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Cytokine expression in response to root canal infection in gnotobiotic mice

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Abstract

Maciel KF, Neves de Brito LC, Tavares WLF, Moreira G, Nicoli JR, Vieira LQ, Ribeiro Sobrinho AP. Cytokine expression in response to root canal infection in gnotobiotic mice. *International Endodontic Journal*, **45**, 354–362, 2012.

Aim To examine cytokine expression profiles during periapical lesion development in response to synergetic human pathogens in a gnotobiotic mouse model.

Methodology Human strains of *Fusobacterium nucleatum* and *Peptostreptococcus prevotii* were inoculated into the root canals of germ-free mice in either mono- or bi-association. Animals were killed 7 and 14 days after infection, and periapical tissues were collected. mRNA expression of the cytokines IFN-γ, TNF-α, Receptor activator of nuclear factor kappa-B ligand (RANKL), IL-10, IL-4 and transforming growth factor β (TGF-β) was assessed using real-time PCR. Levene's test was used to assess the equality of variance of the data, whereas a *t*-test for independent samples was used to evaluate the significance of the differences between groups ($P < 0.05$).

Results The mRNA expression of IFN-γ and TNF-α was up-regulated by *F. nucleatum* during the acute (day 7) and chronic phase (day 14) of periapical lesion development. However, in bi-infection the expression of IFN-γ and TNF-α were effectively absent at both time-points. RANKL mRNA expression was down-regulated during dual infection at the chronic phase. As IL-4 expression was similar at both time-points, IL-4 does not appear to be involved in the periapical response to these bacterial strains. IL-10 was up-regulated during the chronic phase by mono-infection with either *F. nucleatum* or *P. prevotii*. Dual infection increased TGF-β mRNA expression on day 7, which paralleled the decrease in IFN-γ and TNF-α mRNA levels at the same time-point. *F. nucleatum* increased TGF-β mRNA expression during the chronic phase.

Conclusion Cytokine profiles depend on the nature of the bacterial challenge. Both TGF-β and IL-10 appeared to be regulating the proinflammatory cytokine responses at both time-points of the periapical immune response.

Keywords: cytokines, germ-free mice, periapical lesions.

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Introduction

All bacteria that colonize the oral cavity can invade the root canal system (RCS). Currently, studies using molecular biology techniques have demonstrated the polymicrobial aetiology of endodontic infections (Lana *et al.* 2001, Gomes *et al.* 2004, Brito *et al.* 2007, Siqueira *et al.* 2009). Several bacterial species have been associated with the pathogenesis of periradicular diseases, with anaerobic bacteria prevalent in these infections (Lana *et al.* 2001, Peters *et al.* 2002, Gomes *et al.* 2004, Siqueira *et al.* 2004, 2009, Brito *et al.* 2007, Narayanan & Vaishnavi 2010, Tavares *et al.* 2011). Selective environmental pressures (Sundqvist

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1992, Siqueira *et al.* 2009) present in root canals, such as nutrients, oxygen availability and microbial interactions contribute to the anaerobic status of invading bacteria. Additionally, microbial interactions, which can be synergistic or antagonistic (Oliveira *et al.* 1998, Socransky *et al.* 1998, Peters *et al.* 2002, Socransky & Haffajje 2005), are important factors in the establishment and colonization of the root canal (Caires *et al.* 2007, Sundquivst & Figdor 2007). Synergism occurs when the metabolism of a species produces nutrients and growth factors that are reliable food sources for another species, whilst antagonism occurs when a microorganism produces substances that may be toxic to or inhibit the growth of another species (Sundqvist 1992, Caires *et al.* 2007). Moreover, it has been shown, in polymicrobial infections, that the pathogenic effects of the microorganisms are affected by synergistic bacterial relationships (Metzger *et al.* 2009).

In turn, the host response to infection in the RCS is complex and involves the recruitment of inflammatory cells to the periapical tissues and an extensive network of immune mechanisms, including cytokine production (Seymour & Taylor 2004, Prso *et al.* 2007, De Rossi *et al.* 2008, Gazivoda *et al.* 2009). Periapical lesion development depends on the proinflammatory and immunomodulatory cytokines released during infection and the inflammatory restrictions that occur during the chronic phase of lesion development (Ribeiro-Sobrinho *et al.* 2002, Colic *et al.* 2009, 2010, Teixeira-Salum *et al.* 2010). It has been proposed that proinflammatory cytokines, such as IFN-γ, TNF-α and RANKL, play a fundamental role in periapical bone destruction (Menezes *et al.* 2008). IFN-γ is a key mediator of macrophage activation that up-regulates IL-1 and TNF-α expression (Kawashima *et al.* 2007, Colic *et al.* 2009, Gazivoda *et al.* 2009). IFN-γ secretion is primarily triggered by IL-12 and down-regulated by IL-10 (Colic *et al.* 2010). TNF-α is a soluble mediator that plays a role in initiating and coordinating the cellular events associated with the immune response to infection (Prso *et al.* 2007) by up-regulating the host response to bacteria and stimulating bone resorption (Graves *et al.* 2011). Bone resorption depends on the balance between the production of RANKL, which promotes osteoclast differentiation, and its natural decoy receptor, osteoprotegerin (OPG) (Kawashima *et al.* 2007, Menezes *et al.* 2008, Colic *et al.* 2010, Graves *et al.* 2011). Studies performed *in vivo* suggest that IFN-γ and TNF-α influence the regulation of periapical bone resorption by inducing nitric oxide (Fukada *et al.* 2009, Silva *et al.* 2011). Additionally, IFN-γ may interact with TNF-α to induce the overexpression of RANKL and activate osteoclastic bone resorption (Fukada *et al.* 2009, Teixeira-Salum *et al.* 2010). The synergistic effects of RANKL and proinflammatory cytokines are induced in the periapical area in response to bacterial stimuli, which then contribute to periapical lesion expansion (Kawashima *et al.* 2007, De Rossi *et al.* 2008).

Conversely, immunosuppressive mechanisms are responsible for healing and restricting inflammation in periapical lesions (Ribeiro-Sobrinho *et al.* 2002, Colic *et al.* 2009, 2010, Teixeira-Salum *et al.* 2010). Transforming growth factor β (TGF-β) is known to inhibit bone resorption during osteoclast formation and differentiation (Gazivoda *et al.* 2009, Teixeira-Salum *et al.* 2010) and is an important regulatory cytokine with potent immunosuppressive effects. Furthermore, TGF-β inhibits the production of IL-1, TNF-α and IFN-γ whilst also antagonizing the biological activities of these cytokines (Colic *et al.* 2009). However, little is known about the role of TGF-β in the pathogenesis of human periapical lesions. Similarly, IL-4 has been show to exert an inhibitory effect on bone resorption by generating negative effects on osteoclasts whilst stimulating the synthesis and mineralization of bone matrix (Teixeira-Salum *et al.* 2010). IL-4 also stimulates the humoral immune response by inducing antibody production and B cell differentiation (Colic *et al.* 2006, Garlet 2010). IL-10, which is produced by APCs, T regulatory cells and other cell types, is important in the down-regulation of the inflammatory processes in advanced lesions (Gazivoda *et al.* 2009) and plays an important role in bone tissues partly through the induction of OPG synthesis (Sasaki *et al.* 2000, Colic *et al.* 2010).

The aim of this study was to evaluate the cytokine expression profile during periapical lesion development in response to synergetic human pathogens in a gnotobiotic mouse model. The null hypothesis tested was that *Fusobacterium nucleatum* and *Peptostreptococcus prevotii* alone or in association have no effects on IL-4, IL-10, TNF-α, IFN-γ, RANKL and TGF-β mRNA expression.

Materials and methods

Mice

Germ-free mice aged 4–8 weeks (Swiss/NIH, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil) were used and kept in Trexler-type isolators (Class Biologically Clean, Madison, WI, USA). For

experimental procedures, the animals were transferred into microisolators (UNO Roestvastaal BV, Zevenaar, the Netherlands). All manipulations were performed under sterile conditions in a laminar flow hood (Veco, Campinas, Brazil). The animals were fed *ad libitum*. The experimental protocol was approved by the animal ethics committee (151/2010, CETEA/UFMG).

Microorganisms

The microorganisms used were recovered from a patient treated at the Endodontic Clinic of the Dentistry School of the Universidade Federal de Minas Gerais. The bacteria recovered were *F. nucleatum* and *P. prevotii*; their identification has previously been described (Moreira *et al.* 2011). The isolated strains were stored at −70 °C and re-grown in pre-reduced brain heart infusion broth supplemented with haemin and menadione (BHI-SPRAS) (Difco, Detroit, MI, USA). Incubations were performed at 37 °C in an anaerobic chamber containing an atmosphere of 85% N_2, 10% H_2 and 5% CO_2 (Forma Scientific Company, Marietta, OH, USA).

Experimental root canal infection

The experimental procedures were performed with the animals under general anaesthesia; the animals were anaesthetized using 100 mg kg^{-1} ketamine hydrochloride (Dopalen, Division Vetbrands Animal Health, Jacareí, SP, Brazil) and 10 mg kg^{-1} xylazine (Anasedan, Agribrands do Brasil Ltda, Paulínia, SP, Brazil). The pulpal chamber of the maxillary right first molar was accessed under an endodontic operative microscope (Alliance, São Paulo, SP, Brazil) and a one-fourth carbide bur (KG Sorensen, Barueri, SP, Brazil) coupled to a controlled rotation hand piece (Driller, São Paulo, SP, Brazil). The pulp chambers were opened until the orifices of the canals could be visualized and probed with a size 8 K-file. There were no surgical interventions on the maxillary left first molar, which served as the control. For root canal infections, the concentration of the bacterial samples, which were grown in BHI-SPRAS (Difco), was adjusted to be approximately 10^7 CFU per 25 μL in the same broth. Bacterial suspensions were inoculated into the RCS using tuberculin syringes and needles; following inoculation, the cavities were sealed with paraffin (Ribeiro-Sobrinho *et al.* 2001). Three groups of mice were inoculated as follows: group I, *P. prevotii* and *F. nucleatum* strains; group II, *F. nucleatum*; and group III, *P. prevotii*. Six mice were used for each experimental group per time-point; time-points were 7 and 14 days after the surgical procedure.

Sample preparation

The mice were killed at 7 and 14 days after root canal inoculation. The periapical tissues surrounding the root apices and the bone subjected to surgery was aseptically removed, rinsed in phosphate-buffered saline, flash-frozen in a mixture of dry ice and ethanol and stored at −70 °C. Total periapical tissues and bone RNA were isolated using TRIzol reagent (Gibco/BRL Laboratories, Grand Island, NY, USA). After the addition of TRIzol, chloroform was added, and the mixture was centrifuged at 12 000 × *g* at 4 °C for 15 min. The aqueous phase was then collected, and the RNA was precipitated by the addition of isopropanol followed by centrifugation at 12 000 × *g* at 4 °C for 10 min. The precipitated RNA was washed once with cold 75% ethanol, dried, dissolved in RNase-free water, incubated at 55 °C for 10 min and then stored at −70 °C.

Real-time PCR

Complementary DNA was synthesized using 2 μg of RNA by reverse transcription as described by Silva *et al.* (2008). The standard PCR 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. The primer sequences used for the analysis of IL-4, IL-10, TNF-α, IFN-γ, RANKL and TGF-β mRNA expression by quantitative real-time PCR are shown 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 assay primer amplification. The housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) was also amplified and used to normalize mRNA expression levels. All samples were run in duplicate in a 20 μL reaction volume with 1 μg of cDNA. Sequence Detection Software, version v 2.0 (Applied Biosystems), was used to analyse the data after amplification. The results were obtained as threshold cycle (C_t) values, which represent the cycle number at which the fluorescence levels passed a fixed threshold. Expression levels were calculated using the $\Delta\Delta C_t$ method. The C_t values are expressed as the mean of two independent measurements, and the expression levels of mRNA for all samples are expressed as the ratio between the

Table 1 Primer sequences

Gene	Sense and antisense	Length (bp)
HPRT	5′-GTT GGA TAC AGG CCA GAC TTT GTT G-3′	162
	5′-GAT TCA ACT TGC CGT CAT CTT AGG C-3′	
TNF-α	5′-CAT CTT CTC AAA ATT CGA GTG ACA A-3′	171
	5′- TGG GAG TAG ACA AGG TAC AAC CC-3′	
IFN-γ	5′-TCA AGT GGC ATA GAT GTG GAA GAA-3′	90
	5′-TGG CTC TGC AGG ATT TTC ATG-3′	
RANKL	5′-CAT CCC ATC GGG TTC CCA TAA-3′	103
	5′-CCC TTA GTT TTC CGT TGC TTA ACG AC-3′	
IL-4	5′-ACA GGA GAA GGG ACG CCA T-3′	94
	5′-GAA GCC CTA CAG ACG TCA-3′	
IL-10	5′-GGT TGC CAA GCC TTA TCG GA-3′	190
	5′-ACC TGC TCC ACT GCC TTG CT-3′	
TGF-β	5′-TGA CGT CAC TGG AGT TGT ACG-3′	169
	5′-GGT TCA TGT CAT GGA TGG TGC-3′	

HPRT, housekeeping gene hypoxanthine phosphoribosyltransferase; TGF-β, transforming growth factor β.

expression of the gene of interest and the expression of HPRT. All data were analysed using the SPSS statistical programme (SPSS Inc, Chicago, IL, USA). Levene's test was used to assess the equality of variance of the data, whereas a *t*-test for independent samples was used to evaluate the significance of the differences observed between groups. Differences in mRNA expression levels were considered to be statistically significant when the *P*-value was <0.05.

Results

Real-time PCR analyses of IFN-γ, TNF-α, RANKL, IL-10, IL-4 and TGF-β mRNA expression levels were performed with periapical samples from gnotobiotic mice. At day 7 post-inoculation, the mRNA expression of IFN-γ was significantly increased by *F. nucleatum* infection compared to *P. prevotii* infection and infection with both strains. However, at day 14 post-inoculation, IFN-γ mRNA expression in *F. nucleatum* infection was markedly diminished, which resembled the IFN-γ mRNA expression levels present in response to infection with *P. prevotii* or both strains. Conversely, the expression of TNF-α was significantly increased by *F. nucleatum* infection at day 14 compared to *P. prevotii* infection alone or infection with both *F. nucleatum* and *P. prevotii*. RANKL mRNA expression was only down-modulated by dual infection in the chronic phase (Fig. 1).

The immunomodulatory cytokine IL-10 was significantly up-regulated on day 14 post-inoculation by both *F. nucleatum* and *P. prevotii* mono-infections compared with its expression to the same stimuli at day 7 post-inoculation. These results correlate with the observed decrease in IFN-γ mRNA expression at the same point. At day 14 post-inoculation, the bi-association of these bacterial strains induced a decrease in IL-10 expression. IL-4 does not appear to be involved in the periapical immune response to these infections because its expression was similar at both time-points analysed in each infection situation. At day 7 post-inoculation, TGF-β mRNA expression was significantly increased in response to *F. nucleatum* and *P. prevotii* dual infection compared with mono-infection with either strain; this result paralleled the decreased IFN-γ and TNF-α mRNA expression observed for dual infection at the same time-point. It was also observed that the *F. nucleatum* mono-infection induced an increase in TGF-β mRNA expression during the chronic phase (day 14) compared with the acute phase (day 7); however, TGF-β mRNA levels were decreased following *F. nucleatum* mono-infection compared with *P. prevotii* mono-infection at both time-points. Finally, dual infection significantly induced the down-regulation of TGF-β mRNA expression at day 14 post-inoculation (Fig. 2).

Discussion

This study hypothesized that the profile of cytokines expressed in periapical tissues in response to a two-strain synergistic bacterial challenge would be different from an infection with individual species. Several studies have used gnotobiotic animals, which permit the evaluation of a specific microbial challenge as a model to study root canal infections (Kakehashi *et al.* 1965, Ribeiro Sobrinho *et al.* 2005, 2002). In this study, using synergistic bacteria recovered from a human root canal for microbial challenge experiments

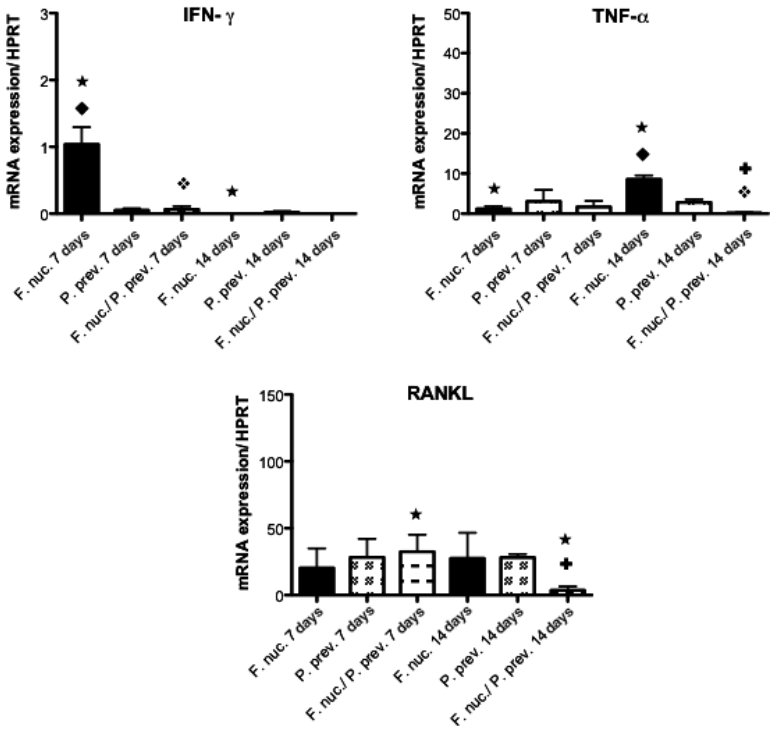

Figure 1 mRNA Expression of IFN-γ, TNF-α, and RANKL cytokines in periapical tissues analysed by real-time PCR. Relative mRNA expression levels were quantified by comparison with the internal control (HPRT). Data are expressed as the mean ± SE for three independent experiments, each with six mice per group. + $P < 0.05$, *Fusobacterium nucleatum*-mono-infected group compared to dual infected group at the same time point; ★ $P < 0.05$, *Peptostreptococcus prevotii*-mono-infected group compared to the dual infected group at the same time point; $P < 0.05$, comparison between both of the mono-infected groups at the same time point; $P < 0.05$, comparison between the same groups at different times points.

(Moreira *et al.* 2011), the cytokine expression profile in the periapical tissues of gnotobiotic mice was evaluated.

IFN-γ is the main activator of macrophages, which subsequently produce cytokines and other mediators that play a significant role in the development of periradicular diseases (Colic *et al.* 2006). Moreover, it has been proposed that IFN-γ is critical for the suppression of pathologic bone resorption (Sasaki *et al.* 2004, Takayanagi *et al.* 2005, Queiroz-Junior *et al.* 2010). In this study, IFN-γ mRNA expression was significantly up-regulated by *F. nucleatum* infection compared to infection with *P. prevotii* or both *F. nucleatum* and *P. prevotii* at day 7 post-infection. The increased IFN-γ mRNA expression on day 7 to *F. nucleatum* infection was paralleled with lower IL-10 expression at this time-point; however, the relative expression of these two cytokines was inverted on day 14 post-inoculation. These results are consistent with previous studies that have shown that Th1 immune responses, mediated by IFN-γ, together with other proinflammatory cytokines, are involved in lesion progression and bone destruction at the beginning of periapical lesion development, whilst the immunosuppressive mechanisms mediated by Th2 cytokines are responsible for healing and the restriction of the inflammatory/immune mechanisms at the later phase of infection (Akamine *et al.* 1994, Kawashima & Stashenko 1999, Lukic 2000). Moreover, the expression of IFN-γ is primarily triggered by IL-12 and down-regulated by IL-10 (Colic *et al.* 2010).

TNF-α is expressed by macrophages in response to infection and in human periapical lesion (Stashenko *et al.* 1998, Prso *et al.* 2007). Huang *et al.* (2011) demonstrated that during Gram-negative polybacterial challenge, bacteria synergize and induce the release of TNF-α, which plays a role in up-regulating the host response to bacteria and in stimulating bone resorption (Graves *et al.* 2011). The results of the present study showed that the expression of TNF-α was increased by mono-infection with *F. nucleatum* at

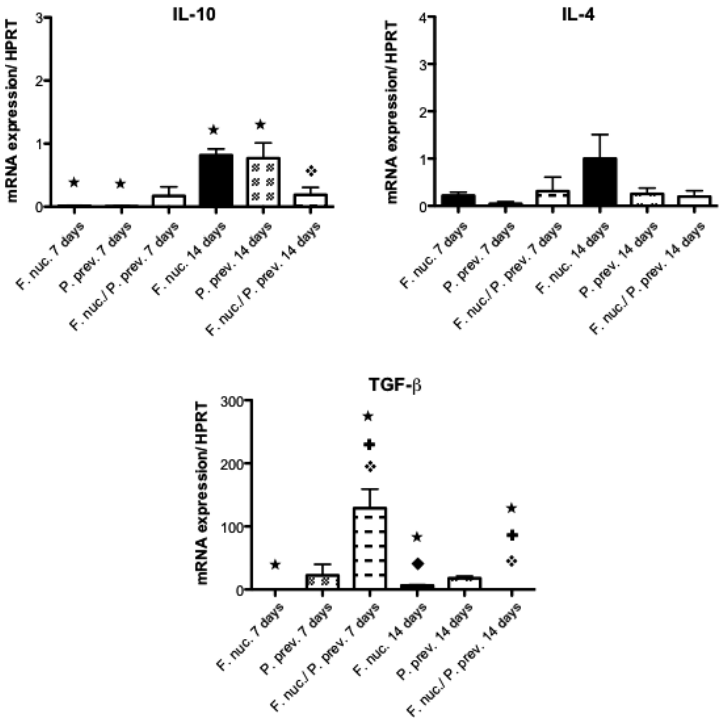

Figure 2 mRNA Expression of IL-10, IL-4, and TGF-β cytokines in periapical tissues analysed by real-time polymerase chain reaction. Relative mRNA expression levels were quantified by comparison with the internal control (HPRT). Data are expressed as the mean ± SE for three independent experiments, each with six mice per group. $P < 0.05$, *Fusobacterium nucleatum*-mono-infected group compared to the dual infected group at the same time point; + $P < 0.05$, *Peptostreptococcus prevotii*-mono-infected group compared to the dual infected group at the same time point; ★ $P < 0.05$, comparison between both of the monoinfected groups at the same time point; $P < 0.05$, comparison between the same groups at different times points.

day 14 post-inoculation. Gazivoda *et al.* (2009) have suggested that the exacerbation of the inflammatory response to infection is most likely triggered by the enhancement of bacterial pathogenicity. Taken together, these results demonstrate that IFN-γ and TNF-α were significantly up-regulated only by mono-infection with *F. nucleatum* in different phases of periapical lesion development. Interestingly, when *F. nucleatum* was associated with *P. prevotii*, both proinflammatory cytokines, IFN-γ and TNF-α, were almost absent at the both phases of infection (days 7 and 14 post-inoculation).

Bone resorption depends on the balance between the production of RANKL and its natural decoy receptor, OPG (Kawashima *et al.* 2007, Menezes *et al.* 2008, Colic *et al.* 2010, Graves *et al.* 2011). When RANKL expression is enhanced, it binds to RANK and favours osteoclast differentiation and bone resorption (Khosla 2001, Colic *et al.* 2010, Queiroz-Junior *et al.* 2010, Graves *et al.* 2011). Although proinflammatory cytokines produced by IFN-γ-activated macrophages increased the expression of RANKL (Khosla 2001, Menezes *et al.* 2008), it was observed that on day 7 post-inoculation the RANKL mRNA expression was similar in both of the mono-infections and in the dual infection. However, RANKL mRNA was down-modulated in chronic phase (day 14). Using a rat mice model, where molar pulps were exposed and left open to the oral environment, Kawashima *et al.* (2007) observed the expression of RANKL on day 7 after pulpal exposure, which was followed by a significant increase in RANKL expression on day 14. The difference between these results and the present study is most likely related to the microbial composition of the root canal infection.

IL-4 has been shown to exert an inhibitory effect on bone resorption (Teixeira-Salum *et al.* 2010) and on macrophage function (Colic *et al.* 2006). However, IL-4 does not appear to be involved in the periapical immune response in our study because its expression was similar at both time-points analysed. This result is

in accordance with previous findings that demonstrated that IL-4 did not exhibit a significant effect on periapical lesion modulation in mice (Sasaki *et al.* 2000, De Rossi *et al.* 2008).

Proinflammatory mechanisms must be controlled to prevent excessive periapical destruction (Gazivoda *et al.* 2009). IL-10 attenuates the progression of bone loss by stimulating OPG production, which inhibits bone resorption by causing unproductive RANK/RANKL interactions (Garlet 2010). In the present study, a significant up-regulation of IL-10 mRNA expression was observed in response to *F. nucleatum* and *P. prevotii* mono-infections on day 14 post-inoculation compared with levels detected on day 7. Interestingly, the expression of IL-10 on day 14 was significantly decreased in dual infections when compared to both mono-infections. Additionally, the predictable immunosuppressive role of IL-10 was also observed, as its up-regulation coincided with decreased IFN-γ mRNA expression at the later phase of lesion development; these results are consistent with the published literature (Kawashima & Stashenko 1999, Lukic 2000, Colic *et al.* 2009). Consistent with the present results, Sasaki *et al.* (2000) demonstrated that IL-10, but not IL-4, suppressed infection-induced bone resorption in experimental periapical lesions in a knock-out mouse model.

Transforming growth factor β inhibits the osteoclast differentiation associated with bone remodelling (Chen & Bates 1993, Menezes *et al.* 2006). In humans, TGF-β expression has been identified in periapical lesions (Tyler *et al.* 1999, Gazivoda *et al.* 2009, Teixeira-Salum *et al.* 2010); however, Colic *et al.* (2009) reported that the concentrations of TGF-β and IL-10 did not significantly differ between granulomas and cysts. In this study, TGF-β mRNA expression on day 7 in response to dual infection was significantly higher compared with both mono-infections. TGF-β mRNA up-expression paralleled the observed decrease in IFN-γ and TNF-α mRNA expression under the same conditions at the same time-point, suggesting that proinflammatory cytokines may be regulated by TGF-β expression. In this study, *F. nucleatum* mono-infection induced an increase in TGF-β expression during the chronic phase (day 14) compared with the acute phase (day 7); however, these changes in TGF-β mRNA expression were more subtle compared the changes observed following *P. prevotii* infection at both time-points. Similar to IL-10, dual infection significantly decreased TGF-β mRNA expression compared with both mono-infections at day 14 post-inoculation. Taken together, it appears that both TGF-β and IL-10 regulate proinflammatory immune responses during the acute and chronic phases of periapical lesion development, respectively. In spite of this, their expression depended on the infection status, as well as if the bacterial stimuli were in mono- or bi-association.

The results of the present study suggest that the cytokine profile expressed in periapical tissues in response to root canal infections depends on the bacterial challenge because both selected species induced specific host immune responses. It has been also demonstrated that the synergistic effects between *F. nucleatum* and *P. prevotii* observed *in vitro* (Moreira *et al.* 2011) did not induce higher proinflammatory cytokine expression *in vivo*. In contrast, at most time-points analysed, dual infection decreased the expression of almost all cytokines. The only exception was TGF-β, which was increased during the acute phase on day 7 post-inoculation. Finally, the gnotobiotic mouse model played an important role in allowing us to address the periapical immune response to a known bacterial challenge.

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