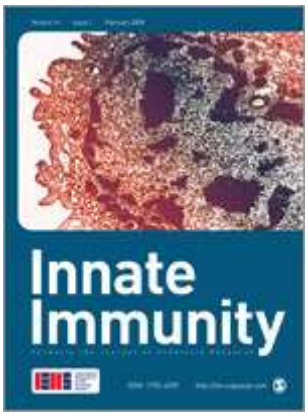

Helicobacter pylori attenuates lipopolysaccharide-induced nitric oxide production by murine macrophages

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Keywords:	Lipopolysaccharide, Nitric oxide, Helicobacter pylori, Macrophage, Nuclear factor (NF)-kappa B
Abstract:	Intragastric growth of Helicobacter pylori (H. pylori) and non-Helicobacter microorganisms is thought to be associated with elevated levels of proinflammatory cytokines and the production of nitric oxide (NO); these effects can lead to chronic inflammation. Microorganisms can activate the expression of inducible nitric oxide

	synthase (iNOS) and the production of NO by macrophages through stimulation with bacterial lipopolysaccharide (LPS). H. pylori can evade these vigorous immune responses, but the underlying mechanism remains unknown. In this study, we used a murine model of macrophage infection to demonstrate that H. pylori inhibits LPS-induced expression of iNOS and production of NO by macrophages. Suppression of LPS-induced NO production by macrophages led to elevated survival of H. pylori in a trans-well system. This effect was abrogated in macrophages from iNOS-/- mice. Analysis of iNOS mRNA and protein levels revealed that H. pylori inhibits iNOS expression at both transcriptional and post-transcriptional levels, and these effects occurred with live bacteria. Furthermore, the effect of H. pylori involved down-regulation of the mitogen-activated protein kinase pathway and the translocation of active nuclear factor (NF)-kappa B into the nucleus. Taken together, our results reveal a new mechanism by which H. pylori modulates the innate immune responses of the host and maintains a persistent infection within the stomach.

Helicobacter pylori attenuates lipopolysaccharide-induced nitric oxide production by murine macrophages

Running title: *H. pylori* modulates LPS-induced NO production

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ABSTRACT

Intragastric growth of *Helicobacter pylori* (*H. pylori*) and non-*Helicobacter* microorganisms is thought to be associated with elevated levels of proinflammatory cytokines and the production of nitric oxide (NO); these effects can lead to chronic inflammation. Microorganisms can activate the expression of inducible nitric oxide synthase (iNOS) and the production of NO by macrophages through stimulation with bacterial lipopolysaccharide (LPS). *H. pylori* can evade these vigorous immune responses, but the underlying mechanism remains unknown. In this study, we used a murine model of macrophage infection to demonstrate that *H. pylori* inhibits LPS-induced expression of iNOS and production of NO by macrophages. Suppression of LPS-induced NO production by macrophages led to elevated survival of *H. pylori* in a trans-well system. This effect was abrogated in macrophages from iNOS$^{-/-}$ mice. Analysis of iNOS mRNA and protein levels revealed that *H. pylori* inhibits iNOS expression at both transcriptional and post-transcriptional levels, and these effects occurred with live bacteria. Furthermore, the effect of *H. pylori* involved down-regulation of the mitogen-activated protein kinase pathway and the translocation of active nuclear factor (NF)-kappa B into the nucleus. Taken together, our results reveal a new mechanism by which *H. pylori* modulates the innate immune responses of the host and maintains a persistent infection within the stomach.

Keywords: Lipopolysaccharide, nitric oxide, *Helicobacter pylori*, macrophage, nuclear factor (NF)-kappa B

INTRODUCTION

Helicobacter pylori is the most common causative agent of gastrointestinal disease in humans. Infection with this pathogen usually occurs in childhood, and the bacteria can persist in the stomach for an individual's lifetime.[1, 2] Persistent infection with *H. pylori* in the gastric mucosa induces the expression of nuclear factor (NF)-κB and the secretion of proinflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-α.[3, 4] Other inflammatory mediators, such as nitric oxide (NO), a bactericidal agent generated by inducible nitric oxide synthase (iNOS) during the conversion of L-arginine to L-citrulline, are activated by *H. pylori* infection in both macrophages [5] and the gastric epithelium.[6] These findings indicate that *H. pylori* is an important factor for the induction of proinflammatory cytokines and NO in the host stomach.

Nitric oxide is derived from iNOS in lipopolysaccharide (LPS)-activated macrophages during inflammatory responses. Following treatment of macrophages with LPS, the NF-κB heterodimer rapidly translocates to the nucleus where it activates the transcription of target genes, including iNOS and several proinflammatory cytokines.[7] In addition, p38 mitogen-activated protein kinase (MAPK), protein kinase C, and extracellular signal–regulated kinase (ERK) are also involved in the activation of NF-κB and the expression of iNOS in response to LPS.[8, 9] Several microorganisms disrupt the activation of MAPKs or the NF-κB signaling pathway in macrophages to evade immune attack.[10-14] The effect of *H. pylori* on the modulation of LPS-activated molecules in macrophages remains unknown.

In addition to *H. pylori*, non-*Helicobacter* microorganisms are found in the gastric

environment.[15, 16] One study reported that *H. pylori*–associated gastritis was associated with the presence of several other microbes in the stomach, including *Enterococcus*, *Pseudomonas*, *Streptococcus*, *Staphylococcus*, and *Stomacoccus*.[17] A more recent study identified 128 phylotypes in 23 gastric biopsy samples; however, the presence of *H. pylori* did not affect the composition of microbiota in the gastric microbial community.[18] These findings indicate that *H. pylori* and non-*Helicobacter* microorganisms are present in the microbiota of the human stomach, and these microbes can elicit proinflammatory mediators and induce vigorous immune responses.[19] These findings also raise the question of how *H. pylori* persists in the microbial ecosystem under the harsh environment of the stomach.

The aim of the present study was to address the question of how *H. pylori* evades the vigorous antimicrobial activities of macrophages. We established an *in vitro* murine model system and an *ex vivo* murine model system to examine whether this bacterium could suppress LPS-induced NO production through the MAPK or the NF-κB signaling pathway. We showed that *H. pylori* inhibits iNOS expression and NO production by murine macrophages stimulated with a high dose of LPS. We further demonstrated that *H. pylori* down-regulates the LPS-induced activation of phosphorylated p38, ERK1/2, and NF-κB, and it subsequently suppresses LPS-induced macrophage responses. Thus, our study reveals that *H. pylori* attenuates LPS-induced NO production in macrophages and consequently evades early host immune responses.

MATERIALS AND METHODS

Antibodies and reagents

Polyclonal rabbit anti-iNOS, anti-phosphorylated c-Jun-N-terminal kinase (p-JNK), and anti-α-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibodies specific for p38 MAPK, stress-activated protein kinase (SAPK)/JNK, and p44/42 (ERK 1/2) were purchased from Cell Signaling (Beverly, MA, USA). Mouse monoclonal anti-phosphorylated p38 MAPK, and the anti-phosphorylated MAPK 1/2 (ERK1/2) (Thr185/Tyr187) antibodies were purchased from Upstate (Billerica, MA, USA). LPS (*Escherichia coli* O55: B5) and aminoguanidine hemisulfate (AG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). SB203580 (p38 inhibitor), PD98059 (ERK inhibitor), and SP600125 (JNK inhibitor) were purchased from Calbiochem (San Diego, CA, USA). The AP-1-Luc and NF-κB-Luc plasmids were purchased from Stratagene (San Diego, CA, USA). The iNOS promoter construct (piNOSLuc) was a kind gift from Dr. E. A. Ratovitski (Johns Hopkins University, Baltimore, MD, USA). The pSV-β-galactosidase vector and the luciferase assay kit were purchased from Promega (Madison, WI, USA). All other reagents were obtained from Sigma-Aldrich.

Bacterial strains, cell culture and mice

H. pylori 26695 (ATCC 700392) was used as a reference strain. The *cagA* or *vacA* isogenic mutants derived from *H. pylori* 26695 were constructed as described.[20] *H. pylori* strains were recovered from frozen stocks on Brucella agar plates (Becton Dickinson, Franklin Lakes, NJ, USA) containing 10% sheep blood. *H. pylori* strains were stored and cultivated as described,[21] and *H. pylori* extracts

were prepared as described.[22] Heated-killed *H. pylori* was obtained by boiling 1×10^9/ml of bacteria suspended in PBS for 30 min. Crude *H. pylori* extracts were prepared by sonicating 1×10^9/ml of bacteria suspended in PBS for 5 min on ice. Crude extracts were then centrifuged at 16,000 × *g* for 5 min at 4° C. The supernatant was filtered through a 0.22-μm filter and used for further analysis.

RAW 264.7 cells (ATCC TIB-71) were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA). De-complement fetal bovine serum (10%; HyClone, Logan, UT, USA) was added to the culture medium. For bacterial infection experiments, the cell culture medium was not supplemented with antibiotics.

Male wild-type C3H/HeN and TLR4-deficient C3H/HeJ mice at ages 6 to 8 weeks were kindly provided by Dr. Ai-Li Shiau (Departments of Microbiology and Immunology, National Cheng Kung University Medical College). C57BL/6 iNOS knockout (C57BL/6-Nos^{tm1Lau}) (iNOS$^{-/-}$) and wild-type mice at ages 6 to 8 weeks were kindly provided by Dr. Ming-Chei Maa (Graduate Institute of Basic Medical Science, China Medical University). Mice were maintained in the animal center of China Medical University (Taichung, Taiwan). All procedures were performed according to the "Guide for the Care and Use of Laboratory Animals" (NRC, USA) and were approved by the animal experiment committee of China Medical University.

Preparation of murine peritoneal exudate macrophages (PEMs)

C57BL/6 iNOS knockout (C57BL/6-Nos^{tm1Lau}) (iNOS$^{-/-}$) and wild-type mice of the same age and gender were used to assess the role of iNOS in *H. pylori*–induced suppression of LPS-induced NO production by macrophages. Murine PEMs were obtained after euthanasia by lavaging each mouse

with 10 ml of cold PBS 3 days after intraperitoneal injection of 2 ml of 3% thioglycolate in PBS. Two hours after seeding the cells in culture plates, the non-adherent cells were removed by washing with PBS, and the adherent cells were used for further experiments.

Mouse inoculations

C3H/HeN (n = 6) and C3H/HeJ (n = 6) mice of 6–8 weeks of age were intragastrically inoculated with *H. pylori*. All mice were maintained under fasting for 24 h before inoculation. The protocol of administration of mouse with LPS was performed as described with slight modifications.[23] Each mouse was administered 1×10^9 CFU/ml of *H. pylori* and purified LPS (75 μg, phenol extracted from *Escherichia coli* O55: B5, Sigma-Aldrich) by intragastric gavage for 3 consecutive days. Six hours after the final inoculation with *H. pylori*, the mice were fed with standard food and water and housed for 1 week. On the 7th day after infection, 6 mice in each group were sacrificed, and the number of *H. pylori* in their stomachs was determined by plating on Brucella blood agar plates and expressed as CFU/g tissue.

Immunoblotting

H. pylori–infected cells were washed three times with PBS and boiled in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer for 10 min. The samples were then resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies and then with horseradish peroxidase–conjugated secondary antibodies (Invitrogen). The proteins of interest were visualized with ECL™ western blotting reagents (GE Healthcare, Buckinghamshire, UK) and were

detected by exposure to X-ray film (Kodak, Boca Raton, FL, USA).

Reverse transcription and quantitative real time-PCR

Total RNA was extracted from PEMs using TRIzol reagent (Invitrogen), and 1 μg of total RNA was reverse transcribed into cDNA using the oligo(dT) primer. Quantitative real-time PCR using SYBR Green I Master Mix and a model 7900 Sequence Detector System was conducted according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). After preincubation at 50ºC for 2 min and 95ºC for 10 min, PCR was performed with 40 cycles of 95ºC for 10 s and 60ºC for 1 min. The threshold was set above the non-template control background and within the linear phase of target gene amplification in order to calculate the cycle number at which the transcript was detected (denoted as C_T). The oligonucleotide primers were: iNOS, forward, 5'-CCCAGAGTTCCAGCTTCTGG-3', and reverse, 5'-CCAAGCCCCTCACCATTATCT-3'; and GAPDH, forward, 5'-CTCAACTACATGGTCTACATGTTCCA-3', and reverse, 5'-CTTCCCATTCTCAGCCTTGACT-3'.

Bacterial survival assay

Bacterial survival was assessed in cultures of *H. pylori*–exposed, LPS-treated macrophages using a trans-well system, as described[22] with slight modification. Briefly, murine PEMs were cultured in the bottom layer of trans-well plates (Corning, Corning, NY, USA). After 48 h, 1×10^6 *H. pylori* were added to the insert membrane (0.1 μm pore size) and co-incubated for an additional 6 h in culture. The bacteria on the insert membrane were then resuspended and cultured by serial dilution onto Brucella blood agar plates. Colonies were counted after 4 to 5 days of incubation. Colony

forming units (CFU) were used to determine anti-bacterial effects.

Determination of nitric oxide production and cell viability assay

NO production was estimated from the accumulation of nitrite (NO_2^-), a stable end product of NO metabolism, in the culture medium, using the Griess reagent (Sigma-Aldrich).[24] The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to measure the effects of LPS and *H. pylori* on macrophage viability.[25] RAW 264.7 cells or PEMs were infected with various multiplicities of infection (MOI) of *H. pylori* for 24 or 48 h, respectively. Cell viability was then measured by examining the ability of viable cells to chemically reduce MTT to formazan**,** which was quantified by measurement of optical density at 570 nm.

Transfection and reporter gene assay

RAW 264.7 cells were grown to 90% confluency in a 12-well plate and transfected with NF-κB-Luc, AP-1-Luc, or iNOS-Luc reporter plasmid using Lipofectamine 2000 (Invitrogen).[26, 27] After 24 h, cells were incubated without or with LPS and then infected with *H. pylori* during an additional 24 h culture. To prepare cell lysates, 100 μl of reporter lysis buffer (Promega) was added to each well, and cells were scraped from dishes. An equal volume of luciferase substrate was added to all samples, and luminescence was measured using a microplate luminometer (Biotek, Winooski, VT, USA). Luciferase activity was normalized to the transfection efficiency as determined by co-transfection of the β-galactosidase expression vector (Promega).[28]

Immunofluorescence labeling of phosphorylated p65

To visualize *H. pylori*–induced inhibition of the translocation of phosphorylated p65 into the

nucleus of macrophages, RAW 264.7 cells were seeded onto cover-slips and treated without or with LPS for 2 h, and then with *H. pylori* for an additional 1 h incubation at 37ºC. Cells were fixed in 3.7% (w/v) paraformaldehyde and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 2 min. For labeling of p65, cells were incubated for 30 min with rabbit polyclonal anti-p65 (H-286; Santa Cruz Biotechnology) and propidium iodide (Calbiochem). Cells were then incubated with a secondary antibody, fluorescein isothiocyanate–conjugated anti-mouse IgG (Chemicon), and they were fixed in paraformaldehyde. Fixed cells were mounted and observed with a confocal laser scanning microscope (Zeiss LSM 510, Carl Zeiss, Göttingen, Germany). The quantification of fluorescence intensity for p65 was analyzed by ZEN software (Carl Zeiss).

Statistical analysis

The Student's *t* test was used to calculate statistical significance; a *P* value of <0.05 was considered significant.

RESULTS

H. pylori inhibits LPS-induced NO production by macrophages

To assess whether *H. pylori* inhibits LPS-induced NO production by macrophages, mouse macrophage RAW 264.7 cells were cultured with LPS (2 μg/ml) and infected with *H. pylori* at MOI of 0 to 100 for 24 h. Nitric oxide production, measured by nitrite levels, was not suppressed when LPS-stimulated RAW 264.7 cells were infected with *H. pylori* at a low MOI of 10 (Fig. 1A). NO production by LPS-stimulated cells was decreased, however, at MOI of 50 to 100. When cells were

infected with *H. pylori* at MOI of 100, LPS-induced NO production was reduced by approximately 50%. Macrophage viability, as determined by the MTT assay, did not change after 24 h of incubation with LPS and *H. pylori* (Fig. 1B).

To further delineate the suppressive effects of *H. pylori* on LPS-induced NO production, peritoneal exudate macrophages (PEMs) were prepared and co-incubated with LPS and *H. pylori* for 48 h. This *ex vivo* approach showed that LPS treatment induced NO production in uninfected PEMs (MOI of 0; Fig. 1C), and LPS-induced NO production was reduced in an MOI-dependent manner when PEMs were infected with *H. pylori* (Fig. 1C). The viability of PEMs, as determined using the MTT assay, was hardly influenced by treatment with LPS and *H. pylori* at different MOI (Fig. 1D). Thus, *H. pylori* inhibited, in an MOI-dependent manner, LPS-induced NO production not only in RAW 264.7 cells but also in murine primary PEMs, and PEMs were more sensitive than the macrophage cell line to the suppressive effects of *H. pylori*.

Live H. pylori is essential for the inhibition of LPS-induced NO production

To determine the functional role of *H. pylori* in inhibiting LPS-induced NO production by RAW 264.7 cells, live bacteria, heat-killed bacteria, and crude bacterial extracts were tested for their ability to inhibit LPS-induced NO production. As shown in Fig. 2A, live *H. pylori* attenuated LPS-induced NO production by RAW 264.7 cells. In contrast to the effects observed with live bacteria, neither heat-killed bacteria nor crude bacterial extracts inhibited LPS-induced NO production. We also used PEMs to study the effects of *H. pylori* on the inhibition of LPS-induced NO production. Consistent with the results for RAW 264.7 cells, LPS-induced NO production by

PEMs was inhibited by live *H. pylori* but not by heat-killed bacteria or crude bacterial extracts (Fig. 2B). We further investigated the *H. pylori*–derived components that are responsible for these effects. Virulence factor isogenic mutants of *H. pylori*, Δ*cagA* and Δ*vacA*, were tested for their ability to inhibit LPS-induced NO production by RAW 264.7 cells. Both isogenic mutants suppressed LPS-induced NO production, similar to that observed with wild-type *H. pylori* (Fig. 3).

To mimic the environment of the bacterial infection, we further analyzed the ability of *H. pylori* to inhibit LPS-induced NO production and to enhance the survival of bacteria adjacent to infected macrophages *in vitro*. Using a trans-well culture system to assay bacterial survival of LPS-stimulated murine PEMs, we found that *H. pylori* at MOI of 100 increased bacterial viability (Fig. 4A). To test the hypothesis that *H. pylori* triggers iNOS activity, which is necessary for NO production, we tested the effects of *H. pylori* in C57BL/6 iNOS$^{-/-}$ mice. *H. pylori* viability was similar without or with LPS and with *H. pylori* at MOI of 0 or 100 in PEMs from iNOS$^{-/-}$ mice, unlike the effects observed in wild-type mice (Fig. 4B). These results revealed that the ability of *H. pylori* to inhibit LPS-induced NO production resulted from a reduction in the antimicrobial activity of macrophages with subsequent enhancement of *H. pylori* survival.

H. pylori inhibits LPS-induced iNOS expression at transcriptional and post-transcriptional levels

To investigate the effects of *H. pylori* on the regulation of iNOS expression, PEMs were treated (or left untreated) with LPS and exposed to *H. pylori* at various MOI for 48 h. Subsequently, iNOS protein levels were analyzed by western blotting. *H. pylori* infection led to a significant decrease, in an MOI-dependent manner, in LPS-induced iNOS level (Fig. 5A). When cells were infected with *H.*

pylori at MOI of 100, LPS-induced iNOS expression decreased by approximately 80%. We then measured expression of iNOS mRNA using reverse transcription and quantitative real-time PCR in PEMs that were co-cultured without or with LPS and with *H. pylori* at various MOI for 6 h. The expression of iNOS mRNA was decreased by *H. pylori* infection at MOI of 50 to 100 (Fig. 5B). Taken together, the results indicated that *H. pylori* inhibited iNOS transcription, which subsequently influenced the translation of iNOS mRNA and NO production by macrophages.

H. pylori attenuates LPS-induced NO production by macrophages through the p38 and ERK1/2 signaling pathways

LPS-induced NO production by macrophages involves several signaling pathways, including p38, ERK 1/2 (p42 and p44), and JNK. We used western blotting to analyze the signal transduction pathways involved in the inhibitory effects of *H. pylori* on NO production by LPS-stimulated PEMs for 60 min of culture. The data showed that without treatment with LPS and *H. pylori*, the phosphorylated molecules involved in the MAPK signaling pathway were expressed at a basal level (Fig. 6A). *H. pylori* infection led to a decrease in phosphorylated p38 in LPS-stimulated macrophages within 5 to 10 min of culture (Fig. 6B). Phosphorylation of ERK1/2 was also inhibited by *H. pylori* infection from 5 to 60 min of culture. In contrast to the results obtained for p38 and ERK1/2, phosphorylation of JNK1/2 in LPS-stimulated PEMs was not affected by *H. pylori* infection. The suppressive effect of *H. pylori* on LPS-induced iNOS expression was augmented by SB203580 and PD98059, specific inhibitors of p38 and ERK1/2, respectively, but not by SP600125, a specific JNK inhibitor (Fig. 6D). Taken together, these results suggested that *H. pylori* inhibited

LPS-stimulated NO production and iNOS expression in macrophages through the p38 and ERK1/2 signaling pathways.

Suppression of LPS-induced NF-κB activation in macrophages by H. pylori

We next investigated the effects of *H. pylori* on the levels of two transcription factors, NF-κB and AP-1, which play essential roles in the regulation of iNOS expression.[29] We first examined the effects of *H. pylori* on NF-κB expression using the luciferase assay in RAW 264.7 cells transfected with the NF-κB-luciferase reporter. *H. pylori* inhibited LPS-stimulated activation of the NF-κB promoter (Fig. 7A). *H. pylori* only slightly inhibited LPS-stimulated AP-1 activity, but the change was not statistically significant (Fig. 7B). We next examined p65 localization and observed that it was primarily located in the cytosol before LPS treatment. After 1 h of stimulation with LPS, p65 translocated into the nucleus of RAW 264.7 cells. When cells were co-cultured with LPS and *H. pylori*, however, p65 remained largely in the cytosol (Fig. 7C). The quantitative data showed that the inhibition of p65 translocation into the nucleus in LPS-treated macrophages upon *H. pylori* infection (Fig. 7D). These data suggested that *H. pylori* alone did not alter the distribution of p65 but rather prevented LPS-induced translocation of p65 into the nucleus.

DISCUSSION

iNOS and NO are two well known factors that serve important roles in the antimicrobial response of macrophages.[30] Our results showed that infection of *H. pylori* elicited a small amount of NO production by macrophages (Fig. 1). Not only live *H. pylori* but also heat-killed *H. pylori* and bacterial lysate also slightly stimulated NO synthesis in the absence of bacterial LPS (Fig. 2). In

addition, without LPS treatment, *H. pylori* stimulated, in an MOI-dependent manner, the expression of iNOS mRNA and protein (Fig. 5). Our results confirm the results of a previous study that showed that *H. pylori* can stimulate iNOS expression and activity in murine macrophages.[5] The release of NO appears to be stimulated by *H. pylori*–derived components because even heat-killed *H. pylori* can stimulate NO production.[31] LPS derived from *H. pylori* produces only low biological activity as a stimulator of NO,[32] with estimates that it is 2,000- to 30,000-fold less potent than LPS derived from *E. coli*.[33] Wilson et al. suggested that *H. pylori* induces iNOS expression through both LPS-dependent and -independent mechanisms.[5] The expression of iNOS and the accumulation of NO have been linked to *H. pylori*–associated gastritis.[6, 34] These results demonstrate that NO production plays an important role in gastric inflammatory responses elicited by *H. pylori*.

Apart from infection with *H. pylori*, the stomach is colonized with non-*Helicobacter* microorganisms. The most common non-*Helicobacter* bacteria of the gastric microflora are *Streptococcus*, *Staphylococcus*, *Neisseria*, *Pseudomonas*, and *Enterobacteriaceae*.[15] In addition, endoscope-transmitted infections have been reported and include *Salmonella* spp., *E. coli*, *K. pneumoniae*, and *P. aeruginosa*.[35] Long-term infection with *H. pylori* [15] or acid-suppressive therapy [16] enables non-*Helicobacter* bacteria to colonize the host stomach. A diverse community of 128 phylotypes was identified, using large-scale 16S rRNA sequencing, in 23 gastric endoscopic biopsy samples, suggesting that the human stomach may be host to a distinct microbial ecosystem.[18] Both LPS and peptidoglycan (PGN), which is found in the cell wall of Gram-negative and Gram-positive bacteria, have been implicated in the production of NO and proinflammatory cytokines by

macrophages.[36-39] Patients with both *H. pylori* and non-*Helicobacter* bacteria in their gastric mucosa have higher levels of proinflammatory cytokines than patients without bacterial infection.[19] The issue that these findings raise, therefore, is how *H. pylori* can survive in a hostile gastric environment surrounded with profound inflammatory responses provoked by other microorganisms. To mimic the hostile gastric environment, with activated macrophages and co-infection of *H. pylori* and non-*Helicobacter* bacteria, purified LPS or PGN were added to our macrophage culture system to determine the effects on NO production. In a preliminary study, we found that 24 h of treatment with PGN (10 μg/ml) resulted in an approximately 4-fold increase in nitrite (8 μM) over basal levels in RAW 264.7 cells (Supplementary Fig. 1). After 24 h of treatment with LPS (2 μg/ml), there was an approximate 8-fold (22 μM) and 150-fold (158 μM) increase in nitrite over basal levels in RAW 264.7 cells and PEMs, respectively (Fig. 1). Murine macrophages were also more sensitive to LPS than PGN. Because of the results of our preliminary studies, we chose LPS for use in the current study.

In the present study, we used *in vitro* and *ex vivo* murine model systems to reveal how *H. pylori* evades LPS-dependent killing by macrophages. To demonstrate that the rational design of our murine model systems mimic a real-life *in vivo* situation, we used wild-type C3H/HeN and TLR4-deficient C3H/HeJ mice to study the role of LPS in killing gastric *H. pylori in vivo*. As shown in Supplementary Fig. 2, *H. pylori* was more significantly eradicated from the stomachs of LPS-administered C3H/HeN mice than from the stomachs of LPS-administered C3H/HeJ mice. The response of LPS-administered C3H/HeN mice was approximately 11-fold greater than that of

LPS-administered C3H/HeJ mice. This result confirmed that LPS enhanced *in vivo* anti-*H. pylori* activity in LPS-responsive mice (C3H/HeN). In addition, the results of this experiment proved our assumption from the *in vitro* and *ex vivo* murine models since the responses were mimicked in the *in vivo* setting.

In this study, we demonstrated that LPS-induced NO production by macrophages was suppressed by *H. pylori* and that this effect was dependent on the presence of live bacteria (Fig. 2). Neither heat-killed bacteria nor crude extracts of *H. pylori* suppressed LPS-induced NO production. The work of von Bothmer et al. likewise demonstrated that *H. pylori* water extract and whole-bacterial suspension produced an L-arginine-sensitive inhibition of NO synthesis.[40] These data raise the issue as to why the bacteria need to be alive. One possibility is that the effect is mediated through the *cag*-pathogenicity island (*cag*-PAI) or VacA. Our results using isogenic mutants of Δ*cagA* or Δ*vacA* indicated that mutant *H. pylori* strains also inhibited LPS-induced NO production in a manner similar to the effects observed with wild-type *H. pylori* (Fig. 3). Despite our results suggesting that the suppressive effect is mediated through direct interaction of *H. pylori* with macrophages, the extent to which virulence factors are associated with the inhibition of NO remains unknown. Future studies also are needed on the genetic analysis of the virulence factors in different *H. pylori* strains.

Several reports have shown that NO can kill *H. pylori* in cell culture systems.[22, 41] Our data indicated that *H. pylori* could survive when *H. pylori* was co-cultured with LPS-activated macrophages in a trans-well system (Fig. 4). This effect might be due to the suppressive effects of *H.*

pylori on LPS-induced NO production by macrophages. This possibility is consistent with the results of previous studies that demonstrated that arginase produced by *H. pylori* [22] and arginase II released from macrophages [42] suppress NO production and lead to immune evasion by the bacteria. Another explanation for the mechanisms through which *H. pylori* inhibits NO production is a decrease in the concentration of L-arginine in culture medium, which interferes with the L-arginine/NO pathway.[40] A reduction in L-arginine availability inhibits iNOS expression and attenuates NO-dependent bactericidal activity.[43] Apart from competition or inhibition of NO production by macrophages, previous studies showed that *H. pylori* induces apoptosis in both macrophages [44] and T lymphocytes.[45] In the present study, we did not add L-arginine to cultures. We used a high dose of LPS to induce iNOS expression, and the effect of LPS was independent of the concentration of L-arginine, as demonstrated previously.[40] Cell viability was not influenced when cells were incubated with *H. pylori* at a high MOI of 100 and in the presence of LPS (Fig. 1). *H. pylori* appears to have intricate mechanisms to regulate the activity of macrophages and to maintain bacterial survival under various infectious conditions.

LPS stimulates iNOS gene expression and NO production. The stimulation is positively regulated by NF-κB, which is normally bound to its inhibitor, IκB in the cytoplasm. Phosphorylation of IκB by IκB kinase results in the degradation of IκB, which dissociates NF-κB and leads to the nuclear translocation of NF-κB and the up-regulation of downstream gene expression. In addition to NF-κB, LPS can activate MAPK pathways in macrophages, including p38, ERK-1/2, and JNK-1/2. A common strategy for pathogens to overcome host defense is

interference with the activity of NF-κB or MAPKs.[10, 12, 13, 46] In the present study, *H. pylori* might have suppressed LPS-induced NO production by macrophages by inhibiting LPS-stimulated NF-κB activation. Our data demonstrated that the phosphorylation of p38 and ERK1/2 was attenuated by *H. pylori* infection at an early stage of infection, whereas JNK-1/2 was not affected. These results suggest that *H. pylori* targets p38 and ERK 1/2, but not JNK1/2. Our data are consistent with previous findings that pathogens can exploit NF-κB to manipulate cellular responses.[10, 13, 14, 47] Together, the findings support our hypothesis that *H. pylori* modulates host signaling to evade the host immune system. In addition, our results provide insight into the molecular mechanisms through which indigenous *H. pylori* survive commensurately in the stomach with non-*Helicobacter* microorganisms that induce potent immune responses.

In conclusion, we demonstrate that infection with live *H. pylori* attenuates LPS-induced iNOS gene transcription and NO production in a mouse macrophage model. We further find that *H. pylori* inhibits LPS-induced MAPK signaling and NF-κB activation in macrophages. Collectively, this study reveals a new mechanism through which *H. pylori* modulates host cell signaling to protect itself from inflammatory responses and to survive in the harsh environment of the stomach.

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Declaration of conflicting interests: None Declared.

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FIGURE LEGENDS

Fig. 1. *H. pylori*–mediated inhibition of LPS-induced NO production in the RAW 264.7 cell line (A) and in murine primary peritoneal exudated macrophages (PEMs; C). Cells were treated without or with LPS (2 μg/ml) and infected with *H. pylori* at various MOI from 0 to 100. After 24 h (RAW 264.7 cells) or 48 h (PEMs) incubation, the culture supernatants were collected for determination of nitrite levels using the Griess reagent. The MTT assay showed that there was no loss of cell viability in RAW 264.7 cells (B) or murine PEMs (D) during the incubation period. The data represent the mean ± standard deviation derived from three independent experiments. Statistical significance was determined using the Student's *t*-test (*$P < 0.05$; **$P < 0.01$). LPS: lipopolysaccharide.

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Fig. 5. *H. pylori*–mediated inhibition of iNOS expression in LPS-treated macrophages. Murine PEMs were treated without or with LPS and infected with *H. pylori* at different MOI. (A) Cell lysates were prepared after 48 h of incubation to measure iNOS protein expression by western

blotting. Protein expression levels were quantified with densitometric analysis and normalized to β-actin (B) iNOS mRNA expression was measured by quantitative real-time PCR after 6 h of incubation, and GAPDH was used as an internal control. The data are presented as the mean ± standard deviation of three independent experiments. Statistical significance was determined using the Student's *t*-test (*$P < 0.05$; **$P < 0.01$). LPS: lipopolysaccharide, MOI: multiplicity of infection.

Fig. 6. Signaling pathways involved in the inhibition of LPS-induced macrophage activation by *H. pylori*. Murine PEMs were incubated without LPS (A) or with LPS (B) and infected (or not infected) with *H. pylori* at an MOI of 100 for the indicated times. Expression levels of phosphorylated p38 (p-p38), p-ERK, and p-JNK were determined by western blotting. Representative western blot results from one of three independent experiments are shown. (C) Protein expression levels were quantified with densitometric analysis, normalized to β-actin, and presented as the mean ± standard deviation derived from three independent experiments. Statistical significance was determined using the Student's *t* test (*$P < 0.05$; **$P < 0.01$ as compared with LPS-stimulated cells in the absence of *H. pylori* infection [LPS+, Hp–], lower panel). (D) Murine PEMs were pretreated for 30 min with SB203580 (p38 inhibitor), PD98059 (ERK inhibitor), or SP600125 (JNK inhibitor), followed by incubation with LPS and infection with *H. pylori* at an MOI of 100 for 48 h. Representative western blot results from one of three independent experiments are shown. The data are expressed as the mean ± standard deviation determined from three independent experiments.

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Supplementary Fig. 1. *H. pylori*–mediated inhibition of PGN-induces NO production by RAW 264.7 cells. Cells were treated with or without PGN (10 μg/ml) and infected with *H. pylori* at an MOI ranging from 0 to 100 for 24 h. Culture supernatants were collected for determination of nitrite release. The data are expressed as the mean ± standard deviation derived from three independent

experiments. Statistical significance was determined using the Student's *t*-test (*$P < 0.05$). PGN: peptidoglycan.

Supplementary Fig. 2. LPS-dependent *in vivo* killing of gastric *H. pylori*. C3H/HeN and C3H/HeJ mice were intragastrically inoculated with *H. pylori* and purified LPS. The amounts of living *H. pylori* in the stomach were determined 7 days after infection. The data are expressed as the mean ± standard deviation (n = 6 for each group). Statistical significance was determined using the Student's *t*-test (**$P < 0.01$). CFU: colony forming units.

FIG. 1

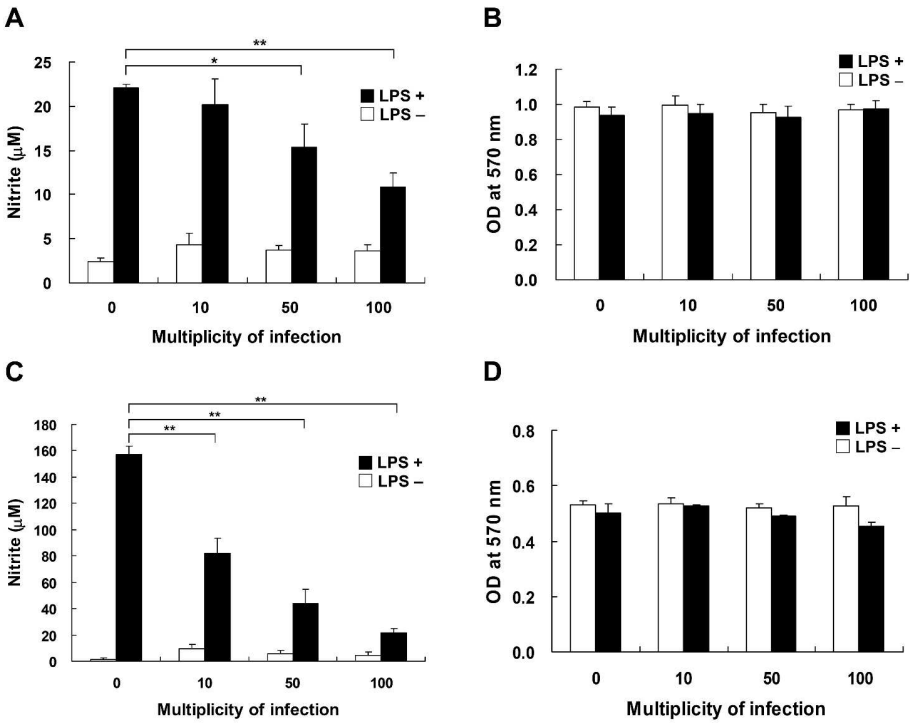

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FIG. 2

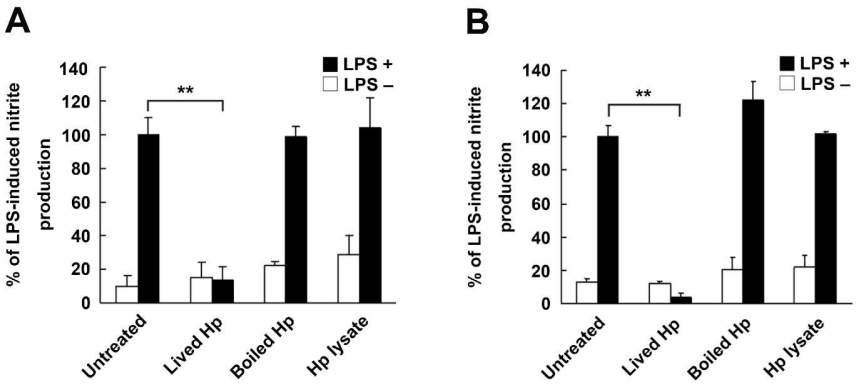

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FIG. 3

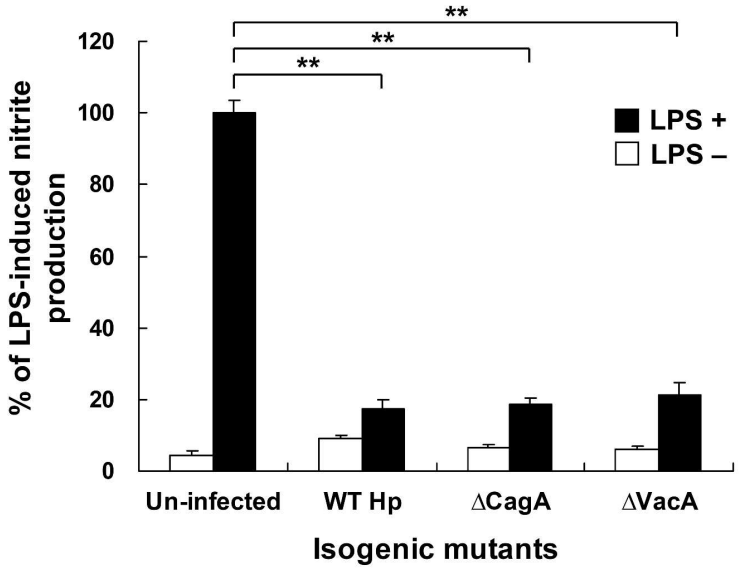

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FIG. 4

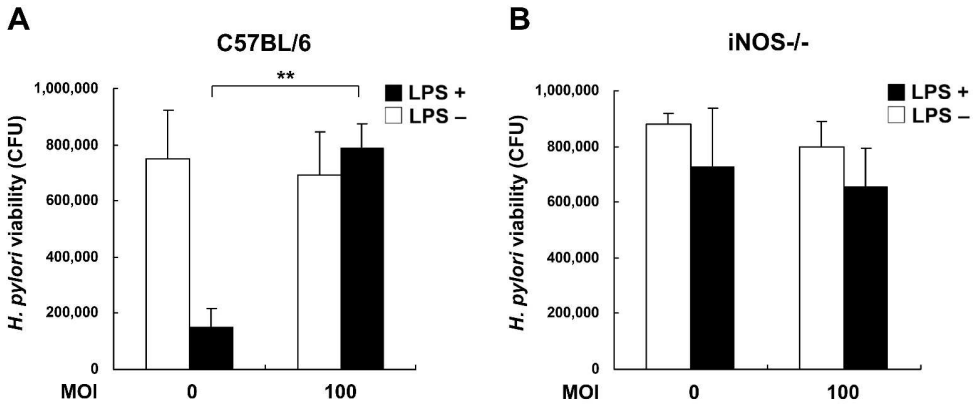

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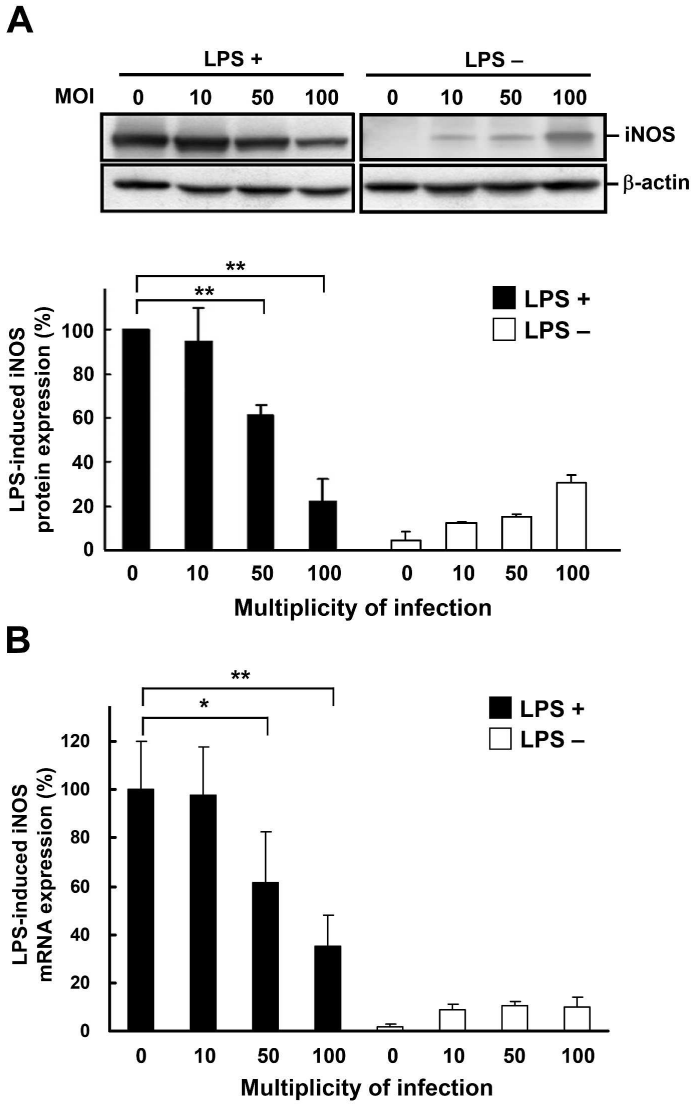
FIG. 5



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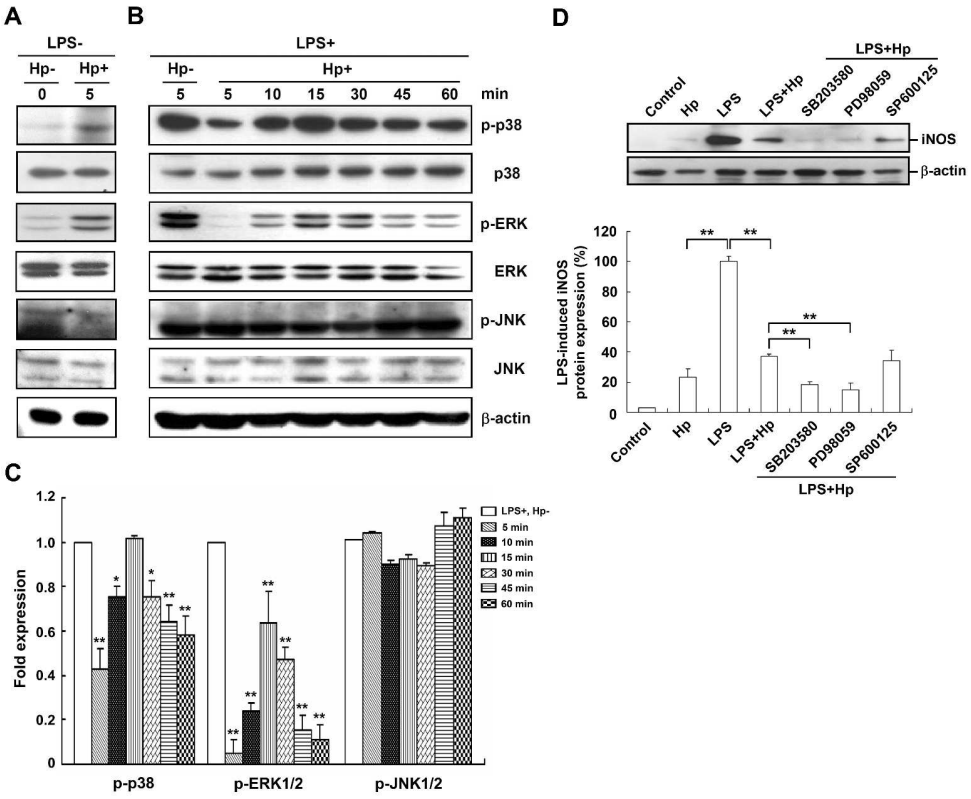

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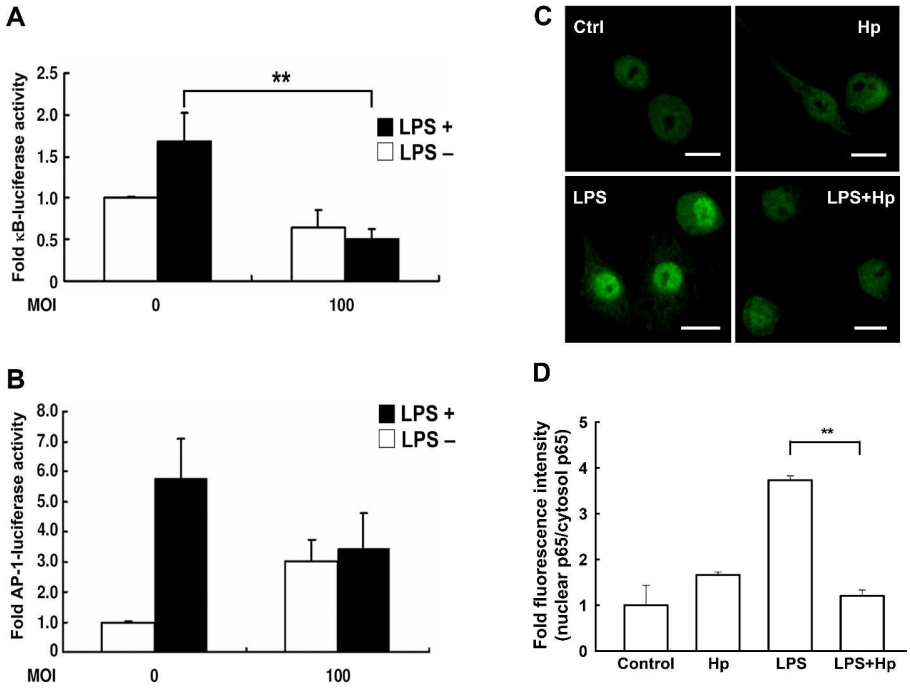

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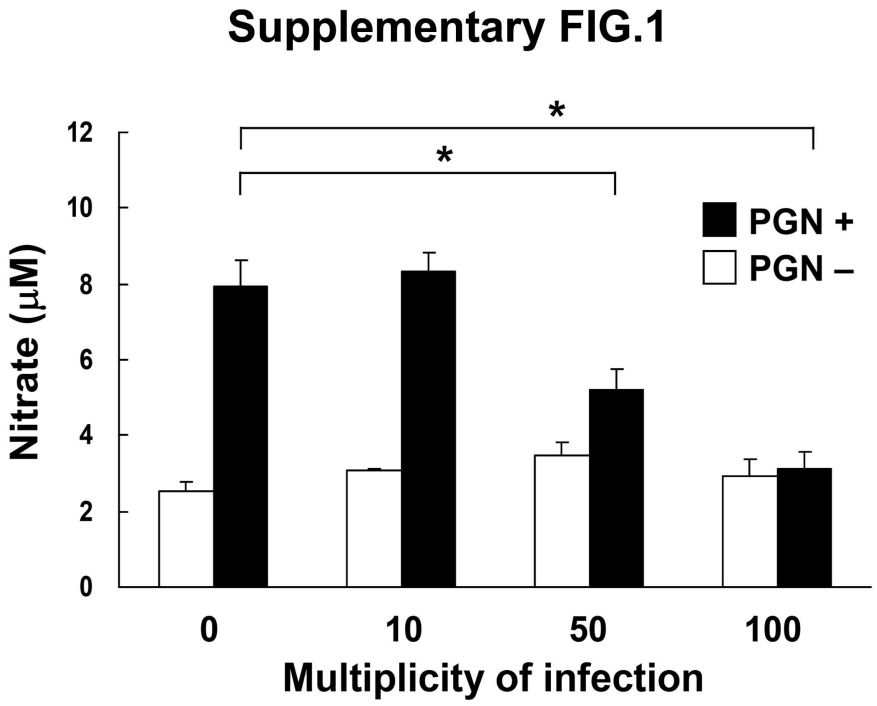

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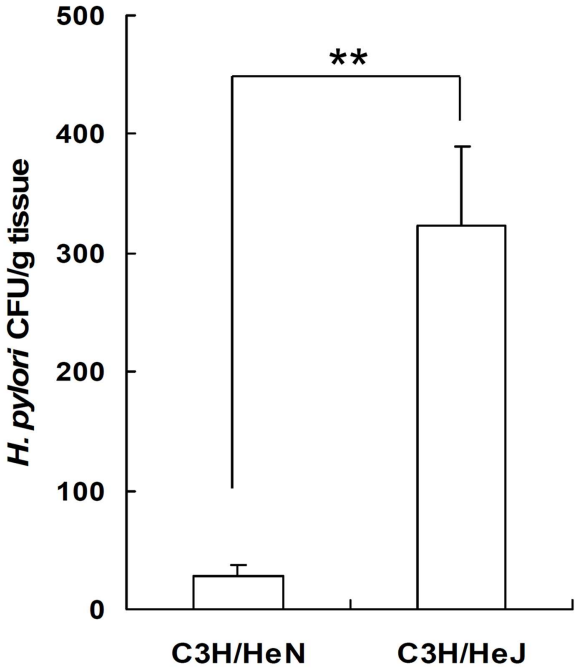
Supplementary FIG. 2



Supplementary Fig. 2. LPS-dependent in vivo killing of gastric H. pylori. C3H/HeN and C3H/HeJ mice were intragastrically inoculated with H. pylori and purified LPS. The amounts of living H. pylori in the stomach were determined 7 days after infection. The data are expressed as the mean ± standard deviation (n = 6 for each group). Statistical significance was determined using the Student's t-test (**P < 0.01). CFU: colony forming units.

Response to the reviewers' comments

Re: *Helicobacter pylori* attenuates lipopolysaccharide-induced nitric oxide production by murine macrophages (manuscript ID: INI-11-0004)

Dear Dr. Lai:

Manuscript ID INI-11-0004 entitled "Helicobacter pylori attenuates lipopolysaccharide-induced nitric oxide production by murine macrophages" which you submitted to the Innate Immunity, has been reviewed. The comments of the reviewer(s) are included at the bottom of this letter.

The reviewer(s) have recommended publication, but also suggest some revisions to your manuscript. Therefore, I invite you to respond to the reviewer(s)' comments and revise your manuscript.

To revise your manuscript, log into http://mc.manuscriptcentral.com/ini and enter your Author Center, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision.

You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript using a word processing program and save it on your computer. Please also highlight the changes to your manuscript within the document by using the track changes mode in MS Word or by using bold or colored text. An specific response to the reviewer(s) letter should be uploaded as Supplementary file.

Once the revised manuscript is prepared, you can upload it and submit it through your Author Center.

When submitting your revised manuscript, you will be able to respond to the comments made by the reviewer(s) in the space provided. You can use this space to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the reviewer(s).

IMPORTANT: Your original files are available to you when you upload your revised manuscript. Please delete any redundant files before completing the submission.

Because we are trying to facilitate timely publication of manuscripts submitted to the Innate Immunity, your revised manuscript should be uploaded as soon as possible (not later than three months time). If it is not possible for you to submit your revision in a reasonable amount of time,

we may have to consider your paper as a new submission.

Once again, thank you for submitting your manuscript to the Innate Immunity and I look forward to receiving your revision.

Sincerely,
Prof. Otto Holst
Editor in Chief, Innate Immunity
otto.holst@googlemail.com

Reviewer(s)' Comments to Author:

Reviewer: 1
Comments to the Author
In this MS the authors make a rather exotic assumption namely that during H. pylori infection in humans, LPS liberated from co-infecting non-Hp Gram-negatives, drives a gastric inflammatory response, culminating in NO production, that kills Hp bacteria….unless Hp counteracts this effect of the non-Hp LPS. This situation is mimicked in a wide variety of in vitro experiments where indeed is shown that live Hp bacteria suppresses the effect of (most likely:enterobacterial) LPS on host cells; an impressive array of assays is done to elucidate signaling routes.

There is nothing wrong with the assays per-se, and most of the work is done well BUT the rationale to do all these in vitro studies is valid only if the assumption mentioned above indeed is correct. However no experiments are done to show that this in vivo mold mimics a real-life in vivo situation. In addition, it is not sorted out which Hp virulence factors are responsible for the immuno-suppressive effect.

I see two way to bring relevance to this paper:

1. Preferably, the authors show in an animal model in vivo that indeed gastric Gram-negative bacteria cause an LPS dependent in vivo killing of gastric Hp. This experiment can be done by gastric co-colonization of Hp with a Gram-negative in both LPS sensitive and LPS resistant mice. This would demonstrate that the assumption made is correct.

2. Alternatively, with a Hp mutant library the authors sort out how Hp suppresses the LPS effect-then one could consider this manuscript as elucidating in detail how a certain virulence factor signals to suppress LPS action-independent from the question whether this is relevant in vivo or not.

Re. We thank the reviewer for the positive comment and critical suggestions. We agree that in the current study, only *in vitro* and *ex vivo* murine model systems were used to demonstrate how *H. pylori* evades LPS-dependent killing by macrophages. In keeping with your suggestions, we used animal models to mimic an *in vivo* setting. We used wild-type C3H/HeN and TLR4-deficient C3H/HeJ mice to study the role of LPS in killing gastric *H. pylori in vivo*. Male C3H/HeN (n = 6) and C3H/HeJ (n = 6) mice of 6–8 weeks of age were intragastrically inoculated with *H. pylori*. All mice were maintained under fasting for 24 h before inoculation. Each mouse was administered 1×10^9 CFU/mL of *H. pylori* and purified LPS (75 µg, phenol extracted from *Escherichia coli* O55: B5, Sigma-Aldrich) by intragastric gavage for 3 consecutive days. Six hours after the final inoculation with *H. pylori*, the mice were fed with standard food and water and housed for 1 week. On the 7^{th} day after infection, 6 mice in each group were sacrificed, and the number of *H. pylori* in their stomachs was determined by plating on Brucella blood agar plates and expressed as CFU/g tissue.

As shown in Supplementary Fig. 2, *H. pylori* was more significantly eradicated from the stomachs of LPS-administered C3H/HeN mice than from the stomachs of LPS-administered C3H/HeJ mice. The response of LPS-administered C3H/HeN mice was approximately 11-fold greater than that of LPS-administered C3H/HeJ mice. This result confirmed that LPS enhanced *in vivo* anti-*H. pylori* activity in LPS-responsive mice (C3H/HeN). In addition, the results of this experiment proved our assumption from the *in vitro* and *ex vivo* murine models since the responses were mimicked in the *in vivo* setting. Data of the *in vivo* animal experiments have been added to the MATERIALS AND METHODS section (page 7, the 2nd paragraph) and described in the DISCUSSION section (page 16, the 2nd paragraph).

Reviewer: 2

Comments to the Author

Manuscript INI-11-0004, entitled "Helicobacter pylori attenuates lipopolysaccharide-induced nitric oxide production by murine macrophages" by Lu, Dah-Yuu; et al.

The authors described the inhibition mediated by H. pylori infection on the production of NO by the RAW264.7 and murine peritoneal exudate macrophages (PEMs) stimulated with LPS. They further demonstrated that this effect requires live bacteria, and is not dependent on the expression of the H. pylori virulence factors CagA and VacA, and it is important to promote bacterial survival.

The inhibitory effect is dependent on the transcriptional downregulation of the iNOS mRNA, which is also resulting in a decreased expression of the iNOS protein. The reduced levels of iNOS observed upon bacterial infection of LPS-stimulated macrophages were associated with decreased activation of the MAPK p38 and ERK1/2 pathways and reduced activity and nuclear translocation of the transcription factor NFkB.

The work is well performed and the results are clearly presented and carefully conducted.

I have fours minor points that need to be addressed:

1. Figure 6. I did not understand what was the logic for performing the experiments presented in this figure. The data don't seem to add any extra value to this work, and I would remove this figure from the manuscript.

Re. We agree that Fig. 6 may confuse the readers, and therefore, we have deleted this figure in the revised manuscript.

2. Figure 5B. Quantification of the qPCR. The quantification of qPCR is not performed by densitometry analysis, as described in the figure legend. I wonder whether the data presented are derived from quantitative (qPCR) or semiquantitative RT-PCR.

Re. We used quantitative-PCR (qPCR) to determine the mRNA levels of iNOS and GAPDH. iNOS mRNA expression was measured by qPCR after 6 h of incubation, and GAPDH was used as an internal control. We have revised the pertinent figure legend to include this information (page 30, legend of Fig. 5).

3. Figure 7A: It would be nice to see the levels of the phosphorylated forms of the MAPK kinases tested also at time 0, meaning without LPS simulation.

Re. In keeping with your suggestion, we conducted the experiment at time 0 without LPS treatment. Fig. 6A (formerly, Fig. 7A) shows that without treatment with LPS and *H. pylori*, the phosphorylated molecules involved in the MAPK signaling pathway were expressed at a basal level. These results have been added in RESULTS section (page 13, the 2nd paragraph) and Fig. 6 of the revised manuscript.

4. Figure 8C. It is very difficult to appreciate the nuclear translocation of p65 from the figures presented. The authors should show only the p65 staining (without the nuclear counterstaining), and quantify the ratio of cytoplasmic versus nuclear fluorescence intensity to clear demonstrate the inhibition of p65 translocation in LPS treated macrophages upon H. pylori infection.

Re. We have presented the results of only the p65 staining (green) without nuclear counterstaining (Fig. 7C). We also have presented the quantitative data of p65 translocation (Fig. 7D), which showed that after *H. pylori* infection, the p65 translocation into the nucleus was inhibited in LPS-treated macrophages. These data clearly demonstrate that *H. pylori* inhibits LPS-induced translocation of p65 into the nucleus. Data of the quantification of fluorescence intensity for p65 was added in RESULTS section (page 14, the 2nd paragraph) and in Fig. 7D of the revised manuscript.