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(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)	長庚大學醫學院微生物及免疫學科				
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	細胞膽固醇參與細菌感染之機制				
本計畫名稱 英文	Molecular mechanism for cellular cholesterol in bacterial infection				
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 106 年 1 月 1 日起至民國 106 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) 03-2118800 ext. 5116 (宅/手機)_____				
通訊地址	桃園市龜山區文化一路 259 號				
傳真號碼	03-2118700	E-MAIL	chlai@mail.cgu.edu.tw		

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	長庚大學醫學院微生物及免疫學科	教授	規劃及推動研究進行、整理文獻背景及撰寫研究成果與論文	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）。

Abstract

Lipid rafts, cholesterol-rich microdomains, which provide platforms for signaling, are thought to be associated with *H. pylori*-induced pathogenesis. The inhibitors of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, commonly known as statins, are widely prescribed for lowering serum cholesterol. Treatment of cells with statins induces autophagy which has been found can contribute to immune defense by degrading invading pathogens. Although the combined use of statins and antibiotics reportedly increases *H. pylori* eradication, the mechanisms of how statin regulates autophagy and mitigates *H. pylori*-associated gastrointestinal disorders remain unclear. Accordingly, this study will investigate how statin reduces *H. pylori* burden in macrophages that mitigates inflammatory response, and evaluate the functional effects of statin in attenuating *H. pylori*-infection in animal models. This study combined a molecular-based study with a nationwide population analysis will reveal that statin use is a feasible approach to prevent *H. pylori*-associated gastrointestinal diseases.

Key words: *Helicobacter pylori*; autophagy; cholesterol; macrophages; inflammation

A. BACKGROUND AND SIGNIFICANCE

Infection of *Helicobacter pylori*

H. pylori, a Gram-negative microaerophilic spiral bacterium, colonizes the human stomach and infects over 50% of the worldwide population (1, 2). Persistent *H. pylori* infection is associated with several gastroenterological illnesses including gastritis, peptic ulcer, and gastric adenocarcinoma (3). *H. pylori* can penetrate the mucosal layer and survive intracellularly in the gastric epithelial cells, thereby escaping host immune response or antimicrobial therapy (4, 5).

H. pylori virulence factors

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 (5). 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) (6). 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 (7).

H. pylori hijacks cellular cholesterol for their benefits

VacA was the first toxin that reported to utilize lipid rafts for its assembly on the cell membrane and intracellular delivery (8). Several studies reveal that depletion of membrane cholesterol significantly reduces the entry of VacA into target cells (8-10). We further show that depletion of cholesterol and mutation of VacA significantly reduces *H. pylori* internalization in gastric epithelial cells (11). 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 (11). Murata-Kamiya *et al.* reported that the initial contact of *H. pylori* with cells induces the phosphotidylserine externalization from inner leaflet to outer leaflet of cell membrane, thus facilitates the translocation of CagA into cytoplasm (12). Our study subsequently demonstrate that CagA C-terminal domain-containing EPIYA regions directly targets to lipid rafts of gastric epithelial cells (13). Wunder *et al.* have been showed that *H. pylori* follows a cholesterol gradient and extracts the lipid from cytoplasmic membranes of epithelial cells for subsequent glucosylation (7). The authors identified the gene HP0421 (*capJ*) as encoding the enzyme cholesterol-α-glucosyltransferase responsible for cholesterol glucosylation (14). These evidence suggest that *H. pylori* possesses 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 (5).

B. Specific aims

Statins, inhibitors for HMG-CoA reductase, have been found to play a protective role in several bacterial infectious diseases. Antimicrobial agents are the most effective means of eradicating an *H. pylori* infection, particularly when applied as a triple therapy regimen consisting of a proton-pump inhibitor, amoxicillin, and clarithromycin (15). Previous studies have reported that combination treatments, including triple therapies prescribed in conjunction with statins, accelerate *H. pylori* clearance and reduce associated inflammation (16-18). However, the clinical relevance and molecular mechanisms underlying the regulating effects of statins on *H. pylori*-induced pathogenesis require further investigation.

Aim 1: Autophagy is a process involving degradation and recycling of intracellular components with the purpose of providing cellular energy and maintaining nutritional support for cell survival (19). Depletion of cell membrane cholesterol could induce autophagy (19, 20), which contributes to immune defense against invading pathogens (21-23). Therefore, this model posits that the use of statin to trigger autophagy activation may reduce pathogenic infection by *H. pylori*.

Aim 2: To validate any meaningful outcomes for statins that are observed in Model 1, an *ex vivo* murine primary macrophages and *in vivo H. pylori*-infected murine models should be performed in this model. Whether prescription of statins enhances autophagy and mitigates *H. pylori*-associated inflammation *in vivo* will be evaluated by using mouse models.

C. Preliminary results

Statin reduces cellular cholesterol

To analyze whether statins affect cellular cholesterol, gastric epithelial cells (AGS cells) were pretreated with simvastatin (0–50 μM) and infected with wild-type *H. pylori* ($VacA^+CagA^+$); the levels of cellular cholesterol were then determined. As indicated in Fig. 1A, simvastatin-treated cells exhibit a significantly reduced level of cellular cholesterol in a concentration-dependent manner. The viability of *H. pylori* and the cells is barely affected by treatment with simvastatin (Fig. 1B). These results indicate that simvastatin does not exhibit toxicity in cells and *H. pylori*, but possesses the activity to reduce the cellular cholesterol.

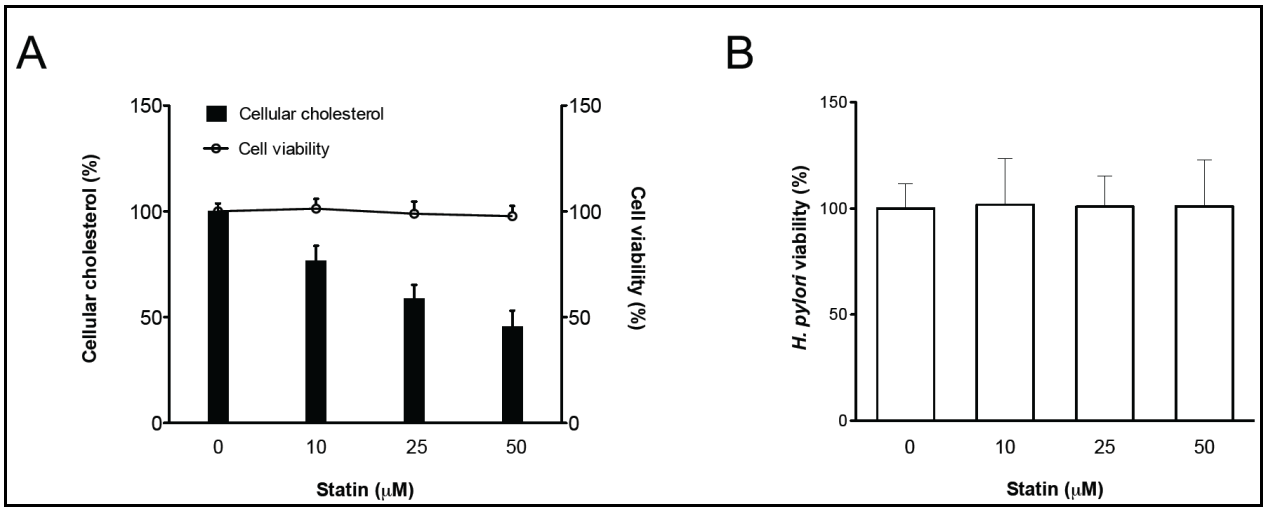

Fig. 1. Statin reduces cellular cholesterol in gastric epithelial cells. AGS cells were treated with various concentrations of simvastatin and infected with *H. pylori* (MOI = 100) for 6 h. (A) Whole cell lysates were then prepared for cholesterol level analysis (black bar). Cell viability was counted (open circle). Cell viability was not influenced by treatment with simvastatin. (B) Viable *H. pylori* was analyzed by counting CFU.

Statin decreases *H. pylori* CagA translocation and phosphorylation

The influence of a lower level of cellular cholesterol on translocation and phosphorylation of CagA in *H. pylori*-infected gastric epithelial cells was then examined. As indicated in Fig. 2, the levels of translocated and tyrosine-phosphorylated CagA are reduced significantly in AGS cells treated with 25 μM simvastatin. This trend is also observed in the other two gastric cancer cell lines, MKN45 and TSGH9201 cells. The results from this study indicate that the reduced levels of cellular cholesterol achieved by simvastatin attenuates CagA translocation and phosphorylation in *H. pylori*-infected cells.

Statin mitigates *H. pylori*-induced pathogenesis

The translocated/phosphorylated CagA in gastric epithelial cells is associated with activation of NF-κB and production of IL-8, followed by induction of hummingbird phenotype formation (24).

To investigate the mechanism responsible for statin-mediated inhibition of *H. pylori* CagA functions, NF-κB luciferase activity and IL-8 production were analyzed. As shown in Fig. 3, treatment of cells with simvastatin and subsequent infection with *H. pylori* reduces the levels of NF-κB activity and secretion of IL-8.

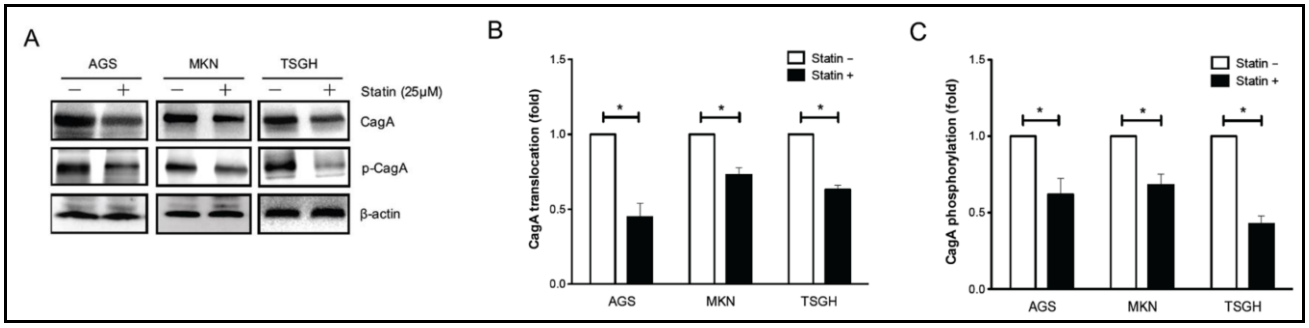

Fig. 2. Statin decreases *H. pylori* CagA translocation/phosphorylation in gastric epithelial cells. Three lines of gastric epithelial cells (AGS, MKN45, and TSGH9201) were pretreated with 25 μM simvastatin, then infected with *H. pylori* at an MOI of 100 for 6 h. (A) Whole-cell lysates were subjected to immunoblot for analysis of CagA translocation/phosphorylation, respectively. The quantitative results of (B) translocated CagA and (C) phosphorylated CagA were determined using densitometric analysis. *, $P < 0.05$ compared with *H. pylori*-infected cells without simvastatin pretreatment.

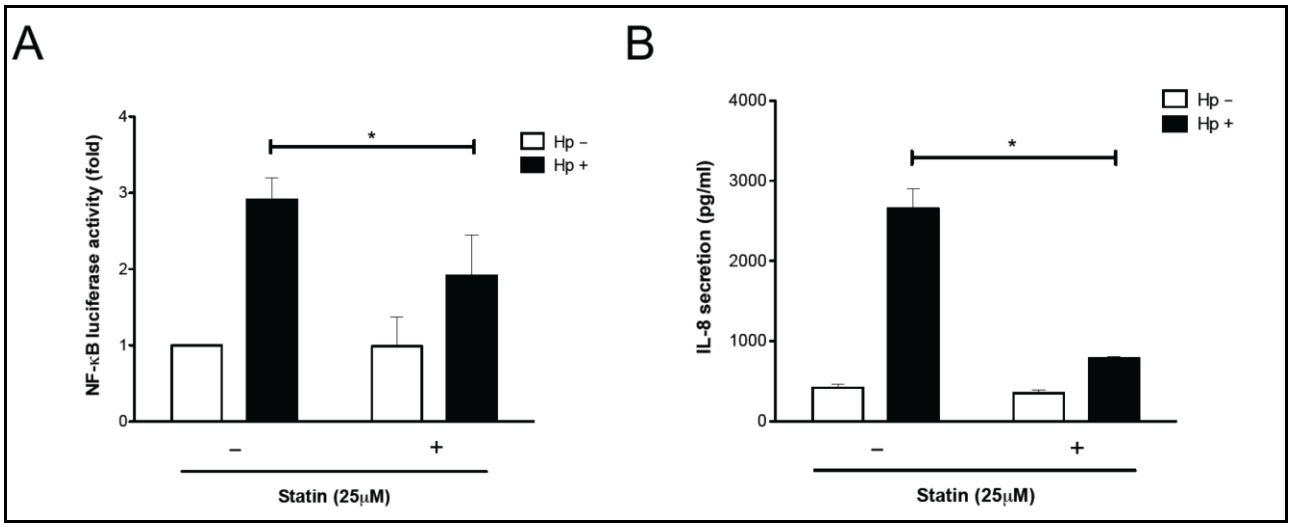

Fig. 3. Statin attenuates *H. pylori* CagA-induced inflammation. (A) AGS cells were transfected with κB-Luc vector and incubated for 24 h. The cells were then treated with 25 μM simvastatin, and infected with *H. pylori* at an MOI of 100 for 6 h. The cells were then prepared for luciferase activity assays. (B) The concentration of IL-8 in the culture supernatant was analyzed using the ELISA method. *, $P < 0.05$.

D. RESEARCH DESIGN AND METHODS

Aim 1. Investigating how statin influences autophagy in regulation of *H. pylori*-induced inflammation

It is now evident that several virulence factors from *H. pylori* are able to exploit cholesterol to gain a foothold in the host niche (5). Those molecules distributing in the cholesterol-rich microdomains sense and respond to *H. pylori* via an orchestrated manner during the persistent infection, which together play roles in disease progression. The depletion of cell membrane cholesterol may stimulate autophagy (20), by which reduces *H. pylori*-induced pathogenesis (25). Our earlier studies showed that reduced cellular cholesterol is found to be successful in attenuation of *H. pylori* VacA-actions and CagA-induced inflammation, as well as decreases bacterial survival in gastric epithelial cells (11, 13, 26). Therefore, it is worthy to investigate whether statin use, which lead to enhancement of autophagy and failure of *H. pylori* infection follow by mitigation of *H. pylori*-associated diseases.

1-1. Investigating *H. pylori* infection enhances autophagy in macrophages

To further ascertain the role of statin in the regulation of autophagy in *H. pylori*-infected macrophages, we will determine the expression levels of effector molecules that are involved in autophagy. A murine macrophage cell line, Raw 264.7, will be treated with various concentrations of simvastatin and infected with *H. pylori* ($VacA^+/CagA^+$) for 2, 6, 16, 36 and 48 h. The expression levels of autophagy-associated proteins (i.e. LC-3 I/II, beclin-1, p62, phospho-mTOR, Atg5, Atg12, and phospho-p70S6 kinase) will be analyzed by using western blotting. Our hypothesis will be supported if we observe increase levels of autophagy-associated proteins in *H. pylori*-infected macrophages following simvastatin treatment.

1-2. Observing co-localization of autophagosome and *H. pylori* in cells by fluorescence microscopy

Raw 264.7 cells will be treated or untreated with simvastatin and then will be infected with *H. pylori* at an MOI of 100. After incubation for 24 and 48 h, cells will be fixed and probed with anti-LC3 (Abcam) and 4’,6-diamidino-2-phenylindole (Invitrogen), and will be analyzed with fluorescence microscopy. The presence of LC3 punctate, which is used as an indication of autophagy (27), will be counted. This study will elucidate whether statin treatment increases autophagy, thus enhancing *H. pylori* resides in autophagosomes.

1-3. Investigating the effect of simvastatin on *H. pylori* intracellular survival

To explore whether statin facilitates autophagosome and lysosome fusion that inhibits *H. pylori* survival in macrophages, RAW 264.7 cells will be treated with various concentrations of simvastatin and infected with *H. pylori* for 24 h. The cells will be treated with gentamicin (100 μg/mL, Sigma-Aldrich) to eradicate extracellular bacteria. The cells will be lysed and plated onto

blood agar plates. The number of viable *H. pylori* colonies will be counted and represented in colony-forming units (CFU). These results will demonstrate that statin enhances autophagy pathway and promotes autophagosomes fused with lysosomes follows by reduces *H. pylori* burden in macrophages.

Aim 2. Validating the functional effects of statin using animal models

Based on our recent findings and preliminary results on the effects of *H. pylori* infection, we posit that statin treatment enhances autophagy and contribute towards immune defense by degrading invading bacteria, therefore, attenuates *H. pylori*-induced inflammation. Because we have established a cell-based assessment platform for this study, we are confident to perform this proposal in Aim 1. One potential issue is that should this trend can be carried out and seen *in vivo*? Therefore, a statin-prescribed animal model will be established and each assay including autophagy formation, *H. pylori*-induced inflammation, and bacterial burden in cells will be further assessed in this specific aim.

2-1. Demonstrating statin enhances *H. pylori*-induced autophagy in primary murine macrophages

To comprehensively validate the effects of statin on *H. pylori*-infected macrophages, an *ex vivo* murine model will be performed. Murine PEMs will be isolated from C57BL/6 mice and will be cultured in RPMI 1640 medium for the following experiments. The association of statin treatment, autophagy machinery, and *H. pylori* will be examined by using western blotting. The observation of autophagosomes and lysosomes will be analyzed by immunofluorescence staining and TEM. The pro-inflammatory cytokine production (i.e. IL-1, IL-6, and TNF-α) will be determined using enzyme-linked immunosorbent assay (ELISA). These results, by using *ex vivo* murine platform, will confirm the real statin functions that are observed in cell-based model in Aim 1.

2-2. Examining whether statin-conferred protection on *H. pylori*-induced inflammation in mice

Our preliminary results show that statin treatment could mitigate *H. pylori*-induced pathogenesis in a cell-based model and this trend is dependent on enhancement of autophagy activation. Here we propose to comprehensively investigate whether this protection against *H. pylori*-associated diseases could be extended to *H. pylori* infection *in vivo*. C57BL/6 mice will be intragastrically treated with various concentration of simvastatin (0, 10, 25, and 50 mg/kg) once daily for 14 days starting from ten-weeks of age. Their body weight and physical examination will be recorded at the beginning of the study period. The mice will be randomly divided into two groups (five mice each) that received either an intragastrical injection with the vehicle alone (PBS) or *H. pylori* (1×10^8) once every 3 days, for a total of five injections. The mice will be sacrificed after an additional 7-days in a normal feed condition and the following experiments will be performed:

(a) Total cholesterol in serum will be analyzed by using Amplex Red cholesterol assay kit (Molecular Probes).

(b) Gastric tissues from mice will be formalin-fixed and then subjected to hematoxylin-eosin (H&E) staining for evaluating the inflammatory scores of the gastric mucosal tissues.

(c) The immunohistochemistry (IHC) staining will be performed to analyze pro-inflammatory cytokine production by using ELISA, including IL-1, IL-6, IL-8, and TNF-α.

(d) The mRNA expression level of *IL-1*, *IL-6*, *IL-8*, and *TNF-α* as the indication for inflammation in the gastric tissues will be further examined by qPCR.

(e) The observation of *H. pylori* intracellular survival, autophagosomes, and lysosomes will be analyzed using TEM.

(f) In addition, the bacterial burden in the gastric tissues will be plated onto selected agar plates and the viable CFU will be counted.

The results from the animal studies will demonstrate statin-treated mice exhibit lower blood cholesterol, much autophagy formation, less gastric inflammation, less leukocyte infiltration, and a decrease *H. pylori* burden in the gastric tissues. Moreover, these findings will delineate the molecular mechanism of how statin mitigates *H. pylori*-induced inflammation.

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