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	探討降血脂藥物對於抑制胃癌之機制				
本計畫名稱 英文	The mechanism of cholesterol-lowering agents in the inhibition of gastric cancer				
整合型總計畫名稱					
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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。				
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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	長庚大學/微生物及免疫學科	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	70%

※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	65,000	65,000	
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	60,000	60,000	
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	35,000	35,000	
消耗性器材	ELISA assay kit、Reporter assay kit	組	4	10,500	42,000	
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	7,000	7,000	

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論文投稿費	期刊雜誌投稿	篇	2	15,000	30,000	
合　　計					300,000	

Abstract

Gastric cancer is the second leading cause of cancer-related deaths in the world. Cholesterol-enriched microdomains (also called lipid rafts), which provide platforms for signaling, are thought to be associated with *H. pylori*-induced pathogenesis and thus lead to gastric cancer progression. Several population-based case-control studies demonstrated that patients treated with statins, which inhibited 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, and reduced the risk for several types of cancers. Therefore, we sought to investigate the molecular mechanism of *H. pylori* hijacks membrane cholesterol and novel approach to eradicate *H. pylori*-related gastric cancer cells. Accordingly, we will analyze the data from National Health Insurance Research Database (NHIRD) and explore whether prescription of statins prevents gastric carcinogenesis and gastritis in *H. pylori*-infected patients. Moreover, we will investigate the role of cholesterol in *H. pylori* virulence factor-induced pathogenesis and how statins reduce *H. pylori*-induced gastric disorders. The results from this study will not only promote the basic and clinical-collaborations but also elucidate the scientific issues related to *H. pylori* pathogenesis and explore statins to therapy the gastric cancer.

Keywords: *Helicobacter pylori*, cytotoxin-associated gene A, gastric cancer, statin

A. BACKGROUND AND SIGNIFICANCE

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 [1]. Several studies have demonstrated that lipid rafts might serve as platforms for entry portals of pathogens, including bacteria [2-5] and viruses [6-8]. 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 [9]. Therefore, suggesting that pathogens might be favor to interact with lipid rafts where provide potential gateways to enter host cells.

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 [10,11]. Additionally, *H. pylori* penetrate across the mucosal layer and that may enable the bacteria to survive in the gastric epithelial cells [12]. 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 [13-15]. 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

The major composition of lipid rafts includes cholesterol, sphingolipids, and phospholipids which interact tightly and create rigid microdomains in the cell membrane [16]. The structure of lipid rafts is known to be stabilized in the cold in non-ionic detergents such as Triton X-100 [17]. 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 [17]. 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 [18]. After depletion of membrane cholesterol by MβCD or filipin, the raft-associated proteins and lipids can be dissociated and rendered the structure becoming nonfunctional [19].

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. 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) [20]. 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 [10].

***H. pylori* hijacks membrane 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 [21]. Several studies revealed that depletion of membrane cholesterol significantly reduces the entry of VacA into target cells [21-23]. 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 [23]. 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 [24]. We further showed that depletion of cholesterol and mutation of VacA significantly reduced *H. pylori* internalization in gastric epithelial cells [4]. 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 [4]. 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 [25]. 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 [26].

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 [10]. Subsequently, this group identified the gene HP0421 (*capJ*) as encoding the enzyme cholesterol-α- glucosyltransferase responsible for cholesterol glucosylation [27]. 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.

B. Specific aims

Gastric cancer is the fourth most common cancer, and the second leading cause of cancer-related deaths, in the world [10]. Cholesterol-enriched microdomains (also called lipid rafts), which provide platforms for signaling, are thought to be associated with the development of various types of cancer [11]. Recently, a population-based case-control study demonstrated that patients treated with statins, which inhibit 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, have reduced risk for gastric cancer [12]. These results suggest that cholesterol-enriched rafts play a crucial role in *H. pylori*-induced pathogenesis and thus lead to

gastric cancer progression. Therefore, we sought to investigate the molecular mechanism of *H. pylori* hijacks membrane cholesterol and novel approach to eradicate cholesterol-enriched gastric cancer cells.

In this proposal, we intend to employ systematic review and meta-analysis studies along with genetic, biochemical, and animal studies to further reveal molecular mechanisms underlying the function of cholesterol, which may serve as a platform for the *H. pylori*-induced host pathogenesis. The Specific Aims of this integral proposal are listing as below:

1. To analyze the correlation between *H. pylori* and cancer risk.
2. To compared the age, sex, and comorbidity of cancer patients with both infected and not infected by *H. pylori.*
3. To compare the prescription of statins in a comorbidity cohort and an *H. pylori* cohort, both taken from the National Health Insurance Research Database (NHIRD).
4. To investigate whether cholesterol involved in response to *H. pylori* infection.
5. To functionally characterize the molecular mechanisms of cholesterol involvement in *H. pylori*-induced pathogenesis.
6. To validate the mobilization of cholesterol to signal *H. pylori* infection using animal study.

C. Preliminary results

1. Comparison between the control and the *H. pylori* cohorts

In this study, we first established a 6022 *H. pylori* cohort and a 24088 comparison cohort with the same average age (mean=51.0 years) and sex ratio (54.7% male; Table 1). In the *H. pylori* cohort, 10.7 % of the patients were without comorbidity; in the comparison cohort, 57.4 % of the patients were without comorbidity. The incidence of peptic ulcers in the *H. pylori* cohort was nearly 4-times higher than in the comparison cohort.

Table 1: Baseline demographic status and comorbidity comparison between the comparison and the *Helicobacter pylori* cohorts

Variable	Comparison cohort N = 24088 (%)	Helicobacter pylori cohort N = 6022 (%)	p-value
Age, years (SD)*	51.0 (15.5)	51.1 (15.4)	0.6516
<40	5852 (24.3)	1463 (24.3)	1.0000
40-49	5604 (23.3)	1401 (23.3)	
50-59	6184 (25.7)	1546 (25.7)	
≧60	6448 (26.8)	1612 (26.8)	
Sex			
Female	10912 (45.3)	2728 (45.3)	1.0000
Male	13176 (54.7)	3294 (54.7)	
Comorbidity			
Without any comorbidity	13108 (54.4)	646 (10.7)	<0.0001
Hypertension	8044 (33.4)	2290 (38.0)	<0.0001
Diabetes	2670 (11.1)	805 (13.4)	<0.0001
Hyperlipidemia	7011 (29.1)	2343 (38.9)	<0.0001
Peptic ulcer	5359 (22.2)	5101 (84.7)	<0.0001

*t-test

2. Statins decreases gastric cancer risk and *H. pylori* infection

Statin, a cholesterol-lowing agent, inhibited HMG-CoA reductase has been found to reduce the risk for several types of cancers [28,29]. In this study by using case-control analysis, we found statin use was associated with decreased risk of gastric cancer in patients adjusted for age, sex, *H. pylori* infection, gastric diseases, gastroesophageal reflux disease, gastric polyp, cirrhosis, and gastritis (Table 2). The adjusted odds ratios of simvastatin and lovastatin were 0.76 (95% CI 0.70 to 0.83, $P < 0.0001$) and 0.79 (95% CI 0.72 to 0.86, $P < 0.0001$), respectively. These results reveal that the prescription of statins is associated with gastric cancer risk of patients with *H. pylori*-infection.

Table 2. Odds ratios and 95% confidence intervals of gastric cancer associated with aimvastatin, lovastatin and covariates

	Crude		Adjusted†	
Variable	OR	(95%CI)	OR	(95%CI)
Medications				
Simvastatin	0.89	(0.82, 0.96)**	0.76	(0.70, 0.83)***
Lovastatin	0.97	(0.90, 1.04)	0.79	(0.72, 0.86)***
Baseline co-morbidities				
H. pylori infection	9.38	(7.37, 11.9)***	5.09	(3.98, 6.51)***
Gastric diseases	4.49	(4.30, 4.69)***	4.00	(3.82, 4.19)***
Gastroesophageal reflux disease	3.24	(3.00, 3.49)***	2.13	(1.97, 2.31)***
Gastric polyp	7.32	(5.73, 9.36)***	5.14	(3.98, 6.62)***
Cirrhosis	1.29	(1.24, 1.35)***	0.95	(0.90, 1.00)
Gastritis	1.72	(1.65, 1.79)***	1.15	(1.10, 1.20)***

† Adjusted for age, sex, helicobacter infection, gastric diseases, gastroesophageal reflux disease, gastric polyp, cirrhosis and gastritis.

**, $P < 0.001$; ***, $P < 0.0001$.

3. *H. pylori* CagA-induced IL-8 promoter activity requires cholesterol

We first evaluated whether the level of endogenous cholesterol influenced the IL-8 transcriptional activation using a human *IL-8* promoter construct (IL8-Luc) which contains AP-1 and NF-κB sites, fused with a luciferase reporter gene (Fig. 3A) [30]. Following transfection with the IL8-Luc, AGS cells were treated with lovastatin to reduce the level of endogenous cholesterol and then infected with wild-type, ΔCagA, or ΔCagE *H. pylori*. Our data show that a significant attenuation in the stimulation of *IL-8* promoter activity in cells infected with the wild-type strain, but not with ΔCagA or ΔCagE *H. pylori* (Fig. 3B). These results suggest that CagA-mediated *IL-8* promoter activity was dependent on host endogenous cholesterol in epithelial cells.

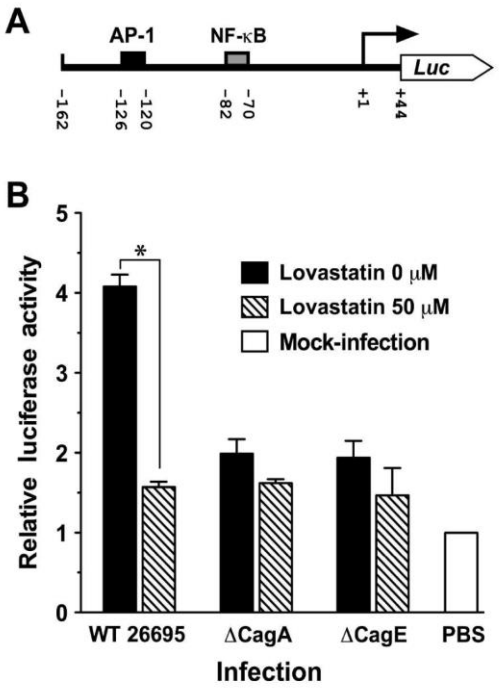

Fig. 3. Cholesterol is required for *H. pylori* CagA-induced *IL-8* promoter activity. (A) A schematic representation of IL8-Luc construct. AP-1, Activator protein-1; NF-κB, nuclear factor-kappaB; Luc, luciferase reporter. In all numbering, the transcription initiation site is denoted by +1. (B) AGS cells were transfected with IL8-Luc vector. After 24 h transfection, the cells were then treated with lovastatin prior *H. pylori* infection. Cells were infected with wild-type (WT 26695), ΔCagA, or ΔCagE mutant *H.*

D. RESEARCH DESIGN AND METHODS

It is now evident that several virulence factors from *H. pylori* are able to exploit or modulate cholesterol to gain a foothold in the host niche. 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 a role in disease progression. Previous study revealed that depletion of cholesterol is found to be successful in anti-HIV activity, particularly in decreasing viral replication and production [31]; these results shed light on the new therapeutic approach that inhibition of cholesterol-enriched binding sites for microbial infection. Therefore, it is worthy to investigate whether statin use, which may lead to failure of *H. pylori* infection in the initial step. In parallel, understanding the molecular mechanism for pathogen-host interaction may provide an insight into development of novel strategies that target cholesterol to control the infection of these pathogens.

In this proposal, we will examine whether the recruitment of cholesterol is triggered by *H. pylori* which engaged the signaling pathways. We then intend to test whether depletion of cholesterol reduce *H. pylori*-induced pathogenesis of host. The following methods will be carried out:

Cell culture

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

Construction of *H. pylori* isogenic mutants

In this proposal, all experiments will be carried out with 26695 (ATCC 700392). An isogenic mutant *H. pylori* Δ*vacA*::*cat* will be generated by insertion of the *cat* fragment derived from pUOA20 [32] into *vacA* gene of *H. pylori* through allelic replacement and selection of chloramphenicol-resistant clones [33]. All isogenic mutants of *H. pylori* will be obtained by following the natural transformation protocol [33]. The genomic DNA of *H. pylori* mutants will be used to check inserted of antibiotics cassette into a target gene. Western blot analysis will be carried out to the abolished expression of each protein.

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 will fix and stain with anti-ceramide (Sigma-Aldrich) or anti-TLR4 (Santa Cruz), the fluorescence intensities will then determine 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℃ for 6 h. Cells will be fixed and stained CTX-B, or DAPI to visualize bacteria and the cell nucleus, and then will be 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, will be transfected into AGS cells. Following transfection, the cells will infect with *H. pylori* and then subject to luciferase activity assays. Luciferase activity will be normalized to transfection efficiency, which will be 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 will be harvested for IL-8 secretion analysis. The concentration of IL-8 will determine by enzyme-linked immunosorbent assay (ELISA). AGS cells will be 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 will be determined using a sandwich ELISA kit (R&D systems) according to the manufacturer's instructions [4].

Animal study and histological examination

Six-week-old male BALB/c mice will obtain from the National Laboratory Animal Center of Taiwan. The mice will be cared for in accordance with the Animal Care and Use Guidelines under

a protocol approved by the Institutional Animal Care Use Committee. Mice will feed a high cholesterol diet (HCD) (60% kcal from fat, TestDiet 58Y1; Purina, Richmond, IN) or a normal diet (ND) for 35 days starting from 11 weeks of age. Their body weight was recorded at the beginning of the study period. For experiments, HCD and ND mice will be randomly divided into two groups (five mice each) that received either an oral injection with the vehicle alone (PBS) or *H. pylori* once every 3 days, for a total of five injections. The mice will then sacrifice after 14 h of fasting. Gastric tissues from mice will formalin-fix and then will subject to hematoxylin-eosin (H&E) or IHC staining. Briefly, tissue sections will be de-paraffinized, rehydrated, blocked with 3% bovine serum albumin, and then stained with rabbit monoclonal antibodies against interleukin (IL)-1β (H-153) (Santa Cruz, CA) for 24 h at 4 ℃. After washing, the samples will probe with a peroxidase-labeled goat anti-rabbit secondary antibody (Epitomics, Burlingame, CA) and will develop with an ABC kit (Vector Laboratories, Burlingame, CA).

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