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	副教授	身分證號碼	
本計畫名稱 中文	細胞膽固醇與醣化磷脂的調控幽門螺旋桿菌感染巨噬細胞的分子機制				
本計畫名稱 英文	Manipulation of cellular cholesterol and glycosphingolipids by Helicobacter pylori benefits their intracellular survival 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 103 年 1 月 1 日起至民國 103 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)			
	BI				
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。				
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二、申請補助經費：

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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						
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國際合作研究計畫國外學者來臺費用						
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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

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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	副教授	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	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	60,000	60,000	
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	65,000	65,000	
消耗性器材	ELISA assay kit、Reporter assay kit	年	1	32,000	32,000	
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	45,000	45,000	
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	42,000	42,000	
消耗性器材	塑膠瓶、吸管、玻璃瓶、及血清瓶等耗材	年	1	12,000	12,000	

消耗性器材	清潔劑、酒精、有機溶劑及化學試劑等	批	1	10,000	10,000	
資訊設備費	碳粉夾、紙張、光碟片等資訊耗材	年	1	5,000	5,000	
論文投稿費	期刊雜誌投稿	篇	2	15,000	30,000	
合　　計					300,000	

Abstract

Persistent infection of *Helicobacter pylori* in the gastric mucosa results in release of several chemokines which attract neutrophile infiltration, macrophage activation, and causes chronic gastritis. Accumulated studies indicate *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. Nevertheless, the molecular interactions between *H. pylori* and macrophages, as well as how this bacterium inhibits innate immunity in such regulations have not been extensively studied. In the first year of the proposal, a murine macrophage infection model system will be established to investigate the molecular mechanism of *H. pylori* evade innate sensing. In the second year, we will functionally characterize the role of cholesterol and the involvement of Toll-like receptor 4 (TLR4)/glycosphingolipids in *H. pylori* survived intracellularly in macrophages. In the third year, we will explore the detailed interactions between *H. pylori* virulence factors hijacked cholesterol and the induction of autophagy. Studies pertaining to this proposal will provide insightful understanding of how *H. pylori* manipulate cellular cholesterol for regulating innate sensing and benefit the bacterial infectious strategy.

Keywords: *Helicobacter pylori*, cholesterol, glycosphingolipids, autophagy, lipid rafts

A. OVERVIEW OF SPECIFIC AIMS

H. pylori infection causes chronic gastritis which was associated with gastric inflammatory and local immune responses. However, immune system was failed to eliminate *H. pylori* from gastric mucosa completely. Persistent *H. pylori* infection in human gastric mucosa induces gastritis and leads to the progression of several types of gastrointestinal diseases, including duodenal and gastric ulcers, and gastric cancer or lymphoma (27). The bacteria may select a safe niche on mucosa where they can reside and evade the immune system as well as the antibiotics. One mechanism by which *H. pylori* escapes immune surveillance is by assimilating and modifying cellular cholesterol (38), an important component of lipid rafts, which are dynamic microdomains in the exoplasmic leaflet of lipid bilayer membranes (4). Of interest, translocation of cytotoxin-associated gene A (CagA), as well as delivery of vacuolating cytotoxin (VacA) into host cells following *H. pylori* infection are also required lipid rafts (19,20,25).

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 (26). 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 are listing as below:

1. To investigate the molecular mechanism of *H. pylori* evade innate sensing.
2. To functionally characterize the role of cholesterol in *H. pylori* survived intracellularly in macrophages.
3. To validate the interaction between *H. pylori* hijacked cholesterol and autophagy formation.

B. BACKGROUND AND SIGNIFICANCE

Infection of H. pylori and multiply intracellularly

H. pylori can evade host immune responses by utilizing its particular strategies to manipulate immune cells in harsh environment of the stomach (22,38). Additionally, *H. pylori* penetrate across the mucosal layer and that may enable the bacteria to survive in the gastric epithelial cells (26). 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 (11,23,28). 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.

Cholesterol-rich microdomains

Lipid rafts are not only a dynamic structure on cell membrane, but also provide an amplified signaling for the activation of the cells (2). Several studies have demonstrated that lipid rafts might serve as platforms for entry portals of pathogens, including bacteria (8,16,19,35) and viruses (3,6,9). 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 (18). Therefore, suggesting that pathogens might be favor to interact with lipid rafts where provide potential gateways to enter host cells.

One of the most famous examples of lipid rafts as a bacterial entry gateway is *Shigella flexneri*. Upon *S. flexneri* attach to host cells, the type III secretion system (TTSS) is activated and the bacterial effctors are directly injected into cytoplasm (31). The machinery of bacterial effectors delivery into host cells is dependent on the interaction of cholesterol with TTSS (14). Similar to *Shigella*, *Salmonella enteritica* employed a TTSS to invade into host cells. Likewise, cholesterol is not only required for *Salmonella* entry but also provide cholesterol-rich vacuoles for bacterial multiply and survives inside the cells (10). Type 1 fimbriated *E. coli* is also found associated with caveolae and raft-components that may facilitate bacterial internalization and enable bacteria to survive intracellularlly (8). Additionally, *Pseudomonas aeruginosa* capitalize on an amplified host inflammatory response by co-opting raft-associated signaling (12).

H. pylori hijacks cellular 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 (29). Several studies revealed that depletion of membrane cholesterol significantly reduces the entry of VacA into target cells (17,29,30). Our collaboration Professor Wen-Ching Wang (National Tsing Hua University) 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 (17). 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 (25). My collaborator Dr. Chih-Ho Lai (China Medical University) and his colleagues further showed that depletion of cholesterol and mutation of VacA significantly reduced *H. pylori* internalization in gastric epithelial cells (19). The authors then demonstrated that 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). Recently, Murata-Kamiya *et al.* reported that the initial contact of *H. pylori* with cells induced the phosphotidylserine externalization from inner leaflet to outer leaflet of cell membrane, thus facilitated the translocation of CagA into cytoplasm (24). 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 (20).

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 (38). Subsequently, this group identified the gene HP0421 (*capJ*) as encoding the enzyme cholesterol-α-glucosyltransferase responsible for cholesterol glucosylation (21). My collaborator Dr. Wen-Ching Wang recently demonstrated that the glycosylated cholesterols synthesized by *H. pylori* partition in cholesterol-rich microdomains around host-pathogen contact sites followed by alters membrane architecture (34). **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**

C. RESEARCH DESIGN AND METHODS

Aim 1. To demonstrate the involvement of TLR4 and glycosphingolipids in *H. pylori* reside in autophagosomes.

***Approach 1*. Validation of *H. pylori* regulates innate sensing using *in vivo* animal model.** To investigate the role of TLR4 in regulation of innate sensing and 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 Figure 1, *H. pylori* were 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 LPS-administered C3H/HeJ mice. Our preliminary results indicated that LPS enhanced *in vivo* anti-*H. pylori* activity in LPS-responsive mice (C3H/HeN). Further study regarding the role of TLR4 in controlling bacterial survived will be carried out by using murine primary macrophages isolated from C3H/HeN or C3H/HeJ mice.

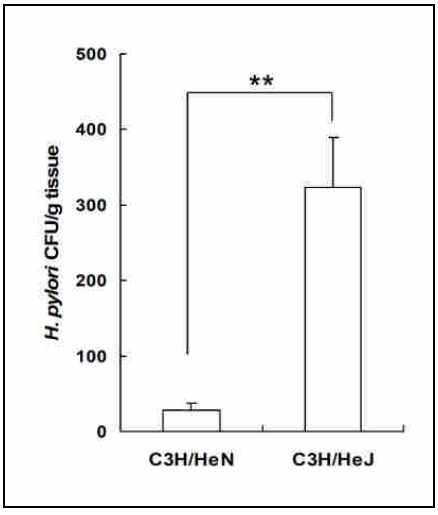

Fig. 1. LPS-dependent *in vivo* killing of gastric *H. pylori*. C3H/HeN and C3H/HeJ mice were intragastrically inoculated with *H. pylori* and purified LPS. The amounts of living *H. pylori* in the stomach were determined 7 days after infection. The data are expressed as the mean ± standard deviation (n = 6, for each group). Statistical significance was determined using the Student's *t*-test (**$P < 0.01$). CFU: colony forming units.

***Approach 2*. Investigation of *H. pylori* uptake, TLR4 mobilization, and their subcellular localization**. We then propose to directly observe whether LPS-elicited TLR4 which were co-localized in lipid rafts at sites of *H. pylori* infection by confocal microscopy. Murine

macrophages were infected with or without *H. pylori* at 37°C for 6 h. Cells were fixed and stained with anti-TLR4 (conjugated with FITC), Alexa 647–conjugated CTX-B to visualize GM1 (red), or DAPI (blue) to visualize bacteria and the cell nucleus. The stained cells were then analyzed under a confocal laser scanning microscope (LSM 510, Carl Zeiss) with a 100× objective (oil immersion, aperture 1.3). The quantification of fluorescence intensity was analyzed by ZEN software (Carl Zeiss) and the intensity was schemed as line intensity histograms.

Aim 2. To investigate the recruitment of TLR4 and glycosphingolipids in *H. pylori* infection of macrophages.

***Approach 1*. Observation of TLR4/ceramide moblilization.** A recent study by using immunofluorescence assay observed that ceramide was co-localized with autophagosomes in activated RAW264.7 cells, suggesting that glycosphingolipids are necessary for formation of autophagosomes, which have been thought to play an important role in the mechanism of innate immunity (32). It remains unclear, however, as to whether ceramide acts as a TLR4 signaling agonist upon *H. pylori* infection in LPS-activated macrophages. Details of the connection between *H. pylori*-induced ceramide/TLR4 and lipid rafts require further research. To this end, the determination of ceramide and TLR4 expression will be analyzed using flow cytometry and western blot. The localization of ceramide and TLR4 in *H. pylori*-infected macrophages will be visualized by confocal microscopy.

Approach 2. **Determination of the trafficking pathway using siRNA.** To examine whether recruitment of ceramide and TLR4 were crucial for *H. pylori*-induced LPS-mediated autophagy, RAW 264.7 cells were transfected with siRNA of *TLR4, atg5, atg12*, or control for 24 h followed by infection of *H. pylori*. For transfection of siRNA, target gene siRNA (ON-TARGET*plus*) and control siRNA were purchased from Thermo Fisher Scientific (Lafayette, CO). RAW 264.7 cells were transfected with siRNAs (100 nM) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The siRNA transfected cells will be infected with *H. pylori* and the autophagy will be observed and analyzed by using confocal microscopy and Western blot analyses, respectively.

***Approach 3*. Animal study and grouping.** To confirm the observation of ceramide and TLR4 are mobilized into membrane rafts in response to *H. pylori* infection in macrophages, a murine 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. PECs from C3H/HeN (n = 10) and C3H/HeJ (n = 10) mice were infected with *H. pylori*, the number of *H. pylori* survived intracellularly was determined by gentamicin protection assay.

Anticipated Outcomes. Pretreatment of cells with imipramine, an acid sphingomyelinase inhibitor, the results will be anticipated to observe the attenuation of LPS-induced co-localization of ceramide

and autophagosomes, and reduction of TLR4 expression. Additionally, in *H. pylori*-infected LPS-activated macrophages, more apparent punctate aggregates of ceramide/autophagosomes, and TLR4 will be showed at sites where *H. pylori* resided when compared with non-infected cells. The results from this specific aim will demonstrate that both ceramide and TLR4 are mobilized into lipid rafts and these interactions are critical for autophagosome formation and may benefit *H. pylori* infection.

It has been reported that depletion of cholesterol results in phagosomes fusion with lysosomes and induced mycobacterium-containing phagolosomes to autonomously undergo autophagy (7). Two *H. pylori* virulence factors, VacA and CagA were thought to be important for bacterial multiplication in macrophages (37). Additionally, infection of *H. pylori* induces autophagosome formation not only in phagocytes but in gastric epithelial cells (36). It has been reported that VacA was sufficient to induce autophagosome formation (33). This observation is in line with the result of cells treatment with VacA that induces autophagosome formation (33,39), and supporting by a previous study demonstrated that internalization of pathogen and stimulation of autophagy are cholesterol-sensitive (1). However molecular mechanisms involved in the interaction of *H. pylori* VacA and CagA, as well as autophagosome formation mediated through cholesterol-dependent manner have not been extensively studied.

Aim 2. To investigate the role of cholesterol in *H. pylori* virulence factors trigger autophagy in macrophages. Cholesterol accumulation by macrophages has been demonstrated that impairs phagosome maturation (15). In contrast, depletion of cholesterol induced autophagy (5). Controversy remains, however, whether the interactions of cholesterol and autophagy are involved in *H. pylori* infection of macrophages. Our preliminary data showed that infection of cells with *H. pylori* increased the expression of LC3-II. Upon treatment of cells with cholesterol depletion agents followed by infection of *H. pylori*, the data indicated that LC3-II expression was significantly increased (Fig. 2). In this specific aim, we will examine whether autophagy is triggered by *H. pylori* virulence factors which engaged the cholesterol-dependent pathway.

***Approach 1*. Analysis of autophagy machinery.** We proposed the hypothesis that *H. pylori* reside in vacuoles with features of lipid rafts immediately recruited the autophagy machinery. RAW 264.7 cells will infect with *H. pylori* and incubate in the presence or absence of LPS. The *H. pylori*-infected cells will be stained with antibodies against to Atg7 and Atg8, either cholera toxin-B (CTX-B, which binds to GM1 ganglioside as a raft marker) or filipin (cholesterol binding protein). The association of autophagy machinery, lipid rafts, and *H. pylori* will be examined by using Western blot and confocal microscope analyses.

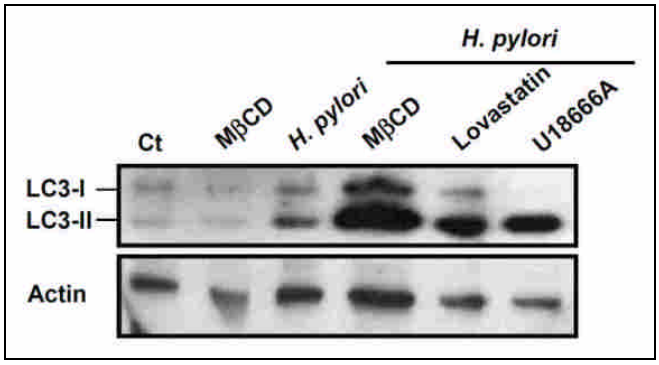

Fig. 2. Cholesterol usurpation synergistically enhances *H. pylori*-induced LC3-II expression. EGFP-LC3 transfected RAW 264.7 cells were pretreated with medium alone, MβCD, lovastatin, and U18666A at 37°C for 1 h. Cells were then washed and incubated *H. pylori* at an MOI of 100 for 24 h. The cell lysates were prepared to measure the LC-3 expression level by western blot analysis.

Approach 2. **Observation of the localization of *H. pylori* and autophagosomes.** Several raft-disruption agents will be employed in this specific aim. RAW 264.7 cells will pre-treat with the chemical reagents prior *H. pylori* infection. The chemical reagents used in this study including: methyl-β-cyclodextrin (a cholesterol depletion agent), lovastatin (which blocks HMG-CoA reductase for inhibition of cholesterol synthesis), U18666A (which prevents cholesterol exit from late endosomes/lysosomes), as well as filipin. The cell lysates will prepare for Western blot analysis of autophagy-associated molecules. The formation of autophagosomes and the association with cholesterol-enriched microdomains in *H. pylori*-infected macrophages will be observed by confocal microscopic analysis.

Approach 3. **Transmission electron microscopic analysis and immunofluorescence staining.** Two major virulence factors of *H. pylori*, VacA and CagA as well as the type four secretion system (TFSS), will also be considered to employ in this study. Various virulence factors knocking-out *H. pylori* strains will be used to examine whether bacterial proteins trigger lipid raft pathway in the formation of autophagosomes. The isogenic mutants will use in this study, including: ΔVacA, ΔCagA, ΔVacACagA, and ΔCagE (a component of TFSS) *H. pylori* mutants. Immunofluorescence staining, transmission electron microscope (TEM), and Western blot analysis will be utilized in this study to support our hypothesis.

Approach 4**. Analysis of the compartment trafficking.** We will examine whether avoidance of lysosomal delivery by depletion of cholesterol or disruption of lipid rafts is essential for *H. pylori* survival within macrophages. U18666A will be used to prevent cholesterol exit from late endosomes/lysosomes prior macrophage infection with *H. pylori*. Macrophages expressing Fc receptors will infect with either opsonized or non-opsonized *H. pylori*, and the CFUs will determine by plating. The expression of MHC-II and in which antigen-presentation, including classical MHC-II molecules (H2-IA, H2-IE), and those involved in antigenic peptide intracellular trafficking (invariant chain, Ii) will further be analyzed for investigation of the role of raft pathway in *H. pylori*-induced autophagosome formation. The proliferation of $CD4^+$ T cells will also be evaluated by using mixed lymphocyte reactions.

Anticipated outcome. Autophagy may orchestrate either recognition by certain Toll-like receptors or proteasome- and TAP-dependent antigen cross-presentation by macrophages (13). Thus, it raises the possibility that biosynthesis of cholesterol-rich autophagosome may trigger intracellular

antigen- presentation pathway. To this end, we will examine the expression of MHC-I (H2-K, H2-D, and H2-L), TAP (an endogenous peptide transporter), as well as activation of $CD8^+$ T cells. The experimental design of cholesterol depletion will demonstrate the recruitment of autophagosome formation, which may enable enhance *H. pylori* survival in macrophages. In contrast, upon replenishment of cholesterol will prevent *H. pylori* from phagolysosomes to autophagic vacuoles and thus decline *H. pylori* survive in macrophages.

C. CONCLUDING REMARKS

The main focus of this grant proposal is to reveal molecular basis of the *H. pylori* virulence factors in modulation immune responses and inflammations. Here, we will provide evidences that LPS-induced nitrite releasing is negatively regulated by *H. pylori*. In addition, we propose to identify the potential molecules, cholesterol-rich microdomains (lipid rafts), including in this manner. Using genetic and biochemical approaches, we expect to gain a clear view of how *H. pylori* has delicate mechanisms in regulation of innate immune response. Worth mentioning, in this proposal, we will address this question by determining *H. pylori*, lipid rafts, and autophagy interactions as well as by identification/characterization of signal molecules in macrophages. Studies from this proposal will enhance us to understand the mechanisms of *H. pylori* regulating innate immune responses and bacterial pathogenesis in host stomachs. Based on our previous results from *H. pylori* as well as understanding of biological functions of cellular cholesterol, we are confident in performing this proposal. We anticipate obtaining research results as follows:

1. **The molecular mechanism of *H. pylori* evade innate sensing.**
2. **Role of cholesterol in *H. pylori* survived intracellularlly in macrophages.**
3. **The interaction between *H. pylori* hijacked cholesterol and induction of autophagy.**

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