Porphyromonas gingivalis K1 and K2 serotypes are associated with periodontitis

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Abstract

Aim: Destructive periodontitis is associated with a Th1-Th17-dominated immune response and the Porphyromonas gingivalis K1 and K2 serotypes induce a strong Th1-Th17 response. This study aimed to investigate whether these P. gingivalis serotypes were associated with bone resorption and periodontitis.

Materials and Methods: The RANKL production and TRAP$^+$ osteoclast induction were quantified when naïve-T-lymphocytes were 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. Upon K1 or K2 stimulation, higher frequency of responding memory-T-lymphocytes was detected in periodontitis patients than controls. Upon K4 or K$^-$ stimulation, higher frequency of responding memory-T-lymphocytes was detected in healthy subjects.

Conclusions: P. gingivalis serotypes K1 and K2 are associated with bone resorption through the increased RANKL production and TRAP$^+$ osteoclast induction. Due to their association with higher frequency of memory-T-lymphocytes in periodontitis patients, these serotypes could be involved in the pathogenesis of periodontitis.
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.
Introduction

Periodontitis is one of the most common human infectious diseases and one of the primary causes of tooth loss in adults (Tonetti and 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 and Kinane 2011, Hernandez et al. 2011). This destructive process is initiated by specific bacteria within the subgingival biofilm and progresses because of host’s immuno-inflammatory mechanisms triggered in responses to these bacteria, which are mainly dependent on the 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 etiologic agent of periodontitis (van Winkelhoff et al. 1988, Slots and 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) signaling (Vernal et al. 2008a, Vernal et al. 2009, Moutsopoulos et al. 2012, Baek et al. 2013, Han et al. 2013, Herath et al. 2013, Vernal et al. 2013).

P. gingivalis possesses multiple virulence factors, which enable this bacteria to evade the host tissue defense mechanisms. Of these key factors is 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 immuno-stimulatory 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 elicited differences in T lymphocyte polarization and function by promoting a Th1 and Th17 pattern of immune response (Vernal et al. 2013). 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, Gemmell et al. 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, Vernal et al. 2006, Vernal and Garcia-Sanz 2008, Dutzan et al. 2009a, Dutzan et al. 2009b, 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 and periodontal disease. The objective of this investigation was to determine whether K1 or K2-primed dendritic cells, when used to stimulate autologous T
lymphocytes, could induce RANKL production and osteoclast differentiation and whether they were associated with activation of K1 and K2 antigen-specific memory T lymphocytes in patients affected with periodontitis.

**Materials and Methods**

**P. gingivalis growth conditions and curves**

The encapsulated *P. gingivalis* strains W83 (serotype K1), HG184 (K2), A7A1-28 (K3), ATCC® 49417™ (K4), HG1690 (K5), and HG1691 (K6) and the non-encapsulated (K') strain ATCC® 33277™ 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. In order 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, Le Pont de Claix, France) 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 10⁶ 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 10¹ to 10³ 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 O111: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. 2013). 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 sec and 60°C for 30 sec, and finally a melt curve of 95°C for 15
sec, 60°C for 1 min and 95°C for 15 sec, 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 1x10^3 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 leukocyte acid phosphatase kit following the manufacturer’s recommendations (Sigma-Aldrich, St. Louis, MO, USA) and observed using a microscope (Axioskop® Plus; Zeiss, Germany). TRAP⁺ cells with three or more nuclei were considered osteoclasts.

**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 8 chronic periodontitis patients and 3 healthy controls by magnetic cell sorting depletion (MACS; Miltenyi Biotec). Memory T lymphocytes were cultured at 10^6 cells/ml with autologous DCs primed with the \textit{P. gingivalis} strains K, K1, K2, or K4 (50:1) and transferred to 96-well round-bottom microtiter 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-³H]thymidine ([³H]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 [³H]dThd incorporation at the end of the cell culture. Wells were considered positive when the incorporated [³H]dThd counts exceeded the mean counts of control wells by more than 3 SD as described (Erard et al. 1985).

\textbf{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., Charlotte, 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

Since 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 analyzed 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) were 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).

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

In order 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 those 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® 33277™) 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 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. Taken together, these data suggest an association between the K1 and K2 serotypes of P. gingivalis with Th17 lymphocyte differentiation and activation and RANKL production.
Frequency of memory T-lymphocyte activation in response to \textit{P. gingivalis} K serotypes

To ascertain whether the differential response of T lymphocytes to the different K serotypes of \textit{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 \textit{P. gingivalis} in memory T lymphocytes purified from healthy and periodontitis affected subjects (Fig. 4). Interestingly, the data showed no significant differences in the frequency of memory T lymphocytes responding to the different strains of \textit{P. gingivalis} analyzed in healthy subjects (frequencies ranging from 1/37627 to 1/18429); however, in the samples from periodontitis patients, the frequencies of responding cells to the different strains of \textit{P. gingivalis} showed a broader range (4-fold), showing significant differences in the frequency of memory T lymphocytes able to respond to K1 or K2 serotypes of \textit{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 \textit{P. gingivalis} were significantly different between healthy subjects and periodontitis patients, being increased in periodontitis patients in response to K1 (1/38490 in healthy subjects vs. 1/25454 in periodontitis patients, \( p = 0.041 \)) and K2 (1/44974 in healthy subjects vs. 1/30433 in periodontitis patients, \( p = 0.054 \)), but decreased in periodontitis patients in response to K4 (1/48429 in healthy subjects vs. 1/111373 in periodontitis patients, \( p = 0.024 \)) and K- (1/37628 in healthy subjects vs. 1/103365 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.
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 and 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 and Heufelder 2001, Takahashi et al. 2005). In this investigation, a differential expression and secretion of RANKL and induction of TRAP\(^+\) osteoclasts was 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 analyzed 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 and Kinane 2011). In addition, it has been established that the increment of RANKL levels in periodontal lesions correlates with local detection of \(P.\ gingivalis\)
(Wara-aswapati 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 and 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 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. 2013). 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.

Extracellular capsule 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 and van Winkelhoff 1998). In this context, differences in the structure of *P. gingivalis* capsule, in particular differences in the polysaccharide composition, are involved in the variable immunogenicity and immune-stimulatory 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. 2013).
During periodontitis, a Th1 and Th17-dominated response has been reported in destructive periodontal disease and, in presence of \textit{P. gingivalis} in the periodontopathogenic subgingival biofilm, the detection of the K1 and/or K2 serotypes could be associated with this immuno-destructive response. A higher frequency of memory T lymphocytes reactive to the serotypes K1 or K2 of \textit{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 \textit{P. gingivalis} K1 or K2 antigens and a proof of the association between these \textit{P. gingivalis} K serotypes and the etiopathogenesis 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. Although we cannot discard that at this moment an additional possibility in which periodontitis would be associated to 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.

The role of the different serotypes of the periodonto-pathogenic bacteria in the host’s immune response and the concomitant connective tissue destruction and alveolar bone resorption is just emerging, particularly in terms of which bacterial serotypes might lead to a protective or destructive T-lymphocyte response in periodontitis. The presented data suggest that, in periodontal lesions, microbiological diagnosis should aim towards determining serotype composition, instead of seeking the identification of pathogenic species. Furthermore, these findings may allow to envisage new therapeutic approaches focusing on modulating the host response against these bacterial serotypes, instead of decreasing the complete bacterial challenge, which is the current clinical periodontal practice. Since it may be possible to discriminate between a protective and a destructive host response in periodontitis, therapeutic immune-intervention could focus
on stimulating the protective and on inhibiting the destructive aspects of the periodontal T-lymphocyte immune response.

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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.

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*Number of the specific FAM dye-labelled probe (Roche).
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® 49417™ (K4) and the non-encapsulated strain ATCC® 33277™ (K') of *P. gingivalis*.

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<td>K' K1 K2 K4</td>
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*p < 0.05*
Figure legends

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 Fig. 1a (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.

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 were used as control. Data are expressed as number of TRAP^+ cells (number/well) and shown
as mean ± SD for 5 independent experiments. Each experiment was performed in triplicate. *p < 0.05.

**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.

**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 8 chronic periodontitis patients and 3 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® 49417™ (K4) and the non-encapsulated strain ATCC® 33277™ (K) of *P. gingivalis*. Data are expressed as frequency of non-responding wells versus number of cultured cells/well and statistically analyzed assuming the Poisson single-hit model. Each experiment was performed in duplicate. *p < 0.05.
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 Fig. 1a (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 show as open circles. *p < 0.05.
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 were used as control. Data are expressed as number of TRAP⁺ cells (number/well) and shown as mean ± SD for 5 independent experiments. Each experiment was performed in triplicate. *p < 0.05.
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