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Porphyromonas gingivalis Lipopolysaccharide Contains Multiple Lipid A Species That Functionally Interact with Both Toll-Like Receptors 2 and 4

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The innate host response to lipopolysaccharide (LPS) obtained from *Porphyromonas gingivalis* is unusual in that different studies have reported that it can be an agonist for Toll-like receptor 2 (TLR2) as well as an antagonist or agonist for TLR4. In this report it is shown that *P. gingivalis* LPS is highly heterogeneous, containing more lipid A species than previously described. In addition, purification of LPS can preferentially fractionate these lipid A species. It is shown that an LPS preparation enriched for lipid A species at *m/z* 1,435 and 1,450 activates human and mouse TLR2, TLR2 plus TLR1, and TLR4 in transiently transfected HEK 293 cells coexpressing membrane-associated CD14. The HEK cell experiments further demonstrated that cofactor MD-2 was required for functional engagement of TLR4 but not of TLR2 nor TLR2 plus TLR1. In addition, serum-soluble CD14 effectively transferred *P. gingivalis* LPS to TLR2 plus TLR1, but poorly to TLR4. Importantly, bone marrow cells obtained from TLR2$^{-/-}$ and TLR4$^{-/-}$ mice also responded to *P. gingivalis* LPS in a manor consistent with the HEK results, demonstrating that *P. gingivalis* LPS can utilize both TLR2 and TLR4. No response was observed from bone marrow cells obtained from TLR2 and TLR4 double-knockout mice, demonstrating that *P. gingivalis* LPS activation occurred exclusively through either TLR2 or TLR4. Although the biological significance of the different lipid A species found in *P. gingivalis* LPS preparations is not currently understood, it is proposed that the presence of multiple lipid A species contributes to cell activation through both TLR2 and TLR4.

A functional innate response system consists of different host components that act coordinately to recognize microbial colonization and elicit appropriate immediate responses (27, 31). Host responses to lipopolysaccharide (LPS), an essential constituent of the cell wall of gram-negative bacteria, can be very potent, such that host sampling of this molecule can contribute significantly to the overall innate response to bacterial infection (6, 60). For example, in vitro studies have confirmed that whole bacteria and their respective isolated LPSs yield similar responses (12, 56), and in vivo studies have validated the important role of LPS in triggering inflammation in response to bacterial infection (25, 30, 55).

A well-characterized innate host recognition pathway is that for *Escherichia coli* LPS, which starts with a series of initial binding and transfer reactions between LPS binding protein (LBP), CD14, and other host proteins (49, 64). CD14 is located in the cell membrane of certain host cells (mCD14) and is also present in serum (sCD14) and gingival crevicular fluid, a serum exudate (28, 45). Transfer of *E. coli* LPS by either mCD14 or sCD14 to a cell-associated Toll-like receptor 4 (TLR4) and MD-2 protein complex (15, 40) initiates host cell activation pathways, leading to innate host defense mediator production.

In the periodontium, innate host responses to microbial colonization are important in both health and disease (14, 59). In clinically healthy periodontal tissue, the highly orchestrated expression of select innate host defense mediators is believed to be associated with commensal microbial colonization (59). These mediators facilitate neutrophil transit through this tissue and into the gingival crevice, where they play a key role in the prevention of disease (24). Periodontitis is an inflammatory disease that is characterized by loss of alveolar bone supporting the tooth root and is the leading cause for tooth loss. Strong evidence for the role of the innate host response to microbial colonization in periodontitis comes from the observation that removal of the dental plaque microbial biofilm remains the most effective treatment for the disease (14). Although the microbial composition of dental plaque associated with health and periodontitis is well characterized (66), little is known about how these different compositions influence the inflammatory response.

Porphyromonas gingivalis is a gram-negative bacterium that is an important etiologic agent of human adult-type periodontitis (54). This bacterium releases copious amounts of outer membrane vesicles containing LPS (19, 20), which can penetrate periodontal tissue (38, 39, 50) and thus participate in the destructive innate host response associated with disease. The potential contribution of *P. gingivalis* LPS to the disease process is not clear, however, due to complex innate host responses to this cell wall component (4). *P. gingivalis* LPS is able to activate human monocytes by a CD14-dependent mechanism (51) and binds sCD14 (9); however, it does not facilitate

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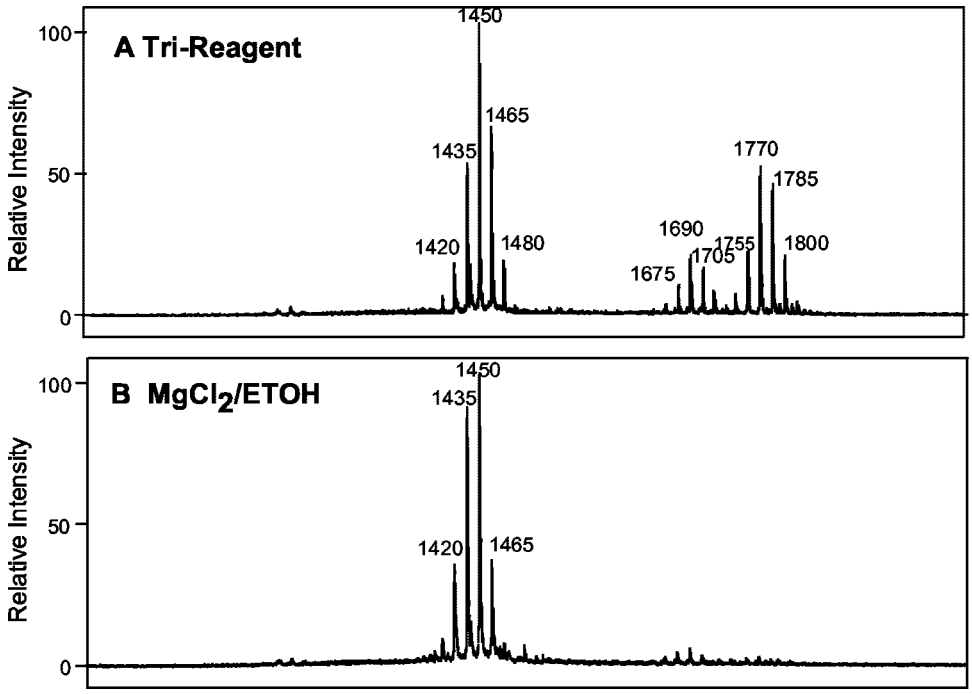

FIG. 1. Characterization of *P. gingivalis* lipid A species by negative ion mass spectrometry. *P. gingivalis* LPS was obtained by either a TRI reagent procedure (67) (A) or a cold $MgCl_2$-EtOH procedure (13) (B). Lipid A was cleaved and separated from the LPS as described by Caroff et al. (7). MALDI-TOF was performed as previously described (21). All values given are averag masses rounded to the nearest whole number for singly charged deprotonated molecules. TRI reagent-extracted LPS yielded two major clusters of lipid A mass ions centered at *m/z* 1,690 and 1,770, which were missing or significantly reduced when the $MgCl_2$-EtOH (B) procedure was used to purify the LPS.

sCD14-dependent E-selectin expression nor interleukin-8 (IL-8) secretion from human umbilical cord vein vascular endothelial cells (11). In fact, this LPS is a natural antagonist for the human endothelial E-selectin and IL-8 responses to *E. coli* LPS and other oral bacteria (11) and has recently been reported to be a TLR4 antagonist in some cell types (8, 10, 68). Furthermore, although several reports have demonstrated that this LPS utilizes TLR2 instead of TLR4 for host cell activation (5, 26, 37), it has also been reported to engage TLR4 (42, 57) to facilitate gingival fibroblast activation through mCD14 (46, 62).

In this report it is shown that a *P. gingivalis* LPS preparation enriched for two major lipid A mass ions at *m/z* 1,435 and 1,450 activates both human and mouse TLR2 and TLR2 plus TLR1, as well as TLR4 in transiently transfected HEK 293 cells coexpressing mCD14. In addition, with the use of primary bone marrow cells obtained from TLR knockout mice, it is shown that this LPS preparation exclusively utilizes either mouse TLR2 or TLR4 to induce tumor necrosis factor alpha (TNF-α) production. The ability of a *P. gingivalis* LPS preparation to activate cells through multiple TLRs helps to reconcile previous possible conflicting observations that have demonstrated that *P. gingivalis* LPS preparations can activate either TLR2 or TLR4.

MATERIALS AND METHODS

Bacterial strains and preparation. *P. gingivalis* ATCC 33277 was obtained from the American Type Culture Collection, Rockville, Md.; it was examined for purity, properly identified, and stored at −70°C. Cultures were made from frozen bacterial stocks to avoid repetitive subculture. Bacterial cells were grown for LPS isolation as follows: *P. gingivalis* was grown anaerobically at 37°C for 2 to 3 days in Trypticase soy broth (30 g/liter) containing yeast extract (1 g/liter; Difco), glucose at 1 g/liter, potassium nitrate at 0.5 g/liter, sodium lactate (Sigma L-1375) at 1 ml/liter, sodium succinate at 0.5 g/liter, and sodium fumerate at 1 g/liter; after autoclaving, filter-sterilized supplements were added (sodium carbonate, 0.4 g/liter; hemin [σ H-2250], 0.005 g/liter; cysteine, 0.4 g/liter; and vitamin K [σ M-5625], 0.001 g/liter). Stationary-phase cells were employed for LPS isolation.

Purification and characterization of LPS. *P. gingivalis* LPS was prepared by the cold $MgCl_2$-ethanol (EtOH) procedure (13) followed by lipid extraction (18) and conversion to sodium salts (43). *E. coli* 0111:B4 LPS (Sigma, St. Louis, Mo.) was subjected to a Folch extraction (18) to remove contaminating phospholipids. All LPS preparations were further treated to remove trace amounts of endotoxin protein as described by Manthey and Vogel (36) with the following modification. Following the final EtOH precipitation, LPS was lyophilized to determine the yield and was resuspended in distilled water to 1 mg/ml without the addition of triethanolamine. LPS was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained for protein by the enhanced colloidal gold procedure, as described in reference 36. The stain was able to detect 100 pg of protein, employing bovine serum albumin as a standard. The presence of nucleic acid was determined by ethidium bromide fluorescence quantification of the amount of double-stranded DNA by using the plastic wrap method (35) and ImageQuant software. The *P. gingivalis* LPS preparation shown in Fig. 1A was obtained with the use of TRI reagent as previously described (67).

GC/MS analysis of LPS fatty acids. Each LPS sample (0.25 mg [dry weight]) and fatty acid methyl ester standards C15:0, C16:OH, C17:OH (Matreya, Inc., Pleasant Gap, Pa.), and C16:0 (Sigma) were analyzed twice: once as their methyl esters in hexane with free alcohol functional groups after derivitization with methanolic HCl as described by the manufacturer (Alltech, Deerfield, Ill.), and then as their trimethylsilyl ethers after transmethylation with *N,O,*-bis(trimethylsilyl)trifluoroacetimide (BSTFA) containing 1% trimethylchlorosilane (Pierce, Rockford, Ill.). Ether formation was accomplished by the transfer of 50-μl aliquots of each sample and standard to 100-μl glass liners followed by the addition of an equal volume of BSTFA-hexane (1:1, vol/vol). The liners were then sealed in autosampler vials and allowed to stand at room temperature for 3 h prior to gas chromatographic-mass spectroscopy (GC/MS) analyses of the methylated fatty acid extracts, performed on a Finnigan Trio 1000 quadrupole

mass spectrometer (Thermo Electron Corp., San Jose, Calif.) fitted with a Hewlett-Packard 5890 series II gas chromatograph equipped with a capillary splitless injector and a Hewlett-Packard 7673A autosampler (Agilent Technologies, Palo Alto, Calif.). A fused silica capillary GC column (30 m by 0.32 mm [inner diameter], 0.25-μm film thickness) was used, coated with the bonded stationary phase (J&W DB-5; Agilent Technologies, Folsom, Calif.), and operated with helium (head pressure, 5 lb/in^2) as carrier gas with a 3-ml min^{-1} septum purge through the injector. Samples were injected in the splitless mode (injector temperature, 250°C) and cold trapped on the column at 40°C. After 1 min, the injector was purged and the column oven temperature was programmed linearly to 280°C at a rate of 10°C/min and held for 5 min. The mass spectrometer was operated in the electron ionization mode, with a filament emission current and electron energy of 150 μA and 70 eV, respectively. The ion source temperature was 200°C, and the GC interface was held at 250°C. Tuning and mass calibration were performed daily using perfluorotributylamine in the repetitive scan mode (scanning from 35 to 450 Da once every second). All data acquisition and processing, including mass spectral database searches against both the Wiley Registry of MS data (6th ed., 1996) and the NIST/EPA/NIH Mass Spectral Library (1992) were performed using Windows-based Finnigan MassLab 1.3 software. GC/MS analyses of the extracted transmethylated fatty acid samples and fatty acid methyl ester standards as well as their trimethylsilyl ether derivatives were carried out using 2-μl injection volumes.

Matrix assisted laser desorption–time-of-flight (MALDI-TOF) MS was performed as previously described (21). Two separate extractions of *P. gingivalis* LPS were produced and analyzed.

Cells and reagents. Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (GibcoBRL, Rockville, Md.) with 10% heat-inactivated fetal calf serum (HyClone, Logan, Utah). The NF-κB reporter construct (ELAM-1 firefly luciferase), the β-actin–*Renilla* luciferase reporter construct, the modified pDisplay expression vector, and the expression constructs for murine TLR2 (pmuTLR2), murine TLR1 (pmuTLR1), murine TLR4 (pmuTLR4), and human TLR4 (phuTLR4) and mCD14 (phumCD14) have been described previously (22, 23). Human TLR1 (phuTLR1) and TLR2 (phuTLR2) open reading frames were cloned into the modified pDisplay expression vector. The human and murine MD-2 plasmids were kindly provided by Kensuke Miyake (The University of Tokyo, Tokyo, Japan). CD14-depleted serum was prepared as described previously (9).

Luciferase assays. HEK 293 cells were transfected by calcium phosphate precipitation and stimulated as described previously (23) with the modifications reported for a 96-well plate assay format (22). Cells were washed twice with medium 3 h after transfection and stimulated 20 to 24 h posttransfection. Stimulations were performed in stimulation medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 10% human serum, or sCD14-depleted normal human serum) for 4 h at 37°C, using concentrations of ligands as indicated below in the text and figure legends. After stimulation, cells were rinsed with phosphate-buffered saline (BioWhittaker, Walkersville, Md.) and lysed with 50 μl of passive lysis buffer (Promega, Madison, Wis.). Reporter gene expression in each lysate (10 μl) was measured using the Dual Luciferase reporter assay system (Promega). Data are expressed as the fold increase in relative light units (which represents the ratio of ELAM-luciferase to β-actin *Renilla*-luciferase expression) relative to that of a no-stimulation control. All experiments were performed a minimum of three separate times with similar results. Results from one experiment performed with data from triplicate wells are presented.

Stimulation of primary bone marrow cells obtained from TLR knockout mice. TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were backcrossed for six generations to C57BL/6 mice and then intercrossed to obtain TLR2/4 double-knockout mice. Bone marrow cells were isolated from femurs and tibias of mice bred at the University of Washington. Following red cell lysis, 1×10^6 to 2×10^6 cells were plated per well in round-bottom 96-well plates. The cells were stimulated with concentrations of ligands as indicated below in the text and figure legends for 5 h in the presence of GolgiStop (Pharmingen). Cells were then stained for surface expression of CD11b (Pharmingen), permeabilized with Cytoperm/Cytofix (Pharmingen), and stained for intracellular TNF-α (Caltag). Cells were analyzed on a Becton Dickinson FACScan flow cytometer using CellQuest software (BD Biosciences).

RESULTS

***P. gingivalis* LPS contains multiple forms of lipid A.** Studies have reported several different structures for the lipid A obtained from *P. gingivalis* (32, 41, 67). Ogawa (41), employing strain 381, reported that *P. gingivalis* LPS contained one predominant lipid A mass ion at *m/z* 1,195, while Kumada et al., examining a clinical isolate designated SU63 (32), reported multiple lipid A mass ions, with the most predominant ones found at *m/z* 1,435 and 1,450, and Yi and Hackett (67), employing strain 33277, reported that a lipid A species at *m/z* 1,691 was the dominant structure found among multiple lipid A species. Consistent with the notion that *P. gingivalis* LPS displays lipid A heterogeneity, our investigators have reported that a purified *P. gingivalis* LPS preparation from strain 33277 contained lipid A mass ions at *m/z* 1,195, 1,435, and 1,450 (3).

In this report, the degree and extent of *P. gingivalis* LPS lipid A heterogeneity was examined with the use of a new crude LPS extraction procedure which employs commercially available TRI reagent (67). It was found that *P. gingivalis* LPS contains more major lipid A mass ions than previously described (Fig. 1A) and that the $MgCL_2$-EtOH LPS purification procedure failed to extract all of the different lipid A mass ions equally (Fig. 1B). TRI reagent extraction of LPS from *P. gingivalis* and subsequent lipid A cleavage (7) revealed numerous major lipid A mass ions that clustered around *m/z* 1,450, 1,690, and 1,770. The lipid A species found clustered around each of these mass ions differed by smaller single methylene units (*m/z* 1,420 and 1,435 adjacent to 1,450; 1,675 adjacent to 1,690; and 1,705 and 1,755 adjacent to 1,770) and larger single methylene units (*m/z* 1,465 and 1,480 adjacent to 1,450; 1,705 adjacent to 1,690; and 1,785 and 1,800 adjacent to 1,770). This pattern of different lipid A mass ions is indicative of fatty acid chain length heterogeneity, accounting for some of the different lipid A species. In contrast, LPS extracted from whole cells by the $MgCl_2$-EtOH method was significantly reduced in both clusters of lipid A mass ions centered at *m/z* 1,690 and *m/z* 1,770. This preparation, designated Pg $LPS_{1435/1450}$ (see below), revealed major lipid A mass ions at *m/z* 1,435 and 1,450 (Fig. 1B); the structures for both of these *P. gingivalis* lipid A species have been previously elucidated (32) (Fig. 2). In addition, two minor peaks at *m/z* 1,420 and 1,465 were observed and are suspected to represent structurally related lipid A mass ions that differ in their fatty acid content.

In this study, the triacylated monophosphorylated lipid A at *m/z* 1,195 was not detected in either the TRI reagent or $MgCl_2$-EtOH LPS preparations. The reasons for the lack of this lipid A mass ion, which was originally described by Ogawa (41) and previously detected by our group (3), are currently not understood. However, initial studies in our laboratory have revealed that different media compositions may influence the appearance of this lipid A mass ion (data not shown).

Characterization of the *P. gingivalis* LPS preparation employed to examine TLR utilization. The $MgCl_2$-EtOH LPS preparation was characterized with respect to possible contaminants that could interfere with the TLR utilization studies. In particular, bacterial lipoproteins that remain tightly associated with LPS during purification have been shown to utilize TLR2 (34). The *P. gingivalis* LPS preparation obtained by the cold $MgCl_2$-EtOH procedure was further treated to remove trace amounts of endotoxin protein as described by Manthey and Vogel (36). Similar to our group's previous results (3), colloidal gold staining (to detect protein) of the *P. gingivalis* LPS preparations before and after phenol extraction revealed that protein was removed from the $MgCl_2$-ETOH *P. gingivalis* LPS preparation (designated Pg0 in Fig. 3) to yield a highly purified

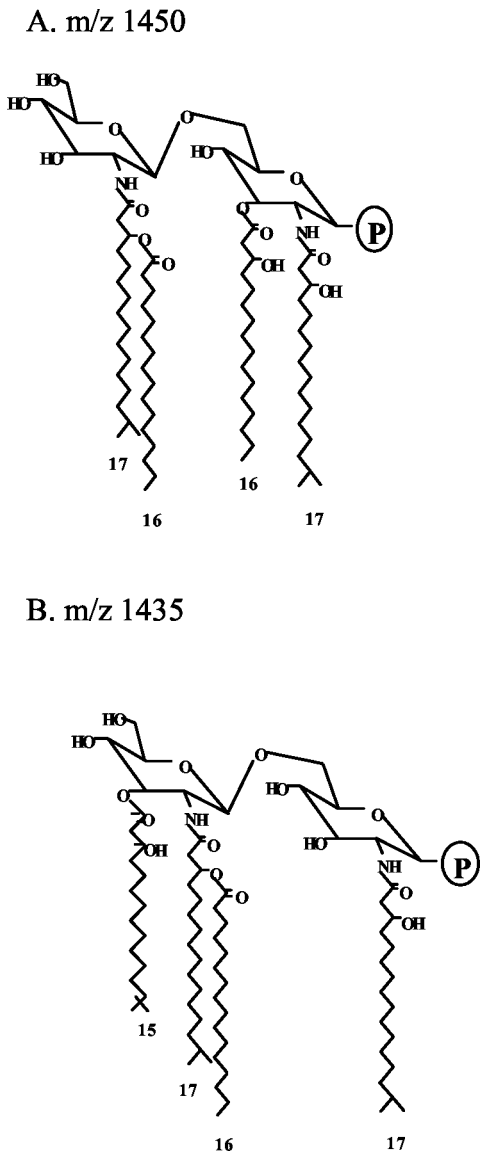

FIG. 2. Structure of *P. gingivalis* lipid A mass ions at *m/z* 1,435 and 1,450 found in purified *P. gingivalis* LPS preparations. Kumada et al. (32) have elucidated the structures of several of the major lipid A mass ions, including the mass ion at *m/z* 1,450 (A) and *m/z* 1,435 (B).

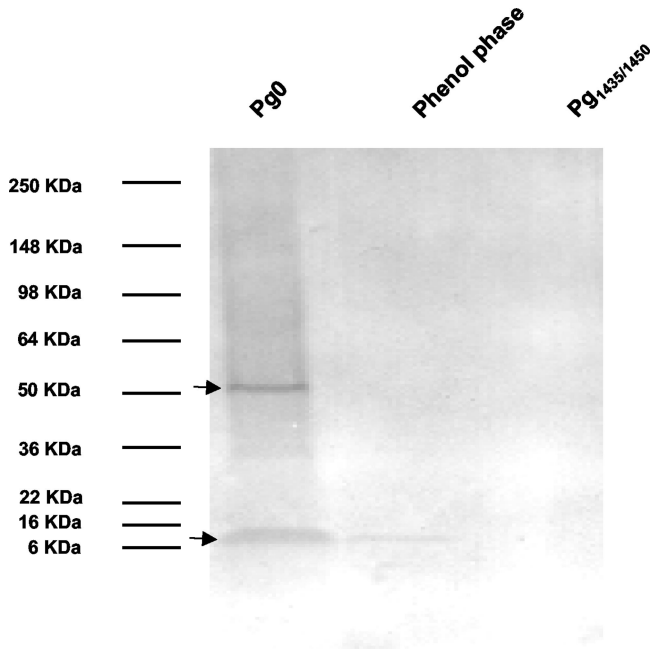

FIG. 3. Colloidal gold staining of purified *P. gingivalis* LPS preparations. *P. gingivalis* LPS was extracted with $MgCl_2$-EtOH as described in the text (this LPS preparation is designated Pg0) and further extracted with phenol (36) to remove contaminating protein (the resulting LPS preparation was designated Pg $LPS_{1435/1450}$). Pg0 (10 μg of LPS), the phenol-soluble fraction (phenol phase [10 μg, dry weight] and Pg $LPS_{1435/1450}$ [10 μg of LPS]) were subjected to SDS-PAGE and stained for protein with the colloidal gold method as described in the text. Note: two proteins found in the Pg0 preparation (indicated by arrows) were removed by the phenol extraction and were not found in the Pg $LPS_{1435/1450}$ preparation. One protein (lower-molecular-mass band) was found in the phenol phase.

Pg $LPS_{1435/1450}$ preparation (Fig. 3). Determination of the relative amount of protein contamination by comparison to known bovine serum albumin standards (based upon the amount of LPS loaded into the gel and the relative intensity of the major protein band) revealed between 0.1 and 1% protein in the *P. gingivalis* LPS preparation before extraction and less than 0.1% protein contamination in the extracted Pg $LPS_{1435/1450}$ preparation.

In addition, GC/MS of fatty acids present in the Pg $LPS_{1435/1450}$ preparation was performed. The identification of all the peaks found in the preparation after transmethylation was performed by identification of the mass spectral patterns. The fatty acids previously identified by Kumada et al. (32) and depicted in Fig. 2 were found along with trace amounts of C14:0 and C18:0. No other fatty acid peaks were detected. These data demonstrate that there was little or no phospholipid, glycolipid, or lipoprotein contamination in the Pg $LPS_{1435/1450}$ preparation. The Pg $LPS_{1435/1450}$ preparation was employed to examine TLR utilization.

***P. gingivalis* LPS enriched in lipid A mass ions of *m/z* 1,435 and 1,450 activates HEK cells through TLR2, TLR2 plus TLR1, and TLR4.** The ability of the Pg $LPS_{1435/1450}$ *P. gingivalis* preparation to activate cells through specific TLRs was examined by employing HEK cell transient transfections with different components of the TLR2 and TLR4 activation complexes. Initially, a control experiment was performed to determine if endogenous receptors on HEK cells were capable of responding to the *P. gingivalis* LPS preparation. HEK cells were cotransfected with the control plasmid (β-actin–*Renilla* luciferase) and the NF-κB-dependent reporter plasmid (ELAM-firefly luciferase), and various concentrations of Pg $LPS_{1435/1450}$ were added. This LPS preparation did not significantly induce the reporter construct, validating HEK 293 cells as a suitable cell line to examine the interactions of *P. gingivalis* LPS with different exogenously added TLRs (data not shown). Therefore, experiments described below report the ability of the Pg $LPS_{1435/1450}$ preparation to activate HEK cells above background levels and compare Pg $LPS_{1435/1450}$ activation to that of known TLR ligands.

The abilities of the Pg $LPS_{1435/1450}$ preparation and the

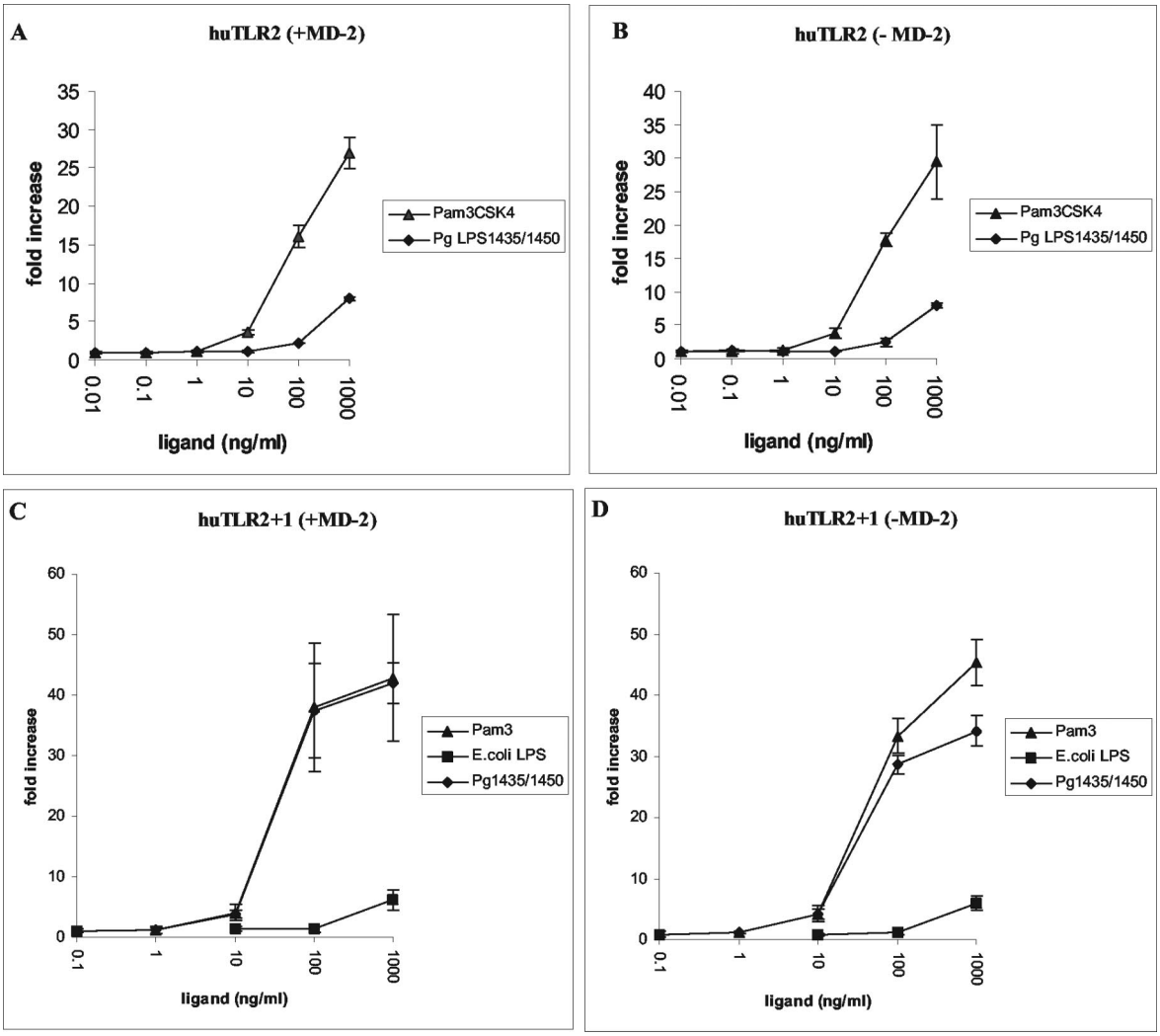

FIG. 4. TLR2 and TLR2 plus TLR1 activation with *P. gingivalis* LPS preparations. HEK 293 cells were transiently transfected with human mCD14 and either huTLR2 (A and B) or huTLR2 plus TLR1 (C and D), with (A and C) or without (B and D) huMD-2. Each transfection experiment also contained the NF-κB reporter (ELAM-1–firefly luciferase) and the transfection control (β-actin–*Renilla* luciferase). Cells were then stimulated with various concentrations of Pam3CSK4, a TLR2 agonist, and Pg $LPS1_{435/1450}$ (some cells were also stimulated with various concentrations of *E. coli* LPS [C and D]) for 4 h and lysed, and the amount of luciferase produced was determined. Values are reported as the fold increase of relative luciferase units (firefly luciferase/*Renilla* luciferase) compared to the nonstimulated control response, which was set at 1. The data presented represent the means and standard deviations from triplicate wells from one experiment and are representative of at least three separate experiments.

synthetic lipopeptide (Pam3CSK4), a known TLR2 agonist (1), to stimulate HEK 293 cells transiently transfected with human TLR2 (huTLR2) were determined (Fig. 4A and B). In these experiments, HEK cells were transfected with human mCD14 with and without MD-2, components of the innate host response known to optimize LPS interactions with TLRs (16, 52, 64). *P. gingivalis* LPS significantly ($P < 0.001$; two-sample *t* test) activated HEK cells through huTLR2 (an eightfold increase at 1 μg of LPS/ml, with or without the addition of MD-2), although it was significantly ($P < 0.001$; two-sample test) less active than Pam3CSK4 (Fig. 4A and B). Additional experiments employing murine TLR2 (muTLR2) with murine MD-2 also demonstrated significant HEK cell activation ($P < 0.001$; two-sample *t* test) that was significantly ($P < 0.001$; two-sample *t* test) less than that with Pam3CSK4 (data not shown).

The contribution of huTLR1 was examined, since a previous report demonstrated that TLR2 can form functional heterodimers with TLR1 (65). In addition, in these experiments *E. coli* LPS was also examined. It was found that the combination of huTLR2 plus TLR1 resulted in greater *P. gingivalis* LPS-dependent HEK cell activation, as evidenced by the observation that the maximal induction of the reporter construct was nearly equivalent for Pg $LPS_{1435/1450}$ and the Pam3CSK4 lipopeptide. There was no significant difference in HEK cell activation at any concentration of ligand tested when MD-2 was present (Fig. 4C) ($P < 0.001$; two-sample *t* test), and a significant difference was observed only at 1,000 ng of li-

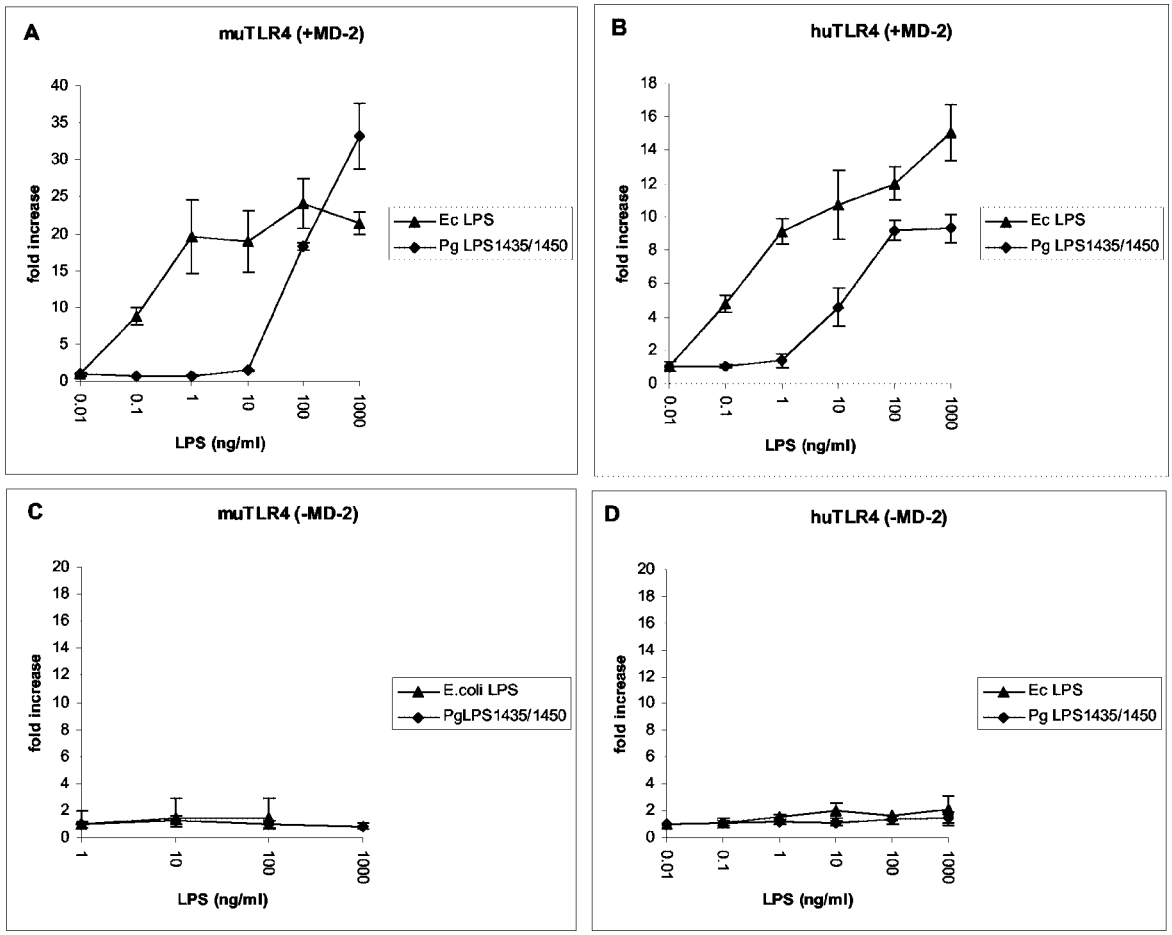

FIG. 5. TLR4 activation with *P. gingivalis* LPS preparations. HEK 293 cells were transiently transfected with mouse mCD14 and muTLR4 (A and C) or human mCD14 and huTLR4 (B and D), with and without mouse and human MD-2, respectively, as indicted in the figure, together with the NF-κB reporter (ELAM-1–firefly luciferase) and the transfection control (β-actin–*Renilla* luciferase). Various doses of *E. coli* and Pg $LPS_{1435/1450}$ were added to the cells for 4 h, the cells were lysed, and the amount of luciferase produced was determined. Values are reported as the fold increase of relative luciferase units (firefly luciferase/*Renilla* luciferase) compared to the nonstimulated control response, which was set at 1. The data presented represent the means and standard deviations from triplicate wells from one experiment and are representative of at least three separate experiments.

gand/ml ($P < 0.05$; two-sample *t* test) in the absence of exogenously added MD-2 (Fig. 4D). *E. coli* LPS slightly activated TLR2 plus TLR1 in HEK cells at 1,000 ng/ml. An analogous pattern of maximal induction of the reporter construct for Pg $LPS_{1435/1450}$ compared to that with the Pam3CSK4 lipopeptide was observed when muTLR2 plus TLR1 was employed (data not shown).

The Pg $LPS_{1435/1450}$ preparation was compared to *E. coli* LPS, a known TLR4 ligand (15, 40, 44), in its ability to stimulate HEK 293 cells transiently transfected with either muTLR4 or huTLR4 (Fig. 5). HEK cells transfected with mouse or human mCD14 and either muTLR4 and muMD-2 (Fig. 5A) or huTLR4 and huMD-2 (Fig. 5B) (either all mouse or all human components) displayed significant increases in activation in response to *E. coli* or *P. gingivalis* LPS preparations ($P < 0.001$; two-sample *t* test for concentrations of 100 and 1,000 ng of LPS/ml). However, the *P. gingivalis* LPS preparation required considerably more LPS to achieve significant activation than the *E. coli* LPS. For example, in Fig. 5A, *E. coli* LPS demonstrated significantly greater-than-fivefold HEK cell activation at 0.1 ng/ml ($P < 0.001$; two-sample *t* test). Both mouse and human TLR4-dependent HEK cell activation in response to *E. coli* or *P. gingivalis* LPS was MD-2 dependent (Fig. 5C and D), consistent with the previously reported key role of this protein in TLR4 LPS responses (40, 52). These data demonstrate that highly purified preparations of *P. gingivalis* LPS are capable of signaling via TLR2, TLR2 plus TLR1, and TLR4.

Role of sCD14 in TLR-mediated responses to *P. gingivalis* LPS. *E. coli* LPS can utilize both mCD14 and sCD14 to facilitate host cell activation (45). However, *P. gingivalis* LPS is unusual in that it is able to activate mCD14-bearing cells, such as monocytes, by a CD14-dependent mechanism (51) and, although it binds sCD14 (9), it does not facilitate sCD14-dependent E-selectin expression nor IL-8 secretion from human umbilical cord vein vascular endothelial cells (11). In the experiments described thus far, HEK cells were transfected with mCD14, since this factor is known to significantly increase cellular responses to LPS compared to its soluble form (sCD14) (40). In the next series of experiments, the ability of

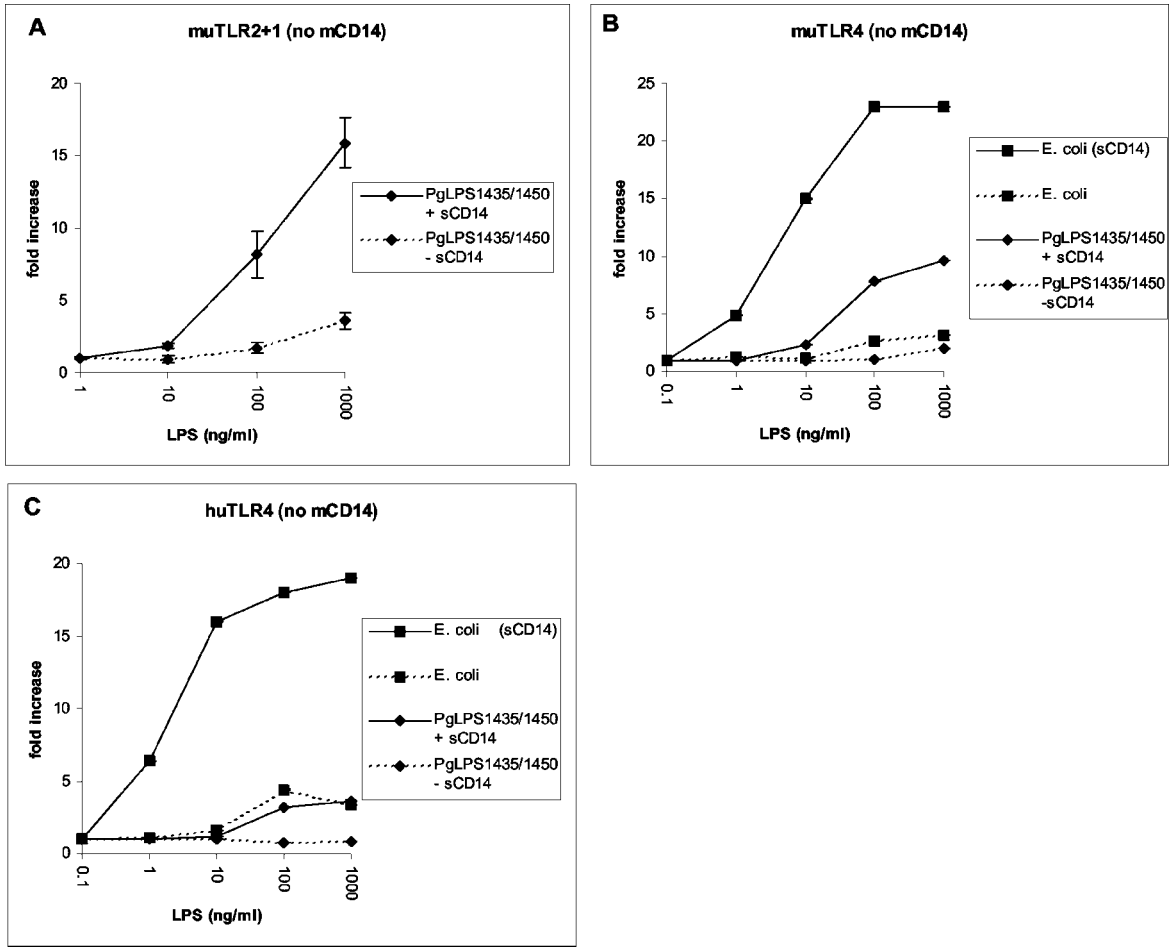

FIG. 6. Contribution of sCD14 for TLR activation by *P. gingivalis* LPS preparations. HEK 293 cells were transiently transfected with MD-2 and with muTLR2 plus TLR1 (A), muTLR4 (B), or huTLR4 (C), together with the NF-κB reporter (ELAM-1–firefly luciferase) and the transfection control (β-actin–*Renilla* luciferase). Various concentrations Pg $LPS_{1435/1450}$ and *E. coli* LPS (B and C) were added to the cells in activation buffer containing either sCD14-depleted human serum as a source for LBP (dotted lines) or FBS as a source for both LBP and sCD14 (solid lines). The HEK cells were incubated with the LPS preparations for 4 h, the cells were lysed, and the amount of luciferase produced was determined. The data presented represent the means and standard deviations from triplicate wells from one experiment and are representative of at least three separate experiments.

P. gingivalis LPS to utilize sCD14 for TLR activation was examined. Previous experiments have shown that FBS has sufficient bovine LBP and sCD14 to facilitate HEK cell activation with both murine and human TLRs (data not shown). The role of sCD14 in facilitating LPS activation of TLR-transfected HEK cells was examined by transfecting HEK cells without mCD14 and employing sCD14-depleted human serum as a source for LBP (Fig. 6).

Human serum depleted of sCD14 demonstrated significantly lower muTLR2-plus-TLR1-dependent HEK cell activation for *P. gingivalis* LPS compared to the same transfection that employed FBS during the cell activation (Fig. 6A). In addition, in other experiments (data not shown) recombinant sCD14 was added back to the sCD14-depleted serum and HEK cell activation was restored to similar levels as observed when FBS was present in the activation buffer. These experiments demonstrated that sCD14 (human and bovine) can efficiently transfer *P. gingivalis* LPS to muTLR2 plus muTLR1 to facilitate HEK cell activation. Next, the contribution of sCD14 for *P. gingivalis* LPS activation of cells through murine and human TLR4 was examined (Fig. 6B and C). When FBS was added as a source of both LBP and sCD14, the Pg $LPS_{1435/1450}$ preparation was unable to mediate effective TLR4-dependent HEK cell activation in comparison to that with *E. coli* LPS (Fig. 6B and C). This difference in HEK cell activation through TLR4 was most dramatic for huTLR4, where *P. gingivalis* LPS yielded less than fivefold activation, compared to approximately 20-fold for *E. coli* LPS (Fig. 6C). These findings are in contrast to the data obtained with mCD14 (Fig. 5), where maximal TLR4 activation was nearly equivalent for both *E. coli* and *P. gingivalis* LPS. Thus, these data reveal a relatively poor ability of *P. gingivalis* LPS to utilize human sCD14 for huTLR4-dependent HEK cell activation.

***P. gingivalis* LPS enriched in lipid A mass ions of *m/z* 1,435 and 1,450 activates primary bone marrow cells obtained from both TLR2 and TLR4 knockout mice.** The HEK transient-transfection experiments with either TLR2 or TLR4 demonstrated that the Pg $LPS_{1435/1450}$ preparation was able to engage

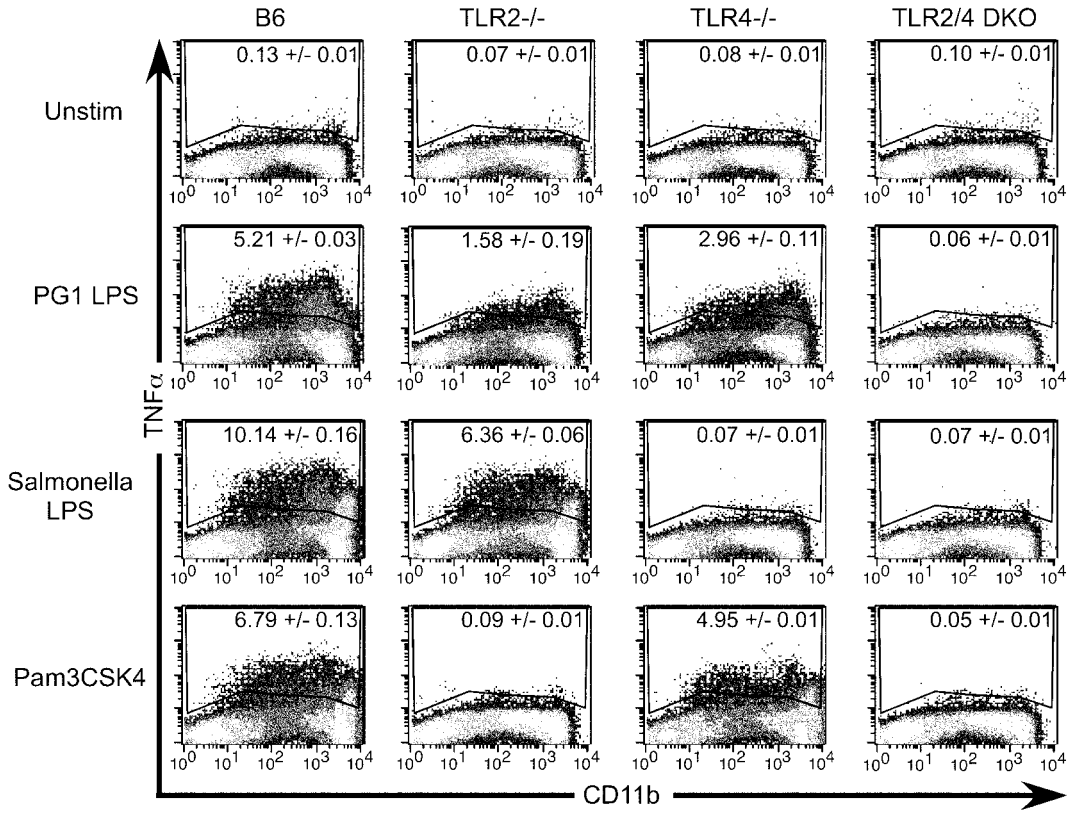

FIG. 7. Pg $LPS_{1435/1450}$ stimulates TNF-α production in primary bone marrow cells via TLR2 or TLR4. Bone marrow cells from the indicated mice were stimulated with 10 μg of Pg $LPS_{1435/1450}$/ml, 10 ng of *S. minnesota* Re595 LPS/ml, or 1 μg of Pam3CSK4/ml. The numbers in each histogram indicate the mean (± range) of the percentage of cells producing TNF-α from duplicate wells from a single mouse. The data are from one experiment, and similar results were obtained in three separate experiments.

either TLR receptor to activate the NF-κB-dependent reporter construct. The ability of this *P. gingivalis* LPS preparation to activate mouse bone marrow cells obtained from $TLR2^{-/-}$, $TLR4^{-/-}$, and $TLR2/4^{-/-}$ knockout mice was examined in order to determine if endogenous TLR2 and TLR4 were utilized. Activation was determined by intracellular TNF-α staining and fluorescence-activated cell sorter analysis (Fig. 7). It was found that bone marrow cells obtained from either $TLR2^{-/-}$ or $TLR4^{-/-}$ mice responded to the Pg $LPS_{1435/1450}$ preparation. These data are consistent with the observations made in HEK cells that demonstrated this *P. gingivalis* LPS preparation could utilize either TLR2 or TLR4 for host cell activation. Furthermore, cells obtained from TLR2/4 double-knockout mice did not respond to the *P. gingivalis* LPS preparation, demonstrating that the LPS activity observed with this cell type was mediated exclusively through TLR2 and TLR4. In contrast, bone marrow cell responses to *Salmonella minnesota* LPS (a TLR4 ligand) and Pam3CSK4 (a TLR2 ligand) demonstrated that these microbial ligands utilized their respective cognate TLR. These data provide good evidence that *P. gingivalis* LPS can utilize TLR2 and TLR4 under conditions where they are expressed at endogenous levels.

DISCUSSION

Different structures have been reported for the lipid A obtained from *P. gingivalis* LPS (32, 41). Ogawa (41) reported the major lipid A species as a triacylated monophosphorylated form with a mass of *m/z* 1,195. In contrast, Kumada et al. (32) reported that *P. gingivalis* LPS preparations contain multiple lipid A species, with the largest being a pentacylated diphosphorylated species displaying a mass ion of *m/z* 1,770. Both of the *m/z* 1,195 and 1,770 lipid A mass ions have been chemically synthesized, and evidence that they activate cells through TLR4 and not TLR2 has been reported (33, 42). However, highly purified native *P. gingivalis* LPS or lipid A preparations consistently demonstrate TLR2 activity (5, 26, 33, 42). Trace amounts of tightly associated endotoxin proteins (34) or another as-yet-undefined *P. gingivalis* component (42) have been proposed as being responsible for the TLR 2 activity in native *P. gingivalis* LPS preparations. In this report, both the protein content and fatty acid composition of the *P. gingivalis* LPS Pg $LPS_{1435/1450}$ preparation did not support contaminants as being responsible for the TLR2 activity that was observed in the *P. gingivalis* LPS preparation. No detectable endotoxin-associated protein was observed in SDS-PAGE. Rather, we propose that the *P. gingivalis* LPS TLR2 activities observed both in the HEK cell and mouse bone marrow cell systems in this study were due to the lipid A mass ions at *m/z* 1,435 and/or 1,450. These mass ions have been reported to be the major lipid A species found in highly purified *P. gingivalis* LPS preparations by both our laboratory (3) and Kumada et al. (32) and represent the most likely candidates for the apparent discrepancy between studies employing chemically synthesized lipid A spe-

cies and native preparations. The structures for the lipid A mass ions at *m/z* 1,435 and 1,450 have been elucidated as being tetra-acylated monophosphorylated species (32). However, in this report, it was also found that the same highly purified *P. gingivalis* LPS preparation was capable of activating HEK and mouse bone marrow cells through TLR4. Both murine and human TLR4/MD-2 systems were capable of responding to *P. gingivalis* LPS. This is unusual, since it has been shown that muTLR4 but not huTLR4 can respond to tetra-acylated *E. coli* LPS (44). Since the major *P. gingivalis* lipid A species examined in this report were tetra-acylated (*m/z* 1,435 and 1,450), it is possible that human TLR4 can detect tetra-acylated LPS from different species of bacteria. Alternatively, since the MALDI-TOF analysis of lipid A was not quantitative, it is not known if the major lipid A mass ions observed were responsible for TLR4 activation or if minor amounts of other lipid A species (e.g., *m/z* 1,770) facilitated activation through this receptor.

The biological significance of the multiple lipid A species found in *P. gingivalis* LPS preparations is not currently understood. A preliminary examination has revealed that several different *P. gingivalis* laboratory strains and clinical isolates display a similar pattern of lipid A heterogeneity as that shown in this work, demonstrating that the major lipid A mass ions are not strain specific (data not shown). Several other bacterial species have been shown to contain multiple lipid A forms that exist in a single bacterial population (2, 47, 48, 53, 58) and that can be regulated by incubation temperature (29) or the concentration of Mg^{2+} in the growth medium (17). In *Salmonella enterica* serovar Typhimurium, additional lipid A species that alter the human endothelial cell response of purified LPS have been shown to be under the control of a phoP/phoQ two-component regulatory system (21). Similarly, *P. gingivalis* may also synthesize multiple lipid A species, accounting for the heterogeneity observed in purified LPS preparations (5, 32). Conversely, *P. gingivalis* lipid A heterogeneity may arise due to isolation and characterization procedures. For example, isolation of LPS often involves heating in phenol, and examination of lipid A requires acid hydrolysis of the lipid A from the 3-deoxy-D-manno-octulosonic acid core sugar, and these procedures may result in cleavage of acid-labile fatty acids and phosphate residues. However, our laboratory has found that *P. gingivalis* LPS preparations contain the 1,435 and 1,450 lipid A species as the major peaks in MALDI-TOF analysis when either the phenol-water (63) or $MgCl_2$ LPS (13) extraction procedures are employed or if the LPS is subjected to two different lipid A hydrolysis procedures (7, 41) (data not shown). This strongly suggests that the lipid A mass ions found at *m/z* 1,435 and 1,450 did not arise due to the type of LPS extraction or lipid A hydrolysis procedures. Additional studies will be required to determine the origin of *P. gingivalis* lipid A heterogeneity; nevertheless, it is clear that purified *P. gingivalis* LPS preparations may contain more than one lipid A species, and this may account for the discrepancy in host cell activation assays observed between chemically synthesized lipid As and native highly purified preparations.

Transient HEK cell transfection experiments provide an excellent means to determine the components of the TLR complex necessary to facilitate host cell responses. In this report, it was found that MD-2 was not required for *P. gingivalis* LPS TLR2-dependent, or TLR2-plus-TLR1-dependent, HEK cell activation but was necessary for activation with mouse or human TLR4. It is interesting that in another HEK system, endogenous levels of MD-2 were sufficient to facilitate TLR4 *E. coli* LPS activation (16). This may explain why in a study employing transiently transfected HEK cells (where the endogenous levels of MD-2 were not reported), TLR4-dependent cell activation with phenol-extracted *P. gingivalis* LPS was not observed (26). The requirement for different TLR cofactors for cell stimulation is consistent with other observations (16, 40, 61) that have shown each bacterial ligand may require different components of the TLR signaling complex to facilitate host cell activation.

Furthermore, sCD14 amply replaced mCD14 to yield highly significant *P. gingivalis* LPS TLR2-plus-TLR1-dependent HEK cell activation, as demonstrated both by significant activation at low LPS concentrations (10 ng/ml) and a greater-than-15-fold increase over background at higher concentrations of LPS. However, huTLR4-dependent HEK cell activation employing sCD14 revealed that the Pg $LPS_{1435/1450}$ preparation only slightly activated these cells above background control levels at 100 and 1,000 ng of LPS/ml. These data demonstrate that the TLR activity of *P. gingivalis* LPS preparations is particularly prone to the presence of key accessory molecules, such as soluble or membrane CD14. In addition, it has previously been shown that *P. gingivalis* LPS can utilize hamster but not human TLR4 when combined with endogenous hamster MD-2 (68), further emphasizing that *P. gingivalis* LPS-dependent host cell activation, in contrast to that with *E. coli* LPS, is more sensitive to the species specificity of the TLR complex components. These factors, combined with the potential for multiple lipid A species in *P. gingivalis* LPS preparations, almost certainly have contributed to the variability reported for TLR utilization by *P. gingivalis* LPS.

One implication of the work presented here is that *P. gingivalis* LPS may activate host cells through either a TLR2- or TLR4-dependent pathway. The results of HEK cell transfection assays and bone marrow cell activation experiments demonstrate that certain *P. gingivalis* LPS preparations have the ability to interact with either TLR2 or TLR4. Another related implication is that the lipid A heterogeneity observed in *P. gingivalis* LPS preparations may reflect an ability of this bacterium to synthesize and express multiple, structurally different forms of lipid A. Alterations in the lipid A structural composition and utilization of multiple TLRs may affect host cell signaling, contributing to the ability of *P. gingivalis* to remain a persistent colonizer of the oral cavity as well as to induce inflammatory disease.

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