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The oral pathogen Porphyromonas gingivalis, as well as its purified fimbriae, are known to activate TLR2 and induce proinflammatory and proadhesive effects. The TLR2 proinflammatory pathway induces NF-κB-dependent inflammatory cytokines, whereas the TLR2 proadhesive pathway is characterized by inside-out signaling that transactivates β2 integrin adhesive activities. In this article, using dominant-negative or pharmacological approaches, we show that the two pathways bifurcate and proceed independently downstream of TLR2. Whereas the proinflammatory pathway is dependent on the adaptor molecules Toll/IL-1 receptor domain-containing adaptor protein (also known as Mal) and MyD88, the proadhesive pathway is Toll/IL-1 receptor domain-containing adaptor protein/MyD88-independent and proceeds through PI3K-mediated signaling. Although the Ser/Thr kinase Akt is a major downstream target of PI3K and was activated by P. gingivalis fimbriae in a TLR2- and PI3K-dependent way, Akt was shown not to play a role in the proadhesive pathway. In contrast, another PI3K downstream target, cytohesin-1, was shown to mediate P. gingivalis fimbria-induced activation of β2 integrin for ICAM-1 binding. Therefore, P. gingivalis fimbriae activate two distinct TLR2 pathways mediating proinflammatory or proadhesive effects. The delineation of these signaling pathways may provide appropriate targets for selectively inhibiting or enhancing specific activities, depending on whether they undermine or promote the host defense. The Journal of Immunology, 2009, 182: 6690–6696.

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Address correspondence and reprint requests to Dr. George Hajishengallis, University of Louisville Health Sciences Center, 501 South Preston Street, Room 206, Louisville, KY 40292. E-mail address: ghajis01@louisville.edu

Abbreviations used in this paper: TIR, Toll/IL-1 receptor; TIRAP, Toll/IL-1 receptor domain-containing adaptor molecule; CR3, complement receptor-3; WT, wild type; PIP3, phosphatidylinositol-(3,4,5)-trisphosphate; MyD88-DN, dominant-negative signaling mutant of human MyD88; TRAM, TRIF-related adaptor molecule; Akt-DN, dominant negative inhibitor of Akt signaling.

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objective to characterize and delineate TLR2 downstream signaling pathways may provide appropriate targets for selectively inhibiting those signaling cascades (e.g., the TLR2 proadhesive pathway) that are exploited by \textit{P. gingivalis} to enhance its survival within the host. Thus, because the innate antimicrobial response to \textit{P. gingivalis} appears to depend on the TLR2/NF-\kappaB pathway (18), it would be inappropriate to design and apply intervention strategies that inhibit TLR2 signaling altogether. The data presented in this article show that TLR2 signaling can indeed bifurcate into two distinct pathways: The standard TLR2 proinflammatory pathway that is dependent upon the signaling adaptors TIRAP and MyD88, and a novel TLR2-mediated but TIRAP/MyD88-independent proadhesive pathway, which stimulates CR3 adhesive activity via Rac1, PI3K, and cytohesin-1.

Materials and Methods

Reagents

mAbs to human CD11b (CBRM1/5) or mouse CD11b (M1/70), and Ig isotype controls were purchased from eBioscience. PMA, wortmannin, LY294002, and LY30351 were from Sigma-Aldrich. Recombinant human or mouse ICAM-1 was obtained from the R&D Systems. Fim fimbriae were chromatographically purified from \textit{P. gingivalis} strain 381, as previously described (21), or from strain ATCC 32377, as described by Yoshimura et al. (27). The strain 32377 fimbriae were provided by Dr. F. Yoshimura, Aichi-Gakken University, Nagoya, Japan. The final fimbrial preparations were free of any contaminating substances on silver-stained SDS-PAGE, and tested negative for endotoxin (<0.7 ng/mg protein) according to quantitative Limulus amebocyte lysate assay (BioWhittaker). Fimbriae from either strain (99.7% identity at the FimA amino acid level) were indistinguishable in the functional assays presented in this study. All reagents were used at effective concentrations determined in preliminary experiments or in previous publications (6, 20, 26).

Cell culture

Human monocytic THP-1 cells stably transfected with human CD14 (THP-1/CD14) (29) were provided by Dr. P. S. Tobias (The Scripps Research Institute, La Jolla, CA) and were cultured at 37°C and 5% CO2 atmosphere, in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 10 mM HEPES, 100 U/ml penicillin RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FBS was added. The cells were cultured overnight and were subsequently washed and used in functional assays.

Cell transfections and inhibition of intracellular signaling

Transfections of THP-1/CD14 cells were performed using the FuGene 6 transfection reagent (Roche Applied Science), as we previously described (20). For inhibition of intracellular signaling, THP-1/CD14 cells were transfected with plasmids expressing dominant negative variants of human TLR2 (pZERO-hTLR2iriless), MyD88 (pDeNy-hMyD88), TIRAP (pDeNy-hTIRAP), TRIF-related adaptor molecule (TRAM) (pDeNy-hTRAM), or empty vector controls (Invivogen). A plasmid expressing a kinase-inactive version of human Akt (K179M), which functions as an inactive control peptide (34), was purchased from Imgenex. To inhibit the expression of cytohesin-1, we followed an antisense strategy developed by Hmama et al., (35, 36) using antisense and control sense phosphorothioate-modified oligonucleotides (S-oligos) specified in previous reports and synthesized by Invitrogen. In brief, THP-1/CD14 cells (106 cells in 0.25 ml serum-free RPMI 1640 medium) were incubated at 37°C with various concentrations (0–5 \muM) of S-oligos in the presence of 2.5% lipofectamine (Invitrogen). After 2 h, the medium was adjusted to 1 ml and 10% FBS was added. The cells were cultured overnight and were subsequently washed and used in functional assays.

Cytokine induction

Primary cells or transfected cell lines were stimulated with \textit{P. gingivalis} fimbriae (1 mg/ml) for 16 h at 37°C and induction of release of TNF-\alpha or IL-6 in culture supernatants was measured by ELISA, using kits from E Bioscience (30). Akt activation. Total and phosphorylated Akt levels were determined using a colorimetric fast-activated cell-based ELISA kit, as instructed by the manufacturer (Active Motif).

Cytokine release assay. The CBRM1/5 epitope induction assay was used to monitor the activation state of human CR3 (CD11b/CD18), as we have previously described (21). The assay is based on the property of the CBRM1/5 mAb to detect a conformational change on human CD11b that signifies the high-affinity binding state of CR3 (37). Activation of mouse CR3 was assessed by monitoring its binding activity for soluble ICAM-1, a ligand that binds activated but not resting CR3 (20, 38). Specifically, biotinylated sICAM-1 was allowed to bind to mouse macrophages for 30 min at 37°C. Subsequently, the cells were washed and incubated on ice with FITC-labeled streptavidin. After washing, binding was determined by measuring cell-associated fluorescence (in relative fluorescence units) on a microplate fluorescence reader (FL600, Bio-Tek) with excitation/emission wavelength settings of 485/530 nm. Background fluorescence was determined in cells treated with medium only and FITC-streptavidin.

Statistical analysis

Data were evaluated by ANOVA and the Dunnett multiple-comparison test using the InStat program (GraphPad Software). Where appropriate (comparison of two groups only), two-tailed \textit{t}-tests were also performed. A \textit{p} value <0.05 was taken as the level of significance. Experiments were performed using triplicate samples and were performed twice or more for verification.

Results

The TLR2 proadhesive pathway is MyD88 independent

Although \textit{P. gingivalis} fimbria-activated TLR2 mediates both proinflammatory and proadhesive effects, it is unknown whether these two types of activities involve common or distinct signaling adaptors. Because MyD88 is a central signaling adaptor for TLR2 proinflammatory signaling (39), we investigated whether treatments that block MyD88 could also inhibit TLR2 inside-out signaling for CR3 activation (monitored by induction of the CBRM1/5 epitope, which signifies the high-affinity binding conformation of CR3; Ref. 37). We found that transfection of THP-1/CD14 cells with a plasmid expressing a dominant-negative signaling mutant of human MyD88 (MyD88-DN) (40) did not affect the ability of the fimbriae to induce the CR3 activation-specific CBRM1/5 epitope, in contrast to transfection with TLR2-DN, which served as positive control (Fig. 1A). However, in the same cells, MyD88-DN significantly inhibited NF-\kappaB activation by fimbriae, as also observed with TLR2-DN (\textit{p}<0.05; Fig. 1A).
Similar results were obtained using a cell permeable peptide, which inhibits dimerization of the MyD88 TIR domains (31). This MyD88 inhibitory peptide had no effect on the ability of *P. gingivalis* fimbriae to induce the CBRM1/5 epitope in monocytic THP-1/CD14 cells, although it significantly suppressed fimbria-induced activation of NF-κB in the same cells (*p* < 0.05; Fig. 1B). Therefore, MyD88 signaling is not required for CR3 activation by *P. gingivalis* fimbriae, although it mediates activation of NF-κB by the same agonist.

These conclusions were confirmed in the mouse model using WT or MyD88-deficient macrophages. The cells were monitored for their adhesive activity for soluble (s)ICAM-1, which binds activated but not resting CR3 (38). We found that *P. gingivalis* fimbriae could activate CR3-dependent binding of sICAM-1 equally well in WT or MyD88-deficient macrophages, although it significantly suppressed fimbria-induced activation of NF-κB in the same cells (*p* < 0.05; Fig. 1B). Therefore, MyD88 signaling is not required for CR3 activation by *P. gingivalis* fimbriae, although it mediates activation of NF-κB by the same agonist.

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gingivalis fimbriae, upon transfection with a TIRAP mutant (P125H) which acts as dominant negative inhibitor (TIRAP-DN) (43). Transfection with TLR2-DN served as a positive control, whereas transfections with empty vector or a dominant negative version (C117H) of the TRAM, which is exclusively involved in TLR4 signaling (44, 45), served as negative controls. We found that TIRAP-DN failed to suppress CBRM1/5 epitope induction by P. gingivalis fimbriae (as seen with the negative controls), although TLR2-DN readily inhibited the same activity (p < 0.05; Fig. 3). However, as expected, both TLR2-DN and TIRAP-DN (but not TRAM-DN) inhibited fimbria-induced NF-κB activation (Fig. 3).

The notion that TIRAP is involved in the TLR2 proinflammatory but not the proadhesive pathway was confirmed using mouse macrophages. Indeed, a TIRAP inhibitory peptide, which blocks TIRAP-dependent signaling (32, 33), significantly (p < 0.05) diminished the ability of P. gingivalis fimbriae to induce IL-6 production, although it had no effect on the ability of fimbria to stimulate CR3 adhesive activity, as measured by sICAM-1 binding (Fig. 4). Treatments with a control peptide had no effect on either assay (Fig. 4). Therefore, TIRAP signaling has no effect on P. gingivalis fimbria-induced proadhesive activities, although it readily inhibits P. gingivalis fimbria-induced proinflammatory activities. These findings, taken together with those from Figs. 1 and 2, indicate that the proadhesive pathway is TIRAP- and MyD88-independent.

Akt is activated in a TLR2/P13K-dependent way but is not involved in the proadhesive pathway

P13K plays an essential role in the P. gingivalis fimbria-induced TLR2 proadhesive signaling pathway (20, 21). Because the Ser/Thr kinase Akt was previously implicated in inside-out proadhesive signaling (46) and is one of the most likely downstream targets of P13K for a variety of cellular functions (47), we examined possible involvement of Akt in the fimbria-activated proadhesive pathway. We first examined whether Akt is activated by P. gingivalis fimbriae in a TLR2- and P13K-dependent way. Indeed, stimulation of THP-1/CD14 cells by fimbriae led to activation (phosphorylation) of Akt, although this activity was significantly (p < 0.05) suppressed in cells transfected with TLR2-DN or pretreated with the P13K inhibitor, LY294002 (but not with its inactive analog, LY303511) (Fig. 5A). None of the treatments influenced total Akt levels (Fig. 5A). We then determined whether transfection of THP-1/CD14 cells with a dominant negative inhibitor of Akt signaling (Akt-DN) could inhibit induction of the CR3 activation-specific CBRM1/5 epitope by P. gingivalis fimbriae. Empty vector and TLR2-DN were used as negative and positive controls, respectively. Akt-DN failed to inhibit CBRM1/5 induction, in contrast to TLR2-DN, which displayed dose-dependent inhibitory action (Fig. 5B). We observed a similar lack of inhibitory effects on CBRM1/5 epitope induction, using a range of concentrations (25–125 μM) of a cell permeable peptide that blocks Akt kinase activity (34) or a pharmacological inhibitor of Akt (5–10 μM; 1L-6-Hydroxymethyl-chiro-inositol2-((R)-2-O-methyl-3-O-octadecylcarbonate)) (data not shown). Therefore, a different P13K downstream effector may link TLR2 to CR3 activation, and we set out to identify it.
Role of cytohesin-1 in *P. gingivalis* fimbria-induced CR3 activation

In addition to Akt, cytohesin-1 is another pleckstrin homology domain-containing cytoplasmic protein that binds to phosphatidylinositol-3,4,5-trisphosphate (PIP3) generated by PI3K. Moreover, cytohesin-1 is also implicated in induction of integrin adhesive capacity (36, 48). We have thus investigated whether cytohesin-1 participates in the *P. gingivalis* fimbria-induced pathway for CR3 activation, using an established antisense strategy for inhibiting cytohesin-1 expression (35). Treatment of THP-1/CD14 cells with antisense S-oligos to cytohesin-1 mRNA (but not with control sense S-oligos) significantly inhibited the ability of fimbria-stimulated cells to induce the CBRM1/5 epitope and bind sICAM-1 (*p < 0.05; Fig. 6). However, the antisense S-oligo treatments had no significant effect on NF-κB activation by *P. gingivalis* fimbriae (Fig. 6). Therefore, cytohesin-1 appears to mediate *P. gingivalis* fimbria-induced activation of the proadhesive but not of the proinflammatory pathway.

**Discussion**

As more information becomes increasingly available on the intricacies of TLR signaling, it is appreciated that the control of adverse host responses in infection-driven inflammatory diseases would require highly selective and precisely targeted intervention. This in turn requires precise delineation of TLR signaling pathways in response to a given pathogen, such as *P. gingivalis*. It is generally thought that TLR4 signaling is more complex than TLR2 signaling, because TLR4 activation initiates two divergent signaling cascades: the TIRAP/MyD88 pathway which activates NF-κB, and the TRAM/TRIF (TIR-domain-containing adapter-inducing IFN-β) pathway, which additionally triggers activation of the IFN regulator factor-3 (8). In this study, we have presented evidence that TLR2 activation also initiates two divergent signaling pathways.

Specifically, TLR2 activation by *P. gingivalis* fimbriae induces a TIRAP/MyD88-dependent proinflammatory pathway and a TIRAP/MyD88-independent but PI3K-dependent proadhesive pathway, as illustrated in the Fig. 7 model. The former pathway induces production of NF-κB-dependent proinflammatory cytokines and the antimicrobial molecule NO, within hours upon cell stimulation with *P. gingivalis* (6, 18). The proadhesive pathway is characterized by inside-out signaling which, within minutes, transactivates the adhesive capacity of CR3 (CD11b/CD18) (21), a β2 integrin which can binds both host and microbial molecules (49). More recently, other agonists were also shown to activate the TLR2 proadhesive pathway (50). In particular, peptidoglycan and the Pam3CysSerLys3 lipopeptide were demonstrated to activate TLR2- and CD11b/CD18-dependent monocyte adhesion to ICAM-1, although intermediate signaling molecules were not examined (50).

Our demonstration that the TLR2/PI3K proadhesive pathway proceeds independently of MyD88 and TIRAP is consistent with earlier findings by another group that the cytoplasmic domain of TLR2 contains two PI3K binding motifs (YXXM/W) that could serve as docking sites for PI3K and play a positive regulatory role in the TLR2 proadhesive pathway (20) (Fig. 7), presumably through...
NF-κB fimbriae interact with the CD14/TLR2 complex leading to TLR2 activation. Activated TLR2 induces TIRAP/MyD88-dependent signaling for NF-κB activation and induction of proinflammatory cytokines (proinflammatory pathway), and TIRAP/MyD88-independent signaling for transactivation of the CR3 adhesive activity (proadhesive pathway). The latter pathway proceeds via Rac1, PI3K, and cytoshein-1 (Cyt-1). Cytoshein-1 is likely to function immediately downstream of PI3K, because it was previously shown to use PI3K-generated PIP3 for membrane docking and interaction with the CD18 cytoplasmic tail (48).

its ability to bind PI3K and augment its activity (53). Despite these earlier developments, the downstream effector of PI3K in the P. gingivalis fimbria-activated proadhesive pathway had remained elusive.

In this study, we identified cytoshein-1, rather than Akt, as the downstream PI3K effector in the TLR2 proadhesive pathway. Interestingly, although PMA induces β2 integrin activation through a distinct pathway involving protein kinase C, this pathway also uses cytoshein-1 as an effector (54, 55). Moreover, the ability of LPS to activate the ligand-binding capacity of leukocyte function-associated Ag-1 (CD11a/CD18) is similarly dependent on cytoshein-1 activity (35). Therefore, cytoshein-1 appears to functionally integrate distinct signaling pathways for β2 integrin activation. Earlier studies have established that the inside-out signaling regulation of integrin binding affinity involves targeting of intracellular proteins to the cytoplasmic tails of the integrin, thereby causing high-affinity conformational changes on its ligand-binding domain (reviewed in Ref. 56). Because cytoshein-1 can use PI3K-generated PIP3 phospholipids as docking sites to interact with the CD18 cytoplasmic tail (48), it can be suggested that cytoshein-1 forms a link between PI3K and CR3 (CD11b/CD18) in the P. gingivalis fimbria-induced TLR2 proadhesive pathway (Fig. 7).

Although a role for Akt in this pathway is not supported by our data, Akt was previously implicated as an essential PI3K downstream effector for glycoprotein Ib-IX-induced inside-out signaling for αIIbβ3 integrin activation in platelets (46).

Monocyte transmigration is mediated by interacting sets of cell adhesion molecules, including the CR3 (CD11b/CD18)-ICAM-1 pair (57). In this regard, activation of TLR2 inside-out signaling by P. gingivalis (or purified fimbriae) leads to CR3-dependent monocyte adhesion to endothelial ICAM-1 and transmigration across endothelial cell monolayers (20). Although this is a potentially protective mechanism that may contribute to the recruitment of monocytes to sites of P. gingivalis infection, adhesion of monocytes to the arterial endothelium and their subsequent migration into the subendothelial area is a hallmark of early atherogenesis (57). Whether this may form a mechanistic basis linking this pathogen to inflammatory atherosclerotic processes, in which it has been implicated (4), is uncertain at the moment. However, there is sufficient evidence that P. gingivalis has co-opted the TLR2/CR3 proadhesive pathway for CR3 binding and entry into macrophages in a way that promotes its virulence (19, 26). Strikingly, the same pathway is exploited by mycobacteria for promoting their uptake by monocytes/macrophages through activated CR3 (36) leading to their survival and intracellular persistence (58), possibly because this receptor is not linked to vigorous microbicidal mechanisms (reviewed in Ref. 59). It is intriguing to speculate that pathogen-induced TLR2 inside-out signaling for CR3 activation may be a universal pathway exploited by a number of pathogens and, therefore, ways to block this pathway could be beneficial in certain infections.

In addition to a better understanding of the signaling pathways activated by P. gingivalis or its purified fimbriae, our current findings may be significant from a translational viewpoint. In this regard, delineation of signaling pathways may provide potential therapeutic targets for selectively enhancing or inhibiting pathway activities, depending on whether they promote or undermine host defense. If TLR2/NF-κB-mediated immunity helps control P. gingivalis infection, whereas exploitation of TLR2-transactivated CR3 favors P. gingivalis persistence, as supported by our recent studies (18, 25, 26, 59), it would make sense to inhibit TLR2 signaling in a way that would inhibit specifically the proadhesive pathway. Indeed, inhibition of the TLR2 proadhesive pathway may prevent CR3-mediated uptake of P. gingivalis, an internalization route that suppresses the ability of macrophages to clear P. gingivalis infection (25, 26). So far, we have identified three targets in the proadhesive pathway downstream of TLR2: Rac1, PI3K, and cytoshein-1. Considering that Rac1 and PI3K are involved in a great number of essential physiological functions (60, 61), it appears that cytoshein-1 may be a more feasible target for intervention. More studies are warranted to determine the usefulness of a cytoshein-based approach as a strategy to counteract immune evasion by P. gingivalis.

Disclosures

The authors have no financial conflict of interest.

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