



# **High and Low Molecular Weight Hyaluronic Acid Affect the Expression of Surface Molecules by Monocytes Stimulated with *Porphyromonas gingivalis* In vitro**

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## **Authors' contributions**

This work was carried out in collaboration among all authors. Author PEAS conceived of the basic idea for this work. Authors ECS, HHOC and NRG designed and carried out the experiments. Authors PPM and LDMF analyzed the microorganisms. Authors LIDS and LRDVA did acquisition of cytometric data. Authors LRDVA and PEAS performed the cytometric analysis. Author PEAS supervised the research and helped to develop the manuscript. Authors PEAS and MCRH analyzed the results. Authors ECS and JDC wrote the first draft of the manuscript. Authors JDC and MCRH provided feedback on the manuscript throughout its development. All authors subsequently took part in the revision process and approved the final copy of the manuscript. All authors read and approved the final manuscript.

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## ABSTRACT

**Aims:** Hyaluronic acid (HA) has been used as an adjunct in the treatment of periodontal disease. HA molecules of different molecular weights act on different cell types, inducing differentiation, activation, migration, and production of inflammatory mediators. The aim of this study was to evaluate the effect of low (LMWHA) and high (HMWHA) molecular weight HA on the expression of costimulatory molecules and toll-like receptors by monocyte subpopulations stimulated *in vitro* with *Porphyromonas gingivalis* (Pg).

**Study Design:** *In vitro* experimental study.

**Place and Duration of Study:** Laboratory of Biology and Immunology of Infectious and Parasitic Diseases, Instituto René Rachou, Fiocruz Minas, between 2018 and 2019.

**Methodology:** Peripheral blood mononuclear cells (PBMCs) from 10 donors were stimulated with Pg during 1 hour and then incubated with 0.2% LMWHA or HMWHA for another sixteen hours. Immunostaining and flow cytometry were performed for detection of CD40, CD80, CD86, HLA-DR, TLR2 and TLR4 expression by CD14+ monocytes and subpopulations of classical (CD14+CD16-) and non-classical/intermediate monocytes (CD14+CD16+).

**Results:** LMWHA or HMWHA increased TLR2 and reduced TLR4 expression, but did not affect CD40, CD80, CD86 or HLA-DR in total and classical monocytes. Considering Pg-stimulated cells, HMWHA increased the expression of CD40, HLA-DR and TLR2 in non-classical/intermediate monocytes.

**Conclusion:** The effect of HA used topically in the periodontal tissues seems to directly involve modulation of surface molecules responsible for cell signaling and control of periodontal immunoinflammatory processes.

**Keywords:** Hyaluronic Acid; monocytes; *Porphyromonas gingivalis*; toll-like receptors; B7 Antigens; CD40 Antigens; HLA-DR Antigens.

## 1. INTRODUCTION

Periodontitis is an inflammation of the supporting tissues of the teeth with progressive attachment loss and bone destruction (Cekici et al., 2014). The inflammation is initiated by complex and diverse microbial biofilms, which form on the teeth, and challenged the host defense system releasing substances such as lipopolysaccharides (LPS), antigens and other virulence factors (Cekici et al., 2014). *Porphyromonas gingivalis* is a Gram-negative bacterium highly pathogenic that has LPS as a major virulence factor and is closely associated with periodontitis (Mysak et al., 2014).

Hyaluronic acid (HA) is a glycosaminoglycan present in extracellular matrix which plays a

crucial role in the post-inflammation tissue repair, facilitating the migration and cell differentiation through cell signaling and interaction with growth factors present in the interstitium (Yamawaki et al., 2009). Studies reveal that most of HA's properties are size-dependent (Yamawaki et al., 2009). High molecular weight HA (HMWHA, >103 kDa) exerts anti-inflammatory, immune-suppressive and wound healing effects, while low molecular weight HA (LMWHA) shows pro-inflammatory properties (Chen et al., 2019).

Studies have shown that topical application of HA gel reduced the inflammation and edema in patients with gingivitis (Jentsch et al., 2003) and reduced inflammation, plaque index, bleeding and probing depth in treating periodontal disease (Fawzy El-Sayed et al., 2012).

The subgingival administration of HA gel as adjuvant to surface root debridement has significant anti-inflammatory effects with a potential role for the treatment of chronic periodontitis through its significant positive impact on clinical periodontal parameters, pro-inflammatory cytokines, and biochemical marker improvement as compared to chlorhexidine (Mohammad et al., 2023). Furthermore, HA gel stimulates the reconstruction of the interdental papilla in the gingival aesthetic zone (Kapoor & Bhardwaj, 2022). Recent systematic review and meta-analysis has shown that the topical application of HA may lead to additional clinical benefits when used as an adjunctive to non-surgical and surgical periodontal therapy (Eliezer et al., 2019).

However, there are no studies evaluating possible effects of HA in the modulation of immunocompetent cells stimulated by periodontopathogens. Considering also that different molecular weights HA act differently in cellular functions, the aim of this study was evaluate the HA effect of high and low molecular weight in the expression of surface molecules involved in the control of inflammatory responses by human monocyte subpopulations stimulated with *P. gingivalis*.

## 2. MATERIALS AND METHODS

### 2.1 Blood Samples

Ten healthy individuals aged from 30 to 60 years (mean 37.9 years), non-smokers, were included in the present study. Exclusion criteria were individuals with systemic infectious disease; immunosuppression; alcoholism; autoimmune disease; chronic inflammatory diseases or who have been taking antibiotic, chemotherapeutic, antineoplastic, immunosuppressive or anti-inflammatory drugs in the last 30 days.

Approximately 20 mL of blood was collected from each donor in heparinized tubes (Becton Dickinson Vacutainer®, Franklin Lakes, NJ, USA). The blood was diluted in phosphate-saline buffer (PBS) in a 1:1 ratio and then carefully overlaid in 10 ml of Ficoll-Paque (GE Healthcare, Uppsala, Sweden). After centrifugation at 20°C, at 200 x g, for 40 minutes, peripheral blood mononuclear cells (PBMCs) were collected, washed in PBS and resuspended in complete Roswell Park Memorial Institute (RPMI 1640) (Sigma Aldrich, St. Louis, MO, USA) medium supplemented with 10% fetal bovine serum

(FBS) (Gibco, ThermoFischer, Waltham, MA, USA). Trypan blue (0.4%) was added to the cells in a ratio of 1:1 for counting in the Countess™ Automated Cell Counter (Invitrogen, ThermoFischer, Waltham, MA, USA) apparatus. PBMCs were then plated at a cell density of  $3 \times 10^5$  cells/well in 96-well U-bottom microplates.

### 2.2 Preparation of Bacteria

*Porphyromonas gingivalis* (Pg) ATCC® 33277™ (Manassas, VA, USA) was cultivated on Brain Heart Infusion Agar (BHI - Difco, Sparks, MD, USA), supplemented with 0.5% yeast extract (Difco), 0.1% hemin (porcine) (Inlab, Diadema, SP, Brazil) and 0.1% menadione (Sigma-Aldrich, St. Louis, MO, USA), and enriched with 5% blood (BHIA-S/E), pH 7.2. Pg was incubated in an anaerobic chamber (Thermo Scientific, Waltham, MA, USA) with 85% N<sub>2</sub>, 5% CO<sub>2</sub> and 10% H<sub>2</sub>, at 37°C, for 72 h. The colonies were transferred to tubes containing saline, homogenized and quantified in a spectrophotometer (Ultrospec 10 Cell Density Meter, Biochrom, Cambridge, UK) at the optical density (OD) of 1, at 600nm. The bacterial suspension was washed with PBS, heated for 30 minutes at 100°C for inactivation and kept at -80°C for use in the experiments.

### 2.3 Hyaluronic Acid

Low molecular weight (LMWHA) (33 kDa) (GLR001, lot # 1467153, R & D Systems) and high molecular weight (HMWHA) (1.46 x 10<sup>6</sup> Da) (GLR002, lot # 1490155, R & D Systems) hyaluronic acid, both with endotoxin level of <0.01EU/ml, were used in this study. HA diluted solutions were prepared in complete RPMI medium supplemented with 10% fetal bovine serum (FBS) immediately before use.

### 2.4 LMWHA and HMWHA Cytotoxicity in PBMCs

To evaluate HA effects on cell viability, PBMCs were incubated with LMWHA or HMWHA at the final concentrations of 0.025, 0.05, 0.1 or 0.2%, at 37°C, 5% CO<sub>2</sub>, for 16 hours. Cells were then subjected to a colorimetric functional assay with methyl tetrazole (MTT) for assessing mitochondrial activity. Methyl tetrazole formazan (MTT, 5mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added to final concentration of 20% (v/v) in RPMI, and the cells were incubated for an additional 3 hours at 37°C, in a humidified atmosphere containing 5% CO<sub>2</sub>. The formazan precipitates were dissolved by adding 100 µL of 10% (w/v) sodium dodecyl sulfate (SDS) solution

(Sigma-Aldrich, St. Louis, MO, USA) overnight, and after homogenization, optical density was read at 570 nm in a microplate reader (Versamax, Molecular Devices, Sunnyvale, CA, USA). The experiments were performed with 5 donor cells in triplicate. Positive control groups were composed of cells in complete RPMI only. Cells heated at 100°C for 15 minutes served as negative control. Data were expressed as percentage of viable cells relative to control.

## 2.5 Stimulation of PBMCs with HA and *P. gingivalis*

Based on the MTT assay that showed no significant changes in the cell viability of PBMC stimulated with different concentrations of HA (Fig. 1) and on the HA concentration used in clinical studies for periodontitis (Casale et al., 2016), the final concentration of 0.2% was defined for further evaluation of the effect of LMWHA and HMWHA on the expression of surface molecules by human monocytes.

PBMCs at  $2 \times 10^5$  cells per well in 96-well U-bottom microplates were incubated with Pg at the multiplicity of infection (MOI) of 0.1 colony forming units (CFU): 1 PBMC at 37°C, 5% CO<sub>2</sub>, for 1 hour. Then, LMWHA or HMWHA at the final concentration of 0.2% were added and the cells incubated for a further 16 hours at 37°C, 5% CO<sub>2</sub>.

The following experimental groups were obtained: Control (RPMI medium), LMWHA (0.2% low molecular weight hyaluronic acid), HMWHA (0.2% high molecular weight hyaluronic acid), Pg (*P. gingivalis*), Pg-LMWHA and Pg-HMWHA.

## 2.6 Immunofluorescence Staining and Flow Cytometry

The protocol for immunostaining was performed based on (Costa et al., 2018), with some modifications. After staining with Live/Dead (Thermo Fisher Scientific, Waltham, MA, USA), cells were incubated with anti-CD14 (ef450 Clone 61D3, eBioscience), CD16 (APCCy7, Clone 3G8, BioLegend), CD40 (FITC, clone 5C3, BioLegend), CD80 (FITC, clone 2D10, BioLegend), CD86 (PE, clone IT2.2, eBioscience), HLA-DR (PeCy5.5 Clone Tu36, BD), TLR2 (PE, clone TL2.1, eBioscience), and TLR4 (APC, clone HTA125, BioLegend) antibodies diluted in 0.015 M PBS, pH 7.4, containing 2% fetal bovine serum (FBS) (Gibco, Life Technologies Corporation, Carlsbad, CA,

USA), for 30 minutes, at room temperature. Cells were then washed with PBS, fixed with 2% formaldehyde in PBS, for 10 minutes, at room temperature, and analyzed by flow cytometry (LSRFortessa™, Becton Dickinson, New Jersey, USA), with acquisition of 70,000 events per tube.

Cytometric analyzes were performed using the FlowJo X software (Tree Star Inc., USA). Based on the location of cells in the granularity versus CD14 plot, total monocyte (CD14+) populations were selected. Considering these gated cells, subpopulations of classical (CD14+CD16-) and non-classical/intermediate (CD14+CD16+) monocytes were selected (Chimen et al., 2017). The percentages of monocytes from each phenotypically identified subpopulation were determined. The percentages of cells expressing CD40, CD80, CD86, HLA-DR, TLR2 and TLR4 and the median fluorescence intensity (MFI) of these molecules were also quantified within the total monocyte (CD14+) population and within each subpopulation of selected monocytes.

## 2.7 Statistical Analysis

The Kolmogorov-Smirnov normality test was used to verify the distribution of the data. In order to verify differences between treatments in cell viability, in the frequencies of surface molecules expressing cells and in the MFI of these molecules, the ANOVA one-way test with repetition followed by the Tukey "post hoc" test or the Friedman test followed by the Dunn's "post hoc" test were used for comparison between pairs, at a significance level of 5%. Analysis was performed using the GraphPad Prism 5.01 software (GraphPad Software, San Diego, California, USA).

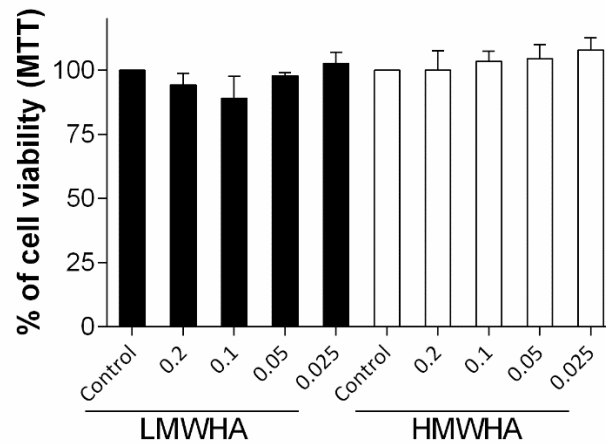
## 3. RESULTS

### 3.1 LMWHA and HMWHA Cytotoxicity in PBMCs

Analysis of PBMC viability by MTT showed that incubation with LMWHA or HMWHA in concentrations ranging from 0.025% to 0.2% did not cause any significant changes (Fig. 1).

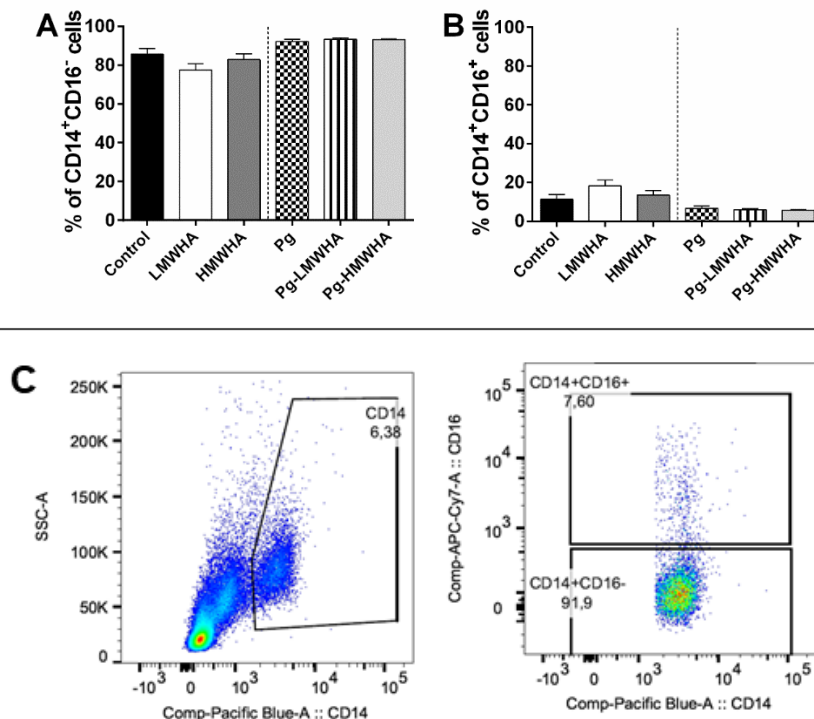
### 3.2 Effect of HA and Pg in the Frequencies of Monocyte Subpopulations

Flow cytometry analysis showed that exposure to LMWHA or HMWHA and bacteria did not significantly affect the frequencies of classical or non-classical/intermediate monocytes in the total CD14+ cells (Fig. 2).



**Fig. 1. Viability of PBMCs assessed by the MTT method after 16 hours of incubation with different concentrations (%) of LMWHA or HMWHA. Results are expressed as mean percentage of mitochondrial activity in relation to the control group**

Data from 5 donors are shown. Experiments were performed in triplicate. Error bars indicate standard error of the mean (SEM). LMWHA (low molecular weight hyaluronic acid); HMWHA (high molecular weight hyaluronic acid); Pg (*Porphyromonas gingivalis*).



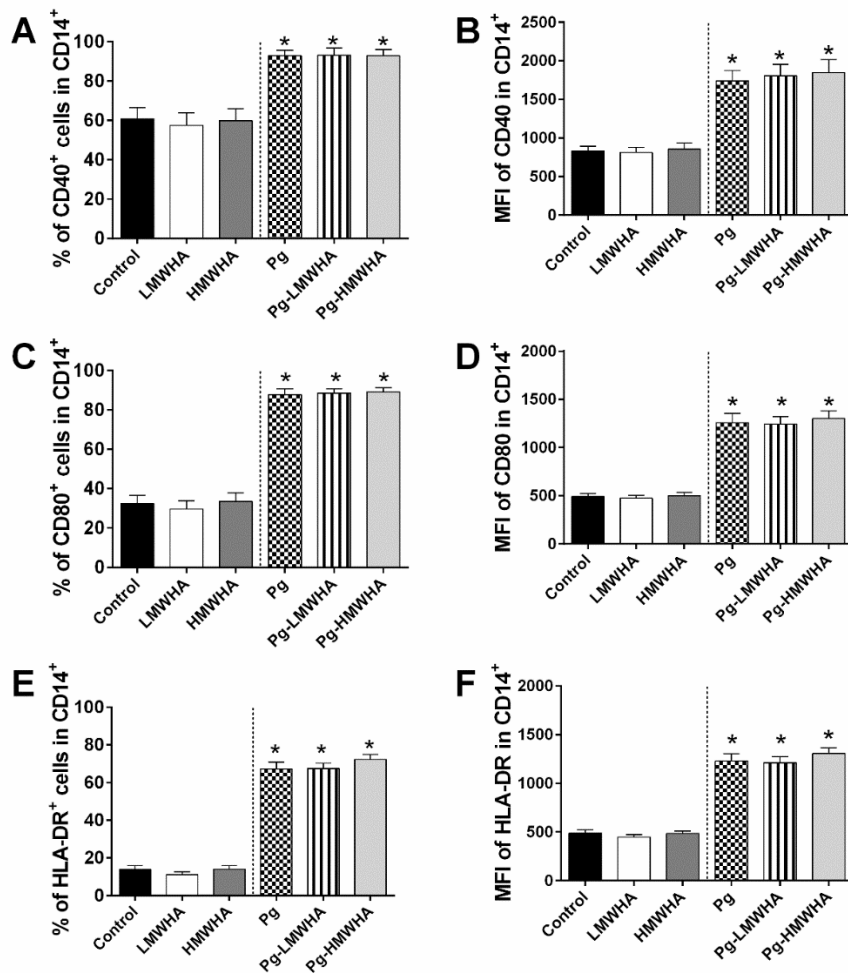
**Fig. 2. The effect of LMWHA or HMWHA and *P. gingivalis* stimulation on the frequencies of classical monocytes (CD14<sup>+</sup>CD16<sup>-</sup>) (A) and non-classical/intermediate monocytes (CD14<sup>+</sup>CD16<sup>+</sup>) (B). PBMCs were stimulated or not with Pg and incubated with 0.2% LMWHA or HMWHA. Total incubation time 17 hours. Dot plot graphs representing the percentage of total monocytes and monocytes from each phenotypically identified subpopulation (C).**

Error bars indicate standard error of the mean (SEM). Data from 10 donors are shown. LMWHA (low molecular weight hyaluronic acid); HMWHA (high molecular weight hyaluronic acid); Pg (*Porphyromonas gingivalis*).

### 3.3 Effect of LMWHA and HMWHA on the Expression of co-stimulatory Molecules and Toll-Like Receptors by Monocytes

Cytometric analyses showed that incubation of PBMC with LMWHA or HMWHA did not significantly alter the frequencies of cells expressing CD40, CD80 and HLA-DR, or the MFI of these molecules in the total monocytes (CD14<sup>+</sup>) (Fig. 3), classical (Fig. 4) or non-

classical/intermediate (Fig. 5) monocyte subpopulations. Similar results were observed for CD86 (data not shown). However, the frequency of CD80<sup>+</sup> cells was significantly higher in the HMWHA group when compared to the LMWHA group only in non-classical/intermediate monocytes (Fig. 5C). Additionally, the frequencies of HLA-DR<sup>+</sup> cells were significantly higher in the HMWHA group when compared to the LMWHA group in classical (Fig. 4E) and non-classical/intermediate (Fig. 5E) monocytes.

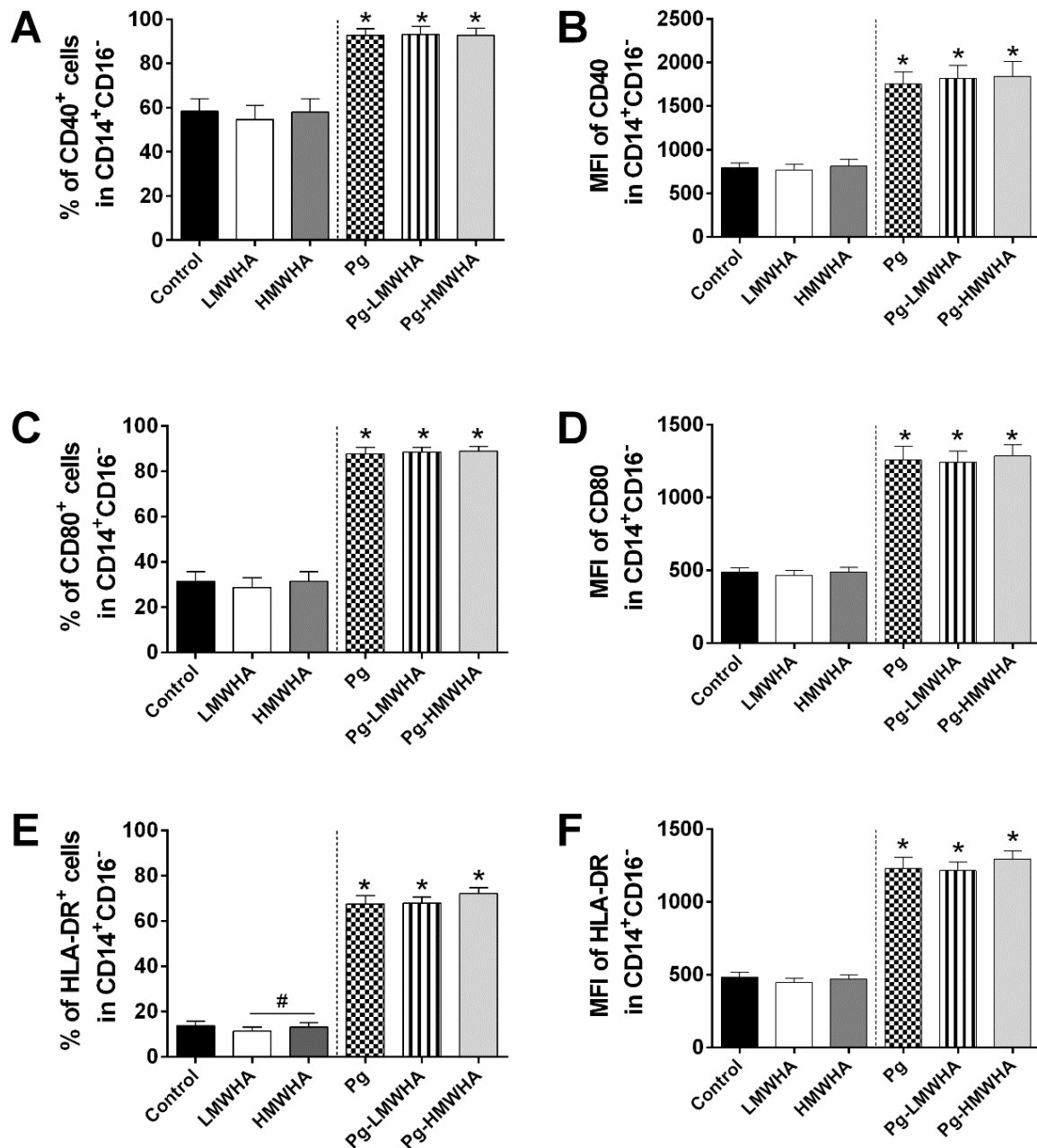


**Fig. 3. The effect of LMWHA or HMWHA and *P. gingivalis* stimulation on the frequencies of positive cells and median fluorescence intensity (MFI) of co-stimulatory molecules in CD14<sup>+</sup> monocytes. PBMCs were stimulated or not with Pg and incubated with 0.2% LMWHA or HMWHA. Total incubation time 17 hours.**

Error bars indicate standard error of the mean (SEM). Data from 10 donors are shown.

LMWHA (low molecular weight hyaluronic acid); HMWHA (high molecular weight hyaluronic acid); Pg (*Porphyromonas gingivalis*).

\* represents a significant difference ( $P < .05$ ) in relation to the control group and connecting lines under # represent significant differences ( $P < .05$ ) between the experimental groups (one-way ANOVA with repeated measures).

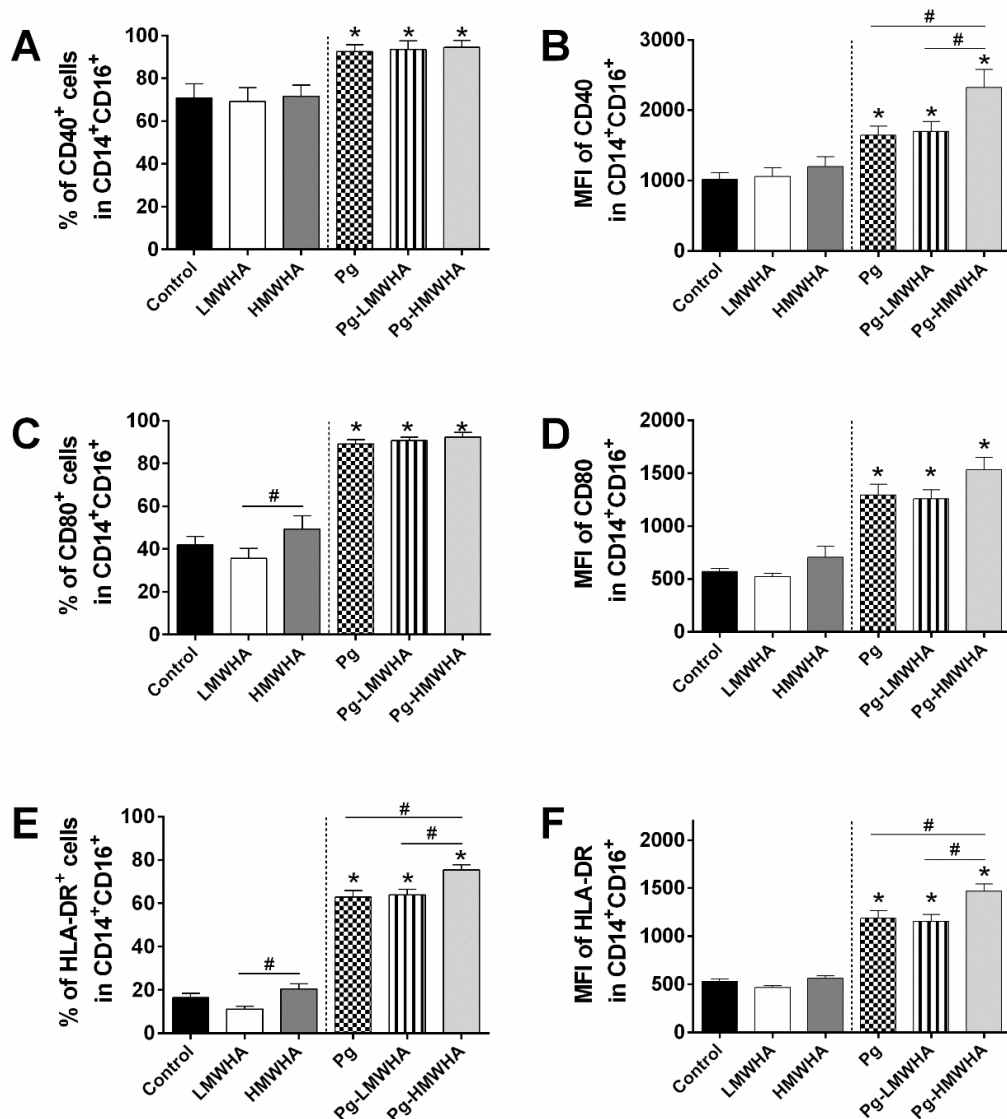


**Fig. 4.** The effect of LMWHA or HMWHA and *P. gingivalis* stimulation on the frequencies of positive cells and median fluorescence intensity (MFI) of co-stimulatory molecules in the classical monocytes (CD14<sup>+</sup>CD16<sup>-</sup>). PBMCs were stimulated or not with Pg and incubated with 0.2% LMWHA or HMWHA. Total incubation time 17 hours.

Error bars indicate standard error of the mean (SEM). Data from 10 donors are shown.

LMWHA (low molecular weight hyaluronic acid); HMWHA (high molecular weight hyaluronic acid); Pg (*Porphyromonas gingivalis*).

\* represents a significant difference ( $P < .05$ ) in relation to the control group and connecting lines under # represent significant differences ( $P < .05$ ) between the experimental groups (one-way ANOVA with repeated measures).



**Fig. 5. The effect of LMWHA or HMWHA and *P. gingivalis* stimulation on the frequencies of positive cells and median fluorescence intensity (MFI) of co-stimulatory molecules in the non-classical/intermediate monocytes (CD14<sup>+</sup>CD16<sup>+</sup>). PBMCs were stimulated or not with Pg and incubated with 0.2% LMWHA or HMWHA. Total incubation time 17 hours.**

Error bars indicate standard error of the mean (SEM). Data from 10 donors are shown.

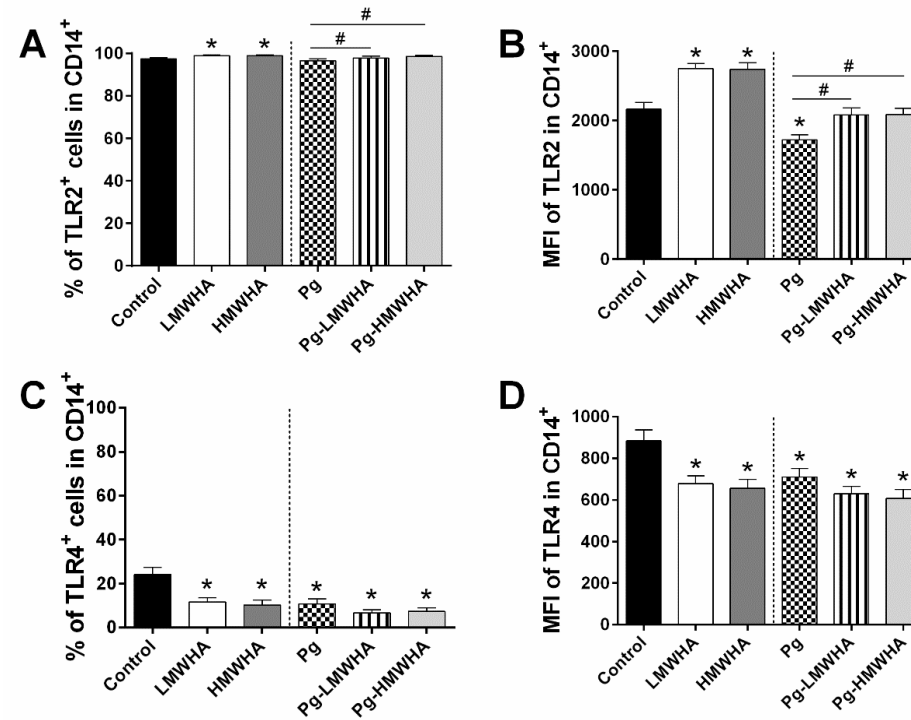
LMWHA (low molecular weight hyaluronic acid); HMWHA (high molecular weight hyaluronic acid); Pg (*Porphyromonas gingivalis*).

\* represents a significant difference ( $P < .05$ ) in relation to the control group and connecting lines under # represent significant differences ( $P < .05$ ) between the experimental groups (one-way ANOVA with repeated measures).

Analysis of TLR expression showed that LMWHA or HMWHA significantly increased the frequencies of TLR2<sup>+</sup> cells and the MFI of TLR2 in the CD14<sup>+</sup> monocyte population (Fig. 6A and B), when compared to control. LMWHA and HMWHA also increased the MFI of TLR2 in classical (Fig. 7B) and non-classical/intermediate

(Fig. 8B) monocytes when compared to control. Conversely, incubation with LMWHA or HMWHA significantly reduced the frequencies of TLR4<sup>+</sup> cells and the MFI of TLR4 in CD14<sup>+</sup> (Fig. 6C and D) and classical (Fig. 7C and D) monocytes, when compared to control.



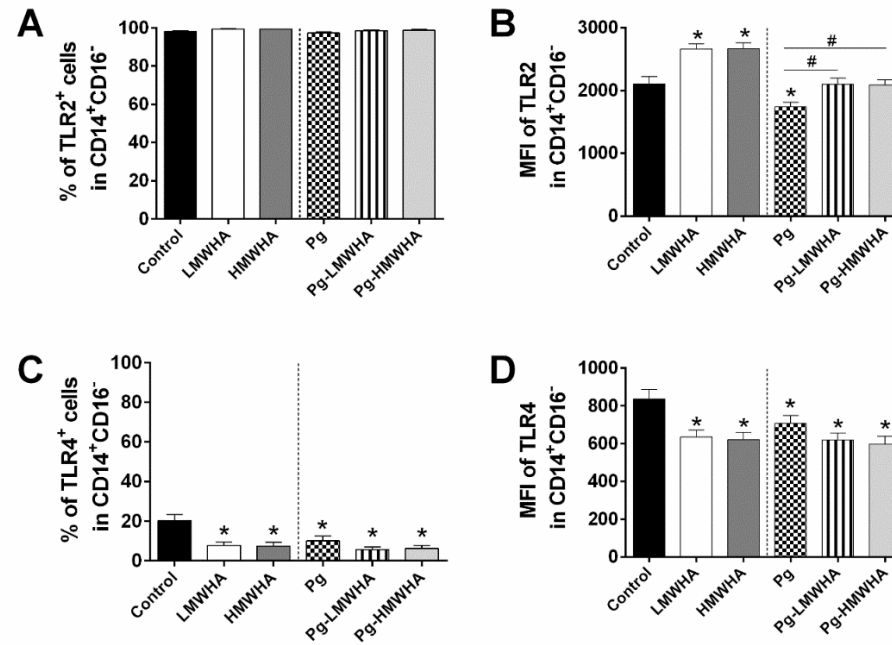


**Fig. 6.** The effect of LMWHA or HMWHA and *P. gingivalis* stimulation on the frequencies of positive cells and median fluorescence intensity (MFI) of toll-like receptors in CD14<sup>+</sup> monocytes. PBMCs were stimulated or not with Pg and incubated with 0.2% LMWHA or HMWHA. Total incubation time 17 hours.

Error bars indicate standard error of the mean (SEM). Data from 10 donors are shown.

LMWHA (low molecular weight hyaluronic acid); HMWHA (high molecular weight hyaluronic acid); Pg (*Porphyromonas gingivalis*).

\* represents a significant difference ( $P < .05$ ) in relation to the control group and connecting lines under # represent significant differences ( $P < .05$ ) between the experimental groups (one-way ANOVA with repeated measures).

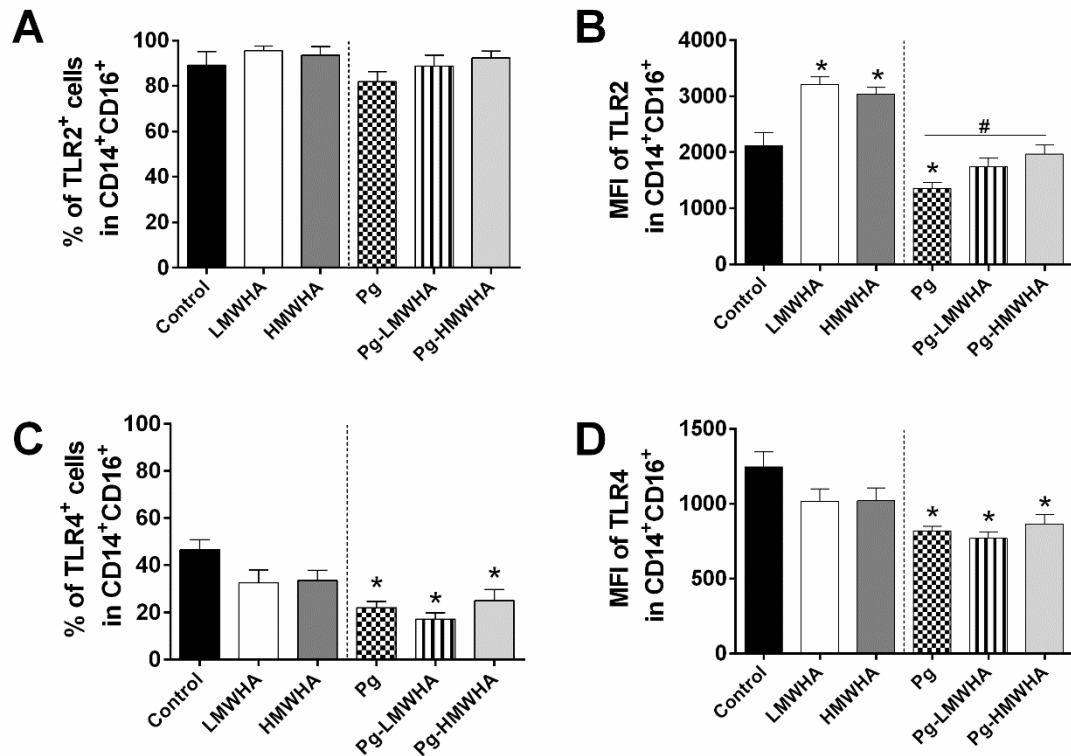


**Fig. 7. The effect of LMWHA or HMWHA and *P. gingivalis* stimulation on the frequencies of positive cells and median fluorescence intensity (MFI) of toll-like receptors in the classical monocytes (CD14<sup>+</sup>CD16<sup>-</sup>). PBMCs were stimulated or not with Pg and incubated with 0.2% LMWHA or HMWHA. Total incubation time 17 hours.**

Error bars indicate standard error of the mean (SEM). Data from 10 donors are shown.

LMWHA (low molecular weight hyaluronic acid); HMWHA (high molecular weight hyaluronic acid); Pg (*Porphyromonas gingivalis*).

\* represents a significant difference ( $P < .05$ ) in relation to the control group and connecting lines under # represent significant differences ( $P < .05$ ) between the experimental groups (one-way ANOVA with repeated measures).



**Fig. 8.** The effect of LMWHA or HMWHA and *P. gingivalis* stimulation on the frequencies of positive cells and median fluorescence intensity (MFI) of toll-like receptors in the non-classical/intermediate monocytes (CD14<sup>+</sup>CD16<sup>+</sup>). PBMCs were stimulated or not with Pg and incubated with 0.2% LMWHA or HMWHA. Total incubation time 17 hours.

Error bars indicate standard error of the mean (SEM). Data from 10 donors are shown.

LMWHA (low molecular weight hyaluronic acid); HMWHA (high molecular weight hyaluronic acid); Pg (*Porphyromonas gingivalis*).

\* represents a significant difference ( $P < .05$ ) in relation to the control group and connecting lines under # represent significant differences ( $P < .05$ ) between the experimental groups (one-way ANOVA with repeated measures).

### 3.4 Effect of LMWHA and HMWHA on the Expression of co-stimulatory Molecules and toll-Like Receptors by Monocytes after Stimulation with Pg

Stimulation of PBMCs with Pg significantly increased the frequencies of positive cells and the MFI of CD40, CD80 and HLA-DR in total (Fig. 3), classical (Fig. 4) and non-classical/intermediate (Fig. 5) monocytes, when compared to control. Conversely, stimulation with Pg significantly reduced the MFI of CD86 (data not shown). Moreover, stimulation with Pg also significantly reduced the frequencies of TLR4<sup>+</sup> cells and the MFI of TLR2 and TLR4 in total (Fig. 6), classical (Fig. 7) and non-classical/intermediate (Fig. 8) monocytes, when compared to control.

Considering cells stimulated with Pg, the addition of LMWHA or HMWHA did not significantly affect the frequencies of positive cells or the MFI of CD40, CD80 or HLA-DR in the CD14<sup>+</sup> (Fig. 3) or classical (Fig. 4) monocytes. Similar results were observed for CD86 (data not shown). However, the addition of HMWHA to Pg-stimulated cells significantly increased the frequency of HLA-DR<sup>+</sup> cells (Fig. 5E) and the MFI of CD40 (Fig. 5B) and HLA-DR (Fig. 5F) in non-classical/intermediate monocytes. Furthermore, comparing HA of different molecular weight, the Pg-HMWHA group showed higher frequency of HLA-DR<sup>+</sup> cells (Fig. 5E) and MFI of CD40 (Fig. 5B) and HLA-DR (Fig. 5F) than Pg-LMWHA group in non-classical/intermediate monocytes.

Analysis of TLR expression in Pg-stimulated cells showed that addition of LMWHA or HMWHA

significantly increased the frequency of TLR2+ cells and the MFI of TLR2 in CD14+ (Fig. 6A and B) and classical (Fig. 7A and B) monocytes. However, in the non-classical/intermediate monocyte subpopulation stimulated with Pg, only HMWHA significantly increased the MFI of TLR2 (Fig. 8B). Also considering Pg-stimulated cells, the addition of LMWHA or HMWHA did not significantly affect the expression of TLR4 in CD14+ (Fig. 6), classical (Fig. 7) or non-classical/intermediate (Fig. 8) monocytes.

## 4. DISCUSSION

Several studies have shown satisfactory clinical effects of HA use as an adjunct in the treatment of gingivitis and periodontitis, reducing tissue inflammation and provided a significant improvement in periodontal parameters (Jentsch et al., 2003; Fawzy El-Sayed et al., 2012; Mohammad et al., 2023; Eliezer et al., 2019; Polepalle et al., 2015; Shah et al., 2016). However, the molecular and cellular mechanisms by which HA acts in inflamed periodontal tissues have not yet been described. In the present study, the cytometric analysis showed that LMWHA or HMWHA were able to increase TLR2 expression in classical monocytes, with or without Pg stimulation, and reduce TLR4 expression in this monocyte subpopulation, without Pg stimulation.

TLRs are involved in the activation of innate and adaptive immune responses and also play roles in tumor progression and inflammation (Gallo et al., 2019). The smaller fragments of HA can bind to the TLR2 and TLR4 receptors of monocytes, dendritic cells and lymphocytes, thus provoking a pro-inflammatory response (Fakhari & Berkland, 2013). Although the direct physical interaction between HA-TLR was not experimentally demonstrated, it is likely that the polyanionic nature of HA mimics the ligands of TLR2 and TLR4, such as lipopolysaccharide (Fakhari & Berkland, 2013). However, the mechanisms by which HA binding affects TLR expression are not known.

We also evaluated the effects of HA on different subpopulations of monocytes and observed that besides the addition of HMWHA or LMWHA did not significantly affect the frequency of subpopulations, HMWHA and LMWHA have different effects on subpopulations of classical and non-classical/intermediate monocytes.

Monocytes are important circulating cells in immunoinflammatory response, being recruited

for bacterial infection sites and participating in combat processes to microorganisms and tissue repair induction (Geissmann et al., 2003). There are different subpopulations of monocytes according to the expression of CD14 and CD16 surface markers: classical monocytes (CD14+CD16-), non-classical (CD14<sup>low</sup>CD16+) and intermediate (CD14<sup>high</sup>CD16+) (Ziegler-Heitbrock, 2014). The classical monocytes are present in larger amounts in human peripheral blood, accounting for about 80 to 95% of total monocytes (Ziegler-Heitbrock, 2014). Here, we have compared classical to non-classical/intermediate monocytes grouped together. This is because the low numbers of isolated intermediate and non-classical monocytes could not allow appropriate functional testing of these subsets individually.

The common role of monocytes in the periodontium is defense against the invasion of pathogens and the maintenance of local homeostasis and tissue integrity. These processes involve cell signaling receptors by microbial products, co-stimulatory molecules and cytokines, which stimulate and modulate the cellular responses to microbial infection (Geissmann et al., 2003). Toll-like receptors (TLRs) are critical components in the innate immune response based on their ability to recognize pathogen associated molecular patterns (PAMPs). The activation of signaling pathways by TLRs represents one of the body's earliest signals that it has been invaded by a foreign microorganism (Teng, 2006). In periodontal disease TLR2 and TLR4 are implicated in the response to *P. gingivalis*, although the contribution of each of these receptors during infection in vivo is uncertain (Burns et al., 2006; Wang & Ohura, 2002; Zenobia & Hajishengallis, 2015). Here we observed that Pg was able to decrease the TLR2 signaling, in line with previous studies that demonstrated inhibition of TLR mediated immune responses by microbial pathogens, often either blocking TLR signals or by downregulating TLR expression levels (Netea et al., 2004). Previous studies also demonstrated that Gram-negative LPS or the whole bacteria can work with infiltrating leukocytes and available cytokines in the microenvironment to block the differentiation of tissue-borne inflammatory monocytes into active Dendritic Cells locally, thereby lowering or inhibiting the development of subsequent adaptive immune responses in vivo (Rotta et al., 2003). This finding suggests that bacteria-mediated signals, via TLRs, can act as negative

regulators of the host's innate and adaptive immunity. Oral pathogens such as *P. gingivalis* have coevolved with the host and, therefore, may possess similar TLR-dependent evasion mechanisms (Teng, 2006). However, the mechanisms by which *P. gingivalis* overcome regulatory barriers are not completely understood.

Our results demonstrated that this downregulation of TLR2 signal by Pg was partially restored with the addition of LMWHA and HMWHA in classical monocytes, while in non-classical/intermediate monocytes only the HMWHA was able to induce an increase in the TLR2 expression. The stimulation of this receptor expression by LMWHA and HMWHA in monocytes might contribute to more efficient fighting microbial colonization in periodontal tissues.

In addition, here we demonstrated that the subset of non-classical/intermediate monocytes shown to be more sensitive to the action of HA in the expression of antigen-presenting and co-stimulatory molecules. The HMWHA-treated group showed higher frequencies of non-classical/intermediate monocytes expressing CD40 and HLA-DR than the LMWHA-treated group. These molecules are directly involved in the activation of cellular acquired immune response. HLA-DR is an isotype of the class II major histocompatibility complex (MHC-II) whose increased expression is indicative of activation of professional antigen presenting cells (APCs) (Teng, 2006). The interaction between MHC-II-antigen to T cell receptor (TCR) is required for lymphocyte sensitization (Koneremann et al., 2012). In addition, CD40 activation is necessary for the immune system to decide between an efficient immune response or tolerance and dysfunction in CD40/CD40L function is reported in autoimmune diseases (Van Kooten & Banchereau, 2000). Some studies show the clinical importance of CD16+ non-classical/intermediate monocytes, which are more activated in inflammatory and infectious processes (Tallone et al., 2011). Although these comprise a minor subpopulation of monocytes, the non-classical/intermediate are possibly more easily recruited into inflammatory sites by increased expression of chemokine receptors (Zimmermann et al., 2011), contributing to the formation of the inflammatory infiltrate.

All these data suggest that subpopulations of monocytes are differently susceptible to HA of

different molecular weights, and probably these molecules interact with receptors on the cell surface, generating signals for the stimulation of the expression of TLR2, CD40 and HLA-DR. Although the literature shows immunosuppressive and anti-inflammatory effects of high molecular weight HA (Wang & Ohura, 2002; Dicker et al., 2014), in the present study, this molecule was able to modulate the expression of receptors involved in the activation of T lymphocytes and monocytes. Thus, it is possible that high molecular weight HA plays immunoinflammatory mechanisms, which can contribute to the microbicidal activity of macrophages and monocytes in the periodontal tissues.

This study has limitations inherent to its in vitro design, which does not fully replicate the complexity of periodontal tissues and their immune interactions. The functional consequences of the observed changes in receptor expression were not assessed, and direct binding of HA to specific receptors such as TLR2 remains to be demonstrated. Future research should explore these mechanisms in ex vivo or in vivo models, including the functional activity of monocyte subsets and their interaction with other immune and resident cells. Understanding these pathways may enhance the therapeutic potential of HA as an immunomodulatory agent in periodontal disease.

## 5. CONCLUSION

The data from this study suggest a role of HA, mainly of high molecular weight, to stimulate monocytes activated by *P. gingivalis*. Thus, the mechanism of HA as an adjunct in the treatment of periodontal disease, appears to directly involve modulation of surface molecules responsible for cell signaling and control of periodontal immunoinflammatory processes. The increased expression of TLR2, primarily induced by high molecular weight HA could keep the cells more responsive to *P. gingivalis*. Studies by functional and cytokine production and other inflammatory mediators by monocytes exposed to HA are essential to confirm this hypothesis. These findings provide additional support for the use of high molecular weight hyaluronic acid as a therapeutic adjunct in periodontal treatment. By modulating monocyte responsiveness and enhancing key surface receptors involved in pathogen recognition and immune activation, HA may contribute to improved resolution of inflammation and better clinical outcomes in patients with periodontal disease.

## CONSENT

As per international standards or university standards, Participants' written consent has been collected and preserved by the author(s).

## ETHICAL APPROVAL

This study was conducted in accordance with all the provisions of the local human subjects oversight committee guidelines and policies of Pontifical Catholic University of Minas Gerais. This study protocol was reviewed and approved by the Research Ethics Committee of the Pontifical Catholic University of Minas Gerais, approval number CAAE: 54227916.0.0000.5137.

## DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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