Oral Microbiology Immunology 2007: 22: 145–151
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ORAL MICROBIOLOGY
AND IMMUNOLOGY

Activation of toll-like receptors 2 and 4 by gram-negative periodontal bacteria

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Kikkert R, Laine ML, Aarden LA, van Winkelhoff AJ. Activation of toll-like receptors 2 and 4 by gram-negative periodontal bacteria.
Oral Microbiol Immunol 2007: 22: 145–151. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard.

Background/aims: Periodontitis is a chronic infectious disease associated with a gram-negative subgingival microflora. Bacterial components stimulate, among other receptors, Toll-like receptor (TLR) 2 and/or TLR4. Accumulating evidence indicates that both qualitatively and quantitatively distinct immune responses result from the triggering of TLR2 as compared to TLR4 triggering. The aim was to study the interaction of *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Tannerella forsythensis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Fusobacterium nucleatum* and *Veillonella parvula* with TLR2 and TLR4. We investigated all known serotypes (K^-, K1–K6) of *P. gingivalis* and *A. actinomycetemcomitans* serotype a–e strains for their potency to stimulate cytokine production.
Methods: Human embryonic kidney (HEK) cells, stably transfected with CD14, CD14-TLR2, or CD14-TLR4 and whole blood were stimulated with bacterial sonicates. Cytokine production (interleukin-6, -8, -10 and -12) was measured in the supernatant by enzyme-linked immunosorbent assay.
Results: All test bacteria stimulated HEK-CD14-TLR2, but only *A. actinomycetemcomitans* and *V. parvula* stimulated HEK-CD14-TLR4. No differences were found in the activation of HEK-CD14-TLR2/4, or cytokine production in whole blood between serotypes of *P. gingivalis* and *A. actinomycetemcomitans*.
Conclusion: Gram-negative periodontal bacteria predominantly stimulated TLR2, which may be of importance for the Th1/Th2 cell orientation of the immune response in periodontitis.

Key words: cytokine; periodontal bacteria; Th1/Th2; Toll-like receptor 2/4; whole blood

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Accepted for publication August 1, 2006

Toll-like receptors (TLRs) are germ-line-encoded pattern recognition receptors expressed on cells of the innate immune system that recognize structural components conserved among classes of microorganisms, also called pathogen-associated molecular patterns. Recognition of pathogen-associated molecular patterns by TLRs initiates signal transduction pathways culminating in the generation of inflammatory cytokines and the expression of B7 costimulatory molecules as well as major histocompatibility complex class II, which instruct the development of antigen-specific acquired immunity (15). At present, 11 TLRs have been discovered in mammals, each of which recognizes a particular pathogen-associated molecular pattern (37), thereby enabling the host to mount a pathogen-specific immune response.

Two members of the TLR family, TLR2 and TLR4, have been identified as the principal signalling receptors for bacterial cell wall components. Both TLR2 and TLR4 are expressed on dendritic cells, the only antigen-presenting cells that are capable of priming naive T cells, including the polarization of naive T cells toward a T helper type 1 (Th1) or Th2 response (22). Engagement of TLRs on dendritic cells by pathogens plays a major role in skewing responses toward Th1 or Th2 (34) and accumulating evidence exists that stimulation of TLR4 leads to the production of Th1-associated interleukin-12 (IL-12), whereas TLR2 triggering promotes Th2 development (14, 33, 34, 38).

Currently, the consensus is that adult periodontitis, a chronic inflammatory disease characterized by deepened periodontal pockets, bleeding after pocket probing, suppuration and alveolar bone and

tooth loss, is initiated by bacteria in dental plaque (16). Clear evidence has been obtained that the gram-negative bacteria *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia*, *Fusobacterium nucleatum* and *Tannerella forsythensis* are major pathogens in adult periodontitis (8, 18, 41). Cell wall components of these periodontal bacteria stimulate, via TLR2 and TLR4, the production of proinflammatory cytokines from the host, such as interleukin-1β and tumour necrosis factor, which induce alveolar bone resorption and the production of matrix metalloproteases (7). In addition, cytokines are produced that may favour the development of a Th1- or Th2-biased adaptive immune response. Several studies have demonstrated that monocytes from periodontitis patients produce cytokines associated with a Th2 immune response (4, 6). Therefore, the hypothesis that progressive periodontal lesions are associated with a Th2-biased immune response (5) has been widely accepted.

Encapsulated bacteria can resist phagocytosis and their polysaccharide capsules are important virulence factors for bacteria (25). Six polysaccharide capsular serotypes (K1–K6) have been described in *P. gingivalis* (19, 40) and subsequent clinical and animal studies have indicated that encapsulated *P. gingivalis* (K1–K6) strains are more virulent than unencapsulated (K^-) strains (20, 21).

The chemical structure of *P. gingivalis* lipid A, the bioactive moiety of lipopolysaccharide, differs remarkably from lipid A produced by enterobacteria such as *Escherichia coli* (27). In addition, *P. gingivalis* lipid A has been shown to induce mitogenic responses in B and T cells isolated from lipopolysaccharide-non-responsive C3H/HeJ mice (30), which possess a point mutation in the TLR4 gene that prevents signalling by enterobacterial lipid A (32). Hirschfeld and coworkers (13, 14) demonstrated that the purification of lipid A is liable to contamination with lipoproteins or other lipid A species. Repurification by phenol re-extraction of lipid A indicated that *E. coli* lipid A signals through TLR4 (13) but *P. gingivalis* lipid A signals through TLR2 (14). Other reports have also unequivocally demonstrated that *P. gingivalis* lipopolysaccharide stimulated TLR2 rather than TLR4 (24, 33, 34, 38). In spite of these numerous data, reports of the activation of TLR4 by *P. gingivalis* lipid A (2, 11, 28) maintain the controversy as to which TLR is involved in *P. gingivalis* signalling.

The purpose of the present study was to determine whether TLR2 and/or TLR4 was involved in the signalling of gram-negative periodontal bacteria such as *P. gingivalis*, *A. actinomycetemcomitans*, *T. forsythensis*, *P. intermedia*, *Prevotella nigrescens*, *F. nucleatum*, and *Veillonella parvula*. To avoid problems of endotoxin contamination during the purification of bacterial substances, we tested sonicates of bacteria for their capability to induce IL-8 production in TLR-transfected human embryonic kidney (HEK) cells. In addition, we sought to determine whether different serotypes of *P. gingivalis* (serotype K^-, K1–K6) and *A. actinomycetemcomitans* (serotypes a–e) have different potency to stimulate cytokine production in TLR-transfected HEK cells and whole blood.

Materials and methods

Bacterial strains and stimuli

Bacterial strains *P. gingivalis* 381 (K^-), W83 (K1), HG184 (K2), A7A1–28 (K3), ATCC 49417 (K4), HG1690 (K5), HG1691 (K6), *T. forsythensis* 92.582 ($prtH^-$) and 92.795 ($prtH^+$), *P. intermedia* HG110, *P. nigrescens* HG65, *F. nucleatum* #313 and *V. parvula* HG318 were grown anaerobically (80% N_2, 10% H_2, 10% CO_2) at 37°C on 5% horse blood agar plates (Oxoid no. 2, Basingstoke, UK) enriched with haemin (5 mg/l) and menadione (1 mg/l) for 4–7 days. *A. actinomycetemcomitans* strains HG569 (serotype a), HG90 (serotype b), HG683 (serotype c), 3381 (serotype d) and HG1650 (serotype e) were grown in a CO_2 incubator (5% CO_2 in air) at 37°C on 5% horse blood agar plates enriched with haemin (5 mg/l) and menadione (1 mg/l) for 3 days. For one experiment, *P. gingivalis* K^- and K1 were also grown anaerobically (80% N_2, 10% H_2, 10% CO_2) at 37°C in brain–heart infusion broth enriched with haemin (5 mg/l) and menadione (1 mg/l) for 2 days. *E. coli* HG101 was grown aerobically at 37°C in brain–heart infusion broth for 4 h.

Bacteria on agar plates were harvested with a sterile cotton-tipped swap immersed in sterile phosphate-buffered saline. All bacteria were washed three times in phosphate-buffered saline, and resuspended in phosphate-buffered saline at a concentration with optical density at 690 nm of 1, corresponding to approximately 2×10^8 colony-forming units/ml. Bacteria were disrupted using a sonicator (Soniprep MSE 150, amplitude 18, 2 min, 5-s intervals) in a sonicating vessel. The vessel was kept on ice during the process. The degree of disruption of the bacteria was assessed by phase-contrast microscopy and with Gram-stain by light microscopy. More than 95% of bacteria were required to be disrupted. Sonicates were stored at 4°C until use. Before use, sonicates were centrifuged (17,900 ***g***, 10 min at 4°C). *Staphylococcus aureus* Cowan I strain cells (SAC, Pansorbin) were obtained from Calbiochem, La Jolla, CA. Lipooligosaccharide (LOS) derived from *Neisseria meningitides* was a generous gift from Dr J. Poolman, RIVM, Bilthoven, The Netherlands.

Cells and cell cultures

HEK 293 cells stably transfected with CD14, CD14-TLR2 or CD14-TLR4 were a kind gift from Drs D. Golenbock and E. Latz, Worcester, MA and have been described elsewhere (39). Transfected HEK cells were cultured in Iscove's modified Dulbecco's medium (BioWhittaker, Verviers, Belgium) supplemented with 5% heat-inactivated fetal calf serum (FCS, Bodinco, Alkmaar, The Netherlands), 100 U/ml penicillin/100 μg/ml streptomycin (Gibco, Merelbeke, Belgium), 50 μM 2-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany) and 5 μg/ml puromycin (Sigma-Aldrich). For stimulation experiments cells were seeded at 5×10^5 cells/well in 96-well flat-bottom microtitre plates (Nunc, Roskilde, Denmark) and stimulated the next day. HEK-CD14-TLR4 cells were stimulated in the presence of an additional 5–10% human serum. After 16–20 h of stimulation, supernatants were harvested for determination of IL-8 production.

Blood samples were collected from eight healthy volunteers using endotoxin-free evacuated blood collection tubes (Greiner, Alphen a/d Rijn, The Netherlands) containing sodium heparin. For whole blood cultures, venous blood was diluted 1/10 with Iscove's modified Dulbecco's medium supplemented with 0.1% heat-inactivated fetal calf serum, 15 U/ml heparin (Leo Pharmaceutical products, Weesp, The Netherlands), 100 U/ml penicillin/100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol. After 16–20 h of stimulation of whole blood cultures with *P. gingivalis* (K^-, K1–K6) and *A. actinomycetemcomitans* (serotypes a–e), supernatants were harvested for the determination of cytokine production.

Both HEK cells and diluted whole blood were cultured in 200-μl wells at 37°C in the presence of 5% CO_2, in a humidified incubator.

Cytokine measurements

IL-6, IL-8, IL-10 and/or IL-12p40 levels were determined using enzyme-linked immunosorbent assay (ELISA) kits (PeliKine-compact, CLB, Amsterdam, The Netherlands), according to the manufacturer's instructions. The plates were read in an ELISA-reader (Labsystems Multiskan Multisoft, Helsinki, Finland) at 450 nm, with 540 nm as a reference.

Statistical analysis

Differences in cytokine production were tested using a one-way analysis of variance test. Differences were considered significant when $P < 0.05$.

Results

Serum requirement and specificity of HEK-CD14-TLR2 and HEK-CD14-TLR4 cells

We observed that IL-8 production following stimulation of HEK-CD14-TLR4 cells with LOS was markedly increased in the presence of human serum (Fig. 1A). In contrast, HEK-CD14-TLR2 cells did not require serum for optimal activation. IL-8 production following stimulation of these cells with the TLR2 agonist SAC in the absence or presence of serum did not differ appreciably (Fig. 1B). Therefore, stimulation of HEK-CD14-TLR4 cells was performed in the presence of 5% or 10% human serum. Specificity of CD14-TLR-transfected HEK cells was checked with LOS and SAC. LOS stimulated IL-8 production in HEK-CD14-TLR4, but not in HEK-CD14-TLR2 (see Fig. 1C), whereas SAC stimulated HEK-CD14-TLR2, but not HEK-CD14-TLR4 cells (see Fig. 1D). Neither LOS nor SAC activated HEK-CD14 cells (Fig. 1C, D).

Stimulation of HEK-CD14-TLR cells with periodontal gram-negative bacteria

E. coli K12 sonicates stimulated both HEK-CD14-TLR2 and HEK-CD14-TLR4 (Fig. 2A). However, unencapsulated *P. gingivalis* (K^-, Fig. 2B) as well as encapsulated *P. gingivalis* strains (K1–K6, Fig. 2C: K1) only stimulated HEK-CD14-TLR2. By contrast, all *A. actinomycetemcomitans* strains tested (serotype a–e) stimulated both HEK-CD14-TLR2 and HEK-CD14-TLR4 (Fig. 2D: serotype a). Two strains of *T. forsythensis*, 92.582 $prtH^+$ (Fig. 2E) and 92.795 $prtH^-$ (data not shown) exclusively stimulated HEK-CD14-TLR2. Also *F. nucleatum* (Fig. 2F), *P. nigrescens* (Fig. 2H) and *P. intermedia* (Fig. 2I) failed to stimulate HEK-CD14-TLR4, and only stimulated HEK-CD14-TLR2. Finally, *V. parvula*, only weakly stimulated both HEK-CD14-TLR2 and HEK-CD14-TLR4. None of the bacterial sonicates tested stimulated HEK-CD14 cells (data not shown).

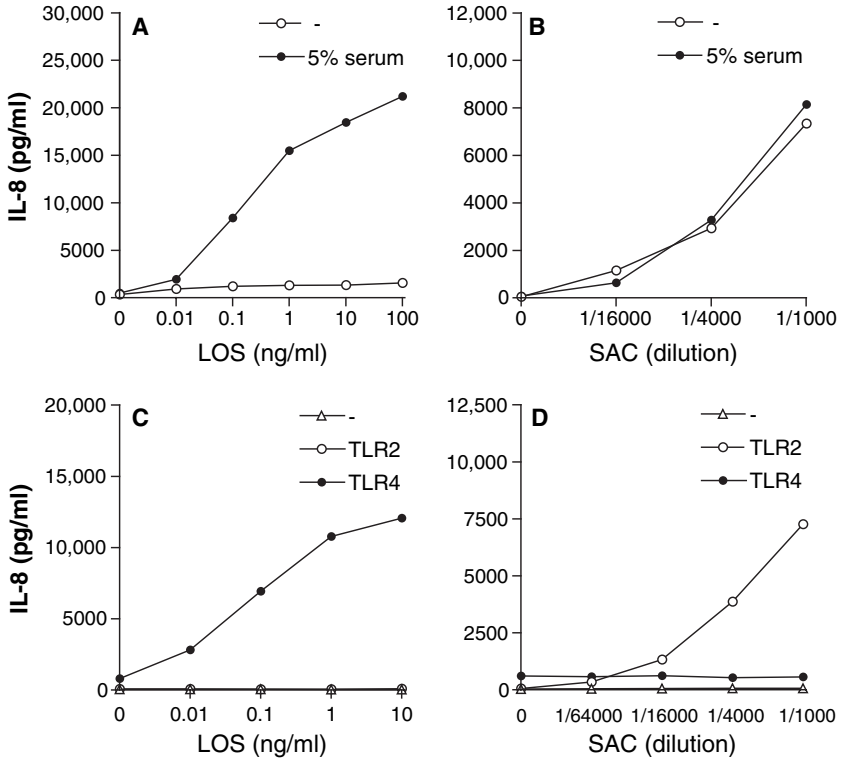

Fig. 1. Serum requirement and specificity of human embryonic kidney (HEK)-CD14-Toll-like receptor (TLR)2 and HEK-CD14-TLR4 cells. HEK-CD14, HEK-CD14-TLR2, and HEK-CD14-TLR4 cells were stimulated with LOS and SAC in the absence or presence of 5% human serum. (A) LOS stimulation of HEK-CD14-TLR4 with or w/o 5% human serum. (B) SAC stimulation of HEK-CD14-TLR2 with or w/o 5% human serum. (C) LOS stimulation of HEK-CD14, HEK-CD14-TLR2 and HEK-CD14-TLR4 cells in the presence of 5% human serum. (D) SAC stimulation of HEK-CD14, HEK-CD14-TLR2, and HEK-CD14-TLR4 cells. One representative of three experiments is shown.

Stimulation of TLRs by different *P. gingivalis* and *A. actinomycetemcomitans* serotypes

When IL-8 induction in HEK-CD14-TLR2 cells by different *P. gingivalis* strains (K^-, K1–K6) was compared, no significant differences between unencapsulated (K^-) and encapsulated (K1–K6) *P. gingivalis* strains were observed (Fig. 3A). Similarly, no differences between serotypes a–e of *A. actinomycetemcomitans* in the stimulation of HEK-CD14-TLR2 (Fig. 3B) and HEK-CD14-TLR4 (Fig. 3C) were found.

Stimulation of whole blood by *P. gingivalis*

To verify the results obtained with the TLR-transfected HEK cells, whole blood of eight healthy donors was stimulated with *P. gingivalis* strains. In accordance with the HEK-CD14-TLR2 results, production of IL-8 (Fig. 4A) and IL-6 (Fig. 4B) in whole blood after stimulation with *P. gingivalis* strains did not show significant differences between unencapsulated (K^-) and encapsulated (K1–K6) strains. Also no significant differences between unencapsulated (K^-) and encapsulated (K1–K6) strains were observed in the production of IL-10 (range 25–100 pg/ml at 1 : 25 dilution) and IL-12p40 (range 100–1200 pg/ml at 1 : 25 dilution) after stimulation (data not shown). In these experiments, stimulation of whole blood with *P. gingivalis* sonicates was characterized by a high interindividual variability in the production of cytokines, as illustrated by the IL-8 production following stimulation with K^- in eight individual donors (Fig. 4C, D). Finally, whole blood IL-8 production after stimulation with serotypes a–e of *A. actinomycetemcomitans* did not exhibit any significant differences (data not shown).

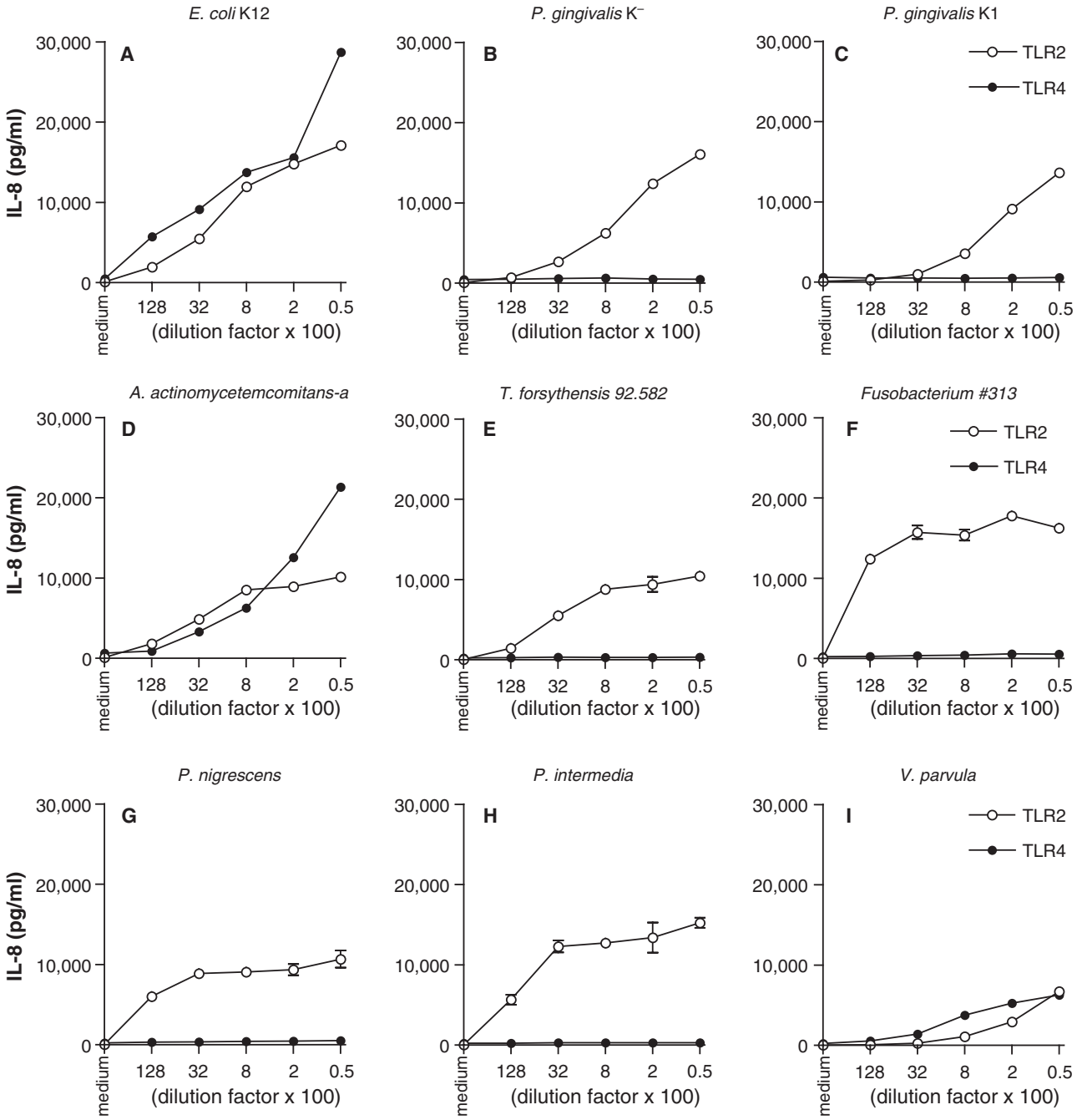

Fig. 2. Stimulation of human embryonic kidney (HEK)-CD14-Toll-like receptor (TLR)2/4 cells with sonicates of gram-negative periodontal bacteria. Induction of IL-8 production in HEK-CD14-TLR4 cells was performed in the presence of 10% human serum. Data indicate single measurements (A–D) or mean ± SEM of duplicate samples (E–I). (A) *Escherichia coli K12*, (B) *Porphyromonas gingivalis* K⁻, (C) *P. gingivalis* K1, (D) *Actinobacillus actinomycetemcomitans* strain a, (E) *Tannerella forsythensis* 92.582, (F) *Fusobacterium* #313, (G) *Prevotella nigrescens*, (H) *Prevotella intermedia*, and (I) *Veillonella parvula*.

Discussion

In the present study we demonstrated that sonicates of *P. gingivalis*, *T. forsythensis*, *P. intermedia*, *P. nigrescens* and *F. nucleatum*, exclusively stimulated TLR2, in comparison to Gram-negative enterobacteria which can stimulate both TLR2 and TLR4 (23). Only *A. actinomycetemcomitans* and *V. parvula* were capable of stimulating both TLR2 and TLR4.

IL-8 production following stimulation of HEK-CD14-TLR4 cells with LOS, a potent agonist of TLR4 (44), was markedly increased in the presence of human serum. Although lipid-binding protein and soluble CD14 play an important role in the activation of TLR4 and are present in human serum, the activation of TLR4 is dependent on the presence of the extracellular adaptor molecule MD-2 (26). Therefore, in experiments with TLR4-transfected cells, either cDNAs for both TLR4 and MD-2 are co-transfected or recombinant soluble MD-2 is supplemented (26). Indirect evidence strongly suggests that MD-2 is also present in human

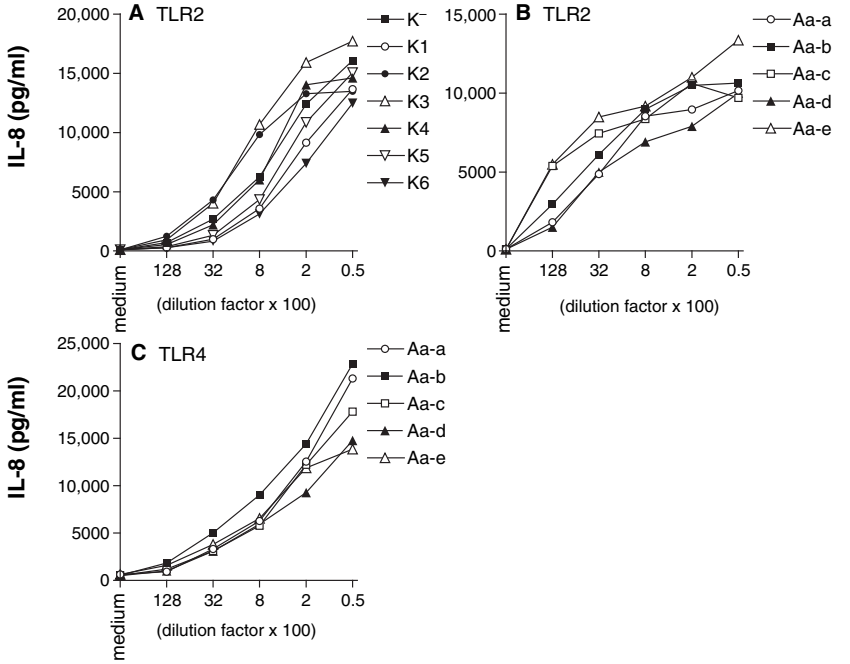

Fig. 3. Differences in virulence between strains within *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* are not correlated with Toll-like receptor (TLR)-stimulating properties. Human embryonic kidney (HEK)-CD14-TLR2 (A, B) and HEK-CD14-TLR4 (C) cells were stimulated with sonicates of *P. gingivalis* (K^-, K1–K6) strains (A), and *A. actinomycetemcomitans* serotype a–e strains (B, C). HEK-CD14-TLR4 stimulations were performed in the presence of 10% human serum. One of two similar experiments is shown.

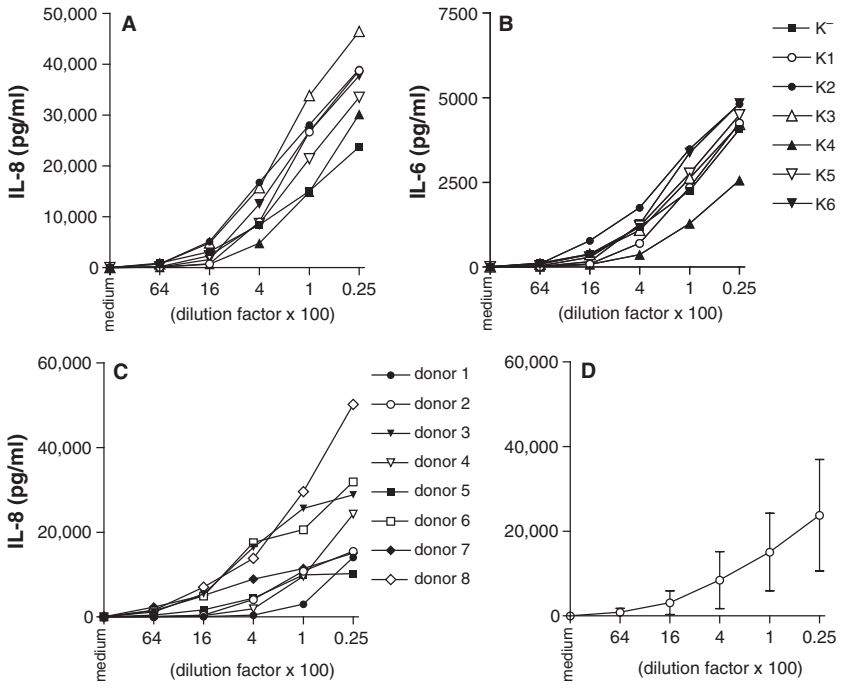

Fig. 4. Stimulation of whole blood with sonicates of *Porphyromonas gingivalis*. Whole blood of eight healthy donors was diluted 1/10 and stimulated with sonicates of unencapsulated (K^-) and encapsulated (K1–K6) *P. gingivalis* strains. (A) IL-8, (B) IL-6, (C) IL-8 production following stimulation with *P. gingivalis* K^- in whole blood of eight individual donors. (D) IL-8 production (mean ± SD) following stimulation of whole blood of eight donors with K^-.

serum (42), which might explain the potentiating effect of serum on LOS-stimulated HEK-CD14-TLR4 cells.

A large body of evidence indicates that *P. gingivalis* lipopolysaccharide stimulates TLR2, and not TLR4 (14, 24, 33, 43). In fact, it has been reported that lipopolysaccharide from *P. gingivalis* is an antagonist for human TLR4 (1, 43). Nonetheless, activation of TLR4 by *P. gingivalis* lipopolysaccharide or lipid A has also been reported (2, 11, 28). Therefore, we sought to determine whether the reported TLR4 activation was *P. gingivalis* strain-specific and whether TLR2 and/or possibly also TLR4 are involved in the recognition of unencapsulated (K^-) and encapsulated (K1–K6) *P. gingivalis* strains. Crude sonicates of washed bacteria were used to circumvent the problem of endotoxin contamination during lipid A purification.

In our study, all the *P. gingivalis* strains tested stimulated HEK-CD14-TLR2, and none stimulated HEK-CD14-TLR4 cells (24, 33, 43). It is noteworthy that in other studies which demonstrated the stimulation of TLR4 by *P. gingivalis*, lipid A was used that was obtained after multiple isolation steps (2, 11, 28). One of the possible explanations for this discrepancy might be that the *P. gingivalis* lipid A used in these experiments was contaminated with other, TLR4-stimulating lipid A species during the elaborate lipid A isolation procedure. Darveau and co-workers (2, 35) reported that *P. gingivalis* ATCC 33277 contained multiple lipid A species that functionally interacted with both TLR2 and TLR4. However, lipid A from the same *P. gingivalis* strain stimulated only TLR2 in a previous study (14). To explain this inconsistency, Darveau and co-workers suggested that lipid A isolated from *P. gingivalis* ATCC 33277 was contaminated with trace amounts of other TLR4-activating lipid A species. In addition, it was suggested that hot phenol-extraction and acid hydrolysis during lipid A isolation procedures might have resulted in the loss of acid-labile fatty acids and/or phosphate groups from the lipid A core sugar, and thereby might have changed the TLR-stimulatory properties of lipid A (2). An alternative explanation for the stimulation of TLR4 by *P. gingivalis* lipid A was provided by Darveau and co-workers, who alleged that TLR4-stimulatory lipid A species in *P. gingivalis* resulted from ingredients in the culture broth of *P. gingivalis* (2, 35). However, also when we cultured *P. gingivalis* (K^-, K1) in culture broth instead of horse blood agar plates, no changes in TLR-stimulating properties were found, and only HEK-CD14-TLR2 was activated, and not HEK-CD14-TLR4 (data not shown). It is clear that further studies are required to elucidate the effects of both medium ingredients and lipid A isolation techniques on

the TLR-stimulatory properties of *P. gingivalis* lipid A. Indeed, preliminary data by Darveau and co-workers (35) suggested that modification of particular ingredients in culture broth affected *P. gingivalis* lipid A species. However, all *P. gingivalis* lipid A species in this study interacted with TLR4, and no explanation was provided for the fact that no TLR4 activation by *P. gingivalis* lipid A was observed by other groups (14, 24, 33, 43).

In addition to *P. gingivalis* lipid A, other components of *P. gingivalis*, such as lipoproteins (11) and fimbriae (29), have also been shown to stimulate TLR2. Apart from TLR2 stimulation, we cannot rule out the possibility that stimulation of TLR4 by *P. gingivalis* lipopolysaccharide or lipid A is masked by inhibitory proteins or lipopeptides present in the *P. gingivalis* sonicates. However, we argue that following infection with periodontal bacteria *in vivo*, the host is also exposed to multiple cell wall components, rather than purified components. Therefore, it can be assumed that *in vivo*, *P. gingivalis* will predominantly stimulate TLR2.

We additionally report here that sonicates of *T. forsythensis*, *P. intermedia*, *P. nigrescens* and *F. nucleatum* exclusively stimulated TLR2, and that *A. actinomycetemcomitans* and *V. parvula* stimulated both TLR2 and TLR4. To the best of our knowledge we are the first to describe the TLR-stimulatory properties of *P. nigrescens*, *F. nucleatum* and *V. parvula*. Lipoproteins of *T. forsythensis* ATCC 43037 (10) and of *P. intermedia* ATCC 25611 (36) have been demonstrated to stimulate TLR2, whereas others reported that highly purified lipid A from *P. intermedia* ATCC 25611 stimulated TLR4 (12). Our data, which demonstrate that *A. actinomycetemcomitans* stimulated not only TLR2 but also TLR4, are in agreement with the literature (17).

The question as to whether or not TLR4 is activated by periodontal bacteria is important. Several studies demonstrated that activation of TLR4 promoted the production of IL-12p70, interferon-γ, and other Th1-associated cytokines, which were produced in much smaller amounts, or not at all in response to TLR2 activation (14, 33, 34, 38). Thus, it can be concluded that TLR2 stimulation, in the absence of simultaneous TLR4 stimulation, favours the development of a Th2-like immune response. It has been generally accepted that Th1 cells are associated with (early) stable periodontal lesions, while Th2 cells are associated with progressive lesions (5, 6). Indeed, stimulated whole blood from periodontitis patients exhibited increased Th2-associated prostaglandin E_2 and decreased Th1-associated IL-12p70 production as compared to controls (3, 4). Our study demonstrated that all the bacteria tested, with the exception of *A. actinomycetemcomitans* and *V. parvula*, exclusively stimulated TLR2, and not TLR4. Therefore, we hypothesize that the continuous stimulation of TLR2 by periodontal bacteria plays an important role in the Th2 orientation of the immune response in chronic periodontitis. In agreement with this hypothesis, *A. actinomycetemcomitans*, an important pathogen in local juvenile periodontitis and aggressive periodontitis that additionally signals through TLR4, induced high levels of Th1-associated interferon-γ (17).

Different *P. gingivalis* (19) and *A. actinomycetemcomitans* (31) serotypes did not differ in their activation of HEK-TLR2/4 cells. The possibility remained that other receptors present on human monocytes and/or neutrophils, but not HEK-CD14-TLR2/4 cells, such as CD11b-CD18, are involved in cell activation by *P. gingivalis* (9) and *A. actinomycetemcomitans*. Therefore, we also stimulated whole blood with *P. gingivalis* and *A. actinomycetemcomitans* strains. However, IL-8, IL-6, IL-10 and IL-12p40 production in whole blood showed only minor differences following stimulation with *P. gingivalis* (K^-, K1–K6), which could not account for the known differences in virulence between the *P. gingivalis* strains. Also, no differences in whole blood IL-8 production were observed following activation with *A. actinomycetemcomitans* serotype a–e strains.

To summarize, in the present study we demonstrated that gram-negative periodontal bacteria mainly stimulate TLR2, which may explain the predominantly Th2 oriented immune response observed in periodontitis. Finally, we demonstrated that differences in virulence between *P. gingivalis* and *A. actinomycetemcomitans* serotypes could not be attributed to differences in HEK-TLR2/4 activation or whole blood cytokine production.

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