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Signaling by Toll-Like Receptor 2 and 4 Agonists Results in Differential Gene Expression in Murine Macrophages

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Lipopolysaccharide (LPS) derived from the periodontal pathogen *Porphyromonas gingivalis* has been reported to differ structurally and functionally from enterobacterial LPS. These studies demonstrate that in contrast to protein-free enterobacterial LPS, a similarly purified preparation of *P. gingivalis* LPS exhibited potent Toll-like receptor 2 (TLR2), rather than TLR4, agonist activity to elicit gene expression and cytokine secretion in murine macrophages and transfectants. More importantly, TLR2 stimulation by this *P. gingivalis* LPS preparation resulted in differential expression of a panel of genes that are normally induced in murine macrophages by *Escherichia coli* LPS. These data suggest that (i) *P. gingivalis* LPS does not signal through TLR4 and (ii) signaling through TLR2 and through TLR4 differs quantitatively and qualitatively. Our data support the hypothesis that the shared signaling pathways elicited by TLR2 and by TLR4 agonists must diverge in order to account for the distinct patterns of inflammatory gene expression.

Lipopolysaccharides (LPS) are among the most potent inflammatory bacterial mediators and have been strongly implicated in the inflammatory response associated with gram-negative sepsis. Most LPS signaling studies have used LPS preparations derived from species within the *Enterobacteriaceae*, which possess relatively well-conserved lipid A structures (reviewed in reference 36). A convergence of data suggest that these prototypic LPS preparations, when highly purified, elicit LPS responses that are restricted in the use of TLR4 as the principal signal-transducing molecule (reviewed in reference 21), which is strongly supported by the finding that synthetic *E. coli* lipid A activated Toll-like receptor 4 (TLR4) and not TLR2 transfectants (8). However, the lipid A of non-enterobacterial species, e.g., *Porphyromonas gingivalis*, which has been implicated in the inflammation associated with chronic periodontitis (reviewed in reference 9), differs both structurally and functionally from enterobacterial lipid A. Specifically, the major species of *P. gingivalis* lipid A is composed of unique branched fatty acids, with longer carbon chains than in enterobacterial lipid A, the absence of a phosphoryl group at position 4′ of the nonreducing glucosamine, as well as other modifications (Fig. 1) (1). Consistent with these structural differences is the finding that *P. gingivalis* LPS activity is poorly inhibited by polymyxin B (12), which has been postulated to inactivate LPS by binding electrostatically to negatively charged phosphate groups, leading to a subsequent interaction of polymyxin B with the hydrophobic fatty acids (25, 33). Although *P. gingivalis*-induced signaling was shown some time ago to be CD14 dependent (34), site-specific mutagenesis of CD14 suggests that the substitution of certain charged amino acids differentially affects the abilities of *Escherichia coli* and *P. gingivalis* LPS to bind CD14 (4, 5). In addition, binding of *P. gingivalis* LPS to LPS binding protein has been reported to be 100-fold less than observed for *E. coli* LPS (9). In vivo, *P. gingivalis* LPS has been reported to be much less toxic than other LPS preparations (reviewed in reference 27). *P. gingivalis* LPS has also been shown to be active on C3H/HeJ macrophages (12, 32), which possess a point mutation in *tlr4* that precludes signaling by enterobacterial LPS (24, 26). In contrast, Tabeta et al. (30) reported that human gingival fibroblasts exhibit a slight increase above basal interleukin-6 (IL-6) production upon stimulation with *P. gingivalis* LPS and that a monoclonal antibody directed against human TLR4 reduced the IL-6 level below that of the medium-treated cells. Differences in cytokine gene expression or secretion by *P. gingivalis* LPS and enterobacterial LPS preparations have also been reported for both myeloid and nonmyeloid cell types (4, 11, 12, 32).

Having purified *P. gingivalis* LPS by the same protocol as used to demonstrate the restricted usage of TLR4 by enterobacterial LPS to mediate signaling (8), we demonstrate herein that a preparation of *P. gingivalis* LPS utilizes TLR2, not TLR4, to mediate inflammatory signaling. In addition, *P. gingivalis* LPS differentially activates a panel of genes relative to *E. coli* LPS, suggesting that TLR2- and TLR4-mediated signaling pathways diverge.

MATERIALS AND METHODS

Mice. C3H/OuJ and C3H/HeJ mice were purchased from the Jackson Laboratory, (Bar Harbor, Maine). Thioglycolate-elicited peritoneal exudate macrophages were cultured as described previously (29).

LPS preparations. *E. coli* K235 LPS was prepared by a modification of the phenol water extraction method of McIntire et al. (20) (<0.008% protein). *E.*

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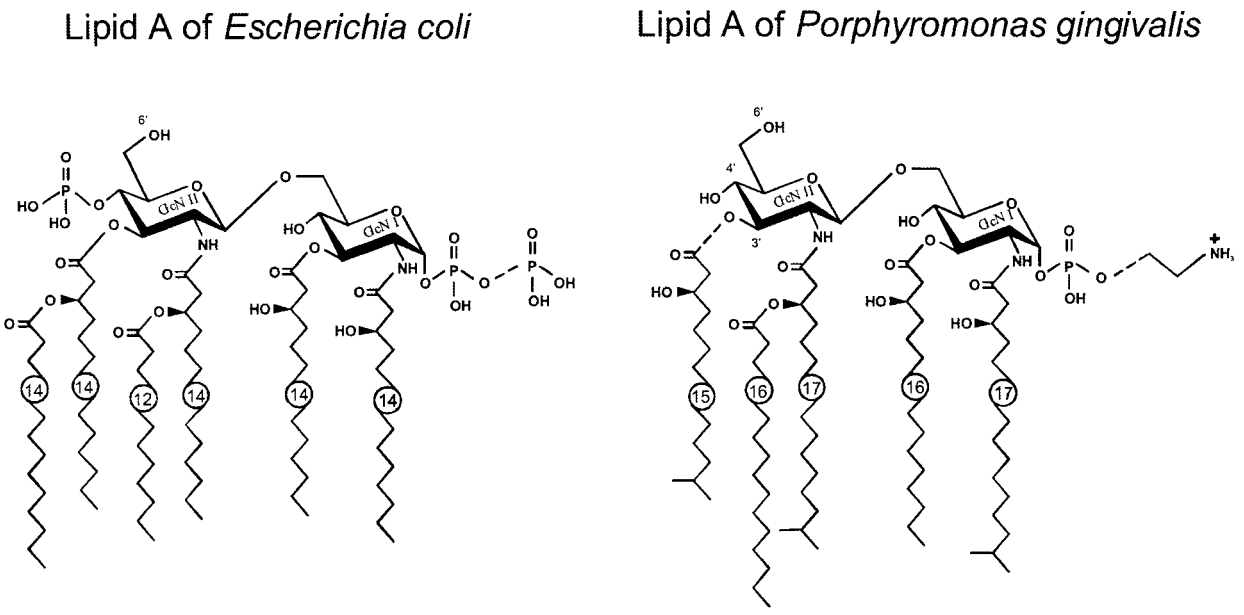

FIG. 1. (A) Chemical structures of the major species of lipid A produced by *E. coli* and *P. gingivalis*. The major structural differences include the nature and number of fatty acids, presence or absence of the second phosphate in position 4′, and substitution of the position 1 phosphate. A more extensive and detailed comparison is presented in reference 36.

coli J5 (Rc) LPS was purchased from List Biological Laboratories (Campbell, Calif.) and was subjected to phenol reextraction (repurified) by a method that results in the elimination of contaminants that are active on C3H/HeJ macrophages (17, 19). This reextraction method, detailed more recently by Hirschfeld et al. (8), results in enterobacterial LPS preparations that utilize TLR4, and not TLR2, for signaling. Briefly, 5 mg of Rc LPS was resuspended in 1 ml of room temperature, endotoxin-free water containing 0.2% triethylamine (TEA). The sample was split into two 500-μl aliquots, and one aliquot was stored at 4°C without further manipulation (unpurified LPS). Deoxycholate was added to the remaining aliquot to a final concentration of 0.5%, followed by the addition of 500 μl of water-saturated phenol. The sample was vortexed intermittently for 5 min, and the phases were allowed to separate at room temperature for 5 min. The sample was placed on ice for 5 min and then centrifuged at 4°C for 2 min at 10,000 × *g*. The top aqueous layer was transferred to a new tube, and the phenol phase was subjected to reextraction with 500 μl of 0.2% TEA–0.5% deoxycholate. The aqueous phases were pooled and reextracted with 1 ml of water-saturated phenol. The pooled aqueous phases were adjusted to 75% ethanol and 30 mM sodium acetate and were allowed to precipitate at −20°C for 1 h. The precipitate was centrifuged at 4°C for 10 min at 10,000 × *g*, washed in 1 ml of cold 100% ethanol, and air dried. The precipitate was resuspended in the original volume (500 μl) of 0.2% TEA. One hundred percent recovery was assumed for the purified LPS sample (19), which will be referred to as purified LPS. *P. gingivalis* 33277 LPS was purified by two rounds of hot phenol extraction (22), followed by phenol reextraction (8, 17, 19). Colloidal gold staining was carried out using a kit (Enhanced Colloidal Gold; Bio-Rad, Hercules, Calif.), which has a lower limit of sensitivity of 10 to 100 pg protein. Twenty micrograms of *P. gingivalis* LPS was analyzed by thin-layer chromatography (TLC) using silica gel H plates, developed with chloroform-methanol-water-ammonium hydroxide (50:25:4:2). The plate was sprayed with dichromate solution and charred. Most of the LPS chromatographed at the origin. When the LPS was acid hydrolyzed (0.1 N HCl at 100°C for 25 min) and analyzed by TLC, most of the lipid A migrated off the origin, consistent with an LPS preparation that is >95% pure.

Isolation of total cellular RNA and RT-PCR. All procedures for detection of cytokine and chemokine mRNA by semiquantitative RT-PCR have been detailed previously (18, 28). Briefly, total cellular RNA was extracted from macrophage cultures and was reverse transcribed. PCR amplifications were performed on the resultant cDNA for the gene of interest, using specific sense and antisense primers for cytokine mRNA, i.e., IL-1β (35 cycles), tumor necrosis factor alpha (TNF-α) (31 cycles), IL-6 (30 cycles), gamma interferon (IFN-γ) (35 cycles), IL-12 p35 (31 cycles), IL-12 p40 (22 cycles), and for chemokine mRNA, i.e., MIP-1α (27 cycles), MIP-2 (26 cycles), IP-10 (30 cycles), JE (32 cycles), and MCP-5 (29 cycles). The gene encoding hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) (24 cycles) was included as a housekeeping gene to control for differences in cDNA for each treatment during the amplification reaction. PCR amplification products were electrophoresed on a 1% agarose gel and blotted overnight onto a Nytran membrane. The DNA was then UV cross-linked onto the membrane and baked at 80°C for 2 h. The amplified PCR products were detected by Southern blot analysis using gene-specific oligonucleotide probes labeled with the Amersham 3-oligolabeling and detection systems (Amersham International, Buckinghamshire, England).

Cell lines and transfections. The human astrocytoma cell lines U87 and U373, were obtained from the American Type Culture Collection (Manassas, Va.). The subclone of the human embryonic kidney epithelial cell line HEK 293 and the constructs for FLAG-tagged human TLR1, TLR2, TLR3, and TLR4, pFLAG control vector, the ELAM-1 luciferase reporter construct, and Rous sarcoma virus–β-galactosidase (RSV-β-Gal) were provided by Tularik (South San Francisco, Calif.) (13). Conditions for transfection of cells have been detailed elsewhere (7, 8). Briefly, 293 cells were cotransfected in six-well plates using a calcium phosphate kit (Clontech, Palo Alto, Calif.) with 2, 0.5, and 0.5 μg of the TLR expression construct, the ELAM-1 luciferase reporter construct, and the RSV-β-Gal construct, respectively, to normalize for transfection efficiency. Cells were grown for 36 h and stimulated with the indicated agonist for an additional 6 h. U87 cells were transfected in 12-well plates using pFx-2 (Invitrogen, Carlsbad, Calif.) with 2 μg of either TLR2 or TLR4 expression construct. Cells were then grown for 24 h in Dulbecco modified Eagle medium (DMEM) with Nutridoma-HU (Boehringer Mannheim, Indianapolis, Ind.) followed by stimulation with agonist for an additional 24 h in DMEM–Nutridoma-HU containing 2% human serum. U373 cells were grown in 24-well plates for 24 h in DMEM with Nutridoma-HU followed by stimulation with agonist for an additional 24 h in DMEM–Nutridoma-HU containing 2% human serum.

Luciferase and cytokine assays. IL-6 (U87 and U373 cells) and IL-8 (HEK 293) levels were measured by enzyme-linked immunosorbent assay (ELISA; Endogen, Woburn, Mass.). ELISAs specific for total and bioactive murine IL-12 have been detailed elsewhere (29). Murine TNF-α was also measured by ELISA (Genzyme, Cambridge, Mass.). To assay for NF-κB-dependent luciferase activity, transfected 293 cells were lysed using reporter lysis buffer (Promega, Madison, Wis.), and 20 μl of lysate was assayed for both luciferase and β-galactosidase activities using a Dynatec MLX luminometer after incubation in luciferase assay reagent (Promega) and Galacto-Light with light emission accelerator (Tropix, Bedford, Mass.), respectively.

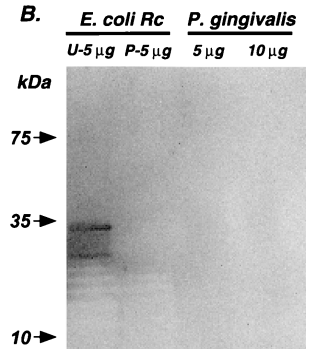

FIG. 2. Repurified *P. gingivalis* LPS is not contaminated with endotoxin protein. A commercial preparation of *E. coli* J5 (Rc) LPS and a preparation of *P. gingivalis* LPS isolated according to Millar et al. (22) were subjected to a modified phenol reextraction protocol previously shown to eliminate trace endotoxin protein contamination (8, 17, 19). Unpurified (U) or repurified (P) Rc LPS samples are indicated. Five micrograms of both Rc LPS samples and 5 and 10 μg of repurified *P. gingivalis* LPS were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Samples were resolved on a 4 to 20% gradient gel and then transferred to a polyvinylidene membrane. Membranes were subsequently stained with colloidal gold.

RESULTS AND DISCUSSION

Previous reports that *P. gingivalis* LPS (i) activates C3H/HeJ macrophages, (ii) differs from enterobacterial LPS in its capacity to elicit a variety of responses, and (iii) possesses a lipid A with a markedly distinct structure led us to evaluate this LPS further upon hot phenol water extraction (22), followed by repurification using a method demonstrated to eliminate TLR2-dependent ligands from several enterobacterial LPS preparations (8). The preparation of *P. gingivalis* LPS used in this study was confirmed to be essentially protein free, as evidenced by a lack of detectable bands in colloidal gold-stained blots of 10 μg of *P. gingivalis* LPS preparation (Fig. 2); i.e., the protein concentration of this preparation is <100 pg of protein/10 μg of LPS, or <0.001%, in contrast to 5 μg of the commercial *E. coli* Rc LPS, which contained clearly detectable protein bands that were eliminated by repurification. Figure 3 illustrates that under conditions where protein-free *E. coli* K235 LPS completely discriminates between LPS-normoresponsive C3H/OuJ and TLR4-defective C3H/HeJ macrophages with respect to induction of IL-1β and MIP-2 mRNA, *P. gingivalis* LPS elicits very comparable levels of dose-dependent induction of both genes in macrophages from both strains. Thus, these data confirm previous evidence supporting the hypothesis that stimulation of murine macrophages with *P. gingivalis* LPS is TLR4 independent (12, 32). Moreover, the potency of the *E. coli* LPS is clearly greater than that of the *P. gingivalis* LPS, as evidenced by a clear diminution of signal in those samples stimulated by *P. gingivalis* versus *E. coli* LPS at 1 ng/ml.

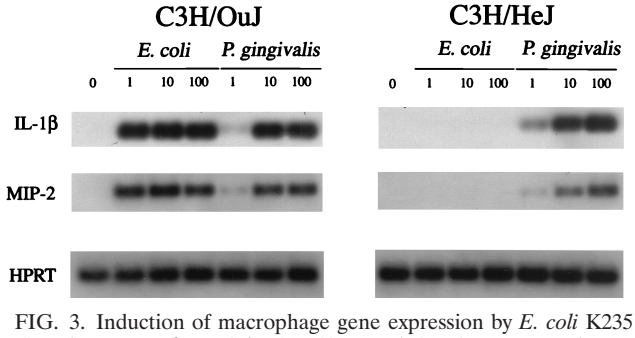

FIG. 3. Induction of macrophage gene expression by *E. coli* K235 LPS and *P. gingivalis* LPS in C3H/OuJ and C3H/HeJ macrophages. Total macrophage RNA was subjected to RT-PCR with Southern blotting for the detection of IL-1β, MIP-2, and *HPRT* mRNA as described in the text. *HPRT* served as the housekeeping gene in these experiments. The data are representative one of three separate experiments.

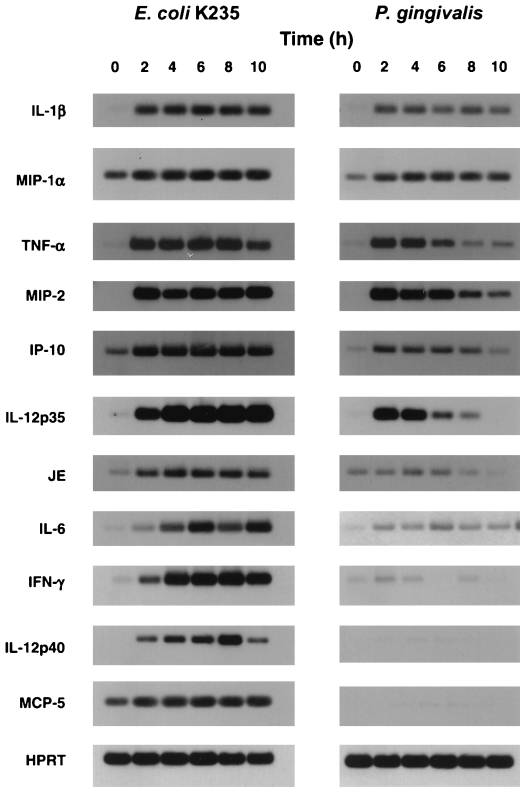

FIG. 4. Differential induction of macrophage gene expression by *P. gingivalis* LPS for a panel of genes normally induced by *E. coli* K235 LPS. Total RNA was subjected to RT-PCR with Southern blotting for the detection of the specific mRNA species as described in the text. The data represent one of two representative experiments carried out in duplicate. *HPRT* was used as the housekeeping gene.

Using 100 ng/ml, a concentration that was superoptimal for induction of expression of both IL-1β and MIP-2 genes by both LPS preparations, we carried out a kinetic analysis and compared induction of 11 cytokine genes in C3H/OuJ macrophages (Fig. 4). Induction of IL-1β and MIP-1α mRNA expression was quite comparable over the time course examined. A second subset of genes, i.e., TNF-α, MIP-2, IP-10, and IL-12 p35, were inducible by the *P. gingivalis* LPS preparation, but steady-state mRNA levels declined more rapidly than when cells were stimulated with *E. coli* LPS. Induction of JE and IL-6 mRNA was poor and steady-state levels declined rapidly, while IFN-γ, IL-12 p40, and MCP-5 gene expression was strongly induced by *E. coli* LPS but not by *P. gingivalis* LPS. Even at a dose of 1 μg/ml, the *P. gingivalis* preparation induced

TABLE 1. Induction of cytokines in C3H/OuJ macrophages stimulated with *E. coli* LPS or with *P. gingivalis* LPS[a]

Cytokine	Level (pg/ml)[b]		
	Medium only	*E. coli* K235 LPS	*P. gingivalis* LPS
TNF-α (6 h)	<23	12,713 ± 3,456	757 ± 62
IL-12 p40 + p70			
6 h	<15	2,701 ± 127	150 ± 32
24 h	<15	7,065 ± 438	328 ± 25
IL-12 p70			
6 h	<15	143 ± 2	<15
24 h	<15	279 ± 20	<15

[a] C3H/OuJ macrophages were cultured at a final density of 4×10^6/well in six-well plates and treated with 2 ml of medium only or medium containing 100 ng of *E. coli* K235 LPS or *P. gingivalis* LPS per ml. Supernatants were harvested at 6 or 24 h and assayed by ELISA for TNF-α, total IL-12 (IL-12 p40 plus p70), or bioactive IL-12 p70 as described previously (29).

[b] Arithmetic mean ± standard deviation of duplicate samples.

MCP-5 only minimally (data not shown). Secretion of TNF-α, total IL-12 (IL-12 p40 plus IL-12 p70), and bioactive IL-12 (IL-12 p70) was markedly attenuated in *P. gingivalis*- versus *E. coli*-stimulated cultures (Table 1). Therefore, not only is the *P. gingivalis*-induced gene expression TLR4 independent, but also such differential gene expression and cytokine secretion implies that there must be qualitative differences in signaling between TLR2 and TLR4.

We recently demonstrated that the apparent TLR2 dependency of many LPS preparations is eliminated by phenol re-extraction, while TLR4 dependency is retained (8, 17, 19). In contrast, our *P. gingivalis* preparation, which was confirmed to be equivalently protein free and exhibited an electrophoretic mobility in TLC consistent with LPS, did not appear to stimulate gene expression through TLR4 since IL-1β and MIP-2 genes were expressed in very comparable, dose-dependent fashions in both normal and TLR4-defective macrophages (Fig. 3). Therefore, to confirm the TLR4 independence of the *P. gingivalis* LPS preparation, the U87 astrocytoma cell line was transiently transfected with either human TLR4 or TLR2 constructs and then stimulated with either *E. coli* Rc LPS (as purchased or repurified) or the *P. gingivalis* LPS preparation, repurified identically to the *E. coli* Rc preparation. Figure 5 illustrates that the *P. gingivalis* LPS induces IL-6 production in U87 cells that overexpress TLR2 (Fig. 5A) but not TLR4 (Fig. 5B). As recently reported (8), the commercially prepared and repurified *E. coli* Rc preparations both stimulated TLR4 transfectants, but only the unpurified preparation was active in TLR2 transfectants. These findings were confirmed using a similar astrocytoma cell line, U373, that has been reported to be responsive to *E. coli* LPS but not to bacterial lipoproteins and to express endogenous TLR4 but not TLR2 mRNA (7). U373 cells secreted IL-6 in response to unpurified or purified *E. coli* Rc LPS but not in response to the *P. gingivalis* LPS preparation (Fig. 5C). TLR2 transfection of HEK 293 cells also conferred sensitivity to *P. gingivalis* LPS, as measured by ELAM-1 luciferase reporter gene expression or IL-8 secretion (Fig. 6), with a dose dependency that closely parallels that seen in the primary macrophages (Fig. 3). In contrast to the *P. gingivalis* sensitivity conferred upon HEK 293 cells by TLR2

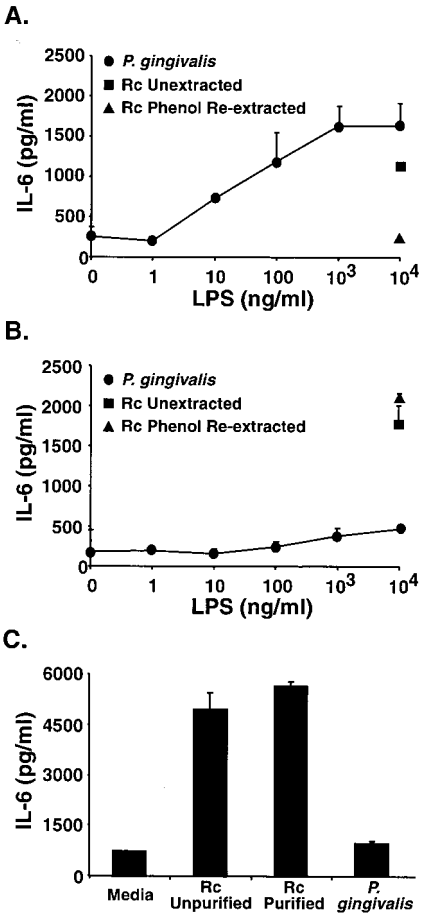

FIG. 5. TLR2, but not TLR4, confers responsiveness to *P. gingivalis* LPS. U87 cells transiently transfected with either human TLR2 (A) or human TLR4 (B) or untransfected U373 cells (C) were stimulated for 24 h with unpurified Rc LPS repurified Rc LPS, or repurified *P. gingivalis* LPS at the indicated concentrations. Supernatants were collected and assayed for IL-6 production by ELISA.

overexpression, transfection with a pFLAG control vector or a TLR1, TLR3, or TLR4 construct failed to render 293 cells sensitive to *P. gingivalis* LPS (data not shown). Finally, Chinese hamster ovary (CHO) cells engineered to express an EL-AM.Tac (CD25) reporter construct and CD14 only (14, 15) failed to respond to this same preparation of *P. gingivalis* LPS or to the synthetic TLR2 agonist, tripalmitoyl-*S*-glycerylcysteine-modified Ser Lys_4 peptide, but did respond to *E. coli* lipid A, presumably via endogenous hamster TLR4. In contrast, CHO cells engineered to express the reporter construct, CD14, and human TLR2 (14, 15) responded to all three stimuli to express Tac antigen (H. Heine, personal communication). Thus, in four separate cell lines (transiently or stably transfected) and in primary macrophages, where functional TLR2 and TLR4 expression levels differ, this *P. gingivalis* LPS preparation initiates intracellular signaling through TLR2, while *E. coli* LPS selectively utilizes TLR4. That our preparation of *P. gingivalis* LPS signals via TLR2 is also consistent with two previous reports that *Rhodobacter sphaeroides* lipid A, which blocks *E. coli* LPS-induced signaling in a TLR4-dependent

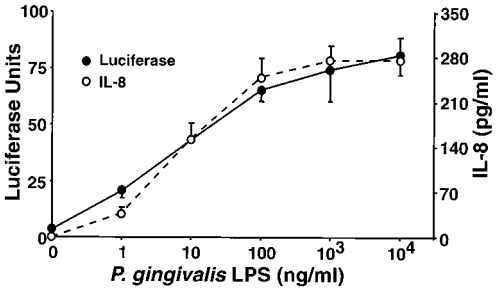

FIG. 6. Transfection of TLR2 confers responsiveness to *P. gingivalis* LPS in HEK 293 cells. HEK 293 cells were transiently transfected with human TLR2 plus the ELAM-1 luciferase reporter construct. Cells were stimulated for 6 h with increasing doses of repurified *P. gingivalis* LPS. Supernatants were analyzed for IL-8 secretion, and cell lysates were analyzed for NF-κB nuclear translocation, expressed in luciferase units.

fashion (15), fails to block *P. gingivalis* LPS-induced TNF production (1, 12).

Our data extend significantly previous observations indicating that *P. gingivalis* LPS activates macrophages differently from enterobacterial LPS preparations. Clearly, the data presented herein indicate that *P. gingivalis* LPS does not signal through TLR4, likely accounting for the comparability of induction of IL-1β and MIP-2 mRNA in C3H/OuJ and C3H/HeJ macrophages (Fig. 3). However, we cannot be certain that the TLR2 stimulatory activity detected in the *P. gingivalis* LPS preparation is attributable to the LPS, despite the fact that the LPS represents the predominant species (>95%). It is possible that a minor contaminant contained within the *P. gingivalis* LPS preparation is responsible for the TLR2 agonist activity of this preparation. Future experiments using synthetic *P. gingivalis* lipid A will be required to confirm whether or not *P. gingivalis* LPS is the true TLR2 agonist.

Faure et al. (6) reported recently that human dermal microvessel or umbilical vein endothelial cells express predominantly TLR4 but very weakly TLR2 and respond vigorously to *E. coli* LPS but not to *Mycobacterium tuberculosis* 19-kDa lipoprotein, a TLR2 ligand. Thus, the earlier observation of Cunningham et al. (4) that human endothelial cells responded to produce E-selectin upon stimulation with *E. coli* but not *P. gingivalis* LPS are likely explained by our observation that *P. gingivalis* requires TLR2, and not TLR4, for activation. However, Cunningham et al. (4) also demonstrated that *P. gingivalis* LPS blocked *E. coli* LPS-induced E-selectin expression in human umbilical vein endothelial cell cultures in a CD14-independent fashion. Thus, it is possible that *P. gingivalis* LPS, like *R. sphaeroides* LPS, is an inactive antagonist of *E. coli* LPS at the level of TLR4. A second possible mechanism for this inhibition is that engagement of TLR2 by the agonist contained within the *P. gingivalis* LPS preparation sequesters or exhausts shared signaling molecules (MyD88, etc.) such that subsequent stimulation by *E. coli* LPS through TLR4 is precluded.

The finding of diminished potency of *P. gingivalis* versus *E. coli* LPS for the induction of IL-1β and MIP-2 in C3H/OuJ macrophages (Fig. 3), coupled with differential gene expression (Fig. 4), implies that signaling pathways through TLR2 and TLR4 are quantitatively and/or qualitatively different. One possibility is that the utilization of TLR2 rather than TLR4 results in a more limited capacity to generate intracellular signals required for the expression of certain genes, either through differences related to the strength of signaling through TLR2 versus TLR4 or, perhaps secondarily, through the difference in capacity of TLR2 and TLR4 to recruit additional signaling molecules to the LPS signaling complex. In this regard, it is possible that TLR2 interacts with other TLRs to elicit signaling by our *P. gingivalis* preparation, as has been recently observed for signaling by *Neisseria meningitidis* (35). Different affinities of *P. gingivalis* LPS and enterobacterial LPS for CD14 (5) could also alter the interaction of CD14, once engaged by a particular LPS, with specific TLRs. Regardless of the mechanism, there appears to be a divergence of signaling pathways that results in differential gene expression distal to or distinct from the engagement of shared upstream signaling molecules. This conclusion is also strengthened by our recent observation that another TLR2 agonist, soluble tuberculosis factor (kindly provided by Matthew Fenton) (21), induces IL-1β but not MCP-5 mRNA (V. Toshchakov, unpublished observations).

It is tempting to speculate that the mitigated toxicity of *P. gingivalis* compared with that of enterobacterial LPS preparations in vivo is secondary to mitigated production of cytokines that have been implicated in endotoxicity (e.g., TNF-α, IL-12, and IFN-γ [reviewed in reference 29). While *P. gingivalis* can invade epithelial cells and replicate intracellularly (14), the failure of this *P. gingivalis* LPS preparation to induce IL-12 and IFN-γ mRNA may also contribute to the chronicity of this agent in the pathogenicity of periodontitis, since IL-12 and IFN-γ are necessary for the elimination of many intracellular pathogens, such as *Mycobacterium* and *Listeria* species (10). Other bacterial pathogens that produce potent proinflammatory molecules that act through TLR2 but not TLR4 have been identified. These include *Borrelia burgdorferi*, the agent of Lyme disease, which causes a chronic infection in mice and humans characterized by inflammatory arthritis and produces numerous distinct tripalmitoyl-*S*-glycerylcysteine-bearing lipoproteins that are TLR2 ligands (2, 3, 7, 16). Several *Mycoplasma* species, which are also associated with chronic infections and arthritis, produce extremely potent diacylated lipoproteins that interact with TLR2 (23, 31). Although both *B. burgdorferi* and *Mycoplasma* species cause chronic diseases, chronicity cannot be attributed solely to signaling through TLR2 since administration of high doses of purified TLR2-dependent bacterial lipoproteins has been associated with acute, toxic-type syndromes (37).

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