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## Periodontal ligament and gingival fibroblasts participate in the production of TGF- $\beta$ , interleukin (IL)-8 and IL-10

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**Abstract:** The aim of this study was to quantify and compare the production of transforming growth factor beta (TGF- $\beta$ ), interleukin (IL)-8 and IL-10 by human cultured periodontal ligament and gingival fibroblasts both obtained from the same donors challenged with lipopolysaccharide (LPS) from *Porphyromonas gingivalis*. Fibroblasts were exposed to 0.1-10  $\mu\text{g/mL}$  of LPS from *P. gingivalis* and after 24 h the supernatants were collected and analyzed by enzyme-linked immunosorbent assay (ELISA). TGF- $\beta$  protein production was upregulated in a concentration-dependent manner, mainly in gingival fibroblasts, which was statistically significant when challenged by 10  $\mu\text{g/mL}$  LPS. Additionally, at this concentration, gingival fibroblasts had almost a two-fold increase in the amount of TGF- $\beta$  when compared to periodontal ligament fibroblasts. Both periodontal ligament and gingival fibroblasts showed an increase in IL-8 production when challenged with 1  $\mu\text{g/mL}$  and 10  $\mu\text{g/mL}$  LPS. IL-10 production remained unaffected when challenged by any of the LPS concentrations tested in either periodontal ligament or gingival fibroblasts. Our results demonstrate that periodontal ligament and gingival fibroblasts when challenged by LPS from *P. gingivalis* with 24 h may play a critical role in producing TGF- $\beta$  and IL-8 but not IL-10.

**Descriptors:** Periodontitis; Fibroblasts; Cytokines; *Porphyromonas gingivalis*.

### Introduction

Periodontitis is a complex chronic infectious disease that leads to the destruction of the tooth-supporting tissues, including alveolar bone, and that leads to tooth loss.<sup>1</sup> The presence of periodontopathogens, such as *Porphyromonas gingivalis*, is considered the major etiologic agent in periodontitis.<sup>2</sup> *P. gingivalis* is a Gram-negative anaerobic bacterium that possesses a number of virulence factors, including lipopolysaccharides (LPS).<sup>3</sup> LPS acts as a potent stimulus to a variety of host cells, which subsequently results in the expression of inflammatory cytokines leading to the development and progression of the related host immune response in periodontal diseases.<sup>4</sup>

Numerous studies have demonstrated that cytokine networks play an important role in the initiation and progression of periodontal disease.<sup>5</sup> Interleukin (IL)-8 is a chemokine produced by a variety of tissues, and it is a potent inducer of neutrophil chemotaxis and activation.<sup>6</sup> The stimu-

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lation of human gingival fibroblasts with highly purified *P. gingivalis* LPS led to mRNA expression of IL-8.<sup>7</sup> In contrast, IL-10, an anti-inflammatory cytokine, plays a major role in suppressing immune and inflammatory responses.<sup>8</sup> Indeed, IL-10 presents a protective role toward tissue destruction, inhibiting both matrix metalloproteinases (MMPs) and receptor activator for nuclear factor- $\kappa$ B (RANK) systems, which leads to the differentiation and activation of osteoclasts.<sup>9</sup> In inflamed gingival tissues, IL-10 potentiates local autoimmune responses characterized by increasing the number of anti-collagen secreting cells.<sup>10</sup>

Interestingly, transforming growth factor beta (TGF- $\beta$ ) is a highly conserved and ubiquitously expressed cytokine, and most cells express members of the TGF- $\beta$  receptor family.<sup>11</sup> During the progression of periodontal inflammation, periodontal ligament and gingival fibroblasts secrete high levels of cytokines and chemokines.<sup>12</sup> In one previous study, it was demonstrated that human gingival and periodontal ligament fibroblasts distinctly responded to a time-course *P. gingivalis* LPS challenge with the production of macrophage inflammatory protein-1 $\alpha$ , stromal derived factor-1 and IL-6.<sup>13</sup> The present study further investigates the interrelationship between both subtypes of fibroblasts collected from the same donors in the production of TGF- $\beta$ , IL-8 and IL-10 when challenged with LPS.

## Materials and Methods

### Cell culture

Fibroblasts were obtained as previously described.<sup>13</sup> Briefly, human periodontal ligament fibroblasts were extracted from three healthy donors. Donors had given written informed consent, and the study was approved by the Ethics Committee of the Bauru School of Dentistry, University of São Paulo. Concurrently, gingival biopsies from the same donors were obtained. Tissue fragments of periodontal ligament and gingiva were individually plated in 100 X 10 mm Petri dishes and covered with 5 mL of Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil) and antibiotics (600  $\mu$ L

mL penicillin, 300  $\mu$ L/mL gentamicin sulfate, 100  $\mu$ L/mL amphotericin B; Invitrogen Life Technologies, Carlsbad, CA, USA). After minced, the explants were placed in 25-cm<sup>2</sup> flasks and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Medium change (DMEM 10% FBS) was performed every second day. Cells were used at the fourth passage.

### LPS challenge

Fibroblasts were seeded at a density of 5 x 10<sup>4</sup> cells/well and incubated in the absence (controls) or presence of 0.1, 1 or 10  $\mu$ g/mL of LPS from *P. gingivalis* (InvivoGen, San Diego, CA, USA) in duplicate as previously described.<sup>14</sup> After 24 h, the supernatant was collected and analyzed by enzyme-linked immunosorbent assay (ELISA).

### ELISA

ELISAs, to determine the protein levels of TGF- $\beta$ , IL-8 and IL-10, were performed using the Ready-Set-Go! ELISA kits (eBioscience, San Diego, CA, USA), according to the manufacturer's protocol. Dilutions were prepared in dilution buffer. Briefly, 96-well plates were coated with anti-human TGF- $\beta$ , IL-8 or IL-10 monoclonal antibodies. After blocking with the assay solution for 2 h to avoid non-specific binding, 100  $\mu$ L of standard TGF- $\beta$ , IL-8 or IL-10 or culture supernatants were added. The cytokines were detected by horseradish peroxidase-labeled monoclonal antibody to each target after 100  $\mu$ L of anti-human biotinylated antibodies were placed in each well and incubated for 2 h at 21°C. The microplate was washed to remove unbound enzyme-labeled antibodies. The amount of horseradish peroxidase bound to each well was determined by the addition of substrate solution. The reaction was stopped by the addition of sulfuric acid and the plates were read at 450 nm (ELISA microplate reader, Bio-Rad, Hercules, CA, USA). The concentrations of each target were determined by interpolation from a standard curve and presented as  $\mu$ g/mL ( $\pm$  one standard error of the mean).

### Statistical analysis

Statistical analysis was performed with GraphPad Prism 5.0 software (GraphPad Prism 5.0,

GraphPad Software, San Diego, CA, USA). Data were compared using a one-way analysis of variance and differences were identified using the Tukey's test, with significance set at  $p < 0.05$ .

## Results

### TGF- $\beta$ is distinctly produced by human gingival and periodontal ligament fibroblasts

When challenged with *P.gingivalis* LPS at a concentration of 10  $\mu\text{g/mL}$ , gingival fibroblasts produced a significantly increased level of TGF- $\beta$  when compared to control (Figure 1). As shown in Figure 1, periodontal ligament fibroblasts treated with any concentration of LPS did not exhibit statistically significant differences when compared among any other LPS concentration or medium. At the highest LPS concentration tested, the TGF- $\beta$  amounts detected in gingival fibroblasts were almost two-fold ( $p < 0.05$ ) increased in comparison to the protein levels for periodontal ligament cells.

### IL-8 levels in gingival and periodontal ligament fibroblasts were significant above 1.0 $\mu\text{g/mL}$

There was no difference in IL-8 production at the lowest concentration of LPS tested (0.1  $\mu\text{g/mL}$ ) (Figure 2). However, when 1  $\mu\text{g/mL}$  of *P. gingivalis* LPS was used, the amount of IL-8 increased signifi-

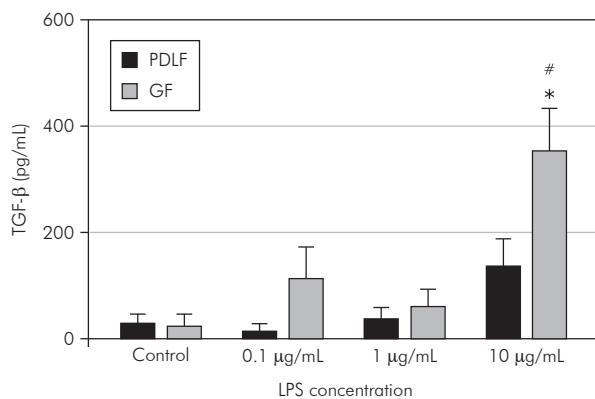
cantly ( $p < 0.05$ ) (Figure 2). Differences between the two subtypes of fibroblasts at any particular concentration were statistically undetectable regarding IL-8 production (Figure 2).

### IL-10 production is unaffected by LPS concentration in either fibroblast subtypes

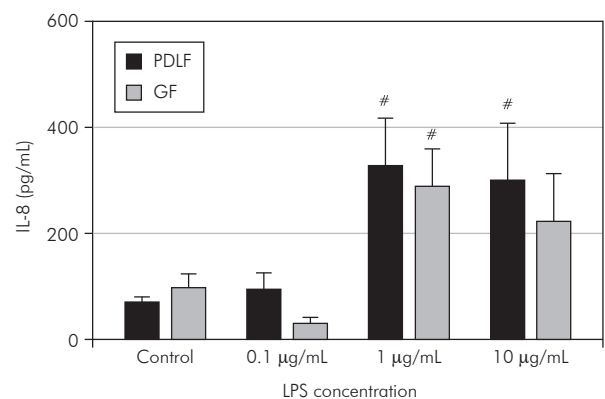
In all concentrations of *P. gingivalis* LPS tested (0  $\mu\text{g/mL}$  to 10  $\mu\text{g/mL}$ ), periodontal ligament and gingival fibroblasts showed no changes in the amount of IL-10 when compared to control (Figure 3). Additionally, there were no differences observed between them at each of the LPS concentrations tested in this study (Figure 3).

## Discussion

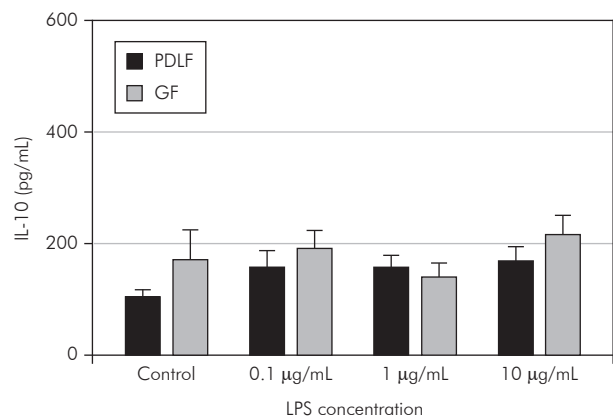
This study investigated the *in vitro* responses of periodontal ligament and gingival fibroblasts provided by the same donors in response to a well-known virulence factor from *P. gingivalis*, LPS, by quantifying important cytokines represented by TGF- $\beta$ , IL-8 and IL-10. These cytokines along with others play a vital role in inflammatory disease such as periodontitis.<sup>15</sup> An earlier work<sup>16</sup> showed that the magnitude of the responses of gingival and periodontal ligament fibroblasts to *P. gingivalis* varies markedly between individuals. Particularly, it is possible that heterogeneity in the responses is caused by the host genetic background of the cells,



**Figure 1** - TGF- $\beta$  production by human cultured periodontal ligament and gingival fibroblasts challenged with LPS from *P. gingivalis*. ELISA was performed for the quantification of the cytokines on the cell culture supernatants. # $p < 0.05$  between the same cell subtype. \* $p < 0.05$  between different subtypes of fibroblasts (gingival X periodontal ligament).



**Figure 2** - IL-8 production by human cultured periodontal ligament and gingival fibroblasts challenged with LPS from *P. gingivalis*. ELISA was performed for the quantification of the cytokines on the cell culture supernatants. # $p < 0.05$  in comparison with the other concentrations of LPS in the same subtype.



**Figure 3** - IL-10 production by human cultured periodontal ligament and gingival fibroblasts challenged with LPS from *P. gingivalis*. ELISA was performed for the quantification of the cytokines on the cell culture supernatant.

and thus periodontal ligament fibroblasts and gingival fibroblasts were collected and compared from the same donors.

Previously, other investigators have shown that viable *P. gingivalis* induces cytokine responses in monocytes and macrophages that are significantly different from those induced by purified LPS or by the major fimbrial protein FimA.<sup>17</sup> For example, viable *P. gingivalis* induced increased IL-6 and IL-8 gene-expression when compared to dead *P. gingivalis* in periodontal fibroblasts.<sup>16</sup> The amount of TGF- $\beta$  production was the only mediator that significantly differed between periodontal ligament and gingival fibroblasts, and this difference was observed at the highest concentration of *P. gingivalis* LPS used. This result may indicate a more activated state for gingival fibroblasts over periodontal ligament cells, given that gingival fibroblasts are more likely to encounter oral pathogens. TGF- $\beta$  is credited for promoting wound fibroblasts to differentiation at sites of inflammation and repair.<sup>18</sup> The unique pattern of production for cytokines by gingival fibroblasts observed could be due to its more pronounced repair characteristic. In a previous study, we demonstrated this distinct ability of cytokine production in these two subtypes of fibroblasts under the same challenge.<sup>13</sup> Since these fibroblasts have different functional characteristics, it may be reasonable to assume that their role in the inflammatory process in periodontitis may also differ. An up-regulation

in IL-6 levels was previously shown,<sup>13</sup> which had a strong positive correlation with the levels of TGF- $\beta$  reported in this work.

Taking this into account, studies have shown that the presence of IL-6, in addition to TGF- $\beta$ , skews the commitment towards a population of CD4<sup>+</sup> T helper cells discrete from Th1 and Th2, known as T helper type 17 cells (Th17).<sup>19,20</sup> Th17 cells are considered an independent T helper cell lineage in human and mouse which can potentially induce tissue inflammation and has been associated with the pathogenesis of many experimental autoimmune diseases and human inflammatory conditions. Moreover, it was found that TGF- $\beta$  together with IL-6 induces massive amounts of IL-17 from naïve T cells and consequently these two cytokines together can differentiate Th 17 lineage. Interestingly, two cytokines with opposing effects have to cooperate to induce the differentiation of Th17 cells.<sup>21</sup> Also, the presence of Th17 cells in the chronic inflammation in human periodontal disease was demonstrated as well as IL-17 expression in the alveolar bone of patients with chronic periodontitis.<sup>22</sup> Studies linking apoptotic cell recognition and clearance report an induction of TGF- $\beta$  secretion, resulting in accelerated resolution of inflammation.<sup>23</sup> In an independent study,<sup>18</sup> TGF- $\beta$  was found to be produced by LPS-treated human gingival fibroblasts and this level of TGF- $\beta$  was unable to decrease IL-8 production, corroborating our findings.

In this study, 1 µg/mL of *P. gingivalis* LPS caused the levels of IL-8 production to increase significantly ( $p < 0.05$ ) for both gingival and periodontal ligament fibroblasts. Even though the concentration of *P. gingivalis* LPS was 10 times greater, IL-8 release was maintained in both fibroblast subtypes. The fact that at least 1 µg/mL of *P. gingivalis* LPS caused IL-8 to significantly increase compared to control ( $p < 0.05$ ) is curious. One could speculate that this may be at or close to the minimum concentration of LPS able to induce these cells to some pro-inflammatory reaction. It is still important to emphasize that IL-8 is an extensively studied chemokine related to neutrophil recruitment,<sup>24</sup> and it is essential in the first line of defense.

It has been reported that IL-10 mediates the

control of periodontal disease progression.<sup>10</sup> Controversy still exists about IL-10's expression profile in periodontal health and disease. It has been shown that IL-10 levels in gingival crevicular fluid were only detected in samples from periodontal patients,<sup>25</sup> and that T-cell lines derived from adult periodontitis subjects but not those from gingivitis subjects could produce IL-10.<sup>26</sup> In contrast, IL-10 expression in periodontitis lesions was less than that in healthy gingival tissue.<sup>27</sup> In the current study, curiously, no differences were found in IL-10 levels between fibroblasts with or without a *P. gingivalis* LPS challenge. In essence, this could be a consequence of an *in vitro* experimental model, which in some way might restrict the results, without considering the micro-environmental factors usually involved *in vivo*. Recent data has shown that in the early phases of inflammation, IL-10 expression was coordinated with other genes expression that had potent pro-inflammatory and chemoattractant properties. Only during later stages, the function of IL-10 did converge into a congruent attempt to limit the tissue damage by dampening the inflammatory process.<sup>28</sup> This finding may indirectly explain the diversity of the IL-10 expression pattern observed in periodontal health and disease. A recent study used gingival fibroblasts treated with *P. gingivalis* LPS for 48 h and reported increased expression of IL-10 when

compared to the untreated control.<sup>29</sup> It is possible that the 48 h time period between the *P. gingivalis* LPS treatment start until the detection of IL-10 levels in the previous study may have allowed gingival fibroblasts to more fully mature than 24 h. Our results demonstrated that human cultured periodontal ligament and gingival fibroblasts significantly increased levels of IL-8 when challenged with *P. gingivalis* LPS and that gingival fibroblasts significantly increased levels of TGF- $\beta$ . Additionally, periodontal fibroblasts produced active TGF- $\beta$ , but not at enough quantities to decrease IL-8 production. On the other hand, the strong positive correlation between IL-6 and TGF- $\beta$  calls one's attention to the possible association of gingival fibroblasts with the maintenance of a Th17 profile at the periodontal inflammatory microenvironment.

## Conclusions

Taken together, these findings suggest that human periodontal ligament and gingival fibroblasts may act as immune-responsive cells, but in a differential manner. Regarding IL-10, no differences were detected in any of the LPS concentrations. Both subtypes of fibroblasts can increase IL-8 levels but only gingival fibroblasts significantly increase TGF- $\beta$  levels when challenged with *P. gingivalis* LPS for 24 h.

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