Receptor Recognition of and Immune Intracellular Pathways for *Veillonella parvula* Lipopolysaccharide

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Veillonella parvula is a low-GC Gram-negative bacterium that is part of the normal flora of the oral, gastrointestinal and genitourinary tracts. Oral V. parvula is involved in the development of early periodontal disease as well as different types of serious infections. Present data on molecular mechanisms responsible for innate immune response against Veillonella are very scanty. The aim of this study was to investigate the Toll-like receptor (TLR) pathways responsible for V. parvula lipopolysaccharide (LPS) and to identify the intracellular pathways induced by this recognition. V. parvula LPS stimulates tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) release in human peripheral blood mononuclear cells (PBMC) in a dose-dependent manner. Pretreatment of cells with a TLR4 antagonist significantly reduced TNF-α and IL-6 production in PBMC stimulated with either Veillonella or Escherichia coli LPS. However, V. parvula LPS was 10- to 100-fold less active than E. coli LPS for cytokine induction. TNF-α, IL-1β, IL-6, and IL-10 were released in wild-type and TLR2<sup>−/−</sup>, but not TLR4<sup>−/−</sup>, mouse macrophage cultures. V. parvula LPS was able to activate the human PBMC p38 mitogen-activated protein kinase (MAPK). A specific p38 MAPK inhibitor strongly inhibited V. parvula LPS-induced TNF-α, IL-1β, IL-6, and IL-10. In conclusion, V. parvula LPS is able to induce cytokine production in both human and murine in vitro models, although it is less effective than Enterobacteriaceae LPS. V. parvula LPS-stimulated cytokine induction, as well as p38 MAPK activation, are TLR4-dependent features.
and washed twice in 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM 2-mercaptoethanol (PPB). Cells were extracted twice with phenol-water (38). Briefly the aqueous layers were combined and dialyzed against 20 liters of distilled water at 4°C. The crude LPS was lyophilized and then dissolved in distilled water and centrifuged once at 80,000 × g for 7 h at 4°C. The pellet was suspended in distilled water and re-centrifuged twice at 105,000 × g for 3 h at 4°C. The LPS was dissolved in PBS with RNase (Sigma, Chemical Co., St. Louis, MO) and DNase (Sigma) at 20 μg/ml each. After 2 h of incubation at 37°C, the solution was centrifuged at 105,000 × g at 4°C. The final pellet was dissolved in distilled water, lyophilized, and stored at −20°C as once-purified LPS. Double-step-purified LPS (repurified LPS) was obtained following the method described by Hirschfeld et al. (17).

Isolation of peripheral blood mononuclear cells and stimulation of cytokine production. Isolation of PBMC was performed as described elsewhere (31), with minor modifications. All volunteers signed an inform consent form, according to the institutional guidelines and procedures. A total of 5 × 10^6 PBMC in a 100-μl volume were added to round-bottom 96-well plates (Greiner) and incubated with either 100 μl of culture medium or the various stimuli: highly purified Veillonella LPS (1 to 10 ng/ml), highly purified E. coli LPS (1 ng/ml; Sigma), or Pam3Cys (10 μg/ml; EM Microcollections). In some experimental sets, cells were pretreated with or without double-extracted Bartonella quintana LPS (1 μg/ml), a TLR4 antagonist (31), 30 min before treatment with V. parvula LPS (100 ng/ml).

Specific signal transduction inhibitors of p38 MAPK (SB202190; 25 μM), ERK1/2 (U0126; 25 μM), JNK1/2/3 kinase (SP600125; 25 μM) (all inhibitors purchased from Superarray Bioscience Corporation, Bethesda, MD) were added to the cultures for 45 min prior to addition of Veillonella LPS. All unstimulated and control samples received an equal amount of inhibitor solvent, dimethyl sulfoxide (0.1% [vol/vol]). After 24 h of culture, the supernatants were collected and stored at −20°C until assayed.

Isolation of peritoneal macrophages and stimulation of cytokine production. For isolation of mouse peritoneal macrophages and spleen cells, groups of five TLR2−/− and TLR4−/− mice (kindly provided by S. Akira, Osaka, Japan) and their control littermates were sacrificed, and resident peritoneal macrophages were harvested by injecting 4 ml of sterile phosphate-buffered saline containing 0.38% sodium citrate. After washing, the cells were resuspended in RPMI 1640 medium containing 1 mM sodium pyruvate, 2 mM l-glutamine, 100 μg/ml gentamicin, and 2% fresh mouse plasma (culture medium). Cells were cultured in 96-well microtiter plates (Greiner, Alphen, The Netherlands) at 10^5 cells per well, in a final volume of 200 μl. The cells were cultured with either control medium or highly purified Veillonella LPS (1 μg/ml), highly purified E. coli LPS (1 ng/ml), or Pam3Cys (10 ng/ml). After 24 h of incubation at 37°C, the plates were centrifuged (500 × g, 10 min), and the supernatant and cell lysate (three freeze-thaw cycles) were collected and stored at −80°C until cytokine assays were performed.

Cytokine determinations. Human tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), IL-6, and IL-10 were measured by using either specific or commercial enzyme-linked immunosorbent assay (ELISA) kits (from R&D Systems, Abingdon, United Kingdom, and the Pelikem Company from Sanquin, Amsterdam, The Netherlands). Sensitivities of both ELISAs were below 5 pg/ml (13). Murine IL-1α and TNF-α were measured with a radioimmunoassay, and murine IL-6 and IL-10 were determined using ELISA kits (R&D Systems, Abingdon, United Kingdom).

Western blot analysis of Veillonella LPS signal transduction. After the isolation of human PBMC, aliquots of the cells were placed in 3-cm sterile tissue culture plates in 1 ml of RPMI 1640 medium complemented with RPMI 1640 containing 10% fetal bovine serum, 10 mM l-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (all obtained from Invitrogen, Carlsbad, CA). The cells were immediately pretreated with or without the TLR4 antagonist B. quintana LPS (1 μg/ml) (31) added 30 min before treatment with V. parvula LPS (100 ng/ml).

At different time points the cells were collected and the proteins were extracted using a lysis buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA (pH 8), 10 mM Na2P2O7, 0.5% NP-40, 10% glycerol, 0.5 mM dithiothreitol, 10 mM NaVO3, 100 mM NaF, 100 μg/ml leupeptin, 0.5 mM phenylmethanesulfonyl fluoride. After incubation for 30 min on ice, lysates were centrifuged at 10,000 × g for 10 min at 4°C. A 30-μg portion of cell extract was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were incubated with anti-p38, anti-phospho-p38, anti-ERK, anti-phospho-ERK, anti-JNK, or anti-phospho-JNK (1:1,000; Cell Signaling), or anti-actin (1:200; Santa Cruz) antibodies and then with anti-rabbit or anti-mouse horseradish peroxidase-coupled secondary antibody (Amersham, Buckinghamshire, United Kingdom). Immune complexes were identified by using an enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom).

Statistical analysis. The data are expressed as mean ± standard errors of the means unless indicated otherwise. Differences between experimental groups were tested using the Mann-Whitney U-test, performed using GraphPad Prism 4.0 software. P values of 0.05 or less were considered significant.

RESULTS

V. parvula LPS is a weak stimulator of cytokine production in human PBMC. Following stimulation with increasing amounts of V. parvula LPS (1 to 100 ng/ml), human PBMC released TNF-α and IL-6 in a dose-related fashion. The highest concentration of V. parvula LPS used (100 ng/ml) released an amount of the above cytokines that was comparable to that produced by 1 ng/ml of the reference E. coli LPS (Fig. 1A and B). A TLR4 antagonist blocks V. parvula LPS-induced cytokine production. When human PBMC were pretreated with a TLR4 antagonist (B. quintana LPS at 1 μg/ml), the release of TNF-α and IL-6 following stimulation with V. parvula LPS (1 to 100
ng/ml), as well as with 1 ng/ml of the reference E. coli LPS, was strongly inhibited (Fig. 2A and B).

**TLR4 is the major receptor for V. parvula LPS-induced cytokine production in mice.** Resident peritoneal macrophages were harvested from TLR2−/− and TLR4−/− mice and their control littermates and stimulated in vitro with either control medium or highly purified *Veillonella* LPS (1 μg/ml), highly purified E. coli LPS (1 ng/ml), or Pam3Cys (10 ng/ml). The three stimuli used were able to produce a cytokine release substantially higher than medium alone in control macrophage cultures. Although *Veillonella* LPS caused a significant release of cytokines in the macrophages from TLR4−/− mice, it was not able to induce a relevant release of cytokines among the macrophages from TLR2−/− mice (Fig. 3A and B and 4A and B).

**Veillonella-induced activation of p38 MAPK, which mediates cytokine production.** *V. parvula* LPS was able to activate the human PBMC p38 MAPK after 60 minutes of incubation. When *B. quintana* double-purified LPS was used to inhibit TLR4 on PBMC, activation of p38 MAPK was hardly inhibited after 60 minutes (Fig. 5A).

The activation of JNK and ERK1/2 after the *V. parvula* LPS stimulus was very inconsistently observed regardless of the time point considered (15, 30, and 60 min) (data not shown). In line with these data, a p38 MAPK inhibitor strongly reduced cytokine production induced by *V. parvula* LPS (Fig. 5B and C). Similar results were observed with an inhibitor of JNK kinase, while inhibition of ERK had a more modest effect (Fig. 5B and C).

**DISCUSSION**

In the present paper we demonstrate that *V. parvula* LPS is recognized by TLR4 in both humans and mice and that the activation of MAP kinases plays an important role in *V. parvula* LPS-stimulated human PBMC signaling.
Previous studies on the in vitro and in vivo activities of the LPS from *Veillonella parvula* demonstrated that such an endotoxin has biological effects comparable to enterobacterial LPS (10, 29). Earlier studies reported on fibroblast collagenase-inducing PBMC cytokines stimulated by *Veillonella* spp. LPS; however, such cytokines were not directly evaluated (16). Formalin-killed *V. parvula* has been demonstrated to induce the release of IL-1β in human blood monocytes at levels comparable to those released by *Porphyromonas gingivalis*, *Bacteroides forsythus*, and *Prevotella intermedia* (27). More recent investigations showed that the concentrations of TNF-α, IL-6, and IL-10 released from umbilical cord and adult mononuclear cells following in vitro challenge with UV-killed *V. parvula* were comparable or even higher than cytokine concentrations evaluated in *Pseudomonas aeruginosa* and *E. coli*-stimulated cord and adult cells (19).

The lower cytokine production of PBMC induced by *Veil-
lonella LPS (approximately 20 to 30% of that induced by E. coli LPS) is mirrored in the type of pathology induced by these microorganisms. On the one hand, gram-negative sepsis with enterobacteriaceae induces a superacute inflammatory reaction, followed sometimes by organ failure and even death. On the other hand, Veillonella is present in the mouth flora and is an important pathogen of periodontal disease, a chronic inflammatory condition. Thus, the low cytokine production by Veillonella is certainly important in inducing the low-grade inflammation in periodontitis.

To the best of our knowledge, very few papers have reported on the role of TLRs for the pathogenesis of Veillonella infection. In one study treatment of dendritic cells with an anti-TLR4 antibody decreased cytokine production induced by UV-treated V. parvula (20), but the nature of the Veillonella ligand recognized by TLR4 was not identified.

Similarly, TLR2- and TLR4-transfected human embryonic kidney cells responded to sonicated Veillonella bacteria stimulation (21). In both these studies, Veillonella seemed to stimulate both TLR4 and TLR2. However, the bacterial product responsible for the TLR engagement was not addressed, and the models used were only from murine species. In the present study, we demonstrate that V. parvalve LPS is recognized by TLR4 in both human and murine cells, and we show the MAPK role in Veillonella LPS-stimulated human PBMC signaling.

Veillonella parvalve has been implicated in both dental/periodontal diseases (3, 18) and joint disorders (26). Periodontal disease and rheumatoid arthritis have remarkably similar inflammatory mediator profiles (22). Within periodontal lesions, activated monocytes, macrophages, and fibroblasts produce cytokines, such as TNF-α, IL-1β, and IL-6, which have been found to be significantly elevated in diseased periodontal sites compared with healthy or inactive sites (11). These cytokines orchestrate the cascade of destructive events that occur in the periodontal tissues and trigger the production of an array of inflammatory enzymes and mediators, resulting in irreversible hard and soft tissue damage (15). By exploiting an in vitro model of periodontal diseases, it has been found that upregulation of IL-6 by lipopolysaccharide treatment is TLR4 dependent. This pattern of gene expression indicates that pathogens may trigger TLR4 signaling and cause periodontitis (35).

Therefore, our present findings may have sound clinical impact. Moreover, due to the similarity of pathogenesis between periodontitis and rheumatoid arthritis, antagonists of TLR4, as well as p38 inhibitors, have the potential to ameliorate progression of periodontal disease (23), rheumatic pathologies, and other chronic inflammatory/degenerative disorders. The TLR4 antagonist B. quintana LPS (31) has been demonstrated to dramatically improve the evolution of experimental arthritis in the mouse (1). Further studies are warranted in order to exploit the modulation of TLR4 and p38 MAPK in the therapy of chronic inflammatory/degenerative diseases.

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