Altered phenotype and function of dendritic cells in individuals with chronic periodontitis

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A B S T R A C T
Objective: To investigate the effects of periodontal bacterial lysates on maturation and function of mature monocyte-derived dendritic cells (m-MDDCs) derived from individuals with chronic periodontitis (CP) or healthy periodontal tissue (HP).

Design: m-MDDCs derived from peripheral blood monocytes, cultured for 7 days in presence of interleukin (IL)-4 and granulocyte-macrophage colony stimulating factor (GM-CSF), were stimulated with lysates of Streptococcus sanguinis, Prevotella intermedia, Porphyromonas gingivalis, or Treponema denticola on day 4, and were then phenotyped. IL-10, IL-12 and IFN-gamma concentration in the supernatant of cultures were measured.

Results: Expression of HLA-DR was lower in bacterial-unstimulated mature m-MDDC from CP compared to HP (p = 0.04), while expression of CD1a and CD123 were higher in CP. The expression pattern of HLA-DR, CD11c, CD123, and CD1a did not change on bacterial stimulation, regardless of the bacteria. Stimulation with P. intermedia upregulated CD80 and CD86 in CP cells (p < 0.05). Production of IL-12p70 by bacterial-unstimulated m-MDDCs was 5.8-fold greater in CP compared to HP. Bacterial stimulation further increased IL-12p70 production while decreasing IL-10. Significantly more IFN-gamma was produced in co-cultures of CP m-MDDCs than with HP m-MDDCs when cells were stimulated with P. intermedia (p = 0.009).

Conclusions: Bacterial-unstimulated m-MDDC from CP exhibited a more immature phenotype but a cytokine profile biased towards proinflammatory response; this pattern was maintained/exacerbated after bacterial stimulation. P. intermedia upregulated co-stimulatory molecules and IFN-gamma expression in CP m-MDDC. These events might contribute to periodontitis pathogenesis.

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1. Introduction

Periodontitis is an infection-driven chronic inflammatory disease affecting the integrity of tooth-supporting tissues.1 Subgingival bacterial pathogens are essential for the initiation and progression of the disease, although it is the resulting host reaction that primarily mediates tissue damage.2 The periodontal host response contains both protective and destructive elements.3 The factors that drive the host anti-bacterial
response towards a destructive or protective response seem not to be understood completely.

Dendritic cells (DCs) are a class of specialized antigen-presenting cells that play an important role in the recruitment and activation of cells of the innate immune system, and deliver co-stimulatory signals to activate naïve T cells, thus triggering the initiation of the adaptive immune response. The cytokines secreted from DCs greatly affect the quality of the innate and adaptive immune responses. Depending on their differentiation and maturation state, DCs can tolerate T cells, or direct their differentiation towards protective or pathogenic immunity. Thus, the interactions between DCs and cells of the innate and adaptive immune system are important in the pathogenesis of many infectious diseases.

DCs are derived from precursor cells present in bone marrow and peripheral blood, mainly monocytes. Then migrate to oral tissues and live there as resident DCs, acting as sentinels in host defense; or differentiate in the sites of infection when they find an invading pathogen. In the immature state, DCs capture antigens efficiently, but as they mature, they undergo phenotypic changes that allow their migration towards lymphoid organs and their unique ability to prime T cells. It is known that bacterial LPS can stimulate the production of chemokines and cytokines, specially GM-CSF, that modulates DC movement and maturation. However, the effects of periodontal bacteria on DC differentiation, maturation and function/activation remain poorly understood. Few studies have been performed and their results are contradictory. Experiments by Jotwani et al. and Aronoen et al. showed that in vitro-generated MDDCs pulsed with Porphyromonas gingivalis underwent maturation (shown as an increase in CD83), regulation of co-stimulatory molecules (CD80, CD86), release of both pro-inflammatory (IL-1β, IL-12p70) and anti-inflammatory (IL-10) cytokines, and secreted immunomodulatory molecules, such as PGE2. In contrast, studies by Cohen et al. and Kanaya et al. suggested that P. gingivalis either inhibited maturation of DCs, which had increased CD1a expression (characteristic of immature DCs) or was only weakly immunostimulatory.

In the present study, we hypothesized that monocyte-derived dendritic cells (MDDCs) from individuals with periodontitis may be more easily directed towards a pro-inflammatory response than DCs from periodontally healthy subjects. We also hypothesized that pathogenic bacteria may influence the pro-inflammatory response to modulate MDDCs maturation. Thus, this study aimed to determine the effects of periodontal bacteria lysates on the phenotype and function of mature-MDDCs derived from individuals with chronic periodontitis or periodontal health.

2. Material and methods

2.1. Preparation of bacterial lysates

Prevotella intermedia (ATCC 49046) and P. gingivalis (ATCC 33277) were cultured for 14 days in blood agar (Vetec, Duque de Caxias, Rio de Janeiro, RJ, Brazil) supplemented with hemin (5 μg/mL), menadione (0.5 μg/mL), and whole sheep blood (5%, vol/vol) at 37°C in an anaerobic jar (Permution, Curitiba, PR, Brazil) by using an anaerobic atmosphere-generating system (Anaerogen, Oxoid, Unipath Inc., Nepean, Ontario, Canada). Treponema denticola (ATCC 35405) was cultured on tryptic soy agar (Biolife, Milano, Italy) at 37°C in an anaerobic jar. Streptococcus sanguinis (ATCC 49296) was cultured on BHl agar (Oxoid, Basingstoke, Hampshire, England) for 48 h at 37°C.

Bacterial lysates were prepared after the growth phase. Bacteria were harvested by centrifugation at 500 × g for 2 min, and the supernatant was discarded. The bacterial pellet was resuspended in sterile Milli-Q water and lysed by heating to 100°C for 10 min. The bacterial lysate was then aliquoted and stored at –20°C until use.

2.2. m-MDDC preparation

The study protocol was approved by the Institutional Ethics Committee, and written informed consent was obtained from each donor from a local blood bank (Hemocenter, State University of Campinas – São Paulo, Brazil). All subjects were white Brazilians, non-smokers in good general health and the periodontal diagnosis was based on the current classification of the American Academy of Periodontology. Age and sex were matched between the groups. Peripheral blood was obtained and standarduffy coats were prepared from eleven white Brazilian volunteer, showing untreated chronic periodontitis (N = 5) or healthy periodontal tissue (N = 6) as follows: Whole blood (WB) was collected in quadruple-pack collection systems with a citrate-based anticoagulant and saline-adenine-glucose-mannitol (SAGM) additive solution for the RBCs. WB was stored at room temperature under the same conditions as whole blood. The blood was allowed to stand infected donor

2.3. Flow cytometry

Bacterial lysate-pulsed m-MDDCs and control m-MDDCs were harvested on day 7 of culture for analysis of surface marker expression. Cells were triple or quadruple-stained with APC, PE, FITC, PE-Cy-5, or PE-Cy-7-conjugated monoclonal antibodies specific for CD80, CD83, CD11c, HLA-DR, CD86, CD14, CD123, and CD1a (BD Biosciences, San Jose, CA, USA). Cells (10⁶/sample) were incubated with antibodies for 20 min at 4°C.
A minimum of 1 × 10⁴ events for each sample were acquired with a FACS Calibur flow cytometer using CellQuest software (BD Biosciences). Cells were gated for size (forward scatter, FSC) and granularity (side scatter, SSC) with dead cells and debris excluded. Cells with the phenotype HLA-DR⁺ CD11c⁺ CD14⁻/low were defined as m-MDDCs. The mean fluorescence intensity (MFI) and percentage of cells positive for each marker were calculated with FlowJo software (TreeStar, Ashland, OR, USA).

2.4. Quantitation of IL-12p70, IL-10, and IFN-gamma

m-MDDCs were harvested on day 4 and either left bacterial-untreated or incubated with 10 µg/ml bacterial lysate for 48 h. Culture supernatants were harvested and IL-12p70 and IL-10 were measured by ELISA using commercially available kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Similarly, IFN-gamma and IL-10 were measured by ELISA in supernatants collected from allogeneic T cells (3 × 10⁶/well) cultured with either bacterial-untreated or bacterial lysate-pulsed m-MDDCs (10⁵/well) for 7 days in 96-well round-bottom tissue culture plates.

3. Statistical analysis

The variables showed a normal distribution (p > 0.05 for Levene test). Therefore, Student’s paired or unpaired t-tests were used to evaluate the effect of the bacterial preparations on the expression of m-MDDC surface molecules and cytokine production. Paired tests were used to determine differences within each group or and unpaired between groups (healthy and periodontitis). The level for significance was set at p ≤ 0.05. Data analysis was performed using a statistical software program (SPSS, SPSS Inc., Chicago, USA).

4. Results

Chronic periodontitis volunteers had a minimum of six teeth with 3 or more sites with probing depth (PD) and clinical attachment level (CAL) ≥5 mm. The volunteers with periodontal health had <20% of sites exhibiting gingival bleeding and/or bleeding on probing (BOP), and did not have any site with PD or CAL measurements >3 mm or history of tooth loss due periodontitis.

4.1. m-MDDC from CP and HP subjects mature differently

m-MDDCs were analyzed after 7 days of culture. As shown in Fig. 1, bacterial-untreated cultures from individuals with CP contained a lower percentage of cells expressing HLA-DR⁺ and CD11c⁺ than did cultures from HP individuals (p = 0.04 and 0.21, respectively, for HLA-DR⁺ and CD11c⁺; Student’s unpaired t-test). If In contrast, there was a non-significant increase in the percentage of immature cells (CD1a +) in CP cultures (p = 0.41; Student’s unpaired t-test), but a significant increase in the surface expression of the same molecule in samples of CP subjects (measured by median fluorescence intensity, MFI; p = 0.02; Student’s unpaired t-test). There was also a tendency for the percentage of plasmacytoid dendritic cells (pDCs, CD123+) to be higher in samples from CP individuals (p = 0.29; Student’s unpaired t-test), and again, with a significantly higher surface expression compared to healthy subjects (p = 0.02; Student’s unpaired t-test).

4.2. m-MDDC from periodontitis and healthy subjects respond differently to bacterial lysates

The expression of HLA-DR, CD11c, CD123, and CD1a, on m-MDDC was regulated in a similar manner by all four bacteria (Fig. 2). Indeed, bacterial stimulation did not change the pattern of differences from that observed in bacterial-untreated cells from HP and CP subjects. The percentage of m-MDDCs (HLA-DR⁺ and CD11c⁺) after bacterial stimulation was lower in cultures from CP subjects compared to healthy subjects (HLA-DR: p = 0.02 for S. sanguinis; CD11c: p ≤ 0.04, for all bacteria; Student’s unpaired t-test). Although not statistically significant, there was a trend to a lower surface expression of HLA-DR and CD11c in cells from CP than HP subjects (Data not shown). In contrast, the percentage of m-MDDCs CD1a⁺ and CD123⁺ was higher in cells from CP individuals stimulated by P. intermedia and P. gingivalis (Fig. 2).

4.3. CD80 and CD86 are distinctly regulated by different bacteria

In bacterial-untreated cultures, CD80 and CD86 expression did not differ between m-MDDC from healthy and periodontitis subjects (p > 0.05; Student’s unpaired t-test). However, stimulation with P. intermedia increased both the percentage of CD80⁺ cells and the MFI of CD80 in cells from CP subjects compared to that of HP (p < 0.008; Student’s unpaired t-test). A similar trend was observed for CD86 (Fig. 2). P. intermedia was the only bacterial lysate to increase CD80 and CD86 surface expression in m-MDDC from CP subjects, while the other bacteria actually downregulated CD80 (p ≤ 0.05; Student’s paired t-test) (Fig. 3).

4.4. IL-10/IL-12 ratio is greater in m-MDDCs supernatants from healthy individuals

In bacterial-untreated cultures, IL-12p70 levels were 5.8-fold higher in the supernatants of m-MDDCs from CP compared to HP, while there was no difference in IL-10 levels (Fig. 4). Bacterial stimulation showed a tendency to down-regulate IL-10 and upregulate IL-12p70 levels in CP compared to HP (p < 0.05 for P. intermedia; Student’s unpaired t-test) (Fig. 4), and to increase the levels of both cytokines in HP compared to bacterial-untreated cells. This tendency was not observed in supernatants of m-MDDCs from CP except for P. intermedia, which showed a tendency to upregulate IL-10 and IL-12p70 levels (Fig. 4). In addition, in cultures from both HP and CP, P. intermedia tended to stimulate more secretion of IL-10 and IL-12 than did the other bacteria (Fig. 5).

The ratio of IL-10 to IL-12 produced by bacterial-untreated and stimulated m-MDDC was on average 3-fold greater for HP compared to CP subjects (Fig. 4: bacterial-untreated 5.5-fold; S. sanguinis 2-fold; P. intermedia 2.6-fold; P. gingivalis 1.6-fold; and T. denticola 2.6-fold).
When co-cultured with T cells, there was no significant difference in IL-10 levels produced by control or stimulated m-MDDCs from either HP or CP subjects. However, significantly more IFN-gamma was produced by m-MDDCs of CP compared to HP subjects after S. sanguinis and P. intermedia stimulation ($p = 0.006$ and $0.009$, respectively; Student’s unpaired t-test) (Fig. 4), with significantly more IFN-gamma produced in response to P. intermedia stimulation than to S. sanguinis (Fig. 4).

**Fig. 1 - (B) Histograms show lower expression of HLA-DR and CD11c in CP cultures (black histograms) compared to HP (grey histograms) and higher CD1a and HLA-DR expression in CP cultures as compared to HP (A).** Mean percentage (±SD) of m-MDDC expressing HLA-DR, CD11c, CD1a, and CD123, and the median fluorescence intensity (MFI) for CD1a and CD123, in cells from HP and CP subjects. * indicates $p \leq 0.05$ significance between HP and CP subjects.
Fig. 2 – Mean percentage (SD) of m-MDDC expressing HLA-DR, CD11c, CD1a, CD123, CD80 and CD86 and the MFI for CD80 and CD86. * indicates $p \leq 0.05$ significance between HP and CP subjects. ] indicates statistically significant differences between control (bacterial-unstimulated) and stimulated cells ($p \leq 0.05$).
5. Discussion

Maturation of MDDCs is accompanied by decreased CD1a and increased cell surface expression of MHC class II (HLA-DR), and co-stimulatory molecules such as CD80 and CD86, which enable antigen presentation and activation of naïve CD4+ and CD8+ T cells, thus promoting the adaptive immune response.7,8 We found that expression of HLA-DR and CD11c were lower in m-MDDCs from CP than HP individuals. In contrast, CD1a and CD123 expression were higher in m-MDDCs than in individuals with periodontitis. These results suggest that differentiation and subsequent maturation of bacterial-unstimulated or bacterially stimulated DCs may be defective in CP individuals, and thus may have their differentiation driven towards pDCs. pDCs express low levels of HLA-DR and co-stimulatory molecules (in agreement with our results) and high level of CD123 molecule and are unable to stimulate antigen-specific T cell proliferation.9 The role of pDCs in periodontitis has not been described. Because CD4+ T helper cells must interact with mature DCs to acquire effector function,17 the lower MDDC maturation and skewing towards pDC differentiation in periodontitis may impair antigen presentation and stimulation of an anti-bacterial response in periodontal tissue. This possibility is supported by our findings with P. intermedia. P. intermedia is the predominant bacteria early in the biofilm, with P. gingivalis and T. denticola becoming more dominant later. Thus, defective DC maturation may already occur in individuals with CP before the colonization of the biofilm by more virulent bacteria. To date, studies of periodontal bacteria effects on DC maturation have yielded contrasting results; there have been reports of both upregulation and downregulation of MDDCs by the bacterium P. gingivalis.9-12,17 These conflicting results may be due in part to the use of different microbial components or to differences in the immunological profiles of the hosts in these studies. In fact, expression of mfa-1 and fimA fimbriae on P. gingivalis negatively and positively, respectively, mediates MDDC maturation.19

Furthermore, strain-specific immune response was induced by three P. gingivalis strains, A7A1-28, W83 and W50. Strains W50 and W83 were shown to induce alveolar bone loss and expression of high levels of interleukin-4 (IL-4), whereas the A7A1-28 strain did not significantly promote bone resorption in mice and stimulated increased IL-10.20 We found that expression of co-stimulatory molecules on m-MDDC from CP and patients was differentially regulated by the bacteria. P. intermedia upregulated CD80 and CD86 only on m-MDDC from CP subjects, while the remaining 3 bacteria downregulated CD80 and CD86. This result suggests that the P. intermedia lysate may be more immunogenic and then induce stronger immune response and more periodontal destruction or the bacteria may be more efficiently eliminated by the response in CP individuals. P. gingivalis has been considered more virulent than P. intermedia,21 which is supported by the differential regulation of CD80 and CD86 by P. intermedia and P. gingivalis. In CP individuals, downregulation of either CD80 or CD86 by S. sanguinis, T. denticola, and P. gingivalis may be part of an immune evasion strategy or, in the case of S. sanguinis, may induce tolerance. Upregulation of co-stimulatory molecule expression on B cells has been described in periodontal disease.22

On average, we found that the ratio of bacterial lysate-induced production of IL-10 to IL-12 was 3-fold greater in the supernatants of m-MDDCs from HP compared to CP subjects. IL-10 exerts an anti-inflammatory effect by reducing the production of inflammatory cytokines (including IL-12) and controlling periodontal bone loss.23 Thus, the tendency of the periodontal bacteria to downregulate IL-10 and upregulate IL-12p70 levels may contribute to poor control of inflammation and increased periodontal tissue destruction in CP subjects. Although the higher expression of IL-12 but lower maturation of DCs in CP patients might seem somewhat contradictory, we suggest that it is not. We speculate that MDDCs from periodontitis individuals may show a more activated basal state, which help to explain why some subjects are more prone to develop periodontitis.
IL-12p70 plays a key role in bacterial clearance through the induction of IFN-gamma, which in turn activates the bactericidal function of macrophages.\textsuperscript{25} We found that \textit{P. intermedia} induced more IL-12p70 and IFN-gamma production by m-MDDC of CP subjects than did other bacteria, and thus \textit{P. intermedia} may be more efficiently eliminated from the host. \textit{S. sanguinis} also stimulated more IFN-gamma production by CP than HP m-MDDC co-cultured with \textit{T} cells. The higher IFN-gamma response in CP subjects compared to HP may mediate a stronger and more destructive inflammatory response. However, the latter explanation may be unlikely as Jotwani et al. found that IFN-gamma levels are not increased significantly in chronic periodontitis patients.\textsuperscript{10}

In conclusion, we show here that m-MDDC from periodontitis subjects differed from those of healthy subjects by exhibiting a more immature phenotype and cytokine profile biased towards a pro-inflammatory response, without increasing IL-10 production. These results clearly indicate a dysregulated immune response in these subjects. This pattern was maintained/exacerbated when cells were stimulated by \textit{P. intermedia}, \textit{P. gingivalis}, \textit{T. denticola}, and \textit{S. sanguinis}. \textit{P. intermedia} significantly upregulated co-stimulatory molecules and IFN-gamma expression in CP m-MDDC. Further studies are required to investigate how such differences between healthy and periodontitis subjects affect the pathogenesis of the periodontal disease.
Fig. 5 – Mean production (±SD) of IL-10 and IL-12p70 by bacterium-stimulated m-MDCs from CP subjects. IFN-gamma production by bacterium-stimulated m-MDCs from CP subjects, co-cultured with T cells.] indicates statistically significant differences between bacteria (p ≤ 0.05).

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Conflict of interest

None declared.

Ethical approval

Ethical Approval was given by the Institutional Ethics Committee (number 05266).

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References


