Bacterial Fimbriae Stimulate Proinflammatory Activation in the Endothelium through Distinct TLRs

Michael Davey, Xinyan Liu, Takashi Ukai, Vishal Jain, Cynthia Gudino, Frank C. Gibson III, Douglas Golenbock, Alberto Visintin and Caroline A. Genco

*J Immunol* 2008; 180:2187-2195; doi: 10.4049/jimmunol.180.4.2187

http://www.jimmunol.org/content/180/4/2187

References

This article cites 51 articles, 24 of which you can access for free at: http://www.jimmunol.org/content/180/4/2187.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Copyright © 2008 by The American Association of Immunologists All rights reserved.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Bacterial Fimbriae Stimulate Proinflammatory Activation in the Endothelium through Distinct TLRs¹

Michael Davey,* Xinyan Liu,* Takashi Ukai,²* Vishal Jain,§ Cynthia Gudino,* Frank C. Gibson III,⁷ Douglas Golenbock,§ Alberto Višntin,§ and Caroline A. Genco³*‡

The major and minor fimbriae proteins produced by the human pathogen Porphyromonas gingivalis are required for invasion of human aortic endothelial cells and for the stimulation of potent inflammatory responses. In this study, we report that native forms of both the major and minor fimbriae proteins bind to and signal through TLR2 for this response. Major and minor fimbriae bound to a human TLR2:Fc chimeric protein with an observed Kₐ of 28.9 nM and 61.7 nM, respectively. Direct binding of the major and minor fimbriae to a human chimeric CD14-Fc protein also established specific binding of the major and minor fimbriae to CD14 with classic saturation kinetics. Using a P. gingivalis major and minor fimbriae mutant, we confirmed that TLR2 binding in whole cells is dependent on the expression of the major and minor fimbriae. Although we did not observe binding with the major or minor fimbriae to the TLR4-Fc chimeric protein, signaling through TLR4 for both proteins was demonstrated in human embryonic kidney 293 cells transfected with TLR4 and only in the presence MD-2. Transient transfection of dominant-negative forms of TLR2 or TLR4 reduced IL-8 production by human aortic endothelial cells following stimulation with major or minor fimbriae. The ability of two well-defined microbe-associated molecular patterns to select for innate immune recognition receptors based on accessory proteins may provide a novel way for a pathogen to sense and signal in appropriate host environments. The Journal of Immunology, 2008, 180: 2187–2195.

The highly sophisticated innate immune system of Ag detection relies upon a family of evolutionarily conserved IL-1 superfamily of transmembrane receptors, known as the TLRs, which play a crucial role in the early host defense against invading microorganisms (1, 2). Microbial activation of these receptors initiates the activation of NF-κB which results in the expression of a large number of inflammatory genes (3–5). TLR4, the receptor for Gram-negative enterobacterial LPS, is the best characterized of the TLR family members. Effective recognition of LPS by the host requires the assembly of a signaling complex which consists of LPS-binding protein, soluble or GPl membrane-linked CD14 and activating protein MD-2. These accessory components, which all lack transmembrane signaling domains, act in concert to bring LPS to TLR4 for the subsequent signaling response (5–7). Another member of the TLR family, TLR2, has been demonstrated to be crucial for the propagation of the inflammatory response to an array of Gram-positive and -negative bacterial components such as peptidoglycan, lipoteichoic acid, lipoproteins, and lipopeptides (8–11). This broader specificity of TLR2 ligands may be attributed to the heterodimerization of TLR2 with either TLR1 or TLR6 (12, 13).

We have previously examined the inflammatory response of two microbial components expressed by the Gram-negative pathogen Porphyromonas gingivalis important for mediating adherence to host tissues and for facilitating invasion by this organism. The major (41-kDa) and minor (67-kDa) fimbriae are distinct types of fimbriae expressed simultaneously by this organism (14, 15). The minor fimbriae of P. gingivalis has been reported to induce TNF (TNF-α), IL-1β, and IL-6 production in human monocytic cell lines and murine peritoneal macrophages (16, 17), although the receptors required for this response have not been defined. The major fimbriae of P. gingivalis are composed of a single subunit protein (fimbrillin), which shares no amino acid sequence homology with any other type of fimbriae (18), and has been shown to be required for adhesion and invasion of fibroblasts and endothelial cells (19–21). In both human and murine monocytes/macrophages or monocytic cell lines, P. gingivalis major fimbriae induce the expression of inflammatory cytokines, including TNF-α and IL-1β (17, 22–24). Using monoclonal blocking Abs in monocytes, it has been reported that the inflammatory response to P. gingivalis major fimbriae is dependent upon stimulation of both TLR2 and TLR4 (17, 18). Likewise, a separate study used anti-TLR2 Ab and human gingival epithelial cells, which predominantly express TLR2, but not TLR4, or CD14, to suggest that P. gingivalis fimbriae are capable of activating human gingival epithelial cells through TLR2 (25). Recently, Hajishengallis et al. (26) demonstrated that the major fimbriae use TLR1 or TLR6 for cooperative TLR2-dependent activation of transfected cell lines. Furthermore, these investigators demonstrated the major fimbriae activation required membrane-expressed CD14.

¹Section of Molecular Medicine and Section of Infectious Diseases, Department of Medicine, School of Medicine, Boston University, Boston, MA 02118; ²Department of Periodontology and Oral Biology, Goldman School of Dental Medicine, Boston University, Boston, MA 02118; ³Section of Infectious Diseases, Department of Medicine, School of Medicine, University of Massachusetts, Worcester, MA 01655; and ⁴Department of Microbiology, School of Medicine, Boston University, Boston, MA 02118

Received for publication June 28, 2007. Accepted for publication November 29, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by National Institutes of Health Grants RO1-HL-080387 (to C.A.G.).

Current address: Department of Periodontology, Unit of Translational Medicine, Course of Medical and Dental Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8588, Japan.

Address correspondence and reprint requests to Dr. Caroline A. Genco, Section of Molecular Medicine, Department of Medicine, School of Medicine, Boston University, 650 Albany Street, Boston, MA 02118. E-mail address: cgenco@bu.edu

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/52.00

www.jimmunol.org
We have been interested in the specific interactions of *P. gingivalis* with human aortic endothelial cells (HAEC) because of the documented association of this organism with an increased risk for inflammatory atherosclerosis and cardiovascular disease (27–29). Using major and minor fimbriae mutants we demonstrated that major fimbriae are required for *P. gingivalis* invasion of HAEC and that only invasive *P. gingivalis* strains induced the production of proinflammatory molecules IL-1β, IL-8, MCP-1, ICAM-1, VCAM-1, and E-selectin (30). Our work demonstrated that following major fimbriae-mediated initial attachment, the minor fimbriae play an important role in more intimate attachment facilitating endocytosis of the bacteria (30). The purified native forms of the major and minor fimbriae induced chemokine and adhesion molecule expression similar to invasive *P. gingivalis*, but failed to elicit IL-1β production. Despite the documented role of these proteins in the robust inflammatory response mounted by this Gram-negative pathogen, the host cell signaling receptors used for the major and minor fimbriae mediated response in endothelial cells have not been definitively defined.

In this study, we used TLR2, TLR4, and CD14 chimeric fusion proteins in a cell-free ELISA to define the binding specificity of both the major and minor fimbriae. Although specific binding of both the major and minor fimbriae to the CD14 and TLR2 chimeric proteins was demonstrated with classical saturation kinetics, binding was not observed with both the major or minor fimbriae and the TLR4-Fc chimeric protein. Signaling through TLR2 and TLR4 for both the major and minor fimbriae was demonstrated in human embryonic kidney 293 cells transfected with TLR2 or TLR4. Importantly, activation in TLR4-expressing cells in response to either the major and minor fimbriae required MD-2 and MD-2-deficient mouse macrophages exhibit a diminished response to both the major and minor fimbriae. Finally, we confirmed that both TLR2 and TLR4 contribute to IL-8 production by HAEC following stimulation with major or minor fimbriae.

### Materials and Methods

**Purification of the major and minor fimbriae of *P. gingivalis***

Wild-type (WT) *P. gingivalis* strain 381, the major fimbriae mutant (DPG3), the minor fimbriae mutant (MFI), and the major and minor fimbriae double mutant (DPGMBF) were maintained anaerobically (10% H2, 10% CO2, and 80% N2) at 37°C in brain heart infusion broth (Difco) supplemented with 0.5% of yeast extract (Difco), 5 μg/ml hemin, and 1 μg/ml menadione. Erythromycin (1.0 μg/ml) and tetracycline (2.0 μg/ml) were added according to the selection requirements of the strains (30). Fimbriae were prepared according to a modification of the method described by Arai et al. (31). Briefly, cell pellets of *P. gingivalis* DPG3 or MFI were disrupted by ultrasonication with a 3-mm microtip at 20-W output on pulse setting with 50% duty cycle for 5 min in an ice bath. The supernatant was obtained by centrifugation and ammonium sulfate was added to precipitate the proteins, which were subsequently dialyzed in 20 mM Tris buffer (pH 7.8). The dialyzed sample containing most of the fimbriae proteins was subjected to further purification by multiple passes through a DEAE Sepharse Fast Flow column (Amersham Biosciences) equilibrated with 20 mM Tris buffer (pH 8.0) and eluted with a linear gradient of 0–0.3 M NaCl. Elution profiles of both major and minor fimbriae were monitored at 280 nm. Fractions were analyzed by 12% SDS-PAGE, and those containing fimbriae were pooled, dialyzed against 5 mM Tris buffer (pH 8.0), concentrated, and quantitated by the Lowry protein estimation method. Fimbriae preparations were analyzed for LPS contamination by PAGE and visualized with Silver Stain Plus (Bio-Rad). In addition, each fimbriae preparation was verified to be LPS free by the Limulus amebocyte lysate assay (<0.1 endotoxin units/ml⁻¹, Cambrex; Table I).

<table>
<thead>
<tr>
<th>Preparationa</th>
<th>Source</th>
<th>Activity of Clotting Enzyme (EU/ml)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAL reagent water</td>
<td>Cambrex LAL kit</td>
<td>0.050 ± 0.006</td>
</tr>
<tr>
<td>LPS standard (0.1 EU/ml) E. coli 011:1B4</td>
<td>E. coli 0111:B4</td>
<td>0.113 ± 0.005</td>
</tr>
<tr>
<td>LPS standard (1.0 EU/ml) E. coli 0111:B4</td>
<td>E. coli 0111:B4</td>
<td>1.063 ± 0.095</td>
</tr>
<tr>
<td>Major fimbriae</td>
<td><em>P. gingivalis</em> 381MF1</td>
<td>0.042 ± 0.003</td>
</tr>
<tr>
<td>Minor fimbriae</td>
<td><em>P. gingivalis</em> DPG3</td>
<td>0.046 ± 0.011</td>
</tr>
</tbody>
</table>

*a A total of 50 μl of each preparation tested was dispensed in triplicate into microplate wells for colorimetric determination of endotoxin contamination of indicated preparations.

*b EU/ml Endotoxin units per milliliter.

Stock preparation of *P. gingivalis* major fimbriae (486 μg/ml) used for all stimulation and binding assays.

Stock preparation of *P. gingivalis* minor fimbriae (595 μg/ml) used for all stimulation and binding assays.

### Biotinylation of major and minor fimbriae

*P. gingivalis* native major and minor fimbriae were biotinylated with EZ-Link Sulfo-NHS-Biotin reagent according to the manufacturer’s instructions (Pierce). Functional activity of the biotinylated major and minor fimbriae was confirmed through comparison of IL-8 production by HAEC stimulated with both unlabeled native and biotin-labeled fimbriae proteins.

### Fimbriae and TLR-Fc chimera-binding assay

To determine an approximate *Kd* for the interaction between TLR2, TLR4, or CD14 and fimbriae, an indirect ELISA-like binding assay, was performed as described (32). MD-2 and Pam2CSK4 were used as controls. Purified chimeric TLR fusion proteins consisting of the entire extracellular domain of either human TLR2 (aa 1–587), human TLR4 (aa 1–632), or human CD14 (aa 1–354) fused in frame with the C-terminal 233-aa Fc portion of mouse IgG2a and modified by the addition of the linker sequence GAAGGG were used for this assay. Fifty microliters of purified TLR2-Fc, TLR4-Fc, or CD14-Fc at 20 μg/ml was added to each well of 96-well microtiter plates and incubated overnight at 4°C. The plates were then washed, blocked with SuperBlock TMB Blocking Buffer (Pierce), for 1 h at 37°C, again washed, and incubated with biotinylated major or minor fimbriae (total binding) or biotinylated fimbriae plus ~1 nM excess unlabeled fimbriae (nonspecific binding) in SuperBlock Blocking Buffer for 2 h at 37°C. Wells were washed extensively followed by incubation with streptavidin-peroxidase (1:2000; Beckman Coulter), and the peroxidase reaction was performed using the TMB Peroxidase ELA Substrate Kit (Bio-Rad). Binding of major or minor fimbriae to TLR2-Fc, TLR4-Fc, or CD14-Fc was detected by measuring absorbance at 450 nm of triplicate samples. The results are presented as specific binding (total binding – nonspecific binding). Approximate *BMax* and *Kd* values were obtained by nonlinear regression analysis of hyperbolic curves using Graph Pad Prism 4.0 software.

To confirm the approximate *Kd* obtained from saturation binding experiments, homologous competitive binding curves were conducted as described above with the following exception: 100 nM of major or minor biotinylated fimbriae was added to each well with increasing concentrations of unlabeled fimbriae as indicated. Approximate *IC50* values were obtained by nonlinear regression analysis of sigmoidal curves using Graph Pad Prism 4.0 software and *Kd* calculated from the value of *IC50* using the equation of Cheng and Prusoff (33).

We also examined *P. gingivalis* strain 381 and the *P. gingivalis* major and minor fimbriae mutant (DPGMBF) whole cells for binding to TLR2-Fc and TLR4-Fc chimeric proteins. After overnight culture, *P. gingivalis* strains were resuspended in HBSS and 105 CFU incubated for 30 min at 37°C with the chimeric proteins at a concentration of 2 μg/ml. After extensive washing with HBSS, the bound chimeric proteins were detected using a FITC conjugated anti-mouse polyclonal Ab (1:200; Sigma-Aldrich) and samples were fixed with 1% PFA on ice for 10 min. Binding was assessed by cytofluorometry.

### Stimulation of mouse macrophages with *P. gingivalis* major and minor fimbriae

The stimulatory activity of major and minor fimbriae on macrophage proinflammatory cytokine production was examined using bone marrow derived...
macrophages (BMDM) harvested from C57BL/6 (WT), TLR2−/−, TLR4−/−, CD14−/−, and MD-2−/− mice as described (34). Bone marrow cells were cultured in RPMI 1640 medium (Cellgro) containing 10% FBS, 100 μg/ml streptomycin, 100 IU of penicillin (Cellgro), and 5 ng/ml mouse recombinant M-CSF (R&D Systems) for 16 h. Nonadherent bone marrow precursor cells (5 × 10^5 cells/0.5 ml/well) were collected and cultured in RPMI 1640 containing 10% FBS, antibiotics, and 30 ng/ml M-CSF in 24-well plates. After 5 days of culture, BMDM were stimulated with P. gingivalis major or minor fimbriae (10 μg/ml), repurified E. coli 0111-B4 (100 ng/ml), Pam3CSK4 (1 μg/ml) (Invivogen), or medium alone for 24 h. Culture medium was harvested for ELISA analysis of IL-6 and MCP-1 as described below.

**Activation of TLR2 or TLR4 signaling in HEK293 cells by P. gingivalis major and minor fimbriae**

NF-κB-luciferase assays were performed essentially as reported in Refs. 32 and 35. Briefly, 293 cells, or 293 cells which stably expressed TLR2 or TLR4, were transiently transfected with a NF-κB-luciferase reporter plasmid and stimulated overnight with fimbriae (0.1–10 μg/ml). P. gingivalis strain 381 LPS (0.005–5 μg/ml), isolated as previously described (30), E. coli 0111:B4 LPS (0.01–0.1 μg/ml), or Pam3CSK4 (0.0001–1.0 μg/ml). For all experiments, 10% FBS was used and served as a source of soluble CD14. In some experiments, MD-2 was added to the system as a conditioned medium from MD-2-expressing 293 cells. Luciferase activity was determined as described (30).

**Aortic endothelial cell culture**

HAEC from multiple donors (Cascade Biologies) were maintained in Medium 200 supplemented with low serum growth supplement (Cascade Biologies) at 37°C in 5% CO2 in tissue-culture flasks. Semiconfluent HAEC were removed from flasks with trypsinization and seeded into 6- or 24-well culture dishes at a cell density of 6–8 × 10^5 or 1.25–1.5 × 10^6 cells/well, respectively. Confluent second- to fifth-passage cells were used in all experiments. For blocking experiments, endothelial cells were pretreated with medium, control IgG2A Ab, TLR2 Ab (TL2.1, IgG2A; eBioscience), or TLR4 Ab (HTA125, IgG2A; eBioscience), for 30 min before the addition of stimulants. HAEC were then incubated in the absence or presence of major or minor fimbriae (1 μg/ml), E. coli LPS (1 μg/ml), or Saccharomyces cerevisiae zymosan (10 μg/ml) for 24 h. Supernatants from stimulated HAEC were analyzed by ELISA as described below.

Resting third-passage HAEC were also analyzed by FACS analysis or by fluorescence microscopy with FITC-conjugated anti-TLR2 or anti-TLR4 as previously described (30). Surface expression of TLR2 and TLR4 was determined on nonpermeabilized HAEC. Confluent second- to fifth-passage cells were used in all experiments. To determine whether a direct interaction between P. gingivalis major and minor fimbriae occurs with TLR2 and/or TLR4, we used TLR2 and TLR4 fusion proteins in a cell-free, ELISA-like binding assay. As expected, the TLR2-Fc fusion protein bound to the positive control, the synthetic diacyl lipopeptide Pam2CSK4. Binding of TLR2-Fc to both the P. gingivalis major and minor fimbriae was also observed (Fig. 1). Although the TLR4-Fc fusion protein bound to the MD-2-positive control, no specific binding to either P. gingivalis major or minor fimbriae was observed (Fig. 1).

**Results**

P. gingivalis major and minor fimbriae exhibit specific interactions with TLR2, but not TLR4

To determine whether a direct interaction between P. gingivalis major and minor fimbriae occurs with TLR2 and/or TLR4, we used TLR2 and TLR4 fusion proteins in a cell-free, ELISA-like binding assay. As expected, the TLR2-Fc fusion protein bound to the positive control, the synthetic diacyl lipopeptide Pam2CSK4. Binding of TLR2-Fc to both the P. gingivalis major and minor fimbriae was also observed (Fig. 1). Although the TLR4-Fc fusion protein bound to the MD-2-positive control, no specific binding to either P. gingivalis major or minor fimbriae was observed (Fig. 1).

To define the specific nature of the interactions between major or minor fimbriae with TLR2:FcTLR2-Fc or TLR4:FcTLR4-Fc, a second ELISA-based assay using biotinylated major or minor fimbriae was used. Biotinylation of major and minor fimbriae was verified by silver stain and immunoblot analyses, respectively (data not shown). ELISA analysis of IL-8 in supernatants from HAEC stimulated with purified native or biotinylated fimbriae preparations demonstrated that biotinylation did not alter the functional ability of either labeled fimbriae to elicit IL-8 production by HAEC (data not shown). To determine an approximate Kd for the interaction between TLR2 and P. gingivalis major or minor fimbriae, purified chimeric TLR2-Fc fusion protein was adsorbed to 96-well microtiter plates followed by addition of the indicated concentration of biotinylated major or minor fimbriae (total binding) or biotinylated major or minor fimbriae plus ~1 nM excess unlabelled homologous fimbriae (to assess nonspecific binding). Specific binding of major and minor fimbriae to chimeric TLR2-Fc was calculated from total and nonspecific binding in classical saturation binding experiments (data not shown). This analysis revealed an apparent Kd of 25 (Fig. 2A) and 53 nM (Fig. 2C) for the interaction between TLR2-Fc and biotinylated major and minor fimbriae, respectively. As expected, ELISA plates adsorbed with Fc-isotype controls IgG2a or uncoated plates displayed no binding to fimbriae (data not shown). In contrast to the results obtained with chimeric TLR2-Fc, no specific or saturable binding of either the major or minor fimbriae to the chimeric TLR4-Fc was observed (Fig. 2, B and D). The failure of either the major or the...
minor fimbriae to bind to TLR4-Fc was not due to inadequate plating of the chimeric receptor, as a biotinylated anti-TLR4 Ab was observed to readily bind the TLR4-Fc-coated ELISA plate (data not shown).

To confirm the specific binding of major and minor fimbriae to chimeric TLR2-Fc, we performed classical homologous competitive-binding experiments. Biotinylated major and minor fimbriae preparations were added to each well with increasing concentrations of their respective unlabeled fimbriae. Using these methods, approximate IC50 values of 1.289 \times 10^{-7} and 1.617 \times 10^{-7} M were obtained for TLR2-Fc binding to the major and minor fimbriae, respectively (Fig. 3). Approximate Kd values were then determined by subtraction of the concentration of labeled fimbriae (100 nM) from the calculated value for IC50. Competitive binding analysis resulted in a calculated Kd of 28.9 nM for major fimbriae binding to chimeric TLR2-Fc and a Kd of 61.7 nM for the minor fimbriae interaction. These results were similar to those obtained in the saturation binding experiments described above. Collectively, these results demonstrate that the interaction between TLR2 and the \textit{P. gingivalis} major and minor fimbriae displays specific, classical saturation kinetics, while these fimbrial proteins do not bind TLR4.

\textit{P. gingivalis} major and minor fimbriae bind to chimeric CD14-Fc

Pattern recognition receptors such as CD14 function as TLR co-receptors for the detection of microbial pathogens or their antigenic components and present them to TLRs for activation of proinflammatory signaling pathways (5). CD14 has been demonstrated to directly bind the major fimbriae of \textit{P. gingivalis}, and mediate the ability of monocytes/macrophages to stimulate TLR-dependent TNF-\alpha release (18). To confirm the direct binding of the major fimbriae to CD14 and establish whether the minor fimbriae could bind CD14, we used purified chimeric CD14-Fc fusion protein in a saturation binding experiment identical with those used for TLR2:Fc and TLR4:Fc constructs. We observed specific binding of the minor fimbriae to CD14 and established that this reaction follows classic saturation kinetics (Fig. 4B). In addition, we confirmed the previously established specific binding of major

\textbf{FIGURE 2.}\ To examine the interaction between TLR2-Fc and TLR4-Fc and \textit{P. gingivalis} major (A and B) or minor (C and D) fimbriae, an indirect ELISA-like binding assay was performed. A purified chimeric TLR2-Fc (A and C) or TLR4-Fc (B and D) fusion protein was adsorbed to each well of 96-well microtiter plate followed by addition of the indicated concentration of biotinylated major (A and B) or minor fimbriae (C and D) (total binding), or biotinylated fimbriae plus -1 nM excess unlabeled fimbriae (nonspecific binding). After incubation with streptavidin peroxidase, the binding of major or minor fimbriae to TLR2-Fc or TLR4-Fc was detected by measuring absorbance at 450 nm of triplicate samples. The results are presented as specific binding (total binding – nonspecific binding) and represent the average ± SE of four independent experiments. Approximate BMax and Kd values were obtained by nonlinear regression analysis of the hyperbolic curve.

\textbf{FIGURE 3.}\ Homologous competitive binding curves were conducted as described in Materials and Methods for saturation curves with the following exception, 100 nM major (A) or minor (B) biotinylated fimbriae was added to each well with increasing concentrations of unlabeled fimbriae as indicated in the graph. An approximate IC50 value was obtained by nonlinear regression analysis of sigmoidal curves and the approximate Kd determined by subtraction of the concentration of labeled fimbriae (100 nM) from the calculated value for IC50 as indicated in the figure. Data presented represents the average ± SE of three independent experiments.
fimbriae to CD14 and established that this reaction appears to follow classic saturation kinetics (Fig. 4A).

**TLR2 binds to P. gingivalis whole cells**

We next examined the ability of *P. gingivalis* strain 381 whole cells to bind to TLR2 and TLR4 chimeric proteins. For these studies, we also used the *P. gingivalis* major and minor fimbriae double mutant DPGMFB (30). Although binding of *P. gingivalis* 381 whole cells was observed to TLR2, we did not observe binding with the *P. gingivalis* double fimbriae mutant strain DPGMFB (Fig. 5). We did not detect binding of the TLR4Fc chimeric protein to whole cells from either *P. gingivalis* strains 381 or DPGMFB. These results suggest that the majority of TLR2 binding exhibited by *P. gingivalis* whole cells may be due to specific binding via the major and minor fimbriae proteins.

*P. gingivalis* major and minor fimbriae signal via TLR2 and TLR4 with a requirement for MD-2

We next confirmed that TLR2 was used for major and minor fimbria mediated signaling using human embryonic kidney 293 cells transfected with fluorescently labeled TLR2 or TLR4. These cells

**FIGURE 4.** To examine the potential interaction between CD14-Fc and *P. gingivalis* major (A) or minor (B) fimbriae, an indirect ELISA-like binding assay was performed. A purified chimeric CD14-Fc fusion protein was adsorbed to each well of 96-well microtiter plate followed by addition of the indicated concentration of biotinylated major or minor fimbriae (total binding) or biotinylated fimbriae plus ~1 nM excess unlabeled fimbriae (nonspecific binding). After incubation with streptavidin-peroxidase, the binding of major or minor fimbriae to CD14-Fc was detected by measuring absorbance at 450 nm of triplicate samples. The results are presented as specific binding (total binding – nonspecific binding) and were repeated once with similar outcomes.

**FIGURE 5.** *P. gingivalis* strain 381 (A) and the major and minor fimbriae double mutant (DPGMFB) (B) whole cells were examined for binding to TLR2-Fc and TLR4-Fc chimeric proteins as detected by FACSscan flow cytometry with FITC-conjugated anti-TLR2 or anti-TLR4 Ab analysis. IgG2a was used as a negative control. Red traces represent the addition of *P. gingivalis* whole cells. Results are representative of three separate experiments.

**FIGURE 6.** HEK 293 cells stably expressing TLR4 (293 TLR4/MD-2, top panel), TLR2 (293TLR2, middle panel), or HEK293 cells alone (bottom panel), were treated with the indicated amount of fimbriae for 16 h and the activity of the NF-κB-luciferase reporter gene determined by luminometry and expressed as relative luciferase units. Plotted are the averages of duplicate experimental samples ± SD. The shown experiment is representative of three separate experiments.

**FIGURE 7.** Bone marrow-derived macrophages from WT C57BL/6 (■), MD-2−/− (□), and CD14−/− (▲) mice were cultured with *P. gingivalis* major or minor fimbriae (10 μg/ml), *E. coli* LPS (100 ng/ml), Pam2CSK4 (PAM3; 2 μg/ml), or medium (Non) for 24 h. Culture medium was harvested for ELISA analysis of MCP-1. Data are shown as the mean and SD. *p < 0.05 compared with the stimulated C57BL/6 macrophages. Results are representative of two experiments.
or presence of major or minor fimbriae (1). In the absence or presence of major or minor fimbriae (1 μg/ml), or E. coli LPS (1 μg/ml), or S. cerevisiae zymosan (10 μg/ml) for 24 h. Supernatants from stimulated HAEC were analyzed by ELISA. All experiments were performed in triplicate with data presented as the mean and SD of three independent experiments. *, p < 0.05 vs control IgG2A Ab.

are known to respond to all known TLR2 ligands as they constitutively express TLR1 and TLR6 (35). As expected, HEK 293 cells which were not transfected with the TLR constructs did not respond to stimulation with any of the ligands examined (Fig. 6, lower panel). NF-κB activation in 293 cells expressing TLR2 was observed in response to both the major and minor fimbriae (Fig. 6 middle panel). As expected, P. gingivalis LPS and Pam2CSK4 also induced NF-κB activation in 293 cells expressing TLR2. We also observed the induction of NF-κB in 293 TLR2/MD-2 expressing cells in response to both the major and minor fimbriae (Fig. 6, upper panel). The induction of NF-κB through TLR4 in response to both the major and minor fimbriae required MD-2 as TLR4-expressing cells alone did not respond to these stimuli (data not shown). Furthermore, the ability of both the major and minor fimbriae to bind an MD-2-Fc fusion protein was confirmed in an ELISA-like binding assay. We observed that both proteins bound to MD-2 in a dose-dependent manner (data not shown). Thus, the ability of the major and minor fimbriae to signal via TLR4 may be facilitated by binding via MD-2.

To further examine the requirement for MD-2 in the recognition of P. gingivalis fimbriae, we tested the stimulatory effect of major and minor fimbriae on macrophage proinflammatory cytokine production using BMDM obtained from MD-2−/− mice. Based on the ability of both the major and minor fimbriae to bind to the CD14-Fc fusion protein, we also included BMDM from CD14−/− mice for these experiments. We observed that the MCP-1 response to both the major and minor fimbriae was significantly reduced in BMDM obtained from MD-2−/− and CD14−/− mice as compared with BMDM obtained from C57 mice; the MCP-1 response to Pam2CSK4 was similar in BMDM obtained from the C57 and MD-2−/− and CD14−/− mice (Fig. 7). As expected, the response to E. coli LPS was diminished in both the MD-2−/− and CD14−/− mice as compared with BMDM obtained from C57 mice; the MCP-1 response to Pam2CSK4 was similar in BMDM obtained from the C57 and MD-2−/− and CD14−/− mice (Fig. 7).

We also observed a diminished IL-6 response to the major and minor fimbriae in BMDM obtained from MD-2−/− and CD14−/− mice as compared with BMDM obtained from C57 mice (data not shown). We also previously observed that the TNF response to both the major (36) and minor (data not shown) fimbriae was diminished in both the TLR2−/− and TLR4−/− mice as compared with BMDM obtained from C57 mice. These results collectively indicate that both the major and minor fimbriae use both TLR2 and TLR4 for a proinflammatory response in mouse macrophages and suggest that signaling via TLR4 occurs via the participation of MD-2 and CD14.

**TLR2 and TLR4 mediate major and minor fimbriae responses in human endothelial cells**

We have previously demonstrated that the purified native forms of the major and minor fimbriae induce chemokine and adhesion molecule expression similar to live P. gingivalis in HAEC (30). To examine the potential for the major and minor fimbriae to engage TLR2 and TLR4 in HAEC, these cell cultures were treated with...
CD14 Ab reduced major and minor fimbriae induction of IL-8 when compared with nontreated cells (data not shown).

FIGURE 10. HAEC were transfected with dnTLR2 (□) or dnTLR4 (■) for 18 h before stimulation with indicated Ags. Nontransfected control HAEC (●) or transfected HAEC were then stimulated with the major or minor fimbriae (1 μg/ml), E. coli LPS (1 μg/ml), or S. cerevisiae zymosan (10 μg/ml). Supernatants from stimulated HAEC were collected 24 h post-stimulation and analyzed by ELISA. All experiments were performed in triplicate with data presented as the mean and SD of three independent experiments. *p < 0.05 vs medium control.

Discussion

Although the repertoire of microbial ligands recognized by TLRs and the concomitant signaling pathways induced by these pathogen-associated patterns are emerging, little is known regarding the specific binding kinetics of these molecules to TLRs. Moreover, it is now apparent that accessory molecules can contribute to a shift in TLR use. Using a chimeric human TLR2 protein in this study, we demonstrate what we believe to be the first direct interaction of the major and minor fimbriae of P. gingivalis with TLR2. These interactions follow classical saturation binding kinetics and were inhibited by competition with excess, nonlabeled major or minor fimbriae. Direct binding of the major and minor fimbriae to CD14 using purified chimeric CD14-Fc fusion protein confirmed the previously established specific binding of major fimbriae to CD14 (26), established specific binding of minor fimbriae to CD14, and established that this reaction follows classic saturation kinetics for both proteins. Furthermore, using P. gingivalis-defined mutants, we observed the specificity of P. gingivalis expressing major and minor fimbriae whole cell binding to TLR2. There appears to be only three other reports of direct ligand binding to TLR2. Massari et al. (35) recently demonstrated binding of the Neisseria meningitidis outer membrane protein, PorB, to the chimeric TLR2 protein. Staphylococcus aureus peptidoglycan was also recently observed to bind a plate-immobilized, recombinant extracellular domain of TLR2 in a concentration-dependent manner (38). In addition, Pam3Cys has been crystallized in complex with a heterodimer formed by the extracellular domains of TLR1 and TLR6, demonstrating direct binding of these TLRs with the microbial ligand (13). Thus, the P. gingivalis major and minor fimbriae represent two additional microbial ligands which have been demonstrated to directly bind to TLR2.

Although we did not observe binding with either the major or minor fimbriae and the TLR4-Fc chimeric protein, both the major and minor fimbriae were shown to signal through a TLR4-mediated mechanism when MD-2 was present. Using mouse macrophages, we observed a requirement for TLR2, as well as TLR4, MD-2, and CD14 in the inflammatory response to major and minor fimbriae. Furthermore, binding of both the major and minor fimbriae to MD-2 was confirmed in an ELISA-based assay. Signaling via TLR2 and TLR4 by both the major and minor fimbriae was also observed in human endothelial cells. Likewise, the involvement of CD14 in the endothelial IL-8 response to both the major and minor fimbriae was also verified. Thus, together with the in vitro-binding assays to chimeric TLR proteins, our results collectively point to an important role for MD-2 and CD14 in the TLR4-signaling response to the major and minor fimbriae in both mouse macrophages and human endothelial cells.

The ability of the P. gingivalis major and minor fimbriae to activate both TLR2 and TLR4 is not unprecedented, as other microbial ligands have been recently reported to engage more than one TLR. Similar to the P. gingivalis major and minor fimbriae,
both heat shock proteins 60 and 70 have been reported to engage both TLR2 and TLR4 (39, 40). Recent studies by Yauch et al. (41) have also demonstrated that the major capsular polysaccharide of Cryptococcus neoformans, glucuronoxylomanan, is recognized by TLR2, TLR4, and CD14. The ability of microbial pathogens to engage multiple TLRs may provide a novel mechanism for diverse pathogens to sense and signal for an inflammatory response in various host environments.

An interesting observation in our studies that deserves further comment was that while TLR4-blocking Ab inhibited major and minor fimbriae-mediated IL-8 response by HAEC, TLR2-blocking Ab did not. Our studies demonstrated that in unstimulated HAEC, TLR4 is detected on the cell surface and thus the ability of TLR4 Ab to block functional responses in HAEC relates to the ability of the Ab to bind to TLR4 on the cell surface. There are several possible explanations for the inability of TLR2 Ab to block functional response in HAEC. First, it is possible that the TLR2 Ab did not interfere with the binding site of TLR2 and fimbrine. Second, we found that in HAEC TLR2 appears to be found intracellularly. Thus, it is also possible that fimbrine-initiated endothelial cell activation of TLR2 occurred intracellularly and thus could not be blocked via Ab treatment. We have previously observed that the IL-6 and IL-8 response of HAEC to both the major and minor fimbrine is dependent on the uptake of fimbrines because cytochalasin D treatment blocked this response (30). It is possible that the intracellular pool of TLR2 that we observed via FACs analysis could potentially contribute to a fimbrine-mediated, proinflammatory response by HAEC. The reduction in IL-8 production by HAEC transfected with nonsignaling forms of TLR2 in response to major and minor fimbrine stimulation supports this contention. One additional point deserves attention regarding the expression of TLR2 in resting HAEC cultures. We have previously demonstrated that although HAEC express low levels of surface TLR2, following incubation with live P. gingivalis, these cells respond by increased surface expression of TLR2 (42). Thus, HAEC appear to regulate the expression of TLR2 in response to microbial stimulation. We speculate that the regulation of surface expression of TLR2 may be important for controlling endothelial inflammation.

The observation of intracellular pools of TLR2 in HAEC is not without precedent because a number of studies have established that TLR2 and TLR4 signaling can occur inside cells and may exhibit cell type specificity (37, 43–47). TLR4 is located intracellularly in human coronary artery endothelial cells where it can function with LPS for activation (37). It was proposed that intracellular location of TLR4 in epithelial and endothelial cells may explain why these cells are generally less sensitive to LPS as compared with monocytes (48). One study demonstrated that TLR2 is enriched in caveolin-1-associated lipid raft microdomains on the apical surface of airway epithelial cells following Gram-positive or -negative bacterial infection (46). Thus, TLR2 and TLR4 have important differences in terms of their localization within the cell and in terms of their ability to stimulate signaling reactions and cytokine production. These properties may enable TLRs to be in the correct cellular locations to optimally encounter their ligands and for customizing the innate immune response to different classes of pathogens.

In this study, we have focused on defining the receptors required for major and minor fimbrine in HAEC because the signaling receptors for P. gingivalis and fimbrine in these specific cells have not been defined. Our results are in overall agreement with the results reported on the major fimbrine response in macrophages (49) and extend these studies by also examining binding and signaling via the minor fimbrine and the response to both proteins in endothelial cells. The chemokine response of monocytes to P. gingivalis major fimbrine has been previously demonstrated to depend upon stimulation of both TLR4 and TLR2, while a similar response to stimulation with the minor fimbrine was reported to only be dependent upon TLR2 (17). Importantly, we found here that both the major and minor fimbrine can stimulate endothelial cells via TLR4. Recent studies have documented that human saliva contains soluble forms of both TLR2 and TLR4 (50, 51) and, thus, in the context of a local infection these innate immune signaling receptors may play a role in P. gingivalis immune activation. The cell specificity of P. gingivalis fimbrine activation may provide a mechanism by which P. gingivalis can signal in an appropriate host environment.

Collectively, this report provides the first evidence of a direct interaction between the major and minor fimbrine of P. gingivalis and TLR2. Although it appears that direct fimbrine stimulation of TLR2 may result in HAEC production of IL-8, the participation of accessory molecules, including MD-2 and CD14, are required for activation of a similar TLR4-mediated response. Based on these results, we propose that the innate immune response to both the major and minor fimbrine in the endothelium occurs through a direct TLR2 mechanism and through a TLR4-mediated mechanism involving CD14, which may facilitate loading of the major or minor fimbrine to MD-2. The ability of two well-defined microbe-associated molecular patterns from this Gram-negative pathogen to select for innate immune recognition receptors based on accessory proteins may provide a novel way for P. gingivalis to sense and signal in appropriate host environments. This has direct implications for the chronic inflammation induced by this pathogen in host cells found at local (oral) and distant (aortic) sites of inflammation.

Acknowledgments

We thank Dr. Paola Massari for critical review of the manuscript, Dr. Chie Hayashi for technical support, and Sulip Goswami for manuscript preparation.

Disclosures

The authors have no financial conflict of interest.

References


Downloaded from http://www.jimmunol.org/ by guest on October 4, 2017


