**Human Cancer Biology**

**Helicobacter pylori** Augments Growth of Gastric Cancers via the Lipopolysaccharide-Toll-like Receptor 4 Pathway whereas Its Lipopolysaccharide Attenuates Antitumor Activities of Human Mononuclear Cells

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**Abstract**

**Purpose:** *Helicobacter pylori* is reportedly involved in the development of gastric cancer. We investigated the mechanisms by which *H. pylori* affects gastric cancer growth and antitumor immune responses in the host, focusing on *H. pylori* – derived lipopolysaccharide (LPS).

**Experimental Design:** *H. pylori* and four gastric cancer cell lines (MKN28, MKN45, NUGC3, and KATOIII) were used. We examined the effect of *H. pylori* or its LPS stimulation on cancer growth and the involvement of the *H. pylori* LPS– toll-like receptor 4 (TLR4) pathway. We also examined the cytotoxicities of *H. pylori* LPS–stimulated human mononuclear cells (MNC) against gastric cancer cells and the effect of *H. pylori* LPS stimulation on cytokine production by MNC.

**Results:** *H. pylori*, as well as its LPS, augmented the growth of gastric cancers, all of which expressed TLR4. Neutralization of TLR4 almost completely abrogated the *H. pylori* – induced proliferative activity of cancer cells. *Escherichia coli* LPS also augmented cancer growth via the LPS–TLR4 pathway. However, only *H. pylori*–derived LPS attenuated the cytotoxicity of MNC against gastric cancer cells. Stimulation with *H. pylori*/LPS also down-regulated perforin production in cancer cells – cocultured CD56+ natural killer cells. *H. pylori* LPS induced neither interleukin-12 nor IFN-γ production by MNC, although *E. coli* LPS did induce production of both significantly. Nevertheless, interleukin-12 stimulation restored the IFN-γ– producing capacity of *H. pylori* LPS – stimulated MNC.

**Conclusion:** *H. pylori* augmented the growth of gastric cancers via the LPS–TLR4 pathway, whereas it attenuated the antitumor activity and IFN-γ–mediated cellular immunity of MNC. *H. pylori* infection might thereby promote proliferation and progression of gastric cancers.

Persistent infection with *Helicobacter pylori* in gastric mucosa might be closely involved in the development of human gastric cancer (1–4). Epidemiologic studies have shown an up to 6-fold increased risk of developing gastric cancer in patients infected with *H. pylori* (1–4). *H. pylori* thereby has been classified as a definite carcinogen by the IARC in 1994 (5). Animal experiments using Mongolian gerbils also showed that infections with *H. pylori* induced gastric cancers (6–9). Recent clinical studies have suggested that *H. pylori* eradication can reduce the risk of developing gastric cancers (10–12).

*H. pylori* is a microaerophilic Gram-negative bacterium and possesses lipopolysaccharide (LPS) as a component of its outer membrane. Recently, toll-like receptor 4 (TLR4), which was identified as a receptor for LPS in 1997 (13, 14), has been found not only on human gastric epithelium but also on gastric cancer cells (15–20). Therefore, LPS derived from *H. pylori* may have certain influences on gastric cancer cells via TLR4. On the other hand, LPS is well known to have antitumor activity in experimental tumor-bearing animals (21–24). LPS, in particular *Escherichia coli*–derived LPS, strongly activates macrophages to produce proinflammatory cytokines. Interleukin (IL)-12 released by macrophages activates lymphocytes, such as natural killer (NK) cells or T cells, to produce IFN-γ, perforin, and/or granzyme B and thereby to acquire cytotoxicity against tumor cells (25). In particular, NK cells can kill tumor cells directly via the perforin-granzyme B pathway (26, 27). However, several investigators reported that *H. pylori* LPS is biologically less active than LPS of *E. coli* or *Salmonella* (28–30).

In the present study, we investigated the effect of *H. pylori* and its LPS on the growth of gastric cancers as well as on the
antitumor cytotoxicity of human mononuclear cells (MNC) in comparison with E. coli LPS. We found that H. pylori, its LPS, and E. coli LPS significantly augmented gastric cancer cell growth via the LPS-TLR4 pathway, whereas only E. coli LPS stimulated human MNC to produce interleukin (IL)-12 and its inducing cytokine, IFN-γ. Therefore, in the presence of H. pylori, gastric cancer cells might evade IFN-γ–mediated antitumor immune responses. Our results suggest that H. pylori may promote proliferation and progression of gastric cancers in the host.

**Materials and Methods**

**Gastric cancer cell lines and human MNC**

Human gastric cancer cell lines MKN28, MKN45, NUGC3, and KATOIII were used. MKN28, MKN45, and NUGC3 were obtained from the Japanese Collection of Research Bioresources Cell Bank. KATOIII cells were obtained from the Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University. To study human MNC, heparinized peripheral blood samples were obtained from healthy adult human volunteers. MNC were separated from peripheral blood by lymphocyte separation medium (ICN Biomedicals, Inc.) gradient centrifugation.

**H. pylori and E. coli – derived LPS**

H. pylori strain TN12, which was clinically isolated and contains cagA and VacA, was a kind gift from Takeda Pharmaceutical Co. H. pylori was grown in brain heart infusion broth (Difco Laboratories) containing 10% horse serum for 150 h under microaerophilic conditions (5% O2, 85% N2, 10% CO2) at 37°C with shaking. The inoculum was prepared by growing cultures for 20 h at 37°C, followed by centrifugation at 3000 × g for 20 min and then resuspension in PBS with glycerol. Samples were adjusted to a concentration of 1 × 10^8 colony-forming units (CFU)/mL and frozen in 1-mL aliquots at −80°C until use. H. pylori was confirmed by a urease test and Gram stain morphology. H. pylori was adjusted to a concentration of 1 × 10^8 CFU/mL with RPMI and cocultured with gastric cancer cells. H. pylori – derived LPS (extracted from the ATCC 43504 strain) was a gift from Ohtsuka Co. (31). E. coli – derived LPS (E. coli 0111: B4) was purchased from Sigma Co.

**Viable cancer cell counts**

After each incubation of gastric cancer cells, the cells (5 × 10^4/mL) on the dish were washed in PBS and treated with trypsin for 5 min at room temperature. After centrifugation to remove the trypsin, the cells were resuspended in RPMI 1640 and mixed with an equal volume of 0.4% trypan blue staining solution for 5 min. Viable cancer cells were then resuspended in RPMI 1640 and mixed with an equal volume of 0.4% trypan blue staining solution for 5 min. Viable cancer cells were counted with a hemocytometer.

**Immunohistochemistry for proliferating cell nuclear antigen**

Gastric cancer cells were plated at a density of 5 × 10^4 cells/cm² in culture dishes and incubated with or without viable H. pylori for 24 h. After incubation, dishes were washed twice with cold saline. Subsequently, the cells in the dishes were fixed in 3.0 mol/L NaCl in 70% ethanol for 30 min. The fixed cells were lysed in 0.12 N HCl in 70% ethanol for 10 min and washed with PBS for 5 min thrice. The cells were incubated with 1% normal horse serum in PBS for 30 min at room temperature. After washing with PBS for 5 min, rabbit polyclonal anti–proliferating cell nuclear antigen (PCNA; FL-261) antibody (Santa Cruz Biotechnology) was used as the primary antibody, followed by the rabbit ABC staining system (Santa Cruz Biotechnology; ref. 32). PCNA-positive cells were counted by light microscopy at ×100 magnification and expressed as the number of positive cells per 1,000 cancer cells.

**Proliferation assay**

The proliferation assay was done using [3H]thymidine incorporation as previously described (33). Briefly, 1 × 10^5 gastric cancer cells were plated in 96-well flat-bottomed plates in 0.2-mL RPMI 1640 containing 10% human serum. After a 24-h stimulation with or without several concentrations of viable H. pylori, heat-killed H. pylori, H. pylori – derived LPS, or E. coli – derived LPS, tumor cells were pulsed with 1 μCi/well [3H]thymidine (Amersham Canada Ltd.). Tumor cells were cultured for an additional 12 h, and incorporation of [3H]thymidine into the DNA of proliferating tumor cells was measured with a liquid scintillation counter.

**Neutralization of TLR4**

Gastric cancer cells were preincubated with 1 ng/mL of the TLR4-neutralizing antibody (HTA125, MONOSAN) for 6 h. Subsequently, the cells were incubated with viable H. pylori, H. pylori LPS, or E. coli LPS for an additional 24 h, and the proliferative activity was examined with [3H]thymidine uptake.

**Analyses of TLR4 expression on cancer cells**

Gastric cancer cell lines were stained with a FITC-conjugated anti-TLR4 monoclonal antibody (mAb; HTA125, Serotec). As an isotype control, mouse IgG2a-FITC (MOPC-31C, PharMingen) was used. Flow cytometry was then done with the ESPICS XL (Coulter) to analyze TLR4 expression on cancer cells.

**RNA extraction and reverse transcription-PCR analysis of cancer cell lines**

**Reverse transcription-PCR.** Total RNA was extracted from the gastric cancer cell lines using TRI Reagent (Sigma) and RNeasy Mini Kit with DNase digestion (Qiagen) according to the manufacturer’s instructions. A cDNA was synthesized with SuperScript II (Invitrogen) using oligo(dT)12–18 primer (Invitrogen). Thirty cycles of PCR with the primers 5′-TGGATACGTTTCTCTTATAAC-3′ and 5′-GAAATGGAGG-CACCCCTTC-3′ for TLR4 and 5′-ACCACTCGCTATGCCATC-3′ and 5′-AGTGTGTTGGACACGACAT-3′ for glyceraldehyde-3-phosphate dehydrogenase were carried out with Taq polymerase (Boehringer Mannheim GmbH). Cycling conditions were 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were subjected to electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and viewed under UV transillumination.

**Real-time PCR.** We obtained total RNA from the gastric cancer cell lines using RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Reverse transcription was done using 1 μg of total RNA. Real-time PCR with the Applied Biosystems ABI PRISM 7700 sequence detector and Assays-on-Demand (TaqMan) were done per manufacturer’s protocol. Real-time PCR was evaluated with the Sequence Detection System software (Applied Biosystems). The threshold cycle (Ct) values were normalized to Cβ values for glyceraldehyde-3-phosphate dehydrogenase. Cycling conditions were 95°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Real-time PCR was carried out on an ABI PRISM 7000 sequence detection system (Applied Biosystems).

**Intracellular staining of perforin**

Cancer cells were stained with a phycoerythrin-conjugated anti–CD56 mAb (NKH-1; Beckman Coulter) and a PC5-conjugated anti–β5 mAb (BA1031; Beckman Coulter). After washes, the cells were fixed and permeabilized in 250 μL of Cytofix/Cytoperm (PharMingen) for 20 min at 4°C. Thereafter, the cells were washed twice and incubated with FITC-conjugated anti-perforin mAb (iG0; Ancell) for 30 min at 4°C. Flow cytometry was then done with EPICS XL (Coulter). An isotype control, FITC-conjugated mouse IgG2b (2810A8; Beckman Coulter), was used.

**Measurements of IFN-γ and IL-12 production by LPS-stimulated MNC and additional stimulation with IL-2 and IL-15**

Human MNC were cultured with H. pylori LPS or E. coli LPS for 24 h to measure IL-12 production. IL-2 (1,000 unit/mL; PeproTech EC) and IL-15 (5 ng/mL; Genzyme) were added to the culture of
LPS-stimulated MNC to maintain the viability of MNC in vitro, and their IFN-γ production was measured. IFN-γ and IL-12 levels in the culture supernatants were determined with ELISA kits commercially available from Endogen. The samples were prepared and tested according to the manufacturer’s protocol.

**Statistics**

The data were expressed as the mean ± SE. Statistical evaluations were conducted with the standard one-way ANOVA followed by the Bonferroni post hoc test. P < 0.05 was considered statistically significant. All analyses were done using statistical software (StatView version 5.0, SAS Institute, Inc.).

**Results**

**H. pylori significantly augments the growth of gastric cancer cell lines.** We examined the effect of *H. pylori* stimulation on the growth of gastric cancers. Gastric cancer cell lines (5 × 10⁴ cells/mL), MKN28, MKN45, NUGC3, and KATOIII, were cultured with or without *H. pylori* (1 × 10⁷ CFU/mL) for 24 and 72 h, and then the numbers of viable cells were counted (A). Gastric cancer cell lines were also cultured with or without *H. pylori* for 48 h, and then evaluated for PCNA index (B) and thymidine uptake (C). Points and columns, mean from three individual experiments with three to four samples per group; bars, SE. *, P < 0.01; †, P < 0.05, versus other groups.

![Graphs showing experimental results](image)

**Fig. 1.** The effect of *H. pylori* stimulation on the number of viable cells (A), PCNA index (B), and thymidine uptake (C) in gastric cancer cell lines. Gastric cancer cell lines (5 × 10⁴ cells/mL), MKN28, MKN45, NUGC3, and KATOIII, were cultured with or without *H. pylori* (1 × 10⁷ CFU/mL) for 24 and 72 h, and then the numbers of viable cells were counted (A). Gastric cancer cell lines were also cultured with or without *H. pylori* for 48 h, and then evaluated for PCNA index (B) and thymidine uptake (C). Points and columns, mean from three individual experiments with three to four samples per group; bars, SE. *, P < 0.01; †, P < 0.05, versus other groups.

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**Results**

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**H. pylori augments the proliferation of gastric cancer cells via the LPS-TLR4 pathway.** We examined which components of *H. pylori* stimulate the proliferation of gastric cancer cells. First, we cultured gastric cancer cell lines with heat-killed *H. pylori* (0.5 or 5 μg/mL) for 24 hours. All cancer cells displayed significantly increased thymidine uptake after stimulation with heat-killed *H. pylori* (Fig. 2A). When gastric cancer cells were cultured with *H. pylori* LPS at 1 μg/mL for 24 hours, their proliferative activity was augmented significantly (Fig. 2B). However, *H. pylori* LPS stimulation at 1 ng/mL (Fig. 2B) and at <1 μg/mL (data not shown) did not induce any significant cancer proliferation. When TLR4 in the culture was neutralized by antibodies, the enhanced proliferative activities induced by *H. pylori* (1 μg/mL) were almost completely abrogated (Fig. 2B). We also examined the participation of the LPS-TLR4 signaling pathway in cancer cell proliferation using *E. coli*–derived LPS. *E. coli* LPS (1 ng/mL) significantly augmented cancer cell proliferation, and the enhanced proliferation was completely abrogated by the neutralizing anti-TLR4 antibody (Fig. 2C).
confirmed that *H. pylori* LPS did not affect the proliferation of any cancer cells at concentrations ≤500 ng/mL (data not shown), whereas *E. coli* LPS induced cancer cell apoptosis at 1 μg/mL (data not shown). Furthermore, neutralization of TLR4 also completely abrogated the proliferation of gastric cancer cells that was enhanced by viable *H. pylori* (Fig. 2D). We confirmed TLR4 expression on the gastric cancer cell lines by flow cytometry (Fig. 3A). We further examined TLR4 mRNA in the gastric cancer cell lines. As expected, all showed significant expression of TLR4 mRNA by reverse transcription-PCR analysis (Fig. 3B). In addition, real-time reverse transcription-PCR analysis revealed that stimulation with heat-killed *H. pylori* up-regulated their TLR4 mRNA levels (Fig. 3C), suggesting the involvement of the LPS-TLR4 signaling pathway.

### Preincubation of gastric cancer cells with *H. pylori* or its LPS suppresses IFN-γ production by MNC.

We examined the effect of preincubation of gastric cancer cells with *H. pylori* or its LPS on the cellular immune responses of MNC, as assessed by IFN-γ production. First, we preincubated gastric cancer cells with *H. pylori* or its LPS for 24 hours. To avoid direct stimulation of MNC with *H. pylori*/LPS, cancer cells were washed thoroughly to remove *H. pylori*/LPS and then cocultured with human MNC for 24 hours. Although human MNC increased their IFN-γ production remarkably when cocultured with cancer cells, preincubation of cancer cells with *H. pylori* significantly suppressed IFN-γ production of MNC (Fig. 4A). Preincubation of cancer cells with *H. pylori* LPS also suppressed IFN-γ production by the cocultured MNC (Fig. 4A). *H. pylori* or its LPS, but not *E. coli* LPS, attenuates the antitumor cytotoxicity of MNC against gastric cancer cells. To examine the viability of cancer cells and IFN-γ production by MNC, cancer cells and MNC were cocultured under stimulation with *H. pylori, H. pylori* LPS, or *E. coli* LPS for 24 hours. Although >90% of the gastric cancer cells were viable when cultured without MNC, coculture with MNC remarkably decreased the proportion of viable cancer cells (Fig. 4B). However, stimulation with *H. pylori* or its LPS significantly increased the proportion of viable cancer cells when cocultured with MNC (Fig. 4B). In contrast, stimulation with *E. coli* LPS did not increase the proportion of viable cancer cells (Fig. 4B), suggesting that *H. pylori* or its LPS, but not *E. coli* LPS, attenuated the antitumor cytotoxicity of MNC. Stimulation with *E. coli* LPS significantly increased IFN-γ production by MNC, which suggests the enhancement of IFN-γ-mediated

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**Fig. 2.** Proliferation of gastric cancer cell lines with stimulation by heat-killed *H. pylori* (A), *H. pylori* – derived LPS (B), *E. coli* – derived LPS (C), and viable *H. pylori* (D).

Gastric cancer cells (5 × 10^4/mL) were cultured with or without heat-killed *H. pylori* (0.5 or 5 μg/mL) for 24 h to measure their thymidine uptake (A). Cancer cells were also cultured with or without stimulation by *H. pylori* – derived LPS (1 ng/mL or 1 μg/mL; B), *E. coli* – derived LPS (1 ng/mL; C), or viable *H. pylori* (1 × 10^8 CFU/mL; D) for 24 h. To examine the effect of TLR4, cancer cells were preincubated with TLR4-neutralizing antibody for 6 h and then cultured with *H. pylori* LPS (B), *E. coli* LPS (C), or viable *H. pylori* (D) for 24 h. Thereafter, thymidine uptake was measured. Columns, mean from three individual experiments with three to four samples per group; bars, SE.

* *, P < 0.01; ** P < 0.05, versus other groups.
cellular immunity (Fig. 4C). However, stimulation with \textit{H. pylori} or its LPS did not increase IFN-\(\gamma\) production by the cancer-cocultured MNC (Fig. 4C).

**H. pylori or its LPS down-regulates perforin production in CD56 NK cells.** We next examined the effect of stimulation with \textit{H. pylori} or its LPS on perforin production in MNC, particularly in CD56 NK cells. The CD56 NK cells were strongly activated to produce a large amount of perforins when cocultured with gastric cancer cells (Fig. 5A and B), compared with those without cocultivation (Fig. 5C). Interestingly, stimulation with \textit{H. pylori} or its LPS apparently down-regulated the perforin production in cancer-cocultured CD56 NK cells (Fig. 5A and B). Neither CD57 NK cells nor CD56/CD57 NKT cells showed remarkable perforin production when cocultured with cancer cells. In addition, their perforin production was not affected by \textit{H. pylori}/LPS stimulation (data not shown).

Stimulation with \textit{E. coli} LPS did not enhance the perforin production by CD56 NK cells, CD57 NK cells, or CD56/CD57 NKT cells that were cocultured with cancer cells (Fig. 5D).

**Inability of \textit{H. pylori} LPS to stimulate human MNC results in a lack of IL-12 secretion and thereby leads to insufficient IFN-\(\gamma\) production.** Finally, we examined the effect of stimulation with \textit{H. pylori} LPS on human MNC, focusing on their cytokine production. Human MNC were cultured with \textit{H. pylori} LPS or \textit{E. coli} LPS for 24 hours. Although \textit{E. coli} LPS stimulated MNC to produce a large amount of IL-12, \textit{H. pylori} LPS did not induce any IL-12 production by MNC (Fig. 6A). Because no IFN-\(\gamma\) production was detected in the culture supernatant of the \textit{H. pylori} LPS– or \textit{E. coli} LPS–stimulated MNC (not shown),
small amounts of IL-2 and IL-15 were added to the culture to maintain MNC viability, and their IFN-γ production at 24 hours was measured. Interestingly, H. pylori LPS did not augment IFN-γ production by MNC, even under additional stimulation with IL-2 and IL-15, although E. coli LPS dose-dependently increased IFN-γ production (Fig. 6B). H. pylori LPS also did not augment IL-12 production by MNC under additional stimulation with IL-2 and IL-15, whereas E. coli LPS did so in a dose-dependent manner (Fig. 6C). Basically, IL-12 is produced exclusively by macrophages, whereas IFN-γ is produced mainly by lymphocytes in response to IL-12 stimulation. Therefore, we next tested the IFN-γ-producing capability of the H. pylori LPS–stimulated MNC in the presence of recombinant IL-12. H. pylori LPS–stimulated MNC increased IFN-γ production remarkably by additional IL-12 stimulation (with IL-2 and IL-15; Fig. 6D). This suggests that insufficient induction of IL-12 in MNC, in particular macrophages, by H. pylori LPS stimulation is one of the major reasons why H. pylori LPS cannot induce human MNC to produce a substantial amount of IFN-γ.

Discussion

Viable H. pylori significantly augmented the growth of gastric cancer cell lines. H. pylori LPS, as well as E. coli LPS, also augmented the proliferation of gastric cancer cells. Gastric cancer cells expressed TLR4, and a neutralizing antibody against TLR4 almost completely abrogated the proliferative activities of cancer cells, which were induced not only by H. pylori LPS and E. coli LPS but also by viable H. pylori. Preincubation of cancer cells with H. pylori suppressed IFN-γ production by the cocultured human MNC. Furthermore, H. pylori or its LPS, but not E. coli LPS, attenuated the antitumor cytotoxicity of MNC against gastric cancer cells by suppressing MNC IFN-γ production. Stimulation with H. pylori/LPS also down-regulated perforin production in CD56 NK cells cocultured with cancer cells. Although E. coli LPS dose-dependently induced IL-12 production by human MNC, H. pylori LPS did not induce any IL-12 production by MNC and thereby could not induce their IFN-γ production even under additional stimulation with IL-2 and IL-15. However, H. pylori LPS–stimulated MNC had the potential to produce IFN-γ, because additional IL-12 stimulation restored a substantial amount of IFN-γ production by those MNC. Insufficient induction of cellular immune responses by H. pylori LPS, which is quite different from E. coli LPS, might thus render the host susceptible to gastric cancer growth.

Many interventional studies have been done to establish a relationship between H. pylori infection and gastric cancer. Wong et al. (11) showed that the overall incidence of gastric cancer was similar between patients who received H. pylori eradication therapy and those who received placebo during a follow-up of 7.5 years, whereas H. pylori eradication obviously delayed the time point of a new cancer detection. Take et al. (12) also showed that detection time points of new cancer lesions were delayed in patients cured by eradication therapy compared with patients with persistent H. pylori infections, although H. pylori eradication did not reduce the occurrence of cancer dramatically. Thus, H. pylori eradication might delay the development of gastric cancers until they grow to a detectable size, despite no remarkable reduction in the overall incidence of gastric cancers. Taken together, H. pylori infection may promote the proliferation and progression of gastric cancer cells, although infection control itself does not affect the carcinogenesis step.

H. pylori is a microaerophilic Gram-negative bacillus. H. pylori and other Gram-negative bacilli possess LPS, but its virulence is lower than that of typical bacterial endotoxins, such as E. coli–derived LPS (28, 30, 34). Generally, LPS is composed of a lipid core (lipid A) and polysaccharide side chains of variable length. Lipid A represents the endotoxic principle of active LPS. Lipid A of H. pylori LPS has a unique structure that contains uncommonly long 3-hydroxy fatty acids and a backbone consisting of a 1-glucosamine disaccharide with a 2-aminoethylphosphate group at position 1 (35, 36). Both
H. pylori– and E. coli–derived LPS significantly augmented the proliferation of gastric cancers, and their LPS-induced proliferative activities on cancer cells were almost completely abrogated by neutralizing anti-TLR4 antibodies (Fig. 2B and C). LPS might thus have the potential for a proliferative effect on cancer cells through its ligand receptor, TLR4. Proliferative activities of gastric cancer cells induced by viable H. pylori might be attributed exclusively to H. pylori–derived LPS because these proliferative activities were also completely suppressed by the neutralization of TLR4 (Fig. 2D). Interestingly, E. coli–derived LPS showed a more remarkable cancer proliferative activity than H. pylori–derived LPS. E. coli LPS augmented cancer proliferation at 1 ng/mL, whereas 1 μg/mL of H. pylori LPS was required to show a similar effect. Nevertheless, there are few reports that E. coli LPS is involved in the growth of colon cancers. Because the host attempts to prevent tumor growth and invasion via lymphocyte-mediated antitumor immunity, we then focused on the difference in antitumor cytotoxicity of human MNC between H. pylori LPS stimulation and E. coli LPS stimulation.

Immunohistochemical studies using biopsy specimens have revealed that the human gastric epithelium expresses TLR4, and that a higher expression of TLR4 is observed in H. pylori–infected gastric epithelium as compared with uninfected gastric epithelium (19, 37–39). Interestingly, the stimulation with heat-killed H. pylori also up-regulated the expression of TLR4 mRNA in gastric cancer cells (Fig. 3C). It has been reported that activation of TLR4 signaling might be important for tumor cells to evade the immune surveillance system (18). In line with this, H. pylori LPS–preincubated cancer cells suppressed IFN-γ production by cocultured lymphocytes (Fig. 4A). These results suggest that activation of the TLR4 signaling pathway in gastric cancer cells by H. pylori LPS stimulation affects the expression of some surface molecules or soluble factors that may help them escape from the attack by NK cells or CTLs via IFN-γ–mediated cellular immune reaction, although further study on the TLR4-related tumor evasion is required.

In the coculture situation, human MNC reduced the proportion of viable cancer cells significantly (Fig. 4B), which suggests a potent antitumor cytotoxicity of MNC. Among those, CD56 NK cells can produce or release substantial amounts of perforin and granzyme B, which directly kill tumor cells

Fig. 6. The effect of stimulation with H. pylori LPS or E. coli LPS on IL-12 production by human MNC (A). Human MNC (1 × 10^6/mL) were cultured with stimulation by H. pylori LPS or E. coli LPS at the indicated concentrations for 24 h to measure their IL-12 production. The effect of stimulation with H. pylori LPS or E. coli LPS on IFN-γ (B) and IL-12 (C) production by human MNC under additional stimulation with IL-2 and IL-15. MNC were cultured with H. pylori LPS or E. coli LPS and further stimulated with a small amount of IL-2 (1,000 unit/mL) and IL-15 (5 ng/mL) for 24 h to measure IFN-γ (B) and IL-12 (C) production. The effect of additional IL-12 stimulation to H. pylori LPS on IFN-γ production by human MNC (D). MNC were cultured by H. pylori LPS stimulation at the indicated concentrations with or without additional IL-12 stimulation (2 ng/mL; adding to IL-2 and IL-15) for 24 h to measure IFN-γ production. Columns, mean from three individual experiments with three to four samples per group; bars, SE. ∗, P < 0.05, versus other groups; †, P < 0.01, versus H. pylori LPS; ‡, P < 0.01, versus without IL-12 stimulation.
(26, 27). Perforin production in CD56 NK cells was apparently up-regulated by coculture with gastric cancer cells (Fig. 5). Generally, LPS can activate or stimulate human MNC to enhance inflammatory immune reactions and antitumor immune responses in the host (24). However, it is noteworthy that H. pylori – derived LPS could not induce IL-12 or IFN-γ production by the human MNC, although E. coli – derived LPS induced these cytokines remarkably (Fig. 6A-C). IL-12 and its inducing cytokine, IFN-γ, are crucially important factors for evoking innate cellular immunities (25, 40–43). Insufficient induction of cellular immune responses by the stimulation of H. pylori LPS may render the host susceptible to cancer progression/proliferation. This finding provides a conceivable explanation on why H. pylori is involved in the development of gastric cancer. It is likely that E. coli LPS stimulates the host cellular immune response to provide enough IFN-γ to prevent cancer progression. Because E. coli inhabits the host colon as normal intestinal flora, antitumor activity may be continuously up-regulated by E. coli LPS, and potent tumor proliferative activity is effectively repressed.

Several investigators reported that H. pylori LPS possesses a lower virulence than E. coli LPS (34, 44). Ismail et al. (45) showed that low doses of outer membrane vesicles shed from the surface of H. pylori induced the proliferation of a gastric epithelial cell line, whereas high doses of outer membrane vesicles induced cytotoxicity. In addition, chronic H. pylori infection often induces alterations in gastric mucosal cell proliferation. Kidd et al. (46) reported that H. pylori LPS exhibits a specific mitogenic effect on gastric enterochromaffin-like cell neoplasia. H. pylori LPS might thus have a unique potential to show a weak virulence to evoke inflammatory responses, but a high potential to augment cell growth, which is quite different from E. coli LPS.

From a clinical standpoint, regulation of the H. pylori LPS-TLR4 pathway may be a new potent therapeutic tool. Because H. pylori is a poor stimulator of macrophages and also impairs the antitumor immune responses of the host NK cells, immunoaivation therapy to restore immune function may be efficacious against H. pylori – mediated gastric cancer development. We hope that our findings will provide a new insight into the mechanisms by which H. pylori is closely involved in the development of gastric cancers and also contributes to the development of new therapeutic strategies against gastric cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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