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Activation of RANKL-induced osteoclasts and memory T lymphocytes by *Porphyromonas gingivalis* is serotype dependant

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Abstract

Aim: Destructive periodontitis is associated with a Th1–Th17 immune response and activation of RANKL-induced osteoclasts. In addition, *Porphyromonas gingivalis* K1 and K2 serotypes induce a strong Th1–Th17 response. This study aimed to investigate whether these *P. gingivalis* serotypes induce higher osteoclasts activation, by increased Th17-associated RANKL production, and an antigen-specific memory T-lymphocyte response.

Material and Methods: The RANKL production and $TRAP^+$ osteoclast induction were quantified on naïve T lymphocytes stimulated with dendritic cells primed with the *P. gingivalis* serotypes. The T-bet, GATA-3, RORC2 and Foxp3 expression was correlated with RANKL production. The frequency of proliferating memory T lymphocytes in response to *P. gingivalis* serotypes was determined in both periodontitis and healthy subjects.

Results: T lymphocytes stimulated by K1 or K2-primed dendritic cells elicited higher levels of RANKL and $TRAP^+$ osteoclasts than cells stimulated with the other serotypes. RANKL positively correlated with RORC2. Whereas periodontitis patients had a higher frequency of memory T lymphocytes responding to K1 or K2, healthy subjects had a higher frequency of memory T lymphocytes responding to K4 or K^-.

Conclusions: *P. gingivalis* serotypes K1 and K2, but not others, are associated with an increased production of the osteoclastogenesis-related factor RANKL. This important information suggests that these serotypes could elicit a greater bone resorption in vivo and have a role in the periodontitis pathogenesis.

MS and JAG-S contributed equally to this work and should be considered as joint last authors.

Key words: bone resorption; periodontitis; *Porphyromonas gingivalis*; RANKL; serotypes; T lymphocytes

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Conflict of interest and source of funding statement

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Periodontitis is one of the most common human infectious diseases and one of the primary causes of tooth loss in adults (Tonetti & Claffey 2005, Cochran 2008). It is characterized by the destruction of periodontal connective tissues and

resorption of the alveolar bone that surrounds the teeth (Cochran 2008, Buduneli & Kinane 2011, Hernandez et al. 2011). This destructive process is initiated by specific bacteria within the subgingival biofilm and progresses because of host's immunoinflammatory mechanisms triggered in responses to these bacteria, which are mainly dependent on T-lymphocyte phenotype and function (Gemmell et al. 2007, Houri-Haddad et al. 2007, Garlet 2010, Graves et al. 2011). Although a number of Gram-negative anaerobic bacteria have been implicated in this disease process, *Porphyromonas gingivalis* is considered a major aetiological agent of periodontitis (van Winkelhoff et al. 1988, Slots & Ting 1999, Herrera et al. 2008). In fact, *P. gingivalis* promotes periodontal inflammation and alveolar bone resorption by stimulating the production of T-helper type 1 (Th1) and Th17-associated pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-12, IL-17 and IL-23, and the activation of nuclear factor kappa B (NFκB)-dependent pro-bone resorptive pathways, through receptor activator of NFκB (RANKL) signalling (Vernal et al. 2008a, 2009, Moutsopoulos et al. 2012, Baek et al. 2013, Han et al. 2013, Herath et al. 2013, Vernal et al. 2014).

P. gingivalis possesses multiple virulence factors, which enable this bacterium to evade the host tissue defence mechanisms. These key factors include its extracellular capsule and depending on the capsular (K) antigenicity different serotypes of this bacterium have been identified: K1–K6 (van Winkelhoff et al. 1993, Laine et al. 1996). Previous investigations from our research group have demonstrated that structural variations in the *P. gingivalis* capsule have a role on dendritic cell (DC) priming and subsequent antigen presentation to T lymphocytes. In fact, a heterogenic immunostimulatory potential on DCs has been detected with the six different K serotypes of *P. gingivalis* (Vernal et al. 2009). Moreover, when T lymphocytes were stimulated by DCs primed with the serotypes K1 or K2, they expressed a Th1 and Th17 pattern of immune response (Vernal et al. 2014). In particular, an increased expression of the transcription factors T-bet and RORC2, which are the master-switch genes for Th1 and Th17 differentiation, respectively, was detected in naïve T lymphocytes exposed to K1 (strain W83) or K2 (strain HG184)-primed DCs as compared with the same cells exposed to other *P. gingivalis* serotypes. These higher levels of master-switch transcription factors correlated with an increment in the production of Th1- and Th17-associated cytokines.

There is strong evidence suggesting that variations in the host immune response, in particular, in the T-lymphocyte phenotype and function, play an important role in the susceptibility, onset and severity of periodontitis (Gemmell et al. 2002, 2007, Garlet 2010, Graves et al. 2011, Hernandez et al. 2011). In general terms, a Th1- and Th17-dominated immune response has been associated with periodontitis and an increased expression of Th1- and Th17-related transcription factors and cytokines has been reported in active periodontal lesions, where alveolar bone resorption is occurring (Takahashi et al. 2005, Vernal et al. 2005, 2006, Vernal & Garcia-Sanz 2008, Dutzan et al. 2009a,b, Ohyama et al. 2009, Garlet 2010, Graves et al. 2011). It can be speculated that the pattern of T-lymphocyte response induced by K1 and K2 serotypes of *P. gingivalis* might be associated with alveolar bone resorption, which is a key factor in the pathogenesis of periodontitis. The objective of this investigation was to determine whether the different serotypes of *P. gingivalis* have a role on the differential activation of RANKL-induced osteoclasts and on the frequency of antigen-specific memory T lymphocytes. We hypothesized that the *P. gingivalis* serotypes K1- or K2-primed DCs, when used to stimulate autologous T lymphocytes, induce higher Th17-associated RANKL production and osteoclast activation as compared with the other *P. gingivalis* serotypes. In addition, we hypothesized that in periodontitis patients, the frequency of memory T lymphocytes able to respond to serotypes K1 and K2 of *P. gingivalis* is higher than in healthy subjects.

Material and Methods

P. gingivalis growth conditions and curves

The encapsulated *P. gingivalis* strains W83 (serotype K1), HG184 (K2), A7A1-28 (K3), ATCC® 49,417™ (K4), HG1690 (K5) and HG1691 (K6) and the non-encapsulated (K^-) strain ATCC® 33,277™ were cultured on 5% horse blood agar (Oxoid Nº2; Oxoid Ltd, Basingstoke, UK), supplemented with haemin (5 mg/l) and menadione (1 mg/l), under anaerobic conditions at 37°C. To obtain a reliable number of colony-forming units for the stimulation of DCs, growth curves were obtained in liquid brain-heart infusion medium (BD, San Agustin de Guadalix, Spain) as described previously (Vernal et al. 2008a).

Dendritic cell differentiation and stimulation

DCs were obtained and stimulated as described previously (Vernal et al. 2008a). From 10 healthy donors, peripheral blood mononuclear cells (PBMCs) were isolated following a Ficoll gradient (Ficoll-Paque Plus; Amersham Pharmacia Biotech, Uppsala, Sweden). Monocytes were purified from PBMCs by magnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured at 10^6 cells/ml in RPMI-1640 containing 10% FCS (Gibco Invitrogen Corp., Grand Island, NY, USA) and 1000 U/ml of rhGM-CSF and rhIL-4 (Immunotools, Friesoythe, Germany) for 6 days at 37°C to differentiate them into DCs. The DCs were then primed with increasing multiplicity of infection (MOI) of 10^{-1} to 10^3 of *P. gingivalis* strains K1–K6 for 2 days. DCs stimulated with increasing MOI of the K^- strain of *P. gingivalis* or 10 ng/ml of *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) (Fluka, Sigma-Aldrich Chemie, Buchs, Switzerland) were used for comparison. Non-induced DCs served as control.

Naïve T-lymphocyte stimulation

For each subject, autologous naïve $CD4^+$ T lymphocytes were purified by magnetic cell sorting depletion (MACS; Miltenyi Biotec) from the

PBMCs as described previously (Vernal et al. 2014). T lymphocytes were cultured at 10^6 cells/ml with primed autologous DCs (50:1) in RPMI-1640 containing 10% FCS for 5 days at 37°C. The experiment was performed separately for each subject. T-lymphocyte cultures devoid of DCs or exposed to non-induced autologous DCs were used as controls.

Expression of transcription factors and RANKL

The total cytoplasmic RNA was isolated from T lymphocytes as described previously (Vernal et al. 2008b) and the reverse transcription was performed using the Transcriptor First-Strand cDNA synthesis kit following the manufacturer's recommendations (Roche Applied Science, Mannheim, Germany). To quantify the mRNA expression for the transcription factors T-bet, GATA-3, RORC2 and Foxp3, the master-switch genes implied in the Th1, Th2, Th17 and Treg differentiation, respectively, and the pro-bone resorptive factor RANKL, 50 ng of cDNA were amplified by quantitative real-time PCR, using the appropriate primers and probes (Table 1) and the FastStart Taqman Probe Master reagent (Roche), in an ABI PRISM 7900 Sequence Detector System (Applied Biosystems, Foster City, CA, USA) as follows: 95°C for 3 min., followed by 40 cycles of 95°C for 3 s and 60°C for 30 s, and finally a melt curve of 95°C for 15 s, 60°C for 1 min. and 95°C for 15 s, for detection of non-specific product formation and false-positive amplification. 18S rRNA expression levels were used as endogenous control.

Secretion of RANKL

Once the T-lymphocyte culture supernatants were collected, the secretion of RANKL was measured by ELISA according manufacturer's protocol (Quantikine; R&D Systems Inc., Minneapolis, MN, USA) using an automatic microplate spectrophotometer at 490 and 630 nm (Labsystem Multiskan, Helsinki, Finland).

Osteoclastogenesis assay

T-lymphocyte culture supernatants from each condition were used to carry out in vitro osteoclastogenesis assays by determining the number of cells expressing the osteoclast-specific marker tartrate-resistant acid phosphatase (TRAP). Briefly, mouse macrophage-monocyte RAW 264.7 (ATCC® TIB-71™) cells were seeded in 96-well plates at a density of 1×10^3 cells/well in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 10 ng/ml rhRANKL (R&D Systems Inc.). After 24 h, cells were washed three times with fresh medium devoid of rhRANKL and cultured in presence of each T-lymphocyte culture supernatant for 5 days. Negative control wells received fresh DMEM. T-lymphocyte cultures devoid of DCs or exposed to non-induced autologous DCs were used for comparison. $TRAP^+$ activity was determined using a leucocyte acid phosphatase kit following the manufacturer's recommendations (Sigma-Aldrich, St. Louis, MO, USA) and observed using a microscope (AxiostarPlus; Zeiss, Germany). $TRAP^+$ cells with three or more nuclei were considered osteoclasts.

Subject selection

Eleven participants were consecutively enrolled at the Diagnosis Center of the Dental School, Universidad de Chile. Among them, eight participants were diagnosed as having chronic periodontitis, whereas three healthy subjects were included as controls. The inclusion criteria were as follows: A minimum of 14 natural teeth, excluding third molars and including at least 10 posterior teeth. Participants had not received any periodontal treatment before the time of examination, did not suffer any relevant systemic disease and had not received antibiotics or non-steroidal anti-inflammatory therapy in the 6-month period before the study. Chronic periodontitis was defined as having at least five teeth with periodontal sites with probing depth (PD) ≥5 mm, clinical attachment level (CAL) ≥3 mm and extensive bone loss determined radiographically. The healthy control group was selected from volunteers with no evidence of periodontal disease determined by the absence of CAL or increased PD. The protocol of the study was clearly explained to all the participants who agreed to participate in the study by signing an internal review board-approved informed consent form.

Frequency of memory T-lymphocyte responses to specific K antigens

For quantifying the frequency of K antigen-specific responding memory T lymphocytes, memory $CD4^+$ T lymphocytes were isolated from the periodontitis and control subjects by magnetic cell sorting depletion (MACS; Miltenyi Biotec). Memory T lymphocytes were cultured at 10^6 cells/ml with autologous DCs primed with the *P. gingivalis* strains K^-, K1, K2, or K4 (50:1) and transferred to 96-well round-bottom microtitre plates and plated at log 3 dilutions in 200 μl/well RPMI-1640 containing 10% FCS for 5 days at 37°C. 1 μCi/well [methyl-^{3}H]thymidine ([^{3}H]dThd; Hartmann Analytic GMBH, Braunschweig, Germany) was added during the last 18 h of culture and beta emission was quantified. T lymphocytes exposed to non-induced autologous DCs were used as control. The frequency of T-lymphocyte responses was measured with an overnight pulse of [^{3}H]dThd incorporation at the end of the cell culture. Wells were considered positive when the incorporated [^{3}H]dThd counts exceeded the mean counts of control wells by more than three SD as described (Erard et al. 1985).

Table 1. Forward primers, reverse primers and PCR probes used for transcription factors and RANKL mRNA and 18S rRNA amplifications by quantitative real-time PCR

mRNA	Forward primer	Reverse primer	PCR probe*
T-bet	tccaagtttaatcagcaccaga	tgacaggaatgggaacatcc	9
GATA-3	ctcattaagcccaagcgaag	tctgacagttcgcacaggac	71
RORC2	agaaggacagggagccaag	caagggatcacttcaatttgtg	21
Foxp3	acctacgccacgctcatc	tcattgagtgtccgctgct	50
RANKL	tgattcatgtaggagaattaaacagg	gatgtgctgtgatccaacga	17
18S rRNA	ctcaacacgggaaacctcac	cgctccaccaactaagaacg	77

*Number of the specific FAM dye-labelled probe (Roche).

Data analysis

The quantitative PCR data were analysed using the ABIPRISM software (Applied Biosystems) and the relative quantification was obtained using the $2^{-\Delta\Delta Ct}$ method and by normalizing the mRNA expression to 18S rRNA. For limiting dilution assays, the frequency of wells with memory T lymphocytes able to proliferate in each experimental condition was determined by assuming a Poisson single-hit model and represented showing the estimated responding cells per well and the frequency of negative cultures (Diaz-Guerra et al. 2007). Data were statistically analysed using the SPSS 15.0 software (Lead Technologies Inc., Charlote, NC, USA). The normality of data distribution was determined using the Shapiro–Wilk test. Differences between groups and within each group regarding the transcription factor mRNA expression, RANKL expression and secretion and $TRAP^+$ osteoclast induction were determined using the Kruskal–Wallis test or ANOVA and Tukey tests. Correlation coefficients were obtained using the Pearson test. Statistical significance was assumed when p-value < 0.05.

Results

RANKL expression and secretion in response to *P. gingivalis* K serotypes

As periodontitis is characterized by the alveolar bone resorption produced by RANKL-induced osteoclasts, the association between the immunogenicity of the K serotypes of *P. gingivalis* and the induction of osteoclast differentiation and activation was analysed by quantifying the mRNA expression and secretion levels of RANKL on stimulated T lymphocytes. A dose-dependent increase in the RANKL mRNA expression levels was elicited on T lymphocytes following DC stimulation with each of the K strains of *P. gingivalis* (Fig. 1a). When the serotype K1 (W83) or K2 (HG184) was used for T-lymphocyte stimulation, higher expressed levels of RANKL were detected compared with the same cells stimulated with the K3–K6 or K^- strains of *P. gingivalis* ($p < 0.05$ at MOIs = 10^{-1}–10^3). This higher expression of RANKL was confirmed at a protein level in the supernatant of the T-lymphocyte cultures, where higher levels of secreted RANKL were detected in T lymphocytes stimulated with K1- or K2-primed DCs compared with the K3–K6 or K^- strains of *P. gingivalis* ($p < 0.05$ at a MOI = 10^3) (Fig. 1b).

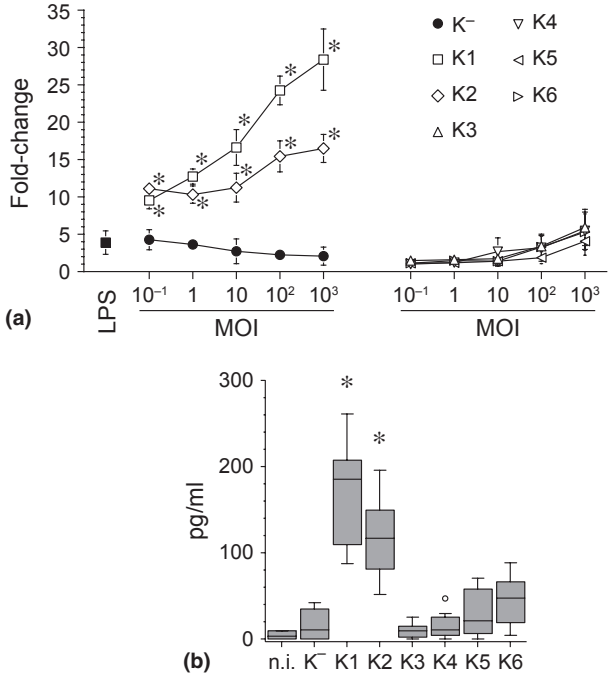


Fig. 1. RANKL mRNA expression and protein secretion levels. (a) Quantitative PCR analysis for the RANKL mRNA expression in T lymphocytes stimulated by autologous dendritic cells (DCs) primed with increasing multiplicity of infection (MOI = 10^{-1}–10^3) of encapsulated (K1–K6) or non-encapsulated (K^-) strains of *Porphyromonas gingivalis*. The black square corresponds to RANKL mRNA expression in T lymphocytes stimulated by autologous DCs primed with lipopolysaccharide of *Escherichia coli*, used as positive control (LPS). For relative expression, the RANKL mRNA expression in T lymphocytes exposed to non-induced DCs was considered as 1, as a reference for fold change in expression (not shown). Data are represented as fold change and shown as mean ± SD for 10 independent experiments. Each experiment was performed in duplicate. *$p < 0.05$, for K1 and K2 with MOIs = 10^{-1}–10^3. (b) Secreted RANKL into the supernatants from the T-lymphocyte cultures used for (a) (MOI = 10^3). Secreted RANKL levels by T lymphocytes exposed to non-induced DCs were used as control (n.i.). Data are represented as cytokine concentration (pg/ml) for 10 independent experiments. The box plots show the medians, 1st and 3rd quartiles as boxes and 10th and 90th percentiles as whiskers. Outliers are shown as open circles. *$p < 0.05$.

$TRAP^+$ osteoclasts induced in response to *P. gingivalis* K serotypes

To confirm the role of the different K serotypes of *P. gingivalis* in osteoclast differentiation and activation, the number of induced $TRAP^+$ osteoclasts in response to each stimulation condition of T lymphocytes was quantified (Fig. 2). After 5 days culture, the number of $TRAP^+$ multinucleated cells was higher in presence of the supernatants of the T-lymphocyte cultures stimulated with K1- or K2-primed DCs as compared with these of T lymphocytes stimulated with K3–K6 or K^- strain of *P. gingivalis*-primed DCs ($p < 0.05$). All the encapsulated strains of *P. gingivalis* induced higher number of $TRAP^+$ cells than the non-encapsulated K^- strain (ATCC® 33,277™) of *P. gingivalis* ($p < 0.05$) and in presence of any of the strains of *P. gingivalis* (K1-K6 or K^- strain) higher number of $TRAP^+$ cells were induced as compared with the non-induced conditions used as controls. No differences were detected in the number of induced $TRAP^+$ multinuclear cells between T lymphocytes exposed to DCs stimulated with K3–K6 serotypes. These

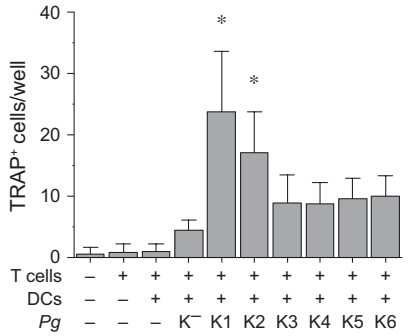


Fig. 2. Number of induced osteoclasts. Number of $TRAP^+$ multinuclear cells induced by T lymphocytes stimulated by autologous dendritic cells (DCs) primed at a multiplicity of infection (MOI) of 10^3 with the encapsulated (K1–K6) or non-encapsulated (K^-) strains of *Porphyromonas gingivalis*. The number of $TRAP^+$ cells induced by T-lymphocytes cultures devoid of DCs or exposed to non-induced autologous DCs was used as control. Data are expressed as number of $TRAP^+$ cells (number/well) and shown as mean ± SD for five independent experiments. Each experiment was performed in triplicate. *$p < 0.05$.

results demonstrate an increment in the osteoclastogenesis induced by T lymphocytes activated in presence of DCs stimulated with serotypes K1 (W83) or K2 (HG184) of *P. gingivalis* compared with the others and this higher induction correlates with the higher levels of expressed and secreted RANKL.

Correlation between transcription factor expression and RANKL secretion

To ascertain whether there was an association between the described RANKL secretion and a specific T-cell phenotype and function, correlation analyses were performed between the expressed mRNA levels of the transcription factors T-bet, GATA-3, RORC2, or Foxp3 and the secreted levels of RANKL (Fig. 3). The correlation analyses yielded positive correlation between either T-bet (Th1) or RORC2 (Th17) expression and RANKL production when T lymphocytes were stimulated with K1- or K2-primed DCs (for K1 $p < 0.05$). On T cells stimulated with K3–K6 or K^- strains of *P. gingivalis* the correlation was negative (not shown), as well as with T cells stimulated with K1- or K2-primed DCs with the transcription factors GATA-3 and Foxp3.

Frequency of memory T-lymphocyte activation in response to *P. gingivalis* K serotypes

To ascertain whether the differential response of T lymphocytes to the different K serotypes of *P. gingivalis* is associated with differences in the frequency of antigen-specific memory T lymphocytes able to respond them, limiting dilution analyses were performed to determine the frequency of cells able to respond to the K1, K2, K4, or K^- strains of *P. gingivalis* in memory T lymphocytes purified from healthy and periodontitis-affected subjects (Fig. 4). Interestingly, the data showed that no significant differences in the frequency of memory T lymphocytes responding to the different strains of *P. gingivalis* analysed in healthy subjects (frequencies ranging from 1/37,627 to 1/18,429); however, in the samples from periodontitis patients, the frequencies of responding cells to the different strains of *P. gingivalis* showed a broader range (fourfold), showing significant differences in the frequency of memory T lymphocytes able to respond to K1 or K2 serotypes of *P. gingivalis* as compared with K^- or K4 (Table 2). Furthermore, the frequencies of memory T lymphocytes able to respond to each of the strains of *P. gingivalis* were significantly different between healthy subjects and periodontitis patients, being increased in periodontitis patients in response to K1 (1/38,490 in healthy subjects *versus* 1/25,454 in periodontitis patients, $p = 0.041$) and K2 (1/44,974 in healthy subjects *versus* 1/30,433 in periodontitis patients, $p = 0.054$), but decreased in periodontitis patients in

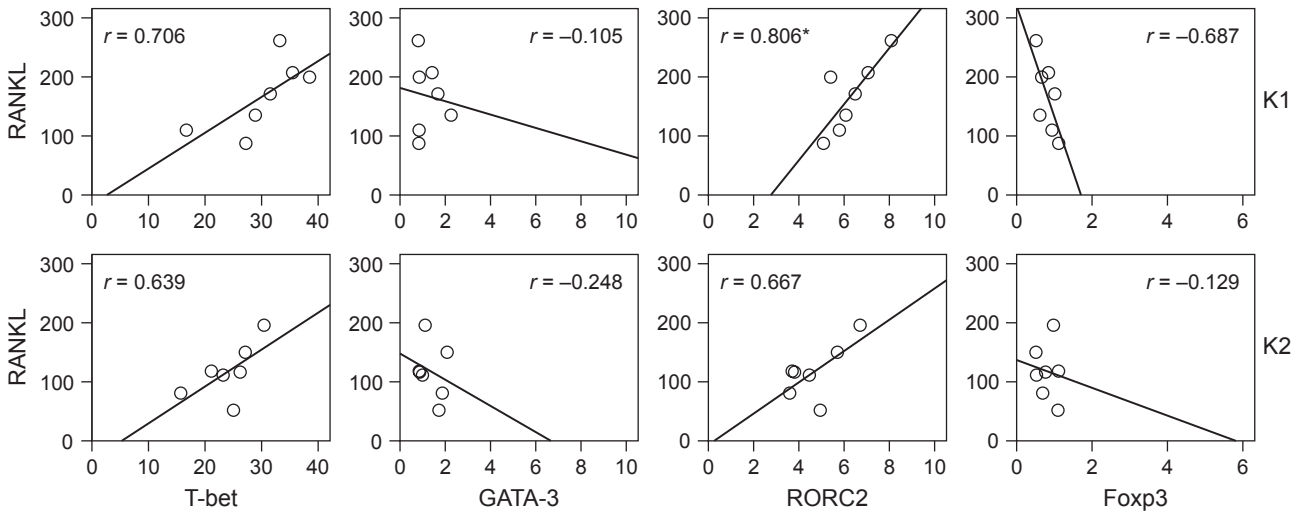
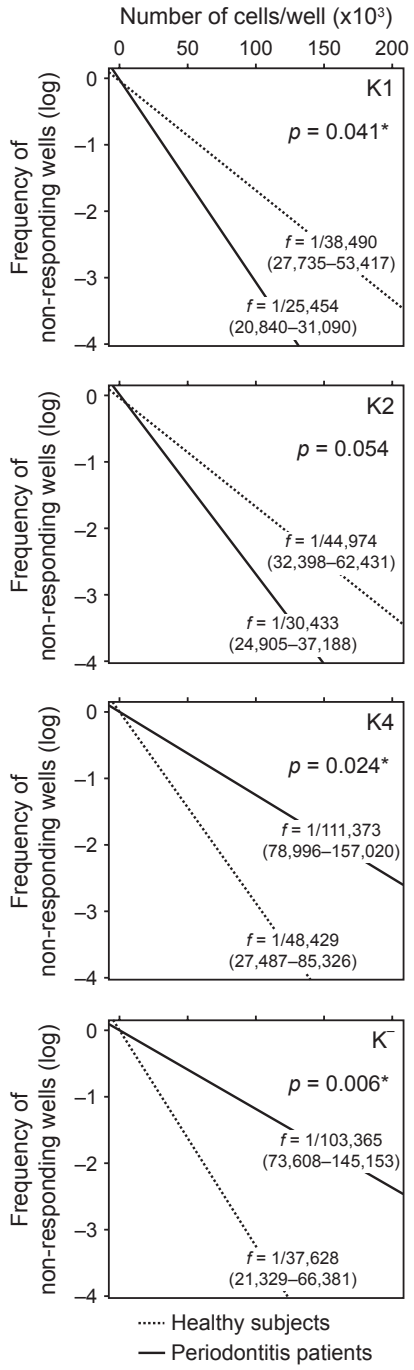


Fig. 3. Correlation between transcription factor expression and RANKL secretion. Correlation between the expressed mRNA levels of the transcription factors T-bet, GATA-3, RORC2 and Foxp3 and the secreted levels of RANKL produced by T lymphocytes stimulated by autologous dendritic cells primed at a multiplicity of infection (MOI) of 10^3 with the encapsulated strains W83 (K1) or HG184 (K2) of *Porphyromonas gingivalis*. The Pearson correlation coefficients (r) were calculated for 10 independent experiments. *$p < 0.05$.



response to K4 (1/48,429 in healthy subjects *versus* 1/11,1373 in periodontitis patients, $p = 0.024$) and K^- (1/3,7628 in healthy subjects *versus* 1/10,3365 in periodontitis patients, $p = 0.006$). Taken together these data demonstrate an association between K1- or K2-induced memory T-lymphocyte activation and periodontitis, and between K^- or K4-induced memory T-lymphocyte activation and periodontal health.

Fig. 4. Frequency of K antigen-specific *Porphyromonas gingivalis* responding memory T lymphocytes. To determine the frequency of memory $CD4^+$ T lymphocytes able to get activated in response to different *P. gingivalis* strains, $CD4^+$ T lymphocytes were isolated from eight chronic periodontitis patients and three healthy controls and then stimulated by autologous dendritic cells primed at a multiplicity of infection (MOI) of 10^3 with the encapsulated strains W83 (K1), HG184 (K2), or ATCC® 49,417™ (K4) and the non-encapsulated strain ATCC® 33,277™ (K^-) of *P. gingivalis*. Data are expressed as frequency of non-responding wells *versus* number of cultured cells/well and statistically analysed assuming the Poisson single-hit model. Each experiment was performed in duplicate. $^*p < 0.05$.

Discussion

Alveolar bone resorption is one of the hallmark pathological events in periodontitis and determines, in the most severe forms of the disease, the loosening of the tooth (Tonetti & Claffey 2005, Cochran 2008). Osteoclasts are the cells responsible for active bone resorption and, during periodontitis, a rise in the detection of $TRAP^+$ osteoclasts coupled with increased levels of RANKL has been reported in periodontal tissues (Teng et al. 2000, Crotti et al. 2003, Liu et al. 2003, Alnaeeli et al. 2006, Vernal et al. 2006, Jin et al. 2007, Han et al. 2013). In fact, RANKL is a key regulator of bone metabolism and an essential element in osteoclast differentiation and activation when pathological bone resorption occurs (Lacey et al. 1998, Hofbauer & Heufelder 2001, Takahashi et al. 2005). In this investigation, a differential expression and secretion of RANKL and induction of $TRAP^+$ osteoclasts were elicited by human naïve T lymphocytes, when stimulated with autologous DCs that were exposed to different *P. gingivalis* K serotypes. In particular, higher levels of RANKL and $TRAP^+$ osteoclasts were detected, when T lymphocytes were exposed to DCs stimulated with the strains W83 (serotype K1) or HG184 (serotype K2) of *P. gingivalis* compared with the same cells stimulated with the other serotypes.

Different studies have analysed the levels of RANKL and osteoprotegerin (OPG), its biological decoy, in periodontal tissues from both periodontitis and healthy subjects. In general, the results are heterogeneous, although the ratio of RANKL/OPG shows a consistent tendency to increase from periodontal health to periodontitis and to decrease following periodontal treatment (Bostanci et al. 2008, Sakellari et al. 2008, Buduneli & Kinane 2011). In addition, it has been established that the increment of RANKL levels in periodontal lesions correlates with local detection of *P. gingivalis* (Waraaswapati et al. 2007, Sakellari et al. 2008) and with the increased levels of pyridinoline cross-linked carboxy terminal telopeptide of type I collagen ICTP, a marker of alveolar bone resorption (Arikan et al. 2011).

In a rat model of oral infection with *P. gingivalis*, it was demonstrated that the detected periodontal bone resorption was RANKL dependent and T lymphocytes were the major source of RANKL in the gingival tissues affected of periodontitis (Han et al. 2013). In this context, the Th17 lymphocytes have the capacity to induce differentiation and activation of osteoclasts by directly acting on their precursors and on mature osteoclasts during periodontitis through synthesis of RANKL (Yasuda et al. 1998, Vernal et al. 2006, Vernal & Garcia-Sanz 2008). Furthermore, many well-known osteotropic factors, including TNF-α, IL-1β, IL-6 and IL-17, exert their osteoclastogenic activity by inducing RANKL expression on osteoblasts and Th17 lymphocytes (Boyle et al. 2003, Sato et al. 2006). Similarly, IL-17 facilitates local inflammation by recruiting and activating immune cells, which leads to an abundance of inflammatory cytokines, such as IL-1β and TNF-α, which enhance the RANKL expression on osteoblasts and Th17 lymphocytes (Dong 2006, Weaver et al. 2006).

P. gingivalis has a potential pathogenic role in periodontal bone resorption by inducing Th17-dependant activities. In fact, *P. gingivalis* promotes Th17-lymphocyte differentiation and function with increments in the production of IL-6, IL-17 and IL-23 and these Th17-phenotype-specific cytokines induce bone resorption in vivo by producing RANKL (Toraldo et al. 2003, Moutsopoulos

Table 2. Significance *p*-values of the comparative frequencies of memory T-lymphocyte activation in response to the encapsulated strains W83 (K1), HG184 (K2), or ATCC® 49,417™ (K4) and the non-encapsulated strain ATCC® 33,277™ (K^-) of *P. gingivalis*

		Healthy				Periodontitis			
		K^-	K1	K2	K4	K^-	K1	K2	K4
Healthy	K^-		–	–	–	–	–	–	–
	K1	0.95		–	–	–	–	–	–
	K2	0.62	0.53		–	–	–	–	–
	K4	0.57	0.52	0.84		–	–	–	–
Periodontitis	K^-	0.006*	8.43E-05*	0.0009*	0.04*		–	–	–
	K1	0.23	0.04*	0.004*	0.05*	2.22E-12*		–	–
	K2	0.52	0.25	0.05*	0.16	1.38E-09*	0.24		–
	K4	0.003*	2.24E-05*	0.0003*	0.0247*	0.77	8.86E-14*	9.10E-11*	

*$p < 0.05$.

et al. 2012, Han et al. 2013). In this context, a differential Th17 response between K serotypes of *P. gingivalis* was recently reported (Vernal et al. 2014). By stimulating with the serotypes K1 or K2 of *P. gingivalis,* there was a higher Th17 differentiation after antigen presentation by DCs when compared with the other *P. gingivalis* serotypes. Moreover, these activated Th17 lymphocytes elicited an increment in the production of Th17-associated cytokines. Hence, the K1 and K2 serotypes of *P. gingivalis* could have a role in the alveolar bone resorption during periodontitis by inducing higher differentiation and activation of RANKL-induced $TRAP^+$ osteoclasts and this RANKL production is associated with the induction of the Th17 phenotype. In fact, in this investigation the detected higher levels of RANKL produced by T lymphocytes stimulated with K1- and K2-primed DCs correlated with the RORC2 mRNA expression, the transcription factor master-switch gene that determine the selective Th17 lymphocyte differentiation.

Pathogenicity of *P. gingivalis* is attributed to an array of potential virulence factors, such as cysteine proteinases (gingipains), haemagglutinins, lipoproteins, fimbriae, LPS and the extracellular capsule, which may activate the host's immune response (Kocgozlu et al. 2009, Wang et al. 2009, Hajishengallis 2011, Kunnen et al. 2012, Firth et al. 2013, Herath et al. 2013, Wilensky et al. 2013). The extracellular capsule has been shown to play an important role in the virulence of Gram-negative bacteria. In *P. gingivalis*, encapsulated strains were highly virulent when subcutaneously inoculated in experimental animal, causing a phlegmonous infection with ulceration and necrosis, whereas the non-encapsulated strain was less virulent, causing localized abscesses (Laine & van Winkelhoff 1998). In this context, differences in the structure of *P. gingivalis* capsule, in particular differences in polysaccharide composition, are involved in the variable immunogenicity and immunostimulatory potential of the different K serotypes of *P. gingivalis* on diverse host's cells (Schifferle et al. 1989, Farquharson et al. 2000, Aduse-Opoku et al. 2006). *P. gingivalis* strains belonging to the serotype K1 elicited a more potent chemokine expression on murine macrophages (d'Empaire et al. 2006) and a higher resistance to phagocytosis and killing by human polymorphonuclear leucocytes than the other K serotypes (Sundqvist et al. 1991). In addition, the K1 serotypes induce higher cytokine production on monocytes (Kunnen et al. 2012) and DCs (Vernal et al. 2009) compared with the other *P. gingivalis* serotypes, and, following the antigenic presentation to T lymphocytes, induce an increment in the Th1 and Th17 differentiation and higher production of Th1- and Th17-related cytokines than the others (Vernal et al. 2014).

During periodontitis, a Th1- and Th17-dominated response has been reported in destructive periodontal disease and in presence of *P. gingivalis* in the periodonto-pathogenic subgingival biofilm, the detection of the K1 and/or K2 serotypes could be associated with this immunodestructive response. A higher frequency of memory T lymphocytes reactive to the serotypes K1 or K2 of *P. gingivalis* was detected in chronic periodontitis patients compared with healthy controls, an evidence of detection of T lymphocytes that previously had been exposed to *P. gingivalis* K1 or K2 antigens and suggestive of the association between these *P. gingivalis* K serotypes and the pathogenesis of periodontitis. A higher frequency of memory T lymphocytes responding to K4 or K^- primed DCs was detected in healthy subjects as compared with periodontitis patients, which suggests an association with periodontal health. We cannot discard that at this moment an additional possibility in which periodontitis would be associated with both an increased frequency of T-cell responses to K1- or K2-specific antigens and a decreased frequency of T-cells responses to K4 or K^- primed DCs.

Current periodontal therapy is focused on reducing the bacterial challenge, either mechanically alone or with adjunctive pharmacological agents, but lacks of strategies addressing the modulation of the patient's immune response. The possible impact of approaches modulating the host's response has gained considerable interest with the current knowledge of the close relationship between the immune system and bone metabolism, in particular, due to the accumulating evidence that alveolar bone resorption can be caused by RANKL production from activated T lymphocytes. This study has shown that a higher production of RANKL and activation of $TRAP^+$ osteoclasts were induced

when T lymphocytes were exposed to DCs stimulated with the strains W83 (serotype K1) or HG184 (serotype K2) of *P. gingivalis* compared with the same cells stimulated with the other *P. gingivalis* serotypes. In addition, a higher frequency of memory T lymphocytes reactive to the serotypes K1 or K2 of *P. gingivalis* was detected in chronic periodontitis patients as compared with healthy controls. These findings may indicate that K1 and K2 serotypes of *P. gingivalis* have a role in the pathogenesis of periodontitis by regulating the T-lymphocyte differentiation and function and thus favouring the osteoclastogenesis and bone resorption.

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Clinical Relevance

Scientific rationale for the study: The *P. gingivalis* K1–K2 serotypes induce a strong Th1–Th17 polarization and function and this immune response pattern is associated with destructive periodontitis. These specific bacteria serotypes, hence, may be associated with the destructive events of periodontitis.

Principal findings: The *P. gingivalis* K1–K2 serotypes induced higher RANKL production, $TRAP^+$ osteoclast induction and frequency of memory T-lymphocyte activation compared with the other serotypes.

Practical implications: The variability in the host response induced by the *P. gingivalis* K serotypes should be taken into account when the role of this bacterial species is considered in the context of the periodontitis pathogenesis.