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Michael Martin; ... et. al

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Differential Induction of Endotoxin Tolerance by Lipopolysaccharides Derived from *Porphyromonas gingivalis* and *Escherichia coli*¹

Michael Martin,* Jannet Katz,[†] Stefanie N. Vogel,[‡] and Suzanne M. Michalek^{2*}

Exposure of mononuclear phagocytes to enterobacterial LPS induces a state of transient hyporesponsiveness to subsequent LPS exposure, termed endotoxin tolerance. In the present study, LPS derived from the oral periodontal pathogen, *Porphyromonas gingivalis*, was compared with that derived from the enterobacterium, *Escherichia coli*, for the ability to induce endotoxin tolerance. Pretreatment of the human macrophage cell line, THP-1, with *E. coli* LPS resulted in a severe reduction in the levels of IL-1 β , IL-6, and TNF- α upon secondary stimulation. In contrast, pretreatment of THP-1 cells with *P. gingivalis* LPS resulted in a mitigation of IL-1 β , but not IL-6 and TNF- α production upon subsequent exposure to *P. gingivalis* LPS: primary or secondary stimulation with ≤ 100 ng/ml *P. gingivalis* LPS resulted in comparable levels of IL-6 and TNF- α , while stimulation of THP-1 cells with ≥ 1 μ g/ml *P. gingivalis* LPS induced a significant enhancement in IL-6 and TNF- α levels upon secondary exposure. To identify possible mechanisms for these differences, changes in the expression of molecules involved in the LPS-signaling pathway were assessed. Pretreatment of THP-1 cells with *E. coli* LPS resulted in a significant reduction in surface Toll-like receptor 4 (TLR4) expression and an inability to degrade I- κ B- α or I- κ B- β proteins upon secondary stimulation. In contrast, pretreatment of THP-1 cells with *P. gingivalis* LPS resulted in a significant enhancement of both CD14 and TLR2, while maintaining the ability to degrade I- κ B- β only upon secondary stimulation. Thus, *E. coli* and *P. gingivalis* LPS differentially affect CD14 and TLR expression as well as secondary LPS-associated responses. *The Journal of Immunology*, 2001, 167: 5278–5285.

A major constituent of the outer membrane of Gram-negative bacteria is LPS, which is considered to be a potent inducer of proinflammatory cytokines by neutrophils, monocytes, and macrophages (1, 2). The ability of LPS to stimulate cellular responses initially involves the complexing of LPS with plasma-derived LPS-binding protein. LPS/LPS-binding protein complexes can then engage either soluble CD14 or CD14 that is membrane bound on the surface of mononuclear phagocytes (3–5). Although CD14 can mediate or enhance LPS responses in various cell types, CD14 is devoid of a transmembrane domain and, thus, incapable of transducing an intracellular signal (5–7). Recently, a family of type I transmembrane Toll-like receptors (TLRs)³ were identified in humans and mice (8). Transfection of otherwise LPS-unresponsive cell lines with certain TLRs conferred LPS responsiveness (8–10), which was enhanced by coexpression of membrane-bound CD14 (10).

To date, 10 human TLRs have been identified and appear to function as innate immune receptors for LPS and other microbial products (8, 9, 11–14). Of these, TLR2 and TLR4 have been reported to function as LPS-signaling receptors (9, 10). It has been shown that LPS-hyporesponsive C3H/HeJ and C57BL/10ScCr

mice possess a point or null mutation, respectively, in the gene that encodes TLR4 (15, 16). In contrast, mice that lack TLR2, but not TLR4, appear to have no significant defects in their ability to respond to enterobacterial LPS (17). Other studies have shown that phenol-water reextraction of enterobacterial LPS to remove endotoxin impurities abrogates TLR2, but not TLR4 agonist activity (18–20). Thus, the ability of commercial enterobacterial LPS preparations to signal via TLR2 appears to be the result of contaminants within these preparations.

Studies assessing the immunostimulatory properties of nonenterobacterial LPS preparations have shown that the LPS from the oral periodontal pathogens *Porphyromonas gingivalis* and *Prevotella intermedia* exhibit biologic activity in C3H/HeJ mice even after phenol-water reextraction (21–25). Furthermore, it has been shown that protein-free *P. gingivalis* LPS utilizes TLR2, and not TLR4, in a study using human cell lines deficient in TLR2 or transfected with human TLR2 or TLR4 (26). This study also demonstrated no quantitative or qualitative differences in the ability of *P. gingivalis* LPS to stimulate C3H/HeJ or C3H/OuJ macrophages. Thus, there appears to be a divergence in the ability of nonenterobacterial and enterobacterial LPS to signal via TLR2 or TLR4 receptors.

Although LPS is a potent inducer of various cellular responses by neutrophils, monocytes, and macrophages, prior exposure of these cells to enterobacterial LPS results in a refractory state upon secondary stimulation. This in vitro state of endotoxin tolerance has typically been associated with a marked reduction in the production of various proinflammatory cytokines in response to a secondary stimulation with LPS (27–29). The molecular mechanisms that underlie induction of LPS tolerance appear to affect proximal signaling events, including down-regulation of TLR4, reduced IL-1R-associated kinase levels, alterations in phospholipase C- γ 1 and phosphatidylinositol-3' kinase, reduced GTP-binding and G protein content, as well as reduced activation of mitogen-activated

Departments of *Microbiology and [†]Oral Biology, University of Alabama, Birmingham, AL 35294; and [‡]Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814

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² Address correspondence and reprint requests to Dr. Suzanne Michalek, Department of Microbiology, University of Alabama, 845 19th Street South, BBRB 258/5, Birmingham, AL 35294. E-mail address: suemich@uab.edu

³ Abbreviations used in this paper: TLR, Toll-like receptor; IL-1ra, IL-1R antagonist.

protein kinases, I- κ B kinases, and the degradation of I- κ B- α and I- κ B- β (30–36).

P. gingivalis has been implicated as an etiologic agent of adult periodontitis, a chronic inflammatory disease characterized by the destruction of the supportive tissue surrounding teeth (37–39). This bacterium possesses a number of potential virulence factors, including LPS, which is important in the disease process (37, 38, 40). *P. gingivalis* LPS has been shown to differ from *Escherichia coli* LPS in structure and various functional activities (37, 41, 42). The purpose of the present study was to examine the ability of *P. gingivalis* LPS to induce endotoxin tolerance. To this end, we compared the in vitro cytokine responses of THP-1 cells following primary and secondary exposure to *P. gingivalis* or *E. coli* LPS. Differential effects on induction of select cytokines, expression of CD14 and TLRs, and dysregulation of I- κ B degradation were observed.

Materials and Methods

Reagents

Protein-free *E. coli* (K235) and *P. gingivalis* (33277) LPS were prepared as previously described (18, 26). Mouse anti-human TLR2 (clone 2392; IgG1) was obtained from Genentech (San Francisco, CA). Mouse anti-human TLR4 (clone HTA125; IgG2a) was kindly provided by K. Miyake (Saga Medical School, Saga, Japan) and was characterized previously (43). Goat anti-mouse IgG1 FITC, IgG2a PE, and mouse anti-human CD14 (clone mem-18; IgG1), and isotype-matched control Abs (IgG1 and IgG2a) were purchased from Caltag Laboratories (Burlingame, CA). Rabbit polyclonal IgG anti-I- κ B- α and anti-I- κ B- β Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Levels of IL-1 β , IL-6, and TNF- α present in cell-free supernatants were determined using cytokine reagents purchased from Caltag Laboratories.

Cytokine analysis

THP-1 cells (1×10^5 /well) were cultured in 96-well plates containing RPMI 1640 supplemented with 10% FBS, 50 μ M 2-ME, 1 mM sodium pyruvate, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 20 mM HEPES, 50 μ g/ml gentamicin, 50 U/ml penicillin, and 50 μ g/ml streptomycin. Cell cultures were incubated at 37°C in a humidified CO₂ incubator. Before stimulation, THP-1 cells were differentiated for 72 h in the presence of 10 ng/ml PMA, washed three times, and rested overnight. To assess the functional role of CD14, TLR2, or TLR4 in cytokine production, THP-1 cells (1×10^5 /well) were incubated with 10 μ g/ml of either anti-CD14 mAb (Caltag Laboratories; clone mem-18), anti-TLR2 mAb, anti-TLR4 mAb, or isotype-matched control Abs for 30 min before stimulation with *P. gingivalis* or *E. coli* LPS. To assess the ability of *P. gingivalis* or *E. coli* LPS to induce endotoxin tolerance in THP-1 cells, differentiated THP-1 cells (2.5×10^5 /ml) were stimulated with 1–10,000 ng/ml LPS for 24 h, washed with serum-free medium, and restimulated with LPS for an additional 24 h. Cytokine levels were quantitated using CLB Pelikine ELISA kits (obtained through Caltag Laboratories), according to the protocol suggested by the manufacturer.

Flow cytometry

Differentiated THP-1 cells were cultured at a concentration of 5×10^5 cells/ml in polypropylene tubes and incubated for 20 h in medium alone or with LPS. Cells were collected by low-speed centrifugation and resuspended in FACS buffer (PBS containing 1% FBS and 0.1% NaN₃). Cells were then incubated with FITC-labeled anti-CD14 or unlabeled anti-TLR2 or anti-TLR4 mAbs for 30 min on ice. Cells were then centrifuged and resuspended in FACS buffer. THP-1 cells initially stained with anti-TLR2 or anti-TLR4 mAb were then incubated with goat anti-mouse IgG1 FITC or goat anti-mouse IgG2a PE, respectively, for 20 min on ice. Cells were washed three times in FACS buffer and resuspended in 2% paraformaldehyde before being analyzed by flow cytometry using a FACStar flow cytometer (BD Biosciences, Mountain View, CA).

Western blot analysis

THP-1 cells (3×10^6 /ml) pretreated with medium or LPS were restimulated for the indicated time periods noted in the figures, washed with PBS, and then lysed on ice for 10 min in 300 μ l lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM sodium

fluoride, 1 mM PMSF, and 1 mg/ml aprotinin). Whole cell lysate was passed through a 20-gauge needle and then incubated on ice for 30 min. Cell debris was pelleted by centrifugation. Supernatants were collected and stored at -80°C until assayed. Thirty micrograms of total cellular protein were suspended in Laemmli buffer, boiled for 3 min, and subjected to SDS-PAGE. Gels were transferred to nitrocellulose membranes and blocked with TBS containing 0.05% Tween 20 (TBS-Tw) and 5% nonfat milk powder for 1 h. After washing in TBS, membranes were probed with rabbit anti-I- κ B- α (1:1000) or anti-I- κ B- β (1:1000) Abs (Santa Cruz Biotechnology) for 2 h at room temperature. Membranes were then washed with TBS-Tw and incubated with a polyclonal secondary goat anti-rabbit IgG HRP Ab (1:2000; Santa Cruz Biotechnology) for 1.5 h at room temperature. Following three washes in TBS-Tw, membranes were developed using ECL, according to the manufacturer's protocol (Santa Cruz Biotechnology).

Statistical analysis

Statistical significance between groups was evaluated by ANOVA and the Tukey multiple-comparison test using the InStat program (GraphPad Software, San Diego, CA). Differences between groups were considered significant at the level of $p < 0.05$.

Results

Comparison of IL-1 β , IL-6, and TNF- α production from THP-1 cells stimulated with *P. gingivalis* or *E. coli* LPS

We first compared the ability of protein-free *E. coli* and *P. gingivalis* LPS to induce primary proinflammatory cytokine responses. PMA-differentiated THP-1 cells were stimulated for 24 h in the presence or absence of varying concentrations of LPS, and cell-free supernatants were collected. *E. coli* LPS was significantly ($p < 0.05$) more potent for the induction of IL-1 β (Fig. 1A), IL-6 (Fig. 1B), and TNF- α (Fig. 1C) than *P. gingivalis* LPS at all concentrations tested. Similar results were observed at earlier time points (6 and 12 h), as well as with elutriated human monocytes (data not shown). These data demonstrate significant differences in the abilities of *P. gingivalis* and *E. coli* LPS to induce these proinflammatory cytokines by human monocytes, consistent with previous observations that *P. gingivalis* LPS is a less potent inducer of inflammatory cytokines (26, 44).

Effects of *E. coli* or *P. gingivalis* LPS pretreatment on secondary cytokine production

To compare the ability of *P. gingivalis* or *E. coli* LPS to induce endotoxin tolerance, THP-1 cells were stimulated for 24 h in the presence of LPS and then restimulated after washing for an additional 24 h with the same LPS concentration and assayed for cytokine production. When THP-1 cells were pretreated with *E. coli* LPS at concentrations from 1 to 10,000 ng/ml, a >90% reduction was observed in the level of IL-1 β , IL-6, and TNF- α production upon secondary stimulation with the same concentration of LPS (Fig. 2, A–C). In contrast, pretreatment of THP-1 cells with *P. gingivalis* LPS resulted in a statistically significant mitigation of IL-1 β only (Fig. 2D). Although the induction of IL-1 β by THP-1 cells was dose dependent, the levels of IL-1 β induced by restimulation with 1 or 10 μ g/ml *P. gingivalis* LPS were significantly lower ($p < 0.05$) than that seen following primary stimulation with *P. gingivalis* LPS (Fig. 2D). Thus, the induction of IL-1 β was reduced, but not completely ablated, as was observed when THP-1 cells were pretreated with as little as 1 ng/ml *E. coli* LPS (Fig. 2A). No significant alterations between the primary or secondary cytokine levels of IL-6 or TNF- α were observed with THP-1 cells pretreated with *P. gingivalis* LPS at concentrations from 1 to 100 ng/ml (Fig. 2, E and F). However, at concentrations of 1 μ g/ml, pretreatment of THP-1 cells with *P. gingivalis* LPS resulted in significantly ($p < 0.05$) enhanced IL-6 and TNF- α levels upon the secondary stimulation (Fig. 2, E and F). Similar results were obtained with elutriated human monocytes (data not shown). As

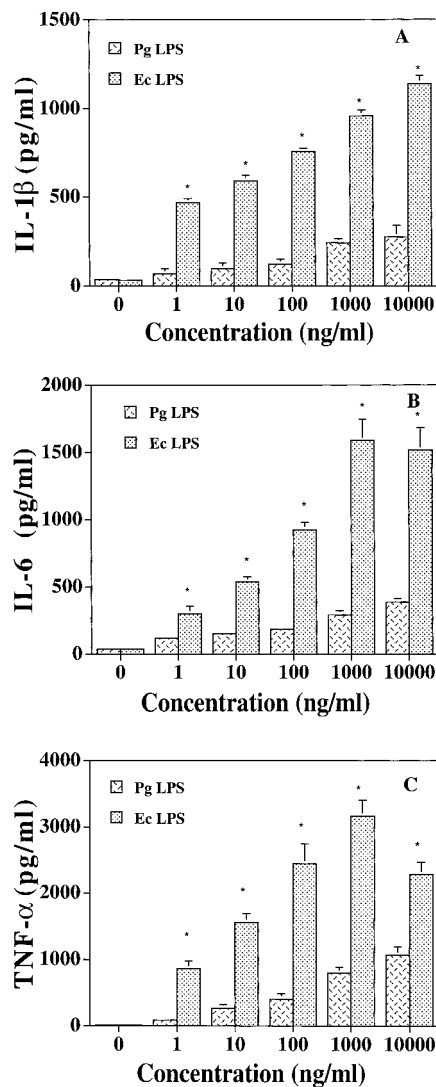


FIGURE 1. IL-1 β (A), IL-6 (B), or TNF- α (C) production by THP-1 cells incubated in the presence or absence of *P. gingivalis* (Pg) or *E. coli* (Ec) LPS. Cell-free supernatants were collected 24 h after stimulation and analyzed by ELISA. *, Significant differences ($p < 0.05$) compared with *P. gingivalis* LPS. Data are expressed as the mean \pm SD ($n = 6$).

shown in Fig. 2, incubation with medium alone following pretreatment with either *E. coli* or *P. gingivalis* LPS resulted in minimal levels of IL-1 β , IL-6, and TNF- α production. These findings demonstrate a major divergence in the ability of *P. gingivalis* and *E. coli* LPS to induce endotoxin tolerance.

Functional role of CD14, TLR2, and TLR4

Previous studies have shown the involvement of CD14 and TLRs in mediating LPS-associated responses (9, 10, 45). Therefore, we next sought to determine the functional involvement of CD14, TLR2, and TLR4 in our observed LPS-induced cytokine responses by THP-1 cells. THP-1 cells were incubated with mAbs against CD14, TLR2, and TLR4 before primary stimulation with LPS. The anti-CD14 mAb had a significant ($p < 0.05$) inhibitory affect on TNF- α production by THP-1 cells stimulated with *E. coli* or *P. gingivalis* LPS (Fig. 3). THP-1 cells preincubated with anti-TLR4 showed significantly ($p < 0.05$) reduced levels of TNF- α upon stimulation with *E. coli* LPS (Fig. 3A). In contrast, pretreatment with anti-TLR2 mAb had no effect on TNF- α production in these cultures (Fig. 3A). Pretreatment of THP-1 cells with anti-TLR4

mAb also resulted in no noticeable reduction in IL-1 β (data not shown) or TNF- α (Fig. 3B) production upon subsequent stimulation with *P. gingivalis* LPS, as compared with the medium-pretreated and isotype control groups. In sharp contrast, preincubation of THP-1 cells with the mAb to TLR2 significantly ($p < 0.05$) inhibited TNF- α production in response to *P. gingivalis* LPS (Fig. 3B). Similar results were obtained with elutriated human monocytes (data not shown). These results demonstrate that *P. gingivalis* and *E. coli* LPS utilize different TLRs in the human THP-1 monocytic cell line, confirming the findings of others (9, 10, 18, 20, 26, 45).

Involvement of TLR2 and TLR4 in endotoxin tolerance

Due to the ability of *E. coli* and *P. gingivalis* LPS to utilize TLR4 and TLR2, respectively, in THP-1 cells to induce in vitro tolerance, we next sought to determine the outcome of abrogating the ability of these LPS to utilize TLR2 or TLR4 during the primary (i.e., tolerizing) exposure, as reflected by the secondary (challenge) responses to *E. coli* or *P. gingivalis* LPS. The addition of anti-TLR2 mAb during pretreatment of THP-1 cells with *E. coli* LPS did not affect its ability to induce endotoxin tolerance (Fig. 4A). However, exposure of THP-1 cells to anti-TLR4 mAb during the primary stimulation with *E. coli* LPS resulted in levels of TNF- α upon challenge that were comparable to those observed in medium-pretreated cells stimulated with *E. coli* (Fig. 4A). As seen in Fig. 2, THP-1 cells pretreated with *P. gingivalis* LPS (1 μ g/ml), followed by a secondary stimulation with *P. gingivalis* LPS, elicited a statistically significant ($p < 0.05$) enhancement in TNF- α production compared with that seen in cultures receiving only a primary LPS stimulation (Fig. 4B). Preincubation of THP-1 cells with anti-TLR4 mAb during the primary stimulation with *P. gingivalis* LPS did not significantly modulate IL-1 β (data not shown) or TNF- α (Fig. 4B) production after secondary challenge. In contrast, preincubation of THP-1 cells with anti-TLR2 during the primary stimulation with *P. gingivalis* LPS abrogated the enhanced secondary TNF- α response and resulted in levels of TNF- α that were similar to those observed after a primary stimulation alone (Fig. 4B). Thus, the altered secondary responses observed with cultures pretreated with *E. coli* or *P. gingivalis* LPS are mediated, in part, via TLR4 or TLR2, respectively.

Surface expression of CD14, TLR2, and TLR4 after a primary LPS exposure

Previous studies assessing the induction of endotoxin tolerance by enterobacterial LPS have demonstrated that pretreatment of peritoneal macrophages results in a down-regulation of a murine cell surface epitope that depends on the interaction of TLR4 with its accessory molecule, MD-2 (36). This decrease in TLR4/MD-2 expression was correlated with a reduction in the production of proinflammatory cytokines upon secondary exposure to LPS. Our results suggest that while *P. gingivalis* LPS induced tolerance with respect to IL-1 β , it did not induce a state of unresponsiveness with respect to TNF- α or IL-6 levels upon a secondary exposure. Therefore, we next wanted to assess the effects of both *E. coli* and *P. gingivalis* LPS on the expression of the proximal signaling molecules, CD14, TLR2, and TLR4. Pretreatment of THP-1 cells with various concentrations of *E. coli* LPS did not significantly affect CD14 expression as compared with medium-treated controls (Fig. 5A). In contrast, pretreatment of THP-1 cells with various concentrations of *P. gingivalis* LPS resulted in a significant ($p < 0.05$) enhancement of surface CD14 expression (Fig. 5A). Assessment of TLR2 and TLR4 expression on THP-1 cells after a 24-h incubation with *E. coli* LPS revealed a significant ($p < 0.05$) reduction in TLR4 expression to near background levels, as represented by the

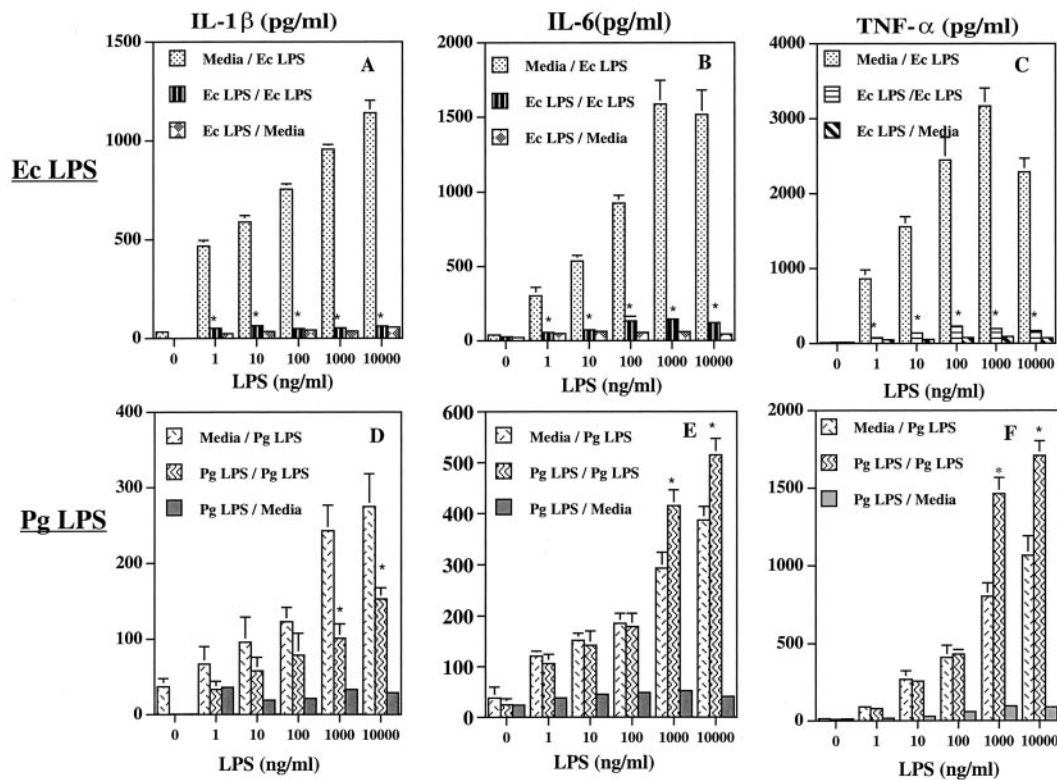


FIGURE 2. Comparison of IL-1β (A and D), IL-6 (B and E), or TNF-α (C and F) production by THP-1 cells that were pretreated with medium, *P. gingivalis* (Pg) LPS, or *E. coli* (Ec) LPS for 24 h, washed, and then incubated with medium, *P. gingivalis*, or *E. coli* LPS for an additional 24 h. Cell-free supernatants were collected and assayed by ELISA. *, Significant differences ($p < 0.05$) compared with medium pretreatment. Data are expressed as the mean \pm SD ($n = 6$).

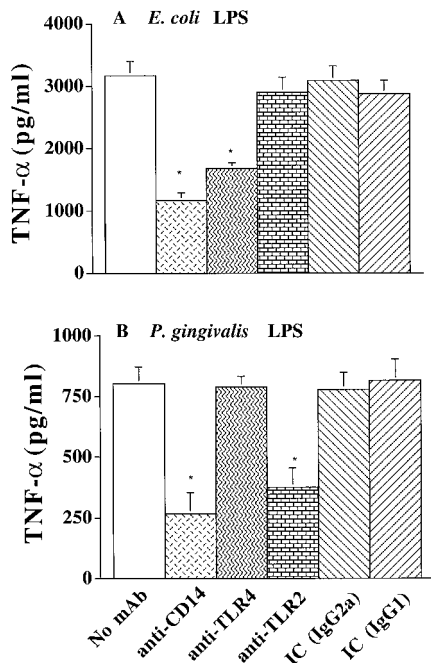


FIGURE 3. Effects of mAbs to TLR-2, TLR-4, or CD14 on the production of TNF-α by THP-1 cells stimulated with *E. coli* (A) or *P. gingivalis* (B) LPS. Before the addition of *P. gingivalis* or *E. coli* LPS (1 μ g/ml), THP-1 cells were pretreated for 30 min with anti-TLR-2, anti-TLR-4, or anti-CD14 mAbs. The levels of TNF-α were determined by ELISA. *, Significant differences ($p < 0.05$) compared with no mAb pretreatment. Data are expressed as the mean \pm SD ($n = 6$).

isotype control group (Fig. 5B). The reduction in TLR4 levels observed with *E. coli* LPS-treated THP-1 cells could partially be overcome by coculturing a blocking Ab to TLR4 (Fig. 5B). However, TLR2 expression on THP-1 cells was essentially identical to medium-treated controls when cultured with *E. coli* LPS (Fig. 5B), while incubation of THP-1 cells with *P. gingivalis* LPS for 24 h resulted in a significant ($p < 0.05$) up-regulation of TLR2 surface expression. This increase could be reduced to levels observed in the untreated control group by concurrent treatment of cells with anti-TLR2 mAb (Fig. 5B). These data demonstrate that *E. coli* and *P. gingivalis* LPS differentially affect CD14 and TLR expression in THP-1 cells.

Differential degradation of I-κB-α and I-κB-β upon secondary exposure to E. coli or P. gingivalis LPS

Activation of NF-κB is achieved via phosphorylation, ubiquitination, and degradation of I-κB proteins (46). This process allows for the translocation of NF-κB to the nucleus and activation of transcription. Previous studies addressing I-κB-α and I-κB-β proteins in LPS-pretreated macrophages have demonstrated that the degradation of these proteins upon a secondary stimulation is severely suppressed in murine peritoneal macrophages (35). Thus, we wanted to determine whether pretreatment of THP-1 cells with *E. coli* or *P. gingivalis* LPS resulted in altered degradation of I-κB proteins upon secondary stimulation. As shown in Fig. 6, *E. coli* LPS was able to induce degradation of both I-κB-α and I-κB-β proteins upon a primary stimulation. In contrast, *P. gingivalis* LPS exhibited noticeable I-κB-β degradation, but not I-κB-α degradation (Fig. 6). Pretreatment of THP-1 cells with *E. coli* LPS resulted in a severe reduction at all time points tested in the ability to

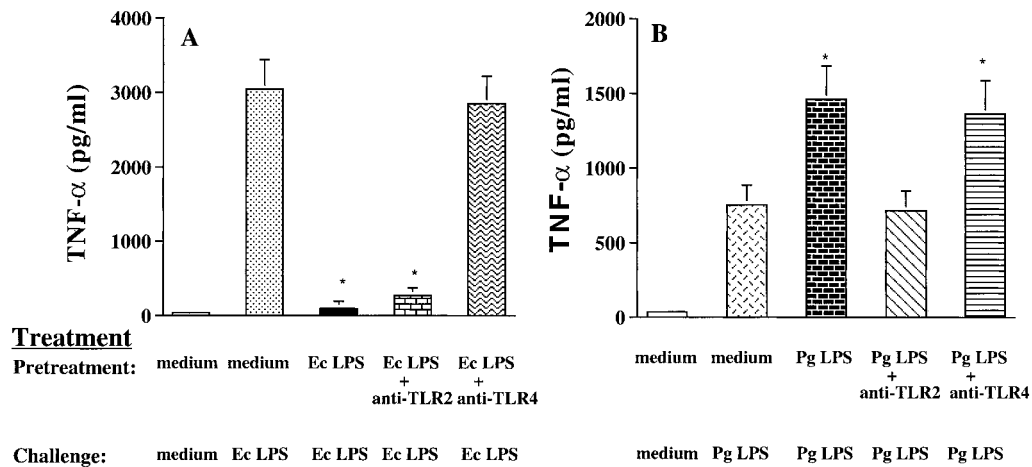


FIGURE 4. Pretreatment of THP-1 cells with mAbs to TLR-2 or TLR-4 during a primary stimulation with *E. coli* (A, Ec) or *P. gingivalis* (B, Pg) LPS affects secondary TNF- α responses to the same stimuli. THP-1 cells were pretreated for 30 min with mAbs to TLR-2 or TLR-4 before the addition of *P. gingivalis* or *E. coli* (1 μ g/ml) LPS for 24 h. Before the secondary stimulation, THP-1 cells were washed and restimulated for an additional 24 h with *P. gingivalis* or *E. coli* LPS. *, Significant differences ($p < 0.05$) compared with primary stimulation. Data are expressed as the mean \pm SD ($n = 6$).

degrade either I- κ B- α or I- κ B- β proteins upon secondary stimulation. In contrast, *P. gingivalis* LPS-pretreated THP-1 cells retained the ability to degrade I- κ B- β , but exhibited no notable degradation of I- κ B- α upon secondary stimulation (Fig. 6).

Discussion

A previous study that assessed the mechanisms of endotoxin tolerance has demonstrated that tolerant macrophages exhibit severe alterations in several LPS-signaling molecules, including the down-regulation of surface TLR4/MD-2 expression (36). Our present findings with *E. coli* and *P. gingivalis* LPS pretreatment of THP-1 cells demonstrate that these two types of LPS differentially

modulate CD14, TLR2, and TLR4 surface expression, as well as primary and secondary cytokine responses.

Studies assessing TLR regulation have shown that treatment of macrophages with LPS or its derivatives, or with cytokines including TNF- α , IL-1 α , and IL-1 β , results in an up-regulation of TLR2, but not TLR4, expression (47, 48). Infection of murine macrophages with *Mycobacterium avium* has also been shown to result in increased TLR2 mRNA and surface expression, and it was suggested that the up-regulation of TLR2 was mediated via the interaction between this bacterium with TLR2 (48). Indeed, these findings are consistent with our observations concerning the ability of *P. gingivalis* LPS to utilize and subsequently up-regulate TLR2 expression. Although the precise mechanism of TLR2 up-regulation is currently unknown, it has been shown that blocking NF- κ B activation abrogates the induction of TLR2 mRNA (47). Analysis of the 5' upstream promoter region of the *tlr2* gene demonstrates the existence of two NF- κ B sites that are important in the ability of LPS to increase *tlr2* gene expression (49). Thus, the ability of *P. gingivalis* LPS to maintain NF- κ B activity and subsequent cytokine production could account for the observed levels of up-regulated TLR2.

In the present study, evidence is provided that demonstrates major differences in the induction of endotoxin tolerance when using a TLR2- compared with a TLR4-specific agonist. Our finding that 1 ng/ml *E. coli* LPS and 10,000 ng/ml *P. gingivalis* LPS induced similar levels of IL-6 and TNF- α from THP-1 cells upon a primary stimulation, while subsequently exhibiting major differences in cytokine production upon secondary stimulation, suggests that divergent pathways utilized by these two types of LPS are responsible for their differential capacities for inducing tolerance. These findings are consistent with previous reports demonstrating an association between TLR usage and differences in the cellular responses induced by some TLR2 and TLR4 agonists (26, 50, 51). However, it has also been reported that there are distinct cellular responses induced by some ligands that utilize the same TLR (52). Reports assessing cross-tolerization and hyporesponsiveness among some TLR2 and TLR4 ligands have also shown that while quantitative differences do exist, their ability to induce tolerance or cross-tolerance was not directly associated with being a TLR2- or TLR4-specific agonist (53, 54). Taken together, while it is unlikely that the differential induction of cellular signaling is solely associated

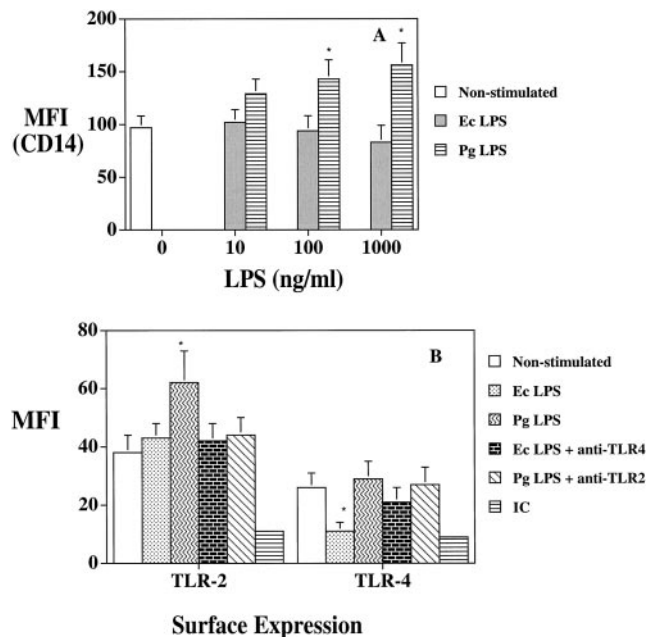
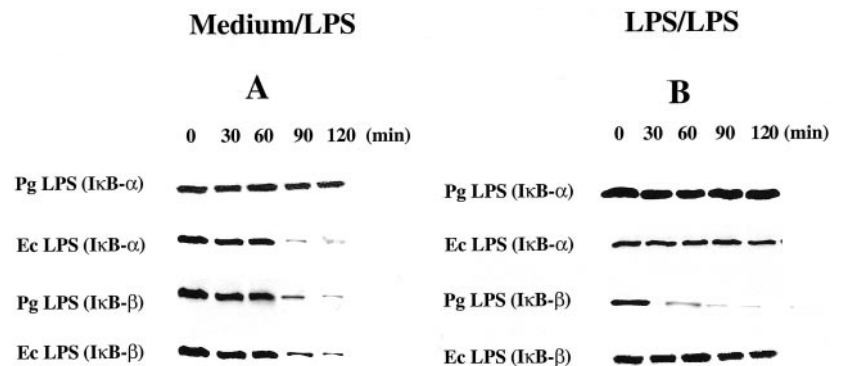


FIGURE 5. Surface expression of CD14 (A) or TLR-2 and TLR-4 (B) on THP-1 cells incubated in medium alone or stimulated with *P. gingivalis* (Pg) or *E. coli* (Ec) LPS for 24 h. The levels of CD14, TLR-2, and TLR-4 expression were determined by flow cytometry. Data are expressed as the mean fluorescence intensity \pm SD ($n = 6$). *, Significant differences ($p < 0.05$) compared with nonstimulated controls.

FIGURE 6. Assessment of I- κ B- α and I- κ B- β protein degradation after primary (A) and secondary (B) exposure of THP-1 cells to homologous LPS. THP-1 cells were incubated in media or pretreated with *E. coli* (Ec) or *P. gingivalis* (Pg) LPS (1 μ g/ml) for 24 h. After washing, cells were restimulated with either *E. coli* or *P. gingivalis* LPS (1 μ g/ml) and harvested at the indicated time points, and cell lysates were analyzed for I- κ B- α or I- κ B- β degradation by Western blot.



with a specific TLR usage, there are clearly divergent pathways activated by various ligands that utilize TLR2 or TLR4. Future studies will be needed to assess how ligand-specific interactions with TLRs result in differential intracellular signaling pathways.

Initial studies assessing the roles of TLR2 and TLR4 suggested that these molecules mediate LPS-associated responses (9, 10). Genetic analysis of hyporesponsive mouse strains demonstrated that the defective LPS responses observed in these mice were the result of a point or null mutation in TLR4 (15). By using TLR2 and TLR4 knockout mice, it was shown that TLR4, and not TLR2, appears to be the functional LPS-signaling receptor for enterobacterial LPS (17). Furthermore, it was demonstrated that the removal of impurities within LPS preparations abrogated the ability of enterobacterial LPS to utilize TLR2, but not TLR4 (18, 20). In contrast, a recent study by Werts et al. (55) assessing the TLR usage of phenol-reextracted *Leptospira interrogans* LPS demonstrated that TLR2, but not TLR4, was involved in the innate immune response to this type of LPS. Additional studies assessing the ability of other nonenterobacterial LPS to signal via TLRs have demonstrated that LPS derived from *P. gingivalis* or *Prevotella intermedia* stimulate mice defective in TLR4 signaling even after repurification and removal of endotoxin contaminants (21). These studies also demonstrated that LPS from *Rhodobacter sphaeroides*, which is known to antagonize enterobacterial LPS, did not inhibit cytokine responses induced by reextracted *P. gingivalis* or *P. intermedia* (21, 56). Evidence for the ability of protein-free *P. gingivalis* LPS to utilize TLR2 and not TLR4 was shown by Hirschfeld et al. (26) using murine macrophages and human cell lines deficient in TLR2 or TLR4. These studies also demonstrated no quantitative or qualitative differences in the ability of *P. gingivalis* LPS to induce cellular responses in mice possessing a defect in TLR4 signaling compared with wild-type controls. Our results confirm and extend these findings by demonstrating that TLR2, and not TLR4, is the predominant TLR utilized by *P. gingivalis* LPS for the induction of cytokine responses by human monocytes, thus providing further evidence that some nonenterobacterial LPS may be utilizing different TLR-signaling pathways than enterobacterial LPS.

The human TLRs have been shown to share considerable structural and functional homology with the IL-1R pathway. The extracellular portion of TLRs contains leucine-rich repeat regions, while the cytoplasmic domain possesses sequence homology to the IL-1 β -like pathway (57, 58). Both the IL-1 β and TLR pathways utilize similar signaling components, including MyD88, IL-1R-associated kinase, and TNFR-activated factor 6, which ultimately leads to the induction of genes via the activation of NF- κ B (59–62). A surprising finding in our study was the reduction for IL-1 β , but not TNF- α or IL-6 production, following a second exposure to *P. gingivalis* LPS. One explanation for these results is that *P. gin-*

gingivalis LPS is a relatively weak inducer of IL-6, TNF- α , and IL-1 β ; however, it is a powerful inducer of IL-1R antagonist (IL-1ra) (44). Thus, if *P. gingivalis* LPS primes cells for enhanced induction of IL-1ra during subsequent challenge, then IL-1ra could inhibit the positive feedback loop for IL-1 production by inhibiting IL-1 binding to its receptor. This could explain the reduced levels of IL-1 β during a secondary challenge with *P. gingivalis* LPS. Furthermore, it could help explain, in part, the inability to induce a refractory state for TNF- α and IL-6 production upon a secondary stimulation. In support of this possibility, Henricson et al. (63) demonstrated that administration of rIL-1ra to mice could reduce the ability of *E. coli* LPS to induce endotoxin tolerance. It has also been reported that pretreatment of macrophages with IL-1 β induces a state of cross-tolerization to LPS (35). Thus, the ability to inhibit or reduce signaling via the IL-1 pathway during LPS exposure may alter the induction of endotoxin tolerance.

Activation of the transcriptional factor, NF- κ B, is regulated by its association in the cytoplasm with I- κ B proteins. These inhibitory proteins mask the nuclear localization signal present in NF- κ B, and thus prevent NF- κ B from translocating into the nucleus. The best-characterized I- κ B proteins are I- κ B- α and I- κ B- β . Although both of these proteins are involved in NF- κ B translocation into the nucleus, the activation of NF- κ B results in a positive feedback loop by which transcription of I- κ B- α is increased and thus serves to inhibit NF- κ B translocation (46). In contrast, I- κ B- β levels remain low until the signal-inducing NF- κ B is attenuated. Our study demonstrated that pretreatment of THP-1 cells with *E. coli* LPS severely decreased the ability of I- κ B- α and I- κ B- β proteins to be degraded upon a secondary stimulation. These findings are in agreement with previous observations demonstrating the inability of endotoxin-tolerant macrophages to degrade I- κ B- α or I- κ B- β proteins and correlate with the down-regulation of upstream LPS-signaling molecules (35). In contrast, THP-1 cells pretreated with *P. gingivalis* LPS exhibited an ability to degrade I- κ B- β , but not I- κ B- α . It has been suggested that I- κ B- α and I- κ B- β proteins can be involved in different NF- κ B-signaling pathways (64). In this regard, Beg et al. demonstrated that I- κ B- α -deficient fibroblasts exhibited normal signal-dependent NF- κ B activation, which correlated with I- κ B- β degradation. It was concluded that I- κ B- β , but not I- κ B- α , was needed for signal-induced NF- κ B activation, while I- κ B- α was required for suppression of NF- κ B activity after stimulation was induced (64). These findings are in agreement with our present findings in which *P. gingivalis* LPS exhibited no significant ability to degrade I- κ B- α upon primary or secondary exposure. Therefore, the ability to differentially degrade I- κ B- α and I- κ B- β upon reexposure of THP-1 cells to *P. gingivalis* LPS suggests that the levels of I- κ B- β may be responsible for the ability to induce secondary LPS-associated cellular responses.

Adult periodontitis is characterized by a chronic inflammatory process that brings about the destruction of the periodontium over a period of years (37, 38). Studies have shown that the LPS of *P. gingivalis* plays an important role in this disease (37, 38, 40). Our study demonstrated that *P. gingivalis* LPS induced much lower levels of the proinflammatory cytokines IL-1 β , IL-6, and TNF- α than *E. coli* LPS, and did not mitigate TNF- α or IL-6 production following secondary stimulation of human monocytes. Interestingly, it has also been shown that *P. gingivalis* LPS fails to induce IL-12 and IFN- γ mRNA production from peritoneal macrophages, which may aid in the clearance of this bacterium (26). Taken together, these observations suggest that the low toxicity of *P. gingivalis* LPS and the pattern of proinflammatory cytokines induced after primary and secondary stimulation could contribute to the chronicity of periodontal infection. Furthermore, our findings reported in this work support the notion that TLR regulation is, in part, one of the mechanisms involved with monocytes acquiring endotoxin tolerance and suggest a novel ability of *P. gingivalis* LPS to circumvent the induction of hyporesponsiveness.

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