*ORIGINAL ARTICLE*

# Induction of CD69 expression by *cag*PAI-positive *Helicobacter pylori* infection

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## Abstract

**AIM:** To investigate and elucidate the molecular mechanism that regulates inducible expression of CD69 by *Helicobacter pylori* (*H. pylori*) infection.

**METHODS:** The expression levels of CD69 in a T-cell line, Jurkat, primary human peripheral blood mononuclear cells (PBMCs), and CD4$^+$ T cells, were assessed by immunohistochemistry, reverse transcription polymerase chain reaction, and flow cytometry. Activation of CD69 promoter was detected by reporter gene. Nuclear factor (NF)-κB activation in Jurkat cells infected with *H. pylori* was evaluated by electrophoretic mobility shift assay. The role of NF-κB signaling in *H. pylori*-induced CD69 expression was analyzed using inhibitors of NF-κB and dominant-negative mutants. The isogenic mutants with disrupted *cag* pathogenicity island (*cag*PAI) and *virD4* were used to elucidate the role of *cag*PAI-encoding type IV secretion system and CagA in CD69 expression.

**RESULTS:** CD69 staining was detected in mucosal lymphocytes and macrophages in specimens of patients with *H. pylori*-positive gastritis. Although *cag*PAI-positive *H. pylori* and an isogenic mutant of *virD4* induced CD69 expression, an isogenic mutant of *cag*-PAI failed to induce this in Jurkat cells. *H. pylori* also induced CD69 expression in PBMCs and CD4$^+$ T cells. The activation of the CD69 promoter by *H. pylori* was mediated through NF-κB. Transfection of dominant-negative mutants of IκBs, IκB kinases, and NF-κB-inducing kinase inhibited *H. pylori*-induced CD69 activation. Inhibitors of NF-κB suppressed *H. pylori*-induced CD69 mRNA expression.

**CONCLUSION:** The results suggest that *H. pylori* induces CD69 expression through the activation of NF-κB. *cag*PAI might be relevant in the induction of CD69 expression in T cells. CD69 in T cells may play a role in *H. pylori*-induced gastritis.

## INTRODUCTION

The leukocyte receptor CD69 is a C-type lectin, disulfide-linked homodimer, type Ⅱ protein that can be induced after activation[1,2]. In healthy subjects, CD69

is not detected in peripheral blood lymphocytes, but is expressed on small subsets of T and B cells in peripheral lymphoid tissues[3]. In addition, CD69 is selectively expressed in chronic inflammatory infiltrates at the sites of active immune responses *in vivo*[4,5]. However, the biological significance of CD69-induced cell activation is poorly understood.

*Helicobacter pylori (H. pylori)* is a Gram-negative bacterium that colonizes the human stomach, as well as areas of gastric metaplasia in the duodenal bulb[6]. The precise role of *H. pylori* in gastric pathology, especially the mechanism responsible for the transition of chronic active gastritis to gastric carcinoma, has been studied by many researchers. The infection triggers a local cellular immune response resulting in chronic cellular infiltration with or without an active component of neutrophils, as well as the development of lymphoid follicles in the lamina propria[7]. Although the exact mechanisms of the induction of various diseases by *H. pylori* infection have not been elucidated, one factor strongly associated with *H. pylori* virulence and the development of peptic ulcers and gastric cancer is the *cag* pathogenicity island (PAI), which constitutes a gene cluster encoding a type Ⅳ secretion system (T4SS)[8].

Enarsson *et al*[9] examined the transendothelial migration of human lymphocytes in response to *H. pylori* with the use of the Transwell system, employing a monolayer of human umbilical vein endothelial cells. *H. pylori* induced a significant T-cell migration and the presence of the *H. pylori cag*PAI increased T-cell transendothelial migration. Overexpression of CD69 was noted on migrating T cells[9]. These results suggest that *H. pylori* infection induces the expression of CD69 on T cells.

The present study was designed to test the hypothesis that *H. pylori* can induce both the surface expression of CD69 antigen and the promoter activity of the CD69 gene in human T cells, and to investigate whether such induction involves the *cag*PAI-coding T4SS and the nuclear factor (NF)-κB pathway. The presence of NF-κB motifs within the proximal promoter region of the CD69 gene may account for the *H. pylori*-inducible promoter activity.

## MATERIALS AND METHODS

### Reagents and bacterial strains
N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) and Bay 11-7082 were purchased from Sigma-Aldrich (St Louis, MO) and Calbiochem (La Jolla, CA), respectively. *H. pylori* ATCC 49503 (American Type Culture Collection, Rockville, MD) was used in most experiments described in this study. An isogenic *H. pylori* mutant lacking the *cag*PAI[9] or *virD4* also was employed, together with the parental wild-type strain (26695). *H. pylori* strains were plated on blood agar plates and incubated at 37 ℃ for 2 d 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 ℃ for 1 d

in a controlled microaerophilic 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 committee and in compliance with the guidelines for biohazards.

### Cell culture
The human T-cell line, Jurkat, was maintained in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin G, and 100 μg/mL streptomycin. Human peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of a healthy donor using Ficoll-Hypaque gradients. PBMCs then were further purified using positive selection with 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
Stomach biopsy specimens from ten patients with *H. pylori* gastritis were examined histopathologically for CD69. The presence of *H. pylori* infection was confirmed by culture, serological analysis (with anti-*H. pylori* immunoglobulin G antibody), rapid urease test, and histological examination with Giemsa staining. Patients with *H. pylori* gastritis showed polymorphonuclear neutrophil infiltration in the gastric epithelium in conjunction with the presence of bacterial forms, which is consistent with *H. pylori* infection. All samples were collected after obtaining informed consent from each patient.

### Reverse transcription-polymerase chain reaction
Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. First-strand complementary DNA was synthesized from 1 μg total cellular RNA using a RNA-polymerase chain reaction (PCR) kit (Takara Bio, Otsu, Japan) with random primers. The specific primers used were as follows: for CD69, 5'-CATAGCTCTCATT-GCCTTATCAGT-3'(forward primer) and 5'-CCTCTC-TACCTGCGTATCGTTT-3'(reverse primer); for β-actin, 5'-GTGGGGCGCCCCAGGCACCA-3'(forward primer) and 5'-CTCCTTAATGTCACGCACGATTTC-3'(reverse primer). Thereafter, cDNA was amplified using 30 and 28 cycles for CD69 and β-actin, respectively. The product sizes were 254 bp for CD69 and 548 bp for β-actin. The PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

### Transfection and luciferase assay
The IκBαΔN- and IκBβΔN-dominant-negative mutants are IκBα and IκBβ deletion mutants lacking the N-terminal 36 and 23 amino acids, respectively[10,11]. The dominant-negative mutants of IκB kinase (IKK)α, IKKα (K44M), IKKβ, IKKβ (K44A), IKKγ, IKKγ(1-305), and

NF-κB-inducing kinase (NIK), NIK (KK429/430AA) have been described previously[12,13]. The CD69 promoter pXP2 luciferase reporter plasmid containing the wild-type sequence (position -255 to position +16), pAIM255-LUC, was described previously[14]. The internal deletion mutants of the NF-κB sites were constructed by deletion of the NF-κB sites of pAIM255-LUC. Jurkat cells were transfected with the appropriate reporter and effector plasmids by electroporation. After 24 h, *H. pylori* was added and incubated for 6 h. The cells were washed in phosphate buffered saline 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.

### Preparation of nuclear extracts and electrophoretic mobility shift assay

Nuclear proteins were extracted and transcription factors bound to specific DNA sequences were examined by electrophoretic mobility shift assay (EMSA) as described previously[15]. The top strand sequence of the oligonucleotide probes or competitors are as follows: for the NF-κB element (κB1) of the CD69 gene, 5'-GATCCAGA-CAACAGGGAAAACCCATACTTC-3'; for the NF-κB element (κB2) of the CD69 gene, 5'-GATCCAGAGTCT-GGGAAAATCCCACTTTCC-3'; for the NF-κB element of the interleukin-2 receptor α chain (IL-2Rα) gene, 5'-GATCCGGCAGGGGAATCTCCCTCTC-3'; and for the AP-1 element of the IL-8 gene, 5'-GATCGTGAT-GACTCAGGTT-3'. The oligonucleotide 5'-GATCT-GTCGAATGCAAATCACTAGAA-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, respectively. 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 (Santa Cruz Biotechnology, Santa Cruz, CA).

### Immunohistochemical analysis

CD69 immunohistochemistry was performed using a mouse monoclonal antibody (clone FN50) to CD69 (BioLegend, San Diego, CA) after pretreatment of the deparaffinized tissue sections with ready-to-use proteinase K (Dako, Carpinteria, CA). The sections were counterstained with methyl green for 10 min, hydrated in ethanol, cleaned in xylene, and mounted. The stained cells were examined under a light microscope (Axioskop 2plus; Zeiss, Jena, Germany) with an Achroplan × 40/0.65 lens (Zeiss). Images were acquired with an AxioCam MRC camera and AxioVision 3.1 software (Zeiss). Gastric lymphocytes and macrophages were identified based on their morphological features.

### Flow cytometry

Cells were washed with cell WASH (Becton Dickinson Immunocytometry Systems, San Jose, CA) and incubated for 30 min with phycoerythrin-labeled mouse monoclonal antibody against CD69 (clone TP1.55.3) or control mouse IgG2b, which were purchased from Beckman Coulter (Fullerton, CA). Cells were analyzed on an Epics XL flow cytometer.

## RESULTS

### Overexpression of CD69 in gastric lymphocytes and macrophages in H. pylori gastritis

We investigated the expression of CD69 by immunostaining in *H. pylori*-positive gastric tissues (*n* = 10). CD69 staining was detected in mucosal lymphocytes and macrophages (Figure 1A). In contrast, only a faint staining for CD69 was detected in the normal mucosa, and the expression level was much weaker than in *H. pylori*-positive gastric tissues (data not shown).

### H. pylori increases CD69 mRNA levels in CD4+ T cells

Using reverse transcription (RT)-PCR, we next examined the effect of coculture of Jurkat T cells (a transformed human T-cell line) with *H. pylori* ATCC 49503 on the induction of CD69 mRNA. Coculture with ATCC 49503 significantly enhanced the steady-state levels of CD69 mRNA in Jurkat cells. CD69 transcript levels clearly increased 1 h after the addition of ATCC 49503 to Jurkat cells (Figure 1B). In another series of experiments, in which Jurkat cells were infected with ATCC 49503 at different concentrations [i.e., the multiplicity of infection (MOI)] for 2 h (Figure 1B), *H. pylori* induced dose-dependent expression of CD69 mRNA. To characterize the effect of *H. pylori* infection on human T cells, we employed RT-PCR to examine CD69 mRNA expression in PBMCs and CD4+ T cells in response to ATCC 49503. After 2-h infection, *H. pylori* induced CD69 mRNA expression in PBMCs and CD4+ T cells, similar to the observation with Jurkat cells (Figure 1C).

To analyze whether the increase of mRNA synthesis results in elevated expression on the cell surface, direct immunofluorescent staining and flow cytometry were performed. Consistent with the RT-PCR analysis, the expression was upregulated in a dose-dependent manner (Figure 2B). The peak expression level of cell surface CD69 was noted at 8 h after infection (Figure 2A). *H. pylori* ATCC 49503 also enhanced cell surface CD69 expression on PBMCs (Figure 2C).

### H. pylori-induced CD69 expression is cagPAI-dependent

The *cag*PAI, a cluster of about 28 genes, is one of the best known virulence factors; it encodes a T4SS that transports CagA protein, peptidoglycan, and possibly other molecules into host epithelial cells[16]. The *cag*PAI also encodes a homologue of the coupling protein *virD4*, which in *Agrobacterium tumefaciens* and conjugation systems is thought to deliver the T4SS substrates to the secretion machinery[17]. In *H. pylori*, *virD4* is necessary for CagA translocation but dispensable for the induction of

**A**

**B**

**C**
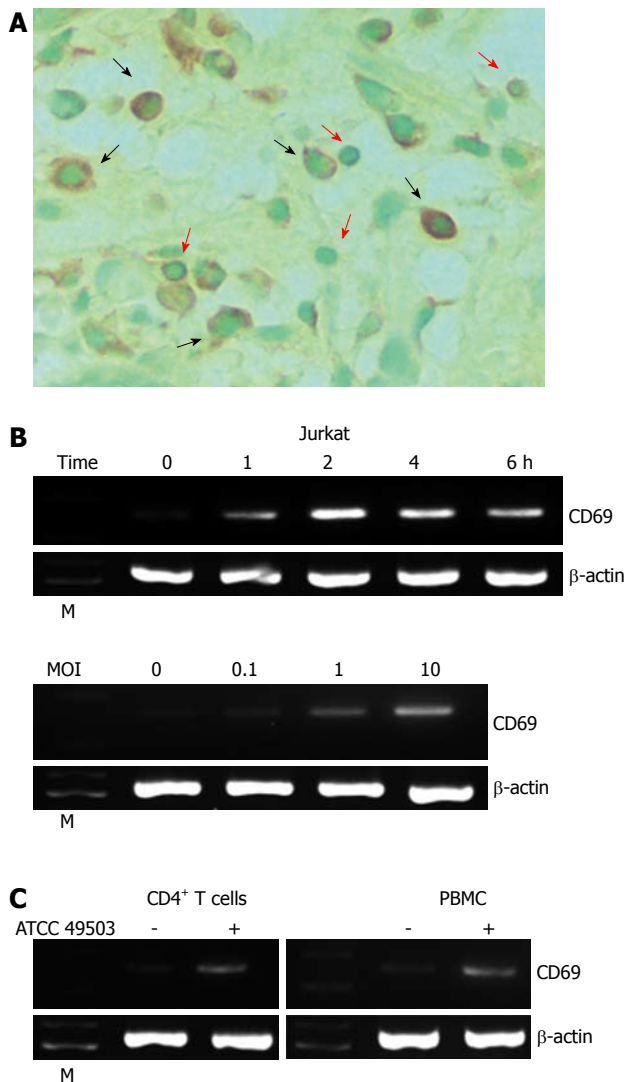
**Figure 1 Expression of CD69 in *Helicobacter pylori*-infected T cells.** A: Immunohistochemical detection of CD69 in tissues of patients with *Helicobacter pylori (H. pylori)*-positive gastritis. Serial sections of gastric biopsy specimens were stained with a mouse monoclonal antibody to CD69 and counterstained with methyl green. Shown is a representative example of mucosa from a patient with *H. pylori*-positive gastritis. Note the positive staining for CD69 in lymphocytes as well as macrophages. Original magnification, × 800. The red and black arrows indicate the surfaces of lymphocytes and macrophages, respectively; B: *H. pylori*-induced CD69 mRNA expression in Jurkat cells. Total RNA was extracted from Jurkat cells infected with *H. pylori* strain ATCC 49503 [the multiplicity of infection (MOI) of 100] for the indicated time intervals and used for reverse transcription-polymerase chain reaction (RT-PCR) (top). Jurkat cells were infected with the indicated concentrations of ATCC 49503 for 2 h. Total RNA was extracted and used for RT-PCR (bottom); C: *H. pylori*-induced CD69 mRNA expression in peripheral blood mononuclear cells (PBMCs) and CD4^{+} T cells. Total RNA was extracted from PBMCs and CD4^{+} T cells infected with ATCC 49503 for 2 h and used for RT-PCR (MOI of 10). β-actin expression served as a control. Lane M: Markers.

IL-8[18,19]. Accordingly, we compared the abilities of the wild-type *H. pylori* strain 26695, an isogenic *cag*PAI mutant (Δ*cag*PAI), and a *virD4* mutant (Δ*virD4*), with regard to the induction of CD69 transcripts and expression of CD69 on the cell surface. Infection with wild-type strain 26695 induced CD69 mRNA expression in Jurkat cells, while the isogenic mutant that lacked *cag*PAI expression

did not induce CD69 mRNA expression (Figure 2D). In contrast, the *virD4* mutant induced CD69 mRNA expression in Jurkat cells (Figure 2D). These results were confirmed by the cell surface expression of CD69 analyzed by flow cytometry (Figures 2B and E).

### Role of NF-κB in H. pylori-induced activation of the CD69 promoter

In the next series of experiments, we investigated whether the *H. pylori*-mediated upregulation of CD69 gene expression directly enhances the activity of its promoter. Jurkat cells were transiently transfected with a reporter gene construct containing a segment from position -255 to position +16 of the CD69 upstream regulatory sequences. Coculture of *H. pylori* strain ATCC 49503 resulted in a dose-dependent increase in the activity of this CD69-driven reporter construct (Figure 3B). The NF-κB signaling pathway is activated in epithelial cells infected with *cag*PAI-positive *H. pylori* but not in cells infected with *cag*PAI-negative strains of *H. pylori*[20-22]. Two potential NF-κB binding sequences were identified at positions -160 (κB1) and -223 (κB2) (Figure 3A). κB1 and κB2 were identical to those found in the gene promoters of c-myc and IL-6, respectively[23]. To test the relative contribution of the NF-κB binding sites to the *H. pylori*-mediated activation of CD69, plasmids with internal deletion mutants of these sites of the CD69 promoter were transfected (Figure 3C). After *H. pylori* infection, single deletion of the κB2 site resulted in marked reduction of the inducible activity. Single deletion of the κB1 site and the combination of double deletions abolished *H. pylori*-mediated activation of this reporter construct. These data clearly indicate that the two NF-κB binding sites in the CD69 promoter regulate CD69-enhanced expression after infection with *H. pylori*.

### H. pylori infection induces binding of NF-κB family proteins to the κB1 and κB2 motifs of the CD69 promoter in T cells

The data presented above indicate that *H. pylori*-induced CD69 expression is mediated by the κB1 and κB2 sites. To analyze whether these two putative NF-κB binding sites of the CD69 promoter could bind NF-κB family members, gel retardation assays were performed using as probes two double-stranded oligonucleotides (CD69 κB1 and CD69 κB2) containing these motifs. To characterize the NF-κB-related proteins that bind to the NF-κB sites of the CD69 promoter in CD69-expressing cells, the two oligonucleotide probes were incubated with nuclear extracts prepared from untreated Jurkat cells and from Jurkat cells infected with *H. pylori*. Jurkat 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 Figure 4A, complexes were induced in these cells within 30 min after infection with *H. pylori* and were detected at 180 min after infection with both oligonucleotide probes. The amounts of these inducible DNA-protein complexes were *H. pylori* dose-dependent.
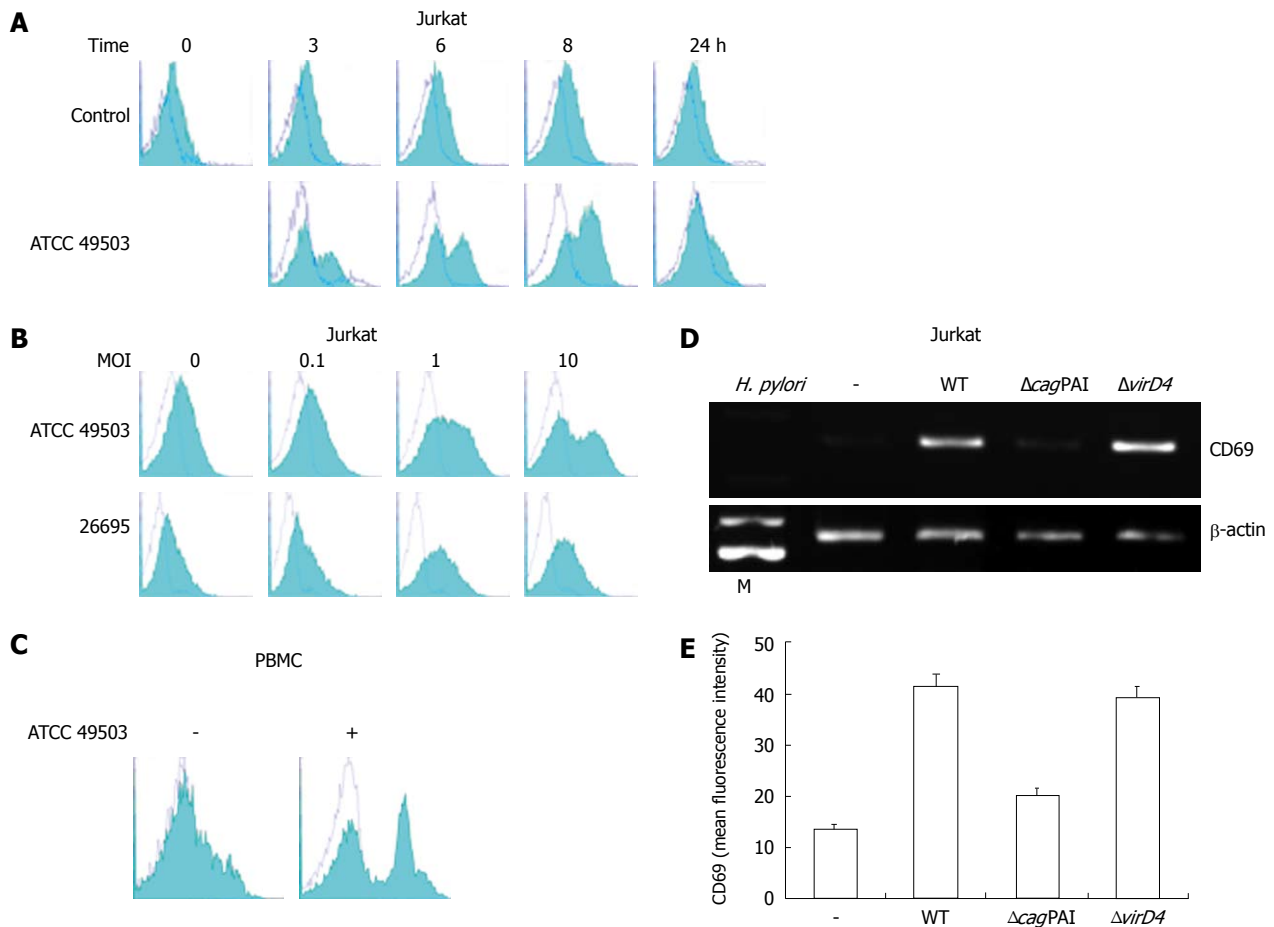
**Figure 2 CD69 expression on Jurkat cells.** A: Time course of cell surface expression of CD69 on Jurkat cells exposed to *Helicobacter pylori (H. pylori)*. Jurkat cells were cultured for the indicated times in culture medium (control) or in the presence of ATCC 49503 [the multiplicity of infection (MOI) of 10]. After cell harvest, CD69 expression on the cells was determined by flow cytometry; B: *H. pylori* infection increases cell surface expression of CD69 on Jurkat cells in a dose-dependent fashion. Jurkat cells were infected with different concentrations of *H. pylori* strains, ATCC 49503 and 26695, and CD69 levels were measured by flow cytometry on cells harvested after 8 h; C: *H. pylori* infection increases cell surface expression of CD69 on peripheral blood mononuclear cells (PBMCs). PBMCs were infected with ATCC 49503 (MOI of 10), and CD69 levels were measured on cells harvested after 8 h; D: *cag* pathogenicity island (*cag*PAI) products of *H. pylori* are required for the induction of CD69 mRNA expression. Total RNA was extracted from Jurkat cells that had been infected with the wild-type strain 26695 (WT) or the isogenic mutants Δ*cag*PAI and Δ*virD4* (MOI of 10) for 2 h and used for reverse transcription-polymerase chain reaction. Lane M: Markers; E: Flow cytometric analysis was carried out for the surface expression of CD69 in Jurkat cells infected with the wild-type strain 26695 (WT) or the isogenic mutants Δ*cag*PAI and Δ*virD4*. Jurkat cells were infected for 8 h with various *H. pylori* strains (MOI of 10). Cells were stained with phycoerythrin-labeled monoclonal antibody. Datas are mean ± SD of three experiments.

In both probes, the addition of an excess of unlabeled κB1 and κB2 oligonucleotides to the binding reaction completely abolished the formation of the inducible DNA-protein complexes (Figure 4B, lanes 3 and 4). Similarly, an equal amount of the oligonucleotide IL-2R κB, which contained the NF-κB motif of the IL-2R α chain gene, efficiently competed with the specific complexes (Figure 4B, lane 5). In contrast, the formation of these DNA-protein complexes was not blocked by the addition of an excess of the unrelated oligonucleotide AP-1 (Figure 4B, lane 6).

To identify the NF-κB family members that bind to the NF-κB motifs of the CD69 gene promoter, the binding reactions were preincubated with antibodies specific to p50, p65, c-Rel, p52, and RelB (Figure 4B). The anti-p50 antibody induced the supershifted bands or reduced the intensity of complexes κB1 and κB2 (Figure 4B, lane 7). The anti-p65 antibody induced supershifted bands or blocked the formation of complexes

κB1 and κB2 (Figure 4B, lane 8). The anti-c-Rel antibody induced the supershifted band and reduced the intensity of only complex κB1 (Figure 4B, lane 9). In contrast, the anti-p52 or anti-RelB antibody did not interfere with the formation of any of these complexes (Figure 4B, lanes 10 and 11). These results indicate that the complexes κB1 and κB2 correspond to p50/p65/c-Rel and p50/p65, respectively. These results suggest that *H. pylori* infection seems to induce CD69 gene expression at least in part through the induced binding of NF-κB family members to the NF-κB sites in the CD69 promoter region.

***NF-κB signal is essential for induction of CD69 expression by H. pylori in T cells***
We also examined whether the *H. pylori*-mediated up-regulation of CD69 gene expression involves signal transduction components in NF-κB activation. Activation of NF-κB requires the phosphorylation of two conserved serine residues of IκBα (Ser-32 and Ser-36)

**A**



**B**



**C**
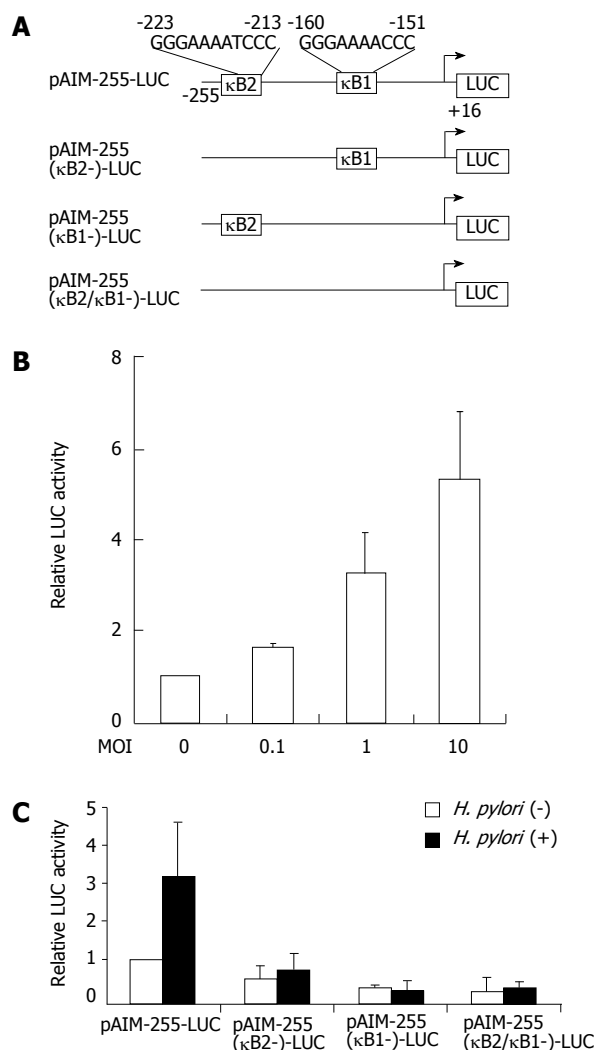


**Figure 3** *Helicobacter pylori* **activates the CD69 promoter through two nuclear factor-κB binding sites.** A: Schematic diagram of the CD69 reporter constructs containing the wild-type (pAIM-255-LUC) and internal deletion mutants of κB1 and/or κB2 motifs. LUC: Luciferase; B: *Helicobacter pylori (H. pylori)* infection increases CD69 promoter activity in a dose-dependent fashion. pAIM-255-LUC was transfected into Jurkat cells, and the cells were subsequently infected with *H. pylori* ATCC 49503 for 6 h; C: The indicated CD69 reporter constructs were transfected into Jurkat cells, and subsequently the cells were infected with ATCC 49503 for 6 h (the multiplicity of infection of 10). The activity is expressed relative to that of cells transfected with pAIM-255-LUC without further *H. pylori* infection, which was defined as 1. Datas are mean ± SD of three experiments.

and IκBβ (Ser-19 and Ser-23) within the N-terminal domain[24]. Phosphorylation leads to the ubiquitination and 26S proteasome-mediated degradation of IκBs, thereby releasing NF-κB from the complex and its translocation to the nucleus and activation of various genes[24]. The IKK complex, which is composed of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ), phosphorylates IκBs[24]. Previous studies indicated that members of the mitogen-activated protein kinase kinase kinase family mediate the physiologic activation of IKK[25]. These kinases include NIK[26]. IκBα-, IκBβ-, and IKKγ-dominant-interfering mutants and IKKα, IKKβ, and NIK kinase-deficient mutants were tested to

**A**



**B**
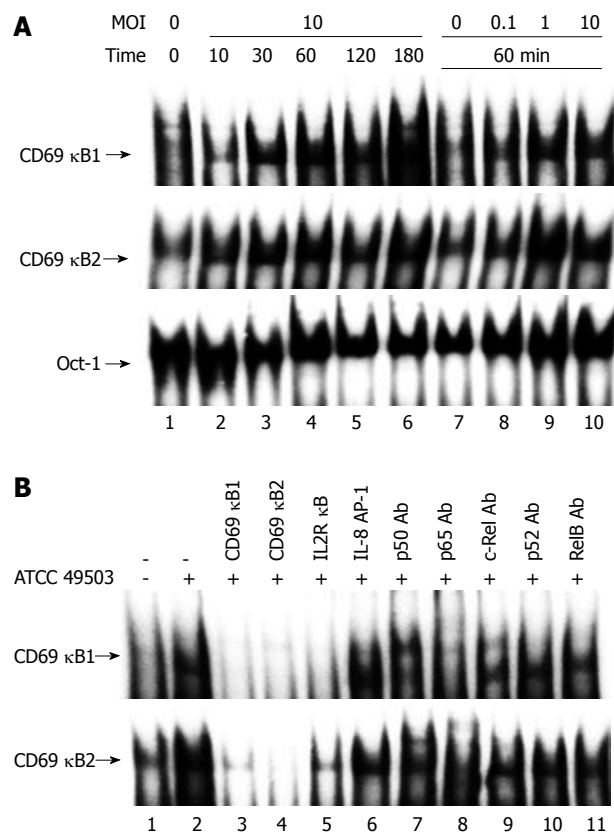


**Figure 4** *Helicobacter pylori* **infection induces nuclear factor-κB binding activity.** A: Nuclear factor (NF)-κB activation in Jurkat cells infected with *Helicobacter pylori (H. pylori)*, as evaluated by electrophoretic mobility shift assay (Oct-1). Nuclear extracts from Jurkat cells infected with different densities [the multiplicity of infection (MOI)] of *H. pylori* ATCC 49503 (lanes 7 to 10) for the indicated times (lanes 1 to 6) were mixed with oligonucleotide probes CD69 κB1 (top) and CD69 κB2 (middle), which contained the putative NF-κB motifs located at positions -160 and -223, respectively; B: Competition assays were performed with nuclear extracts from Jurkat cells infected with ATCC 49503 (MOI of 10) for 180 min. Where indicated, the excess amounts of each specific competitor oligonucleotide were added to the reaction mixture with each oligonucleotide probe (lanes 3 to 6). A supershift assay of NF-κB DNA binding complexes in the same nuclear extracts was also performed. Where indicated, appropriate antibodies (Ab) were added to the reaction mixture before the addition of probe CD69 κB1 (top) or CD69 κB2 (bottom). Arrows indicate the specific complexes, while arrowheads indicate the DNA binding complexes supershifted by the antibodies.

determine their abilities to inhibit the *H. pylori*-mediated activation of the CD69-driven reporter gene. The expression of these various inhibitory mutants abolished *H. pylori*-induced CD69 expression (Figure 5A). These results emphasize the importance of signaling components involved in the activation of NF-κB in *H. pylori*-induced activation of the CD69 promoter.

Because activation of the CD69 promoter by *H. pylori* infection requires the activation of NF-κB, we blocked NF-κB activation with Bay 11-7082, an inhibitor of IκBα phosphorylation[27], or LLnL, a proteasome inhibitor[28]. 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 the *H. pylori*-induced expression of CD69 mRNA (Figure 5B).
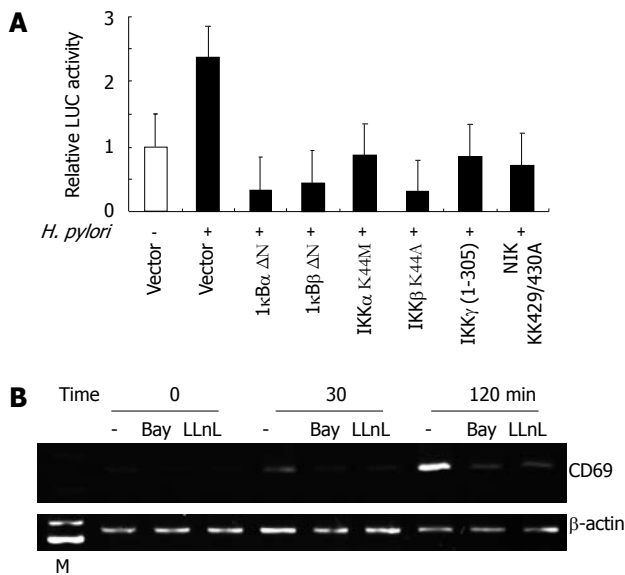
**Figure 5 Nuclear factor-κB signal is essential for the activation of CD69 expression by *Helicobacter pylori* in T cells.** A: Functional effects of IκBα-, IκBβ-, and IKKγ-dominant-interfering mutants and kinase-deficient IKKα, IKKβ, and NIK mutants on *Helicobacter pylori (H. pylori)*-induced activation of the CD69 promoter. Jurkat cells were transfected with the CD69 reporter construct (pAIM-255-LUC) and the indicated mutant plasmids or empty vector (pCMV4) and then infected with *H. pylori* ATCC 49503 for 6 h. Open bar: Luciferase (LUC) activity of the CD69 reporter construct and pCMV4 without *H. pylori* infection. All values were calculated as the change (n-fold) in induction values relative to the basal level measured in uninfected cells. Data are mean ± SD of three independent experiments. B: Bay 11-7082 and N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) inhibit CD69 mRNA expression induced by *H. pylori*. Jurkat cells were pretreated with Bay 11-7082 (20 μmol/L) or LLnL (20 μmol/L) for 2 h prior to *H. pylori* infection and subsequently infected with *H. pylori* ATCC 49503 for 30 or 120 min. CD69 mRNA expression on harvested cells was analyzed by reverse transcription-polymerase chain reaction. Lane M: Markers.

## DISCUSSION

Early studies showed that CD69 regulates the immune response by modulating the expression of various cytokines; CD69-deficient mice show increased anti-tumor and autoimmune responses caused at least in part by increased production of proinflammatory cytokines and chemokines[29,30]. Although the functions of CD69 have been studied extensively, there is little or no information on its role in the immune response against microbial pathogens. Recently, CD69 was reported to be a critical negative regulator of immune activation during intracellular bacterial infection, protecting infected mice against lethal tissue damage[31]. The present study explores the way in which *H. pylori* infection controls the expression of CD69 gene in T cells.

The main findings of the study were: (1) *H. pylori* deregulated the expression of CD69 in T cells; (2) CD69 protein was upregulated in gastric lymphocytes of patients with *H. pylori* gastritis; (3) the importance of *H. pylori cag*PAI in the induction of CD69 expression in T cells; and (4) *H. pylori* stimulates the NF-κB signaling pathway to activate CD69 gene expression and also to activate the CD69 promoter.

This is the first report to demonstrate that CD69

gene expression is regulated by *H. pylori*. Despite the development of immune responses against *H. pylori* infection, the bacteria are rarely eliminated, and colonization generally is persistent. Factors that contribute to the failure of the immune response to clear the organism remain elusive[31]. Recent studies have suggested that CD69 may downregulate the immune response through the production of the pleiotropic cytokine, transforming growth factor-β[32]. Thus, CD69 expressed on T cells may regulate the immune responses against *H. pylori* infection.

It has been reported that the inducible expression of CD69 gene is tightly regulated by transcription factors of the NF-κB, AP-1, EGR, and ATF/CREB families, which are rapidly activated through different signaling pathways[14,33]. However, nothing is known about the regulation of CD69 expression in T cells infected with *H. pylori*. We demonstrate herewith that *cag*PAI-positive *H. pylori* can induce the expression of the CD69 antigen and that this induction is mediated by an increase in the CD69 promoter activity. Deletion of the sequences that contain the κB1 and/or κB2 motifs abolished the response to *H. pylori*. Pharmacologic inhibition of NF-κB, as well as IκBα-, IκBβ-, IKKγ-dominant-interfering mutants and kinase-deficient IKKα, IKKβ, and NIK mutants, determined the role of NF-κB signaling molecules targeted by *H. pylori* to activate CD69 gene expression. Thus, our results suggest that NF-κB is essential for *H. pylori cag*PAI-mediated CD69 induction in T cells, and the two NF-κB sites (κB1 and κB2) appear to play an important role in this process.

Our results also demonstrated that the two NF-κB motifs of the CD69 promoter bind *H. pylori*-inducible NF-κB-related complexes. Antibodies directed against the different NF-κB proteins were used to identify the family members present in the DNA-protein complexes detected with the NF-κB motif-derived probes. These experiments demonstrated that the DNA-binding activities consisted of p50/p65/c-Rel and p50/p65 complexes binding to the κB1 and κB2 motifs, respectively. Although NF-κB clearly plays an important role in *H. pylori*-mediated induction of CD69, the role of CD69 in the control of immune responses against *H. pylori* infection needs to be further clarified. We are planning further studies using CD69-deficient mice to investigate the role of CD69 in the regulation of immune responses against *H. pylori* infection.

## COMMENTS

### Background

*Helicobacter pylori* (*H. pylori*) is regarded as the major cause of various gastric diseases. Despite the development of immune responses against *H. pylori* infection, the bacteria are rarely eliminated. Factors that contribute to the failure of the immune response to clear the organism remain elusive. Recently, the leukocyte receptor CD69 was reported to be a critical negative regulator of immune activation during bacterial infection.

### Research frontiers

CD69 is selectively expressed in chronic inflammatory infiltrates at the sites of active immune responses. However, whether and how *H. pylori* can induce the expression of CD69 has not been addressed in T cells. In this study, the authors demonstrate that *H. pylori* can induce CD69 expression through the activation of nuclear factor-κB and show that *cag* pathogenicity island (*cag*PAI) might be relevant in the induction of CD69 expression in T cells.

### Innovations and breakthroughs

CD69 staining was detected in mucosal lymphocytes and macrophages in specimens of patients with *H. pylori*-positive gastritis. *H. pylori* also induced CD69 expression in peripheral blood mononuclear cells and CD4^{+} T cells *in vitro*. This is the first study to report the regulation of intracellular events leading to CD69 expression by *H. pylori* infection in T cells. The results also demonstrate that the two nuclear factor-κB motifs of the CD69 promoter are important in *H. pylori*-mediated induction of CD69.

### Applications

By understanding how CD69 is induced and the role of CD69 in the control of immune responses against *H. pylori* infection, and by blocking its expression, this study may indicate a future strategy for elimination of *H. pylori*.

### Terminology

CD69 is a C-type lectin, disulfide-linked homodimer, type Ⅱ protein that can be induced after lymphocyte activation. The *cag*PAI is responsible for the secretion of the CagA effector through a type Ⅳ secretion system apparatus as well as transport of peptidoglycan.

### Peer review

This article by Mori *et al* described induction of CD69 expression by *cag*PAI-positive *H. pylori* infection. According to my literature review, this might be the first study reporting to demonstrate that CD69 gene expression is regulated by *H. pylori*. This manuscript is scientific and well-written.

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