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：

欄位	內容				
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	膽固醇-α-葡萄糖基轉移酶在幽門螺旋桿菌誘導發炎反應所扮演的角色				
本計畫名稱 英文	Role of cholesterol-α-glucosyltransferase in *Helicobacter pylori*-induced 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 112 年 1 月 1 日起至民國 112 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：

（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	研究之規劃及推動、實驗設計、整理數據、撰寫研究成果與發表論文	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）。

Abstract

Helicobacter pylori cholesterol-α-glucosyltransferase (CGT) converts cellular cholesterol into cholesteryl α-glucosides (CGs), which contributes to bacterial evasion of immune defense. 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. This study will further investigate how *H. pylori* CGT triggers autophagy by regulation of HMGB1. In addition, we will explore how *H. pylori* CGT enhances the bacterial burden in cells. Understanding the important functions of *H. pylori* CGT and the molecular basis of such particular virulence factor will reveal valuable strategies for treating *H. pylori* chronic infection.

Keywords: *Helicobacter pylori*; cholesterol-α-glucosyltransferase; inflammation

A. Background information

Helicobacter pylori virulence factors

H. pylori persistent infection is associated with several gastroenterological illnesses including gastritis, peptic ulcer, and gastric adenocarcinoma [1]. Although gastric mucosa is well protected against other bacterial infection, *H. pylori* is highly adapted to this ecological niche. 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) [2]. 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 [3].

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 [4]. The autophagy process allows for the formation of autophagosomes that fuse with lysosomes, the contents of which are subsequently lysed by degradative enzymes [5,6]. In the context of degrading intracellular pathogens, autophagy contributes to immune defense to lessen *H. pylori* burden [7-9]. 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 [10].

Involvement of HMGB1 and RAGE in bacteria-induced inflammation

HMGB1 is a ubiquitous nuclear protein that stabilizes nucleosomes, which promotes DNA nicking, and facilitates transcription [11]. It has been shown that HMGB1 functions as a proinflammatory protein that mediates endotoxin-induced lethality, tissue damage, and systemic inflammation [12,13]. RAGE, a single transmembrane-spanning domain protein belonging to the immunoglobulin superfamily, serves as a receptor for HMGB1 in the amplification of proinflammatory signaling [14]. Interaction of RAGE with HMGB1 triggers mitogen-activated protein kinases (MAPKs) and subsequently activates nuclear factor (NF)-κB [15,16], thereby stimulating the release of multiple proinflammatory cytokines [17]. However, the detailed process by which *H. pylori* regulates HMGB1 expression and utilizes autophagy machinery to impair the efficiency of bacterial clearance by host cells remains unclear.

Specific aims

Microbial infection stimulates autophagy that elicits the innate immune defense to eliminate intracellular pathogens [18,19]. However, some pathogens have evolved multipronged strategies to dysregulate autophagy machinery and promote intracellular bacterial survival [20-22]. Our earlier study demonstrated that *H. pylori* infection increases the expression of HMGB1, which subsequently interacts with its receptor RAGE, and is believed to amplify the inflammation

cascade [23]. However, several relevant issues have emerged and required further exploration, including whether CGT is involved in *H. pylori*-induced HMGB1-RAGE-IL-8 axis and that occurred dependently on cholesteryl glucosides. We also will explore role of CGT triggers autophagosome formation which responses for enhancing *H. pylori* burden in gastric epithelial cells. Based on the preliminary results, we propose to extensively investigate the role of CGT in *H. pylori* exploits cellular cholesterol, and how the autophagic response contributes to bacterial survival in cells results in changing microbiota composition and induction of inflammation.

B. Preliminary results

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. 2). These results indicate that *H. pylori* infection induces HMGB1 expression, which in turn elicits the production of RAGE in gastric epithelial cells.

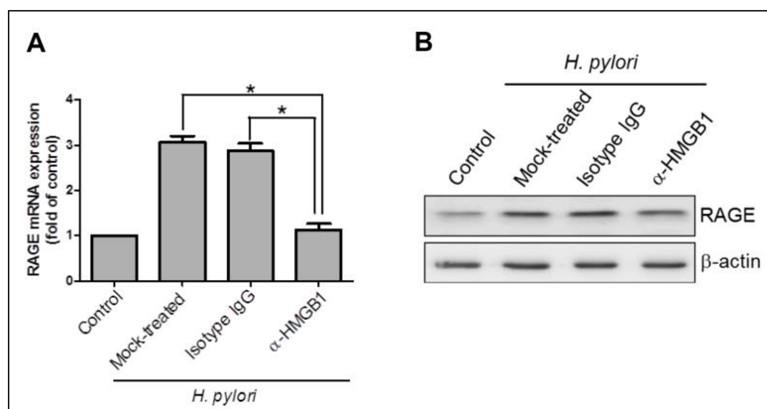

Figure 2. HMGB1 is crucial for RAGE expression in *H. pylori*-infected cells. AGS cells were untreated or pretreated with 1 μg/ml of isotype IgG or anti-HMGB1 at 37°C for 30 min and then infected with *H. pylori* at an MOI of 100 for 6 h. RAGE mRNA and protein expression levels were measured by (A) quantitative real-time PCR, and (B) western blot analysis, respectively. *, $P < 0.05$.

H. pylori CGT manipulates autophagy and increases the bacterial burden in cells

Since cholesterol glucosylation by *H. pylori* delays bacterial internalization and interrupts phagosome maturation [24], we hypothesize that this mechanism dysregulates autophagy processes that enhance bacterial burden in macrophages. We first employed J774A.1 macrophage cells as an assay platform to explore whether *H. pylori* survival was dependent upon CGT-induced autophagy. As shown in Fig. 3, treatment of cells with rapamycin induced microtubule-associated protein light chain 3 (LC3) conversion from LC3-I to LC3-II, which is considered as a marker of autophagy [25]. Interestingly, infection of cells with wild-type (WT) *H. pylori* promoted LC3-II/I conversion, whereas infection with the isogenic Δ*CapJ* mutant did not. We then analyzed expression of the autophagy-related proteins p62 and beclin-1, which participate in the initiation of autophagy with LC3-II [6,26]. Our data showed that infection with the WT *H. pylori* increased

the expression of p62 and beclin-1 as compared with levels observed in either the uninfected control or ΔCapJ-infected groups. These results suggest that infection with *H. pylori*-containing CGT triggers autophagy activation in macrophages. To further explore whether *H. pylori* survival in macrophages is dependent upon CGT-triggered autophagy, cells were treated with 100 nM bafilomycin A1 (BafA1), an autophagy inhibitor, at 37°C for 24 h. As shown in Fig. 3B, inhibition of autophagy by BafA1 dramatically decreased WT *H. pylori* survival in cells. Similar results were observed for the isogenic Δ*CapJ* strain, which showed reduced survival activity upon BafA1 treatment. These results demonstrate that CGT is important for autophagosome maturation and may control the autophagy process, which sustained the survival of *H. pylori* in macrophages. Although *H. pylori* is generally considered as an extracellular pathogen, this organism can reside in both macrophages and gastric epithelial cells [3,27]. Given that our preliminary work is on macrophages not epithelial cells, we will utilize gastric epithelial cells as an experimental model to comprehensively investigate how *H. pylori* CGT activates HMGB1 and manipulates autophagy, which therefore enhances the bacterial burden in cells.

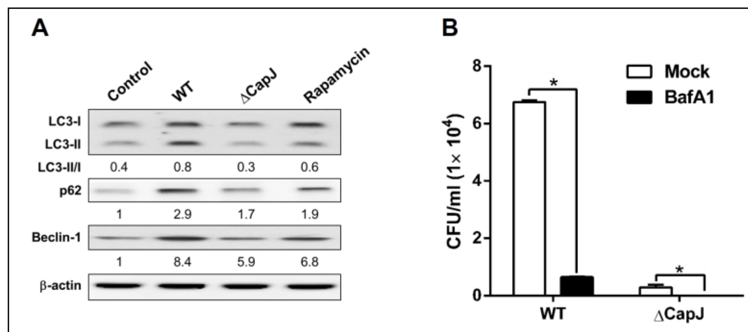

Figure 3. *H. pylori* CGT promotes autophagy maturation and increases bacterial burden in cells. Cells were infected with WT or *capJ*-knockout (ΔCapJ) *H. pylori* for 6 h. (A) The expression of autophagy-related proteins was assessed by western blot analysis. (B) The viable bacteria were determined by gentamicin-protection assay. *, $P < 0.05$.

C. Experimental designs

Activation of HMGB1 is known to induce autophagy in several cell types [28]. A recent study by using conditional ablation of HMGB1 in mice reveals that HMGB1 is required for the initiation of autophagy process [29]. *H. pylori* infection induces HMGB1 expression, suggesting that autophagy is involved in this context. It has also been reported that autophagy regulates the release of HMGB1 in some cell types [30]. However, discrepancies are emerged and showed that HMGB1 acts as a powerful stimulator for autophagy activation [31,32]. Therefore, the mechanism of how *H. pylori* CGT triggers autophagy mediated through HMGB1 activation need to be verified.

Aim 1. Investigating *H. pylori* CGT induces autophagy that is mediated by HMGB1

We will further study the mechanism on how *H. pylori* CGT induces HMGB1 to influence autophagy. Therefore, we will determine the expression of effector molecules that are involved in autophagy pathway. AGS cells will infect with WT or ΔCapJ *H. pylori* for 6 h. The expression of autophagy-associated proteins, including LC-3 I/II, Beclin1, p62/SQSTM1, Atg5, Atg12, and phospho-p70S6 kinase, will be analyzed by western blotting. Our hypothesis will be supported if

we observe increased levels of the expression of autophagy-associated proteins in WT *H. pylori*-infected cells compared to those that are infected by ΔCapJ.

Aim2. Exploring CGT influences autophagy flux mediated through activation of HMGB1

During autophagy, the formation of acidic vesicular organelles (AVO) increases, which can be stained by acridine orange (AO) [33]. AGS cells will be infected with WT *H. pylori* or ΔCapJ mutant at MOI of 100 for 6 h. The cells will be stained with AO (1 μg/ml) for 15 min to visualize AVO. To further observe the process from early to late stages of autophagosome formation, the cells will be probed using a Cyto-ID autophagy detection kit (Enzo Life Sciences) or antibody against p62/SQSTM1. The stained cells will be analyzed under a confocal laser-scanning microscope (Zeiss LSM 780; Carl Zeiss) with a 63× objective (oil immersion, aperture 1.3). We expect to observe an increase of the formation of autophagosomes in cells infected with WT *H. pylori* than that infected with ΔCapJ mutant.

Aim3. Exploring *H. pylori* CGT induces autophagy to increase bacterial survival

We will determine whether *H. pylori* survival in cells is dependent upon CGT-triggered autophagy process. AGS cells will be treated with 100 nM bafilomycin A1 (BafA1) for 24 h, follows by infection with WT *H. pylori* or ΔCapJ mutant at an MOI of 100 for an additional 6 h. The intracellular survival of the bacteria will be determined by gentamicin-protection assay. If CGT is crucial for inducing autophagy, inhibition of autophagy by BafA1 will show a decrease in WT *H. pylori* survival in cells. Similar results will be observed for the isogenic ΔCapJ mutant, which will show a reduced survival activity after BafA1 treatment.

D. Anticipated results

1. Our hypothesis will be supported if we observe the increased levels of HMGB1 expression along with autophagy-associated proteins in cells after infection with WT *H. pylori*. However, the increase will be less noticeable in ΔCapJ mutant-infected cells. In addition, the process of *H. pylori* CGT-induced HMGB1 expression and autophagy activation will be clearly elucidated.

2. We expect that bacterial survival of WT *H. pylori* will be reduced in autophagy-related gene-knockdown cells as compared with the survival observed in shVector controls. By carrying out the studies, we will demonstrate that CGT is essential for *H. pylori*-induced autophagosome maturation and preventing the fusion with lysosomes, which will be crucial for increasing bacterial burden in gastric epithelial cells.

References

[1] Wroblewski LE, Peek RM, Jr., Wilson KT. *Helicobacter pylori* and gastric cancer: factors that modulate disease risk. (2010) *Clin Microbiol Rev* **23**, 713-739.

[2] Amieva MR, El-Omar EM. Host-bacterial interactions in *Helicobacter pylori* infection. (2008) *Gastroenterology* **134**, 306-323.

[3] Wunder C, Churin Y, Winau F, Warnecke D, Vieth M, Lindner B, et al. Cholesterol glucosylation promotes immune evasion by *Helicobacter pylori*. (2006) *Nat Med* **12**, 1030-1038.

[4] Marino G, Niso-Santano M, Baehrecke EH, Kroemer G. Self-consumption: the interplay of autophagy and apoptosis. (2014) *Nat Rev Mol Cell Biol* **15**, 81-94.

[5] Greenfield LK, Jones NL. Modulation of autophagy by *Helicobacter pylori* and its role in gastric carcinogenesis. (2013) *Trends Microbiol* **21**, 602-612.

[6] Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. (2011) *Cell Death Differ* **18**, 571-580.

[7] Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. (2008) *Nature* **451**, 1069-1075.

[8] Zhao Z, Fux B, Goodwin M, Dunay IR, Strong D, Miller BC, et al. Autophagosome-independent essential function for the autophagy protein Atg5 in cellular immunity to intracellular pathogens. (2008) *Cell Host Microbe* **4**, 458-469.

[9] Yang JC, Chien CT. A new approach for the prevention and treatment of *Helicobacter pylori* infection via upregulation of autophagy and downregulation of apoptosis. (2009) *Autophagy* **5**, 413-414.

[10] Wang YH, Wu JJ, Lei HY. The autophagic induction in *Helicobacter pylori*-infected macrophage. (2009) *Exp Biol Med (Maywood)* **234**, 171-180.

[11] Bustin M, Lehn DA, Landsman D. Structural features of the HMG chromosomal proteins and their genes. (1990) *Biochim Biophys Acta* **1049**, 231-243.

[12] Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMG-1 as a late mediator of endotoxin lethality in mice. (1999) *Science* **285**, 248-251.

[13] Yang H, Wang H, Tracey KJ. HMG-1 rediscovered as a cytokine. (2001) *Shock* **15**, 247-253.

[14] Yang H, Wang H, Czura CJ, Tracey KJ. The cytokine activity of HMGB1. (2005) *J Leukoc Biol* **78**, 1-8.

[15] Huttunen HJ, Kuja-Panula J, Rauvala H. Receptor for advanced glycation end products (RAGE) signaling induces CREB-dependent chromogranin expression during neuronal differentiation. (2002) *J Biol Chem* **277**, 38635-38646.

[16] Sappington PL, Yang R, Yang H, Tracey KJ, Delude RL, Fink MP. HMGB1 B box increases the permeability of Caco-2 enterocytic monolayers and impairs intestinal barrier function in mice. (2002) *Gastroenterology* **123**, 790-802.

[17] Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, et al. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. (2000) *J Exp Med* **192**, 565-570.

[18] Birmingham CL, Smith AC, Bakowski MA, Yoshimori T, Brumell JH. Autophagy controls *Salmonella* infection in response to damage to the *Salmonella*-containing vacuole. (2006) *J Biol Chem* **281**, 11374-11383.

[19] Nakagawa I, Amano A, Mizushima N, Yamamoto A, Yamaguchi H, Kamimoto T, et al. Autophagy defends cells against invading group A *Streptococcus*. (2004) *Science* **306**, 1037-1040.

[20] Deretic V, Levine B. Autophagy, immunity, and microbial adaptations. (2009) *Cell Host Microbe* **5**, 527-549.

[21] Ouimet M, Koster S, Sakowski E, Ramkhelawon B, van Solingen C, Oldebeken S, et al. *Mycobacterium tuberculosis* induces the miR-33 locus to reprogram autophagy and host lipid metabolism. (2016) *Nat Immunol*.

[22] Dortet L, Mostowy S, Samba-Louaka A, Gouin E, Nahori MA, Wiemer EA, et al. Recruitment of the major vault protein by InlK: a *Listeria monocytogenes* strategy to avoid autophagy. (2011) *PLoS Pathog* **7**, e1002168.

[23] Lin HJ, Hsu FY, Chen WW, Lee CH, Lin YJ, Chen YY, et al. *Helicobacter pylori* Activates HMGB1 Expression and Recruits RAGE into Lipid Rafts to Promote Inflammation in Gastric Epithelial Cells. (2016) *Front Immunol* **7**, 341.

[24] Du SY, Wang HJ, Cheng HH, Chen SD, Wang LH, Wang WC. Cholesterol glucosylation by *Helicobacter pylori* delays internalization and arrests phagosome maturation in macrophages. (2014) *J Microbiol Immunol Infect*.

[25] Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. (2012) *Autophagy* **8**, 445-544.

[26] Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. (2011) *Nature* **469**, 323-335.

[27] Allen LA, Schlesinger LS, Kang B. Virulent strains of *Helicobacter pylori* demonstrate delayed phagocytosis and stimulate homotypic phagosome fusion in macrophages. (2000) *J Exp Med* **191**, 115-128.

[28] Zhang Q, Kang R, Zeh HJ, 3rd, Lotze MT, Tang D. DAMPs and autophagy: cellular

adaptation to injury and unscheduled cell death. (2013) *Autophagy* **9**, 451-458.

[29] Yanai H, Matsuda A, An J, Koshiba R, Nishio J, Negishi H, et al. Conditional ablation of HMGB1 in mice reveals its protective function against endotoxemia and bacterial infection. (2013) *Proc Natl Acad Sci U S A* **110**, 20699-20704.

[30] Thorburn J, Horita H, Redzic J, Hansen K, Frankel AE, Thorburn A. Autophagy regulates selective HMGB1 release in tumor cells that are destined to die. (2009) *Cell Death Differ* **16**, 175-183.

[31] Messer J, Chang E. Intracellular HMGB1: defender of client proteins and cell fate. (2015) *Oncotarget* **6**, 8432-8433.

[32] Tang D, Kang R, Livesey KM, Cheh CW, Farkas A, Loughran P, et al. Endogenous HMGB1 regulates autophagy. (2010) *J Cell Biol* **190**, 881-892.

[33] Yu L, Tumati V, Tseng SF, Hsu FM, Kim DN, Hong D, et al. DAB2IP regulates autophagy in prostate cancer in response to combined treatment of radiation and a DNA-PKcs inhibitor. (2012) *Neoplasia* **14**, 1203-1212.