Distinct Th responses triggered in human T lymphocytes by different *Porphyromonas gingivalis* capsular serotypes

Rolando Vernal^{1,2}, Eva Diaz-Guerra¹, Augusto Silva¹, Mariano Sanz³ and
Jose. A Garcia-Sanz¹

¹Department of Cellular and Molecular Medicine, Centro de Investigaciones Biológicas (CIB-CSIC), Madrid, Spain. ²Department of Conservative Dentistry, Dental School, Universidad de Chile, Santiago de Chile, Chile. ³Graduate Periodontology, Dental School, Universidad Complutense de Madrid, Madrid, Spain.

Running Title: human T-lymphocyte responses to *P. gingivalis*

Key Words: capsular serotypes; cytokines; *Porphyromonas gingivalis*; T helper lymphocytes; transcription factors.

Corresponding authors:

Jose A. Garcia-Sanz, Centro de Investigaciones Biológicas. CIB-CSIC. Ramiro de Maeztu 9, 28040 Madrid, Spain. Phone: +34 91 8373112, Fax: +34 91 5360432, E-mail: jasanz@cib.csic.es

Rolando Vernal. Departamento de Odontología Conservadora. Facultad de Odontología. Universidad de Chile. Sergio Livingstone Pohlhammer 943, Santiago, Chile. Phone: +56 2 9781833, Fax: +56 2 9781813, E-mail: rvernal@uchile.cl

ABSTRACT

The immune response to *Porphyromonas gingivalis*, one of the key pathogens in periodontitis etiology, was analyzed activating naïve CD4⁺ T cells from healthy individuals with autologous dendritic cells pulsed with different *P. gingivalis* serotypes. Whereas serotypes K1 or K2 triggered a Th1/Th17 response, serotypes K3, K4 or K5 triggered a Th2 response, and K⁻ a Treg phenotype. We found a good correlation between the secreted cytokines and expression of the master switch transcription factors T-bet and RORC2 in response to K1 or K2, or with GATA-3 and the secreted cytokines in response to K3-K5. However, there was no correlation between expression of Foxp3 and secretion of IL-10 or TGF-β1 on response to K⁻. In addition, T cells responding to K1 or K2, but not to the other serotypes led to an increased secretion of RANKL, a key cytokine involved in bone metabolism. Although the data could not be simply explained by differences in the frequency of T cells able to respond to the different serotypes on healthy and periodontitis affected individuals, it allowed to link serotypes K1 and K2 to the Th1/Th17 response and bone resorption characteristic of periodontitis.

Introduction

Periodontitis is an infectious disease characterized by chronic inflammation of the tissues around the teeth, causing destruction of these tissues and as a consequence, teeth loosening. It is a very common disease (prevalence 30-50% of the population in the US), elicited by the bacteria residing at the subgingival biofilm (1). Despite the broad variety of bacterial strains identified within the subgingival biofilm (~500), only a few have been identified as etiological pathogens of periodontitis (2-4). In fact, the Gram-negative bacteria *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*, both present in the subgingival biofilm have been described as the primary etiological agents of periodontitis (5-16). In particular, the significant association of *P. gingivalis* with the most severe forms of periodontitis and of its numbers with periodontal pocket depth (5, 7-10, 17-19), allowed the association of this bacteria with progressive tissue destruction (20, 21). Furthermore, differences in the prevalence and distribution of different *P. gingivalis* capsular (K) serotypes described so far (K1-K6 and K⁻) have also been reported (22, 23).

The interest on analyzing the immune response to periodontitis-associated bacteria comes from the fact that although the bacteria can cause some direct tissue damage, their pathogenicity mainly relies on the induction of a host immuno-inflammatory response, which leads to destruction of periodontal ligament, cementum and alveolar bone, finally leading to tooth loss (24-27). Indeed, an increase in the number of dendritic cells and T lymphocytes has been described within the inflammatory site during periodontal infections (28-30), where dendritic cell maturation and subsequent antigen presentation to naïve T cells has been suggested to take place *in situ* (24). The data regarding the immune response to periodontitis remains, however, controversial. In

some reports, a Th1-type immune response has been associated with active periodontitis, whereas a Th2-type response was associated with remission (31-37). Conversely, other reports associate a stable periodontal lesion with a Th1-type response, whereas active progression was associated with a Th2-type response (38-44). More recently, a Th17-type of response has been associated with periodontitis-induced tissue destruction (45-49) and Treg cells have been suggested to protect from periodontitis (50-52).

We have previously analyzed the response *in vitro* of DCs, generated from peripheral blood monocytes from normal donors to different capsular serotypes of *P. gingivalis*, demonstrating quantitative differences in the response of DCs to different K serotypes (53). In this context, we speculated that different *P. gingivalis* serotypes might lead to a differential T lymphocyte activation and/or to a different type of T-cell immune response generated. In the present investigation, the synthesis of cytokines and the expression of the transcription factors T-bet, GATA-3, RORC2 and Foxp3, the master-switch genes controlling Th1, Th2, Th17, and Treg differentiation, respectively, were analyzed on human T lymphocytes activated with different *P. gingivalis* K serotypes, demonstrating that different serotypes lead to expression of different master-switch genes and secretion of different cytokine patterns.

Results

Antigen-specific activation of T lymphocytes with autologous DCs

Highly purified monocytes (>98% CD14⁺) isolated from peripheral blood were differentiated into DCs upon culture in presence of IL-4 and GM-CSF, obtaining a highly pure population of DCs (>97%), as demonstrated by the expression of the CD1a, the increase in CD86 expression and the concomitant loss of the monocyte-macrophage marker CD14 as quantified by flow cytometry (Figure 1A). These DCs were then stimulated at an MOI of 10³ with *P. gingivalis* serotype K1 (see materials and methods for the complete description of the different K serotypes), and the efficiency of DC maturation (>96%) was confirmed by the increased expression of CD83 and CD80 antigens, associated with an increase in cell size (not shown). These cells were used to activate highly purified populations of autologous naïve T lymphocytes (>98% CD4⁺) isolated from peripheral blood (Figure 1B) with a T-cell:DC ratio of 50:1. As early as 1 day upon T lymphocyte activation, an increase in CD25 and CD69 expression was observed on a subpopulation of T cells, as compared to the same T lymphocytes exposed to non-induced DCs, reaching levels >10% 5 days after. Indeed, expression of the early activation marker CD69 increased >10-fold 1 day after T-lymphocyte induction, reaching plateau levels at day 2. These changes were concomitant with the expression levels of CD25, the inducible α -subunit of the IL-2R, that increased >5-fold 5 days after T-lymphocyte activation. Conversely, the expression levels of CD62L (L-selectin), a leukocyte adhesion molecule member of the homing receptor family that is lost after cell activation, decreased >7-fold 5 day after T-cell activation (Figure 2A). Similar results were found with T cells activated with DCs triggered with *P. gingivalis* strains K1-K6 or K⁻ (not shown). These data were further confirmed by quantitative RT-

PCR analysis of CD25 mRNA expression, where a ≥ 10 -fold increase on CD25 mRNA expression was observed on T lymphocytes activated by *P. gingivalis* serotypes K1-K6 or K⁻ primed DCs as compared with T lymphocytes exposed to non-induced DCs or non-induced T cells. Furthermore, no significant differences were detected when T lymphocytes were activated by DCs primed with any of the encapsulated or non-encapsulated *P. gingivalis* serotypes (Figure 2B).

Cytokine mRNA expression and secretion by *P. gingivalis*-activated T cells

On T lymphocytes activated with autologous DCs primed with different *P. gingivalis* serotypes at different MOI, the expression levels for several cytokine mRNAs was determined by RT-quantitative real-time PCR. The data represented as fold-change for each condition reveal a dose-dependent increase in the expression levels for the different cytokines analyzed (Figure 3). The T cell response against serotypes K1 or K2 was characteristic of a Th1 phenotype, as demonstrated by the over-expression of IL-1 β , IFN- γ , IL-12p35, IL12-p40, TNF- α , and TNF- β mRNAs (at MOIs of 0.1-10³ p<0.05) concomitant with unchanged levels on the expression of cytokines associated to other phenotypes (IL-4, IL-5, IL-13, IL-10 or TGF- β 1). Higher expression levels for IL-2 mRNA (at MOIs of 10² and 10³ p<0.05) were also detected when cells were activated with these serotypes as compared with K⁻ or K3-K6. Interestingly, K1 and K2 serotypes also over-expressed mRNAs for the cytokines characteristic of the Th17 phenotype, namely IL-6, IL-17 and IL-23 mRNAs (at MOIs of 0.1-10³ p<0.05). Conversely, when the same T cells were activated with autologous DCs primed with *P. gingivalis* serotypes K3, K4 or K5, the observed cytokine expression pattern on T-lymphocytes was characteristic of a Th2 phenotype. Indeed, higher expression of IL-4 and IL-5 mRNA levels (at MOIs of 10² and 10³ p<0.05), without changes in the expression of

cytokines of the Th1, Th17 or Treg phenotypes (IL-1 β , IFN- γ , IL-12p35, IL12-p40, TNF- α , TNF- β , IL-10 or TGF- β 1). The expression of IL-13, however, was not increased in any experimental condition.

Overall, upon T lymphocyte activation with DCs primed with capsulated *P. gingivalis* strains K1 or K2, a T cell response biased towards a Th1/Th17 phenotype was detected, whereas a Th2 cytokine expression profile was detected when the same cells were activated with strains K3-K5. Furthermore, the same T lymphocytes activated with DCs primed with the K⁻ strain over-expressed only the mRNAs coding for IL-10 and TGF- β 1 (at an MOI of 10³ p<0.05), denoting a regulatory immune response.

These distinct patterns of response were confirmed at the protein level upon the analysis of cytokines secreted into the culture supernatants (Figure 4). Higher levels of the typical Th1 cytokines IL-1 β , IL-2, IFN- γ , IL-12p40, IL12-p70, TNF- α , and TNF- β were detected when cells were activated at an MOI of 10³ with serotypes K1 or K2 as compared to the other serotypes. Similar results were obtained with these strains for the typical Th17 cytokines IL-6, IL-17 and IL-23, although for the latest, the data was significant only for T cell stimulated with the K1 serotype. Conversely, higher levels of IL-4 and IL-5 were detected in T lymphocytes activated with serotypes K3, K4 or K5 as compared with the others. IL-13 secretion was also higher with cells stimulated with K3, K4 or K5, although it was statistically significant only in T lymphocytes activated with K3. Finally, higher secreted levels of IL-10 were detectable on the supernatants of T cells activated with the K⁻ strain of *P. gingivalis*, as compared to the response to encapsulated serotypes; this response could be associated to a regulatory lymphocyte response.

Transcription factor mRNA expression

Since the data obtained was consistent with a differential induction of T lymphocyte phenotypes triggered upon activation with different *P. gingivalis* serotypes, we decided to confirm these data with the expression levels of the master-switch transcription factors T-bet, GATA-3, RORC2 and Foxp3, determined by quantitative real-time PCR on mRNAs from T lymphocytes activated by autologous DCs primed (MOIs of 10^2 or 10^3) with the different *P. gingivalis* serotypes. The data obtained, represented in Figure 5 show a significant increase of T-bet and RORC2 mRNAs in T lymphocytes activated with serotypes K1 or K2 (MOIs of 10^2 or 10^3 $p < 0.05$), as compared to the same T cells activated with other serotypes. In addition, GATA-3 mRNA levels were increased exclusively upon T cell activation with K3, K4 or K5 serotypes as compared with others, although the differences were only significant for an MOI of 10^3 ($p < 0.05$). Furthermore, Foxp3 mRNA expression was increased in T lymphocyte mRNAs following activation with the K⁻ strain (at MOIs of 10^2 and 10^3 $p < 0.05$).

The correlation analyses between the expression of these transcription factors and the secreted cytokines on each activation condition tested (Table 2) yielded significant positive correlation between T-bet expression and Th1-associated cytokine secretion (IL-1 β , IL-2, IL-12, IFN- γ , TNF- α , and TNF- β) when T lymphocytes were activated with K1 or K2-primed DCs. Under the same conditions, a positive correlation was also observed between RORC2 and Th17-associated cytokines (IL-6, IL-17, and IL-23). A significant positive correlation was also detected between GATA-3 and Th2-associated cytokines IL-4 and IL-5 when T lymphocytes were activated with serotypes K3, K4 or K5, although IL-13 was positively correlated only with K3-activated T lymphocytes.

Conversely, Foxp3 expression did not show any positive correlation with IL-10 or TGF- β 1 secretion.

RANKL mRNA expression and secretion in response to *P. gingivalis*

Since periodontitis progression is characterized by bone tissue destruction, eventually leading to loosening of the teeth, we analyzed the expression levels of RANKL, a cytokine associated with this process. The data obtained showed an increase in RANKL mRNA expression on T cells activated with K1 or K2 (differences significant only for K1), but not with K3-K6, nor with K⁻ (Figure 6A). These data strictly correlate with the levels of secreted RANKL, present in the supernatant of these cultures (Figure 6B), although at the protein level, the differences were statistically significant for both K1 and K2. Correlation analyses between mRNA expression levels of the transcription factors T-bet, GATA-3, RORC2 or Foxp3 and secreted RANKL yielded positive correlation between T cells stimulated with K1 and K2 with the transcription factors T-bet and RORC2, although it was statistically significant only with cells stimulated with K1 (Figure 6C). On T cells stimulated with K3-K6 or K⁻ the correlation was negative (not shown), as well as with cells stimulated with K1 or K2 with the transcription factors GATA-3 and Foxp3. Taken together, these data suggests a link between K1 and K2 serotypes of *P. gingivalis* with cytokines involved in bone resorption.

To ascertain whether there differential response of T cells to the different serotypes were due to differences in the number of T lymphocytes able to respond to them, limiting dilution analyses were performed to determine the frequency of T cells able to respond to K1, K2, K4 or K⁻ in T lymphocytes purified from both healthy and periodontitis affected patients. The data obtained showed a 3-fold lower frequency of T cells able to respond to K4 (p=0.024) and K⁻ (p=0.006) on periodontitis patients as

compared to healthy individuals (frequencies ranging from 1/48429 to 1/37627 in healthy individuals, and 1/111,373 to 1/103,364 in periodontitis patients). The frequencies of T cells able to respond to antigens from K1 or K2 ranged from 1/49,601 to 1/69,688 and did not give, however, significant differences between healthy individuals and periodontitis patients (Figure 7).

Discussion

Although historically disease progression in chronic inflammatory diseases had been ascribed to particular Th phenotypes, emerging data indicate that more than one phenotype can coexist, for example in multiple sclerosis and rheumatoid arthritis, two diseases initially described as typical Th1, the implication of the Th17 phenotype has recently been described (54, 55). In periodontitis, it has been difficult to determine which T cell subset, and specific cytokine profiles are destructive and lead to tissue damage, and which ones are associated with disease resolution (24, 25). In particular since the presence of multiple bacteria, including commensal strains, can modify or even switch from a T cell-type of response to another (56, 57). This has led to conflicting reports, ones suggesting a Th1/Th17 dominated response in periodontal disease (32, 33, 36, 45, 46), whereas in others, a Th2 host response was associated with either progressive disease (38) or remission (35).

Aiming to analyze the role of T-cell immune responses in periodontitis, in particular on T-lymphocyte cytokine profiles at the local inflammation site, we used an *in vitro* system, in which differences between the analyzed samples were restricted to the use of different capsular serotypes of *P. gingivalis*. It is obvious that this system can only be used as a model since it represents an oversimplification of the *in vivo* situation, but fulfilled the requirements to unravel some insights on controversial issues regarding the immune response to periodontitis. Previous data on DC response to different *P. gingivalis* serotypes demonstrated a differential stimulation potential, but did not show significant differences on the type of response (53). In contrast, the data presented here on T cells activated with autologous DCs stimulated with different *P. gingivalis* serotypes has shown that serotype differences led to expression of distinct cytokine sets,

as determined both as mRNA expression and secreted cytokines into the cell culture supernatants (Figures 3,4). These data suggested that activation of T cells with DCs stimulated with either K1 or K2 induced a Th1/Th17 phenotype in CD4⁺ T cells, whereas the serotypes K3-K5 led to the secretion of Th2-type cytokines. The data with K6 and K⁻ were, as discussed below, less straightforward. Further corroboration of the activation of CD4⁺ T cells towards distinct phenotypes came from mRNA expression analyses of the master switch transcription factors T-bet, GATA-3, RORC2 and Foxp3. T-bet and RORC2 were specifically up-regulated on T cells activated with the serotypes K1 or K2, GATA-3 was specifically up-regulated on T cells activated with serotypes K3, K4 or K5, whereas Foxp3 was up-regulated on T cells activated with K⁻. Although the up-regulation of the activation marker CD25 (Figure 2B) or the proliferation (not shown) of T cells activated with K6 was comparable to the levels obtained with other *P. gingivalis* serotypes, this serotype did not lead to up-regulation of any of these transcription factors, nor lead to secretion of any of the cytokines analyzed, and thus, K6 could not be associated to any of the known helper-T cell phenotypes.

Correlation analyses between the levels of secreted cytokines and expression levels of the master switch transcription factors T-bet, RORC2, GATA-3 and Foxp3 were highly informative since they showed that expression of the transcription factor Foxp3 and secreted IL-10 or TGFβ1 were devoid of statistic significance, suggesting that these cells did not represent bona-fide induced-regulatory T cells. Conversely, there was a positive correlation between GATA-3 and Th2-cytokines in T cell samples activated with K3-K5, although the data for IL-13 was significant only for K3. Similarly, there was a positive correlation between T-bet and the classical Th1-cytokines as well as between RORC2 and Th17-cytokines in T cell samples activated with either K1 or K2, but not with K3-K6 nor K⁻. In addition, the lack of correlation between T-bet and Th17

cytokines or between RORC2 and Th1-cytokines (supplementary Figure 1) indicated that the serotypes K1 and K2 were able to activate CD4⁺ T cells towards either a Th1 or a Th17 phenotype, rather than to a dual Th1/Th17 phenotype.

Elevated RANKL levels have been found in the gingival crevicular fluid from periodontitis patients (58, 59). It has also been demonstrated that this cytokine is secreted within the inflammation site by T cells (60). We demonstrated that RANKL mRNA up-regulation and/or secretion correlated with K1 and K2 serotypes, but not with the others. Furthermore, there was a significant correlation between RANKL secretion and RORC2 (for K1), but not with the other master switch transcription factors (the correlation was positive but not significant for K2 and RORC2, as well as for K1 or K2 and T-bet). These data demonstrated that RANKL secretion by T cells was restricted to particular *P. gingivalis* serotypes (namely K1 and K2), and allowed to suggest a link between these serotypes with alveolar bone destruction and teeth loosening, one of the hallmarks of periodontitis.

Limiting dilution analyses to determine the frequency of T lymphocytes able to respond to K1, K2, K4 or K⁻ in both healthy and periodontitis-affected individuals led to identify a significant lower frequency of T cells able to respond to K⁻ and K4 in healthy as compared to periodontitis-affected individuals, but there were no significant differences on the frequency of T cells able to respond to K1 or K2. We do not know the reason for the lack of differences, but might be due to the use of whole bacteria instead of specific antigens for the stimulation, leading to a large number of different antigens being presented, where the antigens of interest might represent only a small fraction.

Since the host-pathogen interaction is highly dynamic, and it is known that the extracellular capsule enables bacteria to withstand phagocytosis (61), it comes to no

surprise that a given pathogen evolves generating changes in its capsule. Six different capsular serotypes (K1-K6) and a strain devoid of capsule (K⁻) have been described from isolates of *P. gingivalis*. These different capsular serotypes induced distinct T cell responses, some leading to a Th1- and Th17-type phenotype whereas others trigger a Th2-type phenotype in the host T cells. These data allow suggesting that *P. gingivalis* by simply changing the ratio of different serotypes within the periodontal lesion can modulate the host immune response to prevent its clearance. Furthermore, it can also be speculated that changes in serotype ratio can be related to the alveolar-bone destructive bursts in specific sites, which could be due to an increase of K1 or K2 and the concomitant RANKL secretion.

Taken together, these data suggest that in active periodontitis sites, 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, instead of decreasing the 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.

Materials and Methods

***P. gingivalis* growth conditions and curves**

P. gingivalis from different serotypes have been used, these are serotype K1 (strain W83), serotype K2 (strain HG184), serotype K3 (strain A7A1-28), serotype K4 (strain ATCC-49417), serotype K5 (strain HG1690), serotype K6 (strain HG1691), and the strain devoid of capsule K⁻ (strain ATCC-33277), from which the different *P. gingivalis* serotypes were initially described. The growth curves were determined in order to obtain a reliable number of colony-forming units for DCs stimulation, as described previously (62).

Human peripheral blood samples.

The research described here was approved by the authors institutional review board. Human peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats (from the Madrid Regional Blood Bank). Healthy donors and periodontitis diagnosed patients, to whom the protocol of the study was clearly explained, agreed to participate by signing an IRB-approved informed consent form.

Monocyte purification and DCs differentiation and stimulation

Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood from healthy donors or periodontitis diagnosed patients over a Ficoll gradient (Ficoll-Paque Plus; Amersham Pharmacia Biotech, Uppsala, Sweden) by using standard procedures. Monocytes were purified from PBMCs by magnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), using an anti-CD14 mAb conjugated to magnetic beads, and immediately subjected to a DC differentiation protocol described

previously (62). Differentiated DCs were then primed with increasing multiplicity of infection (MOI) of $0.1-10^3$ (bacteria/DCs ratio) of the encapsulated *P. gingivalis* strains K1-K6 for 2 days. DCs primed with the same MOIs of the non-encapsulated strain K⁻ of *P. gingivalis* or 10 ng/ml of *E. coli* 0111:B4 LPS (Fluka, Sigma-Aldrich Chemie, Buchs, Switzerland) were used for comparison. Non-induced DCs served as control.

T-lymphocyte purification and activation

CD4⁺ T lymphocytes were obtained from peripheral blood samples by immunomagnetic depletion. In PBMCs, non-T helper and memory T helper cells were indirectly magnetically labeled with a cocktail of biotin-conjugated mAbs and an anti-biotin mAb conjugated to magnetic beads (Miltenyi Biotec) to obtain a purified population of naïve CD4⁺ T lymphocytes. For activation, 10^6 cells/ml T lymphocytes were cultured with autologous primed DCs (50:1) in RPMI-1640 containing 10% FCS for 5 days at 37°C. T-lymphocyte cultures devoid of DCs or cultured with non-induced DCs were used as controls.

Phenotypic analyses

The monocyte purification efficiency, their differentiation towards DCs and their subsequent stimulation were monitored as described previously (60). Cells were stained with PE, FITC or PE/Cy5-labelled anti-CD14, CD1a, CD80, CD83, or CD86 mAbs (BD Biosciences Pharmingen, San José, CA, USA) and analyzed by flow cytometry (EPICS XL, Beckman Coulter, Fullerton, CA, USA). T lymphocyte purity was determined using PE or FITC-labeled anti-CD14 and anti-CD4 mAbs (BD Biosciences Pharmingen).

Kinetics of T-lymphocyte activation

For analysis of T-lymphocyte activation, T lymphocytes (10^6 cells/ml) were stimulated with *P. gingivalis* strain K1-primed DCs (50:1) and analyzed at different times (0-5 days). As control, non-induced DCs were used. Cells were stained with PE- or FITC-labeled anti-CD25, anti-CD62L, or anti-CD69 mAbs (BD Biosciences Pharmingen) and analyzed by flow cytometry.

mRNA expression analyses

Cytoplasmic RNA was isolated from T lymphocytes using the NP-40 method as described previously (63). Reverse transcription was performed using a Transcriptor First Strand cDNA synthesis kit following the manufacturer's recommendations (Roche Applied Science, Mannheim, Germany). To quantify the mRNA expression for the cytokines IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p35 and p40), IL-13, IL-17, IL-23, IFN- γ , TNF- α , TNF- β , and TGF- β 1, the transcription factors T-bet, GATA-3, RORC2, and Foxp3, as well as α chain of the IL2-Rec CD25, 50 ng of cDNA were amplified by quantitative real-time PCR in 384-well plates, using the appropriate primers and probes (Table 1) and the FastStart Taqman Probe Master (Roche), in an ABI PRISM 7900 Sequence Detector System (Applied Biosystems, Foster City, CA, USA), as described previously (63). In all cases, 18S rRNA expression levels were used as endogenous control.

Cytokine secretion

After stimulation for 5 days, T-lymphocyte culture supernatants were collected and the secretion of IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-13, IFN- γ , TNF- α , and TNF- β was measured using a multiplex bead-based assay according the manufacturer's protocols (Linco/Millipore Corp., Billerica, MA, USA). Secretion of IL12p70

(OptEIA™, BD Biosciences, San Diego, CA, USA), IL-17, IL-23, and TGF-β1 (Quantikine®, R&D Systems Inc., Minneapolis, MN, USA) were analyzed by ELISA according manufacturer's protocols and measured with an automatic microplate spectrophotometer (Labsystem Multiskan, Helsinki, Finland) at 492 nm.

Limiting dilution analysis

To determine the frequency of CD4⁺ T lymphocytes able to get activated in response to different *P. gingivalis* strains, CD4⁺ T lymphocytes were isolated from 3 chronic periodontitis patients and 3 healthy controls. 10⁶ lymphocytes/ml were cultured with autologous DCs primed with *P. gingivalis* strain K⁻, K1, K2 or K4. DCs were added to the T-lymphocyte cultures in a 1:50 ratio. T cells were transferred to 96-well round-bottom microtiter plates and plated at log 3 dilutions in 200 µl/well RPMI-1640 for 5 d. After 4 d incubation, 1µCi/well [methyl-³H]thymidine ([³H]TdR; Hartmann Analytic GMBH, Braunschweig, Germany) was added and beta emission was counted 24 h later. T lymphocytes exposed to non-induced DCs were used as control. For LDA, [³H]TdR incorporation was considered positive when the detected counts exceeded the mean counts of control by more than 3 SD as described previously (64).

Data analyses

The flow cytometry data were analyzed using the WinMDi 2.9 software (The Scripps Research Institute, La Jolla, CA, USA), represented as histograms and expressed as percentage of positive cells. The quantitative PCR data were analyzed using the ABIPRISM software (Applied Biosystems) and the relative quantification was obtained using the 2^{-ΔΔCt} method after normalization of the expression from the mRNA of interest with 18S rRNA levels (65). For LDA, the frequency of wells with cells 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 (66). Data were statistically analyzed 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 regarding CD-expression levels analyzed by flow cytometry were determined using the Chi-square test. Differences between groups and within each group regarding the cytokine, transcription factor and CD25 mRNA expression as well as the cytokine secretion were determined using the unpaired Kruskal Wallis test or ANOVA and Tukey tests. Correlation coefficients were obtained using the Pearson test. Statistical significance was assumed when $p < 0.05$.

Acknowledgments

We thank Dr. Arie Jan van Winkelhoff (University Medical Center Groningen, State University of Groningen, The Netherlands) for providing us with the different *P. gingivalis* capsular serotypes, Dr. Rubén León and María del Carmen Sánchez (Dentistry School, Universidad Complutense de Madrid, Spain) for sharing their expertise on bacterial culture and Dr. Ana Isabel Esquifino and Judith Ríos (Medical School, Universidad Complutense de Madrid, Spain) for their generous support with multiplex bead-based assays. This work has been supported by grants from the Spanish Ministry of Science and Education (SAF2007-63631 to JAGS) and the Spanish Ministry of Health (FIS-060181 to MS). RV is recipient of a CONICYT-26080046 fellowship from the Chilean Government. This manuscript was submitted as partial fulfillment of the requirements for a PhD degree in Dentistry Sciences by RV.

Conflict of interest: The authors have declared that no conflict of interest exists.

References

1. Socransky, S.S., and Haffajee, A.D. 2005. Periodontal microbial ecology. *Periodontol 2000* 38:135-187.
2. Tanner, A.C., Paster, B.J., Lu, S.C., Kanasi, E., Kent, R., Jr., Van Dyke, T., and Sonis, S.T. 2006. Subgingival and tongue microbiota during early periodontitis. *J Dent Res* 85:318-323.
3. Moore, W.E., and Moore, L.V. 1994. The bacteria of periodontal diseases. *Periodontol 2000* 5:66-77.
4. Paster, B.J., Boches, S.K., Galvin, J.L., Ericson, R.E., Lau, C.N., Levanos, V.A., Sahasrabudhe, A., and Dewhirst, F.E. 2001. Bacterial diversity in human subgingival plaque. *J Bacteriol* 183:3770-3783.
5. van Winkelhoff, A.J., Loos, B.G., van der Reijden, W.A., and van der Velden, U. 2002. Porphyromonas gingivalis, Bacteroides forsythus and other putative periodontal pathogens in subjects with and without periodontal destruction. *J Clin Periodontol* 29:1023-1028.
6. Darveau, R.P., Tanner, A., and Page, R.C. 1997. The microbial challenge in periodontitis. *Periodontol 2000* 14:12-32.
7. Zambon, J.J. 1996. Periodontal diseases: microbial factors. *Ann Periodontol* 1:879-925.
8. van Winkelhoff, A.J., van Steenberghe, T.J., and de Graaff, J. 1988. The role of black-pigmented Bacteroides in human oral infections. *J Clin Periodontol* 15:145-155.

9. Slots, J., and Ting, M. 1999. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: occurrence and treatment. *Periodontol 2000* 20:82-121.
10. Socransky, S.S., and Haffajee, A.D. 1994. Evidence of bacterial etiology: a historical perspective. *Periodontol 2000* 5:7-25.
11. Genco, R.J. 1996. Current view of risk factors for periodontal diseases. *J Periodontol* 67:1041-1049.
12. Nørskov-Lauritsen, N., and Kilian, M. 2006. Reclassification of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and emended description of *Aggregatibacter aphrophilus* to include V factor-dependent and V factor-independent isolates. *Int J Syst Evol Microbiol* 56:2135-2146.
13. Newman, M.G., Socransky, S.S., Savitt, E.D., Propas, D.A., and Crawford, A. 1976. Studies of the microbiology of periodontosis. *J Periodontol* 47:373-379.
14. Bragd, L., Dahlen, G., Wikstrom, M., and Slots, J. 1987. The capability of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* to indicate progressive periodontitis; a retrospective study. *J Clin Periodontol* 14:95-99.
15. Tonetti, M.S., and Mombelli, A. 1999. Early-onset periodontitis. *Ann Periodontol* 4:39-53.

16. Schacher, B., Baron, F., Ludwig, B., Valesky, E., Noack, B., and Eickholz, P. 2006. Periodontal therapy in siblings with Papillon-Lefevre syndrome and tinea capitis: a report of two cases. *J Clin Periodontol* 33:829-836.
17. Holt, S.C., Kesavalu, L., Walker, S., and Genco, C.A. 1999. Virulence factors of *Porphyromonas gingivalis*. *Periodontol 2000* 20:168-238.
18. Slots, J. 1986. Bacterial specificity in adult periodontitis. A summary of recent work. *J Clin Periodontol* 13:912-917.
19. Socransky, S.S., Haffajee, A.D., Ximenez-Fyvie, L.A., Feres, M., and Mager, D. 1999. Ecological considerations in the treatment of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* periodontal infections. *Periodontol 2000* 20:341-362.
20. Dzink, J.L., Socransky, S.S., and Haffajee, A.D. 1988. The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. *J Clin Periodontol* 15:316-323.
21. Dzink, J.L., Tanner, A.C., Haffajee, A.D., and Socransky, S.S. 1985. Gram negative species associated with active destructive periodontal lesions. *J Clin Periodontol* 12:648-659.
22. Laine, M.L., Appelmek, B.J., and van Winkelhoff, A.J. 1997. Prevalence and distribution of six capsular serotypes of *Porphyromonas gingivalis* in periodontitis patients. *J Dent Res* 76:1840-1844.
23. Califano, J.V., Schifferle, R.E., Gunsolley, J.C., Best, A.M., Schenkein, H.A., and Tew, J.G. 1999. Antibody reactive with *Porphyromonas gingivalis* serotypes

- K1-6 in adult and generalized early-onset periodontitis. *J Periodontol* 70:730-735.
24. Gemmell, E., Yamazaki, K., and Seymour, G.J. 2007. The role of T cells in periodontal disease: homeostasis and autoimmunity. *Periodontol 2000* 43:14-40.
 25. Teng, Y.T. 2003. The role of acquired immunity and periodontal disease progression. *Crit Rev Oral Biol Med* 14:237-252.
 26. Armitage, G.C. 1999. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 4:1-6.
 27. Offenbacher, S. 1996. Periodontal diseases: pathogenesis. *Ann Periodontol* 1:821-878.
 28. Jotwani, R., Palucka, A.K., Al-Quotub, M., Nouri-Shirazi, M., Kim, J., Bell, D., Banchereau, J., and Cutler, C.W. 2001. Mature dendritic cells infiltrate the T cell-rich region of oral mucosa in chronic periodontitis: in situ, in vivo, and in vitro studies. *J Immunol* 167:4693-4700.
 29. Newcomb, G.M., Seymour, G.J., and Powell, R.N. 1982. Association between plaque accumulation and Langerhans cell numbers in the oral epithelium of attached gingiva. *J Clin Periodontol* 9:297-304.
 30. Cirrincione, C., Pimpinelli, N., Orlando, L., and Romagnoli, P. 2002. Lamina propria dendritic cells express activation markers and contact lymphocytes in chronic periodontitis. *J Periodontol* 73:45-52.
 31. Ebersole, J.L., and Taubman, M.A. 1994. The protective nature of host responses in periodontal diseases. *Periodontol 2000* 5:112-141.

32. Salvi, G.E., Brown, C.E., Fujihashi, K., Kiyono, H., Smith, F.W., Beck, J.D., and Offenbacher, S. 1998. Inflammatory mediators of the terminal dentition in adult and early onset periodontitis. *J Periodontal Res* 33:212-225.
33. Baker, P.J., Dixon, M., Evans, R.T., Dufour, L., Johnson, E., and Roopenian, D.C. 1999. CD4(+) T cells and the proinflammatory cytokines gamma interferon and interleukin-6 contribute to alveolar bone loss in mice. *Infect Immun* 67:2804-2809.
34. Hourri-Haddad, Y., Wilensky, A., and Shapira, L. 2007. T-cell phenotype as a risk factor for periodontal disease. *Periodontol 2000* 45:67-75.
35. Pradeep, A.R., Roopa, Y., and Swati, P.P. 2008. Interleukin-4, a T-helper 2 cell cytokine, is associated with the remission of periodontal disease. *J Periodontal Res* 43:712-716.
36. Dutzan, N., Vernal, R., Hernandez, M., Dezerega, A., Rivera, O., Silva, N., Aguilon, J.C., Puente, J., Pozo, P., and Gamonal, J. 2009. Levels of interferon-gamma and transcription factor T-bet in progressive periodontal lesions in patients with chronic periodontitis. *J Periodontol* 80:290-296.
37. Takeichi, O., Haber, J., Kawai, T., Smith, D.J., Moro, I., and Taubman, M.A. 2000. Cytokine profiles of T-lymphocytes from gingival tissues with pathological pocketing. *J Dent Res* 79:1548-1555.
38. Bartova, J., Kratka-Opatrna, Z., Prochazkova, J., Krejsa, O., Duskova, J., Mrklas, L., Tlaskalova, H., and Cukrowska, B. 2000. Th1 and Th2 cytokine profile in patients with early onset periodontitis and their healthy siblings. *Mediators Inflamm* 9:115-120.

39. Fokkema, S.J., Loos, B.G., Slegte, C., and van der Velden, U. 2002. A type 2 response in lipopolysaccharide (LPS)-stimulated whole blood cell cultures from periodontitis patients. *Clin Exp Immunol* 127:374-378.
40. Yun, P.L., Decarlo, A.A., Collyer, C., and Hunter, N. 2001. Hydrolysis of interleukin-12 by *Porphyromonas gingivalis* major cysteine proteinases may affect local gamma interferon accumulation and the Th1 or Th2 T-cell phenotype in periodontitis. *Infect Immun* 69:5650-5660.
41. Gemmell, E., and Seymour, G.J. 1994. Cytokines and T cell switching. *Crit Rev Oral Biol Med* 5:249-279.
42. Manhart, S.S., Reinhardt, R.A., Payne, J.B., Seymour, G.J., Gemmell, E., Dyer, J.K., and Petro, T.M. 1994. Gingival cell IL-2 and IL-4 in early-onset periodontitis. *J Periodontol* 65:807-813.
43. Sigusch, B., Klinger, G., Glockmann, E., and Simon, H.U. 1998. Early-onset and adult periodontitis associated with abnormal cytokine production by activated T lymphocytes. *J Periodontol* 69:1098-1104.
44. Reinhardt, R.A., McDonald, T.L., Bolton, R.W., DuBois, L.M., and Kaldahl, W.B. 1989. IgG subclasses in gingival crevicular fluid from active versus stable periodontal sites. *J Periodontol* 60:44-50.
45. Vernal, R., Dutzan, N., Chaparro, A., Puente, J., Antonieta Valenzuela, M., and Gamonal, J. 2005. Levels of interleukin-17 in gingival crevicular fluid and in supernatants of cellular cultures of gingival tissue from patients with chronic periodontitis. *J Clin Periodontol* 32:383-389.

46. Gaffen, S.L., and Hajishengallis, G. 2008. A new inflammatory cytokine on the block: re-thinking periodontal disease and the Th1/Th2 paradigm in the context of Th17 cells and IL-17. *J Dent Res* 87:817-828.
47. Ohyama, H., Kato-Kogoe, N., Kuhara, A., Nishimura, F., Nakasho, K., Yamanegi, K., Yamada, N., Hata, M., Yamane, J., and Terada, N. 2009. The involvement of IL-23 and the Th17 pathway in periodontitis. *J Dent Res* 88:633-638.
48. Cardoso, C.R., Garlet, G.P., Crippa, G.E., Rosa, A.L., Junior, W.M., Rossi, M.A., and Silva, J.S. 2009. Evidence of the presence of T helper type 17 cells in chronic lesions of human periodontal disease. *Oral Microbiol Immunol* 24:1-6.
49. Takahashi, K., Azuma, T., Motohira, H., Kinane, D.F., and Kitetsu, S. 2005. The potential role of interleukin-17 in the immunopathology of periodontal disease. *J Clin Periodontol* 32:369-374.
50. Nakajima, T., Ueki-Maruyama, K., Oda, T., Ohsawa, Y., Ito, H., Seymour, G.J., and Yamazaki, K. 2005. Regulatory T-cells infiltrate periodontal disease tissues. *J Dent Res* 84:639-643.
51. Cardoso, C.R., Garlet, G.P., Moreira, A.P., Junior, W.M., Rossi, M.A., and Silva, J.S. 2008. Characterization of CD4⁺CD25⁺ natural regulatory T cells in the inflammatory infiltrate of human chronic periodontitis. *J Leukoc Biol* 84:311-318.
52. Okui, T., Ito, H., Honda, T., Amanuma, R., Yoshie, H., and Yamazaki, K. 2008. Characterization of CD4⁺ FOXP3⁺ T-cell clones established from chronic inflammatory lesions. *Oral Microbiol Immunol* 23:49-54.

53. Vernal, R., Leon, R., Silva, A., van Winkelhoff, A.J., Garcia-Sanz, J.A., and Sanz, M. 2009. Differential cytokine expression by human dendritic cells in response to different *Porphyromonas gingivalis* capsular serotypes. *J Clin Periodontol* 36:823-829.
54. Gerli, R., Bistoni, O., Russano, A., Fiorucci, S., Borgato, L., Cesarotti, M.E., and Lunardi, C. 2002. In vivo activated T cells in rheumatoid synovitis. Analysis of Th1- and Th2-type cytokine production at clonal level in different stages of disease. *Clin Exp Immunol* 129:549-555.
55. Hofstetter, H., Gold, R., and Hartung, H.P. 2009. Th17 Cells in MS and Experimental Autoimmune Encephalomyelitis. *Int MS J* 16:12-18.
56. Choi, J., Borrello, M.A., Smith, E., Cutler, C.W., Sojar, H., and Zauderer, M. 2001. Prior exposure of mice to *Fusobacterium nucleatum* modulates host response to *Porphyromonas gingivalis*. *Oral Microbiol Immunol* 16:338-344.
57. Kopitar, A.N., Ihan Hren, N., and Ihan, A. 2006. Commensal oral bacteria antigens prime human dendritic cells to induce Th1, Th2 or Treg differentiation. *Oral Microbiol Immunol* 21:1-5.
58. Mogi, M., Ootogoto, J., Ota, N., and Togari, A. 2004. Differential expression of RANKL and osteoprotegerin in gingival crevicular fluid of patients with periodontitis. *J Dent Res* 83:166-169.
59. Vernal, R., Chaparro, A., Graumann, R., Puente, J., Valenzuela, M.A., and Gamonal, J. 2004. Levels of cytokine receptor activator of nuclear factor kappaB ligand in gingival crevicular fluid in untreated chronic periodontitis patients. *J Periodontol* 75:1586-1591.

60. Vernal, R., Dutzan, N., Hernandez, M., Chandia, S., Puente, J., Leon, R., Garcia, L., Del Valle, I., Silva, A., and Gamonal, J. 2006. High expression levels of receptor activator of nuclear factor-kappa B ligand associated with human chronic periodontitis are mainly secreted by CD4⁺ T lymphocytes. *J Periodontol* 77:1772-1780.
61. Farquharson, S.I., Germaine, G.R., and Gray, G.R. 2000. Isolation and characterization of the cell-surface polysaccharides of *Porphyromonas gingivalis* ATCC 53978. *Oral Microbiol Immunol* 15:151-157.
62. Vernal, R., Leon, R., Herrera, D., Garcia-Sanz, J.A., Silva, and Sanz, M. 2008. Variability in the response of human dendritic cells stimulated with *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans*. *J Periodontal Res* 43:689-697.
63. Vernal, R., Velasquez, E., Gamonal, J., Garcia-Sanz, J.A., Silva, A., and Sanz, M. 2008. Expression of proinflammatory cytokines in osteoarthritis of the temporomandibular joint. *Arch Oral Biol* 53:910-915.
64. Erard, F., Nabholz, M., and MacDonald, H.R. 1985. Antigen stimulation of cytolytic T lymphocyte precursors: minimal requirements for growth and acquisition of cytolytic activity. *Eur J Immunol* 15:798-803.
65. Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻($\Delta\Delta C(T)$) Method. *Methods* 25:402-408.

66. Diaz-Guerra, E., Vernal, R., del Prete, M.J., Silva, A., and Garcia-Sanz, J.A. 2007. CCL2 inhibits the apoptosis program induced by growth factor deprivation, rescuing functional T cells. *J Immunol* 179:7352-7357.

Figure legends

Figure 1. Phenotypic analysis of monocytes, dendritic cells (DCs) and T lymphocytes.

(A) Flow cytometry analyses of CD14, CD1a, CD86, CD83, and CD80 to ascertain the purity of monocytes isolated from peripheral blood mononuclear cells (PBMCs), the efficiency of monocyte differentiation towards DCs in presence of 1000 U/ml IL-4 and GM-CSF and their subsequent maturation after a 48-h stimulation at a multiplicity of infection (MOI) of 10^3 with *Porphyromonas gingivalis* strain K1 W83. (B) Flow cytometry analyses of CD4 and CD14 expression demonstrating the purity of naïve CD4⁺ T lymphocytes isolated from PBMC. The histograms shown are from a representative experiment. The values on each histogram expressed as percentage of positive cells represent the mean $\pm$ SD from 10 independent experiments.

Figure 2. Kinetics of T-lymphocyte activation. (A) T-cell activation was analyzed following expression of cell surface markers CD25, CD62L and CD69 by flow cytometry in T lymphocytes activated by dendritic cells (DCs) primed at a multiplicity of infection (MOI) of 10^3 with *Porphyromonas gingivalis* strain K1 W83 for 5 days (K1). Expression of activation markers in T lymphocytes exposed to non-induced DCs were used as control (n.i.). The histograms shown are from a representative experiment. The values on each histogram expressed as percentage of positive cells represents the mean $\pm$ SD from 5 independent experiments. Similar results were found when T lymphocytes were activated with the different *P. gingivalis* serotypes. (B) RT-quantitative PCR analysis for the CD25 mRNA expression in T lymphocytes activated by dendritic cells (DCs) primed at a multiplicity of infection (MOI) of 10^3 with the encapsulated (K1-K6) or non-encapsulated (K⁻) strains of *P. gingivalis*. For relative expression, the CD25 mRNA expression in T lymphocytes cultured in the absence of

DCs was considered as 1, as a reference for fold-change in expression. Comparisons were done *versus* T lymphocytes exposed to non-induced DCs ($*p < 0.05$). Data are represented as fold-change 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.

Figure 3. Cytokine mRNA expression. Quantitative PCR analysis for the cytokine mRNA expression in T lymphocytes activated by dendritic cells (DCs) primed with increasing multiplicity of infection (MOI) of encapsulated (K1-K6) or non-encapsulated (K⁻) strains of *Porphyromonas gingivalis*. Black squares correspond to cytokine mRNA expression in T lymphocytes activated by DCs primed with *E. coli* LPS, used as positive control (LPS). For relative expression, the cytokine 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 represented as mean $\pm$ SD for 7 independent experiments. $*p < 0.05$.

Figure 4. Cytokine secretion levels. Secreted cytokines into the supernatants from the T lymphocyte cultures used for Figure 3 (the condition represented is MOI of 10^3) with the different K strains. Secreted cytokine levels by T lymphocytes exposed to non-induced DCs were used as control (n.i.). Data are represented as cytokine concentration (pg/ml) from 10 independent experiments. The box plots show the medians, 1st and 3rd quartiles as boxes; 10th and 90th percentiles are represented as whiskers. $*p < 0.05$.

Figure 5. Transcription factor mRNA expression. Quantitative PCR analysis for the T-bet (Th1), GATA-3 (Th2), RORC2 (Th17), and Foxp3 (Treg) mRNA expression in T lymphocytes activated by dendritic cells (DCs) primed at a multiplicity of infection (MOI) of 10^3 with encapsulated (K1-K6) or non-encapsulated (K⁻) strain of

Porphyromonas gingivalis. For relative expression, the transcription factor mRNA expression in T lymphocytes exposed to non-induced DCs was considered as 1, as a reference for fold-change in expression (n.i.). Data are represented as fold-change from 7 independent experiments. The box plots show the medians, 1st and 3rd quartiles represented as boxes and 10th and 90th percentiles represented as whiskers. * $p < 0.05$.

Figure 6. RANKL mRNA expression and secretion levels. (A) Quantitative PCR analysis for the RANKL mRNA expression in T lymphocytes activated by dendritic cells (DCs) primed with increasing multiplicity of infection (MOI) of encapsulated (K1-K6) or non-encapsulated (K⁻) strains of *P. gingivalis*. Black squares correspond to cytokine mRNA expression in T lymphocytes activated by DCs primed with *E. coli* LPS, used as positive control (LPS). For relative expression, the cytokine 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 represented as mean $\pm$ SD for 7 independent experiments. * $p < 0.05$. (B) Secreted RANKL into the supernatants from the T lymphocyte cultures used for Figure 6A (the condition represented is MOI of 10³) with the different K strains. 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) from 10 independent experiments. The box plots show the medians, 1st and 3rd quartiles as boxes; 10th and 90th percentiles are represented as whiskers. Outliers are shown as open circles. * $p < 0.05$. (C) Correlation analyses of RANKL mRNA expression and secretion in T lymphocytes activated by DCs primed at a MOI of 10³ with serotypes K1-K2 of *P. gingivalis*.

Figure 7. Frequency of T lymphocytes able to respond to different *P. gingivalis* serotypes. Frequency of CD4⁺ T lymphocytes able to respond to K1, K2, K4 or K⁻ *P. gingivalis* serotypes in healthy individuals and periodontitis patients determined by limiting dilution analyses. Different T lymphocyte concentrations were cultured with autologous DCs primed with *P. gingivalis* strains K⁻, K1, K2 or K4. The frequency of wells with proliferating cells in each experimental condition was determined by [³H]TdR incorporation and LDS analysis and represented showing the estimated responding cells per well and the frequency of negative cultures.

Table 1. Primers and PCR probes used for quantitative real-time PCR

mRNA	Forward Primer	Reverse Primer	PCR Probe *
IL-1 β	ctgtctcgcgtgttgaaaga	ttgggtaattttgggatctaca	78
IL-2	aagttttacatgcccaagaagg	aagtgaaagttttgctttgagc	65
IL-4	caccgagttgaccgtaacag	gccctgcagaaggtttcc	16
IL-5	ctctgaggattcctgttctgt	cagtacccccctgcacagt	47
IL-6	gcccagctatgaactcettct	gaaggcagcaggcaacac	45
IL-10	tgggggagaacctgaagac	ccttgctctgttttcacagg	30
IL-12p35	cactcccaaacctgctgag	tctcttcagaagtgaagggtta	50
IL-12p40	ccctgacattctgcgttca	aggtcttgctcgtgaagactcta	37
IL-13	agccctcaggagagctcat	ctccataccatgctgccatt	17
IL-17	tgggaagacctcattggtgt	ggatttcgtgggattgtgat	8
IL-23	agcttcatgcctccctactg	ctgctgagtctccagtgg	30
IFN- γ	ggcattttgaagaattggaaag	tttgatgctctggatcatctt	21
TNF- α	cagcctcttctccttctgat	gccagagggtgattagaga	29
TNF- β	ctaccgcccagcagtgtc	gtggtgtcatggggaga	13
TGF- β 1	cacgtggagctgtaccagaa	cagccggttgcctgaggta	72
RANKL	tgattcatgtaggagaattaaacagg	gatgtgctgtgatccaacga	17
T-bet	tccaagttaatcagcaccaga	tgacaggaatgggaacatcc	9
GATA-3	ctcattaagcccaagcgaag	tctgacagttcgacaggac	71
RORC2	agaaggacaggagccaag	caagggatcacttcaatttgtg	21
Foxp3	Acctacgccacgctcatc	tcattgagtgtccgtgct	50
CD25	caagcgagccttcagatt	ggccactgctacctggtact	46
18S rRNA	ctcaacacgggaaacctcac	cgtccaccaactaagaacg	77

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

Table 2. Correlation analyses of cytokine secretion and transcription factor mRNA expression

			Pearson's correlation coefficient (r)**					
		K ⁻	K1	K2	K3	K4	K5	K6
T-bet	IL-1β		0.901*	0.916*				
	IL-2		0.815*	0.882*				
	IFN-γ		0.891*	0.941*				
	IL-12p40		0.845*	0.924*				
	IL-12p70		0.849*	0.886*				
	TNF-α		0.852*	0.969*				
	TNF-β		0.845*	0.813*				
GATA-3	IL-4				0.862*	0.930*	0.799*	0.538
	IL-5				0.828*	0.795*	0.845*	0.435
	IL-13				0.858*	0.746	0.734	0.463
RORC2	IL-6		0.889*	0.936*				
	IL-17		0.900*	0.895*				
	IL-23		0.917*	0.962*				
Foxp3	IL-10	0.348						
	TGF-β1	0.269						

* $p < 0.05$

**Pearson's correlation coefficient (r) between the transcription factors T-bet, RORC2 and Foxp3 and the secreted cytokines from activated T lymphocytes (at an MOI of 10^3 with the different *P. gingivalis* serotypes) (n=7). Analyses that did not show a positive correlation are not shown (blank).

*** Correlations between T-bet and RORC2 with Th1 and Th17 cytokines for K1 and K2 are shown in supplementary Figure 1.

Figure S1. Correlation analyses of cytokine secretion and transcription factor T-bet and RORC2. Table 1 shows a positive correlation between T-bet and RORC2 with Th1 and Th17 cytokines. A detailed analyses of these correlations in K1 and K2 for each of these transcription factors with Th1 and Th17 cytokines show a positive correlation between T-bet and Th1 cytokines or between RORC2 and Th17 cytokines (red), and a lack of correlation between T-bet and Th17 cytokines or between RORC2 and Th1 cytokines (black). These data indicate that K1 and K2 are able to activate both Th1 and Th17 T-cell clones.

Vernal R. *et al.*
Figure 1

Vernal R. *et al.*

Figure 2

Vernal R. *et al.*

Figure 3

Vernal R. *et al.*
Figure 4

Vernal R. *et al.*
Figure 5

Tbet

GATA3

RORC2

Foxp3

Vernal R. *et al.*
Figure 6

Vernal R. *et al.*
Figure 7

Figure 1S