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Role of the Phosphatidylinositol 3 Kinase-Akt Pathway in the Regulation of IL-10 and IL-12 by *Porphyromonas gingivalis* Lipopolysaccharide[1]

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Stimulation of the APC by *Porphyromonas gingivalis* LPS has been shown to result in the production of certain pro- and anti-inflammatory cytokines. However, the signaling pathways that regulate these processes are currently unknown. In the present study, the role of the phosphatidylinositol 3 kinase (PI3K)-Akt pathway in regulating *P. gingivalis* LPS-induced production of IL-10, IL-12 p40, and IL-12 p70 by human monocytes was investigated. *P. gingivalis* LPS selectively activates the PI3K-Akt pathway via Toll-like receptor 2, and inhibition of this pathway results in an abrogation of extracellular signal-regulated kinase 1/2 phosphorylation, whereas the activation of p38 and c-Jun N-terminal kinase 1/2 kinases were unaffected. Analysis of cytokine production following stimulation of monocytes with *P. gingivalis* LPS revealed that inhibition of the PI3K pathway differentially regulated IL-10 and IL-12 synthesis. IL-10 production was suppressed, whereas IL-12 levels were enhanced. Inhibition of *P. gingivalis* LPS-mediated activation of the PI3K-Akt pathway resulted in a pronounced augmentation of NF-κB p65 that was independent of IκB-α degradation. Furthermore, the ability of the PI3K-Akt pathway to modulate IL-10 and IL-12 production appears to be mediated by the selective suppression of extracellular signal-regulated kinase 1/2 activity, as the MEK1 inhibitor PD98059 closely mimicked the effects of wortmannin and LY294002 to differentially regulate IL-10 and IL-12 production by *P. gingivalis* LPS-stimulated monocytes. These studies provide new insight into how engagement of the PI3K-Akt pathway by *P. gingivalis* LPS affects the induction of key immunoregulatory cytokines that control both qualitative and quantitative aspects of innate and adaptive immunity. *The Journal of Immunology*, 2003, 171: 717–725.

The ability of the innate immune system to respond to microbial components has been attributed to a family of type I transmembrane Toll-like receptors (TLRs)[3] (1, 2). Recognition of conserved microbial products by the innate immune system leads to a variety of signal transduction pathways that dictate the subsequent immune response. In this regard, activation of TLRs has been shown to result in the induction and secretion of regulatory cytokines that can positively or negatively influence innate and adaptive immunity (3–7). However, recent evidence also suggests that such stimulation of the innate immune system can result in a TLR-specific repertoire of inducible genes (8–12).

Bioactive IL-12 p70, which is composed of two disulfide-linked subunits (p35 and p40) that are encoded on separate genes, has been shown to be an important mediator of cell-mediated immunity (13–15). Levels of IL-12 produced during the early innate immune response in part dictate macrophage activation, IFN-γ secretion, Th1 effector cell development, and IgG2a production (13–20). Moreover, overproduction of IL-12 has been shown to exert deleterious effects on the development of Th2-type immune responses, as well as contribute to endotoxin shock (14, 15, 21, 22). Although the molecular mechanisms that control IL-12 production are still being resolved, IL-10 has been shown to be a major counter-regulatory cytokine that can affect the immunomodulatory effects of IL-12 directly or indirectly (23–25). The production of IL-10 during bacterial infection has been shown to suppress production of inflammatory mediators and aid in the development of Th2 immunity. Indeed, IL-10-deficient mice develop inflammatory diseases that are associated with pronounced increases in IL-12 production (26, 27). Whereas the ability of IL-10 and IL-12 to regulate qualitative and quantitative aspects of cell-mediated and humoral immunity are well defined, the underlying cellular and molecular mechanisms responsible for dictating their initial induction are an area of intense investigation.

The family of phosphoinositide 3 kinase enzymes is largely responsible for phosphorylation of phosphatidylinositol lipids in response to various stimuli (28). Phosphatidylinositol 3 kinase (PI3K) is a heterodimeric enzyme that consists of a regulatory (p85) and a catalytic (p110) subunit. Activation of PI3K occurs via the regulatory subunit binding to phosphotyrosine residues present within cellular receptors located on the plasma membrane (28, 29). Moreover, direct binding of the catalytic subunit to Ras has also been shown to activate PI3K (28). Once activated, PI3K catalyzes the production of phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$), which allows for recruitment of signaling proteins, including the serine-threonine kinase Akt (30, 31). Binding to $PI(3,4,5)P_3$ results in Akt becoming dually phosphorylated (30–32). Direct evidence for the

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; PI3K, phosphatidylinositol 3 kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; $PI(3,4,5)P_3$, phosphatidylinositol-3,4,5-triphosphate; MEK, mitogen-activated protein kinase kinase; IC, isotype control; MAPK, mitogen-activated protein kinase; LDS, lithium dodecyl sulfate.

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involvement of PI3K in TLR signaling has recently been elucidated by Arbibe et al. (33) who demonstrated that mutations within multiple p85 docking sites of TLR2 resulted in both a loss in the ability of p85 to associate with TLR2 and the concomitant abrogation of NF-κB transcriptional activity. These studies are supported by recent evidence that the PI3K-Akt pathway plays a pivotal role in regulating production of inflammatory mediators in both human and mouse monocytes/macrophages (34, 35). Interestingly, PI3K knockout mice exhibit defective Th2-associated immunity, whereas Th1 responses are elevated (34, 36), including IL-12 production and the development of Th1 immunity. Thus, PI3K activity may be central to the development of cell-mediated immunity by affecting IL-12 synthesis directly, or PI3K may act by exerting an effect on counterregulatory circuits.

P. gingivalis is considered to be one of the primary etiologic agents of adult periodontitis, a chronic inflammatory disease characterized by the destruction of alveolar bone and the supporting connective tissue surrounding teeth (37–43). The LPS of this bacterium has been implicated in both the initiation and progression of disease (34, 38–46). Previous studies have demonstrated that protein-free *P. gingivalis* LPS retains immunostimulatory activity in C3H/HeJ mice (10, 47–51). Furthermore, it has been shown that unlike enterobacterial LPS, *P. gingivalis* LPS uses TLR2 to induce innate immune responses in both human and mouse macrophages (10, 52, 53). Interestingly, it has also been reported that *P. gingivalis* LPS is able to suppress the biological activity of several TLR4 agonists (54). Moreover, the production of bioactive IL-12 induced by *P. gingivalis* LPS has been shown to be negligible when compared with that of *Escherichia coli* LPS (10, 55). In this regard, several studies characterizing the inflammatory response of periodontitis have reported a high degree of Th2- compared with Th1-associated cytokines (55–58). Although activation of TLR2 by *P. gingivalis* LPS has been shown to result in the production of both pro- and anti-inflammatory cytokine production, the associated signaling events that regulate these processes are incompletely understood. Because PI3K has been shown to be activated in response to TLR2 agonists (33) and is important in controlling the production of select proinflammatory cytokines (34, 35), we sought to determine the functional significance of the PI3K-Akt pathway in regulating pro- and anti-inflammatory cytokine production by human monocytes stimulated with *P. gingivalis* LPS. In the present study we show that inhibition of *P. gingivalis* LPS-mediated activation of the PI3K-Akt pathway results in a severe reduction in IL-10 production, with a concurrent augmentation in IL-12 levels. Inhibition of PI3K activity abrogated extracellular signal-regulated kinase (ERK) 1/2 phosphorylation, whereas activation of p38 and c-Jun N-terminal kinase (JNK) 1/2 kinases was unaffected. The ability of the PI3K-Akt pathway to modulate IL-10 and IL-12 production appears to be mediated by the selective suppression of ERK 1/2 activity, because the MEK1 inhibitor PD98059 closely mimicked the effects of wortmannin and LY294002 to differentially regulate IL-10 and Il-12 production by *P. gingivalis* LPS-stimulated monocytes. Thus, activation of the PI3K pathway by *P. gingivalis* LPS can modulate the induction of important immunoregulatory cytokines involved in the development of the immune response.

Materials and Methods

Reagents

Mouse anti-human TLR2 (clone 2392; IgG1) was obtained from Genentech (South San Francisco, CA) and has been previously characterized (52, 53, 59, 60). Mouse anti-human TLR4 (clone HTA125, IgG2a) was purchased from eBioscience (San Diego, CA) and was characterized previously (52, 53, 61). Protein-free *P. gingivalis* LPS was prepared as previously described (10, 62). All isotype-matched control Abs (IgG1 and IgG2a), functional grade (neutralizing) anti-human IL-10 (clone JES3-9D7) mAb, and the anti-human CD14-FITC were purchased from eBioscience. All cell culture reagents were purchased from Life Technologies (Grand Island, NY). Levels of IL-10, IL-12 p40, and IL-12 p70 present in cellfree supernatants were determined using cytokine reagents purchased from eBioscience or R&D Systems (Minneapolis, MN). PD98059, SB203580, and wortmannin were purchased from Calbiochem (San Diego, CA). Abs used for the detection of total and phosphorylated Akt, mitogen-activated protein kinase kinase (MEK) 1/2, ERK 1/2, p38, JNK 1/JNK 2, and IκB-α were obtained from Cell Signaling (Beverly, MA).

Cell culture

Heparinized venous blood from healthy donors was used to obtain PBMC by isolating the buffy coat and eliminating RBC contamination by histopaque (specific gravity-1.077) density gradients. Human monocytes were purified from PBMC by negative selection using a monocyte isolation kit purchased from Miltenyi Biotec (Auburn, CA). Monocytes were isolated from the PBMC by depletion of nonmonocytic cells. The isolation was performed with the aid of an indirect magnetic isolation kit using monoclonal hapten-conjugated CD3, CD7, CD19, CD45RA, CD56, and IgE Abs (Miltenyi Biotec). This procedure routinely resulted in >95% pure $CD14^+$ cells, as shown by flow cytometry. Human monocytes were cultured in 24- (2×10^6/well) or 96- (2×10^5/well) well plates containing RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 mM HEPES, 50 U/ml penicillin, and 50 μg/ml streptomycin. To assess the functional role of TLR2 or TLR4, monocytes were incubated with 20 μg/ml anti-TLR2 mAb, anti-TLR4 mAb, or functional-grade isotype-matched control Abs for 60 min before stimulation with *P. gingivalis* LPS. To assess the functional involvement of ERK 1/2, p38, or wortmannin in LPS-induced cytokine production by monocytes, cells were pretreated for 60 min with the MEK1 inhibitor PD98059, the p38 inhibitor SB203580, or PI3K inhibitors wortmannin or LY294002 at the indicated concentrations.

NF-κB activity

Human monocytes in 24-well polystyrene tissue culture plates were pretreated for 45 min with wortmannin as described in the figure legends and then incubated with medium alone or *P. gingivalis* LPS for 5 h. Cells were collected, washed two times in PBS, and then assayed for p65 NF-κB activity using the TransAM NF-κB kit (Active Motif, Carlsbad, CA). The assay plate has an immobilized oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′). The active form of NF-κB contained in nuclear extracts specifically binds to this oligonucleotide. The primary Ab used to detect NF-κB recognizes an epitope on p65 that is accessible only when NF-κB p65 is activated and bound to its target DNA. The level of nuclear NF-κB p65 was expressed as the optical density emitted at 450 nm from 2 μg of lysate.

Western blot analysis

Human monocytes (2×10^6/ml) in 24-well plates were pretreated with medium, SB203580, PD98059, wortmannin, LY294002, 0.1% DMSO, anti-TLR2, anti-TLR4, or IC Abs before the addition of medium or *P. gingivalis* LPS. At the indicated time points, cells were washed with PBS and then lysed on ice for 10 min in 300 μl of lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM sodium flouride, 1 mM PMSF, and 1 mg/ml aprotonin). The whole-cell lysate was passed through a 20-gauge needle three times and then incubated on ice for an additional 30 min. Cell debris were pelleted by centrifugation, and the supernatants were collected and stored at −80°C until assayed. Twenty micrograms of total cellular protein was suspended in lithium dodecyl sulfate (LDS) buffer, heated for 10 min at 70°C, resolved by LDS-PAGE, and then transferred to polyvinylidene difluoride membranes using the Novex system (Invitrogen, Carlsbad, CA). Probing and visualization of immunoreactive bands were performed by using the Western Breeze Chemiluminescence kit (Invitrogen) and following the manufacturer's protocol. Densitometer scans of the blots were performed using the AlphaImager 2000 documentation and analysis system (Alpha Innotech, San Leandro, CA).

Statistical analysis

Statistical significance between groups was evaluated by ANOVA and the Tukey multiple-comparison test using the InStat program (GraphPad, San Diego, CA). Differences between groups were considered significant at the level of $p < 0.05$.

Results

P. gingivalis *LPS activation of the PI3K-Akt pathway is TLR2-dependent*

Activation of PI3K results in the production of $PI(3,4,5)P_3$, which can recruit signaling proteins possessing pleckstrin homology domains, including the serine-threonine kinase Akt (30–32). After recruitment and activation, Akt becomes phosphorylated at Thr^{308} and Ser^{473} (30–32). Therefore, we initially sought to assess whether *P. gingivalis* LPS activates the PI3K-Akt pathway. Human monocytes were stimulated with *P. gingivalis* LPS for 30 min following pretreatment with medium or the PI3K inhibitors wortmannin or LY294002, and the phosphorylation status of Akt was determined. Stimulation of human monocytes with *P. gingivalis* LPS resulted in the phosphorylation of Akt at Ser^{473} (Fig. 1*A*). Similar results were observed for *P. gingivalis* LPS-mediated phosphorylation of Akt at Thr^{308} (data not shown). Pretreatment of monocytes with the PI3K inhibitor wortmannin or LY294002 significantly ($p < 0.05$) suppressed phosphorylation of Akt induced by *P. gingivalis* LPS (Fig. 1*A*). These data indicate that treatment of human monocytes with *P. gingivalis* LPS results in activation of the PI3K-Akt pathway.

To confirm our previous findings that *P. gingivalis* LPS-activated cells via TLR2 also extended to PI3K activation, phosphorylation of Akt induced by *P. gingivalis* LPS was measured after preincubation of monocytes with a mAb to TLR2 or TLR4, or with an isotype control (IC) Ab. *P. gingivalis* LPS-mediated phosphorylation of Akt was severely ($p < 0.05$) reduced in monocytes pretreated with an anti-TLR2 mAb (Fig. 1*B*). In contrast, no effect on Akt phosphorylation was observed following preincubation of monocytes with a mAb to TLR4 or either IC Ab and then stimulation with *P. gingivalis* LPS (Fig. 1*B*). However, Akt phosphorylation (Ser^{473}) was reduced by pretreatment of monocytes with anti-TLR4 mAb, followed by stimulation with *E. coli* LPS (data not shown). Thus, *P. gingivalis* LPS also mediates activation of the PI3K-Akt pathway in human monocytes via TLR2.

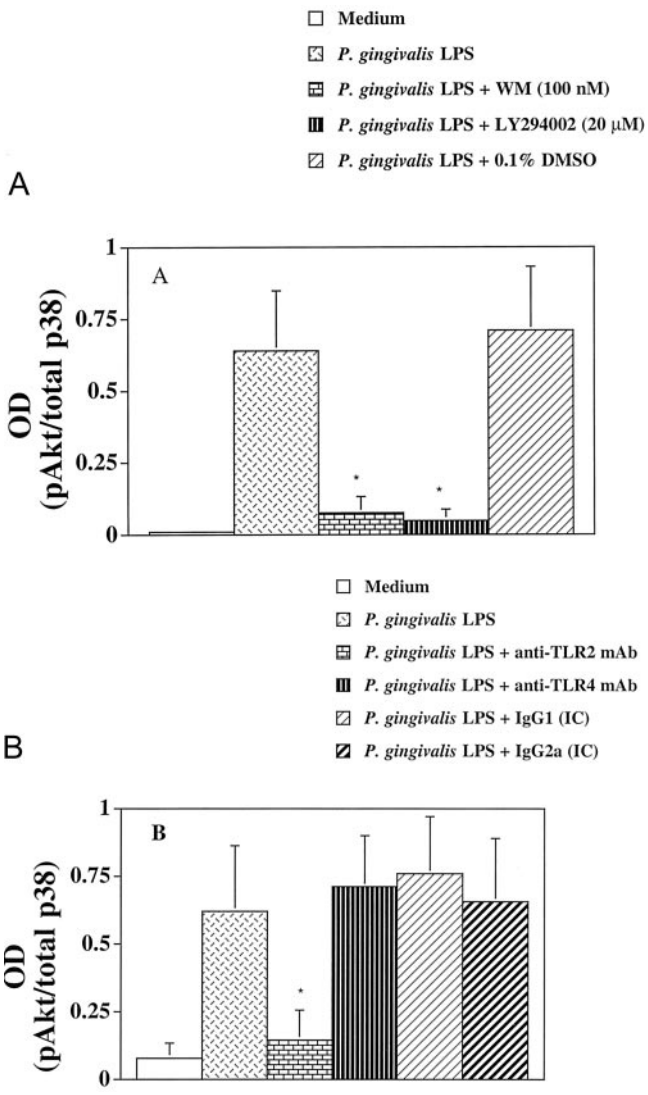

FIGURE 1. *P. gingivalis* LPS activates the PI3K-Akt pathway in human peripheral blood monocytes via TLR2. Human monocytes were preincubated with (*A*) medium only or with 100 nM wortmannin (WM) or vehicle (0.1% DMSO), or with (*B*) mAbs to TLR2, TLR4, or IC Abs (IC) for 60 min before stimulation with 1 μg/ml *P. gingivalis* LPS for 1 h. To assess Akt activation, 20 μg of total cell lysate was resolved on LDS-PAGE and immunoblotted with anti-phospho Akt Ser^{473} followed by ECL detection. Immunoblots were stripped and reprobed with an Ab to total p38 to ensure equal protein loading. Densitometer scans of phosphorylated Akt (Ser^{473}) and total p38 were performed and recorded as the ratio of phosphorylated Akt:total p38. Data are expressed as the mean of five separate experiments ± SEM. *, Significant differences ($p < 0.05$) compared with *P. gingivalis* LPS-stimulated cultures.

Inhibition of PI3K negatively regulates ERK 1/2 activity, but does not affect p38 or JNK 1/2 phosphorylation

The mitogen-activated protein kinase (MAPK) signaling pathway consists of a number of serine/threonine kinases that link signal transduction events from the cell surface to the nucleus via the phosphorylation of transcription factors (63, 64). Three distinct families of MAPK exist in mammalian cells: the p42/44 ERK 1/2, p38, and JNK1/JNK2 (65). To determine whether the ability of *P. gingivalis* LPS to activate PI3K could have regulatory effects on the activation of MAPK, human monocytes were pretreated with or without wortmannin or LY294002, stimulated with *P. gingivalis* LPS, and then assessed for MAPK phosphorylation. Because of the transient activation of both ERK 1/2 and JNK 1/2 by *P. gingivalis* LPS, 10-min time points were used to assess the activation status of these MAPKs. *P. gingivalis* LPS-induced ERK 1/2 phosphorylation was severely ($p < 0.05$) reduced in the presence of wortmannin or LY294002 (Fig. 2*A*). Therefore, to help elucidate the pathway leading to the loss of ERK 1/2 activation, we assessed the phosphorylation status of the upstream kinase, i.e., MEK 1/2 (66). MEK 1/2 activation was observed in response to *P. gingivalis* LPS, and its phosphorylation was strongly blocked ($p < 0.05$) by the PI3K inhibitors wortmannin and LY294002 (Fig. 2*A*). In contrast, *P. gingivalis* LPS-mediated activation of JNK 1/2 was not diminished by inhibiting PI3K activity (Fig. 2*B*). Moreover, like JNK 1/2, p38 activation was not affected by inhibiting the PI3K-Akt pathway (Fig. 2*C*). In this case, because of the observed prolonged phosphorylation of p38 induced by *P. gingivalis* LPS, multiple time points were assessed (Fig. 2*C*). These results show that PI3K activity is required for *P. gingivalis* LPS-induced phosphorylation of ERK 1/2, but not for p38 and JNK 1/2 activation.

PI3K-Akt pathway differentially modulates IL-12 p40, IL-12 p70, and IL-10 production in P. gingivalis *LPS-stimulated monocytes*

Previous studies have shown that activation of the PI3K-Akt signaling pathway can modulate the production of inflammatory mediators by LPS-stimulated macrophage (34–36). Moreover, previous studies using selective inhibitors of MAPK have shown that the inhibition of p38 or ERK 1/2 can positively or negatively affect a variety of cytokines, including IL-10 and IL-12 production by

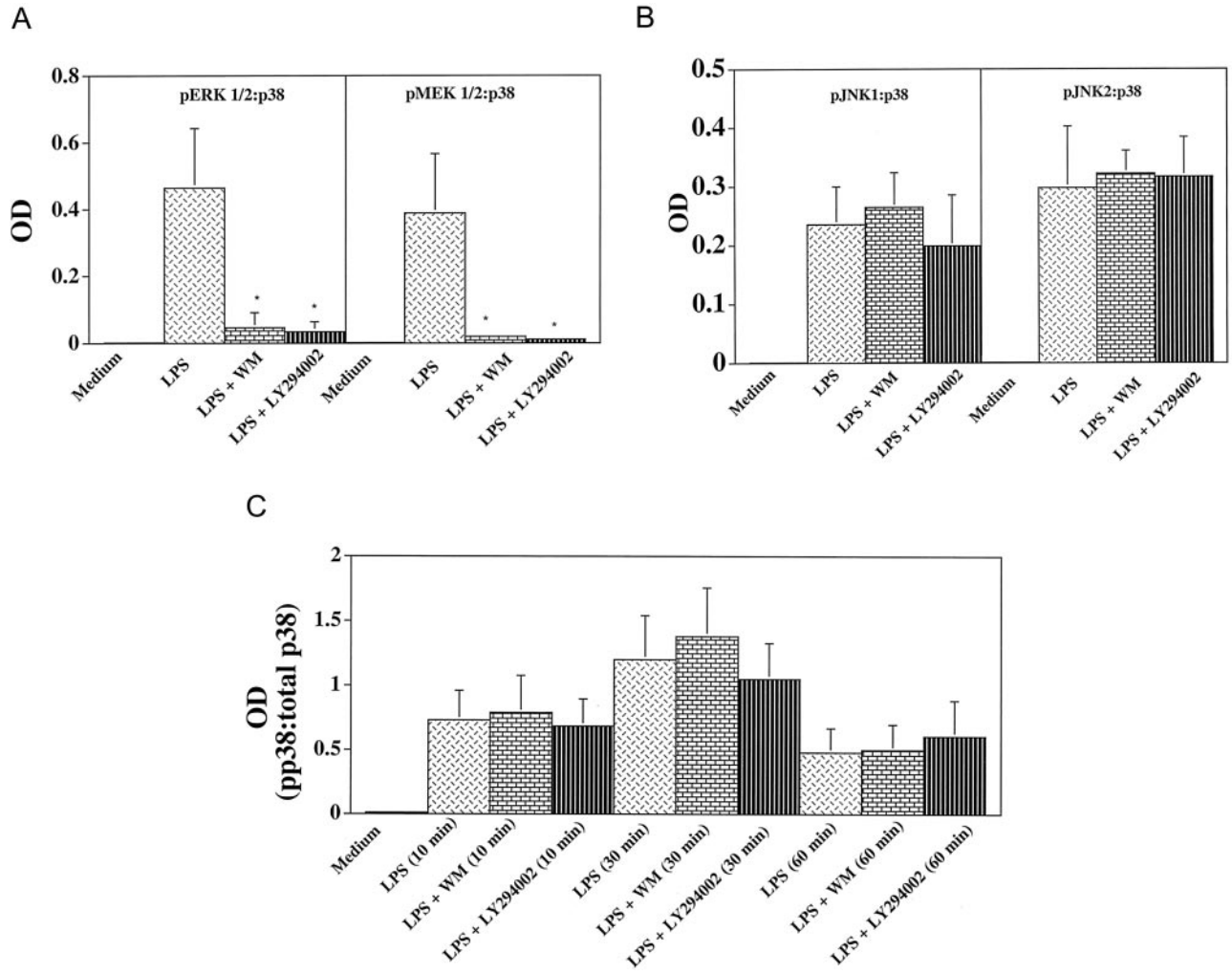

FIGURE 2. The role of PI3K activity in modulating *P. gingivalis* LPS-induced phopshorylation of MEK 1/2 and ERK 1/2 (*A*), JNK 1/2 (*B*), and p38 (*C*). Human peripheral blood monocytes were preincubated with 100 nM wortmannin (WM) for 60 min before the addition of 1 μg/ml *P. gingivalis* LPS. Whole cell lysates were prepared at the given time points, and 20 μg of total protein was analyzed by Western blotting using phospho-specific Abs to MEK 1/2, ERK 1/2, JNK 1/2, and p38. To ensure equal protein loading, blots were stripped and reprobed with an Ab to total p38. Densitometer scans of phosphorylated MEK 1/2, ERK 1/2, JNK 1/2, p38, and total p38 were performed and recorded as the ratio of phosphorylated MEK or phosphorylated MAPK:total p38. Data are expressed as the mean of five separate experiments ± SEM.

mouse or human monocytes/macrophages (24, 67–71). To determine whether the ability of *P. gingivalis* LPS to activate the PI3K-Akt pathway played a functional role in the regulation of cytokine production, we next examined the role of PI3K in modulating *P. gingivalis* LPS-induced IL-12 p35, IL-12 p40, IL-12 p70, and IL-10 production in human monocytes. Because the induction of both IL-10 and IL-12 are predominantly regulated at the transcriptional level, we initially sought to determine whether PI3K-Akt inhibition affected the induction of IL-10, IL-12 p35, and IL-12 p40 production at the level of steady-state mRNA (Fig. 3*A-C*). Levels of both IL-12 p35 and IL-12 p40 mRNA were greatly increased following stimulation of monocytes with *P. gingivalis* LPS in the presence of wortmannin compared with cells stimulated with *P. gingivalis* LPS alone (Fig. 3, *A* and *B*). In contrast, the level of IL-10 mRNA induced following stimulation of human monocytes with *P. gingivalis* LPS and wortmannin was severely suppressed when stimulated (Fig. 3*C*). Additionally, the ability of *P. gingivalis* LPS to activate the PI3K-Akt pathway as well as to regulate the phosphorylation of ERK 1/2 was examined at the level of protein secretion (Fig. 3, *D–F*). Inhibition of PI3K by using wortmannin and LY294002, or inhibiting ERK 1/2 activity by use of the MEK1 inhibitor PD98059, resulted in a significant ($p < 0.05$) increase in the levels of IL-12 p40 secreted by monocytes stimulated with the indicated concentrations of *P. gingivalis* LPS (Fig. 3*D*). Assessment of IL-12 p70 levels revealed that treatment of human monocytes with *P. gingivalis* LPS alone did not result in any detectable IL-12 p70 (Fig. 3*E*). However, monocytes pretreated with wortmannin, LY294002, or PD98059 and then stimulated with *P. gingivalis* LPS exhibited IL-12 p70 production at all concentrations of LPS tested (Fig. 3*E*). In sharp contrast to the highly elevated levels of both IL-12 p40 and IL-12 p70 observed in wortmannin-, LY294002-, or PD98059-treated monocytes, IL-10 production was significantly ($p < 0.05$) diminished by >50% compared with monocytes stimulated with *P. gingivalis* LPS alone (Fig. 3*F*). The vehicle DMSO did not exhibit any discernible effect on the ability of *P. gingivalis* LPS to induce cytokine production by human monocytes (Fig. 3, *D–F*). These data indicate that the ability of the PI3K-Akt pathway to modulate IL-10 and IL-12 production appears to be mediated, in part, by the selective suppression of ERK 1/2 activity, as the MEK1 inhibitor PD98059 closely mimicked the effects of wortmannin and LY294002 to differentially regulate IL-10 and Il-12 production by *P. gingivalis* LPS-stimulated monocytes.

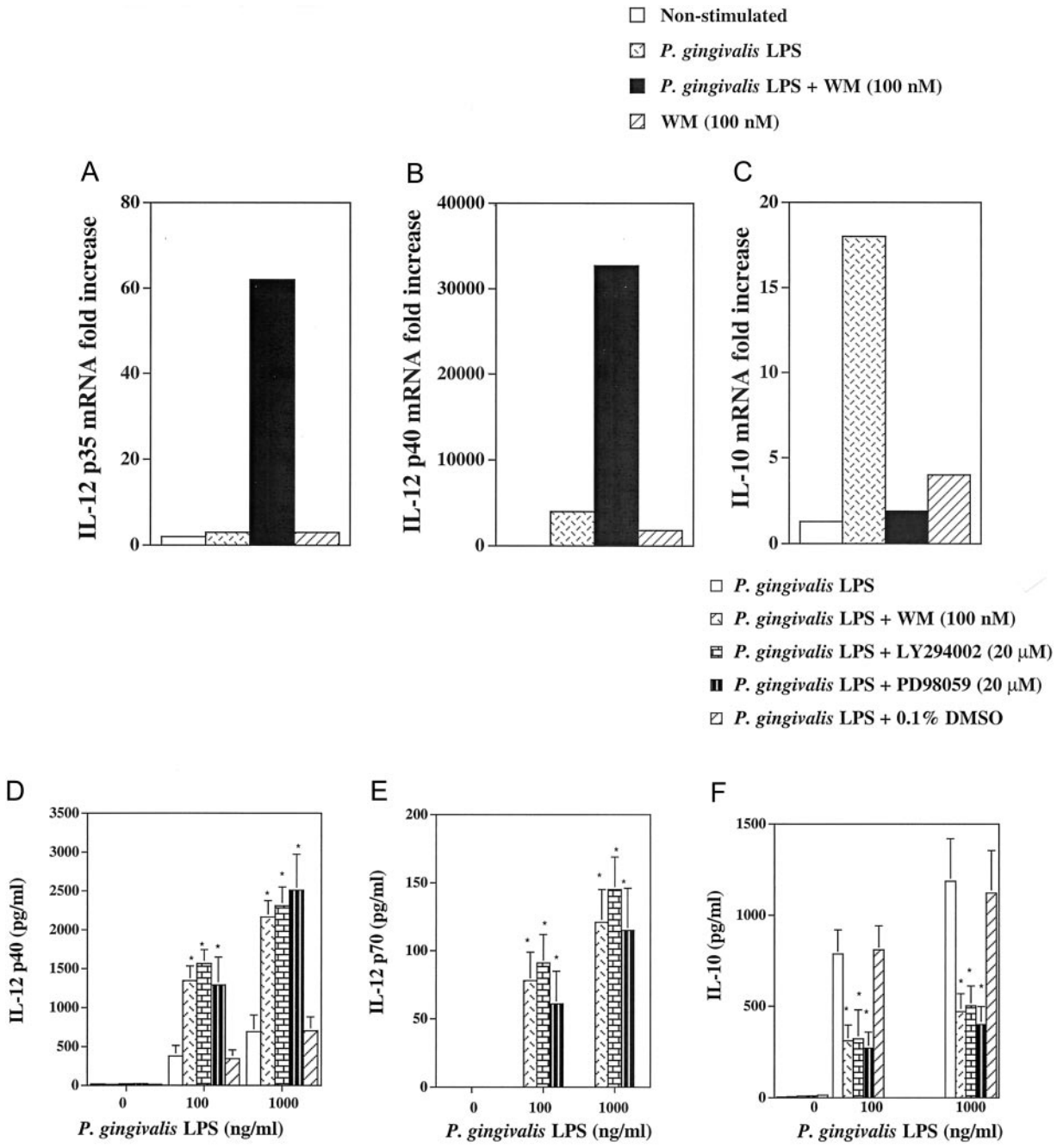

FIGURE 3. Inhibition of the PI3K-Akt pathway differentially affects cytokine production by *P. gingivalis* LPS-stimulated monocytes. Monocytes were stimulated for 5 h with *P. gingivalis* LPS in the presence or absence of wortmannin, harvested, and assayed for cytokine mRNA expression of IL-12 p35 (*A*), IL-12 p40 (*B*), and IL-10 (*C*) by quantitative RT-PCR. Data are expressed as the fold increase over nonstimulated cultures. For cytokine protein determination, cellfree supernatants were collected 20 h after stimulation and assessed for IL-12 p40 (*D*), IL-12 p70 (*E*), and IL-10 (*F*) production by ELISA. ∗, Significant differences ($p < 0.05$) compared with *P. gingivalis* LPS-stimulated cultures. Results represent three to five separate experiments.

Role of IL-10 in PI3K-mediated enhancement of IL-12 p70 production by P. gingivalis *LPS-stimulated monocytes*

It is well documented that IL-10 negatively affects production of inflammatory cytokines, including IL-12 p40 and IL-12 p70, by LPS-stimulated monocytes (23–25). Because inhibition of the PI3K-Akt pathway resulted in a >50% decrease in secreted IL-10 levels (Fig. 3*F*), we next investigated whether enhanced IL-12 production induced by PI3K inhibition was the result of suppressed IL-10 production by *P. gingivalis* LPS-stimulated monocytes. To test this hypothesis, human monocytes were pretreated with a neutralizing mAb to IL-10 in the presence or absence of wortmannin, LY294002, or PD98059 and were then stimulated with *P. gingivalis* LPS. Stimulation of monocytes with *P. gingivalis* LPS that were pretreated with a neutralizing mAb to IL-10 resulted in a demonstrable increase ($p < 0.05$) in the level of IL-12 p70 (Fig. 4). The level of IL-12 p40 was similarly augmented (data not shown). These findings demonstrate that in the absence of PI3K inhibitors IL-10 production by *P. gingivalis* LPS-stimulated monocytes negatively regulates IL-12 production. However, pretreatment of monocytes with either wortmannin, LY294002, or

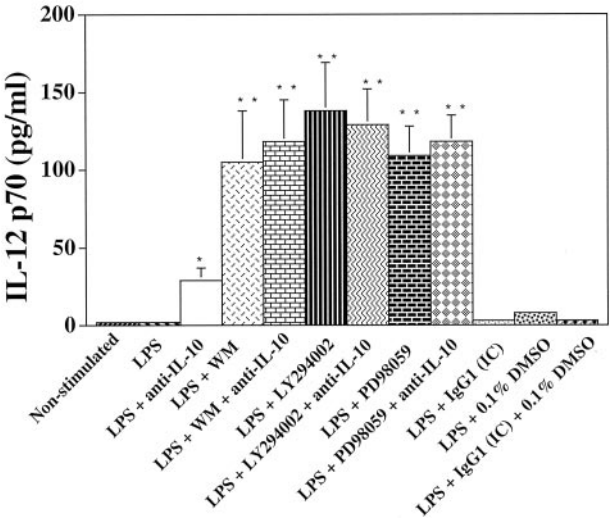

FIGURE 4. Role of IL-10 in PI3K-mediated enhancement of IL-12 production by *P. gingivalis* LPS-stimulated monocytes. Human monocytes were preincubated with a neutralizing mAb to IL-10 (2.5 μg/ml) or IC Ab in the presence or absence of 100 nM wortmannin, LY294002 (20 μM), or 0.1% DMSO control for 1 h. Monocytes were then stimulated with 1 μg/ml *P. gingivalis* LPS for 20 h. Cellfree supernatants were then collected, and the levels of IL-12 p70 were determined by ELISA. *, Significant differences ($p < 0.05$) compared with monocytes stimulated with *P. gingivalis* LPS only. **, Significant differences ($p < 0.05$) compared with cultures preincubated with a neutralizing mAb to IL-10. Data are expressed as the mean of five separate experiments ± SD.

PD98059 in the presence or absence of an anti-IL-10 mAb resulted in a significant ($p < 0.05$) enhancement in IL-12 p70 levels compared with cells pretreated with an anti-IL-10 mAb alone (Fig. 4). Moreover, no significant differences were observed in IL-12 levels between cultures pretreated with wortmannin, LY294002, or PD98059 and with or without an anti-IL-10 mAb (Fig. 4). Taken together, these results reveal that the enhanced IL-12 levels induced in *P. gingivalis* LPS-treated monocytes by inhibiting PI3K and ERK 1/2 activity are not solely attributable to inhibition of IL-10 production.

PI3K regulates NF-κB transactivation in P. gingivalis *LPS-stimulated human monocytes*

The transcription factor NF-κB has been shown to be involved in the regulation of IL-12 (72). It has also been demonstrated that inhibition of PI3K results in the augmentation of NF-κB p65 (35), whereas others have shown that activation of PI3K can lead to activation of NF-κB p65 (33). Therefore, we next tested the potential role of PI3K in modulating NF-κB p65 translocation in *P. gingivalis* LPS-treated monocytes. *P. gingivalis* LPS-induced activation of p65 was significantly ($p < 0.05$) enhanced when PI3K was inhibited (Fig. 5*A*). In contrast, the DMSO vehicle did not exhibit any discernible effect compared with cells stimulated with *P. gingivalis* LPS alone (Fig. 5*A*). Because the activation of NF-κB can be regulated by the phosphorylation and subsequent degradation of IκB proteins (73), we next examined whether the observed enhancement in NF-κB activation was caused by the ability of PI3K to enhance IκB-α degradation or affect its re-synthesis. Analysis of IκB-α degradation induced by *P. gingivalis* LPS was evident at 60 min after exposure; however, inhibition of PI3K failed to alter the rate or extent of degradation or re-synthesis of IκB-α protein (Fig. 5*B*). Similar results were observed for the degradation and re-synthesis of IκB-β (data not shown). These data indicate that PI3K activation by *P. gingivalis* LPS exerts an inhibitory effect on NF-κB p65 translocation that is independent of IκB degradation.

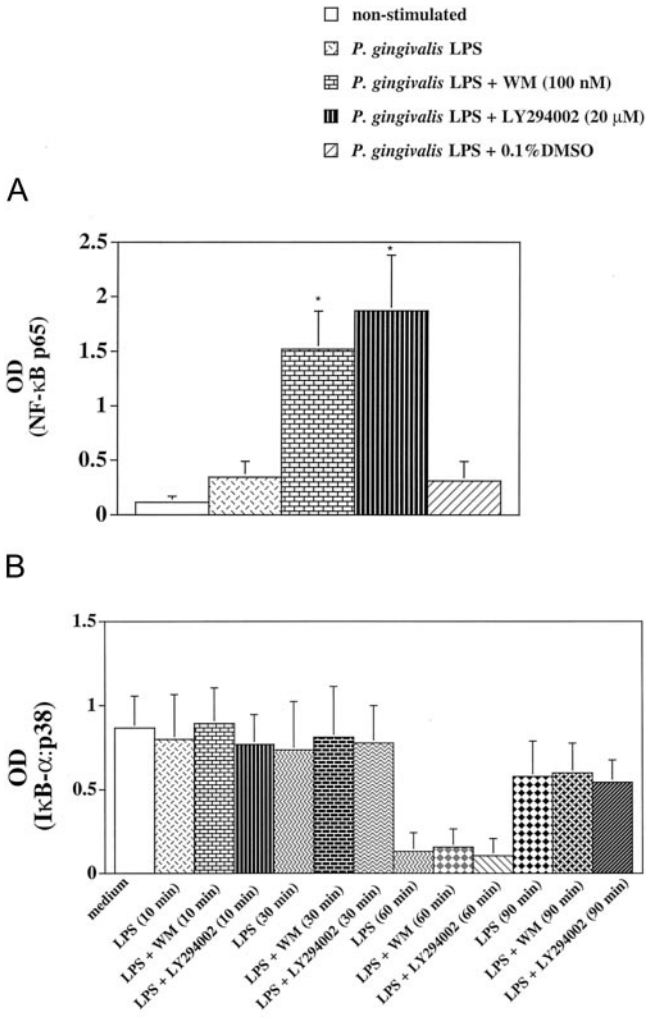

FIGURE 5. Inhibition of the PI3K-Akt pathway enhances *P. gingivalis* LPS-induced NF-κB p65 translocation without affecting IκB-α degradation or resynthesis. *A*, Comparison of NF-κB p65 translocation induced by *P. gingivalis* LPS (1 μg/ml) in the presence or absence of 100 nM wortmannin or LY294002 (20 μM). *B*, Human monocytes were stimulated with 1 μg/ml *P. gingivalis* LPS for the indicated time points in the presence or absence of 100 nM wortmannin or LY294002 (20 μM), and whole-cell lysates were analyzed for degradation of IκB-α by Western blot. Human monocytes were preincubated for 60 min with wortmannin, LY294002, or 0.1% DMSO and then stimulated with *P. gingivalis* LPS for an additional 5 h. *, Significant differences at $p < 0.05$ compared with *P. gingivalis* LPS-stimulated cultures. Data are expressed as the mean of five separate experiments ± SD.

Discussion

A major aim of the current study was to define how engagement of the PI3K pathway affects initial production of both pro- and anti-inflammatory cytokines by human monocytes stimulated with *P. gingivalis* LPS. Our results demonstrate that activation of the PI3K-Akt pathway by *P. gingivalis* LPS resulted in a differential effect on the production of IL-10 and IL-12 at both transcriptional and protein levels. Inhibition of this pathway also dysregulated MEK 1/2, leading to suppression of ERK 1/2 activity and activation of NF-κB p65.

Past studies have shown that activated ERK 1/2 controls production of IL-10 and IL-12 positively or negatively, respectively

(68, 70, 71). Studies by Feng et al. (68) indicated that activation of ERK 1/2 negatively controlled both IL-12 p40 and IL-12 p70 levels, whereas p38 phosphorylation was associated with positive control of IL-12 production. Other studies using a variety of stimuli have extended these observations by showing that ERK 1/2 activation not only negatively regulates production of IL-12, but also positively influences levels of IL-10 (70, 71). Our results indicate that inhibition of the PI3K-AKT pathway results in augmented IL-12 levels, whereas IL-10 production is suppressed in *P. gingivalis* LPS-stimulated monocytes, and this response is associated with a selective failure to activate ERK 1/2. Although a functional role for PI3K in regulating IL-10 and IL-12 levels was unknown at the time these studies were performed, previous studies by Perkinton et al. (74) have shown that the use of specific PI3K inhibitors, including wortmannin and LY294002, prevent ERK 1/2 activation. Our results support the notion that PI3K-mediated activation of ERK 1/2 is responsible for differences in the levels of IL-10 and IL-12, because the use of the MEK1 inhibitor PD98059 similarly affected IL-10 and IL-12 production in *P. gingivalis* LPS-stimulated monocytes pretreated with the PI3K inhibitor wortmannin. These findings indicate that the ability of *P. gingivalis* LPS to activate ERK 1/2 via the PI3K-Akt pathway is largely responsible for the dichotomy in IL-10 and IL-12 production.

Bioactive IL-12 p70 is a key mediator involved in the development of Th1-type immunity (13–15). Interestingly, although *P. gingivalis* LPS stimulated production of IL-12 p40 in culture supernatants, we were unable to detect any IL-12 p70. In contrast, when using the PI3K inhibitor wortmannin we observed a significant increase in IL-12 p35 and IL-12 p40 levels as well as demonstrable levels of IL-12 p70. Whereas some studies have shown that IL-12 production is controlled by the induction of IL-12 p40 (75), others have reported that the IL-12 p35 subunit is actually the regulatory subunit controlling production of heterodimeric IL-12 p70 in human monocytes (25). Thus, it is likely that the ability of PI3K to negatively affect the induction of IL-12 p70 production by *P. gingivalis* LPS-stimulated monocytes is caused by its effect on suppressing IL-12 p35 levels. This would be consistent with the findings of Fukao et al. (34) who showed that PI3K activation negatively controls IL-12 p35 and IL-12 p40 levels in mouse macrophage, as well as the findings by Snijders et al. (25) who demonstrated that the IL-12 p35 subunit is responsible for regulating IL-12 p70 production in human monocytes. Moreover, regulation of IL-12 can also be controlled by autocrine and/or paracrine production of IL-10, which can negatively influence the production of IL-12 p70 (23–25, 76). This was of particular interest because we observed that PI3-kinase inhibition resulted in suppression of IL-10 levels. With the aid of a neutralizing mAb to IL-10, we demonstrated that *P. gingivalis* LPS-induced IL-10 production had an inhibitory effect on IL-12 p70 and that it likely played a role in the ability of wortmannin and LY294002 to augment IL-12 levels. However, PI3K inhibition alone induced significantly enhanced levels of IL-12 p70, which were similar to that seen when wortmannin or LY294002 was used in conjunction with a neutralizing mAb to IL-10. Thus, PI3K inhibition likely enhances IL-12 p70 production via both IL-10-dependent and -independent mechanisms.

The role of PI3K in regulating NF-κB transactivation remains controversial. Our present study is in agreement with those of Guha and Mackman (35), which demonstrated that inhibition of the PI3K-Akt pathway resulted in the augmentation of NF-κB p65 activation in *E. coli* LPS-stimulated monocytes, whereas others have shown that PI3K activation can promote the activation of p65 (33). Although it is presently unclear what factors are responsible for these discrepancies, it is possible that different experimental conditions could account for these observations. Whereas activation of NF-κB can be regulated by the phosphorylation and subsequent degradation of IκB proteins (33, 73), the observed enhancement in NF-κB activation reported in the present study was not caused by enhanced IκB-α degradation or alterations in its re-synthesis. Additionally, the ability of PI3K to influence NF-κB p65 may be related to its ability to activate p38 or ERK 1/2 (77, 78). However, their involvement in the present findings are unlikely because PI3K inhibition resulted in a loss of ERK 1/2 phosphorylation, but no discernible effect on p38 phosphorylation. Another possibility by which inhibition of PI3K may augment *P. gingivalis* LPS-induced NF-κB p65 translocation in human monocytes may be by the loss of Akt activity. Recent studies by Guha and Mackman (35) demonstrated that inhibition of the PI3K-Akt pathway resulted in enhanced NF-κB p65 translocation, which the authors suggest was the result of preventing Akt-dependent inactivation of glycogen synthase kinase-β. However, whether the PI3K-Akt-mediated regulation of glycogen synthase kinase-β activity is directly responsible for NF-κB p65 translocation remains a subject for further study.

Studies comparing the biologic activity of *P. gingivalis* and *E. coli* LPS to induce select proinflammatory cytokine production have demonstrated that the potency of *P. gingivalis* LPS is considerably less or even absent when compared with responses induced by *E. coli* LPS in both human and mouse macrophage cultures (10, 12, 49, 53, 79). It is believed that these differences are likely the result of distinct signaling events occurring within the TLR2- and TLR4-signaling pathways (10, 12, 53). Our present study demonstrates that the ability of *P. gingivalis* LPS to induce IL-12 production is negatively regulated by the PI3K pathway. Furthermore, this pathway also appears to exert a negative effect on the ability of *P. gingivalis* LPS to induce other proinflammatory cytokines, including IL-6 and TNF-α (M. Martin, unpublished observations). Therefore, the induction of several key inflammatory mediators induced by *P. gingivalis* LPS is negatively controlled by the PI3K-Akt pathway, whereas production of the anti-inflammatory cytokine IL-10 is positively controlled. It has been reported that the cytosolic domain of TLR2 contains multiple p85 docking sites that are also present in TLR1 and TLR6, but absent in TLR3, TLR4, and TLR5 (33). However, Arbibe et al. (33) also indicate that a putative p85-docking site is also present within MyD88. Thus, the possibility exists that other TLR-signaling pathways may activate PI3K by the engagement of MyD88. Studies using *E. coli* LPS have shown a functional role for the PI3K-Akt pathway in regulating proinflammatory cytokine production (34, 35). Although no study to date has directly compared the functional significance of the PI3K-Akt pathway in regulating qualitative and quantitative aspects of innate immunity induced by TLR2- and TLR4-agonists, our present study shows that this pathway negatively regulates the production of several cytokines that have been shown to differ from that of the TLR4-agonist *E. coli* LPS (10).

In summary, the present study demonstrates that *P. gingivalis* LPS activates the PI3K-Akt pathway and that this pathway differentially regulates the production of IL-10 and IL-12 production in human monocytes. Previous studies assessing the type of inflammatory response observed in periodontitis reported a high degree of Th2-associated cytokines (55–58). Interestingly, past studies have indicated that several TLR2 agonists are unable to induce detectable levels of IL-12 p70 and favor Th2 development (10, 11, 80). Although the role of PI3K in regulating IL-12 with other TLR2 agonists is not currently known, our current findings demonstrate that the ability of *P. gingivalis* LPS to activate the PI3K pathway via TLR2 is largely responsible for the lack of detectable

IL-12 p70. These findings suggest that activation of the PI3K pathway by *P. gingvalis* LPS may be, in part, responsible for the skewing of Th1- and Th2-type immune responses observed in adult periodontitis.

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