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

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

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) complex, which consists of two catalytically active kinases, IKKe and IKKβ, and an inactive compound, IKKγ (14, 15). Activation of the IKK complex leads to phosphorylation, ubiquitination, and proteolytic degradation of the inhibitory NF-κBα (IκBα), 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 lymphotixin (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). Dierlamm 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/p100 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 cytotoxin-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 (TN2ΔcagA and TN2ΔcagE, 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% FBS, 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 (TN2) LPS was prepared by the method of Galanos et al. (34).

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#### Cell lines and primary mammalian B lymphocytes

The human B lymphoblastoid cell line IM-9 was purchased from the Japanese Collection of Research Resources Cell Bank (National Institute of Health Sciences, Tokyo, Japan) and maintained in Ham’s F12 medium (Invitrogen Life Technologies) supplemented with 10% FBS. The human gastric cancer cell line AGS was purchased from the American Type Culture Collection and maintained in Ham’s F12 supplemented with 10% FBS. The mouse B cell lines, B6.MRL.Aldr-12Smp/5rcr (B6), B6.MRL.Aldr-12Smp/5rcr (B6.129), and B6.MRL.Aldr-12Smp/5rcr (B6.129.2) were obtained from the animal facilities in our division, and were approved by the Ethical Committee of Research and Care of Laboratory Animals in our division, and were approved by the Ethical Committee of Research and Care of Laboratory Animals in our division.
**H. pylori INDUCES ANTIAPOPTOTIC EFFECT ON B CELLS**

**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 IkBα in IM-9 cells and human peripheral blood B cells within 15–20 min; this was followed by rapid degradation over 2 h (Fig. 2A, B, and E), which indicates that *H. pylori* activates NF-κB through the classical 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. 1, A and B), 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), whereby the level of p52 still increased, but that of NF-κB 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 super-shifting with an Ab directed against p52. The blc, eIc, 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, eIc, 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. 4, B 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/6Jcl and ALY/NscJcl-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/6Jcl mice but not in those from ALY/NscJcl-aly/aly mice. In addition, we

**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, eIc, 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-κB/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-κB/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-κB/p100 and p52 (Fig. 7A). NF-κB/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-κB/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 findings, 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-κB/p100, p52, and actin.](http://www.jimmunol.org/)

![FIGURE 6. H. pylori inhibits B cell apoptosis via the alternative pathway. A, Nonsilencing, IKKα-specific, and NF-κB/p100-specific siRNAs were transfected into IM-9 cells. The levels of IKKα, NF-κB/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-κB/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-κB/p100 siRNA, are defined at p < 0.05.](http://www.jimmunol.org/)
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 cytoplasm 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, etc, 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. p52 was not stained in the nucleus of the cells. E, Total RNA was extracted from the gastric mucosa samples of individuals who were either infected (n = 3) or not infected (n = 3) with H. pylori, and RT-PCR for blc, etc, sdf-1α, and gapdh mRNAs was performed.

**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, etc, 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 disadvantageous effect 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 monocytes, 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 such as cytokines or chemokines induced by *H. pylori* are associated with the processing of p100. This possibility has not analyzed even in the case of BAFF or LTβ. Further examination is needed to resolve this query.

In the normal gastric mucosa, B lymphocytes are rarely in direct contact with bacteria in the gastric lumen. *H. pylori* often contacts with gastric epithelial cells and once *H. pylori* infects the mucosa, it induces lymphocytic chemotaxis and infiltration, which facilitate interactions between B cells and *H. pylori*-specific T cells (46, 47), and activate NF-κB in NK-activated B cells. So it may occur that these cells involved in *H. pylori* infection also contribute to the NF-κB activation. However, *H. pylori* also causes gastric erosion or ulcer, then it may occur that *H. pylori* contact directly with infiltrating inflammatory cells including B lymphocytes in the lamina propria. 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.

References


