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基礎醫學研究所
碩士學位論文

幽門螺旋桿菌抑制小鼠巨噬細胞感染模式
抗菌一氧化氮之產生

Helicobacter pylori suppresses antimicrobial nitric oxide

production in a mouse macrophage model

指導教授：賴 志 河 (Chih-Ho Lai, Ph.D.)

研究生：張 佳 嫻 (Chia-Hsien Chang)

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Contents

中文摘要 (Abstract in Chinese)

在幽門螺旋桿菌(*Helicobacter pylori*)存活於胃黏膜組織進而引發慢性胃發炎的反應中，菌體必須具有能在免疫系統活化反應下存活的能力。在胃黏膜組織對於幽門螺旋桿菌的免疫反應及相關的發炎反應中，一氧化氮(nitric oxide, NO)扮演著相當重要的角色。病原體可能會活化巨噬細胞的誘發性一氧化氮合成酶(inducible nitric oxide synthase, iNOS)表現，而 iNOS 在不同細胞中的表現則會經由細菌的脂多醣(lipopolysaccharide, LPS)刺激細胞所調控。有某些病原體具有避免巨噬細胞清除病原的能力，這些能力包括抑制巨噬細胞的活化或引發急速的細胞死亡。在我們的研究中，我們使用了小鼠巨噬細胞的實驗模式，最主要是為了確認幽門螺旋桿菌的感染是否會抑制由 LPS 所誘發產生的一氧化氮及 iNOS 的表現。研究中我們分析了 iNOS 的 mRNA 及蛋白質的表現情形，而結果也證實了幽門螺旋桿菌能抑制 iNOS 的轉錄及轉錄後的表現情形，而這些作用情形主要是由活菌並具有正常功能的菌體所造成的。除此之外，這些現象還包括抑制裂殖原活化蛋白激酶(mitogen-activated protein kinase, MAP kinase)傳遞路徑，而此一路徑最主要是影響 nuclear factor (NF)-κB 活化並進入細胞核。而我們的結果也顯示，幽門螺旋桿菌具有調控先天性免疫反應的能力，而這個調控機制對於菌體存活在宿主胃體並持續進行感染是有益的。

關鍵字：幽門螺旋桿菌，一氧化氮，巨噬細胞，NF-κB

Abstract

The ability of the *Helicobacter pylori* to survive in the interaction of non-phagocytes and phagocytes is postulated to enhance the persistence of this pathogen in the gastric mucosa and then to cause chronic inflammation. Nitric oxide (NO) production plays an important role in the gastric mucosal immune response to *H. pylori* and the associated inflammation. Pathogens might activate macrophage inducible nitric oxide synthase (iNOS) expression. The expression of iNOS is regulated in various cell types and can be enhanced by stimulation of bacterial lipopolysaccharide (LPS). In some pathogens, the ability to evade macrophage killing involves inducing rapid death of macrophages or suppression of macrophage activities. In this study, we used a mouse macrophage infection model to demonstrate that *H. pylori* could inhibit lipopolysaccharide (LPS)-induced NO production and iNOS expression. Analysis of iNOS specific mRNA and protein expression levels after infection revealed that *H. pylori* inhibited iNOS expression at both transcriptional and post-transcriptional levels, and only live and functional bacteria would do. Furthermore, this phenomenon involved down-regulation of mitogen-activated protein (MAP) kinase pathway, which triggered translocation of active nuclear factor (NF)-κB into nucleus. Our data suggested a new mechanism for *H. pylori* regulating innate immune responses of host cells to benefit persistent infection in host stomachs.

Keywords：*Helicobacter pylori*, macrophage, nitric oxide, NF-kappa B

Abbreviations: LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric oxide synthase; MAP, mitogen-activated protein; ERK, extracellular signal-related kinase; JNK, c-Jun-N-terminal kinase; NF-κB, nuclear factor (NF)-κB; IKK, IκB kinase; PEMs, peritoneal excluded macrophages; ODN, oligonucleotide dominate negative.

Introduction

Helicobacter pylori is the most commonly causative agent of duodenal and gastric diseases in human. Infection of the pathogen usually occurs in childhood and can persist in the stomach for a life-time (1-3). The most common outcome of infection is chronic gastritis, but some patients may develop peptic ulcers, gastric carcinoma, and gastric lymphoma. Persistent infection of *H. pylori* in the gastric mucosa results in release of interleukine (IL)-8, which attracts neutrophile infiltration and causes chronic gastritis. It has been demonstrated that CagA$^+$ *H. pylori* can induce NF-κB activation and secrete of IL-8 in gastric epithelial cells (4). It has also been found that employing NOD1-deficient mice reduced control of *H. pylori* densities in the stomach (5). These findings suggest that *H. pylori* is essential for induction of proinflammatory responses in host stomach.

Chronic gastritis is induced not only by epithelium secretion of IL-8 but also by mononuclear cell infiltrates, included of lymphocyte and macrophages. In the study of macrophages, which are major source of the IL-6 present in *H. pylori* induced chronic gastritis, are known to play a critical role in the pathogenesis of mucosal inflammations (6). It was also reported that macrophages secretion increased levels of IL-1β and TNF-α in *H. pylori* infected gastric tissues (7, 8). Analysis of IL-1 gene cluster polymorphisms indicated that IL-1β is associated with an increased risk of gastric cancer (9). Nevertheless, those immune responses is failed to eliminate *H. pylori* from gastric mucosa completely, suggesting

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that this bacteria has the ability to evade host immune elimination.

Another bactericidal agent of macrophages, nitric oxide (NO) is generated by nitric oxide synthase (NOS)-mediated conversion of L-arginine to L-citrulline. It has been reported that *H. pylori* can activate iNOS expression in macrophage (10). *H. pylori*-infected gastritis was shown to have higher expression of iNOS than *H. pylori*-negative patients in gastric epithelium and lamina propria (11). Furthermore, *H. pylori* urease was demonstrated to stimulate of iNOS expression and NO production in a mouse macrophage infection system (12). It is important to know that *H. pylori* can also survive despite marked induction of inducible NOS (iNOS) in macrophages. Gobert *et. al.* reported that *H. pylori* prevents NO production by the bacterial cell envelope gene *rocF*, which encoded an arginase and competed with NOS (13). Mutation of the *rocF* gene results in efficient killing of *H. pylori* in an NO-dependent manner, suggesting that arginase might be important for protection of *H. pylori* from macrophages attack. These evidences suggested that *H. pylori* has a delicate mechanism for the balance between activation of macrophages and protection of the bacteria from immune attack. However, the interaction between macrophages and *H. pylori* to inhibit innate immunity has not been extensively studied.

Although *H. pylori* is known to produce arginase to compete with iNOS for their substrate and regulate NO synthesis (14), the molecular mechanism of *H. pylori* to suppress NO production in macrophages has not been well defined. To address the question of how *H.*

pylori evades antimicrobial activities by macrophage, we established an *in vitro* and *ex vivo* mouse model system to examine whether this bacteria could suppress LPS-induced NO production. Our study reveals that *H. pylori* infection in such a model does inhibit LPS-stimulated iNOS expression and NO production in macrophages. We also demonstrate that *H. pylori* triggers the increase of phophorylated p38, activation of Erk1/2 and NF-κB, subsequently suppresses LPS-stimulated macrophage responses. Thus, study with this model system reveals an important aspect that *H. pylori* has the ability to trigger macrophage activation and subsequently evading of early host immune responses.

Although there is an innate response to the bacteria, one study has shown a attenuated uptake of bacteria into macrophages followed by the formation of megasomes as a result of phagosome fusion (15). These megasomes protect intracellular bacteria from efficient killing. In adaptive immunity, *H. pylori* can specifically block antigen-dependent proliferation of T-cells. This effect is mediated by the virulence factor VacA, which could act as an immunomodulator by interfering with the IL-2 signalling pathway in T-cells by blocking Ca^{2+} mobilization and the activity of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (16). There were some findings support the possibility that VasA is immunosuppressive, but the mechanism involves a direct reaction on T cells rather than in antigen-presenting cells. The toxin inhibits the activation and proliferation of T cells (17).

Even there are so many studies suggest that *H. pylori* could evade host immune

responses by these mechanisms, we hope our team could find some new mechanisms about

immune evasion of *H. pylori*.

Materials and methods

Antibodies and materials

Polyclonal rabbit antibodies specific for iNOS, p-JNK antibody, α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for p38 MAP kinase, SAPK/JNK, p44/42 (Erk 1/2) were purchased from Cell Signaling (Danvers, MA). Phosphorylated p38 MAP kinase, mouse anti-actin antibody, phosphorylated MAP kinase 1/2 (Erk1/2) (Thr185/Tyr187) were purchased from Upstate (Lake Placid, NY). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA). All other chemicals were obtained from Sigma-Aldrich (St Louis, MO).

Bacterial strain and cell Culture

H. pylori 26695 (ATCC700392) reference strain was recovered from frozen stocks on Brucella agar plates (Becton Dickinson) containing 10 % sheep blood, 6 μg/ml vancomycin and 2 μg/ml amphotericin B under microaerophilic conditions for 2–3 days as described previously (18).

To develop *H. pylori* derivative extracts, the following manipulation was performed. Heat-killed *H. pylori* was obtained by boiling the bacteria suspension in PBS (1×10^9/ml) for 30 min. *H. pylori* crude extracts were prepared by sonication on ice for 5 min. Crude extracts were then subjected to centrifugation at 16,000 ×g for 5 min at 4°C, the supernatant was

filtrated through 0.22µm filter and used for the further analysis.

RAW 264.7 cells (ATCC TIB-71, murine macrophage cell line) were cultured in RPMI 1640 (Invitrogene, Grand island, NY) medium. Ten percent of de-complement Fetal bovine serum (Hyclone, UT) was added in culture medium. Penicillin and streptomycin (Invitrogene) were used if needed. In bacteria infection system, cell culture medium was not supplemented with antibiotic reagents.

Preparation of mouse peritoneal excluded macrophages (PEMs)

C57BL/6JNarl male mice were purchased from National Laboratory Animal Center in Taiwan and maintained in the animal center of China Medical University at Taichung. The animal room was maintained on a 12 hr light and 12 hr dark cycle with a standard temperature and humidity. All mice were grown at 8 weeks old, sacrificed under anesthesia, and used to obtain cells from peritoneal exudates. All procedures adhered 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. Mouse peritoneal excluded macrophages (PEMs) were obtained from mice by lavage with 10 ml of cold PBS per mouse at 3 days after intraperitoneal injection of 2 ml 3% thioglycollate (Sigma-Aldrich) in PBS. The PEMs were then seeded in culture plates and incubated at 37°C in humidified 5% CO_2 and 95% air to allow macrophages adherence. After 2 hr, the non-adherent cells were removed by washing

with warmed PBS and the remaining cells (90% macrophages, judged by non-specific esterase stain) were used for further experiments.

Western blot

H. pylori infected cells were washed three times with PBS and then boiled with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.05% brilliant blue R) at 95°C for 10 min. The samples were then resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked with 5% skim milk in PBS buffer containing 0.1% Tween 20 at room temperature for 1 h and then incubated overnight with first antibodies as described in materials (dilution, 1:1,000) at 4°C. The membranes were washed with PBS containing 0.1% Tween 20 and then incubated with HRP-conjugated secondary antibodies (Invitrogen) at a dilution of 1:5,000. The proteins of interest were visualized by using the ECL^{TM} Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK) and were detected by exposure the autoradiograph to X-ray films (Kodak, Rochester, NY).

Semi-quantitative RT-PCR analysis

PEM cells were homogenized with Cyclo-Prep^{TM} Total RNA Purification Kit

(Amresco, Solon, OH) and reverse-transcription assay was performed using a Fast-Run™ HotStart RT-PCR (AMV) kit (Protech Technology Enterprise Co., Ltd, Taiwan). The oligonucleotide primers used corresponded to murine iNOS, forward: 5'-GCCTCGCTCTGGAAAGA-3' and reversed: 5'- TCCATGCAGACAACCTT -3'; GAPDH, forward: 5'-ACTCCCACTCTTCCACCTTC-3', and reversed: 5'-TCTTGCTCAGTGTCCTTGC-3'. All oligonucleotide primers were synthesized by Invitrogen. The experiment was performed as following program: For 1st cDNA synthesis: RNA denaturation for 5 min, 58 °C, 1 cycle; cDNA synthesis for 30 min, 42°C, 1 cycle; AMV RT inactivation & RNA/cDNA/primer denaturation for 2 min, 94°C. For 2nd PCR reaction: denaturation for 30 sec, 94°C; annealing for 1.5 min (iNOS) or 40 sec (GAPDH), 57°C; extension for 1 min, 72°C and the PCR reactions total run 35 cycles. The final elongation at 72°C for 5 min. PCR products were analyzed on 2% agarose gels. The mRNA of GAPDH served as the internal control for sample loading and mRNA integrity.

Bacterial survival assay

Mouse PEMs were cultured in the bottom layer of Trans-well plate (Corning, Lowell, MA) and infection with *H. pylori* at MOI of 0, 50, and 100. After 48 hr, a total of 1×10^6 *H. pylori* were added on the insert membrane (0.1 μm pore size) and co-incubated for a further 6 hr. After which, the bacteria on the insert membrane were suspended and cultured by serial

dilution onto Brucella blood agar plate and colonies were counted after 4–5 days. Colony forming units (CFUs) were enumerated for anti-bacterial effects. Experiments were performed at least three times in triplicates.

Determination of nitric oxide

The production of NO was estimated from the accumulation of nitrite (NO_2^-), a stable end product of NO metabolism, in the medium using the Griess reagent (Sigma-Aldrich) as described previously (19). Briefly, cells were incubated with medium containing various MOI of *H. pylori* wild type strain, in the presence or absence of LPS (2μg/ml) for the indication time. Equal volumes of culture supernatant and Griess reagent were mixed and incubated for 15 min at room temperature. The absorbance was measured at 570 nm on a spectrophotometer, and referred to a nitrite standard curve to determine the nitrate concentration in supernatants.

Cell viability assay

The MTT assay was used to measure the effects of LPS and *H. pylori* to induce death of macrophage cells (20). The RAW 264.7 and PEMs were exposed to various MOI of *H. pylori* during 24 or 48hr incubation periods. Cell viability was measured by the ability of viable cells to reduce MTT (Sigma-Aldrich) to formazan based on the ability of living cells to utilize Thiazolyl Blue and convert it into purple formazan, which absorbs light at 570 nm and could

be analyzed spectrophotometrically. Measurement was performed in triplicate. The absorbance was measured using the BioRad spectrophotometer. The mean OD value of the content of four wells was used for assessing the cell viability expressed as percentage of control.

Transient transfection of NF-κB, iNOS and AP-1 reporter gene

RAW 264.7 cells were grown to 90% confluence in 12-well plate and was transfected NF-κB-Luc, iNOS-Luc and AP-1-Luc by using Lipofectamine 2000 (Invitrogen). After 24 hr incubation, transfection was complete, and cells were incubated with or without LPS and then infected with *H. pylori* strain for 24 hr. 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 in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector obtained from Promega (Madison, MA).

Immunofluorescence labeling of phosphorylated p65 translocation

To visualize the *H. pylori* inhibits phosphorylated p65 translocated into epithelial cells, RAW cells were seeded onto cover-slips for 2hr and treated with or without LPS and *H. pylori*

for a further 1hr at 37°C. Cells were washed and then fixed in 3.7% (wt/vol) paraformaldehyde for at least one hour at 4°C and permeabilized in PBS contained 0.5% (vol/vol) Triton X-100 for 2 minutes. To label p65, cell preparations were then incubated for 30 minutes with NF-κB p65 (H-286) rabbit polyclonal antibody (Santa Cruz Biotechnology), and DAPI (4',6-diamidino-2-phenylindole). The secondary antibody was fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Chemicon). Then, cells were fixed in paraformaldehyde. Preparations were mounted and observed with a confocal laser scanning microscope (Zeiss LSM 510).

Statistical analysis

Student's *t* test was used to calculate the statistical significance of the experimental results for two groups; a *P* value of <0.05 was considered significant.
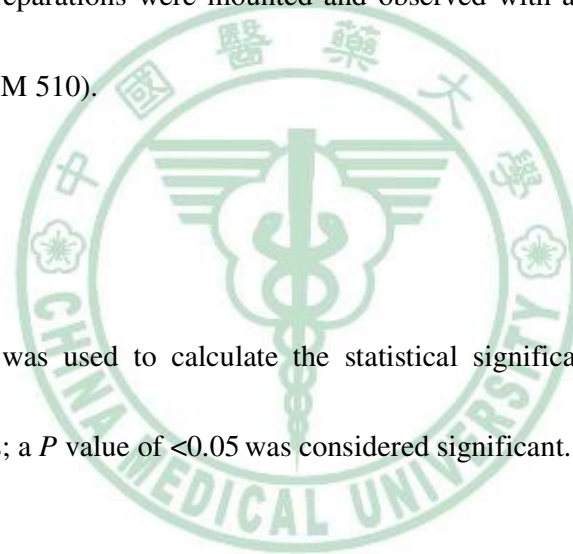
Results

1. *H. pylori* inhibits LPS-mediated nitric oxide production in macrophages

To assess whether *H. pylori* can inhibit LPS-induced nitric oxide production in macrophages, mouse macrophage RAW 264.7 cells were cultured with LPS (2μg/ml) and infected with *H. pylori* at MOI of 0-200 for 24 hr. As shown in **Figure 1A**, nitric oxide production was in an un-effective level when cells were treated with LPS and infected with *H. pylori* at low MOI (0-50). However, NO production in LPS-induced RAW 264.7 cells was suppressed at higher MOI of *H. pylori* infection. When cells were infected with *H. pylori* at MOI of 100, the LPS-induced NO production was reduced near 50%. The NO-production was diminished to basal line which without LPS-induced level after infection with *H. pylori* at MOI of 150 to 200 (**Figure 1A**). To further determine the effects of *H. pylori* suppresses LPS-induced NO production in murine primary macrophage, peritoneal excluded macrophages (PEMs) were prepared as described in material and methods and followed by co-incubated with LPS and *H. pylori* for a further 48 hr. The result of *ex vivo* study showed that while LPS treatment effectively induced NO production in uninfected cells (MOI=0). When PEMs were infected with *H. pylori* at MOI of 10 to 100, the LPS-induced NO production were reduced gradually **(Figure 1B)**. Thus, these results demonstrated that not only *H. pylori* inhibits LPS-induced NO production in a dose-dependent manner in RAW 264.7 cells, but have the same effects on murine primary peritoneal excluded macrophages.

2. Live *H. pylori* is essential for inhibition of LPS-mediated nitric oxide production

To determine the role of functional *H. pylori* can inhibit LPS-induced NO production in RAW 264.7 cells, we analyzed the effects of lived, killed bacteria, and bacterial crude extracts. The results showed that neither heat killed *H. pylori* nor *H. pylori* crude extracts inhibited LPS-induced NO production, in contrast to both live bacteria and water extract **(Figure 2A)**. We also used murine primary macrophage to investigate the effects of *H. pylori* in the inhibition of LPS-stimulated NO production. In consistent with RAW 264.7 cells, the results also showed that live bacteria from *H. pylori* have the effects on inhibition of LPS-induced NO production in PEMs **(Figure 2B)**. Thus, these data suggested that *H. pylori* can inhibit LPS-induced macrophage NO production in a manner that is dependent on a functional live bacteria.

To mimic bacteria infection in a NO-containing condition, we tested whether *H. pylori*-inhibited LPS-induced NO production would enhance the survival of bacteria in a liquid culture environment. The experimental design was described in materials and methods. As shown in **Figure 2C**, our data showed that the higher MOI of *H. pylori* infection of PEMs, the lower anti-microbial activity was found. These data reveal that *H. pylori* have their potential on inhibition of NO production and thus reduced antimicrobial activity, subsequently enhance *H. pylori* survival after countered macrophage attack.

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

To investigate the effects of *H. pylori* on the cytosolic protein levels of iNOS, PEMs were treated with LPS or LPS plus various MOI of *H. pylori* for 48hr and the protein levels of iNOS were analyzed by Western blot. The results showed that infection with *H. pylori* led to a significant decrease in LPS-induced expression of iNOS in a MOI-dependent manner (**Figure 3A**). When cells were infected with *H. pylori* at a MOI of 100, the decrease of LPS-induced iNOS expression approached to 80%. We further detected the effects of *H. pylori* on LPS-induced mRNA expression of iNOS using semi-quantitative RT-PCR analysis. Total RNA were extracted from PEMs after co-cultured with or without LPS and various MOI of *H. pylori* for 48hr. Our results showed that mRNA expression of iNOS was also reduced by *H. pylori* infection at a MOI of 100 (**Figure 3B**). To directly determine LPS-stimulated iNOS promoter activity was suppressed after *H. pylori* infection, RAW 264.7 macrophages were transiently transfected with p-iNOS-luciferase as an indicator of iNOS promoter activation. The result showed that LPS-induced macrophage iNOS luciferase activity was suppressed with *H. pylori* infection (**Figure 3C**). Thus, these data suggest that *H. pylori* may inhibit iNOS promoter activity and subsequently influence NO production in macrophages.

4. Involvement of ERK1/2 and MAPK-signaling pathways in *H. pylori* inhibits LPS-mediated macrophage NO production

As LPS-induced NO production in macrophages has been shown several signaling pathways, including mitogen-activated protein kinase (MAPK), JNK, p42 and p44 (ERK 1/2). We performed Western blot analysis to elucidate the signal transduction mechanisms involved in *H. pylori*-inhibited LPS-induced NO production in PEMs from 0 to 60 min. As shown in **figure 4A**, the phophorylation of p38 induced by LPS was reduced within 0-5 min after *H. pylori* infection. The other signaling pathway, phosphorylation of p42 and p44 (p-ERK1/2), was also inhibited by infection with *H. pylori* during 0-5 min. In contrast to p38 and ERK1/2, phophorylation of JNK1/2 in LPS-stimulated macrophage was not influenced by *H. pylori* infection until 60 min. We further confirmed the inhibition of phosphorylation of p38 and ERK1/2 in LPS-induced macrophages after *H. pylori* infection at MOI of 0 and 100 within 5 min. The data showed that both phosphorylation of p38 and ERK1/2 were decreased when infection with *H. pylori* (**Figure 4B**). These results supported that *H. pylori*-inhibited LPS-stimulated NO production and iNOS expression in macrophages was regulated by p38 and ERK1/2 signaling pathway, while JNK1/2 was not involved in those effects.

5. Suppression of NF-κB activation in *H. pylori* inhibits LPS-mediated macrophage NO production

Consideration of the inhibition of iNOS transcription, we next investigated the effects of *H. pylori* on two essential transcription factors, NF-κB and AP-1, which have been demonstrated to play an essential role in the iNOS protein expression. We then examined the effects of *H. pylori* on transcription factor, NF-κB. The activity of NF-κB was determined by the luciferase assay in RAW 264.7 macrophages transfected with κB-lucifease plasmid. As shown in **figure 5A**, LPS-stimulated a significant activation of NF-κB promoter activity which was inhibited by infection of *H. pylori*. We further examined p65 localization, which showed that p65 was primarily located in the cytosol before LPS-treatment. In response to LPS for 2 hr, the p65 protein was translocated in nuclei. However, the p65 protein was even major in the cytosol when co-cultured with LPS and *H. pylori*. Another transcription factors AP-1 was also analyzed. In parallel, reporter assay driven by AP-1 transactivation in RAW 264.7 macrophages was also revealed the effect of *H. pylori* in inhibition of AP-1. These data suggested that *H. pylori* triggered not only the distribution of p65 but AP-1 transactivation in LPS-stimulated NO production in macrophage.

To evaluate LPS-indcued iNOS promoter activity, the cells were co-treated with p38 ODN (oligonucleotide dominate negative), ERK ODN, IKK α/β ODN or scramble ODN. After transfection, incubated the cells with LPS (2μg/ml) and *H. pylori* (MOI=100) for 24 hr.

The data showed even the cells mutant with P38, ERK or IKK α/β, the iNOS activity could be activated by LPS but not by *H. pylori*. And *H. pylori* had a significant inhibition of LPS-induced iNOS activity (data not show).

Discussion

Expression of iNOS and production of NO is well known to be an important effector molecule in macrophage for responses to bacteria due to antimicrobial activity (21). It has been reported that *H. pylori* can induce iNOS and NO production in macrophages (10, 12, 22). *In vitro* study also revealed that *H. pylori* are killed in an NO-dependent manner (22). However, this response maybe ineffective, as *H. pylori* generally persistent infects for the life of host and results in clinical outcomes. In this study we used a mouse macrophage model system to demonstrate that infection of *H. pylori* can attenuate LPS-induced macrophage expression of iNOS and suppress the production of NO. Our data also revealed that bacteria survived in the environment of co-cultured with *H. pylori* and LPS-induced macrophage in a trans-well model system. This finding was consistent with previous study that *H. pylori* arginase suppresses NO production and leading to immune evasion (13). In addition, *H. pylori* can also activate innate immune responses and induces apoptosis both in macrophage (23) as well as T cells (24). In the discovery of animal infection model, they found that macrophages are the mediators of gastritis of acute *H. pylori* infection in mice (25). Another report indicated that the ineffectiveness of NO-mediated antimicrobial activity maybe absence of local high NO concentration at the site of bacteria infection (26). In order to produce higher level of NO, we used LPS at the higher concentration of 2μg/ml to elevate NO production in macrophage. After treatment of LPS in RAW 264.7 cells for 24 hr or mouse PEMs for 48 hr,

NO concentration was approached to ca. 20μM and 120μM, respectively. However, in the infection model system, co-cultured with *H. pylori* and LPS-induced macrophage still showed that the bacteria has the ability to inhibit NO production at higher bacterial load and resulted in bacteria survived (**Figure 1 and 2C**). Thus, these results reveal that *H. pylori* has a delicate mechanism to regulate the activation of macrophage and suppression of NO production.

In our current study, using Western blot and RT-PCR analysis revealed that *H. pylori* can inhibit iNOS expression in LPS-induced macrophage at the transcriptional and post-transcriptional levels. Accordingly, the effectiveness of inhibitory activity was correlated with increasing of bacterial load (**Figure 3**). The expression of iNOS mRNA was reduced in LPS-induced macrophages infected with *H. pylori* at a MOI of 100. However, *H. pylori* also stimulated macrophage iNOS mRNA expression in the absence of LPS-induced condition. This may be the effectiveness of iNOS mRNA expression in *H. pylori* infection with macrophage for a long period (10).

In this study, *H. pylori* suppression of NO production in LPS-induced macrophage was found that dependent on live bacteria (**Figure 2A and 2B**). Heat-killed and crude extract of *H. pylori* showed absence of LPS-induced NO production, this result was in consistent with previous finding (13), suggesting that this effect is mediated through direct interaction of *H. pylori* and macrophage. Although *H. pylori* has been considered as an extracelluar pathogen, several study revealed that this bacteria has the ability to invade into gastric epithelial cells

(27, 28). In addition, *H. pylori* was found to stimulate megasome formation and leading to delay phagocytosis in macrophage (15). Moreover, a recent report showed that using transmission electron microscopy visualized that *H. pylori* was directly contacted with immune cells of lamina propria (29). Collectively, these findings may explain that the live *H. pylori* have the ability to invade into macrophage and presumably induction of ineffectiveness of immune cells.

There were many research in nitric oxide inhibition, because of nitric oxide over production may cause shocks even lead to death (30, 31). It has well known that LPS stimulate iNOS gene expression and NO production mediated positively regulation of NF-κB, which is normally bound to its inhibitor IκB. Phosphorylation of IκB by IκB kinase (IKK) results in its degradation and leading to NF-κB activation, which then removes from cytosol to the nucleus. In addition to NF-κB, LPS also enable to activate MAP kinase pathway in macrophage, including Erk-1/2, JNK-1/2, and p38 [review in (32)]. During infection, nitric oxide produced is controlled by iNOS, and it could be regulated by TLR4 pathway, included NF-κB, IκB, IKKα/β and MAPK family (22, 33-38). Some research in *Salmonella* found it has similar inhibition of nitric oxide (34, 39, 40). *Yersinia* inhibits MAPK family activation by using Yop protein family (41-49). Here we found the ability of *H. pylori* to suppress LPS-induced NO production in macrophage, this was triggered by the inhibition of p65 nuclear translocation and NF-κB activation. Our current data also demonstrated that

phosphorylation of p38 and Erk1/2 was attenuated by *H. pylori* infection, while JNK-1/2 was

not involved in such interaction. This phenomenon might contribute to the inhibition of p65

phosphorylation and NF-κB activation, subsequently suppress both iNOS expression and NO

production. Our result suggested that the upstream cellular target of *H. pylori* is more directly

interacted with p38 and Erk1/2 but not JNK-1/2.

A study in Drosophila model improve that nitric oxide production contributes to the

induction of innate immune responses to gram-negative bacteria (50). The importance of

iNOS in gastric epithelium cells is the regulation of apoptosis in *H. pylori*-infected cells, this

reaction could prevent preneoplastic transformation (35). During pulmonary infection,

flagellin from gram-negative bacteria is related to the immune response which was activated

by toll like receptor (TLR) 5 (51). There were many studies suggest that lipopolysaccharide

(LPS) from gram-negative bacterium could induce nitric oxide production and iNOS

expression of macrophage. Such reactions could be considered as inflammatory responses as

well as through TLR 4 pathway (52, 53).

H. pylori is a gram-negative bacterium, also there were LPS on its' outer membrane.

Some reports have been discovered that LPS from *H. pylori* could active NF-κB through

TLR2 but not TLR4 (54, 55). In additional, it is thought that LPS from *H. pylori* might be an

antagonist to TLR4 (56). Previous report also suggested that inhibition of nitric oxide by *H.

pylori* infection of macrophage would enhance bacterial viability (13). When *H. pylori*

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infected with macrophage, the inflammation responses was reduced and the inflammatory regulators also down-regulated (22). In gastric epithelium cells, *H. pylori* infection could induce a chronic inflammation by inducing nitrite production (57). Both in human and animal model, nitric oxide synthase expression in epithelium were up-regulated by *H. pylori* infection, and these cause the chronic gastritis (58). In human stomach epithelium, the infection by *H. pylori* would up-regulate endothelial nitric oxide synthase (eNOS) expression and induces angiogenesis in gastric mucosa of dyspeptic patients (59). *Helicobacter pylori* infects to stomach may cause chronic gastritis, gastric ulcer, duodenal ulcer even cause cancer (60). However, *H. pylori* is a pathogen, the host innate immunity may clean the bacterium, but in fact, it could dodge the immune response then survive in tissues (61, 62). Many studies focus on the pathogenesis of *H. pylori* and they confirmed it go through type IV secretion and *cag* pathogenicity island (63-65).

Innate immunity could clean the pathogens then protect body from illness. But during *H. pylori* infection, the innate immunity has lost its' functions (66, 67). Some agreed that *H. pylori* has an immune invasion because of the cholesterol glucosylation (68). Also in other bacterium have the ability of immune evasion, some could escape from immune response by reducing nitric oxide (69). In the infection of *Staphylococcus aureus*, it secretes proteins to inhibit complement activation and neutrophil chemotaxis or that lyse neutrophils, neutralizes antimicrobial defensin peptides, and its cell surface is modified to reduce their effectiveness.

The organism can survive in phagosomes, express polysaccharides and proteins that inhibit opsonization by antibody and complement, and its' cell wall is resistant to lysozyme. Besides, *S. aureus* expresses several types of superantigen that corrupt the normal humoral immune response, resulting in anergy and immunosuppression. In contrast with *Staphylococcus epidermidis, S. epidermidis* must rely primarily on cell-surface polymers and the ability to form the biolfilm to survive in the host (70). In a early discovery, they found *H. pylori* could fuse the phagosomes into megasomes, then proliferated in the megasomes (15). Recently finding is the urease of *H. pylori* could help the organism escape form megasomes (71).

Above these discussions, we know that bacterium have some mechanisms to escape from immune system. The ability of immune evasion is important to pathogens. When pathogens dodge the attack of immune system, they have more chances to survive, proliferate and pathogenesis. *H. pylori* was already known which could survive in host tissues and induce a chronic inflammation. But the mechanisms how *H. pylori* escape from immune system still not clear.

The ability to interfere NF-κB activation is a strategy for pathogen to enhance immune evasion. Several studies have been demonstrated that pathogens can exploit the NF-κB to manipulate cellular responses (72). *Chlamydia* sp. was found to cleavage of p65 protein, subsequently suppress host inflammatory immune response (73). Another report of enteropathogenic *E. coli* was also found to trigger NF-κB activation and result in inhibit iNOS

expression (74). Our current results are consistent with previous finding that bacteria can trigger NF-κB activation, subsequently manipulate immune mediators. This general effect on host signaling may support the hypothesis that *H. pylori* enable to modulate host signaling events and protect bacteria from immune elimination.

In conclusion, in this study we demonstrate that infection with live *H. pylori* can inhibit LPS-induced iNOS gene transcription and NO production in a mouse macrophage model system. *H. pylori* inhibits LPS-induced MAP kinase pathway results in reduced NF-κB transactivation. This phenomenon including suppress antimicrobial mediators in innate immune responses of *H. pylori*. Collectively, our study reveals a new mechanism whereby *H. pylori* protects macrophage inflammation and supports the hypothesis that bacteria modulate host signaling to exert its beneficial effects.
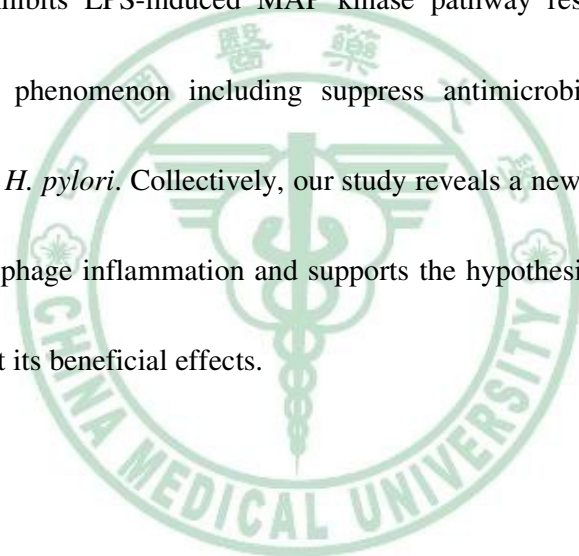
Prospection

In our results, we confirmed *H. pylori* could inhibit LPS-induced nitric oxide production. And the inhibitions of nitric oxide were through iNOS expression inhibition; the phosphorylation inhibition of ERK1/2, P38; and the down-regulation of NF-κB activation. One of our experiments, we did bacteriocied assay (Fig. 2C), the result showed us when *H. pylori* inhibits nitric oxide producing, there were more bacterium survive. This result demonstrates our contention, inhibit nitric oxide production could help more bacterium surviving.

We already know *H. pylori* inhibit nitric oxide producing to raise the survival rate of bacterium, but there still have many questions need to answer. In previously discussions, some suggest *H. pylori* is an antagonist to TLR4, but these discovers were in gastric cancer, vascular endothelial cells and human embryonic kidney cells (54-56). We would like to know how *H. pylori* interact with macrophages. In our research, we used LPS which from *E. coli* to induce the inflammatory reactions of macrophages. When cocultured with *H. pylori* could down-regulate the inflammatory reactions. If it is possible when infect with *H. pylori* could prevent the immune response activating by other bacterium infection? Also, we would like to define the mechanism pathway which is used by *H. pylori* to escape form the attack of immune system. And the interaction of *H. pylori* with other pathogens.

As we showed in introduction, *H. pylori* could cause gastric ulcer, duodenal ulcer even cause cancer. Once infection with *H. pylori*, the bacterium could survive in the host for a long life time. Commonly infection with pathogens could cause the host immune response to clean the pathogens. But in the infection of *H. pylori*, the host response is mild chronic inflammation. And which is the regulating pathway to control the chronic inflammation still unclear. We also have great interest in the *H. pylori* inducing chronic gastric inflammation. To our proposal, we hope to demonstrate the activation of inflammatory factor Interleukin-8 (IL-8) during chronic inflammation if is regulated by Src/p110β PI3K/Akt pathway.

This proposal is the beginning to my research direction in the future. After we find out the mechanism of chronic gastric inflammation, I expect I could study more deeply in how *H. pylori* could prevent the attack of immune systems then survive in host, and the mechanisms that cause the chronic gastric inflammation. In the future, I wish I could study broadly, not only in the pathogenesis of *H. pylori*, but also in other pathogens.

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Figures

A

B
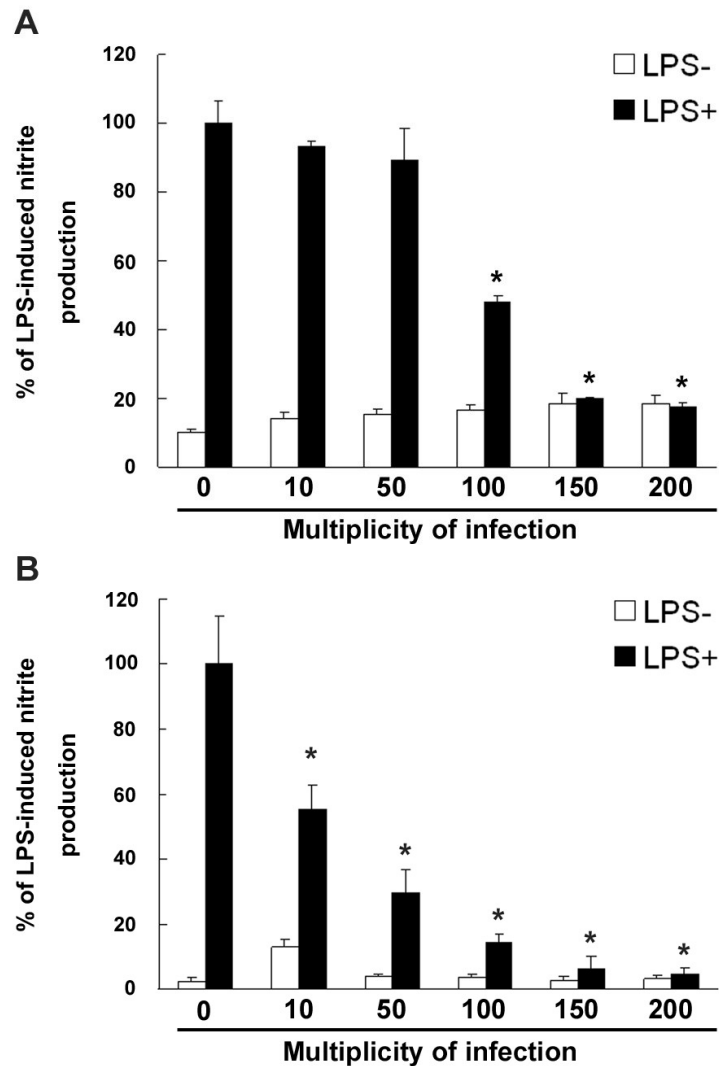
Figure 1. Inhibition by *H. pylori* of LPS-induced NO production in RAW 264.7 cell line and mouse primary peritoneal excluded macrophage. Cells were treated with or without LPS (2μg/ml) and infected with *H. pylori* at various MOI of 0 to 200. After incubation for 24 hr and 48 hr, the culture supernatant of RAW 264.7 cell line (A) and mouse primary PEMs (B) were then collected for the assay of nitric oxide production, respectively. The data are the means and standard deviations of at least three independent experiments performed in triplicate. Statistical significance was evaluated using Student's *t* test (*, $P < 0.05$).
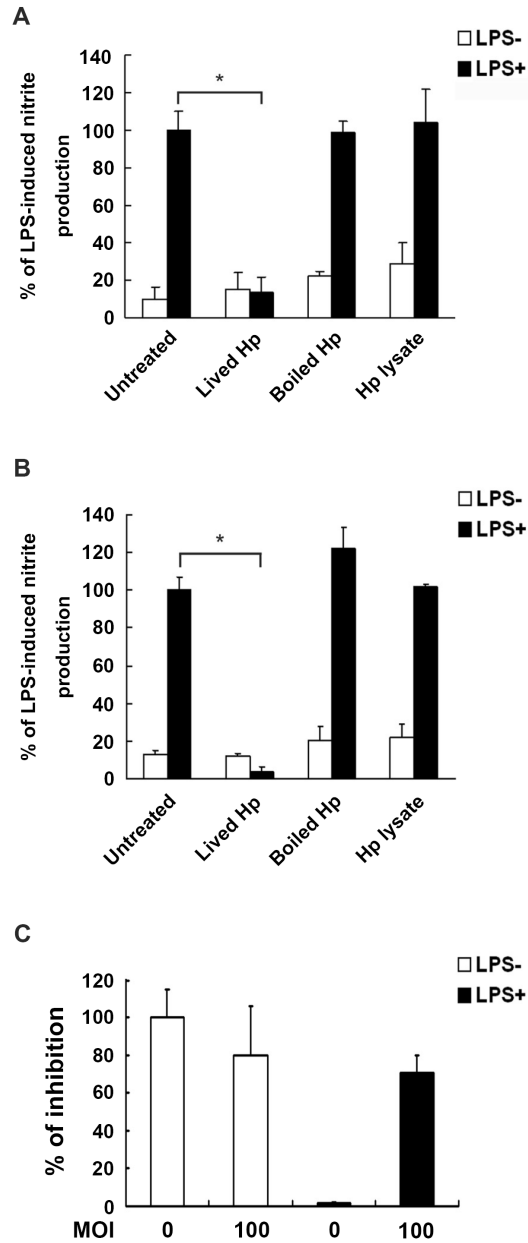
Figure 2. Live *H. pylori* is essential for inhibition of LPS-induced nitric oxide production by macrophage. Cells were treated with or without LPS (2μg/ml) and were left un-infected or infected with lived, heat-killed and crude extract derived from *H. pylori.* After 24 hr and 48 hr, the culture supernatant of RAW 264.7 cell line (A) and mouse primary PEMs (B) was then collected for the assay of nitric oxide production, respectively. (C) Mouse primary PEMs grown on Trans-well bottom layer were infected with *H. pylori* at MOI of 0 to 100 for 48 hr, and then co-incubated with *H. pylori* in the trans-well insert membrane (0.1μm) for a further 6 hr, after which, the CFUs were counted. The bactericidal activity was expressed as the means of at least three independent experiments performed in triplicate. Statistical significance was evaluated using Student's *t* test (*, $P < 0.05$).
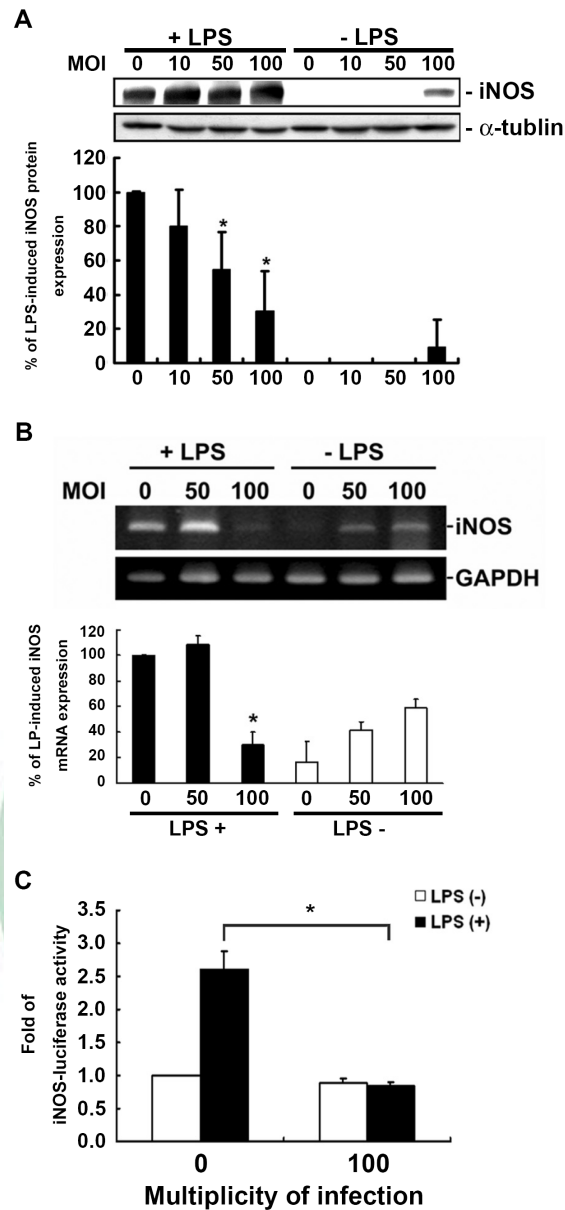
Figure 3. Inhibition by *H. pylori* of expression of protein and gene transcription for iNOS in LPS-induced macrophage activation. Mouse primary PEMs were treated with or without LPS (2μg/ml) and infected with *H. pylori* at different MOI for 48 hr. Cell lysates were prepared for the determination of iNOS in protein levels (A) and mRNA levels (B). The data of protein and mRNA expression were quantified with densitometric analysis and normalized with α–tubulin and GAPDH, respectively. (C) RAW 264.7 cells transfected with reporter gene iNOS and β-gal-lacZ (each at 1μg) were treated with or without LPS (2μg/ml) and infection with or without *H. pylori*. Luciferase activity was normalized to the transfection efficiency with β-gal-lacZ. The data was expressed as the means of at least three independent experiments performed in triplicate. Statistical significance was evaluated using Student's *t* test (*, $P < 0.05$).
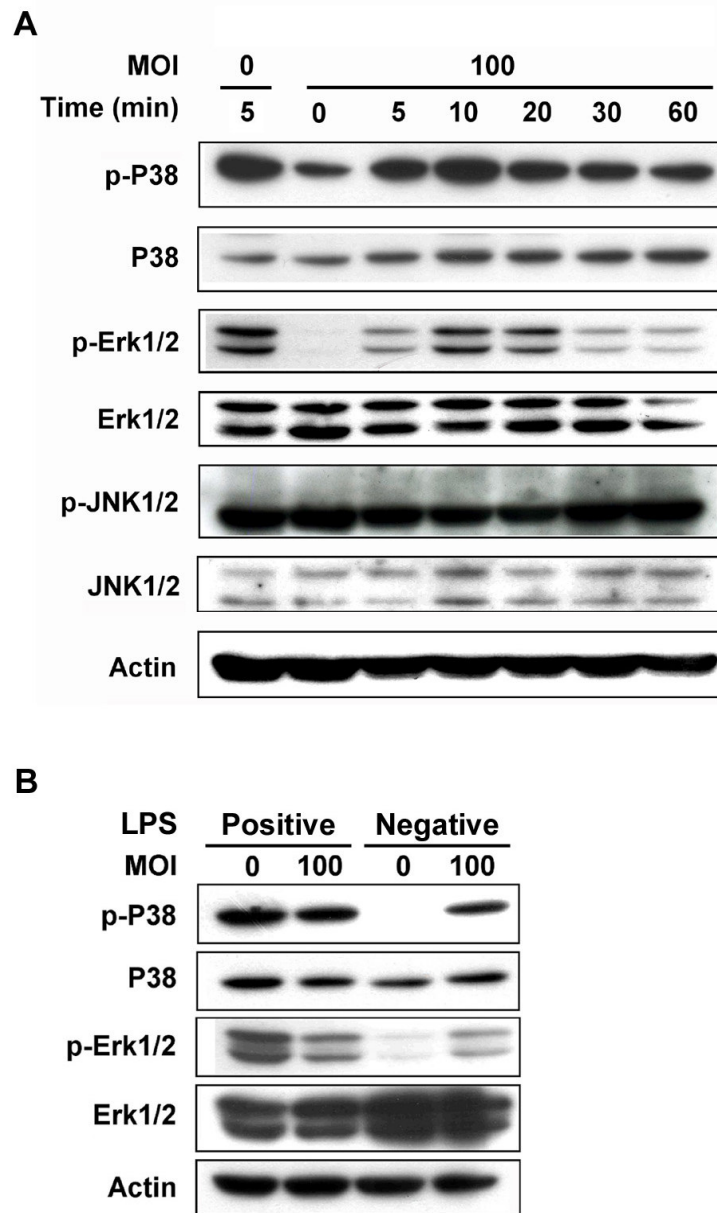
Figure 4. Signal transduction mechanisms involved in attenuation by *H. pylori* for LPS-induced macrophage activation. (A) Mouse primary PEMs were incubated with LPS (2μg/ml) and infection with or without *H. pylori* for indicated time intervals, and p-p38, p-ERK, and p-JNK expression were determined by Western blot analysis. *H. pylori* inhibited LPS-induced p38 and extracellular signal-related kinase 1/2 (ERK1/2) pathway, as evidenced by the decrease in both phosphorylated p38 and phosphorylated ERK at 0-5 min, but not phosphorylated JNK1/2. (B) Mouse primary PEMs were treated with or without LPS (2μg/ml) at MOI of 0 and 100 for 5 min, p-p38 and p-ERK expression were determined by Western blot analysis. Infection of cells with *H. pylori* at a MOI of 100 results in suppression of LPS-induced p38 and ERK1/2 phosphorylation.
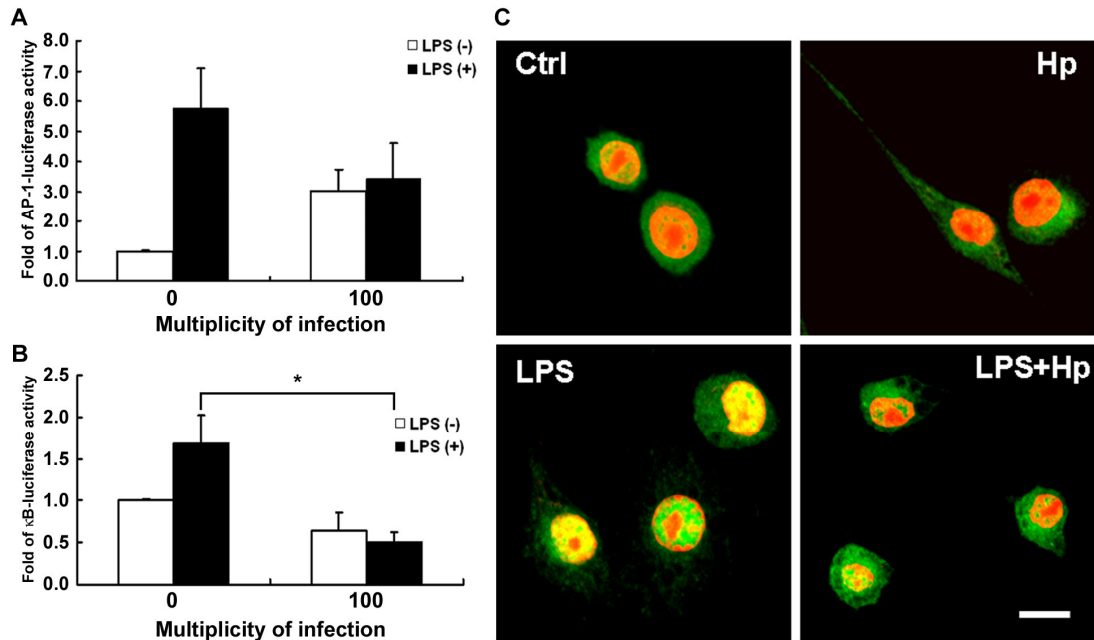
Figure 5. Inhibition of LPS-induced NF-kappaB activation by *H. pylori*. RAW 264.7 cells transfected with reporter gene AP-1 (A), NF-kappaB (B), and β-gal-lacZ (each at 1μg) were treated with or without LPS (2μg/ml) and infection with or without *H. pylori*. Luciferase activity was normalized to the transfection efficiency with β-gal-lacZ. The data was expressed as the means of at least three independent experiments performed in triplicate. Statistical significance was evaluated using Student's *t* test (*, $P < 0.05$). (C) The nuclear translocation of p65 after 1 hr treatment with or without LPS and *H. pylori* was shown by immunofluoresence, which was inhibited by infection with *H. pylori*. Scale bar = 10 μm

Appendix
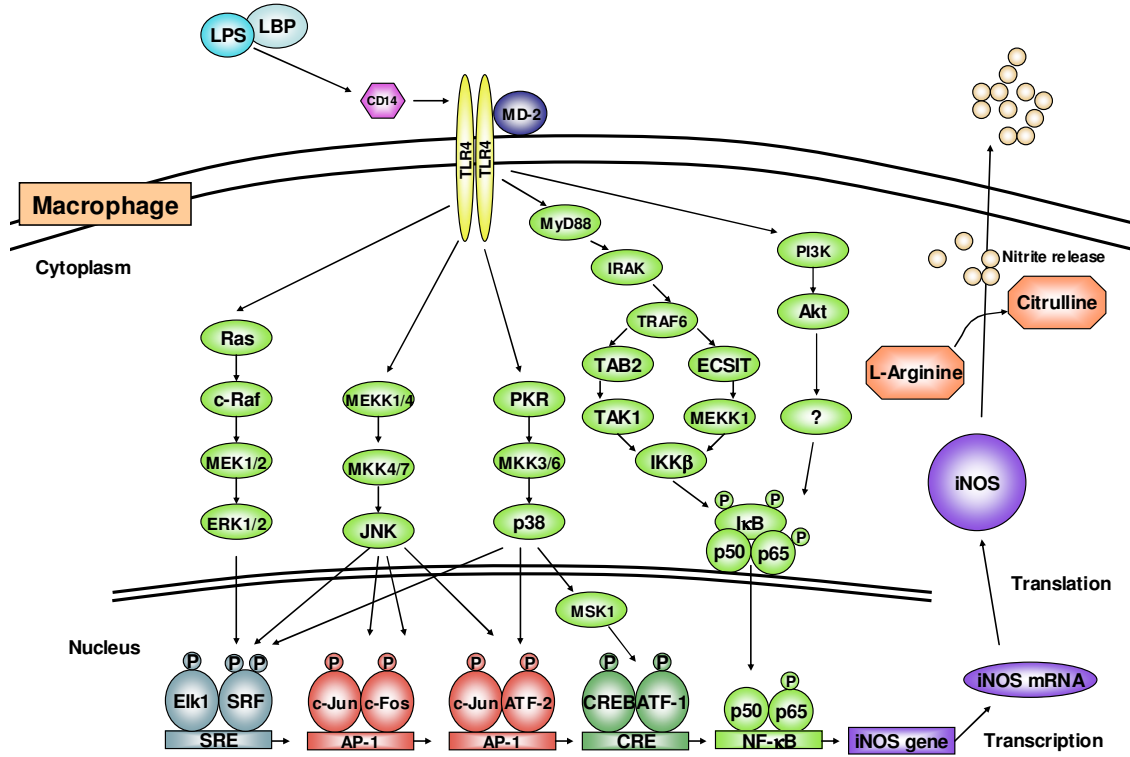
I. The activation of TLR4 and the down-stream pathway.

[This figure was adapted from the article which was published in *Cellular signaling* (2001), Mausumee G *et al*.]
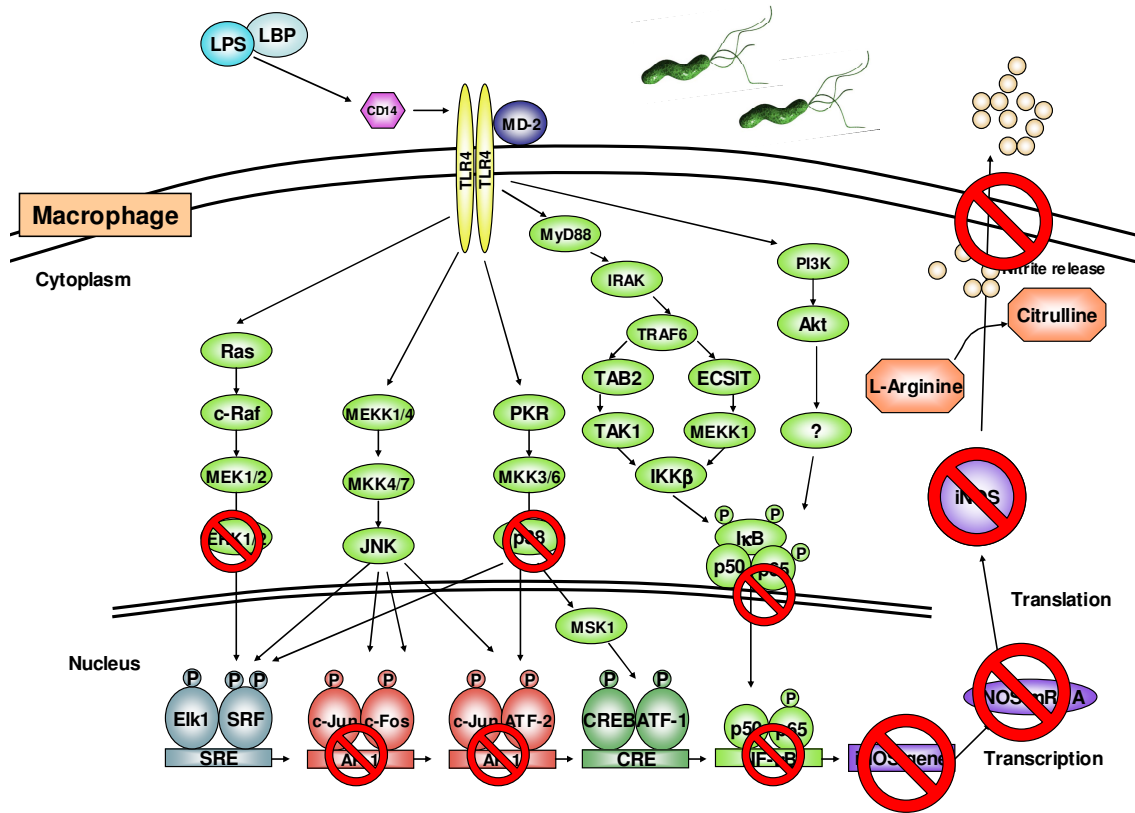
II. *Helicobacter pylori* **infect to macrophage could suppress the inflammatory factors activation.**

To our finding, *H. pylori* could inhibit the nitrite production, iNOS expression. *H. pylori* also suppressed the activation of NF-κB and AP-1. Thus, *H. pylori* could attenuate the phosphorylation of p38 and ERK1/2.