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Distinct human T-lymphocyte responses triggered by *Porphyromonas gingivalis* capsular serotypes

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Abstract

Aim: *Porphyromonas gingivalis* can synthesize an extracellular capsule and different serotypes have been described based on capsular antigenicity. On dendritic cells (DCs), the type of capsule present plays a role on the strength of the developed immune response. This study aimed to investigate the T-lymphocyte responses when stimulated with autologous mature DCs exposed to different *P. gingivalis* K-serotypes.

Materials and Methods: Naïve $CD4^+$ T-lymphocytes were obtained from healthy subjects and stimulated with autologous DCs primed with increasing multiplicity of infections of the different *P. gingivalis* K-serotypes. The Th1, Th2, Th17 and T-regulatory cytokines and transcription factor levels were quantified.

Results: Distinct types of response were detected when T-lymphocytes were stimulated by DCs primed with the different *P. gingivalis* K-serotypes. T-lymphocytes stimulated by K1 or K2-primed DCs elicited higher levels of Th1 and Th17-associated cytokines, T-bet and RORC2 than T-lymphocytes stimulated with DCs primed with the other serotypes. Conversely, the serotypes K3-K5 induced higher levels of Th2-associated cytokines and GATA-3 than the others.

Conclusions: These results demonstrate that DCs primed with the different *P. gingivalis* K-serotypes elicited distinct T-cell responses. Strains K1 (W83) and K2 (HG184) induced a Th1/Th17 pattern of immune response and K3 (A7A1-28), K4 (ATCC®49417™), and K5 (HG1690) a Th2 response.

Key words: cytokines; *Porphyromonas gingivalis*; serotypes; T helper lymphocytes; transcription factors

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Conflict of interest and source of funding statement

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Adaptive immune responses are key for the efficient defence against etiological microorganisms during infectious diseases. $CD4^+$ T lymphocytes are an essential component in these responses by orchestrating the functional activities of both innate and adaptive immune systems. Nowadays, $CD4^+$ T lymphocytes have been divided, on basis of their specific cytokine production profile as well as by the expression of specific transcription factor master-switch genes implicated in T-cell differentiation, into distinct functional lineages, termed: T-helper type 1 (Th1), Th2, Th17 and T regulatory (Treg) lymphocytes. Th1 lymphocytes express the transcription factor master-switch gene T-bet and are characterized by the secretion of interleukin (IL)-1β, IL-2, IL-12, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, and TNF-β; Th2 lymphocytes express

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GATA-3 and produce IL-4, IL-5, and IL-13; Th17 lymphocytes express RORC2 and produce IL-6, IL-17, and IL-23; and Treg lymphocytes express Foxp3 and produce IL-10 and transforming growth factor (TGF)-β1 (Bluestone & Abbas 2003, Harrington et al. 2006, Reiner 2007, Stockinger & Veldhoen 2007, Vernal & Garcia-Sanz 2008). More recently, two new lineages of CD4$^+$ T lymphocytes have been proposed, the Th9 subset, which expresses the transcription factor SPI-B (PU.1) and could display suppressor functions by secreting IL-9, and the Th22 subset, which expresses the transcription factor aryl hydrocarbon receptor and could display pro-inflammatory functions by secreting IL-22 (Trifari et al. 2009, Akdis et al. 2012, Jabeen & Kaplan 2012, Kato-Kogoe et al. 2012, Wolff et al. 2012, Aranha et al. 2013).

Periodontitis is a bacterially induced chronic inflammatory disease that usually affects adults and if untreated causes periodontal tissue destruction and tooth loss. Although the periodonto-pathogenic bacteria may cause direct tissue damage, their pathogenicity mainly depends on the activation of host immuno-inflammatory response. During the periodontal infection, these immuno-inflammatory mechanisms are mainly dependent on the Th-lymphocyte phenotype and function and determine most of the connective tissue destruction and resorption of the tooth-supporting alveolar bone (Gemmell et al. 2007, Houri-Haddad et al. 2007, Garlet 2010, Graves et al. 2011).

Dendritic cells (DCs) are specialized antigen-presenting cells that receive specific signals from bacterial toll-like receptor ligands and stimulate naïve T lymphocytes, thus regulating the development of Th lineages and the outcome of T-cell immune responses (Cutler & Jotwani 2006, Cutler & Teng 2007, Hivroz et al. 2012, Blum et al. 2013). Bacteria, therefore, may determine the type of Th response and the development of a pathogen specific cytokine release pattern during human infections (Iwasaki & Medzhitov 2004, Woehrle et al. 2008, Garlet 2010, Hernandez et al. 2011). In fact, antigens prepared from commensal oral bacteria, such as *Bacteroides fragilis*, *Streptococcus mitis*, or *Propionibacterium acnes*, can prime human DCs to activate T-lymphocytes, although the antigens from each bacteria induce a distinct T-cell phenotype, in particular a Th1, Th2 or Treg, respectively (Kopitar et al. 2006).

Recently, our research group has reported a differential immunogenicity when DCs were stimulated with *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (Vernal et al. 2008a) and, moreover, a differential immune response was also identified when the DCs were stimulated with the different capsular (K) serotypes of *P. gingivalis* (Vernal et al. 2009). It can be, therefore, speculated that these different *P. gingivalis* K serotypes might play a relevant role on the differential stimulation of T lymphocytes, thus driving the pattern of cytokine synthesis and the T-cell immune response. The objective of this investigation is to determine whether DCs maturated by exposure to the different *P. gingivalis* K serotypes, when used to stimulate autologous T lymphocytes, can regulate the pattern of expression and secretion of cytokines, as well as the transcription factors T-bet, GATA-3, RORC2 and Foxp3, which are the master-switch genes implied in Th1, Th2, Th17, and Treg differentiation, respectively.

Materials and Methods

P. gingivalis growth conditions and curves

P. gingivalis strains W83 (K1), HG184 (K2), A7A1-28 (K3), ATCC®49417™ (K4), HG1690 (K5), HG1691 (K6), and ATCC®33277™ (K$^-$) were cultured on 5% horse blood agar (Oxoid Nº2; Oxoid Ltd, Basingstoke, UK), supplemented with 5 mg/l of haemin and 1 mg/l of menadione, at 37°C and under anaerobic conditions (80% N_2, 10% CO_2, and 10% H_2). 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).

Monocyte purification and DCs differentiation and stimulation

Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood from 10 healthy donors 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 monoclonal antibody (mAb) conjugated to magnetic beads, and were immediately subjected to DC differentiation following the previously described protocol (Vernal et al. 2008a). Briefly, monocytes (CD14$^+$ cell fraction) were 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 obtain a population of immature DCs. For maturation, differentiated DCs were then primed with increasing multiplicity of infection (MOI) of 0.1 to 10^3 (bacteria/DCs ratio) of the encapsulated *P. gingivalis* strains K1-K6 for 2 days. DCs stimulated with increasing MOI of the non-encapsulated strain K$^-$ of *P. gingivalis* or 10 ng/ml of lipopolysaccharide of *Escherichia coli* 0111:B4 (Fluka, Sigma-Aldrich Chemie, Buchs, Switzerland) were used for comparison. Non-induced DCs served as control.

T-lymphocyte purification and stimulation

For each subject, a purified population of autologous naïve CD4$^+$ T lymphocytes was obtained by magnetic cell sorting from the CD14$^-$ cell fraction of the PBMCs. Briefly, both non-T helper as well as memory T helper cells were depleted using a cocktail of biotin-conjugated mAbs and anti-biotin mAbs conjugated to magnetic beads (Miltenyi Biotec). For T-cell stimulation, 10^6 T lymphocytes/ml were cultured with autologous DCs (50:1) primed with the different K serotypes of *P. gingivalis* in RPMI-1640 containing 10% FCS for 5 days at 37°C. For each subject, the experiment was performed separately. T-lymphocyte cultures devoid of DCs or exposed to non-induced autologous DCs were used as controls.

Phenotypic cell analysis

The efficiency of the monocyte purification, their differentiation towards

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DCs as well as their subsequent maturation was analysed as described previously (Vernal et al. 2006). Cells were stained with PE, FITC or PE/Cy5-labelled anti-CD14, CD1a, CD80, CD83, and CD86 mAbs (BD Biosciences Pharmingen, San José, CA, USA) and analysed by flow cytometry (EPICS XL, Beckman Coulter, Fullerton, CA, USA). T lymphocyte purity was determined using PE or FITC-labelled anti-CD14 and anti-CD4 mAbs (BD Biosciences Pharmingen).

Kinetics of T-lymphocyte activation

10^6 T lymphocytes/ml were stimulated with *P. gingivalis* strain K1-primed DCs (50:1) for 5 days and analysed at different times (0–5 days). As control, non-induced DCs were used. The T-lymphocyte stimulated cells were stained with PE- or FITC-labelled anti-CD25α, anti-CD62L, and anti-CD69 mAbs (BD Biosciences Pharmingen) and analysed by flow cytometry.

Expression of cytokine, transcription factor and CD25α mRNAs

Cytoplasmic RNA was isolated from T lymphocytes using the NP-40 method as described previously (Vernal et al. 2008b). 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-1β, 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-β1, for the transcription factors T-bet, GATA-3, RORC2, and Foxp3, as well as for the T-cell activation marker CD25α, 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). 18S rRNA expression levels were used as endogenous control.

Secretion of cytokines

After stimulation for 5 days, T-lymphocyte culture supernatants were collected and the secretion of IL-1β, 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 to 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 analysed by ELISA according manufacturer's protocols and measured with an automatic microplate spectrophotometer (Labsystem Multiskan, Helsinki, Finland) at 492 nm.

Data analysis

The flow cytometry data were analysed using the WinMDi 2.9 software (The Scripps Research Institute, La Jolla, CA, USA), represented as histograms and expressed as the percentage of positive cells. 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. 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 regarding CD-expression levels analysed by flow cytometry were determined using the Chi-square test. Differences between groups and within each group regarding the cytokine, transcription factor and CD25α 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 $p < 0.05$.

Results

Phenotypic analysis of monocyte purification, DC differentiation and stimulation

The monocyte and DC purities were demonstrated by the combined staining with mAbs CD14, CD1a, CD86, CD83, and CD80 and analysed by flow cytometry. Similarly to our previous experiments (Vernal et al. 2009), a highly purified (>98%) monocyte population ($CD14^+$ cells) was isolated from peripheral blood. These monocytes differentiated at a high frequency (>97%) into DCs upon culture in presence of IL-4 and GM-CSF, as demonstrated by the expression of the CD1a marker, the increase in the expression of CD86

Table 1. Forward primers, reverse primers and PCR probes used for cytokine, transcription factor, CD25α mRNA and 18S rRNA amplifications by quantitative real-time PCR

mRNA	Forward primer	Reverse primer	PCR probe*
IL-1β	ctgtcctgcgtgttgaaaga	ttgggtaatttttgggatctaca	78
IL-2	aagttttacatgcccaagaagg	aagtgaaagtttttgctttgagc	65
IL-4	caccgagttgaccgtaacag	gccctgcagaaggtttcc	16
IL-5	ctctgaggattcctgttcctgt	cagtaccccttgcacagtt	47
IL-6	gcccagctatgaactccttct	gaaggcagcaggcaacac	45
IL-10	tgggggagaacctgaagac	ccttgctcttgttttcacagg	30
IL-12p35	cactcccaaaacctgctgag	tctcttcagaagtgcaagggta	50
IL-12p40	ccctgacattctgcgttca	aggtcttgtccgtgaagactcta	37
IL-13	agccctcagggagctcat	ctccataccatgctgccatt	17
IL-17	tgggaagacctcattggtgt	ggatttcgtgggattgtgat	8
IL-23	agcttcatgcctccctactg	ctgctgagtctcccagtggt	30
IFN-γ	ggcattttgaagaattggaaag	tttggatgctctggtcatctt	21
TNF-α	cagcctcttctccttcctgat	gccagagggctgattagaga	29
TNF-β	ctaccgcccagcagtgtc	gtggtgtcatggggaga	13
TGF-β1	cacgtggagctgtaccagaa	cagccggttgctgaggta	72
T-bet	tccaagtttaatcagcaccaga	tgacaggaatgggaacatcc	9
GATA-3	ctcattaagcccaagcgaag	tctgacagttcgcacaggac	71
RORC2	agaaggacagggagccaag	caagggatcacttcaatttgtg	21
Foxp3	acctacgccacgctcatc	tcattgagtgtccgctgct	50
CD25α	caagcgagccttccagatt	ggccactgctacctggtact	46
18SrRNA	ctcaacacgggaaacctcac	cgctccaccaactaagaacg	77

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

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antigen and the concomitant loss of the monocyte marker CD14. Upon stimulation at an MOI of 10^3 with *P. gingivalis* strain W83 (K1), the efficiency of DC maturation (>96%) was confirmed by the increased expression of CD83 and CD80 antigens, associated with an increase in cell size. Highly purified (>98%) population of autologous naïve T lymphocytes ($CD4^+$ cells), devoid of monocytes ($CD14^+$ cells), was isolated from peripheral blood.

T lymphocyte activation

T lymphocyte activation kinetics was analysed at different times (0–5 days) following expression of cell surface markers CD25α, CD62L, and CD69 by flow cytometry (Fig. 1). As early as 1 day, a small subset (>2%) of naïve T lymphocytes showed *P. gingivalis*-specific cell activation when stimulated by autologous DCs primed at an MOI of 10^3 with *P. gingivalis* strain W83 (K1), reaching levels >10% 5 days after T-lymphocyte induction due most likely to proliferation of the initially activated T lymphocytes, although we cannot exclude the activation of some additional T-cell clones. 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 increase in the expression levels of CD25α, the inducible α-subunit of the IL-2R, that increased >5-fold by day 5 compared with baseline expression. Conversely, the expression levels of CD62L (L-selectin), leukocyte adhesion molecule member of the homing receptor family that is lost after cell activation, decreased >7-fold 5 days after T-lymphocyte induction. Quantitative PCR analysis showed significant over-expression in CD25α mRNA levels when T lymphocytes where stimulated by autologous DCs primed at an MOI of 10^3 with *P. gingivalis* strains K1–K6 or K^- compared with T lymphocytes exposed to non-induced DCs (Fig. 2). No significant differences were detected when T lymphocytes were stimulated by autologous DCs primed with any of the encapsulated or non-encapsulated *P. gingivalis* strains. Specificity of the response was shown by the lack of induction of CD25 mRNA in T-lymphocytes co-cultured with DCs in the absence of *P. gingivalis* (Fig. 2).

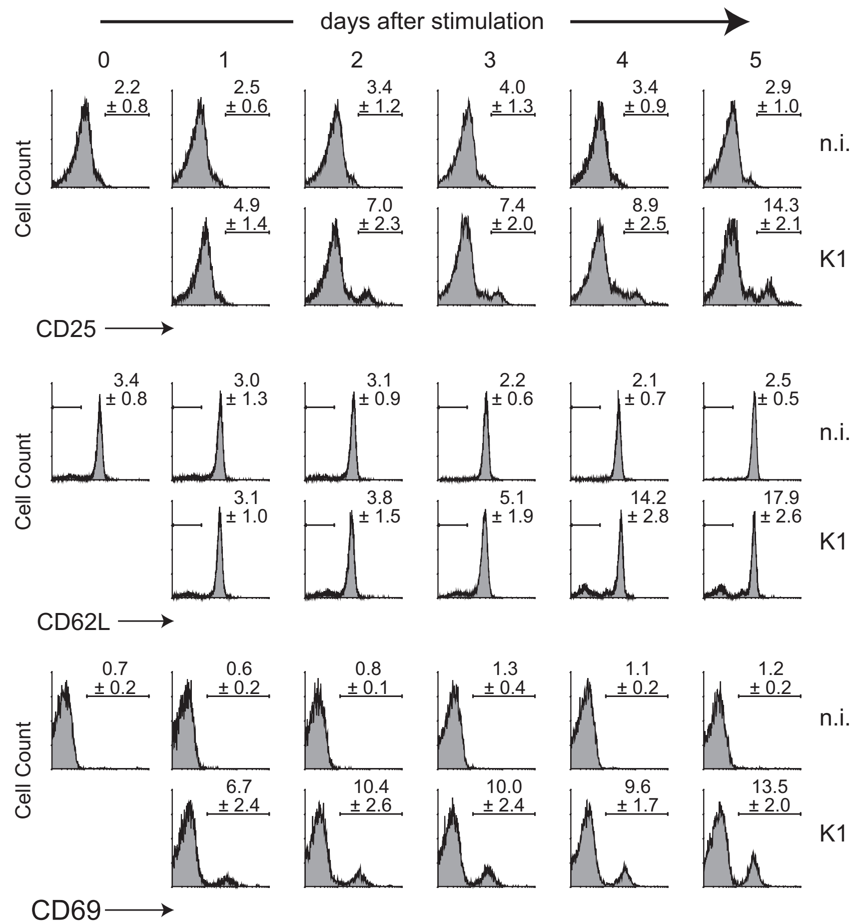

Fig. 1. Kinetics of T-lymphocyte activation. Activation was analysed following expression of cell surface markers CD25α, CD62L and CD69 by flow cytometry in T lymphocytes stimulated by autologous dendritic cells (DCs) primed at a multiplicity of infection (MOI) of 10^3 with *Porphyromonas gingivalis* 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 data from each experiment were expressed as percentage of positive cells over the total and shown as mean ± SD from five independent experiments. CD, cluster of differentiation.

Cytokine mRNA expression

The mRNA expression for the analysed cytokines was determined by quantitative real-time PCR and represented as fold-change for each condition (Fig. 3). A dose-dependent increase in the expression levels was elicited on T lymphocytes following DC stimulation with each of the K strains of *P. gingivalis*. When the serotypes K1 (W83) or K2 (HG184) were used, the observed T-lymphocyte response was characteristic of a Th1-pattern of cytokines, as demonstrated by higher expression levels of IL-1β, IFN-γ, IL-12p35, IL12-p40, TNF-α, and TNF-β mRNAs (at MOIs of 0.1–10^3 $p < 0.05$) than the strains K3-K6 or K^-. Higher expression levels were also detected for IL-2 mRNA (at MOIs of 10^2 and 10^3 $p < 0.05$) when cells were stimulated with the serotypes K1 or K2-primed DCs compared with the others. Similarly, a Th17-pattern of cytokine expression was detected when T lymphocytes were activated with *P. gingivalis* strains K1 or K2-primed DCs. In fact, these serotypes elicited a higher increase in the expression of IL-6, IL-17, and IL-23 mRNAs (at MOIs of 0.1–10^3 $p < 0.05$) as compared with strains K3–K6 or K^-; however, these over-expressed levels were lower than those detected for

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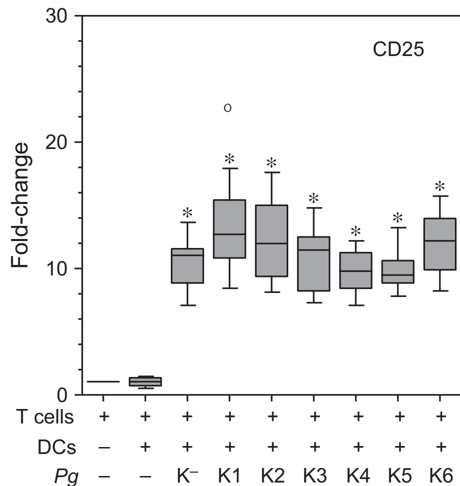

Fig. 2. T-lymphocyte activation. Quantitative PCR analysis for the CD25α mRNA expression in T lymphocytes stimulated by autologous dendritic cells (DCs) primed at a multiplicity of infection (MOI) of 10^3 with the encapsulated (K1–K6) or non-encapsulated (K^-) strains of *Porphyromonas gingivalis*. For relative expression, the CD25α 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. CD, cluster of differentiation; *Pg*, *Porphyromonas gingivalis*.

Th1-associated cytokines. Conversely, when the serotypes K3 (A7A1-28), K4 (ATCC®49417™) or K5 (HG1690) were used for cell stimulation, the observed T-lymphocyte response was characteristic of a Th2-pattern of cytokine expression. Although, IL-13 was not over-expressed in any experimental condition, strains K3-K5-primed DCs elicited on T lymphocytes higher expression levels of IL-4 and IL-5 mRNAs (at MOIs of 10^2 and 10^3 $p < 0.05$) than the strains K1, K2, K6 or K^-. Overall, a T lymphocyte response biased towards a Th1/Th17 or Th2-pattern of cytokine expression was detected under stimulation with capsulated strains K1 and K2 or K3–K5 of *P. gingivalis*, respectively, in contrast to the K^- strain (ATCC®33277™) which fostered a regulatory immune response by eliciting over-expressed levels of IL-10 and TGF-β1 mRNAs (at an MOI of 10^3 $p < 0.05$).

Cytokine secretion

The Th1/Th17 or Th2-pattern of lymphocyte response detected upon stimulation with the serotypes K1 and K2 or K3–K5-primed DCs, respectively, was confirmed when cytokine production was analysed at a protein level (Fig. 4–5). Higher levels of IL-1β, IL-2, IFN-γ, IL-12p40, IL12-p70, TNF-α, and TNF-β were detected when cells were stimulated with serotypes K1 (W83) or K2 (HG184)-primed DCs compared with the other serotypes (at MOIs of 10^2 and 10^3 $p < 0.05$). Similarly, higher levels of IL-6 and IL-17 were secreted by T lymphocytes stimulated by DCs primed with the K1 or K2 strains (at MOIs of 10^2 and 10^3 $p < 0.05$). In addition, the strain K1 induced a higher secretion of IL-23 as compared with the strains K2-K6 or K^- (at an MOI of 10^3 $p < 0.05$). On the other hand, higher levels of IL-4 and IL-5 were detected in T lymphocytes stimulated by DCs primed with serotypes K3 (A7A1-28), K4 (ATCC®49417™) or K5 (HG1690), as compared with the other serotypes (at MOIs of 10^2 and 10^3 $p < 0.05$). In addition, strain K3 elicited higher IL-13 levels than those observed with the strains K1, K2, K6 or K^- (at an MOI of 10^3 $p < 0.05$). Finally, higher secreted levels of IL-10, associated to a Treg response, were detected upon cell activation with the K^- strain (ATCC®33277™) of *P. gingivalis*, compared with all the encapsulated strains (at MOIs of 10^2 and 10^3 $p < 0.05$).

Transcription factor mRNA expression

The mRNA expression for T-bet, GATA-3, RORC2 and Foxp3 was determined by quantitative real-time PCR on T lymphocytes stimulated by DCs primed at MOIs of 10^2 and 10^3 with the different *P. gingivalis* strains (Fig. 6). Interestingly, the T lymphocytes stimulated with serotypes K1 (W83) or K2 (HG184)-primed DCs showed a higher relative expression of T-bet and RORC2 mRNAs (at MOIs of 10^2 and 10^3 $p < 0.05$) than the same cells stimulated with the other strains. For GATA-3, higher mRNA levels were detected upon activation with K3 (A7A1-28), K4 (ATCC® 49417™) or K5 (HG1690) serotypes compared with the others (at an MOI of 10^3 $p<0.05$). Furthermore, Foxp3 mRNA expression was increased upon K^- strain (ATCC® 33277™) stimulation (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 and Fig. S1) yielded significant positive correlation between T-bet expression and Th1-associated cytokine secretion (IL-1β, IL-2, IL-12, IFN-γ, TNF-α, and TNF-β), but not with Th17-associated cytokines, when T lymphocytes were stimulated 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), but not between this transcription factor and Th1-associated cytokines. Similarly, a significant positive correlation was detected between GATA-3 and Th2-associated cytokines IL-4 and IL-5 when T lymphocytes were stimulated with serotypes K3-K5-primed DCs. IL-13 was positively correlated when cells were stimulated with the strain K3-primed DCs. In contrast, Foxp3 expression did not show any positive correlation with IL-10 or TGF-β1 secretion.

Discussion

T-cell phenotype is a key determinant in the pathogenesis of periodontitis and the outcome of this

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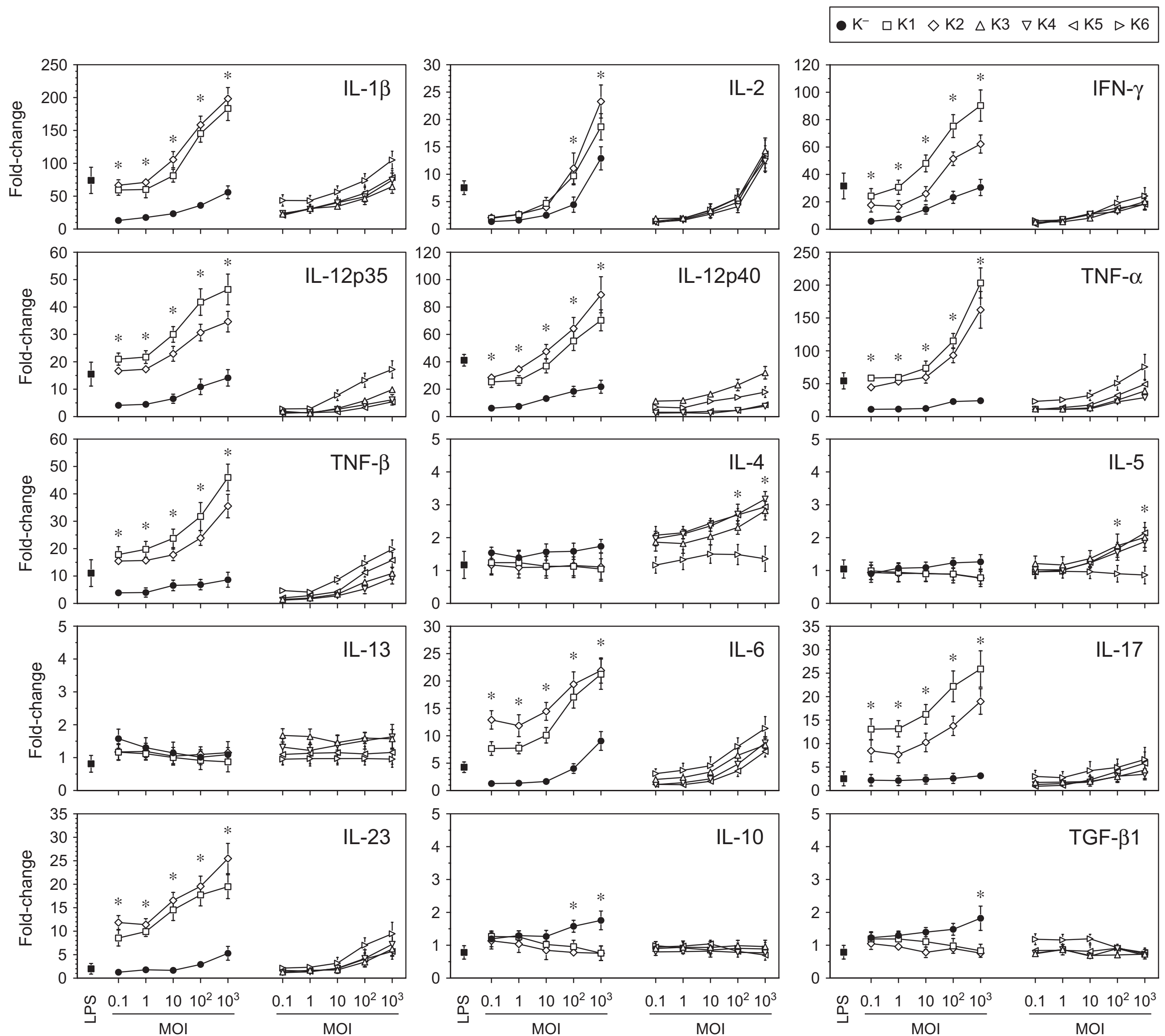

Fig. 3. Cytokine mRNA expression. Quantitative PCR analysis for the cytokine mRNA expression in T lymphocytes stimulated by autologous dendritic cells (DCs) primed with increasing multiplicity of infection (MOI) of encapsulated (K1–K6) or non-encapsulated (K^-) strains of *Porphyromonas gingivalis*. Black squares correspond to cytokine mRNA expression in T lymphocytes stimulated by autologous DCs primed with lipopolysaccharide of *Escherichia coli*, 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 (data not shown). Data are represented as fold-change and shown as mean ± SD for seven independent experiments. Each experiment was performed in duplicate. IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumour necrosis factor. $*p < 0.05$.

disease is usually determined by the T-lymphocyte cytokine profile at the local inflammation site (Gemmell et al. 2007, Houri-Haddad et al. 2007, Garlet et al. 2010, Graves et al. 2011, Hernandez et al. 2011, Di Benedetto et al. 2013). T lymphocytes not only activate, suppress, and/or regulate the adaptive immune responses but are also the responsible of the feedback mechanism that amplifies the innate immune response, hence intensifying chronic inflammation. In chronic periodontitis, however, it has been difficult to determine which T-cell subsets control each process. In general, it has been established that certain T-lymphocytes and their specific Th cytokine profile at the local site of inflammation are more destructive and lead to tissue damage whereas others are more associated with healing (Gemmell et al. 2007, Houri-Haddad et al. 2007, Garlet 2010, Graves et al. 2011). Several studies have, however, evidenced the complexity of the immune response elicited by periodontal pathogens. In a murine experimental periodontitis model, *P. gingivalis* and *Treponema denticola* behaved synergistically and induced alveolar bone destruction through an inflammatory Th1-type response (Choi et al. 2001, Orth et al. 2011). A prior infection with

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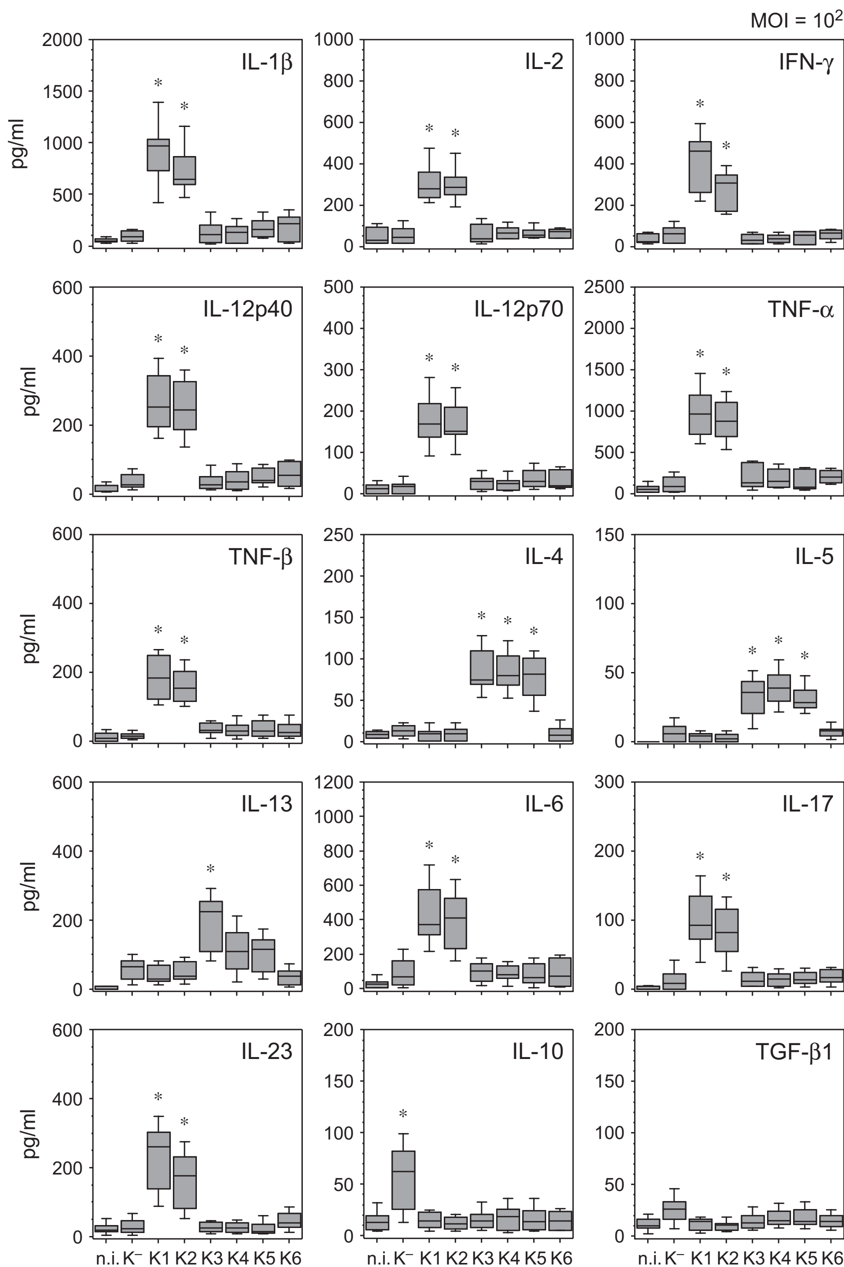

Fig. 4. Cytokine secretion levels. Cytokine secretion in T lymphocytes stimulated by autologous dendritic cells (DCs) primed at a multiplicity of infection (MOI) of 10^2 with encapsulated (K1-K6) or non-encapsulated (K^-) strains of *Porphyromonas gingivalis*. Secreted cytokine levels by T lymphocytes exposed to non-induced DCs were used as control (n.i.). Data are represented as cytokine concentration (pg/ml) for 10 independent experiments. Each experiment was performed in duplicate. The box plots show the medians, 1st and 3rd quartiles as boxes and 10th and 90th percentiles as whiskers. IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumour necrosis factor. *$p < 0.05$.

Fusobacterium nucleatum, however, triggered a switch towards a Th2-type response (Choi et al. 2001). Furthermore, *B. fragilis*, *S. mitis*, and *P. acnes*, commensal oral bacteria usually residents in healthy periodontal tissues, may also induce different T-lymphocyte responses, in particular a Th1, Th2 and Treg pattern of cytokine synthesis, respectively (Kopitar et al. 2006).

In this investigation, a differential T-cell phenotype and expression of distinct cytokine profiles was demonstrated when human naïve T lymphocytes were stimulated with autologous mature DCs that had been exposed to the different *P. gingivalis* K serotypes. A Th1 and Th17 response pattern was evidenced when T lymphocytes were stimulated by DCs primed with the serotypes K1 (W83) or K2 (HG184), while a Th2 response pattern was evidenced when the stimulating *P. gingivalis* belonged to the serotypes K3 (A7A1-28), K4 (ATCC®49417™), or K5 (HG1690).

It has been described that stimulation of human DCs with either *A. actinomycetemcomitans* or *P. gingivalis* induce a Th1-type of response, leading to IL-12 and IFN-γ secretion (Kikuchi et al. 2004, 2005). Intrinsic differences in antigenicity have been also detected among these pathogens and distinct thresholds for the expression of IL-1β, IL-12, IFN-γ, TNF-α, and TNF-β have been reported (Vernal et al. 2008a). In addition, an antagonistic DC response with different *P. gingivalis* strains has been described where *P. gingivalis* strain 381 induced a potent IL-12 response (Jotwani & Cutler 2004), whereas strain O55:B5 triggered a weak IL-12 response (Kanaya et al. 2004). A heterogenic immuno-stimulatory potential on DCs has been also reported with the six different *P. gingivalis* K serotypes. The strains K1 and K2 have shown a stronger cytokine expression than the others what has suggested that the *P. gingivalis* capsule may have a role on the DCs priming and subsequent antigen presentation (Vernal et al. 2009). This investigation has demonstrated a heterogenic T lymphocyte immune response and a differential T-cell polarization when host cells were activated with the different K serotypes of *P. gingivalis*.

Differences in the sugar composition and structure of *P. gingivalis* capsule between the distinct K serotypes could be involved in the detected differential immuno-stimulatory potential on DCs and T lymphocytes and genetic variations in the capsular polysaccharide locus PG0106–PG0120 may determine these differences (Aduse-Opoku et al. 2006, Brunner et al. 2008, 2010a,b). In fact, differences in polysaccharide composition between the strains W50 (serotype K1) and A7A1-28

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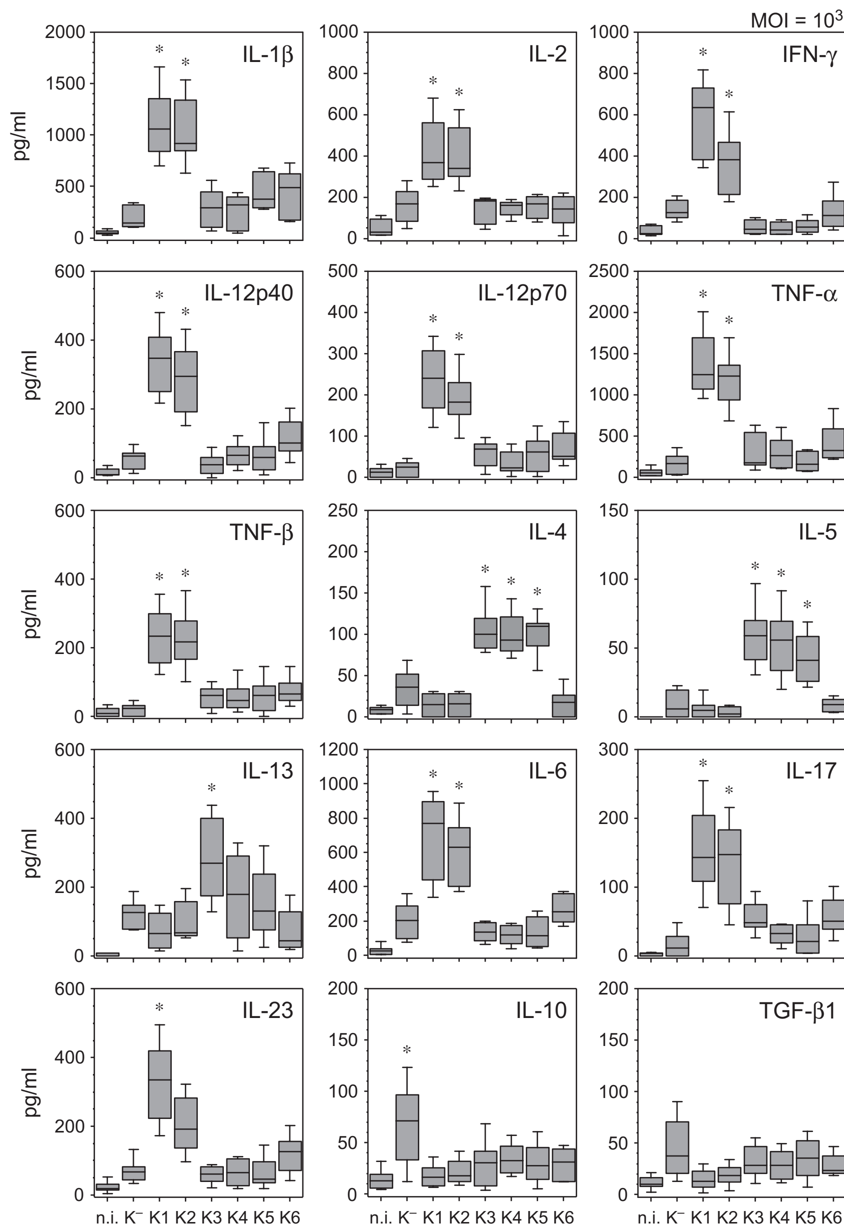

Fig. 5. Cytokine secretion levels. Cytokine secretion in T lymphocytes stimulated by autologous dendritic cells (DCs) primed at a multiplicity of infection (MOI) of 10^3 with encapsulated (K1–K6) or non-encapsulated (K^-) strains of *Porphyromonas gingivalis*. Secreted cytokine levels by T lymphocytes exposed to non-induced DCs were used as control (n.i.). Data are represented as cytokine concentration (pg/ml) for 10 independent experiments. Each experiment was performed in duplicate. The box plots show the medians, 1st and 3rd quartiles as boxes and 10th and 90th percentiles as whiskers. IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumour necrosis factor. *$p < 0.05$.

(serotype K3) have been reported (Schifferle et al. 1989, Farquharson et al. 2000) and deletion of the loci PG0109–PG0118 and PG0116–PG0120 yielded K1 mutants that neither synthesized a capsule nor showed reactivity with anti-K1 antiserum (Aduse-Opoku et al. 2006). Differences in the intensity of the DC maturation, however, could be also involved in the variable T-cell differentiation. In fact, serotypes K1 and K2 of *P. gingivalis* were able to induce a more robust response on DCs compared with the other serotypes and this stronger cell maturation could elicit the Th1/Th17 profile on stimulated T lymphocytes (Vernal et al. 2009). Conversely, a weak DCs maturation, as that induced by the other K serotypes, could elicit a weak co-stimulation during the antigen presentation and, thus, induce the Th2 differentiation (Tao et al. 1997, Badou et al. 2001).

The extracellular capsule enables bacteria to withstand phagocytosis and stimulate the host response in ways that can benefit the colonization of other species of the same microbial community, thus amplifying the destructive inflammatory process (Farquharson et al. 2000, Brunner et al. 2010a,b, Hajishengallis 2011, Singh et al. 2011). A change in the proportions of the different K serotypes of *P. gingivalis* within a periodontal lesion, therefore, could be able to affect the local innate and adaptive immune responses, down or up-regulating the inflammatory response and thus determining the clinical manifestation of periodontitis. In this context, although the genetic diversity of *P. gingivalis* is high in humans, a clonal stability in untreated periodontitis has also been demonstrated (van Winkelhoff et al. 2007, 2008, Rylev & Kilian 2008); however, an association between a specific K serotype of *P. gingivalis* with type, initiation, progression and/or severity of periodontitis has not yet been established.

Although human cytokine profiles rarely fall into exclusive pro-Th1, Th2 or Th17 patterns, over-activation of one pattern can promote disease and down-regulate the other patterns. In this study, a Th1 and Th17 response pattern was evidenced when T lymphocytes were stimulated by DCs primed with the serotypes K1 or K2 of *P. gingivalis*. Correlation analysis of cytokine secretion and transcription factor mRNA expression demonstrated positive correlation between T-bet with Th1-associated cytokines and RORC2 with Th17 cytokines; however, it was also demonstrated a lack of correlation between T-bet with Th17-associated cytokines and RORC2 with Th1-associated cytokines. Thus, strongly suggesting an independent selective differentiation of Th1 and Th17 lymphocyte clones when the stimulating *P. gingivalis* belonged to serotypes K1 or K2. During periodontitis, a

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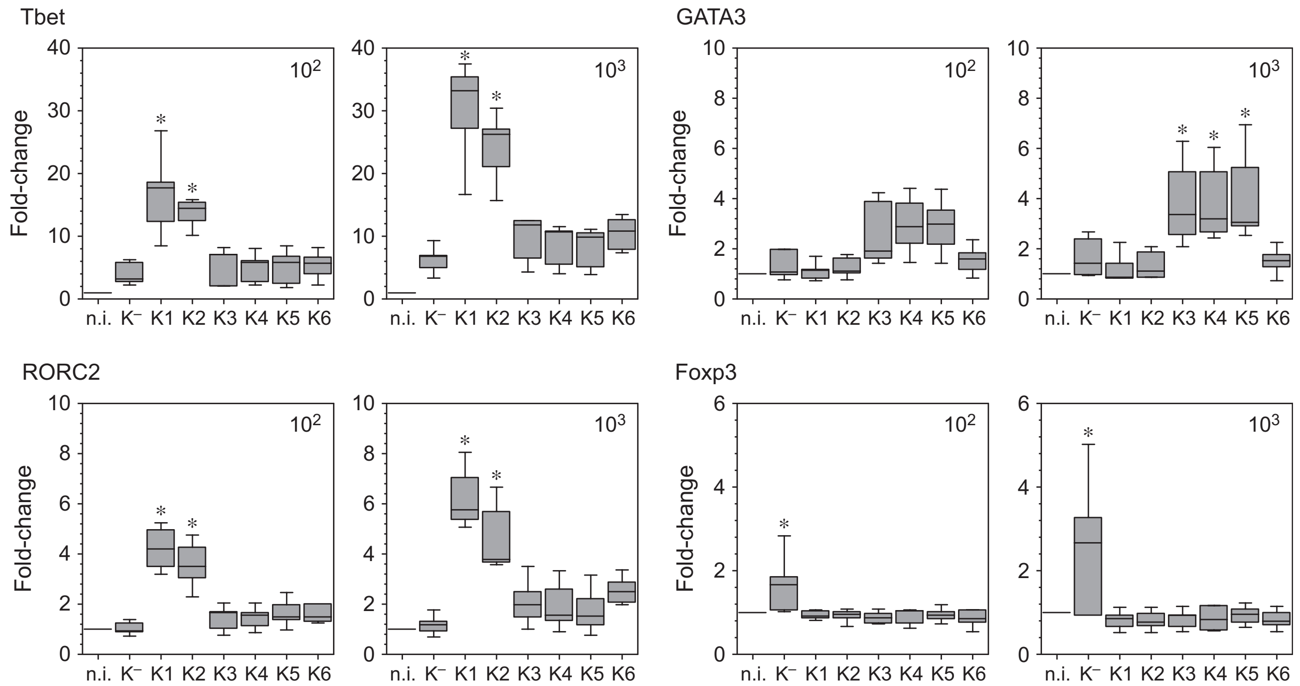

Fig. 6. Transcription factor mRNA expression. Quantitative PCR analysis for the T-bet (Th1), GATA-3 (Th2), RORC2 (Th17), and Foxp3 (Treg) mRNA expression in T lymphocytes stimulated by autologous dendritic cells (DCs) primed at a multiplicity of infection (MOI) of 10^2-10^3 with encapsulated (K1-K6) 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 for seven independent experiments. Each experiment was performed in duplicate. The box plots show the medians, 1st and 3rd quartiles as boxes and 10th and 90th percentiles as whiskers. *$p < 0.05$.

Table 2. Correlation analysis 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						

*$p < 0.05$.
†The Pearson's correlation coefficient between T-bet and Th1-associated cytokines, GATA-3 and Th2-associated cytokines, RORC2 and Th17-associated cytokines and Foxp3 and Treg-associated cytokines were calculated using T lymphocytes activated by dendritic cells primed at an MOI of 10^3 with the different *Porphyromonas gingivalis* serotypes. In blank, transcription factor mRNA expression and cytokine secretion not showing a positive correlation.

Th1 and Th17-dominated response has been reported in destructive periodontal disease (Vernal et al. 2005, Gemmell et al. 2007, Houri-Haddad et al. 2007, Gaffen & Hajishengallis 2008, Dutzan et al. 2009a,b, Garlet 2010, Graves et al. 2011) and a Th2 cytokine pattern have been associated with remission of periodontitis, IL-4 being proposed as a local treatment in the management of this disease (Pradeep et al. 2008). In this context, the presence of the serotype K1 or K2 of *P. gingivalis* in the periodonto-pathogenic biofilm could be associated with a cooperative Th1 and Th17 differentiation and response on periodontal tissues and,

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thus, could play a role in the initiation, progression and/or severity of periodontitis.

According to our data, the non-encapsulated K^- strain (ATCC® 33277™) of *P. gingivalis* could induce a regulatory immune response by fostering a $Foxp3^+$ lymphocyte differentiation and secretion of IL-10 and TGF-β1. In fact, a virulence increase of the encapsulated *P. gingivalis* strains, as compared with the K^- strain, has been proven when these pathogens were inoculated in a mouse model of experimental infection (Laine & van Winkelhoff 1998). In this context, an increased frequency of Treg lymphocytes have been described in healthy periodontal tissues (Ernst et al. 2007); however, it has also been demonstrated an increased frequency of Treg lymphocytes in diseased tissues, suggesting that Treg cell infiltration could reflect an attempt to control tissue destruction, or conversely could be indicative of a destructive role for Treg lymphocytes in periodontitis (Nakajima et al. 2005, Cardoso et al. 2008, Dutzan et al. 2009a,b). Interestingly, treatment with anti-GITR, which inhibits the Treg cell function, induced increased alveolar bone loss and inflammatory cell migration (Garlet et al. 2010), suggesting that the presence of Treg lymphocytes attenuates the severity of experimental periodontitis and the resorption of the alveolar bone that surrounds the teeth. In general terms, Treg lymphocytes play a role in the immuno-pathogenesis of the periodontal tissue destruction and the detection of K^- strains of *P. gingivalis* in the periodontal biofilm could play a role in the Treg activity on periodontal tissues.

The current approach in the management of periodontitis focuses primarily on decreasing the bacterial challenge rather than modulating the host response. The phenotype and symptomatology of periodontitis, however, depend more on the excessive immune and inflammatory responses than on the virulence activity of the pathogens that elicit them. Bacterial pathogens, having co-evolved with the immune defences of their respective hosts, have developed strategies not only to overcome protective host barriers but also to manipulate these defensive systems to their own advantage (Bhavsar et al. 2007, Dethlefsen et al. 2007). The demonstrated variability in the T lymphocyte response triggered by DCs primed with the different K serotypes of *P. gingivalis* shown in the present work is a good evidence of this hypothesis. The differential Th1/Th2/Th17 cell responses dependent on the bacterial colonization patterns and on the presence of virulent clones might give light to what constitutes protective or destructive host responses in periodontitis and thus foster the development of new therapeutic immuno-intervention modalities that maximize the protective and minimize the destructive T-lymphocyte immune responses.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1. Correlation analyses of Th1 and Th17-associated cytokine secretion and transcription factor T-bet and RORC2 mRNA expression.

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Clinical Relevance

Scientific rationale for the study: Dendritic cells regulate the development of T-lymphocyte lineages. The different *P. gingivalis* capsular serotypes induce differential dendritic cell responses, thus, the type of capsule present could also influence the outcome of T-lymphocyte responses.

Principal findings: The different *P. gingivalis* capsular serotypes led to distinct T-lymphocyte responses where K1 and K2 induced a Th1/Th17 pattern of immune response and K3-K5 a Th2 response.

Practical implications: The pathogenic heterogeneity described among *P. gingivalis* capsular serotypes should be taken into account when evaluating the role of this bacterial species in the initiation, progression and/or severity of periodontitis.

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