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				
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本計畫名稱	中文	細菌膽固醇葡萄糖轉移酶干擾自噬活化及誘導發炎反應			
	英文	Bacterial cholesterol-α-glucosyltransferase interferes autophagy activation and induces inflammation			
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 109 年 1 月 1 日起至民國 109 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。				
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三、主要研究人力：

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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、整理實驗數據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

Cholesterol-α-glucosyltransferase (CGT) is encoded by *Helicobacter pylori* type 1 capsular polysaccharide biosynthesis protein J gene (*capJ*), which converts cellular cholesterol into cholesteryl α-glucosides (CGs). *H. pylori* infection induces autophagy, which may facilitate bacterial survival in epithelial cells. We recently reported that *H. pylori* activates high-mobility group box 1 (HMGB1) expression and recruits the receptor for advanced glycation end-products (RAGE) into cholesterol-rich microdomains, which is important for inducing inflammation. However, the association of *H. pylori* CGT-hijacked cholesterol to manipulate autophagy processes, and the interference of innate sensing during such interactions, has not been explored. Therefore, this study will further investigate the role of *H. pylori* CGT in promoting lipid raft formation which contributes to the HMGB1-RAGE-IL-8 inflammation axis. This study will provide insights into the mechanisms underlying how cholesterol glucosylation contributes to autophagy dysregulation and impairs the clearance of *H. pylori* in gastric epithelial cells.

Keywords: *Helicobacter pylori*, cholesterol-α-glucosyltransferase, lipid rafts, inflammation

A. Background and significance

Helicobacter pylori virulence factors

H. pylori, a Gram-negative microaerophilic spiral bacterium, colonizes the human stomach and infects over 50% of the population worldwide (Marshall, 2002). Persistent *H. pylori* infection is associated with several gastroenterological illnesses including gastritis, peptic ulcer, and gastric adenocarcinoma (Wroblewski, et al., 2010). *H. pylori* can penetrate the mucosal layer and survive intracellularly in the gastric epithelial cells, thereby escaping host immune response or antimicrobial therapy (Lai, et al., 2006). Although gastric mucosa is well protected against other bacterial infection, *H. pylori* is highly adapted to this ecological niche. Several virulence factors have been characterized that enable *H. pylori* to survive, multiply, escape from immune surveillance, and eventually lead to persistent infection in a particular niche of the host (Lai, et al., 2013). The ways by which the bacteria support the colonization and persistence in the gastric mucus include polar flagella, urease, adhesins, and two major virulence factors: vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA) (Amieva & El-Omar, 2008). In addition, an earlier study revealed that an *H. pylori* enzyme, cholesterol-α-glucosyltransferase (CGT), which glucosylates cholesterol in macrophages, is thought to be modulated the innate immunity (Wunder, et al., 2006).

Structure and composition of cholesterol-rich microdomains

The major composition of cholesterol-rich microdomains (also refers to lipid rafts) includes cholesterol, sphingolipids, and phospholipids, which interact tightly and create rigid microdomains in the cell membrane (Ikonen, 2001). The structure of lipid rafts is known to be stabilized in the cold in non-ionic detergents such as Triton X-100 (Brown & London, 2000). After treatment of cytoplasmic membrane with cold Triton X-100, insoluble components including lipids and proteins remained in lipid rafts are stabilized in detergent-resistant membrane (DRM) (Brown & London, 2000). Several raft usurping or disruption agents such as methyl-β-cyclodextrin (MβCD), statin, and filipin have been employed in the investigation of lipid raft's functions and compositions (Simons & Toomre, 2000). After depleting membrane cholesterol by MβCD or statin, the raft-associated proteins and lipids are dissociated, causing the structure nonfunctional (Simons, et al., 2002).

H. pylori exploits membrane cholesterol for cellular internalization

VacA is known to utilize lipid rafts for its assembly on the cell membrane and intracellular delivery (Ricci, et al., 2000). We previously demonstrated that depletion of cholesterol and mutation of VacA decrease *H. pylori* internalization in gastric epithelial cells (Lai, et al., 2008). In addition, disruption of lipid rafts attenuates CagA functions, suggesting that the delivery of CagA into epithelial cells is mediated through a cholesterol-dependent manner (Lai, et al., 2008). Our study further demonstrated that the C-terminal EPIYA domain in CagA directs CagA to lipid rafts of gastric epithelial cells (Lai, et al., 2011). Moreover, Wunder *et al.* (Wunder, et al., 2006) showed that *H. pylori* follows a cholesterol gradient and extracts the lipid from cytoplasmic membranes of

epithelial cells for subsequent glucosylation. The study also identified a gene, HP0421 (*capJ*), which encodes cholesterol-α-glucosyltransferase (CGT) and is responsible for cholesterol glucosylation (Lebrun, et al., 2006). Subsequent studies showed that CGT activity reduces the ability of *H. pylori* to evade phagocytosis by macrophages and T cell-mediated immune response (Wunder, et al., 2006; Beigier-Bompadre, et al., 2011). In addition, *H. pylori* acquires phospholipids from cell membrane for cholesteryl glucosides biosynthesis, which is important to promote CagA translocation and phosphorylation (Jan, et al., 2016). Our recent study revealed that CGT partitioned cholesteryl glucosides into lipid rafts around host-pathogen-contact sites and contributed to membrane remodeling involved in bacteria-induced pathogenesis (Wang, et al., 2012). These findings indicate that *H. pylori* orchestrates the exploitation of cholesterol for its intricate infection strategy. However, the association between utilizing cholesterol and triggering autophagy by *H. pylori,* as well as how this bacterium suppresses innate sensing in such interactions are unclear (Fig. 1).

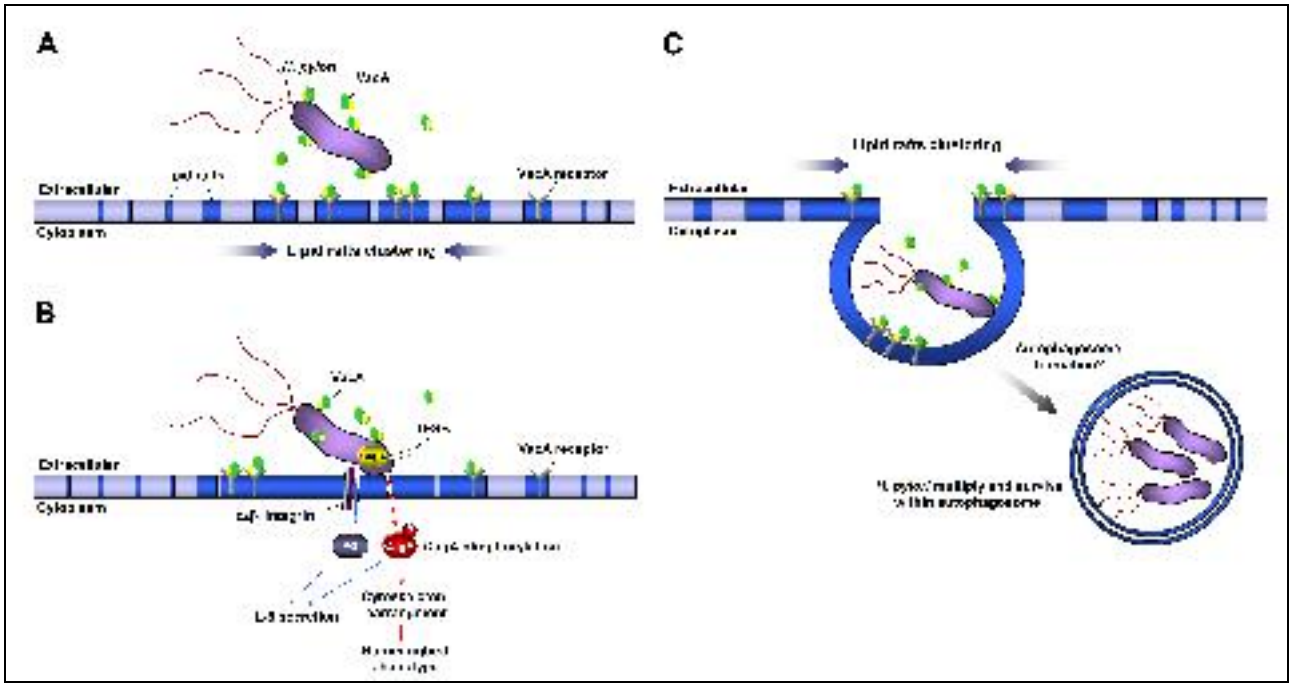

Fig. 1. Proposed mechanism for *H. pylori* virulence factors exploit cellular cholesterol as its infectious strategy. (A) VacA secreted by *H. pylori* binds to receptors, which are localized in the cholesterol-rich microdomains and facilitate raft coalescence into the sites of bacterial infection. (B) The clustering rafts enhance type IV secretion system (TFSS), allowing cytotoxin-associated A (CagA) and peptidoglycan (PGN) injected into cytoplasm and induced downstream signaling events. (C) The raft-associated membrane may extend to form autophagosomes where provide compartments for *H. pylori* to survive intracellularly (Lai, et al., 2013). However, how *H. pylori* cholesterol-α-glucosyltransferase (CGT) triggers autophagosomes that increases bacterial burden in gastric epithelial cells requires further investigation.

Role of autophagy in *H. pylori* infection

Autophagy is a cellular process that functions as a recycling pathway, degrading nonfunctional and unnecessary components, and recycling these components to support cellular survival (Marino, et al., 2014). The human immune system has developed several mechanisms for protecting against

bacterial infections. The autophagy process allows for the formation of autophagosomes that fuse with lysosomes, the contents of which are subsequently lysed by degradative enzymes (Kang, et al., 2011; Greenfield & Jones, 2013). In the context of degrading intracellular pathogens, autophagy contributes to immune defense to lessen *H. pylori* burden (Mizushima, et al., 2008; Zhao, et al., 2008; Yang & Chien, 2009). Contrarily, an earlier study revealed that formation of autophagosomes in cells enhances *H. pylori* survival, suggesting that these autophagic vesicles provide a unique sanctuary for *H. pylori* multiplication (Wang, et al., 2009).

B. Preliminary results

H. pylori infection induces HMGB1 expression in gastric epithelial cells

We first explored whether *H. pylori* increases HMGB1 expression in gastric epithelial cells. AGS and two other gastric epithelial cell lines (SC-M1 and TSGH9201) were infected with *H. pylori* at an MOI of 100 for 6 h, and the expression level of HMGB1 was determined by western blot assay. Our data showed that the expression levels of HMGB1 were elevated in the three *H. pylori*-infected gastric epithelium-derived cell lines (Fig. 2). AGS cells were found to be the most susceptible; therefore, this line was chosen for the following investigations.

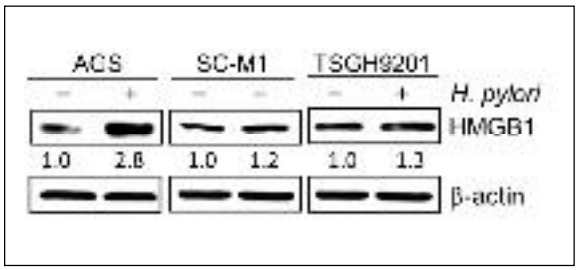

Fig. 2. *H. pylori* induces HMGB1 expression in gastric epithelial cells. Three gastric tissue derived cell lines (AGS, SC-M1, and TSGH9201) were uninfected or infected with *H. pylori* (MOI = 100) for 6 h. Total cell lysates were prepared to evaluate HMGB1 expression by western blot analysis. Protein expression levels were normalized to β-actin.

H. pylori-induced RAGE expression is triggered by HMGB1

We then assessed whether *H. pylori*-induced RAGE expression was elicited by activated HMGB1. AGS cells were mock-treated or pretreated with isotype IgG or neutralizing antibody against HMGB1 (α-HMGB1) for 30 min and then incubated with *H. pylori* for 6 h. Our results showed that blocking of HMGB1 by α-HMGB1 significantly reduced *H. pylori*-induced RAGE mRNA and protein levels, whereas the mock-treated cells or cells treated with isotype IgG showed no such effect (Fig. 3). These results indicate that *H. pylori* infection induces HMGB1 expression, which in turn elicits the production of RAGE in gastric epithelial cells.

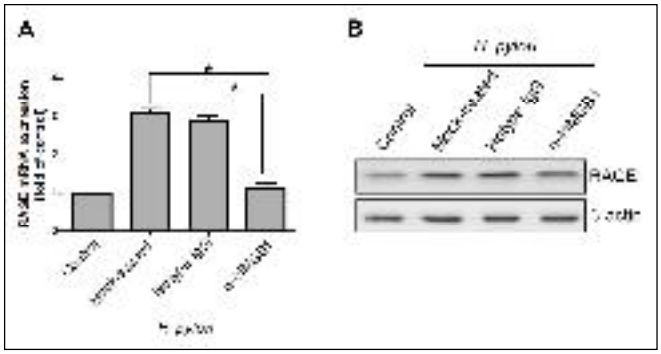

Fig. 3. HMGB1 is crucial for RAGE expression in *H. pylori*-infected cells. AGS cells were untreated or pretreated with 1 μg/ml of isotype IgG or anti-HMGB1 at 37°C for 30 min and then infected with *H. pylori* at an MOI of 100 for 6 h. RAGE mRNA and protein expression levels were measured by (A) quantitative real-time PCR, and (B) western blot analysis, respectively. *, $P < 0.05$.

C. Specific aims and experimental designs

It is now evident that several virulence factors from *H. pylori* are able to exploit cholesterol to gain a foothold in the host niche (Lai, et al., 2013). *H. pylori capJ*-encoded CGT is responsible for host cholesterol glucosylation by converting cholesterol into cholesteryl glucosides, which protect bacteria from phagocytosis (Wunder, et al., 2006; Beigier-Bompadre, et al., 2011; Du, et al., 2014). Recently, we demonstrated that *H. pylori* activates HMGB1 expression and recruits RAGE into lipid rafts in response to promote inflammation in gastric epithelial cells (Lin, et al., 2016). However, whether partitioning cholesterol glucosylation into lipid rafts and remodeling membrane compositions cause HMGB1 to trigger RAGE mobilization around host-bacteria-contact sites and result in inflammation in gastric epithelial cells have yet to be investigated.

Approach 1. Exploring whether CGT activates HMGB1 expression

We will first analyze whether CGT plays an important role in inducing HMGB1 and RAGE expression in gastric epithelial cells. Several *H. pylori* variants including wild-type (WT), isogenic *capJ*-knockout (ΔCapJ), and *capJ*-knock-in (ΔCapJ-in) will be constructed and utilized in this study (Fig. 8). In addition, the catalytically inactive enzyme mutant (E285A) will be constructed to verify the increased expression levels of HMGB1 and RAGE are due to the enzyme activity of CGT. AGS cells will be treated or untreated with *H. pylori* variants at an MOI of 100 for 6 h. HMGB1 and RAGE in cell lysates will be analyzed by western blot analysis. In parallel, culture supernatants from cells infected by *H. pylori* will be assessed for the presence of extracellular HMGB1 by using enzyme-linked immunosorbent assay (ELISA). If the expression of intracellular HMGB1 and RAGE are increased after WT *H. pylori* infection but not after the infection by the ΔCapJ or E285A mutant strains, the results will indicate that CGT activates the expression.

Approach 2. Investigating the role of cholesteryl α-glucosides (CGs) in *H. pylori*-induced HMGB1 expression and recruiting RAGE to lipid rafts

Logically, we cannot rule out the possibility that the activation of HMGB1 and RAGE expression is attributed to an indirect effect by *H. pylori* CGT. The incorporation of membrane phospholipids led to an increase in cholesteryl 6'-acyl α-glucosides (CAGs), one of CGs derivatives, containing fatty acid chains which is important for promoting lipid raft formation (Jan, et al., 2016). We hypothesize that the coalescent lipid rafts provided a platform for enhancing bacterial pathogenesis in the membrane. To test this hypothesis, we will investigate whether CGT is involved in the induction of RAGE mobilization into lipid rafts. AGS cells will be infected with WT or ΔCapJ (MOI = 100) for 6 h. The levels of CAGs with long chain/unsaturated fatty acid will be analyzed by using high-resolution liquid chromatography-tandem mass spectrometric analysis (LC-MS/MS). Meanwhile, *H. pylori*-infected cells will be stained with DAPI (blue) to visualize *H. pylori* and cell nuclei, with Alexa Fluor 488-conjugated cholera toxin subunit B (CTX-B) to visualize a raft marker, GM1, or with antibody against RAGE. The stained cells will be observed by using confocal microscopy. The merged images will be analyzed by CLSM *z*-section (Carl Zeiss)

for visualizing the interactions between bacteria and RAGE in the lipid rafts. Our hypothesis will be supported if we observe that WT *H. pylori*, rather than ΔCapJ, induced an increase level of CAGs and that help to promote lipid raft formation, therefore inducing RAGE mobilization into membrane cholesterol-rich microdomains.

Approach 3. Tracking the subcellular localization of HMGB1

This study will use confocal microscopy to localize intracellular HMGB1 and track its movement in gastric epithelial cells. AGS cells will be infected by *H. pylori* and its mutant derivatives, ΔCapJ, ΔCapJ-in, and E285A mutant strains for 6 h at an MOI of 100. Cells will be fixed and probed with antibody against HMGB1, followed by stain with Alexa Fluor 488-conjugated goat anti-rabbit IgG. The stained cells will be observed under a confocal microscope (Zeiss LSM 780; Carl Zeiss). We expect to observe the translocation of HMGB1 from the nucleus to cytoplasm after infection by WT *H. pylori*. On the other hand, HMGB1 will be restricted in the nucleus upon infecting with the ΔCapJ and E285A mutants; genetic complementation of the ΔCapJ mutation (ΔCapJ-in) should restore the CGT activity and induce HMGB1 expression after infection.

Possible problems and alternatives. Damage-associated molecular patterns (DAMPs) are endogenous danger signals that have been identified elsewhere, including HMGB1, S100A8/9, IL-1α, and IL-33/ST2 (Garlanda & Mantovani, 2013; Kaczmarek, et al., 2013; Stephenson, et al., 2016). Activation of HMGB1 signal is mediated by several pattern-recognition receptors (PRPs), such as RAGE or Toll-like receptors (TLRs) (Torok, et al., 2005; Rojas, et al., 2011; Lu, et al., 2012). Similar to HMGB1, IL-1α and IL-33/ST2 also are types of alarmins, which are abundantly expressed in epithelial and endothelial cells (Chen & Nunez, 2010; Garlanda, et al., 2013). Expression of IL-1α and IL-33/ST2 has been reported in some bacterial infectious diseases, including *Legionella pneumophila* (Barry, et al., 2013) and *Staphylococcus aureus* infections (Li, et al., 2014). However, limited reports indicated that IL-1α and IL-33/ST2 can be upregulated in cells treated with the virulence factors from *H. pylori* (Godlewska, et al., 2008; Shahi, et al., 2015), but their role in *H. pylori*-induced pathogenesis is unclear. Therefore, in addition to HMGB1, other DAMPs will be comprehensively evaluated to demonstrate the exact role of CGT in *H. pylori*-induced inflammation. Meanwhile, the expression of HMGB1 and RAGE produced from several gastric epithelium-derived cell lines such as SC-M1, TSGH9201, MKN-28, and MKN-45 (or MKN-45P) will also be evaluated.

Anticipated results. We expect to demonstrate that *H. pylori* CGT promotes lipid raft formation, which is associated with elevated HMGB1 and recruited RAGE to membrane rafts. The role of CGT in *H. pylori*-induced inflammation will be further validated in an *in vivo* model. Major technical problems are may not occurred, because we are very experienced in most of assessed platforms which have been conducted in our previous studies (Lu, et al., 2012; Hung, et al., 2015; Lin, et al., 2016).

D. Expected results and goals

The main goal of this proposal is to reveal the molecular basis of how *H. pylori* CGT manipulates cellular cholesterol that regulates autophagy pathway in turn promotes bacterial survival in gastric epithelial cells. The role of CGT orchestrates the interactions among HMGB1, autophagy, and RAGE mobilization in lipid rafts which contributes to *H. pylori*-induced inflammation will be comprehensively investigated in this study.

1. Since CGT is responsible for cholesterol glucosylation and lipid raft formation, and *H. pylori* infection can activate HMGB1 to mobilize RAGE in the membrane rafts, we propose that CGT plays a crucial role in *H. pylori*-induced inflammation. If this is the case, *H. pylori*-harboring CGT would recruit RAGE in the lipid rafts and induce more severe inflammation than that infection with ΔCapJ mutant.

2. We expect to demonstrate the increased levels of HMGB1 expression and autophagy activation in WT *H. pylori*-infected cells rather than that in cells infected with ΔCapJ mutant. In addition, the process of *H. pylori*-induced HMGB1 release to activate autophagy will be clearly elucidated.

3. Overall, this investigation will unveil the molecular mechanism of how *H. pylori* CGT exploits lipid rafts and activates HMGB1 to manipulate the autophagy process which contributes to enhance bacterial burden in cells results in induction of inflammation. Therefore, our results combine the cell-based and animal studies will reveal a valuable strategy for treating *H. pylori*-induced inflammation.

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