Lipopolysaccharides From Non-*Helicobacter pylori* Gastric Bacteria Potently Stimulate Interleukin-8 Production in Gastric Epithelial Cells

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BACKGROUND: **Gastric acid secretion is compromised in chronic *Helicobacter pylori* (*H. pylori*) infection allowing overgrowth of non-*H. pylori* gastric bacteria (NHGB) in the stomach.**

METHODS: **NHGB were isolated from gastric mucosa in selective media and further characterized with biochemical methods and 16S rRNA gene sequencing. Human gastric tissues were studied with indirect immunofluorescence with antibodies against *H. pylori* and *Neisseria subflava (N. subflava)*. Gastric epithelial cell lines were cocultured with bacteria or incubated with lipopolysaccharides isolated from NHGB, and interleukin-8 released in the media was measured by enzyme-linked immunosorbent assay. Expression of Toll-like receptor (TLR)2, TLR4, it's coreceptor myeloid differentiation factor 2 (MD2), and CD14 in gastric cells was investigated by immunofluorescence microscopy and reverse transcriptase-polymerase chain reaction.**

RESULTS: ***Haemophilus* species, *Neisseria* species, *Fusobacterium* species, and *Veillonella* species were predominant Gram-negative bacteria coinfected with *H. pylori*. Lipopolysaccharides from *N. subflava* potently stimulated interleukin-8 secretion in MKN45 cells which was cancelled by preincubation with polymyxin B. TLR2, TLR4, CD14, and myeloid differentiation factor 2 were expressed in MKN45 cells, though their levels of expression were low. *N. subflava* adhered to MKN45 cells *in vitro* and colocalized with *H. pylori* in the human gastric mucosa.**

CONCLUSIONS: **Our data suggest that *N. subflava* colonized in the gastric mucosa contribute to gastric inflammation during chronic *H. pylori* gastritis.**

TRANSLATIONAL IMPACT: **NHGB may perpetuate gastric inflammation and accelerate neoplastic progression in the hypochlorhydric stomach.**

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A20

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INTRODUCTION

In the healthy human stomach, gastric juice is highly acidic, with a pH of 1–2. Because of this hostile environment for the growth of bacteria, intragastric milieu was believed to be free of microbiota before the seminal discovery of *Helicobacter pylori* (*H. pylori*) by Warren and Marshall (1). Subsequent studies established that *H. pylori* infection is an important cause of gastroduodenal diseases such as chronic gastritis (2), peptic ulcer (3), mucosa-associated lymphoid tissue lymphoma (4), and gastric cancer (5).

Nevertheless, a number of issues concerning a link between *H. pylori* infection and gastric cancer still remain to be solved. For example, despite high prevalence of *H. pylori* infection in South Asian or African countries, gastric cancer incidence and mortality

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are low (coined as "African enigma" or "Asian enigma") (6,7). Difference in the virulence of *H. pylori* strains may partly explain this phenomenon, but even in Japan where virulent *H. pylori* strains are ubiquitously prevalent, gastric cancer mortality among prefectures varies greatly with a lower tendency in the southern provinces. Moreover, despite long-standing *H. pylori* infection, a significantly lower occurrence of gastric cancer in duodenal ulcer patients as compared to gastric ulcer was reported (8). How do we reconcile this contradictory epidemiological evidence against a direct role of *H. pylori* in gastric carcinogenesis? A potential account for this question may be given if we consider gastric atrophy as an additional key risk factor for gastric cancer development. It was documented that the incidence of gastric atrophy in southern countries is low (9), and acid secretion of patients with duodenal ulcer generally remains high due to antral predominant inflammation. It was also reported that atrophic grades as stratified with pepsinogen I/II ratio in the Japanese prefectures show near complete correlation with age-adjusted gastric cancer incidence (10). Uemura's prospective study also showed that the risk of gastric cancer development was dependent on the grade of atrophy and intestinal metaplasia (11). Collectively, these data support the hypothesis that hypochlorhydria can be an important risk factor for the development of gastric cancer in addition to *H. pylori* infection.

Gastric atrophy allows non-*H. pylori* bacterial overgrowth in the stomach. Indeed, several studies have reported the presence of non-*Helicobacter pylori* gastric bacteria (NHGB) in the stomach in patients with achlorhydria (12) or under proton pump inhibitor therapy (13). Recently, sequencing-based analyses on human gastric microbiota revealed that a variety of NHGB with or without *H. pylori* infection occurs in the human stomach (14–18). Furthermore, Yuan et al. (19) reported the presence of NHGB with *H. pylor*i infection using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Although these reports set a new stage of our understanding of gastric microbiota, their functional roles in gastric pathophysiology remain largely unknown. Coinfection of these NHGB, however, was shown to aggravate gastric inflammation (18), which corroborate with the involvement of NHGB in gastric carcinogenesis in rodent models (20–22). In this report, therefore, we set out to isolate NHGB in patients with *H. pylori* and explored their activities to stimulate interleukin-8 (IL-8) secretion from gastric epithelial cell lines. We also examined the activity of lipopolysaccharides (LPS) isolated from them to stimulate IL-8 secretion. We measured this cytokine as it is a critical cytokine to recruit and activate neutrophils in the mucosa. Activated neutrophils may cause harmful effects to epithelial cells via production of toxic reactive oxygen species leading to genetic changes (23,24). Indeed, a recent report indicated neutrophils might be linked to gastric carcinogenesis (25).

METHODS

Gastric bacterial strains

We performed experiments 3 times for each strain; 3 *H. pylori* strains and 5 strains each of NHGB (*Neisseria spp., Fusobacterium spp., Haemophilus spp., and Veillonella spp.*) were identified by their biochemical properties (ID test HN-20 Rapid, Nissui, Tokyo, Japan; RapID ANA II, KS). Information on the patients from whom strains were isolated is shown in Figure 1 and supplementary Table 1, Supplementary Digital Content 1, http://links.lww.com/CTG/A20. *Fusobacterium spp*., *Neisseria spp.*, and *Veillonella spp*. were further characterized by 16S rRNA gene sequences analyzed with the BigDye Terminator v.3.1 Cycle Sequencing ready Kit (Applied Biosystems, CA). The primers used for polymerase chain reaction (PCR) amplification for identifying bacteria are shown in Supplementary Table 2, Supplementary Digital Content 1, http://links.lww.com/CTG/A20. Written informed consents were obtained from all the patients who participated in this study, and institutional ethical approval for this research was obtained (March 26, 2007).

Extraction of LPS

In order to extract respective LPS from gastric bacteria, large-scale cultures of isolated strains were conducted in appropriate media as shown in Supplementary Table 3, Supplementary Digital Content 1, http://links.lww.com/CTG/A20. Five strains of each non-*H. pylori* bacterial cells and 3 strains of *H. pylori* cultured in broth and agar media were harvested by centrifugation (8,000 rpm, 15 minutes, 4 °C) or scraping of colonies. Approximately 170–220 mg LPS was extracted from the lyophilized bacterial cells (3–5 g dry weight) by a hot phenol–water method (26). In addition, purified LPS from *H. pylori* (GU2 and GC2) and *Escherichia coli* (*E. coli*) (2 lots of O55 and 2 lots of O111) were purchased from Wako Pure Chemicals (Tokyo, Japan). Crude LPS from *Neisseria* was treated with 10 μg/mL of DNase I (Takara Bio, Shiga, Tokyo), 10 μg/mL of RNase A (Sigma-Aldrich, MO), and proteinase K (Sigma-Aldrich) and purified by ultracentrifugation. Pam3CSK4, a TRL2 agonist, was purchased from Novus Bio (CO).

Cell lines and reagents

Human gastric cancer cell lines, MKN45, MKN28, and KATO3, were purchased from the Japanese Collection of Research Bioresources (Osaka, Japan), and AGS was obtained from American Type Culture Collection (Manassas, VA). They were cultured in the media as recommended by the suppliers.

Assay for IL-8 production

IL-8 released in the media by various LPS (0.01, 0.1, 1 and 10 μg/mL) and Pam3CSK4 (0.01, 0.1 μg/mL) was determined with a commercial enzyme-linked immunosorbent assay kit (Quantikine enzyme-linked immunosorbent assay; R&D Systems, MN). Effect of double stimulation with LPS derived from *N. subflava* together with LPS from *H. pylori* at a concentration of 0.1 μg/mL for 24 hrs was also tested. In some experiments, effect of preincubation of LPS from *N. subflava* or *H. pylori* with polymyxin B (10 μg/mL), an LPS inhibitor, was examined. SC-514 (100 μM, Calbiochem, CA), a potent inhibitor of kappa B kinase (IKK)-2 inhibitor, was used to examine the role of nuclear factor-κB (NF-κB) activation in IL-8 production.

To examine the effect of live *H. pylori* or *N. subflava* on IL-8 secretion, MKN45 cells were infected with at the multiplicity of infection of 1, 10, and 100 with different incubation times (6 hours) in the antibiotic-free media.

Immunofluorescence study

We used the non-neoplastic part of the gastric tissues in endoscopically dissected specimens treated for early gastric cancer. This histological study was approved by the ethics committee of the Jichi Medical University (No. A12-61).

The antibody of *N. subflava* was raised by immunization of rabbit with a killed whole-cell (63 °C, 30 minutes) antigen. Immunoglobulin G (IgG) from immunized rabbit sera was purified

Figure 1 Detection rate of microorganisms in the stomach. Twenty-five *H. pylori*-positive patients (17 men and 8 women, aged between 26 and 73 years, mean 55.7 years) and 25 *H. pylori*-negative patients (14 men and 11 women, aged between 26 and 69 years, mean 47 years) who visited Jichi Medical University Hospital for endoscopic examinations were enrolled. *H. pylori* infection in the subjects had been determined by a serological method (E plate, Eiken Chemical, Tokyo, Japan) and culture. Red cells indicate NHGB positive, and grey cells indicate not determined. ENT, *Enterococcus spp*; EUB, *Eubacterium spp*; FUS, *Fusobacterium spp; H. pylori*, *Helicobacter pylori*; HAE, *Haemophilus spp*; NEI, Neisseria spp; NHGB, non-*H. pylori* gastric bacteria; STR, *Streptococcus spp*; VEI, *Veillonella spp*.

with a Sepharose-Protein A Column (Bio-Rad, CA). The specificity of the antibody against *N. subflava* showed no cross-reactivity with flame-fixed cells of *Haemophilus*, *Fusobacterium*, V*eillonella*, or *H. pylori* (data not shown). A prediluted mouse monoclonal anti-*H. pylori*-specific antibody was purchased from Abcam (MA). To visualize immunoreactive materials, Alexa Fluor 488 donkey anti-rabbit IgG (H + L) (1:200; Invitrogen, CA) and Cy3-conjugated AffiniPure donkey anti-mouse IgG (H + L) (1:200; Jackson ImmunoResearch, PA) were added and incubated for 1 hour at room temperature. After rinsing, specimens were examined and digital images were recorded with a Biorevo BZ9000 microscope (Keyence, Osaka, Japan).

To examine the adhesion of *N. subflava* to MKN45 cells, bacteria were cultured with MKN45 for 3 hours with multiplicity of infection 10 and washed 3 times with phosphate buffered saline. After incubation with the anti-*N. subflava* IgG, Alexa Fluor 488 donkey anti-rabbit IgG (1:200) was used as a secondary antibody to visualize attached bacteria.

Mouse monoclonal anti-TL4 (1:100; Abcam), rabbit polyclonal anti-Toll-like receptor (TLR)2 (1:100; Abcam), and Cy3-conjugated AffiniPure donkey anti-mouse IgG (H + L) (1:200; Jackson ImmunoResearch) were used for studying the expression of TLR4 and TLR2 on MKN45.

Nuclear translocation of NF-κB was studied after stimulation of MKN45 cells with *N. subflava* LPS 1 μg/mL for 2 hours. Mouse monoclonal antibody against p65 subunit, an active subunit of NF-κB (clone 12H11; 1:50; Merck Millipore), was used as the primary antibody followed by fluorescent labeling with Cy3-conjugated AffiniPure donkey anti-mouse IgG (H + L) (1:200; Jackson ImmunoResearch), and DAPI (4,6-diamidino-2-phenylinodole, Dihydrochloride; Life Technologies, CA) was used for nuclear staining.

Reverse transcriptase-polymerase chain reaction

Total RNA was prepared from cells using RNAiso Plus (Takara) according to the protocols recommended by the manufacturer. The extracted RNA was reverse-transcribed to first strand cDNA

using PrimeScript Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Kit (Takara). The primers used for amplification in RT-PCR and amplification procedures are provided in Supplementary Table 4, Supplementary Digital Content 1, http://links.lww.com/CTG/A20. Amplified products were separated on 2% agarose gel electrophoresis, stained with Atras ClearSight DNA stain (Bioatras, Tartu, Estonia) and visualized by the Fluor-S Multi-imager (Bio-Rad).

Data analysis

The experiments were performed at least 3 times for each strain and mean and standard error were reported. One-way ANOVA with Tukey's honestly significant difference post hoc analyses was applied to compare difference between groups. The level of significance was set at $P < 0.05$. All statistical analyses were performed with SPSS software (version 11.0; SPSS, IL).

RESULTS

We could demonstrate abundant growth of both aerobic and anaerobic NHGB in the *H. pylori*-infected stomach (Figure 1). Among the 5 *Haemophilus spp*. cultured, 2, 2, and 1 strains were identified as *H. parahaemolyticus*, *H. parainfluenzae*, and *H. influenza*, respectively. All 5 *Neisseria spp*. were identified as *N. subflava* based on a criterion of over 98% homology of 16S rRNA gene sequence (see Supplementary Table 4, Supplementary Digital Content 1, http://links.lww.com/CTG/A20). Similarly, all 5 *Fusobacterium spp*. were identified as *F. periodonticum*, whereas among the 5 V*eillonella spp*., 2, 1, 1, and 1 strains were identified as *V. rogasae*, *V. parvula*, *V. dispar*, and *V. atypia*, respectively.

We determined IL-8 secretion levels with 4 different human gastric cancer cell lines. In our series of experiments, MKN45 cells were mainly used because KATO3 had high background IL-8 secretion, whereas IL-8 secretions from MKN28 were low (data not shown). We also observed that *N. subflava* LPS induce IL-8 secretion in AGS cells to the same degree of *E. coli* LPS (see Supplementary Figure 1, Supplementary Digital Content 1, http://links.lww.com/CTG/A20).

As the first step, we extracted LPS from NHGB and examined their activities in IL-8 secretion from MKN45 cells since culture conditions of MKN45 cells would be hostile to anaerobic bacteria.

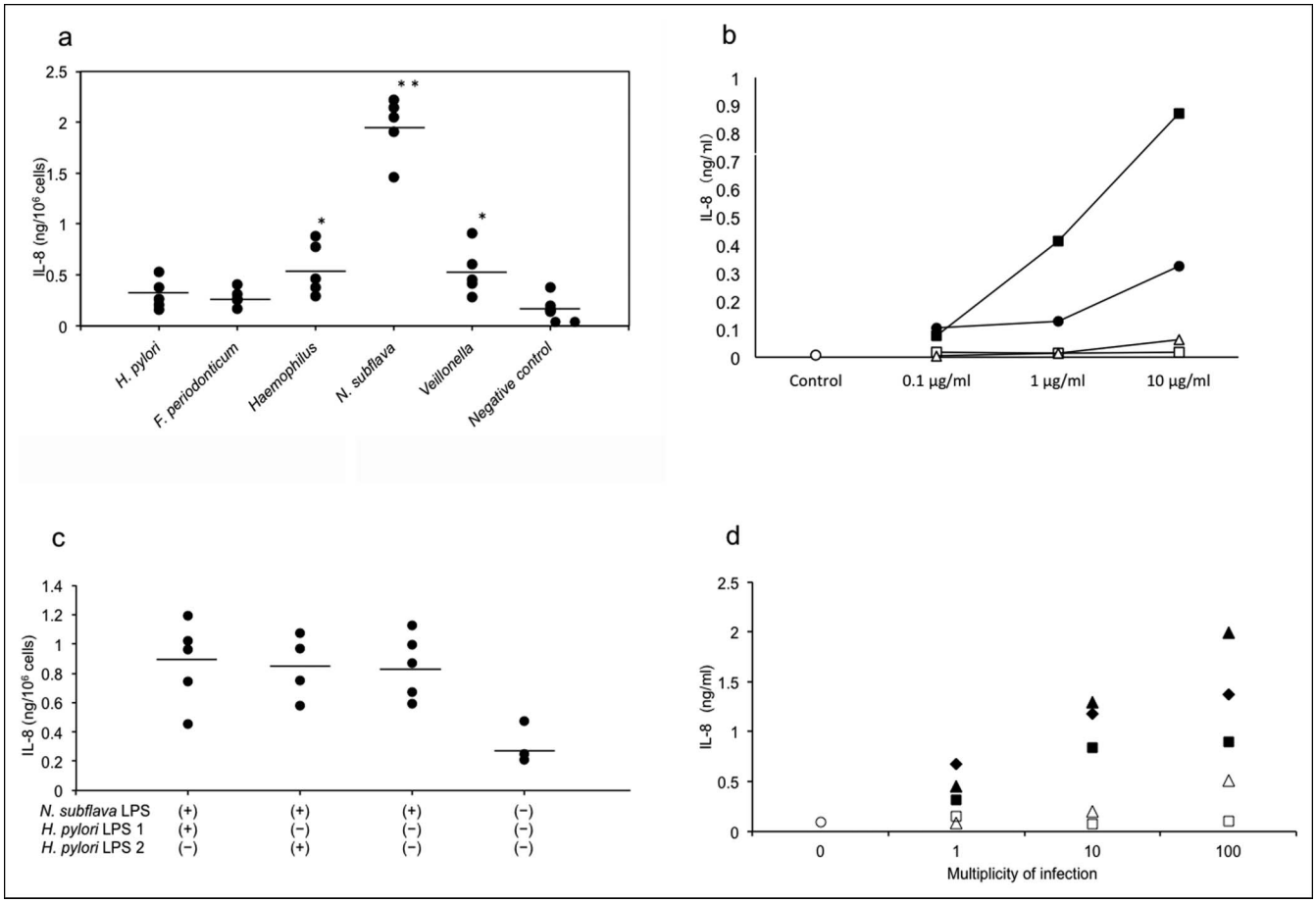

Figure 2 Assay for IL-8 production. IL-8 secretion in human gastric cells. MKN45 cells were treated with LPS from various bacterial species at a concentration of 10 μg/mL for 24 hours. $^{**}P < 0.01$ vs control, *H. pylori, F. periodonticum, Haemophilus spp.*, and *Veillonella spp.* $^{*}P < 0.01$ vs control. *Veillonella, V. atypia, V. rogosae, V. parvula, V. rogosae, V. dispar; Haemophilus, H. parahaemolyticus, H. parainfluenzae, H. influenzae, H. parainfluenzae, H. parahaemolyticus*, in descending order (**a**). IL-8 induction in MKN45 stimulated with crude *N. subflava* LPS (■) or purified *N. subflava* LPS (●) with various concentrations of LPS for 6 hours. ○; control (**b**). IL-8 induction in MKN45 under double stimulation by LPS of *H. pylori* and *N. subflava*. Cells were pretreated with 0.1 μg/mL *H. pylori* LPS for 24 hrs and then stimulated with 0.1 μg/ml *N. subflava* LPS for 24 hours (**c**). IL-8 secretion after infection by live *H. pylori* (△, □) or *live N. subflava* (▲, ♦, ■) in the human gastric cell line MKN45. ○; control. Cells were infected with each bacterium at a multiplicity of infection 1, 10, and 100 for 6 hrs (**d**). *H. pylori*, *Helicobacter pylori*; IL-8, interleukin-8; LPS, lipopolysaccharides; *N. subflava*, *Neisseria subflava*.

LPS from *N. subflava* dose-dependently stimulated IL-8 secretion to the highest levels (Figure 2a,b), whereas LPS of *H. pylori* showed negligible activity. LPS from *Haemophilus* or *Veillonella* moderately stimulated IL-8 secretion. No further increase in IL-8 secretion was observed by pretreatment of *H. pylori* LPS before challenge of *Neisseria* LPS (Figure 2c). Preincubation of *N. subflava* LPS with polymyxin B abolished IL-8 secretion (see Supplementary Figure 2, Supplementary Digital Content 1, http://links.lww.com/CTG/A20). Although purified *N. subflava* LPS showed a statistically significant reduction of IL-8 production compared with that of crude *N. subflava* LPS, it still retained a higher activity than purified LPS from *E. coli* (see Supplementary Figure 3, Supplementary Digital Content 1, http://links.lww.com/CTG/A20).

When whole bacteria were cocultivated with MKN45, all 3 clinical isolates of *N. subflava* showed a higher potency in stimulating IL-8 secretion than clinical isolates of *H. pylori* (Cag A and Cag E positive, data not shown) in each dose tested (Figure 2d).

Pretreatment of MKN45 cells with a potent IKK-2 inhibitor, SC-514, decreased IL-8 levels induced by purified LPS of *N. subflava* (Figure 3a). NF-κB activation in MKN45 by LPS was observed in immunohistochemistry analysis (Figure 3b,c).

MKN45 cells expressed low levels of TLR2 and TLR4 by immunofluorescence staining (Figure 4). Expression of mRNA for TLR2, TLR4, myeloid differentiation factor 2 (MD2), and CD14 was also detected by RT-PCR (see Supplementary Figure 4, Supplementary Digital Content 1, http://links.lww.com/CTG/A20). Although expression levels of TLR2 and TLR4 were low, both Pam3CSK4, an agonist of TLR2, and purified *E. coli* LPS, an agonist of TLR4, could stimulate IL-8 secretion (see Supplementary Figure 5, Supplementary Digital Content 1, http://links.lww.com/CTG/A20).

To verify that *N. subflava* is not simply a passenger organism but stably colonized in the gastric mucosa, we examined the localization of this bacteria in the human gastric mucosa. The presence of *N. subflava* was shown in the surface gastric epithelium as well as in the deeper part of pits, sharing colonization niche with *H. pylori* (Figure 5).

N. subflava show firm adhesion to MKN45 cells (Figure 6), supporting their colonization in the gastric mucosa where they can adhere to the mucosal epithelial cells.

DISCUSSION

We found that *N. subflava* and LPS purified from the bacterium were particularly potent in stimulating IL-8 secretion. Previous studies (12,19) showed that *Neisseria spp.* is one of the predominant Gram-negative species in the human stomach. Although Yuan et al. (19) identified *N. flavescens* as main species of *Neisseria* in the human stomach, our *Neisseria* strains were identified as *N. subflava* based on 16S rRNA gene sequence. An analysis using genomic sequence data shows *N. flavescens* has a close relation to *N. subflava*, so that they might be reclassified into the same species (27) (see Supplementary Table 4, Supplementary Digital Content 1, http://links.lww.com/CTG/A20). According to our immunofluorescence study, *N. subflava* was shown to colonize the gastric mucosa and share their residing

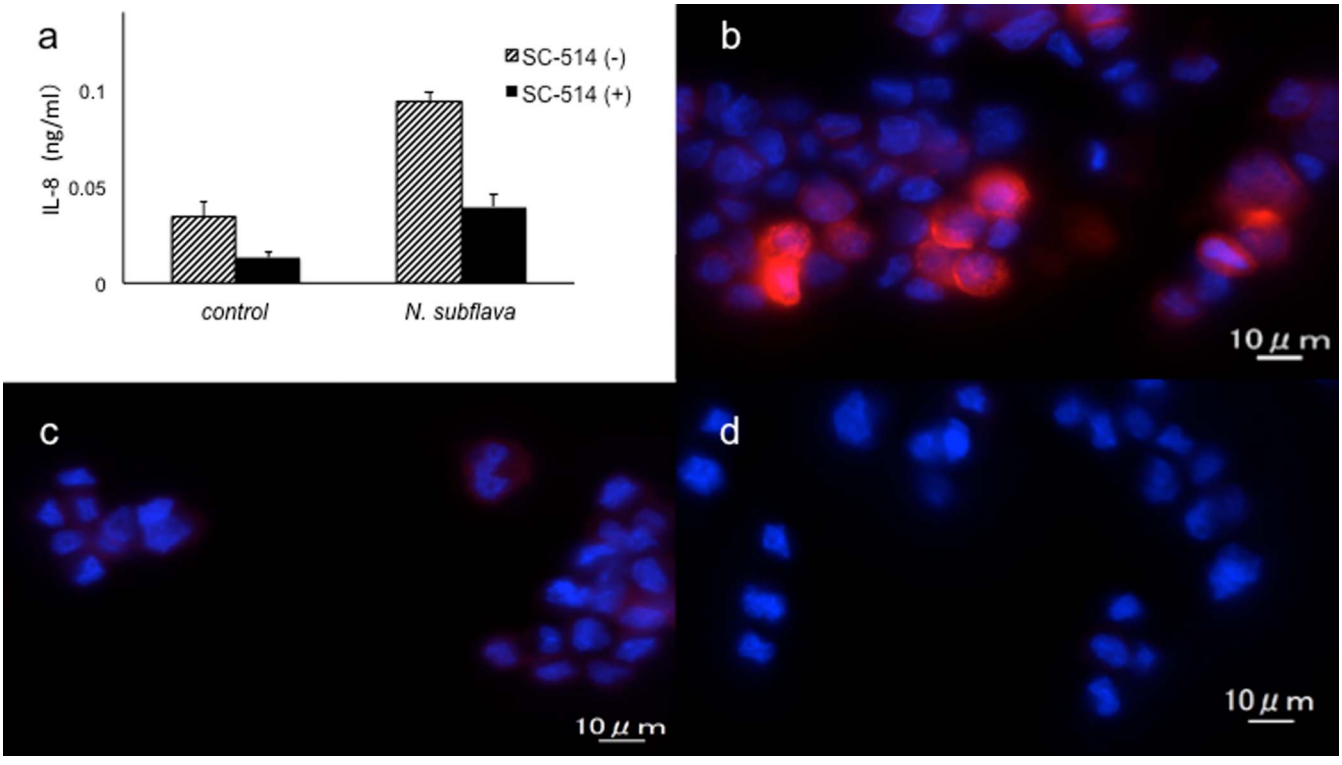

Figure 3 IKK/NF-kB signaling. MKN 45 Cells were treated with purified LPS of *N. subflava* or *E. coli* at a concentration of 1 μg/mL for 6 hrs after pretreatment with 100 μM of SC-514 as IKK inhibitor (**a**). Distribution of p65, an active NF-κB subunit, was detected with anti-p65 antibody (clone 12H11, Merck Millipore). MKN45 cells were stimulated with 1 μg/mL LPS from *N. subflava* for 2 hours (**b**). MKN45 cells were treated with 1 μg/mL LPS of *N. subflava* and 5 μg/mL polymyxin B (**c**). MKN45 cells were treated without LPS (**d**). IKK, inhibitor of kappa B kinase; LPS, lipopolysaccharides; *N. subflava, Neisseria subflava;* NF-κB, nuclear factor-κB.

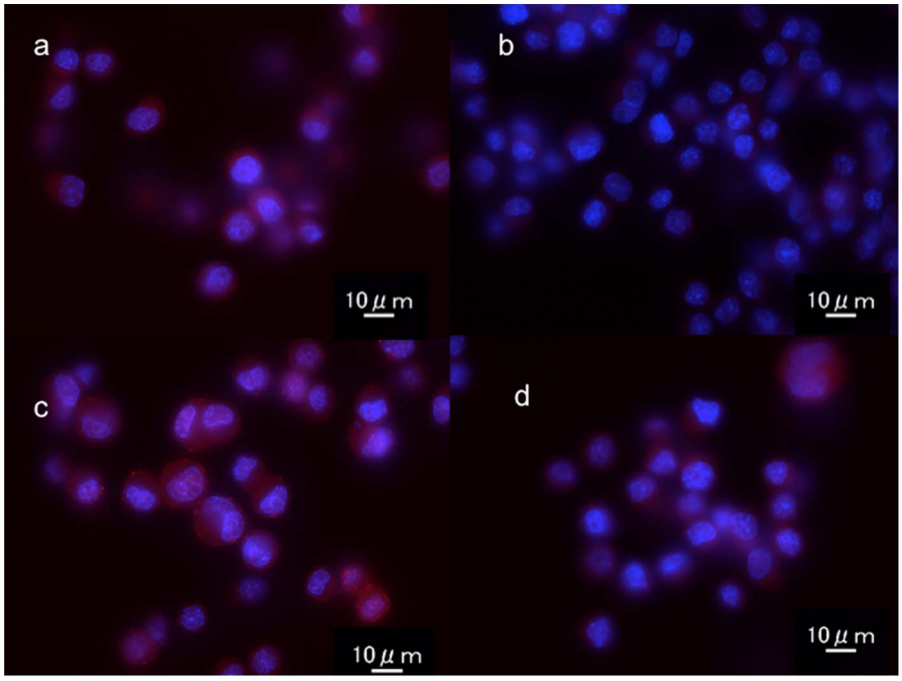

Figure 4 Immunohistochemistry of MKN45. MKN45 cells were with (**a**) or without (**b**) anti-TLR2 antibody. Similarly they were stained with (**c**) or without (**d**) anti-TLR4 antibody. (×1,000). TLR, Toll-like receptor.

niche with *H. pylori*. These findings corroborated with Nakamura's report (28), but contrary to their report, we could not detect *N. subflava* in the submucosal tissues. This may be due to difference in the antibody used in their study (antibody to *N. meningitidis*), whereas we used specific antibody raised to *N. subflava*. It may also be due to the different tissues employed.

Considering their more rapid growth than *H. pylori* together with their potent activity to elicit IL-8 secretion, dual or multiple bacterial coinfection may cause more detrimental effects to the gastric mucosa. *N. subflava* is an indigenous bacterium usually residing in the oral cavity, which would allow continuous infection and colonization in the stomach when the gastric environment is suitable for their growth as seen in hypochlorhydric conditions.

TLRs play pivotal roles in the immune response to bacterial infection (29). Of these, TLR4 is a representative receptor for LPS. The activation of TLR4 by LPS evokes a series of intracellular events and promotes nuclear translocation of NF-κB leading to IL-8 gene expression. Inhibition of IL-8 secretion by polymyxin B indicates that TLR4 activation by LPS from *N. subflava* is a key event to provoke IL-8 secretion through canonical TLR-NF-kB pathway. Inhibition of IL-8 secretion by the IKK-2 inhibitor SC-514 also supports this signaling pathway for LPS from *N. subflava*. We could not ascertain that LPS from *N. subflava* can activate TLR2 in addition to TLR4, similar to the LPS from *H. pylori* (30,31). Structural characterization of LPS from our clinical isolates of *N. subflava* is necessary for gaining more insights into the mechanism of the potent proinflammatory property, since lipid A species from commensal *Neisseria* spp. were reported to be less active in evoking inflammatory signals in a macrophage cell line, THP-1 cells (32).

Whereas MKN45 cells reportedly expressed low levels of TLR2 and TLR4 but not MD2 nor CD14 (30), MKN45 cells used in our experiments did express both TLR2 and TLR4 together with its coreceptors CD14 and MD2, although their levels of expression were low. However, both receptors were functional because Pam3CSK4 and purified LPS from *E. coli* could stimulate IL-8 secretion from our MKN45 cells. In our experiments, LPS from *H. pylori* elicited little activity to stimulate IL-8, consistent with earlier reports (30,31) perhaps due to lower levels of TLR2 expression. Although Yokota et al. (33) reported that *H. pylori* LPS upregulated TLR4 through TLR2 which in turn activated MEK1/2-ERK1/2 signaling pathway in MKN28 and MKN45 cells, we could observe enhanced IL-8 secretion from neither

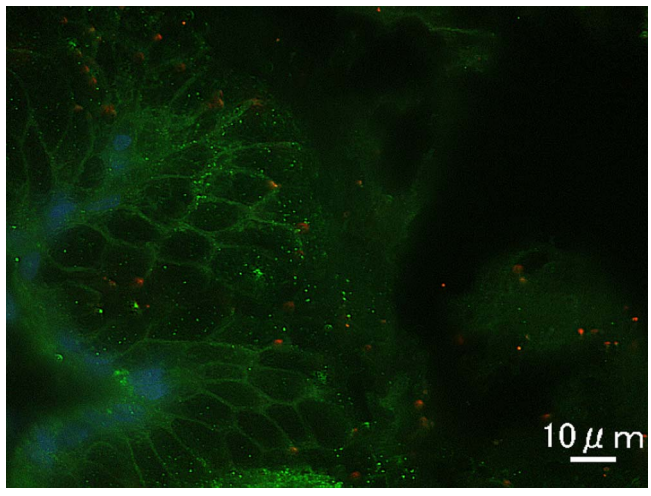

Figure 5 Immunohistochemical staining of the gastric mucosa. *H. pylori* (red dots) and *N. subflava* (green dots) were co-colonized simultaneously on the superficial epithelium as well as in the pit (×1,000). *H. pylori*, *Helicobacter pylori*; *N. subflava*, *Neisseria subflava*.

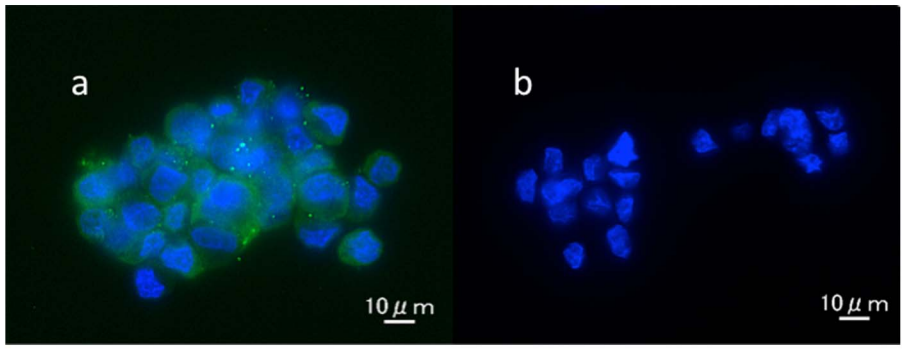

Figure 6 Attachment of *N. subflava* to human gastric cells. Immunohistochemical staining of *N. subflava* (green dots) attached on the cells of MKN45 (**a**). Negative control was stained in the absence of primary antibody (**b**) (×1,000). *N. subflava*, *Neisseria subflava*.

MKN28 nor MKN45 by LPS from *H. pylori*. Moreover, no positive interaction was observed when MKN45 cells were costimulated with LPS from *H. pylori* and that from *N. subflava*.

Varied expression of these TLRs during the subculture of MKN45 cells or different purity levels of LPS and/or lipid A structures of our clinical isolates might be responsible for the difference.

Although this is the first study showing the proinflammatory activity of NHGB and their product of LPS, we acknowledge several limitations in extrapolating our findings to clinical significance. First, we used gastric cancer cell lines for studying IL-8 secretion, which may not share the same signaling mechanism with normal gastric epithelial cells. Second, we did not study the effect of LPS from NHGB on specialized immune cells, such as macrophages and dendritic cells, that may play more important roles in gastric inflammation, though the study is underway. Third, we could not elucidate the mechanism why LPS from *N. subflava* was more potent in stimulating IL-8 than *E. coli* LPS. Structural study of the *N. subflava* LPS molecule as well as a more precise elucidation of independent TLR signaling using cells with monospecific TLR expression is necessary.

In conclusion, we demonstrated that NHGB coexisted with *H. pylori*. These bacteria as well as their LPS could stimulate IL-8 secretion. Among them, *N. subflava* and its LPS exhibited striking stimulation of IL-8 secretion from gastric mucosal cells. Therefore, in coinfection that occurs under a hypochlorhydric environment, NHGB may play an important role in continuation and aggravation of gastric inflammation because of their high growth rate together with higher proinflammatory activities. *N. subflava* colonization might persist as it can be continuously shred from the oropharynx, which in turn perpetuates inflammation and increases gastric cancer risk. Further studies are warranted to elucidate the role of NHGB including *N. subflava* in the progression to neoplastic lesions, which may lead to a new strategy to prevent gastric cancer.

CONFLICTS OF INTEREST

Guarantor of the article: Kentaro Sugano, MD, PhD.

Specific author contributions: Natsumi Miyata, MD, PhD and Yoshikazu Hayashi, MD, PhD, contributed equally to this work. N.M. performed the experiments, analyzed and interpreted findings, and drafted the manuscript. H.Y. performed the experiments and collected the patient data. S.H. planned the study and performed the experiments. K.S., Y.H., and H.Y. cooperated in collecting and interpreting patient data. S.K. planned and conducted the study and edited the manuscript. All authors approved the final manuscript submitted.

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Study Highlights

WHAT IS KNOWN

- ✓ The presence of NHGB in the stomach has been demonstrated by culture, proteomics, and gene sequencing analysis.
- ✓ Influence of these NHGB on gastric pathophysiology is not known.

WHAT IS NEW HERE

- ✓ NHGB stimulate IL-8 secretion from gastric epithelial cells.
- ✓ Several LPS isolated from NHGB, in particular from *N. subflava*, are potent stimulants of IL-8 secretion.
- ✓ *N. subflava* attached to gastric mucosal cells and colocalized with *H. pylori* in the gastric mucosa.

TRANSLATIONAL IMPACT

- ✓ NHGB coinfected with *H. pylori* may contribute to gastric inflammation in hypochlorhydric conditions and accelerate neoplastic progression.

REFERENCES

1. Warren JR, Marshall B. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet 1983;321:1273–5.
2. Sugano K, Tack J, Kuipers EJ, et al. Kyoto global consensus report on *Helicobacter pylori* gastritis. Gut 2015;64:1353–67.

3. Malfertheiner P, Chan FK, McColl KE. Peptic ulcer disease. Lancet 2009; 374:1449–61.
4. Wotherspoon AC, Ortiz-Hidalgo C, Falzon MR, et al. *Helicobacter pylori*-associated gastritis and primary B-cell gastric lymphoma. Lancet 1991; 338:1175–6.
5. International Agency for Research on Cancer Helicobacter pylori Working Group. *Helicobacter pylori* Eradication as a Strategy for Preventing Gastric Cancer IARC Working Group Report. International Agency for Research on Cancer (IARC Working Group Reports): Lyon, France, Vol 8, 2014, pp 8.
6. Holcombe C. African enigma. Gut 1992;33:429–31.
7. Miwa H, Go MF, Sato N. *H. Pylori* and gastric cancer: the Asian enigma. Am J Gastroenterol 2002;97:1106–12.
8. Hansson LE, Nyrén O, Hsing AW, et al. The risk of stomach cancer in patients with gastric or duodenal ulcer disease. N Engl J Med 1996;335:242–9.
9. Matsuhisa T, Matsukura N, Yamada N. Topography of chronic active gastritis in *Helicobacter pylori*-positive Asian populations: Age-, gender-, and endoscopy-matched study. J Gastroenterol 2004;39:324–8.
10. Tsugane S, Kabuto M, Imai H, et al. *Helicobacter pylori*, dietary factors, and atrophic gastritis in five Japanese populations with different gastric cancer mortality. Cancer Causes Control 1993;4:297–305.
11. Uemura N, Okamoto S, Yamamoto S, et al. *Helicobacter pylori* infection and the development of gastric cancer. N Engl J Med 2001;345:784–9.
12. Forsythe SJ, Dolby JM, Webster AD, et al. Nitrate- and nitrite-reducing bacteria in the achlorhydric stomach. J Med Microbiol 1988;25:253–9.
13. Sharma BK, Santana IA, Wood EC, et al. Intragastric bacterial activity and nitrosation before, during, and after treatment with omeprazole. BMJ (Clinical research Ed) 1984;289:717.
14. Monstein HJ, Tiveljung A, Kraft CH, et al. Profiling of bacterial flora in gastric biopsies from patients with *Helicobacter pylori*-associated gastritis and histologically normal control individuals by temperature gradient gel electrophoresis and 16S rDNA sequence analysis. J Med Microbiol 2000; 49:817–22.
15. Bik EM, Eckburg PB, Gill SR, et al. Molecular analysis of the bacterial microbiota in the human stomach. Proc Natl Acad Sci USA 2006;103:732–7.
16. Maldonade-Contreas A, Goldfarb KC, Godoy-Vitorino F, et al. Strucutre of the human gastric bacterial community in relation to *Helicobacter pylori* status. ISME J 2011;5:574–9.
17. Eun CS, Kim BK, Han DS, et al. Differences in gastric mucosal microbiota profiling in patients with chronic gastritis, intestinal metaplasia, and gastric cancer using pyrosequencing methods. Helicobacter 2014;19:407–16.
18. Sanduleanu S, Jonkers D, De Bruine A, et al. Double gastric infection with *Helicobacter pylori* and non-*Helicobacter pylori* bacteria during acid-suppressive therapy: Increase of pro-inflammatroy cytokines and development of atrophic gastritis. Aliment Pharmacol Ther 2001;15:1163–75.
19. Hu Y, He LH, Liu GD, et al. Bacterial flora concurrent with *Helicobacter pylori* in the stomach of patients with upper gastrointestinal diseases. W J Gastroenterol 2012;18:1257–61.
20. Lertpiriyapong K, Whary MT, Muthupalani S, et al. Gastric colonization with a restricted commensal microbiota replicates the promotion of neoplastic lesions by diverse intestinal microbiota in the *Helicobacter pylori* INS-GAS mouse model of gastric carcinogenesis. Gut 2014;63:54–63.
21. Lofgren JL, Whary MT, Zhongming GE, et al. Lack of commensal flora in *Helicobacter pylori*-infected INS-GAS mice reduces gastritis and delays intraepithelial neoplasia. Gastroenterol 2011;140:210–20.
22. Zavros Y, Rieder A, Samuelson LC, et al. Genetic or chemical hypochlorhydria is associated with inflammation that modulates parietal and G-cell populations in mice. Gastroenterol 2002;122:119–33.
23. Kobayashi Y. The role of chemokines in neutrophil biology. Front Biosci 2008;13(1):2400–7.
24. Parkos CA. Neutrophil-epithelial interactions: A double-edged sword. Am J Pathol 2016;186(6):1404–16.
25. Fu H, Ma Y, Yang M, et al. Persisting and increasing neutrophil infiltration associates with gastric carcinogenesis and E-cadherin downregulation. Sci Rep 2016;6:29762.
26. Westphal O, Lüderitz O, Bister F. Über die Extraktion von Bakterien mit Phenol-Wasser. Z Naturforsch 1952;7:148–55.
27. Bennet JS, Jolley KA, Earle SG, et al. A genomic approach to bacterial taxonomy: An examination and proposed reclassification of species within the genus *Neisseria*. Microbiology 2012;158(Pt 6):1570–80.
28. Nakamura M, Matsui H, Serizawa H, et al. Coinfection of *Helicobacter pylori* and *Neisseria subflava* is closely associated with lymph follicle formation in human stomach. Aliment Pharmacol Therap; Symposium Series 2006;2:207–13.
29. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003;21:335–76.
30. Smith MF Jr, Mitchell A, Li G, et al. Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for *Helicobacter pylori*-induced NF-kB activation and chemokine expression by epithelial cells. J Biol Chem 2003; 278:32552–60.
31. Yokota SI, Ohnishi T, Muroi M, et al. Highly-purified *Helicobacter pylori* LPS preparations induce weak inflammatory reactions and utilize Toll-like receptor 2 complex but not Toll-like receptor 4 complex. FEMS Immunol Med Microbiol 2007;51:140–8.
32. John CM, Liu M, Phillips NJ, et al. Lack of lipid A pyrophosphorylation and functional lptA reduces inflammation by Neisseria commensals. Infect Immun 2012;80:4014–26.
33. Yokota SI, Okabayashi T, Rehli M, et al. *Helicobacter pylori* lipopolysaccharides upregulate toll-like receptor 4 expression and proliferation of gastric epithelial cells via the MEK1/2-ERK1/2 mitogen-activated protein kinase pathway. Infect Immun 2010;78: 468–76.