財團法人明日醫學基金會專題研究計畫申請書

本申請案所需經費(單選)	☑A 類(研究主持費及執行計畫所需經費) ☐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				
計畫歸屬	☐自然處　☐工程處　☑生物處　☐人文處　☐科教處　☐永續會				
申請機構/系所(單位)	中國醫藥大學 醫學系 微生物學科				
本計畫主持人姓名	賴志河	職稱	助理教授	身分證號碼	
本計畫名稱 中文	幽門螺旋菌抑制免疫細胞發炎反應之研究				
本計畫名稱 英文	Investigation of suppression of immune responses by *Helicobacter pylori*				
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 97 年 2 月 1 日起至民國 97 年 7 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)			
	BI				
研究性質	☑純基礎研究　☐導向性基礎研究　☐應用研究　☐技術發展				
本年度申請主持國科會各類研究計畫(含預核案)共 1 件。(共同主持之計畫不予計入) 本件在本年度所申請之計畫中優先順序(不得重複)為第 1 。					
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。				
本計畫是否有進行下列實驗：(勾選下列任一項，須附相關實驗之同意文件) ☐人體實驗　☑基因重組實驗　☐動物實驗					
計畫連絡人	姓名：賴志河　電話：(公) 04-22053366 ext 2171　(宅/手機) 0937-936212				
通訊地址	台中市學士路 91 號 醫學系 微生物學科				
傳真號碼	04-22053764	E-MAIL	chl@mail.cmu.edu.tw		

十一、研究計畫中英文摘要：請就本計畫要點作一概述，並依本計畫性質自訂關鍵詞。

（一） 計畫中文摘要。（五百字以內）

幽門螺旋菌被發現是一種重要持續性感染的致病菌已經有二十年了。在大多數感染的個體中，幽門螺旋菌僅導致慢性胃發炎而且通常是無症狀的。但如果是長期持續性的感染，有部分個體可能會引起消化性潰瘍，甚至有些患者還會導致胃癌或淋巴瘤。不論是在非吞噬細胞或吞噬細胞當中，當幽門螺旋菌具有細胞內生存能力的時候，通常會引起細菌在胃黏膜的持續性感染並且導致慢性胃發炎。這可能與幽門螺旋菌刺激巨噬細胞表現誘導性的一氧化氮合成酶有關(inducible nitric oxide synthase, iNOS)。而一氧化氮(nitric oxide, NO)的產生被認為在幽門螺旋菌引起胃黏膜免疫反應與發炎反應中扮演重要的角色。在許多不同型態的細胞皆可被細菌的脂多醣體(lipopolysaccharide, LPS)的刺激而調節 iNOS 表現。但是，有些致病菌不但可以逃避巨噬細胞的吞噬作用而且還會快速殺死細胞。我們先前的研究已探討 VacA (vacuolating cytotoxin A)與 CagA (cytotocin-associated gene A)對胃上皮細胞的分子致病機轉。然而幽門螺旋菌感染巨噬細胞的分子機制仍然有許多不明之處。本多年期研究計畫的目的最主要是探討幽門螺旋菌侵入巨噬細胞的詳細過程，並且分析一些細菌的致病因子與引起細胞致病的細胞內分子訊息之關係。具體的研究目的包括：(一)研究幽門螺旋菌感染巨噬細胞的過程、(二)探討 CagA 對於抑制 NO 產生的的重要性、(三)探討脂質筏在 CagA 誘導巨噬細胞訊息傳遞所扮演的角色。當然還包括偵測一些與細胞死亡有關的細胞激素及細胞內訊息傳遞的分子。這些基礎研究不但可以使我們更清楚瞭解幽門螺旋菌與宿主細胞之相互作用，而且將來在臨床上，更可以促進發展新的策略來防止細菌的持續性感染。

關鍵詞：幽門螺旋菌、巨噬細胞、一氧化氮、脂質筏

（二） 計畫英文摘要。（五百字以內）

H. pylori has emerged as an important persistent bacterial pathogen during the past two decades. In most infected individuals, *H. pylori* infection causes superficial chronic gastritis, which usually showed clinical asymptomatic. During the long-term colonization, some proportions of infected people are at risk of development into gastric and duodenal ulcers, while others may even proceed into gastric cancer or lymphoma. The ability of the *H. pylori* to survive intracellularly within non-phagocytes and phagocytes is postulated to enhance the persistence of this pathogen in the gastric mucosa and cause chronic inflammation. *H. pylori* might induce macrophage inducible nitric oxide synthase (iNOS) expression. Nitric oxide (NO) production plays an important role in the gastric mucosal immune response to *H. pylori* and the associated inflammation. The expression of iNOS is regulated in various cell types and can be enhanced by stimulating the cells with bacterial lipopolysaccharide (LPS). In some pathogens, which the ability to evade macrophage killing involves inducing rapid macrophage cell death. We and others have previously investigated the molecular pathogenesis of VacA (vacuolating cytotoxin A) and CagA (cytotoxin-associated gene A) in gastric epithelial cells. However, the molecular basis how *H. pylori* infect macrophages still remains largely unclear. Our long-term goal is to investigate the infection processes of *H. pylori* into macrophages in detail, several virulence factors involved in bacterial pathogenesis will be studied. The specific aims are to: (i) study the infection process of *H. pylori* in cultured macrophages, (ii) determine the role of CagA in suppressing antimicrobial NO production, and (iii) investigate the role of lipid rafts in CagA-induced signaling in macrophages. This proposal will include to detect inflammatory cytokines released by macrophages during *H. pylori* infection and to determine the expression of intracellular molecules involved in cell death**.** The fundamental understanding of *H. pylori*-macrophage interactions will greatly facilitate the development of new strategies to prevent bacterial persistent infection in clinical therapy.

Keywords: *Helicobacter pylori*, macrophages, nitric oxide, and lipid rafts

十二、研究計畫內容：

（一）近五年之研究計畫內容與主要研究成果說明。（連續性計畫應同時檢附上年度研究進度報告）

（二）研究計畫之背景及目的。請詳述本研究計畫之背景、目的、重要性及國內外有關本計畫之研究情況、重要參考文獻之評述等。本計畫如為整合型研究計畫之子計畫，請就以上各點分別述明與其他子計畫之相關性。

（三）研究方法、進行步驟及執行進度。請分年列述：1.本計畫採用之研究方法與原因。2.預計可能遭遇之困難及解決途徑。3.重要儀器之配合使用情形。4.如為整合型研究計畫，請就以上各點分別說明與其他子計畫之相關性。5.如為須赴國外或大陸地區研究，請詳述其必要性以及預期成果等。

（四）預期完成之工作項目及成果。請分年列述：1.預期完成之工作項目。2.對於學術研究、國家發展及其他應用方面預期之貢獻。3.對於參與之工作人員，預期可獲之訓練。4.本計畫如為整合型研究計畫之子計畫，請就以上各點分別說明與其他子計畫之相關性。

（一）近五年之研究計畫內容與主要研究成果說明。（連續性計畫應同時檢附上年度研究進度報告）

1. The presence of babA2 and cagA in H. pylori clinical isolates

Helicobacter pylori isolates possess unusually high genetic heterogeneity and diversify between geographic regions. Certain *H. pylori* genotypes are indicated to relate to severe gastrointestinal diseases. We have been interested in the genetic studies of several virulence factors, particularly *vacA*, *cagA*, and *babA2*. In our previous study, two virulence markers, *cagA* and *babA2*, were characterized by PCR in 101 *Helicobacter pylori* isolates from a population in Taiwan. *cagA* was detected in 99% of the isolates, while *babA2* was present in all of the isolates. Base deletions and substitutions at the forward *babA2* primer annealing sites were found (20).

2. Lower prevalence of H. pylori infection with vacAs1a, cagA-positive, and babA2-positive genotype in erosive reflux esophagitis disease

We have been interested in the correlation of *H. pylori* virulence factors (i.e *vacA*, *cagA*, and *babA2*) with clinical outcomes. Increased prevalence of esophagitis has been recognized in the West. *H. pylori* infection, particularly virulent strains, is proposed as a protective factor against the development of gastroesophageal reflux disease. Analysis of these relationships shows that esophagitis occurred in a significantly lower rate among *H. pylori*-positive patients with peptic ulcer than those without peptic ulcer. We found that *cagA*, *babA2*, and *vacAs1a* were detected in 100% of 143 isolates. This analysis suggests significantly lower incidence of *H. pylori* infection with the triple-positive virulent genotype in patients with reflux esophagitis in Taiwan (21).

3. The association of antibiotic resistance and higher internalization activity in resistant H. pylori isolates

The current recommended eradication therapies of *H. pylori* consist of a proton pump inhibitor, clarithromycin and amoxicillin or metronidazole, twice daily for a week straight. By use of these treatments, some reports suggest that the cure rate is more than 90%. However, an eradication failure rate ranging from

20-40% is usually found (8, 12, 13). We have been determined *H. pylori* primary resistance and its clinical impact on the efficacy of two lansoprazole-based eradication triple therapies (LAC and LMC). The results show that one-week LAC and LMC are similarly effective therapies. Clarithromycin resistance significantly affected *H. pylori* eradication in both regimens (33).

A new mechanism of bacteria evading antibiotics treatments was addressed where in intracellular *H. pylori* was inaccessible to antibiotics (26). Some reports have also shown that intracellular *H. pylori* could survive in the cytoplasmic vacuoles (1, 3). This intra-vacuolar niche can potentially function as a sanctuary and sustain the survival of bacteria. It is possible that invading into the cells and residing in the multi-vesicular bodies may facilitate the survival of *H. pylori* during antibiotics treatment. To this end, we have been working on characterization and comparison of the strains isolated from the therapy cured and failed patients before and after treatment. We have recently demonstrated that bacterial epithelial internalization may facilitate the survival of *H. pylori* during antibiotics first-line therapy. Moreover, we have characterized the antibiotic resistance of *H. pylori* clinical isolates from patients who failed in therapy before and/or after therapy (19).

（二）研究計畫之背景及目的。請詳述本研究計畫之背景、目的、重要性及國內外有關本計畫之研究情況、重要參考文獻之評述等。本計畫如為整合型研究計畫之子計畫，請就以上各點分別述明與其他子計畫之相關性。

Background and significance

Successful H. pylori infection in stomach

H. pylori was first isolated in pure culture and shown to be associated with severe gastrointestinal diseases in the 1980s (43, 44). *H. pylori* is a spiral-shaped Gram negative bacterium with polar flagella and with powerful urease activity (6). These features enable *H. pylori* to colonize in the gastric mucosa. Subpopulation of bacteria was thought to penetrate across the mucosal layer that covers and protects the gastric epithelial cells (46). Persistent infection with *H. pylori* within human gastric mucosa usually causes several types of gastrointestinal diseases. Recent researches have focused on how several virulence factors of *H. pylori* are invovled in pathogenesis, developing into different clinical sequelea in a specific host niche.

H. pylori virulence factors in pathogenesis

H. pylori contains a set of virulence factors that can make it to survive, multiply, escape from immune surveillance, and eventually cause damage in a special niche of cells. Although gastric mucosa is well protected against other bacterial infection, *H. pylori* is highly adapted to its ecological niche. These manners that support the colonization and persistence of *H. pylori* in the gastric mucus comprise polar flagella, a potent urease, adhesins BabA and SabA, and two major virulence factors CagA and VacA (Table 1).

Table 1. Functions of *H. pylori* virulence factors

Virulence factors	Functions
BabA (blood group antigen-binding adhesin)	(1) Bind to surface mucous cells and their receptors of fucosylated Lewis b (Le^b) (2) Associated with peptic ulcer and gastric adenocarcinoma
VacA (vacuolating cytotoxin)	(1) Induces apoptosis (2) Involved in immunomodulation (3) Colonization of mouse stomach
CagA (cytotoxin-associated gene)	(1) Translocated into host cell by type IV secretion apparatus encoded on *cag*-PAI (2) Phosporylated in host cell (3) Disrupts tight junctions

There are two outer membrane proteins for *H. pylori* adhering of cells, BabA2 (blood group antigen-binding adhesin) (26) and SabA (sialic acid binding adhesion) (40), which bind to surface mucous

cells and their receptors of fucosylated Lewis b (Le^b) and sialyl-Lewis x ($s\text{-}Le^x$), respectively. This promotes *H. pylori* to adhere to the cells and enable the bacteria to efficiently use its secretion systems for delivery of pathogenic mediators to modulate the host. The vacuolating cytotoxin, VacA, is one of the major virulence factors of *H. pylori*. VacA is secreted from the bacteria and is found in the culture supernatant (39, 53), but remains a large portion on the bacterial surface (27). When *H. pylori* colonizing on cells, the bacterial surface-contacted VacA transfers directly from bacteria to the host cells, subsequently followed by uptake and intoxication. Recently, VacA was also reported to inhibit T cell proliferation, suggesting that *H. pylori* might evade the adaptive immune response of host (20). Another major virulence factor of *H. pylori* is the *cag*-pathogenicity island (*cag*-PAI)-encoded type four secretion system (TFSS), which mediates the translocation of CagA (cytotoxin-associated gene) into host cells (53, 65). After injection, CagA is subsequently phosphorylated at one or more tyrosine phosphorylation motifs (EPIYA) and induced host cell pathogenesis. In summary, the above statements discuss the strategies used by *H. pylori* during persistent infections, which allow them to colonize specific sites in the host, then evade immune surveillance, and finally cause host chronic diseases.

Composition of lipid rafts and association of infectious microbes

The structure of lipid rafts is known to be stabilized in the cold in non-ionic detergents such as Triton X-100 (9). After treatment of membrane with cold Triton X-100, insoluble components including lipids and proteins remain in the form of detergent-resistant membrane (DRM), also called detergent-insoluble glycolipid-enriched membrane (DIG), that were considered to be in the lipid rafts (67). Not only cholesterol and phospholipids were consisted in the lipid rafts, but glycosylphosphotidylinositol (GPI) anchored proteins, doule-acylated proteins, and palmitoylated proteins are abundant in rafts (8, 25). It is thought that cholesterol exists in intact cell membranes between rafts and non-rafts phases. In the rafts, cholesterol serves as a spacer for sphingolipids and functions as a dynamic glue that keeps the lipids and proteins assembly together (68). After depletion of cholesterol by methyl-β-cyclodextrin (MβCD), raft-associated proteins and lipids can be dissociated and rendered them nonfunctional (66).

Lipid rafts are not only a dynamic structure on cell membrane but also provide an amplified signaling for the activation of the cells. Those are also known to invaginate the cell membrane and lead to endocytosis mediated through different clathrin-coated internalization (54). Recent studies have demonstrated that lipid rafts might serve as a platform for an entry portal of pathogens, including several bacteria (15, 23, 33), viruses (41, 56), and prions (66). There are two possible advantages 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 entry of bacteria (42). Hence, pathogens might favor interact to lipid rafts as a potential route to enter host cells.

The most interesting example of rafts as a bacterial entry portal is *Shigella flexneri*, which uses the bacterial invasin IpaB interacting with the rafts-associated CD44 within specialized membrane microdomain (33). Moreover, *Shigella* entry is impaired after depletion of cholesterol by methyl-β-cyclodextrin (MβCD). Type 1 fimbriated *E. coli* was also found associated with caveolae and lipid raft components (15). Likewise, bacterial invasion was inhibited by lipid raft disrupting or usurping agents. Some bacteria capitalize on an amplified host inflammatory response by co-opting raft signaling. For instance, *Pseudomonas aeruginosa,*

those rafts are platform for bacteria attack, but also for host counterattack (23).

Significance of H. pylori interaction with lipid rafts

As above described, lipid rafts are platforms of several bacterial toxins. VacA is reported to use lipid rafts microdomains for its assembly on the cell membrane and intracellular delivery (60). Recent reports revealed that depletion of membrane cholesterol significantly reduces the entry of VacA into target cells (31, 60, 64). Our previous study also indicated that GPI-anchor protein, fasciclin I, was required for internalization of VacA, but did not affect the binding of VacA to lipid rafts (31). Another report also revealed that receptor-dependent translocation of VacA to lipid rafts is critical for signaling pathways leading to p38 MAP kinase/ATF-2 activation and vacuolation (50).

More recent report showed that *H. pylori* follows a cholesterol gradient and extracts the lipid from cytoplasmic membranes of epithelial cells for subsequent glucosylation (76). In addition, the authors also identified the gene HP0421 as encoding the enzyme cholesterol-α-glucosyltransferase responsible for cholesterol glucosylation. According to these reports, rafts might have multiple functions for signaling that can be an entry portal for bacteria and that are essential for host protection.

H. pylori evasion of innate immune responses

H. pylori infection causes chronic gastritis associated with gastric inflammatory and local immune response. However, immune system was failed to eliminate the bacteria from gastric mucosa completely. The bacteria may select a safe niche on mucosa where they can reside and evade the immune system as well as the antibiotic. Some studies have demonstrated how the bacteria achieve the long-term persistence. For many other chronic bacterial infections, invasion of cells is one of the major significance in the survival and repopulation of the pathogens. For instance, *Salmonella enterica* serovar Typhi causes persistent infection in reticulum endothelium system of the host, who may become a life-long carrier (46); type I fimbriated *E. coli* can invade into bladder epithelial cells and survive for many weeks in the bladders of mice (48, 62). These findings indicate that the intracellular bacteria were presumably inaccessible to immune cells of the host as well as antibiotics.

Little evidence suggests that *H. pylori* is a dominant intracellular pathogen. However, the infection models *in vitro* and *in vivo* have shown that *H. pylori* could survive and replicate in the intracellular niche (5, 30, 74). In one study, the authors showed delayed uptake of *H. plyori* into macrophages followed by the formation of megasomes as a result of phagosome fusion (1). Previous study also represented that *H. pylori* can be internalization by phagocytosis into LAMP-1 containing phagosomes (32). Likewise, VacA-expressed *H. pylori* promotes the recruitment and retention of the tryptophan-aspartate-containing coat protein (TACO) by phagosomes, thereby disrupting the phagosome maturation in monocytes and promoting enhanced of survival of the bacteria (78). However, it is unclear how intracellular survival of *H. pylori* in macrophage contributes to disease pathogenesis.

Chronic gastritis is induced by mononuclear cell infiltrates, included of lymphocyte and macrophages. In the study of macrophages, which are major source of the IL-6 present in *H. pylori* induced chronic gastritis, are known to play a critical role in the pathogenesis of mucosal inflammations (61). It was also

reported that macrophages secretion increased levels of IL-1 and TNF-α in *H. pylori* infected gastric tissues (24, 77). Nevertheless, the interaction of macrophages with *H. pylori* bacteria or their virulence factors in such interactions are poor understood. Another bactericidal agent of macrophages, nitric oxide (NO) is generated by nitric oxide synthase (NOS)-mediated conversion of L-arginine to L-citrulline. However, *H. pylori* can survive despite marked induction of inducible NOS in macrophages. In a recent study, the authors reported that *H. pylori* prevents NO production by the bacterial cell envelope gene *rocF*, which encoded an arginase and competed with NOS (22). Mutation of the *rocF* gene results in efficient killing of *H. pylori* in an NO-dependent manner, suggesting that arginase might be important for protection of macrophages. Thus, *H. pylori* modulates intracellular signaling pathways in a number of cell types *in vitro* and *in vivo*. However, neither the induction/protection of cell death signaling pathways nor the bacterial factors involved have been fully determined. Therefore, we will focus on how *H. pylori* triggers cell death of macrophage and to delineate the relationship of the bacterial factors and the intracellular signaling pathways involved in cell death.

In the light of the finding of above reports, we propose to extend our previous works and study the interactions between *H. pylori* and host cells. In this proposal, we will first focus our effort on *H. pylori* virulence factors in the bacterial internalization, cell death, and intracellular effects in the infection of macrophages. Furthermore, we wanted to identify which intracellular molecules were contributed to the internalization and induction or protection of cell death in *H. pylori* infection of macrophages. We will also investigate the role of lipid rafts in the protection of *H. pylori* by macrophage phagocytosis. Thus, results from these studies may elucidate new aspects for the pathogenesis of *H. pylori* in macrophages, and provide new approaches for prevention of *H. pylori* infection.

（三）研究方法、進行步驟及執行進度。請分年列述：1.本計畫採用之研究方法與原因。2.預計可能遭遇之困難及解決途徑。3.重要儀器之配合使用情形。4.如為整合型研究計畫，請就以上各點分別說明與其他子計畫之相關性。5.如為須赴國外或大陸地區研究，請詳述其必要性以及預期成果等。

Preliminary results

This proposal is a collaboration between the PI and Dr. Wen-Ching Wang (Department of Life Sciences, National Tsing Hua University) and Dr. Shih-Hua Fang (Department of Microbiology, School of Medicine, China Medical University). The collaboration takes advantage of the expertise of the PI in bacterial pathogenesis of *H. pylori* and the biochemical expertise of Dr. Wang in molecular genetic and protein structure and function of bacteria (11, 13), and the immunological expertise of Dr. Fang in T cell and macrophage regulations (16, 18). The overall goals are (**i) investigation the infection process of *H. pylori* in macrophage, (ii) characterization of the mechanism of CagA in the suppression of antimicrobial nitric oxide (NO) production, and (iii) identification and characterization role of lipid rafts in CagA induced signaling in macrophages.** We have prepared all of the experimental tools required to accomplish this proposal. Specifically, we have (i) generated various *H. pylori* virulence factor isogenic mutants, (ii) observed cellular invasion into epithelial cells by *H. pylori*, (iii) demonstrated that CagA, VacA, and BabA are associated with lipid rafts, (iv) visualized *H. pylori* are colocalized with lipid rafts by macrophage internalization, (v) characterized *H. pylori* inhibits lipopolysaccharide-induced macrophage nitric oxide production. The experiments done to accomplish each of these tasks are described in detail below.

Construction of *H. pylori* isogenic mutants— As a first step toward this goal, we have been generated *cagA* and/or *vacA* knocking out *H. pylori* strain 26695 by homologous recombination (73). Each recombinant plasmid, antibiotic cassette and PCR primers listed in Table 2. Western blot analysis was carried out to the abolished expression of each protein. The isogenic mutants generated in this proposal were listed in Table 3. All of these isogenic mutants will used in our following experiments and compared the results obtained with the wild type.

Table 2. Plasmids and primers used in this study

Plasmid	Description	Primer sequence (5'→3') or restriction sites
pUOA20	*cat* cassette	pUMfeI_F: TGCGTCAATTGGATTGAAAAGTGG
		pUMfeI_R: AGGACGCACAATTGTCGACAGAGA
		pUNheI_F: GAAAAGTGGCTAGCTTTATG
		pUNheI_R: CAGTGCGGCTAGCTGGGATTTT
pE194	Erythromycin resistant gene	pEXbaI_F: CAATAATCTAGACCGATTGCAGTATAA
		pEXbaI_R: GACATAATCGATCTAGAAAAAATAGGCACACG
pACYC177	Kanamycin resistant gene	pANheI_F: GGAAGATGCGCTAGCTGATCCTTCAAC
		pANheI_R: CCCGTCAAGCTAGCGTAATGCTCTGC
p62	*vacA* in pGEM-T	
pGEM-Cag	*cagA* in pGEM-T	Cag_F: ACAAAGAGGAAGCTCGAGATTTAAACC
		Cag_R: GGAAATATCCACGTCGACTTTAAAGAAC
p62-Cat	*cat* inserted in p62	*Mfe*I
pGEM-Cag -Km	Kanamycin resistant gene inserted in pGEM-Cag	*Nhe*I

Table 3. *H. pylori* strains used in this study

H. pylori strains	Description	Source
H. pylori 26695	Wild type, ATCC 700392	ATCC
△VagA	ATCC 700392, *vacA*::*cat*, Chloramphenicol resistant	This study
△CacA	ATCC 700392, *cagA*::*Km*^{r}, Kanamycin resistant	This study
△VacA△CagA	ATCC 700392, *vacA*::*cat* and *cagA*::*Km*^{r}	This study
△BabA	ATCC 700392, *babA*::*ery* and *babA*:: *Km*^{r}	This study
△SabA	ATCC 700392, *sabA*::*cat* and *sabA*:: *Km*^{r}	This study

Observation of cellular invasion into epithelial cells by *H. pylori*— To observe internalization behavior of *H. pylori* strains by cells, gastric epithelial cells AGS were grown on 12-mm round coverslips before being infected by centrifugation. After infection of *H. pylori*, cells were washed three times by PBS. For further fixation, the washed and fixed cells were treated with 2% OsO_4 and then dehydrated fellow by the processes for electron microscopy. Visualization of samples was carried out by a scanning electron microscope (Hitachi). We have observed that cell surface pedestals are seen when *H. pylori* were in tight contact with AGS cells (Figure 1). The epithelial cell model by SEM observation will be used in *H. pylori* infection with macrophages.

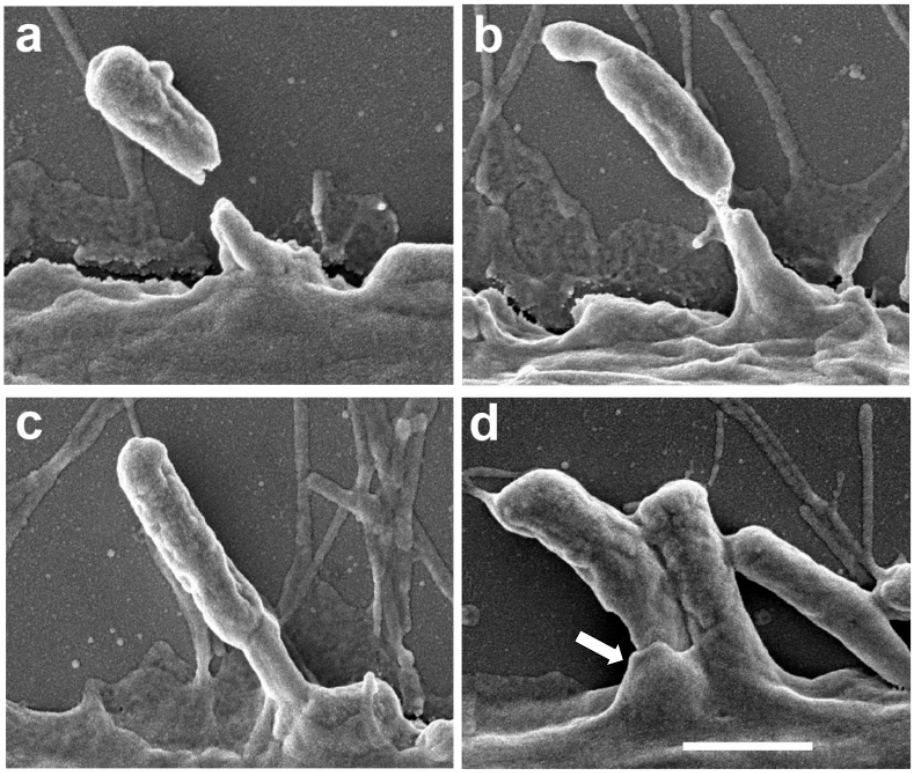

Figure 1. Cellular invasion into AGS epithelial cells by *H. pylori*. After infection of AGS cells by wild-type strain *H. pylori* 26695 for 6 hrs, the rod-shaped bacteria are observed nearby or attaching to the cell surface (a-c). Cell surface pedestals (arrow) are evidently seen when *H. pylori* were in tight contact with AGS cells (d). Scale bar represents 1 μm.

Disruption of lipid rafts block entry of *H. pylori* into AGS—To further demonstrate whether *H. pylori* were associated with lipid rafts, we examined infected AGS cells using CTX-B as a specific marker of lipid rafts. Confocal laser microscope (CLSM) visualized labeling of wild type *H. pylori* 26695 (red color) and CTX-B (green color). After infection of AGS cells with *H. pylori* strain 26695 at 37˚C for 6 hours without pretreatment of MβCD, CTX-B were specifically recruited to sites of bacterial attachment (Figure 2e-h). Merged image of bacteria and CTX-B labeling showed that bacteria were colocalized with lipid rafts on the plasma membrane (Figure 2h). After infection of 12 hours, accumulation of lipid rafts were found appeared around infected *H. pylori* (Figure 2i-l). However, a few internalized bacteria were clearly seen in large vacuoles and changed the morphology to been hummingbird phenotypes. In contrast, in the presence of MβCD, bacteria still attached to AGS cells, but no apparent accumulation of lipid rafts was detected around bacteria (Figure 2m-p). The corresponding images without pretreatment of MβCD and infection of bacteria were shown in Figure 2a-d. These results demonstrated that lipid rafts was associated with internalized *H. pylori*, while disruption of lipid rafts affect bacteria entry into AGS cells. Thus, microscopic examinations represented that the lipid rafts mobilized and colocalized with the intracellular *H. pylori*.

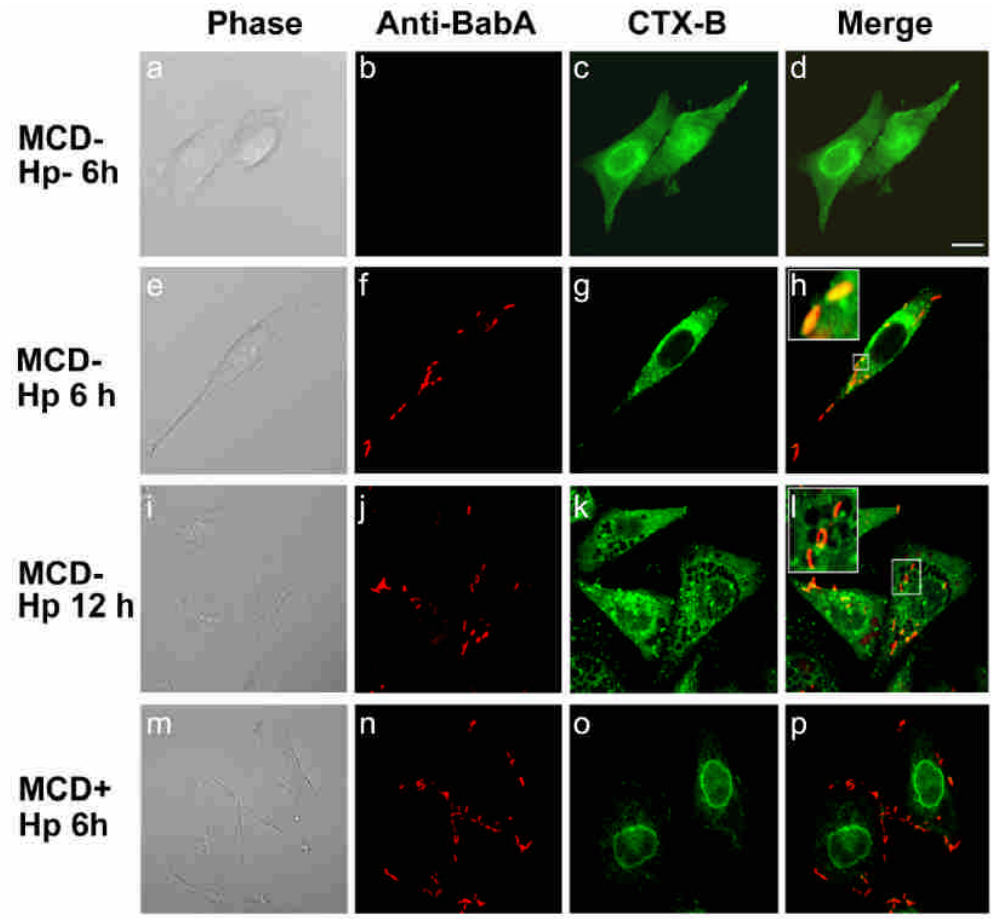

Figure 2. Lipid rafts ere accumulated at the sites of *H. pylori* attachment. AGS cells were untreated (a-l) or pretreated (m-p) with 2.5 mM MCD, exposed to wild-type 26695 bacteria, then probed with anti-BabA and CTX-B and viewed by confocal microscope and phase contrast. CTX-B that recognized lipid-associated GM1 was

recruited around internalized bacteria. The inset in the upper panels showed high magnification. Scale bar =10 μm.

VacA, CagA, and BabA2 are associated with lipid rafts—To further examined the association between virulence factors and lipid rafts during bacterial infection, AGS cells were challenged with *H. pylori* 26695 at 37°C for 6 hours. Samples were solubilized in Triton X-100 and detergent-resistant membranes (DRMs) were isolated on OptiPrep gradients. As shown in Figure 3A, GM1 and caveolin 1 (Cav-1), two markers of DRMs, were enriched in DRMs at the top lighter fractions (5–8), while the transferrin receptor (Tfr), a transmembrane protein localized to clathrin-coated pits, was associated with heavier fractions. When pure bacteria were subjected to the same treatment, CagA, VacA, BabA, and urease (UreA) appeared in heavier fractions, indicating that bacteria were efficiently lysed (Figure 3B). We next analyzed the distribution of those proteins when AGS cells were challenged with bacteria for 6 hours using the DRM fractionation assay. As shown in Figure 3C, VacA, CagA and BabA were primarily isolated in lighter DRM fractions (5–8) with the peak at fraction no. 7. In contrast, urease was found in non-DRM fractions. With 5 mM MCD pretreatment to extract cholesterol, the level of VacA, CagA, and BabA in lighter fractions was significantly reduced. Moreover, the distribution of either protein shifted to heavier fractions, analogous to the pattern seen for GM1 (Figure 3C). Urease remained in the non-raft fractions with MCD pretreatment, like that for the transferrin receptor. It is noted that the distribution of VacA, CagA, and BabA was primarily within DRMs whereas urease within non-DRMs in infected cells without cholesterol depletion. **Along with the shift to non-DRMs for VacA, CagA, and BabA in the cholesterol-depleted cells, these results suggest that VacA, CagA, and BabA might directly interact with the cellular membrane rafts during infection.**

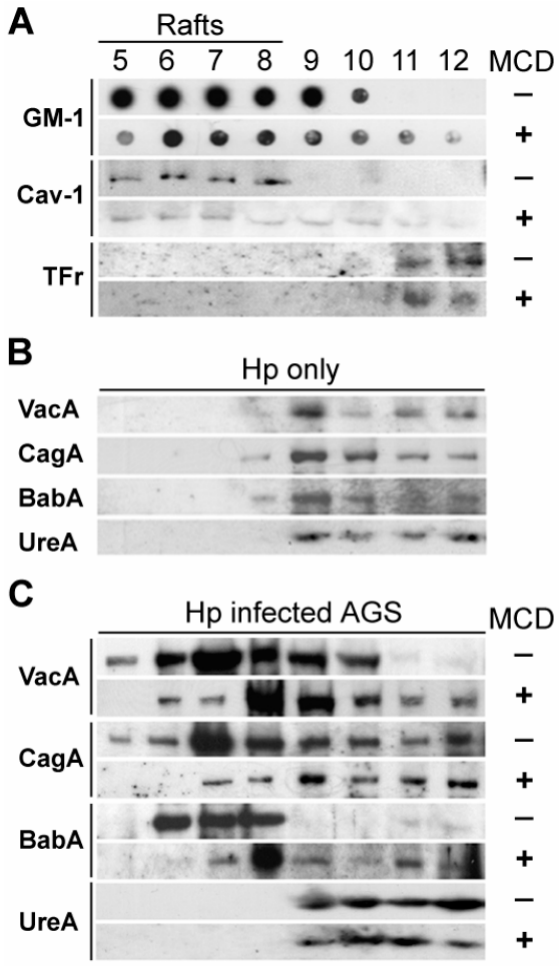

Figure 3. (A) Association of raft markers in detergent-resistant membranes (DRMs). Infected AGS cells were subjected to fractionation in Optiprep density gradient at 4°C. Each fraction was assayed for GM1 with HRP-conjugated CTX-B (0.2 µg/ml) (Sigma-Aldrich) using dot blot analysis, and assayed for caveolin 1 with anti-Cav-1 antibodies or the transferrin receptor with anti-Tfr antibodies using Western blot analysis. (B) Distribution of bacterial surface mediators in non-DRM fractions. Pure bacteria were subjected to fractionation in Optiprep density gradient at 4°C. Each fraction was assayed for VacA, CagA, BabA, and UreA using Western blot analysis. (C) Association of bacterial surface mediators in DRMs during infection. AGS cells infected with *H. pylori* 26695, followed by fractionation in Optiprep density gradient at 4°C. For cholesterol depletion, cells were treated with 5 mM MCD for 1 hour prior to incubation. Each fraction was assayed for VacA, CagA, BabA, and UreA using Western blot analysis.

We hypothesize that *H. pylori* use three virulence factors, VacA, CagA, and BabA2 to interact with lipid rafts, which are known to contain the molecular machinery required for the clustering of raft components, formation of vacuoles and promotion of endocytosis (Figure 4). Thus, modification of lipid rafts and raft-associated proteins might limit the accessibility of *H. pylori* to the antigen processing and presentation machinery as well as antibiotic effects.

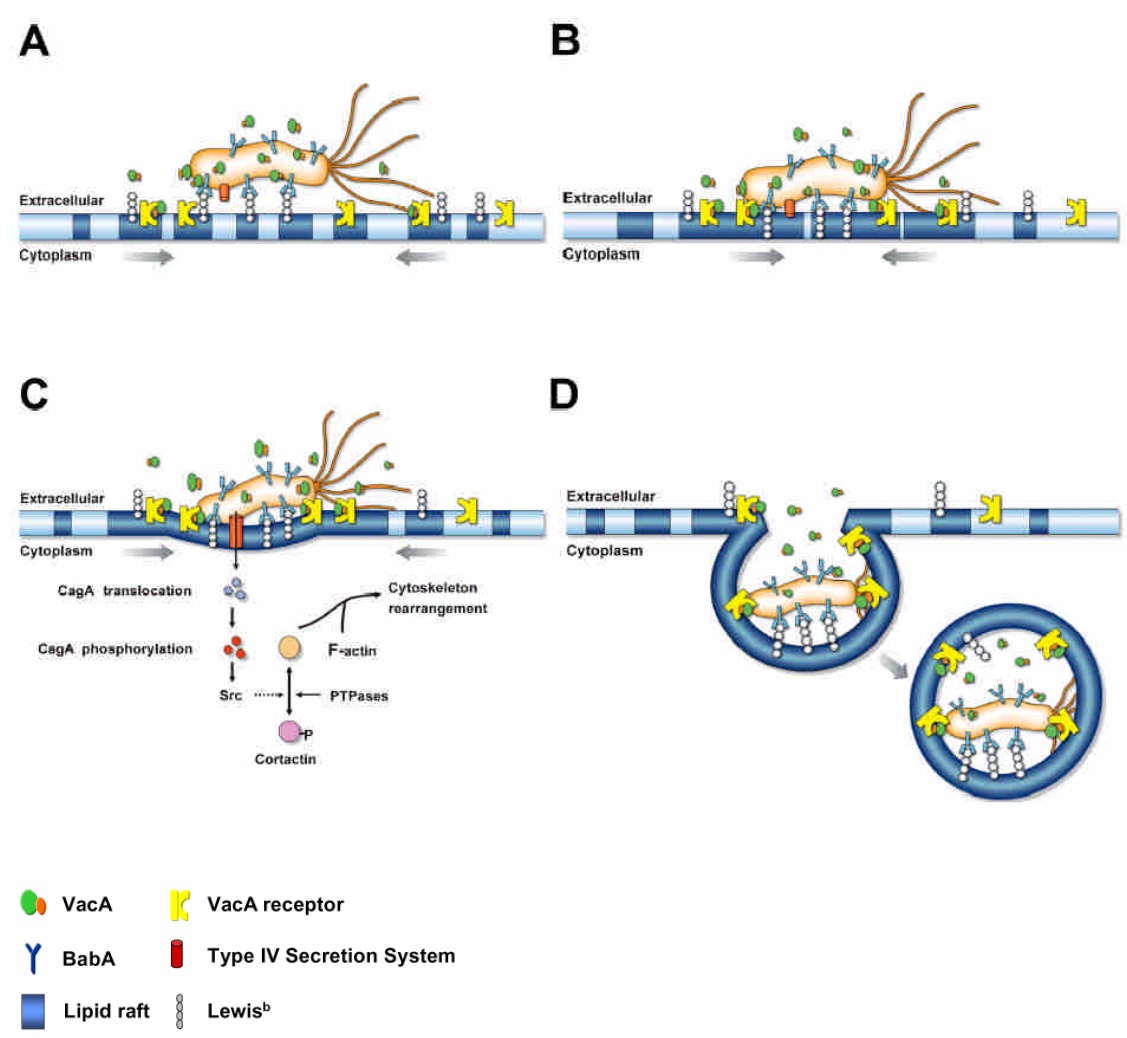

Figure 4. Model depicting *H. pylori* invading AGS cells. (A) A BabA-expressing bacterium specifically couples to counterpart host receptors of Lewis(b) surface epitopes localized within rafts. (B) Bacteria-associated VacA binds to receptors and associates with rafts, triggering fusion of raft-associated membranes from intracellular compartments and promoting raft coalescence into sites of bacterial attachment. (C) The raft-associated bacterium with type IV secretion apparatus injects CagA into cytoplasm, where it is phosphorylated, inducing downstream signaling events. (D) The raft-associated membrane extensions from an intracellular pool eventually form a distinct intracellular compartment.

***H. pylori* infection with macrophage are associated with lipid rafts—** We then tested that whether *H. pylori* are associated with lipid rafts in macrophage infection. As seen in the Figure 5 (first row for 1 hr infections), GM1, a raft marker stained by the CTX-B probe, was evenly distributed in the plasma membrane, displaying a raft-associated punctate pattern in untreated cells. When cells were incubated with *H. pylori* for 6 hr, accumulation of lipid rafts were found appeared around infected *H. pylori* Figure 5 (second row for 6 hr infections). Thus, using confocal laser microscope, we also found that *H. pylori* was internalized and associated with lipid rafts after infection with macrophage.

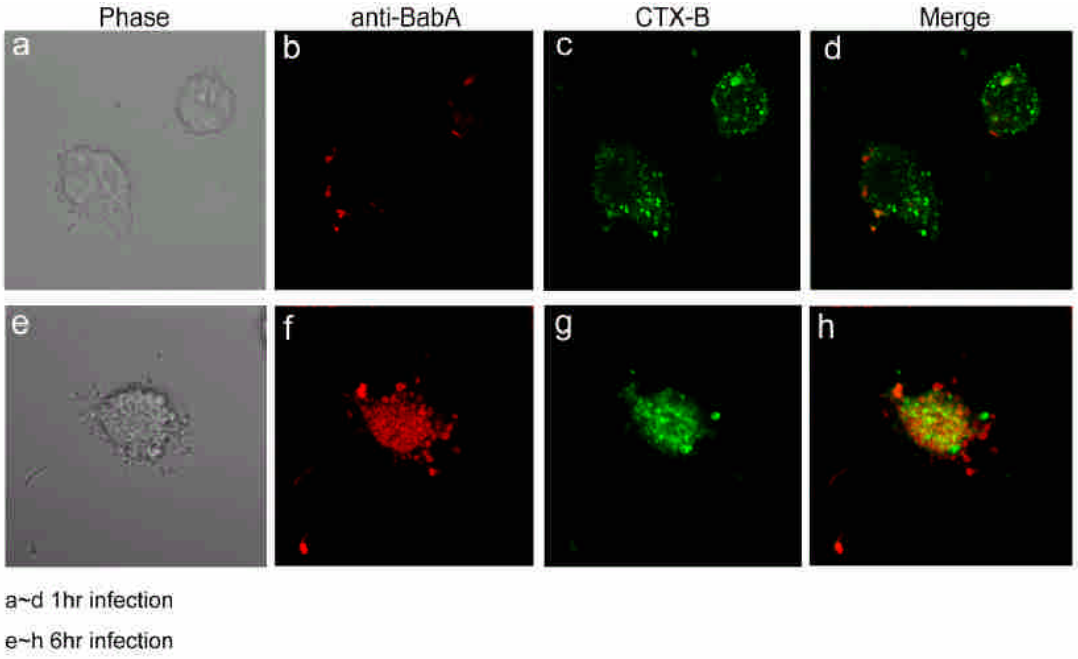

Figure 5. *H. pylori* were colocalized with lipid rafts by macrophage internalization. Confocal laser scanning microscope (CLSM) visualized labeling of wild type *H. pylori* 26695 (red color) and CTX-B (green color), after infection of macrophage cell line J774A.1 with *H. pylori* strain 26695 at 37˚C for 1 hr (a-d) and 6 hr (e-h).

***H. pylori* infection of macrophage suppresses lipopolysaccharide-induced nitric oxide production—** To determine whether *H. pylori* can inhibit LPS–induced NO production, macrophage were infected for 48 hrs with wild-type and *cagA*$^{-}$ *H. pylori*. Macrophages were incubated with medium containing various MOI of *H. pylori* wild type or *cagA*$^{-}$ strains, in the presence or absence of LPS (2 μg/ml) for 48 h. The production of NO was estimated from the accumulation of nitrite (NO_2^-). The results showed that infected with wild-type *H. pylori* reduced the efficacy of LPS-induced NO production with less NO detected in cells infected with higher MOI (Figure 6). In contrast, *cagA*$^{-}$ *H. pylori* represented less effective to suppress LPS–induced NO production than wild-type strain. **Thus, these data demonstrate not only *H. pylori* can inhibit LPS–induced NO production in a dose-dependent manner but also mediate via *cagA*-TFSS regulation.**

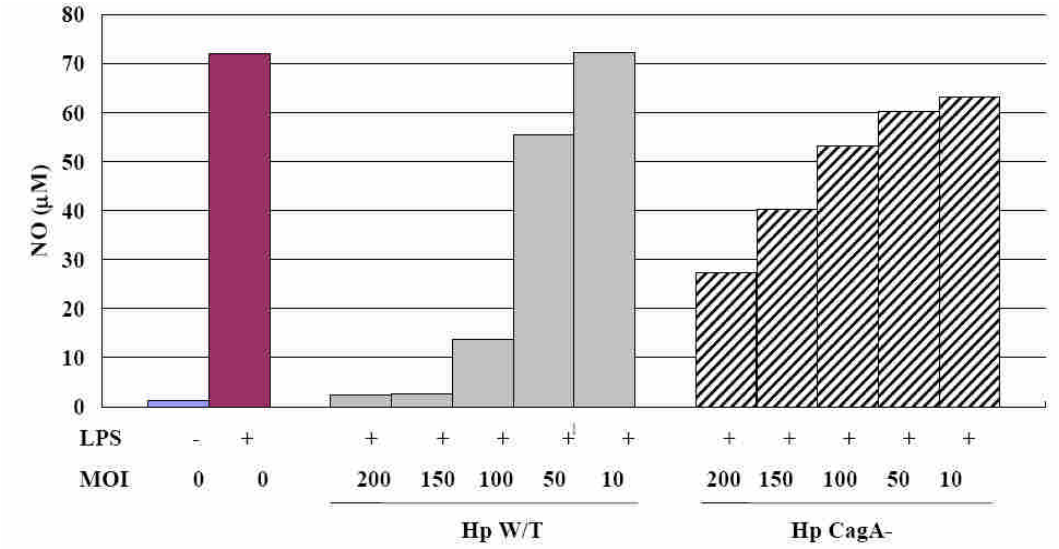

Figure 6. The concentration of NO from pre-treatment of macrophages with various MOI of *H. pylori*. Macrophages were stimulated with LPS (2 μg/ml) in the presence of various MOI of wild type or *cagA*¯ isogenic *H. pylori*. Supernatants were collected after 48 hr and the concentrations of NO were determined by Griess method.

Therapy-failure isolates are associated with increased antibiotic resistance and greater internalization activity—Our recent report showed that bacteria with high antibiotic resistance and/or elevated invading activity escape better during antibiotic treatment of *H. pylori* infections (34). Therapy failure thus resulted in selection and enrichment of strains with both refractory phenotypes, leading to even more resistant strains. These results may support the hypothesis that three mediators VacA, CagA, and BabA essential for bacterial internalization directly partition in membrane rafts during infection. It is likely that intricate pathogen-host interactions take place during the process of internalization, in which BabA binds to receptors of fucosylated blood group antigens to establish the initial contacts, VacA may promote raft coalescence, and CagA injects into host cells for subsequent signaling that may be essential for the second internalization phase. **Most importantly, we believe our preliminary results provide a concept that bacteria with superior intracellular invasion may survive better within a temporary reservoir, protecting them against antibiotic treatment and yielding refractory strains that resist treatment.**

Research design

Macrophages are one of the most powerful professional phagocytes in the immune system, capable of killing the foreign pathogens using the active lysosomal system. However, some pathogens are able to protect the killing mechanisms of the macrophages by several methods. Although we have showed that intracellular *H. pylori* could evade antibiotic therapy and induce resistant strains. Nevertheless, the ability of *H. pylori* to internalize into macrophage and induce macrophage pathogenesis is still unclear. In most *H. pylori*-infected patients, bacterial infection causes early immune response by infiltrated of macrophages and subsequently induces superficial chronic gastritis. However, the molecular basis for infection of macrophage by *H. pylori* still remains poorly understood. Hence, we decided to investigate whether the bacteria had the ability to

direct its own internalization into macrophage and induce the pathogenesis of macrophage. The proposed experiments and research design flowchart (Figure 7) are as follows.

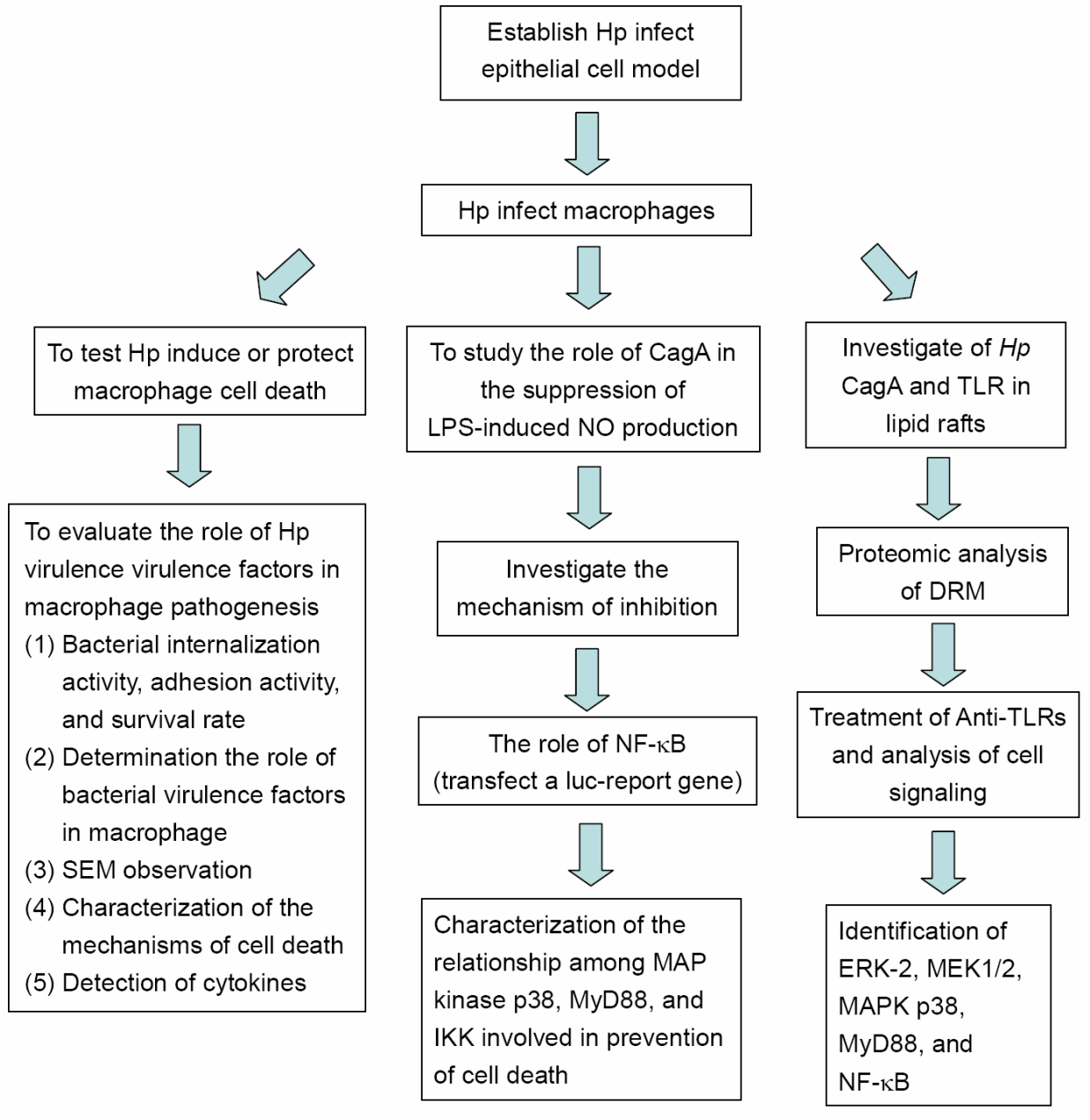

Figure 7. Schematic diagram for the overall research design

Specific aims include:

Year 1

Specific Aim 1. To study the infection process of *H. pylori* in macrophage

Our previous study using epithelial model, we suggested that *H. pylori* have a strategy to penetrate epithelial cells, to survive intracellularly and to evade antibiotic effects as well as host immune responses. However, the innate immune response involved in early gastric inflammation during *H. pylori* infection into

epithelial cells is still unclear. In this proposal, we will first test this hypothesis by investigating the internalization of *H. pylori* in macrophages. In addition, we will aim to study the cell death of macrophages during bacterial infection process. Furthermore, we will extend this study to include murine primary macrophage. The propose experiments are designed as follows:

1.1 To evaluate the adhesion and internalization activity of *H. pylori* with macrophage

A previous study reported that type 1 ($VacA^+/CagA^+$) *H. pylori* strains exhibit delayed phagocytosis by formation of megasome compared with type 2 ($VacA^-/CagA^-$) strains (1). Another study showed that retention of the coat protein tryptophan aspartate-containing coat protein (TACO) prevent type 1 strain-association phagosome trafficking (78). While the authors compared type 1 and type 2 strains with unrelated genetic background. In this proposal, **we will use the wild type strain and its isogenic *cagA* and/or *vacA* mutants with otherwise the same genetic background.**

We will next perform bacterial internalization into macrophages by a standard gentamicin protection assay (57). Our previous studies have investigated the molecular pathogenesis of VacA and CagA in gastric epithelial cells. However, the impact of virulence factors on the interplay between *H. pylori* and macrophages remains elusive. To investigate whether virulence *H. pylori* ($vacA^+$ and $cagA^+$) would able to direct internalization into macrophages, macrophage cell lines such as RAW264.7, J774 and U937 will be infected with wild-type, $cagA^-$ and/or $vacA^-$ strains. By comparison in binding and internalization ability between the wild type and mutant strains, we tried to identify whether these factors participate in the bacterial infection processes. These genetic and microbiological experiments will help to understand the roles of those virulence factors in *H. pylori* infection of host cells.

1.2 To investigate the infection process in macrophages with *H. pylori* infection

It is also well known that *H. pylori* can possesses *cag* PAI encoding of type IV secretion system (TFSS) and assemble the needle-like structure to translocate CagA into host cells (2, 53). Infection with $cagA^+$ *H. pylori* strains is more closely associated with peptic ulceration and gastric cancer than infection with *cagA* mutants. It has been reported that Type I *H. pylori* strains ($VacA^+/CagA^+$) inhibit phagocytosis by macrophages (1). Recent study also demonstrated that VacA-expressing *H. pylori* prevent phagosome maturation thereby promoting enhanced the survival of the bacteria. (78). However, the role of CagA in the interaction of *H. pylori* and macrophages is not well defined. In the present proposal, we will compare the ability to induce large scale membrane extension of macrophages between wild-type and *cagA* mutant infections by scanning electronic microscope (SEM). The result from this study will elucidate the role of CagA for the initiation of bacterial internalization, particular in the formation of lamellipodia and membrane ruffles after infection with *H. pylori* in macrophages.

1.3 To study *H. pylori* induce or prevent cell death in macrophage

Apoptosis macrophages has been implicated in the survival of intracellular bacteria such as *Shigella flexneri* (52) and *Salmonella typhimurium* (38). In contrast, some intracellular bacteria were found to use anti-apoptosis strategies to avoid the loss of their cellular habitat. For instance, *Bartonella henselae* was found to have anti-apoptotic capacity, which mediated via the *virB* type IV secretion system (63). Several

studies have reported that *H. pylori* induced gastric epithelial cells apoptosis (14, 55). Moreover, it has been shown that *H. pylori* induces apoptosis in the macrophage cell line (19, 45). In contrast to the above reports, some studies demonstrated *H. pylori* CagA protein protecting B cell (71) and gastric epithelial cell (4) from apoptosis. More recently, a report showed that spleen B cells were protected from apoptosis by low multiplicity of infection (MOI) of *H. pylori* infection (10). This result indicated that the low levels of *H. pylori* infection that occur *in vivo* are associated with B cell survival, proliferation, and had their potential to evolve into mucosa-associated lymphoid tissue lymphoma. The mechanisms leading to these effects remain ambiguous. Therefore, we will determine whether *H. pylori* capable of inducing or inhibiting cell death of macrophages. To verify if these effects could be extrapolated to primary phagocytic cells, we will also extend our study to include murine macrophages. As macrophages play major roles in innate immunity and act as antigen presenting cells to activate adaptive immunity against bacterial infections. The results of this study will shed light on the role of the interaction between *H. pylori* and immune cells.

1.4 Detection of inflammatory cytokines released by macrophages during *H. pylori* infection

Interaction of macrophages with Gram-negative bacteria results in the production of proinflammatory cytokines such as IL-1 and TNF-α, which enhance phagocytosis and bacterial killing. The increase in cytokines released after infection could also be induce the lysis of cells, thereby releasing intracellular cytokines. To solve this question, cytokines assay will be performed by using enzyme-linked immunosorbent assay (ELISA) kits for IL-1, IL-18, and TNF-α.

Year 2

Specific Aim 2. To study the role of CagA in the suppression of antimicrobial nitric oxide (NO) production

2.1 To investigate *H. pylori* interaction with macrophage inhibits lipopolysaccharide–induced NO production

It has been shown that *H. pylori* might induce macrophage inducible nitric oxide (iNOS) expression (75). Nitric oxide (NO) production plays an important role in the gastric mucosal immune response to *H. pylori* and the associated inflammation. The expression of iNOS is regulated in various cell types and can be enhanced by stimulating the cells with bacterial lipopolysaccharide (LPS) (51). Toll-like receptors (TLRs) have been identified as the main pattern recognition receptors that participate in innate immune responses against bacterial infection (28). A recent report showed that *H. pylori* LPS-induced cell activation is mediated through TLR2 (37). In contrast to TLR2, TLR4 signaling to *H. pylori* LPS does not appear to mediate innate responses in gastric epithelial cells (3). However *H. pylori* LPS was shown to have a 500–1000 fold lower effective than other Gram-negative bacterium, *S. typhimurium* and *E. coli* (21, 49).

Given that *H. pylori* targets gastric epithelial cells and infiltrated innate immune cells, a macrophage model system was required to investigate whether the bacteria possesses the capacity to inhibit NO production and evade the immune response. Thus, experiments were carried out with murine macrophage and preliminary result showed that the cell do not produce NO unless treated with *E. coli* LPS (Figure 6). To

determine whether *H. pylori* can inhibit LPS–induced NO production, macrophage were infected for 48 hrs with wild-type and *cagA*¯ *H. pylori*. Interestingly, the results showed that infected with wild-type *H. pylori* reduced the efficacy of LPS-induced NO production with less NO detected in cells infected with higher MOI (Figure 6). However, *cagA*¯ *H. pylori* represented less effective to suppress LPS–induced NO production than wild-type strain. **Thus, these data demonstrate not only *H. pylori* can inhibit LPS–induced NO production in a dose-dependent manner but also mediate via *cagA*-TFSS regulation.**

Base on our preliminary results, *H. pylori* can inhibit LPS–induced NO production in a manner that is dependent on a functional *cagA*-TFSS. In an aim to understand why *H. pylori* inhibit LPS–induced NO production, several strategies will be used to confirm these effects: (i) to determine whether iNOS expression also inhibit by *H. pylori* infection, (ii) to monitor whether *H. pylori* inhibits iNOS production at the transcriptional or post-transcriptional levels, (iii) to test whether pre-treatment of macrophage with LPS would affect bacteria growth, adherence or internalization. Together with these biochemical experiments, the physiological function of *H. pylori cagA*-TFSS suppress LPS-induced NO production may then be revealed.

2.2 To study the expression of macrophage intracellular molecules

Bacterial LPS activates macrophages by binding to Toll-like receptor 4 (TLR4), leading to production of cytokines, such as TNF-α (58). Engaging TLR4 activate MyD88-dependent and independent pathways, leading to activation of downstream targets (i.e. MAP kinase) (29) **However, a recent report showed that *H. pylori* LPS-induced cell activation was mediated through TLR2 and was able to antagonize TLR4** (37). In gastric cell lines, expression of CagA resulted in translocation of NF-κB into nucleus and activation of IL-8 transcription (7). Although TLRs appears to be directly involved in NF-κB activation, the mechanism of its activations is still remains controversy. To determine the role of *H. pylori* CagA to trigger NF-κB in suppressing LPS-induced NO production, we will first transfect a NF-κB-luciferase reporter gene in to macrophage cell lines for establishing an assay system. The next step will examine in detail the effects of signaling cascade involvement of MAP kinase p38, MyD88, and IKK. To address *H. pylori* suppresses LPS-induced macrophage cell death signaling occurs mediate through TLRs, we will also determine whether antibodies against TLRs would change the induction of macrophage cell death. Results from these experiments will identify intracellular molecules involved in the signal pathway, which may shed light on the role of CagA in *H. pylori* infection of macrophage.

Year 3

Specific Aim 3. Investigation of the role of lipid rafts in CagA induced signaling in macrophages

3.1 Characterization of interaction of *H. pylori* CagA and TLR in lipid rafts

It has been shown that regions of cell membrane known as lipid rafts facilitate LPS-induced cell activation (70). **In addition, TLR2 also recruited within lipid rafts upon stimulation by its ligands** (69). **In our preliminary study in gastric epithelial cell model, disruption of lipid rafts with MβCD may lead to disorganize the association between rafts and *H. pylori* CagA** (Figure 8). Thus, we suggested that CagA was mainly associated with lipid rafts. Using confocal laser microscope, we also found that *H. pylori* was

internalized and associated with lipid rafts after infection with macrophage for 1hr and 6 hr (Figure 5). However, we did not test CagA translocation in macrophages and the interaction with lipid raft-associated molecules. To connect the relationships between *H. pylori*, CagA, TLRs, and lipid rafts as well as signaling cascade molecules, we will determine whether these proteins are distribution in macrophage cell lipid rafts. We will also investigate whether *H. pylori* CagA inhibits LPS-induced NO production was mediated through competition of TLR2 and TLR4. These results will reveal lipid rafts play their role in *H. pylori* CagA– and LPS–induced signaling.

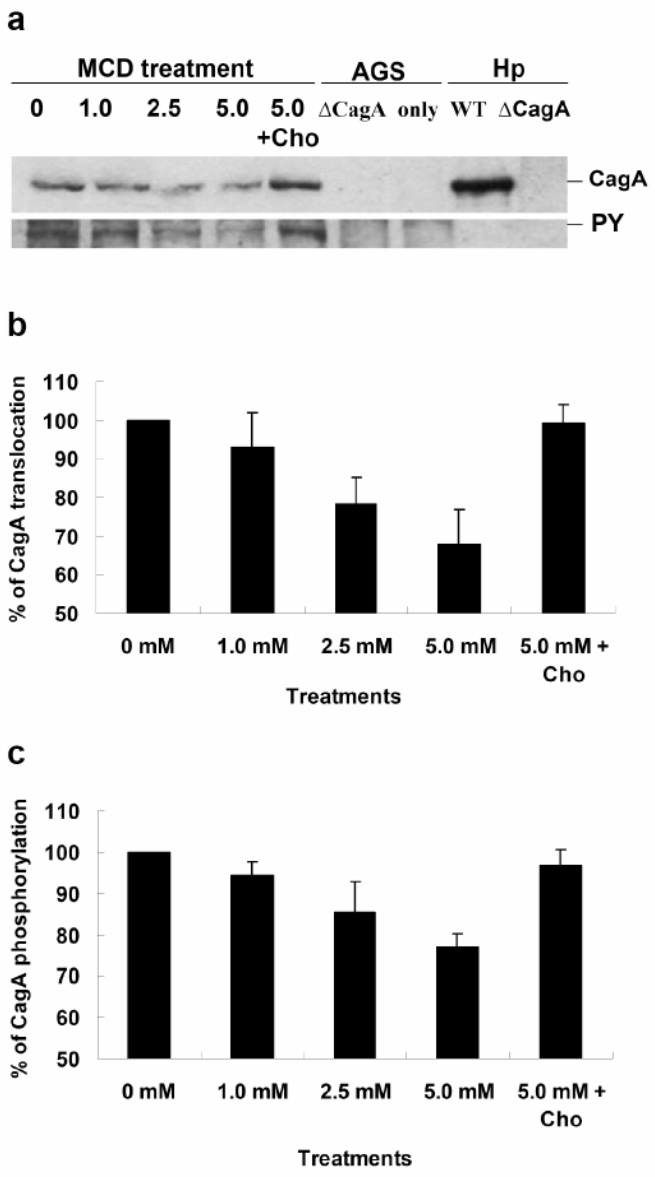

Figure 8. *H. pylori* CagA was associated with lipid rafts. (a) Western blot analysis of *H. pylori* CagA translocation and phosphorylation. AGS cells were pretreated with 0, 1.0, 2.5, and 5.0 mM MβCD, or pretreated with 5.0 mM MβCD and replenished with cholesterol (400 μg/ml), and subsequently infected with *H. pylori* 26695. Densitometry analysis was shown in (b) CagA translocation and (c) CagA phosphorylation. Results shown represent the mean ± SD from triplicate independent experiments.

3.2 Identification of the cellular molecules recruited in lipid rafts in *H. pylori*–infected macrophage

One of the most widely appreciated roles of lipid rafts is in the recruitment and concentration of molecules involved in cellular signaling. To our knowledge, TLR2 was involved in lipid rafts, while TLR4 was not. We proposed that *H. pylori*-LPS binds to TLR2 and enhance clustering of lipid rafts, subsequent translocation of CagA into macrophage as well as regulation of intracellular signaling. Bacterial component LPS can bind to TLR4, which was not recruited in lipid rafts. With this in mind, we will address the possibility that lipid rafts concentrate signaling molecules implicated in *H. pylori* CagA triggered LPS-cellular activation (Figure 9). In order to test this hypothesis, we will investigate whether intracellular molecules were recruited in lipid rafts. The molecules involve in downstream of membrane TLRs, such as ERK-2, MEK1/2, MAPK p38, MyD88, and NF-κB will be determined.

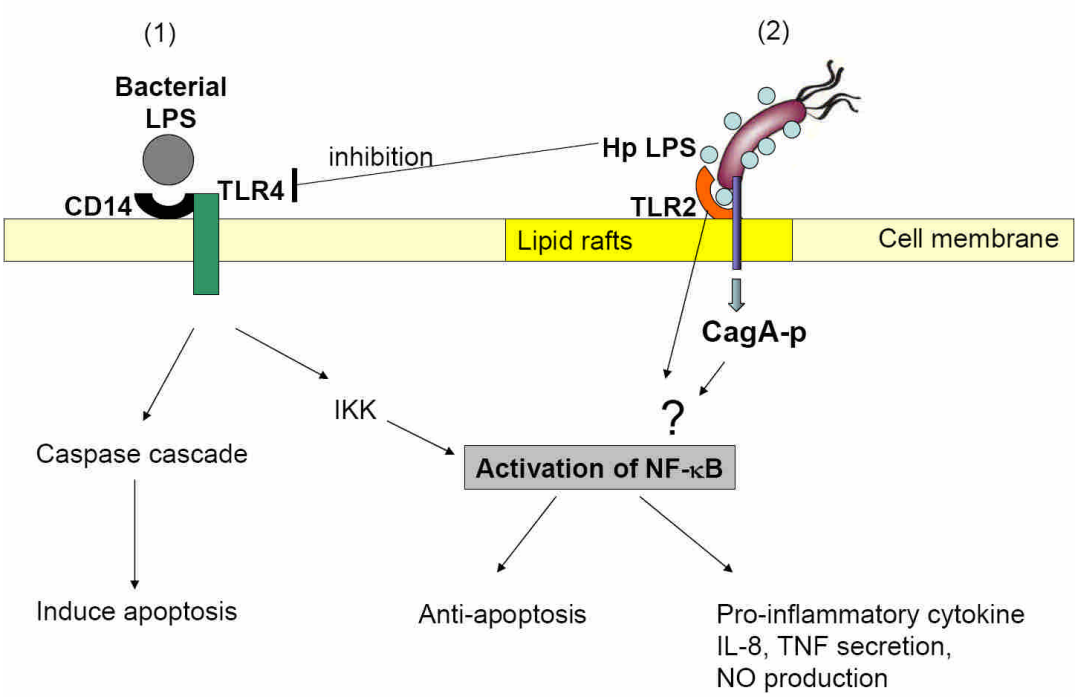

Figure 9. The mechanisms of *H. pylori* induce macrophage pathogenesis

3.3 Analysis of *H. pylori*–macrophage interactions in lipid rafts by a proteomic approach

The study of global protein expression was performed nearly exclusively using two-dimensional gel electrophoresis (2-DE) and mastrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF), thus becoming an emerging discipline. To identify which molecules were up-regulated or down-regulated with *H. pylori*-LPS and CagA within lipid rafts, we will analyze the detergent-resistant membrane (DRM) fractions by a proteomic approach. Various specific antibodies will also be used for probing specific molecules. These results shall reveal how *H. pylori* suppress LPS-induced macrophage death via lipid rafts and enhance them as means of survive intracellularly.

Materials and methods

Cell lines— RAW 264.7 (ATCC TIB-71, murine macrophage cell line), J774A cells (ATCC TIB-67, murine macrophage cell line), were cultured in DMEM (GibcoBRL, NY, USA), U937 (ATCC CRL-1593.2, human

monocyte cell line) was cultured in RPMI 1640 (GibcoBRL) medium. Ten percent of de-complement FBS (Hyclone UT, USA) was added in all cultures. Penicillin and streptomycin (GibcoBRL) were used if needed. In bacteria internalization assay, cell culture medium were not supplemented with antibiotic reagents.

Construction of H. pylori isogenic mutants— In this proposal, all experiments carried out with 26695 (ATCC 700392). An isogenic mutant *H. pylori* Δ*vacA*::*cat* was generated by insertion of the *cat* fragment derived from pUOA20 (73) into *vacA* gene of *H. pylori* through allelic replacement and selection of chloramphenicol-resistant clones (72). Each recombinant plasmid, antibiotic cassette and PCR primers listed in Table 2. All isogenic mutants of *H. pylori* were obtained by following the natural transformation protocol (72). The genomic DNA of *H. pylori* mutants were used to check inserted of antibiotics cassette into a target gene. Western blot analysis was carried out to the abolished expression of each protein. Table 3 lists the isogenic mutants generated in this investigation.

Gentamicin protection assay— To identify the roles of VacA and CagA in *H. pylori* adherence and internalization of macrophage, *H. pylori* 26695 and variant isogenic mutants that are both cultured to logarithmic phase were harvested and resuspended in PBS (pH7.4). The cells were infected with 50 MOI of *H. pylori* and were incubated at 37˚C for different time courses. To determine the number of cells which associated with bacteria per well, infected macrophages were lysed by incubating in distilled water for 10 minutes. To determine the number of internalized bacteria, after being washed with PBS, the infected macrophages were incubated with culture medium containing with 100μg/ml gentamicin (Sigma, MO, USA) at 37˚C for another 1.5 hours. The adherence and internalization activity were calculated as previously described (34).

Bacterial survival assay— To assess the intracellular survival, macrophage cell lines were infected with *H. pylori* wild type or *cagA* mutant at an MOI of 100:1. One hour after infection, cells were centrifuged at 350 *g* for 3 min and supernatant was discarded. Subsequently, cells were washed twice with PBS, resuspended in medium containing 100μg/ml gentamicin (Sigma) and seeded at 1×10^6 /ml. Cells were lysed 1, 4 or 8 hr after infection. Diluted cell lysates were plated on Brucella blood agar plates. Colonies were counted after 4–5 days. Experiments were performed at least three times in duplicates.

Apoptosis assay— Cell Death Detection ELISA kits (Roche) were used to measure cell death. Macrophages were treated with wild-type and *cagA*-deficient knockout strains using the same concentrations and time intervals that were used for getamicin protection assays. Cells were lysed according to the manufacturer's instructions, lysates were used for ELISA, and OD was measured at 405 nm.

Scanning Electron Microscopy— To observe internalization behavior of *H. pylori* strains by macrophages, cells were grown on 12-mm round coverslips before being infected by centrifugation. After infection of *H. pylori*, cells were washed three times by PBS. For further fixation, the washed and fixed cells were treated with 2% OsO_4 and then dehydrated fellow by the processes for electron microscopy. Visualization of samples was carried out by a scanning electron microscope (Hitachi).

Cytokine assay— To detect inflammatory cytokines released by macrophages during *H. pylori* infection, the levels of various cytokines were evaluated. The levels of TNF-α, TNF-β, IL-12, and IL-18, in supernatants from macrophage cultures were determined by an enzyme-linked immunosorbent assay (ELISA) using

antibody from PharMingen, according to the manufacturer's instruction (17). Cells were incubated with LPS in the presence of different MOI of *H. pylori* strains. The supernatants were collected and stored at −80 °C before analysis. Cell viability was determined by trypan blue dye exclusion method and was always greater than 95%.

Nitric Oxide determination— In order to investigate whether *H. pylori* can inhibit LPS–induced nitric oxide (NO) production in a manner that is dependent on a functional *cagA*–TFSS, NO production was determined. The production of NO was estimated from the accumulation of nitrite (NO_2^-), a stable end product of NO metabolism, in the medium using the Griess reagent as described previously (12). Briefly, cells were incubated with medium containing various MOI of *H. pylori* wild type or *cagA*$^-$ strains, in the presence or absence of LPS (2 μg/ml) for 48 h. Equal volumes of culture supernatant or serum and Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% α-naphthylethylenediamine dihydrochloride in distilled water) were mixed and incubated for 15 min at room temperature. The absorbance was measured at 540 nm on a spectrophotometer, and referred to a nitrite standard curve to determine the nitrate concentration in supernatants.

Cell viability assay — Various MOI of *H. pylori* wild type or *cagA*$^-$ strains were co-incubated with macrophages (2×10^6 cells/ml) in a 96-well plate for 48 h. Mitochondrial respiration-dependent MTT assay was employed to determine their cytotoxicity (47). Briefly, MTT in PBS (0.1 mg) was added into each well and then incubated at 37 °C for 4 h. The MTT formazan (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) crystals made due to dye reduction by viable cells were dissolved using acidified isopropanol (0.1 HCl) and mixed at room temperature. After 20 min, index of cell viability was calculated by measuring the optical density (OD) of the color produced by MTT dye reduction with a microplate reader (BIO-RAD, model 3550, USA) at 570 nm ($OD_{570–620}$). The mean OD value of the content of four wells was used for assessing the cell viability expressed as percentage of control.

Transient transfection of NF-κB reporter gene— To investigate NF-κB involvement in *H. pylori* CagA suppresses LPS-induced NO production, we will transfect NF-κB-Luc expression plasmid (given from Dr. Chih-Hsin Tang, Department of Pharmacology, China Medical University). Macrophages were grown to 60% confluence and were transfected using SuperFect (Qiagen). After 24-h incubation, transfection was complete, and cells were incubated with the indicated agents or *H. pylori* strains. To prepare lysates, reporter lysis buffer (Promega) was added to each well, and cells were scraped from dishes. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector.

Immunofluorescence labeling of infected bacteria— To visualize the process of *H. pylori* infected into macrophages and interaction with TLRs within lipid rafts. *H. pylori*-infected cells were fixed and permeabilized with Triton X-100. To label the *H. pylori* outer membrane protein BabA, preparations were then incubated for 30 minutes with an anti-BabA mouse monoclonal antibody (McAb). Antibodies against to TLR2, TLR4, CD14, and various intracellular molecules will be purchased from commercial products (Santa Cruz Biotechnology). After probing with fluorescence-conjugated secondary antibodies, the preparations were mounted and observed with a confocal laser scanning microscope (Zeiss LSM 500).

Isolation of lipid rafts and detergent-resistant fractions— In order to address the role of lipid rafts in the interaction of intracellular molecules during *H. pylori* infection of macrophages, we will determine whether TLR2, TLR4, CD14, NF-κB, and *H. pylori* CagA would associate with lipid rafts. Lipid rafts fractions will be prepared using Optiprep ultracentrifuge method (31) and performed by Western blot analysis. Macrophages were infected with wild type or isogenic mutants *H. pylori*, and then were lyzed with ice cold 1% Triton X 100 in TNE on ice for 1 hour. Cell lysates were added to the solution containing 40% Optiprep (Axis-Shield, Norway) in a total volume of 600 μl and overlaid with 200 μl of 35%, 30%, 25%, 20% and 0% Optiprep in TNE. The gradient solution was centrifuged at 38,000 rpm for 18 hours at 4˚C in a RPS56T rotor (Hitachi, Japan). Fractions were collected from top to bottom and protein were precipitated by 6% trichloroacetic acid (Sigma) and subjected to SDS-PAGE and Western blot analysis.

Proteomics analysis— To identify which molecules were changed after *H. pylori* and TLRs interaction within lipid rafts, we analyze the DRM fractions by proteomics approach. In general, *H. pylori*-infected macrophages were subjected to Optiprep gradients ultracentrifuge to isolate the DRM fractions. The sample were prepared according to Pharmacia's protocol and subjected to 2-DE on a Pharmacia Multiphor II electrohporesis apparatus. In order to identify the molecules, in-gel digestion was carried out and then identified by mass spectrometry (MALDI TOF/TOF). The values for monoisotopic peaks were subjected to blast (http://www.matrixscience.com) against the NCBInr database.

（四）預期完成之工作項目及成果。請分年列述：1. 預期完成之工作項目。2. 對於學術研究、國家發展及其他應用方面預期之貢獻。3. 對於參與之工作人員，預期可獲之訓練。4. 本計畫如為整合型研究計畫之子計畫，請就以上各點分別說明與其他子計畫之相關性。

In this proposal, we anticipate obtaining some valuable results regarding the interaction between *H. pylori* and macrophages. These experiments are designed in the light of our previous study, which shall allow us to reveal *H. pylori* virulence factors in the pathogenesis of macrophages. Results from these experiments do not only provide the fundamental understanding in the pathogen-host interaction but also allow us to develop the new strategies to prevent bacterial persistent infection. In this project, we anticipate obtaining enormous experience in study of pathogen-host interaction, which will be invaluable for the future challengeable works.

In this project, we are confident that we will soon establish the experiment procedures and will also obtain interesting results. The expected progresses are as follows,

Year 1.

To determine the *H. pylori* virulence factors have their important roles for pathogenesis of macrophages, *cagA* and/or *vacA* gene knock out strains were generated. Several biological assays for macrophage infection with bacteria will be assessed. For instance, bacterial survival, adhesion, internalization, cytokine release, and cell apoptosis will be determined in our proposal. The results will be compared with the obtained with the corresponding parent wild type strain. Comparison of the results obtained with the wild type and various virulence factors knock out strains will allow us to assess whether such differences are important for bacteria induce macrophage pathogenesis. Several results will attempt to be obtained as follows.

1. Determination of infection process of *H. pylori* in macrophage

Wild type and isogenic knock-out mutants of *H. pylori* will be tested for the internalization assay in macrophages. These experiments will help to understand the roles of those virulence factors in *H. pylori* persistent infection of host innate immune response.

2. Characterization of internalization behavior in macrophages with *H. pylori* infection

The result from this study will elucidate the role of CagA and VacA for the initiation of bacterial internalization, particular in the intracellular survive and formation of lamellipodia and membrane ruffles after infection with *H. pylori* in macrophages.

3. Investigation of *H. pylori* induce cell death in macrophage

We will determine whether the virulence factors of *H. pylori* capable of inducing or inhibiting cell death of macrophages. We will then investigate which mechanism of *H. pylori*-induced macrophage cell death occurred. If the pathway for *H. pylori* induce cell death can be determined. We will then verify if these effects could be extrapolated to primary phagocytic cells. The results from this study will shed light on the role of *H. plyori* virulence factors in the pathogenesis of macrophages.

4. Inflammatory cytokines released by macrophages during *H. pylori* infection

The study of inflammatory cytokines, such as IL-1, IL-18, and TNF-α will elucidate a mechanism, which used by pathogens to evade killing by macrophages is to kill the macrophages with the cytokines secreted by the infected cells.

Correlation of the results with the biological phenomenon will provide an indication of whether the virulence factors are required for macrophage pathogenesis or enhance *H. pylori* survived intracellularly.

Year 2.

1. Correlation of *H. pylori* infection with inhibition of LPS-induced macrophage NO production

To investigate the role of *H. pylori* suppresses macrophage antimicrobial nitric oxide (NO) production, we will first to set up an assay model. Fortunately, we have currently obtained the assay system for lipopolysaccharide (LPS)–induced macrophage NO production model. Interestingly, our preliminary results showed that macrophage infected with *H. pylori* inhibits LPS–induced NO production. Base on this observation, in the next year, we will determine the expression of NO upstream generator-inducible nitric oxide synthase (iNOS) and know which step was influenced by *H. pylori*. If the inhibition of iNOS is occurred in transcriptional level and depend on *H. pylori* virulence factors, we will propose some intracellular molecules are involved in the processes. Thus, the physiological functions of *H. pylori* suppress LPS-induced NO production will be revealed.

2. Determination the expression of macrophage intracellular molecules

In parallel, we will characterize the properties of molecules downstream of TLRs. Moreover, we will transfect a central molecule NF-κB into macrophage for establishing an assay system. We also will determine the role of *H. pylori* CagA to trigger NF-κB in LPS-induced NO production in macrophage. NF-kB is an important molecule either in inflammation responses or in induction/suppression of cell apoptosis. If our postulates are confirmed with the experiments, we will next examine intracellular molecules which involved in signaling cascade. The results will understand the role of CagA in the interaction with intracellular signal pathway involved in the *H. pylori* infection macrophage.

Year 3.

1. Determination of the role of lipid rafts in CagA induced signaling

Base on our previous experiments effort on lipid rafts, we know *H. pylori* CagA was mainly associated with epithelial cell membrane lipid rafts. In the present proposal, we will change our assay system from epithelial cells to macrophages. If *H. pylori* CagA was interacted with lipid rafts during infection of macrophages, we will determine the relationships between *H. pylori* CagA, LPS, TLRs, and lipid rafts as well as intracellular signaling. Taking these results, that should be possible to identify the role of lipid rafts in *H. pylori* infection with macrophages.

2. Characterization of cellular molecules recruited in lipid rafts in H. pylori-infected macrophages

To more detail investigate the interaction between *H. pylori* and lipid rafts, all experiments will be addressed the molecules involvement in downstream of TLRs. Proteomic approach will also determine the global protein expression. We will then understand the molecular basis of *H. pylori* CagA trigger lipid rafts in macrophage pathogenesis and enhancement of bacterial intracellular survive.

Literature Cited

1. **Allen, L. A., L. S. Schlesinger, and B. Kang.** 2000. Virulent strains of Helicobacter pylori demonstrate delayed phagocytosis and stimulate homotypic phagosome fusion in macrophages. J Exp Med **191:**115-28.
2. **Backert, S., E. Ziska, V. Brinkmann, U. Zimny-Arndt, A. Fauconnier, P. R. Jungblut, M. Naumann, and T. F. Meyer.** 2000. Translocation of the Helicobacter pylori CagA protein in gastric epithelial cells by a type IV secretion apparatus. Cell Microbiol **2:**155-64.
3. **Backhed, F., B. Rokbi, E. Torstensson, Y. Zhao, C. Nilsson, D. Seguin, S. Normark, A. M. Buchan, and A. Richter-Dahlfors.** 2003. Gastric mucosal recognition of Helicobacter pylori is independent of Toll-like receptor 4. J Infect Dis **187:**829-36.
4. **Beswick, E. J., I. V. Pinchuk, G. Suarez, J. C. Sierra, and V. E. Reyes.** 2006. Helicobacter pylori CagA-dependent macrophage migration inhibitory factor produced by gastric epithelial cells binds to CD74 and stimulates procarcinogenic events. J Immunol **176:**6794-801.
5. **Bjorkholm, B., V. Zhukhovitsky, C. Lofman, K. Hulten, H. Enroth, M. Block, R. Rigo, P. Falk, and L. Engstrand.** 2000. Helicobacter pylori entry into human gastric epithelial cells: A potential determinant of virulence, persistence, and treatment failures. Helicobacter **5:**148-54.
6. **Blaser, M. J.** 1993. Helicobacter pylori: microbiology of a 'slow' bacterial infection. Trends Microbiol **1:**255-60.
7. **Brandt, S., T. Kwok, R. Hartig, W. Konig, and S. Backert.** 2005. NF-kappaB activation and potentiation of proinflammatory responses by the Helicobacter pylori CagA protein. Proc Natl Acad Sci U S A **102:**9300-5.
8. **Brown, D. A., and E. London.** 1998. Functions of lipid rafts in biological membranes. Annu Rev Cell Dev Biol **14:**111-36.
9. **Brown, D. A., and J. K. Rose.** 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell **68:**533-44.
10. **Bussiere, F. I., R. Chaturvedi, M. Asim, K. L. Hoek, Y. Cheng, J. Gainor, A. Scholz, W. N. Khan, and K. T. Wilson.** 2006. Low multiplicity of infection of Helicobacter pylori suppresses apoptosis of B lymphocytes. Cancer Res **66:**6834-42.
11. **Cheng, W. C., Y. N. Chang, and W. C. Wang.** 2005. Structural basis for shikimate-binding specificity of Helicobacter pylori shikimate kinase. J Bacteriol **187:**8156-63.
12. **Chi, D. S., M. Qui, G. Krishnaswamy, C. Li, and W. Stone.** 2003. Regulation of nitric oxide production from macrophages by lipopolysaccharide and catecholamines. Nitric Oxide **8:**127-32.
13. **Chiu, W. C., J. Y. You, J. S. Liu, S. K. Hsu, W. H. Hsu, C. H. Shih, J. K. Hwang, and W. C. Wang.** 2006. Structure-stability-activity relationship in covalently cross-linked N-carbamoyl D-amino acid amidohydrolase and N-acylamino acid racemase. J Mol Biol **359:**741-53.
14. **Cover, T. L., U. S. Krishna, D. A. Israel, and R. M. Peek, Jr.** 2003. Induction of gastric epithelial

cell apoptosis by Helicobacter pylori vacuolating cytotoxin. Cancer Res **63:**951-7.

15. **Duncan, M. J., G. Li, J. S. Shin, J. L. Carson, and S. N. Abraham.** 2004. Bacterial penetration of bladder epithelium through lipid rafts. J Biol Chem **279:**18944-51.
16. **Fang, S. H., Y. C. Hou, and P. D. Chao.** 2005. Pharmacokinetic and pharmacodynamic interactions of morin and cyclosporin. Toxicol Appl Pharmacol **205:**65-70.
17. **Fang, S. H., L. H. Hwang, D. S. Chen, and B. L. Chiang.** 2000. Ribavirin enhancement of hepatitis C virus core antigen-specific type 1 T helper cell response correlates with the increased IL-12 level. J Hepatol **33:**791-8.
18. **Fang, S. H., V. Padmavathi, Y. K. Rao, D. R. Venkata Subbaiah, P. Thriveni, M. Geethangili, A. Padmaja, and Y. M. Tzeng.** 2006. Biological evaluation of sulfone derivatives as anti-inflammatory and tumor cells growth inhibitory agents. Int Immunopharmacol **6:**1699-705.
19. **Galgani, M., I. Busiello, S. Censini, S. Zappacosta, L. Racioppi, and R. Zarrilli.** 2004. Helicobacter pylori induces apoptosis of human monocytes but not monocyte-derived dendritic cells: role of the cag pathogenicity island. Infect Immun **72:**4480-5.
20. **Gebert, B., W. Fischer, E. Weiss, R. Hoffmann, and R. Haas.** 2003. Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. Science **301:**1099-102.
21. **Gobert, A. P., J. C. Bambou, C. Werts, V. Balloy, M. Chignard, A. P. Moran, and R. L. Ferrero.** 2004. Helicobacter pylori heat shock protein 60 mediates interleukin-6 production by macrophages via a toll-like receptor (TLR)-2-, TLR-4-, and myeloid differentiation factor 88-independent mechanism. J Biol Chem **279:**245-50.
22. **Gobert, A. P., D. J. McGee, M. Akhtar, G. L. Mendz, J. C. Newton, Y. Cheng, H. L. Mobley, and K. T. Wilson.** 2001. Helicobacter pylori arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. Proc Natl Acad Sci U S A **98:**13844-9.
23. **Grassme, H., V. Jendrossek, A. Riehle, G. von Kurthy, J. Berger, H. Schwarz, M. Weller, R. Kolesnick, and E. Gulbins.** 2003. Host defense against Pseudomonas aeruginosa requires ceramide-rich membrane rafts. Nat Med **9:**322-30.
24. **Harris, P. R., L. E. Smythies, P. D. Smith, and A. Dubois.** 2000. Inflammatory cytokine mRNA expression during early and persistent Helicobacter pylori infection in nonhuman primates. J Infect Dis **181:**783-6.
25. **Hooper, N. M.** 1999. Detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains, lipid rafts and caveolae (review). Mol Membr Biol **16:**145-56.
26. **Ilver, D., A. Arnqvist, J. Ogren, I. M. Frick, D. Kersulyte, E. T. Incecik, D. E. Berg, A. Covacci, L. Engstrand, and T. Boren.** 1998. Helicobacter pylori adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science **279:**373-7.
27. **Ilver, D., S. Barone, D. Mercati, P. Lupetti, and J. L. Telford.** 2004. Helicobacter pylori toxin VacA is transferred to host cells via a novel contact-dependent mechanism. Cell Microbiol **6:**167-74.
28. **Janeway, C. A., Jr., and R. Medzhitov.** 2002. Innate immune recognition. Annu Rev Immunol **20:**197-216.
29. **Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira.** 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity **11:**115-22.
30. **Ko, G. H., S. M. Kang, Y. K. Kim, J. H. Lee, C. K. Park, H. S. Youn, S. C. Baik, M. J. Cho, W. K.**

Lee, and K. H. Rhee. 1999. Invasiveness of Helicobacter pylori into human gastric mucosa. Helicobacter **4:**77-81.

31. **Kuo, C. H., and W. C. Wang.** 2003. Binding and internalization of Helicobacter pylori VacA via cellular lipid rafts in epithelial cells. Biochem Biophys Res Commun **303:**640-4.
32. **Kwok, T., S. Backert, H. Schwarz, J. Berger, and T. F. Meyer.** 2002. Specific entry of Helicobacter pylori into cultured gastric epithelial cells via a zipper-like mechanism. Infect Immun **70:**2108-20.
33. **Lafont, F., G. Tran Van Nhieu, K. Hanada, P. Sansonetti, and F. G. van der Goot.** 2002. Initial steps of Shigella infection depend on the cholesterol/sphingolipid raft-mediated CD44-IpaB interaction. Embo J **21:**4449-57.
34. **Lai, C. H., C. H. Kuo, P. Y. Chen, S. K. Poon, C. S. Chang, and W. C. Wang.** 2006. Association of antibiotic resistance and higher internalization activity in resistant Helicobacter pylori isolates. J Antimicrob Chemother **57:**466-71.
35. **Lai, C. H., C. H. Kuo, Y. C. Chen, F. Y. Chao, S. K. Poon, C. S. Chang, and W. C. Wang.** 2002. High prevalence of cagA- and babA2-positive Helicobacter pylori clinical isolates in Taiwan. J Clin Microbiol **40:**3860-2.
36. **Lai, C. H., S. K. Poon, Y. C. Chen, C. S. Chang, and W. C. Wang.** 2005. Lower prevalence of Helicobacter pylori infection with vacAs1a, cagA-positive, and babA2-positive genotype in erosive reflux esophagitis disease. Helicobacter **10:**577-85.
37. **Lepper, P. M., M. Triantafilou, C. Schumann, E. M. Schneider, and K. Triantafilou.** 2005. Lipopolysaccharides from Helicobacter pylori can act as antagonists for Toll-like receptor 4. Cell Microbiol **7:**519-28.
38. **Lindgren, S. W., I. Stojiljkovic, and F. Heffron.** 1996. Macrophage killing is an essential virulence mechanism of Salmonella typhimurium. Proc Natl Acad Sci U S A **93:**4197-201.
39. **Lupetti, P., J. E. Heuser, R. Manetti, P. Massari, S. Lanzavecchia, P. L. Bellon, R. Dallai, R. Rappuoli, and J. L. Telford.** 1996. Oligomeric and subunit structure of the Helicobacter pylori vacuolating cytotoxin. J Cell Biol **133:**801-7.
40. **Mahdavi, J., B. Sonden, M. Hurtig, F. O. Olfat, L. Forsberg, N. Roche, J. Angstrom, T. Larsson, S. Teneberg, K. A. Karlsson, S. Altraja, T. Wadstrom, D. Kersulyte, D. E. Berg, A. Dubois, C. Petersson, K. E. Magnusson, T. Norberg, F. Lindh, B. B. Lundskog, A. Arnqvist, L. Hammarstrom, and T. Boren.** 2002. Helicobacter pylori SabA adhesin in persistent infection and chronic inflammation. Science **297:**573-8.
41. **Manes, S., G. del Real, R. A. Lacalle, P. Lucas, C. Gomez-Mouton, S. Sanchez-Palomino, R. Delgado, J. Alcami, E. Mira, and A. C. Martinez.** 2000. Membrane raft microdomains mediate lateral assemblies required for HIV-1 infection. EMBO Rep **1:**190-6.
42. **Manes, S., G. del Real, and A. C. Martinez.** 2003. Pathogens: raft hijackers. Nat Rev Immunol **3:**557-68.
43. **Marshall, B. J., D. B. McGechie, P. A. Rogers, and R. J. Glancy.** 1985. Pyloric Campylobacter infection and gastroduodenal disease. Med J Aust **142:**439-44.
44. **Marshall, B. J., and J. R. Warren.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet **1:**1311-5.
45. **Menaker, R. J., P. J. Ceponis, and N. L. Jones.** 2004. Helicobacter pylori induces apoptosis of

macrophages in association with alterations in the mitochondrial pathway. Infect Immun **72:**2889-98.

46. **Monack, D. M., A. Mueller, and S. Falkow.** 2004. Persistent bacterial infections: the interface of the pathogen and the host immune system. Nat Rev Microbiol **2:**747-65.
47. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods **65:**55-63.
48. **Mulvey, M. A., J. D. Schilling, and S. J. Hultgren.** 2001. Establishment of a persistent Escherichia coli reservoir during the acute phase of a bladder infection. Infect Immun **69:**4572-9.
49. **Muotiala, A., I. M. Helander, L. Pyhala, T. U. Kosunen, and A. P. Moran.** 1992. Low biological activity of Helicobacter pylori lipopolysaccharide. Infect Immun **60:**1714-6.
50. **Nakayama, M., J. Hisatsune, E. Yamasaki, Y. Nishi, A. Wada, H. Kurazono, J. Sap, K. Yahiro, J. Moss, and T. Hirayama.** 2006. Clustering of Helicobacter pylori VacA in lipid rafts, mediated by its receptor, receptor-like protein tyrosine phosphatase beta, is required for intoxication in AZ-521 Cells. Infect Immun **74:**6571-80.
51. **Nathan, C., and Q. W. Xie.** 1994. Regulation of biosynthesis of nitric oxide. J Biol Chem **269:**13725-8.
52. **Navarre, W. W., and A. Zychlinsky.** 2000. Pathogen-induced apoptosis of macrophages: a common end for different pathogenic strategies. Cell Microbiol **2:**265-73.
53. **Odenbreit, S., J. Puls, B. Sedlmaier, E. Gerland, W. Fischer, and R. Haas.** 2000. Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. Science **287:**1497-500.
54. **Parton, R. G., B. Joggerst, and K. Simons.** 1994. Regulated internalization of caveolae. J Cell Biol **127:**1199-215.
55. **Peek, R. M., Jr., and M. J. Blaser.** 2002. Helicobacter pylori and gastrointestinal tract adenocarcinomas. Nat Rev Cancer **2:**28-37.
56. **Pelkmans, L., and A. Helenius.** 2002. Endocytosis via caveolae. Traffic **3:**311-20.
57. **Petersen, A. M., J. Blom, L. P. Andersen, and K. A. Krogfelt.** 2000. Role of strain type, AGS cells and fetal calf serum in Helicobacter pylori adhesion and invasion assays. FEMS Immunol Med Microbiol **29:**59-67.
58. **Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler.** 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science **282:**2085-8.
59. **Poon, S. K., C. S. Chang, J. Su, C. H. Lai, C. C. Yang, G. H. Chen, and W. C. Wang.** 2002. Primary resistance to antibiotics and its clinical impact on the efficacy of Helicobacter pylori lansoprazole-based triple therapies. Aliment Pharmacol Ther **16:**291-6.
60. **Ricci, V., A. Galmiche, A. Doye, V. Necchi, E. Solcia, and P. Boquet.** 2000. High cell sensitivity to Helicobacter pylori VacA toxin depends on a GPI-anchored protein and is not blocked by inhibition of the clathrin-mediated pathway of endocytosis. Mol Biol Cell **11:**3897-909.
61. **Sartor, R. B.** 1994. Cytokines in intestinal inflammation: pathophysiological and clinical considerations. Gastroenterology **106:**533-9.
62. **Schilling, J. D., R. G. Lorenz, and S. J. Hultgren.** 2002. Effect of trimethoprim-sulfamethoxazole on recurrent bacteriuria and bacterial persistence in mice infected with uropathogenic Escherichia coli.

Infect Immun **70:**7042-9.

63. **Schmid, M. C., R. Schulein, M. Dehio, G. Denecker, I. Carena, and C. Dehio.** 2004. The VirB type IV secretion system of Bartonella henselae mediates invasion, proinflammatory activation and antiapoptotic protection of endothelial cells. Mol Microbiol **52:**81-92.
64. **Schraw, W., Y. Li, M. S. McClain, F. G. van der Goot, and T. L. Cover.** 2002. Association of Helicobacter pylori vacuolating toxin (VacA) with lipid rafts. J Biol Chem **277:**34642-50.
65. **Segal, E. D., J. Cha, J. Lo, S. Falkow, and L. S. Tompkins.** 1999. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. Proc Natl Acad Sci U S A **96:**14559-64.
66. **Simons, K., and R. Ehehalt.** 2002. Cholesterol, lipid rafts, and disease. J Clin Invest **110:**597-603.
67. **Simons, K., and E. Ikonen.** 1997. Functional rafts in cell membranes. Nature **387:**569-72.
68. **Simons, K., and D. Toomre.** 2000. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol **1:**31-9.
69. **Triantafilou, M., M. Manukyan, A. Mackie, S. Morath, T. Hartung, H. Heine, and K. Triantafilou.** 2004. Lipoteichoic acid and toll-like receptor 2 internalization and targeting to the Golgi are lipid raft-dependent. J Biol Chem **279:**40882-9.
70. **Triantafilou, M., K. Miyake, D. T. Golenbock, and K. Triantafilou.** 2002. Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. J Cell Sci **115:**2603-11.
71. **Umehara, S., H. Higashi, N. Ohnishi, M. Asaka, and M. Hatakeyama.** 2003. Effects of Helicobacter pylori CagA protein on the growth and survival of B lymphocytes, the origin of MALT lymphoma. Oncogene **22:**8337-42.
72. **Wang, Y., K. P. Roos, and D. E. Taylor.** 1993. Transformation of Helicobacter pylori by chromosomal metronidazole resistance and by a plasmid with a selectable chloramphenicol resistance marker. J Gen Microbiol **139:**2485-93.
73. **Wang, Y., and D. E. Taylor.** 1990. Chloramphenicol resistance in Campylobacter coli: nucleotide sequence, expression, and cloning vector construction. Gene **94:**23-8.
74. **Wilkinson, S. M., J. R. Uhl, B. C. Kline, and F. R. Cockerill, 3rd.** 1998. Assessment of invasion frequencies of cultured HEp-2 cells by clinical isolates of Helicobacter pylori using an acridine orange assay. J Clin Pathol **51:**127-33.
75. **Wilson, K. T., K. S. Ramanujam, H. L. Mobley, R. F. Musselman, S. P. James, and S. J. Meltzer.** 1996. Helicobacter pylori stimulates inducible nitric oxide synthase expression and activity in a murine macrophage cell line. Gastroenterology **111:**1524-33.
76. **Wunder, C., Y. Churin, F. Winau, D. Warnecke, M. Vieth, B. Lindner, U. Zahringer, H. J. Mollenkopf, E. Heinz, and T. F. Meyer.** 2006. Cholesterol glucosylation promotes immune evasion by Helicobacter pylori. Nat Med **12:**1030-8.
77. **Yamaoka, Y., M. Kita, T. Kodama, N. Sawai, and J. Imanishi.** 1996. Helicobacter pylori cagA gene and expression of cytokine messenger RNA in gastric mucosa. Gastroenterology **110:**1744-52.
78. **Zheng, P. Y., and N. L. Jones.** 2003. Helicobacter pylori strains expressing the vacuolating cytotoxin interrupt phagosome maturation in macrophages by recruiting and retaining TACO (coronin 1) protein. Cell Microbiol **5:**25-40.