Helicobacter pylori heat-shock protein 60 induces interleukin-8 via a Toll-like receptor (TLR)2 and mitogen-activated protein (MAP) kinase pathway in human monocytes

Ying Zhao, Kenji Yokota, Kiyoshi Ayada, Yumiko Yamamoto, Tomayuki Okada, Lianhua Shen and Keiji Oguma

Department of Bacteriology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Graduate School of Health Science, Okayama University, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

INTRODUCTION

Helicobacter pylori is an important human pathogen that causes gastritis and is strongly associated with peptic ulcer, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma. H. pylori has been found in people worldwide, with over half of the world’s population infected (Mann & Westblom, 1999). One remarkable feature of the infection is that it persists throughout the life of the host in the face of a significant inflammatory response. Only a few carriers suffer peptic ulceration and gastric cancer, because the host immune response may play important roles in clinical outcome; e.g. inflammatory processes might take place within the gastric mucosa via activation of monocytes/macrophages as antigen-presenting cells. Macrophage-derived cytokine production is strongly upregulated during H. pylori infection in non-human primates (Harris et al., 2000). These innate and adaptive responses are principally characterized by increased infiltration of lymphocytes and macrophages into infected gastric tissues (Sarsfield et al., 1996; Suzuki et al., 2002).

A recent study has shown that the 60 kDa heat-shock protein (HSP) HSP60, a potent immune antigen of H. pylori, induces interleukin (IL)-6 secretion from mouse monocytes (Gobert et al., 2004). Not only monocytes/macrophages but also neutrophils have an important role during chronic infection in the early and active stages of inflammation. Neutrophils are directly activated by neutrophil-activating protein (NAP) from the bacteria (Brisslert et al., 2005; Evans et al., 1995). In addition, IL-8 is associated with this phenomenon as a powerful neutrophil chemoattractant. IL-8 is secreted from epithelial cells by bacterial virulence factors such as the cag pathogenicity island (cagPAI) and OipA (Crabtree et al., 1995; Yamaoka et al., 2000). We have previously reported that H. pylori-HSP60 may induce IL-8

Abbreviations: ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; H. pylori-HSP60, Helicobacter pylori heat-shock protein 60; HSP, heat-shock protein; IL, interleukin; JNK, c-Jun N-terminal kinase; LBP, LPS-binding protein; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; rHpHSP60, recombinant H. pylori HSP60; siRNA, small interfering RNA; TLR, Toll-like receptor.
secretion from gastric epithelial cells (Takenaka et al., 2004). In addition, monocytes also secrete IL-8, and this reaction is inhibited by anti-H. pylori-HSP60 mAbs (Lin et al., 2005). These results indicate that the interaction between monocytes and HSP60 may play an important role in gastric inflammation in H. pylori infection.

The mitogen-activated protein kinases (MAPKs) are an important group of serine and threonine signalling kinases that transduce a variety of extracellular stimuli through a cascade of protein phosphorylations, leading to transcription factor activation (Krykiakis & Avruch, 2001). Cag+ H. pylori can induce IL-8 production via the activation of ERK, p38 and c-Jun N-terminal kinase (JNK) MAPKs in gastric epithelial cells (Keates et al., 1999). The role of MAPK in regulating nuclear factor-κB (NF-κB) transactivation remains controversial. Aihara et al. (1997) have shown that both the NF-κB and activating protein 1 (AP-1) DNA binding sites within the IL-8 promoter are required for optimal transcription in response to gastric epithelial cells by H. pylori. MAPK signalling pathways activate NF-κB and/or AP-1 and can result in increased IL-8 secretion from human monocytes in H. pylori infection (Bhattacharyya et al., 2002). Toll-like receptors (TLRs) are a key component of the host innate recognition system against bacterial components (Zhang et al., 2004). TLR2, TLR4 and TLR5 are closely associated with cytokine production and activation of signalling pathways in H. pylori infection (Kawahara et al., 2001; Ishihara et al., 2004; Smith et al., 2003; Takenaka et al., 2004).

Based on these findings, we inferred that the mechanism of IL-8 secretion induced by interaction between HSP60 and monocytes may be important for gastric inflammation. In this study, activation of ERK1/2, p38 and JNK of the MAPK signalling pathway was assessed when stimulated with intact H. pylori, heat-killed H. pylori or recombinant H. pylori HSP60 (rHpHSP60). In addition, we also investigated whether TLR2 is involved in MAPK signalling and NF-κB-mediated activity of IL-8 secretion from rHpHSP60-infected monocytes. Thus, our goal was to describe how HSP60, as a potent H. pylori immune antigen, is responsible for the induction of IL-8 in human monocytes.

**METHODS**

**Bacterial strains.** H. pylori standard strain ATCC 43504 (CagA+, VacA+), obtained from the American Type Culture Collection (ATCC), was used. This strain is commonly used for animal experiments (Yokota et al., 1991; Iwao et al., 1999). Before each experiment, H. pylori was cultured on brain heart infusion agar supplemented with 7% sterile defibrinated horse blood at 37°C in a microaerophilic chamber containing 10% CO2, 5% O2 and 85% N2. After 3 days of incubation, H. pylori was harvested into RPMI1640 (Gibco) without fetal bovine serum (FBS) to an OD600 of 1.0, corresponding to approximately 1 x 10^9 c.f.u. ml^-1. Heat-killed H. pylori was prepared by heating in a 60°C water bath for 30 min.

**Cell culture.** The human monocytic cell lines NOMO1 and U937 were obtained from the Japanese Collection of Research Bioresource (Tokyo, Japan). Both were maintained in RPMI 1640 supplemented with 10% FBS, 50 IU penicillin ml^-1, 50 μg streptomycin ml^-1 (ICN Biomedical) and 2.5 μg amphotericin B ml^-1 (ICN Biomedical) at 37°C in an atmosphere of 5% CO2 and 99% humidity. Cells were washed three times by RPMI1640 without antibiotics before each experiment and used at a final concentration of 1 x 10^5 - 3 x 10^5 cells ml^-1. Cytotoxic effect was determined by the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega), after cells had been cultured with H. pylori, rHpHSP60, several inhibitors and small interfering RNA (siRNA). No mediation of cytotoxicity by these antigens or agents was detected at the concentrations and incubation times employed during this work.

**rHpHSP60 preparation.** rHpHSP60 was prepared as described by Ishii et al. (2001). In brief, the ORF of HSP60 taken from H. pylori ATCC 43504 genomic DNA was amplified and cloned into the vector pGEX-5X3 (Amersham Biosciences). The resultant plasmid was transformed into Escherichia coli DH5a. Cells were cultured in Luria–Bertani broth containing 2% glucose and 100 μg ampicillin ml^-1, harvested by centrifugation, and disrupted by sonication on ice for 5 min with a probe sonicator (Astron). Soluble fusion protein expressed by the HSP60 clone was purified by glutathione–Sepharose 4B affinity chromatography (Amersham Biosciences) according to the manufacturer’s instructions. The purity of the proteins was determined by SDS-PAGE using a 12% running gel, and a 60 kDa protein band was revealed by Coomassie brilliant blue staining. That the 60 kDa protein belonged to H. pylori was confirmed by amino acid sequence analysis. The concentration (mg ml^-1) of the purified protein was determined using the Bio-Rad protein assay kit (based on the Lowry assay), with A655 measured with a microplate reader (Bio-Rad model 680). To exclude the effects of trace amounts of LPS in the recombinant product, a Limulus amoebocyte lysate assay kit (BioWhittaker) was used according to the manufacturer’s instructions. The preparations of rHpHSP60 contained <30 endotoxin units (EU) (mg protein)^-1 (Takenaka et al., 2004; Lin et al., 2005).

**IL-8 secretion from cells.** NOMO1 or U937 cells grown on 96-well plates were stimulated with intact, heat-killed H. pylori, rHpHSP60, glutathione S-transferase (GST), or LPS from Salmonella enteriditis (Sigma) at 37°C in serum-free RPMI1640 for the specified time and at the specified concentration. For inhibition by blocking cell surface molecules, cells were pre-incubated for 30 min with 10 μg ml^-1 anti-TLR2 antibodies (HyCult Biotecnology), 10 μg ml^-1 anti-TLR4 antibodies (Sanbio), 10 μg ml^-1 anti-IL-1 antibodies (Rockland) and 50 μg ml^-1 anti-CD14 antibodies (R&D Systems). For inhibition of MAPK signalling pathways, PD98059 (Promega) and U0126 (Cell Signalling Technology) for ERK1/2 signalling, and SB203580 (Promega) for p38 signalling, were added at the indicated concentrations for 30 min at 37°C before treatment with rHpHSP60. Supernatants and cells were separated by centrifugation at 300 g and stored at ~80°C until assayed. Supernatants were assayed for IL-8 production, and IL-8 was measured by ELISA (IL-8 ELISA development kit, Techne).

**Detection of MAPK phosphorylation.** NOMO1 cells were stimulated with bacterial cells or rHpHSP60, and the cells were harvested by centrifugation. After washing in PBS/phosphatase inhibitor buffer [PIB; 125 mM NaF, 250 mM β-glycerophosphate, 250 mM p-nitrophenyolphosphate (PNPP) and 25 mM NaVO₃], cells were lysed in 50 μl lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM PMSF, 1 mM NaN₃, 1 mM NaF, 1 μg leupeptin ml^-1, 1 μg aprotinin ml^-1 and 1 μg pepstatin A ml^-1). After incubation on ice for 20 min, cells were sonicated, and whole-cell lysates were collected by centrifugation at 10 000 g for 5 min. Cell lysates were mixed with equal volumes of 2× SDS sample buffer and boiled for 5 min. Cell lysates were subjected to SDS-PAGE using 14% running gel. The proteins were then transferred to PVDF membranes (Millipore). The
membranes were washed with Tris-buffered saline (TBS) and blocked with 5% (w/v) non-fat milk in TBS/Tween 20 (1%, v/v) (TBST) for 1 h at room temperature. After three washes of 5 min each with TBST, the membranes were incubated with antibodies to ERK, phospho-ERK, p38 or phospho-p38 MAPK (Cell Signalling Technology) in TBST with 5% (w/v) BSA overnight at 4°C. Following three washes with TBST, the blots were incubated with an anti-rabbit IgG HRP-linked antibody (Wako) for 1 h at room temperature. Bands were visualized by ECL Western Blotting detection reagents (Amersham Pharmacia) and exposure to an LAS-1000 mini Bio-Imaging Analysy System (Fujiﬁlm). The phospho-ERK or phospho-p38 intensities of the bands were analysed with Image Gauge version 4.0 software (Fujiﬁlm) and normalized against ERK or p38 in the corresponding samples.

**Nuclear extract and NF-κB transcription factor assay.** After incubation with an antibody to p65 (RelA), a subunit of the NF-κB dimer, a horseradish peroxidase-conjugated secondary antibody was added, followed by developing solution including MN a 2MoO4 and 0.1 mM glucose-6-phosphate dehydrogenase (G6PDH) primer mix (Roche Diagnostics). The absorbance was read at 450 nm with a reference wavelength of 720 nm, and the absorbance ratio with a Beckman DU-7000. The RNA was extracted from monocytes was assayed, and maximum production from monocytes was assessed by flow cytometry from 72 h post-transfection. Samples were then mixed with anti-TLR2 antibodies or mouse IgG1κ (Sigma), which served as a control, for 1 h and labelled with bovine anti-mouse IgG–phycoerythrin (PE) (Santa Cruz) for 30 min. All staining procedures were completed at 4°C in PBS containing 0.02% azide and 0.2% BSA. Samples were then analysed using ELISA after an additional 3 h. Knockdown of TLR2 after transfection was confirmed by flow cytometry from 72 h post-transfection.

**Flow cytometry analysis.** TLR2 expression on the surface of NOMO1 cells was determined by flow cytometry. Cells were stained with anti-TLR2 antibodies or mouse IgG1κ (Sigma), which served as a control, for 1 h and labelled with bovine anti-mouse IgG–phycoerythrin (PE) (Santa Cruz) for 30 min. All staining procedures were completed at 4°C in PBS containing 0.02% azide and 0.2% BSA. Samples were then analysed using ELISA after an additional 3 h. Knockdown of TLR2 after transfection was confirmed by flow cytometry from 72 h post-transfection.

**Statistical analysis.** Results were expressed as mean ± SD. Data were compared using Student’s t test, and differences were considered significant at P values of less than 0.05.

**RESULTS**

**Intact H. pylori, heat-killed H. pylori and rHpHSP60 induced MAPK activation in human monocytes**

The MAPK pathway has been implicated in the induction of cytokines in human monocytes via bacterial stimulation. We thus assessed the role of MAPK in the secretion of IL-8 by H. pylori-HSP60 stimulation. The time-course of IL-8 production from monocytes was assayed, and maximum production by intact or heat-killed H. pylori-stimulated NOMO1 cells appeared at 6 h; rHpHSP60 induction of IL-8 secretion reached its maximum at 3 h (Fig. 1a). In U937 cells, treatment with intact H. pylori, heat-killed H. pylori or rHpHSP60 for 24 h resulted in peak IL-8 production (Fig. 1b). The time-dependent MAPK phosphorylation was measured to assess the activation of these kinases upon treatment with intact H. pylori, heat-killed H. pylori and 10 min to activate the modified Taq polymerase, followed by 45 cycles of 95°C for 10 s, 68°C for 10 s and 72°C for 16 s, and one cycle of 55°C for 10 s and 40°C for 30 s. The specificity of the PCR product was checked by the melting curve, which showed that the melting temperature was in a very narrow range from 84 to 85°C for IL-8, and 90 to 91°C for G6PDH. The data were analysed by LightCycler software version 3.5 (Roche Diagnostics), and the ratio of IL-8 to G6PDH was calculated.
Fig. 1. Intact *H. pylori*, heat-killed *H. pylori*, and rHpHSP60 induce IL-8 production and stimulate ERK and p38 MAPK activation in a time-dependent manner in monocytes. (a) NOMO1 cells (2 × 10⁶ ml⁻¹) or (b) U937 cells (2 × 10⁶ ml⁻¹) were co-cultured with intact *H. pylori* (1 × 10⁷ cells ml⁻¹), heat-killed *H. pylori* (1 × 10⁷ cells ml⁻¹) or rHpHSP60 (100 μg ml⁻¹) for 1–24 h. The cell supernatants were measured for IL-8 release. Data are expressed as mean ± SD (n=3). (c) NOMO1 cell lysates were analysed by Western blotting using antibodies to phospho-ERK (p-ERK), phospho-p38 (p-p38) or phospho-JNK (p-JNK) at the indicated time points. Antibodies to unphosphorylated ERK, p38 or JNK served as protein loading controls. Image Gauge software was used to calculate relative units to indicate the fold difference between stimulated and non-stimulated cells after normalizing against ERK or p38. (d) NOMO1 cells were stimulated with rHpHSP60 (100 μg ml⁻¹), GST and LPS for 16 h at the indicated concentrations. The cell supernatants were measured for IL-8 release. Data are expressed as mean ± SD (n=3).
rHpHSP60 in NOMO1 cells (Fig. 1c). ERK activation was observed at 3 h, and peaked at 9, 24 and 6 h by stimulation with intact \textit{H. pylori}, heat-killed \textit{H. pylori} and rHpHSP60, respectively. p38 activation by intact \textit{H. pylori} stimulation was observed at 1 h and peaked at 3 h, while activation by heat-killed \textit{H. pylori} and rHpHSP60 peaked at 1 h. However, activation persisted under all treatments for longer periods of up to 24 h. JNK activation was not observed as a result of any stimulation (Fig. 1c). To determine the effects of GST and LPS on IL-8 secretion from NOMO1 cells, GST or LPS at different concentrations was used to treat NOMO1 cells. No effect of GST stimulation was detected, but it was found that LPS induced IL-8 secretion in a dose-dependent manner (Fig. 1d).

IL-8 production and MAPK activation were also found to be dose dependent. IL-8 production from NOMO1 was higher than that from U937 cells for a given number of bacteria. The peak of IL-8 secretion from NOMO1 and U937 appeared at stimulation with $5 \times 10^7$ cells ml$^{-1}$ intact

**Fig. 2.** Intact \textit{H. pylori}, heat-killed \textit{H. pylori} and rHpHSP60 induce IL-8 production and stimulate ERK and p38 MAPK activation in a dose-dependent manner in monocytes. (a) Cells ($2 \times 10^6$ ml$^{-1}$) were treated with intact or heat-killed \textit{H. pylori} for 9 h with the indicated numbers of bacteria. (b) Cells ($2 \times 10^6$ ml$^{-1}$) were treated with rHpHSP60 for 9 h with the indicated concentrations. IL-8 release was measured from the cell supernatants. Data are expressed as mean $\pm$ SD ($n=3$). (c) NOMO1 cell lysates were analysed by Western blotting using antibodies to either phospho-ERK (p-ERK) or phospho-p38 (p-p38) at the indicated concentrations. Antibodies to unphosphorylated ERK or p38 served as protein loading controls. Image Gauge software was used to calculate relative units to indicate the fold difference between stimulated and non-stimulated cells after normalizing against ERK or p38.
H. pylori and 1 × 10⁸ cells ml⁻¹ heat-killed H. pylori. Moreover, the highest concentration of intact H. pylori seemed to influence monocytes; IL-8 secretion declined with stimulation by 1 × 10⁸ cells ml⁻¹ intact H. pylori (Fig. 2a). rHpHSP60 induced IL-8 secretion in a dose-dependent manner from NOMO1 and U937 cells (Fig. 2b). ERK activation increased to an obvious extent with stimulation by 1 × 10⁶ cells ml⁻¹ of both intact and heat-killed H. pylori, and p38 activation induced by rHpHSP60 increased at 5 μg ml⁻¹ (Fig. 2c). These results indicate that intact H. pylori, heat-killed H. pylori and rHpHSP60 induced both IL-8 secretion and ERK and p38 MAPK activation in human monocytes. NOMO1 cells were used in the subsequent experiment because IL-8 production and MAPK activation in NOMO1 cells were comparatively potent.

**Inhibition of rHpHSP60-induced IL-8 production from monocytes**

To identify whether MAPK signalling is responsible for IL-8 secretion, the effects of PD98059, U0126 and SB203580 on ERK and p38 activation were investigated with rHpHSP60 stimulation in NOMO1 cells. ERK1/2-specific inhibitors (PD98059 and U0126) or a p38-specific inhibitor (SB203580) were applied before rHpHSP60 stimulation. PD98059 and U0126 inhibited ERK phosphorylation (Fig. 3a, left), and SB203580 inhibited p38 phosphorylation (Fig. 3a, right). IL-8 production stimulated by rHpHSP60 was also significantly decreased by these specific inhibitors of ERK1/2 and p38 in a dose-dependent manner in NOMO1 cells (Fig. 3b). The results indicated the involvement of ERK and p38 activation in rHpHSP60-induced IL-8 secretion.

Next, we investigated whether cell surface molecules were associated with IL-8 secretion from monocytes. Anti-TLR2, -TLR4, -CD14 and -IL-1 receptor antibodies were employed to pre-treat NOMO1 cells before rHpHSP60 stimulation. Blocking of TLR2 significantly reduced IL-8 secretion from NOMO1 cells; however, treatment with anti-TLR4, -CD14 and -IL-1 receptor antibodies did not affect IL-8 secretion (Fig. 4a). Blocking of TLR2 also inhibited ERK and p38 MAPK activation (Fig. 4b). In addition, to demonstrate that TLR2 participates in the response to rHpHSP60-mediated IL-8 secretion, we used siRNA targeting TLR2 in human monocytes. The knockdown effect was quantified by flow cytometry. The siRNA treatment decreased TLR2 expression by 50 % in the cells compared with the results for negative-control siRNA (Fig. 4c). Cells with TLR2 knockdown (50 %) exhibited a 23 % decrease in IL-8 secretion in response to the TLR2 agonists in rHpHSP60-stimulated NOMO1 cells, compared with production in cells with negative-control siRNA knockdown (Fig. 4d). These results suggest that in human monocytes, TLR2 is involved in IL-8 secretion via activated ERK and p38 MAPK after rHpHSP60 stimulation.

**NF-κB activation and IL-8 mRNA expression via TLR2, ERK and p38 MAPK in NOMO1 cells reacted with rHpHSP60**

The transcription factor NF-κB is a critical regulator of genes involved in inflammation, and it can be activated by intact H. pylori in monocytic/macrophage cells (Maeda et al., 2001). Our experiments showed that an increase in
intranuclear NF-κB was found 10 min after rHpHSP60 stimulation, reaching its maximum level at 1 h after treatment (Fig. 5a). This reaction was significantly inhibited by pretreatment with SB203580, U0126 and anti-TLR2 antibody (Fig. 5b). These results showed that NF-κB may play an important role in response to rHpHSP60-induced IL-8 secretion via ERK and p38 binding in the TLR2 pathway. We have previously reported that rHpHSP60-induced IL-8 mRNA expression reaches its maximum at 1 h in U937 cells (Lin et al., 2005). In the present study, we
investigated IL-8 mRNA expression in NOMO1 cells by rHpHSP60 stimulation. The reaction reached its peak at 3 h (Fig. 5c), and was partially inhibited by the effect of PD98059, SB203580 and anti-TLR2 antibody (Fig. 5d).

**DISCUSSION**

HSPs, also called stress proteins, are a group of proteins present in both prokaryotic and eukaryotic cells. They are induced when a cell undergoes various types of environmental stress. HSPs trigger the immune response through activities that occur both inside (intracellular) and outside the cell (extracellular). Extracellular HSPs are the most powerful ways of sending a ‘danger signal’ to the immune system in order to generate a response that can help the organism manage an infection or disease. HSPs have also been reported that are closely associated with the innate or adaptive immune systems (Ellis, 1990; Young, 1990). Bacterial HSPs have also been reported to have the capability to activate human monocytes and macrophages. HSPs from *E. coli* (GroES and DnaK) induce tumour necrosis factor alpha (TNF-α), IL-6 and granulocyte-monocyte colony-stimulating factor in monocytes (Galdiero et al., 1997). Chlamydial HSP60 enhances adhesion molecules on endothelial cells and induces proinflammatory cytokine IL-6 from macrophages (Bulut et al., 2002; Kol et al., 1998, 1999). Release of TNF-α and IL-1β by human mononuclear phagocytes in response to mycobacterial HSP65 indicates that the protein contributes to both host defence and tissue damage. HSP65 may play a role in the initiation of inflammation that adds to non-species-specific resistance in the early stages of infection (Peetermans et al., 1994, 1995). Mycobacterial HSP65 may also be important in the host immune response and in the development of antigen-specific T-cell-mediated immunity (Friedland et al., 1993). *Legionella* HSP60 also possesses these activities (Retzlaff et al., 1994). These reports indicate that contact between bacterial HSP and human monocytes/macrophages is closely associated with innate or adaptive immune responses to bacterial infection. In this study, we have furthermore suggested that *H. pylori*-HSP60 mediates IL-8 production from human monocytes. The current results indicate that HSP60 may be an important molecule for the IL-8-inducing system in *H. pylori* infection.

The MAPK pathway is an important signal-transduction cascade and may involve the development of inflammation and/or cancer in *H. pylori*-infected mucosa. The MAPK pathway is activated by interaction between *H. pylori* and gastric epithelial cells via a type IV secretion system with the cag pathogenicity island (cagPAI) (Juttner et al., 2003; Shibata et al., 2005). Bhattacharyya et al. (2002) have reported that ERK, p38 and JNK regulate *H. pylori*-mediated...
IL-8 release from macrophages. Recently, the same group has found that IL-1β release induced by H. pylori LPS is associated with the PI-3/Akt/p38 MAPK pathway in macrophages (Basak et al., 2005). In this study, we demonstrated activation of ERK and p38 MAPK by rHpHSP60 as well as by intact H. pylori and heat-killed H. pylori stimulation; we think that H. pylori-HSP60 may play an important role in the activation of ERK and p38 MAPK, because HSP60 may be expressed on the bacterial surface. However, we did not identify activation of JNK by rHpHSP60. In addition, we found that IL-8 production was partially inhibited by ERK- and p38 MAPK-specific inhibitors. MAPK is a complex system involving many pathways; therefore, other pathways may be associated with activation of NF-κB (Fig. 6). Additional studies examining this possibility are needed.

In the innate immune response, HSPs engage signalling receptors that trigger NF-κB activation and cytokine or chemokine release by monocytes/macrophages (Binder et al., 2004). Exogenous HSP70 stimulates cytokine production through a CD14-dependent pathway in HEK293 cells (Asea et al., 2002). CD14 is also a receptor for the LPS and LPS-binding protein (LBP) complex of H. pylori (Cunningham et al., 2000). The IL-1 receptor induces an increase in the expression of many genes with roles in immunity and inflammation by activating NF-κB and p38 MAPK (O’Neill, 2000). HSP70, as an endogenous stimulus for the Toll/IL-1 receptor signal pathway, engages TLR2 and TLR4 (Vabulas et al., 2002). H. pylori activates NF-κB through CD14 and IL-1 receptor-associated kinase (IRAK) in monocytes (Maeda et al., 2001). We have previously reported TLR2 involvement in H. pylori-HSP60-mediated IL-8 secretion in human gastric epithelial cells (Takenaka et al., 2004). In the current study, both anti-TLR2 antibody and siRNA were found to have potentially broad applications in the study of pathogens and TLR2 (Wang et al., 2005; Chen et al., 2006). To determine whether IL-8 secretion decreased in the absence of TLR2, we used siRNA to inhibit TLR2 and found that HSP60-mediated IL-8 secretion was partially suppressed. Anti-TLR4, -CD14 and -IL-1 receptor antibodies did not inhibit the IL-8 secretion in this study, indicating that TLR4, CD14 and IL-1 receptors may not be involved in IL-8 secretion by rHpHSP60 stimulation. It was also shown that there was no contamination in rHpHSP60 preparations. These results demonstrated that not only TLR2 but also another receptor, although not TLR4, CD14 or the IL-1 receptor, may be involved in IL-8 secretion induced by H. pylori-HSP60 (Fig. 6).

The relationship between TLRs and H. pylori infection has been reported in many papers. H. pylori induces NF-κB activation in epithelial cells primarily via TLR2 and TLR5 (Smith et al., 2003). TLR5 or TLR2 can mediate H. pylori-induced IL-8 secretion via p38 MAPK signalling (Torok et al., 2005). H. pylori LPS stimulates TLR4 on gastric epithelial cells and activates NF-κB and MOX1 (mitogen oxidase 1), which are associated with gastritis (Kawahara et al., 2001). Ishihara et al. (2004) have also demonstrated that LPS upregulates the host innate immunity through activation of the TLR–MD2 system in the stomach. We have also reported that H. pylori-HSP60 is recognized by TLR2 on gastric epithelial cells and is associated with gastritis (Takenaka et al., 2004). TLR5 can recognize bacterial flagellin to mediate IL-8 secretion, but H. pylori flagellin evades TLR5-mediated innate immunity (Gewirtz et al., 2004). Recently, Cabral et al. (2006) have reported that the stimulation of TLR2 causes down-regulation of TLR5. Stimulation of TLR4 by LPS might enhance expression of TLR2 (Dr Keniti Amano, Akita University, personal communication). All this implies that the interaction mechanisms of TLR expression may be more complicated than we can imagine. Which TLRs play the main role in gastric mucosa infected by H. pylori is still obscure. This study indicates that TLR2 and H pylori-HSP60 may be
primarily associated with the induction of IL-8 secretion in human monocytes.

Both LPS and HSP60 exist on the surface of *H. pylori*, and these antigens may stimulate the immune cells. The immune activity of LPS differs among various bacteria, and *H. pylori* LPS is 1000 times less effective than that of *E. coli* in inducing cytokine production in monocytes (Bliss et al., 1998). *H. pylori* LPS binds to LBP, and thus CD14-dependent cell activity is lower (Cunningham et al., 1996). The immune activity of HSP60 also differs among various bacteria. HSP60s have been compared for their ability to stimulate human peripheral blood mononuclear cell (PBMC) cytokine synthesis and vascular endothelial cell adhesion protein expression. In spite of their significant sequence homology, *H. pylori*-HSP60 is a more potent PBMC activator than *Chlamydia pneumoniae* and human HSP60 (Maguire et al., 2005). It is possible that LPS and HSP60 are associated with the immune response in *H. pylori* infection, but we still do not know whether one plays a major role or both are associated.

In this study, we focused on the downstream pathway of *H. pylori*-HSP60-induced IL-8 secretion in human monocytic cell lines. We have demonstrated that *H. pylori*-HSP60 induces the secretion of the inflammatory cytokine IL-8 via the ERK and p38 MAPK signalling pathways. In addition, we have found that TLR2, a recognition receptor for *H. pylori*-HSP60, likely plays an important role by initiating the ERK and p38 MAPK cascades in response to IL-8 secretion. TLR2, ERK and the p38 MAPK signal-transduction pathways lead to the activation of NF-kB and expression of IL-8 mRNA. These findings support the idea that *H. pylori*-HSP60 plays an important role in stimulating production of pro-inflammatory cytokines, such as IL-8, in the innate immune response.

**REFERENCES**


