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Murine Experimental Root Canal Infection: Cytokine Expression in Response to *F. nucleatum* and *E. faecalis*

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The aim of this study was to evaluate the gene expression of proinflammatory (RANKL, TNF-α and IFN-γ) and regulatory (TGF-β and IL-10) cytokines as reaction to experimental infection by mono or bi-association of *Fusobacterium nucleatum* (ATCC 10953) and *Enterococcus faecalis* (ATCC 19433). *F. nucleatum* and *E. faecalis*, either in mono- or bi-association were inoculated into the root canal system (RCS) of Balb/c mice. Animals were sacrificed at 10 and 20 days after infection and periapical tissues surrounding the root were collected. The mRNA expression of the cytokines RANKL, TNF-α, IFN- γ, TGF-β and IL-10 was assessed using real-time PCR. The Kruskal-Wallis test was used for statistical analysis. *F. nucleatum* mono-infection induced high expression of RANKL and TNF-α, while its modulation was due to IL-10. High expression of IFN-γ at day 20 was up-regulated by *E. faecalis* and RANKL; TNF-α was up-regulated by an independent mechanism via IL-10 and TGF-β. Bi-association (*F. nucleatum* and *E. faecalis*) stimulated high expression of RANKL, TNF-α and IFN-γ, which seemed to be modulated by TGF-β 20 days later. The gene expression of proinflammatory cytokines was more prominent in the earlier periods of the experimental periapical infection, which concomitantly decreased in the later period. This expression may be regulated by IL-10 and TGF-β in an infection-specific condition

Key Words: cytokines, Gram-positive bacteria, Gram-negative bacteria, periapical lesion.

Introduction

Root canal infections lead to the development of periapical lesions. However, their development depends on the infecting microbiota as well as the host response (1).

In polymicrobial infections, synergistic or antagonistic microbial interactions play a role in the pathogenic effects of microorganisms (2,3). Bacterial relationships involve different associations: among Gram-negative species and also between Gram-negative and Gram-positive species. The peptidoglycan cell wall of Gram-positive bacteria acts synergistically with lipopolysaccharides (LPS) of Gram-negative (4), complicating even more the antigenicity of the endodontic content.

Former researches have clearly demonstrated differences between Gram-positive and Gram-negative bacteria, in mono or bi-association, which induce cytokine and chemokine expression (5).

Periapical inflammatory diseases involve several aspects, such as bacterial ecology and pathogenicity, innate and adaptative immune cells, which in turn produce cytokines (1). Several proinflammatory responses, like those induced by interleukin 1 (IL-1), tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and the receptor activator of the nuclear factor kappa-B ligand (RANKL) may occur in periapical tissues (6,7). On the other hand, anti-inflammatory responses, as those induced by interleukin 4 (IL-4), interleukin 10 (IL-10) and transforming growth factor (TGF-β), modulate the first pro-inflammatory events (6).

LPS from *Fusobacterium nucleatum* is highly cytotoxic (8) whereas lipoteichoic acid from *Enterococcus faecalis* clinical strain promotes TNF-α expression by the nuclear factor kappa-B (RANK) and p38 MAPK signaling pathways in differentiated THP-1 macrophages (9).

This study checked the ability of some bacteria prevalent in human infections, *F. nucleatum* (Gram negative strict anaerobe) and *E. faecalis* (Gram positive facultative anaerobe), experimentally inoculated into root canals of conventional mice either in mono or bi-infection, to induce the proinflammatory (RANKL, TNF-α, IFN-γ) and regulatory cytokines (IL-10 and TGF-β) in the periapical area surrounding these infections.

The tested null hypothesis was that *F. nucleatum* and *E. faecalis* alone or in association have no effect on the expression of RANKL, TNF-α, IFN-γ, IL-10 and TGF-β mRNA.

Material and Methods

Mice

Thirty female 8-week-old mice were used (Balb/c, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil). All manipulations were performed under

sterile conditions in a laminar flow hood (Veco, Campinas, SP, Brazil). The animals were fed *ad libitum*. The animal ethics committee (152/2010, CETEA/UFMG) approved the experimental protocol.

Experimental Root Canal Infection

Previously described (5) experimental procedures were performed. Briefly, the animals were intraperitoneally anesthetised using 100 mg kg^{-1} ketamine hydrochloride (Dopalen, Vetbrands Animal Health, Jacareí, SP, Brazil) and 10 mg kg^{-1} xylazine (Anasedan, Agribrands do Brasil Ltda, Paulínia, SP, Brazil). The pulp chamber of the maxillary right first molar was assessed by an endodontic operative microscope (Alliance, São Paulo, SP, Brazil) and a 1/4 carbide bur (KG Sorensen, Barueri, SP, Brazil), coupled to a controlled rotation hand piece (Driller, São Paulo, SP, Brazil). Afterwards, using #0.8 and #10 K files, the canal was probed.

Three groups of mice were inoculated as follows: group I, *F. nucleatum* strains; group II, *E. faecalis*; and group III, *F. nucleatum* and *E. faecalis*. Ten mice were used for each experimental group per time point; time points were at 10 and 20 days after the surgical procedure.

In the control group, a provisional restorative material composed of zinc oxide and sulphate (Coltosol®; Vigodent, Bonsucesso, RJ, Brazil) sealed the teeth; in the experimental groups, inoculation was performed before this sealing.

Microorganisms

The two selected bacterial strains were *F. nucleatum* (ATCC 10953) and *E. faecalis* (ATCC 19433). The bacteria were maintained at -86 °C; recovery was performed using a brain-heart infusion supplemented with sheep's blood, yeast extract, hemin and menadione (BHI-S; Difco, Detroit, MI, USA). *F. nucleatum* was incubated in an anaerobic chamber containing an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 (Forma Scientific Inc., Marietta, OH, USA) for 48 h and *E. faecalis* was placed in a bacteriological incubator for 24 h. For root canal infections, the concentration of the bacteria grown in BHI-SPRAS (Difco) was adjusted to approximately 10^7 CFU per 25 µL in the same broth in mono-infection. In bi-association, both bacteria were pooled to a total of 10^7 CFU per 25 µL.

Next, the bacterial suspensions were inoculated into the RCS by dripping, using type PPD syringes and needles. Gram staining regularly checked the purity of the microorganisms . The opening and inoculation in animals were performed in the laminar flow hood. Disinfection and antisepsis of the surgical field were performed according to Möller (10). All instruments were sterilized by autoclaving. All the chemicals were pure quality grade.

Animal Sacrifice and Sample Extraction

After 10 and 20 days, the animals were sacrificed by cervical dislocation. The periapical tissue surrounding root apices of the right and left molars was extracted together with the surrounding bone. Gingiva, oral mucosa and crown were removed and discarded (11). Samples were treated with 250 µL of Trizol (GIBCO BRL Laboratories, Grand Island-NY, USA). The samples were crushed using electrical equipment (IKA T10 basic; Merse, Campinas, SP, Brazil) and stored in a -20 °C freezer for RNA extraction.

RNA Extraction

200 µL of chloroform were added to the Eppendorf tubes containing the samples. They were then centrifuged for 15 min at 12,000 g at 4 °C, which produced the precipitation of the RNA. The aqueous phase was transferred to another Eppendorf tube, followed by the addition of 250 µL isopropanol (Merck, SP, Brazil). The mixture was incubated in BOD (Q-315D; Quimis®) at 25 °C for 15 min and centrifuged for 10 min at 12,000 g at 4 °C, producing the precipitation of RNA. The aqueous phase was discarded and the precipitated RNA was washed with 250 µL 75% ethanol. The mixture was then centrifuged at 10,000 g at 4 °C for 15 min. The ethanol-containing tubes were turned over and centrifuged at10,000 g at 4 °C for 15 min. The aqueous phase was discarded and the tubes were overturned for approximately 15 min to remove all liquid. 25 µL of high-quality water treated with DEPC (diethyl pyrocarbonate; SIGMA® Chemical Co., St. Louis, MO, EUA) were added to the solution. The samples were frozen in a -80 °C freezer.

mRNA Quantification

The solution containing the mRNA was read in a spectrophotometer (Nanodrop® /ND 1000, Wilmington, DE, USA) using 260/280 OD wavelength. The quantification of each sample of mRNA was obtained Using the formula [mRNA-mg/mL]=OD260 x dilution x 40.

Obtaining cDNAs

cDNA was synthesised using 2 µg of RNA by reverse transcription, as described by Silva et al. (11). The standard conditions were as follows: a holding stage of 95 °C for 10 min; a cycling stage with 40 cycles of 95 °C for 15 s, 60 °C for 1 min and a melting curve stage of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s.

Cytokine Detection and Quantification

The primer sequences used for the analysis of the cytokines are in Table 1.

Real-time PCR was performed using the Step One Real-time PCR System (Applied Biosystems, Foster

City, CA, USA). In addition, the SYBR-Green detection system (Applied Biosystems) was used to assess primer amplification. The housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) was also amplified and used to normalise mRNA expression levels. All the samples were run in duplicate in a 20 µL reaction volume with 1 µg cDNA. Sequence Detection Software, version v. 2.0 (Applied Biosystems) was used to analyse data after amplification. The results were obtained as threshold cycle (Ct) values, which represent the cycle number at which the fluorescence levels surpassed a fixed threshold. Expression levels were calculated using the ΔΔCt method. The Ct values are expressed as mean of two independent measurements, and the expression levels of mRNA for all samples are expressed as the ratio between the expression of the genes of interest and the expression of HPRT. All data were analysed using the SPSS statistical program (SPSS Inc., Chicago, IL, USA).

Data Analysis

The Kruskal-Wallis test was used due the high variation coefficient (VC), above 30%; this characterized the unstable and heterogeneous sample. Furthermore, several variables showed an asymmetric distribution.

Table 1. Primer sequence

Genes	Sense and antisense	bp*
HPRT	FW: GTTGGATACAGGCCAGACTTTGTT RV: GATTCAACTTGCGCTCATCTTAGGC	162
TNF-α	FW: ATCTTCTCAAAATTCGAGTGACCA RV: TGGAGTAGACAAGGTACAACCC	171
TGF-β	FW: TGACGTCACTGGAGTTGTACG RV: GGTTCATGTCATGGATGGTGC	169
RANKL	FW: CATCCCATCGGGTTCCATAA RV: CCTTAGTTTTCCGTTGCTTAACGAC	103
IL-10	FW: GGTTGCCAAGCCTTATCGGA RV: ACCTGCTCCACTGCCTTGCT	109
IFN-γ	FW: CAAGTGGCATAGATGTGGAAGAA RV: TGGCTCTGCAGGATTTTCATG	90

Results

Genetic expression of RANKL was observed at both evaluation periods (10 and 20 days). *F. nucleatum*, in mono- (Group 1) or bi-association with *E. faecalis* (Group 3) induced statistically higher RANKL expression on day 10 than day 20. On day 10, *F. nucleatum*, in mono- or bi-association with *E. faecalis*, induced higher expression of RANKL than *E. faecalis* alone ($p<0.05$). However, on day 20, there was no difference among the three groups.

Similarly to that of RANKL, TNF-α expression (Fig. 1) was significantly higher on day 10 post-inoculation than on day 20 in *F. nucleatum* mono-infection or bi-infection with *E. faecalis*. On day 10, the gene expression of TNF-α in Groups 1 and 3 was significantly higher than that in Group 2 (*E. faecalis*). However, there was no difference among the groups on day 20. *E. faecalis* induced a baseline cytokine expression in both periods of evaluation.

Bi-association induced increased IFN-γ expression on day 10 compared with both mono-infections at same time. Interestingly, *E. faecalis* mono-infection induced a significant increase in the expression of IFN-γ on day 20 compared to day 10 (Group 2); this increase in expression was also observed in bi-association on day 20 (Group 3).

Mono or bi-associated *F. nucleatum* (Group 1 and 3) induced a statistically

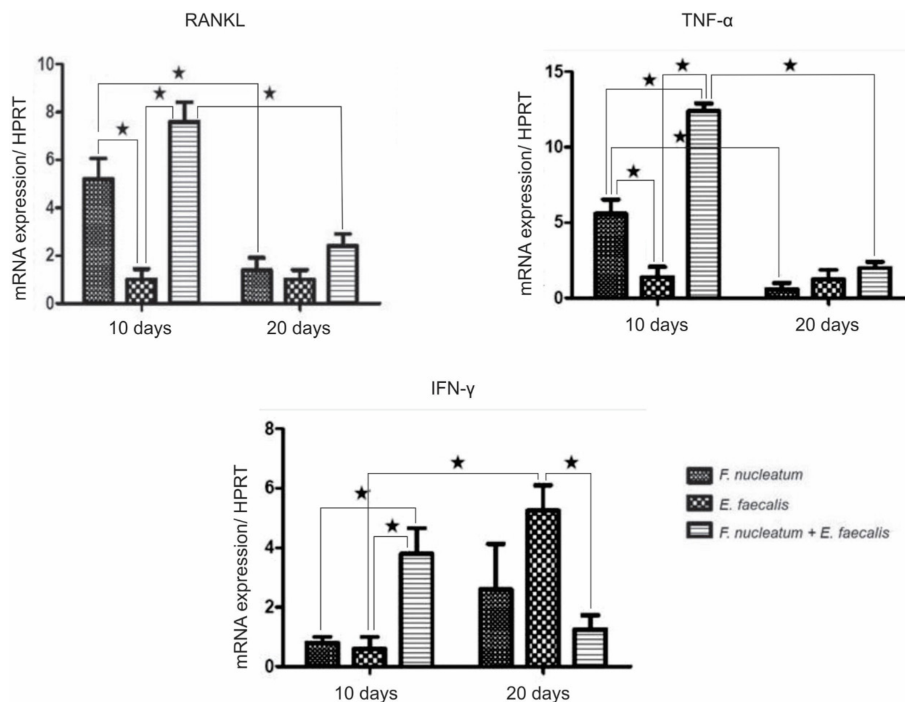

Figure 1. mRNA expression of IFN-γ, TNF-α, and RANKL cytokines in periapical tissues 10 and 20 days after the surgical procedure analyzed by real-time PCR. Relative mRNA expression levels quantified by comparison with the internal control (HPRT). Data expressed as mean±SE for three independent experiments, each with ten mice per group. The star indicates $p<0.05$.

significant increase in TGF-β expression from day 10 to day 20. Additionally, a significant overexpression of TGF-β was observed on day 20 in bi-association samples compared with those of both mono-infections.

Regulatory cytokine IL-10 expression was significantly up-regulated on day 20 in *F. nucleatum* infections (Fig 2) compared to day 10; this was also true of *E. faecalis* mono-association and bi-association during the same time.

Discussion

Previous studies have used animal models to assess the multiple aspects involved in the periradicular disease pathogenesis (1,11,). The tested hypothesis in the present study was that different infections could trigger particular immune responses in the development of periapical lesions. Using two reference strains, *F. nucleatum* and *E. faecalis*, via mono or bi-association, was assessed the immune response in periapical tissues surrounding the infections. Bi-association was chosen since some strict anaerobic bacteria may be more powerful in inducing apical periodontitis when associated with other anaerobic or facultative microorganisms (1). Additionally, it was also shown that both strains associated here did not produce antagonistic factors against each other (12). Moreover, as demonstrated in many laboratories that use rodent models (6), after inducing experimental root canal infection, periapical bone destruction starts 7 days later. From days 7 to 20, rapid destruction occurred followed by slower bone resorption. Based on these findings, were defined both evaluation periods (10 and 20 days) of this study. Pro-inflammatory (RANKL, TGF-α e IFN-γ) and regulatory (IL-10 e TGF-β) cytokine expression were assayed by qPCR.

RANKL is a cytokine whose main role in bone physiology is to stimulate osteoclast differentiation and activation, and to inhibit cell apoptosis (7). This study observed that *F. nucleatum*, in mono (Group 1) or bi- association (Group 3), induced higher RANKL expression on day 10 compared to day 20. These results suggest that the RANKL expression increases in the early stages of periapical lesion expansion; furthermore, *E. faecalis* does not interfere with this process. Although it has been related to a plethora of unidentified microorganisms, both in periapical lesions (7) and the experimental induction of periodontitis (13), RANKL is present in large amounts in lesions in the early stages of development. Blocking of RANKL by OPG (osteoprotegerin) during the experimental induction of periodontal disease decreases alveolar bone loss, as previously shown (14).

TNF-α has activities that affect multiple aspects of the cell migration process, including the induction of adhesion molecules and production of chemokines (15). In this study, it was observed that *F. nucleatum*, in mono or bi-association with *E. faecalis*, induced an increase in TNF-α expression on day 10 compared to day 20. Furthermore, it was determined that *E. faecalis* did not interfere in this expression or the expression of RANKL. These results are similar to those of other studies that demonstrated increased levels of TNF-α and RANKL in gingival fluid and diseased periodontal tissue (15,16). Garlet et al. (16) induced periodontal disease in TNF-α p55 deficient mice and identified a concomitant decrease in the expression of RANKL; consequently, there was a reduction in alveolar bone loss. In this experiment, *F. nucleatum* alone or in association with *E. faecalis* also produced the genetic expression of this cytokine in the evaluated periapical tissues. The lipoteichoic acid isolated from *E. faecalis* and other Gram-positive bacteria strains also stimulate the release of TNF-α by leukocytes in periapical lesion samples, as demonstrated by *in vitro* studies (17). In the present research, despite the results of *E. faecalis* mono-infection, which show induced basal expression of TNF-α at both time points (10 and 20 days), the expression on day 10 was significantly lower than the one observed for *F. nucleatum* alone or in association

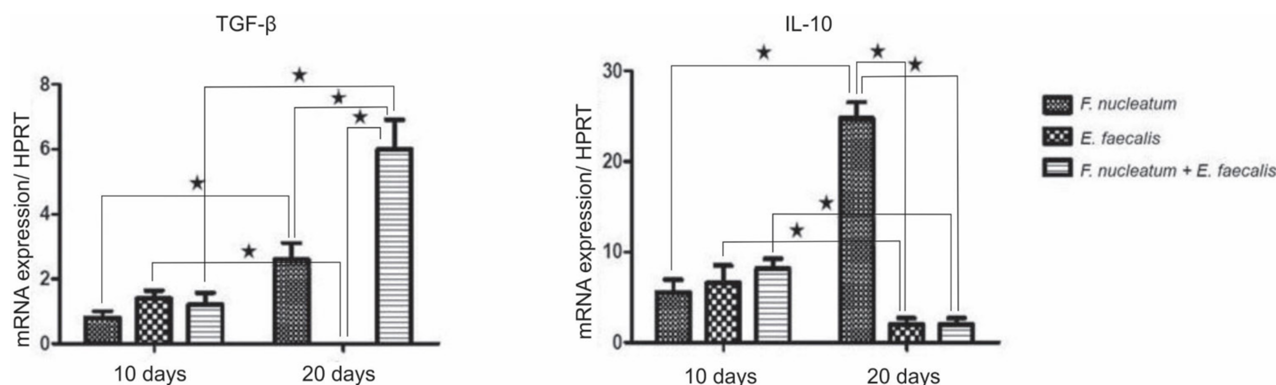

Figure 2. mRNA expression of TGF- , and IL-10 cytokines in periapical tissues 10 and 20 days after the surgical procedure analyzed by real-time PCR. Relative mRNA expression levels quantified by comparison with the internal control (HPRT). Data expressed as mean±SE for three independent experiments, each with ten mice per group. The star indicates $p<0.05$.

with *E. faecalis*.

IFN-γ is a type 1 cytokine that plays an important role in the activation of cell phagocytosis; specifically, it induces the proinflammatory production of cytokines and chemokines (18). Its presence is high in periodontal lesions and it is related to the periradicular response progression in severe forms (19). In this study, the genetic expression of IFN-γ on day 10 before microbial bi-association inoculation was significantly higher than in *F. nucleatum* and *E. faecalis* mono-infection. There was a decrease of IFN-γ expression induced by the bi-infection on day 20, but there was a significant increase in their respective expressions in response to *E. faecalis*. Similarly, Jiang et al. (19) demonstrated that other Gram-positive pathogens, such as *Streptococcus mutans*, stimulated the synthesis of proinflammatory cytokines. Conflicting results in which *G. morbillorum* reduces the expression of cytokines IL-12 and IFN-γ in cell culture supernatant have also been reported (1). IFN-γ is a potent macrophage activator, which leads to increased expression in IL-1 and TNF-α by these cells, modulating RANKL and osteoclastogenesis in periapical inflammatory diseases (20). The increase in the levels of IFN- γ on day 10 in response to bi-infection agrees with these findings. The RANKL and TNF-α were found in high concentrations during the same period and under the same conditions. On day 20, when the decrease in the expression of IFN-γ was correlated with cytokines, confirmed the results. However, an inverse effect of IFN-γ, that it can inhibit osteoclastogenesis has been described (21).

In normal conditions, proinflammatory mechanisms should be controlled because they prevent excessive periradicular tissue destruction. IL-10 and TGF-β are both important immunoregulatory cytokines (22). IL-10 antagonizes the proinflammatory effects, inhibiting the mRNA transcription subgroup of cytokines, which acts as a negative feedback mechanism (22). According to Colic et al. (22), the increased levels of IL-10 induced by *F. nucleatum* mono-infection on day 20 are correlated with the low expression of proinflammatory RANKL, TNF-α and IFN-γ cytokines during the same period; the low expression of IL-10 on day 10 is associated with increased expression of proinflammatory cytokines. These findings are similar to the results of a previous study, which have demonstrated high levels of IL-10 production in inflammatory cells challenged by Gram-negative bacteria (23). However, the proinflammatory response modulation induced by bi-association on day 20 is not associated with IL-10, but with TGF-β; this is primarily due to the fact that overexpression during this period is correlated with the expression of RANKL and TNF-α. TGF-β is a pleiotropic cytokine that regulates growth, differentiation and cell matrix production. Furthermore, TGF-β is a potent immunosuppressive agent that controls the proinflammatory factors related to transcription, like IL-1β and TNF-α (24). Interestingly, the late proinflammatory response observed in the *E. faecalis* mono-infection with high IFN-γ expression on day 20, did not exhibit the negative regulatory effects of cytokines IL-10 or TGF- β. IFN-γ is an important cytokine that mediates B cell isotype changes and leads to an increase in IgG2 production (25). These regulatory effects are also responsible for the reduced RANKL and TNF-α expression during the same period, which invariably lead to an increase in the microbial clearance at the site.

Finally, it is important to highlight the fact that regulatory cytokines, including IL-10 and TGF-β, act directly on osteoclastogenesis. In absence of IL-10, there is reduction in osteoblast and osteoclast expression. In the presence of these cytokines, it is believed that the RANK/RANKL system modulation leads to the induction of OPG production (26). As for TGF-β, its levels in active periapical lesions correlate negatively with RANKL; this may have a protective role against tissue destruction.

This study demonstrated that the genetic expression of periapical cytokines depends on the bacterial community that colonizes root canal systems: mono-infection of *F. nucleatum* promoted high levels of RANKL and TNF-α expression. Moreover, the higher levels of RANKL, TNF-α and IFN-γ were observed when associated with *E. faecalis*. Similarly, the mono-infection of *F. nucleatum* seems to be modulated by IL-10 and TGF-β on day 20, while the bi-association may be regulated by TGF-β at the same time.

Resumo

O objetivo deste trabalho foi avaliar a expressão gênica de citocinas pró-inflamatórias (RANKL, TNF-α e IFN-γ) e regulatórias (TGF-β e IL-10) em resposta à infecção experimental por Fusobacterium nucleatum (ATCC 10953) e Enterococcus faecalis (ATCC 19433) como mono-infecção ou em bi-associação. F. nucleatum e E. faecalis foram inoculados no sistema de canais radiculares de camundongos Balb/c, tanto isoladas como em bi-associação. Os animais foram sacrificados em 10 e 20 dias após a infecção, e os tecidos periapicais foram coletados. As expressões do mRNA das citocinas RANKL, TNF-α, IFN-γ, TGF-β e IL-10 foram analisadas por meio do real-time PCR. O teste de Kruskal-Wallis foi utilizado para análise estatística. A mono-infecção com F. nucleatum induziu alta expressão de RANKL e TNF-α, enquanto sua modulação ocorreu devido à IL-10. A alta expressão de IFN-γ no dia 20 foi regulada positivamente por E. faecalis e RANKL; TNF-α foi regulada positivamente por um mecanismo independente via IL-10 e TGF-β. A bi-associação (F. nucleatum e E. faecalis) estimulou uma alta expressão de RANKL, TNF-α e IFN-γ, que parece ser modulada por TGF-β após 20 dias. A expressão gênica de citocinas pró-inflamatórias foi mais proeminente nos estágios iniciais da infecção periapical experimental, com concomitante redução no período tardio. Este fenômeno pode ser regulado por IL-10 e TGF-β em uma condição de infecção específica.

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