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**Helicobacter pylori** Activates NF-κB via the Alternative Pathway in B Lymphocytes

Tomoya Ohmae,1* Yoshihiro Hirata,* Shin Maeda,** Wataru Shibata,* Ayako Yanai,**† Keiji Ogura,* Haruhiko Yoshida,* Takao Kawabe,* and Masao Omata*1

*Helicobacter pylori* causes various gastroduodenal diseases including gastric MALT lymphoma, but the mechanism underlying *H. pylori*-induced carcinogenesis is not known. The alternative pathway for NF-κB activation, which involves the processing of NF-κB2/p100 to p52, has been implicated in lymphocyte survival, attenuated apoptosis, and secondary lymphoid tissue development. In this study, we investigated *H. pylori*-induced activation of NF-κB through the alternative pathway in B lymphocytes. In immunoblot and EMSA, *H. pylori* induced NF-κB2/p100 processing to p52 and subsequent nuclear accumulation in IM-9 (human B cell line) cells and human peripheral blood B cells, but not in AGS (human gastric cancer cell line) cells. The activation of the alternative pathway was LPS-dependent but not cag pathogenicity island-dependent. Alternative pathway activation by *H. pylori* was associated with attenuated apoptosis. The expression levels of B lymphocyte chemoattractant, EBI-1 ligand chemokine, and stromal cell-derived factor-1α mRNAs were up-regulated in cocultured human B cells and in infected human gastric mucosa. In the infected mucosa, NF-κB2/p100 and p52 were detected immunohistochemically in the cytoplasm and nuclear compartments of lymphocytes, but not in epithelial cells. In summary, *H. pylori* activates the alternative NF-κB pathway in B lymphocytes. The effects on chemokine production and antiapoptosis mediated by *H. pylori*-induced processing of NF-κB2/p100 to p52 may drive lymphocytes to acquire malignant potential. The Journal of Immunology, 2005, 175: 7162–7169.

Gram-negative *Helicobacter pylori* is a bacterium that infects the human gastric mucosa (1). The infection is strongly associated with gastroduodenal diseases, such as chronic active gastritis, gastroduodenal ulcers, gastric adenocarcinoma (2–6), and gastric mucosa-associated lymphoid tissue (MALT) lymphoma, which is a low-grade B cell lymphoma. The etiological association between *H. pylori* infection and gastric MALT lymphoma was established in the early 1990s (7, 8). Subsequently, it has been shown that 50–80% cases of gastric MALT lymphoma regress after *H. pylori* eradication by antibiotics (9–11), and bacterial eradication is now considered to be the first-line therapy for this neoplasm. However, the mechanism by which *H. pylori* contributes to the development of MALT lymphoma remains unclear.

The transcription factor NF-κB regulates the expression of genes that are involved in inflammation, cell proliferation, and apoptosis (12, 13). Five members have been identified in mammals: NF-κB1/p105, NF-κB2/p100, c-Rel, RelA, and RelB. In the classical pathway, NF-κB activation is tightly controlled by the IkB kinase (IKK)2 complex, which consists of two catalytically active kinases, IKKα and IKKB, and an inactive compound, IKKγ (14, 15). Activation of the IKK complex leads to phosphorylation, ubiquitination, and proteolytic degradation of the inhibitory NF-κBα (IkBα), which allows NF-κB homodimers or heterodimers to translocate to the nucleus. Various cell surface receptors, such as TNF-α receptor and IL-1-like/TLR family members, can activate the classical pathway (16).

Recently, it has been reported that the alternative pathway for NF-κB activation contributes to the development, survival, and attenuation of apoptosis of B cells. The alternative NF-κB pathway involves the processing and cleavage of NF-κB2/p100 precursor to p52, which are triggered by p100 phosphorylation by NF-κB-inducing kinase (NIK) and IKKα (17). This pathway is also known to be activated by stimulation of the lymphotoxin (LT)β receptor, BAFF (B cell activating factor belonging to the TNF family) receptor, or CD40, but not by the TNF-α receptor (18–20). The p52 molecule, which forms a heterodimer with another NF-κB subunit, translocates to the nucleus and binds to the NF-κB sites. The activation of the alternative NF-κB pathway and subsequent up-regulation of target genes is reportedly necessary for the secondary development of lymphoid organs, such as the spleen, lymph nodes, and Peyer’s patches (21).

Two major chromosomal translocations, t(11;18)(q21;q21) and t(1;14)(p22;q32), are known to be associated with MALT lymphoma; the former is more common, being observed in approximately one-third of cases (22, 23). Dierlammm et al. (24) have reported that t(11;18)(q21;q21) results in the fusion of API2 (apoptosis inhibitor gene 2) on 11q21 with MALT1 (MALT lymphoma-associated translocation gene 1) on 18q21, which is known as API2-MALT1. The fusion protein produced by API2-MALT1 strongly enhances classical NF-κB pathway activation (25). In contrast, t(1;14)(p22;q32) leads to overexpression of Bcl-10 protein with a frame shift mutation in the CARD (caspase recruitment domain), which also leads to constitutive activation of NF-κB (26–28). Thus, it is now generally recognized that enhanced activation of the classical NF-κB pathway is closely linked to the pathogenesis of MALT lymphoma (29, 30).
Although virulent strains of *H. pylori* are known to activate NF-κB through the classical pathway in epithelial cells (31, 32), NF-κB activation in lymphocytes has not been well defined. In this study, we analyzed the activation of NF-κB in lymphocytes following stimulation with *H. pylori*, and focused on the contribution of the alternative pathway and subsequent phenotypic changes. We demonstrate that *H. pylori* induces the processing of NF-κB to p52 and subsequently the target gene up-regulation in B lymphocytes. *H. pylori* also induces the attenuation of apoptosis via the alternative pathway activation in B lymphocytes.

Materials and Methods

**Bacterial strains**

The TN2 strain, which is positive for cytotoxic-associated gene A (CagA) and for vacuolating cytotoxin A (VacA), was generously provided by Dr. Masafumi Nakao (Takeda Chemical Industries, Osaka, Japan). The TN2-isogenic *cagA*-negative and *cagE*-negative mutants (TN2Δ*cagA* and TN2Δ*cagE*, respectively) were constructed by insertion of the kanamycin-resistant gene, as previously described (33). In the coculture experiments, *H. pylori* was cultured for 24 h in Brucella broth that contained 7.5% PBS, centrifuged, washed with PBS, resuspended in RPMI 1640 that contained 10% FBS, and used immediately thereafter in the assays. The bacterium to cell ratio was 5:1 in all experiments, except in the TUNEL to detect cell death. Heat-killed *H. pylori* were prepared by heating the bacteria at 80°C for 60 min.

**Preparation of *H. pylori* LPS**

*H. pylori* (TN2) LPS was prepared by the method of Galanos et al. (34). Briefly, 20 mg (dry-weight) of *H. pylori* cells were washed in PBS, suspended in cold distilled water (10 mg/ml), and then poured into 10 volumes of cold acetone (−20°C). The sediments were dried under vacuum, ground, and suspended in water (6% w/v at 65°C). An equal volume of phenol solution (90% w/v) was added and, after incubation, the mixture was centrifuged at 10,000 × g for 10 min at 4°C. After removing the upper aqueous layer, the LPS was precipitated by pouring the solution into 10 volumes of cold acetone (−20°C). The precipitate was collected, resuspended in a small amount of distilled water, and ultracentrifuged (100,000 × g) three times for 6 h. The LPS was used in 20 μg/ml.

**Cell lines and primary mammalian B lymphocytes**

The human B lymphoblastoid cell line IM-9 was purchased from the Japanese Collection of Research Bioresources Cell Bank/National Institute of Health Sciences (Tokyo, Japan) and maintained in RPMI 1640 that contained 10% FBS. The human gastric cancer cell line AGS was purchased from American Type Culture Collection and maintained in Ham’s F12 medium, which contained 10% FBS.

Human peripheral blood B lymphocytes were obtained from heparinized 50 ml blood samples drawn from healthy volunteers. Each blood sample was mixed with an equal volume of HBSS (Sigma-Aldrich), and the tube was inverted gently several times. Each sample was layered on 25 ml of Ficoll-Paque Plus (Amersham Biosciences) and centrifuged at 400 × g for 40 min at 20°C. The lymphocyte layer was transferred to a clean tube, centrifuged, washed twice with PBS, and incubated with anti-human CD22 microbeads (Miltenyi Biotec) for 15 min at 10°C, centrifuged at 400 × g for 10 min, and washed with PBS. Then the suspensions were sorted by passage through an MS magnetic column (Miltenyi Biotec). Approximately 5 × 10^6 B cells were collected from one 50-ml blood sample.

Murine splenic B cells were prepared from 6- to 8-wk-old C57BL/6J male mice (CLEA Japan). The experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals in our division, and were approved by the Ethical Committee. Murine splenic cells were isolated and resuspended, and the erythrocytes were lysed with ACK lysis buffer. The cell suspension was incubated with rat anti-mouse CD45R/B220 mAb (1/100 dilution; BD Pharmingen) for 30 min on ice, centrifuged at 400 × g for 10 min, and washed with 10 ml of PBS. Finally, the sample was incubated with MACS goat anti-rat IgG microbeads (Miltenyi Biotec) for 15 min at 10°C, washed with PBS, and sorted by passage through an MS magnetic column (Miltenyi Biotec). Approximately 1 × 10^6 splenic B cells were obtained using this protocol.

**Abs and reagents**

The anti-human NF-κB/p100 and anti-p52 Ab was purchased from Upstate Biotechnology, the anti-mouse NF-κB/p100 and anti-p52 Ab was from Santa Cruz Biotechnology, the anti-human and anti-mouse phospho-IκBα Ab was from Cell Signaling Technology, and the anti-human and anti-mouse actin Abs were from Sigma-Aldrich. Ammonium pyrrolidine dithiocarbamate (APDC, 100 μM; Dojindo Laboratories) was used to inhibit the classical NF-κB pathway.

**Whole cell, cytoplasmic, and nuclear protein extracts**

Cells were washed with PBS and lysed in ice-cold 1% Triton X-100 (Sigma-Aldrich) and Complete Mini EDTA-free (Roche Diagnostics). The lysate was centrifuged at 10,000 × g for 5 min at 4°C, and the supernatant was used as the whole cell protein extract. Cytoplasmic and nuclear extracts were prepared from IM-9 cells using the PARIS kit (Ambion) according to the manufacturer’s instructions.

**RNA interference**

For small interfering RNA targeted to IKKα, we generated 21-base complementary RNAs with two thymidine residues (dTdT) at the 3’ end, i.e., 5′-(AA)GCGACGCUCUUCAGGGAAC-3′, as previously described (35). The nonsilencing RNA sequence 5′-(AA)TTTCGCAAAGCCTGAC-3′ was also used. The NF-κB/p100 small interfering (si)RNA was purchased from Santa Cruz Biotechnology. These siRNAs were transfected with Lipofectamine 2000 (Invitrogen Life Technologies) into 1 × 10^5 IM-9 cells in 3.5-cm dishes. Three hours after the addition of the siRNA, the IM-9 cells were washed, cocultured with *H. pylori*, and used in the following assays.

**Immunoblotting**

The whole cell, cytoplasmic, and nuclear extracts were electrophoresed on SDS-PAGE, transferred to a polyvinylidine difluoride membrane (Amerham Biosciences) and blocked for 1 h with TBST plus 5% dry milk. The membrane was probed overnight at 4°C with the primary Abs, and subsequently washed and incubated with the secondary peroxidase-conjugated Ab. The immunocomplexes were detected with the Enhanced Chemiluminescence Detection kit (ECL Advance; Amerham Biosciences).

**EMSA**

NF-κB DNA-binding activity was analyzed in the nuclear extracts (10 μg) using the EMSA kit (Panomics) according to the manufacturer’s instructions. For the Ab supershift analysis, the nuclear extracts were preincubated for 30 min with rat anti-κB antisera against human NF-κB/p100 and p52 (Upstate Biotechnology).

**Real-time PCR analysis and RT-PCR**

Total cellular RNA samples were isolated using ISOGEN (Nippon Gene) from human peripheral blood B cells or endoscopic gastric biopsy specimens, which were obtained from healthy volunteers and patients with *H. pylori*-induced gastritis. The cDNAs were generated by reverse transcription from 1 μg of total RNA using the ImProm-II Reverse Transcription System (Promega). The mRNA expression levels of B lymphocyte chemotactic factor (BLC), EBI-1 ligand chemokine (ELC), stromal cell-derived factor-1α (SDF-1α), and GAPDH were analyzed by quantitative real-time PCR or RT-PCR. Real-time PCR amplification was performed according to the SYBR Green PCR Master Mix (Applied Biosystems) protocol. Relative quantification of gene expression was obtained using GAPDH mRNA as an internal standard. To perform RT-PCR, the PCR mixtures were incubated for 10 min at 94°C, followed by 30 cycles of 30 s at 96°C, 1 min at 54°C, and 1 min 12°C. The PCR products were analyzed by 1.5% agarose gels. All primer sequences are available upon request.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded gastric biopsy specimens, which were obtained endoscopically from human healthy volunteers and patients with *H. pylori*-induced gastritis, were examined immunohistochemically for NF-κB/p100 and p52. Sections cut at a thickness of 3 μm were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked using 3% H2O2. Immunohistochemistry was performed using the anti-human NF-κB/p100 and anti-p52 Ab (1/1000 dilution) and the avidin-biotin method (DAKO LSAB2 System; DAKO). In immunofluorescent staining, anti-human NF-κB/p100 and anti-p52 Ab and anti-CD20 Ab
Abs. IM-9 cells (C) and human peripheral B cells (D) were treated with H. pylori, BAFF, or TNF-α for the indicated time periods. The cell lysates were analyzed by immunoblotting with the anti-p100/p52 and anti-actin Abs. Data shown are representative results of three independent experiments.

**Apoptosis detection by TUNEL staining**

IM-9 cells that were pretreated for 4 h with the control (nonsilencing), IκBα, or NF-κB2/p100 siRNA were plated in a 12-well plate (1 x 10^5 cells/well) in RPMI 1640 medium that contained 5% FBS, and then stimulated with H. pylori, BAFF, or TNF-α for the indicated time periods.

**Results**

*H. pylori* activates both the classical and alternative NF-κB pathways in B lymphocytes

Initially, we examined whether *H. pylori* activated NF-κB through the classical pathway in B lymphocytes. As determined by immunoblotting, *H. pylori* strain TN2 induced the phosphorylation of IκBα in IM-9 cells and human peripheral blood B cells within 15–20 min; this was followed by rapid degradation over 2 h (Fig. 1A and B), which indicates the involvement of the alternative NF-κB pathway in B cells.

We also examined the alternative pathway, which involves the cleavage of NF-κB2/p100 to p52. The level of p52 protein increased in IM-9 cells and human peripheral blood B cells (Fig. 1C and D), which indicates the involvement of the alternative NF-κB pathway in B cells. In contrast to BAFF stimulation, *H. pylori* stimulation did not decrease the level of NF-κB2/p100. We speculate that this is due to the up-regulation of NF-κB2/p100 through the classical pathway (20). Therefore, we investigated whether *H. pylori* increased the level of p52 in the presence of APDC, which is an inhibitor of the classical NF-κB pathway. APDC suppressed the increase and the subsequent degradation of phospho-IκBα in *H. pylori*-induced IM-9 cells (Fig. 2A), whereas the level of p52 still increased, but that of NF-κB2/p100 did not (Fig. 2B). These results suggest that *H. pylori* activates both the classical and alternative pathways in B lymphocytes. As shown in Fig. 2C, activation of the alternative pathway was not observed in AGS cells.

H. pylori induces nuclear translocation of p52

We assessed the nuclear translocation of p52 by immunoblotting cytoplasmic and nuclear extracts. As shown in Fig. 3A, *H. pylori* induced p52 accumulation in the nuclear extracts of IM-9 cells within 8–24 h. In contrast, there was no increase in the level of p52 in the cytosolic fraction, which confirms the translocation of p52 into the nucleus. We also analyzed the DNA-binding activity of the nuclear NF-κB complex by EMSA. As shown in Fig. 3B, although
the nuclear extract from untreated IM-9 cells showed low NF-κB DNA-binding activity, this activity was strongly enhanced by stimulation with *H. pylori* or BAFF. Binding was confirmed by supershifting with an Ab directed against p52. The *blc*, *elc*, and *sdf-1α* genes are reportedly up-regulated by LTβ1 stimulation via the alternative NF-κB pathway, but not through the classical pathway in splenocytes (20). Indeed, *blc*, *elc*, and *sdf-1α* mRNA were up-regulated by *H. pylori* TN2 by 5.4 ± 1.0-fold, 6.8 ± 0.4-fold, and 23.1 ± 3.4-fold, respectively, in human peripheral blood B cells at 12 h (Fig. 3C).

**Alternative pathway activation does not depend on cag pathogenicity island (cagPAI) gene**

We used the isogenic mutants TN2ΔcagA and ΔcagE to investigate whether the activation of the alternative pathway is dependent on cagPAI of *H. pylori*. Changes in the p52 levels were assessed by immunoblotting. As shown in Fig. 4A, the p52 levels in IM-9 cells were increased by coculture with ΔcagA or ΔcagE, as well as with the wild-type strain. Stimulation of the IM-9 cells with heat-inactivated TN2 bodies or purified LPS enhanced the production of p52, as well as LPS from *S. typhimurium* and *E. coli* (Fig. 4B and C). These findings indicate that *H. pylori* activates the alternative pathway independently of cagPAI, and that LPS is one of the effectors for B lymphocytes.

**Activation of the alternative NF-κB pathway via NIK and IKKα**

We investigated the roles of NIK and IKKα in the activation of the alternative pathway by *H. pylori*. Murine splenic CD45R/B220-positive B cells from C57BL/6JcJ and ALY/NscJcJ-aly/aly mice, the latter having an NIK point mutation (36), were stimulated with *H. pylori* or BAFF. As shown in Fig. 5A, the level of p52 was increased by *H. pylori* or BAFF in B cells from C57BL/6JcJ mice but not in those from ALY/NscJcJ-aly/aly mice. In addition, we

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**FIGURE 3.** Functional analysis of p52 in *H. pylori*-infected lymphocytes. A, Nuclear and cytoplasmic extracts of *H. pylori*-infected IM-9 cells were analyzed by immunoblotting for p100 and p52. TATA box binding protein (TBP) and Sos1 were used as the nuclear and cytoplasmic loading controls, respectively. B, Nuclear extracts from *H. pylori*- or BAFF-treated IM-9 cells were prepared. Equivalent amounts of the nuclear extracts were incubated with the NF-κB probe and anti-p52 Ab, and EMSA was performed. Unstimulated cells (first lane), *H. pylori*-stimulated cells (second lane), *H. pylori*-stimulated cells (third lane) in the presence of the anti-p52 Ab, and BAFF-stimulated cells (last lane). C, Real-time PCR analysis for the expression of *blc*, *elc*, *sdf-1α*. Human peripheral blood B cells were stimulated with *H. pylori* or BAFF for 12 h, and total RNA was prepared. The *gapdh* gene was used as the control for mRNA expression.

**FIGURE 4.** The *H. pylori* virulence factor cagPAI does not play a significant role in the alternative NF-κB pathway. A, IM-9 cells were infected with the TN2 wild-type, cagA mutant, and cagE mutant for 24 h. The levels of p100, p52, and actin were assessed by immunoblot analysis. IM-9 cells were stimulated with heat-killed TN2 (B) or LPS (C) (*H. pylori*, *S. typhimurium*, or *E. coli*) for indicated hours, and immunoblotting was performed.
transfected siRNA for IKKα into IM-9 cells. *H. pylori* treatment increased the level of p52 in IM-9 cells that were transfected with nonsilencing siRNA but not in cells that contained the IKKα siRNA (Fig. 5B). These results suggest that *H. pylori* activates the alternative NF-κB pathway via NIK and IKKα in B cells.

### H. pylori inhibits B cell apoptosis via the alternative pathway

We investigated the potential linkage between alternative NF-κB pathway activation by *H. pylori* and lymphocyte apoptosis. First, we used siRNA for IKKα and NF-κB/p100 to inhibit the alternative pathway, and performed immunoblotting to confirm the RNA interference effects. The siRNA for IKKα effectively suppressed the levels of the IKKα and p52 proteins, and the siRNA for NF-κB/p100 reduced the p100 and p52 protein level (Fig. 6A). Second, we tested apoptosis induction by *H. pylori* using TUNEL staining. As shown in Fig. 6B, apoptotic cells induced by *H. pylori* were further increased by IKKα or NF-κB2/p100 silencing. Indeed, *H. pylori* induced apoptosis in 3.0 ± 0.3%, 7.4 ± 1.3%, and 8.6 ± 0.6% of the nonsilencing, IKKα, and NF-κB2/p100 siRNA affected cells, respectively. Similar results were obtained by cell death detection ELISA analysis (data not shown). These results suggested that the *H. pylori*-induced alternative NF-κB pathway activation was associated with antiapoptotic effects on B lymphocytes.

**Alternative NF-κB pathway activation in the human gastric mucosa**

We examined human gastric biopsy specimens obtained from subjects with or without *H. pylori* infection (as determined by culture) using immunohistochemistry for NF-κB2/p100 and p52 (Fig. 7A). NF-κB2/p100 and p52 were detected in infiltrating inflammatory cells in the *H. pylori*-infected mucosa. Some of the gastric epithelial cells in the *H. pylori*-infected mucosa were also positive for NF-κB2/p100 or p52, although these cells were stained exclusively in the cytoplasm and were so to a lesser extent than in the lymphocytes. To confirm these finding, we performed immunofluorescent double

### FIGURE 5. *H. pylori* activates the alternative NF-κB pathway through NIK and IKKα.

A. Splenic B cells were prepared from C57BL/6 mice and NIK point mutation aly/aly mice. The cells were treated with *H. pylori* or BAFF for 8 h, and protein extracts were subjected to immunoblotting for p100, p52, and actin. B. IM-9 cells were transfected with control siRNA and IKKα siRNA for 6 h, and then infected with *H. pylori* for the indicated time periods. Immunoblotting was performed for IKKα, NF-κB2/p100, p52, and actin.

### FIGURE 6. *H. pylori* inhibits B cell apoptosis via the alternative pathway.

A. Nonsilencing, IKKα-specific, and NF-κB2/p100-specific siRNAs were transfected into IM-9 cells. The levels of IKKα, NF-κB2/p100, and p52 were analyzed by immunoblotting. The siRNA for IKKα decreases the levels of both IKKα and p52 proteins, whereas the siRNA for NF-κB2/p100 reduces the level of p100 and p52 protein. B. IM-9 cells, which were transfected with siRNAs as described in A, were incubated with *H. pylori* for 48 h. The cells were analyzed for apoptosis by TUNEL staining. TUNEL positive cells were visualized by fluorescein (green) and nuclei were counterstained with propidium iodide (red). C. Apoptotic cells per total cell ratio was calculated. The values shown represent the mean ± S.D. from three independent experiments. Statistically significant differences, compared with nonsilencing siRNA and IKKα or NF-κB2/p100 siRNA, are defined at p < 0.05.
staining for NF-κB2/p100 and CD20 B cell surface Ag. The infiltrating inflammatory cells are positive for both NF-κB2/p100 and CD20 (Fig. 7B). Furthermore, in high magnification image, NF-κB/p100 or p52 was positive in both cytoplasms and nucleus of the cell (Fig. 7C). In contrast, in H. pylori uninfected mucosa, B cells in the mucosa were positive for NF-κB2/p100 only in cytoplasm (Fig. 7D), suggesting that the alternative pathway of the NF-κB was not activated. These findings are consistent with the in vitro findings that p52 was increased by H. pylori in nucleus of human B cells. The levels of blc, elc, and sdf-1α mRNA expression in the gastric tissues were assessed by RT-PCR and found to be markedly up-regulated in H. pylori-infected mucosa, but were very low in uninfected mucosa (Fig. 7E). These findings indicated that NF-κB alternative pathway activation and its downstream chemokine expression were actually induced in vivo as well as in vitro experiments.

Discussion
In this report, we have shown that H. pylori activates NF-κB in lymphocytes through both the classical and alternative pathways. As for the classical pathway, chromosomal abnormalities that cause API2-MALT1 fusion protein or Bcl-10 overexpression are reported to activate the NF-κB classical pathway continuously and to contribute to the MALT lymphoma (25–28). This finding may be one of the reasons why the eradication therapy of H. pylori is not effective in patients with these chromosomal abnormalities. Otherwise, it has been reported that mice with a homozygous deletion of the C-terminal ankyrin repeat of NF-κB2/p100, which induces constitutive p52 formation, have marked gastric hyperplasia with lymphocytic infiltration, resulting in early postnatal death (37). Thus, the activation of the alternative pathway may also play a role in lymphocytic infiltration and proliferation in H. pylori-infected gastric mucosa.

We also showed the up-regulation of blc, elc, and sdf-1α expression following exposure to H. pylori. A previous immunohistochemical study has shown that BLC (BCA-1) is expressed in H. pylori-infected gastric MALT and MALT lymphoma tissues (38). BLC, ELC, and SDF-1α are all chemokines that play roles in lymphocytic chemotaxis, recruitment, and lymphoid tissue development (39–41). Thus, these H. pylori-induced chemokines may promote B cell infiltration through autocrine or paracrine mechanisms, leading to the development of MALT lymphoma.

Interestingly, in the present study, NF-κB activation through the alternative pathway by H. pylori was found to attenuate lymphocyte apoptosis. However, in the BrdU ELISA, no advantageous effects on cell proliferation were associated with alternative pathway activation (data not shown). Thus, H. pylori may induce lymphocyte proliferation or malignant transformation by enhancing cell survival through antiapoptotic effects that are mediated by the alternative NF-κB pathway, rather than by direct stimulation of cell proliferation.

In this study, we also discovered that cagPAI does not contribute to the activation of NF-κB in lymphocytes, which is similar to the situation in monocyes, as we have previously reported (42). Recently, it has been reported that ectopic expression of CagA in IL-3-dependent B cells inhibits cell proliferation by suppressing JAK-STAT signaling (43). It seems likely that CagA does not enhance antiapoptotic or proliferative responses in lymphocytes. LPS derived from E. coli, as well as BAFF, LTβ1, and CD40L, reportedly activate the alternative pathway in splenocytes (44). In the current study, we show that LPS derived from H. pylori also activates the alternative pathway, as well as LPS from E. coli or S. typhimurium. This suggests bacteria expressing LPS may activate this pathway as well as H. pylori activation in vitro. However, because H. pylori is the only bacterium that infects in human gastric lumen continuously, we consider that LPS derived from other bacteria cannot be responsible for gastric MALT lymphoma development. Otherwise, H. pylori does not activate the alternative pathway in epithelial cells, as shown in AGS cells and confirmed...
in the immunohistochemical studies. The different roles of the alternative pathway in epithelial cells and lymphocytes may reflect the differential expression of cell surface receptors, especially the LPS receptor TLR4. Indeed, TLR4, which is indispensable for classical pathway activation in lymphocytes, is expressed poorly in gastric epithelial cells (45), whereas it is expressed strongly in lymphocytes and monocytes. Further studies on TLR4 may help us to understand the mechanisms of H. pylori-induced alternative pathway activation.

NF-κB activation through the alternative pathway is slower than through the classical pathway. In general, signal-induced processing of p100 required the new or continued synthesis of a protein, which could explain the slow onset of processing upon stimulation. For example, it was previously reported that the processing of p100 to p52 induced by BAFF (18), LPS (44), LTβ (44), or CD40L (19) was inhibited when cycloheximide was pretreated. We also analyzed whether H. pylori-induced this pathway was inhibited by cycloheximide and found that H. pylori could not induce p100 processing in cycloheximide pretreated IM-9 cells (data not shown). Thus, we consider that H. pylori could activate this pathway via new or continued synthesis of a protein as well as other factors, which could activate the alternative pathway such as BAFF. We cannot exclude a possibility that some indirectly factors in this pathway via new or continued synthesis of a protein as well as components such as dead bacterial bodies and LPS, activates NF-κB. Thus it is likely that H. pylori, and its related components such as dead bacterial bodies and LPS, activates NF-κB classical and alternative pathway in human B cells in the gastric mucosa. In our current study, we focused on the H. pylori-induced activation of NF-κB alternative pathway. We have not assessed whether the direct involvement of Bcl-10 or MALT1 proteins in dysregulated NF-κB activation by classical and alternative pathway in B cells may promote malignant transformation.

In conclusion, H. pylori activates NF-κB in B lymphocytes not only through the classical pathway but also through the alternative pathway. H. pylori LPS is a candidate effector for the alternative pathway activation. The alternative pathway activation by H. pylori may contribute to the development of gastric MALT lymphoma, possibly by suppressing B cell apoptosis.

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Disclosures
The authors have no financial conflict of interest.

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