Over-expression of forkhead box P3 and its association with receptor activator of nuclear factor-κ B ligand, interleukin (IL) -17, IL-10 and transforming growth factor-β during the progression of chronic periodontitis

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Abstract

Aim: T regulatory (Treg) cells have been detected in periodontitis lesions, and forkhead box P3 (Foxp3) expression has been negatively correlated to receptor activator of nuclear factor-κ B ligand (RANKL). The aim of this study was to correlate T-helper type 1 (Th1), Th2, Th17 and Treg transcription factor expressions, in gingival tissues from patients undergoing active periodontal tissue destruction, with bone loss-associated cytokines.

Materials and Methods: In 10 chronic periodontitis patients undergoing disease progression, the mRNA expressions of T-bet, GATA-3, Foxp3, RORC2, interleukin (IL)-1β, IL-10, IL-17, RANKL, interferon (IFN)-γ and transforming growth factor (TGF)-β1 were quantified using real-time reverse transcription-polymerase chain reaction. The levels of these markers were compared between active and inactive periodontal lesions.

Results: In active periodontal lesions, Foxp3, T-bet, RANKL, IL-17, IL-1β and IFN-γ were significantly over-expressed compared with inactive lesions. The expression of IFN-γ was the highest within the active periodontal lesions, similar to that of TGF-β1 within the inactive ones. There was a positive correlation between RANKL and IL-17. Additionally, RANKL and IL-17 were positively correlated with RORC2, but no correlation was detected with Foxp3.

Conclusions: These results lead us to speculate that Foxp3^{+} cells that do not have a regulatory function might have a role in the pathogenesis of active periodontal lesions by down-regulating TGF-β1 and IL-10 synthesis that lead to the over-expression of Th17-associated cytokines RANKL and IL-17.

The immune system has evolved to protect higher organisms from infection by microorganisms and parasites. It functions *via* specialized cells and molecules that are able to distinguish between self-antigens and foreign antigens, being tolerant to self-antigens and responding to foreign antigens in order to control and eliminate them. Tolerance to self-antigens is attained initially by eliminating self-reactive T- and B-lymphocytes during their negative selection in the thymus and the bone marrow, respectively. In addition, the immune system has peripheral mechanisms to deal with lymphocytes that escape this central tolerance system. These peripheral mechanisms are based mainly in T regulatory (Treg) cells (Vernal & Garcia-Sanz 2008).

Treg cells control the induction and activity of effector T cells and thereby prevent and control exacerbated immune responses and the development of autoimmune diseases (Green et al. 1983). These regulatory functions are mediated by the expression of the transcription factor forkhead box P3 (Foxp3) and involve the suppression of the activation of T-, B- and natural killer cells through direct cell–cell mechanisms, mediated by surface molecules, such as cytotoxic T-lymphocyte antigen-4 (CTLA-4), or by the synthesis of immuno-suppressive cytokines, such as interleukin (IL)-10 and transforming growth factor (TGF)-β (McGuirk & Mills 2002, Shevach 2002, Mittrucker & Kaufmann 2004, Horwitz et al. 2008, Shevach et al. 2008).

In fact, increased proportions of $CD4^+CD25^+CTLA-4^+$ cells and an elevated expression of Foxp3 have been detected in periodontitis when compared with that in the gingivitis lesions (Nakajima et al. 2005). Furthermore, a majority ($\sim 90\%$) of T-cell clones derived from gingival tissues with periodontitis lesions expressed Foxp3, which may be induced in $CD4^+$ T cells either by polyclonal or by antigen-specific stimulation (Ito et al. 2005, Okui et al. 2008). The expression of Foxp3 has been positively correlated to both IL-10 and TGF-β1 expression in periodontitis lesions; however, only TGF-β1, but not IL-10, was significantly over-expressed in periodontitis when compared with that in the gingivitis lesions (Nakajima et al. 2005). Conversely, on comparing with healthy gingival tissues, Ernst et al. showed that $CD25^+Foxp3^+$ cells were decreased in periodontitis lesions, while the levels of the receptor activator of nuclear factor-κ B ligand (RANKL), a key factor that mediates the osteoclast differentiation and maturation, were negatively correlated to IL-10 and $CD25^+Foxp3^+$ cells, suggesting that a lower number of Treg cells is associated with over-regulation of the RANKL expression in these affected tissues (Ernst et al. 2007).

In spite of the experimental evidence for the existence of Treg cells in periodontitis-affected tissues, their role during the initiation and progression of this disease still remains unclear. Whether the number and activity of Treg cells are enhanced or diminished in chronic periodontitis compared with healthy or gingivitis tissues still needs to be better clarified, and their presence should be correlated with the activity of periodontal destruction. With this purpose, we have analysed the expression of Foxp3, CTLA-4, IL-10 and TGF-β1 in gingival tissues from patients undergoing periodontitis progression, and associated these results to the T helper type 1 (Th1), Th2 and Th17 transcription master factors, T-bet, GATA-3 and transcription factor orphan nuclear receptor C2 (RORC2), respectively, as well as RANKL and IL-17 bone destruction-associated cytokines.

Materials and Methods
Patients

Seventy-six patients affected with chronic periodontitis were consecutively enrolled at the Center of Diagnosis and Treatment Dra. Eloisa Díaz in the Northern Metropolitan Health Service, Santiago, Chile. The criteria for patient selection were as follows: (a) a minimum of 14 natural teeth, excluding third molars and including at least 10 posterior teeth, (b) no previous periodontal therapy, (c) absence of relevant systemic diseases and (d) no intake of antibiotics or non-steroid anti-inflammatory drugs in the previous 6 months. The protocol of the study was clearly explained to all the participants who agreed to participate in the study by signing an IRB-approved informed consent form.

Clinical measurements and diagnosis of chronic periodontitis

At baseline, after receiving a supragingival prophylaxis to remove gross calculus, all the selected patients underwent a periodontal clinical examination by a single calibrated examiner (J. G.), who registered probing pocket depths (PD), clinical attachment level (CAL) loss and dichotomous measurements of supragingival bacterial plaque (BP) and bleeding on probing (BOP) at six periodontal sites in all teeth, excluding third molars. The diagnosis of moderate to advanced chronic periodontitis was established by having at least six teeth with PD $\geqslant 5$ mm and CAL $\geqslant 3$ mm, together with generalized radiographic bone loss ($\geqslant 30\%$ of the periodontal sites), according to a classification system of periodontal diseases and conditions (Armitage 1999).

Determination of progressive periodontal destruction

Patients were re-examined every 2 months and progression of chronic periodontitis was determined by the tolerance method and when at least two active periodontal lesions were detected in a patient, as described previously (Haffajee et al. 1983, Hernandez et al. 2006, Silva et al. 2008). A periodontal lesion was defined as an active periodontal lesion when, in a patient undergoing progressive periodontitis, the periodontal site exhibited an increase in CAL $\geqslant 2.0$ mm during a 2-month interval. In the same patient, an inactive periodontal lesion had severity in terms of CAL, PD and BOP equivalent to an active lesion, but exhibited an increase in CAL < 2.0 mm during the same time interval. Ten patients undergoing periodontitis progression were included in this study and, within 1–2 weeks of detecting their disease activity, they were provided with appropriate periodontal therapy.

Gingival tissue biopsies

From each patient, specimens of gingival tissue were collected from one active and one inactive periodontal lesion under appropriate local anaesthesia and following the patient's periodontal therapy requirements. Samples were minced in approximately 1 mm^3 fragments, immediately submerged in 500 μl RNA-Safer Stabilizer Reagent (Omega Biotek Inc., GA, USA) and transported at 4°C to be processed for RNA extraction.

Isolation of cytoplasmic RNA

Tissue samples were homogenized in 2 ml of 4 M guanidinium thiocyanate in 0.05 M sodium citrate (pH 7.0), supplemented with 1% sarcosyl and 100 mM β-mercaptoethanol (Fluka, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Homogenized samples were transferred to an RNase-free tube and 200 μl of 2 M sodium acetate (pH 4.5), 2 ml of water-saturated phenol and 400 μl of chloroform-isoamylalcohol (24:1) (Fluka) were added. After incubation for 15 min. at 4°C and centrifugation at 3000 g for 30 min. at 4°C, the aqueous phase was transferred to a fresh RNase-free tube and mixed with 1 volume of isopropyl alcohol (Merck & Co. Inc., Whitehouse Station, NJ, USA) and precipitated overnight at -20°C. Then, samples were centrifuged at 8000 g for 30 min. at 4°C, lysed in 400 μl of ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% SDS, 10 mM EDTA pH 8.0 (Fluka) and 25 $\mu g/ml$ proteinase K (Roche Ltd., Basel, Switzerland) and incubated for 30 min. at 37°C. Subsequently, RNA was extracted with 400 μl of chloropan (phenol:chloroform:isoamylalcohol 25:24:1) and centrifuged for 5 min. at 14,000 g at room temperature (Fluka). The RNA present in the aqueous solution was precipitated overnight at -20°C with 0.3 M Na-acetate, 2.5 volumes of 100% ethanol and 1 μl of glycogen 20 $\mu g/\mu l$ (Roche), recovered by centrifugation performed for 30 min. at 14,000 g at 4°C and resuspended in 20 μl RNase-free H_2O. RNA quality was determined in a bioanalyser (Agilent 2100B, Agilent Tech., Palo Alto, CA, USA). Cytoplasmic RNA was quantified using a spectrophotometer (Nanodrop ND-1000, Nanodrop Tech., Wilmington, ND, USA) and stored at -80°C at a final concentration of 1 $\mu g/\mu l$.

First-strand cDNA synthesis

Reverse transcription was performed using a Transcriptor First Strand cDNA synthesis kit (Roche) following the manufacturer's recommendations. Briefly, 20 μl reaction containing 4 μl 5x RT buffer, 0.5 μl RNase inhibitor (20 U), 2 μl deoxynucleotide mix (1 mM each), 2 μl random hexamer primer (60 μM), 0.5 μl reverse transcriptase (10 U), 10 μl RNA-grade H_2O and 1 μl RNA sample (1 μg) were retro-transcribed under the following conditions: 10 min. at 25°C

and 1 hour at 50°C (Primus 96 plus, MWG Biotech AG, Ebersberg, Germany). The reverse transcriptase was subsequently inactivated by incubating the sample at 85°C for 5 min.

Quantitative real-time polymerase chain reaction (PCR)

The mRNA expression levels of the cytokines IL-1β, IL-10, IL-17, RANKL, interferon (IFN)-γ and TGF-β1; the surface markers CD4, CD8, CD25 and CTLA-4; and the transcription factors Foxp3, T-bet, GATA-3 and RORC2 were quantified using real-time quantitative PCR. As an endogenous control, the expression of 18S rRNA was determined. Fifty nanogram of cDNA were amplified in 384-well plates using a FastStart Taqman Probe Master (Roche) following the manufacturer's recommendations. Forward and reverse primers were designed and a specific FAM dye-labelled probe was selected using the Roche website http://www.roche-applied-science.com (Table 1). Ten microlitre reaction containing 0.1 μl PCR probe (250 nM), 0.1 μl forward primer (900 nM), 0.1 μl reverse primer (900 nM), 5 μl FastStart master, 3.7 μl PCR-grade H_2O and 1 μl cDNA (50 ng) were amplified under the following conditions: 10 min. at 95°C and 40 cycles of 15 s at 95°C and 1 min. at 60°C, in an ABI PRISM 7900 Sequence Detector System (Applied Biosystems, Foster City, CA, USA).

Data analysis

Clinical and experimental data were analysed using the statistical software SPSS 15.0 (Lead Technologies Inc., Charlotte, NC, USA). For each patient, the mean results from each analysed clinical parameter at the baseline and after the longitudinal analysis were generated and expressed as subject means ± SD (Table 2). The Kolmogorov–Smirnov test demonstrated that these clinical data were normally distributed. Comparisons in age, PD and CAL results were made using the two-tailed unpaired Student's t-test, while for BP, BOP and gender data the χ^2 test was used. Quantitative real-time PCR data were analysed with the ABI PRISM Sequence Detector Systems software (Applied Biosystems) and were expressed as C_t (cycle threshold), ΔC_t (C_t mRNA blank – C_t 18S rRNA) and relative quantification (RQ). RQ was obtained using the $2^{-\Delta\Delta C_t}$ method, adjusting the blank mRNA expression to 18S rRNA expression and considering the adjusted expression in inactive periodontal lesions as a reference (RQ = 1) (Livak & Schmittgen 2001). When the RQ levels of IL-10 and TGF-β1 were calculated, the adjusted expression in active periodontal lesions was considered as a reference. The amplification efficiency of each gene reported to 18S rRNA expression was evaluated analysing the ΔC_t variation with template dilutions having a 100-fold range.

Table 1. Forward primers, reverse primers and PCR probes used for cytokine, surface marker and transcription factor amplifications by quantitative real-time PCR

	Forward primers	Reverse primers	Probes*
IL-1β	ctgtcctgcgtgttgaaaga	ttgggtaattttgtgggatctaca	78
IL-10	tgggggagaacctgaagac	ccttgctcttgtttcacagg	30
IL-17	tgggaagacctcattggtgt	ggatttcgtgggattgtgat	8
RANKL	tgattcatgtaggagaattaaacagg	gatgtgctgtgatccaacga	17
IFN-γ	ggcattttgaagaattggaaag	tttggatgctctggtcatctt	21
TGF-β1	cacgtggagctgtaccagaa	cagccggttgctgaggta	72
CD4	ggcagtgtctgctgagtgac	gaccatgtgggcagaacc	18
CD8	cgcctgtgatatctacatctgg	aaacacgtcttcggttcctg	2
CD25	caagcgagccttccagatt	ggccactgctacctggtact	46
CTLA-4	ttcatccctgtcttctgcaa	cacacaaagctggcgatg	66
Foxp3	acctacgccacgctcatc	tcattgagtgtccgctgct	50
T-bet	tccaagtttaatcagcaccaga	tgacaggaatgggaacatcc	9
GATA-3	ctcattaagcccaagcgaag	tctgacagttcgcacaggac	71
RORC2	agaaggacagggagccaag	caagggatcacttcaatttgtg	21
18S rRNA	ctcaacacgggaaacctcac	cgctccaccaactaagaacg	77

*Number of the specific FAM dye-labelled probes selected from the quantitative real-time PCR system of Roche.

IL, interleukin; PCR, polymerase chain reaction; RANKL, receptor activator of nuclear factor κB ligand; IFN, interferon; TGF, transforming growth factor; CTLA-4, cytotoxic T-lymphocyte antigen-4; Foxp3, transcription factor forkhead box P3; RORC2, transcription factor orphan nuclear receptor C2.

Table 2. Clinical characteristics of the chronic periodontitis patients at baseline and of the active and inactive periodontal lesions sampled from patients who underwent progression of chronic periodontitis

	Total subjects	Patients with progressive periodontitis	
	baseline ($n = 76$)	active lesions ($n = 10$)	inactive lesions ($n = 10$)
PD (mm)	3.50 ± 0.68*	4.42 ± 0.46	4.64 ± 0.62
CAL (mm)	3.90 ± 0.48†	6.20 ± 0.76	6.42 ± 0.68
BP (%)	74.1‡	100	100
BOP (%)	57.9§	100	100
Females (%)	56.6	72.2	
Age (years)	46.1 ± 7.7	45.6 ± 6.6	

*PD, total subjects *versus* active and inactive lesions: *p*-value <0.05.

†CAL, total subjects *versus* active and inactive lesions: *p*-value <0.05.

‡BP, total subjects *versus* active and inactive lesions: *p*-value <0.05.

§BOP, total subjects *versus* active and inactive lesions: *p*-value <0.05.

Clinical data are expressed as subject mean $\pm$ standard deviation.

PD, probing depth; CAL, clinical attachment level loss; BP, periodontal sites with supragingival bacterial plaque; BOP, periodontal sites with bleeding on probing.
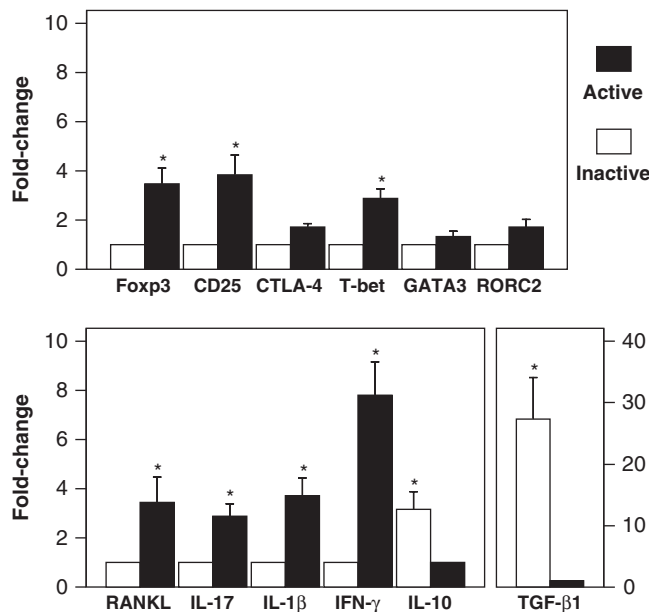

Fig. 1. Gene expressions in active and inactive periodontal lesions from patients who underwent progression of chronic periodontitis. The relative quantification (RQ) of each gene, expressed as fold-change, was obtained using the $2^{-\Delta\Delta C_t}$ method and by normalizing the mRNA expression to 18S rRNA (Livak & Schmittgen 2001). The RQ in active periodontal lesions was obtained considering the normalized expression in inactive periodontal lesions as a reference ($RQ = 1$). When the RQ of IL-10 and TGF-β1 were calculated, the adjusted expression in active periodontal lesions was considered as a reference. Foxp3, transcription factor forkhead box P3; CTLA-4, cytotoxic T-lymphocyte antigen-4; RORC2, transcription factor orphan nuclear receptor C2; RANKL, receptor activator of nuclear factor κB -ligand; IL, interleukin; IFN, interferon; TGF, transforming growth factor. *p-value <0.05.

After determining the normality of the data distribution using the Kolmogorov–Smirnov test, the differences among the mRNA expressions of the analysed transcription factors and cytokines were determined analysing the ΔC_t data and using either the Wilcoxon test or the paired Student's *t*-test. Correlation coefficients were obtained analysing the RQ data and using the Pearson test. A statistical significance was considered when *p*-value <0.05.

Results

Table 2 summarizes the clinical characteristics of all the chronic periodontitis patients evaluated at the beginning of the investigation. Similarly, the clinical characteristics of the active and inactive periodontal lesions sampled for real-time quantitative PCR analysis from patients who underwent progression of chronic periodontitis are given. From a total of 76 patients diagnosed with chronic periodontitis, 10 patients undergoing disease progression were included in this investigation. At the time of surgical therapy and when the biopsy was taken, there were no significant differences in the clinical parameters between active and inactive periodontal lesions.

The expression of mRNA for Foxp3, T-bet, GATA-3, RORC2, IL-1β, IL-10, IL-17, RANKL, IFN-γ, TGF-β1, CD4, CD8, CD25 and CTLA-4 was detected in all the tissue samples of both active and inactive periodontal lesions. The fold-change of RQ for each studied cytokine, cell marker or transcription factor was expressed as the mean and standard deviation (Fig. 1). In active periodontal lesions, Foxp3 was significantly over-expressed (3.4-fold) compared with inactive periodontal lesions. Similarly, CD25 (3.8-fold) and T-bet (2.8-fold) were also significantly over-expressed in active periodontal lesions. CTLA-4, GATA-3 and RORC2 ($\sim$ 1.5-fold) over-expression, however, was not statistically significant.

IFN-γ was the highest over-expressed cytokine detected within active periodontal lesions, with an RQ of 7.8 (Fig. 1). Similarly, in active periodontal lesions, RANKL (3.4-fold), IL-17 (2.8-fold) and IL-1β (3.7-fold) were also significantly over-expressed compared with inactive periodontal lesions. IL-10 and TGF-β1 were, however, down-regulated in the active periodontal lesions (RQ of 0.52 and 0.08, respectively). When the RQ was re-calculated considering the adjusted IL-10 and TGF-β1 expression in the active lesions as $RQ = 1$, the expression of IL-10 and TGF-β1 in the inactive periodontal lesions was 3.1- and 27.3-fold higher, respectively, than the levels detected in the active periodontal lesions (Fig. 1).

The analyses of correlations between the different genes yielded significant positive correlations between Foxp3 and CD25, Foxp3 and CD4 and CD4 and CD25 ($p < 0.001$); however, Foxp3 and CTLA-4, Foxp3 and CD8 and CD4 and CD8 did not show any positive correlation (Fig. 2). In the analysis of cytokine expression, positive correlations were detected between RANKL and IL-1β ($p < 0.001$), and IFN-γ ($p = 0.004$) and IL-17 ($p < 0.001$). In contrast, RANKL was negatively correlated with TGF-β1 ($p = 0.042$) and did not correlate with IL-10 (Fig. 3).
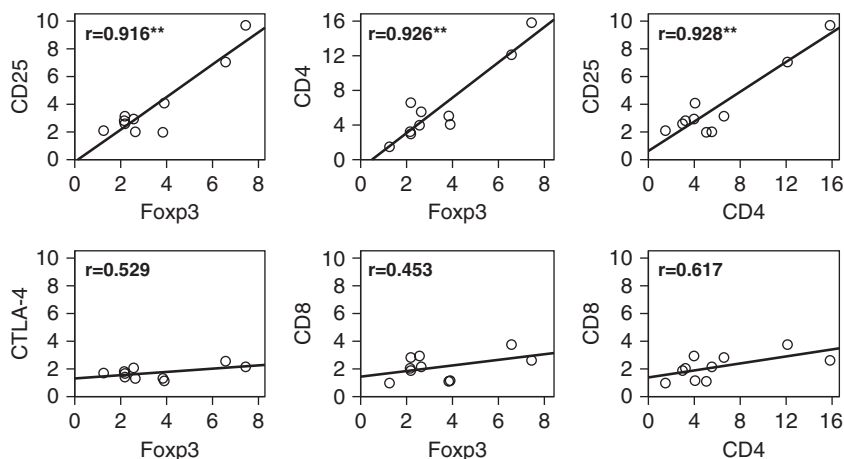
Fig. 2. Correlations of gene expressions of Foxp3, CD25, CTLA-4, CD4 and CD8 in patients who underwent progression of chronic periodontitis. The Pearson correlation coefficients (r) were calculated in 10 chronic periodontitis patients (circles). Foxp3, transcription factor forkhead box P3; CTLA-4, cytotoxic T-lymphocyte antigen-4. **p-value <0.01.
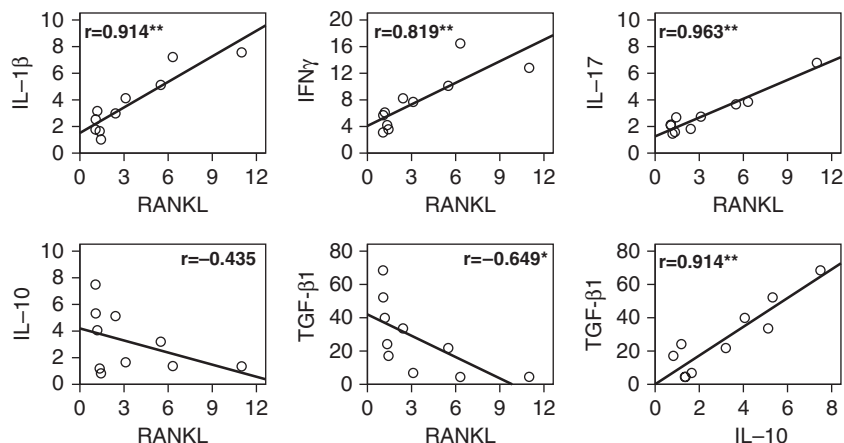

Fig. 3. Correlations of gene expressions of RANKL, IL-1β, IL-10, IL-17, IFN-γ and TGF-β1 in patients who underwent progression of chronic periodontitis. Pearson correlation coefficients (r) were calculated in 10 chronic periodontitis patients (circles). RANKL, receptor activator of nuclear factor κB -ligand; IL, interleukin; IFN, interferon; TGF, transforming growth factor. *p-value <0.05, **p-value <0.01.

The analysis of correlations between RANKL and IL-17 with the different transcription factors studied yielded a positive correlation with RORC2 ($p<0.001$), but not with Foxp3 (Fig. 4). A positive correlation was also detected between RANKL and T-bet ($p = 0.015$) and between T-bet and RORC2 ($p = 0.015$). Foxp3 was not correlated with any of the other transcription factors (Fig. 5); however, there was a negative correlation between T-bet and GATA-3 ($p = 0.002$).

Discussion

The existence, among the T-lymphocyte population, of cells harbouring regulatory functions, able to modulate the immune response during the course of an infection, has been established (Bluestone & Abbas 2003, Mittrucker & Kaufmann 2004). These Treg cells are characterized by the expression of the surface antigen CD25 and the transcription factor Foxp3, which represents a Treg cell lineage-specific marker (Bluestone & Abbas 2003, Hori & Sakaguchi 2004, Mittrucker & Kaufmann 2004, Roncarolo & Gregori 2008). Immunohistological analyses have shown higher numbers of CD4^{+}CD25^{+}CTLA-4^{+} Treg cells in samples from periodontitis-affected patients as compared with gingivitis controls. Furthermore, increased Foxp3 mRNA levels were also detected in these samples (Nakajima et al. 2005).

In our investigation, Foxp3 and CD25 mRNAs were significantly over-expressed in active when compared to inactive periodontal lesions. In addition, these over-expressed Foxp3 levels positively correlated with both CD25 and CD4 mRNA expression. These data confirm previous reports indicating that Foxp3^{+} T cells may be involved in the pathogenesis of chronic periodontitis (Ito et al. 2005, Nakajima et al. 2005, Ernst et al. 2007, Okui et al. 2008). On the other hand, it has been shown that low levels of Foxp3 expression were detected in CD8^{+} T cells (Hori et al. 2003, Okui et al. 2008), and Th1 and Th2 cells generated from CD4^{+}CD25^{−} cells failed to express Foxp3 (Hori & Sakaguchi 2004). These findings are consistent with our results in the chronic periodontitis specimens, where Foxp3 expression was not correlated with CD8, T-bet and GATA-3 expression in active periodontal lesions.

Treg cells function through CTLA-4-mediated cell–cell interactions with dendritic cells (DCs) and effector T cells (McGuirk & Mills 2002, Shevach 2002). CTLA-4-expressing Treg cells induce the expression of the enzyme indoleamine 2,3-dioxygenase, which degrades tryptophan, and in fact, the lack of this essential amino acid inhibits the T-cell activation and promotes T-cell apoptosis (Fallarino et al. 2003). TGF-β1 and IL-10 are also important mediators of these regulatory activities (Mittrucker & Kaufmann 2004, Horwitz et al. 2008, Shevach et al. 2008). TGF-β1-systhesizing Treg cells directly target T cells to ensure immune tolerance to self-antigens, whereas IL-10 inhibits the function of DCs by inhibiting the MHC class II and co-stimulatory-molecule expression (Moore et al. 2001, Li & Flavell 2008). In this investigation, we have shown that CTLA-4 was not significantly over-expressed in active periodontal lesions and this expression was not correlated with Foxp3 over-expression. Furthermore, TGF-β1 and IL-10 expression was down-regulated in active periodontal lesions when compared with their expression in in active ones. Hence, Foxp3-expressing cells do not probably fulfill their regulatory functions through the expression of CTLA-4, TGF-β1 or IL-10 in periodontal lesions showing clinical evidence of periodontal tissue destruction.

It has been previously described that Foxp3^{+} gingival T cells are functionally different from natural Treg cells (Okui
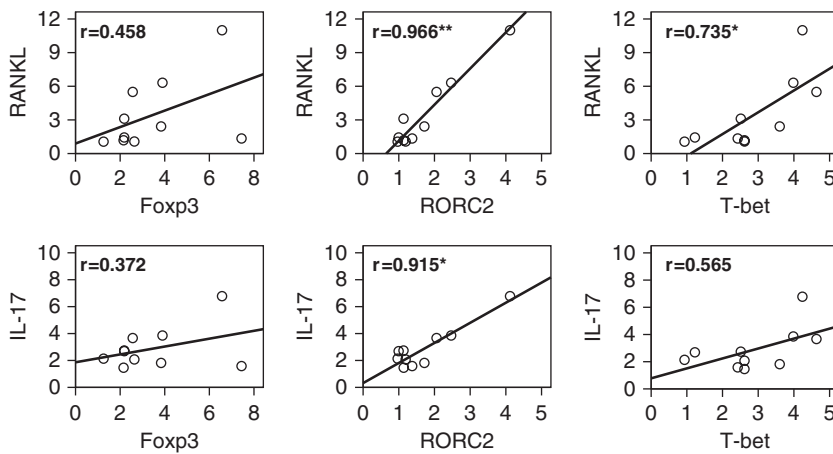
Fig. 4. Correlations of gene expressions of RANKL, IL-17, Foxp3, T-bet and RORC2 in patients who underwent progression of chronic periodontitis. Pearson correlation coefficients (r) were calculated in 10 chronic periodontitis patients (circles). RANKL, receptor activator of nuclear factor κB -ligand; IL, interleukin; Foxp3, transcription factor forkhead box P3; RORC2, transcription factor orphan nuclear receptor C2. *p-value <0.05, **p-value <0.01.
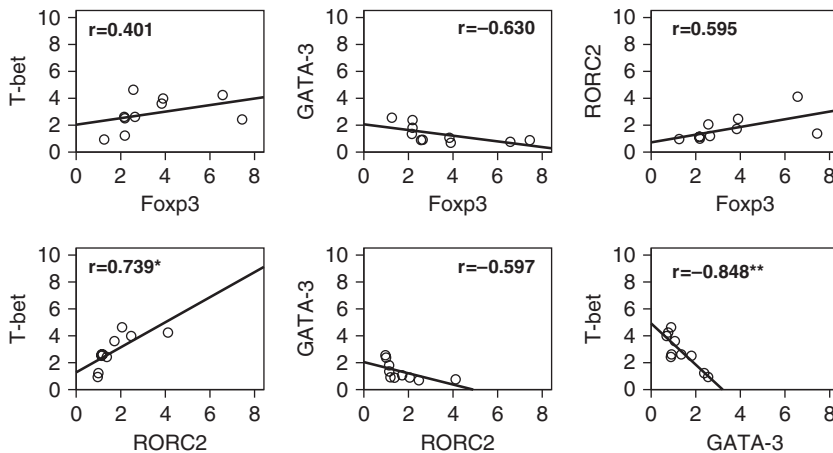

Fig. 5. Correlations of gene expressions of Foxp3, T-bet, GATA-3 and RORC2 in patients who underwent progression of chronic periodontitis. Pearson correlation coefficients (r) were calculated in 10 chronic periodontitis patients (circles). Foxp3, transcription factor forkhead box P3; RORC2, transcription factor orphan nuclear receptor C2. *p-value <0.05, **p-value <0.01.

et al. 2008). In this regard, a transient wave of Foxp3 expression is not sufficient to confer regulatory activity (Allan et al. 2007); rather, a high and sustained Foxp3 expression induced by TCR-stimulation is required to generate functional Treg cells (Passerini et al. 2008). In fact, a combination of strong antigenic signals to TCR and maximal co-stimulation of CD80 is required for Treg-cell development and survival (Salomon et al. 2000, Bensinger et al. 2001). Thus, the absence of CD80 results in a decreased number of Treg cells, which induces autoimmunity (Lenschow et al. 1996, Salomon et al. 2000). A previous study from our research group has demonstrated that CD80 expression by DCs is dependent

on both the type of periodontopathogenic bacteria and the bacterial load (Vernal et al. 2008). Therefore, not only the composition but also the concentration of each strain in the subgingival biofilm may determine the CD80 expression and, thus, the intensity of the stimulus required for Treg-cell development and function. A weak CD80/CD28 signalling could determine a transient Foxp3 expression, which might explain previous reports showing an increased expression of Foxp3 in periodontitis when compared with that in gingivitis and diminished Foxp3^{+} cells in periodontitis when compared with that in healthy controls (Nakajima et al. 2005, Ernst et al. 2007). Similarly, nonfunctional Treg cells characterized by a

transient and weak Foxp3 expression could be responsible for the down-regulation of the TGF-β1 and IL-10 levels detected in active periodontal lesions in this investigation.

One of the main concerns in chronic periodontitis is the definition of active periodontal lesions where tissue destruction is occurring. An active periodontal lesion has been characterized by a prominent infiltration of activated T cells (Okada et al. 1983, Malberg et al. 1992), which express bone destruction-associated cytokines such as IL-17 and RANKL (Takahashi et al. 2005, Vernal et al. 2005, 2006, Kawai et al. 2006). In our study, RANKL expression was positively correlated with IL-17 expression and both of them showed a positive correlation with RORC2, a transcription factor considered the master switch for Th17 differentiation; however, neither RANKL nor IL-17 was correlated with Foxp3 expression. It has been reported that Treg-cell-associated cytokines can suppress the RANKL expression by antigen-specific stimulated peripheral blood cells (Ernst et al. 2007); however, there is scarce information on the relationships between IL-17 and Foxp3^{+} cells.

T-cell phenotype has been proposed as a risk factor for periodontal disease (Houri-Haddad et al. 2007). Studies regarding the role of cytokines in bone tissue have associated Th1 and Th2 cytokines with activation and suppression of bone destruction during periodontitis, respectively; however, further subtypes of T cells, with cytokine profiles and/or immunosuppressive functions distinct from either Th1 or Th2 cells, termed Th17 and Treg cells, have been associated with periodontal bone destruction (Nakajima et al. 2005, Kramer & Gaffen 2007). Although Treg and Th17 cells play different roles during the pathogenesis of infections, they arise from the same precursor cells (Bettelli et al. 2006). Naïve T cells exposed to TGF-β up-regulate Foxp3 and differentiate to Treg cells; however, when cultured with TGF-β and IL-6, naïve T cells transform into Th17 cells (Bettelli et al. 2006, Yamazaki et al. 2007). Thus, when the immune response is not activated, TGF-β favours the generation of Treg cells, which suppress inflammation; however, when an infection is established, IL-6 is synthesized during the innate immune response, inhibiting the generation of Treg cells and inducing the differentiation of proinflamma-

tory Th17 cells in the presence of TGF-β (Bettelli et al. 2007). Therefore, selective T-cell differentiation would depend on the local cytokine milieu during infection, which would determine the predominance of either pathologic (Th1 and/or Th17 cells) or protective activities (Th2 and/or Treg cells) and, thus, the outcome of the periodontal disease.

In summary, our results lead us to speculate that Foxp3^{+} cells that do not bear a regulatory function might have a role in the pathogenesis of active periodontal lesions by down-regulating TGF-β1 and IL-10 synthesis that lead to the over-expression of Th17-associated cytokines RANKL and IL-17.

Acknowledgements

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Clinical Relevance

Scientific rationale for the study: Although periodontopathogens are the primary aetiological agents of periodontitis, the ultimate determinant of disease progression and clinical outcome is the host's immune response. Foxp3^{+} cells are the key element in the regulatory network that controls immune response during an infection.

Principal findings: A Foxp3 over-expression was associated with active periodontal lesions; however, Foxp3^{+} T-cells that do not bear regulatory functions may have a role in periodontal destruction due to the down-regulation of IL-10 and TGF-β1.

Practical implications: Patient susceptibility to periodontal destruction is determined by the balance between effector and regulatory mechanisms developed during the immune response against periodontopathogens. Understanding these regulatory mechanisms should provide a greater insight into the pathogenesis, and this may lead to the development of new immunotherapeutic strategies.