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Serotype-dependent response of human dendritic cells stimulated with *Aggregatibacter actinomycetemcomitans*

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Abstract

Aim: Different serotypes of *Aggregatibacter actinomycetemcomitans* have been described based on the lipopolysaccharide (LPS)-O-polysaccharide antigenicity. In turn, a distinct effect of *A. actinomycetemcomitans* serotypes has been described on cell proliferation and pro-inflammatory cytokine production in different human cells. This study was aimed to investigate the differential dendritic cell (DC) response when stimulated with different bacterial strains belonging to the most prevalent serotypes of *A. actinomycetemcomitans* (a–c).

Materials and Methods: Dendritic cells were obtained from healthy subjects and stimulated with increasing multiplicity of infection (MOI = 10^{-1}–10^{2}) of *A. actinomycetemcomitans,* serotypes a–c, or their lipopolysaccharide (10-50 ng/ml). The levels for interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-5, IL-6, IL-10, IL-12 and IL-23 were quantified by real-time RT-PCR and ELISA.

Results: Variable DC responses were detected when stimulated with the different strains of *A. actinomycetemcomitans*. DCs stimulated with *A. actinomycetemcomitans* strains belonging to the serotype b or their purified LPS expressed higher levels of IL-1β, IL-6, IL-12, IL-23, IFN-γ and TNF-α than DCs stimulated with the other serotypes.

Conclusions: *Aggregatibacter actinomycetemcomitans* strains belonging to the serotype b demonstrated a higher capacity to trigger Th1 and Th17-type cytokine production on DCs. These increased potential is likely explained by a higher immunogenicity of their LPS.

Key words: *Aggregatibacter actinomycetemcomitans*; cytokines; dendritic cells; lipopolysaccharide; LPS; serotypes

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Conflict of interest and source of funding statement

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Aggregatibacter actinomycetemcomitans is a small, non-motile, gram-negative coccobacillus, member from the genus *Aggregatibacter* belonging to the family of *Pasteurellaceae* (Norskov-Lauritsen & Kilian 2006). It grows preferentially in a facultative anaerobic, microaerophilic and capnophilic environment (Sanz et al. 2004, Schacher et al. 2007). Several lines of evidence support its aetiological role as a true periodontal pathogen, mostly in relation to aggressive and syndromic forms of periodontitis, although it can also be found in patients with chronic periodontitis as well as in healthy subjects (Slots & Ting 1999, Socransky & Haffajee 2005, Schacher et al. 2007, Laine et al. 2013). Currently, six *A. actinomycetemcomitans* serotypes (a–f) are recognized based on

the antigenicity of the O-polysaccharide component of the lipopolysaccharide (LPS) (Page et al. 1991, Gmur et al. 1993, Kaplan et al. 2001). Serotypes a, b and c are the most frequent in Caucasians, Asians, Africans and Latin-Americans, being serotype b the most frequently associated with periodontitis (Chen et al. 2010, van der Reijden et al. 2010, Roman-Torres et al. 2010, Sakellari et al. 2011, Aberg et al. 2012, Bandhaya et al. 2012, Cortelli et al. 2012, Jentsch et al. 2012).

The different serotypes of *A. actinomycetemcomitans* differ in their ability to coaggregate with *Fusobacterium nucleatum*. LPS purified from *A. actinomycetemcomitans* strain Y4 (serotype b) interferes with the binding of *A. actinomycetemcomitans* to *F. nucleatum* in a dose-dependent manner, whereas LPS purified from *A. actinomycetemcomitans* strain ATCC® 29523™ (serotype a) has no inhibitory effect on the binding of *A. actinomycetemcomitans* strain Y4 cells to *F. nucleatum* (Rosen et al. 2003). Similarly, a distinct effect of *A. actinomycetemcomitans* serotypes has been described on cell proliferation and pro-inflammatory cytokine production when in contact with different host cells. In fact, bacterial extracts of *A. actinomycetemcomitans* strain ATCC® 29522™ (serotype b) induce a reduction on the human gingival epithelial cell growth and an increment in the expression of interleukin (IL)-8 and intercellular adhesion molecule (ICAM)-1 in these cells compared with serotypes a and c (Shimada et al. 2008). In addition, the serotype b-specific polysaccharide antigen of *A. actinomycetemcomitans* induces higher secretion of IL-1 by murine macrophages than polysaccharide antigen extracted from serotypes a or c (Takahashi et al. 1991) and it has shown an increased resistance to phagocytosis and intra-cellular killing of this microorganism by human polymorphonuclear leucocytes (Yamaguchi et al. 1995). These findings are suggestive that the virulence and pathogenic role of the *A. actinomycetemcomitans* in the aetiology of periodontitis may differ among serotypes and specifically the b serotype of *A. actinomycetemcomitans* may have a differential role in the inflammatory cell activation and recruitment during the innate immune response.

Dendritic cells (DC) link the innate and adaptive immune responses, determining the T-helper (Th) polarization and activation pattern on primed naïve CD4 T lymphocytes, what may determine the disease phenotype, in particular whether in periodontitis there is an enhanced pattern of 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). In this study, we hypothesized that the different *A. actinomycetemcomitans* LPS-O-polysaccharide serotypes trigger variable DC responses with higher levels of cytokine production when they are stimulated with *A. actinomycetemcomitans* strains belonging to the serotype b compared with the others.

Materials and Methods

Bacterial growth conditions and curves

Aggregatibacter actinomycetemcomitans strains ATCC® 43717™ (serotype a), ATCC® 29523™ (serotype a), ATCC® 43718™ (serotype b), ATCC® 29522™ (serotype b), ATCC® 29524™ (serotype b), ATCC® 43719™ (serotype c) and ATCC® 33384™ (serotype c) were cultured on agar brain–heart infusion medium (Oxoid Ltd, Hampshire, England) at 37°C and under capnophilic conditions (8% O_2 and 12% CO_2) using an appropriate microaerobic condition generator (CampyGen™; Oxoid Ltd). To obtain a reliable number of colony-forming units for the stimulation of DCs, growth curves were obtained in liquid brain–heart infusion medium (Oxoid Ltd) as described previously (Vernal et al. 2008a).

LPS isolation and purification

Lipopolysaccharide of the studied bacterial strains was isolated using a modified version of the Tri-reagent protocol as previously described (Al-Qutub et al. 2006). Briefly, bacterial strains were cultured in liquid brain–heart infusion medium until they reached the stationary growth phase and were immediately pelleted by centrifugation at 6000 *g* at 4°C for 10 min. The pellet was washed three times with PBS and then incubated in 3 ml of 5:1 TRIzol reagent (Invitrogen Corp., Barcelona, Spain) and chloroform (Fluka, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) at room temperature for 30 min. The aqueous phase was recovered and lyophilized overnight. LPS was purified with 0.375 M $MgCl_2$ in EtOH 95% and, after washing 5 times with EtOH 95%, with 1% Folch reagent containing 2:1 chloroform and MeOH. LPS was visualized by Tris-glycine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 14% polyacrylamide gel and periodic acid-silver staining, quantified using the malondialdehyde thiobarbituric acid reaction and stored at −20°C.

Dendritic cell differentiation and stimulation

Purified DCs were obtained and stimulated as described previously (Vernal et al. 2008a). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood from 15 healthy donors following a Ficoll gradient method (Ficoll-Paque Plus; GE Healthcare, Uppsala, Sweden) using standard procedures. For generating a population of immature DCs, monocytes were purified from PBMCs by magnetic cell sorting, using an anti-CD14 monoclonal antibody conjugated with magnetic beads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), and cultured at 10^6 cells/ml in 3 ml RPMI-1640 containing 10% foetal calf serum (Gibco Invitrogen Corp., Grand Island, NY, USA) and 20 ng/ml of rhGM-CSF and rhIL-4 (R&D Systems Inc., Minneapolis, MN, USA) for 6 days at 37°C. Immature DCs were then stimulated with increasing multiplicity of infection (MOI) of 10^{-1}–10^2 (bacteria/DCs ratio) of *A. actinomycetemcomitans* strains or 10, 20 or 50 ng/ml of their purified LPS for 2 days. DCs stimulated with 10 ng/ml of the *Escherichia coli* 0111:B4 LPS (Fluka, Sigma-Aldrich Chemie, Buchs, Switzerland) and non-induced DCs served as controls.

Cell analysis by flow cytometry

The monocyte purification and their differentiation towards DCs were

analysed by flow cytometry as described previously (Vernal et al. 2006). Briefly, cells were stained with phycoerythrin (PE) or fluorescein isothiocyarate (FITC)-labelled anti-CD14 and CD1a monoclonal antibodies (BD Biosciences Pharmingen, San José, CA, USA) for 30 min. at 4°C in the dark and then analysed using flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA). To compare the maturation levels of DCs upon stimulation with the different strains of *A. actinomycetemcomitans*, the expression levels of CD83 (marker of DC maturation), CD80 and CD86 (costimulatory signals necessary for T-cell activation during antigen presentation) were determined by flow cytometry using PE or FITC-labelled anti-CD80, CD83 and CD86 monoclonal antibodies (BD Biosciences Pharmingen).

Expression of cytokines by reverse transcription-polymerase chain reaction (RT-PCR)

Total cytoplasmic RNA was isolated from DCs using 400 μl of ice-cold lysis buffer containing 0.5% Igepal® CA-630 (Sigma-Aldrich, Saint Louis, MO, USA), 50 mM Tris-HCl (pH8), 100 mM NaCl and 5 mM $MgCl_2$, supplemented with 10 mM vanadyl-ribonucleoside complex (VRC)-40 (Gibco Invitrogen, Carlsbad, CA, USA), as described previously (Vernal et al. 2008b). RNA was quantified using an spectrophotometer (Bio-Tek, Winooski, VT, USA) and stored at −80°C at a final concentration of 1 μg/μl. Reverse transcription was performed using a First-Strand cDNA Synthesis Super-Mix kit following the manufacturer's recommendations (SuperScrip™III; Invitrogen, Grand Island, NY, USA). The mRNA expression for the cytokines interferon (IFN)-γ, tumour necrosis factor (TNF)-α, IL-1β, IL-5, IL-6, IL-10, IL-12(p35) and IL-23 (p19) was determined by conventional RT-PCR and quantified by real-time RT-PCR using the appropriate primers (Table 1) designed using the Roche website (accessed at https://www.roche-applied-science.com). To determine the mRNA expression for the cytokines, 250 ng of cDNA were amplified by conventional RT-PCR using the GoTaq® Green Master Mix kit (Promega, Madison, WI, USA), following the manufacturer's recommendations. The reaction was run in a 2% agarose gel stained with 1× GelRed (Biotium Inc., Hayward, CA, USA) and photographed using a Gel Logic 2200Pro transiluminator (Carestream Health, Rochester, NY, USA). As positive controls, the cytokine mRNA expressions were determined in Jurkat E61.2 cells stimulated with a combination of anti-CD3 and anti-CD28 monoclonal antibodies. To quantify the mRNA expression for the cytokines, 50 ng of cDNA were amplified by quantitative real-time RT-PCR using the KAPA™ SYBR® Fast qPCR reagent (KAPA Biosystems, Woburn, MA, USA) in a StepOnePlus® real-time PCR system (Applied Biosystems, Singapore) as follows: 95°C for 3 min., followed by 40 cycles of 95°C for 3 s and 60°C for 30 s, and finally a melt curve of 95°C for 15 s, 60°C for 1 min. and 95°C for 15 s, for detection of non-specific product formation and false positive amplification. As an endogenous control, 18S rRNA expression levels were determined.

Table 1. Forward and reverse primers used for cytokine mRNA and 18S rRNA amplifications by conventional and quantitative real-time RT-PCR

mRNA	Ensembl gene ID	Forward primer	Reverse primer
IL-1β	ENSG00000125538.7	ctgtcctgcgtgttgaaaga	ttgggtaatttttgggatctaca
IL-5	ENSG00000113525.5	ctctgaggattcctgttcctgt	cagtacccccttgcacagtt
IL-6	ENSG00000136244.6	gcccagctatgaactccttct	gaaggcagcaggcaacac
IL-10	ENSG00000136634.5	tgggggagaacctgaagac	ccttgctcttgttttcacagg
IL-12	ENSG00000168811.2	cactcccaaaacctgctgag	tctcttcagaagtgcaagggta
IL-23	ENSG00000110944.3	agcttcatgcctccctactg	ctgctgagtctcccagtggt
IFN-γ	ENSG00000111537.3	ggcattttgaagaattggaaag	tttggatgctctggtcatctt
TNF-α	ENSG00000228978.2	cagcctcttctccttcctgat	gccagagggctgattagaga
18S	ENSG00000086189.5	ctcaacacgggaaacctcac	cgctccaccaactaagaacg

IL, interleukin; IFN, interferon; TNF, tumour necrosis factor.

Secretion of cytokines by ELISA

After bacterial stimulation for 2 days, DCs culture supernatants were collected and the secretion of IFN-γ, TNF-α, IL-1β, IL-5, IL-6, IL-10, IL-12(p70) and IL-23 was measured by ELISA (Quantikine®; R&D Systems Inc.) according manufacturer's protocols and using an automatic microplate spectrophotometer (Bio-Tek) at 460 and 560 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 RT-PCR data were analysed using the StepOne Software v2.2.2 (Applied Biosystems) and the relative quantification was obtained by normalizing the cytokine mRNA expression to 18S rRNA expression using the $2^{-\Delta\Delta Ct}$ method. Data were expressed as mean ± standard deviation (SD) and statistically analysed using the SPSS 15.0 software (Lead Technologies Inc., Charlote, NC, USA). The normality of data distribution was determined using the Kolmogorov–Smirnov 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 expression and secretion were determined using the Kruskal–Wallis test or ANOVA and Tukey tests. A statistical significance was considered when $p < 0.05$.

Results

Monocyte purification and dendritic cell differentiation

The monocyte and DC purities were demonstrated by the combined staining with anti-CD14 and anti-CD1a monoclonal antibodies and analysed by flow cytometry (Fig. 1a). Highly purified (>97%) monocyte population ($CD14^+$ cells) was isolated from peripheral blood. These monocytes differentiated at a high frequency (>98%) into DCs ($CD1a^+$ cells) upon culture in presence of rhGM-CSF and rhIL-4, as demonstrated by the appearance of the CD1a antigen and the concomitant loss of the monocyte marker CD14.

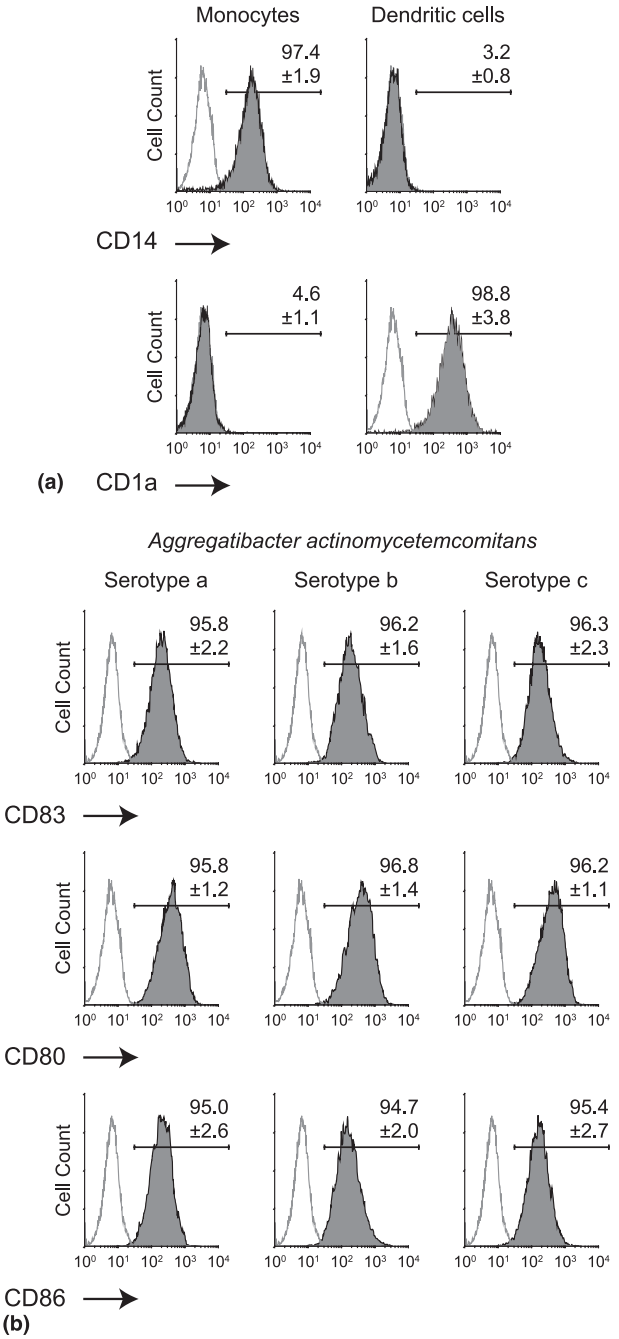

Fig. 1. Monocyte purification and dendritic cell differentiation and maturation. (a) Flow cytometry analysis demonstrating the purity of monocytes isolated from the peripheral blood ($CD14^+$ cells) and the efficiency of differentiation of monocytes towards dendritic cells ($CD1a^+$ cells) in presence of rhGM-CSF and rhIL-4. (b) Flow cytometry analysis of the expression of CD83 (marker of dendritic cell maturation), CD80 and CD86 (costimulatory signals necessary for T-cell activation during antigen presentation) demonstrating the dendritic cell maturation after a 48-h stimulation with the *A. actinomycetemcomitans* strains ATCC® 43717™ (serotype a), ATCC® 43718™ (serotype b) and ATCC® 43719™ (serotype c) at a multiplicity of infection (MOI) of 10^2. The data from each experiment were expressed as percentage of positive cells over the total and shown as mean ± SD from 10 independent experiments. CD, cluster of differentiation.

Maturation of dendritic cells

The efficiency of DC maturation upon stimulation with the different strains of *A. actinomycetemcomitans* was analysed by the combined staining with anti-CD83, anti-CD80 and anti-CD86 monoclonal antibodies analysed by flow cytometry (Fig. 1b). Upon stimulation with *A. actinomycetemcomitans* strains (MOI = 10^2) no differences were detected in the maturation levels of DCs between the different bacterial serotypes, as demonstrated by the increased expression of CD83, CD80 and CD86 antigens (>95%), which was associated with an increase in cell size (not shown), demonstrating that the different *A. actinomycetemcomitans* strains induce DC maturation to a similar extent.

Cytokine mRNA expression in bacteria-stimulated dendritic cells

The mRNA expression for the analysed cytokines was determined by conventional RT-PCR (Fig. 2) and quantified by real-time RT-PCR, plotted as fold-change for each cytokine (Fig. 3). The expression of IFN-γ, TNF-α, IL-1β, IL-12, IL-6, IL-23, IL-5 and IL-10 was induced in DCs from all the analysed subjects; however, IL-5 remained undetectable in one individual under any experimental conditions (Fig. 2).

The DCs stimulated with the strain ATCC® 43718™ (serotype b) of *A. actinomycetemcomitans* had a higher expression of IFN-γ ($p = 0.031$ and 0.02), TNF-α ($p = 0.006$ and 0.007), IL-1β ($p = 0.003$ and 0.01), IL-12 ($p = 0.004$ and 0.001) and IL-23 ($p = 0.009$ and 0.009) than the same cells stimulated with either the serotype a or c respectively (Fig. 3). In addition, the *A. actinomycetemcomitans* serotype b elicited higher expression levels of IL-6 on DCs when compared with the serotype a ($p = 0.01$), but not with *A. actinomycetemcomitans* serotype c ($p = 0.28$). No differences were detected in the mRNA expression for the analysed cytokines in DCs after challenge with *A. actinomycetemcomitans* serotypes a or c. Overall, when the strain ATCC® 43718™ (serotype b) was used, the observed DC response was characteristic of a Th1-pattern of cytokines, as demonstrated by higher expression levels of IL-1β, IL-12, IFN-γ and TNF-α. Similarly, a Th17-pattern of cytokine expression was detected when DCs were stimulated with the *A actinomycetemcomitans* strain ATCC® 43718™ (serotype b). In fact, this bacterial strain elicited a higher

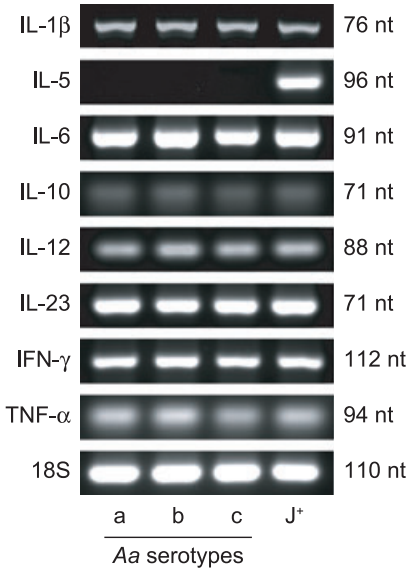

Fig. 2. Cytokine mRNA expression determined by conventional RT-PCR in bacteria-stimulated dendritic cells. The mRNA expression for the cytokines IL-1β, IL-5, IL-6, IL-10, IL-12, IL-23, IFN-γ and TNF-α and for the 18S rRNA was determined in dendritic cells stimulated at a multiplicity of infection (MOI) of 10^2 of *A. actinomycetemcomitans* strains ATCC® 43717™ (serotype a), ATCC® 43718™ (serotype b) and ATCC® 43719™ (serotype c). As positive controls, the cytokine mRNA expressions were determined in Jurkat E61.2 cells stimulated with a combination of anti-CD3 and anti-CD28 monoclonal antibodies (J^+). IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.

increase in the expression of IL-6 and IL-23 and these over-expressed levels were higher than those detected for Th1-associated cytokines.

Cytokine secretion in bacteria-stimulated dendritic cells

The Th1/Th17-pattern of DC response detected upon stimulation with the *A. actinomycetemcomitans* strain ATCC® 43718™ (serotype b) was confirmed when cytokine production (pg/ml) was analysed at a protein level (Fig. 4). Higher levels of IFN-γ (MOI = 10^2 $p < 0.001$ and MOI = 10^1 $p < 0.04$), TNF-α (MOI = 10^2 $p < 0.001$ and MOI = 10^1 $p < 0.002$), IL-1β (MOI = 10^2 $p < 0.05$), IL-12 (MOI = 10^2 $p < 0.05$) and IL-23 (MOI = 10^2 $p < 0.05$ and MOI = 10^1 $p < 0.001$) were detected when DCs were activated with the *A. actinomycetemcomitans* strain ATCC® 43718™ (serotype b), when compared with the others. Furthermore, a dose-dependent increase in the secretion levels for IFN-γ, TNF-α, IL-1β, IL-12, IL-6 and IL-23 was elicited on DCs following stimulation at MOIs of 10^{-1}–10^2 with each of the strains of *A. actinomycetemcomitans*; however, at MOIs of 10^{-1} and 10^0 no differences were detected between them (data not shown). Similarly than detected at a mRNA level, no differences were detected in the secretion for the analysed cytokines in DCs after challenge with *A. actinomycetemcomitans* strains belonging to the serotypes a or c.

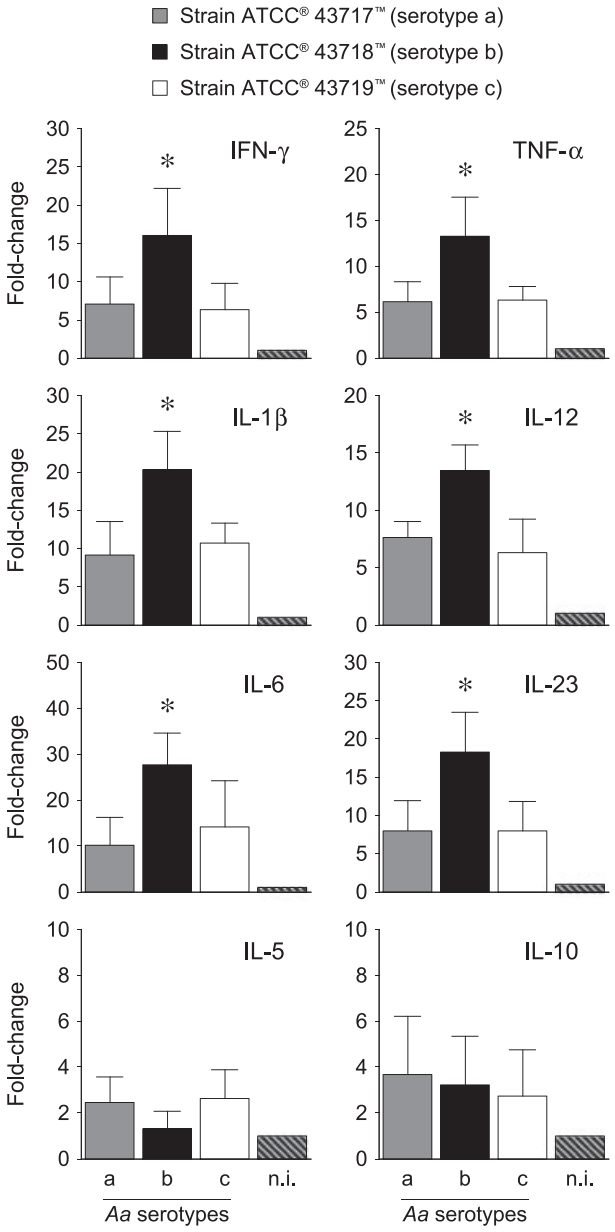

Fig. 3. Cytokine mRNA expression quantified by real-time RT-PCR in bacteria-stimulated dendritic cells. The mRNA expression for the cytokines IFN-γ, TNF-α, IL-1β, IL-12, IL-6, IL-23, IL-5 and IL-10 was determined in dendritic cells stimulated at a multiplicity of infection (MOI) of 10^2 of *A. actinomycetemcomitans* strains ATCC® 43717™ (serotype a), ATCC® 43718™ (serotype b) and ATCC® 43719™ (serotype c). For relative expression, the cytokine mRNA expression in non-induced DCs was considered as 1, as a reference for fold-change in expression (n.i.). 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; TNF, tumour necrosis factor. *$p < 0.05$.

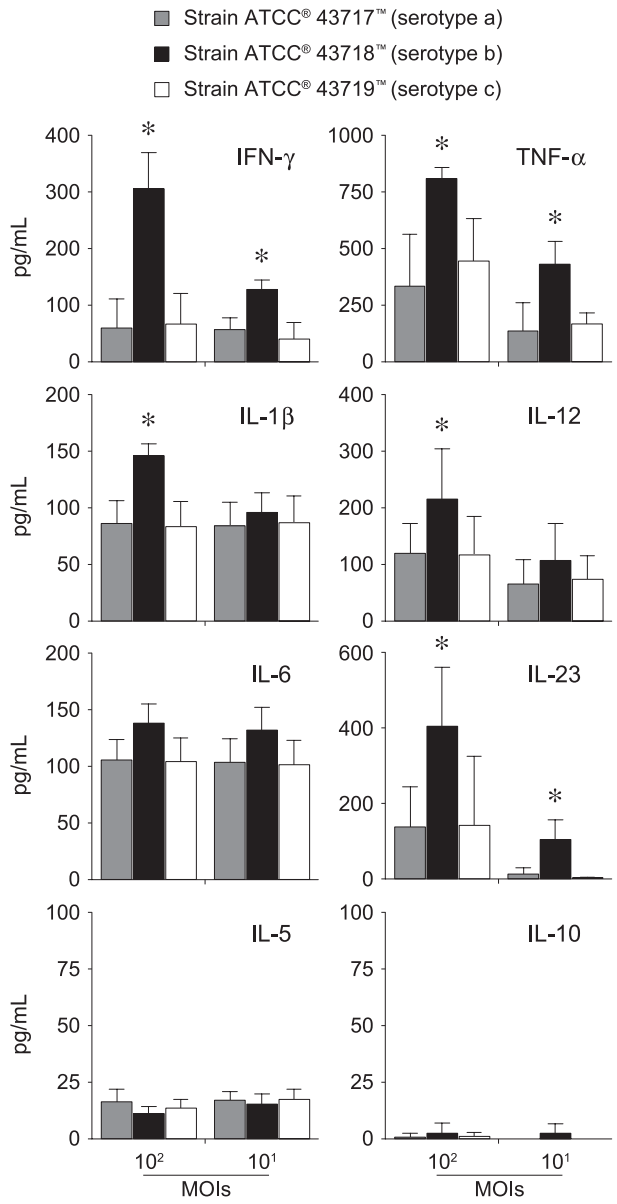

Fig. 4. Cytokine secretion levels in bacteria-stimulated dendritic cells. The secretion for the cytokines IFN-γ, TNF-α, IL-1β, IL-12, IL-6, IL-23, IL-5 and IL-10 was determined in dendritic cells stimulated at different multiplicity of infection (MOI) of 10^1–10^2 of *A. actinomycetemcomitans* strains ATCC® 43717™ (serotype a), ATCC® 43718™ (serotype b) and ATCC® 43719™ (serotype c). Data are represented as cytokine concentration (pg/ml) and shown as mean ± SD for 10 independent experiments. Each experiment was performed in duplicate. IFN, interferon; IL, interleukin; TNF, tumour necrosis factor. *$p < 0.05$.

Cytokine mRNA expression in LPS-stimulated dendritic cells

The increased expression levels of Th1 and Th17-associated cytokines in DC stimulated with the *A. actinomycetemcomitans* strain ATCC® 43718™ (serotype b) was confirmed in LPS-stimulated DCs. The DCs stimulated with 50 ng/ml of LPS purified from the *A. actinomycetemcomitans* strain ATCC® 43718™ (serotype b) had a higher expression of IFN-γ ($p = 0.025$ and 0.04), TNF-α ($p = 0.021$ and 0.012), IL-1β ($p = 0.012$ and 0.018), IL-12 ($p = 0.025$ and 0.022), IL-6 ($p = 0.003$ and 0.004) and IL-23 ($p = 0.014$ and 0.003) than the same cells stimulated with 50 ng/ml of LPS purified from bacterial strains belonging to either the serotype a or c respectively (Fig. 5). In DCs stimulated with 20 ng/ml of LPS, higher expression levels of IL-1β ($p = 0.032$ and 0.039) and IL-23 ($p = 0.045$ and 0.026) were detected in serotype b-stimulated DCs compared with the same cells stimulated with the serotype a or c respectively.

No differences were detected in the mRNA expression for the analysed cytokines in DCs after challenge with 50 ng/ml of LPS between *A. actinomycetemcomitans* strains ATCC® 43717™ or ATCC® 29523™ belonging to the serotype a, between strains ATCC® 43718™, ATCC® 29522™ or ATCC® 29524™ belonging to the serotype b, and between strains ATCC® 43719™ or ATCC® 33384™ belonging to the serotype c (Fig. 6). Overall, the DCs stimulated with 50 ng/ml of LPS purified from all the studied *A. actinomycetemcomitans* strains belonging to the serotype b had a higher expression levels of IFN-γ, TNF-α, IL-1β, IL-6, IL-12 and IL-23 than the same cells stimulated with LPS purified from bacterial strains belonging to either the serotype a or c ($p < 0.05$ for all comparisons).

Discussion

Lipopolysaccharides are amphiphilic glycophospholipids and represent a major constituent of the outer membranes of gram-negative bacteria. The polysaccharide portion consists of a core oligosaccharide region and the O-antigen, which constitutes a chain of O-polysaccharide repeating units that stimulate a wide range of immunological and cell responses in the host, which may have important patho-physiological consequences. In *A. actinomycetemcomitans*, six different serotypes are recognized based on the composition and antigenicity of the O-polysaccharide component of the LPS (Page et al. 1991, Gmur et al. 1993, Kaplan et al. 2001), being the serotypes a, b and c, which were analysed in this study, the most prevalent in the oral cavity in humans (Chen et al. 2010, van der Reijden et al. 2010, Roman-Torres et al. 2010, Sakellari et al. 2011, Aberg et al. 2012, Bandhaya et al. 2012, Cortelli et al. 2012, Jentsch et al. 2012).

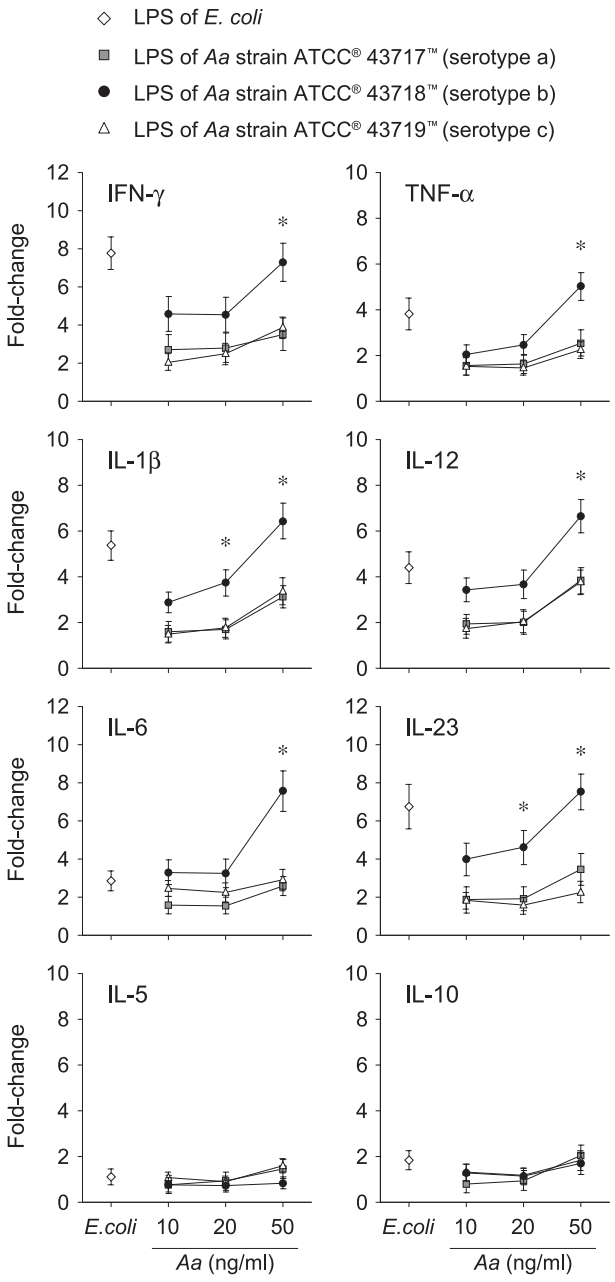

Fig. 5. Cytokine mRNA expression quantified by real-time RT-PCR in lipopolysaccharide (LPS)-stimulated dendritic cells. The mRNA expression for the cytokines IFN-γ, TNF-α, IL-1β, IL-12, IL-6, IL-23, IL-5 and IL-10 was determined in dendritic cells stimulated with 10, 20 and 50 ng/ml of LPS purified from *A. actinomycetemcomitans* strains ATCC® 43717™ (serotype a), ATCC® 43718™ (serotype b) and ATCC® 43719™ (serotype c). DCs stimulated with 10 ng/ml of the *Escherichia coli* LPS were used as positive control. For relative expression, the cytokine mRNA expression in non-induced DCs was considered as 1, as a reference for fold-change in expression. Data are represented as fold-change and shown as mean ± SD for five independent experiments. Each experiment was performed in duplicate. *Aa*, *A. actinomycetemcomitans*; IFN, interferon; IL, interleukin; TNF, tumour necrosis factor. *$p < 0.05$.

Several investigations have studied the *A. actinomycetemcomitans* serotype distribution according to the subject's periodontal status. Serotype b has been found more frequently associated with patients with chronic and aggressive periodontitis when compared with healthy individuals and these patients exhibit elevated serum antibody levels to serotype b-specific antigen (Chen et al. 2010, van der Reijden et al. 2010, Roman-Torres et al. 2010, Sakellari et al. 2011, Aberg et al. 2012, Bandhaya et al. 2012, Cortelli et al. 2012, Jentsch et al. 2012). This association between presence of *A. actinomycetemcomitans* serotype b and periodontitis suggests an increased periodontopathic potential of this species, although the exact mechanisms of this pathogenicity remain unclear.

The host immune response against the bacterial challenge is known to be determinant in the pathogenesis of periodontitis and the amount of alveolar bone resorption and connective tissue destruction that defines this chronic inflammatory disease is dependent on the quality of this immunological response (Gemmell et al. 2007, Houri-Haddad et al. 2007, Garlet 2010, Graves et al. 2011). Specific microbial components that activate antigen-presenting cells, such as DCs, will lead to production of a set of cytokines and the pattern of cytokines produced will determine the subsequent polarization of an antigen-specific lymphocyte response: Th1, Th2 or Th17. It has been suggested that this polarization of the T lymphocytes begins with those cells having the primary contact with antigens and, therefore, DCs likely polarize into type 1, type 2 or type 17 in response to this antigen challenge and this response will then influence outcome of immune response by selectively stimulating the T-cell lineages (Iwasaki & Medzhitov 2004, Cutler & Jotwani 2006, Woehrle et al. 2008).

The present work has demonstrated a differential immune response when DCs were stimulated with different bacterial strains belonging to the serotypes a–c of *A. actinomycetemcomitans*. These differences were described by the quantitative and qualitative variations in the cytokine production when DCs were exposed to increasing amounts of serotype specific *A. actinomycetemcomitans* LPS, thus suggesting their clear role in the activation of DCs.

The association of *A. actinomycetemcomitans* with destructive periodontitis is dependent on its virulence traits, as well as on other

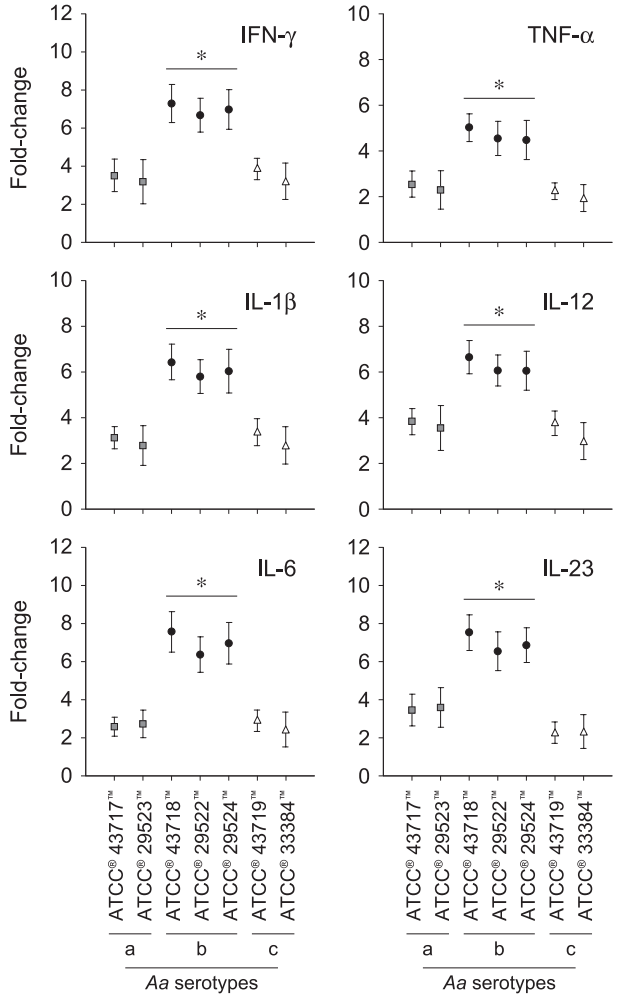

Fig. 6. Cytokine mRNA expression quantified by real-time RT-PCR in lipopolysaccharide (LPS)-stimulated dendritic cells. The mRNA expression for the cytokines IFN-γ, TNF-α, IL-1β, IL-12, IL-6 and IL-23 was determined in dendritic cells stimulated with 50 ng/ml of LPS purified from *A. actinomycetemcomitans* strains ATCC® 43717™ (serotype a), ATCC® 29523™ (serotype a), ATCC® 43718™ (serotype b), ATCC® 29522™ (serotype b), ATCC® 29524™ (serotype b), ATCC® 43719™ (serotype c) and ATCC® 33384™ (serotype c). For relative expression, the cytokine mRNA expression in non-induced DCs was considered as 1, as a reference for fold-change in expression. Data are represented as fold-change and shown as mean ± SD for five independent experiments. Each experiment was performed in duplicate. *Aa*, *A. actinomycetemcomitans*; IFN, interferon; IL, interleukin; TNF, tumour necrosis factor. *$p < 0.05$.

host and environmental factors. The serotype b of *A. actinomycetemcomitans* has also shown to produce cytotoxic membrane microvesicles, in contrast with other known serotypes (Nowotny et al. 1982). In addition, a significant inhibition of cell growth and enhanced expression of IL-8 and ICAM-1 was shown in response to extracts of *A. actinomycetemcomitans* b serotype when in contact with gingival epithelial cells (Shimada et al. 2008). These results suggest that the *A. actinomycetemcomitans* serotype b may alter the protective role of gingival epithelial cells as a mechanical barrier against invasion by pathogenic microorganisms and thus induce the initial stages of the innate immune response through the expression of enhanced levels of chemokines and adhesion molecules that mediate the attraction, migration and activation of neutrophils and macrophages to the periodontal infection focus. In this context, the serotype b of *A. actinomycetemcomitans* have been associated with higher levels of IL-1 secretion by stimulated murine macrophages compared with either the serotypes a or c (Takahashi et al. 1991) and has been implied in an increment on the resistance to phagocytosis and killing by human polymorphonuclear leukocytes (Yamaguchi et al. 1995). Our data are consistent with these results because significant differences in cytokine expression and secretion induced by the different serotypes of *A. actinomycetemcomitans* were demonstrated and they indicate that the strains belonging to the serotype b are able to induce a more robust response on DCs, which might lead to the described increased resistance to phagocytosis (Yamaguchi et al. 1995).

The periodontal tissues contain a large number of professional antigen-presenting cells and DCs play a major role in the periodontal immune response. Abundant immature DCs, including Langerhans cells, have been detected in the junctional epithelium and gingival sulcus, and mature $CD83^+$ DCs have been observed in close proximity to $CD4^+$ T lymphocytes in the gingival connective tissue, increasing in number during periodontal infection (Newcomb et al. 1982, Seguier et al. 2000a,b, Jotwani et al. 2001, Cirrincione et al. 2002, Gemmell et al. 2003, Jotwani & Cutler 2003, Cutler & Jotwani 2006, Cutler & Teng 2007, Wilensky et al. 2014). In fact, the population of gingival DCs is constantly renewed by a continuous cell migration process and it has been shown that local infiltrating monocytes have the capacity to differentiate into DCs when are stimulated with pro-inflammatory cytokines (Winning et al. 1996, Jotwani et al. 2001).

Previous data of our research group have demonstrated a heterogenic immuno-stimulatory potential on DCs when they were stimulated with the six different capsular (K) serotypes described for *Porphyromonas gingivalis*, demonstrating that strains K1 and K2 induce a more robust cytokine expression than the others (Vernal et al. 2009). In the present investigation, similar differences were detected between *A. actinomycetemcomitans* serotypes, suggesting a role of these bacterial serotypes on the DCs priming and

subsequent antigen presentation. In particular, the DC response against the bacterial strains belonging to the serotype b of *A. actinomycetemcomitans* was biased towards a Th1 and Th17-pattern of cytokine response, what may suggest that they stimulate a Th1 and Th17 polarization of naïve T lymphocytes during the antigen presentation, determining a pro-inflammatory immune response and leading to connective tissue destruction and alveolar bone resorption.

To our knowledge, this is the first report identifying quantitative and qualitative variations in the cytokine production on DCs upon stimulation with the different serotypes of *A. actinomycetemcomitans*. These results may explain in part the variances in virulence between the different serotypes of *A. actinomycetemcomitans* and the greater periodontopathic potential of serotype b when compared with the other serotypes, at least between the tested strains. These findings may imply that the bacterial tests looking for the presence of *A. actinomycetemcomitans* may provide us with only partial information. In fact, future microbiological tests should consider that the virulence of *A. actinomycetemcomitans* may vary between the different serotypes and could determine the outcome of the T-lymphocyte immunity during periodontitis.

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Clinical Relevance

Scientific rationale for the study: In *A. actinomycetemcomitans*, different serotypes have been described and it has been hypothesized that the virulence and pathogenic role of *A. actinomycetemcomitans* in the induction of periodontitis may differ among serotypes.

Principal findings: A differential potential of *A. actinomycetemcomitans* serotypes to induce dendritic cell response was demonstrated with bacterial strains belonging to the serotype b triggering higher levels of Th1/Th17-type cytokines.

Practical implications: Not all the pathogens belonging to the *A. actinomycetemcomitans* species have the same immuno-stimulatory potential and this heterogeneity should be taken into account when evaluating their role in the pathogenesis of periodontitis.