**Helicobacter pylori** sensitizes TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in human gastric epithelial cells through regulation of FLIP

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**Helicobacter pylori** infection is associated with chronic gastritis, peptic ulcer and gastric cancer. Apoptosis induced by microbial infections is implicated in the pathogenesis of **Helicobacter pylori** infection. Here we show that human gastric epithelial cells sensitized to **Helicobacter pylori** confer susceptibility to TRAIL-mediated apoptosis via modulation of death receptor signaling. Human gastric epithelial cells are intrinsically resistant to TRAIL-mediated apoptosis. The induction of TRAIL sensitivity by **Helicobacter pylori** is dependent on the activation of caspase-8 and its downstream pathway. **Helicobacter pylori** induces caspase-8 activation via enhanced assembly of the TRAIL death-inducing signaling complex (DISC) through downregulation of cellular FLICE-inhibitory protein (FLIP). Overexpression of FLIP abolished the **Helicobacter pylori**-induced TRAIL sensitivity in human gastric epithelial cells. Our study thus demonstrates that **Helicobacter pylori** induces sensitivity to TRAIL apoptosis by regulation of FLIP and assembly of DISC, which initiates caspase activation, resulting in the breakdown of resistance to apoptosis, and provides insight into the pathogenesis of gastric damage in **Helicobacter pylori** infection. Modulation of host apoptosis signaling by bacterial interaction adds a new dimension to the pathogenesis of **Helicobacter pylori**.

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**Helicobacter pylori**, a common human pathogen that infects about 50% of the world’s population, is associated with duodenal ulcer and peptic ulcer diseases. Recent studies have shown that there is increased apoptosis of gastric epithelial cells during **Helicobacter pylori** infection.1–5 Enhanced gastric epithelial cell apoptosis observed during infection with **Helicobacter pylori** is believed to be significant in the etiology of gastritis, peptic ulcers, and neoplasia. Recent studies have suggested that T cells are selectively increased during infection.4–7 Cytokines like gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) can increase the release of proinflammatory cytokines and augment apoptosis induced by **Helicobacter pylori**.8 **Helicobacter pylori** infection could also induce damage to gastric mucosa by increasing the expression of Fas in gastric epithelial cells, leading to gastric epithelial cell apoptosis through Fas/FasL interaction with infiltrating T cells.9,10 These findings suggest a role for immune-mediated apoptosis of gastric epithelial cells during **Helicobacter pylori** infection.

TRAIL (tumor necrosis factor-related apoptosis-inducing ligand; also called Apo2L), a novel TNF superfamily member with a strong homology to FasL, is capable of inducing apoptosis in a variety of transformed cell lines *in vitro*11,12 but usually not in normal primary cells. It was recently shown that T cells kill target cells via TRAIL/TRAIL receptor interaction,13–15 suggesting that TRAIL might serve as a cytotoxic effector molecule in activated T cells *in vivo*. Recent studies indicate that TRAIL-induced apoptosis occurs through a caspase signaling cascade and that resistance to TRAIL is controlled by intracellular regulators of apoptosis. The molecular events leading to apoptosis induction via TRAIL have recently been analyzed. Crosslinking of the TRAIL receptors leads to the formation of a death-inducing signaling complex (DISC).16 The death adaptor protein FADD (MORT1) and the proteolytic enzymes caspase-8 and -10 are recruited to the TRAIL DISC.17–21 Procaspase-8 is proteolytically cleaved and activated at the DISC. Activated caspase-8 then initiates the apoptosis executing caspase cascade. Previous studies demonstrated that TRAIL-induced apoptosis could be enhanced by *Helicobacter pylori* and hepatitis C virus core protein.22,23 However, the mechanisms leading to induce TRAIL sensitivity by microbes are not clear. Upregulation of the apoptosis-inducing TRAIL receptors after treatment with...
chemotherapeutic drugs and radiation has been implicated in sensitizing human leukemic and glioma cells. It has been shown that chemotherapy-resistant tumor cells can be sensitized for TRAIL-induced apoptosis at the DISC level. Here we report that human gastric epithelial cells sensitized to H. pylori confer susceptibility to TRAIL-mediated apoptosis. H. pylori induces TRAIL apoptosis signaling by downregulation of FLICE-inhibitory protein (FLIP), which enhances the assembly of TRAIL DISC, induces caspase-8 activation, and conveys the death signal to mitochondria, resulting in a breakdown of apoptosis resistance.

Results

H. pylori enhances sensitivity to TRAIL-mediated apoptosis in human gastric epithelial cell lines via activation of caspase-8 and its downstream pathway. TRAIL has been shown to induce apoptosis in a number of different tumor cell types but not usually in normal primary cells. To examine a role for TRAIL-induced apoptosis in gastric epithelial cells, recombinant TRAIL proteins were used to induce apoptosis in human gastric epithelial cell lines (AGS). The results revealed that AGS cells were resistant to TRAIL-mediated apoptosis. We further studied TRAIL-induced apoptosis in gastric epithelial cells after interaction with H. pylori. In the absence of TRAIL, H. pylori induced only mild apoptosis in AGS cells; however, apoptosis was markedly induced after adding TRAIL, and induction of TRAIL-mediated apoptosis by H. pylori was specifically blocked by adding soluble TRAIL receptor, death receptor 4 (DR4)-Fc, indicating that cell death resulted from the interaction between TRAIL and the TRAIL receptor on the cell surface (Figure 1a).

To further delineate the intracellular signal transduction pathway modulated by H. pylori that results in induction of TRAIL-sensitivity, we investigated the activation of caspase pathways following TRAIL engagement and subsequent to H. pylori interaction. In the absence of H. pylori, TRAIL engagement only induced a slight activation of caspase-8 but not caspase-3 in AGS cells. In contrast, during TRAIL engagement, both caspase-8 and caspase-3 were fully activated in AGS cells, resulting in cell apoptosis after exposure to H. pylori (Figure 1b). Furthermore, the ability to induce TRAIL sensitivity in AGS cells by H. pylori was significantly suppressed by the caspase-8 inhibitor, Z-IETD-fmk; pan-caspase inhibitor, Z-VAD-fmk; or the caspase-3 inhibitor, DEVD-fmk. (Figure 1c) In addition, the H. pylori-induced TRAIL sensitivity in AGS cells was significantly suppressed by knocking down caspase-8 (Figure 1d).

To exclude the possibility that the H. pylori-induced sensitivity to TRAIL in gastric epithelial cell lines is due to upregulation of TRAIL DRs, we further examined the expression of TRAIL receptors before and after interaction with H. pylori. The results demonstrated that expression of TRAIL receptors on the gastric cell surface did not appear significantly different before and after interaction with H. pylori (Figure 2). Taken together, the results indicate that H. pylori enhances TRAIL-mediated apoptosis in gastric epithelial cell lines by modulating intracellular death signal transduction by activation of a caspase-8 downstream cascade. In so doing, the pathogen alters the intracellular regulation of resistance to DR-induced apoptosis through a pathway involving the sequential induction of apical caspase-8 activity, caspase cascade, and effector caspase-3 activity.

H. pylori induces the activation of the mitochondrial signaling pathway after TRAIL engagement. For detecting caspase processing events distal to caspase-8 activation, we investigated the activation of mitochondria after exposure to H. pylori in AGS cells. To examine the changes of mitochondrial membrane potential (Δψm) after TRAIL engagement, the Δψm was detected by the uptake of 5,5′,6,6′-tetrachloro-1′,3′,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) dye. The results (Figure 3) demonstrate that TRAIL engagement induced a breakdown in the transmembrane potential of mitochondria after interaction with H. pylori but not in the absence of H. pylori, consistent with the difference in caspase-3 activation. To further confirm the induction of mitochondrial signaling pathway by TRAIL, we also analyzed cytochrome c released from mitochondria into cytoplasm after TRAIL engagement. The results (Figure 3) demonstrate that TRAIL engagement induced the release of cytochrome c from mitochondria to cytosol and activated the mitochondria downstream caspase cascade, caspase-9, in AGS cells in the presence of H. pylori. In addition, the induction of TRAIL sensitivity in AGS cells by H. pylori was significantly inhibited in the presence of the caspase-9 inhibitor Z-LEHD-fmk (Figure 3d), indicating that activation of the mitochondria downstream pathway was required in TRAIL-mediated apoptosis in AGS cells induced by H. pylori.

To investigate the role of the mitochondria apoptosis signaling pathway in H. pylori-induced TRAIL sensitivity, we further transfected Bcl-2 into AGS cells to establish stable transfectants. The results (Figure 4a) demonstrate that the overexpression of Bcl-2 in AGS cells inhibited the induction of TRAIL sensitivity in AGS cells by H. pylori. Taken together, our results indicate that H. pylori induces a sensitivity to TRAIL-mediated apoptosis in gastric epithelial cell lines through a pathway involving the activation of caspase-8 downstream signaling pathway to convey the death signal to mitochondria, leading to activation of the mitochondrial apoptosis signaling pathway and eliminating resistance to apoptosis.

H. pylori induces sensitivity to TRAIL-mediated apoptosis and activates the mitochondrial signaling pathway via Bid cleavage after TRAIL engagement. The finding that TRAIL engagement induces cytochrome c release and caspase-9 activation in AGS cells in the presence of H. pylori indicates that apoptosis signals are conveyed from the TRAIL DR to mitochondria in AGS cells after interaction with H. pylori. For detecting the caspase processing events distal to caspase-8, which connects caspase-8 with mitochondria, we further investigated the processing of Bid after TRAIL engagement. The results (Figure 4b) demonstrate that H. pylori enhanced TRAIL-induced Bid cleavage in AGS cells, which is consistent with activation of the mitochondrial signaling pathway and caspase-3 after TRAIL engagement. In addition, the H. pylori-induced TRAIL sensitivity in AGS
cells was significantly suppressed by knocking down Bid (Figure 4c). Taken together, our results indicate that the induction of TRAIL sensitivity by H. pylori is dependent on the sequential activation of the caspase-8 downstream pathway, enhancing the cleavage of Bid, activating the mitochondrial signaling pathway, and breaking resistance to apoptosis.

**H. pylori enhances the assembly of the TRAIL DISC through reduction of FLIP recruitment.** The expression of DR4 and DR5 DRs was not upregulated by H. pylori, and results indicate that H. pylori enhances TRAIL-mediated apoptosis in gastric epithelial cell lines by modulating intracellular death signal transduction. We then investigated the possible involvement of FLIP in regulating the assembly of TRAIL DISC in the presence or absence of H. pylori. Cells were co-cultured with H. pylori and then stimulated with FLAG-TRAIL. After TRAIL engagement, the cell lysate was subjected to western blotting analysis for detection of caspase activation. The native forms of caspase-8 and caspase-3 are 55/54 and 32 kDa, respectively. The cleaved forms of caspase-8 are 43/41 and 18 kDa, respectively. The cleaved forms of caspase-3 are 19 and 17 kDa, respectively. The expression of actin serves as a loading control.

![Figure 1](image_url)

**Figure 1** H. pylori enhances sensitivity to TRAIL-mediated apoptosis in human gastric epithelial cell lines via activation of caspase-8 and its downstream pathway. (a) Human gastric epithelial cell line AGS were co-cultured with H. pylori for 12 h and incubated with recombinant TRAIL proteins in the presence or absence of soluble TRAIL receptor, DR4-Fc protein, for 12 h. Cell apoptosis was measured by cell death enzyme-linked immunosorbent assay (ELISA). Values represent means ± S.D. of three independent experiments (*P* < 0.05). (b) AGS cells were co-cultured with H. pylori for 12 h and incubated with recombinant TRAIL proteins for the indicated times. Cell lysates were subjected to western blotting analysis for detection of caspase activation. The native forms of caspase-8 and caspase-3 are 55/54 and 32 kDa, respectively. The cleaved forms of caspase-8 are 43/41 and 18 kDa, respectively. The cleaved forms of caspase-3 are 19 and 17 kDa, respectively. The expression of actin serves as a loading control. (c) AGS cells were co-cultured with H. pylori for 12 h in the presence of pan-caspase inhibitor, Z-VAD-fmk (Bachem, Bubendorf, Sweden), specific caspase-8 inhibitor, Z-IETD-fmk, or specific caspase-3 inhibitor, Z-DEVD-fmk (Calbiochem, San Diego, CA, USA) and then were treated with recombinant TRAIL proteins for 4 h. Cell apoptosis was measured by cell death ELISA. Values represent means ± S.D. of three independent experiments (*P* < 0.05). (d) AGS cells were transfected with scramble or caspase-8 siRNA, followed by incubating with H. pylori for 12 h and subsequently treated with TRAIL for 4 h. Cell apoptosis was measured by cell death ELISA. Values represent means ± S.D. of three independent experiments (*P* < 0.05).
To further confirm the role of FLIP in regulating *H. pylori*-induced TRAIL sensitivity in human gastric epithelial cells, AGS cells were transfected with c-FLIPs siRNA to knock down the expression of FLIPs and co-cultured with *H. pylori* followed by treatment with TRAIL. The results (Figure 7) demonstrate that the knockdown of endogenous c-FLIPs markedly enhances TRAIL-induced apoptosis. In addition, the knockdown of c-FLIPs induced the cleavage of caspase-8, Bid, caspase-9, and caspase-3 after TRAIL engagement. Taken together, our results indicate that *H. pylori* enhances TRAIL sensitivity in human gastric epithelial cells via a decrease in FLIP recruitment into the DISC, leading to the activation of caspase-8 and its downstream pathway, thus breaking TRAIL resistance.

**Discussion**

Our studies indicate that *H. pylori* induces TRAIL apoptosis signaling by downregulating FLIP, which enhances the assembly of DISC, induces maximal caspase-8 activation and conveys the death signal to mitochondria, leading to a breakdown in apoptosis resistance. Collectively, our findings uncover a novel mechanism of modulation of TRAIL resistance mediated by *H. pylori* through regulation of the DISC and provides strong evidence that *H. pylori* modulates the transduction of the host cell death signal via downregulation of FLIP.

We demonstrated in previous reports that human gastric epithelial cells sensitized to *H. pylori* confer susceptibility to TRAIL-mediated apoptosis, suggesting a role for immune-mediated apoptosis of gastric epithelial cells by infiltrating T cells during *Helicobacter* infection.6,22 The induction of TRAIL sensitivity by *H. pylori* is independent of the expression of *H. pylori* virulent factors Vac A and Cag A and is dependent on viable bacteria and direct contact with cells.22 The role of TRAIL DR DR5 (TRAIL-R2) in the regulation of TRAIL-induced apoptosis has been evaluated recently in several tumor cell lines.30–34 In our study, the expression of TRAIL receptors did not change after *H. pylori* infection, indicating that the *H. pylori*-induced enhanced sensitivity to TRAIL-mediated apoptosis in gastric epithelial cells is not due to upregulation of TRAIL DRs, DR5 in particular (Figure 2). Thus, instead of the induction of DR upregulation, *H. pylori* induces TRAIL sensitivity in gastric epithelial cells via modulation of DR signal transduction pathways.

*H. pylori* induces sensitivity to TRAIL-mediated apoptosis in gastric epithelial cells through a pathway involving the sequential induction of apical caspase-8 and effector caspase-3 activity via activation of a mitochondrial pathway. Our results suggest that the type II pathway is mainly involved in TRAIL-induced apoptosis in human gastric epithelial cells where mitochondria have an important role in amplifying apoptotic signals. Our results also indicate that *H. pylori* induces the mitochondrial apoptosis pathway via FLIP downregulation, which enhances DISC assembly after TRAIL engagement and further induces the activation of caspase-8 and cleavage of Bid. c-FLIP proteins have an essential role in the regulation of DR-induced apoptosis. Among the three isoforms: c-FLIP long (c-FLIPL, 55 kDa), c-FLIP short (c-FLIPS, 27 kDa), and c-FLIP Raji (c-FLIPR, 25 kDa),35–37
c-FLIPs is a well-described inhibitor of DR-mediated apoptosis. It has been shown that c-FLIPs blocks Fas as well as TRAIL-induced apoptosis via binding to the DISC and inhibiting procaspase-8 processing and activation at the DISC. However, the effect of the c-FLIP\textsubscript{L} isoform on DR-mediated apoptosis is still controversial. Our results
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Figure 4  H. pylori-induced TRAIL sensitivity is dependent on Bid processing. (a) AGS cells were transfected with Bcl-2, followed by incubation with H. pylori and subsequently treated with TRAIL. Cell apoptosis was measured by cell death enzyme-linked immunosorbent assay (ELISA). Values represent means ± S.D. of three independent experiments (*P<0.05). (b) AGS cells were incubated with or without H. pylori followed by treatment with TRAIL at the indicated times. The expression of full-length and truncated Bid (22 and 15 kDa, respectively) was detected by western blotting. Membranes were also blotted with anti-β-actin mAb as a loading control. (c) Cells were transfected with scramble or Bid siRNA, followed by incubation with H. pylori and subsequently treated with TRAIL. Cell apoptosis was measured by cell death ELISA. Values represent means ± S.D. of three independent experiments (*P<0.05)

Figure 5  H. pylori downregulates FLIPs expression and enhances the TRAIL-induced DISC assembly. (a) AGS cells were incubated with increasing amounts of H. pylori (MOI 25, 50, 100, 200) for 12 h and subsequently treated with TRAIL. Cell lysates were analyzed by western blotting to detect the activation level of caspase-8. Actin expression was used as a protein loading control. (b) Cells were co-cultured with H. pylori for 12 h, stimulated with 1 μg/ml FLAG-TRAIL combined with 2 μg/ml anti-FLAG antibody or normal mouse IgG for 1 h, and cell lysates were precipitated with protein A agarose. Isolated receptor complexes (IP) were assessed for caspase-8, FLIPs, FADD, and DR5 by western blotting

Recently, several bacterial pathogens have been found to trigger apoptosis in host cells in vitro or in vivo, and several types of mechanisms have been elucidated.44 Recent reports have shown that H. pylori can secrete CagA into gastric epithelial cell by Type IV secretion, inducing intracellular protein phosphorylation in host cells,45–48 suggesting that H. pylori can modulate intracellular signaling via factors secreted into host cells. However, in our previous study we demonstrated that induction of TRAIL sensitivity by H. pylori is independent of H. pylori virulent factors Vac A and Cag A.22 Therefore, the bacterial factors leading to induction of TRAIL sensitivity by H. pylori is still not clear, and it awaits further investigation. Taken together, our results indicate that H. pylori modulates the sensitivity to TRAIL-mediated apoptosis in gastric epithelial cells by the induction of FLIP downregulation, which enhances DISC assembly, further induces caspase-8 activation, and conveys the death signal to mitochondria, resulting in a breakdown of apoptosis resistance and causing gastric mucosal damage during inflammation. Thus, modulation of host cell death signal transduction by bacterial interaction adds a new dimension to the immune pathogenesis in chronic Helicobacter infection.

Materials and Methods

Bacterial strains, cell lines, and media. H. pylori strains ATCC 43504 were obtained from American Type Culture Collections (ATCC, Manassas, VA, USA). Before each experiment, H. pylori was passaged on 5% sheep blood agar plates by incubation in an atmosphere consisting of 5% CO₂, 15% CO₂, and 80% N₂.
before incubation with *H. pylori*.

Cell apoptosis was measured by cell death enzyme-linked immunosorbent assay. Values represent means ± S.D. of three independent experiments (*P* < 0.05).

(b) Cells were pretreated with 2 μM MG132, a proteosome inhibitor, for 30 min before incubation with *H. pylori* (MOI 50, 100, 200). After co-culture for 12 h, c-FLIPs was determined by western blotting. The expression of actin serves as a loading control.

Expression and purification of recombinant TRAIL protein and soluble TRAIL receptor DR4-Fc.

The recombinant TRAIL proteins were expressed in the E. coli expression system and purified with a Ni column. In brief, the coding portion of the extracellular domain of TRAIL (amino acids 123–314) was PCR-amplified, subcloned into pSET B vector (Invitrogen, Groningen, Netherlands), and expressed in the *E. coli* expression system. The purification of recombinant His-TRAIL fusion protein was performed by metal affinity chromatography using Ni-NTA resin according to the manufacturer’s recommendations. (Qiagen, Hilden, Germany).

His-TRAIL was purified by the Bradford method and protein assay reagent (BioRad, Richmond, CA, USA). To generate soluble recombinant DR4-Fc fusion molecules, the coding sequence for the extracellular domain of human DR4 was isolated by RT-PCR using the forward primer, 5’-CGATTTTATGCGCCACCCACCA-3’ and the reverse primer, 5’-GAA GATCATAGATGCTCATTGCC-3’. The amplified product was ligated in-frame into BamHI-cut pUC19-IgG1-Fc vector containing the human IgG1 Fc coding sequence. The fusion gene was then subcloned into pBacPAK9 vector (Clontech, Palo Alto, CA, USA). DR4-Fc fusion protein was recovered from the filtered supernatants of the recombinant virus-infected Sf21 cells using protein G-Sepharose beads (Pharmacia, Piscataway, NJ, USA). The bound DR4-Fc protein was eluted with glycine buffer (pH 3) and dialyzed into PBS.

*Flow cytometry.* AGS cells were resuspended in 90 μl FACS staining buffer (1% FCS in PBS), incubated with various fluorochrome-conjugated antigen-specific antibodies (anti-DR4, -DR5, -DcR1 (decoy receptor 1) and -DcR2) (eBioscience, San Diego, CA, USA) at 4 °C for 30 min, and followed by washing twice with 1 ml of FACS staining buffer. Cell fluorescence intensity was detected by a FACSScan and analyzed by the CellQuest software.

*Detection of Δψm.* To detect the changes in Δψm, AGS cells were seeded at 104 cells/ml into a 6-well plate and grown in DMEM supplemented with 10% fetal calf serum in a 95% air-5% CO2 atmosphere at 37 °C in a humidified incubator overnight. Cells were co-cultured with HP strain 51932 at a concentration of 108 CFU/ml for 12 h and treated with 1.5 μM TRAIL recombinant protein for 4 h, and harvested by centrifugation at 200 × g. Cells were lysed by incubation with lysis buffer for 30 min, followed by centrifugation at 200 × g for 10 min at room temperature. The supernatant was collected and incubated with immunoreagent that was prepared according to the manufacturer’s protocol (Cell Death Detection ELISA PLUS; Roche Mannheim Biochemicals, Mannheim, Germany). This was cultured overnight in a 96-well plate at 104 gastric epithelial cells/well, treated with TRAIL recombinant protein for 4 h, and harvested by centrifugation at 200 × g.

The detection of Δψm was performed with a sensitive ELISA assay, which detects cytoplasmic histone-associated DNA fragments, was performed according to the manufacturer’s protocol (Cell Death Detection ELISAPLUS; Roche Mannheim Biochemicals, Mannheim, Germany). This was cultured overnight in a 96-well plate at 104 gastric epithelial cells/well, treated with TRAIL recombinant protein for 4 h, and harvested by centrifugation at 200 × g.

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mitochondrial transmembrane potential. Thus, red fluorescence indicates intact mitochondria, whereas green fluorescence shows monomeric JC-1 that remained unprocessed due to breakdown of the mitochondrial transmembrane potential. After trypsinization by TEG buffer (0.125% trypsin, 0.05% EDTA, 0.05% glucose in PBS), the cells were resuspended in DMEM and incubated with JC-1 10 µg/ml for 15 min at 37°C. Cells were washed with PBS twice and resuspended in PBS. Analysis was performed by a FACS scan, and mitochondrial function was assessed as JC-1 green (uncoupled mitochondria) or red (intact mitochondria) fluorescence. For confocal laser scanning microscopy analysis of mitochondrial function, the JC-1-treated cells were excited at 488 nm, and emissions were simultaneously recorded at 527 and 590 nm into an independent detector.

Preparation of cytosols, analysis of cytochrome c release, and Bid cleavage. After incubation with TRAIL, cells were washed twice with ice-cold PBS. They were suspended in 100 µl of extraction buffer (50 mM PIPES-KOH, pH 7.4, 220 mM mannitol, 50 mM sucrose, 50 mM KCl, 5 mM EDTA, 2 mM MgCl2, 1 mM dithiothreitol, and protease inhibitors) and allowed to swell on ice for 30 min. Cells were homogenized by passing the suspension through a 25-gauge needle (10 strokes). Homogenates were centrifuged at 14 000 × g at 4°C for 10 min. The resultant pellets were incubated with RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate or, 10 mg/ml phenylmethylsulfonyl fluoride, and 10 mg/ml aprotinin) for 30 min on ice. After incubation with TRAIL, cells were washed twice with ice-cold PBS, subjected to SDS-PAGE and transferred onto PVDF. The membrane was blocked in 1% BSA in PBS and 0.05% Tween 20 with 5% dry milk. Blots were probed in PBS and 0.05% Tween 20, with anti-cytochrome c mAb (bIESiobio) or anti-Bid polyclonal Ab (Cell Signaling, Beverly, MA, USA) and secondary antibodies. The immunoblots were checked with anti-β actin mAb to ensure equal loading.

Immunoblot analysis. Cells (5 × 10^5) were washed twice with ice-cold PBS, scraped from their 6-cm culture dishes into 1 ml ice-cold PBS, and centrifuged at 1000 × g for 5 min. The resultant pellets were incubated with RIPha lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 10 mg/ml phenylmethylsulfonyl fluoride, and 10 mg/ml aprotinin) for 30 min on ice. The cell lysates were centrifuged, and the supernatant was stored at −80°C until used. The protein concentrations of the extracts were measured with protein assay reagent. The extracted cell lysates (50 µg) were subjected to SDS-PAGE and transferred onto PVDF. The membrane was blocked in 5% nonfat skim milk or 5% BSA in TBST (100 mM Tris-HCl at pH 7.4, 9% NaCl, 2% Tween-20) at room temperature for 30 min and incubated with mouse mAb against caspase-3 (Imgenex, San Diego, CA, USA), caspase-8 (Cell Signaling Technology, Danvers, CO, USA), FLIP (ENZO Life Sciences, Farmingdale, NY, USA), and actin (Chemicon International, Temecula, CA, USA) or rabbit polyclonal antibody against caspase-9, cytochrome c, Bid (Cell Signaling Technology), FADD (ENZO Life Sciences), and DR5 (Chemicon International). Bound primary antibodies were detected with an anti-mouse IgG or anti-rabbit IgG conjugated with HRP (Cell Signaling Technology), and the bands were visualized using the enhanced chemiluminescence method (Perkin-Elmer, Waltham, MA, USA). The expression of actin was used to verify equal loading in all studies.

DISC immunoprecipitation. For co-immunoprecipitation of TRAIL DISC, 5 × 10^5 A543 cells were used per sample. Cells were seeded onto 10-cm dishes, incubated overnight with H. pylori cells for 12 h, and harvested at 13000 r.p.m. for 5 min. Cells were resuspended in 500 µl RIPA medium, treated with 0.5 µg Flag-TRAIL (ENZO Life Sciences) combined with 1 µg anti-Flag mAb, M2 (Sigma-Aldrich, St. Louis, MO, USA) or normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and the mixture was incubated on an end-over-end rotator at room temperature for 1 h. PBS (5 ml) was added to the mixture with 10 × volume of ice, and the cells were collected by centrifugation at 13000 r.p.m. at 4°C. The cells were lysed with 1000 µl cold lysis buffer with 10 µM Z-VAD-fmk for 30 min at 4°C. The resulting protein complexes were precipitated from the lysates by adding protein A agarose and then incubated for 1 h at 4°C. Each sample was washed three times with lysis buffer at 4°C. Isolated receptor complexes (IP) were assessed for caspase-8, FLIPs, FADD, and DR5 by western blotting.

Conflict of Interest
The authors declare no conflict of interest.
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