Impaired Immune Tolerance to *Porphyromonas gingivalis* Lipopolysaccharide Promotes Neutrophil Migration and Decreased Apoptosis*\(^\text{V}\)

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Periodontitis, a chronic inflammatory disease of the tissues supporting the teeth, is characterized by an exaggerated host immune and inflammatory response to periopathogenic bacteria. Toll-like receptor activation, cytokine network induction, and accumulation of neutrophils at the site of inflammation are important in the host defense against infection. At the same time, induction of immune tolerance and the clearance of neutrophils from the site of infection are essential in the control of the immune response, resolution of inflammation, and prevention of tissue destruction. Using a human monocyte cell line, we demonstrate that *Porphyromonas gingivalis* lipopolysaccharide (LPS), which is a major etiological factor in periodontal disease, induces only partial immune tolerance, with continued high production of interleukin-8 (IL-8) but diminished secretion of tumor necrosis factor alpha (TNF-\(\alpha\)) after repeated challenge. This cytokine response has functional consequences for other immune cells involved in the response to infection. Primary human neutrophils incubated with *P. gingivalis* LPS-treated naïve monocyte supernatant displayed a high migration index and increased apoptosis. In contrast, neutrophils treated with *P. gingivalis* LPS-tolerized monocyte supernatant showed a high migration index but significantly decreased apoptosis. Overall, these findings suggest that induction of an imbalanced immune tolerance in monocytes by *P. gingivalis* LPS, which favors continued secretion of IL-8 but decreased TNF-\(\alpha\) production, may be associated with enhanced migration of neutrophils to the site of infection but also with decreased apoptosis and may play a role in the chronic inflammatory state seen in periodontal disease.

Adult periodontitis is a chronic inflammatory disease of the tissues supporting the teeth, which is characterized by a constant interaction of the microorganisms in subgingival plaque and host defense mechanisms. Most of the tissue destruction in periodontitis is caused not directly by bacteria but by indirect mechanisms (35), such as an exacerbated host response manifesting itself as excessive inflammation, ultimately leading to tissue destruction (4). The inflammation caused by the activation of Toll-like receptors (TLRs), a group of pathogen recognition receptors (PRRs), is very important for detection and removal of the invading pathogen and should benefit the host in the short term by the recruitment and activation of cells and factors that aid in pathogen clearance. However, a prolonged or excessive immune response which leads to chronic inflammation can be detrimental for the host (12).

The resolution of an inflammatory response is a complex process and includes induction of immune tolerance and apoptosis of effector cells (5). Immunologic tolerance is defined as transient unresponsiveness to an antigen that is induced by previous exposure to the same or a different antigen. Induction of immune tolerance leads to a reduced immune response to repeated challenge by microbial antigens (27). The innate oral mucosal immune response becomes tolerant during sustained exposure to bacterial structures, and this may be one mechanism for the regulation of the local immune response (29). Apoptosis is a form of regulated, physiologic cell death in which the nucleus undergoes condensation and fragmentation, mitochondria swell and lose their transmembrane potential, and the apoptotic cells are rapidly phagocytosed without their contents being released (36). This process contrasts with necrosis, a type of cell death in which the nuclear and plasma membranes break down and cellular contents often spill out, inducing a local inflammatory reaction (18). Many inflammatory conditions, such as rheumatoid arthritis and systemic lupus erythematosus, are considered to result from defective apoptosis of immune cells (11).

*Porphyromonas gingivalis* is a Gram-negative anaerobic bacterium implicated as a major periodontal pathogen. Surface components of *P. gingivalis*, such as lipopolysaccharide (LPS), interact with host-expressed TLRs, which are key control elements in the innate immune response (34). TLR activation triggers release of chemokines and cytokines that are responsible for accumulation of inflammatory cells and clearance of infection. It has been well established that as a consequence of...
stimulation with bacterial products, monocytes and macrophages synthesize a broad spectrum of cytokines that have important proinflammatory effects and are related to periodontal tissue destruction (17).

Elevated secretion of various cytokines, including interleukin-1β (IL-1β), IL-6, and IL-8, and tumor necrosis factor alpha (TNF-α), by host cells has been observed following stimulation with P. gingivalis LPS (30). IL-8 is one of the principal mediators of the inflammatory response and a potent chemoattractant of neutrophils. IL-8 attracts leukocytes to the site of infection, leading to neutrophil infiltration which, if not controlled, may culminate in host tissue damage. Therefore, downregulation of IL-8 production is vital in the prevention of chronic inflammation and tissue destruction caused by an influx of neutrophils (31). During the course of inflammatory processes, monocytes, lymphocytes, and mast cells produce TNF-α, a powerful priming agonist for neutrophils (22). It increases neutrophil adhesion to endothelium and promotes release of reactive oxygen species and phagocytosis. However, TNF-α has also been recognized as a critical player in the control of neutrophil life span and induction of apoptosis (24).

In the gingival crevice, neutrophils are the predominant line of defense against invading pathogens (28). They ingest microbes and destroy them by the combination of reactive oxygen species and cytotoxic components of their granules. These cytotoxic molecules cause significant damage to the tissues if the life span and clearance of neutrophils are prolonged (20). The localized accumulation of functional neutrophils at the site of inflammation is important in the host defense against infection, but the proper elimination of neutrophils is equally important in the resolution of the inflammatory response (16).

The aim of this study was to examine the induction of immune tolerance by P. gingivalis LPS and the effect of cytokines produced bytolerized human monocytes on neutrophil chemotaxis and apoptosis. Escherichia coli LPS was used as a positive control LPS, as it is a well-described inducer of immune tolerance (14). In this study, we show that P. gingivalis LPS is able to induce only partial immune tolerance in human monocytes compared to E. coli LPS. Production of TNF-α was significantly decreased in tolerized cells after stimulation with P. gingivalis LPS, but IL-8 production remained as high as in naïve cells. As a consequence, we postulated that P. gingivalis may contribute to tissue destruction by promoting influx of neutrophils and their survival at the site of infection.

MATERIALS AND METHODS

Reagents. LPS from E. coli O55:B5 was obtained from Sigma-Aldrich, Dorset, United Kingdom. LPS from P. gingivalis ATCC 33277 was obtained from InvivoGen, San Diego, CA. Mass spectrometry analysis of its lipid A showed a predominant peak at m/z 1770, which corresponds to the penta-acylated diphasophoryl glycerol isoform.

Cell culture. Human THP-1 monocytes were obtained from the European Collection of Cell Cultures (ECACC) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen, Paisley, United Kingdom). Cells were cultured at 37°C, 100% humidity, and 5% CO2 at a density of 5 × 104 cells/ml. Neutrophils were isolated from venous blood from three healthy donors by dextran sedimentation (Dextran 500; Sigma-Aldrich) and Ficoll-Hypaque gradient centrifugation as described elsewhere (7). Contaminating erythrocytes were removed by hypotonic lysis. Purified neutrophils were resuspended at a concentration of 1 × 106 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen, Paisley, United Kingdom) and maintained in RPMI 1640 medium.

Induction of endotxin tolerance and cytokine measurement. A THP-1 mono-

ocyte sepsis model was used for induction of endotoxin tolerance (15). Briefly, monocytes were treated with 1 μg/ml of E. coli or P. gingivalis LPS (which corresponds to 109 bacteria) for 24 h, washed three times with serum-free medium, and retreated once with the same concentration of LPS for 4 h. Cell-free supernatants from tolerized and naïve cells were collected after 4 h, and IL-8 and TNF-α were measured by using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions (R&D Systems, Abingdon, United Kingdom, and PeproTech EC Ltd., London, United Kingdom, respectively). Cell viability after 24 h pretreatment was confirmed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (3).

Chemotaxis assay. Freshly isolated polymorphonuclear leukocytes (PMNs) were resuspended at a density of 106 cells/ml in RPMI 1640 medium containing 10% bovine serum albumin (Sigma-Aldrich). Chemotaxis was assayed in a 12-mm well modified Boyden chamber (Corning, NY). Resuspended PMNs in a 0.5-ml volume of fresh medium were added to the upper wells, and 0.5 ml of supernatants from naïve or tolerized THP-1 cells were added to the lower wells. Wells containing recombinant human IL-8 alone (R&D Systems) (100 ng/ml) were included as a positive control for neutrophil chemotaxis. Wells containing both forms of LPS alone were included to exclude the influence of LPS on neutrophil chemotaxis. The chamber was incubated at 37°C and cells allowed to migrate for 30 min. Following incubation, the number and viability of cells that migrated to the lower chamber were assessed using trypan blue exclusion. The chemotactic index, i.e., the ratio of the cells in the lower chamber in the presence versus the absence of chemoattractant (random migration induced only by fresh medium), was determined for each sample. The experiment protocol was performed three times, and the mean chemotactic index values are presented.

Apoptosis assessment. Neutrophil apoptosis was assessed by caspase-3 activity and by examining mitochondrial transmembrane potential. Neutrophils were incubated with naïve or tolerized THP-1 supernatants or only with 1 μg/ml of E. coli and P. gingivalis LPS for 3 h, and a fluorometric assay specific for caspase-3 activity was performed. Mitochondrial transmembrane potential was assessed using the MitoCapture kit (BioVision), and results were visualized by laser scanning confocal microscopy. Recombinant human TNF-α (R&D Systems) at a 10 ng/ml final concentration was used as a positive control for induction of neutrophil apoptosis.

(i) Caspase-3 activity determination by Ac-DEVD-AMC cleavage. Caspase-3 activity was measured by a kinetic assay using the caspase-3 fluorogenic substrate Ac-DEVD-AMC (Enzo Life Sciences). Briefly, cultured cells were washed with ice-cold PBS (pH 7.4) and lysed in lysis buffer (10 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM Na2PO4) for 30 min on ice, and the protein concentration was determined according to the bicinchoninic acid (BCA) method. Cell lysates (containing 100 μg of protein) and substrate Ac-DEVD-AMC (20 μM) were combined in a standard reaction buffer (20 mM HEPES [pH 7.5], 10% glycerol, 2 mM dithiothreitol [DTT]). The fluorescence of AMC liberated from Ac-DEVD-AMC was determined every minute over a 45-min period in a Genios Tecan spectrofluorimeter (excitation, 360 nm; emission, 465 nm). The activity of caspase-3 was expressed in relative fluorescence units (RFU) by subtracting the reading at 0 min from that at 45 min. Blanks were measured in the absence of cell lysate to determine background fluorescence.

(ii) Mitochondrial transmembrane potential. After 3 h of incubation in THP-1 supernatants, neutrophils were incubated with MitoCapture reagent (BioVision Inc.) for 15 min at 37°C according to the manufacturer’s instructions and then immediately observed under a laser scanning confocal microscope (Leica TCS SP2). In healthy cells, MitoCapture accumulates in mitochondria, giving off a bright red fluorescence. In apoptotic cells, MitoCapture cannot aggregate in the mitochondria due to the altered mitochondrial transmembrane potential and thus remains in the cytoplasm in its monomer form, fluorescing green. The results are representative of three separate experiments using neutrophils isolated from three different healthy donors.

Statistical analysis. Differences between the means of treatments were ana-

lyzed by the Student t test using GraphPad Prism version 4 (GraphPad Software, San Diego, CA). Differences between multiple treatments were compared by one-way analysis of variance (ANOVA) followed by Tukey’s post test. Values are expressed as the mean± the standard error of the mean (SEM). A P value of <0.05 was considered to represent a statistically significant difference (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
RESULTS

Differential regulation of cytokines/chemokines during E. coli and P. gingivalis LPS-induced endotoxin tolerance. Following initial challenge with 1 μg/ml of E. coli LPS, naïve THP-1 cells reacted by producing high levels of IL-8 and TNF-α. Repeated challenge of THP-1 cells with the same concentration of E. coli LPS significantly decreased production of both examined cytokines (P < 0.01) (Fig. 1). Naïve THP-1 cells treated with 1 μg/ml P. gingivalis LPS reacted in a similar manner as naïve cells treated with E. coli LPS. However, repeated challenge with P. gingivalis LPS almost completely abolished production of TNF-α (P < 0.01), while the IL-8 concentration remained as high as it was after the first challenge (Fig. 2).

Chemotactic potency of THP-1 supernatants. Bacterial products, cytokines, and chemokines can modulate neutrophil migratory potential during the inflammatory response via induction of cytoskeletal changes, neutrophil-endothelial cell interactions, and expression of G protein-coupled receptor. As illustrated in Fig. 3, supernatants collected from naïve THP-1 cells treated with E. coli LPS showed high neutrophil migratory potential, expressed as the number of neutrophils which migrated through the membrane toward the supernatant in the bottom chamber. The number of neutrophils that migrated toward the supernatant collected from E. coli LPS-tolerized THP-1 cells was significantly less than that for supernatant collected from naïve cells (P < 0.05). This finding correlates with the observed decreased IL-8 production in E. coli LPS-tolerized supernatants (Fig. 2). In contrast to the effects obtained with E. coli LPS, supernatants collected from both naïve and tolerized THP-1 cells treated with P. gingivalis LPS showed an equally high level of chemotactic potency, once again correlating with elevated IL-8 levels present in both supernatants. Neither E. coli nor P. gingivalis LPS on its own had any significant effect on neutrophil chemotaxis (Fig. 3). In order to further demonstrate that the IL-8 component of the monocyte supernatants was responsible for the increased neutrophil migration, we incubated monocyte supernatants with IL-8 neutralizing antibodies (R&D Systems) prior to a chemotaxis assay. As a result of this treatment, the supernatants containing IL-8 neutralizing antibodies recruited significantly fewer neutrophils (about 65% fewer) than the media containing the correspondent isotype control antibody (data not shown).

Reduction of neutrophil apoptosis by tolerized THP-1 supernatants. The previous findings suggest that E. coli and P. gingivalis endotoxin tolerances differentially affect cytokine production, and this has significant repercussions for the chemotactic potency of monocyte supernatants in recruiting neutrophils. Given that the tolerized monocytes produce significantly less TNF-α than naïve cells, we investigated the effect of monocyte supernatants on neutrophil apoptosis.

(i) Quantitation of neutrophil apoptosis by caspase-3 activity. Neutrophils incubated in supernatant collected from naïve
THP-1 cells treated with both E. coli and P. gingivalis LPS showed significantly elevated levels of caspase-3 activity, indicating promotion of neutrophil apoptosis compared to that for neutrophils incubated in nontreated THP-1 supernatants (control) (Fig. 4). In neutrophils incubated with supernatant from P. gingivalis LPS-tolerized THP-1 cells, caspase-3 activity was significantly lower \((P < 0.01)\), indicating prolonged neutrophil survival, which correlated with a decreased TNF-\(\alpha\) level in this supernatant. E. coli and P. gingivalis LPSs on their own did not promote neutrophil apoptosis. As expected, recombinant TNF-\(\alpha\) at concentration of 10 ng/ml strongly promoted caspase-3 activity (Fig. 4). In order to confirm the role of TNF-\(\alpha\) in the induction of neutrophil apoptosis by monocye supernatants, macrophage supernatants were treated with TNF-\(\alpha\) neutralizing antibodies (R&D Systems) prior to a caspase-3 activity assay. Treatment of supernatants with a TNF-\(\alpha\) neutralizing antibody significantly reduced neutrophil apoptosis (caspase-3 activity was reduced by 75\%) compared to that for the supernatants containing the corresponding isotype control antibody (data not shown).

(ii) Changes in mitochondrial transmembrane potential. As illustrated in Fig. 5, confocal analysis of neutrophils revealed that the “apoptotic” green signal was more intense in cells treated with naïve THP-1 supernatants. These findings suggest that disrupted mitochondrial transmembrane potential, which is one of the earliest intracellular event that occurs following induction of apoptosis, correlates with the elevated level of TNF-\(\alpha\) in these samples. Neutrophils treated with P. gingivalis LPS-tolerized THP-1 supernatant fluoresced bright red, indicating healthy, active cells with intact mitochondrial transmembrane potential.

**DISCUSSION**

The innate immune system represents the first line of defense against pathogens. TLR activation provides the “danger signal” required for clearance of infection by innate immune cells. While detection of pathogens triggers a robust and essential inflammatory reaction, this process needs to be tightly regulated to prevent extensive tissue damage (6).

Endotoxin tolerance is a protective phenomenon in which prolonged exposure of immune cells to endotoxin causes their progressive inability to respond to subsequent endotoxin challenge. Endotoxin tolerance is important in maintaining homeostasis in the gut and liver, which are exposed daily to a range of microbial components from commensal organisms in the gut (1). Downregulation of the innate immune response promotes host-microbe homeostasis and highly orchestrated expression of certain host defense mediators and cytokines is associated with healthy periodontal tissues (13).
High concentrations of IL-8 have been found in gingival crevicular fluid from periodontitis patients, suggesting an important role for this potent chemokine in disease pathogenesis (21). In our study we observed a high level of IL-8 production by naïve and tolerized human monocytes treated with E. coli LPS. After repeated challenge with P. gingivalis LPS, we observed a decline in TNF-α production, but the IL-8 concentration remained at the same high level as seen after the first challenge. In contrast, endotoxin tolerance induced by E. coli LPS abolished production of both cytokines following a second challenge. Mechanisms involved in the differential induction of endotoxin tolerance by E. coli and P. gingivalis LPS include enhancement of CD14 and TLR2 surface expression and the inability to block degradation of IkBα after pretreatment with P. gingivalis LPS, while pretreatment with E. coli LPS results in a significant reduction in TLR4 expression and reduced degradation of IkBa and IkBβ (26). Although LPS is a major virulence factor of P. gingivalis, the cytokine profile of human monocytes induced by live P. gingivalis may differ from those of its bacterial components (38).

In our study we used penta-acylated diphosphorylated P. gingivalis LPS, as it is the most potent and prevalent isoform of LPSs extracted from clinical isolates (2). Other P. gingivalis LPS isoforms have been reported to have much lower bioactivity, and some of them may even be antagonists for TLRs (10). It is difficult to compare our results with other studies because either different isotypes of P. gingivalis were used or there is no information regarding the lipid A structure of LPS used in those studies. Martin et al. observed a decline in IL-1β but not in IL-6 and TNF-α production in THP-1 cells tolerized with P. gingivalis LPS, while production of all three cytokines was diminished in cells tolerized with E. coli LPS (26). Shimauchi et al. found downregulation of IL-6 production but upregulation of IL-8 in P. gingivalis pretreated cells. Those authors observed that downregulation of IL-6 was dependent on secretion of the anti-inflammatory cytokine IL-10, which did not affect IL-8 production (32). Muthukuru et al. reported downregulation of TLR expression in gingival tissue samples from chronic periodontitis patients, but IL-8 production by monocytes from the same patients was more resistant to downregulation following repeated challenge with both P. gingivalis LPS and E. coli LPS, compared to other cytokines (29). Disrupted cytokine production by P. gingivalis- tolerized monocytes could have consequences for other immune cells, such as neutrophils, involved in the host response to infection.

Neutrophil recruitment to sites of infection is a critical element of the innate immune response. IL-8 has been the most widely acknowledged neutrophil chemotractant. It has been shown that respiratory failure and airway damage in cystic fibrosis patients result from increased production of IL-8, neutrophil infiltration, and an excessive immune response (23). Inflammatory bowel disease (IBD), Crohn’s disease (CD), and ulcerative colitis are disorders of unknown etiology characterized by chronic relapsing-remitting inflammation of the gastrointestinal tract. A pathological hallmark of active IBD is a strong migration of neutrophils into the mucosa, which can be characterized found in the lamina propria and in the epithelial layer in IBD patients. Increased and deregulated recruitment and overactivation of neutrophils have been shown to contribute to tissue damage in chronic inflammatory disorders and constitute a key pathological feature of both IBD and colitis (33). Our results show that supernatants collected from E. coli-tolerized THP-1 monocytes have much less chemotactic potential than supernatant collected from naive THP-1 cells treated with E. coli LPS. In contrast, supernatants collected from P. gingivalis-tolerized cells retain a high neutrophil migratory potential. This may, at least partially, be a result of the persistently high production of IL-8 in P. gingivalis LPS-tolerized cells.

The homeostasis of neutrophils maintained by the balance between production and death is considered very important for an appropriate and correct immune response (25). The prolonged life span of neutrophils in the periodontium results in the release of numerous tissue-damaging enzymes, such as collagenase, elastase, and hyaluronidase, which may contribute to epithelial and connective tissue destruction and eventually alveolar bone resorption characteristic of periodontitis (37).

Apoptosis of neutrophils and their subsequent removal by macrophages facilitates the resolution of inflammation (19). Caspase-3 is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the cascade of events associated with apoptosis. The presence of caspase-3 in cells of different lineages suggests that caspase-3 is a key enzyme required for the execution of apoptosis (9). Mitochondria combine life-supporting functions with participation in apoptosis by controlling caspase activity (23). Decreased production of TNF-α and delayed neutrophil apoptosis have a significant effect on pathogenesis of periodontitis. Gamonal et al. reported reduced levels of TNF-α in the gingival crevicular fluid in periodontitis patients compared to that in healthy subjects. In addition, those authors observed lower numbers of apoptotic neutrophils in gingival tissue sections from periodontitis patients than in healthy biopsy specimens (16). Brown et al. reported delayed apoptosis in inflammatory cells and deficient production of anti-inflammatory cytokines in patients with chronic granulomatous disease (8), while Murray and Wilton showed that P. gingivalis LPS has the ability to prolong the life span of neutrophils while causing their inappropriate activation (28).

We observed elevated caspase-3 activity in neutrophils incubated with supernatants from naïve THP-1 cells treated with either E. coli or P. gingivalis LPS. Supernatant from P. gingivalis-tolerized THP-1 cells did not have any proapoptotic properties, and caspase-3 activity was reduced to the level of spontaneous cell death. Promotion of apoptosis was a consequence of secreted products present in supernatant from THP-1 cells, as both LPSs on their own failed to induce neutrophil apoptosis at the time point examined. In agreement with these data, mitochondrial transmembrane potential was disrupted in neutrophils treated with naïve THP-1 supernatant, suggesting induction of cell death, while it was completely preserved in neutrophils incubated with P. gingivalis-tolerized THP-1 supernatant. A delay in neutrophil apoptosis may be a consequence of the diminished production of TNF-α in P. gingivalis LPS-tolerized human monocytes, since recombinant TNF-α, used as a positive control, strongly induced caspase-3 activity and changes in mitochondrial transmembrane potential.

In conclusion, our findings suggest that impaired induction of immune tolerance by P. gingivalis LPS may lead to a disrupted cytokine response important for maintaining periodon-
tal health. High production of chemokines (e.g., IL-8) in combination with diminished secretion of proapoptotic signals such as TNF-α by human monocytes may cause persistent neutrophil migration to the site of infection with a delay in their physiological clearance. Such a delay in neutrophil apoptosis would prolong the inflammatory response, with a consequent increased potential for tissue damage. Induction of endotoxin tolerance and cross-tolerance by LPNs from other Gram-negative species involved in pathogenesis of periodontal disease should be examined to determine if this effect on neutrophil chemotaxis and apoptosis is restricted to P. gingivalis or is shared with other species.

REFERENCES


