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NF-κB- and C/EBPβ-driven Interleukin-1β Gene Expression and PAK1-mediated Caspase-1 Activation Play Essential Roles in Interleukin-1β Release from *Helicobacter pylori* Lipopolysaccharide-stimulated Macrophages*

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Chaitali Basak, Sushil Kumar Pathak‡, Asima Bhattacharyya, Debabrata Mandal, Shresh Pathak, and Manikuntala Kundu§

From the Department of Chemistry, Bose Institute, 93/1 Acharya Prafulla Chandra Road, Kolkata 700009, India

Helicobacter pylori **is a Gram-negative microaerophilic bacterium that causes chronic gastritis, peptic ulcer, and gastric carcinoma. Interleukin-1β (IL-1β) is one of the potent proinflammatory cytokines elicited by *H. pylori* infection. We have evaluated the role of *H. pylori* lipopolysaccharide (LPS) as one of the mediators of IL-1β release and dissected the signaling pathways leading to LPS-induced IL-1β secretion. We demonstrate that both the NF-κB and the C/EBPβ-binding elements of the *IL-1β* promoter drive LPS-induced *IL-1β* gene expression. NF-κB activation requires the classical TLR4-initiated signaling cascade leading to IκB phosphorylation as well as PI-3K/Rac1/p21-activated kinase (PAK) 1 signaling, whereas C/EBPβ activation requires PI-3K/Akt/p38 mitogen-activated protein (MAP) kinase signaling. We observed a direct interaction between activated p38 MAP kinase and C/EBPβ, suggesting that p38 MAPK is the immediate upstream kinase responsible for activating C/EBPβ. Most important, we observed a role of Rac1/PAK1 signaling in activation of caspase-1, which is necessary for maturation of pro-IL-1β. *H. pylori* LPS induced direct interaction between PAK1 and caspase-1, which was inhibited in cells transfected with dominant-negative Rac1. PAK1 immunoprecipitated from lysates of *H. pylori* LPS-challenged cells was able to phosphorylate recombinant caspase-1, but not its S376A mutant. LPS-induced caspase-1 activation was abrogated in cells transfected with caspase-1(S376A). Taken together, these results suggested a role of PAK1-induced phosphorylation of caspase-1 at Ser^{376} in activation of caspase-1. To the best of our knowledge our studies show for the first time that LPS-induced Rac1/PAK1 signaling leading to caspase-1 phosphorylation is crucial for caspase-1 activation. These studies also provide detailed insight into the regulation of *IL-1β* gene expression by *H. pylori* LPS and are particularly important in the light of the observations that *IL-1β* gene polymorphisms are associated with increased risk of *H. pylori*-associated gastric cancer.**

Helicobacter pylori is a Gram-negative microaerophilic bacterium that causes chronic gastritis and also peptic ulcer, gastric carcinoma, and gastric lymphoma. *H. pylori*-associated gastritis is characterized by severe infiltration of neutrophils and mononuclear cells in the gastric mucosa (1). Accumulation and activation of these cells is induced by the local production of chemokines and cytokines. Recent studies have demonstrated that mucosal levels of interleukin (IL)[1]-1β, IL-6, and IL-8 are significantly higher in *H. pylori*-positive patients than in *H. pylori*-negative patients (2, 3). ELISA and RT-PCR analyses suggest that IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α play important roles in gastric inflammation caused by *H. pylori* infection. Several potential soluble virulence factors derived from *H. pylori* are considered to attract or activate neutrophils and mononuclear cells, and there is evidence that *H. pylori* strain genotype as well as host factors determine the clinical outcome. *H. pylori* does not invade mononuclear cells. Evidence from our own laboratory (4) as well as other laboratories (5) suggests that *H. pylori* lipopolysaccharide (LPS) mediates release of cytokines and chemokines from human monocytes. The biological effects of these cytokines may result in the recruitment, influx, and activation of neutrophils in gastric mucosa during *H. pylori* infection. *H. pylori* LPS differs from that of *Enterobacteriaceae* (6) in its ability to activate inflammatory cells possibly because of structural differences between the lipid A molecules. *H. pylori* LPS activates inflammatory cells to produce IL-1β, IL-8, and TNF-α (7). IL-1β, TNF-α, and IL-8 are increased in the antral mucosa of individuals with *H. pylori* gastritis (8, 9). However, the molecular mechanisms associated with *H. pylori* LPS-induced cytokine/chemokine production have not been extensively studied.

IL-1β is a potent inflammatory cytokine that is released as a component of the host response against bacterial infection. It is primarily expressed by activated monocytes/macrophages. IL-1β is produced as a precursor molecule, pro-IL-1β, in the cytosol of macrophages. Pro-IL-1β is a 31–34-kDa inactive form of the cytokine, which is later cleaved by caspase-1 to active

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‡ A Junior Research Fellow of the Council of Scientific and Industrial Research, Government of India.

§ To whom correspondence should be addressed. Fax: 91-33-23506790; E-mail: manikuntala@vsnl.net.

[1] The abbreviations used are: IL, interleukin; LPS, lipopolysaccharide; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; PI-3K, phosphatidylinositol 3-kinase; IRAK, IL-1 receptor-associated kinase; TRAF, tumor necrosis factor receptor-associated factor; IKK, IκB kinase; PAK, p21-activated kinase; MAPK, mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; z-YVAD-FMK, z-Tyr-Val-Ala-Asp(OMe)-CH_2F; z, benzyloxycarbonyl; DTT, dithiothreitol; NTA, nitrilotriacetic acid; C/EBP, CCAAT/enhancer-binding protein; ELISA, enzyme-linked immunosorbent assay; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GST, glutathione *S*-transferase; RT, reverse transcription; GSK, glycogen synthase kinase; ERK, extracellular signal-regulated kinase; dn, dominant-negative.

17-kDa IL-1β (10). The active IL-1β is released and exhibits its diverse biological functions.

Soluble mediators of *H. pylori* are known to induce IL-1β. Of particular significance is the finding that *IL-1β* gene cluster polymorphisms suspected of enhancing production of IL-1β are associated with an increased risk of gastric cancer (11). This makes it worthwhile to explore the mechanism of induction of IL-1β by *H. pylori*, and in particular, the role of LPS. The expression of IL-1β is regulated at the level of transcription (12–14), mRNA stabilization, and post-translational proteolytic processing (15). C/EBPβ (13)- and NF-κB (14)-binding sites have been characterized in the human *IL-1β* promoter.

Toll-like receptors (TLRs) play central roles in innate immunity by recognition and discrimination of specific conserved patterns of molecules derived from bacteria, fungi, or viruses (16–18). Activation of TLRs results in stimulation of signaling pathways widely involving recruitment of the adaptor molecule myeloid differentiation factor 88 (MyD88) (19–21). The serine/threonine kinase IL-1 receptor-associated kinase 1 (IRAK1) is subsequently recruited, becomes phosphorylated, dissociates from the complex and associates with tumor necrosis factor receptor-associated factor 6 (TRAF6) (22–24) leading to the activation of mitogen-activated protein kinases (MAPKs), transcription factors such as NF-κB and concomitant production of cytokines (25, 26). MAPKs comprise an important group of serine/threonine signaling kinases that transduce a variety of extracellular stimuli through a cascade of protein phosphorylations, which lead to the activation of transcription factors (27–29). There are three principal MAPK family members: (i) p46 and p54 c-Jun N-terminal kinase or stress-activated protein kinase (JNK or SAPK, respectively) with multiple subisoforms, (ii) p38 MAPK with α, β, γ, and δ isoforms, and (iii) p42 and p44 extracellular signal-regulated kinase (ERK). MAPKs are activated by specific upstream MAPK kinases (MKKs) (30).

Since TLR- and MAPK signaling lead to the control of gene transcription, we explored the role of these signaling pathways in *H. pylori*-LPS-mediated IL-1β release, and its regulation at the level of transcription. We also explored the pathways regulating proteolytic processing of pro-IL-1β. The study described here provides evidence that *H. pylori* LPS signals through TLR4 to regulate IL-1β transcription driven by NF-κB and C/EBPβ elements in the *IL-1β* promoter. Whereas regulation at the level of NF-κB involves the canonical TLR4-directed phosphorylation of IκB-α leading to NF-κB activation, regulation of C/EBPβ involves a TLR4/phosphatidylinositol 3-kinase (PI-3K)/Akt/p38 MAPK pathway. We observed a direct interaction between phospho- p38MAPK and C/EBPβ, suggesting that p38MAPK is the immediate upstream kinase responsible for activating C/EBPβ. In addition, PI-3K/Rac1/p21-activated kinase 1(PAK1) signaling also regulates NF-κB, as well as the caspase-1-mediated processing of pro-IL-1β to -IL-1β. We present the novel finding that PAK1 interacts with caspase-1, and that this interaction is blocked in dominant-negative (dn)-Rac1-transfected cells. PAK1 immunoprecipitated from *H. pylori* LPS-challenged cells, phosphorylated caspase-1 at Ser^{376} and activation of caspase-1 was abrogated in caspase-1 (S376A)-transfected cells. We hypothesize that PAK1 is a key upstream kinase regulating the activation of caspase-1 at least in some cell types.

EXPERIMENTAL PROCEDURES

Chemicals—Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate), SB203580, U0126, SP 600125, z-Tyr-Val-Ala-Asp(OMe)-CH_2F (z-YVAD-FMK) *N*-acetyl-Tyr-Val-Ala-Asp-(7-amino 4-trifluoromethylcoumarin) (Ac-YVAD-AFC), phorbol 12-myristate 13-acetate (PMA), and wortmannin, were products of EMD Biosciences, San Diego, CA. Protease inhibitors were from Roche Applied Science. All other reagents were of analytical grade. The human interleukin-1β ELISA kit was from Amersham Biosciences. *Escherichia coli* LPS was purchased from Sigma. [γ-^{32}P]ATP was from Jonaki, BRIT, Hyderabad, India.

Antibodies—Anti-p38 MAPK, -PAK1, -phospho-p38MAPK, -phospho-PAK1, -interleukin-1β, and -p-Tyr were from Cell Signaling Technology, Beverly, MA. Anti-p85 and supershift antibodies against p50, p65, and C/EBPβ were from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Anti-Rac1 and anti-caspase-1 were from BD Biosciences, San Jose, CA. Neutralizing TLR-4 antibody (HTA 125) was purchased from Imgenex Biotech, Bhubaneswar, India.

LPS Preparations—*H. pylori* 26695 was cultured for 3 days on horse blood agar plates in a microaerophilic milieu (10% CO_2, 5% O_2, and 85% N_2) at 37 °C. The bacterial cells were harvested; LPS was prepared by the hot phenol-water method described by Westphal and Jann (31), dialyzed, and freeze-dried. LPS was estimated using the E-TOXATE amoebocyte lysate assay kit from Sigma.

Cell Culture—THP1 cells (derived from a patient with acute monocytic leukemia) are mature cells from the monocyte/macrophage lineage. These were obtained from the National Center for Cell Science (Pune, India). The cell line was maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 20 mM sodium bicarbonate. The cells were incubated at 5% CO_2 and 95% humidity in a 37 °C chamber. THP-1 cells were treated with PMA to induce maturation of the monocytes to a macrophage-like adherent phenotype (32). Cells were washed thrice with culture medium and cell viability was determined to be >95% by the trypan blue dye exclusion method. The inhibitors U0126, SB203580, SP600125, Akt inhibitor, and wortmannin, were dissolved in Me_2SO. Appropriate vehicle controls were maintained in all experiments in which these inhibitors were used.

Plasmids and Transient Transfections—TLR4 was amplified from RNA isolated from THP-1 cells using the sense and antisense primers 5′-ATGGATCCGCATGGAGCTGAATTTCTA-3′ and 5′-ATAAAGCTTCTAAGATGTTGCTTCCTG-3′, respectively and cloned between the BamHI and HindIII sites of pcDNA3.1. TLR4-(1–643) (TLR4-dn) encoding the first 643 amino acids of TLR4, was generated using the same sense primer and the antisense primer 5′-ATAAAGCTTCTACTGGCTTGAGTAGAT-3′. RNA was isolated from THP-1 (using the Qiagen RNeasy Mini kit) and used as template to amplify the cDNA of the coding sequence for caspase-1 by RT-PCR using the sense and antisense primers 5′-TAGAATTCATGGCCGACAAGGTCCTG-3′(a)and 5′-TTGGATCCATGTCCTGGGAAGAGGTA-3′(d), respectively. Mutant caspase-1 (S376A) was generated by overlap extension PCR. The initial rounds of PCR were carried out with primer pairs a and b (5′-GTTCGATTTGCATTTGAGCAG-3′), and c (5′-CTGCTCAAATGCAAATCGAAC-3′) and d. The products of each PCR were purified and used as templates for the second round of PCR using the primers a and d. The caspase-1 gene was cloned between the EcoRI and BamHI of pTrc6His. For transfection experiments, caspase-1 constructs were generated by cloning between the EcoRI and BamHI sites of pFlagCMV6c. Myc-tagged IRAK1-(1–215) (IRAK1-dn) in pcDNA3.1 was obtained from Klaus Ruckdeschel, Max von Pettenkofer Institute for Hygiene and Medical Microbiology, Munich, with the consent of Marta Muzio. MyD88-(152–196) (MyD88-dn) in pcDNA3.1 was obtained from Marta Muzio and Alberto Mantovani, Mario Negri Institute for Pharmacological Research, Milan, Italy. pRK5-FLAG-TRAF6, pRK5-Flag TRAF6-(289–522) (TRAF6-dn), pRK5-FLAG-IKKβ, pRK5-FLAG-IKKβ K44A (IKKβ-dn) were gifts from Tularik Inc. FLAG-tagged p38 and JNK MAPKs and their dominant-negative mutants (p38 (agf) and JNK (afp), respectively), were obtained from Roger Davis, University of Massachusetts Medical School, Worcester, MA. The dominant negative mutant of the phosphatidylinositol 3-kinase p85 subunit deleted in the inter-SH2 region of wild-type p85α (PI-3KΔ85) was a gift from Robert Farase, J. A. Haley Veterans Hospital, Tampa, FL and the kinase-deficient mutant of Akt (Akt-Kd) carrying the mutation K179M was a gift from Kenneth Walsh, Molecular Cardiology/Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, MA. pCMV-PAK1(wt) and pCMV-PAK1 (K299R) (PAK1-K_D) were gifts from Jeffrey Frost, University of Texas Southwestern Medical Center, Dallas, TX. GST-C/EBPβ-(22–227) was a gift from Jean-Rene Cardinaux, University of Lausanne, Switzerland. Transient transfections were carried out using FuGENE 6 (Roche Applied Science) according to the manufacturer's protocol. β-Gal reporter plasmid was used to normalize transfection efficiencies.

Treatment with LPS and Preparation of Cell Lysates—THP-1 cells were treated with PMA and cultured in 24-well tissue culture plates at 5×10^5 cells per well followed by treatment with *H. pylori* LPS (or *E. coli* LPS where indicated). The wells were washed with ice-cold phosphate-buffered saline and lysed with lysis buffer (20 mM Tris-HCl

pH 7.4, 1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 137 mM NaCl, 20 mM NaF, 1 mM EDTA, 40 mM Na-β-glycerophosphate, 4 μg/ml each of leupeptin, pepstatin, and aprotinin, 1 mM Na_3VO_4, 1 mM pefabloc, 1 mM benzamidine) (buffer A) on ice for 15 min. Cell lysates were boiled for 5 min after the addition of 5× Laemmli sample buffer and subjected to Western blotting. Where necessary, THP-1 cells were first treated with pharmacological inhibitors or vehicle (Me_2SO) alone, prior to incubation with LPS.

Western Blotting—Proteins were separated by SDS-PAGE and then transferred electrophoretically to polyvinylidene difluoride membranes. The blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature and subsequently incubated overnight at 4 °C with primary antibodies (in TBS-Tween 20 (1%, v/v) (TBST) with 5% (w/v) bovine serum albumin). Following three washes of 5 min each with TBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) or appropriate secondary antibodies) in blocking buffer for 1 h at room temperature. After three washes with TBST, the blots were developed by chemiluminescence using the phototope-HRP Western Detection kit (Cell Signaling Technology) and exposed to x-ray film (Kodak XAR5).

Western Analysis of Caspase-1 Activation—Transfected or non-transfected THP-1 cells after treatment with *H. pylori* LPS (20 ng/ml) were pelleted and freeze-thawed three times in 20 μl of cell extraction buffer (50 mM PIPES/NaOH, pH 6.5, 2 mM EDTA, 0.1% (w/v) CHAPS, 5 mM DTT, 20 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM pefabloc). The lysates were centrifuged at 10,000 × *g* for 5 min at 4 °C, and the supernatants were boiled for 5 min after addition of 5× Laemmli sample buffer before subjecting to Western blotting using anti-caspase-1 antibody.

Akt Kinase Assay—The activity of Akt protein kinase was determined by using the Akt kinase assay kit (Cell Signaling Technology) using glycogen synthase kinase (GSK)-3 as substrate, according to the manufacturer's instructions. Briefly, lysates were incubated with immobilized Akt monoclonal antibody. The beads after washing, were incubated with 2 μg of GSK-3 fusion protein as substrate in 20 μl of kinase reaction buffer containing 200 μM ATP. After incubation for 30 min at 30 °C, the reaction was terminated by the addition of 5× Laemmli sample buffer. Phosphorylated proteins were resolved by SDS-PAGE followed by Western blotting. Blots were probed with anti-phospho-GSK-3 antibody and visualized by chemiluminescence.

Affinity Precipitation Assay for Rac—This was done as described by Benard *et al.* (33). Cell extracts after treatment were incubated with GST-PBD (the p21-binding domain (PBD) of PAK1 fused to GST) in a maximal final volume of 500 μl for 1 h at 4 °C. The bead pellet was then centrifuged for 2 min at 2,000 rpm at 4 °C and washed three times with washing buffer (25 mM Tris-HCl pH 7.6, 1 mM DTT, 30 mM $MgCl_2$, 40 mM NaCl, 1% Nonidet P-40) and twice with the same buffer without Nonidet P-40. The bead pellet was finally suspended in 20 μl of Laemmli sample buffer. Proteins bound to the beads were separated on SDS-polyacrylamide (12%) gels, transferred onto nitrocellulose membrane and blotted for Rac1.

Assays of C/EBPβ-p38 MAP Kinase Interaction—In order to study the *in vivo* interaction between C/EBPβ and p38 MAP kinase, C/EBPβ was immunoprecipitated from lysates of cells treated without or with *H. pylori* LPS. The immunoprecipitate was boiled in Laemmli buffer, separated on SDS-PAGE, transferred onto polyvinylidene difluoride membrane and blotted for p38 MAPK. In separate experiments, lysates from cells treated without or with *H. pylori* LPS were incubated with immobilized phospho-p38 MAP kinase (Cell Signaling Technology) overnight at 4 °C. Bead-bound proteins were separated by SDS-PAGE, electrotransferred, and probed with anti-C/EBPβ antibody.

In an alternative approach, GST-C/EBPβ-(22–227) was expressed in *E. coli* BL21, purified over glutathione-Sepharose beads and GST-C/EBPβ-bound beads were incubated for 2 h at 4 °C with lysates from cells treated without or with *H. pylori* LPS as described by Kovacs *et al.* (34). The bead pellets were washed, suspended in Laemmli buffer, separated on SDS-polyacrylamide (10%) gels, transferred onto nitrocellulose membrane, and blotted for p38 MAP kinase.

Affinity Precipitation Assay to Study the Interaction of Caspase-1 and PAK1—His-tagged caspase-1 was expressed in *E. coli* DH5α by inducing cells using 200 μM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 2 h and immobilized on Ni^{2+}-NTA-agarose. The agarose beads were incubated for 2 h at 4 °C with lysates from cells treated without or with *H. pylori* LPS. The bead pellets were washed, suspended in Laemmli buffer, separated on SDS-polyacrylamide (7.5%) gels, transferred onto nitrocellulose membranes and blotted for PAK1. In order to study the *in vivo* interaction between PAK1 and caspase-1, lysates from cells treated without or with *H. pylori* LPS were incubated overnight with PAK1 antibody at 4 °C, followed by incubation with protein A/G-agarose for 3 h. Agarose-bound proteins were separated by SDS-PAGE, electrotransferred, and probed with anti-caspase-1 antibody.

Caspase-1 Activity Assays—Transfected or non-transfected THP-1 cells were either left untreated or were treated with LPS (20 ng/ml) and then lysed by incubating with 25 μl of lysis buffer (25 mM HEPES, pH 7.5, 2 mM EDTA, 0.1% (w/v) CHAPS, 5 mM DTT, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM pefabloc) on ice for 15 min. The supernatant was kept frozen at −70 °C until used. Lysates (20 μg of protein) were taken in 100 μl of assay buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM DTT, 10% (v/v) glycerol, 0.1% CHAPS, and 200 μM caspase-1 substrate Ac-YVAD-AFC. Fluorescence of the released AFC was measured using excitation and emission wavelengths of 400 nm and 505 nm, respectively.

Phosphorylation of Caspase-1 in Vitro—PAK1 was immunoprecipitated from lysates of cells challenged with *H. pylori* LPS by incubating with PAK1 antibody overnight followed by incubation with protein A/G-agarose for another 3 h. The immunoprecipitate was washed twice in kinase buffer (300 mM Tris, 1 M KCl, 1 mM $CaCl_2$, 60 mM $MgCl_2$, 1 mM Na_3VO_4, 10 mM DTT, pH 7.5) and incubated with 5 μg of purified His-tagged caspase-1 as substrate in 20 μl of kinase buffer containing 1 μCi of [γ^{32}P]ATP and 7.5 μM cold ATP for 15 min. The reaction was terminated by the addition of 5× Laemmli buffer and phosphorylated caspase-1 was detected by autoradiography of proteins or by Western blotting using phosphoserine-specific antibody.

RNA Isolation and Reverse Transcription (RT)-PCR—Total RNA was prepared from cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. 100 ng of RNA was reverse-transcribed using the Titan 1-tube RT-PCR kit (Roche Applied Sciences). The sense primer 5′-AAA CAG ATG GCT TAT TAC AGT-3′, and the antisense primer 5′-TGG AGA ACA CCA CTT GTT GCT CCA-3′, were used to amplify 391 bp of IL-1β mRNA. Glyceraldehyde-3-phosphate dehydrogenase was amplified using the following primers: sense, 5′-CCA TCA ATG ACC CCT TCA TTG ACC-3′; and antisense, 5′-GAA GGC CAT GCC AGT GAG CTT CC-3′ to generate a 604-bp product. The PCR conditions for IL-1β mRNA were: denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min, extension at 68 °C for 1 min for 35 cycles.

Electrophoretic Mobility Shift Assay—To prepare nuclear extracts, cells after treatment were washed twice with ice-cold phosphate-buffered saline and scraped in ice-cold TNE buffer (40 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA), pelleted by centrifugation at 600 × *g* for 1 min, resuspended in 400 μl of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM pefabloc, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.5 mg/ml benzamidine) and lysed by the addition of 12.5 μl of 10% (v/v) Nonidet P-40. The nuclear pellet was suspended in 25 μl of ice-cold extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM pefabloc, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.5 mg/ml benzamidine). The supernatant (nuclear extract) was immediately frozen and stored at −70 °C. Typically, 15 μg of nuclear extract was incubated on ice for 5 min in a volume of 20 μl containing 4 μl of 5× binding buffer (125 mM HEPES, pH 8, 2.5 mM EDTA, 2.5 mM DTT, 5% Nonidet P-40, 20 mM NaCl, 20% (v/v) glycerol) and 0.75 μg of poly (dI-dC) as nonspecific competitor DNA. ^{32}P-end-labeled double-stranded oligonucleotides (80,000 counts per minute per reaction) containing the *IL-1β* gene-specific C/EBP-binding site (5′-TTTTGAAAG-3′) or the mutated oligonucleotide with the mutated sites underlined (5′-<u>G</u>TTT<u>T</u>AA<u>G</u>G-3); IL-1β-specific Spi1 site (5′-TACTTCTGCTTT-3′) or the mutated oligonucleotide with the mutated sites underlined (5′-TACTT<u>AG</u>-GCTTT-3′); *IL-1β* gene-specific NFκB site (5′-GGGAAAATCC-3′) or the mutated oligonucleotide with mutated sites underlined (5′<u>TCTA</u>-<u>GA</u>ATCC-3′), was added and incubated for 15 min at 25 °C. The DNA-protein complex that formed was separated from the oligonucleotide on a 4% native polyacrylamide gel, which was dried and analyzed by autoradiography. For supershift assays nuclear extracts were incubated for 1 h at 4 °C with rabbit polyclonal antibodies against p50, p65 (for NF-κB), or C/EBPβ or no antibody, before incubation with the radiolabeled probe.

IL-1β Promoter Reporter Gene Constructs and Luciferase Reporter Assays—The *IL-1β* promoter carrying the sequence from −356 to +12 of the human *IL1β* gene (harboring the NF-κB- and C/EBPβ-binding elements) was cloned into the vector pGL3-Basic between the KpnI and BglII sites to give construct C1. Separate reporter constructs containing either the NF-κB (−356 to −131) (C2) or the C/EBPβ-binding element (−131 to +12) (C3) were also constructed. For luciferase assays, cells were transfected with luciferase reporter plasmids, treated with LPS (20 ng/ml), washed once with phosphate-buffered saline, and scraped into luciferase lysis buffer (25 mM Tris-phosphate, pH 7.8, 4 mM EGTA,

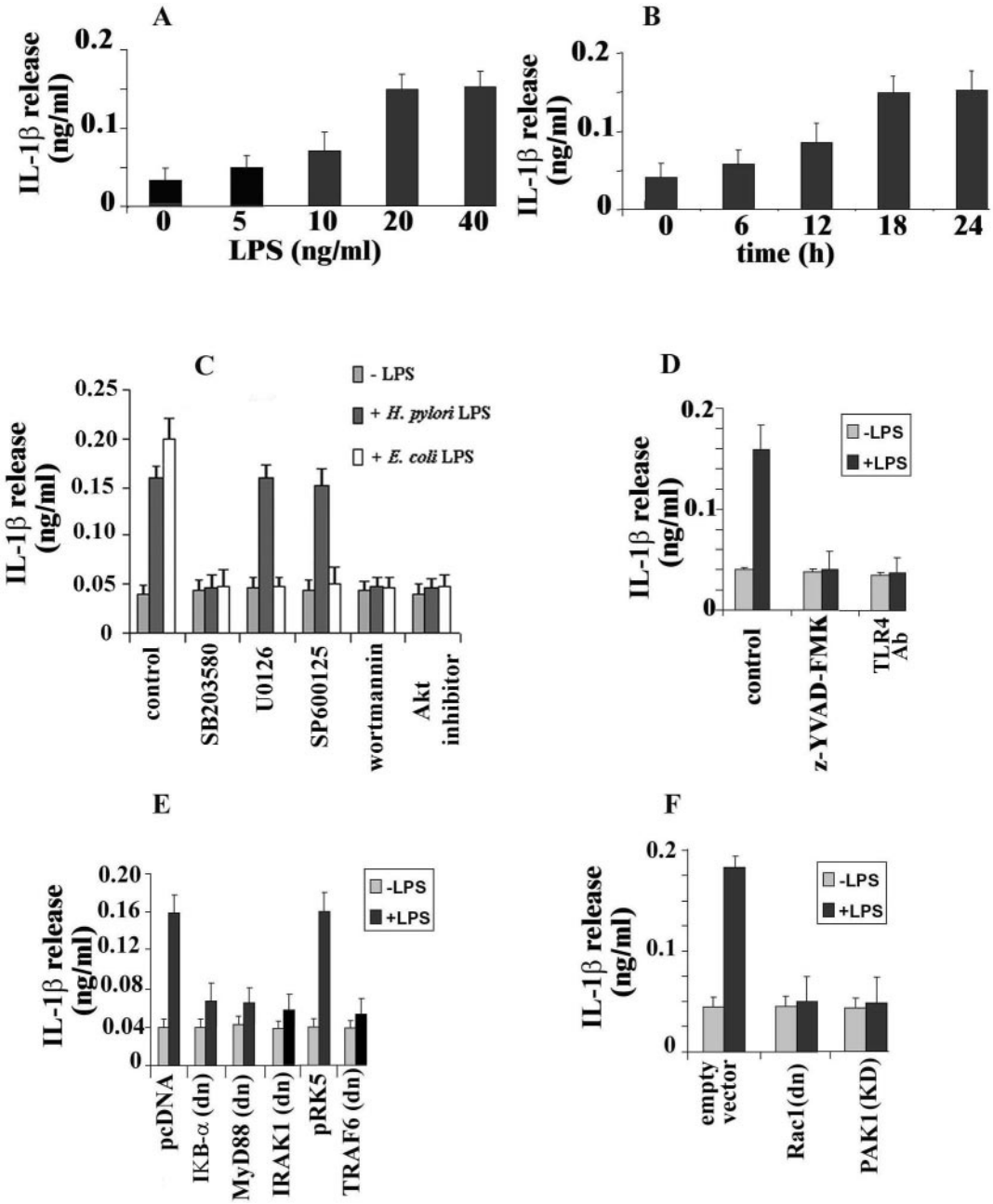

FIG. 1. ***H. pylori* LPS stimulates release of IL-1β from THP-1 cells.** Cells were cultured in 96-well plates and either left untreated or treated with different concentrations of LPS for 20 h (*A*), or with LPS (20 ng/ml) for different time periods (*B*). Where indicated (*C* and *D*) cells were pretreated with different inhibitors for 30 min or with neutralizing anti-TLR4 antibody prior to treatment with LPS (20 ng/ml) for 20 h. For *E* and *F*, cells were transfected with different constructs as indicated, prior to LPS treatment. The supernatant was assayed for IL-1β release using an IL-1β-specific ELISA. Data represent the means ± S.E. of three different experiments.

1% (v/v) Triton X-100, 10% glycerol, 2 mM DTT, 10 μg/ml leupeptin). After centrifugation, the supernatant was stored at −70 °C. For promoter activation analysis, luciferase activity assays were performed in a luminometer, and the results were normalized for transfection efficiencies by assay of β-galactosidase acitivity.

RESULTS

IL-1β Induction by H. pylori LPS—The ability of *H. pylori* LPS to stimulate the release of IL-1β was studied in THP-1 cells. ELISA showed that LPS stimulated the release of IL-1β in a dose-dependent manner (Fig. 1*A*). IL-1β release peaked at 18 h (Fig. 1*B*).

Role of the Mitogen-activated Protein Kinases (MAPKs) in IL-1β Induction by H. pylori LPS—MAPKs are known to regulate the upstream signaling events that control cytokine induction. We therefore evaluated the role of MAPKs in LPS-induced IL-1β production by THP-1 cells using pharmacological inhibitors of MAPKs. *E. coli* LPS-triggered IL-1β release has been reported to be inhibited by inhibitors of ERK (35). This finding was reproduced in our present study (Fig. 1*C*). On the other hand, we observed that *H. pylori* LPS-mediated secretion of IL-1β was unaffected by the MEK inhibitor U0126 (10 μM) (Fig. 1*C*) ruling out the involvement of the MEK/ERK pathway in IL-1β production in this case. IL-1β production was inhibited by the p38 MAPK inhibitor SB203580 (5 μM) (Fig. 1*C*) suggesting a role of the p38 MAPK pathway in IL-1β production. *H. pylori* LPS-mediated secretion of IL-1β was not inhibited by the JNK inhibitor, SP 600125 (25 μM). The lack of involvement of JNK was again unlike what has been reported in the case of *E. coli* LPS (35) and also observed by us in this study (Fig. 1*C*).

The TLR4 Signaling Pathway in LPS-stimulated IL-1β Production in THP-1 Cells—The TLR signaling pathway is known to play a role in regulation of cytokine production through a signaling cascade leading to the activation of NF-κB (19, 20, 38). LPS is known to signal through TLR4. We therefore investigated the involvement of the TLR4 signaling pathway in LPS stimulated IL-1β secretion by THP-1 cells. Pretreatment of cells with neutralizing antibodies against TLR4 prior to challenge with *H. pylori* LPS, blocked IL-1β secretion (Fig. 1*D*) supporting a role of TLR4 signaling in the present instance. Transfection with dominant-negative TLR2 had no effect on LPS-mediated IL-1β release (data not shown).

In the canonical pathway of NF-κB activation, TLR4 signals along the MyD88/IRAK/TRAF6 axis, leading to the phosphorylation of IκB-α and its subsequent degradation by the proteasome and releasing NF-κB facilitating its movement into the nucleus. The likely involvement of this classical pathway in *H. pylori* LPS-induced IL-1β production was tested by transfecting cells with dominant-negative constructs of MyD88, or IRAK1 or TRAF6 or mutated IκB-α(S32A, S36A) in which the two critical serine residues, which are phosphorylated by the IKKs were mutated. An abrogation of IL-1β release (Fig. 1*E*) in each case, confirmed the likely role of the classical pathway of NF-κB activation in this case.

Role of the PI-3K/Akt Signaling Pathway in IL-1β Induction by H. pylori LPS—PI-3K is known to signal downstream of TLR4 (39). We examined the role of the PI-3K/Akt signaling pathway using the PI-3K inhibitor wortmannin. Both *E. coli* and *H. pylori* LPS-stimulated IL-1β production was inhibited by wortmannin (200 nM) (Fig. 1*C*) or by using the Akt inhibitor (15 μM) prior to stimulation with LPS. In both cases, LPS-stimulated IL-1β release was abrogated by these inhibitors indicating the involvement of PI-3K and Akt in LPS-stimulated IL-1β release.

Activation of heterodimeric (p85-p110) PI-3K is most often promoted by tyrosine phosphorylation of p85 and recruitment to the plasma membrane (40). Therefore PI-3K activation was assessed by preparing cell lysates after treatment with LPS, immunoprecipitating with anti-p85 and analyzing the immunoprecipitates by Western blotting with anti-phosphotyrosine antibody. Prior treatment with neutralizing antibody against TLR4, abrogated the activation of PI-3K (Fig. 2*A*), suggesting the likely role of TLR4-dependent signaling in LPS-stimulated IL-1β production. A putative PI-3K-binding site (amino acids 257–260, YKAM) has been identified in the C terminus of MyD88 and LPS stimulation has been shown to result in the formation of the PI-3K-MyD88 complex (41). We therefore tested whether activation of PI-3K is dependent on MyD88. Dominant-negative MyD88 could indeed abrogate the activation of PI-3K (Fig. 2*A*).

p38 MAPK Activation Is Dependent on Akt Kinase—Since both Akt kinase and p38 MAPK were required for LPS-stimulated IL-1β release, we sought to examine whether the activation of either of these kinases was dependent on the other. The activation of p38 MAPK as assessed by Western analysis of phospho-p38 MAPK was abrogated in cells pretreated with neutralizing antibodies against TLR4 or with wortmannin or Akt inhibitor (Fig. 2, *B* and *D*). On the other hand LPS-stimulated Akt kinase activity as assessed on Akt immunoprecipitates of treated cells using GSK3 as substrate, was not affected by transfecting cells with dn-p38 MAPK (data not shown), but abrogated in cells pretreated with wortmannin (Fig. 2*E*). Finally, activation of p38 MAPK was independent of PAK1, since transfection of cells with PAK1 (KD) did not abrogate LPS-stimulated p38 MAPK activation (Fig. 2*C*). Taken together, these data suggested that LPS triggers TLR4/MyD88/PI-3K/Akt/p38 MAPK signaling.

Role of Rac1/PAK1 Signaling in LPS-Stimulated IL-1β Production—Exposure of macrophages to LPS activates the transcription of many cytokine genes in an NF-κB-dependent manner (42). In macrophages, Rac1/PAK1 signaling mediates NF-κB activation in response to *E. coli* LPS, leading to cytokine gene transcription (43). PAK family kinases were discovered by their ability to selectively bind the Rho GTPase family members such as Rac (44–47). Thus we felt it necessary to elucidate the role of Rac1 and PAK1 in *H. pylori* LPS-induced IL-1β release. Transfection of cells with dn-Rac1 or kinase-dead PAK1 prior to challenge with LPS, led to the abrogation of IL-1β release (Fig. 1*F*), suggesting a role of both Rac1 and PAK1 in LPS-mediated IL-1β production. Pretreatment with wortmannin or transfection of cells with dn-Rac1 abrogated PAK1 activation assessed using phospho-PAK antibody (Fig. 2, *F* and *G*). PAK1 activation was therefore dependent on PI-3K and Rac1 in the present instance.

LPS-mediated Activation of Rac1 Is Dependent on PI-3K—In order to assess the activation of Rac1 and formation of Rac1-GTP in response to stimulation with LPS, we performed affinity precipitation experiments with a GST fusion protein corresponding to the p21-binding domain (PBD) of PAK1 (GST-PBD) that specifically binds to and precipitates Rac-GTP from cell lysates (33). The presence of Rac1 in the precipitate was assessed using Rac1 antibody. As shown in Fig. 2*H*, GST-PBD effectively interacted with the active GTP-bound form of Rac1 in cell lysates obtained from LPS-treated cells. However, no interaction was observed between GST-PBD and GTP-Rac1 in cells treated with wortmannin, indicating that Rac1 activation is dependent on PI-3K in the present instance. Immunoblotting analysis of total lysates revealed no changes in the expression levels of Rac1. PI-3K has been demonstrated to be upstream of Rac activation in several instances such as Rac-dependent platelet-derived growth factor and insulin-stimulated membrane ruffling (48, 49).

LPS Stimulates Caspase-1 Activity in THP-1 Cells—Post-transcriptional regulation and processing of pro-IL-1β protein into mature IL-1β involves caspase-1 (10, 51). Preincubation of cells with caspase-1 inhibitor, z-YVAD-FMK followed by treatment with LPS, diminished mature IL-1β production to control levels (Figs. 1*D* and 3*A*), suggesting that caspase-1 is involved in LPS-induced IL-1β maturation. We examined whether LPS could stimulate caspase-1 activity in THP-1 cells. Caspase-1 activity of LPS-treated cells was maximum after 8 h (data not shown). LPS-stimulated caspase activity was blocked by transfection of cells with kinase-dead PAK1 or with dn-Rac1 (Fig. 3*B*) suggesting the involvement of Rac1/PAK1 signaling in the activation of caspase-1.

We next attempted to evaluate whether PAK1 interacts with caspase-1. PAK1 was immunoprecipitated from lysates of *H. pylori* LPS-challenged cells. The immunoprecipitates when subjected to Western analysis, showed the presence of caspase-1 (Fig. 4*A*), suggesting a direct interaction between PAK1 and caspase-1 following *H. pylori* LPS challenge of THP-1 cells. Control experiments where immunoprecipitation was carried out with isotype IgG did not show any band indicating the specificity of the interaction between caspase-1 and PAK1. In an alternative approach, caspase-1 was expressed as a recombinant His-tagged fusion protein immobilized on Ni^{2+}-NTA agarose. When lysates of THP-1 cells challenged with *H. pylori* LPS were incubated with immobilized caspase-1-agarose, Western blotting showed that PAK1 could be pulled down from the lysate. Control experiments using Ni^{2+}-NTA agarose alone, failed to pull down PAK1 (Fig. 4*B, lane a*). These results definitively demonstrated that PAK1 interacts with caspase-1. The interaction was abrogated when lysates from

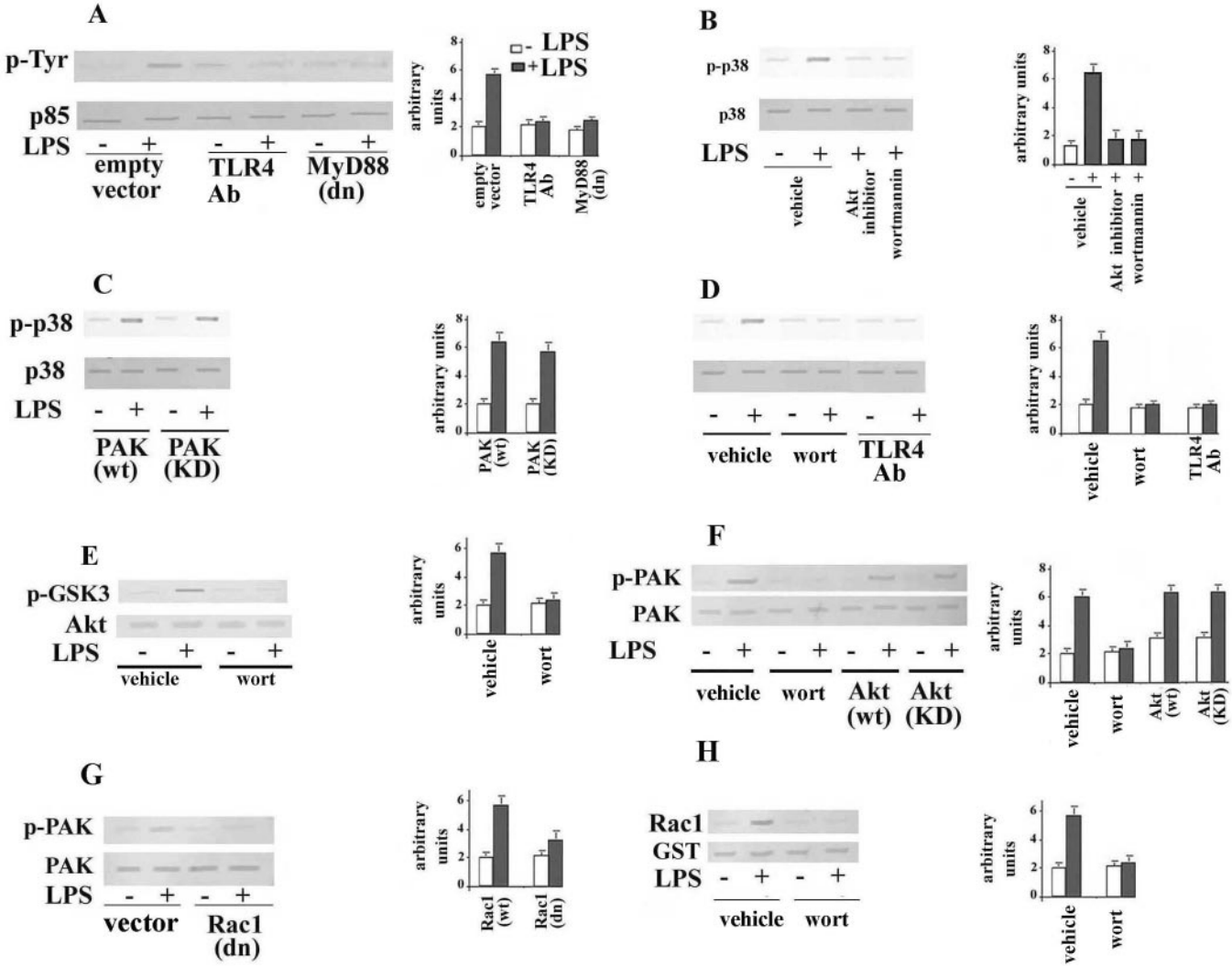

FIG. 2. ***H. pylori* LPS induced activation of PI-3K, p38 MAPK, Akt, Rac1, and PAK1 in THP1 cells.** Cells were transfected with vector alone or with different constructs as indicated (*A*, *C*, *F*, *G*) or with different inhibitors or with neutralizing anti-TLR4 antibody, prior to treatment with LPS (20 ng/ml). PI-3K activation was measured on PI-3K immunoprecipitates by Western blotting using anti-phosphotyrosine antibody (*A*). Cell extracts were prepared and phosphorylation of p38 MAPK and PAK was assessed by Western blotting using anti-phospho-p38 MAPK (*B–D*) and anti-phospho PAK (*F* and *G*) antibody, respectively. Akt kinase activity of cell extracts was assessed by immunoprecipitation with immobilized Akt kinase antibody followed by phosphorylation of GSK3 as substrate (*E*). Activation of Rac1 in cellular extracts was assessed by affinity precipitation of the Rac1·GTP complex from whole cell lysates using PAK1-PBD (*H*) followed by Western blotting using anti-Rac1 antibody as described under "Experimental Procedures." Reprobing with p-85 (*A*), p38 MAPK (*B–D*), Akt (*E*), PAK (*F* and *G*), and GST (*H*) antibodies was performed to verify equal loading in all lanes. Blots are representative of results obtained from three separate experiments. Bar diagrams on the *right side* of each blot represent the results obtained after densitometric scanning from three different experiments. *Bars* represent means ± S.D.)

cells transfected with dn-Rac1 were used (Fig. 4*C*). We next attempted to evaluate whether interaction of PAK1 with caspase-1 was associated with the phosphorylation of caspase-1. We observed that immunoprecipitated PAK1 from lysates of cells treated with *H. pylori* LPS, was able to phosphorylate recombinant caspase-1 (Fig. 4*D*). Some studies have examined the substrate specificity of the PAK family of kinases (52, 53). Based on these studies, the optimal minimal sequence for PAK1 appeared to be (R/K)(R/K)R*X*S. Scanning the caspase-1 sequence, it appeared possible that RKVRFS^{376} could represent a PAK1-recognizable motif, with Ser^{376} representing the site of phosphorylation. This was supported by our observations that PAK1-mediated phosphorylation performed as described above, was abrogated when the S376A mutant of caspase-1 was used as a substrate, suggesting that PAK1 phosphorylates caspase-1 at serine 376. The relevance of this phosphorylation in the activation of caspase-1 was evident from the fact that caspase-1 activation was abrogated in *H. pylori* LPS-challenged THP-1 cells transfected with caspase-1 (S376A) (Fig. 3*B*).

LPS Regulates IL-1β Gene Expression in THP-1 Cells—Semi-quantitative RT-PCR analysis for IL-1β mRNA expression was performed on total cellular RNA from THP-1 cells treated with LPS. LPS induced IL-1β mRNA expression (Fig. 5*A*). In order to determine whether PI-3K and p38 MAPK signaling influence *IL-1β* gene expression, cells were pretreated with wortmannin (200 nM) or SB203580 (5 μM) before stimulation with LPS. IL-1β mRNA levels were assessed by RT-PCR. We observed that SB203580 and wortmannin both suppressed IL-1β mRNA levels (Fig. 5*A*), suggesting a role of PI-3K and p38 MAPK signaling in maintaining steady state levels of IL-1β mRNA.

NF-κB- and C/EBPβ-Binding to the IL-1β Promoter in THP-1 Cells Treated with LPS—The *IL-1β* gene is regulated by an inducible promoter element. These cis-acting DNA sequences are the ultimate targets of numerous transcription factors activated by distinct signal transduction pathways. Monocyte/macrophage-specific expression of the *IL-1β* gene depends upon its promoter located between positions −131 and +12 (36, 37).

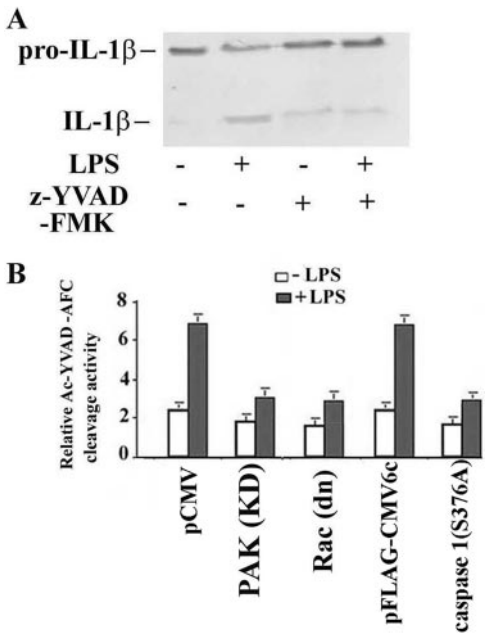

FIG. 3. ***H. pylori LPS*-mediated IL-1β maturation is dependent on caspase-1 activation.** *A*, cells were either left untreated or treated with z-YVAD-FMK (50 μM) prior to treatment with *H. pylori* LPS (20 ng/ml) for 8 h. Cells were lysed, and Western blots were performed with anti-IL-1β antibody. The blot shown is representative of results obtained in three independent experiments. *B*, cells after transfection were treated with or without LPS (20 ng/ml) for 8 h, lysed as described under "Experimental Procedures," and caspase-1 activity was assayed using the fluorescent substrate Ac-YVAD-AFC. Results represent the mean ± S.D. of three independent experiments.

The *IL-1β* promoter contains three important transcription factor-binding motifs, namely NF-IL6 (or C/EBPβ) which is the β-form of the C/EBP of the basic leucine zipper family, NF-κB and Spi-1 (PU.1), a myeloid and B-cell-specific winged helix-turn helix transcription factor that belongs to the ETS family of proteins. In order to determine whether *H. pylori* LPS- inducible expression of the *IL-1β* gene depends on transcription factor binding to these motifs regulated by upstream signal transduction pathways, we performed electrophoretic mobility shift assays (EMSAs) using oligonucleotides containing the NF-κB, the C/EBPβ and Spi-1 DNA-binding elements of the IL-1β promoter. Electrophoretic mobility shifts were observed using the *IL-1β* gene-specific NF-κB- and C/EBPβ-binding oligonucleotides (Fig. 5, *B* and *D*) following stimulation with LPS while no shift was observed in the case of Spi-1-specific oligonucleotide (data not shown). Lack of binding with the mutated NF-κB- and C/EBPβ- specific DNA probes showed that the complexes were specific. Supershift of the band obtained with the NF-κB-specific oligonucleotide with anti-p65 or anti-p50 suggested that p65 and p50 are the components of the protein complex that binds to the NF-κB element of the *IL-1β* promoter. Anti-C/EBPβ supershifted the band obtained with the C/EBPβ-specific DNA probe. Electrophoretic mobility shift of the NF-κB-specific oligonucleotide was inhibited in cells pretreated with wortmannin but not with SB203580 (Fig. 5*C*), suggesting the involvement of PI-3K but not p38 MAPK in NF-κB activation. On the other hand, electrophoretic mobility shift of the C/EBPβ-specific oligonucleotide was inhibited by both wortmannin and SB203580 (Fig. 5*E*), suggesting that both PI-3K and p38 MAPK are involved in C/EBPβ binding to specific elements of the *IL-1β* promoter.

p38 MAP Kinase and C/EBPβ Interact in H. pylori-challenged THP-1 Cells—To study whether p38 MAP kinase interacts with C/EBPβ *in vivo*, we immunoprecipitated lysates from *H. pylori*-LPS-challenged cells with C/EBPβ-specific antibodies from THP-1 cells treated with *H. pylori* LPS. Western analysis showed that p38 MAP kinase coimmunoprecipitated in the complex (Fig. 6*A*). Isotype IgG failed to precipitate detectable p38 MAP kinase confirming the specificity of the immunoprecipitation procedure. In order to study the interaction between C/EBPβ and p38MAPK, C/EBPβ was expressed as a recombinant GST fusion protein and immobilized on glutathione-Sepharose. When lysates of THP1 cells challenged with *H. pylori* LPS were incubated with immobilized C/EBPβ-GST Sepharose, Western blotting showed that p38 MAPK could be pulled down from the cell lysate (Fig. 6*B*). Control experiments with GST alone (*lane a*) did not show any band when Western blotting was carried out with p38 MAPK-specific antibody. Prior treatment with Akt inhibitor could abrogate the interaction between C/EBPβ and p38MAPK. The converse experiments were also performed. Immunoprecipitation was carried out using anti-phospho-p38 MAPK antibody immobilized on agarose. Immunoprecipitates showed the presence of C/EBPβ on Western analysis, whereas appropriate controls did not (Fig. 6*C*). These results supported the view that phospho-p38MAPK interacts with C/EBPβ in an Akt kinase-dependent manner.

Luciferase Reporter Gene Assays—The three luciferase reporter constructs C1 (encompassing both the NF-κB- and the C/EBPβ-binding sites, C2 (harboring only the NF-κB-binding site) and C3 (harboring only the C/EBPβ-binding site) were used to test luciferase expression following challenge with LPS for 3 h. When cells were transfected with the luciferase reporter construct C1, pretreatment of cells with Akt inhibitor prior to LPS challenge led to a 50% reduction in the fold-stimulation of luciferase activity (Fig. 7*A*). Akt inhibitor had no effect on LPS-stimulated luciferase activity when construct C2 was used as the luciferase reporter construct (Fig. 7*B*), whereas it completely inhibited LPS-stimulated luciferase activity when the reporter construct C3 was used (Fig. 7*C*). These results suggested that Akt kinase signaling regulates *IL-1β* promoter activity at the level of the transcription factor C/EBPβ but not at the level of NF-κB-driven promoter activation. On the other hand, transfection of cells with the kinase-inactive mutant of IKKβ (IKKβ-dn), or kinase-dead PAK1 (PAK-K_D) led to a partial inhibition of luciferase activity driven by the NF-κB- and C/EBPβ-binding element-containing C1 (Fig. 7*A*), a complete inhibition of luciferase activity driven by the NF-κB-binding element-containing C2 (Fig. 7*B*), and to no inhibition of luciferase activity driven by the C/EBPβ-binding element-containing C3 (Fig. 7*C*). These results suggested that PAK1 and IKKβ signaling leads to NF-κB activation and stimulation of *IL-1β* gene expression. TLR4 was central to signaling leading to *IL-1β* gene expression, since pretreatment of cells with neutralizing antibodies against TLR4 abrogated LPS-induced luciferase acitivity driven by the presence of both NF-κB- and C/EBPβ-binding elements in the luciferase reporter construct.

DISCUSSION

The modulation of host macrophage cell signaling by *H. pylori* has not been studied in detail. The host response to bacteria is initiated when the microorganism interacts with cell surface receptors including TLRs. Recognition through TLRs largely occurs through pathogen-associated molecular patterns of which the best known is LPS, a potent activator of macrophages. This leads to the release of several cytokines including IL-1β. Although *H. pylori* infection is associated with increased levels of proinflammatory cytokines especially IL-1β (8, 9), the exact mechanism of the release of IL-1β by cells of monocytic lineage is not clear as yet. Understanding this mechanism is of particular relevance in the light of the observations that *IL-1β* gene polymorphisms are associated with increased risk of gas-

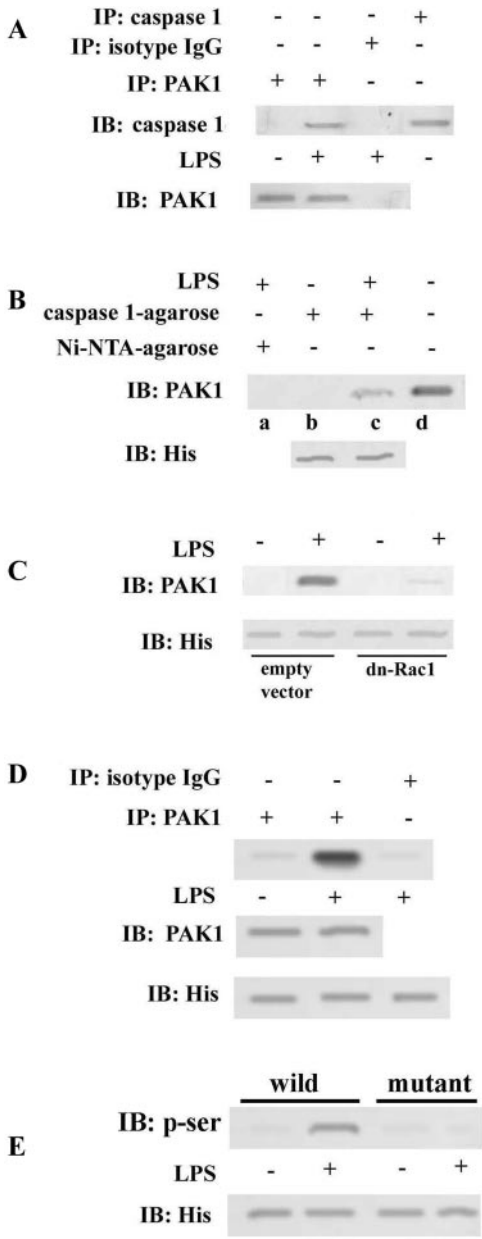

FIG. 4. ***H. pylori* LPS-driven interaction between PAK1 and caspase-1.** *A*, THP1 cells were left untreated or treated with *H. pylori* LPS followed by immunoprecipitation with anti-PAK1 antibody. The immunoprecipitate was blotted with anti-caspase-1 antibody. Negative control for the immunoprecipitation was performed in lysates from treated cells using anti-isotype IgG. As a positive control (*last lane*), caspase-1 was immunoprecipitated from the cell lysate, followed by blotting with anti-caspase-1 antibody. Reprobing was performed with anti-PAK1 antibody as a control for equal loading on the gels. *B*, cell lysates obtained before (*lane b*) and after (*lanes a* and *c*) treatment with LPS were incubated with Ni-NTA-agarose (*lane a*, negative control) or caspase-1 bound to Ni^{2+}-NTA-agarose (*lanes b* and *c*). The bound proteins were then subjected to Western analysis with anti-PAK1 antibody. Reprobing was carried out with anti-His antibody. *Lane d* is a positive control where PAK1 was detected by immunoblotting of the cell lysate using anti-PAK1 antibody. *C*, cells were transfected either with empty vector or with dominant-negative Rac1 (dn-Rac1). Cells were then either left untreated or treated with *H. pylori* LPS. Cell lysates were then treated with caspase-1-bound -Ni^{2+}-NTA-agarose. The bound proteins were then subjected to Western analysis with anti-PAK1 antibody, followed by reprobing with anti-His antibody. *D*, THP1 cells were left untreated or treated with *H. pylori* LPS followed by immunoprecipitation with anti-PAK1 antibody. The immunoprecipitate was used to phosphorylate recombinant wild-type caspase-1 in the presence of [γ^{32}P]ATP. The autoradiogram is representative of three independent experiments. Half of the above reaction mixture was subjected to SDS-PAGE followed by Western blotting with anti-PAK1 as well anti-His antibody in order to ensure equal loading in all lanes. *E*, PAK1 was immunoprecipitated from untreated and *H. pylori* LPS-treated cell lysates and used to phosphorylate either wild type or mutant (S376A) recombinant His-tagged caspase-1 separately. The phosphorylated caspase-1 was detected by Western blotting using anti-phosphoserine antibody. The blot was reprobed with anti-His-antibody.

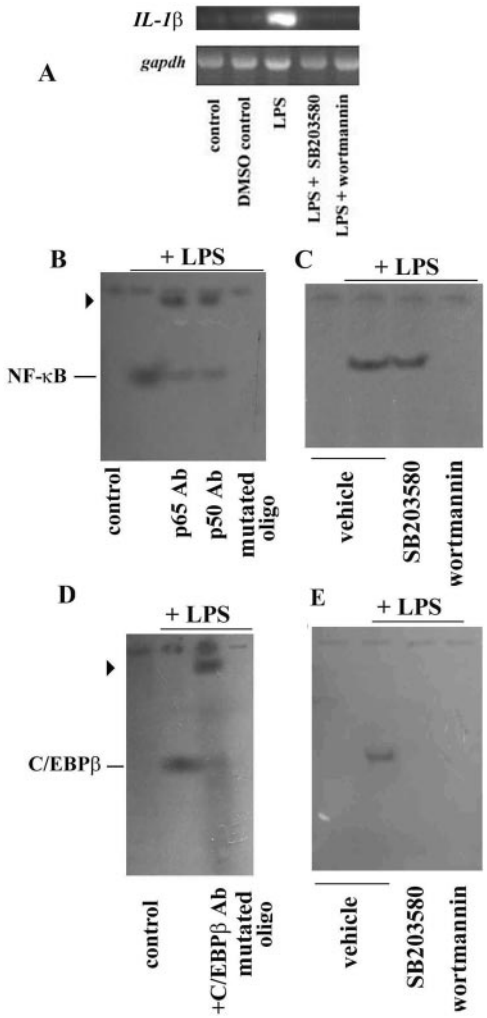

FIG. 5. ***H. pylori* LPS stimulates *IL-1β* gene expression and NF-κB and C/EBPβ binding to the *IL-1β* promoter in THP-1 cells.** Cells were left untreated or pretreated with the inhibitors SB203580 (5 μM) or wortmannin (200 nM) for 30 min, prior to challenge with LPS (20 ng/ml) for 3 h. *A*, total RNA was isolated and RT-PCR was performed using primers specific for the *IL-1β* or the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) genes. The products were visualized on agarose gel by ethidium bromide staining. The data shown are representative of results obtained with three independent experiments. For electrophoretic mobility shift assays (EMSA), the nuclear extracts were subjected to EMSA using labeled IL-1β-specific NF-κB (*B* and *C*) or C/EBPβ (*D* and *E*) DNA probes. Where required, the binding reaction was performed in the presence of mutated oligonucleotides or supershift antibodies as indicated. The *arrowheads* indicate the position of supershifted bands. Gels are representative of results obtained from three independent experiments.

tric cancer. Since it has been reported that aqueous extracts of *H. pylori* could induce IL-1β release, we explored whether LPS, one of the components of the aqueous extract, could be responsible for IL-1β release. We observed that *H. pylori* LPS mediates the secretion of IL-1β by THP1 cells. We report in-depth studies on *H. pylori* LPS-mediated IL-1β release. We observed that LPS stimulation leads to electrophoretic mobility shifts of *IL-1β* gene-specific NF-κB- and C/EBPβ-binding sequences. Since TLR signal transduction has been linked to inflammatory

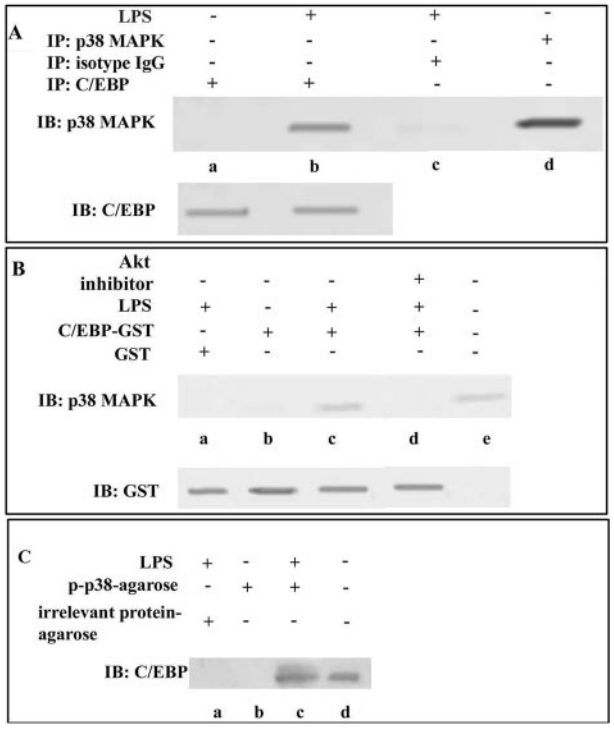

FIG. 6. ***H. pylori* LPS-driven interaction between p38 MAPK and C/EBPβ.** *A*, cells were left untreated (*lane a*) or treated with H. *pylori* LPS (*lanes b* and *c*) followed by immunoprecipitation with anti-C/EBPβ antibody. The immunoprecipitate was blotted with anti-p38 MAPK antibody. Negative control for the immunoprecipitation was performed in lysates from treated cells using anti-isotype IgG. As a positive control, p38 MAPK was immunoprecipitated from the cell lysate, followed by blotting with anti- p38 MAPK antibody (*lane d*). Reprobing was performed with anti-C/EBPβ antibody as a control for equal loading on the gels. *B*, cell lysates obtained after treatment without (*lane b*) or with *H. pylori* LPS (*lanes a–d*) were incubated with glutathione-Sepharose (*lane a*, negative control) or C/EBPβ bound to glutathione-Sepharose (C/EBPβ-GST) (*lanes b–d*). Resin-bound proteins were then subjected to Western analysis using anti-p38 MAPK antibody. Reprobing of the blot was carried out with anti-GST antibody to confirm equal loading. *Lane e* is the positive control where p38 MAPK was immunoprecipitated from cell lysates followed by immunoblotting with anti-p38 MAPK antibody. *C*, cell lysates obtained after treatment without (*lane b*) or with (*lanes a* and *c*) LPS were immunoprecipitated with anti-phospho-p38 MAPK coupled to agarose (*lanes b* and *c*) or with an irrelevant protein coupled to agarose (*lane a*). The agarose-bound proteins were then subjected to Western analysis with anti-C/EBPβ antibody. *Lane d* is a positive control where C/EBPβ was detected by immunoblotting of the cell lysate using anti- C/EBPβ antibody.

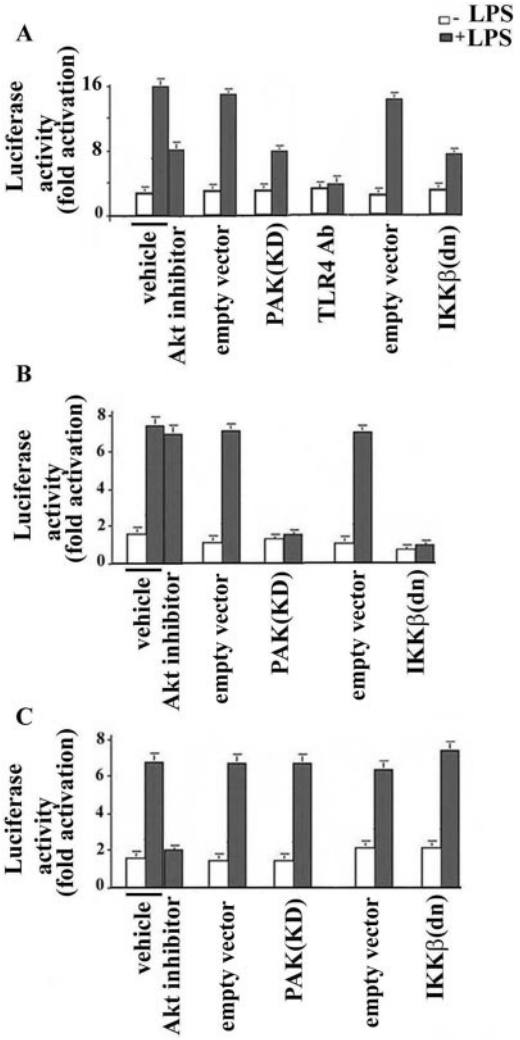

FIG. 7. ***H. pylori* LPS drives luciferase expression from luciferase constructs containing the *IL-1β* gene-specific promoter.** The *IL-1β* promoter luciferase reporter constructs were transfected into THP1 cells along with empty vectors or PAK1 (Kd) or IKKβ (dn) as indicated. In each case cells were cotransfected with β-galactosidase reporter plasmid and luciferase activities were normalized to β-galactosidase activity from contransfection. Where indicated cells cotransfected with luciferase construct and β-galactosidase reporter plasmid were treated with Akt inhibitor for 1 h or with neutralizing anti-TLR4 antibody. Transfected cells were then treated with LPS for 3 h, lysed, and luciferase as well as β-galactosidase activities were measured. Fold activation refers to the increase in luciferase activity over that found in cells transfected with the reporter and a control plasmid without any treatment. *A*, *IL-1β* promoter region (−356/+12)-luciferase (C1); *B*, *IL-1β* promoter region (−356/−131)-luciferase (C2); *C*, *IL-1β* promoter region (−131/+12)-luciferase (C3). The data represent the means ± S.D. of three separate experiments. *Ac-YVAD-AFC*, *N*-acetyl-Tyr-Val-Ala-Asp-(7-amino 4-trifluoromethylcoumarin); *PBD*, p21-binding domain.

cytokine gene induction in macrophages, we evaluated the role of TLR signal transducers in the regulation of *IL-1β* gene expression and IL-1β release by *H. pylori* LPS. Pretreatment of cells with neutralizing antibodies against TLR4-abrogated LPS-stimulated release of IL-1β. Transfection with dominant-negative IκBα abrogated NF-κB-driven luciferase expression in response to stimulation with LPS, suggesting that the canonical TLR4 signaling pathway leading to IκBα phosphorylation is associated with NF-κB activation in response to stimulation of THP1 with *H. pylori* LPS. Our studies extended to further detailed dissection of the LPS-driven signaling pathways leading to *IL1–1β* gene expression. Considering that PI-3K has been reported to be associated with proinflammatory cytokine gene induction, we explored the role of the PI-3K pathway in the present instance. PI-3K was found to be downstream of TLR4/MyD88 signaling. Our detailed studies demonstrate that PI-3K signals along two divergent pathways: PI-3K → Rac1 → PAK1 and PI-3K → Akt → p38 MAPK. PAK1 was found to be necessary for NF-κB-dependent luciferase reporter activation, whereas Akt → p38 MAPK signaling was upstream of C/EBPβ-driven luciferase expression. We demonstrated that C/EBPβ and p38 MAPK interact *in vivo* and that GST-C/EBPβ can immunoprecipitate p38MAPK in its phosphorylated form from *H. pylori* LPS-challenged THP1 cell lysates. p38 MAPK has indeed been found to phosphorylate C/EBPβ in *H. pylori* LPS-challenged THP-1 cells.[2]

Whether PAK1 signaling is dependent or independent of IKKβ-mediated IκB-α phosphorylation is presently under investigation. Expression of active PAK1 in macrophages stimulates the nuclear translocation of the p65 subunit of NF-κB without activation of IKKs (43). Whether stimulation of macro-

[2] M. Kundu, unpublished observations.

phages with *H. pylori* is associated with PAK1-dependent, IKK-independent nuclear translocation of p65, is also presently under investigation.

Our study does not suggest that the signaling pathways described here provide a complete scheme of regulation of *IL-1β* gene expression. Luciferase reporter plasmids harboring the *IL-1β* gene-specific NF-κB- and C/EBPβ-binding elements showed that inhibiting either the NF-κB or the C/EBPβ activation pathways described here, led to a partial inhibition of luciferase expression. On the other hand, IL-1β release was brought down almost to control levels when either of these pathways was inhibited. Thus, the endogenous *IL-1β* promoter locus is likely to be controlled by additional mechanisms such as nucleosome remodeling. Nevertheless, we demonstrate that TLR4/MyD88 signaling leads to activation of the classical IRAK/TRAF6/IKK pathway as well as to activation of PI-3K both of which are necessary for IL-1β release. LPS-mediated signaling drives *IL1–1β* gene expression by activation of both NF-κB and C/EBPβ. The TLR4/MyD88/PI-3K signaling axis is critical in LPS-mediated IL-1β secretion It controls Rac1/PAK1-associated NF-κB activation and Akt/p38 MAPK-associated C/EBPβ activation.

Most interestingly, we observed that PAK1 regulates mature IL-1β production by activating caspase-1. We demonstrated that following *H. pylori* LPS challenge, PAK1 associates with caspase-1 *in vivo*. In addition, recombinant caspase-1 was able to immunoprecipitate PAK1 from *H. pylori* LPS-treated cell lysates. This association was inhibited in cells transfected with dn-Rac1. Cells transfected with dn-PAK1 failed to show *H. pylori* LPS-induced activation of caspase-1. To the best of our knowledge, we demonstrate for the first time that in addition to its role in NF-κB activation, Rac1/PAK1 signaling is upstream of caspase-1 activation, Our studies provide evidence of a novel role of PAK1-mediated phosphorylation of S376 of caspase-1 in its activation. Caspase-1 activation has been reported to be mediated by formation of a molecular platform known as the inflammosome comprising caspase-1, Pycard/Asc, and NALP1 (50). However, the molecular events triggering assembly of the inflammasome remain unclear. Our observations suggest that Rac1/PAK1 signaling is likely to be involved upstream of inflammasome assembly. We have also observed that recombinant, constitutively active PAK1 can phosphorylate recombinant caspase-1 *in vitro*.[2] The significance of PAK1-mediated caspase-1 phosphorylation is under active investigation.

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Chaitali Basak, Sushil Kumar Pathak, Asima Bhattacharyya, Debabrata Mandal, Shresh Pathak and Manikuntala Kundu

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