財團法人明日醫學基金會專題研究計畫申請書

一、基本資料： 申請條碼：

本申請案所需經費(單選)	■A 類(研究主持費及執行計畫所需經費) ☐B 類(研究主持費，限人文處計畫，不須填寫表 C002 及 C004 至 C009)				
計畫類別(單選)	■一般型研究計畫 ☐特約研究計畫 ☐新進人員研究計畫 ☐其他				
研究型別	■個別型計畫 ☐整合型計畫				
申請機構/系所(單位)	中國醫藥大學 醫學系				
本計畫主持人姓名	賴志河	職稱	教授	身分證號碼	
本計畫名稱 中文	自噬路徑於幽門螺旋菌誘導巨噬細胞致病之機制				
本計畫名稱 英文	Involvement of autophagic pathway in *Helicobacter pylori*-induced pathogenesis in macrophages				
整合型總計畫名稱					
整合型總計畫主持人				身分證號碼	
全程執行期限	自民國 104 年 1 月 1 日起至民國 104 年 12 月 31 日				
研究學門(請參考本申請書所附之學門專長分類表填寫)	學門代碼	名稱(如為其他類，請自行填寫學門)			
研究性質	■純基礎研究 ☐導向性基礎研究 ☐應用研究 ☐技術發展				
本計畫是否為國際合作計畫	■否； ☐是，合作國家：_____，請加填表 I001～I003				
本計畫是否申請海洋研究船	■否； ☐是，請務必填寫表 C014。				
計畫連絡人	姓名：賴志河 電話：(公) 04-22052121 ext 7729 (宅/手機) 0937-936212				
通訊地址	台中市學士路 91 號 醫學系 微生物學科				
傳真號碼	04-22053764	E-MAIL	chl@mail.cmu.edu.tw		

二、申請補助經費：

金額單位：新台幣元

執行年次 / 補助項目		第一年（101 年 01 月～101 年 12 月)	第二年（___年___月～___年___月）	第三年（___年___月～___年___月）	第四年（___年___月～___年___月）	第五年（___年___月～___年___月）
業務費		300,000				
研究人力費						
耗材、物品及雜項費用						
國際合作研究計畫國外學者來臺費用						
研究設備費						
國外差旅費						
國外或大陸地區差旅費						
出席國際學術會議差旅費						
國際合作研究計畫出國差旅費						
管理費						
合計		300,000				
貴重儀器中心使用額度						
博士後研究	國內、外地區	共_____名	共_____名	共_____名	共_____名	共_____名
	大陸地區	共_____名	共_____名	共_____名	共_____名	共_____名

申請機構或其他單位（含產業界）提供之配合項目（無配合補助項目者免填）

配合單位名稱	配合補助項目	配合補助金額	配合年次	證明文件

三、主要研究人力：

（一）請依照「主持人」、「共同主持人」、「協同研究人員」及「博士後研究」等類別之順序分別填寫。

類 別	姓名	服務機構/系所	職稱	在本研究計畫內擔任之具體工作性質、項目及範圍	*每週平均投入工作時數比率(%)
主持人	賴志河	中國醫藥大學/醫學系	教授	統籌及推動本研究計畫、參與主要實驗工作、整理文獻背景及撰寫研究成果	80%

※註：每週平均投入工作時數比率係填寫每人每週平均投入本計畫工作時數佔其每週全部工作時間之比率，以百分比表示（例如：50%即表示該研究人員每週投入本計畫研究工作之時數佔其每週全部工時之百分五十）。

（二）如申請博士後研究，請另填表 CIF2101 及 CIF2102(若已有人選者，請務必填註人選姓名，並將其個人資料表併同本計畫書送本會)。

五、耗材、物品及雜項費用：

（一） 凡執行研究計畫所需之耗材、物品及雜項費用，均可填入本表內。

（二） 說明欄請就該項目之規格、用途等相關資料詳細填寫，以利審查。

（三） 若申請單位有配合款，請於備註欄註明。

（四） 請分年列述。

金額單位：新台幣元

項 目 名 稱	說明	單位	數量	單價	金額	備註
消耗性器材	細胞培養耗材：胎牛血清、無菌培養皿、無菌吸管、培養基、液態氮、CO_2 氣體及離心管等	年	1	80,000	80,000	
消耗性器材	微生物培養耗材：綿羊血、無菌吸管、培養基、培養皿、洋菜膠、棉棒等	年	1	45,000	45,000	
消耗性器材	ELISA assay kit、Reporter assay kit	年	1	42,000	42,000	
消耗性器材	蛋白質電泳試劑與耗材：電泳配製試劑、protein marker、PVDF membrane 及濾紙等	年	1	35,000	35,000	
消耗性器材	分子生物學實驗藥品與耗材：限制酶、聚合酶、電泳膠、緩衝液及 DNA marker 等	批	1	32,000	32,000	
消耗性器材	塑膠瓶、吸管、玻璃瓶、及血清瓶等耗材	年	1	22,000	22,000	

消耗性器材	清潔劑、酒精、有機溶劑及化學試劑等	批	1	8,000	8,000	
資訊設備費	碳粉夾、紙張、光碟片等資訊耗材	年	1	7,000	7,000	
論文投稿費	期刊雜誌投稿	篇	2	15,000	30,000	
合　　計					300,000	

Abstract

Helicobacter pylori infection is highly associated with the incidence of gastric inflammatory diseases. Previous studies have indicated *H. pylori* can survive in the macrophages with massive induction of innate immune responses. These evidences suggested that *H. pylori* have a delicate mechanism to orchestrate the host immune responses either to activate it or to evade killing. Autophagy pathway has been reported to play critical roles in disease progression, immune defense, and cancer development. However, the molecular interactions between *H. pylori* and macrophages, as well as how this bacterium triggers autophagy pathway in such regulations have not been well investigated. In this proposal, (i) a murine macrophage infection model system will be established to investigate the molecular mechanism of *H. pylori* evade innate sensing. (ii) We will functionally characterize the role of autophagy and cholesterol in *H. pylori* survived intracellularly in macrophages. (iii) We will explore the detailed interactions between *H. pylori* virulence factors hijacked cholesterol and the induction of autophagy. The results from this proposal will provide insightful understanding of how *H. pylori* manipulate autophagy pathway for regulating innate sensing and benefit the bacterial infectious strategy.

Keywords: autophagy; cholesterol; *Helicobacter pylori*; inflammation; macrophages

A. Background introduction

Infection of *H. pylori*

H. pylori can evade host immune responses by utilizing its particular strategies to manipulate immune cells in harsh environment of the stomach [1,2]. Additionally, *H. pylori* penetrate across the mucosal layer and that may enable the bacteria to survive in the gastric epithelial cells [3]. Numerous reports have focused on identification of *H. pylori* virulence factors involved in its pathogenesis and the underlying mechanisms that lead to different clinical sequelea in a specific host niche [4-6]. It is interesting that mounting evidence suggested that *H. pylori* exploits cholesterol-rich microdomains (also called lipid rafts) for their internalization of cells, as many other pathogens.

Virulence factors of *H. pylori*

H. pylori contains a set of virulence factors that enable it to survive, multiply, escape from immune surveillance, and eventually lead to persistent infection in a particular niche of host. Although gastric mucosa is well protected against other bacterial infection, *H. pylori* is highly adapted to its ecological niche. These fashions that support the colonization and persistence of *H. pylori* in the gastric mucus including polar flagella, urease, adhesins, and two major virulence factors: vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA) [7]. In addition to VacA and CagA, an important study by Wunder *et al*. who revealed that the *H. pylori* enzyme, cholesterol-α-glucosyltransferase, which is responsible for cholesterol glucosylation in macrophages and is thought to be modulated the innate immunity [1].

Formation of autophagy by *H. pylori*

Autophagy was known as a preserved cell process which functioned as recycle pathway to degrade un-functioned and unnecessary components, and re-arrange these components to supports cellular survival [8]. Upon bacterial infection, human immune system may have different mechanisms for opposing to the infection. Recent studies have indicated autophagy may function as an immune defense system to degrade invaded pathogens [9,10]. indicating the possibility of preventing *H. pylori* pathogenesis by stimulate cellular autophagy formation [11].

Lipid raft-microdomains

The major composition of lipid rafts includes cholesterol, sphingolipids, and phospholipids which interact tightly and create rigid microdomains in the cell membrane [12]. The structure of lipid rafts is known to be stabilized in the cold in non-ionic detergents such as Triton X-100 [13]. After treatment of membrane with cold Triton X-100, insoluble components including lipids and proteins remain in the composition of detergent-resistant membrane (DRM), that were considered to be in the lipid rafts [13]. Several raft usurping or disruption agents including methyl-β-cyclodextrin (MβCD), filipin, lovastatin, and nystatin have been extensively employed

in the investigation of their particular functions and compositions [14]. After depletion of membrane cholesterol by MβCD or filipin, the raft-associated proteins and lipids can be dissociated and rendered the structure becoming nonfunctional [15].

Involvement of cholesterol in microbial infection

Lipid rafts are not only a dynamic structure on cell membrane, but also provide an amplified signaling for the activation of the cells [16]. Several studies have demonstrated that lipid rafts might serve as platforms for entry portals of pathogens, including bacteria [17-20] and viruses [21-23]. There are two possible benefits for pathogens entry through rafts: one is prevention of intracellular degradation, and the other is triggering of signaling that causes membrane fission and cytoskeleton rearrangement, which are both required for infection of bacteria [24]. Therefore, suggesting that pathogens might be favor to interact with lipid rafts where provide potential gateways to enter host cells.

***H. pylori* hijacks cholesterol for their benefits**

VacA was the first toxin that reported to utilize raft-microdomains for its assembly on the cell membrane and intracellular delivery [25]. Several studies revealed that depletion of membrane cholesterol significantly reduces the entry of VacA into target cells [25-27]. Our previous study had been found that GPI-anchor protein, fasciclin I, was required for internalization of VacA, but did not affect the binding of VacA to lipid rafts [27]. It has also demonstrated that receptor-dependent translocation of VacA to lipid rafts is critical for signaling pathways leading to p38 MAP kinase/ATF-2 activation and vacuolation [28]. We further showed that depletion of cholesterol and mutation of VacA significantly reduced *H. pylori* internalization in gastric epithelial cells [19]. Disruption of lipid rafts attenuates CagA translocation, hummingbird phenotype, and IL-8 secretion, suggesting that the delivery of CagA into epithelial cells is mediated through a cholesterol-dependent manner [19]. Moreover, the CagA C-terminal domain-containing EPIYA regions directly targeted to lipid rafts of gastric epithelial cells was further determined by our recent study [29]. Wunder *et al.* have been showed that *H. pylori* follow a cholesterol gradient and extracts the lipid from cytoplasmic membranes of epithelial cells for subsequent glucosylation [1]. These evidences suggested that *H. pylori* harbor a delicate mechanism for the orchestration between activating macrophages and protecting the bacteria from immune attack. However, the association of *H. pylori* exploits lipid rafts and triggers autophagy, as well as how this bacterium inhibits innate immunity in such interactions have not yet been studied extensively (Fig. 1).

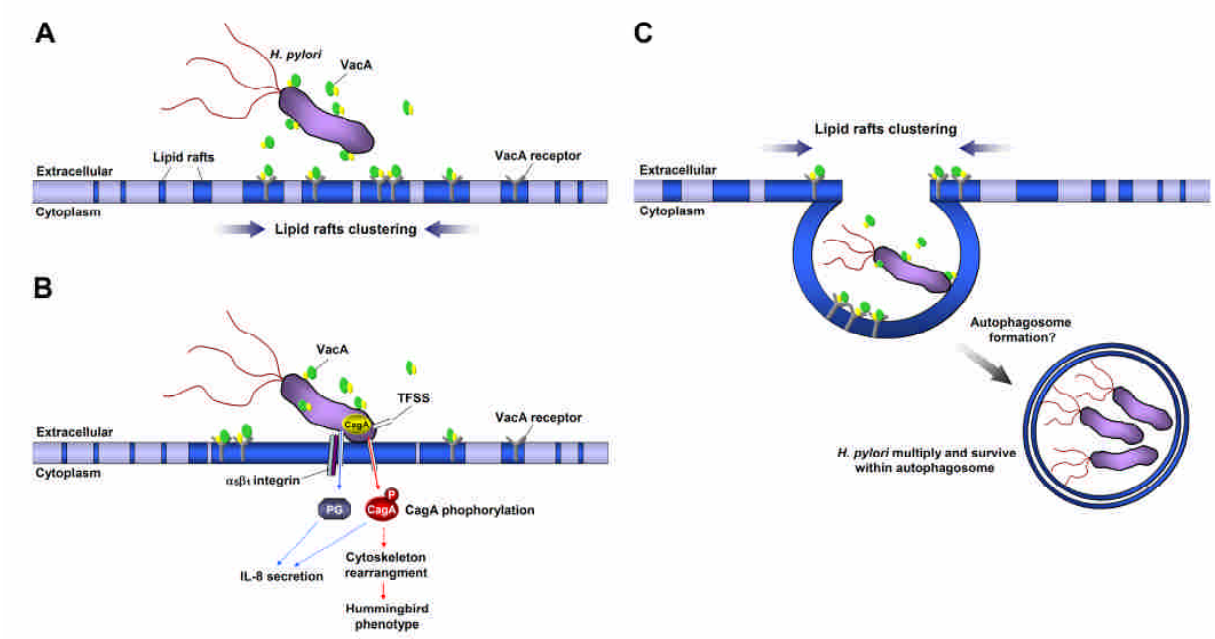

Fig. 1. Model depicting *H. pylori* manipulates host cholesterol as its infectious strategy. (A) VacA secreted by *H. pylori* binds to receptors which are localized in the cholesterol-rich microdomains and facilitated rafts coalescence into the sites of bacteria infection. (B) The clustering rafts enhance type IV secretion system (TFSS) injects CagA and peptidoglycan (PGN) into cytoplasm and induce downstream signaling events. (C) The raft-associated membrane may extend to form autophagosomes where provide compartments for *H. pylori* survives intracellularly.

B. Specific aims

Intracellular survive of *H. pylori* has been indicated association with severe clinical diseases which were presumably inaccessible to immune cells of the host as well as antibiotics [3]. Although several studies have highlighted some critical issues of pathogen evade immune attack, the molecular interactions of *H. pylori* orchestrate activation or suppression of immune responses remain unclear. In this proposal, we propose to employ genetic, biochemical and animal studies to unveil molecular mechanisms underlying the function of cellular cholesterol, which may serve as a platform for *H. pylori* infection and evasion of innate immunity. The specific aims of this proposal are listing as below:

Aim 1. To investigate the molecular mechanism of *H. pylori* evade innate sensing.

Aim 2. To functionally characterize the role of autophagy and cholesterol in *H. pylori* survived intracellularly in macrophages.

Aim 3. To validate the interaction between *H. pylori* hijacked cholesterol and autophagy formation.

C. Preliminary results

H. pylori infection induces autophagy pathway

The results of our case-control study indicated that patients prescribed statins were associated with significantly reduced incidence of PUD. We thus established an *in vitro* assay platform to investigate the involved pathways. Following the induction of autophagy, microtubule-associated protein light chain 3 (LC3) is converted from LC3-I to LC3-II, and the expression of LC3-II is considered a marker of autophagy [30]. As shown in Fig. 2A, our results indicated that *H. pylori*-induced LC3-I/II conversion was associated with increased MOI. We selected an MOI of 100 as our infection model condition and performed a time course analysis. As shown in Fig. 2B, the increase in LC3-I/II conversion correlated with the duration of infection time. We then examined the autophagy-related proteins beclin-1 and p62, which are known to participate in the initiation of autophagy with LC3-II [31,32]. Our data indicated that after the infection of cells with *H. pylori*, beclin-1 and p62 expression was upregulated in a time-dependent manner (Fig. 2C). These results indicated that *H. pylori* infection activates the autophagy pathway in macrophages.

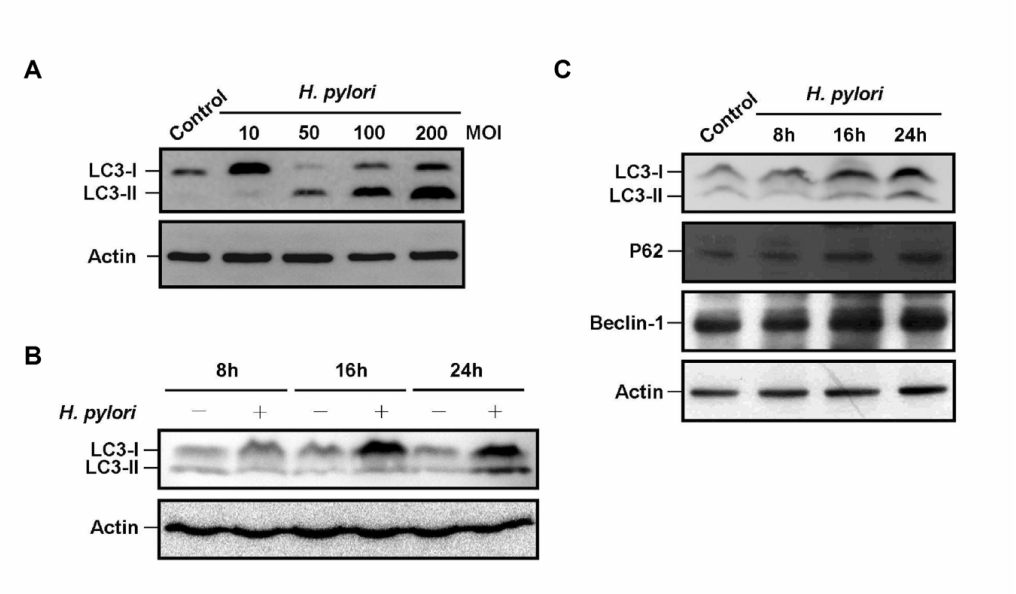

Fig. 2. *H. pylori* infection induces autophagy in macrophages. RAW264.7 cells were incubated with *H. pylori* (A) at various MOIs (0–200) for 16 h or (B) at an MOI of 100 for the indicated times. Western blot analysis was then performed to determine LC3-I/II protein expression. Upregulation of LC3-II expression indicated autophagosome formation. (C) The autophagy-related proteins p62 and beclin-1 were analyzed. Results shown are representative of one of 3 independent experiments performed.

Cholesterol depletion increases *H. pylori*-induced autophagy

We then investigated the effects of cellular cholesterol on autophagy. We treated the cells with various cholesterol-depleting/usurping agents, including MβCD (which removes cholesterol

from the cell membrane) [19], U18666A (which inhibits the biosynthesis of cholesterol in cells) [33], and simvastatin (which is an 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor) [34]. As shown in Figs. 3A and 3B, the upregulation of LC3-II expression was greater in *H. pylori*-infected cells treated with cholesterol-depleting/usurping agents than in untreated *H. pylori*-infected cells. We then analyzed the effects of simvastatin on cells by using immunofluorescence, and observed autophagosome formation by using a Cyto-ID autophagy green dye. Our result indicated that untreated cells and cells treated with simvastatin alone exhibited low-intensity Cyto-ID fluorescence. In comparison with in untreated *H. pylori*-infected cells, *H. pylori*-infected cells treated with simvastatin exhibited increased autophagy (Fig. 3C). These results indicated that simvastatin reduces cellular cholesterol, leading to accelerated *H. pylori*-induced autophagy.

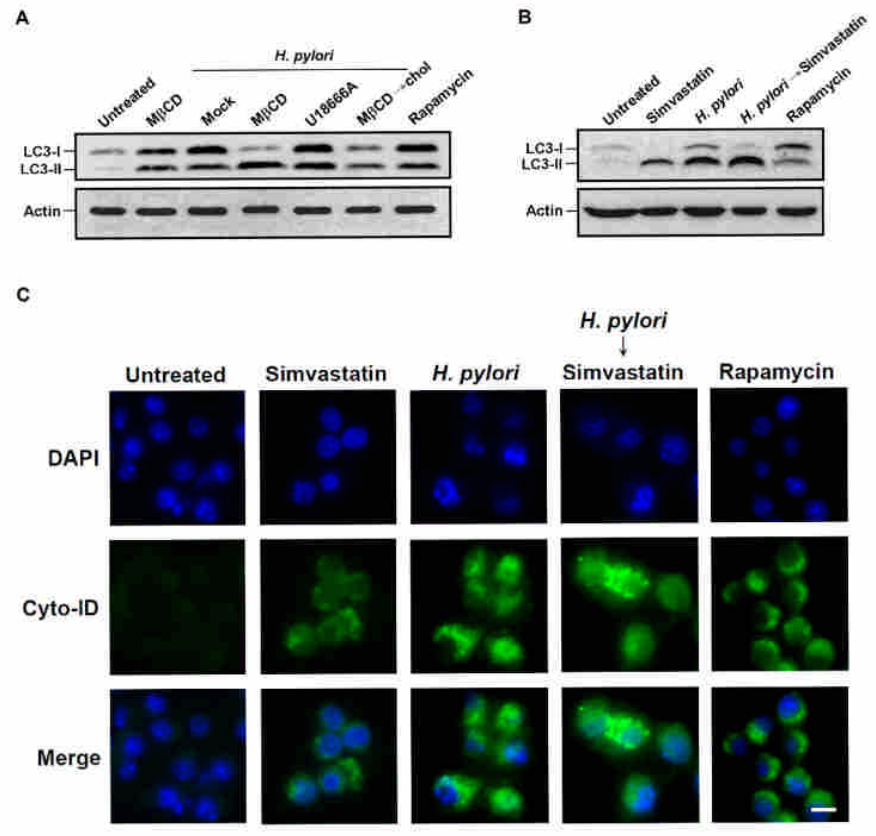

Fig. 3. Reducing cellular cholesterol increases *H. pylori*-induced autophagy. RAW264.7 cells were infected with *H. pylori* (MOI = 100) for 1 h, then treated with the assigned cholesterol depleting/usurping agents (2.5 mM MβCD, 1 μM U18666A, or 20 μM simvastatin). After 16 h of incubation, (A, B) western blot was performed to analyze LC3-I/II expression. (C) The treated cells were probed with 4',6-diamidino-2-phenylindole (DAPI, blue) to visualize nuclei or Cyto-ID (green) to visualize autophagosomes. The stained cells were then analyzed using fluorescence microscopy. Rapamycin (100 nM) was used as a positive control for autophagosome formation. Results shown are representative of one of 3 independent experiments performed. chol, cholesterol. Bar, 10 μm.

D. Materials and methods

Cell culture

AGS cells (human gastric epithelial cells) will culture in F12 (GibcoBRL, NY). Ten percent of de-complement FBS (Hyclone UT, USA) was added in all cultures. Penicillin and streptomycin (GibcoBRL) were used if needed. In bacteria internalization assay, cell culture medium were not supplemented with antibiotic reagents.

Bacterial survival assay

To assess the intracellular survival, AGS cells will be infected with *H. pylori* wild type or *cagA* mutant at an MOI of 100:1. One hour after infection, cells will be centrifuged at 350 *g* for 3 min and supernatant was discarded. Subsequently, cells will be washed twice with PBS, re-suspended in medium containing 100μg/ml gentamicin (Sigma) and seeded at 1×10^6 /ml. Cells will be lysed 1, 4 or 8 hr after infection. Diluted cell lysates will be plated on Brucella blood agar plates. Colonies will be counted after 4–5 days. Experiments will be performed at least three times in duplicates.

Flow cytometry analysis

It remains unclear, however, as to whether ceramide acts as a TLR4 signaling agonist upon *H. pylori* infection. Details of the connection between *H. pylori*-induced ceramide/TLR4 and cholesterol require further research. To this end, the determination of ceramide and TLR4 expression will be analyzed using flow cytometry. AGS cells were pretreated with imipramine (10 μM) which is an inhibitor for acid sphingomyelinase, MβCD (2.5 μM), or lovatatin (10 μg/ml) for 1 h, followed by infection with *H. pylori* at an MOI of 100 for 6 h. The cells were fixed and stained with anti-ceramide (Sigma-Aldrich) or anti-TLR4 (Santa Cruz), the fluorescence intensities were then determined by flow cytometry.

Immunofluorescence labeling and confocal microscopy

The localization of cholesterol in *H. pylori*-infected AGS cells will be visualized by confocal microscopy. AGS cells were infected with *H. pylori* (or not infected) at 37°C for 6 h. Cells were fixed and stained CTX-B, or DAPI to visualize bacteria and the cell nucleus, and then analyzed by confocal microscopy. The adhered *H. pylori* (stained with DAPI) co-localized with ceramide and TLR4 images will obtain using confocal microscopy *z*-section analysis.

Transient transfection of NF-κB reporter gene

To investigate the involvement of cholestrol and ceramide in *H. pylori*-induced IL-8 activation, the human *IL-8* promoter-Luc construct, IL-8/wt containing both AP-1 and NF-κB sites, was transfected into AGS cells. Following transfection, the cells were infected with *H. pylori* and then subjected to luciferase activity assays. Luciferase activity was normalized to

transfection efficiency, which was determined by the β-galactosidase activity generated from a co-transfected β-galactosidase expression vector (Promega).

Cytokine assay

Culture supernatants of *H. pylori*-infected cells were harvested for IL-8 secretion analysis. The concentration of IL-8 was determined by enzyme-linked immunosorbent assay (ELISA). AGS cells were pre-treated with simvastatin, lovastatin, anti-TLR4, or anti-ceramide and then infected with *H. pylori* at a MOI of 100 for 24 h. The IL-8 concentration in AGS cell culture supernatants was determined using a sandwich ELISA kit (R&D systems) according to the manufacturer's instructions [19].

Small interference RNA

To examine whether recruitment of ceramide, TLR4 and cholesterol integrity were crucial for *H. pylori*-induced IL-8 production, AGS cells were pretreated with simvastatin or lovastatin, which can reduce the concentration of endogenous cholesterol and thereby disrupt the rafts [35], prior to infection with *H. pylori*. AGS cells were transfected with TLR4 or control siRNA for 24 h followed by infection of *H. pylori*, the *IL-8* activity and IL-8 production were analyzed using luciferase activity assay and ELISA, respectively. For transfection of siRNA, TLR4 (ON-TARGET*plus* siRNA 7099) and control siRNA (sc-37007) were purchased from Thermo Fisher Scientific (Lafayette, CO) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. AGS cells were transfected with siRNAs (100 nM) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For investigation of ceramide and TLR4, AGS cells were grown to 90% confluency in 12-well plates and transfected with IL-8/wt reporter construct using Lipofectamine 2000 (Invitrogen). After 16 h, cells were infected with *H. pylori* in the absence or presence of anti-TLR4 or anti-ceramide for 6 h. To prepare total 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 (Promega) was added to all samples, and luminescence was measured using a microplate luminometer (Biotek, Winooski, VT). Luciferase activity was normalized to transfection efficiency, which was determined by the β-galactosidase activity generated from a co-transfected β-galactosidase expression vector (Promega).

Bacterial colonization assay

To confirm the observation of ceramide and TLR4 are mobilized into membrane rafts in response to *H. pylori* infection in gastric epithelial cells, a mice *in vivo* model system will be established. Male wild-type C3H/HeN and TLR4-deficient C3H/HeJ mice at ages 6 to 8 weeks 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. C3H/HeN (n = 20) and C3H/HeJ (n = 20) mice 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* 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, 20 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.

Statistical analysis

For experimental data analysis, the results were expressed as mean ± SEM and student's *t*-test was used to calculate the statistical significance of the experimental results between two groups; a *p* value of <0.05 was considered significant.

E. Anticipated results

The main focus of this grant proposal is to reveal molecular basis of host factors which involvement in *H. pylori*-induced inflammatory responses. Using genetic and biochemical approaches, we expect to gain a clear view of how *H. pylori* has delicate mechanisms in regulation of immune responses. Worth mentioning, in this proposal, we will validate this question by determining *H. pylori* and autophagy interactions as well as by identification/characterization of signal molecules in a murine model system. Studies from this proposal will enhance us to understand the mechanisms of *H. pylori* regulating innate immune responses and bacterial pathogenesis in host stomachs.

We plan to sustain and develop a program of international quality science**s**, integrating experimental and theoretical means to cross-field science research. We will address pathogen-cell interactions at the molecular level. *In vivo* experiments by these newly developed techniques will be also performed for the application of studying in this proposal. The molecular mechanisms of *H. pylori* manipulates immune responses will subsequently identify in a mouse model system. Based on our previous results in *H. pylori* pathogenesis, as well as understanding of clinical outcomes in *H. pylori*-related diseases, we are confident in the execution of this proposal.

References

1. Wunder C, Churin Y, Winau F, et al. Cholesterol glucosylation promotes immune evasion by *Helicobacter pylori*. Nat Med 2006;12:1030-1038.
2. Lu DY, Tang CH, Chang CH, et al. *Helicobacter pylori* attenuates lipopolysaccharide-induced nitric oxide production by murine macrophages. Innate Immun 2012;18:406-417.
3. Necchi V, Candusso ME, Tava F, et al. Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by *Helicobacter pylori*. Gastroenterology 2007;132:1009-1023.
4. Gebert B, Fischer W, Weiss E, et al. *Helicobacter pylori* vacuolating cytotoxin inhibits T lymphocyte activation. Science 2003;301:1099-1102.
5. Ramarao N, Gray-Owen SD, Backert S, Meyer TF. *Helicobacter pylori* inhibits phagocytosis by professional phagocytes involving type IV secretion components. Mol Microbiol 2000;37:1389-1404.
6. Mahdavi J, Sonden B, Hurtig M, et al. *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. Science 2002;297:573-578.
7. Amieva MR, El-Omar EM. Host-bacterial interactions in *Helicobacter pylori* infection. Gastroenterology 2008;134:306-323.
8. Marino G, Niso-Santano M, Baehrecke EH, Kroemer G. Self-consumption: the interplay of autophagy and apoptosis. Nat Rev Mol Cell Biol 2014;15:81-94.
9. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature 2008;451:1069-1075.
10. Zhao Z, Fux B, Goodwin M, et al. Autophagosome-independent essential function for the autophagy protein Atg5 in cellular immunity to intracellular pathogens. Cell Host Microbe 2008;4:458-469.
11. Yang JC, Chien CT. A new approach for the prevention and treatment of Helicobacter pylori infection via upregulation of autophagy and downregulation of apoptosis. Autophagy 2009;5:413-414.
12. Ikonen E. Roles of lipid rafts in membrane transport. Curr Opin Cell Biol 2001;13:470-477.
13. Brown DA, London E. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J Biol Chem 2000;275:17221-17224.
14. Simons K, Toomre D. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 2000;1:31-39.
15. Simons M, Kramer EM, Macchi P, et al. Overexpression of the myelin proteolipid protein leads to accumulation of cholesterol and proteolipid protein in endosomes/lysosomes: implications for Pelizaeus-Merzbacher disease. J Cell Biol 2002;157:327-336.
16. Bini L, Pacini S, Liberatori S, et al. Extensive temporally regulated reorganization of the lipid raft proteome following T-cell antigen receptor triggering. Biochem J 2003;369:301-309.
17. Duncan MJ, Li G, Shin JS, et al. Bacterial penetration of bladder epithelium through lipid rafts. J Biol Chem 2004;279:18944-18951.
18. Kowalski MP, Pier GB. Localization of cystic fibrosis transmembrane conductance regulator to lipid rafts of epithelial cells is required for *Pseudomonas aeruginosa*-induced cellular activation. J Immunol 2004;172:418-425.
19. Lai CH, Chang YC, Du SY, et al. Cholesterol depletion reduces *Helicobacter pylori* CagA translocation and CagA-induced responses in AGS cells. Infect Immun 2008;76:3293-3303.

20. Wang M, Hajishengallis G. Lipid raft-dependent uptake, signalling and intracellular fate of *Porphyromonas gingivalis* in mouse macrophages. Cell Microbiol 2008;10:2029-2042.
21. Favoreel HW, Mettenleiter TC, Nauwynck HJ. Copatching and lipid raft association of different viral glycoproteins expressed on the surfaces of pseudorabies virus-infected cells. J Virol 2004;78:5279-5287.
22. Chung CS, Huang CY, Chang W. Vaccinia virus penetration requires cholesterol and results in specific viral envelope proteins associated with lipid rafts. J Virol 2005;79:1623-1634.
23. Bremer CM, Bung C, Kott N, et al. Hepatitis B virus infection is dependent on cholesterol in the viral envelope. Cell Microbiol 2009;11:249-260.
24. Lafont F, van der Goot FG. Bacterial invasion via lipid rafts. Cell Microbiol 2005;7:613-620.
25. Ricci V, Galmiche A, Doye A, et al. High cell sensitivity to *Helicobacter pylori* VacA toxin depends on a GPI-anchored protein and is not blocked by inhibition of the clathrin-mediated pathway of endocytosis. Mol Biol Cell 2000;11:3897-3909.
26. Schraw W, Li Y, McClain MS, et al. Association of *Helicobacter pylori* vacuolating toxin (VacA) with lipid rafts. J Biol Chem 2002;277:34642-34650.
27. Kuo CH, Wang WC. Binding and internalization of *Helicobacter pylori* VacA via cellular lipid rafts in epithelial cells. Biochem Biophys Res Commun 2003;303:640-644.
28. Nakayama M, Hisatsune J, Yamasaki E, et al. Clustering of *Helicobacter pylori* VacA in lipid rafts, mediated by its receptor, receptor-like protein tyrosine phosphatase beta, is required for intoxication in AZ-521 Cells. Infect Immun 2006;74:6571-6580.
29. Lai CH, Wang HJ, Chang YC, et al. *Helicobacter pylori* CagA-mediated IL-8 induction in gastric epithelial cells is cholesterol-dependent and requires the C-terminal tyrosine phosphorylation-containing domain. FEMS Microbiol Lett 2011;323:155-163.
30. Klionsky DJ, Abdalla FC, Abeliovich H, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 2012;8:445-544.
31. Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ 2011;18:571-580.
32. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. Nature 2011;469:323-335.
33. Poh MK, Shui G, Xie X, et al. U18666A, an intra-cellular cholesterol transport inhibitor, inhibits dengue virus entry and replication. Antiviral Res 2012;93:191-198.
34. Lin H, Xiao Y, Chen G, et al. HMG-CoA reductase inhibitor simvastatin suppresses Toll-like receptor 2 ligand-induced activation of nuclear factor kappa B by preventing RhoA activation in monocytes from rheumatoid arthritis patients. Rheumatol Int 2011;31:1451-1458.
35. Endo A. 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors. Methods Enzymol 1981;72:684-689.