Published in final edited form as:

Periodontol 2000. 2010 October ; 54(1): 53–70. doi:10.1111/j.1600-0757.2009.00333.x.

Contribution of *Porphyromonas gingivalis* lipopolysaccharide to periodontitis

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Lipopolysaccharide is an essential macromolecule that comprises the outer surface of gram-negative bacteria. Lipopolysaccharide, recognized by the human host as a foreign molecule, elicits an immune response that is designed to eliminate the bacterial intruder. Lipopolysaccharide from enteric bacteria such as *Escherichia coli* provokes a strong immune response. On the molecular level this is because *E. coli* lipid A, the bio-active component of lipopolysaccharide, is a strong agonist of the innate immune receptor, Toll-like receptor 4 (TLR4). However, lipopolysaccharide from the periodontopathogen, *Porphyromonas gingivalis*, elicits a strikingly weak immune response. Additionally, *P. gingivalis* is capable of synthesizing a heterogeneous population of lipid A molecules, which are subtly different from each other in structure. One of these lipid A structures has the capacity to dampen the immune response elicited by lipid A agonists at TLR4. The weak agonistic and antagonistic lipid A structures of *P. gingivalis* are proposed to play an important role in disrupting the oral immune homeostasis required for maintaining oral health, and thereby promote periodontal disease.

Importance of lipopolysaccharide to both bacteria and host

Lipopolysaccharide is the major macromolecule found on the outer surface of gram-negative bacteria (Fig. 1a). The hallmark of gram-negative bacteria is its envelope, which consists of two membranes, the inner and outer membrane, with an intervening periplasmic space. Each membrane is a lipid bilayer consisting of two leaflets. Both leaflets of the inner membrane, which surrounds the cytoplasm, are composed of phospholipids. The outer membrane is an asymmetric bilayer that consists of phospholipids on the inner leaflet and the lipid anchor domain of lipopolysaccharide, lipid A, on the outer leaflet. Lipopolysaccharide is critical to the bacterium for maintaining its structural integrity, and for establishing a selective permeability barrier that limits entry of hydrophobic molecules and toxic chemicals such as detergents and antibiotics (104). Lipopolysaccharide is also required for the proper folding and insertion of many outer membrane proteins. In light of these important functions of lipopolysaccharide, it is perhaps not surprising that lipopolysaccharide is essential for the survival of nearly all gram-negative bacteria (128).

Lipopolysaccharide is typically composed of three domains: lipid A, a short core oligosaccharide, and an O-antigen that may be a long polysaccharide (Fig. 1a) (104,128). Lipid A is the inner-most component of lipopolysaccharide. It is conserved in structure and forms the outer leaflet of the outer membrane. The O-antigen is the outer-most component of the lipopolysaccharide and forms the external surface of the bacterium. The structure of O-antigen is highly variable and highly immunogenic leading to the use of O-serotyping to distinguish between sub-species of bacteria that have different O-antigen compositions.

In mammalian hosts, lipopolysaccharide provokes a strong innate immune response. Lipid A, also known as endotoxin, is the bioactive region of lipopolysaccharide that is recognized by the innate immune system (77). Lipid A from *E. coli*, either commensal or pathogen, is highly immunostimulatory even at low concentrations. Immune detection of lipid A is so sensitive and robust that a bloodstream infection can cause a severe complication called endotoxic shock, or septic shock, a major clinical problem that leads to approximately 200,000 deaths in the United States each year (2).

An enormous amount of research in the last two decades has led to the elucidation of the mechanism by which lipopolysaccharide elicits this potent immune response. The mammalian lipopolysaccharide receptor is a protein complex consisting of Toll-like receptor 4 (TLR4), MD-2 and CD14. This protein complex is present on many cell types including the immune cells, macrophages and dendritic cells (58,120,126,139,140,164). An accessory protein, lipopolysaccharide-binding protein, is required for recognition of lipopolysaccharide. Lipopolysaccharide-binding protein converts oligomeric micelles of lipopolysaccharide to monomeric form and delivers it to CD14, which in turn concentrates lipopolysaccharide for binding to the MD-2-TLR4 complex. Binding of lipopolysaccharide to this complex leads to an intracellular signaling cascade that ultimately results in the release of a number of pro-inflammatory cytokines, including tumor necrosis factor- α , interleukin-1 β , interleukin-6 and interleukin-8 (34,150). Release of these cytokines has been implicated as the etiological agents of a variety of pathologies ranging from mild (fever) to lethal (septic shock, organ failure and death).

The purpose of the innate defense proteins released following recognition of lipopolysaccharide by the TLR4-MD-2-CD14 complex is either bacterial killing or bacterial removal. For example, interleukin-1 β release contributes to increase in the expression of adhesion factors on endothelial cells to enable transmigration of leukocytes, and interleukin-8 is a neutrophil chemoattractant. It is only when the innate immune response becomes over-active or uncontrolled that the host sustains damage, and this runaway response can lead to many disease pathologies.

The general mechanism by which the innate immune system detects microbial invaders is by recognizing molecular patterns that are common to a variety of different microbes. This concept of pattern recognition, which was first put forward by Janeway (90,91), proposes that the host has evolved receptors that recognize common conserved structures, termed Pathogen Associated Molecular Patterns (PAMPs). Recognition of PAMPs results in the immediate, efficient and non-specific innate immune response mentioned above and there is accumulating evidence that it plays a critical role in triggering antigen-specific adaptive immunity as well (92). Lipid A satisfies the requirements of a PAMP in that it is (1) present on almost all known gram-negative bacteria, (2) required for viability of the bacterium (*Neisseria meningitidis* is an exception (146)) and (3) has a conserved structure (see below). Additionally, lipid A is found only on gram-negative bacteria and is, therefore, considered a foreign structure by the host. Consistent with its proposed role as a major PAMP that is recognized by bacteria-monitoring immune cells, *in vitro* studies have confirmed that purified lipid A from a given bacteria can trigger immune responses similar to that triggered by the whole bacteria (143). Moreover, *in vivo* studies with knock-out mice have validated the important role lipopolysaccharide serves in host recognition of bacterial infection (see below). Clearly, innate host recognition of lipopolysaccharide is a key initiating event for the subsequent clearance of gram-negative bacteria from infected host tissues.

The receptors on immune cells that bind PAMPs are called Pattern Recognition Receptors (PRRs). Toll-like Receptor 4 (TLR4) is a founding member of the PRR family of molecules (90) which include other TLRs, found in cell membranes, and Nod-like receptors, which are

intracellular pathogen detectors (147). A total of 10 TLRs are expressed in humans. Each TLR recognizes a different PAMP (of bacterial, viral or fungal origin) and, upon engaging it, triggers a signaling cascade designed to counter the microbial intruder (15). There are two main routes that engaged PRRs use to eliminate infection (67,159). The first is a transcriptional response: TLRs signal via engagement of adaptor molecules, notably MyD88 and TRIF, leading to activation of the NF- κ B and IRF-3/7 families of transcription factors, which in turn activate the transcription of pro-inflammatory cytokines. The second is a post-translational response, elicited mainly by cytosolic PRRs that appear to stimulate the inflammasome, a multiprotein complex that triggers the cleavage and activation of the cysteine protease caspase-1 (37), which in turn cleaves and activates the secretion of a variety of substrates including the pro-inflammatory cytokines interleukin-1 β and interleukin-18.

The importance of the pattern recognition receptor, TLR4, and CD14 and MD-2 proteins in lipopolysaccharide recognition is highlighted by the lack of response of immune cells from mice carrying knockout mutations in any of these genes (49,55,58,97). Also, a lack of these components in mice leads to increased susceptibility to infection by the gram-negative pathogen *Salmonella enterica* serovar *typhimurium*, illustrating the general principle that functional innate immune recognition is important for protection against bacterial infections. Additionally, polymorphisms in these genes in humans reduce the magnitude of the lipopolysaccharide response (3,49).

Structure of lipid A

Of the three regions that constitute lipopolysaccharide, lipid A, core oligosaccharide and O-antigen, it is the lipid A component that forms the outer leaflet of the outer membrane and elicits an inflammatory response from mammalian cells. It is this region of the lipopolysaccharide that will be the focus of this review. The prototypical lipid A structure, also referred to as endotoxin, is the lipid A structure of the enteric bacterium *E. coli* and is shown in Fig. 1b (128). The *E. coli* lipid A molecule consists of a disaccharide (two sugar subunits) backbone to which is attached four fatty acid, or acyl, chains. Two additional acyl chains are subsequently added to two of the primary acyl chains, resulting in a total of six acyl chains. The disaccharide moiety is a β -1',6-linked glucosamine unit. Each sugar unit has six carbon (C) atoms, labeled C1-C6 for the reducing sugar and C1'-C6' for the non-reducing sugar. The four primary acyl chains are bonded to C2, C3, C2' and C3', via an amide linkage to C2 and C2' and via an ester linkage to C3 and C3'. These reactions are catalyzed by distinct acyl transferase proteins LpxD and LpxA respectively. The four primary acyl groups are 3-hydroxy myristic acid chains, i.e. saturated hydrocarbon chains comprising 14 C atoms and an -OH group at position C3 of the chain. The secondary acyl chains are added, via ester links, to the 3-hydroxy groups of the C2' and C3' acyl chains. While the secondary acyl chain added to the C2' chain is lauric acid (12 Cs), the one added to the C3' chain is myristic acid (14 Cs). Finally, both "ends" of the disaccharide backbone, the C1 and C4' positions, are phosphorylated. We shall refer to this structure as 6-acyl, di-P or 6-acyl, 1,4'-P.

The above prototypical structure of lipid A is conserved throughout most enteric bacteria. This structure, recognized by the TLR4-MD-2 complex, triggers the robust host immune response mentioned earlier. Subtle differences in this otherwise conserved structure, however, can alter this immune response. For example, the loss of a secondary acyl chain, generated by mutation of a gene called *msbB* (143) not only fails to activate host innate immune responses but also has the capacity to block or down-regulate the response to the prototypical lipopolysaccharide structure. In other words, this lipid A molecule is capable of antagonizing the response elicited by the *E. coli* lipid A agonist. A second subtly different

lipid A structure is mono-phosphoryl lipid A, which lacks the C1 phosphate, and is derived from degradation of *Salmonella minnesota* lipid A (125). This lipid A structure selectively activates immune cells to induce immune responses without triggering the production of toxic levels of inflammatory mediators, thereby making it a candidate as a vaccine adjuvant (89).

Lipid A structure and, by extension, other PAMP structures are conventionally defined as invariant or highly constrained structures that are extremely difficult for microbes to alter. However, an increasing body of evidence has demonstrated that the extent of lipid A (and other PAMP) structural plasticity is considerable. A pathogen is a microorganism that usually employs strategies such as manipulation or avoidance of host innate immunity to cause disease, and it is presumed that pathogens must be able to avoid innate immunity to some extent. Certainly, the identification in a wide variety of bacteria of lipid A structures that differ from the prototypical structure, and which elicit an altered innate immune response, fits this notion. Examples include lipid A from *Helicobacter pylori* (95,106,111,148), *Francisella tularensis* (160,163) and *Rhizobium* species (11,122,123). It is now also recognized that a single bacterial population may contain more than one lipid A structural type, and that environmental conditions can regulate the switching from one lipid A type to another. Examples of bacteria that switch from one lipid A type to another include (i) *Yersinia pestis*, in response to temperature changes (23,66,131), (ii) *Salmonella enterica* serovar *typhimurium*, in response to low magnesium (48) and (iii) *Pseudomonas aeruginosa*, in response to the altered environment it encounters when it enters the lungs of cystic fibrosis patients (35). Most lipid A structures identified so far that are different from the prototypical lipid A structure, or that are produced in response to an environmental cue, are less potent stimulators of the innate immune response.

Lipid A heterogeneity in the periodontopathogen *Porphyromonas gingivalis*

Dental plaque is an oral microbiological consortium that forms a biofilm on the surface of the tooth and the root of the tooth (76,85). There is a well documented shift in microbial numbers and composition of dental plaque during transition from health to periodontal disease (141). Periodontitis is a chronic inflammatory disease that results in loss of the bone supporting the root of the tooth, eventually leading to tooth loss. Removal of dental plaque by scaling and root planing usually results in excellent clinical outcomes, which indicates that periodontitis is microbial associated. While etiology is not due to one specific bacterial species, the amount and composition of dental plaque is considered a major determinant for occurrence of this chronic inflammatory condition. Microbiological analyses have revealed that in health, the dental plaque biofilm consists mostly of gram-positive species such as *Streptococcus* and *Actinomyces*, and approximately 15% gram-negative species (152). The microbial load is low; 10^2 to 10^3 isolates may be cultured from an individual healthy periodontal pocket. In contrast, diseased sites in the periodontium show a higher microbial load; 10^5 – 10^8 bacteria may be cultured from an individual pocket. Gram-negative species also increase to 15–50% (24,152). Extensive microbial analyses and molecular techniques that identify non-cultivable oral bacteria (72) have revealed the presence of periodontopathogenic bacteria, which are associated with periodontal diseased sites, and prominent among these are the gram-negative anaerobic bacteria *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Prevotella intermedia*. *P. gingivalis*, *T. forsythia* and *T. denticola* have been named the ‘red-complex’ periodontopathogens due to their propensity to group together and due to their association with deep periodontal pockets (142). The mechanisms underlying the shift in microbial composition associated with disease are not currently understood. Neither is it understood how this shift leads to periodontitis.

A crucial early discovery was that gram-negative oral anaerobic bacteria produce lipid A that is a weaker stimulator of innate immune responses than the prototypical lipid A of enteric bacteria (26,38,83,121). The lipid A structure of the oral bacteria *P. gingivalis*, *P. intermedia*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* have been published. The most extensive lipid A structural analysis of any oral bacterium is that of *P. gingivalis*. Numerous studies have shown that *P. gingivalis* lipopolysaccharide is less potent than that of *E. coli* (26,38,82,107,108) and also elicits a different repertoire of inflammatory mediators in innate immune cells (107,151). Lipid A from *P. gingivalis* is unique in that it exhibits remarkable structural heterogeneity (20,27,74,109,130). Four lipid A structural sub-groups have been identified in *P. gingivalis* which, remarkably, can be stimulatory, inert or antagonistic with respect to TLR4 activation.

The *E. coli* lipid A, which is a TLR4 agonist, has a molecular weight (m/z) of 1797. When analyzed by MALDI-TOF MS (Matrix Assisted Laser Desorption/ Ionization - Time of Flight Mass Spectrometry) a single peak is seen at m/z 1797. *P. gingivalis* lipid A, in stark contrast, can show a cluster of peaks at m/z 1688, m/z 1449, m/z 1768 and m/z 1368 depending upon the environmental conditions in which the bacteria are grown. More than a decade of research from many laboratories has identified the lipid A structures corresponding to these peaks, which are as follows (Fig. 2):

1. m/z 1688 is penta-acylated mono-phosphorylated (5-acyl, mono-P) lipid A,
2. m/z 1448 is tetra-acylated mono-phosphorylated (4-acyl, mono-P) lipid A,
3. m/z 1768 is penta-acylated, di- phosphorylated (5-acyl, di-P) lipid A and
4. m/z 1368 is tetra-acylated, non- phosphorylated (4-acyl, non-P) lipid A.

The fully phosphorylated and fully acylated *P. gingivalis* lipid A structure, m/z 1768, is different from that of the prototypical *E. coli* lipid A structure. It lacks the secondary acyl chain that 'piggy-backs' on the C3' primary acyl chain, and its five acyl chains are longer than that of *E. coli* lipid A. The amide-linked chains at C2 and C2' have 17 Cs while the ester linked chains at C3 and C3' have 16 Cs and 15 Cs respectively, in contrast to *E. coli*'s 14 Cs. The odd-chain fatty acids are also called branched chains. The fifth acyl chain, which piggy-backs on the -OH group of the C2' acyl chain is a saturated 16 C chain, palmitic acid, as opposed to the 12 C lauric acid in *E. coli* lipid A. *P. gingivalis* lacks an *msbB* homolog and, therefore, does not make a hexa-acylated lipid A molecule.

Structural heterogeneity was first proposed to reconcile disparate reports from many laboratories that described different lipid A structures for *P. gingivalis*. It was eventually established that all reported lipid A structures could be isolated from *P. gingivalis* depending upon growth conditions and lipopolysaccharide extraction procedure (1,7,27).

One level of *P. gingivalis* lipid A heterogeneity was revealed by the cluster of 4–5 peaks seen around each of the major peaks listed above during analysis of lipid A by MALDI-TOF MS. These multiple peaks differ by 14 atomic mass units, which corresponds to the size of a -CH₂ group. These clusters arise biosynthetically due to the relaxed acyl chain specificity displayed by the *P. gingivalis* fatty acid acyl transferases LpxA and LpxD (8). In other words, the acyl chains added by these transferases may differ in length by one or more saturated carbon units.

A second level of *P. gingivalis* lipid A heterogeneity was revealed by the presence of lipid A species that differed in the number of acyl chains and phosphate groups, as listed above. A key finding was the identification of hemin concentration as an environmental factor that triggers alteration in *P. gingivalis* lipid A structural composition (1). Hemin is an iron source for *P. gingivalis* (113). It is the oxidized form of heme, the prosthetic group attached to

many mammalian proteins, notably hemoglobin found in blood. When grown in the laboratory, an iron source such as hemoglobin or heme is essential for growth of *P. gingivalis*. Accumulation of heme on the surface of the bacterium when grown on blood agar plates leads to its characteristic black colony phenotype. Heme is a relevant physiological microenvironmental factor for *P. gingivalis* since its concentration is low in healthy tissue and high in diseased sites (50,51). Increased heme concentrations in diseased sites are due to an increase in vascularity during inflammation and vascular ulceration that leads to the release of blood into gingival tissue, yielding bleeding-upon-probing-positive sites, a clinical measure of disease. Multiple investigations have revealed that the heme concentration in the growth medium can affect the virulence of *P. gingivalis* (84,113) highlighting the potential relevance of heme in the progression of periodontal disease.

When grown in low heme concentrations of 1 $\mu\text{g/ml}$, which is sufficient for growth of *P. gingivalis*, the 5-acyl, mono-P (m/z 1688) lipid A structure is observed. Application of MALDI-TOF tandem MS revealed that the phosphate group is at the C4' position (20). However, when grown in high heme concentrations of 10 $\mu\text{g/ml}$, the tetra-acyl, mono-P (m/z 1448) lipid A structure is the predominant species, though variable amounts of the 5-acyl, di-P (m/z 1768) lipid A are observed as well. Interestingly, tandem mass spectrometry identified the phosphate group in the m/z 1448 species as present on the C1 position. The above three lipid A structures, m/z 1448, 1688 and 1768, have at least one phosphate group in each moiety. The negatively charged phosphate group enables the observation of lipid A using MALDI-TOF MS in the 'negative mode'. A recent advance was made by the observation of a novel lipid A species that is devoid of phosphate groups (130) at both the C1 and C4' positions. The technique of 'positive mode' MALDI-TOF MS facilitates the observation of this lipid A species (20), which is 4-acyl, non-P lipid A and has a molecular weight (m/z) of 1368. This non-phosphorylated lipid A structure is observed when *P. gingivalis* is grown at either low or high heme concentrations (Figure 3), albeit in a greater proportion relative to other lipid A structures at low heme conditions (1 $\mu\text{g/ml}$) (20).

Strikingly, the distinct *P. gingivalis* lipid A sub-types described above elicit substantially different immune responses. This observation underscores the notion that a subtle change in the lipid A structure results in the lipid A molecule interacting differently with the host innate immune system, and eliciting a different response. Through selective bacterial growth conditions, lipopolysaccharide extraction procedures and mutational analyses, *P. gingivalis* lipopolysaccharide preparations enriched in the various lipid A sub-types have been tested for their ability to elicit an immune response using in vitro cell-culture based methods. Lipopolysaccharide enriched for m/z 1688 and m/z 1768 lipid A are TLR4 agonists (20,132) but elicit an immune response that is not as pronounced as that elicited by *E. coli* lipid A. A lipopolysaccharide preparation comprising m/z 1368 lipid A in addition to a minor amount of m/z 1688 lipid A is a very weak TLR4 agonist. This suggests that the m/z 1368 lipid A, which lacks phosphate groups, is inert with respect to TLR4 activation (20). The non-phosphorylated (m/z 1368) lipid A is the major lipid A form seen in wild-type *P. gingivalis* grown under low heme conditions (Fig. 3A and 3b). In stark contrast, lipopolysaccharide enriched for m/z 1448 lipid A is a TLR4 antagonist (22,26,132,165). This naturally made antagonist has the ability to dampen the immune response elicited by *E. coli* lipid A or *P. gingivalis* m/z 1688 lipid A. The m/z 1448 antagonist lipid A is the significant lipid A species made by *P. gingivalis* during high heme growth conditions (Fig. 3C and 3D).

Many recent structural studies of the human TLR4-MD-2 complex have revealed that lipid A binds to the MD-2 component (21,19,112,117). *P. gingivalis* lipid A antagonist acts by competitively binding the lipid A binding site on MD-2 and, therefore, inhibiting agonist lipid A species from binding MD-2 (19).

In summary, the tetra-acyl non-P lipid A is inert with respect to TLR4 activation, potentially leading to immune evasion, whereas the tetra-acyl 1-P lipid A is a TLR4 antagonist leading to immune suppression. This provides the structural basis for the low immune stimulatory capability of *P. gingivalis* lipid A. The penta-acyl 4'-P and penta-acyl di-P lipid A forms are enriched only in defined *P. gingivalis* mutants and therefore these strong TLR4 agonists are not the predominant species found in wild-type *P. gingivalis* under any observed growth conditions. The immune evasion and immune suppression strategies of *P. gingivalis* via modulation of lipid A structure are exhibited when grown under low and high hemin conditions respectively (Figure 4).

Genetic determinants of *P. gingivalis* lipid A structural heterogeneity

Evidence to date indicates that many environmental modifications of different lipid A structures occur by alteration of a completely synthesized (hexa- or penta-acylated, bi-phosphorylated) lipid A (127). In bacteria that have been examined, all of the genes necessary to synthesize a complete lipid A structure are found, and lipid A structural heterogeneity arises by a remodeling of the complete structure. Consistent with this, lipid A structures that contain fewer fatty acids arise by removal of fatty acids by lipid A deacylases (41,127,135,145). Likewise, lipid A phosphates are removed by lipid A phosphatases after incorporation of the phosphate groups on the lipid A (65,127,157,162). The simplest explanation for modification of an existing completed lipid A structure is that lipid A biosynthesis is sequential and the enzymes for each step demonstrate substrate specificities for subsequent additions on the nascent molecule (127). Modification of the lipid A structure by alteration of the completed molecule increases the number of different lipid A structures that can be generated, presumably conferring survival advantages to the bacterium. *P. gingivalis* appears to be similar to other gram-negative bacteria in that it synthesizes a complete lipid A that initially contains five fatty acids and bears phosphate moieties at both the C1- and C4' position of the di-glucosamine backbone (m/z 1768) (74). Therefore, the mono-phosphorylated, non-phosphorylated and tetra-acylated lipid A structures observed in *P. gingivalis* lipopolysaccharide preparations most likely arise from the action of lipid A phosphatase and lipid A deacylase enzymes.

While the *P. gingivalis* deacylase gene has not been identified, both C1 and C4' phosphatase genes were recently identified (20). The C1-phosphatase is encoded by PGN_1713 in *P. gingivalis* 33277 (PG1773 in *P. gingivalis* W83) (98,99). The C4' phosphatase is encoded by PGN_0524 in isolate 33277 (PG1587 in isolate W83). Since PGN_1713 is required to remove the lipid A C1-phosphate group, a *P. gingivalis* strain that lacks the PGN_1713 gene is expected to generate lipid A species that retains a phosphate group at the C1 position. Indeed, the major lipid A species in a *P. gingivalis* Δ PGN_1713 mutant is tetra-acyl, 1-P (m/z 1448), when grown in low hemin conditions (Figure 3G and 3H) (20). Interestingly, a considerable amount of tetra-acyl non-P species (m/z 1368) is also observed, suggesting the presence of a second, perhaps promiscuous, lipid A 1-phosphatase encoding gene. The phenotype of the Δ PGN_1713 mutant is virtually identical to that of wild-type *P. gingivalis* grown in high hemin conditions. Importantly, they both make the antagonist m/z 1448 lipid A structure, which is capable of dampening the immune response elicited by lipid A from *E. coli* or by *P. gingivalis* lipid A preparations enriched for agonist species (20). This suggests that the PGN_1713 protein is not functional in wild-type *P. gingivalis* when grown in high hemin conditions (Fig. 4).

Focusing on the other end of the lipid A molecule, PGN_0524 encodes the lipid A C4' phosphatase. *P. gingivalis* mutated for PGN_0524 is expected to generate lipid A species that retains a phosphate group at C4'. The major lipid A species made by a Δ PGN_0524 mutant is penta-acyl, 4'-P (m/z 1688) (Figure 3E and 3F) (20). This is the minor penta-

acylated lipid A species observed in wild-type *P. gingivalis* grown under low hemin conditions. Remarkably, very little tetra-acylated species is observed in the Δ PGN_0524 mutant, indicating that removal of the C4' phosphate is a prerequisite for removal of an acyl chain from the penta-acylated species. This observation is consistent with similar observations from other bacteria (162). Lipid A from the Δ PGN_0524 mutant is a TLR4 agonist. It is a stronger agonist than wild-type *P. gingivalis* lipid A from low hemin conditions but not as strong as the prototypical *E. coli* lipid A agonist. A *P. gingivalis* double mutant that lacks both PGN_1713 and PGN_0524 is enriched for a di-phosphorylated penta-acylated lipid A species (Figs. 3I and 3J) and, as expected, is the strongest *P. gingivalis* agonist lipid A species identified so far (20). The fact that the double mutant still makes mono-P penta-acyl (m/z 1688) lipid A species also suggests that there exist additional, as yet unidentified, lipid A phosphatases in the bacterium.

Impact of lipid A structure on resistance to cationic antimicrobial peptides

Modification of lipid A structure has been shown to significantly impact susceptibility of the bacterium to cationic antimicrobial peptides such as polymyxin B (68,158,162). In fact, many genes that play a role in lipid A biosynthesis were originally identified in *Salmonella* based on conferral of a phenotype of polymyxin resistance (46,47). Polymyxin B is a cyclic peptide with a net charge of +4 and a fatty acid tail (104). The optimal distribution of positive charges and detergent-like activity of the fatty acid tail makes it a very powerful outer membrane perturbant and greatly increases its permeability. Polymyxin B resistance arises from modifications of lipid A that result in a decreased electrostatic attraction between positively charged polymyxin B and negatively charged lipopolysaccharide. These modifications include substitution of the negatively charged C1 and C4' phosphate groups with positively charged groups such as 5-aminoarabinose and ethanolamine (104). *P. gingivalis* is highly resistant to polymyxin B, surviving exposure to as much as 200 μ g/ml of this cationic antimicrobial peptide (20), likely due to the lack of phosphate groups at both C1 and C4' in a majority of the lipid A species. Perhaps not surprisingly, a Δ PGN_0524 mutant that does not make non-phosphorylated lipid A species, is very sensitive to polymyxin B, in concentrations as low as 2 μ g/mls (20). Therefore, synthesis of the unusual non-phosphorylated brand of lipid A by *P. gingivalis* (by action of lipid A phosphatases and deacylase) confers to it a distinct survival advantage against cationic anti-microbial peptides such as polymyxin B and, presumably, to physiologically relevant ones such as β -defensins and cathelicidins.

TLR4 vs TLR2

It is well established that lipid A engages the TLR4 complex, triggering the pro-inflammatory cytokine-producing signaling cascade. However, the assertion that *P. gingivalis* lipopolysaccharide can also engage a different Toll-like receptor, TLR2, has been a controversy in the field (27,57,86,110). TLR2 is known to recognize bacterial PAMPs such as lipoproteins, lipoteichoic acid and peptidoglycan. The TLR4-independent mechanism for eliciting an immune response was first proposed for *P. gingivalis* lipopolysaccharide because of its ability to activate lymphocytes and macrophages from C3H/HeJ mice, which do not respond to lipopolysaccharide due to a mutation in TLR4 (for a review, see (110)). The unique structural features of *P. gingivalis* lipid A, such as long acyl chains, fewer acyl chains and fewer phosphate groups, were thought to mediate the proposed interaction with TLR2. *P. gingivalis* is also unusual in that it synthesizes complex lipids other than lipopolysaccharide such as phosphoglycerol dihydroceramides with unusually long linear and branched acyl chains (100,101), as well as phosphoethanolamine-containing lipids (102), which could contribute to TLR2 activity. It was also suggested that minute

amounts of contaminating lipopeptides in lipopolysaccharide preparations could account for TLR2 activation.

Definitive evidence that *P. gingivalis* lipid A acts only via TLR4 came from chemically synthesized *P. gingivalis* lipid A analogues, which activate only via TLR4 and not TLR2 (75,137,168). Additionally, *P. gingivalis* is known to make many lipoproteins, which could potentially be co-isolated with lipopolysaccharide, and at least one of which has been shown to activate TLR2 (110). While it is now established that interaction of *P. gingivalis* lipopolysaccharide with the host's innate immune system is via TLR4, it is also clear that TLR2 plays a significant role in initiating a destructive immune response. In studies employing TLR2 or TLR4 knock-out mice in *P. gingivalis* induced bone loss models it was found that TLR2, not TLR4, mediated bone loss (14). This finding was a surprise considering that TLR4 engagement is believed to be a more potent inflammatory stimulator, and supports the notion that TLR2, a host receptor normally associated with gram-positive oral commensal responses, can initiate a destructive inflammatory response.

Innate immune response to lipopolysaccharide from oral bacteria

Potency of the endotoxic property of lipopolysaccharide has been historically quantified through the use of host response assays such as the local Schwartzman reaction, the rabbit pyrogenicity assay and the chick embryo lethality test (33). We mentioned earlier that many studies have reported the low endotoxic potencies of lipopolysaccharide, or the constituent lipid A, from oral bacteria in comparison to lipopolysaccharide derived from enteric bacteria (31). As elaborated in the previous section, extensive studies of *P. gingivalis* lipid A have shed light into the structural basis for this weak activity, notably the synthesis of lipid A molecules that enable immune evasion (via synthesis of inert non-phosphorylated species) and immune suppression (via synthesis of antagonistic lipid A species). The structure of lipid A of *P. intermedia*, another gram-negative anaerobic periodontopathogen, is similar to that of *P. gingivalis* in that it synthesizes a m/z 1688 penta-acyl 4'-P species, and it is also a weak stimulator of cytokine response in murine immune cells (54).

We also mentioned earlier that there is a marked shift in bacterial population and composition in dental plaque during the transition from periodontal health to disease. Commensal bacteria, found in healthy sites, are mostly gram-positive and include relatively few gram-negative species. By contrast, diseased periodontal sites have a higher microbial load and higher prevalence of gram-negative species. Deep periodontal pockets, a marker of advanced chronic periodontitis, show a marked association with the red-complex bacteria, *P. gingivalis*, *T. forsythia* and *T. denticola* (142). Other commonly found oral bacteria have also been grouped according to their degree of association with diseased sites. The red-complex shows greatest association with chronic periodontitis followed by the orange, yellow, green and, finally, the purple-complex bacteria, which show the least association with chronic periodontal diseased sites (142).

The lipid A structure of two gram-negative oral bacteria that are not commonly associated with chronic periodontitis, *A. actinomycetemcomitans* and *F. nucleatum*, have been published (4,88). *F. nucleatum*, an orange complex bacterium, has been proposed to be a 'bridge' species between commensal and pathogenic colonizers. It is the most numerous species at healthy sites and its numbers increase significantly at diseased sites (94). *A. actinomycetemcomitans*, which belongs to the green complex, is a late colonizing species. It is the etiological agent of localized aggressive periodontitis (93) but has very little correlation with chronic periodontitis, unlike the red-complex periodontopathogens. Lipid A from both these bacteria are hexa-acylated, and are quite similar in structure to that of *E. coli*. The *A. actinomycetemcomitans* lipid A differs from that of *E. coli* only in the

replacement of the 12C piggy-back chain with a 14C acyl chain on the C2'-linked primary fatty acid chain. The *F. nucleatum* lipid A also is hexa-acylated but has longer carbon chains than *E. coli* lipid A. Of note, lipopolysaccharide from both *A. actinomycetemcomitans* and *F. nucleatum* induce the Schwartzman reaction (87,88,114,118,119), unlike lipopolysaccharide from the periodontopathogens *P. gingivalis*, *T. forsythia*, and *P. intermedia* (13,38,39,63,64,87,109,138). Lipopolysaccharide from other oral bacteria that are positive for the Schwartzman reaction include *Veillonella* (12), *Campylobacter rectus* (73), *Leptotrichia buccalis* (69) and *Eikenella corrodens* (87). Interestingly, the lipid A profile of the purple complex *Veillonella*, green complex *Capnocytophaga sputigena* and *Capnocytophaga ochracea*, and orange complex *C. rectus* also show them to be hexa-acylated (33). In vitro cell culture assays showed each of the above four lipid A species to be TLR4 agonists; however, they do not elicit as potent a response as *E. coli* lipid A (33). With this limited evidence in mind, it is tempting to speculate that the differences in lipid A structure partially distinguish commensal from periodontopathogenic bacteria. Commensal bacteria, which are more prevalent in healthy sites, possess lipid A structures that are hexa-acylated and mild activators of innate immunity, while bacteria associated with chronic periodontal disease synthesize lipid A with fewer than six acyl chains and do not induce an appreciable innate immune response.

Innate immune status in healthy vs. diseased periodontal tissue

It is increasingly evident that in clinically healthy periodontal sites the innate immune defense system is maintained in an active state. Select innate host defense mediators, such as E-selectin, ICAM and interleukin-8, are expressed in a carefully orchestrated manner in healthy periodontal tissue (40,96,105,154,155,156). Interleukin-8, for example, is expressed in a gradient that is most concentrated at the superficial junctional epithelial cell layers (155). However, a disruption of this pattern of expression is observed with a change in microbial composition and load. While greater levels of interleukin-8 are seen in tissue from diseased periodontal sites, the expression pattern is patchy with loss of the characteristic gradient (156).

Production of the above-mentioned immune mediators in healthy tissue facilitates the transit of neutrophils from highly vascularized gingival tissue to the gingival crevice where they form a barrier between the host tissue and the dental plaque biofilm (70). This barrier presumably contributes to controlling the magnitude and composition of the plaque and, hence, maintaining oral health. Indeed, individuals with deficiencies in either neutrophil numbers (neutropenia) or transit, whether by congenital defects or chemical induction, invariably develop periodontitis (6,16,52,56,115,136,161,166).

Periodontal tissue also expresses numerous other innate host mediators such as human β -defensin molecules 1, 2, and 3 (78,79,80) as well as soluble (61) and membrane bound CD14 (62) and lipopolysaccharide-binding protein (133). Clinically healthy human gingival tissue also expresses low levels of TLR2 (134,149) and, while expression of TLR4 was reported in one study (149), a different study did not observe expression of this innate host defense receptor in healthy tissue (134). Another study describes the expression of TLR's 1–10 in both clinically healthy and diseased tissue (10). These data are all consistent with the notion that innate immune mediator expression in clinically healthy tissue is key to the maintenance of periodontal health.

It is also increasingly recognized that the activated, controlled inflammatory state of the innate immune system found in healthy periodontal tissue is a response to resident commensal bacteria (30) that are found immediately adjacent to periodontal tissue and, therefore, provide a constant microbial challenge to the host innate immune system. For

example, in vitro studies have shown that commensal oral bacteria induce the production of host defense proteins interleukin-8 and β -defensins (18,25,53,59,71,80,156), which are found on the outermost layers of the gingival epithelium. While the contribution of commensal bacteria to the development of innate immunity in the oral cavity is poorly understood, recent studies utilizing germ-free mice have established that commensal bacteria in the intestine play an active role in establishing innate protective mechanisms (36,81,129,144). These studies demonstrate that commensal bacteria contribute to the establishment of a controlled inflammatory state in the intestine, similar to that observed in clinically healthy periodontal tissue. One preliminary investigation revealed that interleukin-1 β level in the periodontium was higher in conventionally reared mice compared to age matched germ-free mice (32). This is the first evidence demonstrating that periodontal tissue responds to commensal colonization.

Yet another major conceptual advance concerning the etiology of periodontitis is the recognition that the host significantly contributes to the tissue destruction and alveolar bone resorption that are hallmarks of the disease (116). This stands in contrast to the notion that periodontopathogens are the sole and direct instigators of disease. Many lines of evidence support this hypothesis. For example, gingival crevicular fluid from diseased sites contains concentrations of cytokine and inflammatory mediators significantly higher than gingival crevicular fluid from healthy sites (17,42,43,153). Studies involving the administration of select anti-inflammatory compounds in animal models of disease (5,29,44), and studies using interferon- γ , interleukin-6, and tumor necrosis factor receptor knock-out mice (9,39), all demonstrated significant attenuation of bone loss in mouse models of periodontitis. This suggests that an aggressive host inflammatory response contributes to periodontitis. P- and E-selectin double knock-out mice exhibit impaired neutrophil transit in the periodontium. These mice spontaneously develop periodontitis, indicating the importance of neutrophil transit in maintenance of oral health. Moreover, when these mice were administered antibiotics, they no longer succumbed to disease, confirming the contribution of oral bacteria to disease (103). Further evidence demonstrating that increased host mediated inflammation contributes to disease was obtained from an interleukin-1 β overproducing transgenic mouse strain. These mice developed periodontitis with or without the administration of antibiotics (28), demonstrating that it is possible to develop periodontitis in the absence of a bacterial component during excessive host inflammatory mediator production.

Periodontitis, therefore, can result from a number of different perturbations of host homeostasis, whether due to a shift in microbial composition or shift in host immune mediator production. It seems likely that a shift in dental plaque numbers and composition plays a critical role in initiating destructive innate immune inflammatory responses, which results in disease. For example, it was shown that while both low and high plaque samples activate both TLR2 and TLR4, plaque obtained from individuals with higher plaque scores elicit significantly more TLR4 activation (167). It is clear that physical removal of dental plaque by scaling and root planing results in a decrease of red-complex periodontopathogens, restoration of innate immune homeostasis, tissue healing and favorable clinical outcomes.

Importance of being antagonist

Combining the above observations, we hypothesize that lipopolysaccharides from oral commensal bacteria are mild TLR4 agonists that, by presenting a constant low-level challenge, help the innate immune system establish and maintain a controlled inflammatory state, the hallmark of oral health. On the other hand, lipopolysaccharide from the periodontopathogen, *P. gingivalis*, is composed of lipid A species that are predominantly

either inert or antagonistic with respect to TLR4 activation. A major outcome of synthesis of inert lipid A species is the failure to induce production of innate immune mediators such as interleukin-8, E-selectin and interleukin-1 α , which are important to maintain the controlled inflammatory state. This failure, for instance, can lead to disruption in neutrophil transit and, hence, lack of control of a constantly expanding microbial population in dental plaque.

P. gingivalis antagonistic lipid A has a structure that is subtly distinct from the inert lipid A structure. It is produced when *P. gingivalis* is exposed to high concentrations of hemin, an environmental condition that is known to exist in severely inflamed periodontium. Antagonistic lipid A, crucially, is capable of inhibiting the production of these mediators made in response to agonist lipopolysaccharide from other oral microbes. Recently, it was shown that *P. gingivalis* agonist lipid A structures induced the expression of human β -defensin-1, -2 and -3, while *P. gingivalis* antagonist lipid A species down-regulated their expression (78). Since *P. gingivalis* releases lipopolysaccharide in the form of easily diffusible vesicles (45), the presence of the *P. gingivalis* lipid A TLR4 antagonist in the gingiva has the potential to dampen TLR4 responses for the entire microbial community. Interestingly, TLR4 antagonist can also suppress host signaling pathways involved in maintaining host homeostasis that, ostensibly, have little bearing on innate immune signaling. For example, gingival fibroblasts, which are involved in continuous remodeling of the periodontal tissue matrix, exhibit proliferative responses to epidermal growth factor that is released by the gingival epithelium. This signaling involves the MAPK protein phosphorylation cascade. It has been shown that the *P. gingivalis* TLR4 lipid A antagonist inhibits epidermal growth factor-mediated signaling at the level of ERK1/2, p38 and CREB (124), thereby having the potential to disrupt tissue development and, hence, tissue homeostasis.

When whole bacterial cells were tested for their ability to trigger immune responses, as opposed to their isolated lipopolysaccharide, it was shown that all three red-complex periodontopathogens, *T. forsythia*, *T. denticola* and *P. gingivalis*, elicited reduced interleukin-8 production by gingival epithelial cells (60) in contrast to oral bacterial species *F. nucleatum* and *A. actinomycetemcomitans* (25,53,59). While reduction of interleukin-8 levels, produced in response to commensal bacteria, by *P. gingivalis* whole cells is well documented (25,61,62), and likely multi-factorial (55), the role of antagonistic lipopolysaccharide in this process is likely considerable. It is not known whether the other red-complex periodontopathogens also make antagonistic lipid A under certain environmental conditions.

In summary, the oral red-complex periodontopathogen, *P. gingivalis*, synthesizes a variety of lipid A structures in response to specific environmental conditions. These lipid A species have the astonishing capacity of being either agonistic, inert or antagonistic with respect to TLR4 activation. Antagonistic lipid A can potentially disrupt the delicate balance of innate immune mediators produced in response to commensal bacteria. Presence of a TLR4 antagonist may, therefore, affect the entire microbial community's interaction with the host. Surprisingly little is known about how bacterial modulation of host immune mediator production may lead to destructive inflammation. Research in this field should continue to improve our understanding of periodontitis and potentially other chronic inflammatory diseases.

Acknowledgments

We greatly appreciate the help of Dr. Stephen R. Coats and Roger W. Kramer for critical reading of the manuscript. This work was supported by NIH grant T32 DE07132-25 to S.J. and NIH grant DE12768 to R.P.D.

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Figure 1.

(a) Schematic of the outer membrane of gram-negative bacteria. Lipopolysaccharide (LPS) forms the outer leaflet of the outer membrane. It is composed of three domains namely the lipid A anchor (red), the core oligosaccharide and the outer-most O-antigen (green), which is variable in structure and immunogenic. (b) Schematic of the prototypical *E. coli* lipid A structure, whose molecular mass is m/z 1797. Two glucosamine residues are linked by a β -1',6 linkage. The enzymes LpxA and LpxD add four primary acyl chains (red and blue respectively) to the C2, C3, C2' and C3' positions, while MsbB (green) and HtrB (purple) add secondary acyl chains to the primary chains.

Figure 2.

Heterogeneity in *P. gingivalis* lipid A structures. The four main groups of lipid A structures that have been isolated from *P. gingivalis* under different environmental conditions are depicted. The m/z 1768 structure undergoes dephosphorylation and/or deacylation to give rise to the m/z 1688, m/z 1448 and m/z 1368 lipid A structures. The m/z 1688 structure can also have the phosphate group at the C1 position. Removal of the acyl chain from the C3 position of the m/z 1688 structure gives rise to an m/z 1435 structure (not shown). The acyl chains in red are added by the enzyme LpxD, the chains in blue by LpxA and the secondary acyl chain in purple by HtrB. *P. gingivalis* lacks a *msbB* homolog.

Figure 3.

MALDI-TOF MS analysis of *P. gingivalis* lipid A isolated from the wild-type (A-D) when grown in either low (WT lo) or high (WT hi) hemin conditions, and of lipid A isolated from mutants that lack either the C4'-phosphatase PGN_0524 (E, F) or the C1-phosphatase PGN_1713 (G, H) or both (I, J). The column on the left shows the MALDI-TOF MS profile in negative mode, which display negatively charged ions, while those on the right are of the same samples in positive mode, which display uncharged or positively charged ions. Molecular weight (m/z) of ions is plotted on the x-axis and relative intensity of ions on the y-axis. Adapted from (20).

Figure 4.

Model of differential 1- and 4'-phosphatase activities, encoded by PGN_1713 and PGN_0524 respectively, under low (left) or high (right) hemin conditions lead to the accumulation of lipid A species that either evade or antagonize innate immune responses. It is proposed that the activity of the lipid A 1-phosphatase, PGN_1713, is inhibited under high hemin conditions. It is also proposed that penta-acylated lipid A species, while being detected by MALDI-TOF MS and thin layer chromatography, are functionally dominated by the inert (low hemin) or antagonistic (high hemin) tetra-acylated lipid A species that are present in wild-type *P. gingivalis* grown in low and high hemin conditions respectively. Adapted from (20).