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BH3-only protein Bim is associated with the degree of *Helicobacter pylori*-induced gastritis and is localized to the mitochondria of inflammatory cells in the gastric mucosa

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Abstract

BH3-only protein, Bim, is a pro-apoptotic protein that mediates mitochondria-dependent cell death. However, the role of Bim in *Helicobacter pylori*-associated gastritis remains unclear. This study aimed to assess the cellular localization of Bim and its possible role in *H. pylori*-induced gastritis. The study was conducted on biopsy specimens obtained from 80 patients who underwent upper gastrointestinal endoscopy (*H. pylori*-negative: n = 30, positive: n = 50). Association between Bim mRNA expression and severity of gastritis was evaluated and the localization of Bim was examined by immunofluorescence. Bim mRNA expression was positively correlated with the degree of gastritis, as defined by the Sydney system. Immunohistochemical analysis confirmed increased Bim expression in *H. pylori*-infected gastric mucosa compared with uninfected mucosa in both humans and mice. Bim localized in myeloperoxidase- and CD138-positive cells of *H. pylori*-infected lamina propria and submucosa of the gastric tract, indicating

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Disclosures

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that this protein is predominantly expressed in neutrophils and plasma cells. In contrast, Bim did not localize in CD20-, CD3-, or CD68-positive cells. Bim was expressed in the mitochondria, where it partially co-localized with activated Bax and cleaved-PARP. In conclusion, Bim is expressed in neutrophils and plasma cells in *H. pylori*-associated gastritis, where it may participate in the termination of inflammatory response by causing mitochondria-mediated apoptosis in specific leucocytes.

Keywords

BH3-only protein; Bim; Bcl-2; Bax; apoptosis; gastritis; mitochondria; *Helicobacter pylori*

Introduction

Helicobacter pylori is a gram-negative spiral bacterium that infects more than 50% of the world's population (1). *H. pylori* infection triggers inflammation of the gastric mucosa, leading to the development of gastritis, peptic ulcer, gastric cancer, and lymphoma of mucosa-associated lymphoid tissue (1–5). *H. pylori* infection induces neutrophil and monocyte infiltration in the gastric mucosa, and the extent of mucosal injury is directly proportional to the degree of infiltration of these cells (6–10). Neutrophils, which are considered the first line of cellular defense in cases of infection or injury, are required for the onset of *H. pylori*-induced gastritis and are responsible for inducing inflammatory reactions upon contact with bacteria (7, 9). The long life span of neutrophils may contribute to the severity of gastritis (11). Therefore, it is crucial to understand the apoptotic pathway activated in inflammatory cells during chronic gastritis. Neutrophils that accumulate at the site of injury primarily express cytosolic myeloperoxidase (MPO), a multi-functional heme protein, in azurophilic granules. MPO plays a role in antimicrobial responses, and it also contributes to *H. pylori*-related gastric ulcers through an oxidative process (12). Interestingly, MPO also inhibits neutrophil apoptosis, while a membrane marker of the azurophilic granule is downregulated during neutrophil apoptosis (13). Moreover, previous studies have demonstrated that MPO localizes to the nucleus and binds to DNA, where it repairs DNA strand breaks during conditions of stress (14).

Mitochondria-induced apoptotic pathway, which is regulated by the Bcl-2 family of proteins, plays an important role in leucocyte death (15). The pro-apoptotic proteins, Bcl-2-associated X protein (Bax) and Bcl-2 antagonistic killer (Bak), located on the mitochondrial membrane, function as “executioners” by mediating the release of cytochrome c into the cytosol (16). Anti-apoptotic proteins such as B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xL), and myeloid cell leukemia 1 (Mcl-1) prevent cytochrome c release from the mitochondria. BH3-only proteins such as BH3 interacting-domain death agonist (Bid), Bcl-2-associated death promote (Bad), Bcl-2 interacting mediator of cell death (Bim), Bcl-2-interacting killer (Bik), Bcl2 modifying factor (Bmf), p53 upregulated modulator of apoptosis (Puma), Harakiri (Hrk), and Noxa are activated by apoptotic stimuli (17, 18), which, in turn, triggers the activation of Bax/Bak, most likely by replacing anti-apoptotic group members (19, 20). Recent studies have demonstrated that suppression of Bim, a BH3-only protein, provided at least partial protection from spontaneous apoptosis of neutrophils

(17, 20, 21). Therefore, Bim is thought to play an important role in the hematopoietic system during apoptosis (22).

H. pylori infection initiates both the pro-apoptotic and pro-survival pathways (23, 24). However, the role of BH3-only proteins during *H. pylori* infection remains unclear. Bim mRNA is upregulated in *H. pylori*-infected gastric mucosa. Moreover, exposure to VacA toxin induced endoplasmic reticulum stress-mediated upregulation of Bim in cultured gastric cells (25). Importantly, previous observations have suggested that overexpression of pro-apoptotic proteins and decreased expression of anti-apoptotic Bcl-2 family proteins might play an important role in regulating *H. pylori*-induced apoptosis in both gastric epithelial cells and neutrophils. However, to our knowledge, the role of BH3-only proteins in these conditions has not been elucidated (11, 26). The current study investigated the association of Bim with the degree of *H. pylori*-mediated gastritis, as well as the localization and possible role of this protein in the gastric mucosa. Elucidating these relationships will provide a better understanding of the regulatory mechanisms in play during *H. pylori* infection.

Methods

Human biopsy samples

Patients who underwent upper gastrointestinal endoscopy between June 2007 and May 2013, and who provided written consent to participate in the study, were enrolled in this study. Upper gastrointestinal endoscopy was performed at the Nagasaki University Hospital and its related branches. Endoscopies were performed for medically indicated reasons and not for research purposes only. Written informed consent was obtained from the patients prior to their enrollment in the study, in agreement with the Declaration of Helsinki. The study was assessed and approved by the Ethics Committee of the Nagasaki University Hospital (Office of Human Subjects Protection, Registration number: IORG0007678). The following criteria were used to exclude patients from the study: age <15 or >85 years, body mass index >30 kg/m², pregnancy, diabetes mellitus, systemic infection, renal impairment, liver disease, alcohol abuse, drug addiction, use of medications effective against *H. pylori* during the preceding 3 months, and chronic corticosteroid or nonsteroidal anti-inflammatory drug use. During endoscopic examination of the patients, two biopsy specimens were obtained from the lesser curvature of the gastric antrum. One sample was used for RNA isolation. The other sample was fixed in 10% formalin and embedded in paraffin for histopathological examination. The status of *H. pylori* infection was assessed by the rapid urease test (Helicocheck; Otsuka Pharmaceutical, Tokushima, Japan), and histological examination of the tissue samples was performed by Giemsa staining. Patients were considered positive for *H. pylori* infection when either of these test results was positive, and they were defined as *H. pylori*-negative when both test results were negative.

Mouse samples

Experiments involving animal models were approved by the Keio University Animal Research Committee. Male C57BL/6 mice were infected with *H. pylori* for 40 weeks, as described previously (27). Briefly, the mice were administered Sydney Strain 1 (SS1) (28) *H. pylori* (CagPAI-positive, VacA s2-m2 type) at a concentration of 10⁶ colony-forming

units/mL or vehicle (0.5% carboxymethyl cellulose solution) by per oral injection at 6 weeks of age (27). Forty weeks after infection, the mice were sacrificed and their stomachs were collected (27). *H. pylori* infection was confirmed by histological examination, as well as by micro-aerobic culturing of the bacteria present in the stomach homogenates on NISSUI Plate *Helicobacter* Agar for all samples (Nissui, Tokyo, Japan).

RNA isolation and quantitative reverse-transcription polymerase chain reaction (PCR)

The biopsy samples were stored in 1 mL of RNAlater® (Applied Biosystems, Foster City, CA). Total RNA was subsequently extracted using a GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Munich, Germany). The quantity and quality of the extracted RNA were measured using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA was then reverse-transcribed into complementary DNA using random primers (Invitrogen, Grand Island, NY). The complementary DNA was quantified by quantitative PCR (LightCycler® 480; Roche Applied Science, Penzberg, Germany) using SYBR® green (Molecular Probes). The PCR primers designed for human Bim were as follows: forward (5'-AGA TCC CCG CTT TTC ATC TT-3') and reverse (5'-TCT TGG GCG ATC CAT ATC TC-3'). 18S rRNA was used (Ambion, Austin, TX) as an internal control. The relative mRNA expression level for each sample was expressed as a ratio of Bim mRNA to 18S rRNA.

Immunofluorescence

After antigen retrieval by microwave treatment in a commercially available solution (Target Retrieval Solution, Citrate pH 6; DAKO, Glostrup, Denmark), deparaffinized sections were incubated with rabbit monoclonal anti-Bim antibody (Cell Signaling, catalog # 2933) at a dilution of 1:100. The specificity of the anti-Bim antibody has been confirmed previously by Xie et al (29). In selected samples, tissues were co-stained with mouse monoclonal anti-CD3 (Nichirei Bioscience, catalog #413241), mouse monoclonal anti-CD20 (DakoCytomation, catalog # N1502), mouse monoclonal anti-MPO (Santa Cruz, catalog #sc-52707), mouse monoclonal anti-CD68 (DakoCytomation, catalog #M1577), mouse monoclonal anti-CD138 (DakoCytomation, catalog #M7228), mouse monoclonal anti-Cox IV (Cell signaling, catalog #11967), mouse monoclonal anti-cleaved PARP (Abcam, catalog # ab110315), and mouse monoclonal anti-Bcl-2 (Leica Microsystems, #NCL-bcl-2) antibodies to determine their co-localization with Bim. Mouse monoclonal 6A7 antibody (Santa Cruz, catalog # sc-23959) was employed to visualize activated Bax. The samples were subsequently incubated with Alexa Fluor® 488-conjugated goat anti-rabbit antibody (1:100 dilution; Invitrogen, Carlsbad, CA, USA) and Alexa Fluor® 568-conjugated goat anti-mouse antibody (1:100 dilution). The specimens were then counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI-I; Vysis Inc., Downers Grove, IL, USA) and analyzed using a high standard, all-in-one fluorescence microscope (BIOREVO BZ-9000; Keyence Japan, Osaka, Japan), by employing the Z-stack function.

Histopathological assessment according to the updated Sydney system

The paraffin-embedded biopsy specimens were cut into 4-µm-thick sections and stained with hematoxylin and eosin. Intestinal metaplasia was defined by the presence of goblet cells in

the glandular mucosa, as assessed Alcian blue (pH 2.5) periodic acid-Schiff staining. The degree of infiltration of neutrophils and mononuclear cells, glandular atrophy, and intestinal metaplasia in the histological sections were scored from zero to three, corresponding to none, mild, moderate, or marked, respectively, in accordance with the updated Sydney system. The density of *H. pylori* colonization of epithelial cells was evaluated histologically by Giemsa-staining and was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe), also based on the above-mentioned system of classification. The specimens were evaluated in a blinded manner by an independent pathologist.

Statistical analysis

Differences between groups were compared using Welch's *t*-test. The Jonckheere–Terpstra test was used to analyze the associations between Bim expression and the scores obtained based on the Sydney system of classification. The PHREG procedure in the SAS 8.2 software (SAS Institute, Cary, NC, USA) was employed for statistical analysis. All tests were two-tailed and a *p*-value of <0.05 was considered statistically significant.

Results

Correlation of Bim with neutrophil and monocyte infiltration according to the Sydney system of classification

In total, 80 patients (mean age = 59 ± 13.1 years) were initially included in the study. Eight subjects were excluded because of inadequate biopsy sampling. Forty-five patients with confirmed *H. pylori* infection (24 men, 21 women; mean age = 59 years) were shortlisted for further analysis. The remaining 27 individuals who tested negative for *H. pylori* infection (13 men, 14 women; mean age = 56 years) were used as controls. There were no significant differences in baseline characteristics such as gender and age between the *H. pylori*-negative and *H. pylori*-positive individuals (Table 1). Initially, expression of Bim mRNA in *H. pylori*-infected and uninfected human mucosa was compared. Consistent with the results of a previous study conducted with a smaller sample size (25), Bim mRNA expression levels were significantly elevated in *H. pylori*-infected mucosa compared with uninfected controls, as assessed by Welch's *t*-test (Figure 1). Next, the association between Bim mRNA expression and the degree of chronic gastritis was evaluated. Thirty-six of 45 *H. pylori*-positive biopsy samples were available for assessment of pathological gastritis scoring by the updated Sydney system. A statistically significant association was found between Bim mRNA expression levels and the degree of infiltration of inflammatory cells in *H. pylori*-positive biopsy mucosa samples. Bim mRNA increased proportionately with the rise in the grade of neutrophil and monocyte infiltration, as assessed by the Jonckheere–Terpstra test (Figure 2A, B). Bim mRNA expression also had a tendency to increase as the density of *H. pylori* increased (Figure 2C; Jonckheere–Terpstra test). In contrast, Bim mRNA levels increased as the grades of intestinal metaplasia decreased (Figure 2D; Jonckheere–Terpstra test). The degree of glandular atrophy of the antrum and corpus in the histological samples was not significantly correlated with the expression of Bim, although Bim expression tended to decrease as the atrophy grade increased (Supplemental figure 1). Given that lesser infiltration of neutrophils and monocytes was observed in cases with severe intestinal

metaplasia (Supplemental figures 2A and B), expression of Bim was deduced to be associated with enhanced inflammation in *H. pylori*-infected gastric mucosa.

Localization of Bim in human and mouse gastritis

Next, the localization of Bim protein was investigated by immunofluorescence in the human samples. Hematoxylin and eosin staining revealed infiltration of inflammatory cells, edema, and congestion within the lamina propria (all of which are symptoms of mild gastritis) in *H. pylori*-infected gastric mucosa (Figure 3A). Strong Bim protein expression was observed in the cells within the lamina propria of *H. pylori*-infected gastric mucosa, but not in the epithelial cells (Figure 3B). Bim expression in the *H. pylori*-infected mouse mucosa was then examined. Similar to human gastritis, surface erosion and inflammatory cell infiltration was observed in *H. pylori*-infected mouse gastric mucosa (Figure 4A). Bim was also expressed within the inflammatory cells in the lamina propria of *H. pylori*-infected gastric mucosa of mice (Figure 4B).

Localization of Bim within the neutrophils and plasma cells in *H. pylori*-infected mucosa

Given the above results, the precise localization of Bim within cells was examined by immunostaining for markers of T cells (CD3), B cells (CD20), plasma cells (CD138), macrophages (CD68), and neutrophils (MPO). Bim protein was localized within the cytoplasm of MPO-positive neutrophils, and the percentage of Bim-positive neutrophils was 38%. (Figure 5A, Supplemental Figure 3). Interestingly, Bim was present within cells expressing nuclear MPO, and it did not co-localize with neutrophils expressing abundant MPO in their granules (Figure 5A, Supplemental Figure 3). Bim was also present in 33% of CD138-positive plasma cells (Figure 5B, Supplemental Figure 3). In contrast, Bim did not localize in CD3-, CD20-, or CD68-positive cells (Figure 5C, Supplemental Figure 3). These results suggested that Bim is expressed uniquely in some populations of neutrophils and plasma cells, but not in other inflammatory cells.

Localization of Bim in the mitochondria in gastritis patients

To investigate the possible roles of Bim in inflammatory cells, the subcellular localization of Bim was investigated. In both the human and murine samples, Bim protein expression in most cells showed a perinuclear punctate pattern in the cytosol (Figure 3, 4), suggesting that Bim may be expressed within specific organelles. Previous studies have shown that, under certain conditions, Bim is present in an inactive state within the cytosol, and that it migrates to the mitochondria upon activation of the apoptotic pathway (30, 31). In line with this concept, Bim co-localized with the mitochondrial marker Cox-4 in *H. pylori*-infected human gastric mucosa (Figure 6). Moreover, Bim partially co-localized with activated Bax, a direct mediator of mitochondrial dysfunction, as assessed by co-immunofluorescence analysis using 6A7 monoclonal antibody, which only detects activated Bax following conformational change (Figure 6). Finally, Bim was also partially co-localized with cleaved PARP-expressing cells (Figure 6). Taken together, these results suggested that Bim is partially expressed in the mitochondria in cells that are undergoing apoptosis.

Discussion

The current study demonstrated that the BH3-only protein, Bim, might regulate a subset of inflammatory cells in the gastric mucosa during *H. pylori*-mediated gastritis. Expression of Bim protein is positively correlated with the extent of neutrophil and monocyte infiltration and negatively related to the degree of intestinal metaplasia. Bim is predominantly expressed in the mitochondria of neutrophils and plasma cells of *H. pylori*-infected mucosa, but not within other inflammatory cells. Lastly, Bim co-localizes with activated Bax, an essential player in mitochondria-mediated apoptosis.

Bim plays a key role in modulating the fate of inflammatory and/or host cells during infection. For example, *Mycobacterium tuberculosis* infection induces apoptosis of macrophages through upregulation of Bim (32). These data, together with our observations, imply that modulation of Bim might represent a common mechanism exploited by different types of infectious organisms, and that Bim-modulation response by the host could determine the outcome of infection.

Our results demonstrated that the expression of Bim mRNA in the gastric mucosa was associated with neutrophil/monocyte infiltration. The negative correlation between Bim mRNA expression and intestinal metaplasia can be attributed to the fact that mucosal inflammation is generally reduced during intestinal metaplasia, resulting in lower Bim mRNA expression. Our data support the hypothesis that Bim expression is associated with the severity of inflammation. Bim expression can be enhanced both transcriptionally and post-transcriptionally (18, 33). Consistent with the mRNA expression patterns, the immunofluorescence assay demonstrated that Bim protein is predominantly expressed in *H. pylori*-positive mucosa in both humans and mice. Bim is selectively expressed in a population of neutrophils and plasma cells in the *H. pylori*-infected gastric mucosa, but not in T cells, B cells, and macrophages. Perhaps, different cell types require different BH3-only proteins to activate apoptosis (33). It is noteworthy that Bim expression in resting granulocytes is reported to be below the limit of detection of immunofluorescence staining, although low concentrations of Bim could be detected by immunoblotting (34). In line with these observations, we could not detect Bim expression in granulocytes freshly isolated from the blood vessel (data not shown). In our study, Bim expression in both neutrophils and plasma cells exhibited a punctate pattern, at least partially in the mitochondria of *H. pylori*-infected granulocytes and plasma cells. These findings are in line with prior observations that Bim expression increases once the neutrophils are exposed to inflammatory signals (35, 36). Proteins such as granulocyte colony-stimulating factor paradoxically enhance Bim expression (20), as part of a self-defense mechanism to limit inflammation. Inflammatory cytokines induced by *H. pylori* infection may also trigger Bim activation, promoting apoptosis and clearance of neutrophils as well as plasma cells. Thus, activation of Bim in these cells may result in prompt resolution of inflammation, whereas Bim inhibition may prolong neutrophil survival, leading to persistent gastric injury. Notably, Bim co-localized within cells expressing nuclear-MPO and not with granulocytes that abundantly express cytosolic MPO. MPO plays a crucial role in killing bacterial cells. In addition, secreted MPO binds to the membrane of granulocytes, preventing mitochondrial dysfunction and caspase-3 activation of granulocyte apoptosis in autocrine and paracrine processes. Thus,

cytosolic MPO activity indicates the presence of functioning neutrophils that are able to kill bacterial cells, whereas low MPO levels may imply loss of neutrophil function (37). Nuclear MPO, like cytosolic MPO, is mainly expressed in a subset of granulocytes and not in lymphocytes; however, nuclear and cytosolic MPOs have distinct roles. Inter-nuclear MPO is tightly bound to DNA, and it protects DNA from double-strand breaks induced by oxidative stress (14). Bim was found to be expressed in cells containing nuclear MPO, suggesting that Bim is mainly present in granulocytes that are under cellular stress and no longer functionally competent to combat bacteria (14).

Proapoptotic Bcl-2 protein, Bax, is directly responsible for mitochondrial dysfunction, causing caspase-3-mediated apoptosis in the cells. Previous studies have demonstrated that Bax expression and its translocation to the mitochondria occurs only in pre-apoptotic neutrophils (38). Our study revealed partial co-localization of Bim with activated Bax. Furthermore, Bim also co-localized with cleaved PARP, which is direct evidence of ongoing apoptosis. These additional evidences support the concept that Bim contributes to apoptosis in neutrophils and plasma cells during *H. pylori* infection. Upon activation during inflammation, Bim translocates to the mitochondria and subsequently induces apoptosis of immune-reactive cells by triggering apoptosis. Interestingly, a previous study by Kim et al. demonstrated transient upregulation of anti-apoptotic Bcl-2 family proteins and downregulation of caspase-3 activity in isolated healthy neutrophils exposed to water-soluble proteins expressed by *H. pylori* (expression of Bim was not examined) (11). The difference between our data and those of Kim et al. may be attributed to variations in the experimental designs; Kim et al. conducted a short *ex-vivo* study, while our study focused on assessing the expression of pro-apoptotic proteins in human chronic gastritis. Moreover, Kim et al. reported that water-soluble proteins expressed by *H. pylori* led to decreased caspase-3 activity, which was resolved within 24 h. Thus, infection with *H. pylori* may exert both anti- and pro-apoptotic effects, depending on the duration of infection and culture conditions. The precise role of Bim in *H. pylori*-induced gastritis remains contentious and warrants further studies (39).

Although Bim is a crucial pro-apoptotic protein in hematopoietic cells, other proteins may contribute to the apoptotic signal (21). Kirschneck et al. demonstrated that, in addition to Bim, another BH3-only protein, Noxa, might be required to induce apoptosis in neutrophils (21). In the immunofluorescence analysis, not all the activated Bax co-localized with Bim. Although differential mRNA expression of other pro-proteins between healthy and *H. pylori*-infected mucosa was not observed in the current study, we cannot exclude the possibility that other BH3-only proteins play a role in *H. pylori* infection.

In conclusion, the current study revealed the presence of Bim in the mitochondria of neutrophils and plasma cells in *H. pylori*-infected gastric mucosa. Although further studies are necessary to elucidate the mechanism of activation of Bim in *H. pylori* infection, the current data suggest that Bim possibly plays a role in apoptosis of inflammatory cells during *H. pylori* infection. Therefore, Bim might serve as a therapeutic target to inhibit the progression of *H. pylori*-mediated inflammation and other *H. pylori*-mediated diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

<i>H. pylori</i>	<i>Helicobacter pylori</i>
Bax	Bcl-2-associated X protein
Bak	Bcl-2 antagonistic killer
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
Mcl-1	Myeloid cell leukemia 1
Bid	BH3 interacting-domain death agonist
Bad	Bcl-2-associated death promote
Bim	Bcl-2 interacting mediator of cell death
Bik	Bcl-2-interacting killer
Bmf	Bcl2 modifying factor
Puma	p53 upregulated modulator of apoptosis
Hrk	Harakiri

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Figure 1. Expression of Bim mRNA in *Helicobacter pylori*-positive and -negative mucosa mRNA was extracted from biopsy specimens of human *H. pylori*-negative and -positive gastric mucosa, and quantitative polymerase chain reaction (qPCR) for Bim mRNA expression was performed. $P < 0.01$, *H. pylori*-negative samples ($n = 27$) vs. *H. pylori*-positive samples ($n = 45$). Data are presented as mean $\pm$ standard deviation (SD). Each dot represents an individual data point.

Figure 2. Relationship between the expression levels of Bim mRNA and severity of gastritis

The association between Bim mRNA expression and severity of gastritis was evaluated according to the updated Sidney classification system from grades 0 to 3 (A: infiltration of neutrophils, B: infiltration of monocytes, C: density of *H. pylori*, and D: degree of intestinal metaplasia). *H. pylori*-positive human gastric mucosa. $n = 36$. Data are presented as mean $\pm$ SD. Each dot represents an individual data point. *H. pylori* (+), $n = 36$. Grade 0 monocyte infiltration was not detected in any of the *H. pylori*-positive samples.

Figure 3. Expression of Bim in human gastric mucosa

(A) Representative hematoxylin and eosin staining of *H. pylori*-positive and -negative human gastric mucosa. Original magnification was 10×. (B) The presence of Bim is indicated by green staining in *H. pylori*-infected gastric mucosa (strongly positive Bim-staining in the lamina propria) and *H. pylori*-negative gastric mucosa (less intense Bim-staining). Blue color indicates nuclear staining by 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Original magnification was 40×. The inset shows magnified view of the Bim-positive area in *H. pylori*-positive mucosa.

Figure 4. Bim expression in mouse gastric mucosa

(A) Hematoxylin and eosin staining of control and *H. pylori*-infected mouse gastric mucosa after 40 weeks of infection. Erosion of gastric mucosa and infiltration of inflammatory cells in the lamina propria are seen in *H. pylori*-infected samples. (B) The presence of Bim is indicated by green staining. The right panel shows *H. pylori*-infected gastric mucosa (strongly positive Bim-staining in the lamina propria, indicated by arrows), and the left panel shows healthy *H. pylori*-negative gastric mucosa. Blue color indicates nuclear staining by DAPI. Asterisk indicates non-specific staining in red blood cells. Original magnification was 40 $\times$. The figure shows one experimental result representative of four *H. pylori*-negative and -positive samples.

A

B

Figure 5. Localization of Bim in *H. pylori*-infected human gastric mucosa

Co-localization of Bim and the expression of hematopoietic markers (A: MPO and B: CD138), and (C: CD20, CD3, and CD68) were assessed by immunofluorescence in *H. pylori*-positive gastric mucosa. Blue color indicates nuclear staining by DAPI. Original magnification was 100×. Circles in A indicate Bim (cytosol) expression in MPO (nucleus)-positive cells. Arrows in B indicate Bim localization in CD138-positive cells. The figure shows one experimental result representative of four *H. pylori*-negative and -positive samples.

Figure 6. Subcellular localization of Bim in inflammatory cells

Bim was co-stained with COX4 (upper panel), Bax (middle panel), and cleaved PARP (lower panel). Blue color indicates nuclear staining by DAPI. Original magnification was 100×. Arrows in middle and lower panel indicate Bim localization with Bax and cleaved PARP, respectively. The figure shows one experimental result representative of four *H. pylori*-negative and -positive samples.

Table 1

Baseline characteristics of the two groups (*H. pylori*-negative and -positive) evaluated in the study.

		H.pylori (–)	H.pylori (+)	Statistical significance
Number of patients		27	45	—
Age, yr (SD)		56 (13.1)	59 (14.1)	n.s
Gender	Male (%)	13 (48.1)	24 (46.7)	n.s
	Female (%)	14 (51.9)	21 (53.3)	n.s