# 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：

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|---|---|---|---|---|---|
| 本申請案所需經費(單選) | ■A 類(執行計畫所需經費)<br>☐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<br>☐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 | Statin 誘導自噬作用之機制 | | | |
| | 英文 | The mechanism of statin in inducing autophagy | | | |
| 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 107 年 1 月 1 日起至民國 107 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) | | | |
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| 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 | 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

Statins, inhibitors of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, commonly known as are widely prescribed for lowering serum cholesterol. Activation of 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 activates autophagy pathway and reduces *H. pylori* burden in macrophages that mitigates inflammatory response. This study combined a molecular-based study will reveal that statin use is a feasible approach to prevent *H. pylori*-associated gastrointestinal diseases.



**Keywords:** *Helicobacter pylori*, statin, autophagy, cholesterol

## A. Background and significance

### *Helicobacter pylori* infection and pathogenesis

Persistent *H. pylori* infection is associated with several gastroenterological illnesses including gastritis, peptic ulcer, and gastric adenocarcinoma (Wroblewski, et al., 2010). *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 (Lai, et al., 2013). 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) (Amieva & El-Omar, 2008). 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 (Wunder, et al., 2006).

### Statins reduce the risk of bacterial infections

The inhibitors of HMG-CoA reductase, commonly known as statins, are widely prescribed for lowering serum cholesterol (Armitage, 2007). In addition, statins are shown to reduce the risk of severe bacterial infections, including *Chlamydia pneumonia* (Erkkila, et al., 2005), *Clostridium difficile* (Motzkus-Feagans, et al., 2012), *Streptococcus pneumonia* (Boyd, et al., 2012), and *Staphylococcus aureus* (Chow, et al., 2010). However, the immunomodulatory properties of statins only partly explain the potential anti-infection mechanism (Jain & Ridker, 2005).

### Role of autophagy in anti-microbial activity

The human immune system has various mechanisms for opposing bacterial infections. Autophagy may contribute to immune defense by degrading invading pathogens (Mizushima, et al., 2008; Zhao, et al., 2008), indicating that stimulating cellular autophagy may reduce *H. pylori* pathogenesis (Yang & Chien, 2009). Antimicrobial agents are the most effective means of eradicating *H. pylori* infection, particularly a triple therapy regimen consisting of a proton-pump inhibitor, amoxicillin, and clarithromycin (O'Connor, et al., 2013). Although the cure rate varies in different countries, the triple therapy regimen remains the recommended treatment for *H. pylori* infection (O'Connor, et al., 2013). Studies have reported that combination treatments, including triple therapies and prescribed with statins, accelerate the clearance of *H. pylori* and reduce *H. pylori*-related inflammation (Tariq, et al., 2007; Yamato, et al., 2007; Nseir, et al., 2012), suggesting that statins may influence the immune response, leading to upregulate autophagy and attenuate *H. pylori*-induced inflammation. However, the molecular mechanisms underlying the regulating effects of statins on *H. pylori*-induced pathogenesis require further investigation.

## B. Specific aims

Cholesterol-rich rafts play a crucial role in *H. pylori*-induced pathogenesis and its progression to peptic ulcer diseases and gastric cancer (Gupta, et al., 2008; Zeaiter, et al., 2008). Given the evidence from our recent studies that statins, the rate-limiting enzyme in cholesterol biosynthesis, reduced *H. pylori*-induced pathogenesis of gastric epithelial cells and exhibited reduced risk of gastric cancer (Lin, et al., 2016), this proposal will explore the molecular mechanism underlying the conversion. We will further investigate how statin regulates the autophagy pathway to manipulate immune response against *H. pylori* in macrophages by using both cell-based and animal models. We will also examine the effect of statins on the risk of *H. pylori*-associated gastric disorders using the nationwide population-based case-control study. This study, combination of a molecular basis study with clinical database analysis, will reveal the molecular mechanism how statins promotes autophagy pathway that decreases *H. pylori* burden in macrophages and therefore attenuates the incidence of *H. pylori*-related gastroenterological illnesses.

## C. Preliminary results

### Statin increases *H. pylori*-induced autophagy

Since treatment of cells with a cholesterol-lowering agent induces autophagy activation (Cheng, et al., 2006; de Chastellier & Thilo, 2006), we posit that statins may influence the immune response, leading to upregulate autophagy and attenuate *H. pylori*-induced inflammation. We therefore established a macrophage infection model to investigate the mechanisms which are involved in the inhibition of *H. pylori*-induced gastric inflammation by statins. Following the induction of autophagy, microtubule-associated protein light chain 3 (LC3) is converted from LC3-I to LC3-II, and the expression of LC3-II is considered a marker of autophagy (Klionsky, et al., 2012). As shown in Fig. 1, the upregulation of LC3-II expression is greater in *H. pylori*-infected Raw 264.7 cells than in uninfected cells. Additionally, an increase in LC3-II/LC3-I conversion is correlated with the elevating simvastatin treatment in *H. pylori*-infected cells. We further examine the autophagy-related proteins, beclin-1 and p62, which are known to participate in the initiation of autophagy with LC3-II (Kang, et al., 2011; Levine, et al., 2011). Our results show that after treatment of *H. pylori*-infected cells with simvastatin, the expression levels of beclin-1 and p62 are slightly upregulated in the concentrations of 5 and 10 μM (Fig. 1A). We then analyze the effects of simvastatin on peritoneal exudate macrophages (PEMs) which were isolated from C57BL/6 mice. As shown in Fig. 1B, the upregulation of LC3-II expression is greater in *H. pylori*-infected PEMs treated with simvastatin than in untreated cells.

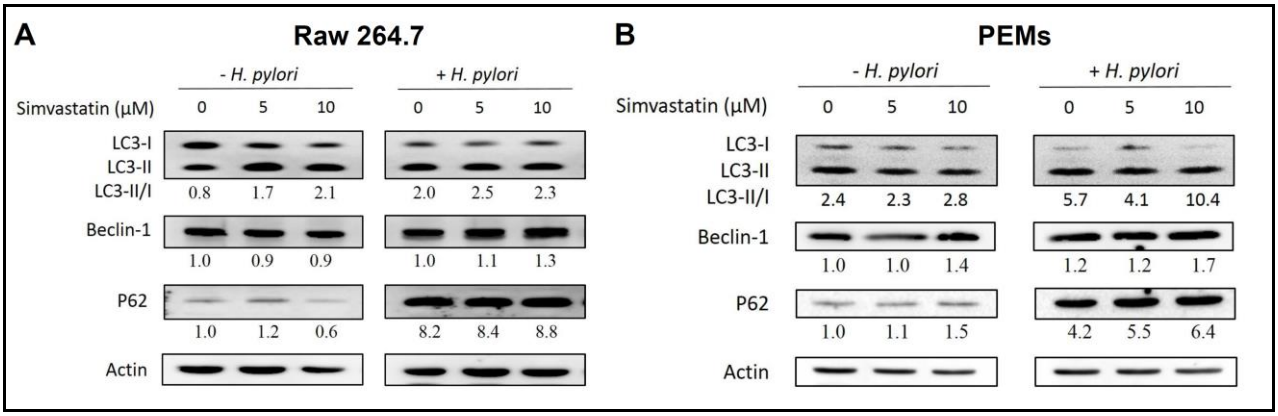


**Fig. 1. Statin promotes *H. pylori*-induced autophagy in macrophages.** (A) Raw 264.7 cells and (B) peritoneal exudate macrophages (PEMs) were pretreated with simvastatin (0, 5, 10 μM) for 8 h followed by infection of *H. pylori* for an additional 16 h. Cell lysates were prepared for detection of autophagy-associated proteins by using western blot analysis. Actin was used as the loading control. The expression level of each protein was quantified by signal intensity and indicated at the bottom of each lane.

### Statin enhances autophagosome formation

We further analyze the effects of simvastatin on macrophages by using immunofluorescence, and observe the formation of autophagosome by using Cyto-ID autophagy green dye. As shown in Fig.

2A, simvastatin-untreated cells and *H. pylori*-infected cells show faint Cyto-ID green fluorescence. However, in the cells treated with simvastatin follows by infection with *H. pylori*, exhibit significantly increased autophagy (punctate-formation) when compared to simvastatin-untreated cells (Fig. 2B). These results again demonstrate that simvastatin enhances *H. pylori*-induced autophagy in both Raw 264.7 cells and murine primary macrophages.

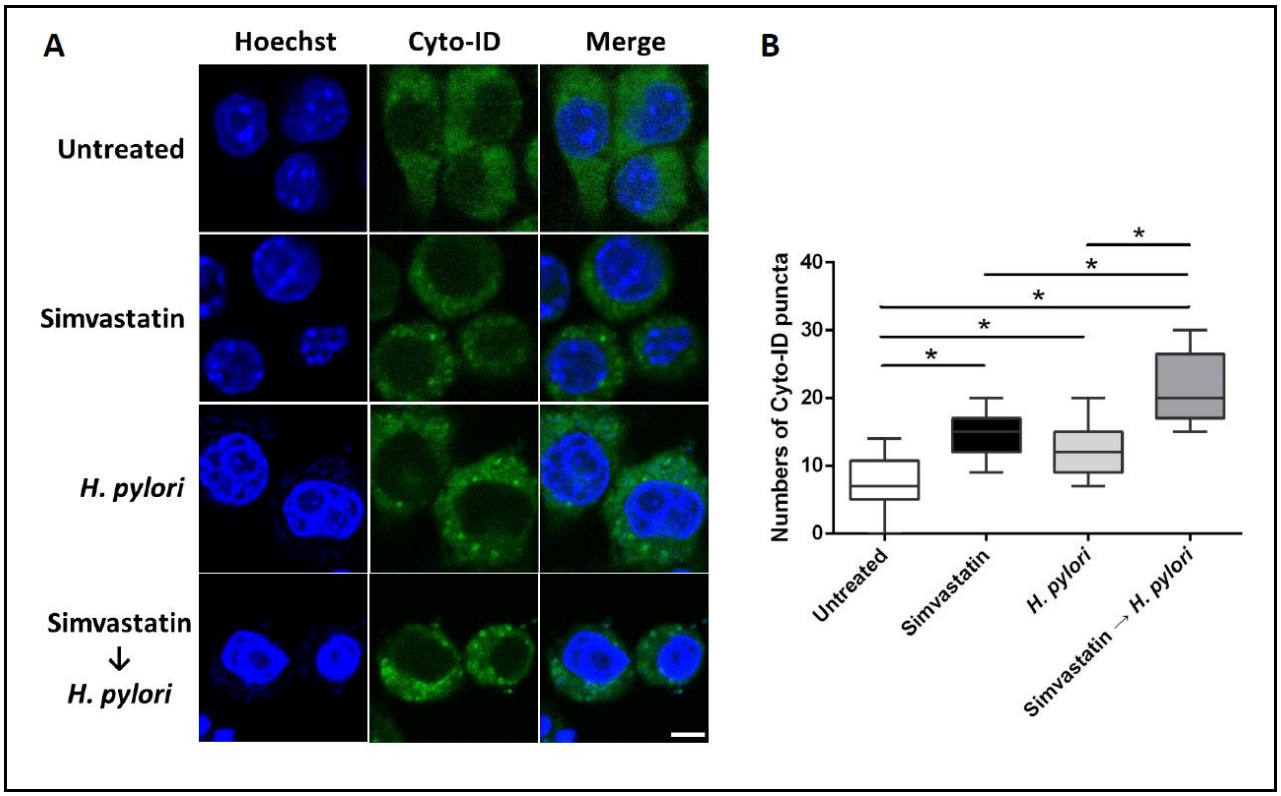


**Fig. 2. Statin enhances autophagosome formation in *H. pylori*-infected macrophages.** (A) Raw 264.7 cells were untreated or treated with simvastatin and infected with or without *H. pylori*. After incubation for 16 h, the cells were fixed and stained with Cyto-ID for detection autophagosome (green), and Heochst 33342 for visualizing the nucleus (blue). The stained cells were then analyzed by confocal microscopy. Scale bar, 5 μm. (B) The number of Cyto-ID puncta in each cell was counted and represented as box-plot. Fifty cells from each sample were counted for evaluation of Cyto-ID punctates. Statistical significance was evaluated using Student's *t*-test (*, $P<0.01$).

## D. research design and methods

The virulence factors 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 (Cheng, et al., 2006), by which reduces *H. pylori*-induced pathogenesis (Yang & Chien, 2009). 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 (Lai, et al., 2008; Lai, et al., 2011; Wang, et al., 2012). 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. **Investigating how statin influences autophagy in regulation of *H. pylori*-induced inflammation**

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 (Xu, et al., 2007), will be counted. This study will elucidate whether statin treatment increases autophagy, thus enhancing *H. pylori* resides in autophagosomes.

2. **Demonstrating statin facilitates autophagosome and lysosome fusion in *H. pylori*-infected cells**

We will further investigate whether statin promotes the fusion of autophagosome and lysosome in *H. pylori*-infected macrophages that may reduce the bacterial survival in the cells. Raw 264.7 cells will be treated or untreated with simvastatin and then will be infected with *H. pylori* for 24 h. *H. pylori*-infected cells will be fixed and probed with LAMP1 (Invitrogen) for detection of lysosomes and stained with Cyto-ID (Enzo Life Sciences) for detection autophagosomes. The fluorescence intensity of Cyto-ID and LAMP1, and the co-localization of autophagosomes and lysosomes will be analyzed by using a confocal microscope (Carl Zeiss). Alternatively, the formation of autophagy in simvastatin treated *H. pylori*-infected macrophages will be investigated and observed using transmission electron microscopy (TEM). We don't expect any major technical problem because we are experienced in most of techniques.

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.

4. **Exploring whether statin confers protection against *H. pylori* infection through autophagy pathway**

Small interfering RNA (siRNA) targets to several autophagy-related genes (*beclin-1*, *atg5* or *atg12*) or chemical inhibitors against autophagy will be employed to inhibit autophagy pathway.

The effects of the knockdown on macrophages will be examined after treatment with simvastatin. Raw 264.7 cells will be transfected with siRNA against target genes (ON-TARGET*plus*) and control siRNA (Thermo Fisher Scientific) by using Lipofectamine 2000 (Invitrogen). After transfection, cells will be treated various concentrations of simvastatin and will be infected with *H. pylori*. The expression levels of autophagy-related molecules (i.e. LC3, Atg proteins, and phospho-p70S6 kinase) will be analyzed by using western blot analysis. In addition, the bacterial survival in macrophages will be evaluated. Whether regulation of autophagy pathway by statin could provide the protection effect in *H. pylori* infection will be validated by using siRNA strategy. Alternatively, chemical inhibitors against autophagy activation can be used to confirm this trend.

## E. Anticipated results

The main goal of this proposal is to reveal molecular basis of how statins inhibit the *H. pylori* virulence factors in modulation immune responses and inflammations. Worth mentioning, in this proposal, we will validate this question by determining *H. pylori* and cholesterol interactions *in vitro*. We will integrate the scientist and physician who are major in infectious diseases, cell biology, and microbiology to form a research team for fundamental scientific problems on pathogen-host clinical issues. Studies from this proposal will demonstrate the use of statins may be able to attenuate *H. pylori*-induced pathogenesis in host stomachs and reduce the risk of gastrointestinal diseases as well.

## References


Amieva, M. R. & El-Omar E. M. (2008). Host-bacterial interactions in *Helicobacter pylori* infection. *Gastroenterology.* 134, 306-323.

Armitage, J. (2007). The safety of statins in clinical practice. *Lancet.* 370, 1781-1790.

Boyd, A. R., Hinojosa C. A., Rodriguez P. J. & Orihuela C. J. (2012). Impact of oral simvastatin therapy on acute lung injury in mice during pneumococcal pneumonia. *BMC Microbiol.* 12, 73.

Cheng, J., Ohsaki Y., Tauchi-Sato K., Fujita A. & Fujimoto T. (2006). Cholesterol depletion induces autophagy. *Biochem Biophys Res Commun.* 351, 246-252.

Chow, O. A., von Kockritz-Blickwede M., Bright A. T., Hensler M. E., Zinkernagel A. S., Cogen A. L., et al. (2010). Statins enhance formation of phagocyte extracellular traps. *Cell Host Microbe.* 8, 445-454.

de Chastellier, C. & Thilo L. (2006). Cholesterol depletion in *Mycobacterium avium*-infected macrophages overcomes the block in phagosome maturation and leads to the reversible sequestration of viable mycobacteria in phagolysosome-derived autophagic vacuoles. *Cell Microbiol.* 8, 242-256.

Erkkila, L., Jauhiainen M., Laitinen K., Haasio K., Tiirola T., Saikku P., et al. (2005). Effect of simvastatin, an established lipid-lowering drug, on pulmonary *Chlamydia pneumoniae* infection in mice. *Antimicrob Agents Chemother.* 49, 3959-3962.

Gupta, V. R., Patel H. K., Kostolansky S. S., Ballivian R. A., Eichberg J. & Blanke S. R. (2008). Sphingomyelin functions as a novel receptor for *Helicobacter pylori* VacA. *PLoS Pathog.* 4, e1000073.

Jain, M. K. & Ridker P. M. (2005). Anti-inflammatory effects of statins: clinical evidence and basic mechanisms. *Nat Rev Drug Discov.* 4, 977-987.

Kang, R., Zeh H. J., Lotze M. T. & Tang D. (2011). The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ.* 18, 571-580.

Klionsky, D. J. & Abdalla F. C. & Abeliovich H. & Abraham R. T. & Acevedo-Arozena A. & Adeli K., et al. (2012). Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy.* 8, 445-544.

Lai, C. H., Hsu Y. M., Wang H. J. & Wang W. C. (2013). Manipulation of host cholesterol by *Helicobacter pylori* for their beneficial ecological niche. *BioMedicine.* 3, 27-33.

Lai, C. H., Chang Y. C., Du S. Y., Wang H. J., Kuo C. H., Fang S. H., et al. (2008). Cholesterol depletion reduces *Helicobacter pylori* CagA translocation and CagA-induced responses in AGS cells. *Infect Immun.* 76, 3293-3303.

Lai, C. H., Wang H. J., Chang Y. C., Hsieh W. C., Lin H. J., Tang C. H., et al. (2011). *Helicobacter pylori* CagA-mediated IL-8 induction in gastric epithelial cells is cholesterol-dependent and requires the C-terminal tyrosine phosphorylation-containing domain. *FEMS Microbiol Lett.* 323, 155-163.

Levine, B., Mizushima N. & Virgin H. W. (2011). Autophagy in immunity and inflammation.

*Nature.* 469, 323-335.

Lin, C., Liao W., Lin H., Hsu Y., Lin C., Chen Y., et al. (2016). Statins attenuate *Helicobacter pylori* CagA translocation and reduce incidence of gastric cancer: *in vitro* and population-based case-control studies. *PLoS One.* Accpeted and in press.

Mizushima, N., Levine B., Cuervo A. M. & Klionsky D. J. (2008). Autophagy fights disease through cellular self-digestion. *Nature.* 451, 1069-1075.

Motzkus-Feagans, C. A., Pakyz A., Polk R., Gambassi G. & Lapane K. L. (2012). Statin use and the risk of *Clostridium difficile* in academic medical centres. *Gut.* 61, 1538-1542.

Nseir, W., Diab H., Mahamid M., Abu-Elheja O., Samara M., Abid A., et al. (2012). Randomised clinical trial: simvastatin as adjuvant therapy improves significantly the *Helicobacter pylori* eradication rate--a placebo-controlled study. *Aliment Pharmacol Ther.* 36, 231-238.

O'Connor, A., Molina-Infante J., Gisbert J. P. & O'Morain C. (2013). Treatment of *Helicobacter pylori* infection 2013. *Helicobacter.* 18 Suppl 1, 58-65.

Tariq, M., Khan H. A., Elfaki I., Arshaduddin M., Al Moutaery M., Al Rayes H., et al. (2007). Gastric antisecretory and antiulcer effects of simvastatin in rats. *J Gastroenterol Hepatol.* 22, 2316-2323.

Wang, H. J., Cheng W. C., Cheng H. H., Lai C. H. & Wang W. C. (2012). *Helicobacter pylori* cholesteryl glucosides interfere with host membrane phase and affect type IV secretion system function during infection in AGS cells. *Mol Microbiol.* 83, 67-84.

Wroblewski, L. E., Peek R. M., Jr. & Wilson K. T. (2010). *Helicobacter pylori* and gastric cancer: factors that modulate disease risk. *Clin Microbiol Rev.* 23, 713-739.

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

Xu, Y., Jagannath C., Liu X. D., Sharafkhaneh A., Kolodziejska K. E. & Eissa N. T. (2007). Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity.* 27, 135-144.

Yamato, M., Watanabe T., Higuchi K., Taira K., Tanigawa T., Shiba M., et al. (2007). Anti-inflammatory effects of pravastatin on *Helicobacter pylori*-induced gastritis in mice. *Dig Dis Sci.* 52, 2833-2839.

Yang, J. C. & Chien C. T. (2009). A new approach for the prevention and treatment of *Helicobacter pylori* infection via upregulation of autophagy and downregulation of apoptosis. *Autophagy.* 5, 413-414.

Zeaiter, Z., Cohen D., Musch A., Bagnoli F., Covacci A. & Stein M. (2008). Analysis of detergent-resistant membranes of *Helicobacter pylori* infected gastric adenocarcinoma cells reveals a role for MARK2/Par1b in CagA-mediated disruption of cellular polarity. *Cell Microbiol.* 10, 781-794.

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