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Recognition of *Candida albicans* by gingival fibroblasts: The role of TLR2, TLR4/CD14, and MyD88

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ABSTRACT

Recent evidence indicates that nonprofessional immune cells such as epithelial cells, endothelial cells, and fibroblasts also contribute to innate immunity via secretion of cytokines. Fibroblasts are the principal type of cell found in the periodontal connective tissues and they are involved in the immune response during periodontal disease. The role of fibroblasts in the recognition of pathogens via Toll-like receptors (TLRs) has been established; however, few studies have been conducted concerning the involvement of innate immune receptors in the recognition of *Candida albicans* by gingival fibroblast. In the current study, we investigate the functional activity of TLR2, cluster of differentiation 14 (CD14), and myeloid differentiation primary response gene 88 (MyD88) molecules in the recognition of *C. albicans* by gingival fibroblast. First, we identified that gingival fibroblasts expressed TLR2, TLR3, and TLR4. Our results showed that TLR agonists had no effect on these receptors' expression by TLR2, MyD88, and CD14-deficient cells. Notably, *C. albicans* and a synthetic triacylated lipoprotein (Pam3CSK4) induced a remarkable increase of TLR3 expression on MyD88-deficient gingival fibroblasts. TLR4 expression levels were lower than TLR2 and TLR3 levels and remained unchanged after TLR agonist stimulation. Gingival fibroblasts presented morphological similarities; however, TLR2 deficiency on these cells leads to a lower proliferative response, whereas the deficiency on CD14 expression resulted in lower levels of type I collagen by these cells. In addition, the recognition of *C. albicans* by gingival fibroblasts had an effect on the secretion of cytokines and it was dependent on a specific recognition molecule. Specifically, tumor necrosis factor-α (TNF-α) production after the recognition of *C. albicans* was dependent on MyD88, CD14, and TLR2 molecules, whereas the production of interleukin-1β (IL-1β) and IL-13 was dependent on TLR2. These findings are the first to describe a role of gingival fibroblast in the recognition of *C. albicans* and the pathways involved in this process. An understanding of these pathways may lead to alternative treatments for patients with periodontal disease.

1. Introduction

Fibroblasts are the major mesenchymal cell type in connective tissue and deposit the collagen and elastic fibers of the extracellular matrix [1]. Fibroblasts exhibit considerable functional diversity [2]. Evidence suggests that fibroblasts are not a homogeneous population even within a single tissue, but there are different subsets of cells such as tissue macrophages and dendritic cells [3]. Recent evidence indicates that nonprofessional immune cells such as fibroblasts, epithelial cells, and endothelial cells also contribute to innate immunity via Toll-like receptor (TLR) activation and production of cytokines [4].

The immune system uses a variety of innate immune receptors to sense infectious agents. These receptors can be expressed on the cell surface or on intracellular compartments, or be segregated to blood and tissue fluids [5]. The best known innate immune receptors involved in the recognition of pathogens are pattern recognition receptors (PRRs) [6]. Among the PRRs, TLRs are the most characterized family [7]; however, other PRRs (e.g., integrins, C-type lectin receptors, nucleotide-binding oligomerization domain-like receptors, and inflammasomes) are equally important pathogens sensors [6]. Similar to immune

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cells, studies have suggested that fibroblasts might utilize TLRs for sense pathogens, resulting in the production of many mediators contributing to the control of the infection [8,9]. Gingival fibroblasts constitutively express TLR2, TLR3, and TLR4 [10], and also the adhesion molecules ICAM-1 (CD54) and CD44 [11,12]. Activation of gingival fibroblast with microbial products induced the production of proinflammatory cytokines [13,14]. These findings indicate that these sensors molecules in gingival fibroblasts are important to recognize different microorganisms and regulate the immune response via specific cytokine production.

Fibroblasts are the principal type of cell found in the periodontal connective tissues presenting a unique phenotype according to their origin, being not only involved in tissue healing, but also with immune response [15]. Periodontal tissues are frequently injured by bacterial species that exist in complexes in subgingival tissue [16]. These bacterial complexes are considered the main etiological factor of periodontal diseases. Recently, the relation of *Candida albicans* with periodontal disease has also been investigated [17], and the association of subgingival colonization of *C. albicans* with severity of chronic periodontitis was established [18]. In recent years, the role for TLRs to sense *C. albicans* and other fungal species had been investigated [18].

Thus, we hypothesized that these cells will have a particular response during the fungal infection, resulting in a modulation of the cytokines via the recognition of TLR2, CD14, and MyD88 molecules.

2. Material and methods

2.1. Mice

C57BL/6 mice [wild-type (WT)], TLR2 (TLR2-KO), CD14 (CD14-KO), and MyD88-deficient (MyD88-KO) mice (6–8 weeks old) were obtained from Ribeirão Preto Medical School, University of São Paulo. Each mouse was housed in an isolated cage, and food and water were provided ad libitum. All experimental procedures involving animals in this study were reviewed and approved by the Animal Research Ethics Committee of the Bauru School of Dentistry, University of São Paulo (CEEPA-Proc. No 039/2009).

2.2. Candida albicans and growth conditions

C. albicans (ATCC 10231) was grown overnight to generate yeast cells in Sabouraud dextrose broth (Difco Becton, Dickinson and Company, Le Pont de Claix, France) at 29 °C. Cells were harvested by centrifugation, washed twice with PBS, and re-suspended in culture medium (RPMI 1640). Yeast cell morphology was confirmed using the Gram method and a stereomicroscope (MS 23358; Wild Heerbrugg, Romanshorn, Switzerland). Cell counts and concentrations were determined using a Neubauer chamber (Propper Manufacturing, Long Island City, NY, USA) as described [19]. *C. albicans* yeasts were heat-killed for 30 min at 95 °C.

2.3. Murine gingival fibroblast

Fibroblasts were isolated from gingival tissue, as described [20]. Gingival tissue was removed under aseptic conditions and minced with scissors in DMEM containing 10% fetal calf serum. Gingival tissue from a single animal was placed in 15 ml medium in tissue falcon 50 mL centrifuge tubes (Corning, New York, NY, USA). Cells were allowed to grow out of the minced tissue, and when cells reached 70% confluence, they were passaged following trypsinization. Fibroblasts was cultivated in 75 cm^2 flasks (Corning, New York, NY, USA) and maintained at 37 °C, under 5% CO^2, changing the culture medium every 2 days. Cells were grown for 14 days (3–4 passages) before being used.

2.4. Culture of gingival fibroblasts

Cells were obtained by explants and cultivated in DMEM (Gibco™) supplemented with 15% fetal calf serum (Gibco™), 100 UI/L penicillin (Gibco™), 100 µg/L streptomycin (Gibco™), and 250 ng/L amphotericin B (Gibco™), and they were used between the third and fourth passages for all the analysis in the current study. The cells were cultured in six-well plates (5×10^5 fibroblasts/well) or in eight-well Lab-Tek (Hatfield, PA, USA) plates (2×10^5 fibroblasts/well) for immunofluorescence staining. After overnight attachment, they were stimulated by live or heat killed *C. albicans* 5:1(E:T) and 10:1(E:T), *Escherichia coli* lipopolysaccharide (LPS; 100 ng/mL) (InvivoGen, San Diego, CA, USA) or with a synthetic ligand of TLR2, PAMP3CSK4 [1 µg/mL] (InvivoGen). After 24, 48, and 96 h, supernatants were collected and stored at −20 °C until used to measure cytokine levels and the cells analyzed by flow cytometry.

2.5. Flow cytometry analysis

For immunostaining, APC-, PerCP-, PE-, and FITC conjugated Abs anti-CD282 (TLR2), CD283 (TLR3), CD284 (TLR4), and isotype-matched control monoclonal antibodies were used (BD Biosciences). The intracellular detection of TLR3 in fibroblasts was performed using Cytofix/Cytoperm and Perm/Wash buffer from BD Biosciences according to the manufacturer's instructions. Fibroblasts were acquired according to forward and side scatter parameters. Data acquisition was performed using a FACS Callibur™ (BD Immunocytometry Systems, Franklin Lakes, NJ, USA) and the data were analyzed using CellQuest software (BD Biosciences).

2.6. Immunofluorescence

All immunofluorescence staining was performed in eight-well chamber slides (Lab-Tek), as described [21]. Fibroblasts were fixed using acetone at −20 °C for 10 min. Fixed cells were washed with phosphate buffered saline (PBS) 1× and stained overnight at 4 °C with anti-type I collagen (Millipore Corporation, Billerica, MA, USA), anti-actin α-smooth muscle (Sigma- Aldrich, St. Louis, MO, USA) in PBS containing 1% (wt/vol) BSA, followed by 1 h with Alexa 488-labeled anti–mouse immunoglobulin G (Vector Laboratories, Burlingame, CA, USA), then 10 min of staining with 4′6-diamidino 2 phenylindole (DAPI) (Vector Laboratories). The antibodies were diluted in 1:200 phosphate-buffered saline (PBS). Subsequently, the slides were analyzed by confocal laser scanning microscopy TCS-SPE with 63× magnification (Leica Microsystems, Koblenz, Wetzlar, Germany).

2.7. Proliferation assay

Gingival fibroblasts were first labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) following the manufacturer's instruction (Invitrogen, Carlsbad, CA), as described previously [20]. Fibroblasts (5×10^5/well) were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 48 h and 96 h culture, the cells were collected using trypsin (Sigma-Aldrich), and analyzed using flow cytometry. Fibroblast proliferation was characterized by sequential halving of CFSE fluorescence, generating equally spaced peaks on a logarithmic scale. Data was analyzed using CellQuest software (BD Biosciences).

2.8. Cytokine detection

Murine IL-13, IL-6, IL-1β, TGF-β and TNF-α levels were measured in the supernatants of cultured gingival fibroblasts using ELISA kits (BD Biosciences), according to the manufacturer's instructions.