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Variability in the response of human dendritic cells stimulated with *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans*

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Background and Objective: Dendritic cells are able to prime and polarize naïve T cells towards either a T helper 1 or a T helper 2 response, depending on the antigen type and concentration, the costimulatory signals and the local cytokine millieu. In this investigation, we analyzed the response of human dendritic cells to stimulation with different concentrations of *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans*.

Material and Methods: Using different concentrations of *P. gingivalis* ATCC 33277 and *A. actinomycetemcomitans* ATCC 33384, we determined the expression of the maturation markers CD80 and CD86 from purified human dendritic cells by flow cytometry. We also evaluated the mRNA expression levels for the cytokines interleukin-1β, interleukin-2, interleukin-5, interleukin-6, interleukin-10, interleukin-12, interleukin-13, interferon-γ, tumor necrosis factor-α and tumor necrosis factor-β by quantitative real-time reverse transcription–polymerase chain reaction.

Results: Both *P. gingivalis* and *A. actinomycetemcomitans* led to dendritic cell maturation, but the expression of CD80 was higher when the dendritic cells were stimulated with *A. actinomycetemcomitans*. Although both pathogens induced a T helper 1 pattern of cytokine expression, *A. actinomycetemcomitans*-stimulated dendritic cells expressed interleukin-1β, interleukin-12, interferon-γ, tumor necrosis factor-α and tumor necrosis factor-β at lower bacterial concentrations than *P. gingivalis*. While 10^6 bacteria/mL of *P. gingivalis* or 10^4 bacteria/mL of *A. actinomycetemcomitans* induced expression of interleukin-12p40, the expression of other cytokines required 10 to 100-fold higher concentrations of bacteria.

Conclusion: These results demonstrate that *A. actinomycetemcomitans* is a more potent immunogen than *P. gingivalis* because, at least *in vitro*, it induces stronger differentiation and activation of dendritic cells. In addition, our data also show that for a given strain, the bacterial load determines the pattern of cytokines that are expressed.

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The immune response in periodontal tissues against bacterial challenges involves very specialized and organized cellular and molecular mechanisms (1,2). Dendritic cells are antigen-presenting cells with the ability to prime naïve $CD4^+$ T cells (3,4) and induce their polarization towards a specific T helper (T helper 1 or T helper 2) phenotype, depending on the local cytokine millieu and the costimulatory signals (5,6). T helper 1 cells secrete interferon-γ, interleukin-2, interleukin-12, tumor necrosis factor-α and tumor necrosis factor-β, whereas T helper 2 cells produce interleukin-4, interleukin-5, interleukin-6 and interleukin-13 (7).

Several studies have evidenced the complexity of the immune response elicited by periodontal pathogens. Indeed, whereas animals infected with *Porphyromonas gingivalis* elicited a T helper 1-type response, a prior infection with *Fusobacterium nucleatum* was found to trigger a switch towards a T helper 2-type response (8). In addition, it has been reported that *Aggregatibacter actinomycetemcomitans* lipopolysaccharide induces a stronger up-regulation of the activation and differentiation marker CD86 on dendritic cells than the lipopolysaccharide from *Escherichia coli* (9). This complexity in the response is strengthened by studies showing antagonistic dendritic cell responses with different *P. gingivalis* strains. *P. gingivalis* strain 381 induced a potent interleukin-12 response (10), whereas strain O55:B5 triggered a weak interleukin-12 response (11). Finally, it has also been reported that stimulation of human dendritic cells with either *A. actinomycetemcomitans* (12) or *P. gingivalis* (9) induces a T helper 1-type response, leading to the secretion of interleukin-12 and interferon-γ.

With the goal of clarifying several aspects of the immune response elicited against periodontal pathogens, we analyzed the expression of the maturation markers CD80 and CD86, which act as costimulatory signals for the activation of T lymphocytes, as well as the expression levels of cytokine mRNAs, in human dendritic cells stimulated *in vitro* with different concentrations of *A. actinomycetemcomitans* or *P. gingivalis*. We hypothesized that both pathogens activate dendritic cells to secrete cytokines, leading to a T helper 1-type response, although with intrinsic differences in the magnitude of the response to each pathogen, together with distinct thresholds for the synthesis of the different cytokines.

Material and methods

Bacterial growth conditions and determination of growth curves

P. gingivalis ATCC 33277 and *A. actinomycetemcomitans* ATCC 33384 were cultured on nonselective 5% horse blood agar (Oxoid Nº2; Oxoid Ltd, Basingstoke, UK), supplemented with 5 mg/mL of hemin and 1 mg/mL of menadione, and incubated at 37°C under anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2). Growth curves were obtained in liquid brain–heart infusion medium (BD, Le Pont de Claix, France) supplemented with 5 mg/mL of hemin and 1 mg/mL of menadione. Bacterial samples were inoculated in 10 mL of medium until reaching an optical density of 0.05, as measured by spectrophotometry at a wavelength of 550 nm (Spectronic 20; Bausch & Lomb, Rochester, NY, USA). The spectrophotometry readings were taken at different time-points and the experiment was stopped when the bacteria reached the stationary growth phase. With each optical density measurement, a sample was taken, serially diluted ($\log_{10}$) in phosphate-buffered saline and 100 μL of each dilution was plated onto nonselective medium and incubated at 37°C under anaerobic conditions. After 3–7 d, the number of bacterial colonies counted from each sample was plotted against the corresponding optical density reading, thus obtaining a reliable number of colony-forming units.

Monocyte purification

Human peripheral blood mononuclear cells, obtained from the buffy coats of 10 normal donors, were isolated over a Ficoll gradient (Ficoll–Paque Plus; Amersham Pharmacia Biotech AB, Uppsala, Sweden) following standard procedures. Monocytes were then purified from peripheral blood mononuclear cells by magnetic cell sorting (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Peripheral blood mononuclear cells were washed twice and incubated with microbeads-conjugated monoclonal anti-CD14 for 15 min at 4°C. After being washed once, the cells were resuspended, loaded onto large size (LS) separation columns and separated by applying a magnetic field. The positive cell fraction was flushed out, counted, stained with monoclonal anti-CD1a, -CD14, -CD80, -CD83 and -CD86 conjugated to phycoerythrin, fluorescein isothiocyanate or phycoerythrin/cyanine 5 (BD Biosciences Pharmingen, San José, CA, USA) and evaluated using flow cytometry (EPICS XL; Beckman Coulter, Fullerton, CA, USA).

Dendritic cell differentiation and stimulation

For generating the dendritic cells, $CD14^+$ cells were cultured at 10^6 cells/mL in 3 mL of RPMI-1640 containing 10% fetal calf serum (Gibco Invitrogen Corp., Grand Island, NY, USA), 1000 U/mL of recombinant human granulocyte–macrophage colony-stimulating factor and 1000 U/mL of recombinant human interleukin-4 (Immunotools GmbH, Friesoythe, Germany), for 6 d at 37°C (13). The resulting dendritic cells were then stimulated with increasing concentrations (10^1–10^9 bacteria/mL) of *A. actinomycetemcomitans* and *P. gingivalis* or with 10 ng/mL of lipopolysaccharide from *E. coli* 0111:B4 (Fluka, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) for 2 d. Unstimulated dendritic cells were used as a control. The differentiation and maturation of dendritic cells were evaluated by flow cytometry.

Isolation of cytoplasmic RNA

Cytoplasmic RNA was isolated as previously described (14). Briefly, dendritic cells were washed twice in phosphate-buffered saline and lysed in 400 μL of ice-cold lysis buffer containing 0.5% Nonidet-P40, 50 mM

Tris–HCl, pH 8, 100 mM NaCl and 5 mM $MgCl_2$, supplemented with 10 mM vanadyl-ribonucleoside complex (VRC)-40 (Gibco). Complete cell lysis was obtained by pipetting the dendritic cells up and down 10 times. The nuclei were removed by centrifugation for 10 s at 14,000 g and 400 μL of the supernatant was then digested with 25 μg/mL of proteinase K (Roche Ltd, Basel, Switzerland), in the presence of 1% sodium dodecyl sulfate and 15 mM EDTA (20°C) (Fluka), for 30 min at 37°C. Subsequently, RNA was extracted with an equal volume (400 μL) of chloropan (phenol : chloroform : isoamylalcohol, 25:24:1) and centrifuged for 10 min at 14,000 g at room temperature (20°C) (Fluka). The RNA present in the aqueous solution was precipitated for 1 h at −20°C with 0.3 M sodium acetate, 2.5 volumes of 100% ethanol and 1 μL of glycogen (20 μg/μL) (Roche), recovered by centrifugation for 30 min at 14,000 g and resuspended in 20 μL of RNase-free H_2O. RNA quality was determined in a bioanalyzer (Agilent 2100B; Agilent Technologies, Palo Alto, CA, USA). Cytoplasmic RNA was quantified using a spectrophotometer (Nanodrop ND-1000; Nanodrop Technologies, Wilmington, ND, USA) and stored at −80°C at a final concentration of 1 μg/μL.

First-strand cDNA synthesis

Reverse transcription was performed using a Transcriptor First Strand cDNA synthesis kit (Roche) following the manufacturer's recommendations. Briefly, 20 μL of reaction containing 4 μL of ×5 reverse transcription (RT) buffer, 0.5 μL of RNase inhibitor (20 U), 2 μL of deoxynucleotide mix (1 mM of each deoxynucleotide), 2 μL of random hexamer primer (60 μM), 0.5 μL of reverse transcriptase (10 U), 10 μL of RNA-grade H_2O and 1 μL of RNA sample (1 μg) was retrotranscribed under the following conditions: 10 min at 25°C and 1 h at 50°C (Primus 96 plus; MWG Biotech AG, Ebersberg, Germany). The reverse transcriptase activity was subsequently inactivated by incubating the sample at 85°C for 5 min.

Quantitative real-time polymerase chain reaction

To examine the mRNA expression of the cytokines interleukin-1β, interleukin-2, interleukin-5, interleukin-6, interleukin-10, interleukin-12, interleukin-13, interferon-γ, tumor necrosis factor-α and tumor necrosis factor-β, 50 ng of cDNA was amplified by quantitative real-time polymerase chain reaction (PCR) in 384-well plates, using a FastStart Taqman Probe Master (Roche), as previously described (15). Forward and reverse primers and specific 6-carboxyfluorescein (FAM) dye-labelled probes were designed using the Roche website (http://www.roche-applied-science.com). The primers and FAM-labelled probes are listed in Table 1. Ten-microlitre reactions containing 0.1 μL of PCR probe (250 nM), 0.1 μL of forward primer (900 nM), 0.1 μL of reverse primer (900 nM), 5 μL of FastStart master, 3.7 μL of PCR-grade H_2O and 1 μL of cDNA (50 ng) were amplified under the following conditions: 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C, in an ABI PRISM 7900 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). As an endogenous control, 18S rRNA expression levels were determined.

Data analysis

The flow cytometry data were analyzed using the WinMDI 2.8 software (The Scripps Research Institute, La Jolla, CA, USA) and are represented as histograms. The results for each staining were expressed as the percentage of positive cells over the total number of cells. Real-time PCR data were analyzed using the ABI PRISM Sequence Detector Systems software (Applied Biosystems, Foster City, CA, USA). Relative quantification was obtained by adjusting the cytokine mRNA expression to 18S rRNA expression and considering the adjusted expression in uninduced dendritic cells as a reference. Data were expressed as mean ± standard deviation and analyzed using SPSS software, version 13.0.1 (Lead Technologies Inc., Charlote, NC, USA). After determining the normality of the data distribution using the Shapiro–Wilk test, the differences between groups regarding the cytokine mRNA expression were determined using the unpaired Student's *t*-test. To compare cytokine mRNA expression in each group, the unpaired analysis of variance and Tukey *post hoc* tests were used. The CD80 and CD86 expression data were analyzed using the chi-square test. A statistical significance was considered when the *p*-value was < 0.05.

Results

For this study, highly purified ($> 95\%$) populations of monocytes isolated from buffy coats were used. The population purity was demonstrated by the com-

Table 1. Forward primers, reverse primers and polymerase chain reaction (PCR) probes used for cytokine amplifications by quantitative real-time PCR

Cytokine	Forward primer	Reverse primer	PCR probe[b]
Interleukin-1β	ctgtcctgcgtgttgaaaga	ttgggtaatttttgggatctaca	78
Interleukin-2	aagttttacatgcccaagaagg	aagtgaaagtttttgctttgagc	65
Interleukin-5	ctctgaggattcctgttcctgt	cagtacccccttgcacagtt	47
Interleukin-6	gcccagctatgaactccttct	gaaggcagcaggcaacac	45
Interleukin-10	tgggggagaacctgaagac	ccttgctcttgttttcacagg	30
Interleukin-12p35	cactcccaaaacctgctgag	tctcttcagaagtgcaagggta	50
Interleukin-12p40	ccctgacattctgcgttca	aggtcttgtccgtgaagactcta	37
Interleukin-13	agccctcagggagctcat	ctccataccatgctgccatt	17
Tumor necrosis factor-α	cagcctcttctccttcctgat	gccagagggctgattagaga	29
Tumor necrosis factor-β	ctaccgcccagcagtgtc	gtggtgtcatggggaga	13
Interferon-γ	ggcattttgaagaattggaaag	tttggatgctctggtcatctt	21
18S[a]	ctcaacacgggaaacctcac	cgctccaccaactaagaacg	77

[a]18S, 18S ribosomal RNA, used as housekeeping gene for relative quantification.
[b]6-carboxyfluorescein dye-labelled probe.

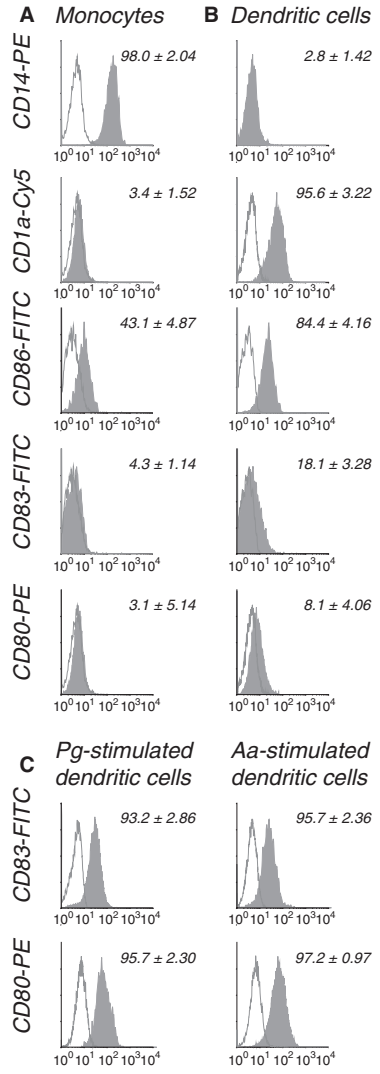

Fig. 1. Monocyte isolation and dendritic cell differentiation and activation. Monocytes were isolated from peripheral blood mononuclear cells and cultured in the presence of interleukin-4 and granulocyte–macrophage colony-stimulating factor to induce their differentiation towards dendritic cells, which were subsequently activated with either *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans*. Flow cytometry analyses demonstrating (A) the purity of the isolated monocyte populations, (B) the efficiency of differentiation of monocytes to dendritic cells and (C) the activated phenotype of the dendritic cells 48 h after stimulation with periodontal pathogens (10^9 bacteria/mL) are shown. The percentage of cluster of differentiation (CD)-positive cells from three independent experiments is expressed as mean ± standard deviation. Aa, *Aggregatibacter actinomycetemcomitans*; Cy5, cyanine 5; FITC, fluorescein isothiocyanate; PE, phycoerythrin; Pg, *Porphyromonas gingivalis*.

bined staining with monoclonal anti-CD14, -CD1a, -CD83, -CD80 and -CD86 (Fig. 1A). These monocytes differentiated at a high frequency (> 95%) into dendritic cells upon culture in the presence of interleukin-4 and granulocyte–macrophage colony-stimulating factor, as shown by the appearance of CD1a antigen, concomitant with the loss of the monocyte–macrophage marker CD14 and the increase in the fraction of CD86-positive cells (Fig. 1B). The activation of dendritic cells by *P. gingivalis* or *A. actinomycetemcomitans* was confirmed by the increased expression of CD83 and CD80 (Fig. 1C).

P. gingivalis and *A. actinomycetemcomitans* induce dendritic cell aggregation in a dose-dependent manner, an effect readily observed by phase-contrast microscopy (Fig. 2). Interestingly, whereas uninduced dendritic cells (negative control) were devoid of any visible aggregation, clear differences were visible between *A. actinomycetemcomitans*-stimulated and *P. gingivalis*-stimulated dendritic cells. Indeed, in cells stimulated with *A. actinomycetemcomitans*, aggregation was already detectable at the lowest bacterial concentration (10^1 bacteria/mL), whereas a similar aggregation pattern was only observed in dendritic cells stimulated with 10^5 bacteria/mL of *P. gingivalis*. At concentrations of 10^8–10^9 bacteria/mL of both bacterial species, the aggregation pattern was similar to that obtained with lipopolysaccharide-stimulated dendritic cells (the positive control).

These morphologic changes in the dendritic cell cultures were consistent with the changes detected in the expression of CD80 and thus were used as an indicator of dendritic cell activation. Figure 3 shows a dose-dependent increase in the percentages of CD80 expression, ranging from 22.63 ± 2.32% to 95.75 ± 2.30% and from 26.10 ± 3.30 to 97.24 ± 0.97 when the dendritic cells were stimulated with *P. gingivalis* and *A. actinomycetemcomitans*, respectively. *A. actinomycetemcomitans* induced a higher level of CD80 expression than *P. gingivalis*, these differences being statistically significant at concentrations between 10^3 and 10^6 bacteria/mL. CD80 expression reached a plateau with concentrations of 10^8–10^9 bacteria/mL for both bacteria. At these concentrations, the expression levels of CD80 were similar to those of lipopolysaccharide-stimulated dendritic cells (the positive control).

In dendritic cells, the expression of interleukin-1β, interleukin-5, interleukin-6, interleukin-10, interleukin-12p35, interleukin-12p40, interferon-γ, tumor necrosis factor-α and tumor necrosis factor-β mRNAs was determined by quantitative reverse transcription-PCR. The data obtained, plotted as fold-change for each cytokine against the different stimulation conditions (Fig. 4), showed a dose-dependent increase in the expression levels for each cytokine. Overall, the response was biased towards a T helper 1 pattern of cytokine expression in both *P. gingivalis* and *A. actinomycetemcomitans*-stimulated dendritic cells. Interestingly, the dendritic cells stimulated with *A. actinomycetemcomitans* had a higher relative expression of interleukin-1β, interleukin-12p35, interleukin-12p40, interferon-γ and tumor necrosis factor-α mRNAs than the same cells stimulated with *P. gingivalis*. In all experiments, interleukin-2 expression remained undetectable, confirming the absence of contaminating T lymphocytes in the cultures (data not shown). Furthermore, whereas interleukin-5 was not induced under any experimental conditions, interleukin-6, interleukin-10 and interleukin-13 mRNAs were weakly induced at very high bacterial concentrations (10^8 and 10^9 bacteria/mL). In addition, whereas a higher induction of tumor necrosis factor-β mRNA was observed in *A. actinomycetemcomitans*-stimulated dendritic cells at loads of 10^4–10^7 bacteria/mL, tumor necrosis factor-β mRNA expression was higher in *P. gingivalis*-stimulated dendritic cells at loads of 10^8 and 10^9 bacteria/mL.

From these data, distinct thresholds for the expression of the different cytokines were detected (Fig. 4). While in *P. gingivalis*-stimulated dendritic cells the induction of interleukin-12p35 and interleukin-12p40 became detectable with 10^6 bacteria/mL, expression

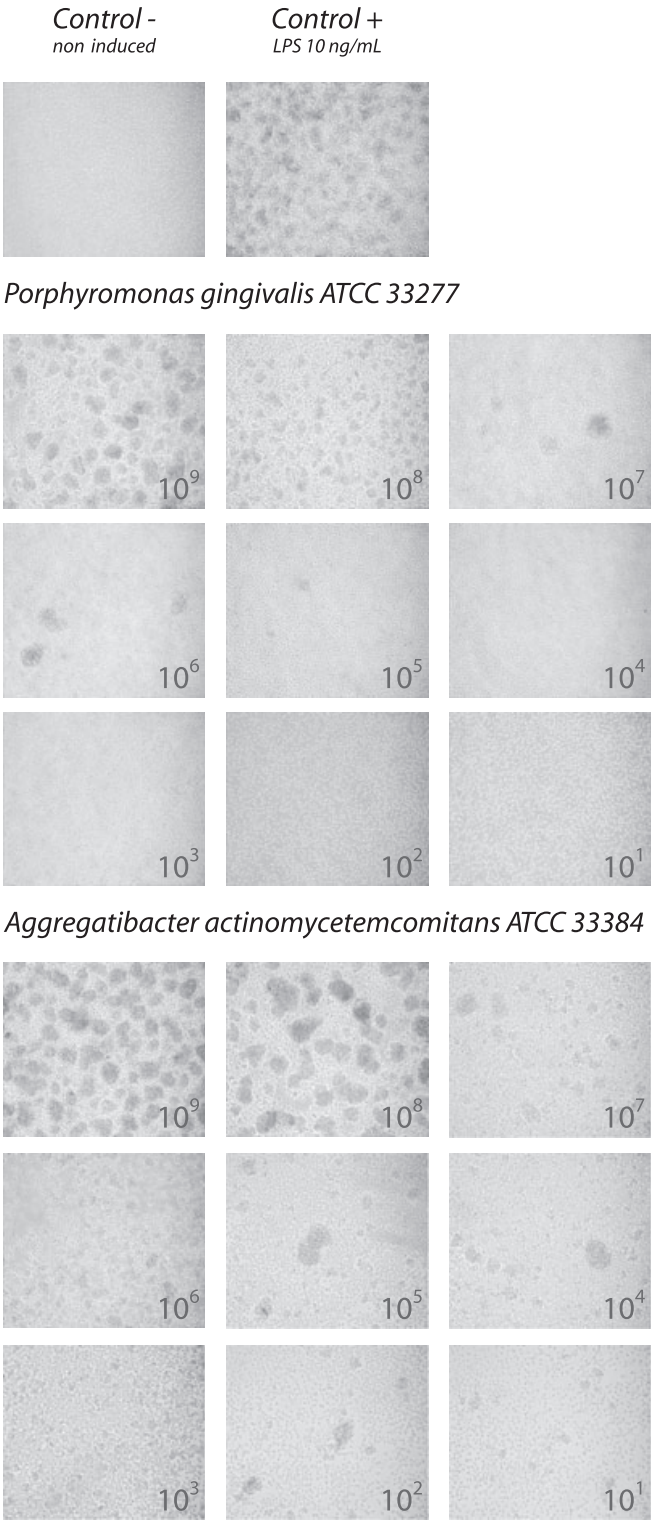

Fig. 2. Dendritic cell aggregation induced by antigen stimulation. Phase-contrast microscopic images of a representative experiment showing dendritic cells stimulated with 10^1–10^9 bacteria/mL of *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans*, with (as controls) 10 ng/mL of *Escherichia coli* lipopolysaccharide (control +) and uninduced dendritic cells (control −).

of other cytokine mRNAs, such as interleukin-1β, interferon-γ, tumor necrosis factor-α and tumor necrosis factor-β, required 100-fold higher bacterial concentrations. Similar results were obtained in *A. actinomycetemcomitans*-stimulated dendritic cells, in which the induction of interleukin-1β, tumor necrosis factor-β and interleukin-12p40 mRNAs became detectable with 10^4 bacteria/mL, whereas interleukin-12p35, interferon-γ and tumor necrosis factor-α mRNAs required 10- to 100-fold higher bacterial concentrations.

Discussion

Dendritic cells represent a large family of antigen-presenting cells that circulate through the bloodstream and are scattered in nearly all tissues (2,3). They are key components of the innate immune response and serve as a bridge for the adaptative immune response (3). Dendritic cells capture microbial antigens while in an immature state. They then mature and stimulate T-cell clones with the ability to recognize these antigens (3,4). During this process, the antigen must be first recognized and captured by dendritic cells, then intracellularly processed into small peptides that are subsequently conjugated to major histocompatibility complex proteins and then presented to the T lymphocytes, which bear in their surface receptors, called the T-cell receptor, with the ability to recognize specifically this antigen–major histocompatibility complex protein complex (2). These signals, although sufficient to stimulate memory T cells, fail to stimulate naïve T lymphocytes, unless additional costimulatory signalling events take place (16,17). The type I integral membrane glycoprotein B7.1 (CD80) is a costimulatory molecule expressed by dendritic cells in response to microbial antigens and it is usually used as a marker of dendritic cell maturation and activation (16).

In our investigation, monocytes from normal donors were purified and then differentiated *in vitro* into dendritic cells to analyze the effects of *P. gingivalis* and *A. actinomycetemcomitans*. Although we cannot disre-

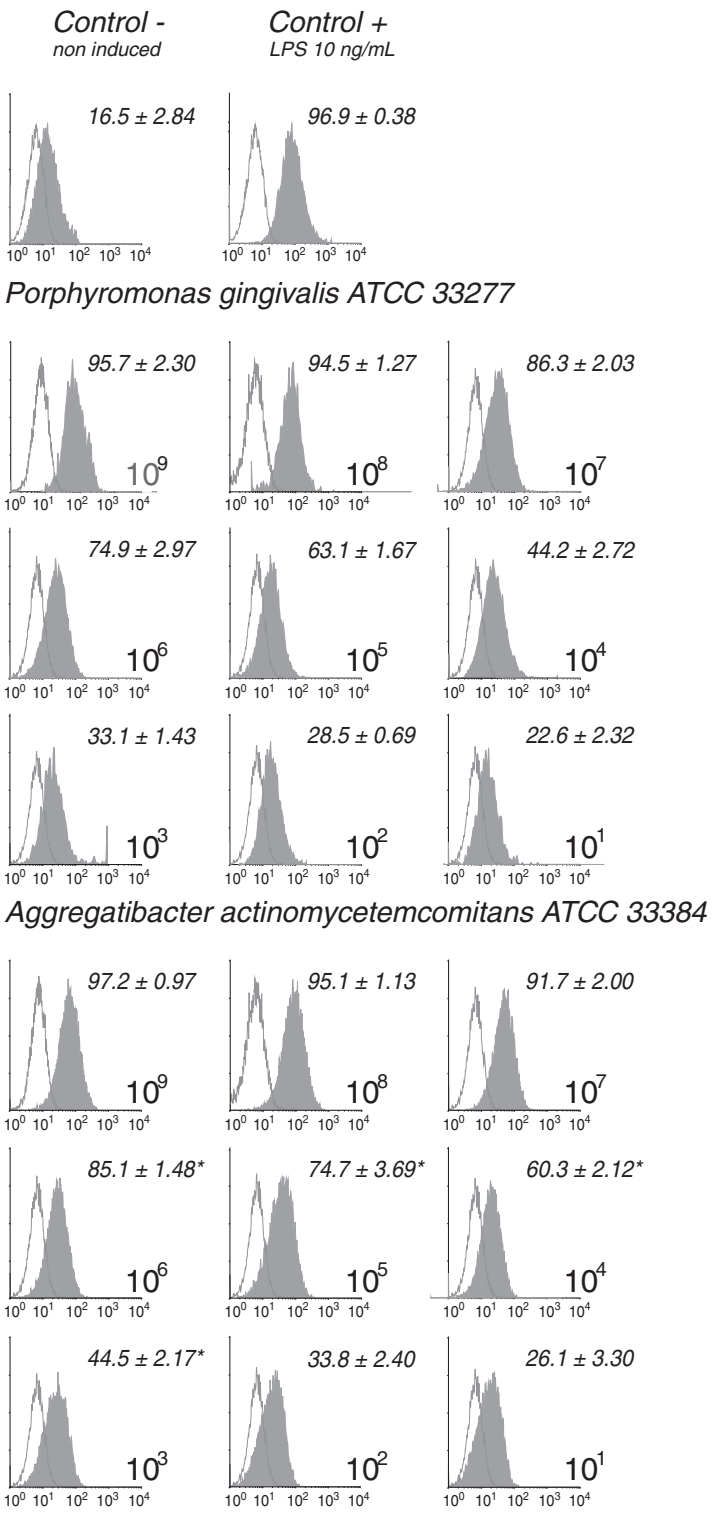

Fig. 3. Dendritic cell activation with different concentrations of either *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans*. $CD80^+$ expression was determined by flow cytometry analyses of dendritic cells stimulated with different concentrations (10^1–10^9 bacteria/mL) of either *P. gingivalis* or *A. actinomycetemcomitans*. As a positive control, dendritic cells stimulated with 10 ng/mL of *Escherichia coli* lipopolysaccharide (control +) were used. As a negative control, uninduced dendritic cells (control −) were used. The percentage of $CD80^+$ expression from three independent experiments is expressed as mean ± standard deviation. *$p < 0.05$. LPS, lipopolysaccharide.

gard differences between the response of dendritic cells from healthy subjects and individuals with periodontitis, we believe that the purification of monocytes and their *in vitro* differentiation, instead of directly purifying dendritic cells, minimizes these differences. The differentiation and activation of dendritic cells was associated with an increase in cell size, loss of the monocyte–macrophage marker, CD14, and increase in CD86, CD1a, CD83 and CD80 expression, a pattern consistent with that established on tumor necrosis factor-α-stimulated dendritic cells (18).

For the cytokine analysis, rather than to quantify the proteins directly, we decided to determine the mRNA expression levels by real-time reverse transcription-PCR because this is the most sensitive method for quantifying low expression levels (19) and for fast and reliable screening. For normalization, 18S rRNA expression levels were selected as a result of their small fluctuations in nucleated cells (20). Indeed, no differences were detected in the 18S rRNA expression in dendritic cells, either unstimulated or stimulated with different concentrations of either *A. actinomycetemcomitans* or *P. gingivalis* (data not shown). Thus, the data obtained, after normalization, allowed the quantification of cytokine levels (19).

Since the mid-1980s several authors have hypothesized that stable periodontitis is associated with a T helper 1 host response, while a progressive disease state is associated with a T helper 2 response (7,21–23). Other authors have even suggested that periodontitis is a T helper 2-associated disease (24,25). However, data from different studies have shown a predominance of T helper 1 vs. T helper 2-type cells in inflammatory infiltrates from periodontitis-affected tissues (26,27). Recently, the involvement of both T helper 1 and T helper 2 cells has also been proposed in the pathogenesis of periodontitis (28,29). In our investigation we used T-cell-depleted dendritic cells and showed that, although with different intensity, both *A. actinomycetemcomitans* and *P. gingivalis* are able to induce a predominant T helper 1 cytokine expression, as

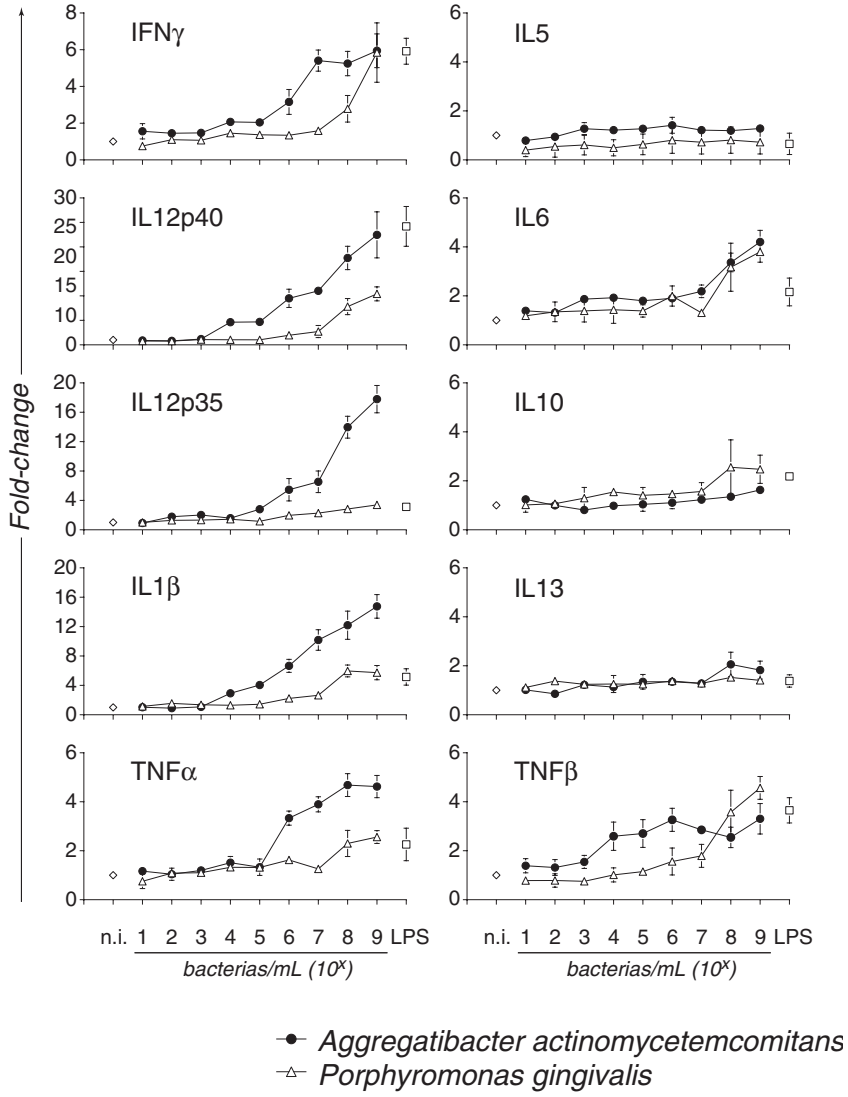

Fig. 4. Cytokine mRNA expression determined by quantitative reverse transcription–polymerase chain reaction. Quantification of interleukin-1β, interleukin-5, interleukin-6, interleukin-10, interleukin-12p35, interleukin-12p40, interleukin-13, interferon-γ, tumor necrosis factor-α and tumor necrosis factor-β is represented as fold-change in dendritic cells stimulated with 10^1–10^9 bacteria/mL of *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans*. Circles and triangles correspond to the cytokine mRNA expression associated with each bacterial concentration in *A. actinomycetemcomitans*-stimulated and *P. gingivalis*-stimulated dendritic cells, respectively. Squares correspond to cytokine expression in *Escherichia coli* lipopolysaccharide-stimulated dendritic cells, used as a positive control, and the diamonds correspond to cytokine expression in uninduced dendritic cells considered as 1 for relative quantification, as a reference for the fold-change in cytokine expression. Data from three independent experiments are shown as mean ± standard deviation. Non-induced; LPS, lipopolysaccharide.

demonstrated by the high levels of interleukin-12 and interferon-γ, essential for a T helper 1-type response (16). Interleukin-12 is a 70-kDa heterodimeric protein, formed by a heavy chain (p40) and a light chain (p35) that are independently regulated. Most cell types express p35 either constitutively or after being stimulated; however, p40 is expressed in a more restricted manner, primarily by antigen-presenting cells (30). Functional interleukin-12 (interleukin-12p70) is secreted only when cells co-express both heavy and light chains and, therefore, the ability of a given cell type to secrete interleukin-12 depends on the p40 expression; however, its absolute amount depends on the p35 induction (31). In agreement with previous studies (9,12), our data show that *A. actinomycetemcomitans* was a stronger interleukin-12 inducer than *P. gingivalis*. This weak ability of *P. gingivalis*-stimulated dendritic cells to express interleukin-12p35 might explain the results from previous investigations reporting that dendritic cells respond to *P. gingivalis* lipopolysaccharide synthesizing lower levels of tumor necrosis factor-α and no interleukin-12p70, when compared with *E. coli* lipopolysaccharide (32). It may also explain the skewed polarization of T cells toward a T helper 2 rather than a T helper 1 phenotype in *P. gingivalis*-stimulated BALB/c mice previously exposed to *F. nucleatum* (8).

Natural killer cells, $CD8^+$ T cells and T helper 1 $CD4^+$ T cells are the major sources of interferon-γ (33). Natural killer-derived interferon-γ stimulates dendritic cells to increase interleukin-12 secretion, which, in turn, leads to more interferon-γ production by natural killer cells, forming an amplification loop that results in the generation of substantial quantities of interleukin-12 (12). It has been shown that antigen-presenting cells can also secrete interferon-γ, but the role of this secretion remains unclear (34). Our data demonstrate induction of interferon-γ in dendritic cells stimulated with either *A. actinomycetemcomitans* or *P. gingivalis*.

Two mechanisms have been proposed for the dendritic cell response. The first is an instructive model, in which a single dendritic cell subset can transduce distinct microbial antigens and cytokines, eliciting any T helper response, and the second is a selective model, in which different dendritic cell subsets recognize particular microbial antigens resulting in a given T helper response, different from that induced by another antigen activating a different dendritic cell subset (35). Our data support the instructive model for the dendritic cell response, as different cytokine expression levels dependent on the bacterial concentration were detected in dendritic cells from the same origin

(monocyte-derived dendritic cells), which were stimulated with live periodonto-pathogenic bacteria harboring their whole antigenic potentiality. Further support of this hypothesis comes from the fact that dendritic cells stimulated with either *P. gingivalis* or *A. actinomycetemcomitans*, harboring their whole antigenic potential, reach activation levels similar to cells stimulated with *E. coli* lipopolysaccharide, rather than a different activation plateau. From these data, we cannot conclude whether the lipopolysaccharide from these bacteria is the main immunogen, and the available data do not allow for an 'educated guess' regarding the nature of the main immunogen. Furthermore, we cannot exclude immunogenic variability in the different strains of *P. gingivalis* and *A. actinomycetemcomitans*. For this study we selected the ATCC strains of each bacterium because they have been highly characterized and are widely available.

The comparison between the dendritic cell responses induced by *P. gingivalis* and *A. actinomycetemcomitans* shows distinct thresholds for the expression of interleukin-1β, interleukin-12, interferon-γ, tumor necrosis factor-α and tumor necrosis factor-β. In general, induction of cytokine expression was detectable with concentrations of 10^4–10^6 bacteria/mL of *A. actinomycetemcomitans* and of 10^7–10^8 bacteria/mL of *P. gingivalis*, which demonstrates that in addition to the intrinsic antigenicity differences between these pathogens, cytokine expression is also a function of the antigenic load. Similar clonal expansions of ovalbumin-specific OT-2 T-cell receptor $CD4^+$ T cells and OT-1 T-cell receptor $CD8^+$ T cells have been observed when stimulating with 25 μg of both *P. gingivalis* lipopolysaccharide and *E. coli* lipopolysaccharide; however, a different cytokine profile was detected. While *P. gingivalis* lipopolysaccharide induced a T helper 2 response with significant levels of interleukin-5, interleukin-10 and interleukin-13, but lower levels of interferon-γ, *E. coli* lipopolysaccharide induced a T helper 1 response with a predominance of interferon-γ synthesis. Similarly, C57BL/6 $CD11c^+$ $CD8\alpha^+$ dendritic cells stimulated with *E. coli* lipopolysaccharide synthesized interleukin-12p70, whereas interleukin-12p70 was not detected after stimulation with *P. gingivalis* lipopolysaccharide (35). Furthermore, it has been reported that dendritic cells stimulated with 10^5 and 10^6 bacteria/mL of *A. actinomycetemcomitans* synthesized higher levels of interleukin-12 and similar levels of interleukin-10 than dendritic cells stimulated with 10^5 and 10^6 bacteria/mL of *E. coli*. In contrast, the interleukin-12 and interleukin-10 synthesis in dendritic cells stimulated with 10^6 bacteria/mL of *P. gingivalis* was weak and absent, respectively (9,12). Conversely, our data demonstrate that similar levels of cytokine expression can be induced by both *A. actinomycetemcomitans* and *P. gingivalis* when used at different bacterial loads. When the microbial stimulatory concentration was the same, a higher T helper 1-type response was detected with *A. actinomycetemcomitans*. The variability in cytokine synthesis and T helper response reported in periodontitis therefore might be explained by the differences in the concentrations of the pathogens or antigens.

Our data demonstrate a higher immunogenic capacity of *A. actinomycetemcomitans*, which could be compensated for a higher bacterial load of *P. gingivalis*. This fact may be relevant because in the subgingival biofilm, different concentrations of pathogenic bacteria can be detected depending on the local micro-environmental conditions (36). Microorganisms generally grow as members of extensive microbial communities in unique niches (37). The dental plaque contains more than 10^8 microorganisms/mg and the subgingival microbial community may contain more than 700 distinct phylotypes (38). The concentrations and compositions of the subgingival biofilm vary not only between healthy and periodontitis subjects, but also between healthy and periodontitis sites in the same subject (39). Thus, the analysis of the immune response developed during periodontitis by evaluating only specific concentrations of bacteria or antigens must be carefully considered. How the host responds to the presence of a polymicrobial community (such as the subgingival biofilm), how different concentrations and compositions of pathogens determine the developed immune response and how normal commensals interact with opportunistic pathogens modifying this response, are questions for future consideration.

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