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Mechanism for *Helicobacter pylori* stimulation of interleukin-8 production in a gastric epithelial cell line (MKN 28): roles of mitogen-activated protein kinase and interleukin-1β

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Abstract

Although it is known that the pathogenic mechanism of *Helicobacter pylori* involves the stimulated production of interleukin-8 (IL-8) as an inflammatory mediator, the details of the pathway remain unclear. The role of mitogen-activated protein kinase (MAPK) in IL-8 production by *H. pylori* has been examined in an *in vitro* study. IL-8 mRNA expression in gastric epithelial cells (MKN 28) was determined by reverse transcriptase–polymerase chain reaction (RT–PCR). IL-8 production was examined by ELISA. The activation of p38 MAPK was assessed by western blotting. Neither IL-8 mRNA nor activated p38 MAPK or p44/42 MAPK was detected in cells not treated with *H. pylori*. In contrast, incubation of cells with *H. pylori*, or IL-1β, or both, clearly stimulated the expression of IL-8 mRNA within 60 min in a concentration-dependent manner. Phosphorylation of p38 MAPK and p44/p42 MAPK, as well as IL-8 production, occurred within 30 min and 24 hr after co-culturing MKN 28 cells with *H. pylori* and IL-1β, respectively. Pretreatment of cells with MAPK inhibitors [1-[7-(4-fluorophenyl)-1,2,3,4-tetra-hydro-8-pyridylpyrazolo[5,1-*c*][1,2,4]triazin-2-yl]-2-phenylethanedione sulfate monohydrate (FR167653), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole (SB203580), or 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059)] significantly inhibited IL-8 production stimulated by *H. pylori* or IL-1β or both. The combination of *H. pylori* and IL-1β additively stimulated IL-8 production. The additive effect of *H. pylori* and IL-1β on IL-8 production was inhibited by treatment with a p38 MAPK inhibitor. It was revealed that the culturing of MKN 28 cells with *H. pylori* significantly stimulates IL-8 production to a degree sufficient for induction of neutrophil chemotaxis via activation of p38 and p44/42 MAPK.

Keywords: H. pylori; IL-1β; IL-8; MAPK; MKN 28 cells

1. Introduction

Helicobacter pylori is recognized as an important risk factor for gastritis, peptic ulcer diseases, and gastric carcinoma [1–4]. *H. pylori*-infected gastric mucosa is always associated with a mucosal inflammatory reaction involving the infiltration of a large number of polymorphonuclear and mononuclear cells [5,6]. It is considered that such a response is related to the expression of proinflammatory cytokines, such as IL-8, IL-1β, IL-6, TNF-α, and IFN-γ, in both the surface epithelium and macrophages/monocytes [7–11]. Among the above cytokines, IL-8 has been found to play a pivotal role in the induction of neutrophil infiltration in *H. pylori*-associated diseases. It is of interest that human gastric epithelial cells produce a large amount of IL-8 *in vitro* upon co-culturing with *H. pylori* [12–14]. The new members of the MAPK family, p38 MAPK and stress-activated protein kinase, are both activated by cytokines and various forms of cellular stress, ultimately resulting in a stress response and apoptosis [15]. In addition, MAPK modulates cytokine production [16]. The present study was performed to determine whether or not MAPK is involved in the IL-8 production pathway in MKN 28 cells induced by

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Abbreviations: MAPK, mitogen-activated protein kinase; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; FBS, fetal bovine serum; CFU, colony-forming units; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT–PCR, reverse transcriptase–polymerase chain reaction; PDTC, pyrrolidine-carbodithioic acid; NF-κB, nuclear factor-κB; IL-1RA, IL-1 receptor antagonist; MEK, MAP kinase-ERK kinase; ATF-2, activating transcription factor-2; PTK, protein tyrosine kinase; and MAPKK, MAPK kinase.

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H. pylori. We also examined in this study whether or not IL-1β released from *H. pylori*-induced cells is involved in the IL-8 production pathway.

2. Materials and methods

2.1. Cell culture

Human gastric cancer MKN 28 cells (RIKEN Cell Bank) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (GIBCO BRL), 10 mM HEPES-NaOH (pH 7.4), 100 U/mL of penicillin, 100 U/mL of streptomycin, and 0.25 μg/mL of amphotericin B. The cells were inoculated into either 60-mm dishes (3×10^6 cells in 2 mL) or 24-well plates (3×10^5 cells in 0.5 mL of medium), and then cultured at 37° under 5% CO_2 in air.

2.2. H. pylori

A *cagA*- and *vacA*-positive standard strain of *H. pylori* (ATCC 43504; American Type Culture Collection) was used in this study. The bacteria were incubated overnight in a brain–heart infusion broth (Difco Laboratories) supplemented with 10% FBS at 37° under a microaerophilic atmosphere and were allowed to grow to a concentration of approximately 2.0×10^8 CFU/mL. The bacteria were harvested by centrifugation at 1000 *g* for 15 min at room temperature and then resuspended, at the indicated concentration, in antibiotic-free RPMI 1640 medium.

2.3. RT–PCR

MKN 28 cells were cultured in complete medium in 60-mm dishes at 37° under 5% CO_2. After the cells had reached subconfluency, bacteria were added to a final concentration of 1×10^8 CFU/mL. Recombinant human IL-1β was added to a final concentration of 1 ng/mL. Total cellular RNA was isolated from MKN 28 cells by means of the acid guanidinium thiocyanate-phenol-chloroform method [17], using TRIzol Reagent (GIBCO BRL). First strand cDNA was prepared from 10 μg of the total RNA with Moloney murine leukemia virus reverse transcriptase (GIBCO BRL), according to the instructions of the manufacturer. PCR was performed with a PCR thermal cycler (TP3000; TaKaRa). The primers used were as follows: human IL-8 (sense: 5′-ATGACTTCCAAGCTGGCCGTGGC-3′; antisense: 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′) [18], and human GAPDH (sense: 5′-CCACCCATGGCAAATCCATGGC-3′; antisense: 5′-TCTAGACGGCAGGTCAGGTCCACC-3′) [19]. After denaturation at 94° for 5 min, PCR was performed for 30 cycles, each of which consisted of denaturation at 94° for 30 sec, annealing at 65° for 45 sec, and extension at 72° for 45 sec. The amplification was terminated after a 15-min final extension at 72°. These procedures yielded cDNA products of 289 bp for IL-8 and 593 bp for GAPDH. After aliquots of the reactants had been subjected to agarose (2%) gel electrophoresis, the PCR products were visualized by ethidium bromide staining.

2.4. Western blotting

The cells were pretreated with inhibitors 2 hr before stimulation. Following appropriate treatment, cells were processed for SDS–PAGE and western immunoblotting [20], with the following modifications. Cell pellets were solubilized with 250 μL of ice-cold lysis buffer [50 mM HEPES (pH 7.6), 300 mM NaCl, 2 mM EDTA, 2 mM $MgCl_2$, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium orthovanadate, 10 mM sodium fluoride, 10 μg/mL of leupeptin, 2 μg/mL of aprotinin, 1 mM dithiothreitol, and 0.5% Triton-X 100]; 2 × sample buffer [125 mM Tris–HCl (pH 6.8), 10% dithiothreitol, 4% SDS, 10% sucrose, 0.01% bromophenol blue] was then added, and the samples were boiled for 5 min. The samples (5 μg protein) were subjected to 12.5% SDS–PAGE, and the separated proteins were electrophoretically transferred to Immobilon-P membranes (Millipore), as described by Towbin *et al.* [21]. The membranes were incubated with antibodies for p38, phospho-p38, p44/p42, phospho-p44/p42, or phospho-ATF-2 (New England Biolabs), after nonspecific binding sites had been blocked. The immune complexes were visualized with chemiluminescence reagents (New England Biolabs).

2.5. Determination of IL-8 protein

MKN 28 cells were cultured in complete medium in either 60-mm dishes or 24-well plates at 37° under 5% CO_2. The cells were pretreated with inhibitors or vehicle (antibiotic-free RPMI 1640 medium) 2 hr before stimulation. After 24 hr, cell supernatants were obtained by centrifugation at 10,000 *g* for 15 min at 4° to remove bacteria, and then were stored at −40° until used for analysis. The concentration of human IL-8 in the culture supernatants was determined by ELISA and expressed as picograms per 10^4 cells.

2.6. Determination of neutrophil chemotactic activity

MKN 28 cells were cultured in complete medium in 24-well plates at 37° under 5% CO_2. After the cells had reached subconfluency, bacteria were added to obtain a final concentration of 1×10^8 CFU/mL. Recombinant human IL-1β was added to a final concentration of 1 ng/mL. The cells were pretreated with inhibitors 2 hr before stimulation. After incubation for 24 hr at 37° under 5% CO_2, cell supernatants were obtained by centrifugation at 10,000 *g* for 15 min at 4° to remove bacteria, and then were stored at −40° until used for analysis.

Purified neutrophils were prepared from heparin-treated venous blood of healthy adult human volunteers and separated by dextran sedimentation followed by Ficoll-Paque® gradient centrifugation, as described by Boyum [22]. In brief, human neutrophils were separated from mononuclear leukocytes by centrifugation on Ficoll-Paque® cushions at 400 *g* for 25 min at room temperature. The resulting pellets were suspended in hypotonic buffer to remove contaminating erythrocytes. After isolation, the purity and viability of the neutrophils were both more than 95%, as determined by Diff-Quick® (International Reagents) staining and the trypan blue exclusion method, respectively.

The neutrophil chemotaxis assay was performed by the Boyden method as slightly modified by Falk *et al.* [23], using a membrane filter of 8 μm pore size (Neuro Probe). Culture medium (25 μL) was added to the lower compartment of the chemotactic chamber. Neutrophils (7.5×10^4 cells) were placed in the upper compartment. The chamber was allowed to sit for 45 min at 37° to permit neutrophil migration. Subsequently, the filter was removed, and the cells were stained with hematoxylin and Diff-Quick®. The number of neutrophils that had migrated to the lower face of the filter was determined in five, randomly chosen fields under a light microscope (×400).

2.7. Reagents

Recombinant human IL-1β was provided by the Otsuka Pharmaceutical Co. FR167653 (1-[7-(4-fluorophenyl)-1,2,3,4-tetra-hydro-8-pyridylpyrazolo[5,1-*c*][1,2,4]triazin-2-yl]-2-phenylethanedione sulfate monohydrate), an inhibitor of the production of IL-1, TNF-α [24–26], and p38 MAPK, was provided by the Fujisawa Pharmaceutical Co. PDTC, which inhibits the activation of NF-κB [27], was purchased from Nacalai Tesque. IL-1RA was purchased from R & D Systems Inc. The above agents were dissolved in distilled water. 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole (SB203580, a specific inhibitor of p38 MAPK) [28] and 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059, a specific inhibitor of MEK) [29] were purchased from Calbiochem and dissolved in dimethyl sulfoxide. All other chemicals used in the study were of reagent grade.

2.8. Statistical analysis

The data are presented as means ± SEM. Statistical differences in the concentration–response studies were evaluated by means of Dunnett's multiple comparison test. Student's *t*-test was used for comparisons between any two groups. A P value of < 0.05 was regarded as significant.

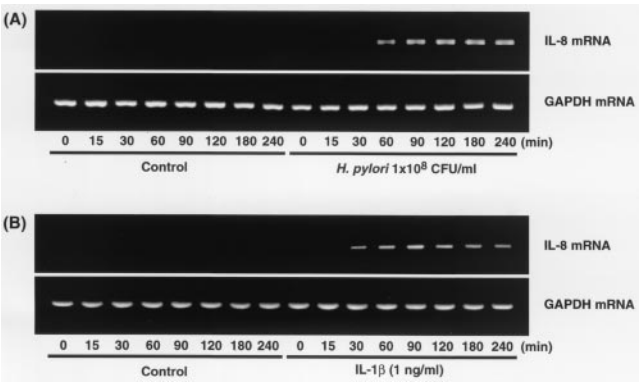

Fig. 1. Time–course changes of IL-8 mRNA expression in MKN 28 cells stimulated with *H. pylori* (A) and IL-1β (B). Cells were incubated with *H. pylori* (1×10^8 CFU/mL), IL-1β (1 ng/mL), or the vehicle (control) for up to 4 hr; total mRNA was isolated at the indicated times. Both IL-8 cDNA (289 bp) and GAPDH cDNA (593 bp) (as an internal standard) were amplified and detected by RT–PCR.

3. Results

3.1. H. pylori induction of IL-8 mRNA expression in MKN 28 cells

IL-8 mRNA was not expressed in unstimulated MKN 28 cells. *H. pylori* co-cultured with MKN 28 cells, however, clearly stimulated the expression of IL-8 mRNA in a concentration-dependent manner. The highest IL-8 mRNA expression level was observed after stimulation with 1×10^8 CFU/mL. Heat-killed *H. pylori* (1×10^8 CFU/mL) failed to elicit IL-8 mRNA expression. GAPDH mRNA was detected to a similar degree, regardless of the treatment. Time–course analysis demonstrated that MKN 28 cells co-cultured with *H. pylori* (1×10^8 CFU/mL) clearly expressed IL-8 mRNA beginning 60 min after infection and lasting for 4 hr (Fig. 1). IL-1β (0.1 to 1 ng/mL) also stimulated the expression of IL-8 mRNA in a concentration-dependent manner (data not shown). It should be noted that IL-1β (1 ng/mL) induced marked expression of IL-8 mRNA 30 min after treatment.

3.2. Time course of p38 MAPK activation in MKN 28 cells induced by H. pylori

Phosphorylated p38 MAPK was not observed in unstimulated MKN 28 cells (Fig. 2A). Co-culturing of the cells with *H. pylori* (1×10^8 CFU/mL) clearly stimulated p38 MAPK activation beginning 30 min after application and continuing for 2 hr (Fig. 2B). Pretreatment with FR167653 (10 μM) for 2 hr prior to *H. pylori* stimulation did not affect p38 MAPK activation for the following 60 min. p38 MAPK was also activated with heat-killed *H. pylori*, but the degree of activation was distinctly lower than that observed with live bacteria. Similarly, IL-1β (1 ng/mL) induced p38 MAPK phosphorylation, although the activa-

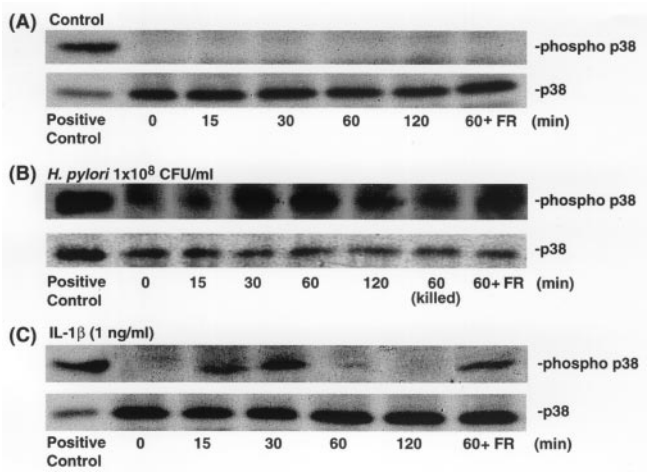

Fig. 2. Time–course changes of the phosphorylation of p38 MAPK in MKN 28 cells stimulated by *H. pylori* or IL-1β. Cells were incubated with the vehicle (control) (A), *H. pylori* (B), or IL-1β (C) for up to 2 hr. Cells were harvested with lysis buffer at the indicated times. Pretreatment was performed with 10 μM FR167653 (FR) 2 hr prior to the stimulation. Both phosphorylated p38 MAPK and total (phosphorylated plus nonphosphorylated) p38 MAPK in MKN 28 cells were detected by western blot analysis. The lower immunoblots in panels A–C represent total p38.

tion was rapid and transient compared with that induced by *H. pylori* (Fig. 2C). In addition, FR167653 had no effect on IL-1β-stimulated MAPK phosphorylation.

3.3. p38 MAPK involvement in H. pylori-induced IL-8 production in MKN 28 cells

Although the production of IL-8 in unstimulated MKN cells was negligible (0.6 $\pm$ 0.1 pg/10^4 cells), co-culturing of the cells with *H. pylori* (1×10^8 CFU/mL) markedly enhanced the production of IL-8 to 56.3 $\pm$ 12.2 pg/10^4 cells (Fig. 3). Both FR167653 and SB203580 inhibited the *H. pylori*-induced IL-8 production in a concentration-dependent manner (Fig. 3, A and B, respectively). Significant inhibition was observed with 1, 3, and 10 μM FR167653 (41, 62, and 79%), and 0.3, 1, 3, and 10 μM SB203580 (30, 54, 62, and 69%).

3.4. ATF-2 activation in H. pylori- and IL-1β-induced MKN 28 cells

Phosphorylated ATF-2 was observed in a small amount in unstimulated MKN 28 cells. ATF-2 was

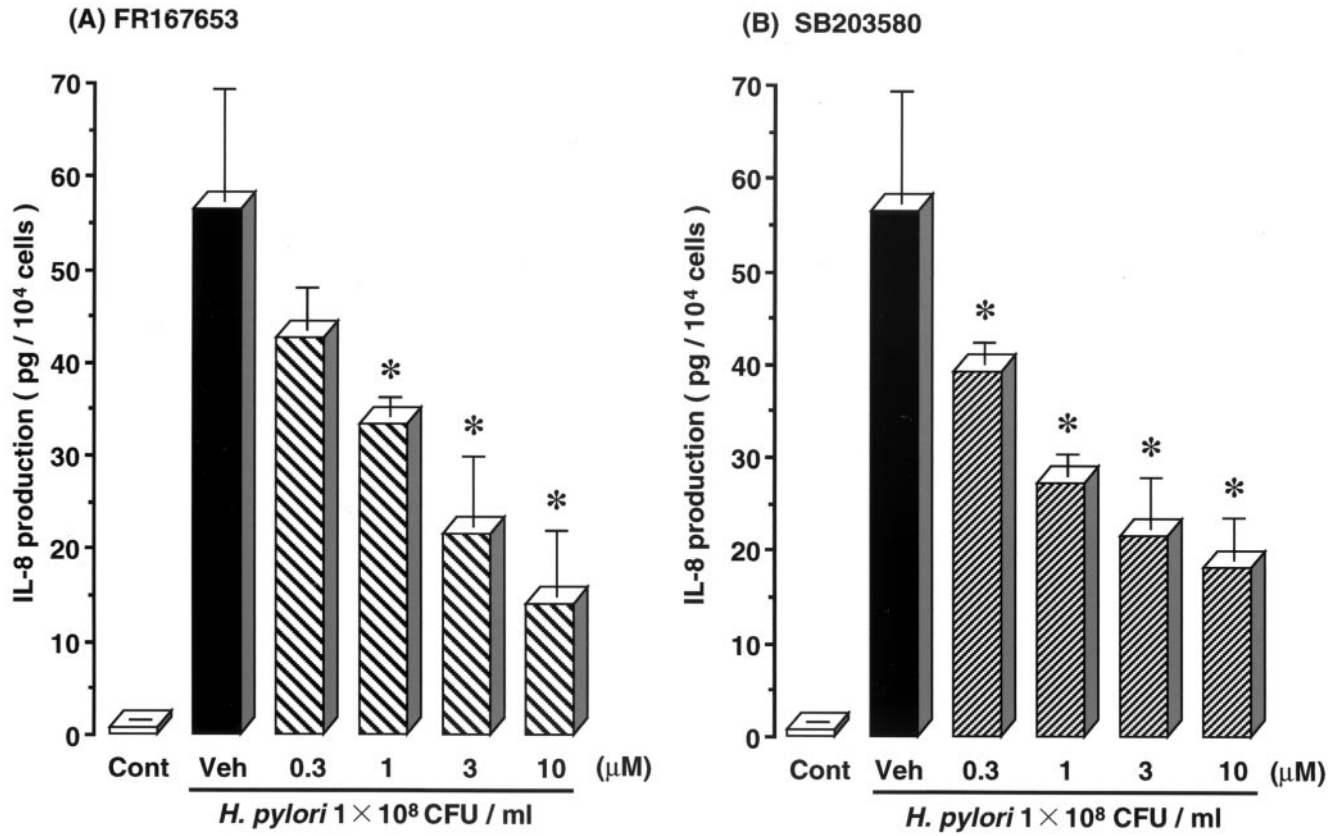

Fig. 3. Effects of FR167653 (A) and SB203580 (B) on the production of IL-8 in MKN 28 cells stimulated by *H. pylori*. Cells were incubated with *H. pylori* or the vehicle (control) for 24 hr. Pretreatment was performed with FR167653 or SB203580 2 hr prior to the stimulation with *H. pylori*. Data are presented as means $\pm$ SEM (N = 4). Key (*) significantly different from the vehicle-treated group, $P < 0.05$.

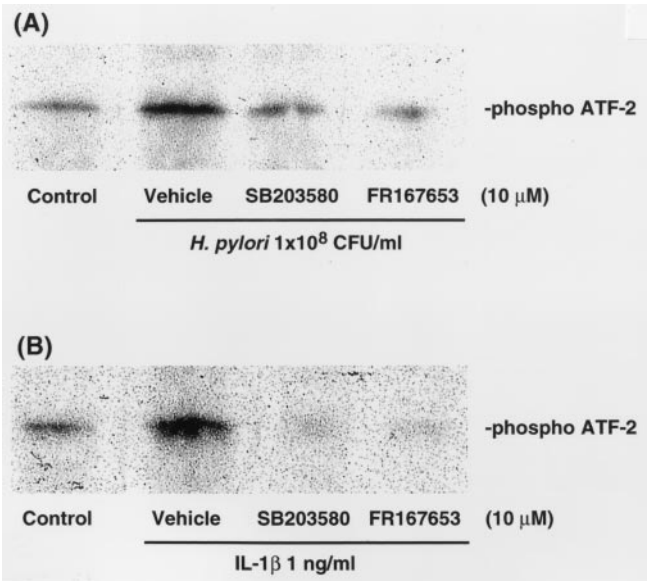

Fig. 4. Phosphorylation of ATF-2 in MKN 28 cells stimulated by *H. pylori* (A) or IL-1β (B). Cells were pretreated with SB203580 (10 μM) and FR167653 (10 μM) 2 hr prior to stimulation with *H. pylori* (A) or IL-1β (B). Phosphorylated ATF-2 in MKN 28 cells was detected by western blot analysis.

strongly activated by *H. pylori* and IL-1β stimulation (Fig. 4, A and B, respectively). Pretreatment of MKN 28 cells with SB203580 (10 μM) or FR167653 (10 μM) potently suppressed the activation of ATF-2 induced by *H. pylori* and IL-1β.

3.5. Time course of p44/p42 MAPK activation in H. pylori-induced MKN 28 cells

Low levels of phosphorylated p44/p42 MAPK were observed in unstimulated MKN 28 cells. It should be noted that p44/p42 MAPK was clearly activated beginning 15 min after *H. pylori* and IL-1β stimulation (Fig. 5, A and B, respectively). Pretreatment of MKN 28 cells with SB203580 or FR167653 had no effect on p44/p42 MAPK activation in response to *H. pylori* and IL-1β. In contrast, PD98059 (10 μM) potently suppressed such activation by *H. pylori* and IL-1β (Fig. 5, C and D, respectively).

3.6. Effects of MAPK inhibitors on H. pylori-induced IL-8 production

Both FR167653 (10 μM) and SB203580 (10 μM) significantly reduced the *H. pylori*-induced production of IL-8 (33.1 $\pm$ 4.1 and 46.4 $\pm$ 3.6 pg/10^4 cells vs 81.1 $\pm$ 7.1 pg/10^4 cells in the vehicle group), the inhibition levels being 59 and 43%, respectively (Fig. 6A). PD98059 (10 μM) also significantly inhibited the *H. pylori*-induced production of IL-8 to 51.1 $\pm$ 4.0 pg/10^4 cells (37% inhibition). In addi-

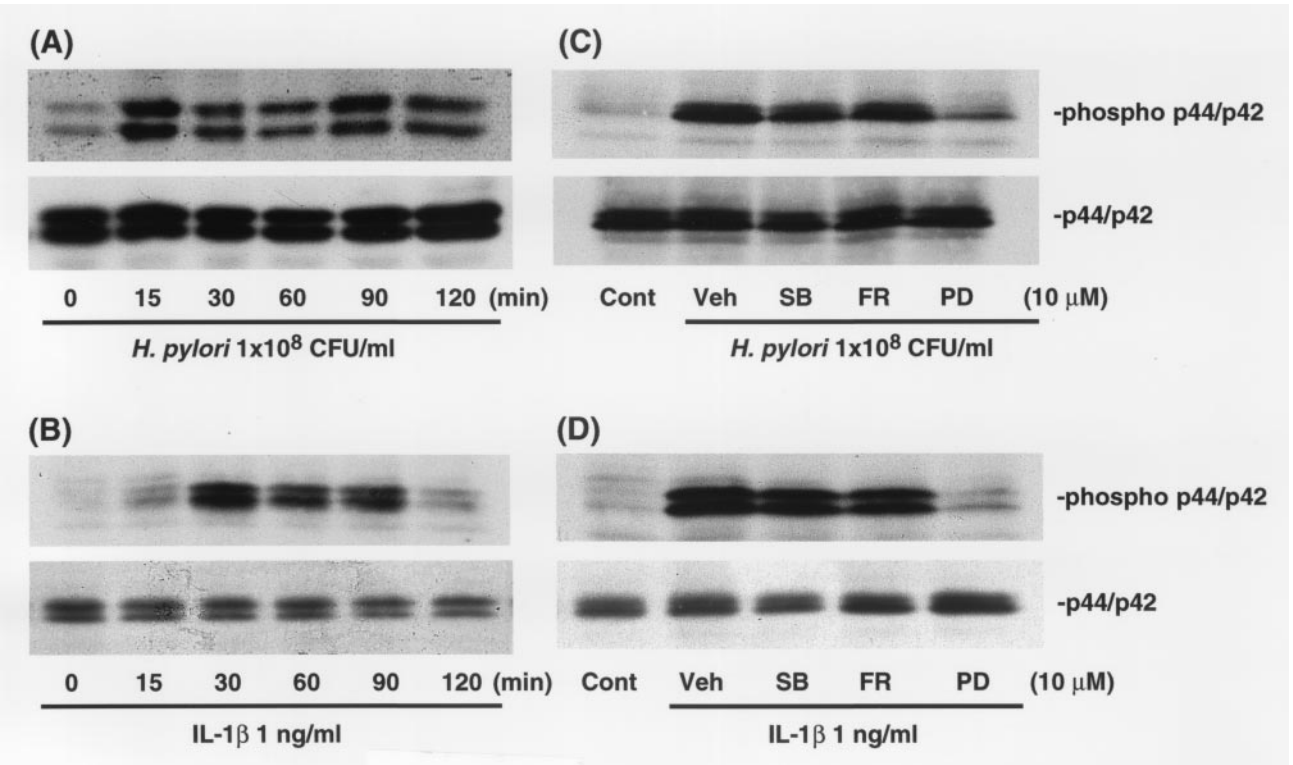

Fig. 5. Time–course changes of the phosphorylation of p44/p42 MAPK in MKN 28 cells stimulated by *H. pylori* (A) or IL-1β (B). Cells were harvested with lysis buffer at the indicated times. Cells were also pretreated with 10 μM SB203580 (SB), 10 μM FR167653 (FR), and 10 μM PD98059 (PD) 2 hr prior to the stimulation with *H. pylori* (C) and IL-1β (D). Both phosphorylated p44/p42 MAPK and total (phosphorylated plus nonphosphorylated) p44/p42 MAPK in MKN 28 cells were detected by western blot analysis. The lower immunoblots in panels A–D represent total p44/p42.

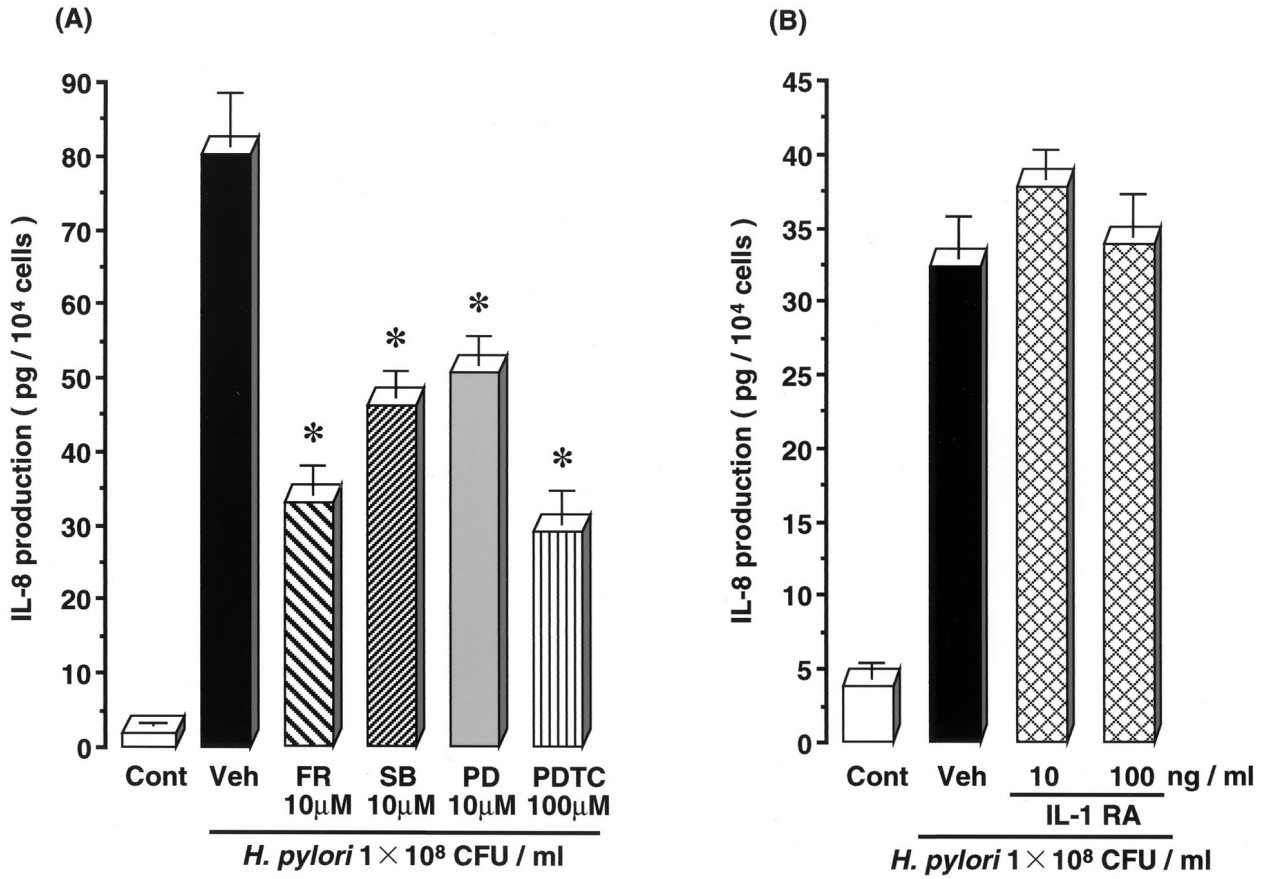

Fig. 6. Effects of FR167653, SB203580, PD98059, and PDTC (A), and IL-1RA (B) on the production of IL-8 in MKN 28 cells stimulated by *H. pylori*. Cells were incubated with *H. pylori* or the vehicle (control) for 24 hr. All reagents were applied 2 hr prior to *H. pylori* treatment. Data are presented as means ± SEM (A: N = 6, B: N = 4). Key: (*) significantly different from the vehicle-treated group, $P < 0.05$.

tion, PDTC (100 μM) significantly reduced *H. pylori*-induced IL-8 production to 29.3 ± 4.6 pg/10^4 cells (64% inhibition). It was of interest that IL-1RA (10 and 100 ng/mL) did not inhibit the stimulated production of IL-8 in response to *H. pylori* (Fig. 6B).

3.7. Effects of MAPK inhibitors on IL-1β-induced IL-8 production

Similar to the case of *H. pylori*, the production of IL-8 by MKN 28 cells was stimulated markedly by IL-1β (1 ng/mL) to approximately 31–47 pg/10^4 cells (Fig. 7). Both FR167653 (10 μM) and SB203580 (10 μM) significantly reduced the IL-1β-induced production of IL-8 to 13.0 ± 0.8 and 19.7 ± 1.9 pg/10^4 cells from 47.1 ± 1.7 pg/10^4 cells in the vehicle group; the inhibition was thus 72 and 58%, respectively. This IL-8 production was also inhibited by PD98059 (10 μM) to 16.4 ± 1.5 pg/10^4 cells (65% inhibition). As expected, IL-1RA (1, 3, 10, 30, and 100 ng/mL) inhibited IL-8 production in response to IL-1β in a concentration-dependent manner (Fig. 7B); the inhibition with 100 ng/mL was complete.

3.8. Effect of FR167653 on the production of IL-8 stimulated by H. pylori + IL-1β

Both *H. pylori* (1×10^8 CFU/mL) and IL-1β (1 ng/mL) significantly increased the production of IL-8 by MKN 28 cells to 80.1 ± 7.3 and 46.9 ± 4.5 pg/10^4 cells, respectively (Fig. 8A). Combination treatment of cells with *H. pylori* + IL-1β significantly and additively increased the production of IL-8 to 148.2 ± 1.5 pg/10^4 cells (Fig. 8B). Treatment with FR167653 (10 μM) significantly inhibited this stimulation of IL-8 production to 49.4 ± 7.0 pg/10^4 cells (67% inhibition). The neutrophil chemotactic activity in the above medium was increased 386 ± 37% by *H. pylori* alone and 161 ± 31% by IL-1β alone (Fig. 8C). Combined treatment with *H. pylori* + IL-1β also significantly increased the activity by 406%. Thus, the degree of the increase after combined treatment was similar to that observed with *H. pylori* alone. It was found that FR167653 decreased the neutrophil chemotactic activity in the medium by 57% upon stimulation with *H. pylori* + IL-1β, yet the difference was not significant. There was no observed chemotactic activity in the culture medium that was incubated

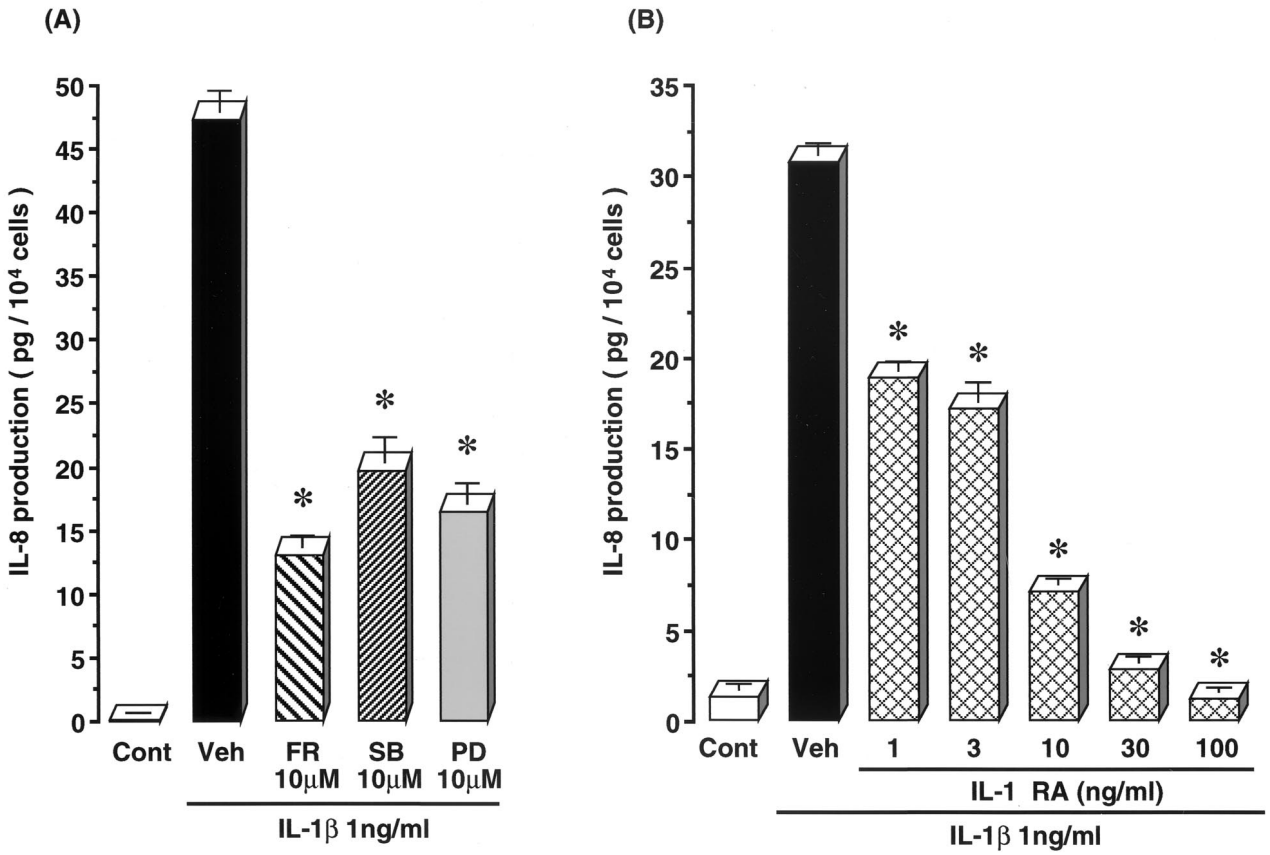

Fig. 7. Effects of FR167653, SB203580, and PD98059 (A), and IL-1RA (B) on the production of IL-8 in MKN 28 cells stimulated by IL-1β. Cells were incubated with IL-1β or the vehicle (control) for 24 hr. All reagents were applied 2 hr prior to stimulation with IL-1β. Data are presented as means ± SEM (N = 4). Key: (*) significantly different from the vehicle-treated group, $P < 0.05$.

with *H. pylori* alone or IL-1β alone (no MKN 28 cells) (data not shown).

4. Discussion

This study demonstrated that live, but not killed, *H. pylori* clearly stimulates MKN 28 cells, resulting in marked expression of IL-8 mRNA and, thus, IL-8 production. These results are consistent with the original findings of Crabtree et al. [7] and Sharma et al. [18], who reported that co-culturing of live *H. pylori* with human gastric cancer cell lines enhanced the expression of IL-8 mRNA and IL-8 production. In addition, Keates et al. [30] found that a clinically isolated *H. pylori* strain markedly stimulated the activities of p38 MAPK, p44/p42 MAPK, and c-jun N-terminal kinase (JNK) in AGS gastric epithelial cells. Accordingly, they suggested that such an MAPK might be involved in the mechanism by which *H. pylori* stimulates IL-8 production. In this study, we also confirmed that p38 MAPK and/or p44/p42 MAPK are potently activated within 30 min after treatment with *H. pylori*, as evidenced by the phosphorylated kinases in MKN 28 cells.

As expected, two kinds of p38 MAPK inhibitors, FR167653 and SB203580, significantly inhibited IL-8 production induced by *H. pylori.* In addition, PD98059 also clearly inhibited the phosphorylation of p44/42 MAPK and IL-8 production due to *H. pylori.* These results strongly suggest that p38 MAPK and p44/p42 MAPK play crucial roles in IL-8 production induced by *H. pylori.* Since both FR167653 and SB203580 clearly inhibited the phosphorylation of transcriptional factor ATF-2 in MKN 28 cells, it is most likely that the stimulated p38 MAPK activated ATF-2, which eventually led to the production of IL-8.

On the other hand, Aihara *et al.* [13] reported that herbimycin, a PTK inhibitor, repressed *H. pylori*-induced IL-8 production in MKN 45 cells, suggesting that a tyrosine kinase-mediated pathway might be involved in the mechanism underlying IL-8 production. Since MAPKK exhibits tyrosine kinase activity, it remains possible that the inhibitory effect of herbimycin on IL-8 production results from inhibition of MAPKK.

H. pylori activation of epithelial cell chemokines is associated with NF-κB activation [30,31]. An immunochemical study clarified that IL-8 gene transcription was indeed induced by a heterodimer of p50 and p65 binding to the NF-κB site in the promoter region of the IL-8 gene. Recently, Aihara et al. [13] demonstrated that both NF-κB and

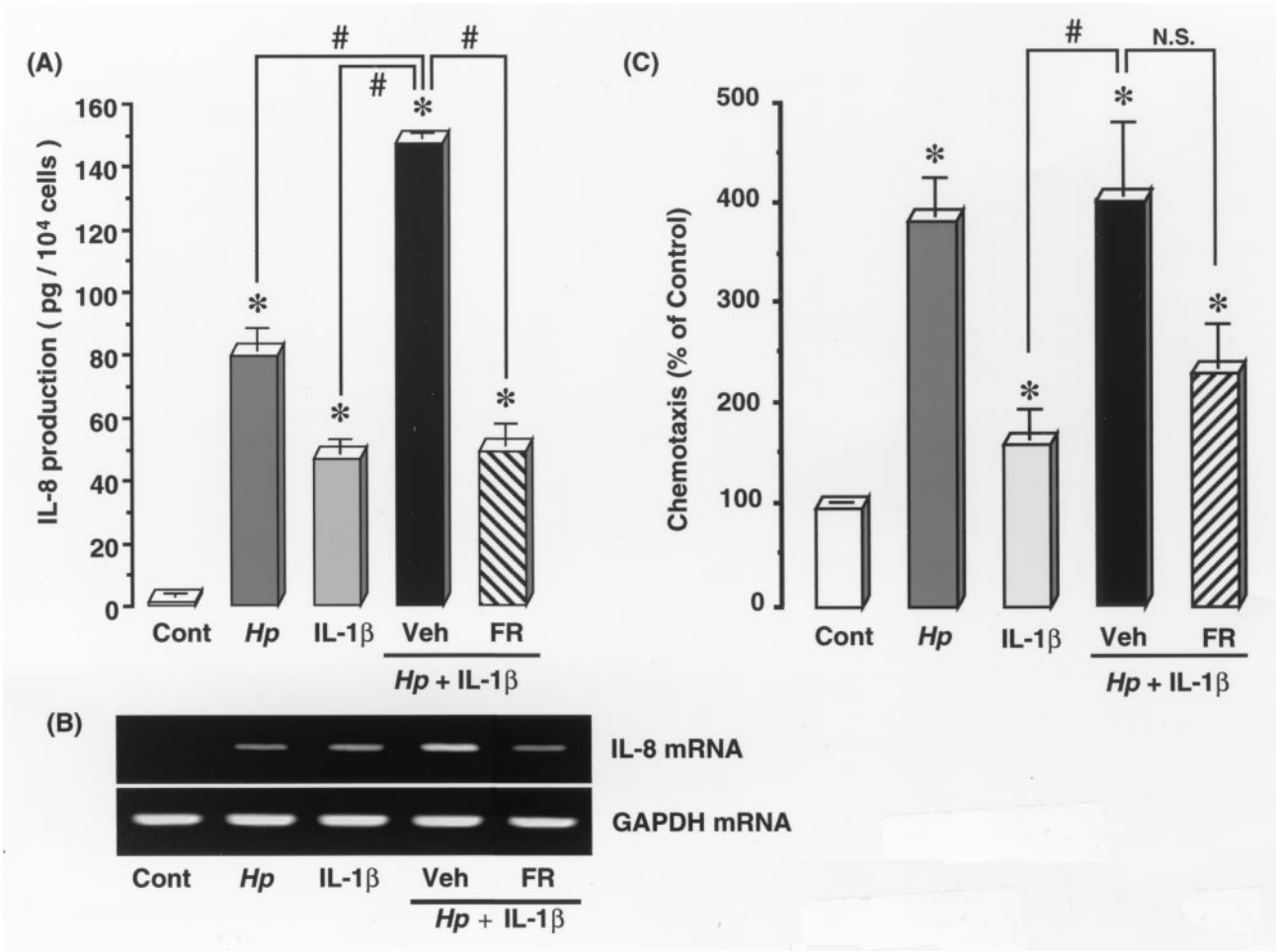

Fig. 8. Effects of FR167653 on the production of IL-8 (A), IL-8 mRNA expression (B), and neutrophil chemotactic activity (C) in MKN 28 cells stimulated by *H. pylori* + IL-1β. Cells were incubated with *H. pylori* (1×10^8 CFU/mL), IL-1β (1 ng/mL), *H. pylori* (1×10^8 CFU/mL) + IL-1β (1 ng/mL), or the vehicle (control) for 24 hr. FR167653 was applied 2 hr prior to the stimulation with *H. pylori* or IL-1β or both. The chemotactic value in the control group was 13.8 $\pm$ 2.1 neutrophils/45 min. Data are presented as means $\pm$ SEM (A: N = 3–4; B: N = 4). Key: (*) significantly different from the *H. pylori* + IL-1β-treated group, $P < 0.05$.

activating protein-1 (AP-1) act as essential transcriptional factors in *H. pylori*-induced IL-8 gene transcription in MKN 45 cells. We also confirmed that PDTC suppresses IL-8 production stimulated by *H. pylori*. A number of recent studies have demonstrated the presence of cross-talk between the MAPK and NF-κB pathways in HEK 293T and HeLa cells [14,32–34]. Keates *et al.* [14] demonstrated that p38 MAPK and MEK were not required for *H. pylori*-mediated NF-κB activation in AGS cells. Based upon such results, Keates suggested that the MAPK and NF-κB pathways act independently to regulate IL-8 production in AGS cells following *H. pylori* infection. Although both MAPK and NF-κB are definitely involved in the pathway for IL-8 production in the cells examined in this study, it remains unclear whether these two intracellular factors act dependently or independently.

It is of interest that similar to *H. pylori* stimulation, IL-1β treatment of MKN 28 cells resulted in the expression of IL-8 mRNA 30 min earlier than that observed with the bacteria. Subsequently, the production of IL-8 in the cells was elevated beginning at 24 hr after IL-1β treatment. Such increased IL-8 production was clearly inhibited by treatment with FR167653, SB203580, or PD98059. These results suggest that the IL-1β-induced IL-8 production pathway resembles the *H. pylori*-induced pathway. It should be noted that the stimulated IL-8 production was inhibited significantly by IL-1RA, even at a concentration of 1 ng/mL. In contrast, IL-8 production in response to *H. pylori* was unaffected by IL-1RA, even with 100 ng/mL. These results indicate that *H. pylori* stimulates IL-8 production in MKN 28 cells through a pathway that is independent of the IL-1 receptor.

In addition, an additive effect of *H. pylori* and IL-1β was observed on IL-8 production. This suggests that the increased IL-8 production observed in *H. pylori*-infected patients is a direct effect of the *H. pylori* on epithelial cells and an indirect effect of IL-1β released from inflammation-related cells, such as macrophages/monocytes, resulting from bacterial infection [35,36]. The combination of two pathways, in such a manner, resulting from *H. pylori* infec-

tion, would suggest that inflammatory diseases are more serious and complicated than previously thought.

As expected, the increased IL-8 production was inhibited significantly by FR167653, although the inhibition was limited to approximately 70%. As mentioned earlier, the stimulated IL-8 production resulting from either *H. pylori* or IL-1β was inhibited markedly by FR167653. Consequently, the fact that the increased IL-8 production in the presence of FR167653 was found to be nearly identical to the levels observed with *H. pylori* alone and IL-1β alone escapes simple explanation. Combined treatment with *H. pylori* and IL-1β might result in different, more complicated responses, rather than a simple additive effect of the two factors.

In addition, it was found that the medium upon stimulation with *H. pylori*, IL-1β, or both contained significantly increased chemotactic activity. Despite the fact that combined treatment with *H. pylori* + IL-1β resulted in an additive effect on IL-8 production, the combination did not induce any potentiated chemotaxis. It is most likely that under the present experimental conditions, chemotaxis is maximally stimulated by *H. pylori* alone. The chemotaxis in response to the combination treatment was suppressed insignificantly by FR167653, suggesting that the chemotactic response results from not only IL-8, but also additional factors present in the medium with the bacteria.

In conclusion, this study revealed that: (a) co-culturing of *H. pylori* and MKN 28 cells significantly stimulates IL-8 production to a degree sufficient to induce neutrophil chemotaxis via activation of p38 MAPK and p44/p42 MAPK, and (b) *H. pylori* and exogenous IL-1β can additively stimulate IL-8 production.

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