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TLR2 Transmodulates Monocyte Adhesion and Transmigration via Rac1- and PI3K-Mediated Inside-Out Signaling in Response to *Porphyromonas gingivalis* Fimbriae

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We present evidence for a novel TLR2 function in transmodulating the adhesive activities of human monocytes in response to the fimbriae of *Porphyromonas gingivalis*, a pathogen implicated in chronic periodontitis and atherosclerosis. Monocyte recruitment into the subendothelium is a crucial step in atherosclerosis, and we investigated the role of *P. gingivalis* fimbriae in stimulating monocyte adhesion to endothelial cells and transendothelial migration. Fimbriae induced CD11b/CD18-dependent adhesion of human monocytes or mouse macrophages to endothelial receptor ICAM-1; these activities were inhibited by TLR2 blockade or deficiency or by pharmacological inhibitors of PI3K. Moreover, this inducible adhesive activity was sensitive to the action of *Clostridium difficile* toxin B, but was not affected by *Clostridium botulinum* C3 exoenzyme, pertussis toxin, or cholera toxin.

Accordingly, we subsequently showed through the use of dominant negative signaling mutants of small GTPases, that Rac1 mediates the ability of fimbria-stimulated monocytes to bind ICAM-1. A dominant negative mutant of Rac1 also inhibited the lipid kinase activity of PI3K suggesting that Rac1 acts upstream of PI3K in this proadhesive pathway. Furthermore, fimbriae stimulated monocyte adhesion to HUVEC and transmigration across HUVEC monolayers; both activities required TLR2 and Rac1 signaling and were dependent upon ICAM-1 and the high-affinity state of CD11b/CD18.

The interactions of CD11b/CD18 with fibrinogen and ICAM-1 can be induced upon stimulation of other surface receptors, such as chemotactic receptors (1, 10) or TLRs (11, 12). Inside-out signaling pathways for CD11b/CD18 activation can be induced upon stimulation of other surface receptors, such as chemotactic receptors (1, 10) or TLRs (11, 12).

The potential of CD11b/CD18 for vascular cell interactions by binding to ICAM-1 or to endothelial-associated matrix proteins, such as fibrinogen, may contribute to cardiovascular inflammation (5, 13). In this context, the adhesion of bloodborne leukocytes to the arterial endothelium, followed by their migration into the subendothelial area is a hallmark of early atherogenesis (14). The transmigratory process is mediated by interacting sets of cell adhesion molecules, including the CD11b/CD18-ICAM-1 pair, which has been experimentally implicated in atherosclerosis and other inflammatory conditions (13, 15–17). It is thought that infectious agents contribute to vascular inflammation and certain bacterial pathogens such as *Chlamydia pneumoniae*, *Helicobacter pylori*, and *Porphyromonas gingivalis* have been implicated as accessory factors in the development or acceleration of atherosclerosis (14, 18, 19). In this regard, infection-driven chronic inflammatory diseases, including periodontitis, are associated with increased risk for cardiovascular disease (20–22).

*P. gingivalis* is a Gram-negative oral bacterium that is strongly associated with chronic periodontitis (23). This pathogen may disseminate from periodontal lesions into the systemic circulation and *P. gingivalis*-specific DNA has been detected in human atherosclerotic plaques (24). Studies in animal models of periodontitis or atherosclerosis have established the *P. gingivalis* fimbriae (filamentous appendages on the cell surface) as a major virulence factor of this pathogen (25, 26). Although both wild-type *P. gingivalis* and an isogenic non-fimbriated mutant are detected in the blood and aortic arch tissue of orally infected hyperlipidemic mice, only
the presence of wild-type *P. gingivalis* is associated with periodontal disease and increased atherosclerotic plaque formation (26). In this study, we have identified a plausible inflammatory mechanism whereby *P. gingivalis* fimbriae may contribute to the atherosclerotic process. Based on earlier findings that *P. gingivalis* fimbriae bind CD14 and stimulate TLR2/P3K-mediated inside-out signaling for CD11b/CD18 activation (11, 27), we now show that activation of this pathway leads to increased monocyte adhesion to fibrinogen, ICAM-1, and endothelial cells. This inductive proadhesive pathway is distinct from other CD11b/CD18 activation pathways stimulated by FMLP or PMA, as shown by differential toxin sensitivity. On the basis of toxin sensitivity data and additional experiments using dominant negative (DN) signaling mutants of small GTPases, we found that Rac1 acts upstream of PI3K and is essential for the ability of fimbria-stimulated monocytes to bind CD11b/CD18 ligands, endothelial cells, and transmigrate across endothelial monolayers. The property of *P. gingivalis* fimbriae to induce CD11b/CD18-dependent adhesive interactions may contribute to the role of monocytes in the process of atherosclerosis or other inflammatory conditions.

Materials and Methods

Reagents

mAbs to human TLR2 (clone TL2.1), TLR4 (HTA125), CD11b (CBRM1/5, FITC-labeled), MHC class I (W6/32), and Ig isotype controls (IgG1, IgG2a) were purchased for eBioscience. FITC-labeled mAb to human CD11b (Bear-1) and mAbs to human CD14 (MEM-18), mouse CD11b (M1/70), and its IgG2b isotype control were from Caltag Laboratories. mAbs to human CD11b (2LP19c) was from DakoCytomation. mAb to ICAM-1 (BBIG-I1) and human rICAM-1 plus rIL-1 receptor; PTx, pertussis toxin; CTx, cholera toxin; CdTxB, C. difficile toxin B; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate.

**Cell culture**

Monocytes were purified from the peripheral blood of healthy human volunteers by positive selection using a magnetic cell separation system (Miltenyi Biotec, Auburn, CA). Monocytes were cultured in RPMI-1640 (Invitrogen Life Technologies) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.05 mM 2-ME in a humidified incubator with 5% CO2 atmosphere.

**Transmigration assay**

To assess monocyte transmigration across a HUVEC monolayer, we used the Transwell plate system (6.5-mm Transwell inserts with 8.0-μm pores; Corning Costar) and a modification of a previously described protocol (33). Briefly, HUVECs were seeded at 5 × 104 cells/well in complete RPMI. Cell lines were provided by Dr. P. S. Tobias (The Scripps Research Institute, La Jolla, CA). HUVEC (PromoCell) were cultured at 37°C and 5% CO2 atmosphere in PromoCell Endothelial Cell Growth medium (2% FCS, 0.1 ng/ml epidermal growth factor, 1.0 ng/ml basic fibroblast growth factor, 1.0 μg/ml hydrocortisone, 0.4% endothelial cell growth supplement/heparin, 10 μg/ml gentamicin, 50 ng/ml amphotericin B), according to the supplier’s recommendations. Thiglycollate-elicted macrophages were isolated from the peritoneal cavity of mice deficient in CD14, TLR2, TLR4, both TLR2 and TLR4, or from wild-type control mice, as previously described (27, 32). The mice deficient in CD14, TLR2, or TLR4 were of C57Bl/6 genetic background, whereas mice harboring homozygous TLR2 and TLR4 mutations were 9-fold backcrossed toward the C3H genetic background (kindly donated by Dr. C. Kirschning, Technical University of Munich, Munich, Germany). Mouse macrophages were cultured in complete RPMI as described. The use of animals was reviewed and approved by the Institutional Animal Care and Use Committee. Human or mouse cell viability was monitored using a CellTiter-Blue Cell Viability assay kit (Promega). The use of fimbriae and other agonists as well as treatments with blocking mAbs or other antagonists did not affect cell viability as compared with medium-only control treatments.

**THP-1/CD14 cell transfections**

Transfections of THP-1/CD14 cells were performed using the FuGene 6 transfection reagent (Roche Applied Science) at a reagent to DNA ratio of 3:1, according to the manufacturer's instructions. The DN versions of the human Rac1, Cdc42, and RhoA genes (Rac1/T17N, Cdc42/ T17N, and RhoA/T19N, respectively) as well as the empty control vector pUSEamp3 were obtained from Upstate Biotechnology. A plasmid expressing a DN mutant of human TLR2 (pZERO-hTLR2tirless) and empty vector control (pZERO-mcs) were obtained from Invivogen. The cells were used in functional assays 48 h posttransfection. Transient transfection efficiency was 40–45% as determined by GFP reporter plasmid transfection and fluorescence microscopy to detect cells presenting green fluorescence.

**Monocyte adhesion to immobilized ligands or endothelial cells**

The 96-well microtiter plates were coated with 10 μg/ml human fibrinogen or rICAM-1. Following overnight incubation at 4°C, remaining uncoated sites on the plates were blocked with 5% skim milk (Sigma-Aldrich) for 1 h at room temperature. Monocytes were labeled with the fluorescent dye calcine AM (2.5 μM; Molecular Probes) for 30 min, washed, and resuspended in assay buffer (HBSS, 10 mM HEPES (pH 7.4), 1 mM MgCl2, and 0.4 mM CaCl2). Labeled cells were added to the ligand-coated wells (5 × 104 cells/well) at 37°C in the absence or presence of 30-min stimulation with native fimbriae or rFimA (1 μg/ml) or positive control agonists (FMLP, 10−7 M; PMA, 10−7 M) in the presence of 20% FCS. Monocytic adhesion was assessed at 30 min with blocking mAbs or pharmacological inhibitors before stimulation. At the end of the 30-min binding time, nonadherent cells were removed by careful washing repeated four times. Cell adhesion was quantified using a fluorescence microscope plate reader (FL600, Bio-Tek Instruments) with excitation/emission wavelength settings of 485/530 nm, and was expressed as a percentage of total cells expressing fluorescent microscopy fluorescence (added × 100). HUVEC (between passages 2 and 5) were seeded on 96-well plates at 5 × 104 cells/well and cultured for 2 days to form a confluent monolayer. Calcine AM-labeled monocytes were added at 2 × 105 cells per well of confluent HUVEC monolayer and incubated for 30 min at 37°C and 5% CO2 atmosphere. Nonadherent monocytes were gently washed off and monocyte adhesion was assessed as described using a Bio-Tek fluorescence microplate reader. Wells containing only HUVEC were used to determine background fluorescence, which was minimal and subtracted from each experimental value.

**Transmigration assay**

To assess monocyte transmigration across a HUVEC monolayer, we used the Transwell plate system (6.5-mm Transwell inserts with 8.0-μm pores; Corning Costar) and a modification of a previously described protocol (33). Briefly, HUVEC was seeded at 5 × 104 cells/well in complete RPMI. Cell lines were provided by Dr. P. S. Tobias (The Scripps Research Institute, La Jolla, CA). HUVEC (PromoCell) were cultured at 37°C and 5% CO2 atmosphere in PromoCell Endothelial Cell Growth medium (2% FCS, 0.1 ng/ml epidermal growth factor, 1.0 ng/ml basic fibroblast growth factor, 1.0 μg/ml hydrocortisone, 0.4% endothelial cell growth supplement/heparin, 10 μg/ml gentamicin, 50 ng/ml amphotericin B), according to the supplier’s recommendations. Thiglycollate-elicted macrophages were isolated from the peritoneal cavity of mice deficient in CD14, TLR2, TLR4, both TLR2 and TLR4, or from wild-type control mice, as previously described (27, 32). The mice deficient in CD14, TLR2, or TLR4 were of C57Bl/6 genetic background, whereas mice harboring homozygous TLR2 and TLR4 mutations were 9-fold backcrossed toward the C3H genetic background (kindly donated by Dr. C. Kirschning, Technical University of Munich, Munich, Germany). Mouse macrophages were cultured in complete RPMI as described. The use of animals was reviewed and approved by the Institutional Animal Care and Use Committee. Human or mouse cell viability was monitored using a CellTiter-Blue Cell Viability assay kit (Promega). The use of fimbriae and other agonists as well as treatments with blocking mAbs or other antagonists did not affect cell viability as compared with medium-only control treatments.
**PI3K activation assay**

PI3K activity was measured as enzymatic production of phosphatidylinositol-3,4,5-trisphosphate (PIP3) from phosphatidylinositol 4,5-bisphosphate (PIP2) substrate by means of a PI3K ELISA kit (Echelon Biosciences) as previously described (27). Briefly, PI3K was immunoprecipitated from cell lysates using anti-PI3K Ab and protein A-agarose beads, and the bead-bound enzyme was subsequently incubated with PIP2 substrate in kinase reaction buffer for 2 h at room temperature. The generation of PIP3 product was determined by competitive ELISA.

**CD11b/CD18 activation assay**

The CBRM1/5 epitope induction assay was used to monitor the activation state of CD11b/CD18, as we have previously described (11). The assay is based on the property of the CBRM1/5 mAb to detect a conformational change on CD11b that signifies the high-affinity binding state of CD11b/CD18 (34).

**Statistical analysis**

Data were evaluated by ANOVA and the Dunnett multiple-comparison test using the InStat program (GraphPad). Where appropriate (comparison of two groups only), two-tailed t tests were also performed. Statistical differences were considered significant at the level of \( p < 0.05 \). Experiments were performed using triplicate samples and were performed twice or more to verify the results.

**Results**

P. gingivalis fimbriae induce CD11b/CD18-dependent cell adhesion to immobilized ICAM-1 or fibrinogen via TLR2-mediated inside-out signaling

We have previously shown that P. gingivalis fimbriae induce an activation-specific neoepitope (CBRM1/5) on CD11b, via a novel inside-out signaling pathway involving CD14, TLR2, and PI3K (11). We now investigated the functional significance of this signaling pathway. First, we examined the ability of fimbria-stimulated monocytes to bind well-characterized ligands, such as ICAM-1 and fibrinogen, which are bound by CD11b/CD18 only when this integrin is activated (6). Upon 30-min stimulation at 37°C with 1 μg/ml native fimbriae or rFimA, human monocytes bound efficiently to ICAM-1- or fibrinogen-coated microtiter wells, in contrast to medium-only-treated monocytes that bound poorly (6–16% of the binding activity of stimulated cells; Fig. 1, A and B). When monocytes were pretreated with anti-CD11b mAb (2LPM19c, 10 μg/ml) or with an allosteric antagonist of CD11b/CD18 (XVA143, 1 μM) before stimulation with fimbriae, their ability to bind immobilized ICAM-1 (Fig. 1A) or fibrinogen (Fig. 1B) was significantly (\( p < 0.05 \)) diminished. Pretreatment with IgGl isotype control or a mAb to an unrelated surface Ag (MHC class I) had no effect in this regard (Fig. 1, A and B). These data indicate that P. gingivalis fimbriae (in native or recombinant form)
stimulate monocyte adhesion to immobilized ICAM-1 or fibrinogen in a CD11b/CD18-dependent way.

However, the data described do not necessarily show that the observed fimbria-induced monocyte adhesion is mediated via the CD14-TLR2-PI3K inside-out signaling pathway (11). We thus pretreated monocytes with mAbs to CD14 or TLR2, and determined their ability to bind immobilized ICAM-1 or fibrinogen upon activation with native fimbrae or rFimA. In contrast to isotype controls or mAbs with irrelevant specificities (TLR4 or MHC class I), anti-CD14 or anti-TLR2 significantly inhibited adhesion (p < 0.05; Figs. 1, C and D). The effect of combined anti-TLR2 and anti-TLR4 treatment was not significantly different from the use of anti-TLR2 alone (Fig. 1, C and D). We next determined the effect of PI3K inhibitors on fimbria-stimulated monocyte adhesion. Specifically, pretreatment with wortmannin or LY294002 (but not with its inactive analog, LY303511) resulted in significantly (p < 0.05) reduced cell adhesion to immobilized ICAM-1 or fibrinogen (Fig. 1, E and F, respectively). The inhibitory action of wortmannin or LY294002 could not be attributed to nonspecific toxic effects, because both compounds had no influence on PMA-stimulated cell adhesion, which was however inhibitable by GF109203X, a PKC inhibitor (Fig. 1, E and F).

The involvement of CD14 and TLR2, but not of TLR4, in P. gingivalis fimbra-induced cell adhesion was conclusively shown using pattern-recognition receptor (PRR) knockout mouse macrophages. We found that wild-type or TLR4-deficient macrophages could readily bind to immobilized ICAM-1 or fibrinogen upon stimulation with native fimbrae or rFimA (Fig. 2, A and B). In stark contrast, similarly stimulated macrophages deficient in CD14 or TLR2 or macrophages with combined TLR2 and TLR4 deficiencies failed to display enhanced binding to the same molecules (Fig. 2, A and B). FMLP and PMA were used as controls and their stimulatory effect on macrophage adhesion was not influenced by any PRR deficiency tested (Figs. 2, A and B). The observed cell adhesion to ICAM-1 or fibrinogen was dependent upon CD11b/CD18 as shown by the inhibitory effect of treatments with anti-CD11b or with XVA143 (Fig. 2, C and D). The data from Figs. 1 and 2 collectively indicate that fimbrae interact with CD14 and TLR2 leading to activation of PI3K-mediated intracellular signaling for enhanced CD11b/CD18-dependent cell adhesion.

**Toxin sensitivity of fimbra-stimulated monocyte adhesion**

The ability of P. gingivalis fimbrae to induce the CD11b activation-specific CBRM1/S5 neoeptope is not affected by the G protein inhibitor, pertussis toxin (PTx), in contrast with PTx-sensitive induction of CBRM1/S5 by FMLP (11). Similarly, PTx had no effect on the ability of fimbrae to stimulate monocyte adhesion to immobilized ICAM-1 or fibrinogen, although the same toxin significantly inhibited (p < 0.05) FMLP-stimulated cell adhesion (Figs. 3A and 4A). To further characterize the fimbra-induced proadhesive pathway, we have investigated three additional toxins that inhibit integrin activation by interfering with activation signals, namely cholera toxin (CTx), Clostridium difficile toxin B (CdtxB), and Clostridium botulinum C3 transference exoenzyme (C3 exoenzyme) (35–37). Following pretreatment with various doses of the toxins, human monocytes were allowed to bind immobilized ICAM-1 (Fig. 3) or fibrinogen (Fig. 4) for 30 min at 37°C in the absence or presence of stimulation with P. gingivalis native fimbrae, FMLP, or PMA. CTx did not significantly influence monocyte adhesion to ICAM-1 (Fig. 3B) or fibrinogen (Fig. 4B) regardless of the agonists used for cell stimulation. However, there was a statistically significant linear trend (p < 0.05) regarding the effect of CTx on cell adhesion induced by fimbrae or FMLP, but not with PMA. Specifically, CTx appeared to have a modest enhancing effect on fimbra-induced cell adhesion, whereas the opposite trend was observed for FMLP-induced cell adhesion (Figs. 3B and 4B). Interestingly, within the same dose range we tested, CTx acts as a potent inhibitor of P. gingivalis fimbra-induced cytokine release (38).

In contrast to CTx, CdtxB had a profound inhibitory effect (p < 0.05) on both fimbra- and FMLP-stimulated monocyte adhesion to immobilized ICAM-1 or fibrinogen (Figs. 3C or 4C).

*FIGURE 2.* Adhesion of P. gingivalis fimbra-stimulated mouse macrophages to ICAM-1 or fibrinogen is inhibited by CD14 or TLR2 deficiency. Fluorescently labeled macrophages from wild-type mice or mice deficient in CD14, TLR2, TLR4, or both TLR2 and TLR4 (TLR2/4) were added to 96-well plates coated with ICAM-1 (A and C) or fibrinogen (B and D). The cells were allowed to bind for 30 min at 37°C in the absence or presence of stimulation with P. gingivalis native fimbrae or rFimA (both at 1 μg/ml), or with control agonists (FMLP, 10⁻⁷ M; PMA, 0.1 μg/ml). Before the assays (C and D), wild-type macrophages were pretreated for 30 min with 10 μg/ml IgG2b isotype control or anti-CD11b, or with 1 μM XVA143. Cell adhesion was assessed fluorometrically as outlined in the legend to Fig. 1. Data are presented as the mean ± SD (n = 3) from a typical set of experiments that were repeated yielding similar results. Significantly reduced macrophage adhesion (+, p < 0.05) due to receptor deficiency (A and B) or due to CD11b/CD18 blockade (C and D) is shown.
respectively). However, suppression of PMA-induced cell adhesion by CdTxB did not reach statistical significance, despite a significant linear trend with increasing toxin dose (Figs. 3C and 4C). The C3 exoenzyme had no effect, whatsoever, on fimbria-stimulated cell adhesion, although the same toxin significantly inhibited \((p < 0.05)\) the activity of FMLP and PMA (Figs. 3D and 4D). The toxins did not influence basal cell adhesion, which was \(8\%\) of that seen in the presence of agonists (data not shown). These data suggest that the signaling pathway involved in \(P.\) gingivalis fimbria-stimulated monocyte adhesion to a ligand (fibrinogen) or a counterreceptor (ICAM-1) of CD11b/CD18 is distinct from those stimulated by FMLP or PMA. The fimbria-stimulated pathway is sensitive to the action of CdTxB but is not affected by PTx, CTx, or C3 exoenzyme.

**Rac1 is involved in \(P.\) gingivalis fimbria-induced cell adhesion and is acting upstream of PI3K**

CdTxB is known to inhibit the small molecular weight GTPases, Rho, Rac, and Cdc42, whereas C3 exoenzyme specifically inhibits Rho (RhoA, B, and C), but not Rac or Cdc42 (39). Therefore, on the basis of the toxin sensitivity data (Figs. 3 and 4), we hypothesized that the proadhesive pathway activated by \(P.\) gingivalis fimbriae involves participation of Rac or Cdc42, but not of Rho GTPase. To test this hypothesis, we determined the ability of human monocytic THP-1/CD14 cells to bind immobilized ICAM-1 or fibrinogen, upon cell transfection with empty vector control or with DN inhibitors of Rac1, Cdc42, or RhoA. We found that THP-1/CD14 cells transfected with Rac1-DN, but not with Cdc42-DN or RhoA-DN, displayed significantly reduced adhesion to ICAM-1 in response to fimbriae \((p < 0.05)\), compared with empty vector-transfected cells (Fig. 5A). Similar results were obtained when adhesion was tested on fibrinogen-coated plates (data not shown). In a parallel experiment using similarly transfected but FMLP-stimulated THP-1/CD14 cells, cell adhesion to ICAM-1 was significantly inhibited \((p < 0.05)\) by RhoA-DN but not by Rac1-DN or Cdc42-DN (Fig. 5A). Therefore, Rac1 appears to be a second signaling intermediate, in addition to PI3K, involved in \(P.\) gingivalis fimbria-stimulated cell adhesion to ICAM-1. These data further support that \(P.\) gingivalis fimbriae and FMLP activate distinct intracellular signaling pathways, involving Rac1 and RhoA, respectively, for CD11b/CD18 activation.

Rac1 and PI3K regulate cellular function through various, often overlapping, signaling pathways and either of the two intracellular enzymes can activate the other, depending on the specific pathway involved (40). We examined whether Rac1-DN could additionally inhibit fimbria-induced PI3K activation to determine whether Rac1 acts upstream of PI3K. Specifically, we examined whether Rac1-DN inhibits the lipid kinase activity of PI3K, monitored
through the generation of PI3K from PIP2 substrate. TLR2-DN and RhoA-DN were used as positive and negative controls, respectively. We found that Rac1-DN and TLR2-DN (but not RhoA-DN) could significantly inhibit \( (p < 0.05) \) the ability of fimbriae to activate PI3K (Fig. 5B). Therefore, Rac1 appears to be a signaling intermediate, acting between TLR2 and PI3K, in the \( P. \) gingivalis fimbria-stimulated pathway for CD11b/CD18-dependent cell adhesion.

Our findings that \( P. \) gingivalis fimbriae stimulate monocyte adhesion to ICAM-1, a major endothelial receptor, suggested that fimbriae may similarly up-regulate monocyte adhesion to HUVEC. To investigate this possibility, monocytes were added with or without fimbriae (1 \( \mu \)g/ml) to a HUVEC monolayer that was previously either activated (by 1 ng/ml IL-1\( \beta \) for 16 h) or maintained unstimulated (medium only). Unstimulated or IL-1\( \beta \)-stimulated HUVECs were washed before addition of monocytes to remove IL-1\( \beta \) from the coculture system. We found that the ability of monocytes to adhere to the HUVEC was significantly higher (1 \( \mu \)g/ml) in the presence of fimbriae than in the presence of medium only, regardless of whether the HUVECs were pretreated with IL-1\( \beta \) (Fig. 6A). However, monocyte adhesion to IL-1\( \beta \)-stimulated HUVECs was significantly enhanced (1 \( \mu \)g/ml) compared with unstained HUVECs (Fig. 6A). When monocytes were preincubated with anti-CD11b mAb or the XVA143 allosteric antagonist of CD11b/CD18 before being exposed to fimbriae and added to the HUVEC monolayer, their adhesive activity was significantly reduced (1 \( \mu \)g/ml; Fig. 6B). In contrast, an IgG1 isotype control or a mAb to an unrelated surface Ag (MHC class I) were without effect in this regard (Fig. 6B). Furthermore, when unstained or IL-1\( \beta \)-stimulated HUVECs were preincubated with anti-ICAM-1 mAb before addition of monocytes and fimbriae, their ability to support monocyte adhesion was significantly decreased (1 \( \mu \)g/ml; Fig. 6C). In contrast, control treatments (isotype control or irrelevant mAb) had no effect (Fig. 6C). These data collectively suggest that \( P. \) gingivalis fimbriae promote monocyte adhesion to HUVEC in a CD11b/CD18- and ICAM-1-dependent way.

P. gingivalis fimbriae promote monocyte transmigration through HUVEC monolayer

We next examined the ability of fimbria-stimulated monocytes for transmigration across HUVEC monolayers. For this purpose, monocytes were added with or without fimbriae (1 \( \mu \)g/ml) to the upper chamber of Transwells containing HUVEC monolayers, and the incubation was conducted for 3 h. We found that the ability of monocytes for transmigration was significantly enhanced (1 \( \mu \)g/ml) in the presence of fimbriae than in the presence of medium only (Fig. 7A). However, when monocytes were preincubated with...
FIGURE 5. Rac1 is involved in P. gingivalis fimbria-induced cell adhesion to ICAM-1 (A) and stimulation of the lipid kinase activity of PI3K (B). A. THP-1/CD14 cells transfected with empty vector control or with DN mutants of Rac1, Cdc42, or RhoA (at the indicated microgram amounts of plasmid DNA per 2 x 10⁵ cells) were fluorescently labeled and added to 96-well plates coated with ICAM-1. The cells were allowed to bind for 30 min at 37°C in the absence or presence of stimulation with 1 μg/ml P. gingivalis fimbriae or 10⁻⁷ M FMLP. After washing to remove nonadherent monocytes, cell adhesion was assessed fluorometrically and was expressed as a percentage of total cells added. B. THP-1/CD14 cells transfected with DN mutants of TLR2, Rac1, or RhoA were stimulated for 30 min with 1 μg/ml fimbriae. Subsequently, PI3K was immunoprecipitated from cell lysates and its enzymatic activity was assessed as described in Materials and Methods. Data are presented as the mean ± SD (n = 3), from one of two (A) or three (B) independent sets of experiments that yielded similar results. Statistically significant (*, p < 0.05) inhibition of cell adhesion (A) or of PIP₃ production (B) due to transfection with DN mutants is indicated.

anti-CD11b mAb or the XVA143 antagonist before being exposed to fimbriae and added to HUVEC, their ability for transendothelial migration was significantly inhibited (p < 0.05; Fig. 7B). On the contrary, an IgG1 isotype control or mAb with irrelevant specificity (MHC class I) did not affect monocyte transmigration (Fig. 7B). Furthermore, pretreatment of HUVEC with anti-ICAM-1 mAb before addition of monocytes and fimbriae resulted in significantly reduced (p < 0.05) monocyte transmigration, whereas control treatments had no effect in this regard (Fig. 7C). These data jointly suggest that P. gingivalis fimbriae promote transendothelial migration of monocytes in a CD11b/CD18- and ICAM-1-dependent mode.

Under the experimental conditions used, it was possible that fimbriae also activated the HUVEC. Indeed, the incubation time (3 h) appeared adequate for fimbria-induced up-regulation of ICAM-1 expression in HUVEC (41). We therefore set out to confirm that fimbria-induced transendothelial migration of monocytes was mediates, at least in part, by direct effects of fimbriae on monocytes (i.e., through induction of CD11b/CD18 activation). To this end, we compared the transmigration activity of monotypic THP-1/CD14 cells to that of CD14-nonexpressing THP-1/RSV cells. The rationale was that fimbriae would not effectively induce CD11b/CD18 activation in the latter cell line (due to diminished CD14 expression, required for fimbria-induced inside-out signaling (11)), resulting in reduced transmigration activity of THP-1/RSV cells compared with THP-1/CD14 cells. Indeed, fimbriae readily induced CD11b/CD18 activation in THP-1/CD14 cells (but not in THP-1/RSV cells), as evidenced by induction of the CD11b activation-specific CBRM1/5 neoepitope (Fig. 8A). Moreover, the fimbria-induced transendothelial migration activity of THP-1/CD14 cells was significantly higher (p < 0.05) compared with that of THP-1/RSV cells (Fig. 8B), confirming that CD11b/CD18 activation plays an important role in the transmigration process. The relative inability of THP-1/RSV cells to respond to fimbriae was due to any inherent defects in this cell line. Indeed, when PMA was used instead as an agonist, both cell lines could equally well induce the CBRM1/5 neoepitope (Fig. 8A) and stimulate transmigration (Fig. 8B).

Rac1 is involved in fimbria-stimulated monocyctic cell adhesion to HUVEC and transendothelial migration

Rac1, but not RhoA, is required for enhanced THP-1/CD14 adhesion to ICAM-1 in response to P. gingivalis fimbriae (Fig. 5). We now determined whether Rac-1 is similarly involved in the ability of fimbria-stimulated THP-1/CD14 cells to adhere to HUVEC and transmigrate across the HUVEC monolayer, and moreover, examined whether these activities correlate with CD11b/CD18 activation. For this purpose, we used THP-1/CD14 cells transiently transfected with Rac1-DN. Cells transfected with TLR2-DN or RhoA-DN were used as positive or negative controls, respectively. We found that cells transfected with Rac1-DN or TLR2-DN showed significantly reduced ability (p < 0.05) for CD11b/CD18 activation (CBRM1/5 epitope induction), adhesion to HUVEC, and transendothelial migration in response to P. gingivalis fimbriae, as compared with untransfected cells or cells transfected...
with empty vector control (Fig. 9). Transfection with Rac1-DN or TLR2-DN had no effect on surface expression of CD11b/CD18, as shown by staining with Bear-1, a mAb that detects CD11b regardless of its activation state (data not shown). In contrast to THP-1/CD14 cells transfected with Rac1-DN or TLR2-DN, transfection with RhoA-DN did not influence their ability for CD11b/CD18 activation and adhesion to HUVEC (Fig. 9). However, the transmigrating activity of RhoA-DN-transfected cells was similarly affected and was significantly diminished \( p < 0.05 \) relative to untransfected or empty vector-transfected cells (Fig. 9). Therefore, RhoA may be involved in fimbria-induced monocyte transmigration by acting on a process that is independent of CD11b/CD18 activation and adhesion to HUVEC. These data demonstrate that

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We then determined whether fimbriae could activate monocyte transendothelial migration in cell-associated form. This examination used whole cells of *P. gingivalis* from wild-type strains 381 and AT436 or their respective isogenic non-fimbriated mutants. We found that the wild-type strains were significantly more potent (*p* < 0.05) than their non-fimbriated mutants in stimulating monocyte adhesion to HUVEC and transmigration across HUVEC monolayers (Fig. 10). These data suggest that *P. gingivalis* has the potential to contribute to the inflammatory processes in atherosclerosis by stimulating monocyte recruitment into subendothelial areas.

**Discussion**

It has been speculated that inside-out signaling mechanisms evolved to resolve two competing objectives (42). On the one hand, there is need for highly mobile leukocytes to roam and detect potential threats; in contrast, these wandering cells should hold fast to sites of infection they encounter and thus need to be rapidly reprogrammable. In this context, we consider that TLRs are appropriate transmodulators of the adhesive activities of leukocytes because these PRRs can both detect infection and transduce activating intracellular signals (43). Our identification of a novel, TLR2-mediated inside-out signaling pathway (11) and, most importantly, the current demonstration that this pathway regulates the adhesive and transmigrating activities of human monocytes clearly support this notion. CD11b/CD18 and β2 integrins in general are appropriate effectors of such proadhesive pathways due to their ability to engage, once activated, diverse ligands or counterreceptors implicated in the inflammatory response (1, 2, 4, 6). The significance of inside-out signaling in the regulation of leukocyte adhesion was emphasized by the discovery of an alternative form of leukocyte adhesion deficiency (44). In this type of deficiency, β1, β2, and β3 integrins are normally expressed on the cell surface but fail to be activated by intracellular signaling pathways to bind ligands (44).

This study showed that *P. gingivalis* fimbriae stimulate monocyte adhesion to β2 integrin ligands (Fig. 1) or to endothelial cells (Fig. 6) and activate monocyte transendothelial migration (Figs. 7 and 8B) through induction of inside-out signaling. On the basis of previous (11) and current data, this proadhesive signaling pathway involves the sequential participation of CD14, TLR2, Rac1, PI3K, and the CD11b/CD18 (Fig. 11). Initial evidence that this mechanism of CD11b/CD18 activation involves a distinct intracellular signaling pathway from those induced by FMLP or PMA came from data of differential toxin sensitivity (Figs. 3 and 4). The novel pathway activated by *P. gingivalis* fimbriae was sensitive to the action of CdTxB, but was not influenced by C3 exoenzyme, PTx, or CTx. These findings pointed to a possible participation of Rac1 in this pathway because Rac1 is inhibitable by CdTxB but not by C3 exoenzyme (39). At the same time, these data suggested that RhoA was not a likely signaling molecule candidate in the fimbria-induced pathway because RhoA is inhibitable by both CdTxB and C3 exoenzyme (39). Experiments examining the effects of DN signaling mutants on the ability of fimbriae to induce CD11b/CD18-dependent monocyte adhesive activities confirmed the importance of Rac1 and the irrelevance of RhoA, although the latter was important for the FMLP-induced proadhesive pathway. An additional difference regarding the mechanisms whereby fimbriae and FMLP stimulate monocyte adhesion involves the lack of PI3K requirement in the case of FMLP. Specifically, although FMLP activates PI3K in our experimental system, the use of specific PI3K inhibitors (wortmannin or LY294002) does not inhibit the ability of FMLP to stimulate monocyte adhesion (unpublished observation). Therefore, PI3K is not a point of convergence in the proadhesive pathways activated by fimbriae or FMLP. FMLP-activated PI3K may mediate other effector functions in monocytes, such as induction of NF-κB activation (45). The ability of fimbriae to stimulate monocyte transendothelial migration was inhibited not only by the Rac1-DN but also by the RhoA-DN mutant (Fig. 9C). Because RhoA is not involved in fimbria-induced activation of CD11b/CD18 and monocyte adhesion to endothelial cells, this finding appeared somewhat unexpected. However, RhoA was previously found to be essential for the retraction of the tail of the migrating monocyte to complete diapedesis (33) and this may account for the inhibitory effect of the RhoA-DN mutant in our transmigration model.

Rac1 and other small GTPases of the Rho family are regulated by GTP/GDP exchange and function as molecular switches that control signaling pathways involved in kinase regulation, gene transcription, cytoskeleton organization, cell motility, and other functions. The study of Rac1 and its role in leukocyte adhesion and transmigration is relevant to several aspects of leukocyte biology, including the regulation of leukocyte adhesion to endothelial cells and the development of new therapeutic strategies for atherosclerosis.
cellular processes (46, 47). The property of Rac1 to function as a molecular on-off switch is consistent with its involvement in P. gingivalis fimbria-induced inside-out signaling pathway, which needs to be rapidly and transiently activated. PI3K is also a component of this signaling pathway and appears to act downstream of Rac1 (Fig. 11). In this regard, it was shown that Rac1, but not RhoA, can bind PI3K and augment its activity (48). These findings are in line with our observations that a DN inhibitor of Rac1, but not of RhoA, inhibits PI3K activity (Fig. 5B). Two PI3K binding motifs are present on the TLR2 cytoplasmic tail and PI3K is recruited to TLR2 upon activation with heat-killed Staphylococcus aureus (49). This pathway proceeds downstream of PI3K through the Ser/Thr kinase Akt and results in NF-kB-dependent transcription (49), whereas stimulation of alternative PI3K effectors may result in activation of CD11b/CD18-dependent adhesion as seen in the present study.

The ability of CD14/TLR2 to detect P. gingivalis fimbriae and initiate inside-out signaling for CD11b/CD18 activation is a potentially protective mechanism, which can contribute to monocyte recruitment to sites of P. gingivalis infection. Fimbriae could stimulate this proadhesive pathway in bacterial cell-associated form or more effectively as free molecules shed from the bacterial cell surface or as components of released outer membrane vesicles that can readily infiltrate tissues (50). However, the ability of P. gingivalis fimbria-stimulated TLR2 to transmodulate the adhesive activity of CD11b/CD18 may also constitute a potentially harmful mechanism contributing to destructive inflammation in conditions associated with P. gingivalis. Evidence from biopsy studies on human carotid endarterectomy specimens or from experimental

FIGURE 9. Rac1 is involved in induction of CD11b/CD18 activation, adhesion to HUVEC, and transendothelial migration of fimbria-stimulated monocytic cells. THP-1/CD14 cells, transfected with empty vector control (EVC) or with DN mutants of TLR2, Rac1, or RhoA (at the indicated microgram amounts of plasmid DNA per 2 x 10⁶ cells), were stimulated with 1 μg/ml P. gingivalis fimbriae and tested for induction of CD11b/CD18 activation, adhesion to HUVECs, and transendothelial migration. Results were normalized to the activity of untransfected THP-1/CD14 cells, and are presented as the mean ± SD (n = 3) from one of two independent sets of experiments that yielded similar findings. Statistically significant inhibition (*, p < 0.05) of cell activity due to transfection with DN mutants is indicated.

FIGURE 10. The ability of P. gingivalis to promote monocyte adhesion (A) and transmigration through HUVEC monolayers (B) correlates with its fimbriation state. Fluorescently labeled monocytes (2 x 10⁵) were added to confluent HUVEC monolayers and assayed for adhesion or transmigration. The monocytes were added in the presence or absence of wild-type (Wt) P. gingivalis strains 381 or A7436 or their non-fimbriated isogenic mutants (Mt) at the indicated multiplicity of infection. Results are presented as the mean ± SD (n = 3) from one of two independent experiments that yielded similar findings. Statistically significant enhancement (*, p < 0.05) of monocyte adhesion or transmigration by wild-type P. gingivalis compared with corresponding mutant is shown.
monocyte-derived macrophages with pendent on the presence of fimbriae (41). An additional mecha-

nism, characteristic of atherosclerotic lesions (55). In addition to endothelial cells (58). This pathogen also induces foam cell for-

mocytes (56, 57) or through up-regulation of ICAM-1 expression in cytoskeleton-driven monocyte accumulation into the subendothelium.

In conclusion, innate recognition of P. gingivalis fimbriae results in activation of TLR2, which transmodulates the adhesive activity of CD11b/CD18 via Rac1 and PI3K (Fig. 11). Activated CD11b/CD18 renders the monocytes capable of binding endothelium ICAM-1 and transmigrating across endothelial cells. Because monocyte recruitment into subendothelial areas plays an important role in the early steps of atherogenesis, the inducible proadhesive pathway described in this study may form a mechanistic basis linking P. gingivalis to inflammatory atherosclerotic processes.

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Disclosures

The authors have no financial conflict of interest.

References


