Mitogen-activated protein kinases and nuclear factor-κB regulate *Helicobacter pylori*-mediated interleukin-8 release from macrophages

Asima BHATTACHARYYA*, Shresh PATHAK*, Simanti DATTA†, Santanu CHATTOPADHYAY†, Joyoti BASU* and Manikuntala KUNDU*[1]

*Department of Chemistry, Bose Institute, 93/1 Acharya Prafulla Chandra Road, Kolkata 700009, India, and †National Institute of Cholera and Enteric Diseases, P-33 CIT Road, Scheme XM, Beleghata, Kolkata 700010, India

Gastric infection, as well as inflammation, caused by *Helicobacter pylori*, activates the production of cytokines and chemokines by mononuclear cells; interleukin-8 (IL-8) is one of the major inflammatory chemokines. Since *H. pylori* does not invade mucosal tissue, we observed the effect of the water extract of *H. pylori* (HPE), containing shed factors, on the production of IL-8 by human peripheral blood monocytes and the human monocyte cell line THP-1. HPE-treatment induced activation of the mitogen-activated protein kinases (MAPKs) ERK (extracellular signal-regulated kinase), p38 and JNK (c-Jun N-terminal kinase), an effect which was not dependent on the presence of the *cag* pathogenicity island. p38 MAPK activation was sustained. The specific inhibitors, U0126 (for ERK1/2 signalling) and SB203580 (for p38 MAPK signalling), both abrogated IL-8 secretion from HPE-treated THP-1. Dominant-negative mutants of the up-stream kinases MEK1 (MAPK/ERK kinase 1), MKK (MAPK kinase) 6 and MKK7 also inhibited IL-8 secretion, pointing to a role of all three MAPKs in HPE-mediated IL-8 release. The inhibitory effects of polymyxin B and anti-CD14 antibody suggested that the effect of HPE on MAPKs was mediated by *H. pylori* lipopolysaccharide (LPS). By analysis of IL-8-promoter-driven luciferase gene expression, we observed that the effects of HPE-induced nuclear factor-κB (NF-κB) activation and MAPK signalling were mediated at the level of the IL-8 promoter. While ERK1/2 activation could be linked to enhanced DNA binding of activator protein-1 (AP-1), p38 MAPK signalling did not affect AP-1 DNA binding. Taken together, these results provide the first evidence that LPS from *H. pylori* stimulates IL-8 release from cells of the monocytic lineage through activation of NF-κB and signalling along MAPK cascades. The stimulation of MAPK signalling in macrophages by LPS of *H. pylori* amplifies the inflammatory response associated with gastric *H. pylori* infection and needs to be taken into consideration when developing therapeutics based on these signalling pathways.

Key words: activator protein-1 (AP-1), chemokine, gastric ulcer, transcription factor, lipopolysaccharide (LPS).

INTRODUCTION

Helicobacter pylori is a Gram-negative bacterium which causes chronic gastritis and has important roles in peptic ulcer disease, gastric carcinoma and gastric lymphoma. *H. pylori* has been found in humans in all parts of the world, with over half of the world's population infected with *H. pylori*; the stomach being its principal reservoir. In developing countries, 70–90 % of the population carries *H. pylori*. In 20–30 % of cases, the end result of the infection can be life-threatening [1]. The increasing realization that *H. pylori* is associated with duodenal and gastric ulcers and gastric carcinoma highlights the need to understand the basic cellular mechanisms in the pathogenesis of *H. pylori* gastritis at the molecular level. The mechanisms associated with the epithelial hyperproliferation observed in chronic *H. pylori* infection and the inflammatory response need to be understood.

Cytokines and chemokines have been proposed to play an important role in *H. pylori*-associated gastroduodenal diseases, but the exact mechanism of the cytokine or chemokine induction remains unclear. Chemokines are a superfamily of closely related chemoattractant cytokines which specialize in mobilizing leuco-cytes to areas of immune challenge [2]. The family of chemokines characterized by the motif Cys-Xaa-Cys (where Xaa is any amino acid) includes interleukin-8 (IL-8), which is one of the principal mediators of the inflammatory response [3,4]. When *H. pylori* is co-cultured with gastric epithelial cell lines, it induces secretion of IL-8 [5,6]. The *cag* pathogenicity island (PAI) of *H. pylori* harbours a large number of genes that encode pathogenicity factors [7]. *H. pylori*-induced IL-8 expression in gastric epithelial cells is associated with the CagA$^+$ phenotype [8]. Isogenic mutants, altered in one of six *cag* genes (*cagE*, *cagG*, *cagH*, *cagI*, *cagL* or *cagM*) failed to elicit IL-8 secretion from gastric epithelial cells [9]. Differential activation of mitogen-activated protein kinases (MAPKs) in gastric epithelial cells by *cag*$^+$ and *cag*$^-$ *H. pylori* is related to IL-8 secretion [10].

In spite of recent reports, the mechanism by which *H. pylori* (which has little or no invasive activity) induces gastric-tissue inflammation and injury is not fully understood. In particular, the role of mononuclear cells in this process deserves investi-gation. The inflammatory lesions associated with *H. pylori* gastritis and duodenitis contain large numbers of mononuclear cells. The close proximity of *H. pylori* to the gastric mucosa

---

suggests that the organism interacts with mononuclear cells, thereby modulating the inflammatory response. Neutrophil infiltration of the gastric lamina propria is characteristic of *H. pylori*-associated gastritis [11]. Among the host cell factors responsible for neutrophil infiltration are the pro-inflammatory cytokines and chemokines, such as IL-1, tumour necrosis factor (TNF)-α and IL-8, all of which are increased in the antral mucosae of individuals with *H. pylori* gastritis [12]. Monocytes and macrophages are a source of IL-8 production [13]. Since the bacterium is minimally invasive, we have investigated the mechanisms of IL-8 induction by soluble mediators from *H. pylori* in order to understand better the pathogenesis of the inflammatory response.

The MAPKs comprise an important group of serine and threonine signalling kinases that transduce a variety of extracellular stimuli through a cascade of protein phosphorylations that lead to the activation of transcription factors [14–16]. There are three principal MAPK family members: (i) p46 and p54 c-Jun N-terminal kinase (JNK), or stress-activated protein kinase, 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): (i) MKK4 and MKK7 activate JNK [17–19], (ii) MAPK/ERK kinase (MEK)1 and MEK2 activate the ERKs [20], and (iii) MKK3, MKK6 and, sometimes, MKK4 activate p38 MAPK [21].

To investigate the signalling pathways involved in IL-8 synthesis by macrophages that are exposed to soluble mediators of *H. pylori*, we examined the ability of shed factors from the water extract of *H. pylori* (HPE) to trigger phosphorylation of different MAPKs and to effect IL-8 release from macrophages through a MAPK-dependent signalling pathway. In the present study, we demonstrate that shed factors from *H. pylori* are capable of activating nuclear factor-κB (NF-κB) and triggering phosphorylation of ERK1/2, p38 and JNK MAPKs. HPE stimulated luciferase gene expression, driven by the IL-8 promoter through activation of NF-κB and activator protein-1 (AP-1). By using specific inhibitors and transfections with dominant-negative (dn) mutants, we demonstrate the contribution of MAPKs in IL-8 secretion in macrophages stimulated by *H. pylori*. Our results suggest that the activation of NF-κB, and ERK1/2, p38 and JNK MAPKs contribute to the secretion of IL-8 from human monocytes or macrophages mediated by HPE. We hypothesize that NF-κB activation and MAPK signalling are important elements controlling IL-8 synthesis by macrophages exposed to shed factors from *H. pylori*. Our studies demonstrate that contact with the organism is not required for stimulating IL-8 secretion from macrophages. The inhibitory effects of polymyxin B and anti-CD14 antibodies suggest a role of lipopolysaccharide (LPS) in eliciting these effects. The identification of *H. pylori*-specific signalling pathways, leading to the induction of a pro-inflammatory immune response, is likely to lead to strategies for therapeutic intervention to overcome gastric inflammation associated with *H. pylori* infection.

EXPERIMENTAL

Reagents

PMA was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PD98059, U0126 and SB203580 were from CN Biosciences (San Diego, CA, U.S.A.). Phosphorylation state-specific antibodies, total antibodies against MAPKs and the Phototope®-HRP Western Detection kit were from Cell Signaling Technology (Beverly, MA, U.S.A.). The oligonucleotides cor-

responding to the NF-κB- and AP-1-binding sequences in the IL-8 promoter were from MWG Biotech AG (Ebersberg, Germany). All other chemicals were from Sigma Chemical Co.

H. pylori strains

Three *H. pylori* strains have been used in this study: strain 26695 (reference strain) and strain 18A (clinical isolate) were positive for the *cag* PAI, and strain 80A (clinical isolate) lacked the complete *cag* PAI. The status of the *cag* PAI was determined by PCR using primers described by Mukhopadhyay et al. [22].

HPE preparations

H. pylori strains (*cag*$^+$ and *cag*$^-$) were grown on brain heart infusion agar plates supplemented with 10% heat-inactivated bovine serum (GibcoBRL, Grand Island, New York, NY, U.S.A.) and incubated at 37 °C in a microaerophilic environment. Bacteria (in exponential phase) were harvested in PBS (pH 7.4) and resuspended to a D_{600} of 1.5, a value corresponding to 3.5×10^8 colony forming units/ml. After 20 min incubation at room temperature, the bacteria were pelleted at 4000 *g* for 10 min. The supernatant was centrifuged at 16000 *g* for 10 min and filtered through a 0.22 μm pore-size filter. Total protein content was measured by the Bradford assay (Bio-Rad, Hercules, CA, U.S.A.). The resulting HPE (200–250 μg/ml) was stored in aliquots in pyrogen-free polycarbonate tubes at -70 °C until required. The stock was diluted to 0.1% (v/v) in medium before use in all the experiments (i.e. equivalent to 10^5 bacterial cells/ml). This concentration was first determined to be optimal for activation of all three MAPKs, and for IL-8 release (results not shown).

Isolation of human peripheral blood monocytes (PBMs)

Blood was drawn from healthy adult volunteers into heparinized (100 units/ml) syringes, layered on Ficoll-Hypaque (Amersham Biosciences, Little Chalfont, U.K.) and isolated by centrifugation according to the manufacturer's instructions. Cells were incubated in dishes containing RPMI 1640 medium supplemented with 25 mM Hepes, 2% penicillin and streptomycin, 1% glutamine and 10% foetal bovine serum for 1 h at 37 °C in a humidified CO_2 (5%) incubator. Dishes were washed to remove non-adherent cells. Adherent cells were $\geqslant 90\%$ viable as determined by Trypan Blue dye exclusion.

Cell culture

THP-1 cells (derived from a patient with acute monocytic leukaemia) are mature cells from the monocyte/macrophage lineage. These were obtained from the National Centre for Cell Science (Pune, India). The cell line was maintained in RPMI 1640 medium containing 10% heat-inactivated foetal 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. Cells were washed three times with culture medium and cell viability was determined to be $> 95\%$ by the Trypan Blue dye exclusion method. The inhibitors PD98059 and SB203580 were dissolved in DMSO. Appropriate vehicle controls were maintained in all experiments in which these inhibitors were used.
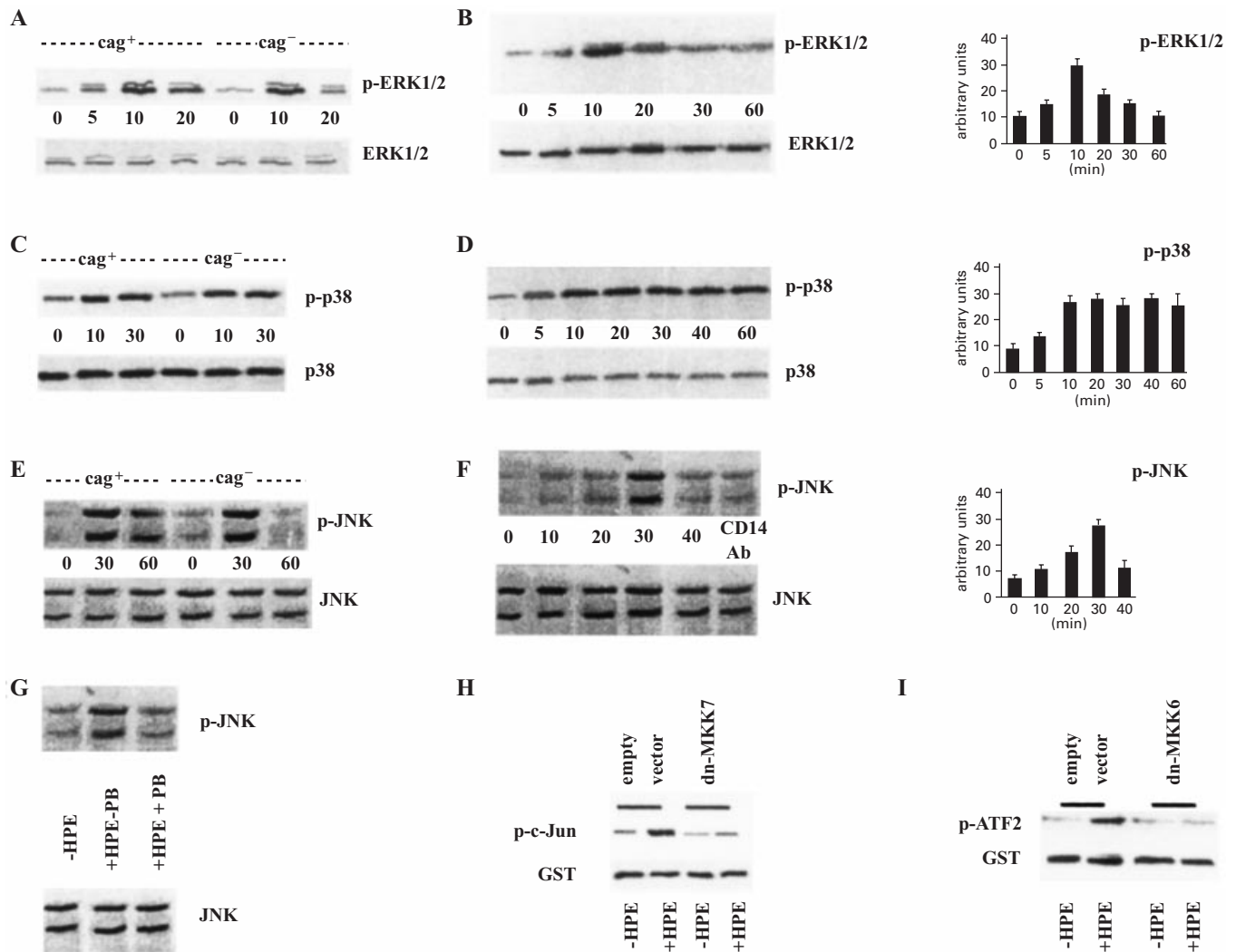
Figure 1   ERK, p38 and JNK MAPKs are activated in HPE-treated PBMs and THP-1 cells

Cellular extracts from PBMs (**A**, **C** and **E**) or THP-1 cells (**B**, **D** and **F**) treated for various lengths of time (indicated in min) with HPE were analysed by Western blotting for the presence of phospho-MAPKs. *cag*$^+$ and *cag*$^-$ refer to treatments with HPE from strains 26695 and 80A respectively. Similar results were obtained with strain 18A. With each strain, experiments were performed three times. The results shown are from a representative set of experiments. (**A**) and (**B**) show results obtained after probing with anti-phospho-ERK1/2 antibodies; (**C**) and (**D**) show results obtained after probing with anti-phospho-p38 MAPK antibodies and (**E**) and (**F**) show results obtained after probing with anti-phospho-JNK antibodies. Each of the blots was also probed with antibodies against total ERK1/2, p38 and JNK to ensure that these were present in equal amounts in all lanes. The histograms in (**B**), (**D**) and (**F**) show the results obtained after densitometric scanning of the blots from three independent experiments (bars represent means $\pm$ S.D.) The last lane in (**F**) shows the results obtained when THP-1 cells were pre-incubated for 30 min with anti-CD14 antibodies (1 μg/ml) before treatment with HPE (for 30 min) and probing with anti-phospho-JNK. (**G**) shows the effect of polymyxin B on HPE-induced JNK activation. Cellular extracts from THP-1 cells were either left alone ($-$HPE) or treated with HPE [pre-incubated in the absence or presence of polymyxin B (10 μg/ml)] for 30 min. Lysates were analysed by Western blotting with anti-phospho-JNK antibodies. (**H**) and (**I**) show the effects of HPE on JNK and p38 MAPK activity in THP-1 cells. THP-1 cells were transfected with vector alone or dn-MKK7 (**H**) or dn-MKK6 (**I**) followed by treatment with medium alone or with HPE for 30 min. Cellular extracts were prepared and activated JNK and p38 MAPKs were pulled-down with immobilized GST–c-Jun (**H**) and immobilized anti-phospho-p38 MAPK (**I**). Complexes were added to an *in vitro* kinase assay. Purified GST–ATF-2 was used as substrate in (**I**). Western blotting was performed using anti-phospho-c-Jun and anti-phospho-ATF-2 antibodies for (**H**) and (**I**), respectively. Probing was performed with anti-GST antibody to verify equal loading in all lanes. Results shown are representative of three independent experiments performed with three different HPE preparations from strain 26695.

Plasmids and transient transfections

The full length wild-type (wt) MEK1 and dn mutant MEK2A in a pCMV expression vector were gifts from Dr D. J. Templeton, Case Western Reserve University, Cleveland, OH, U.S.A.; dn-MKK3, dn-MKK4, wt-MKK6 dn-MKK6, cloned in Flag-tagged pcDNA3.1 were kindly provided by Dr Roger Davis, University of Massachusetts, Worcester, MA, U.S.A.; wt- and dn-MKK7 were gifts from Dr Pamela Holland, Immunex Corporation, Seattle, WA, U.S.A. Transient transfections were carried out using the Effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The β-gal reporter plasmid was used to normalize transfection efficiencies.

Treatment with HPE and preparation of cell lysates

PBMs or THP-1 cells that were cultured in 24-well tissue culture plates (5×10^5 cells per well) were treated with HPE. Where indicated, THP-1 cells were preincubated for 30 min with anti-CD14 antibodies (1 μg/ml) or polymyxin B (10 μg/ml) before treatment with HPE. The wells were then washed with ice-cold PBS. Cells were lysed with lysis buffer [20 mM Tris/HCl
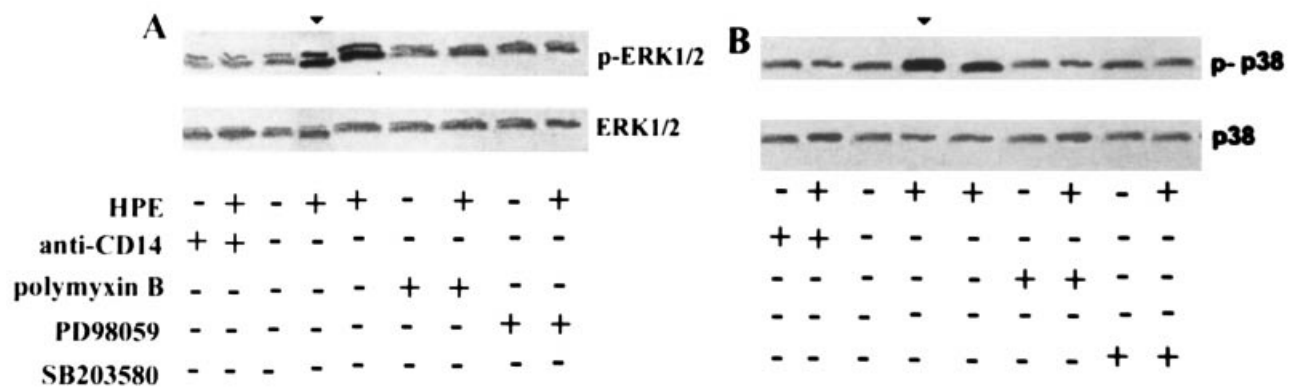
Figure 2   Effect of inhibitors on HPE-induced MAPK activation

THP-1 cells were either left untreated or treated with anti-CD14 antibody (1 μg/ml) or the MAPK inhibitors PD98059 or SB203580 for 30 min before incubation with HPE (from strain 26695) for 10 min (in **A**) or 30 min (in **B**). Where indicated, HPE was preincubated without (▼) or with polymyxin B (other lanes) before addition to THP-1 cells as described for Figure 1(G). Cells were lysed and subjected to Western blotting with anti-phospho-ERK1/2 (**A**) and anti-p38 MAPK (**B**) antibodies. Each of the blots was also probed with antibodies against total ERK1/2 and p38 MAPK to ensure that these were present in equal amounts in all lanes. The results shown are representative of three independent experiments obtained with three different preparations of HPE from strain 26695.

(pH 7.4), 1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 137 mM NaCl, 20 mM NaF, 1 mM EDTA, 40 mM sodium β-glycero-phosphate, 4 μg/ml each of leupeptin, pepstatin and aprotinin, 1 mM Na_3VO_4 1 mM Pefabloc® and 1 mM benzamidine] 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 pharmaco-logical inhibitors or vehicle (DMSO) alone, before incubation with HPE.

Western blotting

Whole cell lysates were separated by SDS/PAGE and the proteins were transferred on PVDF membranes. The blots were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature and subsequently incubated overnight at 4 °C with primary antibodies diluted (1:1500) in TBST [TBS, 1% (v/v) Tween 20 and 5% (w/v) BSA]. Following three washes of 5 min each with TBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology) in blocking buffer for 1 h at room temperature. After three washes with TBST, the blots were developed with chemiluminescence reagent and exposed to X-ray film (Kodak XAR5, Eastman Kodak, Rochester, NY, U.S.A.).

In vitro p38 MAPK assay

The activity of p38 MAPK was determined by solid-phase kinase assays using glutathione S-transferase–activating transcription factor 2 (GST–ATF-2; Cell Signaling Technology) as substrate. After treatment, cells were lysed in lysis buffer [20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM sodium β-glycerophosphate and 1 mM Na_3VO_4] for 5 min on ice. After centrifugation at 12000 ***g*** (Sorvall SS-34, Sorvall, Newtown, CT, U.S.A.) for 5 min at 4 °C, supernatants were incubated with immobilized phospho-p38 MAPK monoclonal antibody (Cell Signaling Technology), according to the manufacturer's protocol, in order to assay for p38 kinase activity. The beads were washed twice with lysis buffer, followed by two washes with kinase reaction buffer [25 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM

sodium β-glycerophosphate, 2 mM dithiothreitol (DTT) and 100 μM Na_3VO_4]. The beads were then incubated with 2 μg GST–ATF-2 fusion protein in 20 μl 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 (10% gel) followed by Western blotting. Blots were probed with anti-phospho-ATF-2 antibody and visualized using a chemiluminescence detection kit (Phototope®-HRP Western Detection kit, Cell Signaling Technology).

In vitro JNK activity assay

To assay JNK activity, equal amounts of total protein were incubated with glutathione S-transferase–c-Jun (amino acids 1–89) fusion protein beads coupled to agarose for 2 h at 4 °C. The beads were washed twice with lysis buffer and twice with kinase reaction buffer [20 mM Hepes (pH 7.6), 20 mM $MgCl_2$, 20 mM sodium β-glycerophosphate, 10 mM NaF, 0.2 mM Na_3VO_4 and 0.2 mM DTT]. The kinase reaction was performed in kinase buffer containing 200 μM ATP and terminated after incubation at 25 °C for 30 min by the addition of 2 × protein sample buffer, and analysed by SDS/PAGE followed by Western blotting with phosphospecific anti-c-Jun antibody.

Electrophoretic mobility-shift assay (EMSA)

In order to prepare nuclear extracts, plates with adhered cells were washed twice with ice-cold PBS and scraped in ice-cold TNE buffer (40 mM Tris/HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA). Cells were pelleted at 600 ***g*** for 1 min and pellets were resuspended in 400 μl of lysis buffer A (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 and 0.5 mg/ml benzamidine). Cells were lysed by the addition of 12.5 μl of 10% (v/v) Nonidet P-40 and 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 and 0.5 mg/ml benzamidine). The supernatant (nuclear extract) was

immediately frozen and stored at $-70\,^{\circ}\mathrm{C}$. Typically, $10\,\mu\mathrm{g}$ of nuclear extract was incubated on ice for 5 min in the presence or absence of competitor DNA in a $20\,\mu\mathrm{l}$ volume containing $4\,\mu\mathrm{l}$ of $5\times$ 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 $0.75\,\mu\mathrm{g}$ of poly(dI-dC) as non-specific competitor DNA. ^{32}P-end labelled double-stranded oligonucleotides (60 000 counts per minute per reaction) containing the IL-8 gene-specific NF-κB binding site (5$'$-**GTGG**AATTTCC-3$'$) or the mutated oligonucleotide with the mutation sites shown in bold, or the IL8-specific AP-1 binding site (5$'$-GTGAT**GA**CTCAGGT-3$'$) or the mutated oligonucleotide with the mutation sites shown in bold, was added and incubated for 15 min at $25\,^{\circ}\mathrm{C}$. The DNA–protein complex that formed was separated from the oligonucleotide on a 4% native polyacrylamide gel, which was dried and analysed by autoradiography. For supershift assays, nuclear extracts were incubated for 1 h at $4\,^{\circ}\mathrm{C}$ with rabbit polyclonal antibodies against p50, p65 (for NF-κB) or c-fos, c-jun (for AP-1) or no antibody, before incubation with the radiolabelled probe.

Luciferase reporter assays

The luciferase reporter plasmids incorporating the IL-8 promoter (bp -135 to $+46$) in the vector pGL-2 Basic [23] were obtained from Dr Andrew Keates, Beth Israel Deaconess Medical Center, New York, NY, U.S.A. The binding elements are shown in bold type with the mutations underlined: NF-κB, 5$'$-CG**TTAAC**TTTCCTCT-3$'$; AP-1, 5$'$-GTGAGATCTGAAGT-GTGAT**AT**CTCAGG-3$'$. Cells were transfected with luciferase reporter plasmid. Transfected cells were treated with HPE, washed once with PBS and scraped into luciferase lysis buffer (50 mM Tris, pH 8, 70 mM K_2HPO_4, 0.1% Nonidet P-40, 2 mM $MgCl_2$, 1 mM DTT, $20\,\mu\mathrm{g/ml}$ aprotinin, $10\,\mu\mathrm{g/ml}$ pepstatin and $10\,\mu\mathrm{g/ml}$ leupeptin). The lysates were rapidly mixed and insoluble material was pelleted by centrifugation at $4\,^{\circ}\mathrm{C}$. The supernatant was removed, frozen in liquid nitrogen and stored at $-80\,^{\circ}\mathrm{C}$. 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 activity.

IL-8 measurement in culture supernatants

Transfected or non-transfected THP-1 cells were either left untreated or were treated with inhibitors of MAPK signalling, followed by incubation with HPE. The conditioned medium was removed and assayed for IL-8 by ELISA using the human BIOTRAK IL-8 assay kit (Amersham Biosciences) according to the manufacturer's protocol. The limit of detection was 10 pg/ml IL-8.

Statistical analysis

Results obtained from independent experiments are represented as means $\pm$ S.D. and were compared by Student's t test. Differences were considered significant for $P < 0.05$.

RESULTS

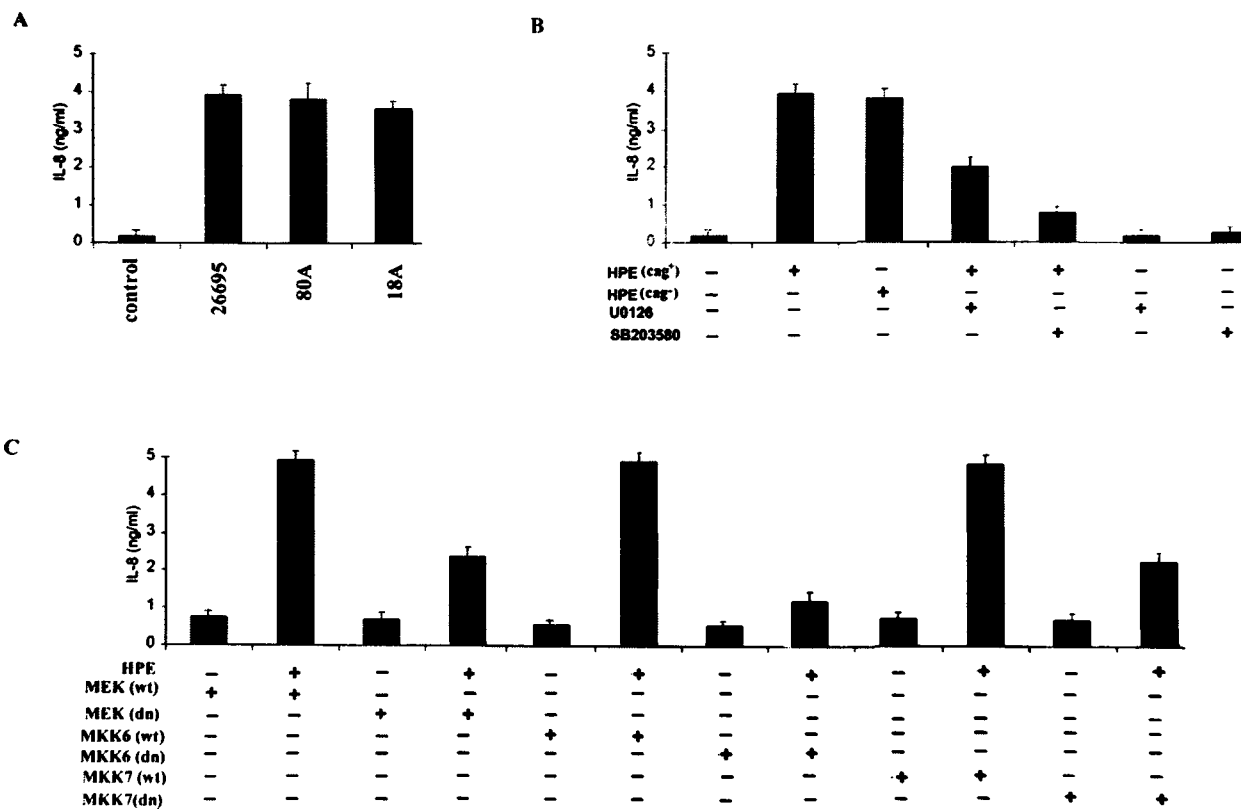### HPE-treatment results in activation of ERK1/2 p38 and JNK MAPKs

Because MAPKs regulate cytokine and chemokine induction in response to external stimuli, including soluble bacterial mediators such as LPS, time-dependent MAPK phosphorylation was measured to assess the activation of these kinases upon treatment
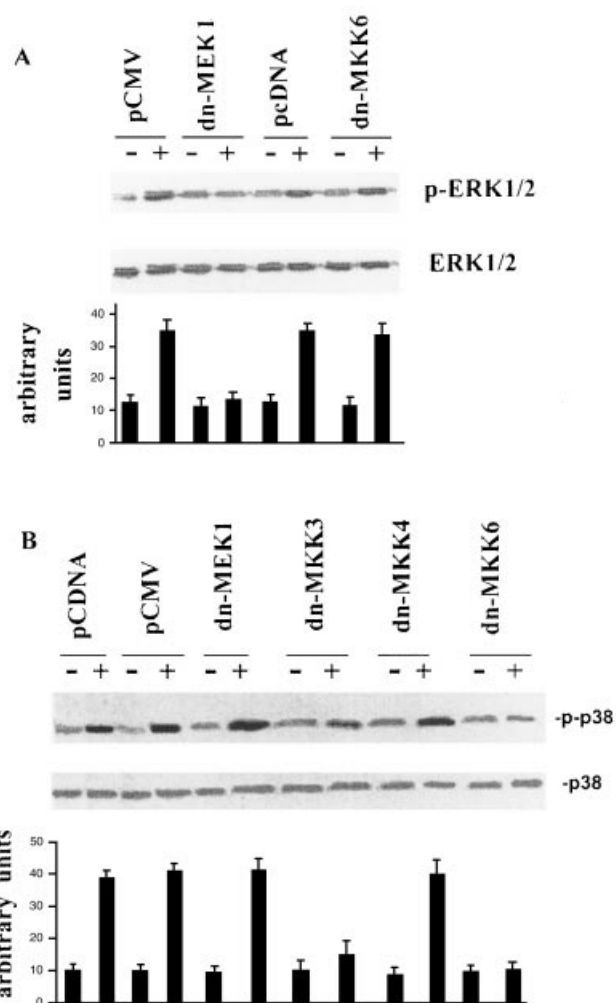
Figure 3   dn-MEK-1 and dn-MKK6/3 inhibit HPE-stimulated activation of MAPKs

(**A**) Cells were transfected with control vector (pCMV and pcDNA 3.1) or dn-MEK1 or dn-MKK6, followed by incubation with medium ($-$) or with HPE (from strain 26695) ($+$) for 10 min. Cell extracts were prepared and phosphorylation of ERK1/2 was visualized by Western blotting using anti-phospho-ERK1/2 antibody as described for Figure 1(A). (**B**) Cells were transfected with control vector (pCMV and pcDNA) or dn-MEK1, dn-MKK3, dn-MKK4, dn-MKK6, followed by incubation with medium ($-$) or with HPE (from strain 26695) ($+$) for 30 min. Cells were lysed and subjected to Western blot analysis with anti-phospho-p38 MAPK antibody as described for Figure 1(B). Each of the blots was also probed with antibodies against total ERK1/2 and p38 MAPK to ensure that these were present in equal amounts in all lanes. For all transfection experiments, co-transfection with β-galactosidase reporter plasmid (transfection control) was used to normalize transfection efficiencies by assaying for β-galactosidase activity. The bottom panels represent results obtained after densitometric scanning from three different experiments using three different preparations of HPE (bars represent means $\pm$ S.D.).

with HPE. Human PBMs showed a time-dependent increase in phosphorylation of ERK1/2, p38 and JNK MAPKs upon HPE-treatment. (Figures 1A, 1C and 1E). A similar pattern of MAPK activation by HPE was also observed in the human macrophage-like THP-1 cells. ERK1/2 activity peaked at 10 min (Figure 1B), while JNK activity peaked at 30 min (Figure 1F). On the other hand, p38 MAPK activation was sustained (Figure 1D). HPE from *cag*$^+$ (26695 and 18A) and *cag*$^-$ (80A) strains elicited similar effects. JNK activation was further confirmed by an *in vitro* kinase assay using GST–c-Jun as substrate. HPE-mediated GST–c-Jun phosphorylation was abrogated by transfection with

A

IL-8 (ng/ml)

control 26695 80A 18A

B

IL-8 (ng/ml)

HPE (cag⁺)	−	+	−	+	+	−	−
HPE (cag⁻)	−	−	+	−	−	+	−
U0126	−	−	−	+	−	−	−
SB203580	−	−	−	−	+	−	+

C

IL-8 (ng/ml)

HPE	−	+	−	+	−	+	−	+	−	+	−	+
MEK (wt)	+	+	−	−	−	−	−	−	−	−	−	−
MEK (dn)	−	−	+	+	−	−	−	−	−	−	−	−
MKK6 (wt)	−	−	−	−	+	+	−	−	−	−	−	−
MKK6 (dn)	−	−	−	−	−	−	+	+	−	−	−	−
MKK7 (wt)	−	−	−	−	−	−	−	−	+	+	−	−
MKK7(dn)	−	−	−	−	−	−	−	−	−	−	+	+

Figure 4   HPE stimulates IL-8 secretion from PBMs and THP-1 cells in a MAPK-dependent pathway

(**A**) PBMs were either left untreated or treated with HPE from different strains of *H. pylori* as indicated before measuring IL-8 release in the conditioned medium. (**B**) Monolayers of THP-1 cells were cultured alone or with HPE from a *cag⁺* (26695) or a *cag⁻* (80A) strain of *H. pylori*, with or without pre-treatment (for 30 min) with the MEK1 inhibitor U0126 (10 μM) or the p38 MAPK inhibitor SB203580 (5 μM). THP-1 cells were also treated with inhibitors only, as controls. (**C**) Monolayers of THP-1 cells were transfected with wt-MEK or dn-MEK, wt-MKK6 or dn-MKK6, or wt-MKK7 or dn-MKK7 and left untreated or treated with HPE from strain 26695. In all experiments, the release of IL-8 was measured in the conditioned medium harvested after 14 h. Bars represent the means ± S.D. for three independent experiments performed with three different HPE preparations.

dn-MKK7, the upstream kinase responsible for JNK activation (Figure 1H).

Specific inhibitors block HPE-stimulated MAPK activation

PD98059 [24,25] specifically inhibits MEK/ERK signalling without inhibiting other MAPK pathways. THP-1 cells were treated with PD98059 (10 μM) for 30 min before treatment with HPE. Extracts were then analysed by Western blotting for phosphorylated ERK1/2. PD98059 blocked HPE-mediated ERK1/2 phosphorylation (Figure 2A). The effect of SB203580, which specifically inhibits p38 MAPK [26], was examined. Pre-treatment of cells with SB203580 (5 μM) caused inhibition of the phosphorylation of p38 MAPK (Figure 2B).

Role of LPS in HPE-mediated MAPK activation

By binding to lipid A, polymyxin B is a well-known inhibitor of LPS [27]. To determine whether the effect of HPE on MAPK activation was mediated by LPS, HPE was pre-incubated with polymyxin B (10 μg/ml) for 30 min at room temperature before adding these preparations to THP-1 cells. Pre-incubation with polymyxin B inhibited the ability of HPE to activate the MAPKs ERK1/2, p38 (Figure 2) and JNK (Figure 1G). HPE that was pre-incubated alone for 30 min at room temperature was able to activate these MAPKs. In order to confirm the role of the LPS receptor, THP-1 cells were treated with anti-CD14 antibody

before treatment with HPE. This resulted in the abrogation of phosphorylation of ERK1/2, p38 (Figure 2) and JNK (Figure 1F), mediated by HPE. These results indicate that the activation of MAPKs in macrophages by HPE is LPS-mediated. Since LPS is heat-stable, we tested the ability of heat-treated (100 °C for 15 min) HPE to activated MAPKs. Heat treatment did not affect HPE-mediated MAPK activation (results not shown).

HPE-induced ERK1/2 activation is mediated by MEK1

Cells were transfected with control vectors (pCMV, pcDNA 3.1) or dn-MEK2A or dn-MKK6. Cells were then treated with HPE and the phosphorylation status of ERK1/2 was evaluated. Although dn-MEK1 abrogated the HPE-induced phosphorylation of ERK1/2 (Figure 3A), dn-MKK6 had no effect on ERK1/2 phosphorylation, suggesting that HPE-mediated ERK1/2 activation occurs in a MEK-dependent manner.

HPE-induced p38 MAPK activation is induced by MKK6 and, to a lesser extent, MKK3

Having demonstrated the sustained activation of p38 MAPK by HPE, we investigated the involvement of upstream MKKs in the HPE-mediated activation of p38 MAPK. Cells were transfected with control vectors or dn forms of MEK1, MKK3, MKK4, or MKK6. Cells were then treated with HPE and activation of
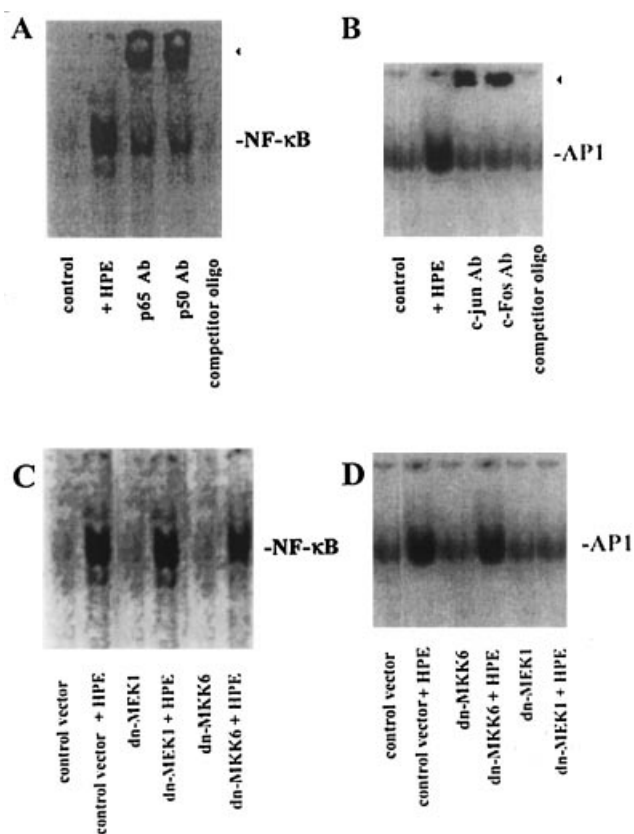
Figure 5   EMSA of NF-κB and AP-1 after treatment of THP-1 cells with HPE

Monolayers of THP-1 cells were cultured alone or with HPE from strain 26695. The nuclear extracts were subjected to EMSA using a labelled NF-κB (**A**) or AP-1 (**B**) DNA probe. Where indicated, the binding reaction was performed in the presence of a 1:10 surplus of unlabelled oligonucleotides (competitor) or supershift antibodies. The arrowheads indicate the positions of the supershifted bands. THP-1 cells transfected with vector alone, dn-MEK1 or dn-MKK6 were left untreated or treated with HPE from strain 26695 and EMSAs were carried out using labelled NF-κB (**C**) or AP-1 (**D**) DNA probes as described above.

p38 MAPK was assessed by evaluating the phosphorylation status of p38. Although dn-MKK6 strongly inhibited the ability of HPE to activate p38 MAPK (Figure 3B), dn-MKK3 had a smaller effect. MKK4 has also been reported to act as a p38 MKK; however, transfection of cells with dn-MEK1 or dn-MKK4 did not inhibit HPE-induced p38 MAPK activation (Figure 3B), suggesting that kinase activities of MKK6 and, to a lesser extent, MKK3 are necessary to induce p38 phosphorylation by HPE. The role of MKK6 in p38 MAPK activity was further supported by an *in vitro* kinase assay in which active p38 MAPK was precipitated using immobilized anti-phospho-p38 MAPK and its ability to phosphorylate ATF-2 fusion protein (a substrate of p38 MAPK) was assayed. HPE stimulated p38 MAPK activity, as assessed by phosphorylation of ATF-2 (Figure 1I). This was abrogated by transfection of cells with dn-MKK6.

HPE-induced JNK activation involves MKK7

Cells were transfected with dn-MKK7 before stimulation with HPE. JNK activity was inhibited by dn-MKK7 (Figure 1H), as assessed by a pull-down assay of activated JNK with immobilized
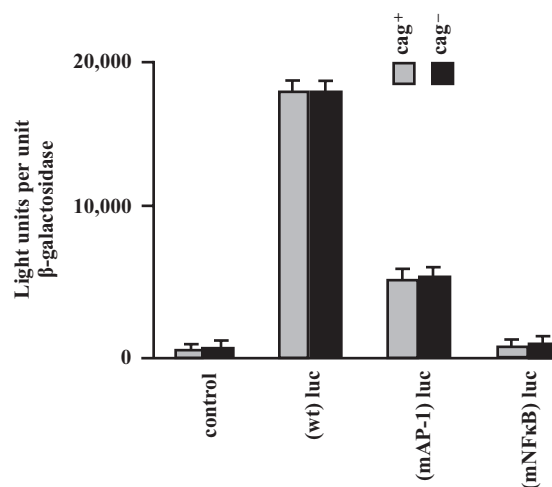
Figure 6   Stimulation of IL-8 promoter activity by HPE

THP-1 cells were transfected with control vector or with the wt IL-8 promoter construct [(wt)luc] as well as those containing mutations of the NF-κB [(mNF-κB)luc] or AP-1 [(mAP-1)luc] binding elements, along with β-galactosidase reporter plasmid. Cells were then treated with HPE prepared from *cag*$^-$ (80A) or *cag*$^+$ (26695) strains for 2 h. Bars represent the means $\pm$ S.D. for three determinations (with three different preparations of HPE) normalized for transfection efficiency by assaying β-galactosidase activity.

GST–c-Jun followed by an *in vitro* kinase assay with GST–c-Jun acting as substrate, suggesting that HPE signals along the MKK7/JNK pathway.

Induction of IL-8 release in THP-1 cells by HPE

MAPKs are known to regulate the upstream signalling events that control chemokine induction. We therefore hypothesized that stimulation of MAPKs by shed factors of *H. pylori* in THP-1 was involved in IL-8 induction. IL-8 release was activated by treatment of both PBMs (Figure 4A) and THP-1 cells (Figure 4B), with HPE from *cag*$^+$ and *cag*$^-$ strains; optimal IL-8 release occurring between 12 and 14 h of treatment (results not shown). To define the role of MAPKs in HPE-mediated IL-8 release in THP-1 cells, the effects of specific inhibitors on IL-8 release were evaluated. HPE-mediated IL-8 release was inhibited when cells were pre-treated for 30 min with either U0126, a MEK1 inhibitor [28], or SB203580, a specific inhibitor of p38 MAPK. Both these MAPK pathways therefore appear to be involved in HPE-mediated IL-8 release from THP-1 cells. These results were further supported by transfecting cells with wt or dn MEK1 and MKK6 (Figure 4C). The role of JNK signalling in IL-8 release was supported by the observation that transfection with dn-MKK7 abrogated HPE-mediated IL-8 release (Figure 4C).

NF-κB and AP-1 factors are bound to their IL-8-specific DNA elements in THP-1 cells treated with HPE

Expression of the IL-8 gene is primarily controlled at the transcriptional level. Nucleotide sequence analysis of the 5′ regulatory region of the IL-8 gene has shown the presence of binding sites for several transcription factors, including NF-κB and AP-1, in different types of cells, including THP-1 cells [29]. To determine whether the ERK1/2 and p38 pathways were acting at a transcriptional level in THP-1 cells exposed to HPE, the activation of the transcription factors NF-κB and AP-1 were

studied by EMSAs using oligonucleotides containing the IL-8-specific NF-κB and AP-1 DNA-binding elements. NF-κB was activated by treatment with HPE (Figure 5A). Competition with the non-labelled NF-κB DNA probe showed that the complexes were specific. Supershift of the band with anti-p65 or anti-p50 suggested that p65 and p50 are components of the protein complex that binds to the NF-κB element of the IL-8 promoter. In order to determine if the increased NF-κB is mediated through activation of the MEK1-ERK1/2 or the MKK6/p38 MAPK pathways, cells were transfected with dn-MEK1 or dn-MKK6 before treatment with HPE and EMSA. No change in NF-κB DNA binding was observed, discounting the involvement of these MAPK pathways in NF-κB DNA binding.

The DNA binding ability of AP-1 was also stimulated by HPE (Figure 5B). Competition with the non-labelled AP-1 DNA probe showed that the complexes were specific. Supershift of the band with anti-c-fos or anti-c-jun suggested that c-fos and c-jun are components of the protein complex that binds to the AP-1 element of the IL-8 promoter. In order to assess the role of ERK1/2 and p38 MAPKs in AP-1 DNA binding, cells were transfected with dn-MEK1 or dn-MKK6 before HPE-treatment. Dn-MEK1 abrogated HPE-driven AP-1 DNA binding, suggesting a direct role of ERK1/2 in this process. Since JNK is involved in c-jun synthesis, JNK activation by HPE probably exerts its effects at the level of c-jun. As dn-MKK6 had no effect, the involvement of p38 MAPK in AP-1 DNA binding was discounted.

HPE-induced IL-8 gene transcription through activation of NF-κB and AP-1

THP-1 cells were transfected with luciferase expression vectors either carrying the wt IL-8 promoter region, or with mutated NF-κB and AP-1 binding sequences. Following stimulation with HPE for 2 h, intracellular activities were determined by measurement of luciferase activities, normalized for transfection efficiency using β-galactosidase. A mutated NF-κB sequence completely abrogated luciferase expression, whereas a mutated AP-1 sequence partially abrogated luciferase expression (Figure 6). The effects were the same when using HPE derived from either a cag^- or a cag^+ strain.

DISCUSSION

The modulation of host macrophage cell signalling by *H. pylori* has not been studied in detail. Since MAPKs are involved in signalling, leading to the synthesis of pro-inflammatory cytokines and chemokines, we sought to investigate whether shed factors from *H. pylori* influence MAPK signalling. We observed that shed factors were sufficient to activate the MAPKs ERK1/2, p38 and JNK, and the *cag* PAI was not involved in the process; p38 MAPK activation was sustained. Inhibition of MAPK activation by both anti-CD14 antibody and polymyxin B suggested that *H. pylori* LPS is primarily responsible for the effects elicited by HPE. IL-8 is one of the chemokines that mediates gastric inflammation associated with *H. pylori* infection. The molecular mechanism by which *H. pylori* induces IL-8 secretion in cells of the monocytic lineage is poorly understood. We hypothesized that MAPK signalling could possibly be linked to IL-8 release triggered by *H. pylori*. We observed that the secretion of IL-8 that is mediated by *H. pylori* in PBMs and in THP-1 cells occurs in a contact-independent manner and is inhibited by transfections with dn mutants of the upstream kinases involved in ERK1/2, p38 and JNK phosphorylation. These results confirmed the role of MAPK signalling in HPE-mediated IL-8 release. In contrast,

IL-8 release from gastric epithelial cells requires contact of the host cells with the whole bacterium [30].

The IL-8 promoter contains three elements known to be involved in transcriptional up-regulation. The NF-κB element is believed to be the main regulator of inducible expression of the IL-8 gene. However, for certain signals, the expression of the IL-8 gene has been reported to depend on the activation of a second transcription factor, such as AP-1 [29]. By measuring IL-8 promoter-driven luciferase gene expression, we observed that HPE-enhanced IL-8 gene transcription was critically dependent on NF-κB and partly dependent on AP-1. Shed factors from *H. pylori* were sufficient to trigger activation of NF-κB and AP-1. Maeda et al. [31] have reported that NF-κB activation in THP-1 by *H. pylori* is independent of contact and *cag* PAI, but depends on CD14 and TLR4. Transfection with dn-MEK1 or dn-MKK6 suggested that neither the ERK1/2 nor the p38 MAPK pathway regulates NF-κB DNA binding stimulated by HPE. dn-MEK1 abolished AP-1 DNA, which suggests a link between ERK1/2 activation and AP-1 DNA binding. However, AP-1 DNA binding activated by HPE was not affected by transfection with dn-MKK6, which suggests that p38 MAPK does not have a role at the level of AP-1 DNA binding. Since NF-κB activation is probably the main regulator of HPE-mediated IL-8 gene expression, we speculate that p38 MAPK may exert its effect on NF-κB-driven IL-8 gene expression through regulation of basal transcription factors, such as TATA-binding protein; a role attributed to p38 MAPK in THP-1 [32]. A recent report has shown that FR167653, a potent p38 MAPK inhibitor, prevents *H. pylori*-induced gastritis in Mongolian gerbils [33]. The sustained activation of p38 MAPK in THP-1 cells by HPE and the marked inhibition of IL-8 secretion by transfection with dn-MKK6 argues in favour of a detailed investigation of the potential of MAPK inhibitors and, in particular, p38 MAPK inhibitors as anti-inflammatory agents in *H. pylori*-associated gastritis. This is the first report of the crucial role of shed factors of *H. pylori* in stimulation of MAPK signalling, leading to IL-8 release from human macrophages. *H. pylori* LPS is reportedly much less potent in eliciting cytokines or chemokines than LPS from *Salmonella enterica* or *Escherichia coli* [34,35]. However, our results corroborate the findings of Innocenti et al. [36] that IL-8 release from macrophages is stimulated in a contact-independent manner involving *H. pylori* LPS. This is unlike the effect of *H. pylori* on IL-8 induction from epithelial cells, which requires direct contact of the bacterial cell with the host cell [30].

Finally, our observations suggest that the recruitment of mononuclear cells into the gastric mucosa associated with the development of gastritis serves to amplify the induction of pro-inflammatory cytokines and chemokines such as IL-8, without contact with, or invasion by, the organism.

REFERENCES

1   Westblom, T. U., Czinn, S. J. and Nedrud, J. G. (1999) Gastroduodenal disease and *Helicobacter pylori*: pathophysiology, diagnosis and treatment. Curr. Top. Microbiol. Immunol. **241**, 301–308

2   Ben-Baruch, A., Michiel, D. F. and Oppenheim, J. J. (1995) Signals and receptors involved in recruitment of inflammatory cells. J. Biol. Chem. **270**, 11703–11706

3 Mukaida, N., Harada, A. and Matsushima, K. (1998) Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. Cytokine Growth Factor Rev. **9**, 9–23

4 Mulligan, M. S., Jones, M. L., Bolanowski, M. A., Baganoff, M. P., Deppeler, C. L., Meyers, D. M., Ryan, U. S. and Ward, P. A. (1993) Inhibition of lung inflammatory reactions in rats by an anti-human IL-8 antibody. J. Immunol. **150**, 5585–5595

5 Crabtree, J. E., Wyatt, J. I., Trejdosiewicz, L. K., Peichl, P., Nichols, P. H., Ramsay, N., Primrose, J. N. and Lindley, I. J. (1994) Interleukin-8 expression in *Helicobacter pylori* infected, normal, and neoplastic gastroduodenal mucosa. J. Clin. Pathol. **47**, 61–66

6 Sharma, S. A., Tummuru, M. K., Miller, G. G. and Blaser, M. J. (1995) Interleukin-8 response of gastric epithelial cell lines to *Helicobacter pylori* stimulation in vitro. Infect. Immun. **63**, 1681–1687

7 Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Rappuoli, R. and Covacci, A. (1996) *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc. Natl. Acad. Sci. U.S.A. **93**, 14648–14653

8 Crabtree, J. E., Covacci, A., Farmery, S. M., Xiang, Z., Tompkins, D. S., Perry, S., Lindley, I. J. and Rappuoli, R. (1995) *Helicobacter pylori* induced interleukin-8 expression in gastric epithelial cells is associated with CagA positive phenotype. J. Clin. Pathol. **48**, 41–45

9 Segal, E. D., Lange, C., Tompkins, L. S. and Falkow, S. (1997) Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. Proc. Natl. Acad. Sci. U.S.A. **93**, 14648–14653

10 Keates, A. C., Warny, M., Peek, R. M., Murray, P. G. and Kelly, C. P. (1999) Differential activation of mitogen-activated protein kinases in AGS gastric epithelial cells by *cag*$^+$ and *cag*$^-$ *Helicobacter pylori*. J. Immunol. **163**, 5552–5559

11 Ernst, P. B. and Gold, B. D. (2000) The disease spectrum of *Helicobacter pylori*: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. Annu. Rev. Microbiol. **54**, 615–640

12 Yoshida, N., Granger, D. N., Evans, Jr, D. J., Evans, D. J., Graham, D. Y., Anderson, D. C., Wolf, R. E. and Kvietys, P. R. (1993) Mechanisms involved in *Helicobacter pylori*-induced inflammation. Gastroenterology **105**, 1431–1440

13 Bliss, Jr, C. M., Golenbock, D. T., Keates, S., Linevsky, J. K. and Kelly, C. P. (1998) *Helicobacter pylori* lipopolysaccharide binds to CD14 and stimulates release of interleukin-8, epithelial neutrophil-activating peptide 78, and monocyte chemotactic protein 1 by human monocytes. Infect. Immun. **66**, 5357–5363

14 Kyriakis, J. M. and Avruch, J. (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol. Rev. **81**, 807–869

15 Karin, M., Liu, Z. and Zandi, E. (1997) AP-1 function and regulation. Curr. Opin. Cell Biol. **9**, 240–246

16 Ip, Y. T. and Davis, R. J. (1998) Signal transduction by the c-Jun N-terminal kinase (JNK) — from inflammation to development. Curr. Opin. Cell Biol. **10**, 205–219

17 Derijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Han, J., Ulevitch, R. J. and Davis, R. J. (1995) Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science **267**, 682–685

18 Lin, A., Minden, A., Martinetto, H., Claret, F. X., Lange-Carter, C., Mercurio, F., Johnson, G. L. and Karin, M. (1995) Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. Science **268**, 286–290

19 Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M. and Zon, L. I. (1994) Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature (London) **372**, 794–798

20 Winston, B. W., Remigio, L. K. and Riches, D. W. H. (1995) Preferential involvement of MEK1 in the tumor necrosis factor-induced activation of p42$^{MAPK/Erk2}$ in mouse macrophages. J. Biol. Chem. **270**, 27391–27394

21 Herlaar, E. and Brown, Z. (1999) p38 MAPK signalling cascades in inflammatory disease. Mol. Med. Today **5**, 439–447

22 Mukhopadhyay, A. K., Kersulyte, D., Jeong, J.-Y., Datta, S., Ito, Y., Chowdhury, A., Chowdhury, S., Santra, A., Bhattacharya, S. K., Azuma, T., Nair, G. B. and Berg, D. E. (2000) Distinctiveness of genotypes of *Helicobacter pylori* in Calcutta, India. J. Bacteriol. **182**, 3219–3227

23 Wu, G. D., Lai, E. J., Huang, N. and Wen, X. (1997) Oct-1 and CCAAT/Enhancer-binding protein bind to overlapping elements within the interleukin-8 promoter. J. Biol. Chem. **272**, 2396–2403

24 Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J. and Saltiel, A. R. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. U.S.A. **92**, 7686–7689

25 Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995) PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. J. Biol. Chem. **270**, 27489–27494

26 Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W., Strickler, J. E., McLaughlin, M. M., Siemens, I. R., Fisher, S. M., Livi, G. P., White, J. R., Adams, J. L. and Young, P. R. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature (London) **372**, 739–746

27 Morrison, D. C. and Kline, L. F. (1977) Activation of the classical and properidin pathway of complement by bacterial lipopolysaccharide (LPS). J. Immunol. **118**, 362–369

28 Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F., Copeland, R. A., Magolda, R. L., Scherle, P. A. and Trzaskos, J. M. (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J. Biol. Chem. **273**, 18623–18632

29 Murayama, T., Ohara, Y., Obuchi, M., Khabar, K. S. A., Higashi, H., Mukaida, N. and Matsushima, K. (1997) Human cytomegalovirus induces interleukin-8 production by a human monocytic cell line, THP-1, through acting concurrently on AP-1- and NF-κB-binding sites of the interleukin-8 gene. J. Virol. **71**, 5692–5695

30 Rieder, G., Hatz, R. A., Moran, A. P., Walz, A., Stolte, M. and Enders, G. (1997) Role of adherence in interleukin-8 induction in *Helicobacter pylori*-associated gastritis. Infect. Immun. **65**, 3622–3630

31 Maeda, S., Akanuma, M., Mitsuno, Y., Hirata, Y., Ogura, K., Yoshida, H., Shiratori, Y. and Omata, M. (2001) Distinct mechanism of *Helicobacter pylori*-mediated NF-κB activation between gastric cancer cells and monocytic cells. J. Biol. Chem. **276**, 44856–44864

32 Carter, A. B., Knudtson, K. L., Monick, M. M. and Hunninghake, G. W. (1999) The p38 mitogen-activated protein kinase is required for NF-κB-dependent gene expression. The role of TATA-binding protein (TBP) J. Biol. Chem. **274**, 30858–30863

33 Takahashi, S., Keto, Y., Fujita, T., Uchiyama, T. and Yamamoto, A. (2001) FR167653, a p38 mitogen-activated protein kinase inhibitor, prevents *Helicobacter pylori*-induced gastritis in Mongolian gerbils. J. Pharmacol. Exp. Ther. **296**, 48–56

34 Muotiala, A., Helander, J. M., Pyhala, L., Kosunen, T. U. and Moran, A. (1992) Low biological activity of *Helicobacter pylori* lipopolysaccharide. Infect. Immun. **60**, 1714–1716

35 Perez-Perez, G. I., Shepard, V. L., Marrow, J. D. and Blaser, M. J. (1995) Activation of human THP-1 cells by *Helicobacter pylori* lipopolysaccharide. Infect. Immun. **63**, 1183–1187

36 Innocenti, M., Svennerholm, A. M. and Quiding-Jarbrink, M. (2001) *Helicobacter pylori* lipopolysaccharides preferentially induce CXC chemokine production in human monocytes. Infect. Immun. **69**, 3800–3808