analysis measured protein expression of cementum-osteogenic biomarkers. **I, J,** Representative dot plots of ChemR23^{+} hPDLSCs populations after 7-days treatment with MaR1 and/or TNF-α in normal α-MEM medium and OMD, respectively. **K, L,** Percentage of ChemR23^{+} cells after 7-days treatment with MaR1 and/or TNF-α in α-MEM and OMD, respectively. (*), $p<0.05$ versus control (non-stimulated); (§) $p<0.05$ versus group treated with Il-1β; (Δ) $p<0.05$ versus group treated with TNF-α; (ø) $p<0.05$ versus group treated with Il-1β +TNF-α. Results are given as the mean ± SEM.




I
α-MEM treatment
Control
MaR1
TNF-α
MaR1+TNF-α
18.9%
12.6%
98.7%
36.1%
FSC
ChemR23
J
OMD treatment
Control
MaR1
TNF-α
MaR1+TNF-α
10.1%
7.9%
11.9%
8.7%
FSC
ChemR23
K
%ChemR23+ Cells – αMEM
MaR1 - + - +
TNF-α - - + +
L
%ChemR23+ Cells – OMD
MaR1 - + - +
TNF-α - - + +

4.3.5 hPDLSCs lose expression of ChemR23 under osteogenic differentiation medium but MaR1 and TNF-α differently regulate this receptor which may lead to their distinct cementum-osteogenic activities

Several G-protein coupled receptors have been studied with the aim of evaluating the mechanisms by which SPMs can perform their functions *in vivo*, and among them the Chemerin receptor 23 (ChemR23) stands out [17]. However, this receptor is recognized for its promiscuity of interactions, and can be activated by proinflammatory cytokines and pro-resolving mediators, which makes it perform diametrically opposite functions [48,49]. In this context, while models of *in vivo* overexpression of this receptor have shown that it can mediate an increase in bone preservation [17], others point out that the overexpression of this receptor in stem cells may decrease the process of osteoblastogenesis and increase the osteoclastogenic activity [18,50]. Still, in addition to ChemR23-expressing cells being recruited in human inflammatory diseases, a synergistic response of pro-inflammatory mediators with its natural ligand chemerin is also known [51]. Additilionally, stem cells express ChemR23 and secrete its ligand chemerin. The synthesis of this molecule is dependent of culture conditions and ChemR23 expression increases under stimulation with inflammatory cytokines [19]. In fact, ChemR23 can perform different functions and its expression on stem cells under different stimuli still needs to be better investigated, especially in view of the cementum-osteogenesis process which, as shown above, is strictly regulated by the balance of mediators such as MaR1 and TNF-α.

We observed that under osteogenic differentiation medium (OMD) naturally occurs a reduction in the percentage of ChemR23^{+} hPDLSCs when compared to cells grown in normal culture medium α-MEM (10.1% vs 18.9%, $p<0.01$) (Fig. 11I, Fig. 11J). Such reduction in OMD treated cells was observed independent of the experimental group with MaR1 and/or TNF-α. However, when evaluating the effect of the treatment conditions on hPDLSCs cultivated in α-MEM, an increase in the number of ChemR23^{+} cells under TNF-α stimulus (98.7%) and a reduction of this percentage to 36.1% during TNF-α/MaR1 co-treatment were found (Fig. 11K). We may argue that this phenomenon could be understood as an attempt to reverse the phenotypic alteration of hPDLSCs relative to ChemR23 under pro-inflammatory stimulation, bringing it to a level similar to those found in the MaR1 (12.6%, Fig. 11I) or non-stimulated (18.9%, Fig. 11I) groups. At the same time, although in the α-MEM group treated

only with MaR1 there is no change in the % ChemR23^{+} cells compared to the unstimulated control, morphological changes can be observed according to the FSC axis.

In summary, hPDLSCs lost the expression of ChemR23 when cultured in cementum-osteogenic medium, which in consonance with other studies [18,50] reveal a reduction in the expression of this receptor in the process of osteoblastogenesis. However, the inflammatory environment with TNF-α naturally upregulate this receptor which may trigger a reduction in the osteogenic activity of hPDLSCs treated with this mediator. On the other hand, this deleterious effect seems to be reversed by the resolution phase of the inflammation, since MaR1 counterbalance this process by reducing the percentage of ChemR23^{+} cells, which appears to restore the cementum-osteogenic potential of hPDLSCs.

4.4 Discussion

In the present study, we provided evidence that Il-1β/TNF-α reduce the stemness of hPDLSCs by altering the proportion of Oct4^{+}Sox2^{+} cells, reduce the expression of periodontal regenerative biomarkers by hPDLSCs and impair *in vitro* wound healing by inducing apoptosis. However, MaR1/RvE1 reverse such deleterius effect increasing Oct4^{+}Sox2^{+} hPDLSC population, improving cell proliferation, and reducing cell death. Moreover, Il-1β/TNF-α reduce the expression of cementum-osteogenic biomarkers by hPDLSCs, but MaR1/RvE1 rescue this activity and increase it, which at least in relation to MaR1-TNF-α interaction can be associated with a different modulation of ChemR23. Altogether, these results elucidate how the interaction of pro-inflammatory and pro-resolving mediators can affect important properties of hPDLSCs, helping to unravel the intricate signaling network in the inflammatory environment and how it can affect the stem cell response, shedding light on new therapeutic approaches.

Inflammation is emerging as an important regulator of stem cells and plays an intricate role in health and disease. Once an inflammatory program has already been initiated the production of cytokines, interferons etc. by local immune populations can further impact the behavior of stem cells. In some cases, even the differentiated cells de-differentiate in response to inflammation, acquiring stem-like characteristics and increasing cellular plasticity [52,53].

Previous studies pointed out that TNF-α can enhance stem cell phenotype by increasing positive cells to Oct4, and even their ability to form cell colonies, to migrate, and to differentiate into odontogenic lineages [54]. Still, TNF-α/Il-1β upregulated Sox2 expression in gingival stem cells [55]. Paradoxically, the double $Oct4^{+}Sox2^{+}$ SCs have an immunomodulatory effect by reducing the expression of both cytokines and has already been proposed as an alternative for the treatment of inflammatory diseases [56]. Additionally, TNF-α also inhibit the osteogenic activity of hPDLSC [46]. In fact, the literature is much more consistent in evidence that the inflammatory environment may increase stemeness by inducing overexpression of pluripotency transcription factors (e.g. Oct4, Sox2) in the context of carcinogenesis, especially in cases of tumor-initiating and -propagating cells [42,57,58]. However, when stem cells were harvested from a healthy environment of healthy individuals, we observed in our study the opposite, and IL-1β/TNF-α reduced expression of $Oct4^{+}Sox2^{+}$ hPDLSCs while a typically pro-resolving environment with MaR1/RvE1 acts in the opposite way.

To date, the enzyme 15-lipoxygenase which is involved in the generation of SPMs that play essential roles in resolution responses may restore tissue homeostasis, stem cell viability, and decrease the synthesis of inflammatory mediators [59]. Interestingly, SPMs through autocrine mechanisms can induce the release of more SPMs by hPDLSCs leading to an increase in their prohealing properties [22]. Overall, these findings show that a more global view of the self-renewal process needs to be kept in focus to assess the real impact of this intricate network of mediators on stem cells. In fact, our results indicate that in the cascade of the inflammatory response, mediators of resolution play a decisive role in the process of regeneration of tissues lost to the pro-inflammatory environment and in the return to a homeostatic state.

Although SPMs, such as RvE1 and MaR1, are not yet widely studied in relation to their effect on stem cell biology, other derivatives of polyunsaturated fatty acids may provide some clues in relation to their possible activities. In this sense, lipoxins attenuate the inflammatory response in stem cells of the apical papila [60], promote the resolution of acute lung injury through activation of tissue specific stem cells [61], and regulate neural stem cell proliferation and differentiation [62]. It is clear that the tissue regeneration is achieved via the priming of resident slow-cycling stem cells to adopt a proliferative state and yield transit-amplifying cells which will differentiate to restore tissue architecture. In this context, RvE1 promotes bone preservation under inflammatory conditions [63] and induces neo-formation of

alveolar bone, periodontal ligament and cementum in a model of periodontitis induced by *Porphyromonas gingivalis* infection in rabbits [10]. In addition, MaR1 promotes neuroprotection and functional neurological recovery after spinal cord injury [64], and bridges resolution of infectious inflammation to tissue regeneration by increasing the phagocytic capacity of macrophages from patients with incisor-molar pattern periodontitis [65] and by accelerating post-surgical regeneration in *Dugesia tigrina* [8,9]. These findings are corroborated by our study that evidenced an important role of these SPMs in increasing cementum-osteogenic activity and expression of periodontal ligament biomarkers (e.g. α-SMA, TNMD, TRSTN) by hPDLSCs under Il-1β/TNF-α stimulus, although the mechanisms by which this effect is achieved still need to be elucidated.

Several structures are determinant for the activity of periodontal regeneration but the cementum is still little studied. In a simplistic way, cementum can be described as the mineralized tissue that covers the roots of teeth and serves to attach the tooth to alveolar bone via collagen fibers of the periodontal ligament. However, the more we study this tissue, the more we understand the complexity of its structures and functions. Nowadays, there is the idea that not only does cementum act as a barrier to delimit epithelial growth that can impair fiber attachment but also that a continuous cementum layer acts as a microbial barrier and that defects in this tissue could result in periodontitis [66]. The extracelular matrix of this tissue share many similarities with overall mineralized tissues, including calcium deposition by alkaline phosphatase activity. Thus, in our study both processes were evaluated concomitantly under the same differentiation conditions. Notwithstanding, cementum-specific proteins CAP and CEMP-1 [66-68] were also evaluated in parallel. In agreement with other studies [69,70], our results pointed out that the inflammatory environment inhibit cementoblast differentiation, but as a novelty we present the argument that a balance of proinflammatory cytokines with resolution mediators seems to be essential to restore cementogenic activity, which raises new insights into cell therapy of periodontal regeneration.

Nevertheless, given the different contexts in which they are synthesized, SPMs may act through activation of specific pathways. RvE1 is synthesized primarily in the early stages of inflammation as a product from the eicosapentaenoic acid and its 18-HpEPE product during the increase in neutrophil influx; on the other hand, the synthesis of MaR1 requires a macrophage phenotype shift that occurs at a later stage and it is derived from the metabolism of docosahexaenoic acid and its product 13S, 14S-epoxy-maresin [71,72]. Our results show differences between MaR1 and RvE1 in relation to the control of the gene expression of

biomarkers such as periostin, CEMP1, and CAP and even in relation to osteogenic activity measured by ARS. To understand such differences, future mechanistic studies need to be delineated. For now, we have evidence that RvE1 selectively interacts with ChemR23 and BLT1 receptors in hematopoietic cell lines [3], whereas the interaction pathways for MaR1 and RvE1 in stem cells remain unknown. Accordingly, previous studies have shown that both SPMs can regulate various cellular processes through phosphorylation of proteins that determine an increase in cell proliferation and survival [73-75], as raised by our study.

4.5 Conclusion

The main finding of this study is that stem cells isolated from the periodontal ligament of healthy patients suffer the effects of an *in vitro* artificial inflammatory environment, which changes their phenotype and properties so that pro-resolving lipid mediators improve their periodontal regerative-related activities under pro-inflammatory stimilus. In a future and complementary perspective, we emphasize that phosphoproteomic studies could help to decipher related activated pathways, as well as enzymes essential for the metabolism of these lipids into stem cell biology. Also, evaluations of the interactome should be performed in order to uncover the interaction of organelles under SPM stimulus, in order to remove hPDLSCs from a state of quiescence to another of cellular differentiation. A more integrated view of these techniques may lead to overcoming the existing barriers in stem cell therapy, increasing its effectiveness and predictability in the treatment of chronic inflammatory diseases such as periodontitis.

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4.6 References

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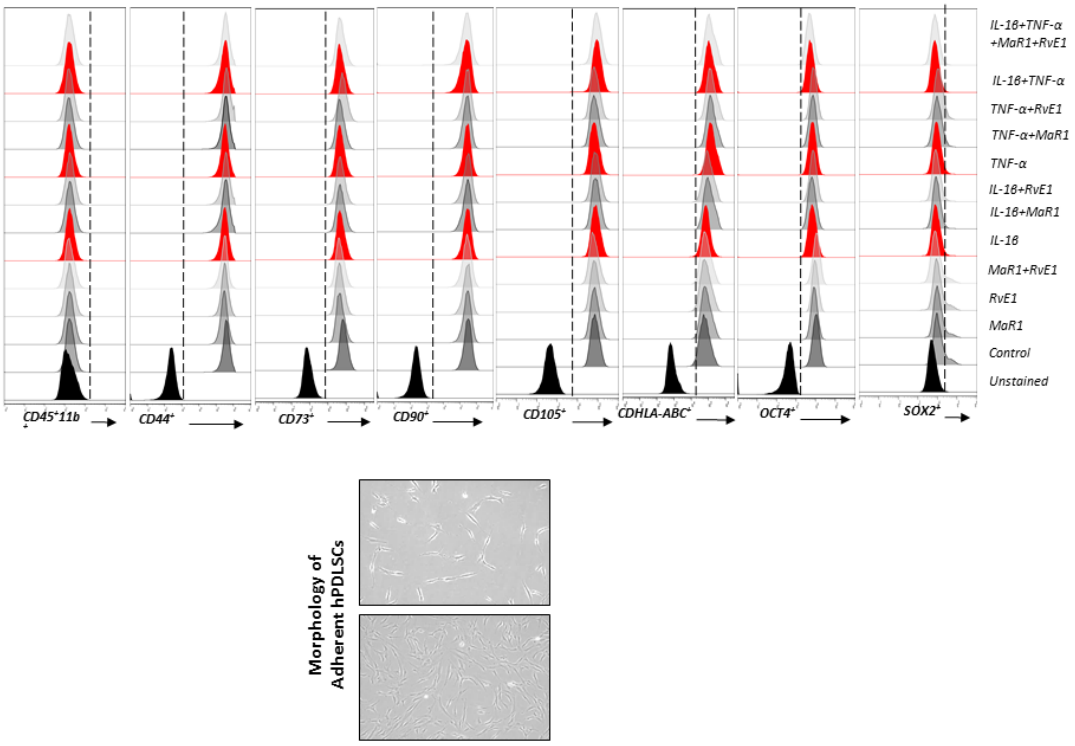
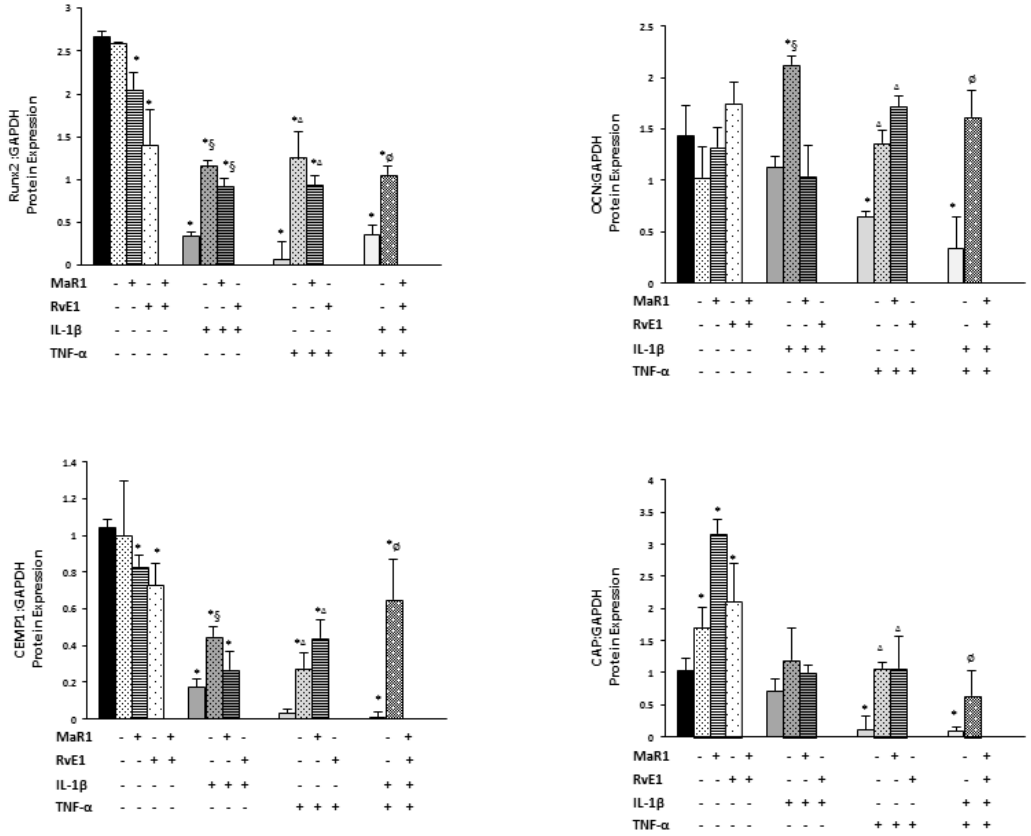
Supporting information Figure S1. Light microscopy image of Passage 3. Original magnification: x10 showing a fibroblast-like morphology of adherent cells. Still, histograms showing the flow cytometry analysis of stem cell antigens in hPDLSCs.



Supporting information Figure S2. Protein relative expression in hPDLSCs submitted to cementum-osteogenic differentiation after 7-days treatment in osteogenic medium (OMD) with different experimental conditions. GAPDH was used as control. Osteogenic (Runx2, Osteocalcin/OCN) and cementogenic (CEMP1, CAP) biomarkers were evaluated by WB.



5 CHAPTER III: A hypothetical role of probiotics in the control of inflammation and mucosal homeostasis by improving the synthesis of pro-resolving lipid mediators *

ABSTRACT

Endogenous specialized pro-resolving lipid mediators (SPMs) are synthesized during a self-limited inflammatory response in order to drive the resolution phase of the inflammation. Recent evidence also points them as important components for the maintenance of mucosal integrity. Since SPMs are produced in the human mucosa, where they limit persistent inflammation and regulate its natural process of self-renewal, we hypothesized that commensal bacteria may play a central role in coordinate the synthesis of bioactive SPMs, such as lipoxins and resolvins. Through mechanisms of direct interaction with epithelial cells, the commensal microbiota, modified or not by the use of probiotics, can aid in the synthesis of oxylipins derivatives that can be subsequently manipulated by host cells in the synthesis of SPMs. Modifying the expression of key regulators, such as prostaglandins, cyclooxygenases and P450 enzymes, resident bacteria can enhance the synthesis of bioactive SPMs by epithelial cells as well as underlying recruited neutrophils. In addition, probiotics and commensal microorganisms act by altering the synthesis of pro- and anti-inflammatory cytokines, which could lead to modifications in the phenotype of macrophages that are indispensable cell types in the synthesis of others bioactive lipids such as maresins. This integrated microbiome-lipid metabolism perspective imply in a broader approach of the etiopathogenesis of chronic inflammatory diseases microbial dysbiosis-associated.

5.1 Introduction

Inflammatory responses are shaped by a delicate balance between positive and negative feedback loops, regardless of their septic or aseptic pathogenesis. In fact, for the past 40 years investigators have focused on identifying factors that initiate and perpetuate inflammation, and only more recently emphasis has shifted to the end of this spectrum [1]. Events at the onset of acute inflammation establish biosynthetic circuits for a series of chemical mediators that later not only work as antagonists but also play an agonist role as they not only inhibit the inflammatory cascade but also actively dismantle it leading to the restoration of tissue homeostasis and function. Collectivelly coined specialized pro-resolving

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mediators (SPMs), endogenous lipid derivatives produced in the resolution phase of the inflammation are part of a fine and orchestrated network of self-regulated factors that ideally initiate and close the inflammatory cycle [2,3].

However, given that SPMs are produced in the human gut mucosa, where they limit persistent inflammation [4], and the intrinsic regulation of the epithelial barrier by the commensal microbiota [5], it is conceivable to argue that both host components may be interconnected in order to maintain the mucosal integrity. The intestinal epithelium, for example, is in permanent contact with luminal contents and the variable dynamic enteric microbiota, and, not surprisingly, a compilation of studies yielding evidence of inflammatory disease amelioration and immunomodulation suggest probiotics communicate with the host in multiple ways [6]. Many of these studies have yielded promising results regarding the use of exogenous and commensal probiotic species in the treatment of acute gastroenteritis, *Clostridium difficile*-associated diarrhea or colitis, irritable bowel syndrome, necrotizing enterocolitis (NEC), and other chronic inflammatory diseases associated with microbial dysbiosis such as periodontitis [7-11].

Therefore, this article raises hypotheses about how the commensal microbiota, modulated or not by the adjuvant use of probiotics, can directly or indirectly corroborate with the beneficial effect of the synthesis of bioactive SPMs on the modulation of the microbiome-epithelial barrier immunological axis.

5.2 Specialized pro-resolving mediators as key regulators of the host immunity

Temporal analyses of eicosanoids produced during the inflammatory cascade, as found in clinical and experimental exudates, show early coordinate appearance of leukotriens (e.g. LTB_4) and prostaglandins (e.g. PGE_2) via cyclooxygenase activation (COX-1, COX-2) that amplify acute inflammation by increasing polymorphonuclear (PMN) leukocyte influx through neutrophil (Nφ) recruitment. However, peripheral blood PMNs exposed to PGE_2 switch eicosanoid biosynthesis in a dose-time dependent manner from predominantly LTB_4 derived from the action of the enzyme 5-lipoxygenase (5-LO) on arachidonic acid (AA) to lipoxin A_4 (LXA_4), a 15-LO product that limit PMN infiltration [12-14]. In a previous study,

PGE_2 or PGD_2 added to isolated human PMN increased 15-LO type I translation from mRNA stores in a cAMP-dependent process, increasing LXA_4 biosynthesis which was therefore considered to be a first-class SPM [13]. Consecutively, this mediator stimulates non-phlogistic monocyte recruitment, that amplifies ω-3 polyunsaturated fatty acids (PUFAs) metabolism via eicosapentaenoic acid (EPA) and docosahexaenoic (DHA), which will give rise to E and D series resolvins, respectively [15]. Still, in the last stages of the resolution either DHA or its derivatives can be converted to maresin [macrophage mediator in resolving inflammation (MaR1)] by activated macrophages [Mϕ] that shifts their pro-inflammatory phenotype M1 to a pro-resolving M2 [16]. On this basis, M2 Mϕ produces more MaR1 from its intermediate precursor 13S,14S-eMaR and incubation of M1 Mϕ with either 13S,14S-eMaR or MaR1 leads to significant reductions in the M1 lineage markers CD54 and CD80 expression and a concomitant upregulation of the M2 lineage markers CD163 and CD206 [17]. Altogether, these findings indicate that besides SPMs being responsible for changes in the cell types present in the inflammatory environment, they appear to regulate and being tightly regulated by changes in the phenotype of these cells.

In this sense, it is necessary to keep in mind some concepts that are considered essential to fulfill pro-resolving mediators criteria that include: a) be produced *in vivo* at levels comensurate with their actions; b) be able to reduce PMN chemotaxis and infiltration; c) be able to enhance Mϕ phagocytosis and efferocytosis; d) be able to accelerate resolution; e) be able to reduce pro-inflammatory mediators (e.g. TNF-α and Il-1β); and f) be able to increase anti-inflammatory cytokines (e.g. Il-10) and lipid mediators (e.g. LXA_4) [2]. Additionally, SPMs also stimulate antimicrobial activities of mucosal epithelial cells by upregulate the expression of bactericidal/permeability-increasing protein (BPI). Epithelial cells of wide origin (oral, pulmonary, and gastrointestinal mucosa) express BPI that is similarly regulated by lipoxins. This protein dominantly localizes to epithelia in human mucosal tissues, and it was identified as an unappreciated "molecular shield" for protection of mucosal surfaces against Gram-negative bacteria and their endotoxins [18]. In mucosa, lipoxins can be generated by Nφ from 15-hydroxyeicosatetraenoic acid (15-HETE) precursor provided by epithelial cells. In addition, LXA_4 is produced in the human gut mucosa, which is important given the continuous exposure of this organ to commensal bacteria [1,19]. Such structures are coated by a dense layer of beneficial microorganisms that live in symbiotic interaction with the host giving it a critical role in the evolution of the immune system [20] and, considering their physiological nature for regeneration as well as the intimate contact of

the lining mucosa of the digestive tract with all kinds of metabolites exogenous to the organism that trigger a certain type of continuous homeostatic inflammation state, it is natural to raise the argument that metabolic interactions between the synthesis of SPMs and probiotic bacteria should occur, although such association is still unknown or neglected.

5.3 Probiotics as key regulators of the mucosal immunity

Probiotic bacteria induce immunological alterations very similar to those functions attributed to SPMs. For example, *Bifidobacterium breve* can induce a transient increase in inducible BPIs [21], which shape host-microbe interactions [22]. Probiotic-derived microbe-associated molecular patterns, secreted products and metabolites selectively ameliorate mucosal inflammation by supressing M1 subsets and increase M2 Mϕ, increasing Mϕ and Nφ phagocytic capacity, drecreasing Nφ recruitment and tissue infiltrated, reducing Il-1β and TNFα release and at the same time increasing the synthesis of Il-10 by monocytes, epithelial cells and Mφ, as observed in studies with strains of *Lactobacillus plantarum*, *L. casei*, *L. gasseri, L. rhamnosus, B. breve*, and *B. lactis* [23-27]. *L. rhamnosus* strains GG and GR-1 have been shown to elicit the release of G-CSF from Mϕ, a growth factor that has a paracrine effect on neighboring Mφ and can suppress inflammatory responses [28]. Overall, various signaling pathways are the target of probiotic modulation on Mϕ, Nφ, and epithelial cells including NF-κB and ERK1/2, p38 and JNK MAPKs, activation of negative regulators of TLR-4 pathways, and induction of supressor of cytokine signaling (SOCS) via JAK/STAT/SOCS [29].

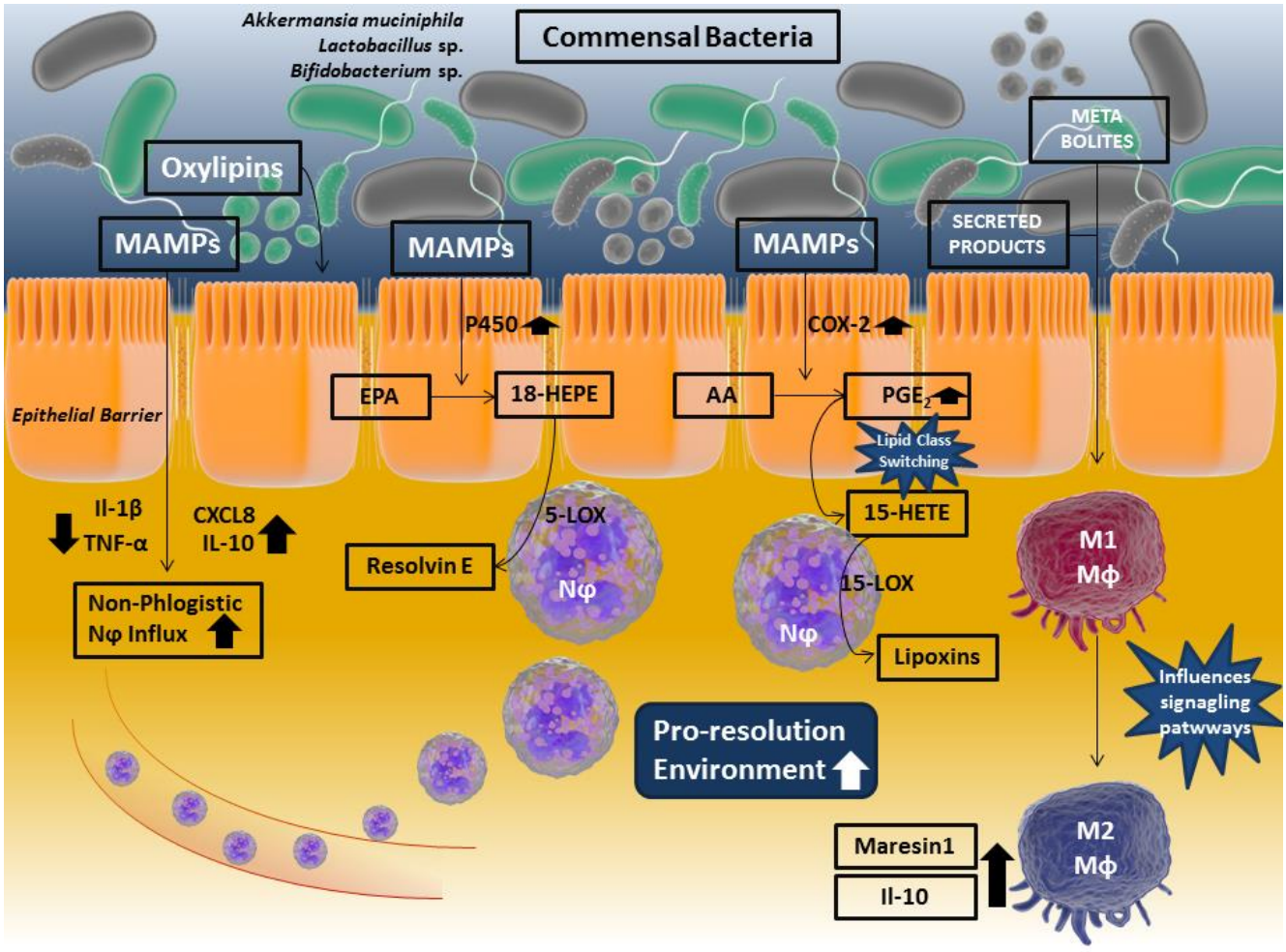
In the context of the mucosal homeostasis, there is a fine balance between epithelial cell proliferation, differentiation and cell death, allowing this dynamic cellular barrier to continually replace itself, and protect from infectious pathogenic agents. For example, intestinal epithelial cells (IEC) represent a physical barrier that maintains the segregation between luminal microbes, digesta and the mucosal immune system [30], and probiotics and commensals can modulate IEC function in a variety of ways, including indirect effects on microbial biofilms and direct effects on IECs via enhancement of barrier function by enhancing tight junctions; induction of antimicrobial peptides; and control of pro-

inflammatory and immunoregulatory cytokines [31-33]. Although in general the effect of probiotics on the functions of an intact epithelial barrier is strain-specific, it is the control of chemokine and cytokine synthesis as well as presumably SPMs by epithelial cells that can ultimately determine the effect of these bacteria on the mucosal integrity. Not coincidentally, probiotics and their constituents and products are defined mainly by their capacity to ameliorate inflammatory bowel disease (IBD), such as Crohn's disease and ulcerative colitis [6]. Curiously, oral administration of *B. bifidum* OLB 6378 to rats with NEC stimulates TLR2 expression in the ileal epithelium, enhances epithelial expression of COX-2, increases intestinal production of PGE_2, and reduces the severity and incidence of NEC. However, inhibition of COX-2 in the rat model of NEC suppresses the effect of *B. Bifidum*, which strongly points to a beneficial effect dependent on the metabolism of lipid mediators for some probiotic strains [34].

5.4 Hypothesis

Considering the intrinsic modulatory relationship between microbiota and the host immune response and that mucosal surfaces are continuously exposed to microorganisms *in vivo*, we hypothesize that commensal bacteria may play an active role on the synthesis of SPMs. In a direct way, oxygenated products of the metabolism of these bacteria can be incorporated by underlying epithelial cells and subsequently used in the synthesis of bioactive SPMs. In addition, these microorganisms also increase the epithelial expression of COX-2 and PGE_2 so that SPMs are synthesized or ω3-derived are released for neutrophils to process and produce bioactive SPMs compounds. Indirectly, commensal probiotics aid in the change of Mϕ phenotype, favoring in the conversion to resolution-phase macrophages when some pathogenic stimulus occurs or even in maintaining a Mϕ physiological phenotype related to the synthesis of SPMs such as maresins. Therefore, commensal bacteria may act by regulating possible pathological alterations or even maintaining a physiological inflammatory balance state in mucosal tissues by regulating the lipid metabolism of bioactive SPMs (Fig. 5.1).

Figure 5.1 - Probiotics and commensal bacteria, such as *Akkermansia muciniphila*, *Lactobacillus* sp., and *Bifidobacterium* sp. control the synthesis of specialized pro-resolving lipid mediators (SPMs) (e.g. lipoxins, resolvins, and maresins) by multiple pathways and by communicating with a variety of cell types. Commensal lactic acid bacteria produce oxylipins derivatives through transient formation of hydroxylated intermediates by fatty acids hydratases. Oxylipins may eventually be used in the lipid cascade of SPM synthesis. Microbe-associated molecular patterns (MAMPs), secreted products and metabolites can increase the expression of key molecules in the epithelial barrier. Beneficial bacteria may increase the expression of cyclooxygenase-2 (COX-2) in epithelial cells which leads to an increase in the synthesis of prostaglandin E_2 (PGE_2) from arachidonic acid (AA). An increase in PGE_2 concentration is essential for lipid class switching. AA is then converted to 15-Hydroxyeicosatetraenoic acid (15-HETE) and eventually can be used by neutrophils (Nφ) in the synthesis of lipoxins. Up-regulation of P450 cytochrome enzymes increases the conversion of ω-3 derived eicosapentaenoic acid (EPA) to 18-hydroxyeicosapentaenoic acid (18-HEPE), which can be used by Nφ in the synthesis of resolvins. In pathogenic challenge conditions, probiotics and commensal bacteria may also increase non-phlogistic recruitment of Nφ by reducing the synthesis of proinflammatory cytokines (IL-1β and TNF-α) and increasing levels of CXCL8 and IL-10, which increases the phagocytic capacity of Nφ without up-regulating the pro-inflammatory response. Eventually, these Nφ can be used in the synthesis of SPMs. In addition, these microorganisms can influence signaling pathways in macrophages (Mϕ) which leads to a conversion of M1 Mϕ (pro-inflammatory) to M2 Mϕ (anti-inflammatory) and a consequent increase in IL-10 release and in the synthesis of the macrophage-derived SPM, Maresin1. The modulation of the synthesis of bioactive SPMs by the microbiome reverses pathological alterations and guarantees tissue homeostasis.



5.5 Evaluation of the hypothesis

During the synthesis of SPMs the participation of oxylipins is essential. Conceptualized as oxygenated products from the dioxygen-dependent oxidation of PUFAs by the activity of COX and LOX enzymes or by cytochrome P450 epoxygenase, oxylipins are widespread in all kingdoms of life [35]. The biological roles of oxylipins have been extensively studied in animals, plants, algae and fungi, but remain largely unidentified in prokaryotes. Overall, human EPA-derived and DHA-derived oxylipins are the precursors-masters of the cascade of resolution of inflammation [36,37]. On this basis, microbial and mammalian cytochrome P450 enzymes convert EPA into 18-HEPE, which can be transformed to resolvin E1 and resolvin E2 [1,2,38]. Another oxylipin, 15-HETE can be metabolized via 15-LOX to produce lipoxins, and curiously *Pseudomonas aeruginosa* encodes the first identified secretory lipoxygenase that converts host AA to 15-HETE for local LXA_4 production [39]. Yet, *L. bulgaricus* OLL1181 induced the mRNA expression of cytochrome P450 family 1A1 (CYP1A1) in human colon cells. In addition, mice treated orally with OLL1181 showed an increase in CYP1A1 mRNA expression in the large intestine and amelioration of DSS-induced colitis [40]. Hence, it is likely that microorganisms at inflamed sites or in the gastrointestinal tract can contribute to production of SPMs in humans.

In a related context, molecules such as lactones, saturated and unsaturated aldehydes, alcohols and short-chain fatty acids, which belong to the oxylipins family have been detected in cell-free supernatants of late exponential phase cultures of several bacteria. In particular, lactic acid bacteria (LAB) such as *L. helveticus*, *L. plantarum* and *L. sanfranciscensis* are reported to release such molecules in culture media or fermented foods [41,42]. Since lipoxygenase, dioxygenase and cytochrome P450 genes have never been found in *Lactobacillus* sp., a possible pathway for linoleic conversion and oxylipins formation could include, as a first step, the transient formation of hydroxylated intermediates by fatty acids hydratases [43], although the role of these probiotics in the metabolism of ω-3 derivatives still needs to be elucidated. Due to the multiple roles played by oxylipins which are flavouring agents, antimicrobial compounds and interspecific signalling molecules, the knowledge of the mechanisms involved in their biosynthesis in food related bacteria could have an important biotechnological impact, also allowing the overproduction of selected bioactive molecules.

Indeed, the gut microbiome may be beneficial to the host and recently arose as a promising strategy to manage PUFA metabolites. For example, *fat-1*-transgenic mice produce and store higher levels of EPA and DHA in their tissues and as a result generate increased levels of resolvins and protectins [44]. In a previous study with *fat-1* mice it was observed that these animals displayed greater phylogenic diversity in the cecum and a peculiar high relative abundance of Verrucomicrobia. The mucus-degrading bacterium *Akkermansia muciniphila* is the only identified member of this phylum, and fecal microbiota transplantation from *fat-1* to wild type mice with metabolic syndrome was able to reverse weight gain and to normalize intestinal permeability [45]. Moreover, *fat-1*-transgenic mice show reduced gastrointestinal inflammation [44]. So, ω3 PUFA mediate alterations of gut microbiota in a process that appears to be a two-way street. This is an important conjecture considering that *A. muciniphila* has been recently proposed as a hallmark of healthy gut due to its anti-inflammatory and immunostimulant properties and its ability to improve gut barrier function and endotoxinemia [46,47].

Among the next-generation beneficial microbes that have been identified, *A. muciniphila* is a promising candidate. Indeed, this species is inversely associated with obesity, diabetes, cardiometabolic diseases and low-grade inflammation [48]. Besides the numerous correlations observed, a large body of evidence has demonstrated the causal beneficial impact of this bacterium in a variety of preclinical models. This microorganism is an intestinal anaerobe which has been proposed as a new functional microbe with probiotic properties in the light of the observation that most, if not all, healthy subjects are known to carry this intestinal microbe [46-49]. Studies based on fluorescent *in situ* hybridisation combined with flow cytometry reported that *A. muciniphila* is a common representative of the human microbiota in healthy adults as well as in babies, for 1-4% of the intestinal microbiota [50]. Acordingly, dietary fats influence the growth of *A. muciniphilia* relative to other bacterium in the dietary tract. In a study in which mice were fed diets which varied in fat composition but were otherwise identical; one group received lard while the other received fish oil, a known source of ω-3 PUFAs. After 11 weeks, the group receiving a fish oil diet had increased levels of *A. muciniphila* and bacterium of genus *Lactobacillus*, while the group receiving a lard diet had decreased levels of *A. muciniphila* and *Lactobacillus* and concomitant increased levels of inflammation [51]. In addition, a reduction in the number of these Verrucomicrobia is closely related to the occurrence of IBD [52,53].

The hallmark of IBD is chronic persistent inflammation of the colonic mucosa (UC) or full thickness intestinal wall (Crohn's disease). Under normal circumstances, an inflammatory response precipitated by an external event like bacteria and gut antigens would be up regulated and then resolved. So, an imbalance in the cellular immune system between pro- and anti-inflammatory mechanisms could explain a state of chronic inflammation, which is characteristic of IBD. The IL-10 anti-inflammatory mediator seems to be involved in its pathogenesis since IL-10 knockout mice spontaneously develop the disease, which is similar to human IBD [54,55]. However, the role of SPMs in this process is less clear. Notwithstanding, preivous work pointed out a defect in the biosynthesis of native lipoxins in UC patients due to altered enzyme levels or altered activity of either 15-LOX or 5-LOX [4]. A major precept of IBD is that it occurs due to a dysregulated and excessive mucosal immune response to potentially antigenic components in the resident bacterial microbiota, which can conceivably be treated by the introduction of microorganisms that somehow change the effect of the commensal bacteria on the epithelial cell barrier or else change the overall response of the mucosal immune system so that it inhibits rather than promotes inflammation. Not surprisingly, *Bifidobacterium* sp. and *Lactobacillus* sp. have shown long-term beneficial effects in the treatment of UC patients [56]. On provocation, a similar immune paradigm can be raised from other forms of mucosal dysbiosis-triggered inflammatory diseases, such as periodontitis.

On the basis of the current hypothesis, another effect of the use of probiotics is their indirect action on the synthesis of SPMs by modulating cellular influx and phenotypic cellular changes. Probiotics are thought to inhibit pathogen colonization by increasing CXCL8 syhthesis in isolated Nφ at the same time that they decrease their percentage of apoptosis and necrosis, modulating the entry and activity of PMNs migrating from peripheral blood [57]. In fact, both effects can be observed and probiotics can either act by decreasing or increasing the synthesis of CXCL8 by epithelial cells, with consequent reduction or increase of Nφ influx, respectively [29,58-60]. Noteworthly, however, is that these beneficial bacteria seem to help in the an inflammatory pro-resolution environment depending on the present stimulus. For example, although bifidobacteria and lactobacilli may act by increasing the release of CXCL8 by epithelial cells under stimulation with pathogens that possess sophisticated means of evading the immune system such as *P. gingivalis*, they concomitantly reduce the production of IL-1β and TNF-α by these cell types of the innate immunity [61], which favors a typical non-phlogistic recruitment profile essential in the mechanisms of resolution of inflammation.

Given that in the synthesis of SPMs a close relationship exists between oxylipin precursors produced by epithelial cells, such as 15-HETE and 18-HEPE, and their subsequent metabolism by Nφ for the formation of resolvins and lipoxins [1,28,62], it would be important to point out this indirect effect of probiotics stimulating non-phlogistic recruitment of PMNs to the epithelial barrier.

As alluded to above, probiotics and their secreted products and metabolites also modulate Mϕ signaling pathways. Short-chain fatty acids, such as butyrate, produced by probiotics and commensal bacteria are potent inhibitors of IL-12 and up-regulators of IL-10 production on human monocytes [63]. Mϕ are highly plastic cells and the M1 and M2 subsets are considered the two extremes of a huge range of cell phenotypes. M2 Mϕ are associated with mucosal homeostasis and tolerance, mediated by anti-inflammatory/regulatory cytokines including IL-10, TGF-β and IL-1Ra. M1 Mϕ, on the other hand, are associated with immune activation and pro-inflammatory responses driven by cytokines such as TNF-α, IL-1β, and IL-12 [64,65]. The potential of probiotic bacteria to regulate Mϕ subsets, however, appears to be strain-specific and does not necessarily follow the classical M1-M2 patterns, in addition contradictorily both profiles can be induced by these microorganisms [25,27]. Importantly, it was described a previously unknown resolution-phase macrophage denoted (rM) that possesses a hybrid phenotype of alternative activation, mannose receptor expression and synthesis of IL-10 and arginase 1 (classically M2, anti-inflammatory) with high COX-2 and iNOS expression (classically M1, pro-inflammatory) [66,67]. This rM phenotype, inducible by elevating intra-cellular cAMP, is vital in the synthesis of potent SPMs derived-Mϕ, named maresins [16,17,66]. Thus, it is useful to discuss the ability of probiotics to control Mϕ plasticity and, consequently, SPM synthesis.

5.6 Testing the hypothesis

The hypothesis presented here can be tested by performing relevant *in vitro*, and *in vivo* studies. Commensal bacteria, e.g. *A. muciniphila,* could be grown in the presence or absence of PUFAs, such as ω3 and linoleic acids. Then, bacterial-free supernatant could be evaluated by metabolome analysis for the presence of oxylipins and related enzymes. The

bacterial supernatant could subsequently be used to stimulate epithelial cells in which the expression of PGE_2, COX-2 and P450 enzymes would be evaluated, as well as lipidomic analyzes could be used to evaluate the synthesis of bioactive SPMs and the presence of oxylipins derived, such as 15-HETE and 18-HEPE, besides the evaluation of key pro-resolving mediators (CXCL8, IL-1β, TNFα, and IL-10). The supernatant of epithelial cells previously stimulated with bacterial oxylipin-enriched supernatant could also be used to stimulate neutrophils, with subsequent measurement of their migratory and phagocytic activities and on the synthesis of SPMs, as well as to stimulate macrophages and, comprehensively, to evaluate their subsequent phenotypic changes by the evaluation of signature cytokines and the production of anti-inflammatory mediators.

In vivo studies with models of chronic inflammatory diseases of the digestive tract associated with microbial dysbiosis, such as IBD and periodontitis, using transgenic *fat-1* mice that store higher levels of EPA and DHA in their tissues or knockout animals for SPM receptors with subsequent application of probiotic bacteria should also be delineated in order to measure the impact of these microorganisms on the lipid metabolism under pathological conditions.

5.7 Implications of the hypothesis

Tissues under persistent microbial stimuli are characterized by their high rate of self-renewal and SPMs have been identified as essential regulators of the tissue regeneration process after inflammatory injury or even the physiological restorative activity indispensable for the mucosal homeostasis [1-4]. Thus, an important consequence of regulating the synthesis of SPMs by probiotics would be its effect on the regenerative properties of adult tissue-specif stem cells. Lipoxins, for example, significantly enhance proliferation, migration, and wound healing capacity of periodontal ligament stem cells, and when encapsulated in microparticles accelerate wound healing of skin ulcers [68,69], as well as maresins amplify the regenerative activity in planaria [70]. On the basis of these concepts, as we harbor 10-fold more bacterial cells than human cells [20], explorations into how the microbiota may influence bioactive lipid production might redefine how we view our ‘microbial selves’.

In addition, we emphasize that if this integrated vision microbiome-lipid metabolism addressed in the present hypothesis materializes, implications in the understanding of mucosal inflammatory diseases may arise. Failures in the activation of the synthesis of an appropriate content of bioactive SPMs components by commensal bacteria could be the core of the etiopathogenesis of chronic inflammatory diseases of the digestive tract associated with microbial dysbiosis such as IBD and periodontitis.

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6 CONCLUSIONS

i. Probiotics regulate the innate immune response mediated by gingival epithelial cells (GECs) preventing cell death induced by *P. gingivalis,* reducing pathogen adhesion and invasion at the same time as they increase their own adhesion to GECs. The control of the interaction of GECs with P. gingivalis results in a reduction in the synthesis of IL-1β and TNF-α with concomitant increase in the release of CXCL8, which may occur due to a modulation of the transcription of TLR2/TLR4. Probiotics also altered the transcription of genes encoding antimicrobial peptides and apoptosis regulatory genes, overall in a strain-specific manner. Among the twelve species of bifidobacteria and lactobacilli evaluated, *L. acidophilus* La-5 emerges as an important probiotic with immunomodulatory activities in the control of the immune response linked to the etiopathogenesis of periodontitis.

ii. The ratio of pro-inflammatory mediators and pro-resolving lipid mediators seems to control periodontal regenerative activities since a predominantly inflammatory environment under IL-1β/TNF-α stimulus reduced the stemness of periodontal ligament stem cells (hPDLSCs), downregulated the expression of their regenerative biomarkers, impaired their in vitro wound healing, and decreased their related osteo-cementogenic activities. On the other hand, the induction of an milue related to the resolution of inflammation with the addition of MaR1/RvE1 reverse this process by partially rescuing biomarkers of stemness and ameliorate hPDLSCs regenerative-related activities. Therefore, an adequate control of the inflammatory environment seems to be fundamental for an improvement in the process of tissue regeneration that must overcome the destruction of periodontal tissues lost in periodontitis.

iii. Since probiotic bacteria appear to exert an important immunomodulatory activity on epithelial barriers we hypothesize that these beneficial microorganisms can control tissue homeostasis and alter the immune response triggered by pathogens by modulating the synthesis of bioactive specialized pro-resolving lipid mediators.

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