의학박사 학위논문

위 상피세포에서 IL-8의 분비를 증가시키는 헬리코박터 파일로리 이외의 위 내 세균의 동정 및 병원성 발현의 기전 규명

Identification of non-*Helicobacter pylori* gastric bacteria which stimulate interleukin-8 production in gastric epithelial cells and revealing the pathogenic mechanism

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A thesis of the Degree of Doctor of Philosophy

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ABSTRACT

Introduction: The role of non-*H. pylori* gastric bacteria (NHGB) in gastric inflammation is largely unknown. The aim of this study was to investigate their ability of inducing interleukin-8 (IL-8) production from gastric epithelial cells and to demonstrate the underlying mechanism.

Methods: Gastric juice was obtained from 13 patients with gastric cancer. After culture, isolated NHGB were identified by 16S rRNA gene sequencing. Human gastric epithelial cells (AGS) were co-cultured with the bacteria, and IL-8 concentrations in cell culture supernatants were quantified by enzyme-linked immunosorbent assay. We additionally performed inhibition studies using inhibitors to TLR4, NOD1, ERK, p38, JNK, and NF-κB, to reveal the mechanism of pathogen recognition, intracellular signal transduction and translational regulation of IL-8 by the bacteria.

Results: Sixteen species of NHGB were isolated from gastric juice. After inoculation to AGS cells, *Neisseria perflava* potently stimulated IL-8 secretion in a time- and dose- dependent manner, which was attenuated when concurrently treated with NOD1 inhibitor. When AGS cells were co-treated with *N. perflava* and inhibitors of ERK, p38 or JNK, respectively, to determine the role of mitogen-activated protein kinases (MAPKs) in IL-8 production, a significant reduction in IL-8 production was observed after treatment with p38 inhibitor. The cells pretreated with NF-κB inhibitor produced significantly reduced level of IL-8 when stimulated with *N. perflava*.

Conclusions: This is the first study demonstrating that *N. perflava* induces MAPK

phosphorylation and NF-κB activation via a NOD1-dependent mechanism in gastric epithelial cells. *N. perflava* may contribute to the inflammation in gastric mucosa.

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Keywords: Gastric microbiome; *Neisseria*; Gastritis; Inflammation; Interleukin-8.

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LIST OF ABBREVIATIONS

NHGB: non-*Helicobacter pylori* gastric bacteria

IL-8: interleukin-8

MAPK: mitogen-activated protein kinase

PPI: proton-pump inhibitor

T4SS: type IV secretion system

TLR: toll-like receptor

NOD: nucleotide-binding oligomerization domain

NLR: NOD-like receptor

NF-κB: nuclear factor kappa B

AP-1: activator protein-1

BLAST: Basic Local Alignment Search Tool

PRR: pattern recognition receptor

JNK: c-Jun N-terminal kinase

MEK: mitogen-activated protein kinase kinase

IκB: inhibitor of NF-κB

IKK: IκB kinase

MOI: multiplicity of infection

ELISA: enzyme-linked immunosorbent assay

HRP: horseradish peroxidase

OD: optical density

PBS: phosphate buffered saline

EGC: early gastric cancer

AGC: advanced gastric cancer

WD: well differentiated adenocarcinoma

MD: moderately differentiated adenocarcinoma

PD: poorly differentiated adenocarcinoma

SRC: signet ring cell carcinoma

SD: standard deviation

ERK: extracellular signal-regulated kinase

INTRODUCTION

Gastric cancer is a major health problem, as the fifth most common cancer and the fourth leading cause of cancer-related death worldwide.[1] *Helicobacter pylori* is well recognized as a major risk factor for upper gastrointestinal tract diseases, including chronic gastritis, peptic ulcer, mucosa-associated lymphoid tissue lymphoma, and gastric cancer.[2] *H. pylori* was designated as a class I carcinogen by the International Agency for Research on Cancer in 1994.[3] Chronic inflammation of the gastric mucosa after *H. pylori* infection is the predominant mechanism for the development and progression of premalignant gastric lesions such as atrophic gastritis and intestinal metaplasia. It is widely accepted that this premalignant cascade caused by chronic inflammation precedes development of intestinal type gastric cancer, which is the end result of progressive changes in the gastric mucosa.[4] In the initial carcinogenesis process, *H. pylori* infection plays an important role, leading to subsequent long-lasting chronic inflammation through various pathways. It leads to diffuse histopathological changes of the gastric epithelium, as well as production of cytokines which attract inflammatory cells, inducing oxidative stress to cause damage to the host DNA of gastric epithelial cells.[5]

Although the concept of *H. pylori* as an unrivaled factor in gastric cancer development has been widely accepted, the pathogenesis is still largely unknown. There are also several issues to be addressed regarding the association between *H. pylori* infection and gastric cancer. For example, only a small portion of the population infected with *H. pylori* develop gastric cancer, and even successful

eradication treatment does not completely prevent this deadly disease.[6,7] In Africa, despite the high prevalence of *H. pylori* infection, the incidence of gastric cancer is relatively low, which is called the "African enigma".[8] This phenomenon is contrary to the situation of East Asia, where the incidence of gastric cancer is one of the highest in the world.[1] These contradictory findings suggest the association of several factors other than *H. pylori* infection, such as various virulence factors, or dietary, genetic, and racial differences with gastric cancer. There are some evidences that suggest subsequent atrophic change, rather than *H. pylori* itself, is related to gastric cancer. Indeed, previous studies have reported increased risk of gastric cancer in patients with mucosal atrophy or long-term use of proton-pump inhibitors (PPIs).[9,10] It is well known that increased extent and severity of gastric atrophy and intestinal metaplasia, as well as decreased serum pepsinogen I/II ratio, is associated with higher risk of gastric cancer.[11-13] Decreased gastric acidity as the consequence of extensive atrophy or PPI use, might be an important factor in this association. One of the possible links to explain the association between an increase in gastric pH and the occurrence of gastric cancer is the microbiome, as bacterial overgrowth is very common under these changes in the gastric environment.

The healthy human holds a complex of diverse microbes whose microbial richness contributes to maintain local immune homeostasis. Gastrointestinal microbiome constantly interacts with its environment and host, thereby being involved in the inflammatory response, immune reaction, and metabolism of the host.[14] Infection with *H. pylori* brings a huge change to the gastric microbial environment. Until the discovery of *H. pylori*, research on gastric microbiota laid dormant for many years. As the intragastric environment was thought to be very

harsh for microorganisms, and also due to the technical difficulties, stomach was once thought to be a sterile organ. Thanks to the recent development of molecular biology techniques, this paradigm has now been completely changed. Several sequencing-based metagenome analyses on human gastric microbiome has revealed the presence of numerous bacteria in the stomach.[15-17] There are now increasing interest in the role of gastric microbiome in the carcinogenesis process. In the initial stage of *H. pylori* infection, the bacteria predominantly proliferate in the stomach, leading to dysbiotic change represented by decreased diversity and altered composition of microbiota.[18] They recruit immune cells that secrete inflammatory cytokines, which eventually breaks immune homeostasis leading to chronic inflammation.[19] Gastric atrophy progresses as a consequence of ongoing inflammation and mucosal damage, resulting in decreased parietal cells and reduced acidity in the stomach.[20] Eventually, the increased pH in the stomach allows overgrowth of non-*H. pylori* gastric bacteria (NHGB). This vicious cycle perpetuates chronic inflammation, which in turn seems to induce gastric carcinogenesis.[21]

Indeed, previous studies have shown that NHGB overgrows in patients with mucosal atrophy or long-term PPI use.[22,23] Recent researches have also revealed that there are differences in the composition of gastric microbiome between the patients with or without gastric cancer.[18,24,25] The composition of gastric microbiota changes into that of oral cavity-type or intestinal-type microbiota, in the stomach with dominant metaplastic change.[24] In a recent gastric microbiota network analysis in Korean patients, Park *et al.* demonstrated that microbial modules correlated with a higher risk of gastric cancer development included nitrosating/nitrate-reducing bacteria or type IV secretion system (T4SS) protein

gene-contributing bacteria.[26] In another randomized controlled study to investigate microbes related to progression of inflammation and premalignant conditions after *H. pylori* eradication, a specific cluster of oral bacteria was associated with development of gastric atrophy and intestinal metaplasia.[27] In the group of subjects with these premalignant changes, functional pathways including amino acid metabolism were enriched. Previously, we have also observed similar changes in functional pathways during the gastric carcinogenesis process.[28] In an animal study conducted using transgenic mice, the absence of gastric colonization with enteric microbes significantly inhibited the development of gastric neoplasm, even in the presence of *H. pylori* infection.[29] These increasing evidences suggest that NHGB might also play a certain role in the development of gastric cancer. These bacteria could induce and persist the carcinogenesis process in the hypoacidic stomach, via inducing inflammation, promoting cell proliferation, affecting stem cell dynamics, and producing metabolites that affect DNA integrity and immune response.[30]

However, the limitation of current microbiome studies in the field of gastric diseases seems to be quite clear. Most metagenome studies are limited to revealing the difference in bacterial composition between the disease group and the control group, only providing us indirect evidences for the possible role of microbiome in gastric carcinogenesis. In addition, most of the researches intended to elucidate the molecular pathogenesis of gastric cancer in association with microbiome, are still limited to *H. pylori*.

Although not thoroughly understood, the inflammatory process induced by *H. pylori* in the gastric mucosa is roughly explained as follows. T4SS encoded by *cag* pathogenicity island can inject cytotoxic virulence factors such as CagA or peptidoglycan directly into the gastric epithelial cell.[31,32] Various pattern

recognition receptors, including toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), are also involved in the recognition of *H. pylori*.[33-35] The stimulation initiated by *H. pylori* then activates the intracellular signaling pathways. Mitogen-activated protein kinase (MAPK) phosphorylation is induced in a NOD1 dependent mechanism, and transcription factors nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) are also activated, which consequently increase the intranuclear transcription of several inflammatory cytokines.[36,37] Among the cytokines induced by gastric epithelial cells in response to *H. pylori* infection, interleukin-8 (IL-8) is a potent inducer of migration and infiltration of neutrophils, lymphocytes, and inflammatory mediators to the gastric mucosa.[38,39] IL-8 gene has binding sites for NF-κB and AP-1 in the promoter region, and this cytokine is directly associated with the severity of gastritis.[38,40]

On the other hand, very little is known about the functional roles of NHGB in the gastric carcinogenesis yet. Although there are increasing number of studies dealing with NHGB, they are mostly limited to only revealing different microbial compositions between gastric cancer and control groups, or indirect functional studies. Even though these studies give us insight to the possible role of NHGB involved in the gastric carcinogenesis, these evidences are not enough to show the causal relationship between the microbiome and gastric cancer. Actually, there are very few studies focused on the role of NHGB in inducing inflammation of gastric mucosa, and the exact underlying mechanism or pathogenesis of the inflammatory process are still largely unknown.[41,42] It is therefore necessary to investigate the role of NHGB on the gastric inflammation, and to understand the mechanism of their pathogenicity on a molecular biological perspective. In this study, we aimed

to isolate NHGB from patients with gastric pathology, to investigate their ability of inducing IL-8 secretion from gastric epithelial cells. We also examined the underlying molecular pathogenesis of IL-8 secretion induced by the isolated bacteria.

MATERIALS AND METHODS

1. Patients and sample collection

We obtained gastric juice from 13 patients newly diagnosed with gastric cancer, who were planned for surgical gastrectomy, to isolate NHGB. Patients' demographic data included age, sex, and tumor characteristics. Resected gastric specimens were fixed in 10% formalin for overnight. Then, the specimens were dissected and embedded into paraffin blocks. Tissue sections from formalin fixed paraffin embedded blocks were stained using hematoxylin-eosin. The histopathologic features of the non-neoplastic stomach were analyzed by the updated Sydney System (0, none; 1, mild; 2, moderate; and 3, severe) to assess the severity of glandular atrophy and intestinal metaplasia.[43] *H. pylori* were identified microscopically in sections with Giemsa staining. Giemsa staining was performed on antrum, body, and adjacent tumor tissues using Ventana BenchMark Special Stains system (Ventana Medical Systems, AZ, USA). Gastric juice (7-30mL) was collected during the surgery, right after an incision was made in the stomach, in an aseptic way to avoid contamination. Gastric juice was then temporarily stored in 4℃ before transferring to the laboratory for further experiment. We additionally planned to investigate whether the cultured NHGB with pathogenicity would also be enriched in the early phase of neoplastic progression, considering that sustained inflammation by NHGB might play an important role in the early carcinogenesis process. Since gastric adenoma is a well-known premalignant lesion, often progressing into gastric cancer, we assumed that patients with gastric adenoma would be suitable for this purpose. We therefore used gastric juice from 20 patients

with gastric adenoma and 9 control subjects without atrophic gastritis, to compare the composition of gastric microbiome between the two groups by metagenome analysis. This study was approved by the Institutional Review Board of Chung-Ang University Hospital (IRB No. 1772-001-290, C201604), and written informed consent was obtained from all the subjects involved in the study.

2. Culture and isolation of bacteria from gastric juice

In order to separate the microorganisms, gastric juice stored in 4℃ was immediately transferred to the laboratory. The gastric juice sample was gently shaken with a vortex mixer, and 100 μL of the suspension was inoculated on a blood agar plate (SPL Life Sciences, Gyeonggi-do, Korea). After incubation for 48 h at 37℃ in 5% CO_2, the plates were checked for growth of bacterial colonies. Single colonies with different morphologies were isolated from the plate and sub-cultured on a blood agar plate to finally obtain pure colonies of single bacterial strain. The pure culture of isolates was then stocked for further characterization and identification.

3. Identification of bacteria with 16S rRNA gene sequencing

Two sequencing methods were used for bacterial identification of single bacterial colonies and microbiome of clinical specimens, respectively. For the identification of cultured bacteria, the single bacterial colonies were incubated at 100℃ for 15

min, and the suspension was centrifuged at 13,200 rpm for 30 min at 4℃ to obtain a supernatant. After bacterial genomic DNA was extracted using Expin PCR SV kit (GeneAll, Seoul, Korea), the hypervariable region V1-V9 was amplified using universal primers targeting the conserved region of the 16S rRNA gene. The extracted genomic DNA was sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Bionics, Seoul, Korea) on an ABI 3730XL DNA analyzer (Applied Biosystems, MA, USA) according to the manufacturer's instructions. The nucleotide sequence data was identified using the Basic Local Alignment Search Tool (BLAST) provided for sequence alignment by the National Center for Biotechnology Information (NCBI). For taxonomic assignment, the strain was confirmed as the one with the highest rank among the strains with sequence homology of 99% or more.

For the identification of microbiome in the gastric juice samples of the adenoma group and the control group, clinical samples were separated into pellet and supernatant by centrifugation (10,000 × g for 10 min at 4℃). The pellet was then further diluted with 200μL of phosphate buffered saline (PBS) to make a suspension, and boiled for 40 min under 100℃. After bacterial genomic DNA was extracted using DNeasy PowerSoil kit (QIAGEN, Hilden, Germany), the hypervariable region V3-V4 was amplified using universal primers targeting the conserved region of the 16S rRNA gene, and the amplicon libraries were prepared. Library preparation for sequencing followed 16S Metagenomic Sequencing Library Preparation (Part # 15044223 Rev. B). All amplicons were sequenced using a MiSeq (Illumina, CA, USA), according to the manufacturer’s instructions. For the taxonomic assignment, the representative sequences of the operational taxonomic units were finally classified using SILVA 138 database, under a threshold of 97%

sequence similarity.

4. Gram stain

Bacterial suspension made from stocked single bacterial isolate was smeared with a loop on a glass slide to make a thin film. The smear was air dried and heat-fixed by passing it over a flame for a few times. Heat-fixed smear was then stained with crystal violet for 60 s, followed by gentle rinsing with water and drying. The slide was then covered with Gram's iodine solution for 60 s, followed by gentle rinsing with water and drying. A few drops of 95% ethanol were applied as a decolorizing solution for 30 s, followed by gentle rinsing with water and drying. The slide was then covered with safranin counterstain for 60 s, before rinsing with water and drying. After applying a drop of mounting media onto the slide, a cover glass was mounted. Finally, the slide was examined with a microscope under oil immersion objective (×1000).

5. API NH assay

API NH (BioMérieux SA, Marcy-l'Etoile, France) experiment was performed according to the manufacturer's instructions. Briefly, the incubation box was prepared and the strip was put on the tray filled with sterile distilled water. Colonies of *Neisseria* were obtained with a loop and mixed in API NaCl 0.85% medium to prepare a suspension with a turbidity equivalent to 4 McFarland. The prepared bacterial suspension was distributed into the cupules. Only the tube part was filled for the first 7 microtubes (PEN to URE), while the tube and cupule was

both filled for the last 3 microtubes ([LIP/ProA], [PAL/GGT], [ßGAL/IND]). For the first 7 microtubes, the cupule was filled with mineral oil. After incubation for 2 h at 36 ± 2℃ in aerobic conditions, the reactions were read by referring to the reading table in the package. We then added 1 drop of ZYM B reagent to [LIP/ProA] and [PAL/GGT] microtubes, and 1 drop of JAMES reagent to [ßGAL/IND] microtube. After 3 min, the reactions were read by referring to the reading table in the package. If the [LIP] reaction was positive (blue pigment), [ProA] reaction was interpreted as negative, regardless of addition of ZYM B reagent. If several reactions were doubtful after the 2-h incubation period, the strip was re-incubated for another 2 h and the reactions were read again.

6. Cell lines and treatment

Human gastric epithelial cell line, ATCC CRL-1739, (ATCC, VA, USA) was cultured in Roswell Park Memorial Institute (RPMI) 1680 medium supplemented with 10% fetal bovine serum. Cell monolayers were cultured on petri dishes at 37.5℃ in a humidified atmosphere of 5% CO_2 in air. To quantify IL-8 levels, AGS cells were aliquoted into a 24-well plate (1×10^5 cells per well) for 24 h. Isolated bacteria were cultured with the cells under microaerophilic conditions (37℃, 5% O_2, 10% CO_2, 85% N_2). Afterwards, in the following experiments to investigate the signaling pathways related to *N. perflava* infection, the cells were co-treated with *N. perflava* and chemical agents. In the *N. perflava*-treated group, cells were inoculated with bacteria at a multiplicity of infection (MOI) of 100:1 (bacterium-to-cell ratio of 100:1). After incubation at 37 ℃ for 24 h, the cell culture medium from each well was harvested for measurement of IL-8 level.

For the experiments to confirm the pattern recognition receptor (PRR) involved in the signaling pathway, the cells were treated with ML130 (NOD1 inhibitor, Abcam, Cambridge, UK) and TAK-242 (TLR4 inhibitor, Santa Cruz Biotech, Texas, USA). To investigate the involvement of MAPK or NF-κB signaling pathways, a p38 inhibitor SB203580 (Cell Signaling, MA, USA), a c-Jun N-terminal kinase (JNK) inhibitor SP600125 (Tocris, MN, USA), a mitogen-activated protein kinase kinase (MEK) inhibitor PD0325901 (Sigma-Aldrich, MA, USA), and an inhibitor of NF-κB (IκB) kinase (IKK) inhibitor BAY 11-7082 (Abcam, Cambridge, UK) were used.

7. Enzyme-linked immunosorbent assay (ELISA) for cyto kine production

The amount of IL-8 secreted in the cell culture medium was measured with ELISA, using a monoclonal anti-human IL-8 antibody (Endogen, Woburn, MA, USA) for capture. This antibody was cultured on a Nunc-immuno plate at room temperature for overnight and subsequently cultured for 2 h with blocking buffer and then washed three times. After washing, a biotin-labeled detecting antibody and streptavidin-horseradish peroxidase (HRP) conjugate were added to the plate, and cultured sequentially for 1 h at room temperature. After washing three times, the plate was supplemented with tetramethylbenzidine substrate solution and incubated for 30 min at room temperature. The reaction was stopped by adding 0.18 M H_2SO_4. The amount of IL-8 was estimated by measuring absorbance at 450 nm using ELISA reader (Molecular Devices, San Jose, CA, USA) and expressed in pg/mL. Secretion of other cytokines including IL-1β, IL-6, TNF-α, and TGF-β in response

to bacterial infection were also quantified with ELISA in the same manner, using following kits: Human/Mouse TGF-beta1 Uncoated ELISA kit, Lot 221477-009 (Invitrogen, USA), Human TNF-alpha DuoSet ELISA (R&D Systems, MN, USA; Cat No. DY210, 15 plate), Human IL-1 beta DuoSet ELISA (R&D Systems; Cat No. DY201, 15 plate), and Human IL-6 DuoSet ELISA (R&D Systems; Cat No. DY206, 15 plate). All experiments were performed in duplicate and repeated two times.

8. Cytotoxicity assay

Cell viability was estimated using a D-Plus CCK cell viability assay kit (Dongin LS, Seoul, Korea) in accordance with the manufacturer's protocol. AGS cells were seeded in 96-well plates (5×10^4 cells/well). After 24 h of culture, the cells were treated with bacteria, to assess their cytotoxicity. The cells were treated with different concentrations of bacteria (MOI, 100:1, 500:1) for 24 h. The cell culture medium was replaced with 100 μL of CCK working solution, and the cells were further incubated for 4 h. The optical density (OD) was measured at 450 nm using a VersaMax Microplate Reader (Molecular Devices, San Jose, CA, USA). The OD value of control cells was considered to represent 100% viability.

9. Western blotting

To determine the NF-κB activity, IκB-α phosphorylation and degradation in AGS cells were assessed by western blotting. Harvested cells were suspended in RIPA

buffer for 30 min with periodical vortex mixing at 10-min intervals. After incubation, the cell lysates were centrifuged at 13,200 rpm for 30 min at 4℃, to remove cell debris. Extracted proteins were quantified with Bradford assay and then injected to a 12% polyacrylamide gel. After separation at 80V in stacking gel and at 100V in separation gel, proteins were transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany). Nonspecific binding was blocked with 5% non-fat milk for 2 h at room temperature. Primary antibodies, anti-IκB-α antibody (1:200, Santa Cruz Biotech, Dallas, Texas, USA) and anti-phospho-IκB-α antibody (1:200, Santa Cruz Biotech, Dallas, Texas, USA), were diluted with 5% non-fat milk. After incubation of membranes with primary antibodies, membranes were washed three times with PBS including 0.05% Tween-20. The membrane was then incubated with secondary anti-mouse IgG HRP-conjugated antibody (1:2000, Santa Cruz Biotech, Dallas, Texas, USA) diluted with 5% non-fat milk, for 2 h. Following three washes with PBS, the protein bands were visualized with enhanced chemiluminescence reagent (Santa Cruz Biotech, Dallas, Texas, USA) by using ChemidocXRS+ System (Bio-Rad, CA, USA). Then, the band densities were quantified using Image Lab software version 6.0.1 (Bio-Rad Laboratories, CA, USA).

10. Statistical analysis

Statistical analysis was performed using SPSS software version 15.0 (SPSS Inc., Chicago, IL, USA) and R Statistical Software version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). Continuous variables were compared

between the groups using Mann-Whitney U-test or Kruskal-Wallis test. Regarding next-generation sequencing (NGS) analysis, we compared relative abundances of microbiome between the adenoma group and the control group. Wilcoxon rank-sum test was performed to identify differentially abundant taxa between the two groups. Multiple testing correction was performed to control the false discovery rate, using the Benjamini–Hochberg procedure. Total-Sum Scaling method was used for normalization. P-value < 0.05 was considered statistically significant.

RESULTS

1. Identification of NHGB cultured from gastric juice

As a result of bacterial culture in gastric juice samples, a total of 16 strains were isolated. Information on the patients with gastric cancer, from whom the samples were obtained, is shown in Table 1. Glandular atrophy and intestinal metaplasia of the gastric body and antrum were evaluated using the resected gastric specimen (Table 2). *H. pylori* infection was present in 23.1%, and atrophy and intestinal metaplasia was evident in antrum of all of the enrolled patients. Isolated strains were identified through 16S rRNA gene sequencing, and the list of isolated strains for each patient is shown in Fig. 1. Cultured *Neisseria* spp. were identified as *N. perflava*, *N. subflava*, and *N. mucosa*. *Streptococcus* spp. were identified as *S. salivarius*, *S. mitis*, *S. anginosus*, and *S. parasanguinis*, and *Rothia* spp. as *R. mucilaginosa*, *R. dentocariosa*, and *Rothia* sp. In addition, *Staphylococcus capitis*, *Escherichia coli*, *Pseudomonas plecoglossicida*, and *Actinomyces* sp. were identified, and the other 2 strains were not determined.

Table 1. Demographic characteristics of the enrolled patients (n=13)

Variables	Values
Mean age ± SD (years)	66.4 ± 10.7
Sex (male : female)	10 : 3
Disease status	
EGC	5 (38.5%)
AGC	8 (61.5%)
Cancer stage	
I	7 (53.8%)
II	2 (15.4%)
III	3 (23.1%)
IV	1 (7.7%)
Tumor location	
Upper third	3 (23.1%)
Middle third	6 (46.2%)
Lower third	4 (30.8%)
Histology	
WD/MD/papillary adenocarcinoma	6 (46.2%)
PD/SRC	7 (53.8%)
H. pylori infection	3 (23.1%)

Values are number (%) or mean ± SD unless stated otherwise. EGC, early gastric cancer; AGC, advanced gastric cancer; WD, well differentiated adenocarcinoma, MD, moderately differentiated adenocarcinoma; PD, poorly differentiated adenocarcinoma; SRC, signet ring cell carcinoma; SD, standard deviation.

Table 2. Histologic evaluation of the background gastric mucosa of the enrolled patients (n=13)

Variables	Severity			
	Normal	Mild	Moderate	Severe
Glandular atrophy				
Antrum	0 (0%)	6 (46.2%)	6 (46.2%)	1 (7.7%)
Body	2 (15.4%)	6 (46.2%)	4 (30.8%)	1 (7.7%)
Intestinal metaplasia				
Antrum	0 (0%)	4 (30.8%)	4 (30.8%)	5 (38.5%)
Body	2 (15.4%)	7 (53.8%)	3 (23.1%)	1 (7.7%)

Values are expressed as number (%).

	Patients													
	1	2	3	4	5	6	7	8	9	10	11	12	13	total
Neisseria perflava	○					○								2/13
Neisseria mucosa		○					○	○			○			4/13
Neisseria subflava								○				○	○	3/13
Streptococcus salivarius	○						○						○	3/13
Streptococcus mitis	○			○									○	3/13
Streptococcus anginosus		○												1/13
Streptococcus parasanguinis					○									1/13
Staphylococcus capitis		○												1/13
Rothia mucilaginosa				○				○	○	○	○			5/13
Rothia dentocariosa		○	○	○								○	○	5/13
Rothia sp.			○	○										2/13
Escherichia coli		○	○											2/13
Pseudomonas plecoglossicida		○												1/13
Actinomyces sp.					○									1/13
Unidentified bacterium(1)						○	○						○	3/13
Unidentified bacterium(2)	○							○	○	○	○	○		6/13

Figure 1. List of isolated bacteria from the gastric juice of enrolled patients. Sixteen strains were isolated from 13 patients with gastric cancer. Many strains were commonly identified in several different patients.

2. Evaluation of IL-8 inducing ability of cultured strains

We determined IL-8 secretion levels with AGS cell line, when stimulated by various NHGB isolated from the gastric juice. After liquid culture of the 8 bacterial strains, the AGS cells were infected with each bacterial strain at an MOI of 10:1, 50:1, and 100:1, respectively. After 24 h of incubation, IL-8 concentrations in cell culture supernatants were quantified with ELISA. Only two strains, namely *N. perflava* and *N. mucosa*, potently stimulated IL-8 production from the AGS cells, compared to other bacteria (Fig. 2a). This increase in IL-8 production showed a positive correlation with the bacterial numbers. As *N. perflava* showed the most prominent induction of IL-8, we also evaluated the secretion of other cytokines (IL-1β, IL-6, TNF-α, and TGF-β) upon stimulation by *N. perflava* infection (Fig. 2b). However, there was no significant change in the levels of IL-1β, IL-6, TNF-α, and TGF-β, after treatment with *N. perflava*.

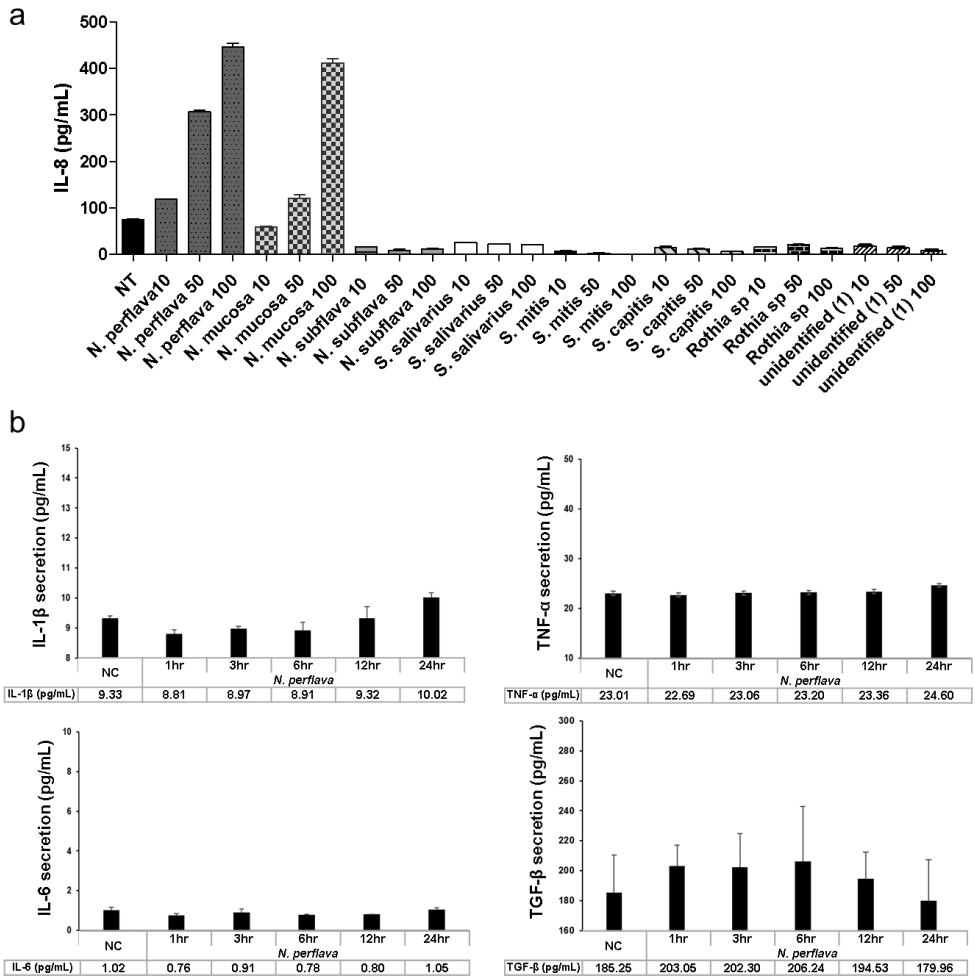

Figure 2. Analysis of cytokine production in AGS cells after stimulation with isolated bacteria. (a) Induction of IL-8 production by microorganisms isolated from gastric juice. After co-culture of isolated strains and AGS cells, IL-8 concentrations in cell culture supernatants were measured with ELISA. The numbers (10, 50, and 100) represent MOI of 10:1, 50:1, and 100:1, respectively. (b) Secretion of other cytokines (IL-1β, IL-6, TNF-α, and TGF-β) were further evaluated upon stimulation by *N. perflava* infection. Mean ± SEM are shown.

3. Changes of the microbial composition in the patients with premalignant gastric lesions

We tried to further investigate whether *Neisseria* genus is more abundant in the early carcinogenesis process. Therefore, we compared the relative abundance of gastric microbiome between two groups of subjects: 1) patients with gastric adenoma, which are well-known as premalignant lesions; 2) control subjects without evidence of atrophic gastritis. At the phylum level, *Firmicutes*, *Campylobacterota*, and *Cyanobacteria* were significantly more abundant, whereas *Proteobacteria* were significantly less abundant in the adenoma group, compared to the control group (Fig. 3a). At the genus level, relative abundances of some bacterial genera were also different between the two groups. Especially, *Neisseria* were significantly more abundant in the adenoma group than the control group ($q < 0.05$, Fig. 3b).

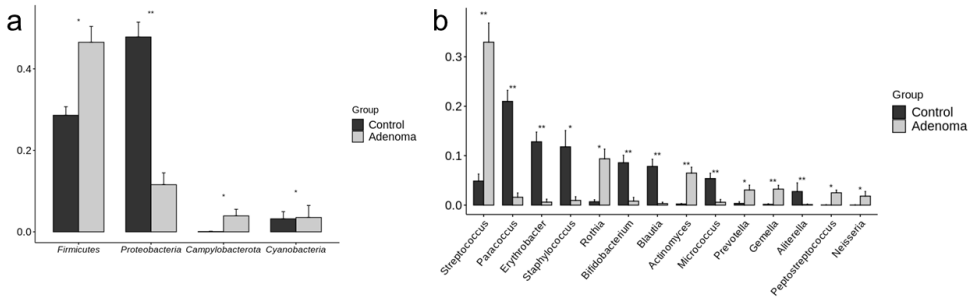

Figure 3. Differences in the relative abundance of gastric microbiome between the gastric adenoma group and the control group. (a) Comparison of the relative abundances of gastric bacteria at the phylum level between the two groups, demonstrating the differentially abundant taxa with clinical significance. (b) Comparison of the relative abundances of gastric bacteria at the genus level between the two groups, demonstrating the differentially abundant taxa with clinical significance. * $q < 0.05$, ** < 0.01.

4. Characteristics of *N. perflava*

We chose *N. perflava* for further experiments, as it showed the most potent IL-8 inducing ability. When the strain was observed under a light microscope after pure culture and gram staining, gram-negative cocci were confirmed. Notably, there was an outer membrane around the gram-stained cells (Fig. 4a). The biochemical characteristics of this strain was further analyzed with API NH, which revealed that *N. perflava* utilizes glucose, fructose, maltose and sucrose to produce acid. It was positive for proline arylamidase and gamma glutamyl transferase, while negative for penicillinase, ornithine decarboxylase, urease, lipase, alkaline phosphatase, β-galactosidase and indole production (Fig. 4b).

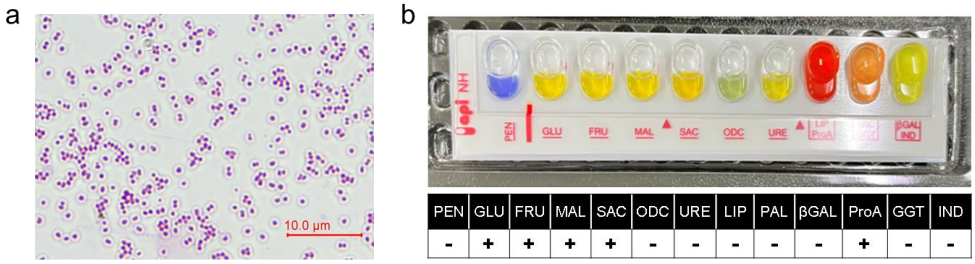

Figure 4. Morphological and biochemical characterization of *N. perflava*. (a) The light microscope image of *N. perflava* (gram stain, ×1000). (b) Biochemical characterization of the *N. perflava* using API-NH. PEN, penicillinase; GLU, glucose; FRU, fructose; MAL, maltose; SAC, saccharose; ODC, ornithine decarboxylase; URE, urease; LIP, lipase; PAL, alkaline phosphatase; βGAL, β-galactosidase; ProA, proline arylamidase; GGT, gamma glutamyl transferase; IND, indole.

5. Stimulation of IL-8 secretion by *N. perflava* in AGS cells

To assess the cytotoxicity of *N. perflava* to gastric epithelial cells, the cells were infected with *N. perflava* for 24 hours at an MOI of 100:1 and 500:1, respectively. Cell viability assay demonstrated that there was no reduction in the number of viable cells in both concentrations compared to the uninfected control cells (Fig. 5a).

Next, we planned to confirm whether the ability of *N. perflava* to induce IL-8 is related to the concentration or infection time of *N. perflava*. The amount of IL-8 secreted by AGS cells was measured while increasing the MOI from 100:1 to 500:1. The IL-8 level continuously increased as the cells were treated with a higher concentration of *N. perflava* (Fig. 5b). In addition, when the amount of IL-8 level was quantified over time after treating AGS cells with *N. perflava* at an MOI of 100:1, it was confirmed that the IL-8 level increased in a time-dependent manner from 3 h (Fig. 5c). Therefore, subsequent in vitro experiments with *N. perflava* were conducted at an MOI of 100:1.

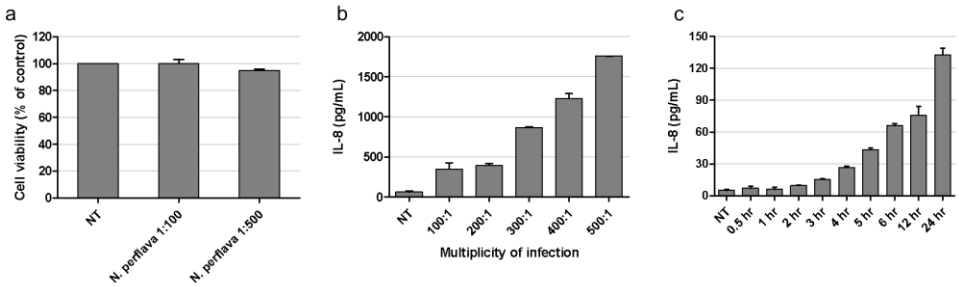

Figure 5. Assay of IL-8 production by AGS cells treated with *N. perflava*. (a) Cell cytotoxicity assay of *N. perflava* over AGS cell line. Quantification of IL-8 level in AGS cell culture medium after infection with *N. perflava*, in a (b) dose- and (c) time- dependent manner. The dose response was evaluated after 24 h of incubation. Mean ± SEM are shown.

6. Gastric epithelial cells recognize *N. perflava* via NOD1

We intended to demonstrate how *N. perflava* is recognized by gastric epithelial cells to induce IL-8 expression. Given that NOD1 and TLR4 are representative receptors which can recognize pathogen-associated molecular patterns of gram negative bacteria, we performed experiments targeting these receptors. Either inhibitor for NOD1 or TLR4 was co-treated with *N. perflava* in AGS cells. The inhibitor assay confirmed that IL-8 secretion was reduced in a dose-dependent manner when treated with the NOD1 inhibitor, ML130 (Fig. 6a), while it did not decrease when treated with the TLR4 inhibitor, TAK-242 (Fig. 6b).

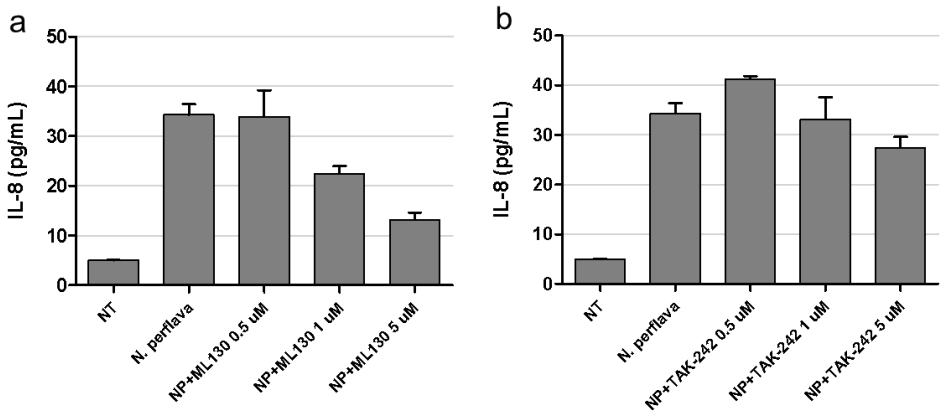

Figure 6. Recognition of pathogen associated molecular patterns of *N. perflava* via NOD1, a cytoplasmic receptor of the AGS cell line. The level of IL-8 was measured in AGS cell culture medium co-treated with *N. perflava* and (a) ML130 (NOD1 inhibitor), or (b) TAK-242 (TLR4 inhibitor). The cells were treated with each inhibitor at a concentration of 0.5, 1, and 5 uM. Mean ± SEM are shown.

7. *N. perflava* induces IL-8 expression via p38 dependent signaling

To determine which intracellular signaling pathway is involved in the NOD1-dependent IL-8 induction by *N. perflava*, we examined the activities of extracellular signal-regulated kinase (ERK), p38, and JNK MAPKs, which are rapidly activated after recognition by the PRRs, and involved in the initiation of downstream phosphorylation cascades. Regarding ERK MAPK, we used MEK inhibitor, which is a potent inhibitor of ERK phosphorylation, to investigate the effect of ERK inhibition on the signaling cascade. MAPK phosphorylation was analyzed at 15 min, 30 min, 1 h, 2 h, and 3 h following stimulation with *N. perflava*. The results showed that phosphorylation of ERK and p38 increased at 3 h and at 15 min post-stimulation, respectively. Phosphorylation of JNK, on the other hand, was not evident (Fig. 7a).

To investigate the role of MAPKs in IL-8 production, this time we treated AGS cells with inhibitors of ERK, p38, and JNK, before stimulation with *N. perflava*. IL-8 level was quantified in the cell culture supernatant after 24 h of incubation. The IL-8 production was suppressed only in cells treated with the p38 inhibitor, SB203580, while inhibition of JNK or ERK did not significantly reduce the level of IL-8 production (Fig. 7b).

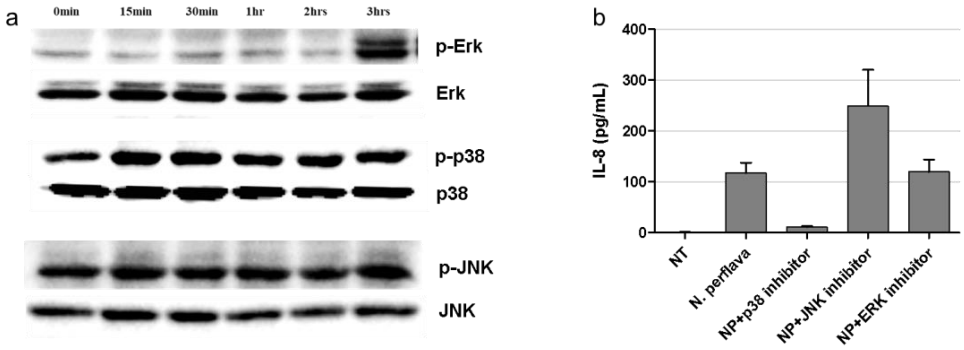

Figure 7. The early phosphorylation signaling cascade induced by *N. perflava* was dependent on p38 MAPK activation. (a) Quantification of p-ERK, p-p38 and p-JNK in AGS cells treated with *N. perflava* over time. (b) Quantification of IL-8 level in AGS cell culture medium co-treated with *N. perflava* and MAPK inhibitors. Mean ± SEM are shown.

8. *N. perflava* up-regulates IL-8 expression via the transcription factor NF-κB

NF-κB is an important transcription factor involved in the expression of most inflammatory cytokines, and it has also been reported to be related to the IL-8 expression in gastric epithelial cells by *H. pylori* infection.[36,44,45] To confirm whether the IL-8 expression induced by *N. perflava* was also NF-κB-dependent, we evaluated the activity of IκB-α, which inhibits NF-κB activation by binding to NF-κB and inducing its sequestration in the cytoplasm. As a result, degradation of IκB-α was induced in AGS cells when stimulated by *N. perflava*. We also demonstrated that the *N. perflava*-induced IκB-α degradation increased in a time-dependent manner (Fig. 8a).

Next, since IKK complex, which induces phosphorylation and degradation of IκB-α, plays a pivotal role in NF-κB signaling cascade, we intended to investigate whether inhibition of IKK could diminish the *N. perflava*-induced IL-8 production. When BAY 11-7082, a chemical inhibitor of IKK, was co-treated with *N. perflava* in AGS cells, reduced IL-8 production was observed (Fig. 8b). These findings show that the IL-8 production induced by *N. perflava* is mainly regulated by the transcription factor NF-κB.

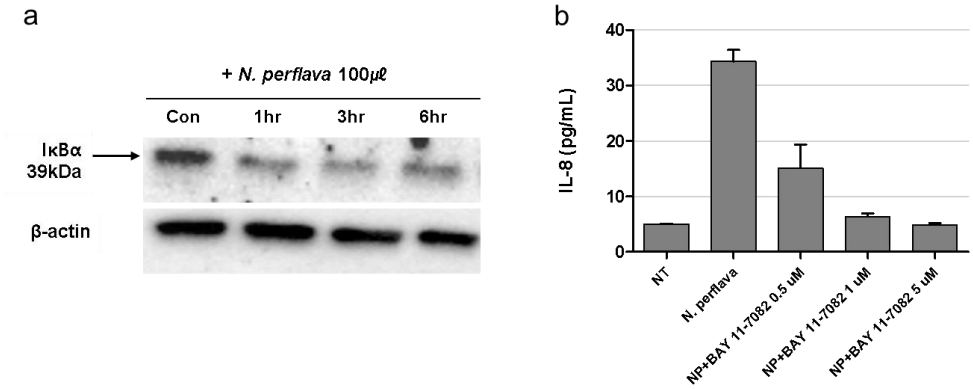

Figure 8. Involvement of NF-κB activation in IL-8 responses to *N. perflava* infection. (a) Western blotting analysis to assess the level of IκB-α in AGS cells treated with *N. perflava* over time. (b) Quantification of IL-8 level in AGS cell culture medium co-treated with *N. perflava* and BAY 11-7082 (NF-κB inhibitor). Mean ± SEM are shown.

9. *N. perflava*-induced NF-κB activation is dependent on NOD1 signaling and p38 phosphorylation

To further investigate the molecular signaling mechanism by which *N. perflava* induces IL-8 production in gastric epithelium, we next planned to evaluate the effect of NOD1 inhibition and p38 MAPK inhibition on NF-κB activation. Since IκB-α regulates nuclear translocation of NF-κB and its activity, which controls the transcription of IL-8 gene, the expression level of IκB-α was evaluated after treatment with a NOD1 inhibitor and a p38 inhibitor, respectively.

N. perflava and a NOD1 inhibitor were simultaneously treated on AGS cells, and co-cultured. As we have already shown that degradation of IκB-α was clearly present 3 h after treatment with *N. perflava*, the cells were harvested after 3 h to assess the expression of IκB-α. Compared to the positive control, IκB-α degradation was not evident when NOD1 was inhibited (Fig. 9a). This suggests that *N. perflava* induces NF-κB activation through a NOD1 dependent pathway. Similarly, *N. perflava* and p38 MAPK inhibitor were simultaneously treated on AGS cells, and co-cultured for 3 h. The cells were then harvested to assess the expression of IκB-α. Compared to the positive control, IκB-α degradation was diminished when p38 MAPK was inhibited (Fig. 9b). This suggests that p38 phosphorylation is involved in *N. perflava*-induced NF-κB activation.

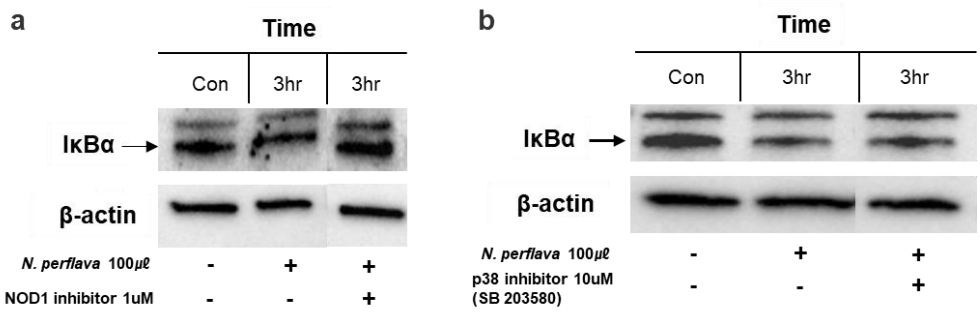

Figure 9. Inhibitors of NOD1 and p38 attenuate IκB-α degradation induced by *N. perflava* infection. (a) Quantification of IκB-α in AGS cells treated with *N. perflava* and a NOD1 inhibitor. (b) Quantification of IκB-α in AGS cells treated with *N. perflava* and a p38 inhibitor.

DISCUSSION

We isolated various NHGB from the patients with gastric cancer by using both culture-based and culture-independent methods, and demonstrated that *N. perflava* could potently stimulate IL-8 secretion in gastric epithelial cells. This *N. perflava*-induced IL-8 response was achieved via p38 MAPK phosphorylation and NF-κB activation in a NOD1-dependent mechanism.

There has always been a question about the role of NHGB in the pathogenesis of gastric diseases. It is a matter of interest whether the NHGB, especially the microbes known as oral commensals, only temporarily exist in the stomach during the gastric transit, or actually constitute the gastric microbial niche and affect the microbial community, interacting with host cells. It is also not well-established whether the microbial community differs in luminal and mucosal niches of the gastrointestinal tract. There are accumulating evidences, although mostly indirect ones, that gastritis can be induced and perpetuated by NHGB.[27,41,46] This can be especially an important concept in *H. pylori*-negative gastritis and chronic gastritis after spontaneous regression or eradication of *H. pylori*. Thanks to the recent advances in NGS technologies, many studies repeatedly revealed that there are differences in the composition of gastric microbiome between the patients with or without gastric cancer.[18,24,25] According to literature, *Streptococcus* and *Neisseria* have been widely detected from the patients with gastritis, premalignant gastric conditions, and gastric cancer.[25,28,47] These findings raised the suspicion of their contribution to the pathogenesis of gastritis or gastric cancer. Similar findings were also reported in a culture-based study using gastric samples, as well as studies with

culture-independent methods. Liu *et al*. once reported that these two taxa were more frequently isolated in the gastric juice and tissues of gastritis group compared to healthy group, suggesting these bacteria may be playing an active role, rather than a simple bystander, in the process of gastritis development.[46] In our study, we also demonstrated that the relative abundance of *Neisseria* in the gastric juice was significantly increased in the gastric adenoma group, compared to the control group. These findings lead to the inference that some NHGB might become enriched in the early carcinogenesis process, acting their possible role in the chronic inflammatory process. However, there is no consensus on whether or which NHGB might play pivotal roles in gastric carcinogenesis yet.

The genus *Neisseria* is well known as a major constituent of the salivary microbiome in adults, and one of the common flora colonizing human oral cavity, nasopharynx, and upper airway.[48-50] *Neisseria* species are generally non-pathogenic commensal inhabitants of the mucosa, except for *N. gonorrhoeae* and *N. meningitidis*, which are representative pathogenic species causing serious diseases such as gonorrhea or meningitis.[51] Several non-pathogenic *Neisseria* species including *N. sicca*, *N. flava*, and *N. mucosa* are known as inhabitants of dental plaque.[52] These bacteria are believed to be primarily involved in early formation of plaques, along with several *streptococcus* species.[53] Recently, culture-based and sequencing-based studies have repeatedly reported frequent identification of *Neisseria* in the stomach.[25,28,46,54] It is one of the representative gastric bacteria that constitute the microbial milieu of patients with chronic gastritis.[25,55] There are a few studies reporting that *N. flavescens* or *N. subflava* accounts for the majority of *Neisseria* identified in the stomach.[41,55] Although some suspect that it could be contaminants or transient bacteria originating from swallowed saliva, *Neisseria* has

been repeatedly identified in gastric samples from multiple studies. Also, *Neisseria* shows a clearly different relative abundance in patients with or without gastric diseases, as well as stable colonization in gastric tissues, making it considered as one of the NHGB.[26,41,46]

There have been only a few studies on non-pathogenic *Neisseria* species, providing indirect evidences of the association between gastric diseases and these NHGB. A recent study confirmed culture and identification of *N. flava* from patients with gastritis, as a non-*H. pylori* strain showing urease activity.[54] Another study reported that *N. perflava* or *N. flavescens* was more frequently isolated in the gastritis group compared to the healthy group, implying an association between these bacteria and gastric inflammation.[46] It has been reported that coinfection of *H. pylori* and *N. subflava* may be associated with formation of lymphoid follicles in the human stomach.[56] Miyata *et al.* showed that *N. subflava* as well as its lipopolysaccharides could also stimulate IL-8 production in gastric epithelial cell lines, suggesting their ability of promoting gastric inflammation, which may lead to neoplastic progression.[41] They also showed that *N. subflava* is a stable colonizer of the gastric mucosa, which is located deep in the pits as well as in the superficial epithelium, and not only a passenger microbe. Similarly, we have shown that a *Neisseria* strain can induce IL-8 secretion in gastric epithelial cells in the present study. These findings altogether suggest that some so-called non-pathogenic *Neisseria* species could show pro-inflammatory activities and potentially express pathogenicity in human gastric mucosa. In a randomized controlled trial designed to identify gastric microbes associated with progressive inflammation and premalignant conditions after *H. pylori* eradication, 16S rRNA gene analysis demonstrated the depletion of *Haemophilus*, *Neisseria* and *Actinobacillus* in the

gastric mucosa after eradication therapy.[27] *Neisseria* genus was represented by *N. subflava* in this study, and this change in the microbiome might be related to the reduced inflammation after eradication. There was also a study demonstrating a possible pathogenic role of another *Neisseria* species in celiac disease. Metagenome analysis of duodenal biopsy samples revealed that *Neisseria* genus were significantly more abundant in active celiac disease compared to control, and the most abundant *Neisseria* species was *N. flavescens*.[57] They also demonstrated that *N. flavescens* isolated from patients with active celiac disease had the ability to escape from lysosomal compartment in a human intestinal cell line, and to induce inflammatory responses in dendritic cells and duodenal mucosal explants. These findings imply potential pathogenicity of *N. flavescens* in association with celiac disease. Interestingly, they also showed differences in gene structure and function between the *N. flavescens* isolates obtained from the subjects with and without active celiac disease, respectively. This could partially explain how particular strains of *N. flavescens*, commonly known as a non-pathogenic commensal species, could contribute to the immune response by promoting the release of pro-inflammatory cytokines.

We have discovered a *Neisseria* isolate showing potential pathogenicity in the gastric epithelium, which was identified as *N. perflava*. There are very few data on the clinical significance of *N. perflava* in human, which is known as a member of normal flora colonizing the oral cavity and nasopharynx. A study on immunocompromised patients showed pharyngeal carriage of *Neisseria*, and *N. perflava* accounted for the majority of it.[58] Although they normally do not exhibit clinically significant pathogenicity, there are a few reports of opportunistic infections caused by this species, such as meningitis, endocarditis, peritonitis,

abscesses, and complicated bacteremia.[51,59,60] Meanwhile, there have been some changes in the classification of *Neisseria* species over time. Although the commensal *Neisseria* can be classified into discrete groups of related species, the intra- and inter-group relationships have been distorted by frequent DNA exchange and recombination events. This leads to the genetic diversity and heterogeneity of commensal *Neisseria*, even including the 16S rRNA gene, obscuring the phylogenetic relationships among the *Neisseria* species.[61-64] Recent advances in molecular biology techniques have shown that the species *N. subflava*, *N. perflava*, *N. flava*, and *N. flavescens* are genetically very closely related, and they were considered as biovars. These bacterial groups are now condensed altogether into a single species *N. subflava*.[61,65] Considering this reclassification, above mentioned evidences seem to imply possible pathogenicity of *N. subflava* and its closely related isolates in upper gastrointestinal tract. In line with this, *N. perflava* was also addressed in our study, to induce an inflammatory response in gastric epithelial cells.

It is well known that pathogenic *Neisseria* species such as gonococcus and meningococcus induce IL-8 production in endothelial and epithelial cells.[66,67] However, it is still unclear how *N. perflava*, a representative normal flora, can express its virulence in AGS cells. Our knowledge on the genomic information of *N. perflava* is very limited, not to mention its pathogenicity. One possible inference is that high genetic diversity promoted by frequent genetic recombination and mutation may be one of the reason for the virulence of some isolates. In fact, comparative genomic analysis shows that non-pathogenic *Neisseria* species share many of the genes, including so-called virulent genes, with the pathogenic *Neisseria* species.[68-71] Meanwhile, high genetic variability was also discovered

among *N. perflava* clinical isolates obtained from immunocompromised patients.[72] The fact that pathogenic and commensal *Neisseria* share multiple gene families and that horizontal gene transfer and genetic variation actively occur between the species may partly explain the virulence caused by some isolates.[70,71,73-75] It is therefore important to conduct a study using clinical isolates specifically obtained from the patients with gastric cancer, to investigate the possible relationship between the bacteria and gastric carcinogenesis, as we did in our study. Recently, a study on gastric microbiome and its metagenomic function has reported an increase of *Neisseriaceae* as the abundance of *H. pylori* decreased.[76] Interestingly, they also suggested that there might be transfers of genes encoding T4SS protein between *Neisseriaceae* and *H. pylori*, which could eventually facilitate gastric carcinogenesis. Thereby, it might be inferred that genetic transfer between pathogenic bacteria and commensal *Neisseria* species could lead some non-pathogenic strains to acquire the ability of IL-8 induction, although not proven.

Now that we have demonstrated that *N. perflava* can elicit the pro-inflammatory response in gastric epithelial cells in vitro, a question arises as to whether *N. perflava* can clinically show virulence in the human stomach. First of all, for this to happen, the bacteria will have to stay stably on the gastric mucosa to interact with the host cells. One possible explanation is that, in chronic gastritis, the pro-inflammatory response by *N. perflava* may be perpetuated, since the inflammatory condition and hypoacidic environment itself may provide a favorable milieu for the colonization of the bacteria. Of the virulent factors of pathogenic *Neisseria*, the outer membrane adhesins Opa and Opc are important in colonization and invasion of human cells.[77] Previous studies showed that some commensal *Neisseria* species, *N. lactamica*, *N. sicca*, and *N. perflava*, also express pathogen-

like Opa adhesins, which were similar to those of pathogenic *Neisseria* species in structure and function.[78-80] These findings might suggest how commensal *Neisseria* can colonize the gastric mucosa, and exhibit subsequent host-bacterial interaction. It is also worth noting that *Neisseria* has a much faster growth rate than *H. pylori*. In addition, a previous study reported that *N. subflava* shares a residing niche with *H. pylori* and colonizes the gastric mucosa.[41,81] Similarly, it will be easier for *N. perflava* to settle in such a hypoacidic mucosal environment created by the urease activity of *H. pylori*, and its pro-inflammatory effect could thereby be amplified. In addition, since *N. perflava* is one of the constituents of normal oral microflora, it can be continuously supplied into the stomach. Indeed, the bacterial communities of the saliva and gastric aspirates in the individuals without *H. pylori* infection showed very similar structures, suggesting a continuous migration of oral bacteria into the stomach, implying that the oral cavity is the major source of live bacteria.[82]

In our study, we demonstrated that *N. perflava* can induce IL-8 production in gastric epithelial cells in a dose- and time-dependent manner. In the stomach, *H. pylori* is a representative pathogen which induces IL-8 production in gastric epithelial cells.[37,83-85] This *H. pylori*-induced IL-8 production has an important role in recruiting and activating leukocytes, such as neutrophils and monocytes, in gastric mucosa.[38,86,87] These responses are crucial in the regulation of inflammatory and immune processes in the pathogenesis of chronic gastritis. Actually, the level of IL-8 in gastric mucosa correlates well with histological severity in *H. pylori*-associated gastritis.[39] Regarding gastric cancer, the association between IL-8 genetic polymorphism and risk of gastric cancer has been repeatedly suggested in literature.[88-90] Previous studies have also shown that IL-8 not only plays a crucial role in the proliferation, invasion and migration of cancer cells in the context of *H.*

pylori infection, but also affects the response to anticancer drugs by promoting chemoresistance in gastric cancer.[91-93] Although indirect, these evidences might imply that the induction and perpetuation of chronic inflammation by IL-8 could be one of the links between IL-8 and increased risk of gastric cancer. This is why we specifically selected IL-8 as the target inflammatory cytokine to evaluate the possible pathogenicity of the bacteria in our study, among various cytokines related to gastric inflammation. Similar to *H. pylori* infection, IL-8 production induced by *N. perflava*, which was formerly known as a nonpathogenic *Neisseria* species, might imply its role in the perpetuation of gastric inflammation, which could act as an important factor in the early carcinogenesis process.

PRRs on the epithelial cells or immune cells can recognize highly conserved microbial structures of pathogens, namely pathogen-associated molecular patterns, thereby activating microbicidal and pro-inflammatory responses.[94] This pathogen recognition mechanism is considered an important part of the innate immune system, which modulates the barrier function of intestinal epithelium.[95,96] Current evidences show that *H. pylori* is recognized by various PRRs, including TLRs, NLRs, C-type lectin receptors, and RIG-1 like receptors, and their association with gastric carcinogenesis has been also suggested.[97] TLR4 is deeply involved in the recognition of gram-negative bacteria, as it is the primary receptor for lipopolysaccharide recognition.[98] Meanwhile, among the NLRs existing in the intracellular cytoplasm as cytosolic PRRs, NOD1 and NOD2 can detect peptidoglycan-derived peptides present in both gram-positive and gram-negative bacteria.[99,100] Especially, NOD1 has been widely studied in association with *H. pylori* infection, and NOD1 is expected to have an important role for the induction of both NF-κB and AP-1 activation during *H. pylori* infection.[36]

Therefore, we conducted our experiments targeting TLR4 and NOD1 to reveal how *N. perflava*, a gram-negative bacterium, is recognized by gastric epithelial cells. In our study, *N. perflava*-induced IL-8 production was decreased in a dose-dependent manner when the AGS cells were treated with the NOD1 inhibitor, while this effect was not reproduced when treated with the TLR4 inhibitor. These findings indicate that NOD1 is required for IL-8 production during *N. perflava* infection. However, the main mechanism of NOD1 activation in *H. pylori* infection is direct injection of virulence factors into the cytoplasm through T4SS, whereas it is still unclear how *N. perflava* is recognized by intracellular NOD1.[32,101] Moreover, the *N. perflava*-induced IL-8 response was not completely abolished, although markedly reduced, by NOD1 inhibition. This implies that other signal recognition mechanisms may also act in a complex manner in the IL-8 secretion pathway activated by *N. perflava*. Previous studies have reported that *Moraxella catarrhalis* can be recognized by NOD1 after invading the epithelial cells, and ERK and p38 MAPK were involved in the subsequent signaling of IL-8 production.[102,103] Interestingly, *Listeria monocytogenes* also showed NOD1-dependent activation of p38 MAPK and NF-κB pathway, leading to IL-8 production in endothelial cells, which was surprisingly similar to the findings in our study.[104] Although *L. monocytogenes* is a gram-positive bacterium, NOD1 seems to be triggered by its meso-diaminopimelic acid-containing peptidoglycan.[105,106] Therefore, it can be inferred that there would be a mechanism by which NOD1 recognizes *N. perflava* in the epithelial cell, and further research is warranted on this subject.

To further elucidate if MAPK cascade is involved in *N. perflava*-induced IL-8 production, we conducted an experiment to verify the activity of ERK, p38, and JNK MAPKs. These three are well-known subfamilies of the MAPK family, which

transduces, amplifies, and integrates signals from various stimuli, such as inflammatory cytokines secreted by pathogen invasion or growth factors, and provokes appropriate physiologic responses in mammalian cells, such as proliferation, differentiation, development, inflammatory responses, and apoptosis.[107,108] According to the literature, it is known that the downstream MAPK pathways of NOD1 are different depending on the bacteria.[104,109] We could verify the activation of ERK and p38 MAPK pathways in gastric epithelial cells during *N. perflava* infection using western blotting. Upon stimulation with *N. perflava*, p38 was rapidly activated in 15 min, while ERK activation was noted after 3 h. These findings imply that p38 is involved in early MAPK phosphorylation after NOD1-dependent recognition of *N. perflava*. While ERK is generally activated by growth factors, and is mainly involved in cell proliferation and differentiation, p38 usually has an important role in inflammatory responses or apoptosis in response to stress.[110-112] In particular, p38 is well known as an essential modulator of the early transcriptional responses to stress stimuli.[113,114] Indeed, previous studies have shown that p38 plays an important role in the inflammatory response in *H. pylori*-associated gastritis.[115,116]

In addition, when each of the MAPK inhibitors was treated on the cells, the concentration of IL-8 decreased upon p38 inhibition. On the other hand, inhibition of ERK did not reduce the IL-8 concentration. Taking these findings together, activation of the p38 MAPK signaling pathway seems to have a key role in *N. perflava*-induced IL-8 secretion of gastric epithelial cells. There would be some possible explanations for why inhibition of ERK and p38 showed different inhibitory effects on IL-8 secretion. The fact that ERK phosphorylation was confirmed 3 h after *N. perflava* infection suggests that ERK may not play a major

role in rapid MAPK activation, in the IL-8 secretion pathway initiated by NOD1-dependent recognition of *N. perflava*. The phosphorylation of ERK may have occurred as a delayed response to other signals, or as a secondary response to the preceding reaction of the cell. Another reason may be the different mechanisms by which ERK and p38 activate NF-κB, which has been previously reported in *H. pylori*-infected gastric epithelial cells. This may have caused a difference in reduction of IL-8 secretion during inhibitor treatment.[117] In addition, MAPKs might have some antagonistic effects on each other, and inhibition of one may increase phosphorylation of the others. Actually, previous studies have elicited this possible cross-talk of MAPKs. Inhibition of one of the MAPKs, p38 or ERK1/2, induced a cross-activation of the other in corneal epithelial cells.[118] Similarly, p38 inhibitor increased ERK and JNK activation in gastric epithelial cells.[119] In our study, as well, the IL-8 level increased upon JNK inhibition. In this case, p38 and ERK phosphorylation may have been enhanced as a countermeasure to JNK inhibition, resulting in an increase in IL-8 secretion. This also might be the reason why IL-8 reduction was not evident when ERK was suppressed in our study.

Since IκB-α is an intracytoplasmic inhibitor of NF-κB, and IKK phosphorylates and degrades IκB-α, IκB-α degradation can increase the NF-κB activity while inhibition of IKK reduces the activity of NF-κB. We found that IκB-α was degraded in a time-dependent manner upon *N. perflava* treatment. In addition, inhibition of IKK led to the attenuated IL-8 response by *N. perflava*, also indicating that NF-κB activation is important in *N. perflava*-induced IL-8 secretion. NF-κB is also a core factor in *H. pylori*-induced pro-inflammatory responses in epithelial cells.[120] In *H. pylori*-gastritis, recognition of its peptidoglycan by NOD1 in the cytoplasm leads to NF-κB activation.[32] Activated NF-κB complexes are then

translocated into the nucleus, upregulating the expression of genes encoding the pro-inflammatory cytokines and chemokines, including IL-8. Other than NF-κB, AP-1 is another important transcription factor which regulates the expression of many pro-inflammatory cytokines in *H. pylori* infection.[36,121] The inflammatory response by *H. pylori* activates the IL-8 gene by various signaling pathways. And in particular, signaling through NF-κB can rapidly increase the IL-8 expression, since NF-κB is already present in the cytoplasm. Along with other cytokines, IL-8 is a very important cytokine involved in the development of gastritis, and its possible association with growth and spread of tumor has been suggested.[91,122] Similarly, *N. perflava*-induced IL-8 induction through NF-κB activation may also be associated with the development of gastritis. *N. perflava* may play a meaningful role especially in the stomach without *H. pylori* infection, or in a post-eradication state. Considering that the effect of promoting IL-8 secretion by *N. perflava* almost disappeared with inhibition of NF-κB pathway, this seems to be the most important transcription factor responsible for *N. perflava*-induced IL-8 secretion. Further research on the role of other transcription factors, such as AP-1, is also needed in the *N. perflava*-induced inflammatory cascades.

In summary, the findings from our study demonstrate that NOD1-dependent recognition of *N. perflava* induces p38 phosphorylation and subsequent NF-κB activation in gastric epithelial cells, leading to IL-8 secretion. We have shown that a clinical isolate of NHGB can elicit a pro-inflammatory response, implying a potential role of microbiome in gastric inflammation, and revealed its underlying molecular mechanism.

This research though is subject to some limitations. First, there is a controversy that bacteria in gastric juice could just be transient microorganisms,

and they do not accurately reflect the microbial environment interacting with the host cells of gastric mucosa.[123] Some studies suggested that the microbial composition of gastric juice differs from that of gastric mucosa, and a significant proportion of the gastric microbiota overlaps with the oral microbiome.[15,124] Nevertheless, gastric juice was directly obtained from the stomach in the operating room in our study, without passing through the mouth or esophagus, to reduce contamination as much as possible. We also proved that a live *Neisseria* strain, which is actually frequently discovered and cultured in the stomach, shows a pro-inflammatory effect on gastric epithelial cells. Colonization in the gastric mucosa and firm adhesion to gastric epithelial cells of another *Neisseria* species was also proved in a previous study.[41] Second, gastric cancer cell lines were used to investigate the IL-8 induction. There might be some discrepancies between the findings we have shown and the signaling pathways in normal gastric epithelium. We also did not study the effect of *N. perflava* on immune-related cells such as lymphocytes, dendritic cells, or macrophages. Neutrophils act as key cells of innate immunity and inflammation, by producing significant amount of reactive oxygen and nitrogen species.[125] Similarly, the reaction of various immune cells against *N. perflava* infection may also contribute significantly to the inflammatory responses. Also, we did not perform in vivo study. It is very hard to accurately recapitulate the microenvironment of human organ under in vitro conditions. Therefore, in vivo studies using animal models are warranted, which would further elucidate the relationship between NHGB and inflammatory responses in the actual stomach. Lastly, although we have demonstrated the pro-inflammatory process in response to *N. perflava* infection, this does not necessarily prove that the bacteria are directly involved in the development of gastric cancer. We should therefore be

careful in interpreting and generalizing this result to the actual process of gastric inflammation and carcinogenesis.

In conclusion, we have shown that NHGB may induce inflammatory responses in gastric epithelial cells, and elicited its underlying mechanism. There have been very little studies, if any, revealing the role of specific NHGB in the pathogenesis of gastritis or gastric cancer. Our study clearly demonstrated that *N. perflava* isolated from the patients with gastric cancer can induce a pro-inflammatory cytokine IL-8 in gastric epithelial cells, which is closely related to the development and perpetuation of gastritis. In particular, *N. perflava* is a common oral flora which can be continuously supplied into the stomach, which might be an important issue especially in a stomach with chronic acid suppression due to diffuse mucosal atrophy. As far as we know, this is the first study to demonstrate not only that *N. perflava* can stimulate IL-8 production in gastric epithelial cells, but also that this response is induced through p38 MAPK phosphorylation and NF-κB activation, via NOD1-dependent mechanism. More studies on the role of NHGB including *Neisseria* in the process of gastric inflammation and carcinogenesis are warranted, which will give us a new insight on the pathogenesis of gastric diseases, focusing on its association with microbiome.

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국문 초록

서론: 위 내의 염증 반응에서 헬리코박터 파일로리 이외의 세균이 가지는 역할에 대해서는 아직 잘 알려져 있지 않다. 본 연구에서는 이러한 세균들이 위 상피 세포에서 IL-8의 생성을 유도하는 능력과 그 기전을 살펴보고자 하였다.

방법: 13명의 위암 환자에게서 채집한 위액을 이용해 헬리코박터 파일로리 이외의 세균을 배양, 분리한 뒤, 이를 16S rRNA 유전자 염기서열 분석을 통해 동정하였다. 인간 상피세포주 (AGS)를 세균과 함께 배양한 뒤, 배양상청액의 IL-8 농도를 효소면역측정법으로 측정하였다. 또한, 병원체의 인식 및 세포내 신호 전달 기전, 세균에 의한 IL-8 전사 조절 기전을 밝히기 위해 TLR4, NOD1, ERK, p38, JNK 및 NF-kB에 대한 억제제를 이용한 저해 시험을 진행하였다.

결과: 위액에서 총 16 종의 비-헬리코박터 파일로리 균주가 배양되었다. 이를 각각 AGS 세포에 접종하여 배양하였을 때, *Neisseria perflava*는 시간 및 용량 의존적으로 IL-8의 분비를 뚜렷하게 촉진하였고, NOD1 억제제를 함께 처리하였을 때에는 해당 반응이 약화되었다. IL-8 생성에 있어 MAPKs의 역할을 확인하기 위해 AGS 세포에 *N. perflava*와 함께 ERK, p38, JNK 억제제를 각각 처리한 결과, p38 억제제 처리시 IL-8의 생성이 유의하게 감소된 것을 확인하였다. AGS 세포에 NF-kB 억제제를 처리 후 *N. perflava* 배양시, IL-8의 생성이 유의하게 감소하

였다.

결론: 본 연구는 *N. perflava*가 위 상피 세포에서 NOD1 의존적으로 MAPK 인산화와 NF-kB 활성화를 유도함을 처음으로 보여주었다. 이를 통해 *N. perflava*는 위 점막의 염증 반응에 기여할 가능성이 있음을 알 수 있다.

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주요어: 위 마이크로바이옴; 나이세리아; 위염; 염증; 인터루킨-8

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