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Helicobacter pylori-Induced Interleukin-12 p40 Expression

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Running title: MECHANISM OF *H. PYLORI*-INDUCED IL-12 EXPRESSION

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ABSTRACT

Interleukin-12 (IL-12) is a heterodimeric cytokine produced by antigen-presenting cells that promotes the development of T-helper lymphocyte 1 (Th1). Chronic gastritis induced by *Helicobacter pylori* is considered a Th1-mediated process. IL-12 levels in gastric biopsy samples of *H. pylori*-infected patients are higher than in those of uninfected individuals, but the cellular source of IL-12 remains elusive. IL-12 staining was detected in mucosal epithelial cells, lymphocytes, and macrophages in specimens of patients with *H. pylori*-positive gastritis. Therefore, we investigated IL-12 p40 mRNA induction by *H. pylori* in gastric epithelial cells and T cells. Although *cag* pathogenicity island (PAI)-positive *H. pylori* induced IL-12 p40 mRNA expression, isogenic mutant of *cag* PAI failed to induce it in both cell types. Supernatants from *H. pylori* cultures and *H. pylori* VacA induced IL-12 p40 mRNA expression in T cells but not in epithelial cells. Activation of IL-12 p40 promoter by *H. pylori* was mediated through NF-κB. Transfection of IκB kinase and NF-κB-inducing kinase dominant negative mutants inhibited *H. pylori*-induced IL-12 p40 activation. Inhibitors of NF-κB, phosphatidylinositol 3-kinase, p38 mitogen-activated protein kinase, and Hsp90 suppressed *H. pylori*- and VacA-induced IL-12 p40 mRNA expression. The results indicate that *H. pylori* induces IL-12 p40 expression by activation of NF-κB, phosphatidylinositol 3-kinase, and p38 mitogen-activated protein kinase. Hsp90 is also a crucial regulator of *H. pylori*-induced IL-12 p40 expression. In addition to *cag* PAI, VacA might be relevant in induction of IL-12 expression and Th1 polarized response only in T cells.

INTRODUCTION

Helicobacter pylori is a Gram-negative, spiral-shaped, microaerophilic bacterial pathogen found in the gastric mucosa of >50% of the world population. In 10-20% of
50 infected individuals, the *H. pylori*-induced chronic gastric inflammation progresses to peptic ulcer, gastric cancer, or gastric mucosa-associated lymphoid tissue lymphoma (22, 23, 55). Despite the development of immune responses against *H. pylori* infection, the bacteria are rarely eliminated, and colonization is generally persistent. Factors that contribute to the failure of the immune response to clear the organism
55 remain elusive (2). Bacterial, environmental, and host genetic factors may affect the progress and outcome of gastric disease. One such factor responsible for severe disease is the virulence of individual *H. pylori* strains. Several virulence factors have been described and include the presence of a *cag* pathogenicity island (PAI) and vacuolating cytotoxin (VacA) (11, 42, 46). *H. pylori* strains that carry *cag* PAI genes,
60 called type I strains, are highly prevalent in patients with peptic ulcer and gastric cancer (4, 9, 13). *H. pylori* strains that express higher activity of VacA correlate with increased severity of gastritis (26, 30, 51). VacA has been reported to have immunosuppressive activity, including the inhibition of T-cell proliferation (5, 18). However, VacA also has proinflammatory activities in immune cells (40, 56).

65 There is abundant evidence that T lymphocytes play a pivotal role in the pathogenesis of *H. pylori*-induced chronic gastric inflammation (50). This pathological state is considered a T-helper lymphocyte 1 (Th1)-mediated process characterized by increased production of gamma interferon (IFN-γ), which is

implicated in perpetuating the inflammatory changes that lead to disease (15, 29).
70 Interleukin-12 (IL-12) is a heterodimeric molecule (p70) consisting of a heavy chain (p40) and a light chain (p35) that promotes the development of Th1 cells and stimulates proliferation, cytolytic activity, and IFN-γ production by T and natural killer cells (8, 38). The expression of the p40 gene is specific to IL-12 producing cells, while the p35 gene expression is constitutively expressed in different cell types.

75 Although antigen presenting cells such as macrophages and dendritic cells are the primary producers of IL-12 p40 after microbial challenge (58), we have found that *H. pylori* induces the expression of IL-12 p40 in both gastric epithelial cells and T cells in this study. We analyzed the molecular mechanism of *H. pylori*-mediated IL-12 p40 induction in gastric epithelial cell lines, MKN45, MKN28, and AGS, and in a
80 T-cell line, Jurkat, and isolated $CD4^+$ T cells. Although *cag* PAI-positive *H. pylori* induced IL-12 p40 mRNA expression, isogenic mutant of *cag* PAI failed to induce it in both cell types. The results showed that *H. pylori* induced IL-12 p40 expression by activating NF-κB. Hsp90 acted as a crucial regulator in *H. pylori*-induced IL-12 p40 expression. Our results also indicate that the mechanism of IL-12 p40 induction is
85 different in the two cell types and that *H. pylori*-mediated IL-12 p40 induction in T cells involves both *cag* PAI and VacA.

MATERIALS AND METHODS

90 **Antibodies and reagents.** Mouse monoclonal antibodies to IL-12 and IL-23 were purchased from R&D Systems (Minneapolis, MN) and BioLegend (San Diego, CA),

respectively. Rabbit polyclonal antibodies to phospho-Akt (Thr-308), phospho-Akt (Ser-473), and NF-κB subunits p50, p65, c-Rel, p52, and RelB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to actin
95 was purchased from NeoMarkers (Fremont, CA). Mouse monoclonal antibody to phospho-IκBα (Ser-32 and Ser-36) and rabbit polyclonal antibodies to p38 and phospho-p38 (Thr-180 and Tyr-182) were purchased from Cell Signaling Technology (Beverly, MA). IL-1α and tumor necrosis factor α (TNF-α) were purchased from Peprotech EC, Inc. (London, UK). *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL)
100 and Bay 11-7082 were purchased from Sigma-Aldrich and Calbiochem (La Jolla, CA), respectively. 17-Allylamino-17-demethoxygeldanamycin (17-AAG) was purchased from Alomone Labs (Jerusalem, Israel). Phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 and p38 inhibitor SB203580 were obtained from Calbiochem (La Jolla, CA).

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Bacterial strains. *H. pylori* ATCC 49503 (American Type Culture Collection, Rockville, MD) was used in most experiments described in this study. Isogenic *H. pylori* mutant lacking the *cag* PAI (1) or VacA was also studied together with their parental wild-type strain (26695). For generation of the *vacA* (*hp0887*) deletion
110 mutant of *H. pylori* 26695, the *vacA* upstream sequence was amplified with F1 (forward) primer containing a *Xho*I site and R1 (reverse) primer containing a *Sma*I site, and cloned in pBluescript II (Stratagene, La Jolla, CA), resulting in plasmid pVacAu. The *vacA* downstream sequence, which was amplified with F2 primer containing a *Sma*I site and R2 primer containing *Bam*HI site, was cloned in pVacAu,

115 yielding plasmid pVacAud. The *aphA-3* (the kanamycin resistance gene) cassette, specifically designed for the construction for non-polar mutants (39) was ligated between the fragments at the *Sma*I site of pVacAud in the correct orientation, resulting in plasmid pVacAdel. The transformants were grown on 5% sheep blood agar plates supplemented with 4 μg/ml of kanamycin. The resulting kanamycin-resistant

120 transformants were analyzed for formation of vacuoles on the infected AGS cells, and the location of the *aphA-3* gene was analyzed by PCR. The sequences of the primers are as follows: F1, 5'-CCGCTCGAGCTTTAATCCTTCGCAAGTCTTTTCGC; R1, 5'-TCCCCCGGGGCGCCAAACTTTATCGGGTTTATCTG; F2, 5'-TCCCCCGGGTATTATTATGGGGACACTTC; and R2,

125 5'-CGGGATCCATGGCGATAGCGGTAGTGGAGT. *H. pylori* strains were plated on blood agar plates and incubated at 37°C for 2 days under microaerophilic conditions. Using inoculating needles, bacteria harvested from the plates were suspended in 50 ml of brucella broth containing 5% fetal bovine serum (FBS) and then cultured in a liquid medium at 37°C for 1 day in a controlled microaerophilic

130 environment. Bacteria were harvested from the broth culture by centrifugation and then resuspended at the concentrations indicated below in antibiotic-free medium. All procedures were performed with the approval of the appropriate institutional biosafety review committees and in compliance with their guidelines for biohazards.

135 **Purification of VacA.** ATCC 49503 was the source of VacA for purification as described previously (43). Purified VacA was activated immediately before use on

cells. Acid activation of VacA was accomplished by dropwise addition of HCl to the purified toxin.

140 **Cell culture.** Human gastric epithelial cells (MKN45, MKN28, and AGS) and T cells (Jurkat) were maintained in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Human peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy donors using Ficoll-Hypaque gradients. PBMC were then further purified using positive selection with
145 immunomagnetic beads specific for CD4 (Miltenyi Biotec, Auburn, CA). On the day of the experiment, cells were refed with fresh antibiotic-free medium and cocultured with *H. pylori* for the time intervals indicated below.

Tissue samples. Five histopathologically normal stomach biopsy specimens from
150 control patients that underwent esophagogastroscopy for other reasons and stomach biopsy specimens from five patients with *H. pylori* gastritis were used for reverse transcription (RT)-PCR analysis and examined histopathologically for IL-12. The presence of *H. pylori* infection was confirmed by culture, serological analysis (with anti-*H. pylori* immunoglobulin G antibody), rapid urease test, and histological
155 visualization with Giemsa staining. Patients with *H. pylori* gastritis showed polymorphonuclear neutrophil infiltration in the gastric epithelium in conjunction with the presence of bacterial forms, consistent with *H. pylori* infection. All samples were obtained after informed consent was received from the subjects.

160 **RT-PCR.** Total cellular RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. First-strand cDNA was synthesized from 1 μg total cellular RNA using an RNA PCR kit (Takara Bio Inc., Otsu, Japan) with random primers. Thereafter, cDNA was amplified using 35 and 28 cycles for IL-12 p40 and for β-actin, respectively. The specific primers used were as
165 follows: IL-12 p40, forward primer 5'-CATTCGCTCCTGCTGCTTCAC-3' and reverse primer 5'-TACTCCTTGTTGTCCCCTCTG-3'; and for β-actin, forward primer 5'-GTGGGGCGCCCCAGGCACCA-3' and reverse primer 5'-CTCCTTAATGTCACGCACGATTTC-3'. The product sizes were 267 bp for IL-12 p40 and 548 bp for β-actin. The thermocycling conditions for the targets were
170 as follows: 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. The PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

Plasmids. The IκBαΔN and IκBβΔN dominant negative mutants (kindly provided by D. W. Ballard, Vanderbilt University School of Medicine, Nashville, TN) are IκBα
175 and IκBβ deletion mutants lacking the NH_2-terminal 36 and 23 amino acids, respectively (6, 37). The IKKα dominant negative mutant IKKα (K44M) and the IKKβ dominant negative mutant IKKβ (K44A), as well as the NF-κB-inducing kinase (NIK) dominant negative mutant NIK (KK429/430AA), have been described previously (19). The IL-12 p40 promoter pXP2 luciferase reporter plasmid containing
180 the wild-type sequence (position –292 to position +56) or the internal deletion mutant of NF-κB site was used to map *H. pylori*-responsive regions (14). To construct the human p40 promoter/luciferase reporter construct, we generated p40 promoter

fragments by PCR of genomic DNA obtained from THP-1 cells. The resulting PCR products were ligated in pCR^{TM} II (Invitrogen) and subsequently excised and

185 relegated in *Bam*HI/*Xho*I sites of the pXP2 luciferase vector. To create the internal deletion of the NF-κB site, a PCR product extending from −106 to +56 bp was ligated in *Bam*HI/*Xho*I sites of pXP2. Subsequently, a second PCR product extending from −111 to −292 bp and flanked by *Bam*HI sites was ligated into *Bam*HI site of the −106/+56 p40-pXP2 vector.

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Transfection and luciferase assay. MKN45 cells were transfected with 1 μg of the appropriate reporter and 2 μg of effector plasmids using Lipofectamine (Invitrogen). Jurkat cells were also transfected with 10 μg of the reporter plasmid using electroporation. After 24 h, *H. pylori* was added and incubated for 6 h. The ratio of

195 bacteria to cells (multiplicity of infection; MOI) was 20. The cells were washed in phosphate-buffered saline (PBS) and lysed in reporter lysis buffer (Promega, Madison, WI). Lysates were assayed for reporter gene activity with the dual luciferase assay system (Promega). Luciferase activities were normalized relative to the *Renilla* luciferase activity from phRL-TK.

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Preparation of nuclear extracts and EMSA. NF-κB binding activity with the NF-κB element was examined by electrophoretic mobility shift assay (EMSA) as described previously (41). To examine the specificity of the NF-κB element probe, we preincubated unlabeled competitor oligonucleotides with nuclear extracts for 15 min

205 before incubation with probe. The probe or competitors used were prepared by

annealing the sense and antisense synthetic oligonucleotides as follows: for the NF-κB element of the IL-12 p40 gene, 5’-GATCCTTGAAATTCCCCCAG-3’; for the NF-κB element of the IL-2 receptor (IL-2R) α-chain (IL-2Rα) gene, 5’-GATCCGGCAGGGGAATCTCCCTCTC-3’; and for the AP-1 element of the IL-8 gene, 5’-GATCGTGATGACTCAGGTT-3’. The oligonucleotide 5’-GATCTGTCGAATGCAAATCACTAGAA-3’, containing the consensus sequence of the octamer binding motif, was used to identify specific binding of the transcription factor Oct-1. The above underlined sequences are the NF-κB, AP-1, and Oct-1 binding sites. To identify NF-κB proteins in the DNA-protein complex shown by EMSA, we used antibodies specific for various NF-κB family proteins, including p50, p65, c-Rel, p52, and RelB, to elicit a supershift DNA-protein complex formation. These antibodies were incubated with the nuclear extracts for 45 min at room temperature before incubation with radiolabeled probe.

Western blot analysis. Cells were lysed in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 6% 2-mercaptoethanol, and 0.01% bromophenol blue. Equal amounts of protein (20 μg) were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, followed by transfer to a polyvinylidene difluoride membrane and sequential probing with the specific antibodies. The bands were visualized with an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

IL-12 p40, IL-12 p70, and IL-23 measurements. The IL-12 p40, IL-12 p70, and IL-23 contents in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) (Biosource International, Camarillo, CA). MKN45 and AGS cells were cultured in RPMI 1640 supplemented with 10% FBS in 24-well plates. Subconfluent monolayers of cells were cocultured with *H. pylori* for 24 h. The supernatants were then collected after centrifugation to remove bacteria and stored at –80°C until they were assayed for IL-12 p40, IL-12 p70, and IL-23 by ELISA. The concentrations of these cytokines were determined using a standard curve constructed with recombinant cytokines.

Immunohistochemical analysis. Serial sections were deparaffinized in xylene and dehydrated using a graded ethanol series. For better detection, sections were pretreated with ready-to-use proteinase K (Dako, Inc., Carpinteria, CA) for 10 min at 37°C. This procedure increased the number of antigenic sites available for binding by the antibody. Sections were washed four times in PBS for 5 min each. In the next step, the tissues were placed in 3% hydrogen peroxide and absolute methanol for 5 min to reduce endogenous peroxidase activity, followed by four washing in PBS for 5 min each. The tissue sections were incubated with mouse anti-human IL-12 (1:100) or anti-human IL-23 monoclonal antibody (1:100) or a control mouse immunoglobulin G for 3 h at 37°C. After four washing with PBS for 5 min each, the sections were covered with EnVision plus (Dako, Santa Barbara, CA) for 40 min at 37°C and washed four times in PBS for 5 min each. Antigenic sites bound by the antibody were identified by reacting the sections with a mixture of 0.05% 3,3’-diaminobenzidine

tetrahydrochloride in 50 mM Tris-HCl buffer and 0.01% hydrogen peroxide for 7 min. Sections were washed three times in distilled water for 5 min each and then counterstained with methyl green for 10 min, hydrated in ethanol, cleaned in xylene, and mounted. The stained cells were examined using a light microscopy. Gastric epithelial cells, lymphocytes, and macrophages were identified morphologically.

Statistical analysis. Data were analyzed by using the Student *t* test. *P* values of < 0.05 were considered significant.

RESULTS

Increased expression of IL-12 p40 in gastric mucosa of patients with *H. pylori* gastritis. RT-PCR showed the presence of IL-12 p40 transcripts in specimens of patients with *H. pylori* gastritis (*n* = 2) (Fig. 1A). Analysis of *H. pylori*-negative control specimens (*n* = 2) showed undetectable levels of IL-12 p40 mRNA. We also investigated the presence of IL-12 protein in *H. pylori*-positive gastric diseases and determined its cellular source. For this purpose, we immunostained *H. pylori*-positive gastritis tissues (*n* = 5). Interestingly, IL-12 staining was detected in mucosal epithelial cells (Fig. 1B to D) and lymphocytes as well as macrophages (Fig. 1E). This antibody shows no cross-reactivity with IL-12 p40 homodimer or with IL-23. Therefore, the biologically active heterodimeric IL-12 p70 was detected in inflamed *H. pylori* gastritis tissues. In contrast, only a faint staining for IL-12 was detected in the normal mucosa, but the level of its expression was much weaker than that in *H.*

pylori-positive gastritis tissues (Fig. 1F and G). A p19 protein was identified which combines with IL-12 p40 to form IL-23, with similar but discrete functions from IL-12 (32). We also investigated whether IL-23 protein was increased in *H. pylori*-positive gastritis tissues. IL-23 staining was also detected in epithelial cells (Fig. 1H), macrophages, and lymphocytes (Fig. 1I) of specimens of *H. pylori*-positive gastritis. In contrast, IL-23 staining was not detected in the normal mucosa (Fig. 1J and K).

***H. pylori* increases IL-12 p40 mRNA levels in gastric epithelial cells.** Using RT-PCR, we next examined whether coculture of gastric epithelial cell lines, MKN45, MKN28, and AGS, with *H. pylori* results in the induction of IL-12 p40 mRNA. Coculture with *H. pylori* significantly enhanced the steady-state levels of IL-12 p40 mRNA in all three cell lines (Fig. 2A). IL-12 p40 transcript levels clearly increased 2 h after the addition of *H. pylori* to MKN45 cells (Fig. 2A). In the next step, we examined whether IL-12 p40 was secreted into the culture media of MKN45 and AGS cells cocultured with *H. pylori*. ELISA indicated that IL-12 p40 was secreted into the media of MKN45 and AGS cells cocultured with *H. pylori* over a 24-h period (Fig. 2B). IL-12 p70 was also secreted into the media of MKN45 cells cocultured with *H. pylori*. However, IL-23 was not secreted (data not shown).

The levels of production of IL-1 and TNF-α were reported to be significantly higher in mucosa of *H. pylori*-positive patients compared with those in normal mucosa (44). Because previous studies have shown that proinflammatory cytokines alter another cytokine expression, we further investigated whether gastric epithelial cells

respond to *H. pylori* and proinflammatory cytokines to induce IL-12 p40 expression. MKN45 cells were stimulated with IL-1α, IL-8, or TNF-α alone for 12 h and then infected with *H. pylori*. The results in Fig. 2C demonstrate that the proinflammatory
300 cytokines IL-1α and TNF-α induced MKN45 cells to express IL-12 p40, and the expression of IL-12 p40 was further upregulated in response to *H. pylori* when there was stimulation by IL-1α and TNF-α. In contrast, IL-8 failed to induce IL-12 p40 expression (data not shown).

305 ***H. pylori*-induced IL-12 p40 mRNA expression is *cag* PAI-dependent.** Recent studies indicated that the expression of multiple genes in the *cag* PAI is necessary for cytokine production in gastric epithelial cells *in vitro* (9, 59). Accordingly, we examined the abilities of a wild-type *cag* PAI-positive *H. pylori* strain (26695) and an isogenic *cag* PAI mutant (Δ*cag* PAI) to induce IL-12 p40 mRNA expression.
310 Infection with wild-type strain 26695 induced IL-12 p40 mRNA expression in MKN45 and MKN28 cells, while the isogenic mutant that lacked *cag* PAI expression did not induce IL-12 p40 mRNA expression (Fig. 3A). These results suggest that the *H. pylori cag* PAI plays an important role in the induction of IL-12 p40 mRNA expression.

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IL-12 p40 gene transcription regulated by *H. pylori* is *cag* PAI-dependent. In the next series of experiments, we investigated whether *H. pylori*-mediated upregulation of IL-12 p40 gene expression could directly enhance the activity of its promoter. MKN45 and AGS cells were transiently transfected with a reporter gene construct

320 containing the segment from position –292 to position +56 of the IL-12 p40 upstream regulatory sequences. Coculture of wild-type strain 26695 resulted in a dose-dependent increase in the activity of this IL-12 p40-driven reporter construct (Fig. 3B). We further investigated the involvement of *cag* PAI in the induction of IL-12 p40 promoter activity in MKN45 cells. Activation of IL-12 p40-driven reporter 325 was not observed by the isogenic mutant Δ*cag* PAI (Fig. 3B). These findings indicate that *cag* PAI is required for activation of the IL-12 p40 promoter.

NF-κB is essential for *H. pylori*-induced activation of IL-12 p40 promoter. The NF-κB signaling pathway is activated in epithelial cells infected with *cag* 330 PAI-positive *H. pylori* but not in cells infected with *cag* PAI-negative strains of *H. pylori* (17, 35, 54). To test the relative contribution of the NF-κB binding site to *H. pylori*-mediated activation of IL-12 p40, the plasmid with internal deletion mutant of this site of the IL-12 p40 promoter was transfected (Fig. 4). Deletion of the NF-κB binding site (ΔκB) abolished *H. pylori*-mediated activation of this reporter construct. 335 Therefore, the NF-κB binding site contributes to the activation of IL-12 p40 promoter induced by *H. pylori*.

***H. pylori* infection of gastric epithelial cells induces binding of NF-κB family proteins to the NF-κB element of the IL-12 p40 promoter.** Because the internal 340 deletional analysis of the IL-12 p40 promoter indicated that *H. pylori* infection activated transcription through the NF-κB site, it was important to identify the nuclear factors that bind to this site. The NF-κB sequence derived from the IL-12 p40

promoter was used as a probe in EMSA. MKN45, MKN28, and AGS cells were infected with *H. pylori* at different times after challenge, and nuclear protein extracts were prepared and analyzed to determine NF-κB DNA binding activity. As shown in Fig. 5A, a complex was induced in these cells within 10 min after infection with *H. pylori* and detectable 180 min after infection. This NF-κB binding activity to IL-12 p40 promoter was reduced by the addition of either cold probe or a typical NF-κB sequence derived from the IL-2Rα enhancer but not by an oligonucleotide containing the AP-1 binding site (Fig. 5A, lanes 2 to 4). Next, we characterized the *H. pylori*-induced complexes identified by the IL-12 p40 NF-κB probe. These complexes were supershifted by the addition of anti-p50 or anti-p65 antibody (Fig. 5A, lanes 5 to 9), suggesting that *H. pylori*-induced IL-12 p40 NF-κB complexes are composed of p50 and p65. Based on these results, *H. pylori* infection seems to induce IL-12 p40 gene expression at least in part through induced binding of p50 and p65 to the NF-κB site in the IL-12 p40 promoter region.

As described above, *cag* PAI-positive strains induced more IL-12 p40 mRNA than *cag* PAI-negative *H. pylori* strain. Next, we determined whether *cag* PAI-positive *H. pylori* strains preferentially induce NF-κB than its negative counterpart. Markedly increased NF-κB DNA-binding activity was induced by wild-type strain 26695 compared with the activity induced by the isogenic *cag* PAI mutant (Fig. 5B). These results indicate that better activation of NF-κB binding by *cag* PAI-positive strains is the underlying mechanism of the observed activation of the IL-12 p40 promoter by these bacterial strains. Considered together, these results indicate that *H. pylori* infection induces IL-12 p40 gene expression at least in part

through the induced binding of p50 and p65 NF-κB family members to the NF-κB element of the IL-12 p40 promoter and that this effect is dependent on *cag* PAI products.

370 **NF-κB signal is essential for induction of IL-12 p40 expression by *H. pylori* in gastric epithelial cells.** We next examined whether *H. pylori*-mediated upregulation of IL-12 p40 gene expression involves signal transduction components in NF-κB activation. The activation of NF-κB requires phosphorylation of two conserved serine residues of IκBα (Ser-32 and Ser-36) and IκBβ (Ser-19 and Ser-23) within the 375 NH_2-terminal domain (28). Phosphorylation leads to ubiquitination and 26S proteasome-mediated degradation of IκBs, thereby releasing NF-κB from the complex to translocate to the nucleus and activate genes (28). The IKK complex, which is composed of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ), phosphorylates IκBs (28). Previous studies indicated that members of the 380 mitogen-activated protein (MAP) kinase kinase kinase family, mediate physiologic activation of IKK (64). These kinases include NIK (61). IκBα and IκBβ dominant interfering mutants and IKKα, IKKβ, and NIK kinase-deficient mutants were tested to determine their abilities to inhibit *H. pylori*-mediated activation of the IL-12 p40-driven reporter gene. Expression of these various inhibitory mutants abolished *H.* 385 *pylori*-induced IL-12 p40 expression (Fig. 6A). These data show that signaling components involved in the activation of NF-κB are necessary for *H. pylori* activation of the IL-12 p40 promoter.

Because activation of the IL-12 p40 promoter by *H. pylori* infection required activation of NF-κB, we blocked NF-κB activation with Bay 11-7082, an inhibitor of

390 IκBα phosphorylation (48), or LLnL, a proteasome inhibitor (25). The latter is known to inhibit the activation of NF-κB by blocking the degradation of the IκBα protein. Both Bay 11-7082 and LLnL markedly inhibited *H. pylori*-induced NF-κB DNA-binding and expression of IL-12 p40 mRNA (Fig. 6B and C).

Hsp90 also plays a critical role in the inflammatory response and the

395 requirement of Hsp90 for activation of NF-κB has been suggested (7, 57). We evaluated the effect of Hsp90 inhibitor, 17-AAG, on *H. pylori*-induced IL-12 p40 expression. MKN45 cells constitutively express Hsp90 protein, but *H. pylori* infection did not affect its expression (57). Pretreatment with 17-AAG completely inhibited *H. pylori*-induced IL-12 p40 expression (Fig. 6D). Next, we tested the direct influence of

400 17-AAG on *H. pylori*-induced transcriptional activity of NF-κB using EMSA. Pretreatment with 17-AAG decreased the retardation of gel mobility through inhibition of the DNA binding activity of the NF-κB complex, indicating repression of the transcriptional activity of NF-κB (Fig. 6E). Of note, no differences in binding to the octamer motif on DNA were noted in the absence or presence of 17-AAG. These

405 findings suggest that Hsp90 may be involved in *H. pylori*-induced NF-κB-dependent IL-12 p40 signaling.

***H. pylori* increases IL-12 p40 mRNA expression in T cells.** Because IL-12 staining was detected in lymphocytes of specimens from patients with *H. pylori*-positive

410 gastritis, we investigated the induction of IL-12 p40 mRNA expression by *H. pylori* in

Jurkat T cells. IL-12 p40 mRNA expression was induced in Jurkat cells within 1 h after infection with *H. pylori* (Fig. 7A). *H pylori* infection also resulted in an increase in the activity of IL-12 p40-driven reporter construct (Fig. 7B). To characterize the effect of *H. pylori* infection on human T cells, IL-12 p40 mRNA expression in $CD4^+$
415 T cells in response to *H. pylori* was examined by RT-PCR. After infection for 2 h, *H. pylori*-stimulated induction of IL-12 p40 mRNA expression in $CD4^+$ T cells was observed, similar to the observations with Jurkat cells. Furthermore, Jurkat and $CD4^+$ T cells cocultured with *H. pylori* over a 24-h period produced low quantity, but significantly higher in comparison with spontaneous IL-12 p70 (Fig. 7C). However,
420 IL-23 was not secreted (data not shown). Interestingly, the supernatant of *H. pylori* induced IL-12 p40 mRNA expression in Jurkat cells but not in MKN45 cells (Fig. 7D). This result indicates that direct interaction is not essential for induction of IL-12 p40 mRNA expression in Jurkat cells. Furthermore, the induction of IL-12 p40 mRNA expression by isogenic *vacA* mutant (ΔVacA) and *cag* PAI mutant (Δ*cag* PAI)
425 was inhibited in Jurkat cells compared with the wild-type *H. pylori* 26695 (Fig. 7E). In contrast, *vacA* mutant induced IL-12 p40 mRNA expression in MKN45 cells (Fig. 7E). *H. pylori* separated by a permeable membrane induced IL-12 p40 mRNA expression in Jurkat cells, although its level was less than that induced by *H. pylori* without a membrane (data not shown). These results suggest that both VacA in the
430 culture supernatant and *cag* PAI through direct interaction are essential for IL-12 p40 induction in Jurkat cells. In addition, induction of IL-12 p40 expression was observed after challenge using the supernatant of *H. pylori* 26695, but not that of ΔVacA (data

not shown). These observations indicate that both *cag* PAI and VacA are responsible for induction of IL-12 p40 mRNA expression in Jurkat cells.

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VacA induces IL-12 p40 mRNA expression and NF-κB activation in Jurkat cells. Because the induction of IL-12 p40 mRNA expression by isogenic *vacA* mutant was inhibited in Jurkat cells compared with the wild-type strain, we next examined IL-12 p40 mRNA expression in Jurkat cells stimulated with VacA protein purified from *H.*

440 *pylori* ATCC 49503. Overexpression of IL-12 p40 mRNA was observed in Jurkat cells (Fig. 8A). In contrast, no VacA-induced IL-12 p40 expression was observed in MKN45 cells, implying that VacA-induced IL-12 p40 expression is limited to certain cell types. To determine whether VacA could activate NF-κB signals in Jurkat cells, immunoblot analysis was performed using cell lysates after stimulation with VacA.

445 Stimulation of Jurkat cells with VacA increased IκBα phosphorylation in a time-dependent manner (Fig. 8B).

We further investigated the roles of p38 MAP kinase and PI3K/Akt in VacA-induced IL-12 p40 expression in T cells. The p38 MAP kinase is a family of serine/threonine kinases that form an integral component of proinflammatory

450 signaling cascades in various cell types (31). Blocking p38 with specific inhibitors diminishes NF-κB-driven transcriptional activity and attenuates the expression of NF-κB target genes (3, 60). Previous studies showed that VacA induced activation of p38 in gastric adenocarcinoma cell line AZ-521 and monocytic cell line U937 (20, 21). Accordingly, we examined whether VacA activates p38 in Jurkat cells.

455 Phosphorylation of p38 was evident after a 10-min induction with VacA (Fig. 8B).

There was no significant change in total p38 protein levels in VacA-treated Jurkat cells. Recent evidence suggests that PI3K/Akt pathway also modulates NF-κB signaling pathway (27). Therefore, Akt activation was checked with antibodies that specifically recognize phosphorylated Akt. As shown in Fig. 8B, VacA treatment
460 increased phsphorylated Akt levels in a time-dependent manner. A p38 MAP kinase inhibitor (SB203580) and a PI3K inhibitor (LY294002) attenuated VacA-induced IL-12 p40 expression (Fig. 8C). These findings suggest that VacA seems to activate p38 MAP kinase and PI3K/Akt, and ultimately induces IL-12 p40 expression in T cells.

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DISCUSSION

During *H. pylori* infection, there is a pronounced specific acquired immune response, characterized by generation of antibodies, and differentiation and activation of
470 effector T cells. Although the latter includes both a Th1 and Th2 components, mucosal cytokine profiles imply Th1 predominance (15, 29), and the number of cells producing IFN-γ, the key Th1 cytokine, in the *H. pylori*-infected gastric mucosa correlates with the severity of gastritis (33). IL-12 is supposed to be one of the major Th1-inducing factors in *H. pylori*-colonized gastric mucosa (47). It is produced
475 primarily by antigen-presenting cells and exerts immunoregulatory effects on T and natural killer cells (58). However, the cellular source of IL-12 has not been investigated in the *H. pylori*-infected gastric mucosa. We found that IL-12 and IL-23 protein levels were markedly upregulated in gastric epithelial cells, lymphocytes, and

macrophages in *H. pylori*-infected gastritis compared with normal controls. Our
480 present data clearly show that *H. pylori* induces IL-12 p40 mRNA expression in gastric epithelial cells and T cells.

The regulation of intracellular events leading to successful IL-23 p19/IL-12 p40 heterodimerization and IL-23 production are not well understood. IL-12 p70 but not IL-23 was secreted into the media of MKN45, Jurkat, and $CD4^+$ T cells cocultured
485 with *H. pylori*. Furthermore, it has been reported that IL-12 p40 but not IL-23 was produced in the activated monocytes (16). These discrepancies suggest the presence of additional intracellular regulating mechanism driving the successful p19/p40 heterodimerization and IL-23 production.

We demonstrated that the expression of IL-12 p40 mRNA is further
490 upregulated in response to *H. pylori* when there is stimulation by the proinflammatory cytokines IL-1α and TNF-α. Although IL-12 p70 was secreted into the media of MKN45 cells cocultured with *H. pylori*, the amount was small. In contrast, IL-12 p70 protein was clearly detected in inflamed *H. pylori* gastritis tissues. Importantly, these studies indicate that in the presence of proinflammatory cytokines, *H. pylori* can
495 induce an amount of IL-12 sufficient to induce the Th1 cell response. Thus, induction of IL-12 p40 expression by gastric epithelial cells upon interaction with *H. pylori* might be mediated through induction of the proinflammatory cytokines IL-1α and TNF-α from *H. pylori*-activated macrophages or other cells. IL-23 production in *H. pylori*-infected gastritis might also be mediated through the similar mechanism.

500 *H. pylori*-induced gastritis is triggered primarily by *H. pylori* attaching to gastric epithelial cells (36). Once attached, it injects effector molecules into gastric

epithelial cells or the lamina propria via a type IV secretion system. The products of *cag* PAI genes are supposed to form a type IV secretion system (2). *H. pylori* is considered non-invasive and to rarely infiltrate the gastric mucosa, even though there
505 is an active Th1 immune response in the lamina propria of the *H. pylori*-infected stomach (55). However, Ito et al. (24) suggested that *H. pylori*-induced gastric epithelial damage allows the bacteria to invade the lamina propria and reach the gastric lymph nodes, which could result in chronic stimulation of the immune system.

Regulation of IL-12 p40 is fulfilled primarily at the transcription level and is
510 likely to be controlled by regulatory elements in the promoter region that can influence the transcriptional activity of the gene. The promoter of the IL-12 p40 gene contains multiple response elements that act as protein binding sites. For instance, NF-κB and Ets2 are the two most important transcription factors in IL-12 expression regulation, which bind to the −116/−107 and −211/−206 regions (14, 34). It has been
515 reported that C/EBPβ increases IL-12 gene transcription by interacting with the −80/−74 region of the promoter (49). Moreover, Sp1 and AP-1 were found to contribute equally to the regulation of IL-12 expression (10). In the present study, we demonstrated that *H. pylori* challenge of gastric epithelial cells and T cells induces NF-κB activation, and that this event plays a critical role in the induction of IL-12
520 p40. *H. pylori* induces IL-12 p40 expression in T cells even without direct interaction with the cell surface and does not always require the presence of *cag* PAI. There is virtually no infiltration of T cells in the mucosal layer in the absence of gastritis. However, once gastritis develops, numerous T cells infiltrate the mucosa (45). Our hypothesis is that in *H. pylori* infection, initially a small number of organisms come in

525 direct contact with epithelial cells, activate NF-κB, and induce IL-12 p40. This step requires *cag* PAI. However, once T cells infiltrate the mucosal layer, *H. pylori* is able to activate NF-κB without direct contact, independently of *cag* PAI. This step amplifies the Th1 response in the gastric mucosa. It is interesting that the time to reach the maximal levels of IL-12 p40 mRNA expression in T cells was more early
530 than that in gastric epithelial cells. The influence of T cells toward IL-12 p40 expression may limit at relatively early phase after infection.

VacA, a protein toxin produced by *H. pylori*, has multiple effects on susceptible cells (e.g., epithelial and lymphatic cells), including vacuolation with alterations of endo-lysosomal function, mitochondrial damage, and inhibition of T-cell
535 proliferation (5, 12, 18). These different effects of VacA appear to result from activation of different signal transduction pathways. Interestingly, in T cells, we found that VacA enhanced IL-12 p40 expression via NF-κB, p38 MAP kinase, and PI3K/Akt activation. p38 activates not only NF-κB (3, 60) but also ATF-2 or CREB, which can bind to the AP-1 region in the promoter. In addition to the NF-κB site of
540 the IL-12 p40 promoter, the AP-1 site may also be a functionally critical site in T cells. Interestingly, VacA-induced IL-12 p40 expression was not observed in gastric epithelial cells. Three cell surface proteins on epithelial cells have been implicated so far as specific receptors for VacA. These include the epidermal growth factor receptor (52) as well as receptor-like tyrosine phosphatase α (62) and β (63). Recently, the β2
545 (CD18) integrin subunit was identified as a leucocyte-specific receptor for VacA on human T cells (53). Defective CD18 expression may be responsible for the lack of induction of IL-12 p40 expression by VacA in the epithelial cells.

In this study, we demonstrated the involvement of NIK and IKKs in the induction of IL-12 p40 expression. We also investigated the role of Hsp90 and found it plays a role in *H. pylori*-induced NF-κB activation.

In conclusion, we have shown in the present study that the mechanism of *H. pylori*-mediated IL-12 p40 expression is different in gastric epithelial cells and T cells. IL-12 p40 expression is induced through a *cag* PAI-dependent NF-κB pathway in gastric epithelial cells but through *cag* PAI- and VacA-dependent NF-κB pathways in T cells. Thus, the host response to *H. pylori* infection might involve VacA in addition to *cag* PAI, leading to Th1 response.

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FIGURE LEGENDS

FIG. 1. Expression of IL-12 p40 in *H. pylori*-infected gastric mucosa. (A) RT-PCR analysis of IL-12 p40 in representative human gastric tissues. Lanes 1 and 2, normal mucosa; lanes 3 and 4, *H. pylori*-positive gastritis; lane M, markers. β-Actin expression served as a control. Immunohistochemical detection of IL-12 (B to G) and IL-23 (H to K) in tissues of patients with *H. pylori*-positive gastritis. Serial sections of gastric biopsy specimens were stained with mouse monoclonal antibodies to IL-12 and IL-23, and counterstained with methyl green. Representative examples of mucosa from patients with *H. pylori*-positive gastritis (B to E, H, and I) and normal mucosa (F, G, J, and K). Note the positive staining for IL-12 and IL-23 in epithelial cells and lymphocytes as well as macrophages from patients with *H. pylori*-positive gastritis. (B to D and F) Original magnification, ×170. (E and G) Original magnification, ×430. (H, J, and K) Original magnification, ×140. (I) Original magnification, ×360. The white, black, and red arrows indicate surface of epithelial cells, lymphocytes, and macrophages, respectively. The asterisks indicate deeper structures of epithelial cells.

FIG. 2. *H. pylori*-induced IL-12 p40 mRNA expression and secretion in gastric epithelial cells. (A) Dynamics of *H. pylori*-induced IL-12 p40 mRNA expression. Total RNA was extracted from the indicated cells infected with *H. pylori* ATCC 49503 for the indicated times and used for RT-PCR. MOI was 20. Representative results of three similar experiments in each panel. (B) Increased secretion of IL-12 p40 and p70 into the supernatants of MKN45 and AGS cell cultures in response to *H.*

pylori ATCC49503 infection at 24 h. IL-12 p40 and p70 concentrations in the

810 supernatants were determined by ELISA. Data are mean ± standard deviation of three experiments. **, $P < 0.01$; determined by the Student *t* test. (C) Expression of IL-12 p40 mRNA was further upregulated in response to *H. pylori* when there was stimulation by the proinflammatory cytokines IL-1α and TNF-α in MKN45 cells. MKN45 cells were stimulated with IL-1α (100 ng/ml) or TNF-α (100 ng/ml) for 12 h

815 and then incubated in the presence or absence of *H. pylori* for 2 h, and the expression of IL-12 p40 mRNA was assessed by RT-PCR. Representative results of three similar experiments in each panel.

FIG. 3. *cag* PAI products of *H. pylori* are required for induction of IL-12 p40 mRNA

820 expression. (A) Total RNA was extracted from the indicated cells infected with the wild-type strain 26695 (WT) or the isogenic mutant Δ*cag* PAI (Δ*cag*) for the indicated times and used for RT-PCR. Representative results of three similar experiments in each panel. (B) *H. pylori* infection increased IL-12 p40 promoter activity in a dose-dependent fashion. Luciferase reporter construct was transfected into MKN45

825 and AGS cells, and the cells were subsequently infected with WT or Δ*cag* PAI for 6 h. The activities are expressed relative to that of cells transfected with reporter construct without further *H. pylori* infection, which was defined as 1. Data are mean ± standard deviation of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; determined by the Student *t* test.

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FIG. 4. *H. pylori* activates the IL-12 p40 promoter through the NF-κB binding site. *Left:* Schematic diagram of the IL-12 p40 reporter constructs containing the wild-type (−292) and internal deletion mutant of NF-κB site (ΔκB). *Right:* Either IL-12 p40 reporter construct or that bearing an internal deletion of NF-κB binding site was
835 transfected into MKN45 cells, and the cells were subsequently infected with *H. pylori* ATCC 49503 for 6 h. MOI was 20:1. The activity is expressed relative to that of cells transfected with each construct without further *H. pylori* infection, which was defined as 1. Data are mean ± standard deviation of three independent experiments. *, $P <$ 0.05; determined by the Student *t* test.

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FIG. 5. *H. pylori* infection induces NF-κB binding activity. (A) Time course of NF-κB activation in MKN45, MKN28, and AGS cells infected with *H. pylori*, evaluated by EMSA (left panels). Nuclear extracts from the indicated cells infected with *H. pylori* ATCC 49503 for the indicated times were mixed with ^{32}P-labeled
845 NF-κB probe. MOI was 20:1. Competition assays were performed with nuclear extracts from these cells infected with *H. pylori* ATCC 49503 for 30 min (right panels). Where indicated, 100-fold excess amounts of each specific competitor oligonucleotide were added to the reaction mixture with labeled probe NF-κB (lanes 2 to 4). A supershift assay of NF-κB DNA binding complexes in the same nuclear
850 extracts was also performed. Where indicated, appropriate antibodies (Ab) were added to the reaction mixture before the addition of the ^{32}P-labeled probe (lanes 5 to 9). Arrows indicate the specific complexes, while arrowheads indicate the DNA binding complexes supershifted by antibodies. (B) *cag* PAI products of *H. pylori* are required

for induction of NF-κB binding activity in MKN45 cells. Nuclear extracts from

855 MKN45 cells infected with different densities (MOI) of wild-type strain 26695 or isogenic mutant Δ*cag* PAI for 1 h were analyzed for NF-κB. Representative results of three similar experiments in each panel.

FIG. 6. NF-κB signal is essential for activation of IL-12 p40 expression by *H. pylori*

860 in gastric epithelial cells. (A) Functional effects of IκBα and IκBβ dominant interfering mutants and kinase-deficient IKKα, IKKβ, and NIK mutants on *H. pylori*-induced activation of the IL-12 p40 promoter. MKN45 cells were transfected with IL-12 p40 reporter construct and the indicated mutant plasmids or empty vector (pCMV4) and then infected with *H. pylori* ATCC 49503 for 6 h. *Open bar:* luciferase

865 activity of IL-12 p40 reporter construct and pCMV4 without *H. pylori* infection. All values were first calculated as fold induction values relative to the basal level measured in uninfected cells. Data are mean ± standard deviation of three independent experiments. (B) Bay 11-7082 and LLnL inhibit IL-12 p40 mRNA expression induced by *H. pylori.* MKN45 cells were pretreated with Bay 11-7082 (20 μM) or LLnL (20

870 μM) for 1 h prior to *H. pylori* infection, and subsequently infected with *H. pylori* ATCC 49503 for 6 h. IL-12 p40 mRNA expression on harvested cells was analyzed by RT-PCR. (C) Bay 11-7082 and LLnL inhibit *H. pylori*-induced NF-κB DNA binding. MKN45 cells were pretreated with Bay 11-7082 (20 μM) or LLnL (20 μM) for 1 h prior to *H. pylori* infection, and subsequently infected with *H. pylori* ATCC

875 49503 for 1 h. Nuclear extracts from harvested cells were analyzed for NF-κB. (D) Inhibitory effects of 17-AAG on *H. pylori*-induced IL-12 p40 expression. MKN45

cells were incubated with 1 μM 17-AAG for 16 h prior to infection with different densities (MOI) of *H. pylori* for 6 h. RT-PCR was performed to check for the effects of 17-AAG treatment on IL-12 p40 mRNA expression in *H. pylori*-infected MKN45

880 cells. (E) Attenuation of *H. pylori*-induced NF-κB DNA binding by 17-AAG treatment. MKN45 cells were treated with (+) or without (−) 17-AAG for 16 h prior to infection with *H. pylori* for 1 h. Nuclear extracts were isolated from MKN45 cells infected with *H. pylori* and analyzed for NF-κB. Representative results of three similar experiments in each panel.

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FIG. 7. *H. pylori*-induced IL-12 p40 mRNA expression in T cells. (A) Total RNA was extracted from Jurkat cells and $CD4^+$ T cells infected with *H. pylori* ATCC 49503 for the indicated times and used for RT-PCR. MOI was 10. (B) *H. pylori* activates the IL-12 p40 promoter in T cells. IL-12 p40 reporter construct was transfected into Jurkat

890 cells, and the cells were subsequently infected with *H. pylori* ATCC 49503 for 6 h. MOI was 20. The activity is expressed relative to that of cells transfected with construct without further *H. pylori* infection, which was defined as 1. Data are mean ± standard deviation of three independent experiments. **, $P < 0.01$; determined by the Student *t* test. (C) Increased secretion of IL-12 p70 into the supernatants of Jurkat and

895 $CD4^+$ T cell cultures in response to *H. pylori* ATCC49503 infection at 24 h. IL-12 p70 concentrations in the supernatants were determined by ELISA. Data are mean ± standard deviation of three experiments. *, $P < 0.05$; **, $P < 0.01$; determined by the Student *t* test. (D) Jurkat and MKN45 cells were incubated with the indicated concentrations of culture supernatants from *H. pylori* ATCC 49503 for 2 h. Note the

900 supernatant-induced IL-12 p40 mRNA expression in Jurkat cells but not in MKN45 cells. (E) VacA and *cag* PAI of *H. pylori* is required for induction of IL-12 p40 expression in Jurkat cells, but VacA is not essential for induction of IL-12 p40 in MKN45 cells. Total RNA was extracted from Jurkat and MKN45 cells infected with *H. pylori* (wild-type strain 26695 or the isogenic mutants ΔVacA and Δ*cag* PAI) for 2
905 h and used for RT-PCR. Representative results of three similar experiments in each panel.

FIG. 8. VacA-induced IL-12 p40 mRNA expression in T cells. (A) Dynamics of VacA-induced IL-12 p40 mRNA expression. Total RNA was extracted from the
910 indicated cells treated with VacA (20 μg/ml) for the indicated times and used for RT-PCR. (B) Jurkat cells were incubated with VacA for the indicated times. Cell lysates were prepared at the indicated incubation times and subjected to immunoblotting with the indicated antibodies. (C) Effects of PI3K and p38 MAP kinase inhibitors on VacA-mediated IL-12 p40 expression in Jurkat. Cells were
915 pretreated with LY294002 (6.25 μM) or SB203580 (6.25 μM) for 1 h followed by VacA treatment for 2 h, and then analyzed IL-12 p40 mRNA expression by RT-PCR. Representative results of three similar experiments in each panel.