Variability of the Dendritic Cell Response Triggered by Different Serotypes of *Aggregatibacter actinomycetemcomitans* or *Porphyromonas gingivalis* Is Toll-Like Receptor 2 (TLR2) or TLR4 Dependent

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Background: Different serotypes of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* have been shown to induce differential dendritic cell (DC) responses. This study investigates whether cytokine and CC-chemokine receptor (CCR) production by DCs stimulated with different serotypes of *A. actinomycetemcomitans* or *P. gingivalis* is Toll-like receptor 2 (TLR2) and/or TLR4 dependent.

Methods: DCs were obtained from healthy individuals and primed at a multiplicity of infection (MOI) of 10^2 with different *A. actinomycetemcomitans* or *P. gingivalis* serotypes in the presence or absence of anti-TLR2 or anti-TLR4 blocking antibodies. TLR2 and TLR4 expression, CCR5 and CCR6 expression, and interleukin (IL)-1β, IL-10, IL-12, and IL-23 expression and secretion were quantified by flow cytometry, real-time reverse-transcription polymerase chain reaction, and enzyme-linked immunosorbent assay.

Results: When DCs were stimulated with serotype b of *A. actinomycetemcomitans* or serotype K1 of *P. gingivalis*, higher levels of TLR2 or TLR4, respectively, were detected compared to DCs stimulated with the other serotypes. Similarly, higher levels of cytokines and CCRs were detected in serotype b– or serotype K1–primed DCs compared to the others, and these increased levels positively correlated with levels of TLR2 or TLR4. When TLR2 signaling was blocked using a specific anti-TLR2 monoclonal antibody, serotype b–induced cytokine and CCR expression was inhibited; when TLR4 signaling was blocked, serotype K1–induced response was inhibited.

Conclusions: These results demonstrate that the variability of secretion of cytokines and expression of CCRs detected in DCs stimulated with different serotypes of *A. actinomycetemcomitans* or *P. gingivalis* is TLR2 or TLR4 dependent, respectively. *J Periodontol 2015;86:108-119.*

KEY WORDS

***Aggregatibacter actinomycetemcomitans*; dendritic cells; interleukins; *Porphyromonas gingivalis*; receptors, ccr; Toll-like receptors.**

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Toll-like receptors (TLRs) constitute a family of receptors expressed in various cell types on the innate immune system. They are composed of leucine-rich transmembrane proteins, which function by discriminating pathogens and initiating host immunoinflammatory responses.[1] At least 12 human TLRs have been described, with the ability to recognize a variety of molecules, many being bacterial virulence factors, such as polysaccharides of the extracellular capsule, lipopolysaccharide (LPS), flagellin, and fimbriae.[1,2]

In periodontal tissues, TLR2 and TLR4 recognize subgingival biofilm bacteria and participate in the initiation and progression of the immuno-inflammatory events that mediate the connective tissue breakdown and alveolar bone resorption characteristic of periodontitis.[3] In fact, increased expression of TLR2 and TLR4 was observed in diseased periodontal tissues, mostly associated with antigen-presenting cells, such as macrophages and dendritic cells (DCs).[4,5] The signaling of TLR2 and TLR4 on DCs stimulates their activation, production of cytokines, including interleukin (IL)-1β, IL-12, IL-23, tumor necrosis factor (TNF)-α and TNF-β, and expression of CC-chemokine receptors (CCRs). These receptors, including CCR5 and CCR6, interact with specific chemokine ligands, thus mediating DC migration via the afferent lymph vessels to the draining lymph nodes, facilitating antigen presentation to T lymphocytes.[6-8]

Aggregatibacter actinomycetemcomitans and *Porphyromonas gingivalis* cell wall components stimulate TLR2 and TLR4. In human cells, *A. actinomycetemcomitans* induces overexpression of TLR2 and TLR4 messenger RNAs (mRNAs),[3] and TLR2 signaling mediates the apoptosis of human monocytes.[9] Similarly, *P. gingivalis* induces overexpression of TLR2 and TLR4 mRNA levels in human and mouse cells,[10] and the increased mRNA expression levels of these TLRs reported in periodontitis-affected tissues positively correlates with the numbers of *P. gingivalis* detected in local subgingival plaque.[11]

Different serotypes of *A. actinomycetemcomitans* and *P. gingivalis* have been described based on the differential antigenicity of *O*-polysaccharide component of the LPS[12-14] and the extracellular capsule,[15,16] respectively. A differential immune response was described when DCs were stimulated with these different bacterial serotypes. In particular, strains ATCC 43718, ATCC 29522, and ATCC 29524, belonging to serotype b of *A. actinomycetemcomitans*, and strains W83 and HG184, belonging to serotypes K1 and K2 of *P. gingivalis*, show a stronger cytokine production by DCs.[17]

Because TLR2 and TLR4 signaling mediates the pattern of cytokine production by DCs, it may be speculated that the differential DC response when exposed to different serotypes of *A. actinomycetemcomitans* and *P. gingivalis* could be due to different TLR2 or TLR4 expression. The objective of this investigation was to determine whether the variability in cytokine secretion and CCR expression in DCs stimulated with the different serotypes of *A. actinomycetemcomitans* and *P. gingivalis* is TLR2 and/or TLR4 dependent.

MATERIALS AND METHODS

Culture of A. actinomycetemcomitans *and* P. gingivalis

A. actinomycetemcomitans strains ATCC 43717 (serotype a), ATCC 43718 (serotype b), and ATCC 43719 (serotype c) were cultured on agar brain–heart infusion medium‡ at 37°C and under capnophilic conditions (8% O_2 and 12% CO_2) using an appropriate micro-aerobic condition generator.§ Encapsulated *P. gingivalis* strains W83 (serotype K1), HG184 (serotype K2), A7A1-28 (serotype K3), ATCC 49417 (serotype K4), HG1690 (serotype K5), and HG1691 (serotype K6) and the non-encapsulated (K^-) strain ATCC 33277 were cultured on 5% horse blood agar‖ supplemented with 5 mg/L hemin and 1 mg/L menadione at 37°C and under anaerobic conditions (80% N_2, 10% CO_2, and 10% H_2) using an appropriate anaerobic condition generator.¶

Bacterial Growth Curves

To obtain a reliable number of colony-forming units for the stimulation of DCs, growth curves were obtained in liquid brain–heart infusion medium# as described previously.[18] Live bacteria, having their whole antigenic potentiality, were obtained at the exponential growth phase of the bacterial culture and used for these experiments.

Blood Donors

Blood cells were obtained during platelet-apheresis processes from healthy donors consecutively enrolled at the Blood Bank of the Hospital Luis Calvo Mackenna in the Eastern Metropolitan Health Service, Santiago, Chile. The study group consisted of 12 adults (seven males and five females, aged 21 to 38; mean age 28.3 ± 5.10 years) who did not have periodontal disease as determined by absence of gingival inflammation, clinical attachment loss, or increased probing depths. Further exclusion criteria were: 1) a positive test for human immunodeficiency virus and hepatitis B or C virus; 2) presence of manifest infections during the last month; 3) fever;

‡ Oxoid, Hampshire, England.
§ CampyGen, Oxoid.
‖ Oxoid.
¶ AnaeroGen, Oxoid.
# Oxoid.

4) symptomatic allergies; 5) abnormal blood cell counts; 6) increased liver enzymes; or 7) medication of any kind except vitamins and oral contraceptives. The protocol of the study was clearly explained to all the participants, who agreed to participate in the study by signing an institutional review board–approved informed consent. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000.

Isolation of Monocytes and Differentiation to DCs

For each individual, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation** using standard procedures. Monocytes were positively purified from PBMCs using an anti-CD14 monoclonal antibody (mAb) conjugated to magnetic beads by magnetic cell sorting.†† Monocytes ($CD14^+$ cells) were then differentiated to DCs by culture at 10^6 cells/mL in RPMI-1640 containing 10% fetal calf serum‡‡ and 20 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and rhIL-4§§ for 6 days at 37°C.

DC Stimulation

Differentiated DCs were primed at a multiplicity of infection (MOI) of 10^2 (bacteria/DC ratio) with different *A. actinomycetemcomitans* and *P. gingivalis* strains for 2 days. DCs stimulated with 10 ng/mL *Escherichia coli* strain 0111:B4 LPS‖‖ and non-induced DCs were used as controls. To determine TLR2- or TLR4-dependent DC signaling and response, 20 μg/mL anti-TLR2 or anti-TLR4 blocking antibodies¶¶ were added to culture medium 30 minutes before and 24 hours after DC stimulation. For each individual, the experiments were performed separately.

Flow Cytometry Analysis

The efficiency of the monocyte purification and differentiation toward DCs, as well as DC maturation under bacterial stimulation with the different strains of *A. actinomycetemcomitans* or *P. gingivalis*, were analyzed by flow cytometry as described previously.[19] Cells were stained with fluorescein isothiocyanate, phycoerythrin (PE), or PE-cyanine 5 (Cy5)-labeled anti-CD14 (monocyte marker), anti-CD1a (immature DC marker), anti-CD83 (mature DC marker), anti-CD80, and anti-CD86 (costimulatory signals necessary for T-cell activation during antigen presentation) mAbs## for 30 minutes at 4°C in the dark and then analyzed by flow cytometry.*** Similarly, the expression of TLR2 and TLR4 on the surface of the differentiated and matured DCs was analyzed using the following mAbs: anti-TLR2/CD282-AlexaFluor-647 and anti-TLR4/CD284-PE-Cy7.†††

Total Cytoplasmic RNA Isolation and First-Strand cDNA Synthesis

Total cytoplasmic RNA was isolated from DCs using 400 μL ice-cold lysis buffer containing 0.5% cell detergent,‡‡‡ 50 mM Tris-HCl (pH 8), 100 mM NaCl, and 5 mM $MgCl_2$, supplemented with 10 mM vanadyl-ribonucleoside complex-40,§§§ as described previously.[20] RNA was quantified using a spectrophotometer‖‖‖ and stored at –80°C. Reverse transcription was performed using a cDNA synthesis kit¶¶¶ following the manufacturer's recommendations.

Expression of TLRs, Cytokines, and CCRs mRNAs

To associate the expression of TLR2 and TLR4 with DC function after bacterial stimulation, the mRNA expression levels for TLR2 and TLR4, the cytokines IL-1β, IL-10, IL-12, and IL-23, and the chemokine receptors CCR5/CD195 and CCR6/CD196 were quantified by real-time reverse-transcription polymerase chain reaction (RT-PCR) using the appropriate forward and reverse primers: TLR2, ctctcggtgtcggaatgtc and aggatcagcaggaacagagc; TLR4, ccctcccctgtacccttct and tccctgccttgaataccttc; IL-1β, ctgtcctgcgtgttgaaaga and ttgggtaatttttgggatctaca; IL-10, tgggggagaacctgaagac and ccttgctcttgttttcacagg; IL-12, cactcccaaaacctgctgag and tctcttcagaagtgcaagggta; IL-23, agcttcatgcctccctactg and ctgctgagtctcccagtggt; CCR5, cttgggtggtggctgtgt and gaccagccccaagatgacta; and CCR6, actgtggctgttggtttgtg and tcagccccttcagctcac. Fifty nanograms of cDNA were amplified using a quantitative PCR reagent### in a real-time PCR system**** as follows: 95°C for 3 minutes; followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds; and finally a melt curve of 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds, for detection of non-specific product formation and false-positive amplification. As an endogenous control, 18S ribosomal RNA (rRNA) expression levels were determined using the following forward and reverse primers: ctcaacacgggaaacctcac and cgctccaccaactaagaacg.

** Ficoll-Paque, Amersham Pharmacia Biotech, Uppsala, Sweden.
†† Miltenyi Biotec, Bergisch Gladbach, Germany.
‡‡ Gibco Invitrogen, Thermo Fisher Scientific, Grand Island, NY.
§§ R&D Systems, Minneapolis, MN.
‖‖ Fluka, Sigma-Aldrich Chemie, Buchs, Switzerland.
¶¶ eBioscience, San Diego, CA.
BD Biosciences Pharmingen, San José, CA.
*** FACScan, Becton Dickinson, Franklin Lakes, NJ.
††† eBioscience.
‡‡‡ Igepal CA-630, Sigma-Aldrich, St. Louis, MO.
§§§ VRC-40, Gibco Invitrogen, Thermo Fisher Scientific.
‖‖‖ Bio-Tek, Winooski, VT.
¶¶¶ Roche Applied Science, Mannheim, Germany.
KAPA SYBR Fast qPCR, KAPA Biosystems, Woburn, MA.
**** StepOnePlus, Applied Biosystems, Thermo Fisher Scientific, Singapore.

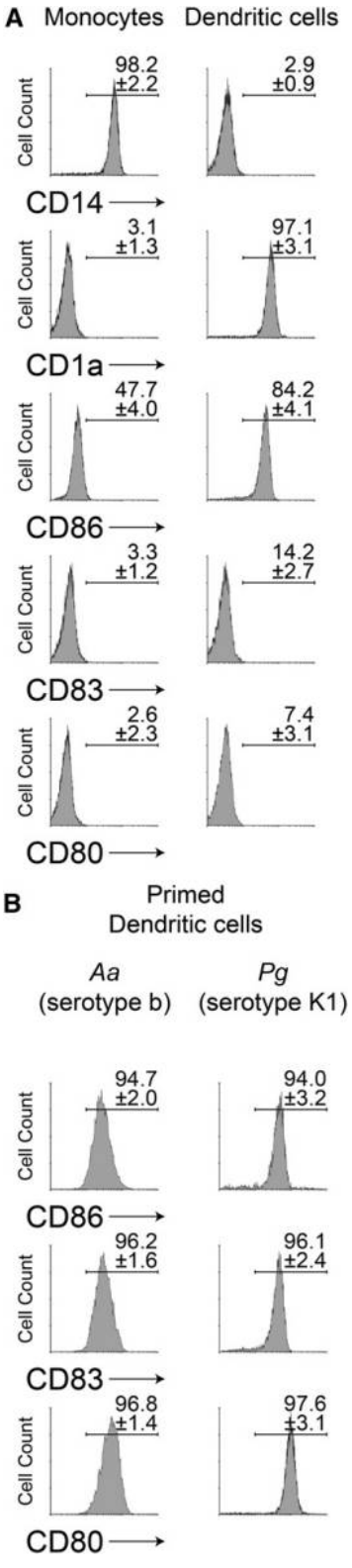


Figure 1.
Monocyte purification and DC differentiation and maturation. ***A)*** *Purity of monocytes (CD14) isolated from PBMCs and efficiency of monocyte differentiation toward DCs (CD1a).* ***B)*** *DC maturation (CD80, CD83, and CD86) after stimulation with serotype b of* A. actinomycetemcomitans *(Aa) or K1 of* P. gingivalis *(Pg).*

Secretion of Cytokines

To associate the expression of TLR2 and TLR4 with DC cytokine production, the secreted levels of IL-1β, IL-10, IL-12, and IL-23 were quantified. After cell stimulation for 2 days, DC culture supernatants were collected, and the secretion of cytokines was measured by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's protocols†††† and using an automatic microplate spectrophotometer.‡‡‡‡

Statistical Analyses

Flow cytometry data were analyzed and represented as histograms§§§§ and expressed as the percentage of positive cells over the total. The quantitative RT-PCR data were analyzed,|||||| and the relative quantification was obtained using the $2^{-\Delta\Delta Ct}$ method and by normalizing the mRNA expression to 18S rRNA. Data were expressed as mean ± SD and statistically analyzed.¶¶¶¶ The normality of data distribution was determined using the Kolmogorov-Smirnov test. Differences regarding CD and TLR expression levels analyzed by flow cytometry were determined using the χ^2 test. Differences among groups and within each group regarding TLR, cytokine, and CCR mRNA expression as well as cytokine secretion were determined using the analysis of variance and Tukey post hoc tests. To ascertain whether there was an association among TLR2 and TLR4 expression and DC function after exposure to the different bacterial serotypes, correlation coefficients were obtained using the Pearson test. Statistical significance was considered when $P < 0.05$.

RESULTS

Monocyte Purification and DC Differentiation and Maturation

Similar to previous experiments,[17,18,20] monocytes ($CD14^+$ cells) were purified at a high efficiency (>98%) from PBMCs and then differentiated at a high frequency (>97%) into DCs ($CD1a^+$ cells) (Fig. 1A). The number of isolated PBMCs ranged from 5×10^8 to 1×10^9 cells for each independent experiment, and the number of purified monocytes and differentiated DCs upon culture in the presence of rhGM-CSF and rhIL-4 ranged from 5×10^7 to 1×10^8 cells. The efficiency of DC maturation upon stimulation at an MOI of 10^2 with the strain ATCC 43718 of *A. actinomycetemcomitans* (serotype b) or the strain W83 of *P. gingivalis* (serotype K1) was analyzed by combined staining with anti-CD86, anti-CD83, and

†††† Quantikine, R&D Systems.
‡‡‡‡ Bio-Tek.
§§§§ WinMDi 2.9, The Scripps Research Institute, La Jolla, CA.
|||||| StepOne Software, v.2.2.2, Applied Biosystems, Thermo Fisher Scientific.
¶¶¶¶ SPSS, v.15.0, IBM, Charlotte, NC.

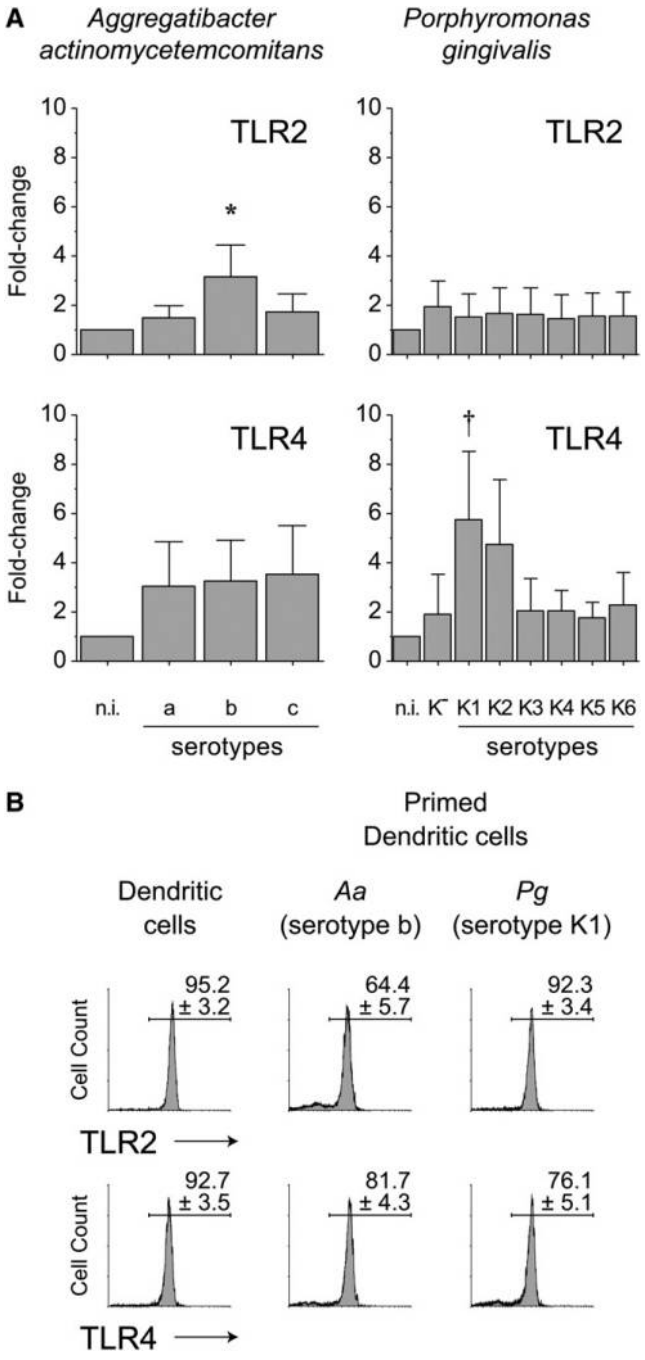


Figure 2.
TLR2 and TLR4 expression. ***A)*** *TLR2 and TLR4 mRNA expression in DCs stimulated with serotypes a, b, and c of* A. actinomycetemcomitans *or K1 through K6 of* P. gingivalis. *For relative expression, the TLR expression in non-induced DCs was considered as 1, as a reference for fold-change in expression (n.i.).* ***** *P <0.05* A. actinomycetemcomitans *serotype b compared to serotypes a and c.* † *P <0.01* P. gingivalis *serotype K1 compared to serotypes K3 through K6 and strain K⁻.* ***B)*** *TLR2 and TLR4 expression in differentiated and matured DCs after stimulation with serotype b of* A. actinomycetemcomitans *(Aa) or K1 of* P. gingivalis *(Pg).*

anti-CD80 mAbs (Fig. 1B). No differences were detected in the maturation levels of DCs among these bacterial serotypes, as demonstrated by the increased expression of CD86, CD83, and CD80 antigens (>94%, >96%, and >97%, respectively), demonstrating that serotype b of *A. actinomycetemcomitans* and serotype K1 of *P. gingivalis* induce DC maturation to a similar extent.

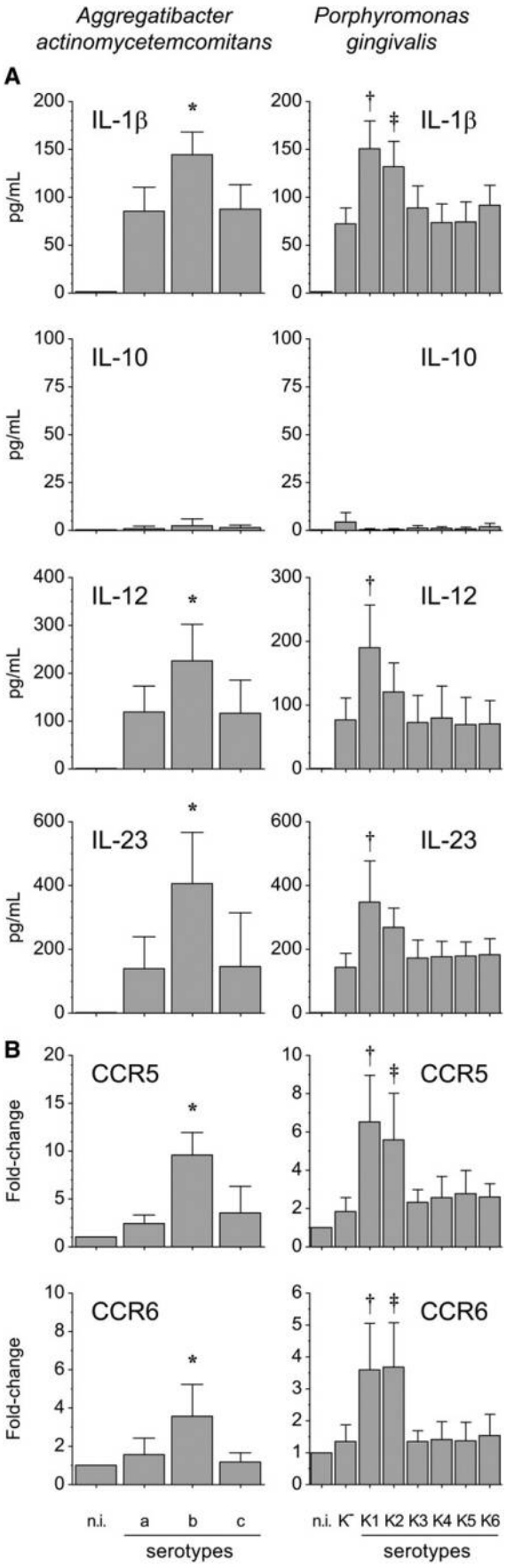
TLR Expression

mRNA expression for TLR2 and TLR4 determined by quantitative real-time PCR was represented as fold-change for each condition (Fig. 2A). An increment in the expression levels for TLR2 and TLR4 mRNAs was shown when DCs were stimulated with the different serotypes of *A. actinomycetemcomitans* compared to non-induced cells. When strain ATCC 43718 (serotype b) was used for DC stimulation, higher expressed levels of TLR2 mRNA were detected compared to using strains ATCC 43717 (serotype a, $P = 0.013$) or ATCC 43719 (serotype c, $P = 0.034$). The levels of TLR4 mRNA, however, were no different when the same DCs were stimulated with *A. actinomycetemcomitans* serotypes a, b, or c. When *P. gingivalis* was used to stimulate DCs, an increment in the expressed levels for TLR4 mRNA was detected in cells primed with the different bacterial serotypes in comparison to non-induced DCs. When strain W83 (serotype K1) was used for DC stimulation, higher expressed levels of TLR4 mRNA were detected in comparison to the same cells stimulated with strains ATCC 33277 (strain K⁻, $P = 0.009$), A7A1-28 (serotype K3, $P = 0.014$), ATCC 49417 (serotype K4, $P = 0.014$), HG1690 (serotype K5, $P = 0.006$), or HG1691 (serotype K6, $P = 0.026$). No differences were detected in the expressed levels for TLR4 mRNA with *P. gingivalis* serotypes K1 and K2. Levels of TLR2 mRNA, however, were no different when the same DCs were stimulated with different serotypes of *P. gingivalis* in comparison to non-induced controls.

The expression of TLR2 and TLR4 was also determined on the surface of the differentiated and stimulated DCs using flow cytometry (Fig. 2B). Differentiated DCs express TLR2 and TLR4 at a high frequency (>95% and >92%, respectively). When DCs were stimulated with serotype b of *A. actinomycetemcomitans* or serotype K1 of *P. gingivalis*, a non-significant decrement in the expression of TLR2 or TLR4 was detected.

Cytokine Secretion

The secretion levels of the cytokines determined by ELISA were expressed as picograms per milliliter for each condition (Fig. 3A). Higher levels of IL-1β, IL-12, and IL-23 were measured when DCs were stimulated with serotype b of *A. actinomycetemcomitans*



in comparison to serotypes a or c. No differences were detected between serotypes a and c. Similarly, higher levels of IL-1β, IL-12, and IL-23 were measured when DCs were stimulated with serotype K1 of *P. gingivalis* in comparison to the same cells stimulated with the other serotypes. When DCs were stimulated with serotype K2 of *P. gingivalis*, higher secreted levels for IL-1β were detected compared to serotypes K3 through K6. No differences in cytokine secretion levels were detected when serotypes K3 through K6 or strain K^- of *P. gingivalis* were used to stimulate the DCs. When IL-10 was measured, no differences were detected when DCs were stimulated with the different serotypes of *A. actinomycetemcomitans* or *P. gingivalis*.

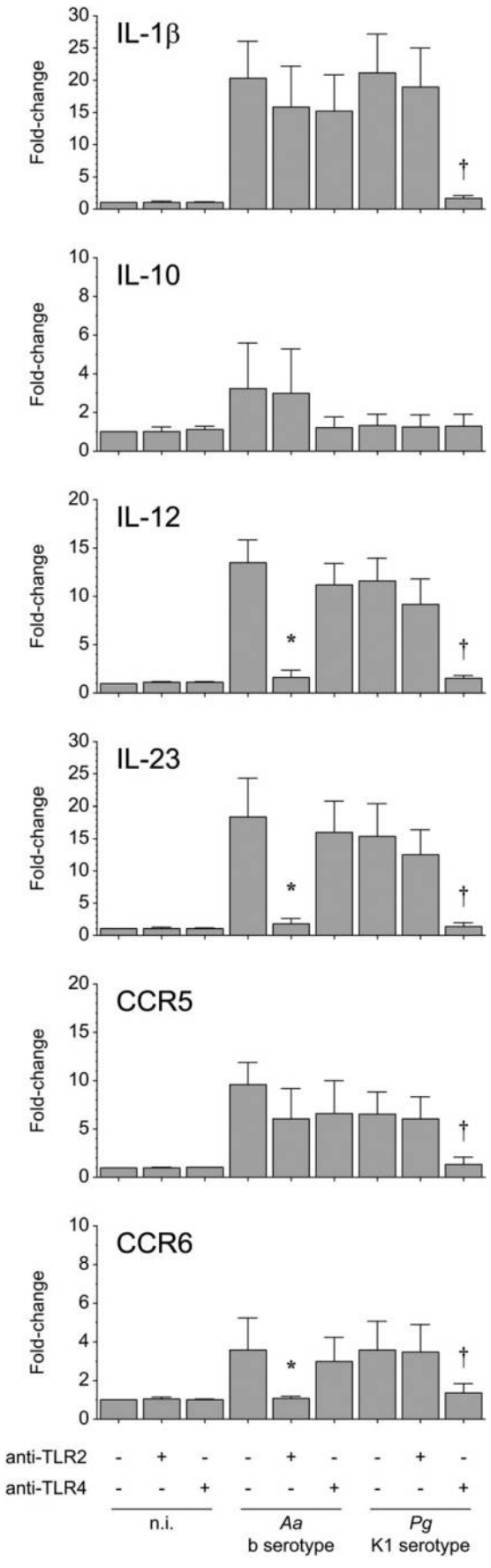
CCR Expression

mRNA expression patterns for CCR5 and CCR6 determined by quantitative real-time PCR were represented as fold-change for each condition (Fig. 3B). Higher expressed levels of CCR5 and CCR6 were measured when DCs were stimulated with serotype b of *A. actinomycetemcomitans* in comparison to serotypes a or c. No differences were detected when comparing serotypes a and c. Similarly, serotypes K1 and K2 of *P. gingivalis* induced a higher expression of CCR5 and CCR6 compared to serotypes K3 through K6. No differences in CCR expression levels were detected when serotypes K3 through K6 or strain K^- of *P. gingivalis* were used to stimulate the DCs.

Inhibition of Cytokine and CCR Expression

mAbs against TLR2 or TLR4 inhibited the cytokine and CCR mRNA expression induced by serotype b of *A. actinomycetemcomitans* or serotype K1 of *P. gingivalis* (Fig. 4). Treatment of DCs with anti-TLR2 blocking antibody significantly inhibited the IL-12 (P <0.001), IL-23 (P <0.001), and CCR6 (P = 0.005) mRNA expression induced by serotype b of *A. actinomycetemcomitans*, whereas treatment with anti-TLR4 significantly inhibited the IL-1β (P<0.001), IL-12 (P<0.001), IL-23 (P<0.001), CCR5 (P=0.005),

Figure 3.
Cytokine secretion and CCR expression. ***A)*** *IL-1β, IL-10, IL-12, and IL-23 secretion in DCs stimulated with serotypes a, b, and c of* A. actinomycetemcomitans *or K1 through K6 of* P. gingivalis. *Secreted cytokine levels by non-induced DCs were used as control (n.i.).* ***B)*** *CCR5 and CCR6 mRNA expression in DCs stimulated under the same conditions described in A. For relative expression, the CCR expression in non-induced DCs was considered as 1, as a reference for fold-change in expression (n.i.).* ***** P <*0.05* A. actinomycetemcomitans *serotype b compared to serotypes a and c.* † P <*0.02 and* ‡ P <*0.05* P. gingivalis *serotypes K1 and K2, respectively, compared to serotypes K3 through K6 and strain* K^-.



and CCR6 (P <0.018) mRNA expression induced by serotype K1 of *P. gingivalis*.

Correlation Among TLRs and Cytokines or CCRs in A. actinomycetemcomitans*–Primed DCs*

Figures 5A and 5B depict the correlation analysis between TLR2 and TLR4 expression, respectively, and DC function after stimulation with different serotypes of *A. actinomycetemcomitans*. A positive correlation was observed among the mRNA levels of TLR2 and IL-1β, IL-12, or IL-23 when using serotype a, among the mRNA levels of TLR2 and IL-23 or CCR6 when using serotype b, and among the mRNA levels of TLR2 and IL-1β or IL-12 when using serotype c. Similarly, a positive correlation was detected among the mRNA levels of TLR4 and IL-1β, IL-12, IL-23, CCR5, or CCR6 when using serotypes a or c. When the DCs were stimulated with serotype b, however, the positive correlation was detected only among the mRNA levels of TLR4 and IL-1β, IL-12, or CCR5.

Correlation Among TLRs and Cytokines or CCRs in P. gingivalis*–Primed DCs*

Figures 6A and 6B depict the correlation analysis between TLR2 and TLR4 expression, respectively, and the DC function after stimulation with the different serotypes of *P. gingivalis*. There was no positive correlation among the mRNA levels of TLR2 and either IL-1β, IL-12, or IL-23 secretion as well as CCR5 or CCR6 expression in any experimental condition. The correlation analysis, however, resulted in a positive correlation among the mRNA levels of TLR4 and IL-1β, IL-12, IL-23, CCR5, or CCR6 when DCs were stimulated with the different serotypes of *P. gingivalis*.

DISCUSSION

The innate immune system plays an essential role in the host's first line of defense against bacterial challenge by recognizing distinct molecular patterns from different pathogens through pattern recognition receptors, such as the TLRs. Activation of these receptors triggers different cell signaling pathways that results in the production of pro-inflammatory cytokines and CCRs, which actively intervene in the antimicrobial defense mechanisms mediated through the inflammatory response and the development of

Figure 4.
Inhibition of cytokine and CCR expression. IL-1β, IL-10, IL-12, IL-23, CCR5, and CCR6 expression in DCs stimulated with serotype b of A. actinomycetemcomitans *(Aa) or K1 of* P. gingivalis *(Pg) in the presence (+) or absence (–) of anti-TLR2 or anti-TLR4 blocking antibodies. For relative expression, the cytokine and CCR expression in non-induced DCs was considered as 1, as a reference for fold-change in expression (n.i.).* * P <*0.005* A. actinomycetemcomitans *serotype b stimulation with anti-TLR2 versus without anti-TLR2.* † P <*0.005* P. gingivalis *serotype K1 stimulation with anti-TLR4 versus without anti-TLR4.*

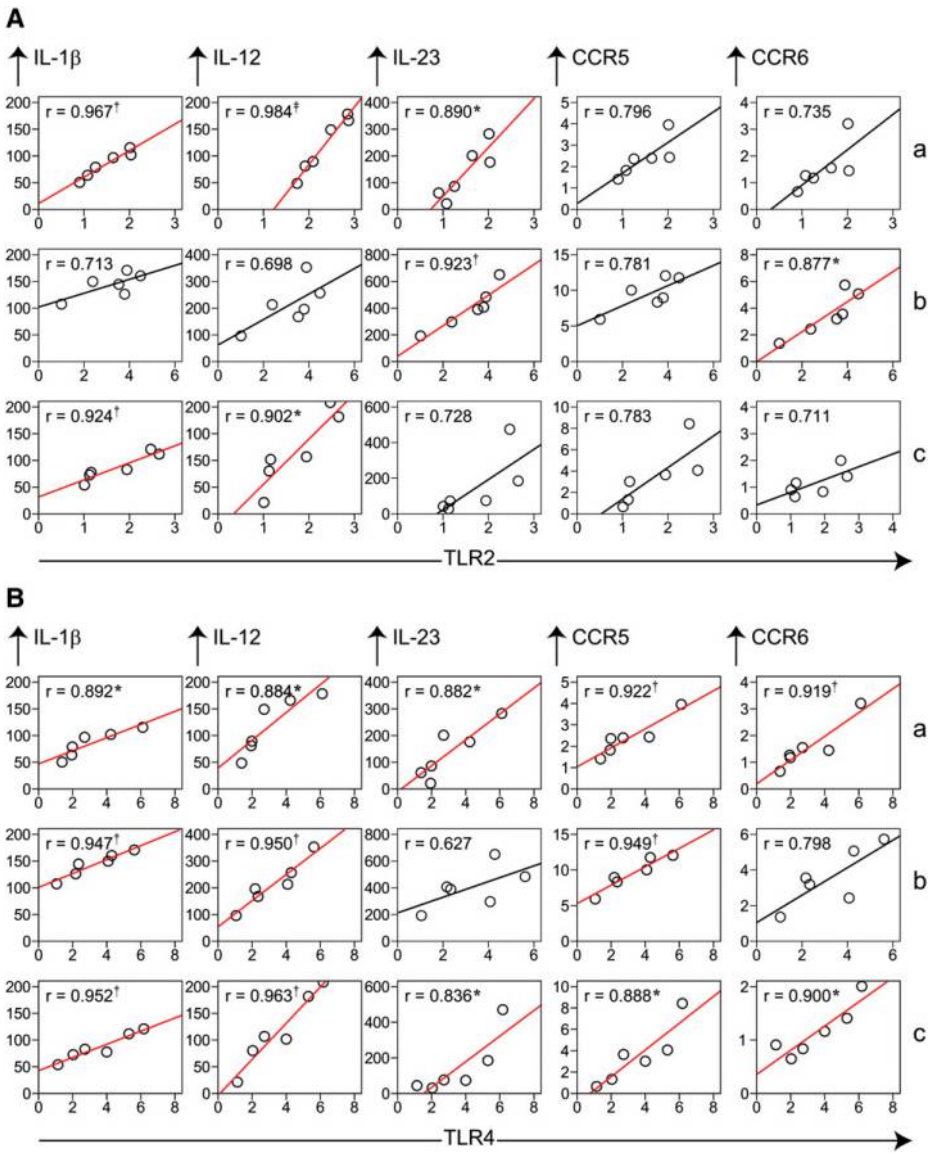


Figure 5.

Correlation among TLRs and cytokines or CCRs in A. actinomycetemcomitans*–primed DCs.* ***A)*** *Correlation among the expressed mRNA levels of TLR2 and the secreted levels of IL-1β, IL-12, and IL-23 or the expressed mRNA levels of CCR5 and CCR6 in DCs stimulated with serotypes a, b, and c of* A. actinomycetemcomitans. * P <0.05, † P <0.01, ‡ P <0.001. ***B)*** *Correlation among the expressed mRNA levels of TLR4 and the secreted levels of IL-1β, IL-12, and IL-23 or the expressed mRNA levels of CCR5 and CCR6 in DCs stimulated at the same conditions described in Fig. 5A.* * P <0.05, † P <0.01.

appropriate pathogen-specific adaptive immune response.[4,5]

In periodontitis, an adaptive immune response dominated by T helper 1 (Th1) and Th17 has been demonstrated in the periodontal lesions where connective tissue breakdown and alveolar bone resorption are occurring. This response is characterized by the production of Th1- and Th17-related cytokines, as well as the expression of CCRs that mediate in pro-osteoclastogenic leukocyte chemoattraction and activation.[19,21-26]

The periodontopathogens *A. actinomycetemcomitans* and *P. gingivalis* have been shown to have specific virulent factors to induce these Th1- and Th17-dependent destructive pathways.[27,28] Furthermore, different serotypes of both *A. actinomycetemcomitans* and *P. gingivalis* have shown a differential potential to induce DC and T lymphocyte responses, particularly serotype b of *A. actinomycetemcomitans* and serotypes K1 or K2 of *P. gingivalis*, which showed a higher capacity to trigger Th1- and Th17-related cytokine production.[17,18,20,29,30] In this investigation, increased production of IL-1β, IL-12, and CCR5, associated with a Th1-type of response, as well as increased production of IL-23 and CCR6, associated with a Th17-type of response, were detected in DCs when stimulated with serotype b of *A. actinomycetemcomitans* or serotypes K1 or K2 of *P. gingivalis*, compared to the other serotypes.

Serotype b of *A. actinomycetemcomitans* has demonstrated a higher immuno-stimulatory potential in different immune and non-immune cells compared to the other serotypes.[20,31-36] In this study, *A. actinomycetemcomitans* induced overexpression of TLR2 and TLR4 mRNAs in DCs, with higher TLR2 expression levels when cells were stimulated with serotype b in comparison to the other serotypes. The increased levels of TLR4 mRNA after stimulation by serotype b positively correlated with the increased production of the Th1-type factors, IL-1β, IL-12, and CCR5. In addition, the increased levels of TLR2 mRNA after stimulation by serotype b positively correlated with the increment in the production of the Th17-type factors, IL-23 and CCR6. On the other hand, when TLR2 signaling was blocked before bacterial stimulation, a significant inhibition in the expression levels for IL-12, IL-23, and CCR6 mRNAs was detected in serotype b–induced DCs. Hence, the higher immunostimulatory potential

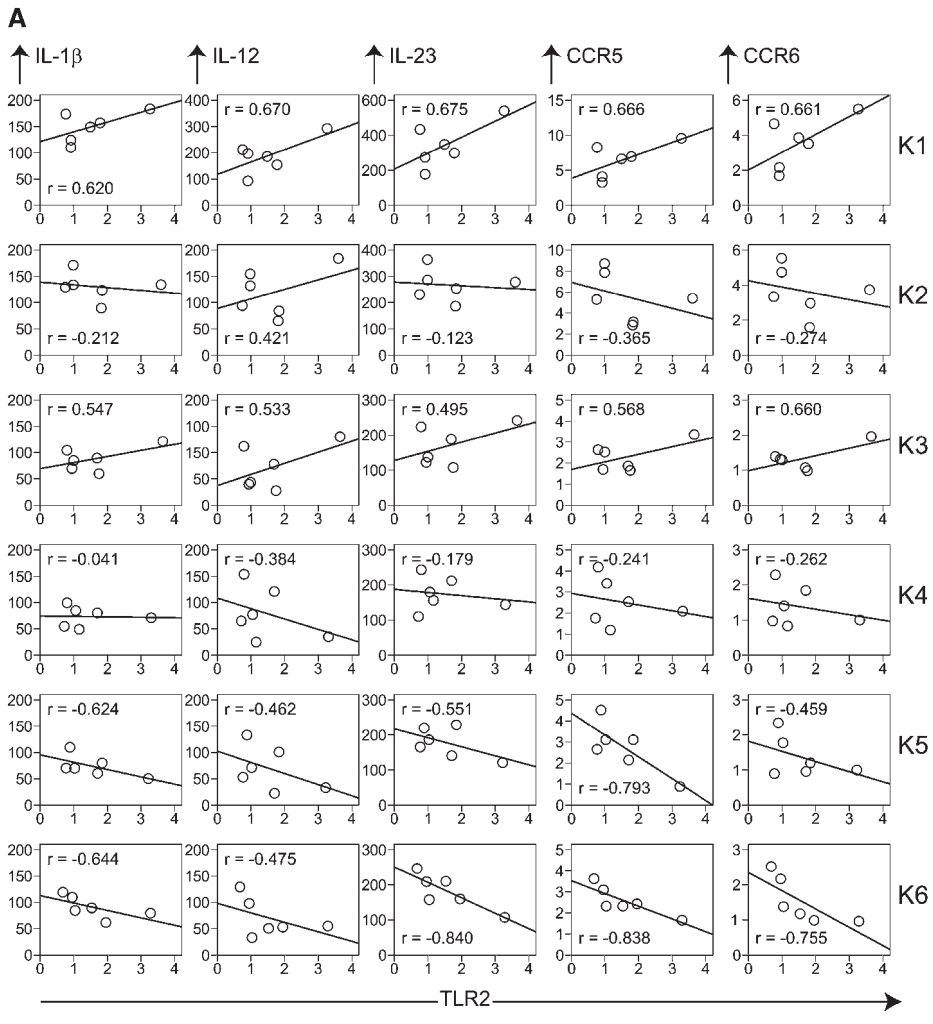


Figure 6.
Correlation among TLRs and cytokines or CCRs in P. gingivalis*–primed DCs.* ***A)*** *Correlation among the expressed mRNA levels of TLR2 and the secreted levels of IL-1β, IL-12, and IL-23 or the expressed mRNA levels of CCR5 and CCR6 in DCs stimulated with serotypes K1 through K6 of* P. gingivalis. ***B)*** *Correlation among the expressed mRNA levels of TLR4 and the secreted levels of IL-1β, IL-12, and IL-23 or the expressed mRNA levels of CCR5 and CCR6 in DCs stimulated at the same conditions described in Fig. 6A.* ****** *P <0.05,* † *P <0.01.*

of serotype b of *A. actinomycetemcomitans* demonstrated in this investigation by the increased levels of the pro-osteoclastic IL-23 and CCR6 could be explained by a TLR2-dependent DC stimulation.

Previous studies have shown that the phagocytic capacity of human monocytes against *A. actinomycetemcomitans* serotype b (strain Y4) is TLR2 dependent, and TLR2 signaling also mediates the induction of cell apoptosis.[9] Similarly, in TLR2$^{-/-}$ mice infected with *A. actinomycetemcomitans* serotype b (strain JP2), macrophages and neutrophils showed lower phagocytic and migratory activity compared to wild-type mice, and this decreased migratory capacity was associated with lower production of IL-1β and CCL5, which are potent pro-inflammatory cell chemoattractants.[37] These results are consistent with the present findings that have shown that the increased Th17-type response of DCs primed with serotype b of *A. actinomycetemcomitans* was mainly TLR2 dependent and therefore, the increased TLR2 signaling triggered by *A. actinomycetemcomitans* may play an important role in the inflammatory and destructive events characteristic of destructive periodontitis.

Similarly, serotype K1 of *P. gingivalis* has shown a higher immunogenic capacity on murine macrophages, DCs, and T lymphocytes and increased resistance to phagocytosis and killing by human neutrophils.[17,29,30,38,39] The current investigation has shown that the increased Th1- and Th17-type responses of DCs primed with serotype K1 of *P. gingivalis* were mainly TLR4 dependent and hence, the TLR4 signaling triggered by *P. gingivalis* may have a role in the pathogenesis of destructive periodontitis.

Previous studies have suggested that TLR2 signaling is important in the development of inflammatory and antimicrobial mechanisms aimed at the elimination of *P. gingivalis* infections, but also results in osteoclast differentiation and bone resorption.[40,41] In fact, TLR2 expression, but not that of TLR4, was shown to be upregulated in human gingival fibroblasts after *P. gingivalis* infection.[11] In contrast, in the present study, only the overexpression of TLR4 was demonstrated when DCs were stimulated with different serotypes of *P. gingivalis*. These differences could be explained by the different strains of *P. gingivalis* used in the studies. When the *P. gingivalis* strain ATCC 33277 (K^{-}) was used, because it is devoid of extracellular capsule, it induced lower Th1- and Th17-related cytokine production, osteoclast activation, and the described TLR2 overexpression in fibroblasts, in comparison to the encapsulated strains of *P. gingivalis* used in this study.[17,29,30]

The response to LPS from *P. gingivalis* has been described as heterogeneous because it has demonstrated both affinity and antagonism for TLR2 and

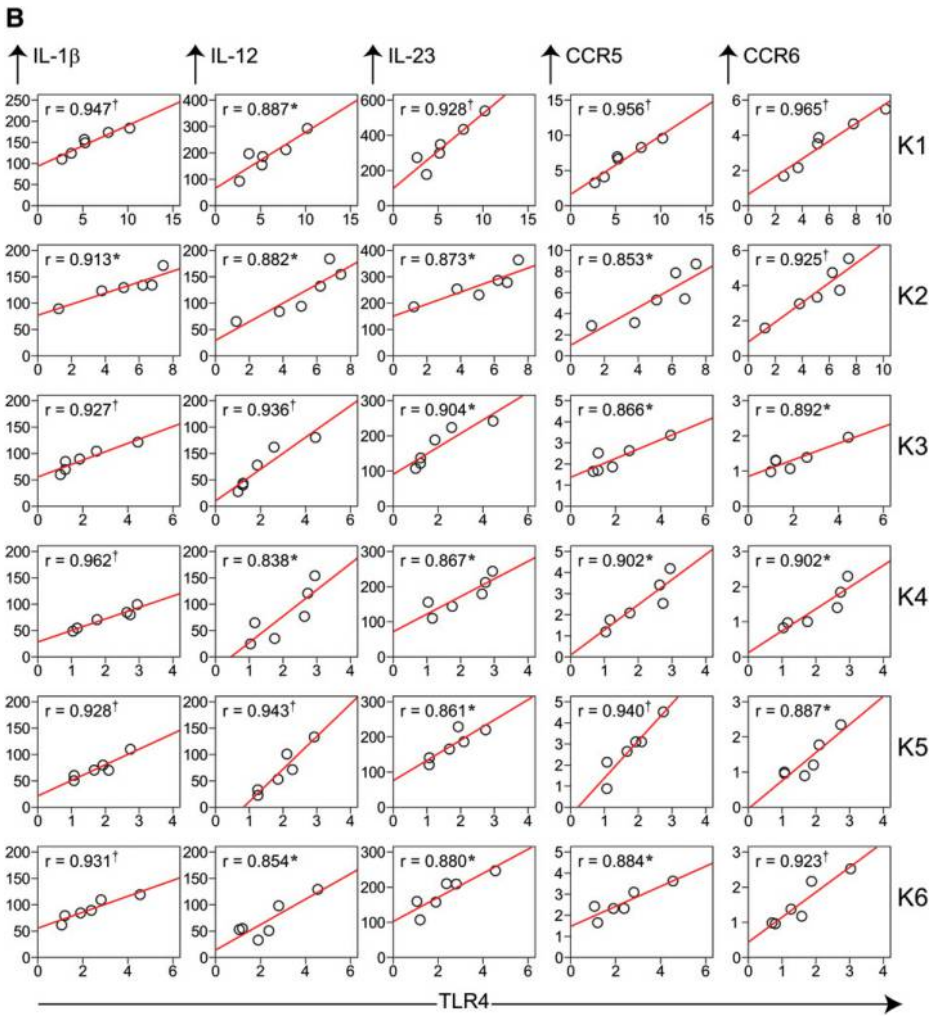


Figure 6.
(continued)

TLR4.[10,42,43] Conversely, fimbriae from *P. gingivalis* have demonstrated induction of DC and monocyte function via TLR2 signaling. In fact, mAbs against TLR2 inhibit the chemokine production by fimbriae-stimulated DCs; however, antibodies against TLR4 do not have the same effect.[44] In addition, TLR2 signaling was associated with TNF-α secretion in monocytes stimulated with fimA fimbriae purified from the *P. gingivalis* strain ATCC 33277 (K^-),[45] and this TLR2 and TNF-α-dependent mechanism has been implied in *P. gingivalis*–induced inflammatory bone loss in vivo.[46] In this study, DCs were stimulated with live *P. gingivalis* obtained from the exponential growth phase, which have their whole antigenic potentiality, including not only LPS and fimbriae, but also all the immune-stimulatory antigens implicated in the activation of DCs and in TLR4-dependent cytokine and CCR production.

TLR4 forms a homodimer that recognizes LPS from Gram-negative bacteria that in turn leads to activation of Th1 and Th17 pro-inflammatory cytokine production and periodontal breakdown.[42,43,47] This investigation has confirmed that TLR4 is involved in the production of cytokines and CCRs when DCs are stimulated with *A. actinomycetemcomitans* or *P. gingivalis*.

TLR2 is able to recognize the most diverse set of pathogen-associated molecular patterns from Gram-negative bacteria and, therefore, can mediate immune responses against these pathogens.[48,49] With *P. gingivalis*, one cannot completely discard TLR2 signaling after DC stimulation since the current authors analyzed the mRNA expression levels for TLR2 after bacterial induction, and it has been proposed that TLR2 forms heterodimers with TLR1 and TLR6; these heterodimers could pre-exist and not be induced by the ligands. In fact, upon stimulation by specific ligands, TLR2/TLR1 and TLR2/TLR6 heterodimers could be recruited in a ligand-non-induced way.[50]

LPS consists of a hydrophobic domain known as lipid A, a core oligosaccharide region, and a distal *O*-antigen presenting a chain of *O*-polysaccharide repeating units. When DCs are exposed to bacterial antigens, TLR4 responds against lipid A, whereas TLR2 responds against *O*-polysaccharide.[51] Similarities in the structure of lipid A among the different serotypes of *A. actinomycetemcomitans* could explain the similar overexpression of TLR4 shown in this investigation. The *O*-polysaccharide from the LPS produced by serotype b of *A. actinomycetemcomitans*, however, is structurally distinct from the *O*-polysaccharides from other serotypes. In fact, the *O*-polysaccharide from serotype b consists of a repeating trisaccharide unit composed of α-D-fucose, α-L-rhamnose, and β-D-*N*-acetyl-galactosamine residues and the *O*-polysaccharide from serotypes a and c is composed of 6-deoxy-α-D-talose and 6-deoxy-α-L-talose.[52,53] These structural differences may help to explain the variability in the overexpression of TLR2 and the concomitant differences in the Th1- and Th17-type

immune responses generated in DCs stimulated with the different serotypes of *A. actinomycetemcomitans*.

CONCLUSIONS

Taken altogether, these data lead us to propose that the variability of the Th1- and Th17-type of immune response generated in DCs stimulated with different serotypes of *A. actinomycetemcomitans* or *P. gingivalis* is TLR2 or TLR4 dependent, respectively. In DCs, serotype b of *A. actinomycetemcomitans* induces higher expression levels for TLR2 and serotype K1 of *P. gingivalis* induces higher expression levels for TLR4 compared to the other serotypes, and these increased levels are associated with the production of Th1- and Th17-related cytokines and CCRs. Functional studies are necessary to confirm this hypothesis, and a more comprehensive understanding of the regulatory mechanisms involved in TLR2 and TLR4 signaling is needed.

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