Porphyromonas gingivalis lipopolysaccharide: an unusual pattern recognition receptor ligand for the innate host defense system

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Lipopolysaccharide (LPS) is a key inflammatory mediator. Due to its ability to potently activate host inflammatory and innate defense responses, it has been proposed to function as an important molecule that alerts the host of potential bacterial infection. However, although highly conserved, LPS contains important structural differences among different bacterial species that can significantly alter host responses. For example, LPS obtained from *Porphyromonas gingivalis*, an etiologic agent for periodontitis, causes a highly unusual host innate host response. It is an agonist for human monocytes and an antagonist for human endothelial cells. Correspondingly, although it activates p38 MAP kinase in human monocytes, *P. gingivalis* LPS does not activate p38 nor ERK MAP kinase in endothelial cells. In fact, *P. gingivalis* LPS is an effective inhibitor of *Escherichia coli* LPS induced p38 phosphorylation. These data show that *P. gingivalis* LPS modulates host defenses in endothelial cells by interfering with MAP kinase activation. In addition, *P. gingivalis* LPS is unusual in that it engages TLR-2 but not TLR-4 when examined in stably transfected CHO cell lines. We propose that, since LPS is a key ligand for the human innate host defense system, these unusual properties of *P. gingivalis* LPS are associated with the bacterium's role in the pathogenesis of periodontitis. □ *Inflammation; innate defense; lipopolysaccharide;* Porphyromonas gingivalis

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Periodontitis is a bacterially induced chronic inflammatory disease that is the major cause for tooth loss in the adult population (1). The disease has been associated with cardiovascular disease (2) and pre-term delivery of low birth weight infants (3). It represents a significant impairment to the quality of life of the adult population as a whole and potentially may serve as a bacterial or inflammatory reservoir for more significant health risks. The current disease paradigm for the manifestation of periodontitis involves both a bacterial and a host component (4). Bacterial virulence factors, in conjunction with the host response to bacterial infection, are believed to be responsible for the connective tissue destruction and alveolar bone resorption characteristic of the disease. In this report interactions between the inflammatory component of the innate host defense system and lipopolysaccharide (LPS) obtained from *Porphyromonas gingivalis*, a periodontopathogen, will be discussed.

Innate defense status of the periodontium

The inflammatory response is a fundamental component of the innate host defense system. The response facilitates the movement of serum-soluble and cellular components out of the vasculature and into the specific location in tissue where microbial invasion is detected. In the periodontium the highly vascularized tissue surrounding the tooth root surface provides an abundant source of serum components and in fact is the source of gingival crevicular fluid, a serum exudate. Although inflammation is normally thought of as a response to infection and is associated with disease, there is good evidence that in healthy tissue, bacteria and their antigens are recognized, and a low-level, potentially protective response is generated (5–11). Early histological studies (9) showed that clinically healthy tissue contains a 'wall' of neutrophils precisely located between bacteria and the junctional epithelium, the periodontal tissue in closest proximity to the colonized tooth surface (6). More recent molecular characterization has shown that interleukin (IL)-8, E-selectin, and ICAM-1 are expressed in clinically healthy tissue (5, 8, 10–12). Indeed, it has been shown that a gradient of IL-8 and ICAM-1 expression exists in clinically healthy tissue (13). IL-8 expression is greatest at the most superficial junctional epithelial cell layers, and the levels of ICAM-1 increased toward areas exposed to bacterial challenges. These inflammatory mediators are necessary for leukocyte diapedesis from the vasculature and direct movement through tissue. Gingival epithelial cells secrete IL-8, a potent neutrophil chemokine, and these cells represent the most likely source for this inflammatory mediator found in healthy tissue, although both endothelial cells and fibroblasts can secrete this molecule. E-selectin expression on endothelial cells facilitates a tethering interaction between the leukocyte and the endothelial cell wall initiating the 'rolling' stage required for leukocyte exit (14). It is likely that the highly regulated and ordered expression of these molecular mediators of inflammation facilitates the orderly exit of neutrophils out

of the vasculature and into the gingival crevice, where they are crucial in protecting the host from bacterial challenge (13).

The highly ordered inflammatory status of healthy individuals is necessary to prevent periodontitis. The loss of the protective neutrophilic barrier function either by congenital deficiency (15–18) or by chemical induction with antimitotic agents such as cyclophosphamide (19–22) invariably leads to disease. In addition, there is a strong correlation between a congenital neutrophil chemotaxis defect and certain forms of early onset of disease (early-onset periodontitis (EOP)) (15, 23). Studies have also shown that the lack of an intact innate host defense system may be responsible for the significantly increased incidence of severe periodontitis observed in both diabetic patients (type I and type II) and tobacco users (24–28). These data are all consistent with the notion that diminished neutrophil function or localization is the key to the development of periodontitis.

The bacterial population and the nature of the inflammatory response associated with periodontitis are markedly different than those found in the clinically healthy individual. The most common forms of periodontitis are invariably associated with large numbers of bacteria (up to and possibly exceeding 10^8/site) living on or adjacent to the tooth root surface (29). These bacteria form subgingival plaque that consists of a consortium of at least 200 different species of bacteria (30). The bacteria live in a highly organized biofilm community (31–33) that firmly entrenches itself onto the tooth root surface and becomes recalcitrant to host defense removal. Subgingival plaque's role in disease includes providing a platform for bacterial antigen release and deeper invasion of periodontal tissues by periopathogens. Bacterial antigens, such as LPS, have been shown to penetrate periodontal tissue, exposing nearly all cell types to this often potent inflammatory mediator (34–38). Bacterial antigen interaction with the host immune system is believed to be the basis for the destructive inflammatory response found in active disease (39). Consistent with more bacterial components being released and deeper penetration of these components into periodontal tissue, there are marked changes in inflammatory mediators found in diseased tissue. Both increased levels of the same inflammatory mediators present in healthy tissue (such as E-selectin, ICAM-1, and IL-8) and new mediators such as IL-1b and IL-1b receptor antagonist and prostaglandin E_2 (PGE_2) are found both in tissue and gingival cervical fluid (40–43). In addition, increased local concentrations of inflammatory mediators are found deep within periodontal tissue (44). The production of inflammatory mediators deep within periodontal tissue can have devastating effects on the highly organized innate host defense molecular architecture found in clinically healthy tissue. For example, PGE_2 has been shown in vitro (45) and a by at least one clinical correlation in vivo (46) to block the ability of leukocytes to respond to chemotactic stimuli. The disorientation of this neutrophil property may severely hinder the ability of the host to maintain a protective state and may contribute to premature degranulation contributing to the host tissue destruction characteristic of disease.

Recognition of bacterial components by the innate host defense system

Although it is now clear that inflammation is active both in oral health and in disease (29) and that the microbial composition changes during the transition from health to disease (1, 30), little is known about how these changes influence the inflammatory response. Recently, it was proposed that one mechanism the host uses to regulate the inflammatory response to different bacteria is by recognition of select bacterial structures. This concept, termed pattern recognition, was originally proposed by Janeway (47) and suggests that the host has evolved receptors (termed pattern recognition receptors) that recognize common conserved structures found in various different microbes. The 'pattern recognition' hypothesis describes the characteristics of microbial pattern recognition receptor ligands as being shared by a large group of microbes and being conserved essential structures that are distinct from self-antigens. LPS clearly contains these characteristics and has a long history of interacting with the host immune system. For example, more than two hundred years ago it was observed that neoplasms often regressed after acute infections (48). Therapeutic studies with bacterial fractions led to the discovery of 'hemorrhagic necrosis' of tumors (49), with bacterial LPS being identified as the active component, and the eventual discovery of tumor necrosis factor (TNF)-α (50), a potent cytokine. Therefore it is not surprising that LPS is the best characterized pattern recognition receptor ligand and that numerous studies have shown that the host uses this molecule to detect both microbial colonization and infection.

Lipopolysaccharide activation of host cells

Lipopolysaccharide binding protein (LBP), CD14, and Toll-like receptors (TLRs) are part of a major innate host defense inflammatory activation pathway that recognizes and facilitates the host inflammatory response to LPS (47, 51–54). It has been shown that LPS, initially binds LBP, which is an acute-phase serum protein; LBP then facilitates the transfer of LPS to either mCD14, a membrane-bound form, or sCD14, a soluble form found in serum (55–57). Myeloid cells contain mCD14 as a glycosyl-phosphatidylinositol-anchored membrane protein, whereas the sCD14/LPS complex is required for non-myeloid cell activation by LPS. In vivo, non-myeloid cells may be activated directly by the sCD14/LPS complex or indirectly by LPS interaction with myeloid cells containing

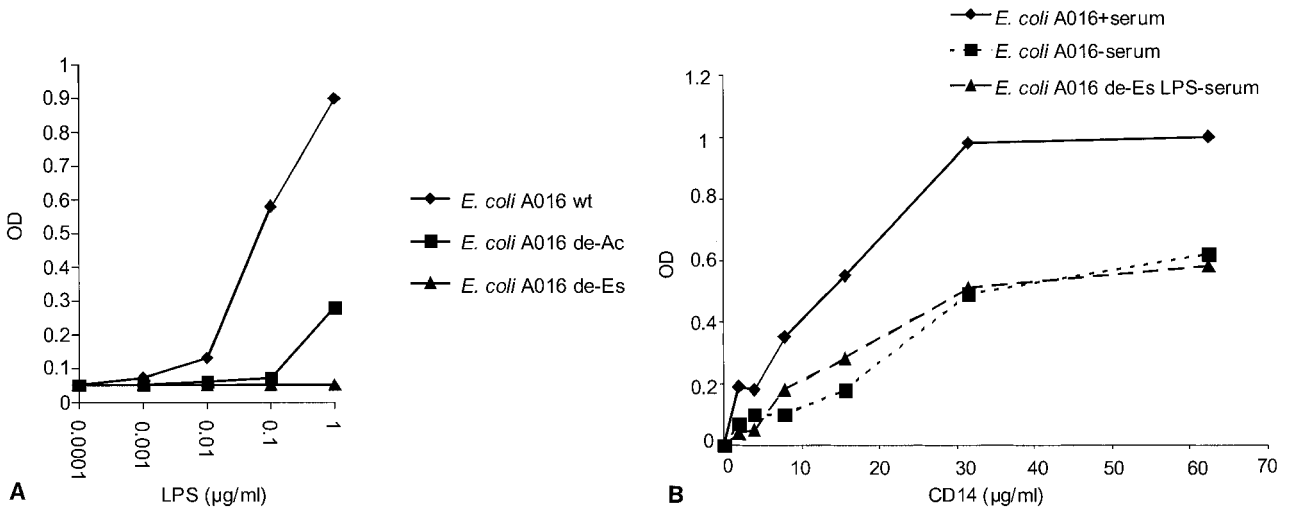

Fig. 1. The effect of fatty acid acylation on lipopolysaccharide (LPS) binding to LPS-binding protein (LBP) and transfer to sCD14. The ability of *Escherichia coli* LPS with differing numbers of fatty acids attached to lipid A to bind LBP and transfer to sCD14 was determined with the enzyme-linked immunosorbent assay (ELISA)-based LBP and the CD14 binding assay as described (82). In panel A, LBP binding of *E. coli* A016 LPS (wt), A016 LPS that was treated with acyloxyacyl hydrolase (de-Ac, kind gift of Dr. Robert Munford), and A016 LPS that was treated with base to remove ester-linked fatty acids (de-Es) was determined. In panel B, the ability of *E. coli* A016 LPS (wt), and de-Es A016 LPS to transfer to sCD14 with and without serum (as a source of LBP) was examined. Various concentrations of sCD14 were captured on an ELISA plate (indicated on the x-axis), and 1 µg/ml of the different LPS preparations were added; binding was detected with anti *E. coli* LPS monoclonal antibody 3B4 as described (82). Each experiment was performed on three separate occasions with similar results; a representative experiment is shown. OD = optical density.

mCD14 (monocytes, neutrophils), promoting the release of pro-inflammatory cytokines. The importance of CD14 in inflammation has been validated in mice, where CD14 was shown to be required for the development of sepsis, a systemic acute inflammatory event in response to *Escherichia coli* LPS (58). In addition, in a primate model of respiratory infection anti-CD14 therapy was able to reduce *E. coli* LPS-induced inflammation (59).

One role of CD14 is to concentrate microbial components such as LPS on the host cell surface for further recognition by the innate host response system (57). Two lines of evidence have pointed out that recognition of specific lipid A structural details most likely occurs after CD14 binding (60, 61). Kitchens & Munford (61), by careful titration of LBP and LPS, have shown that deacylated LPS (dLPS) inhibits wild-type LPS activation at an uncharacterized site after CD14 binding in the LPS recognition pathway. Further, Delude et al. (60) conclusively showed that inhibition of LPS responses by *Rhodobacter sphaeroides* lipid A and lipid IV_A was not due to LPS recognition by CD14. Both of these studies suggested that host cells recognize LPS antagonists after CD14 binding by an uncharacterized LPS recognition protein. Recently, the TLR family of proteins has been identified that represents a crucial component of the mammalian innate host defense microbial recognition pathway that is able to functionally recognize different microbial ligands. A series of structural (62–64) and functional (62–67) mammalian homologues of the *Drosophila melanogaster* Toll protein have been described that act with CD14 as a co-receptor to facilitate activation of host defense cells. In *Drosophila* Toll regulates anti-fungal responses (68), whereas a Toll homologue designated 18-wheeler is responsible for responses to gram-negative bacteria (69). In a similar fashion different mammalian TLRs regulate responses to different microbial components (70, 71). For example, it has been shown that TLR-4 is a host protein responsible for recognition of the specific structural features of *R. sphaeroides* lipid A and lipid IV_A (70, 72). In addition, TLR-2, when transfected into CHO cells, responds to both gram-positive and gram-negative cell wall components, whereas TLR-4 in the same system does not respond to gram-positive components (71).

Different activities of *Porphyromonas gingivalis* LPS on the innate host inflammatory response

P. gingivalis LPS elicits several unusual host responses when compared with the better-known activity of *E. coli* LPS. Since LPS is a key pattern receptor recognition ligand, these observations suggest that the host 'sees' *P. gingivalis* bacteria differently and modulates its response. Specifically, there are three activities of *P. gingivalis* LPS that are significantly different from that observed for *E. coli* LPS. The first is that *P. gingivalis* LPS is not as potent an activator of human monocytes as *E. coli* LPS (73). The second is that endothelial cells do not express E-selectin in response to *P. gingivalis* LPS (73), and the third is that *P.*

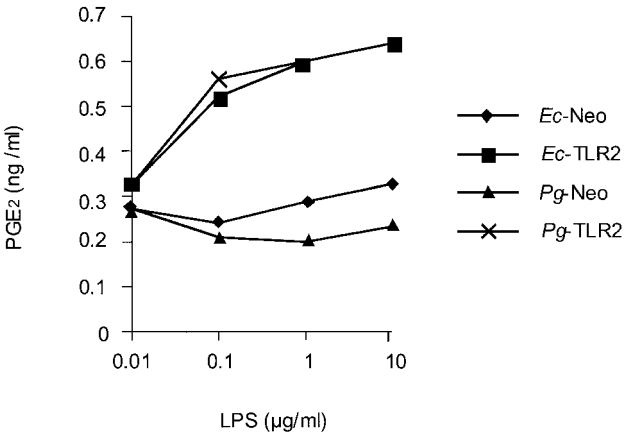

Fig. 2. CHO cell lines were transfected with either pcDNA3 (vector) or Toll-like receptor (TLR)-2 as described by Yoshimura et al. (71). The ability of these cell lines to respond to *Escherichia coli* (Ec) or *Porphyromonas gingivalis* (Pg) lipopolysaccharide (LPS) by the secretion of prostaglandin E_2 (PGE_2) was determined after 18 h of incubation. Cell lines with and without the addition of thrombin-cleaved rsCD14 (1 µg/ml) and rLBP (1 µg/ml) were examined. Recombinant sCD14:Ig and LBP:Ig were cleaved overnight with 1 µg of human thrombin per 5 µg Ig fusion protein to yield rsCD14 and rLBP as described. Three experiments were performed with similar results; a representative experiment is shown.

gingivalis LPS is a natural antagonist of human endothelial cells (73).

Human monocytes are capable of responding to exceedingly low concentrations of *E. coli* LPS by producing a wide array of inflammatory mediators and indirectly activating many other cell types. Some studies have suggested that *P. gingivalis* LPS has an ability to stimulate cytokine production comparable to that of *E. coli*, whereas our work and that of others suggest a much lower potency (74–81). Studies utilizing purified *P. gingivalis* lipid A or the corresponding synthetic structure have indicated that *P. gingivalis* lipid A has low activity in stimulating some cytokines (IL-1, TNF-α) but a comparable activity in stimulating others (IL-6, IL-1ra) (75, 77). We have proposed that microbial component LBP binding affinities can explain, in part, the poor ability of human monocytes to respond to *P. gingivalis* LPS (82). LPS obtained from different bacteria differs significantly in binding affinities for LBP, and this is one mechanism by which the host may be able to differentiate between LPS molecules (82). For example, LPS from both *Helicobacter pylori* and *P. gingivalis* bind LBP 10 to 100 times less efficiently than *E. coli* (82). We have proposed that poor LBP binding accounts for the lower transfer rates to CD14 and the less efficient ability of these LPS species to activate human monocytes (82). Less efficient LBP binding provides one mechanism for the low biological reactivity of these LPS species (76, 82). Consistent with this, reports of LPS activity in in vitro systems have shown that different concentrations of serum (serum is the major source of both LBP and sCD14) or no serum, serum from various species, or heat-inactivated serum affect the level of observed activation (54, 55, 74). The mechanisms by which LPS binds LBP are not known; however, recently a bactericidal/permeability-increasing protein (BPI), a protein closely related to LBP, has been crystallized (83). The structural data show that BPI contains two apolar pockets that interact with the acyl chains of phosphatidylcholine. Accordingly, it was suggested that LBP may interact with the fatty acid chains of LPS. Consistent with this, we have shown that removal of both the secondary fatty acids of *E. coli* lipid A by acyloxyacyl hydrolase enzyme treatment significantly reduced the binding of *E. coli* LPS to LBP (Fig. 1A). Further removal of four ester-linked fatty acids (de-Es LPS) by selective base treatment resulted in no binding to LBP (Fig. 1A). These data indicate that the relative binding affinity of LPS to LBP is significantly influenced by LPS fatty acid acylation. However, although de-esterified LPS binds poorly to LBP (Fig. 1A), it is transferred to both sCD14 and wild-type LPS at increased sCD14 concentrations in the absence LBP (Fig. 1B). This indicates that LPS binding to CD14 is not influenced by fatty acid acylation in the absence of LBP. These data may explain in part how the number and type of fatty acyl chains are key factors influencing the strength of the inflammatory response.

The second unusual property of *P. gingivalis* LPS is that, unlike *E. coli* LPS, it does not cause E-selectin expression on human endothelial cells. However, the lack of efficient LBP-mediated transfer of *P. gingivalis* to CD14 does not explain its failure to elicit E-selectin expression. Since LPS is transferred to CD14 by LBP in an enzymatic fashion (82, 84), comparisons of the ability of different LPS species to interact with CD14 have been performed by measuring the K_m and the $\frac{1}{2}\,Sat_{max}$ (the concentration of LPS required to obtain half the maximum amount of binding to CD14) (82). As mentioned above, these comparisons for LPS obtained from *E. coli* and *P. gingivalis* showed significant differences that correlated with the ability of the LPS species to bind LBP (82). The differences observed, however, were not of sufficient magnitude to explain the inability of *P. gingivalis* LPS to induce CD14 dependent E-selectin expression on endothelial cells. For example, the $\frac{1}{2}\,Sat_{max}$ for *P. gingivalis* LPS binding to CD14 was 22 nM, and for *E. coli* LPS it was 2 nM. This 11-fold difference in binding is not of sufficient magnitude to account for the observation that 50,000 times more *P. gingivalis* LPS (when compared with *E. coli* LPS) did not elicit E-selectin expression. Since the lack of E-selectin expression could not be explained by poor transfer to CD14, it was concluded that either these LPS species bound CD14 sufficiently differently such that additional interactions necessary for E-selectin expression did not occur, or some component of the activation pathway after CD14 presentation of LPS to the endothelial cell did not respond to these LPS species. We have shown that *P. gingivalis* and *E. coli* LPS bind CD14 differently (85). We are currently determining whether this difference in binding may account in part for the failure to activate E-selectin.

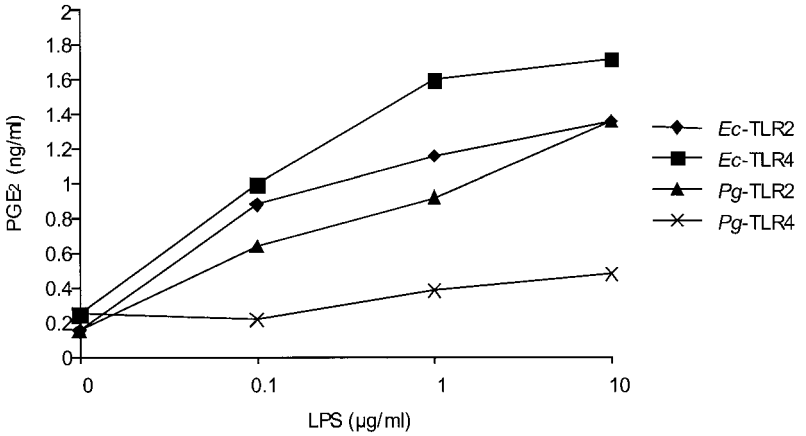

Fig. 3. CHO cell lines were co-transfected with mCD14 and either TLR-2 or Toll-like receptor (TLR)-4 as described in Yoshimura et al. (71). The ability of these cell lines to respond to *Escherichia coli* and *Porphyromonas gingivalis* lipopolysaccharide (LPS) by the secretion of prostaglandin E_2 (PGE_2) was determined after 18 h of incubation.

In addition, however, we have also determined that *P. gingivalis* LPS does not interact with TLRs in the same manner as *E. coli* LPS (Figs. 2 and 3), and this may also partially explain the lack of E-selectin expression in response to this LPS. The experiment shown in Fig. 2 shows that CHO cells transfected with TLR-2 respond to *P. gingivalis* LPS. Various concentrations of both *P. gingivalis* and *E. coli* LPS consistently (three separate experiments were performed) yielded an increase in the amount of PGE_2 secreted into the culture supernatant when compared with control cells transfected with vector only and TLR-2 transfected cells without added LPS. Next, the ability of CHO cell lines co-transfected with either TLR-2 or TLR-4 and mCD14 to respond to *E. coli* and *P. gingivalis* LPS was examined (Fig. 3). Consistent with the data obtained with CHO cells transfected with TLR-2 alone, both *E. coli* and *P. gingivalis* LPS dose-dependent increases in PGE_2 secretion were observed in cells co-transfected with mCD14 and TLR-2. Moreover, consistent with published data, the mCD14 and TLR-4 co transfected cell line responded to *E. coli* LPS. However, the mCD14 and TLR-4 co transfected cell line yielded a minimal response to *P. gingivalis* LPS. The lack of a TLR-4 response to *P. gingivalis* LPS is consistent with the observation that LPS non-responder mice, which have a defect in TLR-4, typically do not respond differently than wild-type mice to *P. gingivalis* LPS (86–88) and shows that *P. gingivalis* LPS uses different TLRs for activation of host cells. This is especially relevant considering that it has been shown that endothelial cells express more TLR-4 than TLR-2 and that transient transfection of human endothelial cells with TLR-2 can facilitate responses to bacterial components that use this TLR (89). Additional work is planned to determine the role of the TLR repertoire in determining endothelial cell responses to *P. gingivalis* LPS.

Finally, *P. gingivalis* LPS inhibits E-selectin expression and neutrophil adherence on HUVEC in response to *E. coli* LPS and cell wall preparations of gram-negative bacteria found in clinically healthy supragingival plaque (76). E-selectin inhibition occurred at the level of mRNA, as shown by the absence of E-selectin transcripts in RNA blot hybridization analysis (76). However, E-selectin antagonism did not occur when TNF-α was combined with *P. gingivalis* LPS, suggesting that *P. gingivalis* LPS is restricted to inhibiting bacterial but not cytokine mediated E-selectin activation. In addition, it was found that *P. gingivalis* LPS did not activate p38 nor ERK MAP kinase. In fact, *P. gingivalis* LPS was an effective inhibitor of *E. coli* LPS-induced p38 phosphorylation. These data show that *P. gingivalis* LPS modulates host defenses in endothelial cells by interfering with MAP kinase activation.

Role of *P. gingivalis* LPS in periodontitis

As mentioned above, in the clinically healthy periodontium there exists a highly orchestrated innate host inflammatory response that serves to protect the host from infection. One possible role of *P. gingivalis* LPS is to disrupt this healthy host/bacterial dynamic. *P. gingivalis* may play a role in the disruption of innate host surveillance by interfering with the ability of the host to position sufficient leukocytes in close proximity to bacterial colonization. *P. gingivalis* LPS blocks E-selectin expression in response to LPS from other bacteria, an adhesion molecule necessary for efficient leukocyte exit from the bloodstream (76). In addition, after *P. gingivalis* invasion of gingival epithelial cells the cells no longer secrete IL-8 in response to other oral bacteria (90). *P. gingivalis* LPS has also been shown to have reduced cytokine-inducing activity both in human monocytes in vitro and in a murine inflammation model in vivo (75, 76, 81, 91, 92). The ability to block both endothelial and epithelial cell responses to other bacteria combined with poor activation of myeloid cells is

consistent with an innate host impairment strategy of pathogenesis. In the absence of an effective innate host defense leukocyte barrier, bacterial numbers of multiple species could increase dramatically, leading to more bacterial antigen release. An increase in the bacterial population surrounding the tooth root surface accompanied by the failure of the host to remove them is consistent with the etiology of periodontitis. Bacterial stimulation without adequate homing to bacterial infection could account for the accumulation of activated inflammatory cells in diseased tissues rather than at the site of bacterial colonization.

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