HELICOBACTER PYLORI ATTENUATES PROLIFERATION OF RAW264.7 MONOCYTIC MACROPHAGE CELLS BY DOWNREGULATING CELL CYCLE GENES

GRACE TAN MIN YI

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2016

HELICOBACTER PYLORI ATTENUATES PROLIFERATION OF RAW264.7 MONOCYTIC MACROPHAGE CELLS BY DOWNREGULATING CELL CYCLE GENES

GRACE TAN MIN YI

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF MEDICAL SCIENCE

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2016

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **Grace Tan Min Yi** [redacted]

Registration/Matric No: **MGN 130053**

Name of Degree: **Master of Medical Science**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

***Helicobacter pylori* attenuates proliferation of RAW264.7 monocytic macrophage cells by downregulating cell cycle genes.**

Field of Study: **Bacteriology/ Immunology**

I do solemnly and sincerely declare that:

1. I am the sole author/writer of this Work;
2. This Work is original;
3. Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
4. I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
5. I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
6. I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature **Date:**

Subscribed and solemnly declared before,

Witness's Signature **Date:**

Name: Dr. Wong Won Fen

Designation: Senior lecturer

ABSTRACT

Helicobacter pylori is a Gram-negative, microaerophilic bacteria that colonizes the gastric epithelium in nearly half of the human population. It is well-established as an etiological agent for gastritis, peptic ulcer and gastric cancer. *H. pylori* infection begins with the invasion gastric epithelial layer. Subsequent damage of the epithelial layer enables *H. pylori* penetration into the lamina propria where it encounters macrophages. At present, the effect of low-MOI *H. pylori* infection on macrophage has not been fully elucidated. In this study, genome-wide transcription regulation of *H. pylori*-infected RAW264.7 murine monocytic macrophage cells was investigated at MOI 1, 5, 10 and 100. *H. pylori*-infected RAW264.7 cells displayed enlarged cell size and complexity, and expressed surface activation markers such as F4/80, CD11b, CD83 as well as CD86. Moreover, *H. pylori*-infected RAW264.7 cells displayed attenuation in cells proliferation through Ki67 cell proliferation assay. However, *H. pylori*-infected cells (MOI 1, 5, 10) do not displayed cellular apoptotic properties as shown in Annexin V assay. RNA of the non-infected control and *H. pylori*-infected RAW264.7 cells were subjected for microarray analysis, revealing up and downregulation of 1341 genes and 1591 genes respectively. Quantitative real time-PCR was performed on top 10 upregulated genes and top 10 downregulated genes for results validation. In total, KEGG pathway analysis showed induction in 8 significant pathways while 16 pathways were significantly suppressed. The activation of immune response-related genes in *H. pylori*-infected RAW264.7 cells, including colony stimulating factors (*Csf1, Csf2, and Csf3*), pro-inflammatory cytokines *(Il-1α, Il1-β, Tnf* and *Il-23α*) was confirmed by quantitative real time PCR and activated surface markers *(CD40, CD44, CD200)* was confirmed by flow cytometry analysis respectively. Furthermore, KEGG pathway analysis also showed repression of expression in multiple genes encoding for DNA replication and cell cycle. Interestingly, the list of top 10 downregulated genes also

comprised *Aurora-B kinase (Aurkb),* an essential cell cycle regulator responsible in augmenting phosphorylation and activation of cell cycle molecules. Along with this, cell cycle analysis showed that *H. pylori* infection inhibited cell cycle progression at G1-to-S phase as well as G2 to M phase transitions. The expression pattern of the cell cycle-associated genes was further validated using quantitative real time PCR and confirmed their suppression at the transcription level. Furthermore, immunoblot analysis verified the decreased protein expression of Aurkb and downstream phosphorylation of cyclin-dependent kinase 1 (Cdk-1), centromere protein A (Cenp-A) and also cyclin D caused by *H. pylori* infection. Lastly, *H. pylori* infection in primary bone marrow-derived macrophages (BMDM) was performed. Similar cell proliferation inhibition was observed, as in the RAW264.7 cell line. In summary, *H. pylori* disrupts expression of cell cycle-associated genes, thereby impeding proliferation of RAW264.7 cells, and such disruption may be an immuno-evasive strategy utilized by *H. pylori*.

ABSTRAK

Helicobacter pylori (H. pylori) adalah sejenis bakteria Gram-negatif, organisma mikroaerofilik yang menjajahi gastrik epitelium manusia. Ia menjangkiti hampir separuh populasi dunia dan adalah penyebab gastritis, ulser duodenum serta kanser gastrik. Infeksi *H. pylori* bermula pada permukaan epitelium gastrik, dan bertemu dengan makrofaj gastrousus dan monosit yang menyusup dalam lamina propria mukosa. Kini, penyiasatan kesan infeksi *H. pylori* pada kegandaan jangkitan rendah (MOI) terhadap makrofaj masih belum disiasati. Dalam kajian ini, ekspresi genom sel RAW264.7 yang diinfeksi *H. pylori* pada MOI 1,5 10 dan 1000 disiasati. Sel RAW264.7 yang diinfeksi menunjukkan pembesaran saiz dan peningkatan kompleksiti, diikuti pengaktifan penanda permukaan makrofaj seperti F4/80, CD11b, CD83 dan CD86. Selain itu, sel RAW264.7 memaparkan atenuasi sel proliferasi melalui asai sel proliferasi Ki67. Akan tetapi, proses apoptotik tidak dipaparkan oleh sel RAW264.7 yang diinfeksi *H. pylori* dalam keputusan asai apoptotik Annexin V. Pengekstrakan RNA dilakukuan terhadap sel RAW264.7 tanpa infeksi dan diinfeksi untuk dijalankan analisa mikrotatasusunan (*microarray*). Keputusan analisa menunjukkan peningkatan ekspresi 1341 gen dan penurunan ekspresi 1591 gen. Pengekspresan 10 gen yang tertinggi dan terendah dikenalpasti melalui tindak balas berantai polimerase masa bernyata (*real time-PCR*). Secara keseluruhan, 8 tapak jalan sel RAW264,7 menunjukkan peningkatan signifikan manakala 16 tapak jalan ditindas dalam infeksi *H. pylori*. Pengaktifan gen sel RAW264.7 diinfeksi berkaitan respons imun seperti faktor perangsang koloni (*Colony stimulating factors*) (*Csf1, Csf2* dan *Csf3*), dan sitokin pro-keradangan (*pro-inflammatory cytokines*) (*Il-1α, Il1-β, Tnf and Il-23α*) dikenalpasti melalui tindak balas polimerase masa bernyata manakala pengaktifan penanda permukaan *CD40*, *CD44* dan juga *CD200* dikenalpasti melalui asai sitometri pengaliran (*flow cytometry analysis*). Analisis tapak jalan KEGG juga menunjukkan ekspresi gen

pereplikaan DNA dan kitar sel dikurangkan. Senarai sepuluh gen yang paling dikurangkan dalam analisa mikrotatasusunan menunjukkan keputusan menarik, iaitu penurunan signifikan pada gen yang bernama *Aurora kinase b (Aurkb),* salah satu pengawal selia kitar sel yang utama. *Aurkb* bertanggungjawab dalam tindak balas pemfosforilan dan pengaktifan gen kitar sel. Untuk mendalamkan tapak jalan kitar sel, tindak balas berantai polimerase semasa juga dijalankan untuk pengenalpasti keputusan, dan kesemua ekspresi gen kitar sel dikurangkan pada tahap transkripsi. Bersama dengan ini, analisa sitometri kitar sel juga dijalankan untuk menunjukkan perencatan janjangan sel pada peralihan fasa G1 ke S, dan G2 ke M. Bukan sahaja itu, analisa pemblotan Western juga dijalankan terhadap sel RAW264.7 diinfeksi *H. pylori*. Pengurangan ekspresi protein Aurkb dan protein hilirannya phospho-Cdk 1, protein sentromer A dan siklin D diperhatikan pada sel RAW264.7 diinfeksi *H. pylori* berbanding dengan sel RAW264.7 tidak diinfeksi. Akhirnya, untuk pengenalpastian kesesuaian RAW264.7 sel sebagai model *in vitro*, pengekstrakan sum-sum tulang dan pembezaan kepada makrofaj (BMDM) dilakukan. BMDM yang diinfeksi dengan *H. pylori* menunjukkan proliferasi menurun dan ia adalah selaras dengan keputusan sel RAW264.7. Secara rumusan, sebagai satu strategi mengelak sistem hos perumah, *H. pylori* menganggu gen kitar sel makrofaj, menyebabkan perencatan proliferasi sel RAW264.7.

ACKNOWLEDGEMENTS

I dedicate my thesis

To my mentor and supervisor- Dr. Wong Won Fen, for teaching me the lab skills and providing me guidance required to be a true researcher;

My co-supervisors, Dr. Chang Li-Yen and Prof. Sazaly Abu Bakar, for advice and constant supports in my postgraduate journey;

Dr. Loke Mun Fai, for enlightment and constructive suggestions in *H. pylori* research;

My parents on continuous mental support during difficult times;

My younger brother Simon who always prays and cares for me;

My lab mates and ex-lab mates- Elaheh, Yeow, Keith and Alicia, for assistance in my laboratory work without hesitation;

My batch mates from Department of Medical Microbiology- for the companion and words of encouragements during this postgraduate journey;

University Malaya Fellowship, for the financial support throughout my Master studies;

Last but not least, Daniel Chong, for being my pillar of strength despite the ups and downs.

TABLE OF CONTENTS

CHAPTER 3: MATERIALS AND METHOD

CHAPTER 4: RESULTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviations	Description
%	percent
℃	Degree celcius
CFU/mL	colony forming unit/ mililiter
h	hours
kDa	kilo Dalton
mL	mililiter
mM	mili Molar
ng	nanogram
nm	nanometer
U/mL	unit per mililiter
v/v	volume/ volume
α	alpha
β	beta
µg/mL	microgram per mililiter
µm	micrometer
APC	Antigen presenting cell
APC	Allophycocyanin
ATCC	America Type Culture Control
BEC	S-(2-boronoethyl)-L-cysteine
BHI	Brain Heart Infusion
BMDM	bone marrow derived macrophages
bp	base pair
cagPAI	Cag Pathogenicity Island
Ccl2	CC-chemokine ligand 2
Cd	Cluster of differentiation
Cdk	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
CO_2	carbon dioxide
cRNA	complementary ribonucleic acid
CSF	colony stimulating factor
Ct	cycle threshold
Cx3cr1	CX3 chemokine receptor 1
DC	Dendritic cell
DMEM	Dulbecco's Modified Essential Medium
EPIYA	glutamate-proline-isoleucine-tyrosine-alanine
FAK	focal adhesion kinase
FBS	fetal bovine serum
FC	fold change
FcR	Fc receptor
FCS	fetal calf serum
FDR	false discovery rate

FE	fold enrichment
FITC	Fluorescein isothiocyanate
FSC	forward scatter
GC	gastric cancer
G-CSF	granulocyte colony stimulating factor
H. bilis	*Helicobacter bilis*
H. felis	*Helicobacter helis*
H. muridarum	*Helicobacter muridarum*
H. pylori	*Helicobacter pylori*
H. rappini	*Helicobacter rappini*
H. trogontum	*Helicobacter trogontum*
HLA-DR	human leukocyte antigen-D related
hpi	hours post infection
HP-NAP	*H. pylori* neutrophil-activating protein
IFN- γ	Interferon-γ
IL	interleukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAMP	lysosome-associated membrane protein
LPS	Lipopolysaccharide
MALT	mucosa-associated lymphoid tissue
Mbp	mega base pairs
MHC	Major Histocompatibility Complex
MIF	migration inhibitory factor
MMLV	Moloney Murine Leukemia Virus
MOI	multiplicity of infection
mRNA	messenger RNA
Na_3VO_3	sodium orthovanadate
NCBI	National Center for Biotechnology
NH_3	ammonia
NK cells	natural killer cells
nm	nanometer
NO	nitric oxide
OD	optical density
PAMPs	pathogen-associated molecular patterns
PBS	Phosphate Buffer Saline
PD1L	programmed cell death-1 ligand
PE	phycoerythirin
PI	propidium iodide
PLC-γ	phospholipase C-gamma
PMSF	phenlymethylsulfonyl fluoride
PRRs	pathogen recognition receptors
PVDF	polyvinylidenedifluoride
Rb	Retinoblastoma
RIN	RNA integrity number
RIPA	Radioimmunoprecipitation assay

RNA	ribonucleic acid
ROI	reactive oxygen intermediates
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SS1	Sydney Strain 1
SSC	side scatter
SD	standard deviation
TBST	Tris buffered saline with Tween-20
TCR	T cell receptor
T_H0	naïve $CD4^+$ T helper cells
T_H1	T Helper 1
T_H17	T Helper 17
T_H2	T Helper 2
TLR	toll-like receptor
TLR2	toll-like-receptor 2
TNF-α	tumor necrosis factor
Vac A	Vacuolating toxin A
WT	wild type

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Introduction

Helicobacter pylori (*H. pylori*) is a micro-aerophilic, *Gram*-negative, spiral shaped organism that colonizes human gastric epithelium and duodenal mucosal (Pathak *et al.*, 2013). It is one of the most successful human pathogen as it colonizes the gut of half of the world's population (Chaturvedi *et al.*, 2007). The epidemiology of *H. pylori* has been declining in prevalence in most of the countries (Torres *et al.*, 2000). However, the rate of *H. pylori* infection is still high in under-developed countries. In Asia, the studies showed high prevalence rates of *H. pylori* infection ranging from 54 % to 76 % (Eusebi *et al.*, 2014). In Malaysia, the overall seroprevalence rate of *H. pylori* infection is 35.9 %, with Indians having the highest infection rate (45.4 %), followed by Chinese (36.8 %) and the west were seen in Malays (18.3 %) (Goh, 2009).

H. pylori is the first bacterial species to be classified as a group I carcinogen by the International Agency for Research on Cancer (Salih, 2009). It is the main causative agent of gastritis and duodenal ulcer which may further progresses into gastric adenocarcinoma in a substantial proportion of the infected individuals. According to the most recent Malaysia Cancer Statistics, gastric cancer ranks as the 8th most frequent cancers in Malaysia, comprising up to 3.8 % of all cancer cases (Registry, 2011). Among the three major ethnics, ethnic Chinese population consistently show a high gastric cancer age standardized incidence rate (ASR) comparatively to ethnic Indians and Malays (Goh, 2009). Survival of patients with gastric cancer is dependent on the stage at which diagnosis is made. Thus, early diagnosis is a crucial factor in influencing the outcome of the infection. Without treatment, the infection is often incompletely eradicated and can persist through life despite eliciting vigorous innate and adaptive immune response of the host (Chaturvedi *et al.*, 2007).

The normal gastric mucosa of *H. pylori*-negative adults and children is populated by very few macrophages. During *H. pylori*-mediated infection, the bacterium starts in the gastric epithelium, which triggers activation of residual macrophages and large influx of immune cells that include monocytes and neutrophils. Monocytes attracted to the site of infection will further differentiate into dendritic cells (DC) or macrophages. Findings also suggested gastritis in the acute *H. pylori* infection is predominantly mediated by macrophages (Kaparakis *et al.*, 2008). Moreover, the number of macrophages increases with the severity of gastritis and the duration of the infection (Krauss-Etschmann *et al.*, 2005). Evidence of this statement was also shown when gastric biopsy or tissue RNA specimens from infected patients demonstrate that both M1 and M2 types of macrophages existed (Fehlings *et al.*, 2012). In general, M1 macrophage functions to elicit inflammatory responses, while M2 in contrast limits excessive inflammatory response to avoid damage to epithelial layer. Activation of macrophages by *H. pylori* infection causes increased CD11b, CD32, CD80, CD86 and HLA-DR surface activation markers, accompanied by secretion of cytokines including IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, IL-23 and migration inhibitory factor (MIF) (Fehlings *et al.*, 2012). Besides, macrophages secrete IL-12p70 and IL-23 to stimulate T_H1 and T_H17 responses, respectively in the *H. pylori*-infected gastric mucosa or mesenteric lymph nodes (Fehlings *et al.*, 2012; Quiding-Järbrink *et al.*, 2010). Furthermore, clinical data on gastric cancer patients also suggest that macrophage infiltration is correlated with the advanced stage and poor survival of patients (Wu *et al.*, 2015). All these researches suggest that *H. pylori* is often able to evade the host cellular and humoral immune response through macrophages; successfully establishing a chronic infection by achieving a balance between inducing immune responses and surviving the inflammatory milieu (Lina *et al.*, 2014).

In order to maintain the persistent infection in the host, *H. pylori* develop various immune evasion strategies to resist the elimination by host immune system, for example: utilizing its virulence factors to delay macrophage-mediating phagocytosis (Allen *et al.*, 2000; Ramarao *et al.*, 2000). Antigen presentation properties of macrophages are also proven to be attenuated as *H. pylori* delays phagosome maturation through retention of endosome markers (Rab7 and CD63) (Borlace *et al.*, 2011). Besides, *H. pylori* causes apoptosis of macrophages through activation of Arginase 2 (Arg2) (Lewis *et al.*, 2010) or mitochondrial-dependent pathways (Menaker *et al.*, 2004). Despite all these investigations showing *H. pylori* promotes cell death of macrophages; there are also contradicting evidence showing the presence of a large number of macrophages at the infection site during *in vivo* examination (Fehlings *et al.*, 2012; Quiding-Järbrink *et al.*, 2010). This study hypothesizes that *H. pylori* are present in the stomach at low multiplicity of infection (MOI), triggering activation of host immune response but not sufficient to trigger apoptosis in host macrophages. However, at present, the crosstalk of macrophages and *H. pylori* is not fully described. Moreover, the mechanism as to how *H. pylori* escape the immune response in its gastric niche is clinically important. In this study, this research question is elucidated using RAW264.7 murine cell line as *in vitro* model representing the monocytic macrophage population of the host. As monocytic macrophage cell line, they are able to internalize *H. pylori* SS1 (a well-established mouse-adapted pathogenic strain) and display features of activated macrophages, eliciting robust immune response after maturation (Gobert, Alain P. *et al.*, 2002).

1.2 Objectives

It is important to understand the response of macrophages against *H. pylori* infection at different stages, particularly during initial and chronic stages. The overall objective of this study is to investigate the genome-wide gene expression and cellular responses of *H. pylori*-infected RAW264.7 monocytic macrophages. The specific objectives are as follow:

1. To infect macrophages with low MOIs of *H. pylori* and monitor the cell responses
2. To investigate the transcriptional modifications of macrophages during *H. pylori* infection
3. To further analyze and validate the up or downregulated genes mediated by *H. pylori* infection
4. To understand the interaction between host macrophages responses during *H. pylori* infection

CHAPTER 2: LITERATURE REVIEW

2.1 Background of *Helicobacter pylori*

In the late 19th and early 20th centuries, researchers discovered the existence of spiral bacteria in both stomach of animals as well as humans. It was speculated that these microorganisms cause gastric cancer and peptic ulcer disease, but was ignored because most of the patients have no clinical symptoms (Kusters *et al.*, 2006). Only in year 1982, *H. pylori* was discovered by two Australian researchers, Drs. Barry Marshall and Robbin Warren (Marshall & Warren, 1984). The organism was initially named "*Campylobacter*-like organism" "*Campylobacter pyloridis"* and "*Campylobacter pylori"* but later changed to *H. pylori* in recognition that it is distinctly different from the genus of *Campylobacter* (Goodwin *et al.*, 1989). Before the discovery, it was a long-standing belief in medical teaching that stress and lifestyle factors were the major causes of peptic ulcer diseases. After they rebutted that dogma, it was soon clear that *H. pylori* causes more than 90 % of duodenal ulcers and 80 % of gastric ulcers (Marshall *et al.*, 1985). The infection in certain patients further develops into malignant diseases such as gastric cancer (GC), pancreatic cancer (Uemura *et al.*, 2001), and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Campuzano-Maya, 2014). The incidences of gastric carcinoma may vary dramatically among geographical areas due to the diversity of *H. pylori* strains and polymorphisms among individuals (Uemura *et al.*, 2001)

2.2 Epidemiology of *H. pylori* infection in Malaysia

Due to the multiracial background, Malaysia exhibits a wide variability of *H. pylori* prevalence among racial groups. Among the three major racial groups in Peninsular Malaysia, Malay community was found to have a lower prevalence of 10.0 to 25.0 % comparatively to 35.0 to 55.0 % among ethnic Chinese and 50.0 to 60.0 % among ethnic Indians (Goh & Parasakthi, 2001). Among the indigenous community, *H. pylori* prevalence was reported 37.7 % in Penan community (East Malaysia) and 37.9 % in the Seletar community (Southern Malaysia) (Musa *et al.*, 2014). These studies indicate overcrowding in poor socioeconomic conditions encourages the spread of infection, leading to an increase in infection among a particular population. Moreover, a theory of "racial cohort" has been proposed, which explains that *H. pylori* infection had remained confined to a particular racial group due to the low level of intermarriages between races. Thus, the transmission and perpetuation of infection takes place within a racial group rather than between races (Goh, 2009). Furthermore, as *H. pylori* infection requires close transmission and is normally acquired during early childhood, the infection remains closely confined to predefined cohorts.

Across the globe, there are six *H. pylori* populations which are designated as hpAfrica1, hpAfrica2, hpNEAfrica, hpEurope, hpEastAsia, and hpAsia2 (Linz, B. *et al.*, 2007). The most recent phylogeny studies in Malaysia report that *H. pylori* isolated strains from local Chinese individuals are clustered closely with hspEAsia (subpopulation of hpEastAsia), while most strains from the local Malay and Indian subjects were found clustered together to form the hspIndia branch (subpopulation of hpAsia2) (Gunaletchumy *et al.*, 2014). However, previous research reports that the isolates from Malay patients were a mixed group. They hypothesize that the ethnic Malay population was originally free of *H. pylori* and have more recently acquired from others most likely the Indians (Goh, 2009) while *H. pylori* isolates from the Chinese

individuals and Indian individuals were divided along their ethnic origin (Tay *et al.*, 2009).

Studies on gastric cancer and *H. pylori* have proven even more interesting. Despite the higher prevalence of *H. pylori* in ethnic Indian, they have a relatively low incidence of peptic ulcer disease and gastric cancer. In fact, Cancer registries from Peninsular Malaysia and Singapore consistently show a higher gastric cancer in ethnic Chinese compared to Indians and Malays (Curado *et al.*, 2007; Lim, G. C. C. *et al.*, 2002). One of the speculations is the 'Indian Enigma' (Misra *et al.*, 2014). Dietary factor such as consumption of curcumin as gastroprotective and anti-cancer property may be one of the reason for the low prevalence among ethnic Indians (Sintara *et al.*, 2010; Yadav *et al.*, 2013). However, the exact reasons for these observations are still undefined.

2.3 Clinical symptoms and manifestations of *H. pylori* infection

H. pylori specifically colonizes the gastric epithelium of human where they can lead to spreading and persistence, thus resulting in severe inflammatory and malignant neoplastic disorders (Hagymási & Tulassay, 2014; Testerman & Morris, 2014). Natural colonization of *H. pylori* is restricted to human, primates and domestic animals such as cat (Cover & Blaser, 2009; Neiger & Simpson, 2000; Simpson *et al.*, 2001). It can be transmitted by direct human-to-human transmission, via oral-oral route, fecal-oral route or both (Konno *et al.*, 2005; Rowland *et al.*, 2006). The classic inflammatory disorders are gastritis and dyspepsia.

Gastritis is defined as an irritation of the stomach lining and is usually determined by the Sydney System (Dixon *et al.*, 1996). This system requires five histology location of the stomach: the greater and lesser curvature of antrum, the greater and lesser curvature of corpus, and the incisura. With these five specimens, it is graded

on 0-4 scale on a few variables: *H. pylori* density, neutrophil infiltration, mononuclear infiltration, atrophy (decline of organ due to degeneration of cells) and intestinal metaplasia. A simpler system of categorizing gastritis is based on Table 2.1.

Table 2.1: Morphologic determinants of gastritis, adapted from (Owen, 2003)

Location	Antral predominant, corpus predominant or pangastritis
Focality	Focal or diffuse
Depth	Superficial or full thickness
Atrophy	Present or absent
Metaplasia	Present of absent
Inflammation	Active, chronic or both
H. pylori	Present or absent

Majority of patients diagnosed with *H. pylori* gastritis have chronic gastritis for years. Acute gastritis affects the entire stomach and is accompanied by the loss of acid secretion. This will then recruit neutrophils to the lamina propria and epithelium, resulting in epithelial layer damage due to reactive oxygen species and other neutrophil products. The intensity of neutrophil infiltration is considered proportionate to the degree of activity of gastritis (Owen, 2003). Meanwhile, chronic gastritis depends on the exact location of *H. pylori* in the stomach lining. It can be antrum-predominant, corpus-predominant or diffuse (Figure 2.1).

.

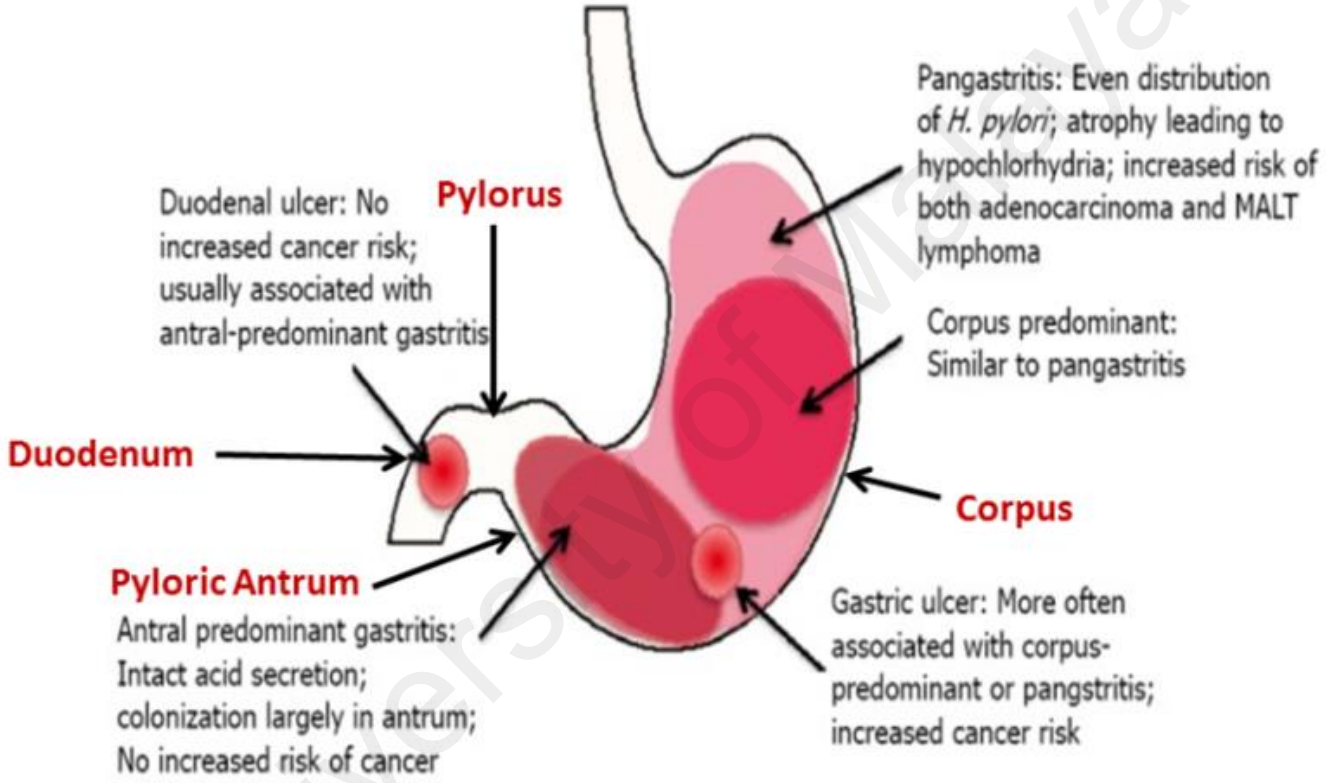


Figure 2.1: The typical pathology for *H. pylori*-associated pathology. Gastritis may be antral-predominant, corpus-predominant or diffuse. Figure adapted from (Testerman & Morris, 2014)

For antrum-predominant cases, *H. pylori* is limited at the antrum, thus does not affect the acid secretion (McColl *et al.*, 1998). Antral gastritis favors development of duodenal ulcer while corpus-predominant often develops into gastric ulcer and sometimes progressing into metaplasia and adenocarcinoma (Kusters *et al.*, 2006; Lochhead & El-Omar, 2007). Till date, *H. pylori* has been reported to be responsible for 70.0 to 85.0 % of gastric ulcers and 90.0 to 95.0 % of duodenal ulcer. Interestingly, only corpus-predominant cases show increased risk of cancer development (Konturek *et al.*, 2009; Testerman & Morris, 2014). Diffuse gastritis, which is also known as multifocal gastritis, causes acid secretion impairment, allowing *H. pylori* to further colonize the whole corpus.

Dyspepsia is defined as pain associated with the stomach or upper abdomen. There is evidence that suggests *H. pylori* infection contributes to some cases of dyspepsia, even though many patients have no evidence of gastric damage or ulceration. In a clinical trial involving 585 dyspepsia patients, *H. pylori* eradication has successfully reduce epigastric pain syndrome in these patients. The symptoms of dyspepsia do not always reduced after *H. pylori* eradication therapy, but the risk of future ulcer and gastric cancer development were reduced (Xu, S. *et al.*, 2013).

H. pylori infection causes gastric cancer in 2.9 % of *H. pylori* patients (Uemura *et al.*, 2001). There are three main types of stomach cancers, which are gastric adenocarcinoma, non-Hodgkin's lymphoma including mucosa-associated lymphoid tissue (MALT) lymphoma, and gastrointestinal stromal tumor (Castaño-Rodríguez *et al.*, 2014). In year 2012, 95.0 % diagnosed cases are GC, resulting it to be the fifth most common cancer worldwide, accounting 6.8 % of the total cancer cases. In relation to location, GC may occur in the cardia or non-cardia region of the stomach. Although *H. pylori* infection association with cardia cancer is uncertain, it's infection is responsible for approximately 75.0 % of all non-cardia gastric cancers and 63.4 % of all stomach

cancers in the world. According to Lauren classification, non-cardia gastric cancer is further categorized as two histological variants: intestinal-type and diffuse-type GC (Fuchs & Mayer, 1995).

Intestinal-type GC, often starts from precancerous phase with a transition of normal mucosa into multifocal atrophic gastritis. It starts from chronic gastritis and further progresses to atrophic gastric mucosa, intestinal metaplasia, dysplasia and finally GC (Figure 2.2). It is characterized by the formation of gland-like structures, distal stomach localization and is more frequent in males (2:1 ratio) (Fuchs & Mayer, 1995). On the other hand, diffuse type GC is less differentiated, characterized by scattered tumor cells without gland formation, and affects younger individuals. The mechanism for the development of diffuse-type GC is poorly understood, but abnormal DNA methylation is likely involved (Nasri *et al.*, 2008; Yamamoto *et al.*, 2011). The prevalence of *H. pylori* infection is statistically higher in patients with intestinal-type (89.2 %) compared to diffuse-type (31.8 %) GC (Testerman & Morris, 2014). The diagnosis for gastric cancer is often delayed, as the early specific symptoms are often absent, causing patients seeking treatment only after the infection had invaded the muscularis propria. This maybe one of the reason why the survival rate of gastric cancer is less than 15.0 % in United States in year 2005 (Correa, 2004).

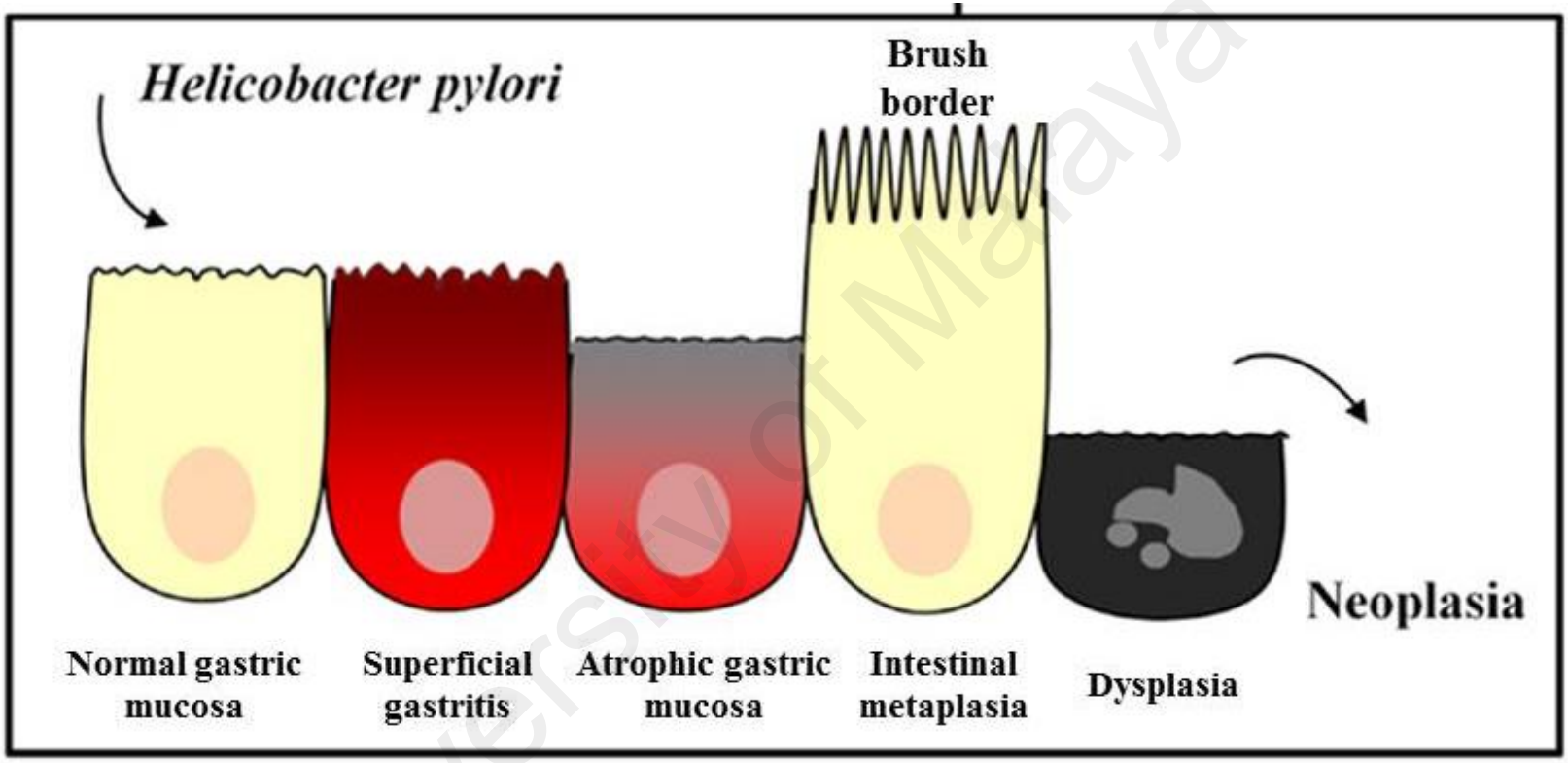


Figure 2.2: Progression of gastric etiology by *H. pylori* infection. According to Correa's cascade, the histological variants commences as chronic gastritis and progresses to atrophic gastritis, intestinal metaplasia, dysplasia and finally leads to gastric cancer. Adapted from (Castaño-Rodríguez *et al.*, 2014)

2.4 Genome of *H. pylori*

To date, according to National Center for Biotechnology (NCBI), there are 75 complete sequenced *H. pylori* genomes that are approximately 1.7 Mbp, with a G+C content of 35.0 to 40.0 %. The genome of all the strains has less than 1600 genes (Tomb *et al.*, 1997). Comparatively to other Gram-negative bacteria such as *Esherichia coli* which have approximately 4000 genes, *H. pylori* possesses a relatively small genome (Blattner *et al.*, 1997). All genomes of *H. pylori* have their own copies of 5S, 16S and 23S of rRNA genes.

H. pylori strains are genetically heterogeneous and lack of clonality; resulting in distinct strains isolated from every *H. pylori*-positive patients (Kansau *et al.*, 1996). The genetic heterogeneity is thought to occur via DNA rearrangement and deletion of foreign sequences (Achtman & Suerbaum, 2000; Falush *et al.*, 2003; Suerbaum & Achtman, 2004) It is strongly suggested that this is possibly an adaptation skill of the bacterium, to adjust itself in different gastric conditions and immune responses of the host (Kuipers *et al.*, 2000).

2.5 Basic morphology of *H. pylori*

In *in vivo* and *in vitro* conditions, *H. pylori* is in an S-shaped bacterium with 1 or 3 turns. It is 0.5 × 5 μm in length, spiral in shape and possesses 3 to 5 polar flagella that are used for motility (Goodwin *et al.*, 1989; Jones *et al.*, 1985) (Figure 2.3A). Generally, it is considered that the spiral morphology and flagella are essential for bacterial colonization. However, Helicobacter genus “ball up” and form coccoid cells as they aged. When this form is dominating, there will be a decrease in cultivability as determined by colony forming unit (CFU) counts. *H. pylori* flagella are mostly sheathed; with configurations ranging from a single polar flagellum through to bipolar tufts of up to 20 flagella (DePamphilis & Adler, 1971). They are approximately 30 nm

in diameter, consisting of an internal filament ~12 nm, where their outer membrane is connected with the outer membrane of the cell (Goodwin *et al.*, 1989; Jones *et al.*, 1985). Other than flagella, periplasmic fibers are also found on Helicobacter species such as: *H. felis, H. muridarum, H. bilis, H. trogontum* and *H. rappini* (Figure 2.3B) (Jones *et al.*, 1985). It has been suggested that periplasmic fibers are involved in motility. They are different from flagella, as they have different insertion points to flagella (Lee & O'Rourke, 1993). *H. pylori* is also microaerophilic, urease, catalase and oxidase positive.

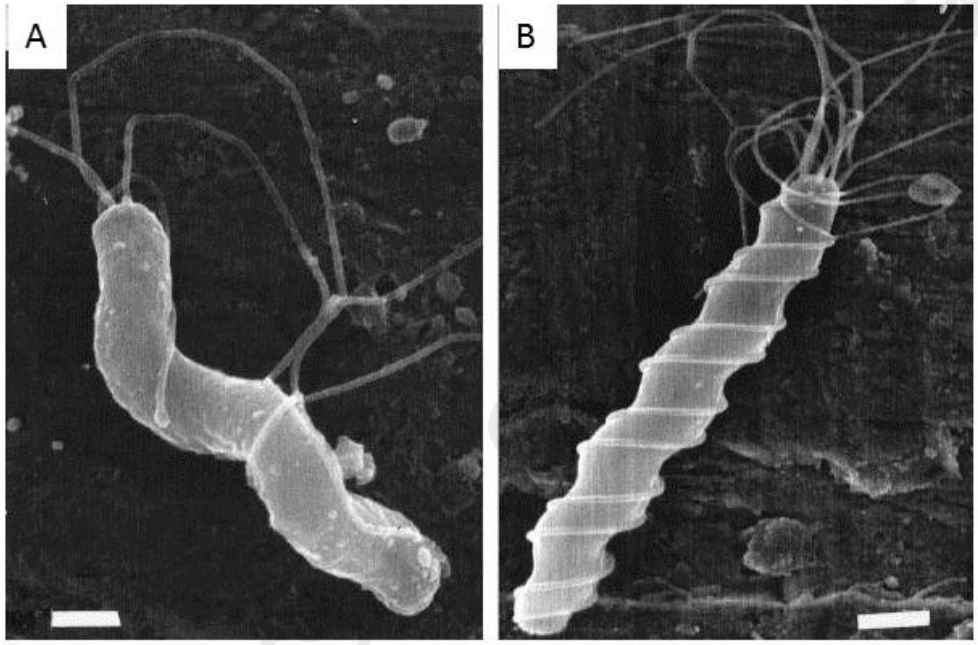


Figure 2.3: Morphology of Helicobacters. (A) S-shaped *H. pylori* with sheathed polar flagella. Field emission SEM bar= 0.5 μm (B) Detail of periplasmic fibers. Figure adapted from (Bode *et al.*, 1993)

2.5.1 Internal organization of *H. pylori*

As a Gram-negative bacterium, *H. pylori* possesses typical cell wall detail of outer and inner membrane separated by periplasmic space (Costa *et al.*, 1999). However, its peptidoglycan is found to have a unique muropeptide composition, which is less complex comparatively to other Gram-negative bacteria. Researchers also found intracellular granules in different location within the bacterium: the cytoplasm, the flagella pole and in the cell membrane (Bode *et al.*, 1993). The granules in the cytoplasm are the largest (0.05 to 0.2 μm) as they act as an energy source and phosphorus reservoir; the granules near the flagella pole are smaller (0.02 μm) yet compact, indicating its function to provide energy for motility. Lastly, the granules located in the cell membrane are the smallest in size (<0.01 μm), and responsible for the maintenance in the cell membrane (Bereswill *et al.*, 1998).

2.6 Virulence factors of *H. pylori*

H. pylori colonizes the gut of approximately 50 % of world's population. However only a small proportion of people infected with *H. pylori* develop gastrointestinal diseases. Of those infected, those who will develop this disease are influenced by host genetics and environmental factors, most importantly by the different virulence factors that determine the pathogenicity of *H. pylori.* Bacterial virulence factors are characteristics present in some bacteria which enable them, rather than others to cause disease. These important virulence factors of *H. pylori* are discussed below.

2.6.1 Urease

The unique feature of *H. pylori* is its ability to survive extreme acidic environment of the stomach (Marshall & Langton, 1986). This can be succeeded due to its ability in synthesizing cytosolic urease, which enables the hydrolysis of urea into

ammonia (NH_3) and carbon dioxide (CO_2), creating a neutral layer for its own survival (Hu & Mobley, 1990; Labigne *et al.*, 1991). Urease-defective *H. pylori* mutants show their inability in colonizing the stomach (Eaton *et al.*, 1991). Urease is formed by two subunits, UreA (26.5 kDa) and UreB (60.3 kDa) (Labigne *et al.*, 1991). The reaction starts with urea being taken up by *H. pylori* through a proton gated channel (Weeks *et al.*, 2000). This channel is regulated positively by protons, opening at acidic pH to increase urea uptake; and closing it at neutral pH to avoid over-alkalinization, as the alkaline environment is toxic towards the bacterium (Clyne *et al.*, 1995). Moreover, urease does not only contribute to the pathogenesis of *H. pylori* but also help in the recruitment of neutrophils and monocytes, resulting stimulation of pro-inflammatory cytokines (Harris *et al.*, 1996). The end product of the reaction, ammonia also causes damage to the host cells. It can cause swelling of intracellular acidic compartments, alterations of vesicular membrane transport, depression of protein synthesis and cell cycle arrests (Suzuki, M. *et al.*, 1992). It can also react with intermediates released by neutrophil (myeloperoxidase) to form carcinogenic agents that are responsible for stomach adenocarcinoma (Megraud *et al.*, 1992).

2.6.2 Vac A

2.6.2.1 Polymorphism and structure of VacA

H. pylori synthesizes Vacuolating toxin A (VacA) as an approximately 140 kDa pre-protoxin, which undergoes sequential proteolytic processing to become an 88 kDa auto-transporter protein (Cover *et al.*, 1993). The mature protein is secreted in a two-step process involving p33, the signal peptide which directs secretion from the cytoplasm to the periplasm, and p55, carboxy-terminal auto transporter which directs export across the outer membrane (Figure 2.4B). The differences in vacuolating activity between strains are due to the diversity of sequence found in several defined regions of

VacA (Forsyth *et al.*, 1998). The isolates identified carried non-sense mutations, internal duplications, deletions or 1 base pairs (bp) insertion with the gene (Ito *et al.*, 2008). There are three regions in the gene, signal (s) region, middle (m) region and intermediate region (i) (Figure 2.4A). The m region of *VacA* encodes an approximately 800 bp region in the carboxyl terminal of p55 domain of VacA. The entire m region is apparently essential for cell surface binding and vacuolating activity of the toxin (Skibinski *et al.*, 2006; Wang, W. C. *et al.*, 2001). Within the m region, there are two primary alleles, m1 and m2 (Atherton *et al.*, 1997; Ji *et al.*, 2000; Pagliaccia, C. *et al.*, 1998). S region is the amino-terminal end of VacA, which is the signal sequence that directs secretion across the bacterial inner membrane into amino terminus of the processed mature toxin (Atherton *et al.*, 1997; Strobel *et al.*, 1998; Van Doorn *et al.*, 1999). This region also shown considerable sequence diversity, with two primary allelic groups referred as s1 and s2. The i region has been identified in the carboxyl-terminal half of p33 between the s and m regions. However, the function of this region and the structure has not been identified (McClain *et al.*, 2001).

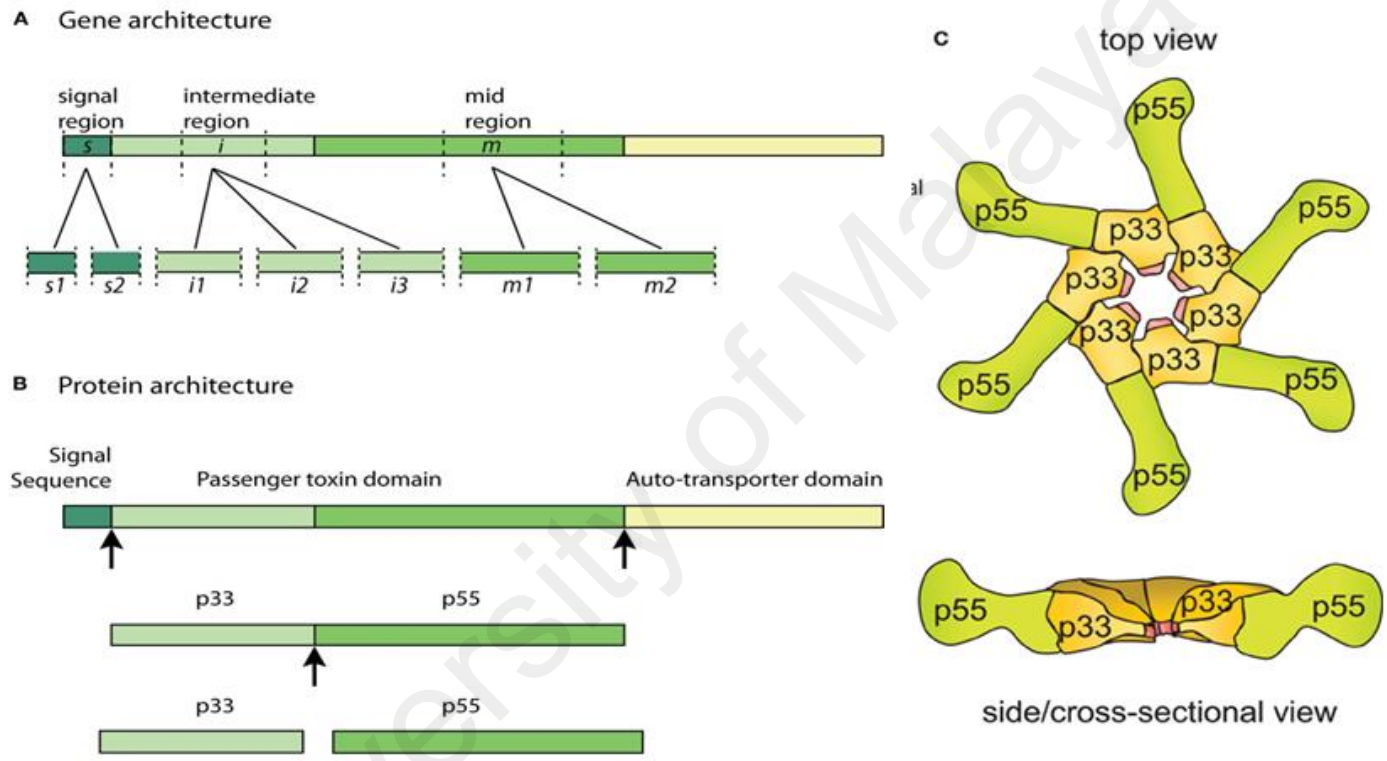


Figure 2.4: Schematic of VacA structure. (A) described the polymorphic nature consist of three major allele families, which are located in the signal region (s region), the intermediate region (i region), and the mid region (m). (B) described Figure adapted from (Kim & Blanke, 2012; Palframan, S. L. *et al.*, 2012)

Epidemiology studies have correlated VacA s, m and i region allelic types with the occurrence and severity of *H. pylori*-related diseases. Many studies have provided evidence for a higher association of disease in individuals infected with *H. pylori* strains possessing s1 VacA alleles. It displays greater cellular activity, higher risk for gastric carcinoma than strains harboring VacA s2 alleles (Letley *et al.*, 2003; McClain *et al.*, 2001).

2.6.2.2 Function of VacA

VacA is one of the most important virulent factors of *H. pylori*. The cytotoxin gene *VacA* is present in all strains of *H. pylori*, although there are considerable differences in vacuolating activities attributed to the variations in gene structure (Atherton *et al.*, 1995; Van Doorn *et al.*, 1999). The unique feature of VacA is the unusual resistance to pepsin digestion, and not denatured at pH 1.5. In fact, it is activated by short exposure to acid or alkaline (de Bernard, M. *et al.*, 1995). The main cellular activity of VacA is the capacity to induce the biogenesis of large intracellular vacuoles (Leunk *et al.*, 1988; Pagliaccia, Cristina *et al.*, 1998; Smoot *et al.*, 1996). It also disrupts gastric epithelial barrier function, disturbs late endosomal compartments and modulates the inflammatory response (de Bernard, M. *et al.*, 2002). VacA can also bind to receptor-type protein tyrosine phosphatase (RPTPβ) to increase cell proliferation, differentiation and adhesion, which all play a role in ulcerogenesis (Xu, Y. & Fisher, 2012).

2.6.3 Cag Pathogenicity Island and CagA

Cag Pathogenicity Island (Cag PAI) is the 40-kb DNA insertion element, containing approximately 32 genes that encode building blocks of type IV secretion apparatus. It enables *H. pylori* to exhibit a high degree of genetic heterogeneity due to its genomic arrangement, gene insertions and/or deletion (Alm *et al.*, 2000; Go *et al.*, 1996; Salama *et al.*, 2000; Tomb *et al.*, 1997). The gene *CagPAI* codes for the building blocks of type IV secretion apparatus; delivering CagA protein (120-140 kDa) that is translocated into host cells after bacterial attachment (Odenbreit *et al.*, 2000). Once inside the cell, CagA is tyrosine phosphorylated by the host Abl and Src kinases at glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) motifs and induces cell morphological changes (called "hummingbird phenotype") (Neel *et al.*, 2003). Phospho-CagA interacts with β-

catenin, disrupts the apic-junctional complexes, and causes the cell to lose its polarity. It also will interact with other intracellular effector such as SHP-2, that resulting in dephosphorylation and inactivation of focal adhesion kinase (FAK), resulting in cellular elongation. Non-phosphorylated CagA also exerts effect within the cell. The translocation of CagA targets E-cadherin (cell adhesion protein), hepatocyte growth factor receptor c-Met, phospholipase C-gamma (PLC-γ), and the adaptor protein Grb2. This leads to pro-inflammatory and mitogen responses, disruption of cell-cell junction and loss of cell polarity. Moreover, CagA is proven to induce apoptotic signal through the p53, tumor-suppressor pathway, but the exact mechanism remains not fully elucidated (Backert *et al.*, 2001; Higashi *et al.*, 2002; Stein *et al.*, 2002).

H. pylori strains are frequently segregated into CagA-positive ($CagA^+$) and CagA-negative ($CagA^-$) strains, depending on the presence of the terminal gene product CagA. Studies had proven that $CagA^+$ amplifies the risk for severe gastritis, atrophic gastritis and distal gastric cancer in comparison to $CagA^-$ *H. pylori* (Wroblewski *et al.*, 2010). Almost all $CagA^+$ strains are classified as VacA s1 genotypes, whereas $CagA^-$ strains are classified as the VacA s2/m2 strain.

2.6.4 HP-NAP

Other than the well-known virulence factors above, *H. pylori* neutrophil-activating protein (HP-NAP) is also an important pathogenesis factor. The name was given because it stimulates high production of oxygen radicals from neutrophil, thus promoting neutrophil adhesion to endothelial cells (Boncristiano *et al.*, 2003). In structure, HP-NAP is a 150 kDa decamer which is localized in the bacterial cytosol and released upon autolysis (Evans *et al.*, 1995; Zanotti *et al.*, 2002). During its release into the host, it mediates the binding of *H. pylori* to the cell surface via interaction with carbohydrates (Teneberg *et al.*, 1997). First of all, it is chemotactic for neutrophils and

monocytes (Satin *et al.*, 2000). It binds to toll-like-receptor 2 (TLR2) on neutrophil via its C-terminal region, stimulating a cascade of intracellular events, such as: increase in cytosolic Ca^{2+} concentration, phosphorylation, assembly of cytosolic subunits of NADPH oxidases, which finally induces reactive oxygen intermediates (ROI) production (de Bernard, M. & D'Elios, 2010; Dundon *et al.*, 2002; Satin *et al.*, 2000). Research also found that HP-NAP is able to increase the expression of IL-12p35, IL-12p40, and IL-23p19 in both neutrophil and monocytes, creating cytokine milieu enriched in IL-12 and IL-23, driving the differentiation of antigen-stimulated T cells towards T_H1 phenotype. Other pro-inflammatory cytokines such as tumor necrosis factor (TNF) and IL-8 are also secreted along with Major Histocompatibility Complex (MHC) class II upregulation in HP-NAP incubated monocytes. These monocytes were further investigated, where expression of CD80, 86 and human leukocyte antigen-D related (HLA-DR) marker significantly increased (D'Elios *et al.*, 2007).

2.7 Macrophages

2.7.1 Monocytes subsets and chemokine receptors

The main role of monocytes is to replenish the pool of tissue macrophages and dendritic cells. In order to response to different environment stimuli, they are heterogenic and can be divided into subsets that display specific functions (Yang *et al.*, 2014). Classic inflammatory monocytes are equipped with pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs) recognizing pathogen-associated molecular patterns (PAMPs). They are derived from $CD34^+$ myeloid progenitor cells which originate from the bone marrow and traffic via bloodstream to peripheral tissue throughout the body, where they seed as immature monocytes. These bloodstream monocytes are subdivided into subsets that differ in size, trafficking, innate immune

receptor expression and their ability to differentiate when stimulated with cytokines or microbial molecules (Auffray *et al.*, 2009).

In mouse, monocytes are divided into three subsets according to their differential expression of chemokine receptor Ly6C (Gr1): $LY6C^{high}$, $LY6C^{medium}$, and $LY6C^{low}$ (Geissmann *et al.*, 2003). Ly6C is an inflammatory monocyte receptor that is enriched on monocytic myeloid lineages (Si *et al.*, 2010) It is a useful marker that is use to determine relative amounts of granulocytes and monocytes or macrophages (Murray & Wynn, 2011). $LY6^{+}$ cells including ($LY6C^{high}$ and $LY6C^{medium}$) have high levels of CC-chemokine receptor 2 (Ccr2), CD11b, CD115 and low levels of CX3 chemokine receptor 1 (Cx3cr1). On the contrary, $LY6^{-}$ has high level of Cx3cr1, CD115 but low levels of Ccr2 and CD11b (Serbina *et al.*, 2008). The second subset of circulating monocytes which has high levels of LY6C has high expression of Ccr2 but low levels of Cx3cr1. This group has a patrolling function in and around the vascular endothelium (Auffray *et al.*, 2007). Ccr2 is the receptor that is responsible for the binding of CC-chemokine ligand 2 (Ccl2) and Ccl7 (Tsou *et al.*, 2007). Cx3cr1 is a chemokine receptor mediating resident monocyte accumulation. For patrolling monocytes-subset $LY6C^{low}$ does not response to Ccl2 as it lack of Ccr2 expression; however it can rapidly mobilized subset $LY6C^{high}$ for monocyte recruitment (Hanna *et al.*, 2011).

Human monocytes express a different subset of surface markers comparatively to mouse. The subsets are divided into three groups, $CD14^{+}CD16^{-}$, $CD14^{++}$ $CD16^{+}$and $CD14^{+}CD16^{++}$. CD14 is a common monocyte marker whereas CD16 is Fc receptor (FcR) that binds to the Fc region of antibody. The markers and function of monocyte subsets are summarized below (Table 2.2).

Table 2.2: Summary of monocyte subsets with different markers, chemokine receptors and function. Adapted from (Shi & Pamer, 2011)

Subset	Markers	Chemokine receptors	Function
LY6C^{+}	CD11b^{+}CD115^{+}LY6C^{high}	Ccr2^{high} Cx$_3$cr1^{low}	Pro-inflammatory and phagocytosis
	CD11b^{+}CD115^{+}LY6C^{medium}	Ccr2^{high} Cx3cr1^{low}	Pro-inflammatory
LY6C^{-}	CD11b^{+}CD115^{+}LY6C^{low}	Ccr2^{low}Cx3cr1^{high}	Patrolling
Classical	CD14^{++}CD16^{-}	Ccr2^{medium}Cx$_3$cr^{high}Ccr5^{+}	Phagocytosis
Intermediate	CD14^{++}Cd16^{+}	Ccr2^{medium}Cx3cr1^{high}Ccr5^{+}	Pro-inflammatory
Non-classical	CD14^{+} CD16^{++}	Cx3cr1^{high}Ccr2^{low}	Patrolling

2.7.2 Monocytes recruitment and macrophage fate

Monocytes quickly differentiate into macrophages and dendritic cells at the tissue site. At steady state, tissue macrophages have intrinsic anti-inflammatory functions. It is predicted that regulatory macrophages secrete a large amount of IL-10) in suppressing the inflammatory activity of macrophages to the gut flora (Barnes & Powrie, 2009). At this level, these myeloid-derived suppressor cells which are $CD11b^{+}Ly6C^{+}$ negatively regulate T cell function through direction interactions. Hypothesis speculates that there are two populations of mononuclear phagocytes in the gut: first would be the resident macrophages which is characterized by the expression of CD103, and the second would be derived from the circulating monocyte pool (Murray & Wynn, 2011).

2.7.3 Activation of macrophages

Immature macrophages are characterized by low oxygen consumption, low protein synthesis rate and modest cytokine production (Grage-Griebenow *et al.*, 2001; Hume *et al.*, 2002). If they are not activated, they will die shortly after production by a process of programmed cell death (Xaus *et al.*, 2001). In the tissues, macrophages can be activated due to tissue damage or infection. During infection, monocytes are recruited during "emergency myelopoiesis", a process where large pools of monocytes and neutrophils are generated from bone marrow beyond the normal requirement due to tissue stress. In this process, cytokines such as granulocyte colony stimulating factor (G-CSF), Ccl1 and Ccl5 are released (Serbina *et al.*, 2008). As a consequence, macrophages will acquire microbicidal effector functions by increasing of chemokines and cytokines production, in order to recruit more monocytes. The specificity of immune response is differentiated by the cytokine milieu, where it compels the

mononuclear phagocytes to express specialized and polarized functional properties (Gordon, 2003; Mantovani, A. *et al.*, 2005; Mantovani, A. *et al.*, 2004).

The common microbial stimulant example, lipopolysaccharide (LPS), is a well-known potent activator of macrophages. It binds directly to CD14, the pathogen recognition receptor, which is known as the chaperone LPS molecules to activate the TLR4/MD2 signaling complex (da Silva Correia *et al.*, 2001; Gioannini *et al.*, 2004; Moore *et al.*, 2000). Another important stimulant is interferon-γ (IFN-γ), the obligatory cytokine that activates macrophages which are produced by professional APC, $CD4^+$, $CD8^+$ and Natural Killer (NK) cells (Bach *et al.*, 1997; Young, 1996). IFN-γ is able to upregulate the class II antigen presenting pathway and also promotes peptide-specific activation of $CD4^+$ T cells (Boehm *et al.*, 1997; Mach *et al.*, 1996). IFN-γ activates transcription factors, STAT1/2, which bind to gamma-activated sequences (GAS) in several immune effector genes. Other stimuli also include cytokines such as TNF and granulocyte macrophage-colony stimulating factor (GM-CSF) (Gordon, 2003).

After stimulation, besides expressing high levels of class II MHC molecules and costimulatory molecules, CD80 and CD86; macrophages cause CD40-mediated activation of T lymphocytes by two signals (Figure 2.5). First signal involves recognition by T cells via T cell receptor (TCR), of an antigenic peptide presented by macrophages via class II MHC. Second will be the interaction between costimulatory molecules with T-cell co-receptor CD28. In response to combined stimulation with antigen, co-stimulators and particular cytokines, naïve $CD4^+$ T helper cells (T_H0) will further differentiate into T_H1, T_H2 and T_H17 effector cells (O'Shea & Paul, 2010).

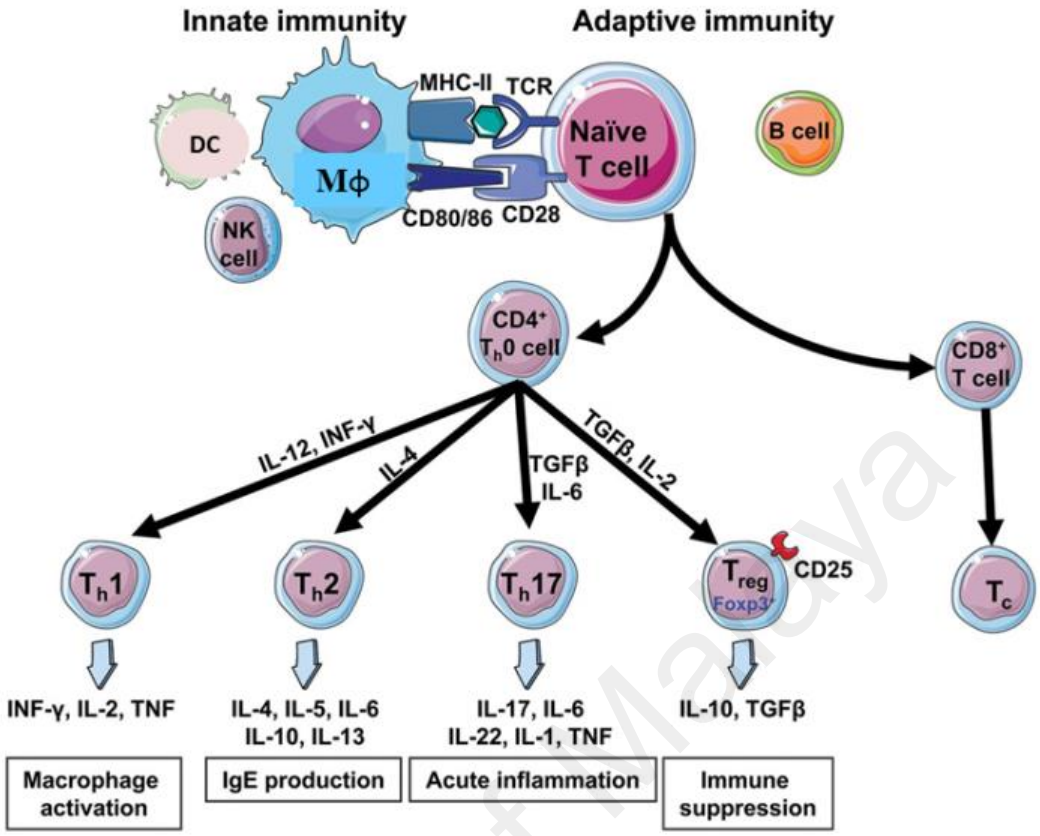


Figure 2.5: The activation of naïve T_H0 by macrophages. Macrophages present antigens on MHC-II to naïve T cells (T_H0), leading to T-cell clonal expansion and further differentiated to T_H1, T_H2 and T_H17 according to the stimulation of cytokines. Figure taken from (Idris-Khodja *et al.*, 2014)

2.7.4 Polarization of macrophages into M1 and M2 macrophages

Mirroring the T_H1/ T_H2 nomenclature, polarized macrophages are distinguished as M1 and M2 macrophages. Macrophages are capable of differentiating into M1 and M2 macrophages in respond to various environmental cues, for example: microbial stimulants, damage cells and activated lymphocytes. M1 and M2 are two distinct populations, both with opposing functional phenotypes polarization for example, killing or repairing; inhibiting or promoting cell proliferation (O'Shea & Paul, 2010; Wang, N. *et al.*, 2014). Moreover, they have different receptors, cytokine and chemokine expressions. There are many definitions to differentiate M1 and M2 based on their phenotypic similarities and differences. Mantovani *et al.* (2004) grouped the macrophages into M1 and 3 subsets of M2 (Figure 2.6) (Mantovani, A. *et al.*, 2004) M1 is activated by IFN-γ, LPS or TNF, whereas M2 can be further divided into M2a, M2b and M2c. M2a is induced by IL-4, IL-13; M2b is activated by Fc receptors and immune complexes while M2c is induced by IL-10.

Besides, Martinez and Gordon (2014) propose macrophages classification according to their role in the immune response (Martinez & Gordon, 2014). They classified them into four levels: growth and survival factors, interaction with lymphoid and myeloid cytokines, interaction with pathogens, and resolution (Figure 2.7).

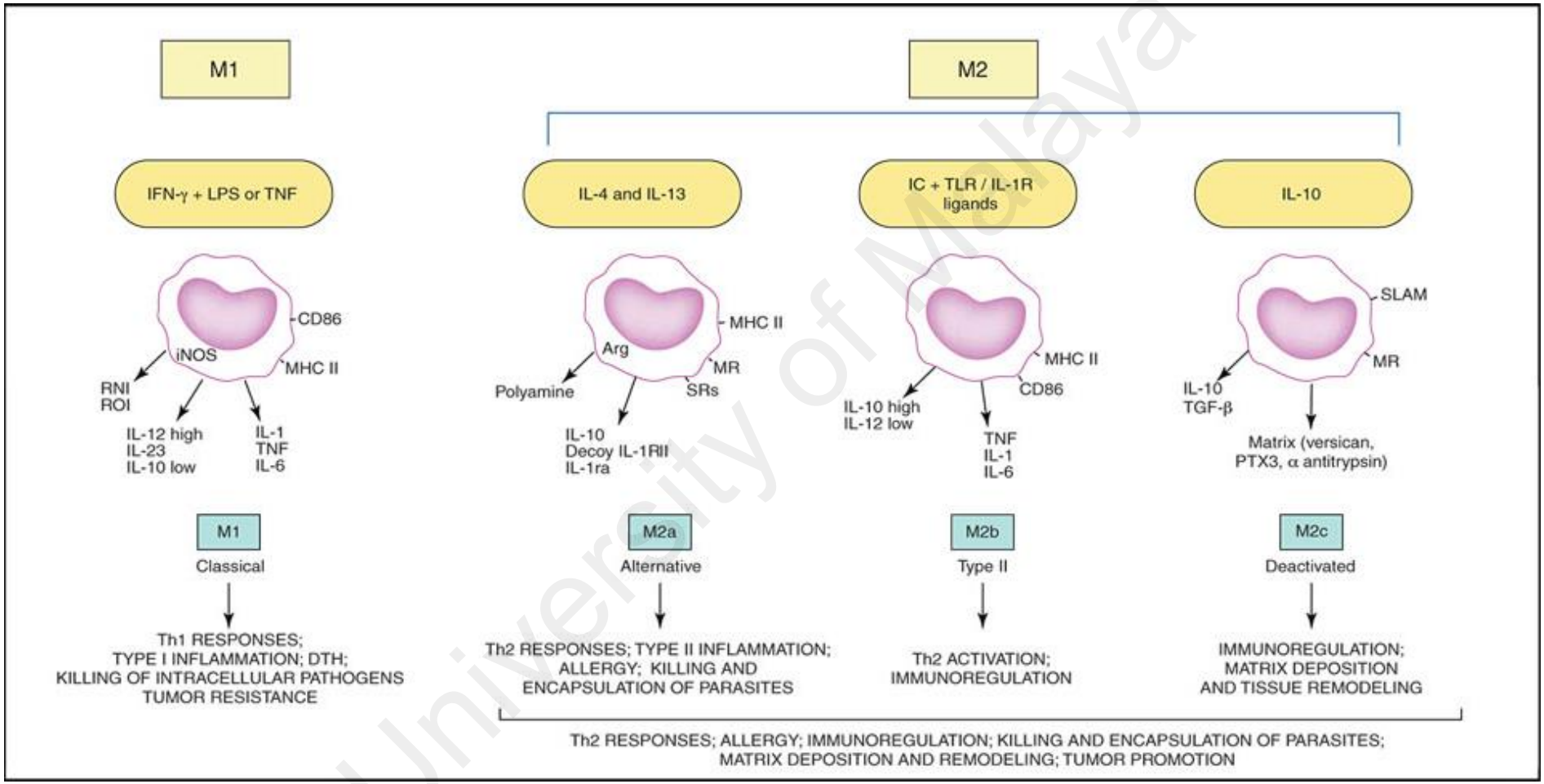


Figure 2.6: Grouping of macrophages into M1 and M2 subtypes based on applied stimuli and the induced transcriptional changes by (Mantovani, A. *et al.*, 2004)

Growth and survival factors	**Lineage determining cytokines (CONVENTIONAL MATURATION)**		**Survival, recruitment and retention**	**Other**
	M-CSF R	GM-CSF R	Adhesion molecules Chemokines	VitD3, Retinoic acid, PPRgamma ligands
Lymphoid and myeloid Cytokines	**Classical and Alternative activation**		**Pro and antinflammatory**	
	IFN-γ	IL-4, IL-13	TNF, IL-6, IL-1ß	IL-10, TGF-ß,
Interaction with Pathogens	**Direct interaction**		**Humoral**	
	TLRs, NODs, NLRs, RLRs, Nucleic acid sensors		IgG, IgE, IgA	Complement, Lectins, Ficolins
Resolution	**Systemic mechanisms**		**Local mechanisms**	
	Glucocorticoids		ECM Proteoglycans, ATP and sugar nucleoCdes, Resolvins, Maresins, etc	

Figure 2.7: A multipolar view of macrophages activation paradigm (Martinez & Gordon, 2014)

M1 is commonly characterized by the expression of high level of pro-inflammatory cytokines, high production of reactive nitrogen and oxygen intermediates, promotion of T_H1 response and strong microbicidal and tumoricidal activity. M1 macrophage secretes IL-1 and TNF pro-inflammatory cytokines, and releases nitric oxide (NO) to kill the invading organisms by producing reactive oxygen and nitrogen intermediates. Activated M1 cell shows significant increased expression of surface markers such as CD14 and CD32. The upregulation of CD14 might affect the sensing of microbial by the cells, while CD32 might be expressed as inhibitory Fcγ-RIIB. On the contrary, expression of CD11b and HLA-DR decrease in comparison to uninfected M1 macrophages. Activated M1 cell also releases large amounts of pro-inflammatory cytokines IL-1β, IL-6, IL-12, IL-12p40, IL-23 and MIF but not IL-12p70, in which IL-12 and chemokines such as Cxcl9 and Cxcl10 drives the polarization and recruitment of T_H1 response. Moreover, it has a higher expression of MHC class II, mediating resistance to intracellular pathogens and eliciting tissue-disruptive reactions (Gordon & Taylor, 2005). The secretion of IL-1β and IL-6 will recruit $CD4^+$ T_H1 while the secretion of IL-23 by M1 macrophages might contribute to the induction and maintenance of T_H17 responses.

Unlike M1, M2 macrophage is anti-inflammatory (Gordon, 2003, 2007; Gordon & Martinez, 2010) and has important roles in wound healing and fibrosis. It also antagonizes M1 macrophage responses, which may be crucial for the activation of the wound healing response and for recovery of tissue homeostasis. In contrast to M1, M2 macrophage is induced by IL-4/IL-13 released by mast cell, T_H2 cell, neutrophil and basophil (Loke *et al.*, 2002; Raes *et al.*, 2005). As a result, M2 releases anti-inflammatory cytokine, IL-10 (Biswas *et al.*, 2006; Hagemann *et al.*, 2008; Torroella-Kouri *et al.*, 2009). IL-4 is a cytokine that is produced by T_H2 cells, eosinophils,

basophils and macrophages. It also releases chemokines Ccl17, Ccl22, Ccl24 and Ccr4/3 to recruit T_H2 pathway (Mantovani, A. *et al.*, 2004).

2.7.5 *H. pylori* infection activates M1 and M2 macrophages

Previous studies have proven that M1 and M2 macrophages present in the gastric biopsy or tissue RNA specimens from *H. pylori*-infected patients (Quiding-Järbrink *et al.*, 2010). In fact, gastric mucosa from *H. pylori*-positive individuals contain twice as many M1 macrophages, and three times as many M2 macrophages when compared to control patients (Fehlings *et al.*, 2012). The differentiation of macrophages was verified by CD68 (M1) and CD163, stabilin-1 (M2). M1 macrophages expressed increase expression of inducible nitric oxide synthase (iNOS) and Cxcl11 after 8 weeks of infection. This is supported by another research, as cytokines IL-1β, IL-6, TNF-α, IL-8, IL-21, IFN-γ and TGF-β were found increased in *H. pylori*-gastritis patients (Caruso *et al.*, 2007; Crabtree *et al.*, 1991; Lindholm *et al.*, 1998). In contrast, *H. pylori* also induces M2 macrophages, where they have increased expression of CD14, CD206 and MIF. Expression of CD32, CD11b, CD16, CD64, CD80, CD60 and HLA-DR remain unchanged. Similarly in Quiding-Järbrink *et al.*, (2010), *H. pylori*-infected M2 macrophages display elevated cytokines such as Ccl17, Ccl18 and increase surface expression of CD206 comparatively to healthy patient samples (Quiding-Järbrink *et al.*, 2010). These indicate the importance of M1 and M2 macrophages in host defense against *H. pylori.*

2.8 Immune evasion strategies of *H. pylori* towards macrophages

2.8.1 *H. pylori* attenuates iNOS translation in macrophages via Arginase II

Nitric oxide (NO) is a central component of innate immunity and an effective antimicrobial agent. When macrophages are activated by cytokines; microbial

compounds or both, *iNOS* gene is readily upregulated, and it converts NO from L-arginine and functions as tumoricidal and antimicrobial molecule (Lowenstein *et al.*, 1993; Mantovani, B. *et al.*, 1972). The reaction first starts from the conversion of L-arginine and molecular oxygen to N-hydroxyl-L-arginine, where this intermediate will further converts to citrulline and NO. NO as free radicals in the phagosome can direct kill or reduced replication of pathogens (Hashimoto *et al.*, 2011; Murray & Wynn, 2011). Macrophage-derived NO is likely to be an important component of the host defence against *H. pylori*, since lamina propria macrophages from *H. pylori*-infected patients express iNOS and NO can kill *H. pylori in vitro*. It is also reported that the generation of NO in response to *H. pylori* is entirely dependent on the availability of L-arginine and is occurred in a concentration-dependent manner (Auffray *et al.*, 2009). Multiple reports have demonstrated that *H. pylori* successfully invade the mucosa, enabling this pathogen to have direct contact with the lamina propria immune cells including macrophages (Geissmann *et al.*, 2003; McGaha *et al.*, 2011; Palframan, R. T. *et al.*, 2001)

Lewis *et al.*, (2010) predicts that *H. pylori* infection releases Arginase enzyme; a type of NO scavenger that can attenuate iNOS of macrophage (Lewis *et al.*, 2010). The induction of arginase activity has been reported to modulate macrophage NO production by substrate competition. Arginase enzyme is the antagonist to iNOS, as they both compete for the same substrate L-arginine. However, Arginase metabolizes L-arginine to urea and L-ornithine; where it is further metabolize by ornithine decarboxylase to produce polyamines putrescine, spermidine and spermine (Pegg, 2006). *H. pylori*-stimulated macrophages are found to upregulate Arg2 that is localized in the mitochondria. When *H. pylori*-infected macrophage cells were treated with arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC), it further enhances the NO production, suggesting the relationship between arginase and NO. This is further

confirmed by Arg2 knockdown in RAW264.7 cells, where significant increase of *H. pylori*-stimulated NO production can be observed.

To determine the clinical importance role of Arg2, Arg2 was further knockout in C57BL/6 mice ($Arg2^{-/-}$). Compared to wild type (WT) mice, $Arg2^{-/-}$ mice had increased histologic gastritis and decreased bacterial colonization. Moreover, real time PCR results showed upregulation in the iNOS mRNA expression of WT mice, indicating the relationship between Arg2 and iNOS. More gastric macrophages were isolated in $Arg2^{-/-}$ mice, and produce more NO comparatively to WT. With immunohistochemistry, the $F480^{+}$ gastric macrophages of WT mice were confirmed to express Arg2. Besides, cytokines production of the chronic *H. pylori* infection model was also examined. IL-12p40, IL-17a and IFN-γ were increased in WT mice, but further upregulated in $Arg^{-/-}$ mice; while IL-10 which is the hallmark of counter regulatory response to T_H1 and T_H17 was only expressed in WT.

2.8.2 *H. pylori* delays phagosome maturation in macrophages

Phagocytosis is a process of macrophage internalization of large particulate material destined to be degraded by lysosomal enzymes. This process can be divided into three distinct steps: (i) attachment of the particle onto receptors at the surface of macrophage; (ii) engulfment, a process of plasma membrane surrounding around the particle and (iii) formation of phagolysosome (Silverstein, 1989). This process can only happen if macrophages successfully undergo phagosome maturation. Phagosome maturation is described as a step-wise interaction of phagosomes with early endosome markers, late endosome markers and lysosomes. In summary, newly formed phagosomes recruit early endosome markers such as Rab5 and EEA1. EEA1 is a Rab5 effector protein that is responsible for the docking of endocytic vesicles, providing direction to early endosomal fusion. It interacts with GTP-bound Rab5 to mediate early

endosome fusion (Wilson *et al.*, 2000). After 10-15 minutes, these early endosome markers are lost, and then they are replaced with late endosome markers such as Rab7 and CD63. Rab7 is responsible for the recruitment of downstream effectors that enable endosomes and lysosomes for microtubules trafficking (Vieira *et al.*, 2002). CD63 is a well-established component of late endosomal and lysosomal membranes, also known as lysosome-associated membrane protein 3 (LAMP-3) (Weibel & Palade, 1964). Once the late endosome markers are lost, a full complement of lysosomal hydrolytic enzymes will fuse with phagosome, ready for phagocytosis process. Their maximal degradative capacity can be characterized by LAMP-1 and LAMP-2 markers. LAMPs are transmembrane proteins that maintain the structural integrity of the lysosomal membrane (Eskelinen *et al.*, 2003). LAMP-2 is also reported as a receptor for chaperone-mediated autophagy (Cuervo & Dice, 1996) and involved in MHC class II antigen presentations (Zhou, D. *et al.*, 2005).

Loss of late endosome markers enables the fusion of lysosome with phagosome. Primary human macrophages showed abnormal retention of late endosome markers such as Rab7 and CD63 when they are *H. pylori*-infected. (Borlace *et al.*, 2011). As a result, macrophages form megasomes that contain multiple viable *H. pylori* within. This is also proven in previous studies which show that the infected-macrophages (RAW264.7, J774, THP-1 and primary mouse peritoneal macrophages) have no or limited amounts of LAMP-1 marker (Schwartz & Allen, 2006; Zheng & Jones, 2003). The absence of this lysosome marker causes the formation of hybrid phagosome-endosome-lysosome compartments, resulting less degradative properties of the macrophage. Therefore, *H. pylori*-infected macrophages demonstrate impaired antigen presentation property and cannot activate a strong humoral immune response, which explains the long term persistency of *H. pylori* infection in the host (Borlace *et al.*, 2011).

2.8.3 *H. pylori* induces apoptosis of macrophages in association with alterations in the mitochondrial pathway

Apoptosis is a programmed cell death mechanism to dismantle intracellular components and eliminate cells without triggering inflammation or causing damage to the surrounding cells (Ashkenazi & Dixit, 1998; McIlwain *et al.*, 2013). It can be signaled through death receptor and mitochondrial pathways, where it involves an intracellular proteolytic cascade that coordinates their activities to demolish structural proteins (Ashkenazi & Dixit, 1998; Green & Reed, 1998). This machinery consists of a family of proteases called caspases. They are synthesized in the cell as inactive precursors (procaspases), and usually activated when they are cleaved. They are divided into two big families: initiator caspases and executioner caspases. Initiator caspases-8 and 9 are normally in the form of inactive monomers and are activated by dimerization (Boatright *et al.*, 2003; Chang *et al.*, 2003; Muzio *et al.*, 1998). The activation of initiator caspases lead to cleavage of executioner caspases (3, 6, 7), where the active sites of executioner caspases will be dimerized and create functional proteases (Riedl & Shi, 2004).

The activation of procaspases is regulated by the Bcl-2 family of intracellular proteins (Figure 2.8). They are divided into two groups, for example, pro-apoptotic, and anti-apoptotic proteins. Pro-apoptotic Bcl-2 family members such as Bax and Bak stimulate the release of cytochrome c from the mitochondria. Other than that, Bad, Bid and Bim act as activator to directly induce Bax/Bak. In contrast, Bcl-2, Bcl-xL and Bcl-w act as apoptotic inhibitors by blocking the release of cytochrome c (Alberts *et al.*, 2002).

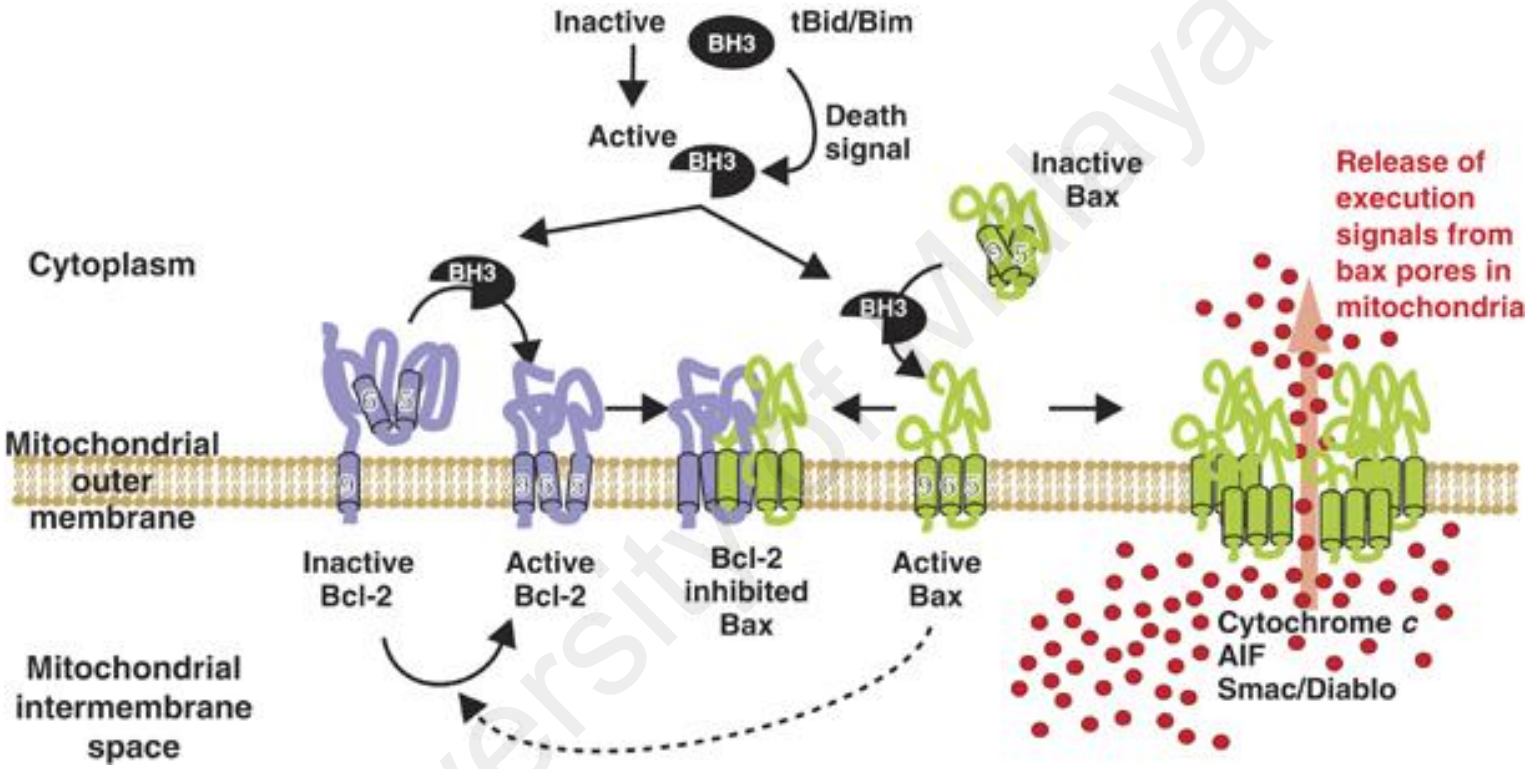


Figure 2.8: Dead signals activate inactive form of Bid (tBid/Bim) by cleavage, which causes the conformational changes of Bax and Bcl-2. In the absence of conformational change of Bcl-2, membrane integrated Bax monomers (purple) form large oligomers (green) and release cytochrome c from mitochondria. Vice versa, conformational changed Bcl-2 (active) will inhibit oligomerization of membrane-embedded Bax. Adapted from (Dlugosz *et al.*, 2006)

Menaker *et al*. (2004) demonstrates that *H. pylori* induced apoptosis in RAW264.7 macrophages at a multiplicity of infection (MOI) 50 (Menaker *et al.*, 2004) Apoptosis in macrophages were proven with different assays: transmission electron microscopy (TEM), annexin V assay and acridine orange-ethidium bromide staining. *H. pylori*-infected macrophages show apoptotic morphological features such as membrane blebbing, cytoplasmic vacuolation and condensed nuclear chromatin. A significant increase percentage of apoptotic cells and nonviable cells are reported in the infected macrophage population. Additionally, they propose *H. pylori*-induced apoptosis is signaled through caspase 8 activation, which attenuates the cleavage of Bid protein. Bid is the specific substrate of caspase 8 and is strongly dependent upon cleavage for its pro-apoptotic activity. With decreased level of uncleaved Bid, an increase of mitochondria-membrane permeability and cytochrome c release can be observed. This suggests that *H. pylori* induces apoptosis of macrophages with alterations in the mitochondrial pathway of the host.

CHAPTER 3: MATERIALS AND METHODS

3.1 Bacteria strains cultivation

The *H. pylori* strains used in this study were summarized in Table 3.1. Mouse-adapted strain of *H. pylori,* Sydney Strain 1 (SS1) was provided by *Helicobacter pylori* Research laboratory at University of Western Australia. J99 strain (ATCC-700824) was purchased from America Type Culture Control (ATCC) while 298 strain was derived from a local clinical isolate, UM032 as described (Khosravi *et al.*, 2013). All strains of *H. pylori* were grown on chocolate agar supplemented with 7 % laked horse blood (Oxoid, UK) under microaerophilic conditions at 37 °C, 10% CO_2, in a humidified incubator and were subcultured every 3 days. For the storage of bacterial stock, fresh colonies from the agar plate were resuspended in Brain Heart Infusion (BHI) supplemented with 0.4 % (w/v) yeast extract, 10 % (v/v) fetal calf serum (FCS) (Gibco, Life Technologies, USA) and 10% (v/v) glycerol (Acros Organics, Belgium) and stored at -80 °C. All preparation of culture media, reagents and chemicals are listed in detail in Appendix A.

Table 3.1: Bacteria strains used in this study

***H. pylori* strain**	**Genotype or description**	**Source/ Reference**
SS1	$CagA^+$, VacA s2m2	(Lee *et al.*, 1997)
J99	$CagA^+$, $VacA^+$	(Marshall & Warren, 1984) (Goodwin *et al.*, 1989)
298	$CagA^+$, VacA s2m2	(Khosravi *et al.*, 2013)

3.1.1 Bacteria colony forming unit determination

Bacterial CFU of *H. pylori* was done using Miles and Misra method (Akashi *et al.*, 2003), *H. pylori* inoculum was first resuspended in 1 mL BHI broth. The bacterial suspension was serially diluted in 1.5mL microcentrifuge tubes (Corning, USA), using two-fold serial dilution in BHI broth as diluent, from 10^{-1} to 10^{-8}. Reading of each dilution was measured and recorded at OD_{625nm}. Immediately, 20 µL of each dilution of

bacterial was dropped on the surface of chocolate agar in triplicate. After three days of incubation at 37 °C, 10 % CO_2, the full size discrete colonies of *H. pylori* were counted. The bacteria titer was determined by the following calculation:

$$\text{Bacterial titer, CFU/ml} = \frac{\text{number of colonies} \times \text{dilution}}{\text{innoculum volume}}$$

The standard curve of *H. pylori* is plotted using OD_{625nm} reading against bacterial titer (CFU/mL) (Appendix B), where the graph was used to determine the viable colony count by measuring the turbidity of the suspension.

3.2 Culture of RAW264.7 cells

RAW264.7 cells (ATCC TIB-71) (Hartley *et al.*, 2008), which are murine monocytic macrophage cell line are suitable host to examine the cellular responses against *H. pylori* SS1. RAW264.7 cells were cultured in Dulbecco's Modified Essential Medium (DMEM) (Gibco-Life Technologies, USA) supplemented with 10 % heat inactivated fetal bovine serum (FBS) (Gibco-Life Technologies, USA) and incubated at 37 °C, 5 % CO_2. For subculture, old medium was removed, replaced with 5 mL Phosphate Buffer Saline (PBS) (Oxoid) before scraping all cells using a cell scraper (Corning, USA). Supernatant was then collected and centrifuged for 5 minutes at 1,500 rpm. One day prior to inoculation, cells were seeded in a T25 flask (Corning, USA) at 5.0×10^5 cells/mL and infected with *H. pylori* SS1, J99 or 298 at a multiplicity of infection (MOI) of 1:1, 5:1 10:1 or 100:1 for 24 h .

3.3 Cell count

Using a live cell movie analyzer (JuLi Br, NanoEntek, Korea), real time cell growth was plotted against time. Cell count and cell confluency was plotted against hour post infection (hpi), with 1 h interval. The experiment was run for 24 h.

3.4 RNA extraction

Non-infected RAW264.7 cells and infected RAW264.7 (MOI 10:1) cells were scraped, before centrifuging at 1,500 rpm for 5 minutes. All medium from the cells were completely removed before adding 1 mL of TRIzol Reagent (Invitrogen, CA) into the cells. Cells were lysed on ice by pipetting the cells up and down. After that, cells were incubated at room temperature for 5 minutes, before adding 0.2 mL of chloroform for each sample. The cells were vortex vigorously, followed by incubation at room temperature for 2-3 minutes. The cells were then centrifuged at 13,000 rpm for 15 minutes at 4 °C. Using a blunt tip, upper layer of the aqueous phase was transferred to a new tube. The newly transferred samples were then added with 0.4 mL of Isopropanol. Each mixture was inverted 10 times, followed by 10 minutes of incubation at room temperature. The samples were centrifuged again at 13,000 rpm for 10 minutes at 4 °C. After centrifugation, the supernatant of each samples were discarded. 1 mL of 70 % ethanol was added to each sample for RNA washing. Samples were vortexed briefly before centrifugation at 13,000 rpm for 5 minutes at 4 °C. Supernatant were discarded again, and the tubes containing sample were inverted to air dry the RNA pellet. RNA isolated was resuspended in 12 μL of RNAse-free water. Nanodrop was used to ensure the quality and integrity of total RNA samples by checking the A260/A280 ratio of each sample.

3.5 Microarray analysis.

Two biological replicate samples of non-infected control (C1, C2) and infected RAW264.7 (MOI 10:1) (S1, S2) were sent for microarray analysis. RNA integrity of each samples were ensured using Bioanalyzer 2100, where RNA integrity number (RIN) was >9.5). For each samples, 100 ng of sample RNA was labeled with Low Input Quick Amp Labeling Kit, One-Color (Agilent p/n 5190-2305) following manufacturer

instruction. Briefly, RNA was first converted into double-stranded cDNA by priming with an oligo-dT primer containing recognition site for T7 RNA polymerase to produce cyanine 3'-CTP labeled cRNA. cRNA (600 ng) was hybridized onto 8-array slide at 10 rpm for 17 h at 65 °C. Slide was washed and scanned on Agilent High Resolution Microarray Scanner (C-model). 600 ng of cRNA was hybridized onto 8-array slide (Agilent SurePrint Human GE Microarray, which contains 55,821 probes (Design ID: G4851A, Lot: 0006097429) for 17 h at 65 °C, 10 rpm in Agilent hybridization oven. After hybridization, the microarray slide was washed in gene expression wash buffer 1 for one minute at room temperature and wash again in gene expression buffer 2 for another minute at 37 °C before scanning on Agilent High Resolution Microarray Scanner (C-model). The results were visualized in a gel image and electropherogram using the Agilent 2100 Bioanalyzer expert software. Raw signal data were extracted from the TIFF image with Agilent Feature Extraction Software (V107.1.1).

3.5.1 Microarray data processing and statistical analysis

Raw signal values of samples were processed in Genespring GX. The signal of intensities value was normalized by shifting their minimum to 1, before converting it to log2 notation. Normalized signal values were then transformed to median of all samples (per gene normalization), so that data can be further processed by statistical analysis. The total data were further filtered with fold changes (FC>2 or FC<-2), and subjected to paired sample *t*-test (P<0.05) so that the extreme outliers were excluded from further analysis. Hierarchical clustering for significant probes was also executed with Pearson Correlation distance metric and average linkage.

Data were analyzed with unpaired two-tailed Student's *t*-test or Benjamini-Horchberg False Discovery Rate (FDR) multiple testing correction. Samples were

considered significant if $P < 0.05$. Top 20 differentially expressed genes were calculated after comparison is made between 2 conditions (S1, S2 and C1, C2).

3.5.2 Pathway analysis

Pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al. 2016). Significantly modulated pathways relative to control of fold enrichment (FE) less than -2 or FE more than 2, $P< 0.05$ were selected. Heat maps were generated for the significant pathways with multi experimental viewer (MeV) software

3.6 Quantitative Reverse-transcriptase polymerase chain reaction (qRT-PCR)

cDNA of non-infected control and infected RAW264.7 cells was prepared using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen, USA) according to manufacturer's protocol. In each 2 μg of RNA sample, 0.5 μg of random primers (dNTPs) was added before heating the samples at 70 °C for 5 minutes, in order to melt secondary structure within the template. After that, the samples were cooled immediately on ice to prevent secondary structure from reforming. The samples were then added with 5μL of MMLV 5× reaction buffer, 25 units of recombinant RNAs in Ribonuclease inhibitor, 200 units of MMLV reverse transcriptase and top up to 10 μL with RNAse free water. The samples were flicked gently, and further incubated at 60 minutes at 37°C for extension. The samples were kept at -20 °C prior used.

In order to validate the expression of genes in microarray analysis, top 10 upregulated, top 10 downregulated and cell cycle pathway-associated genes were selected for validation. Primers were designed based on the exon sequence of each gene, according to the Ensemble database. The primers design criteria were inserted into Primer3 were as follow: 20 bp long, annealing temperatures at 60 °C, CG at 50 %,

generation of 180-250 bp PCR products with no hair pins and 3' complementarity. All designed primers were synthesized by First Base (Singapore). Lyophilized PCR primers were dissolved in DNAse/RNAse free water in stock concentration of 100 μM according to manufacturers' instructions. The stock primers were further diluted to a working concentration of 10 μM prior use. All PCR primers used in this study were summarized below (Table 3.2).

Table 3.2: Primer sequences for qRT-PCR analysis. List of the forward and reverse primer sequences (5'-3') of the genes selected for qRT-PCR analysis.

Gene Symbol	Forward primer	Reverse primer
Alox5	ACAGGAAGTACAGGAAGGGAAC	GGGTTACTCTCTCCAGGGGT
Ankle1	CTCTTCCCTCACACTGGCTG	AGACCTGTTTCCTTGGCTGG
Aurkb	CTGGAGAATGGCTCAGAAGG	ACAGTGATGGGGGAGAAATG
Aurkb	CTGGAGAATGGCTCAGAAGG	ACAGTGATGGGGGAGAAATG
Avil	CGCTAATGACAAAAGGCTGC	CCTCCGCTCCTATCCACAAG
Car6	CGTGGGAGAAAGTCAGTGGT	GCCTAGCTGTGTTAGCCGTC
Ccl7	TCTGTGCCTGCTGCTCATAG	CATTCCTTAGGCGTGACCAT
Ccnb1	CTCCCTTTCATCCACAGGAA	TTGCAGTCTGCCTTCTCTCA
Ccnb2	CAGTTCCCAAATCCGAGAAA	GAACAAGTATGCCAGCAGCA
Ccne1	ACTTTCTGCAGCGTCATCCT	CTATGTCAACGACACGGGTG
Ccne2	GACTGGATGGTGCCTTTTGT	GATGCACAAAATCCTGGGTT
Cdk1	CCCACCTACGGACAGTGTTT	CGTATCGCTGTGCTGCTTAG
Cdk2	TAAGTGCTGTGGGGAACACA	TCACCCTTTCTTCCAGGATG
Cenpa	CCAGCCACTGAGAGTCACAA	TTGGGAACTGTAGGTGAGGGC
Csf1	CCCTGGCTACTGGGTCAATA	AAAGCTCAGAGGGAGCACTG
Csf3	CTCAACTTTCTGCCCAGAGG	GCTCTATCGGGTATTTCCCC
Cx3cr1	GGAGACTGGAGCCAACAGAG	CAG GACACAGCCAGACAAGA
Cxcl2	GCCTGGATCGTACCTGATGT	GGAAGGAGTGTGCATGTTCA
Dhcr24	GACATCCAGAAACAGGTCCG	ATAGACACCAAGGGCTCCAC
Espl1	CTCTTGAACCTTGGCTGTCC	CATCATTCTGCTCATGGGAA
Fgd2	TCTGCCTGACCTGCTACA	GTGAGCCTTTGTGTCCTGG
Il1b	TGCCACCTTTTGACAGTGATG	ACGGGAAAGACACAGGTAGC
Kbtd11	ACAGGTGCGTGGAAGGT	CTATGGGCGAGTGTCTGGAA
Lcn2	AATGTCACCTCCATCCTGGTC	GCTCCTTGGTTCTTCCATACAG
Lrp8	CACACGGATTGGTTTCACTG	CAGAAGGAATTCAGTCCCCA
Mad1l1	TAGACCGGTGCGAGAGAGAT	AAAACAGCAGAGTCAGGGGA
Pappa2	GAAGTGCTGGCTGAGATTCC	GCTCTTTAATCCTGGGAGGG
Ptgs2	GCAGGAAGTCTTTGGTCTGG	TCCTCCTGGAACATGGACTC
Zwilch	TTGCCCCATCACAAACTACA	TTTAATTCCAGCACTTGGGG

Quantitative real-time PCR (qPCR) was carried out with the preparation of reverse transcriptase mastermix containing 5 µL SsoAdvanced SYBR Green Supermix (Biorad, CA), 1 µL of diluted forward and reverse primer (10 nm) respectively, 2 µL of DNAse/RNAse free water and 1 µL of samples' cDNA template. The qRT-PCR was performed using Real-Time PCR 7500 (Applied Biosystems, CA) with the following cycling conditions: initial denaturation at 95 °C for 2 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 1 minute, followed by another round of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 1 minute and lastly denaturation at 95 °C for 15 seconds again. The cycle threshold (Ct) value of each gene was compared with the Ct value of endogenous control, β actin. Data were analyzed using the comparative Ct method ($2^{-\Delta\Delta CT}$ method). Fold change of each gene is calculated as follow:

$$2^{-\Delta\Delta \mathrm{Ct}} = 2\text{^} - ((\mathrm{Ct\ infected} - \mathrm{Ct\ endogenous}) - (\mathrm{Ct\ non\ infected} - \mathrm{Ct\ endogenous}))$$

All reactions were run in triplicate and fold change of each gene was presented as mean ± standard deviation (SD).

3.7 Flow cytometry analysis

For flow cytometry analysis, non-infected control was compared with *H. pylori* SS1-infected RAW264.7 cells at different MOI (1, 5, 10, and 100). To eliminate variation of infection among different strains, we also compared non-infected control with Cag A^+ (J99, 298) and Cag-deficient strains (SS1). All flow cytometry analyses were carried out in a FACS Canto II cytometer (BD Biosciences, USA). Data were analyzed on FACS Diva software (BD Biosciences, USA). Instrument settings optimization were done by adjusting the photomultiplier tubes (PMTs) setting. Filters used for fluorescence listed below:

Table 3.3 Filters used for fluorescence dye in flow cytometry analysis

Wavelength (nm)	PMT position (nm)	Intended dyes	Range of wavelength (nm)
488 (blue)	D	PE/PI	564-606
	E	FITC	515-545
633 (red)	C	APC	650-670

Population of interest was gated to eliminate cellular debris from each analysis using a gate on forward and side scatter. Voltage for FSC and SSC were adjusted to let the population of cells to shift to the gated area (Figure 3.1A). Separate tubes of unstained cells (1.0×10^6 cells) were prepared as negative reference point to eliminate false positive results (Figure 3.1B). For multiple staining markers, each single stain cells (1.0×10^6) were prepared for compensation purposes.

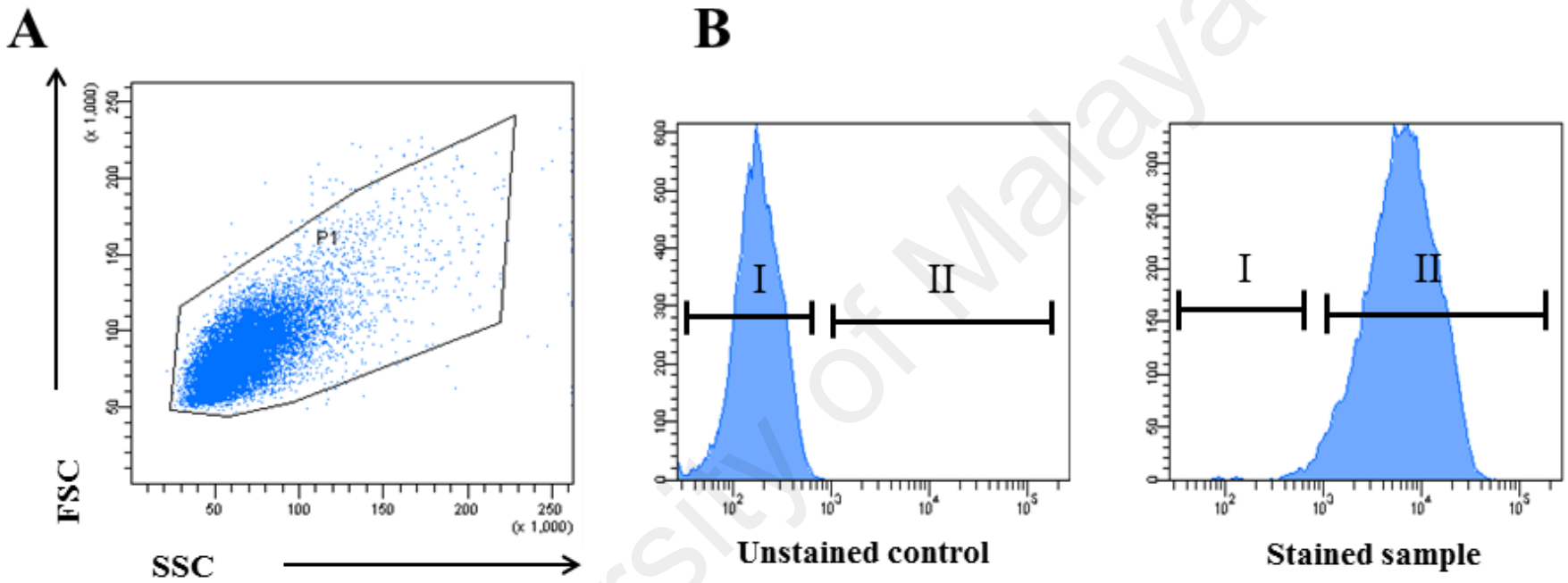


Figure 3.1 Instrument settings for flow cytometry. 3.1(A): Example of gating for RAW264.7 cells according to FSC and SSC scatter. 3.1(B): Histogram of unstained controls was prepared as negative reference point, gate I indicates unstained cells, and gate II indicates stained samples.

3.7.1 Cell proliferation assay

Cell proliferation assay was performed to understand the effect of *H. pylori* against the proliferation of RAW264.7 cells. For cell proliferation assay, 1.0×10^6 of non-infected control and infected cells (MOI 1, 5, 10 and 100) were centrifuged at 1,500 rpm for 5 minutes and supernatant was completely removed. For intracellular staining, fresh Foxp3 fixation working buffer (eBioscience, CA) was prepared by diluting 1 part of Foxp3/perm concentrate with 3 parts of Foxp3 fixation/perm diluent, while one times perm buffer is prepared by diluting with nine times distilled water. Each sample (non-infected control and infected) were stained and vortexed with 1 mL fixation working buffer in Facs tubes (BD Biosciences, USA) for 30 minutes. Without washing, 2 mL perm buffer was added and samples were centrifuged at 1,500 rpm for 5 minutes. Samples were then stained with FITC conjugated anti-Ki67 antibodies for 20 minutes, before repeating the step of adding perm buffer, followed by centrifugation at 1,500 rpm again. Finally, the supernatant was removed and replaced with 500 µL new Foxp3 fixation buffer. The samples were then ready for analysis.

3.7.2 Cell cycle assay

Cell cycle assay was performed on non-infected control and *H. pylori*-infected RAW264.7 cells (MOI 1, 5, 10 and 100). For cell cycle assay, 1.0×10^6 of non-infected control and infected cells (MOI 1, 5, 10 and 100) were centrifuged at 1,500 rpm for 5 minutes and supernatant was completely removed. For fixation of sample, 5 mL of 70 % cold ethanol was added dropwise to each sample while vortexing before incubating at -80 $^{\circ}$C overnight. The next day, samples were centrifuged at 1,500 rpm, 5 minutes to remove all supernatant. Samples were then washed twice with 5 mL of 1 × PBS, before staining with 500 µL of propidium iodide (PI)/RNase staining buffer (BD Biosciences, USA). The samples were incubated for 15 minutes at room temperature and analyze the

samples within 1 h. Singlet cells were gated according to SSC and FSC to prevent dublets and clumps (Figure 3.2).

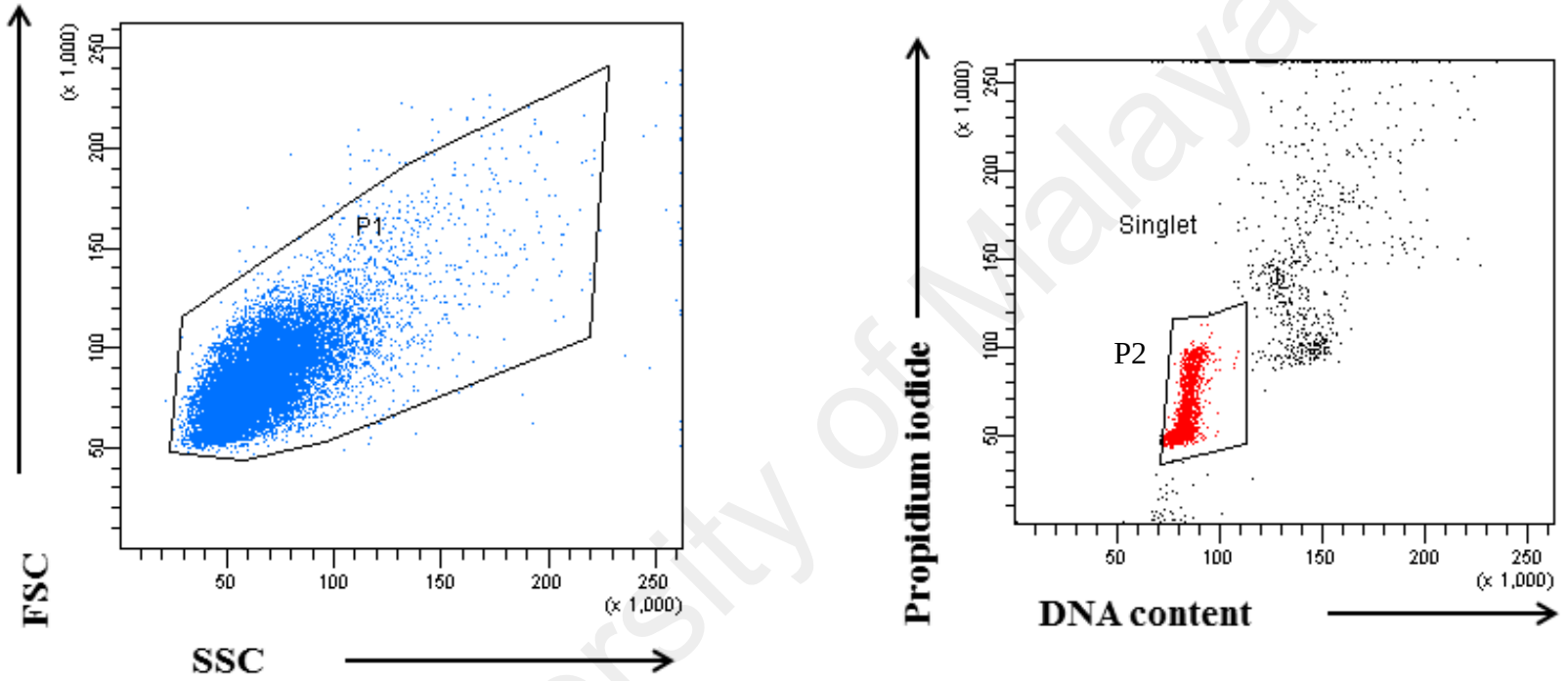


Figure 3.2 Gated RAW264.7 singlet cells in cell cycle analysis to prevent doublet discrimination in flow cytometry analysis. The P1 gate (blue) indicates cell gated using FSC and SSC (cell size and complexity), while the second gate P2 (red) gates on singlet, eliminating clumps and doublets.

3.7.3 Apoptosis assay (annexinV)

1.0×10^6 of non-infected control and infected cells (MOI 1, 5, 10 or 100) were centrifuged at 1,500 rpm for 5 minutes and supernatant were completely removed. 1× annexin V binding buffer (BD Biosciences, USA) was prepared by mixing 1 part of 10× annexin V binding buffer to 9 parts of distilled water. First, each pellet was resuspended in 100 µL of binding buffer, where 5 µL of FITC (Fluorescein isothiocyanate) annexin V (BD Biosciences, USA) and 5 µL PI were added. Samples were gently vortex prior to 15 minutes incubation in the dark. After incubation, 400 µL binding buffer was added and filtered through cell strainer for analysis.

3.7.4 Cell surface marker analysis

Cell surface marker analysis was performed on non-infected RAW264.7 cells and *H. pylori*-infected RAW264.7 cells. 1.0×10^6 cells/ sample were scraped from the flask and centrifuged at 1,500 rpm for 5 minutes and supernatant were completely removed. For surface marker analysis, cells were stained with antibodies for 30 min in dark, washed with 1 mL of PBS supplemented with 3 % FBS, before resuspending each sample in 1 mL of PBS/ 3 % FBS and finally passing through cell strainer for flow cytometrical analysis. For multiplex staining, single stains of each fluorochrome were prepared for compensation purposes. Antibodies used were *APC* (Allophycocyanin)-conjugated CD11b (Mac-1), CD86, PE (Phycoerythrin)-conjugated CD44, PE-conjugated CD274 and PE-conjugated CD83 (Biolegend, CA).

3.8 Immunoblot analysis

Non-infected and *H. pylori*-infected RAW264.7 cells (MOI 1,5 or 10) were seeded at the same concentration (5.0×10^6 cells/mL) for protein extraction. Protein lysates were prepared from using Radioimmunoprecipitation assay buffer (RIPA) lysis buffer (1×

TBS, 1.0 % NonidetP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 0.004 % sodium azide) supplemented with 1 mM sodium orthovanadate (Na_3VO_3), 2 mM phenlymethylsulfonyl fluoride (PMSF) and 1× protease inhibitor cocktail (Santa Cruz Biotech, CA). Concentration of each protein samples were measured and adjusted to 1000 μg/mL by BCA assay (Pierce, USA). Laemmli sample buffer was added to protein lysate at a ratio of 1:3 and denatured at 95 °C for 5 minutes. Samples were then immediately transferred on ice. 20 μL of each samples and protein ladder (Pierce) were separated by NuPage 4-12% Bis-Tris SDS PAGE gel (Invitrogen) and blotted onto polyvinylidenedifluoride membranes (PVDF) using iBlot dry blotting system (Invitrogen) for 7 minutes according to manufacturer's protocol. Membranes were blocked with 5.0 % bovine serum albumin in Tris Buffered Saline with 0.2 % Tween (TBS-T). After blocking, membranes were incubated overnight at 4 °C with primary antibodies at 1:1000 dilutions. Membranes were then washed for three times, 15 minutes each and incubated with respective secondary antibodies at 1:5000 dilutions. The membranes were then washed again three times, 15 minutes before proceed to staining. Primary antibodies used were antibodies against β-actin, Cyclin D1 (DCS6), phospho-Cdc2 (Tyr15), Cenp-a (Cell Signaling Technologies, Beverly, MA) and Aurora B kinase (Abcam, UK). β-actin was used as a loading control to normalize the levels of protein detected across all samples loaded. Secondary antibodies used were alkaline phosphatase-conjugated mouse or rabbit anti-IgG (Promega, USA). Membranes were developed using colorimetric nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT-BCIP) substrate in staining buffer (Promega, USA). All buffers and reagents prepared were written in Appendix A3.

3.9 Primary macrophage cells isolation

The isolation of primary macrophages from C57BL/6 mice was adapted from (Weischenfeldt & Porse, 2008). C57BL/6 mice were purchased (Jackson Laboratory, Bar Harbor, ME). Preparation of primary macrophages was adapted from (Weischenfeldt & Porse, 2008). Before the experiment, lymphocyte medium containing Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, USA) supplemented with 10 % heat-inactivated FBS, 100 µg/mL streptomycin and 100 U/mL penicillin, 1× non-essential amino acids, 1 mM HEPES and stimulated with 20 ng/mL M-CSF (Biolegend, CA) was prepared. Two male mice at 8–12 weeks old were euthanized and both femurs were excised at both ends from the mice. The excess muscle of the mice was cut off using scissors. RPMI medium were injected into the middle of the bone marrow to flush out the cells. The cells were gently crushed in between two frosted glass slides before passing through a 70 µm cell strainer to bring the cells into single cell suspension. All cells were then cultured in 6-well plates in lymphocyte medium, differentiated in a humidified incubator with 5% CO_2 at 37^{o}C. After 3 days, non-adherent cells were collected and cultured in freshly prepared lymphocyte medium for another 3 days to obtain BMDM. At day 7, fully differentiated macrophage progenitors were infected with *H. pylori* SS1 at MOI 10 for 24 h. The BMDM non-infected control and infected BMDM were scraped with a cell scraper and further analyzed for cell proliferation assay as described in (3.7.1).

3.10 Statistical analysis

Data were analyzed with unpaired two-tailed Student's *t*-test or Benjamini-Horchberg FDR multiple testing correction. Samples were considered significant if $P < 0.05$.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 *H. pylori* activates RAW264.7 monocytic macrophage cells

In this study, *H. pylori* SS1 strain was used for infection, as it is a well-established mouse-adapted pathogenic strain, which infects RAW264.7 murine monocytic macrophage cells (Gobert, Alain P. *et al.*, 2002). After 24 hpi at MOI 1, 5 and 10, *H. pylori*-infected RAW264.7 cells looked grossly enlarged (indicated with dotted arrows) compared to the non-infected RAW264.7 cells. Furthermore, *H. pylori*-contained phagosomes were observed among the infected RAW264.7 cells (indicated with black arrows) (Figure 4.1A). With increasing MOI, cells also displayed enlarged cell size and complexity, as proven by increased intensity of forward scatter (FSC) and side scatter (SSC) by flow cytometry analysis (Figure 4.1B). From the gated FSC and SSC histogram, RAW264.7 cells showed an increase in population; where FSC showed an increased from 11 % to 56 %; while SSC increased from 11 % to 27 % respectively. This indicated infected-RAW264.7 cells displayed bigger cell size (FSC) and complexity (SSC).

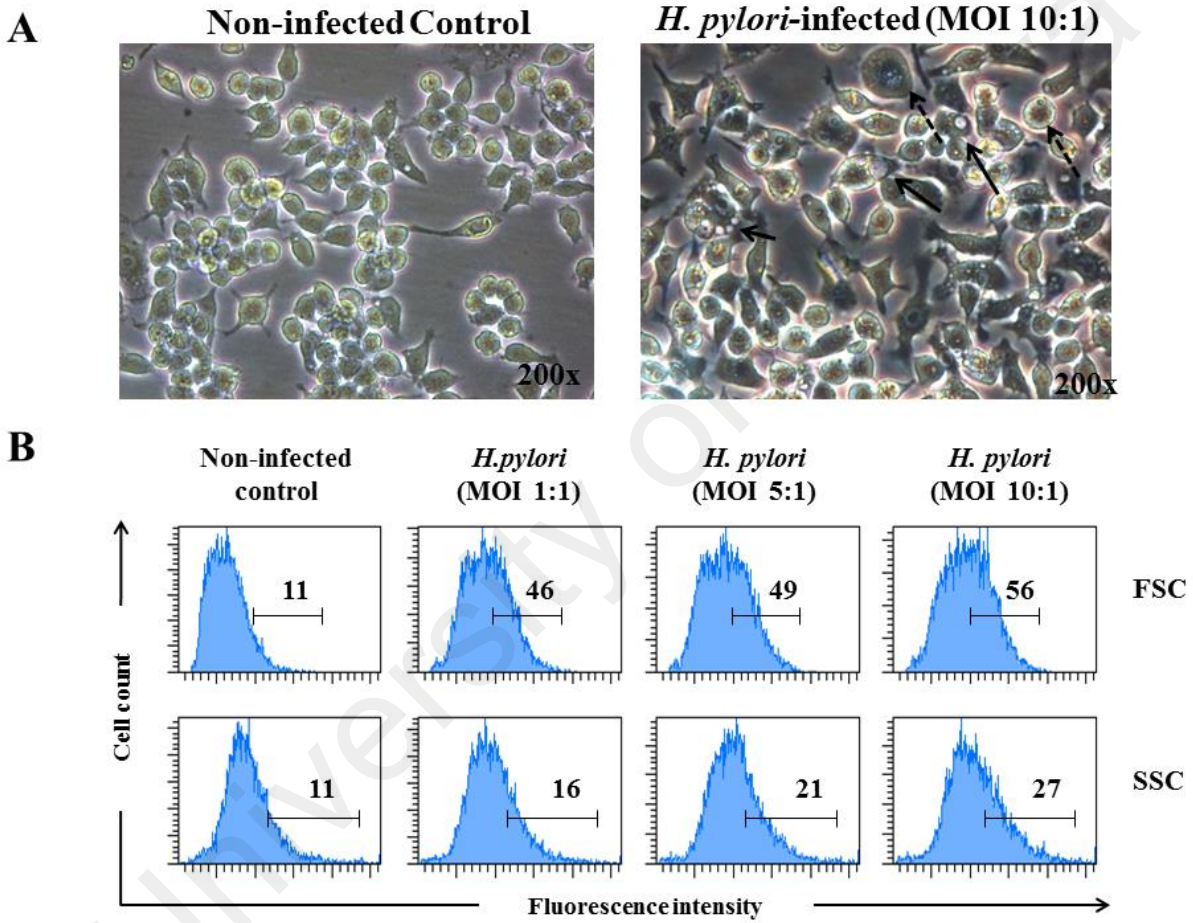


Figure 4.1: *H. pylori*-infected RAW264.7 cells showed enlargement in cell size. (A) Microscopic images of non-infected RAW264.7 cells and *H. pylori*- infected cells at MOI 10 after 24 hpi. Black arrows indicate phagosomes and dotted arrows represent enlargement.(B) Flow cytometry analysis showing FSC and SSC of the non-infected and infected RAW264.7 cells. Numbers represent the percentage of cells at the gated area.

Besides, we observed increased surface expression of monocytic marker F4/80 and CD11b compared to non-infected control, suggesting that the *H. pylori*-infected RAW264.7 cells experienced monocytic-to-macrophage differentiation (Figure 4.2) F4/80 glycoprotein, is one of the most established surface markers that is constitutively expressed on most of the resident tissue macrophages (Austyn & Gordon, 1981; Morris *et al.*, 1991). The expression of F4/80 is tightly regulated according to the physiological state of macrophages (McKnight & Gordon, 1998); where native monocytes express lesser intensity comparatively to mature macrophages (Gordon *et al.*, 1992). Functions of F4/80 include complement receptors, mannose receptors and scavenger receptors (Platt *et al.*, 1999; Stahl, 1992).

CD11b, also known as Mac1, is a receptor that expressed on monocytes, granulocytes, macrophages and NK cells (Ho & Springer, 1982). It is responsible for adhesion, migration, phagocytosis, chemotaxis, cellular activation and cytotoxicity (Solovjov *et al.*, 2005). Additionally, it participates in inflammatory diseases (Morrison *et al.*, 2008), and mediating activation of phagocyte NADPH oxidase in macrophages (Zhou, H. *et al.*, 2013). The *H. pylori*-infected RAW264.7 cells showed higher expression of F4/80 and CD11b phenotypes, indicating their robust cellular activity. Cell surface markers CD83 and CD86 were moderately increased comparatively to non-infected control (Figure 4.2); further suggests that *H. pylori* infection activates monocytic macrophage cells.

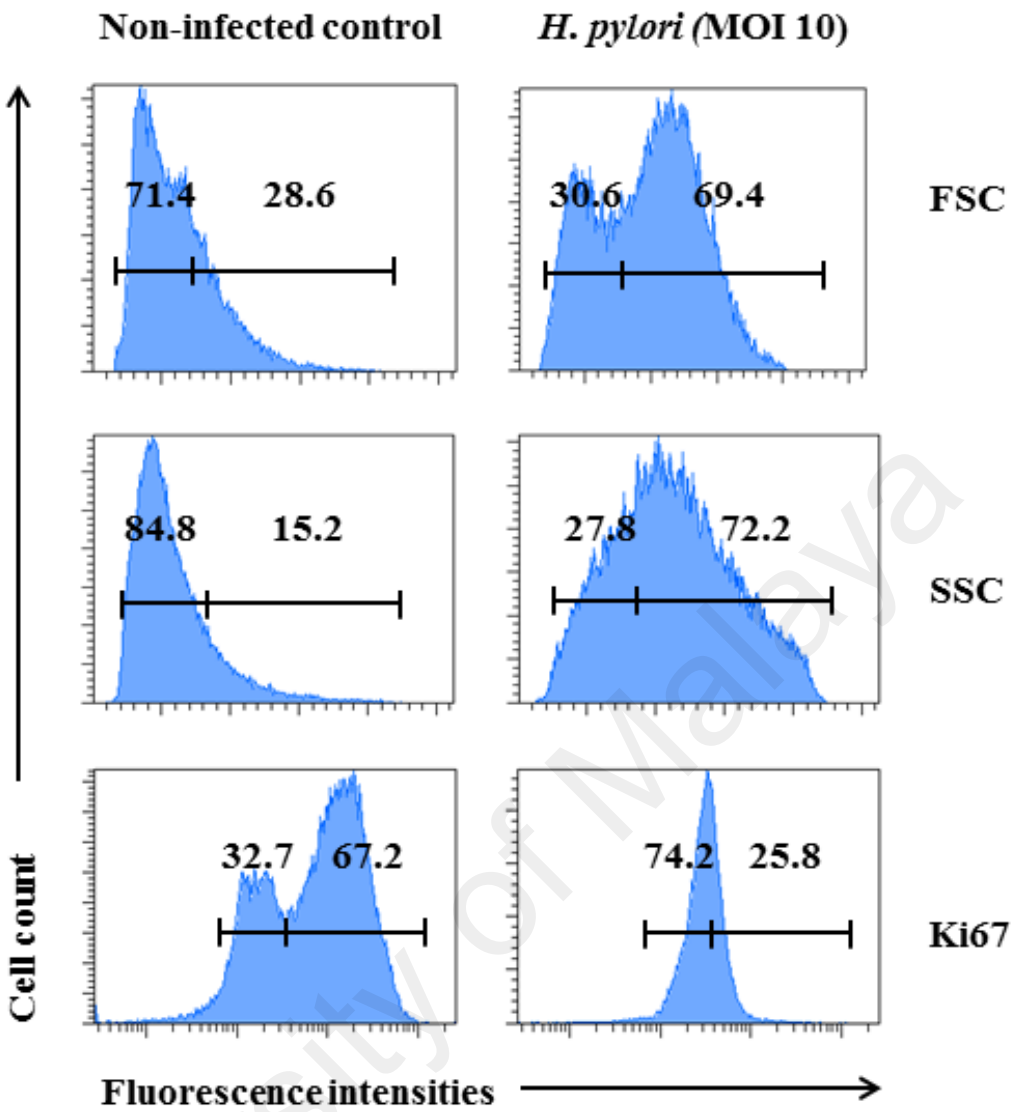


Figure 4.2: Flow cytometry analysis of cell surface markers on non-infected control and *H. pylori* (MOI 10, 24 Hpi)-infected cells: Numbers represent the percentage of cells in the gated area.Surface markers examined include CD11b, F480, CD83 and CD86. Data shown were representative of two independent experiments.

4.2 *H. pylori* infection does not induce apoptosis at low MOI (MOI 1, 5 and10)

H. pylori-infected RAW264.7 cells did not display apoptotic features at MOI 1 to MOI 10, suggesting that at low MOI, *H. pylori* was sufficient to activate the monocytic-macrophages yet inadequate in causing cell death in RAW264.7 cells. This was proven with annexin V-FITC apoptosis assay (Figure 4.3), where majority (~98%) of the cell population were viable (quartile I: annexin V^-PI^-), and <1% were at early apoptotic (quartile II: annexin $V^+PI^{-),}$ late apoptotic (quartile III: annexin V^+PI^+) or necrotic (quartile IV: annexin V^-PI^+). On the contrary, when *H. pylori*-infection was further increased to MOI 100, approximately 30% of the cells shifted from viable cells to apoptotic: 22.8% were early-stage apoptotic, 1.8 % were necrotic and 7.4% were late apoptotic dead cells.

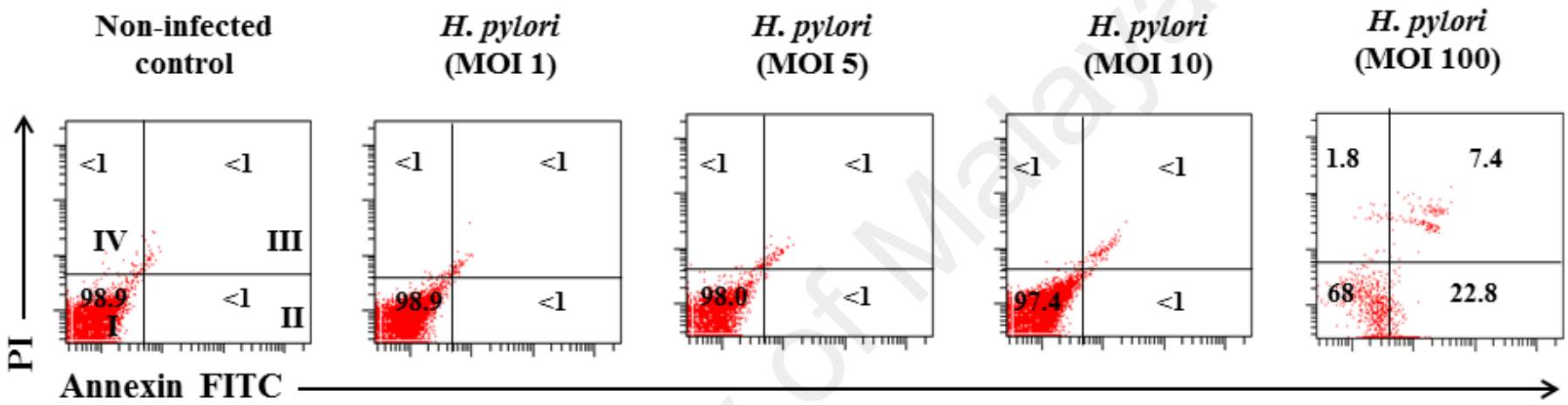


Figure 4.3: Annexin V-FITC flow cytometry analysis of non-infected control and (*H. pylori* (MOI 1, 5, 10,100)-infected cells. Cells were stained with annexin V-FITC and propidium iodide (PI). Number represents the percentage of cells at different quartile, I: Live cells, II: early apoptotic cells, III: necrotic cells and IV: late apoptotic/dead cells. Data shown were representative of two independent experiments

4.3 *H. pylori* infection attenuates proliferation of RAW264.7 cells

Cell proliferation of *H. pylori*-infected RAW264.7 cells was analyzed by cell count and Ki67 staining. After 24 hpi, microscopic images of *H. pylori*-infected RAW264.7 cells showed reduced in cell numbers at MOI 10 and MOI 100 (Figure 4.4A). Thus, cell count was done to quantify the reduced proliferation (Figure 4.4B). At 0 hpi, cell count of non-infected RAW264.7 cells and *H. pylori*-infected RAW264.7 cells were 1.0 x 10^6 cells. At 12 hpi, macrophages count decreased from 7.098 ±1.68 million cells to 3.223 ±1.059 million cells at MOI 10 (P=0.008); and 2.688 ± 0.403 million cells at MOI 100 (P=0.007); revealing 42 % reduction for MOI 10 and 60.7 % reduction for MOI 100. At 24 hpi, cell count further reduced from 13.938 ± 1.80 million cells to 6.88 ± 2.130 million cells for MOI 10 (P=0.002); and 4.039 ± 1.263 million cells for MOI 100 (P=0.0005). The reduction increased to 43 % and 71 % for MOI 10 and MOI 100 respectively.

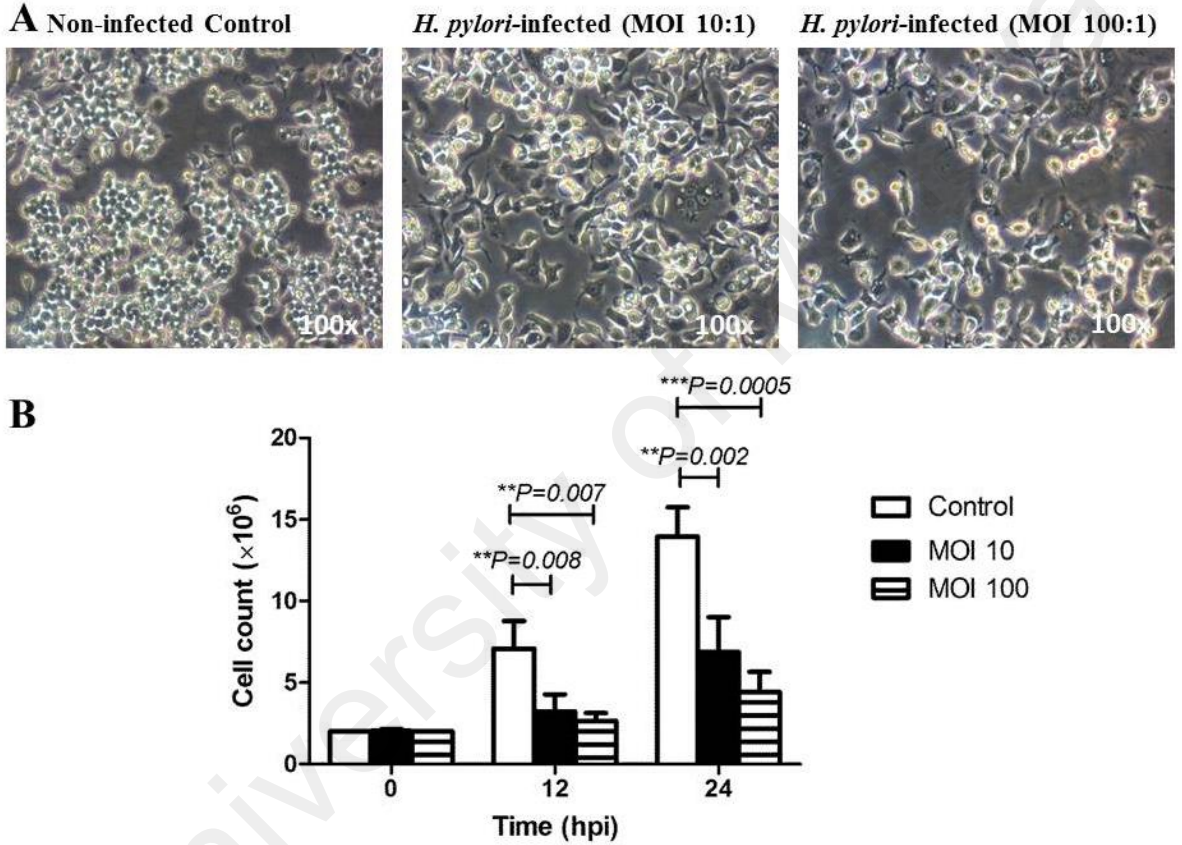


Figure 4.4: Microscopic images and cell count of non-infected control cells and *H. pylori*-infected cells at indicated MOIs. (A) Microscopic images of non-infected control cells and *H. pylori*-infected cells at MOI 10 and 100. (B) Bar chart shows absolute cell count of the non-infected and *H. pylori*-infected cells at 12 hpi and 24 hpi. Data were shown as mean ± SD from one experiment run in duplicate, and were representative of two independent experiments.

Antigen Ki67 is a nuclear protein which is used as a marker for cellular proliferation. It presents during all active phases of the cell cycle but not in resting cells. Using Ki67 cellular proliferation assay, *H. pylori* was further confirmed to attenuate the proliferation of RAW264.7 cells. Figure 4.5 shows non-infected control consisted 61 % of Ki67 ^{low} cells and 38 % of Ki67^{high} cells. The Ki67 ^{high} population shrunk to 31 %, 20 %, 15 % and 9 % respectively when the cells were inoculated in increasing MOI of 1, 5, 10 and 100 respectively. On the contrary, Ki67 ^{low} cell population increased steadfastly to 69 %, 80 %, 85 % and 90 % at MOI 1, 5, 10 and 100. These two experiments proved that *H. pylori* infection inhibits proliferation of RAW264.7 cells.

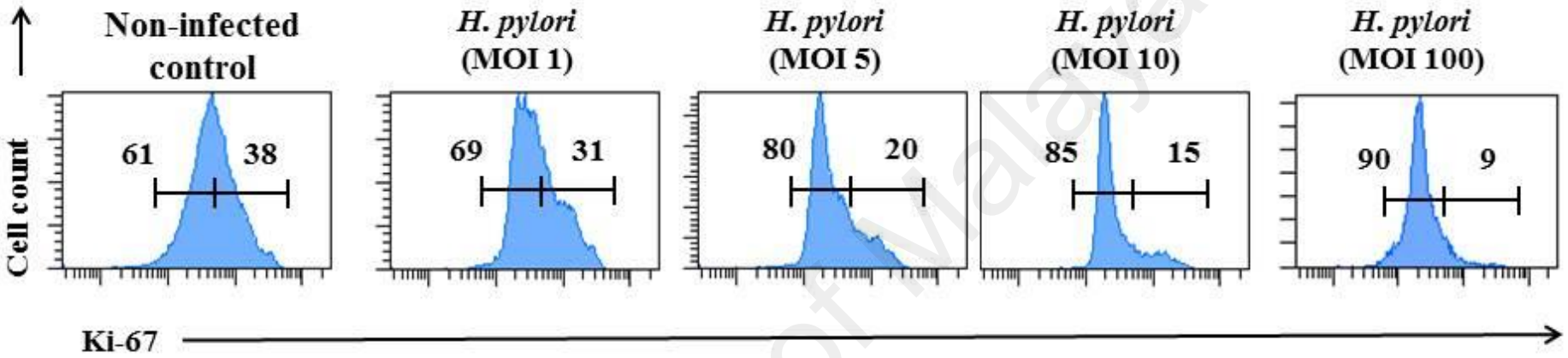


Figure 4.5: Flow cytometry analysis of intranuclear expression of Ki67 cell proliferation marker in the non-infected control versus different MOI *H. pylori*-infection (1, 5, 10 and 100) for 24 h. Numbers represent the percentages of cells in the gated area. Shown were representative data from two independent experiments.

4.4 *H. pylori* activates large scale gene transcription machinery in RAW264.7 cells

In order to understand the effect of *H. pylori* infection towards the transcription profile of monocytic macrophages, genome wide microarray analysis was performed using RNA samples derived from non-infected control and *H. pylori*-infected RAW264.7 cells at MOI 10. Two biological replicates were prepared for each sample. The concentration of RNA samples extracted were as below (Table 4.1), with the ratio of 260/280, 260/230 and indicated RNA integrity number (RIN). From the table, ratio of 260/280 (~2.0) ensured there was no protein contamination in the samples, while the ratio of 260/230 confirmed no genomic DNA was present in the samples range of 2.0-2.2. All samples with a RIN number ~10.0 ensured no degradation of RNA samples. The band of 28S, 18S of extracted RNA samples as well as the chromatograms can be seen in Figure 4.6. Electropherogram of all samples included a clear visible 28S/18S rRNA peak ratio and a small 5S RNA.

Table 4.1: Quality of RNA samples sent for microarray analysis. Control-1 and control-2 are biological replicates of non-infected control, whereas sample-1 and sample-2 are biological replicates of *H. pylori*-infected RAW264.7 cells at MOI 10. Ratio 260/280 indicates the purity of RNA (~2.0); ratio 260/230 shows the purity of nucleic acid (range of 2.0-2.2) and RNA integrity number (RIN) measures the integrity/quality of RNA samples.

Sample	Concentration (ng/µl)	260/280	260/230	RIN
Control-1	1171.6	2.03	2.19	10
Control-2	1407.0	2.00	2.14	10
Sample-1	942.3	2.01	1.97	9.8
Sample-2	892.5	1.98	2.07	9.7

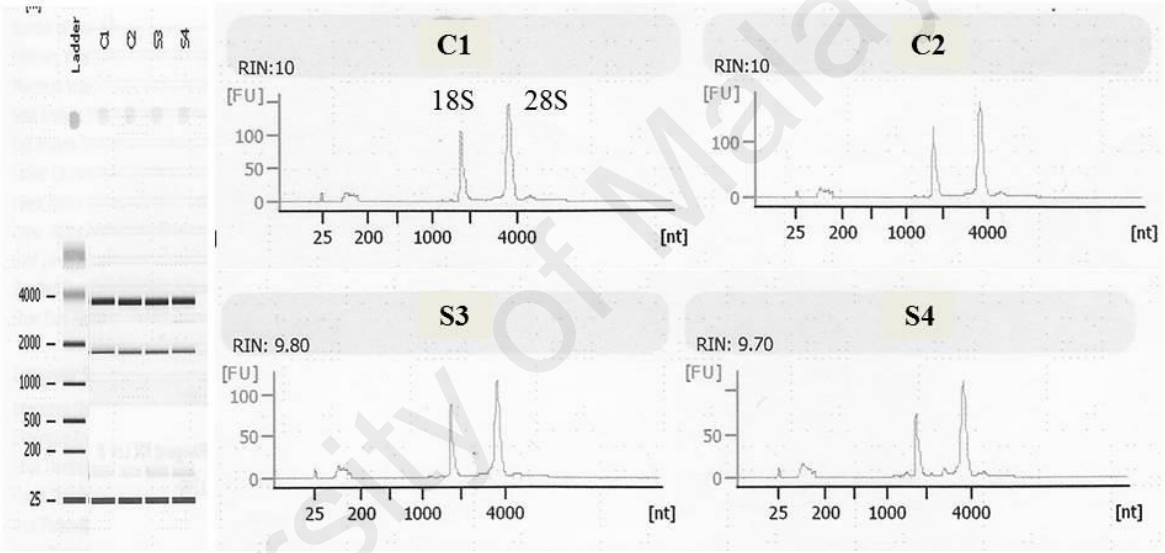


Figure 4.6: Electrophoresis and electropherogram of extracted RNA samples. C1, C2 are biological replicates of non-infected controls; S3 and S4 are biological replicates of *H. pylori*-infected RAW264.7 cells. Bands and peaks at 1524 bps represent 18S, while 3249 bps represents 28S.

From the raw data generated from the probes, scatter plot was generated based on normalized (Log2) expression levels of total probes or differentially expressed probes with a threshold FC of more than 2 or FC less than -2 (Figure 4.7A). In total, 11327 probes and 10923 probes were detected for non-infected control and *H. pylori*-infected samples respectively. After filtering with FC>2, FC<-2, P<0.05, 1341 genes (2471 probes) were significantly upregulated, while 1591 genes (2651 probes) (Figure 4.7A) were significantly downregulated. This proved that *H. pylori* infection elicited vital transcription regulation in host cells. Quality control of the results using hierarchical clustering revealed a total of 5122 probes were executed, and other extreme outliers were excluded from further analysis (Figure 4.7B).

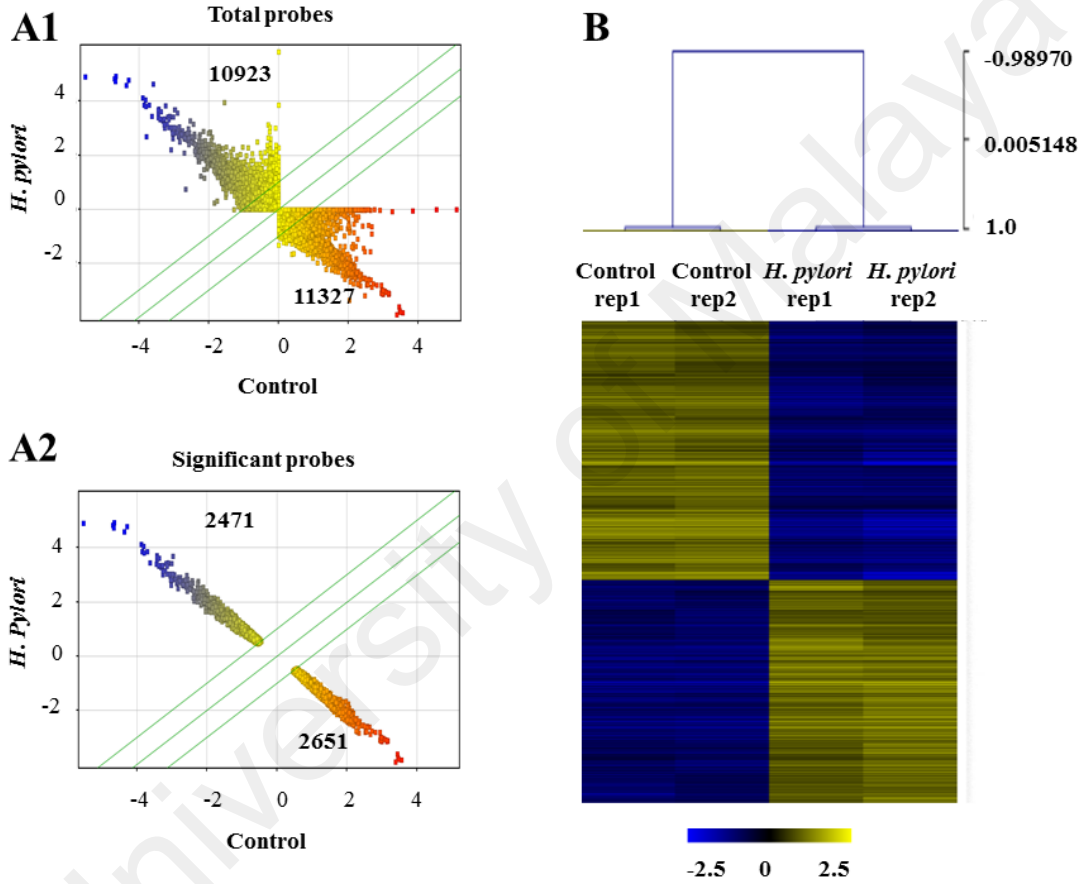


Figure 4.7: Transcriptome analysis and hierarchical clustering of microarray probes. Transcriptome analysis (A1) unfiltered probes and (A2) probes that are subjected to filter of FC>2, FC<2 and P<0.05.Both scatter plots showing the comparison of probe expressions for the non-infected control against the *H. pylori*-infected cells. X and Y axis show normalized log2 values. (B) Hierarchical clustering for significant 5122 probes (including upregulated and downregulated genes) - 2932 genes was executed with Pearson Correlation distance metric and average linkage.

4.5 Top 10 upregulated genes in *H. pylori*-infected RAW264.7 cells

From the microarray analysis the top 10 upregulated genes were identified (Table 4.2). The expression levels of these top 10 genes were further verified by qRT-PCR (Figure 4.8). The qRT-PCR fold change is listed in (Appendix C).

The top up-regulated gene was *Car6* (*Carbonic anhydrase* 6), that was induced at +1396-fold (P=0.0036) Besides, *Pappa2* (*Pappalysin* 2) was upregulated at 233-fold (P=0.0036), and *Ptgs2* (*Prostaglandin endoperoxidase synthase* 2) was increased 541-fold (P=0.0041); all of these genes are related to cell stress management (Chen, J. *et al.*, 2005). However, there is no research that relates these genes with *H. pylori* infection.

Among the top 10 upregulated genes, five of them were immune response-related genes that encode for cytokines, for example: *Csf1*, *Csf3, Il-1β* and chemokines *Ccl7* and *Cxcl2*. Colony stimulating factors (CSF) were important growth factors which regulated the growth and differentiation of macrophages upon infection (Cheers *et al.*, 1988). *Csf1* and *Csf3* was upregulated at +208-fold (P=0.02) and +771-fold (P=0.03) respectively. *Cxcl2,* also known as macrophage inflammatory protein 2α (MIP-2α), and *Ccl7* (also known as monocyte-specific chemokine 3) are chemo-attractants of monocytes and lymphocytes (Salanga *et al.*, 2014). They are secreted as an inflammatory response in order to recruit immune effector cells to site of infection (Schluger & Rom, 1997). Microarray results also showed that *Cxcl2* and *Ccl7* were upregulated by 682-fold (P=0.0003) and 496-fold (P=0.0008) respectively.

Other genes in the list of top 10 upregulated genes were *Lcn2* and *Avil*, which were induced 746-fold (P=0.0014) and 196-fold (P=0.0003) respectively. *Lcn2* is a negative regulator for inflammation, which is mainly known as an antimicrobial defense mediator that response to Toll-like receptor activation during infection (Flo *et al.*, 2004; Warszawska *et al.*, 2013). *Avil* is an actin regulatory protein that has unique functions in

the morphogenesis of neuronal cells; however, its function in macrophages is still yet unknown (Marks *et al.*, 1998).

Table 4.2: List of top 10 genes that were significantly upregulated in *H. pylori*-infected RAW264.7 cells compare to non-infected RAW264.7 cells. Significance analysis was performed with Student's *t*-test and Benjamini-Horchberg FDR multiple testing correction

Genbank Accession (NM)	Description	Folds	P	FDR
009802	Carbonic anhydrase 6 (Car6)	1396	0.0036	0.0291
009971	Colony stimulating factor 3(Csf3)	771	0.0031	0.0279
008491	Lipocalin 2 (Lcn2)	746	0.0014	0.0238
013654	Chemokine (C-C motif) ligand 7 (Ccl7)	682	0.0003	0.0209
011198	Prostaglandin-endoperoxide synthase 2 (Ptgs2)	541	0.0041	0.0984
009140	Chemokine (C-X-C motif) ligand 2 (Cxcl2)	496	0.0008	0.0216
008361	Interleukin 1 beta (Il1b)	257	0.0064	0.0345
001085376	Pappalysin 2 (Pappa2)	233	0.0028	0.0273
007778	Colony stimulating factor 1 (Csf1)	208	0.0002	0.0209
009635	Advillin (Avil)	196	0.0003	0.0209

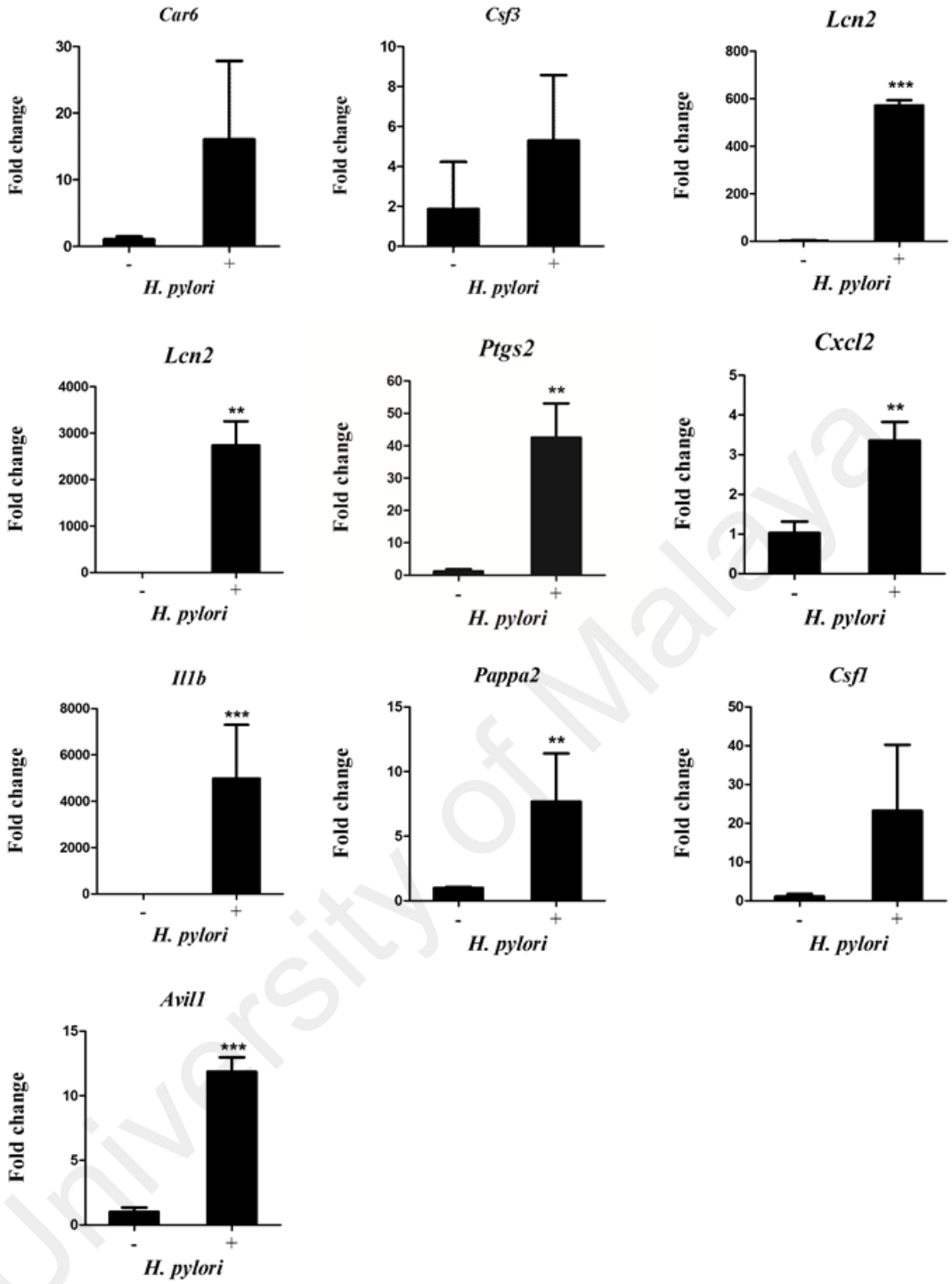


Figure 4.8: Validation of top 10 upregulated genes by qPCR. Relative fold change shows expression of each gene relative to internal control β-actin. -: Non-infected control; +: *Hp*-infected cells. Data were shown as mean ± SD, from one experiment run in triplicate. Statistical significance was analyzed with unpaired Student's *t*-test (*P<0.05, **P<0.01, ***P<0.001)

4.6 Top 10 downregulated genes in *H. pylori*-infected RAW 264.7 cells

Top 10 downregulated genes in *H. pylori*-infected RAW264.7 cells consist of genes with various functions (Table 4.3). qRT-PCR analysis of the verified genes are illustrated in Figure 4.9, except *Crip1* and *Mybl2* due to the sequence not found for *Mus musculus* species. The top downregulated gene is *Cx3cr1* which was reduced 157-fold (P=0.0022). *Cx3cr1* is an important chemokine receptor that regulates macrophage homeostasis and bacterial clearance, while limiting T_H17 responses (Medina-Contreras *et al.*, 2011). The reduction of *Cx3cr1* expression in infected-RAW264.7 cells could contribute to the impaired pathogen recognition. The second top downregulated gene was *Fgd2*, which was downregulated 146-fold (P=0.0255). It is a CDC42-specific exchange factor that is usually expressed in professional antigen presenting cells which functions in controlling the organization and dynamics of actin cytoskeleton (Huber *et al.*, 2008). Interestingly, previous research also indicates *Fgd2* of BMDMs was strongly suppressed by the infection of *H. pylori* (Weiss et al., 2013). *Dhcr24* which was downregulated 78-fold (P=0.0001) is an enzyme of cholesterol synthesis and has a pro-survival role during oxidative stress (Crameri *et al.*, 2006; Kuehnle *et al.*, 2008). Its suppression is linked to the inhibition of Sterol regulatory element-binding protein (SREBP) target genes, which leads to the repression of inflammatory response genes (Spann *et al.*, 2012).

Other than that, *Alox5* and *Lrp8* were downregulated at 78-fold (P=0.0002) and 70-fold (P=0.0008) respectively. *Alox5* in macrophages is responsible in synthesizing 5-lipoxin A4 (LXA4) (Bafica *et al.*, 2005). LXA4 works together with prostaglandin E2 (PGE2), where these two groups of eicosanoid regulate programmed macrophage cell death. LXA4 promotes termination of inflammation, while PGE2 oppose the effects of LXA4 (Chen, M. *et al.*, 2008). It is reported that mice deficient of *Alox5* (LXA4 deficiency) are more resistant to chronic infection when infected by *M. tuberculosis*

(Levy et al., 2001). Furthermore, the deleted gene had resulted higher secretion of pro-inflammatory cytokines such as IL-12 and IFN-γ (Divangahi *et al.*, 2010). We hypothesize that the diminished in *Alox5* was a result of macrophage activation, in reducing *H. pylori* invasion.

Interestingly, there were two cell cycle-associated genes in the downregulated list; which is *Aurkb* -75-fold (P=0.0017) and *Mybl2*, -66-fold (P=0.0008). *Aurkb* is essential in chromosomal alignment and cytokinesis (Fu *et al.*, 2007). Moreover, it is found to regulate G1-to-S phase progression in T lymphocytes (Song *et al.*, 2007). *Mybl2* is expressed in all proliferative cells, and is one of 39 critical transcription factors expressed in stem cells (Muller *et al.*, 2008). It regulates both proliferation and differentiation of cell and its expression can overcome G1 arrest (Lin *et al.*, 1994). Moreover, its activity directly affects the transcription of various cell cycle associated genes such as *Cyclin D1, Cyclin B2, C-myc* and *Cdc25b* (Papetti & Augenlicht, 2011). *Ankle1* which is an endonuclease responsible in DNA cleavage and damage response was reduced by 71-fold (P=0.0002) (Brachner *et al.*, 2012). Lastly, *Kbtbd11* and *Crip1* which were downregulated at 133-fold (P=0.0007) and 67-fold (P=0.0006) has no well-defined function yet.

Table 4.3: List of top 10 significantly downregulated genes comparatively to non-infected RAW264.7 cells. Significance analysis was performed with Student's *t*-test and Benjamini-Horchberg FDR multiple testing correction.

Genbank Accession	Description	Folds	P	FDR
nm009987	Chemokine (C-X3-C) receptor 1 (Cx3cr1)	-157	0.0022	0.0259
nm013710	FYVE, RhoGEF and PH domain containing 2 (Fgd2)	-146	0.0021	0.0255
nm029116	Kelch repeat and BTB (POZ) domain containing 11 (Kbtbd11)	-133	0.0007	0.0211
nm053272	24-dehydrocholesterol reductase (Dhcr24)	-78	0.0001	0.0145
nm009662	Arachidonate 5-lipoxygenase (Alox5)	-78	0.0002	0.0210
nm011496	Aurora kinase B (Aurkb)	-75	0.0017	0.0246
nm172756	Ankyrin repeat and LEM domain containing 1 (Ankle1)	-71	0.0002	0.0210
nm001080926	Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor (Lrp8)	-70	0.0008	0.0213
nm007763	Cysteine-rich protein 1 (intestinal) (Crip1)	-67	0.0006	0.0145
nm008652	Myeloblastosis oncogene-like 2 (Mybl2)	-66	0.0008	0.0216

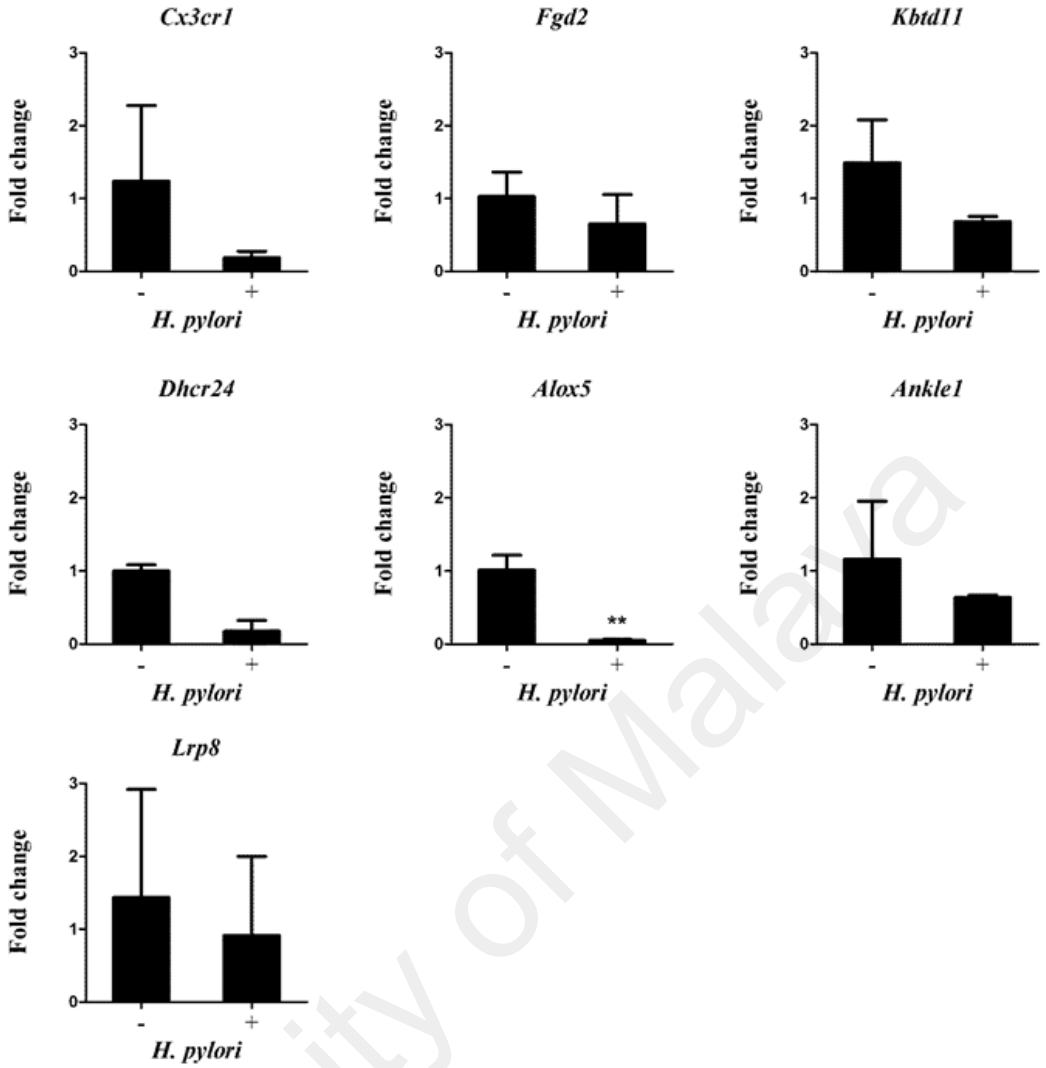


Figure 4.9: Validation of the selected genes from the list of top 10 downregulated genes by qRT-PCR. The gene sequence for *Crip1 and Mybl2* is unknown, while *Aurkb* will be discussed below. Relative fold change shows expression of each gene relative to internal control β-actin. -: Non-infected control; +: *Hp*-infected cells. Data were shown as mean ± SD, from one experiment run in triplicate. Statistical significance was analyzed with unpaired Student's *t*-test (*P<0.05, **P<0.01, ***P<0.001).

4.7 *H. pylori* infection altered dysregulation of multiple signaling pathways

Pathway enrichment analysis of the significant 1341 upregulated and 2651 downregulated genes was performed to extract the biological significance of the differentially expressed genes between non-infected control and infected RAW264.7 cells. After inserted the significantly up- and down-regulated genes altered by *H. pylori* infection into the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, the fold enrichment (FE) for each pathway was calculated. In total, 25 biological pathways with changes more than 2-fold ($P<0.05$) showed a potential link to *H. pylori* infection (Figure 4.10). From 25, 9 pathways were significantly induced with lysosome pathway (mmu04142) showing the highest enrichment score (FE=3.94, $P<0.00001$) (Table 4.4), followed by cytokine-cytokine receptor interaction pathway (FE1.8, $P<0.0001$). The full list of genes by each pathway is listed at Appendix D.

On the contrary, 16 pathways were significantly suppressed (Table 4.5). Out of the 16 pathways, DNA replication pathway (mmu03030) was the most suppressed pathway with a FE of -8.27 ($P<0.0001$). Mismatch (mmu03430) and nuclear excision (mmu03420) DNA repair mechanism was identified at FE=-7.30 ($P<0.0001$) and FE=-3.49 ($P<0.0001$), respectively. The full gene list is listed at the Appendix D

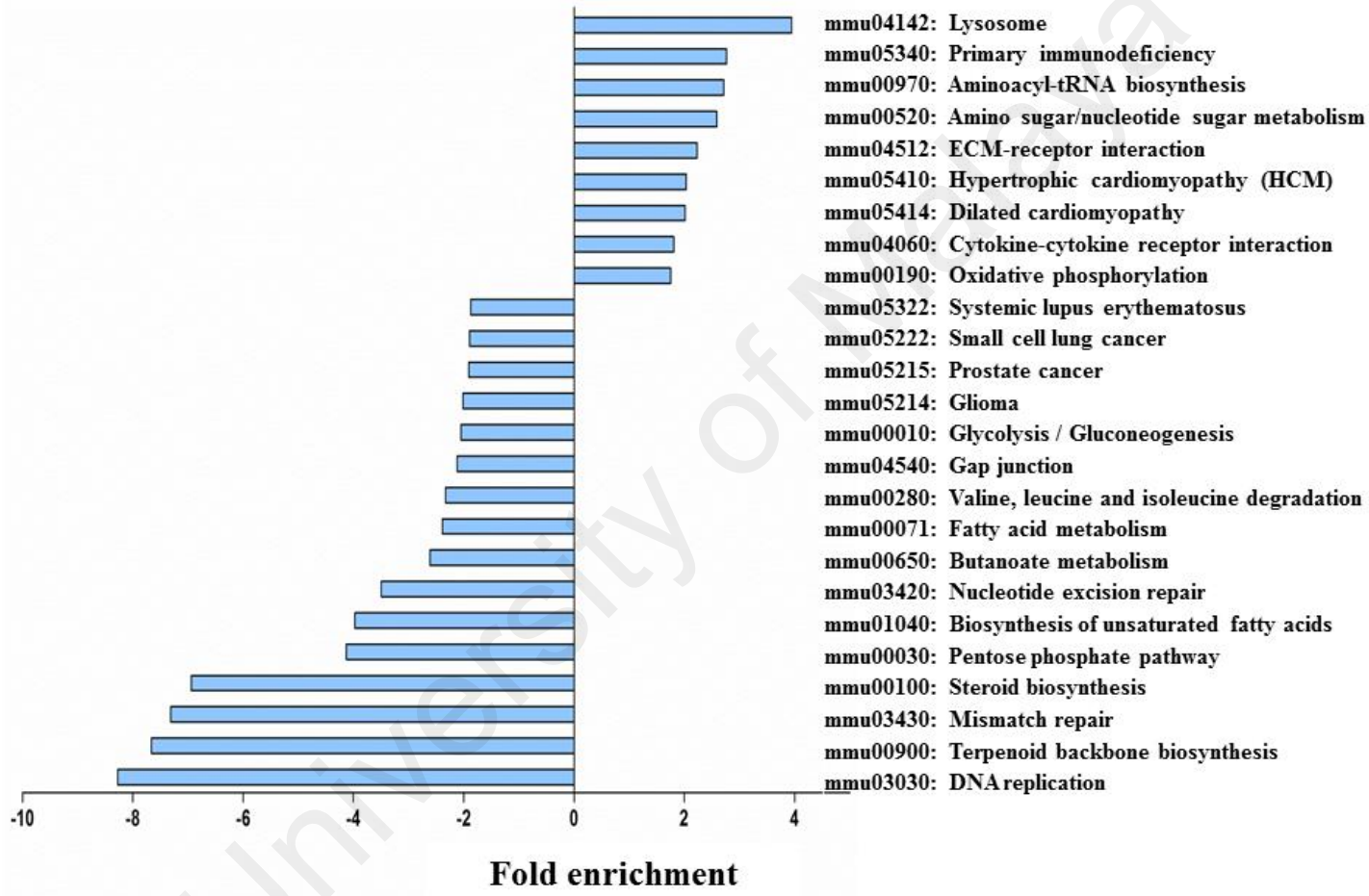


Figure 4.10: KEGG pathway analysis. Bar chart showing the FE of significantly modulated pathways in the *H. pylori*-infected RAW264.7 cells relative to control (FE<-2 or FE>2, P<0.05). In total, 8 pathways showed induction while 16 pathways were reduced.

Table 4.4: List of induced pathway from KEGG pathway analysis. mmu represents *Mus musculus* (mouse), % represents the count over total of 403 genes. FE represents the fold enrichment of the pathway. P value was filtered at P<0.05. Significance analysis was performed with Benjamini-Horchberg False Discovery Rate (FDR) multiple testing correction.

mmu	Term	Count	%	FE	P value	Bonferroni	Benjamini	FDR
04142	Lysosome	33	2.14	3.95	1.22E-11	2.05E-9	2.05E-9	1.47E-8
05340	Primary immunodeficiency	7	0.45	2.77	0.0369	1.00	0.32	36.86
00970	Aminoacyl-tRNA biosynthesis	8	0.52	2.71	0.0251	0.99	0.33	26.67
00520	Amino sugar and nucleotide sugar metabolism	8	0.52	2.59	0.0316	1.00	0.29	32.45
04512	ECM-receptor interaction	13	0.84	2.23	0.0122	0.88	0.23	13.94
05410	Hypertrophic cardiomyopathy (HCM)	12	0.78	2.03	0.0314	1.00	0.31	32.30
05414	Dilated cardiomyopathy	13	0.84	2.01	0.0260	0.99	0.31	27.50
04060	Cytokine-cytokine receptor interaction	31	2.01	1.81	0.0016	0.24	0.09	1.94
00190	Oxidative phosphorylation	16	1.04	1.75	0.0373	1.00	0.30	37.16

Table 4.5: List of suppressed pathway from KEGG pathway analysis. mmu represents *Mus musculus* (mouse), % represents the count over total of 535 genes. FE represents the fold enrichment of the pathway. P value was filtered at P<0.05. Significance analysis was performed with Benjamini-Horchberg False Discovery Rate (FDR) multiple testing correction.

mmu	Term	Count	%	FE	P value	Bonferroni	Benjamini	FDR
03030	DNA replication	27	1.625	-8.27	2.68E-20	4.78E-18	2.39E-18	3.30E-17
03430	Mismatch repair	15	0.903	-7.31	4.93E-10	8.77E-08	2.92E-08	6.06E-07
00100	Steroid biosynthesis	11	0.662	-6.94	4.78E-07	8.52E-05	2.13E-05	5.88E-04
00900	Terpenoid backbone biosynthesis	10	0.602	-7.66	6.41E-07	1.14E-04	2.28E-05	7.89E-04
03420	Nucleotide excision repair	14	0.842	-3.49	9.02E-05	0.016	0.002	0.111
00030	Pentose phosphate pathway	10	0.602	-4.13	3.60E-04	0.062	0.005	0.441
01040	Biosynthesis of unsaturated fatty acids	10	0.602	-3.97	4.96E-04	0.084	0.006	0.608
04540	Gap junction	17	1.023	-2.12	0.005	0.603	0.053	6.175
05322	Systemic lupus erythematosus	18	1.083	-1.87	0.013	0.911	0.104	15.369
05215	Prostate cancer	16	0.963	-1.91	0.018	0.961	0.126	20.047
00650	Butanoate metabolism	9	0.542	-2.61	0.018	0.962	0.123	20.280
00071	Fatty acid metabolism	10	0.602	-2.38	0.021	0.976	0.134	22.704
00010	Glycolysis / Gluconeogenesis	13	0.782	-2.05	0.022	0.979	0.134	23.489
00280	Valine, leucine and isoleucine degradation	10	0.602	-2.33	0.024	0.986	0.142	25.560
05222	Small cell lung cancer	15	0.903	-1.89	0.024	0.987	0.139	25.873
05214	Glioma	12	0.722	-2.01	0.032	0.997	0.172	33.265

4.7.1 *H. pylori* infection activates transcription of immune response-related genes in RAW264.7 cells

Heat maps were generated for a few selected pathways. From the induced pathway, cytokine –cytokine receptor interaction (mmu04060) with a total of 31 genes were selected. The genes were divided into a few subgroups: cytokines, surface markers, chemokines and intergrins (Figure 4.11A). From the heatmap, most of the genes were induced (induced genes were shown as yellow, reduced genes were shown as blue), including colony stimulating factors (*Csf1*, *Csf2*, *Csf3*), pro-inflammatory cytokines (*Il-1α, Il1β, Tnf* and *Il23a*) suggesting that *H. pylori* infection activates macrophage and granulocyte differentiation and promote robust inflammatory responses (Hamilton, 2008). Early *in vitro* studies also shown CSFs regulate the expansion of a given hematopoietic cell lineage to functionally mature peripheral blood cell (Frumkin, 1997) . It also has a broad range of immune functions, such as inducing production of IL-1 and TNF, stimulating monocyte cytotoxicity against tumor targets (Caldwell & Emerson, 1994). Moreover, the induction of *Il23a* by RAW264.7 cells during the infection may accelerate the differentiation of T_H17 cells to combat *H. pylori*, where IL-23 plays a fundamental role in stabilizing phenotypic features of T_H17 lineage.

Important activation surface markers such as CD40, CD44, and CD200 were also induced during the infection. CD40 and CD86 are receptor ligands for T cell CD40L and CD28/CTLA4 respectively. CD44 is homing receptor that participates in the migration of inflammatory cells (Alstergren *et al.*, 2004; Aziz, 2003; Hollingsworth *et al.*, 2007). Interestingly, the activation of CD44 in gastric mucosa is linked to progression to more advance gastric lesions (Garay *et al.*, 2016). These transcriptional regulations were verified by translating to protein levels (Figure 4.11B). Comparing with non-infected control; *H. pylori*-infected RAW264.7 cells displayed a higher amount of these molecules. After infection, $CD44^{low}$ population decreased from 29% to

9%, $CD44^{medium}$ population increased from 56% to 63% while $CD44^{high}$ increased from 8% to 28%. CD274 is also known as programmed cell death-1 ligand (PD1L). PD1L and its ligands belong to B7:Cd28 family and are responsible in regulating T cell activation and peripheral tolerance (Keir *et al.*, 2008). PD1L act as a negative regulator, delivering inhibitor signals to T cells, affecting T cell proliferation and cytokine production, especially secretion of IL-10 (Said *et al.*, 2010; Sharpe *et al.*, 2007). The protein expression of CD274 was verified by flow cytometry analysis, whereby 51% population of infected RAW264.7 cells expressed high intensity of CD274 compared to only 3.7% of the uninfected cells.

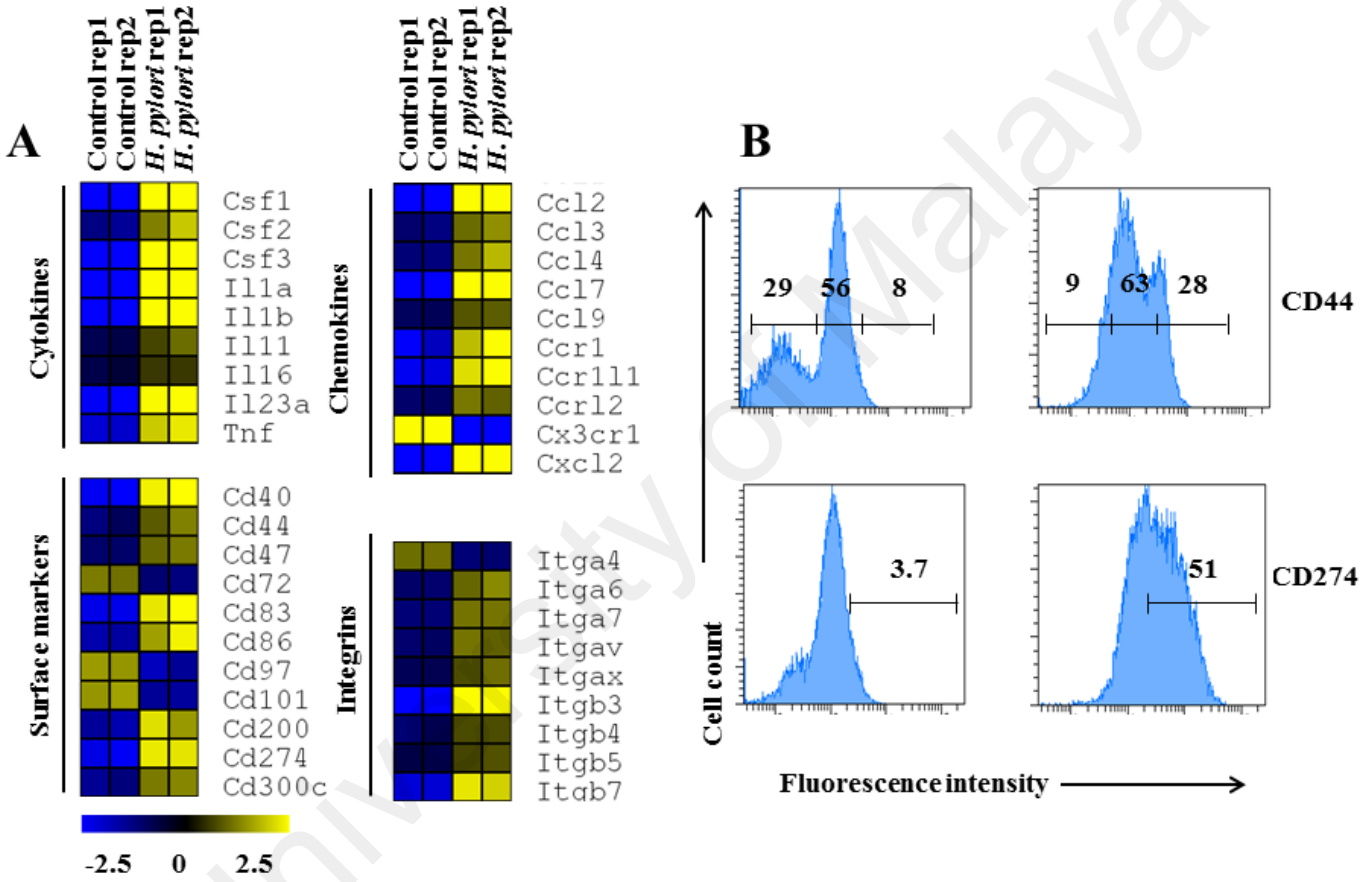


Figure 4.11: Heatmap and flow cytometrical analysis for immune response-related genes. (A) Heatmap of significant genes encoded for cytokines, surface markers, chemokines and intergrins. Color intensity reflects the normalized $\log_2$ values of RNA abundance. Yellow: increase, blue: decrease, dark: no change. (B) Flow cytometrical analysis of CD44 and CD274 on the non-infected control and *H. pylori* (MOI 10, 24 hpi)-infected cells. Number represents the percentages of cell in the gated area.

4.7.2 *H. pylori* infection suppresses transcription of genes with various pathways

In contrast to the enhancement of expression of immune response-related genes, various pathways were also suppressed. A few pathways was selected to be represented in heat maps (Figure 4.12). For DNA repair pathway (Figure 4.12A), only *Gadd45a* and *Gadd45b* were upregulated, whereas others were suppressed. *Gadd45a* and *Gadd45b* are nuclear proteins that are induced during terminal differentiation and hematopoietic cytokines (Vairapandi *et al.*, 1996; Zhan *et al.*, 1994; Zhang *et al.*, 1999). It has been implicated that it plays a central role interacting with partner protein *PCNA* in nucleotide excision repair (Zhan *et al.*, 1999), which in our study was downregulated in the microarray analysis result. Moreover, it suggests that *Gadd45a* and *Gadd45b* act as pro-survival genes, protecting hematopoietic cells against DNA damage agents and mediating G2/M cell cycle checkpoint in murine cells (Liebermann & Hoffman, 2007; Zhan *et al.*, 1999). Contrasting to that, excision repair complementing (ERCC) and X-ray repair cross complementing (XRCC) were both greatly diminished by *H. pylori* infection. XRCC is key factors involved with DNA double strand break repair (Thacker & Zdzienicka, 2003) while ERCC is essential for nucleotide excision repair mechanism (Bohr *et al.*, 1988).

Steroid biosynthesis and terpenoid backbone synthesis are the main cellular anabolic pathways. Both pathways were diminished during *H. pylori* infection (Figure 4.12B, C). Therefore, *H. pylori* modulate lipid metabolism and biosynthesis of hormones to leading to metabolic shifts (Eisenreich *et al.*, 2013). This may be one of the *H. pylori's* immune evasion strategies masking pathogen-induced response. Another highlight of this pathway was *Dhcr24*, one of the top 10 downregulated genes in microarray analysis.

Furthermore, majority of genes involved in DNA replication such as members of *Mcm* (minichromosome maintenance), *Pol* (DNA polymerase) and *Rfc* (replication factor C) families were substantially downregulated (Figure 4.12D).

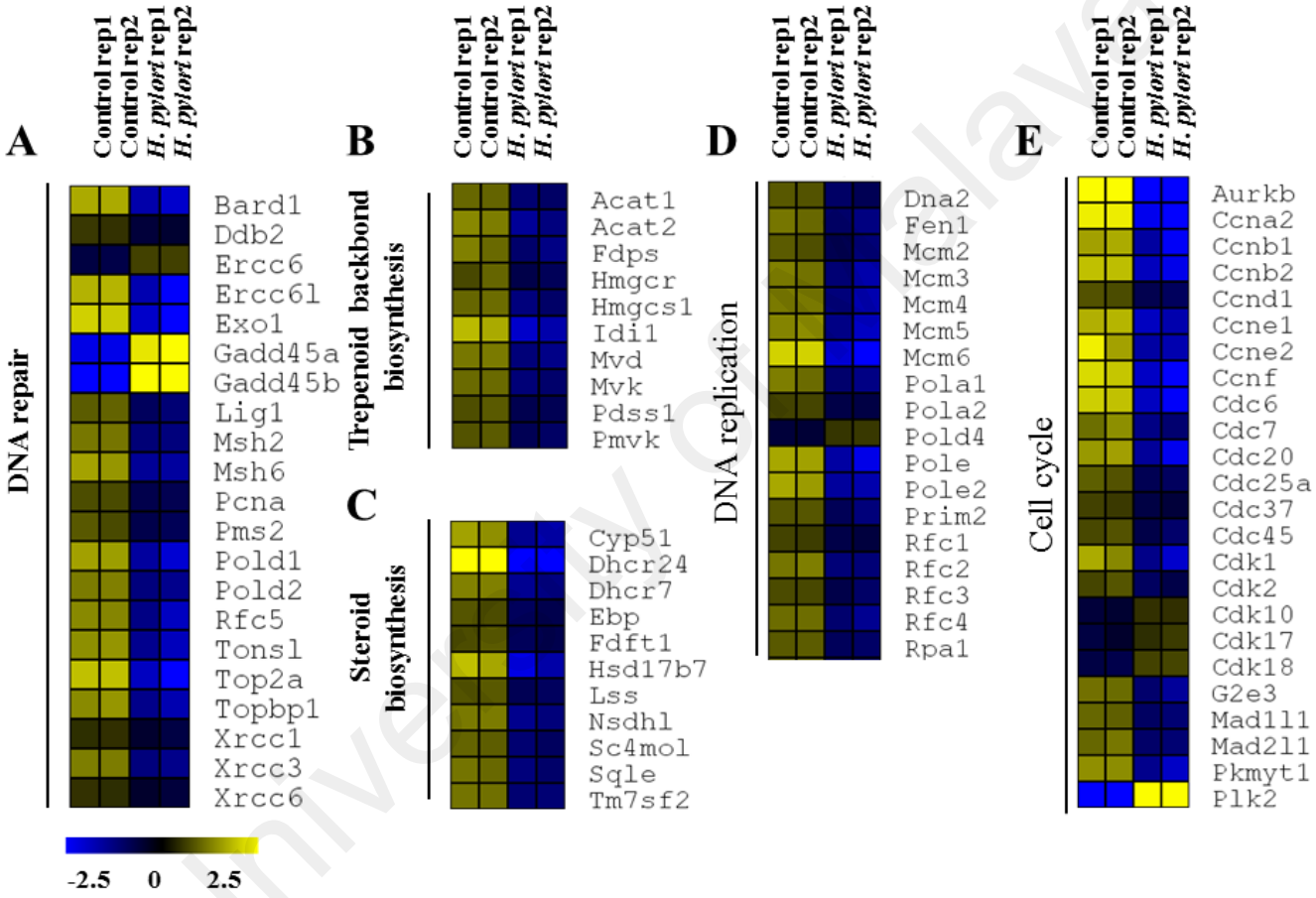


Figure 4.12: Heat maps of significant genes encoded for downregulated pathways. The pathways include: (A) DNA replication (B) terpenoid biosynthesis (C) steroid biosynthesis, (D) DNA replication and (E) cell cycle. Color intensity reflects the normalized $\log_2$ values of RNA abundance. Yellow: increase, blue: decrease, dark: no change.

4.7.3 *H. pylori* infection suppresses transcription of DNA replication as well as cell cycle-associated genes

Among all the pathways, cell cycle pathway was targeted for further investigation. The expression of selected DNA synthesis and cell cycle molecules were validated through qPCR. All genes were downregulated consequent to *H. pylori* infection, consistent with microarray results. *Aurkb* expression was reduced by 3.4 times (P=0.051) upon *H. pylori* infection. The mRNA levels of cyclins that are responsible for G1/S phase in cell cycle such as *Ccnb1, Ccnb2, Ccne1* and *Ccne2* were reduced by 201.7 (P=0.045), 2.15 (P=0.131), 16.8 (P=0.03) respectively. Moreover, transcription level of cyclin *Ccnf* which controls centrosome homeostasis and mitotic fidelity was also diminished by 3.42 (P=0.172) fold comparing to non-infected RAW264.7 cells. Cyclin dependent-kinases (Cdks) such as *Cdk1, Cdk2* are both downregulated for 112.68-fold (P=0.005) and 27.2-fold (P=0.077) respectively. Other than that, gene *Mad1l1* which encodes components for spindle-assembly as well as *Cenpa* which encodes centromere protein were also reduced by -1.82 (P=0.547) and -3.43 (P=0.172) respectively. *Espl1* which encodes separase was reduced by 1.72 (P=0.448) while *Zwilch* that is responsible for mitotic checkpoint was inhibited by 4.06-fold (P=0.194). All of the downregulated genes associated to cell cycle were listed in Table 4.6 and illustrated in Figure 4.13.

Table 4.6: Fold changes of cell cycle associated genes. Experiments were done in triplicate, in two different experiments. Shown were representative data of two independent experiments P values were calculated by Student's *t*-test.

Gene	Mean fold change	P value
Aurkb	-3.5	0.051
Ccnb1	-201.7	0.045
Ccnb2	-16.7	0.030
Ccne1	-2.15	0.131
Ccne2	-9.43	0.029
Ccnf	-2.62	0.130
Cdk1	-112.68	0.005
Cdk2	-27.2	0.077
Cenpa	-3.43	0.172
Espl1	-1.72	0.448
Mad1l1	-1.82	0.547
Zwilch	-4.06	0.194

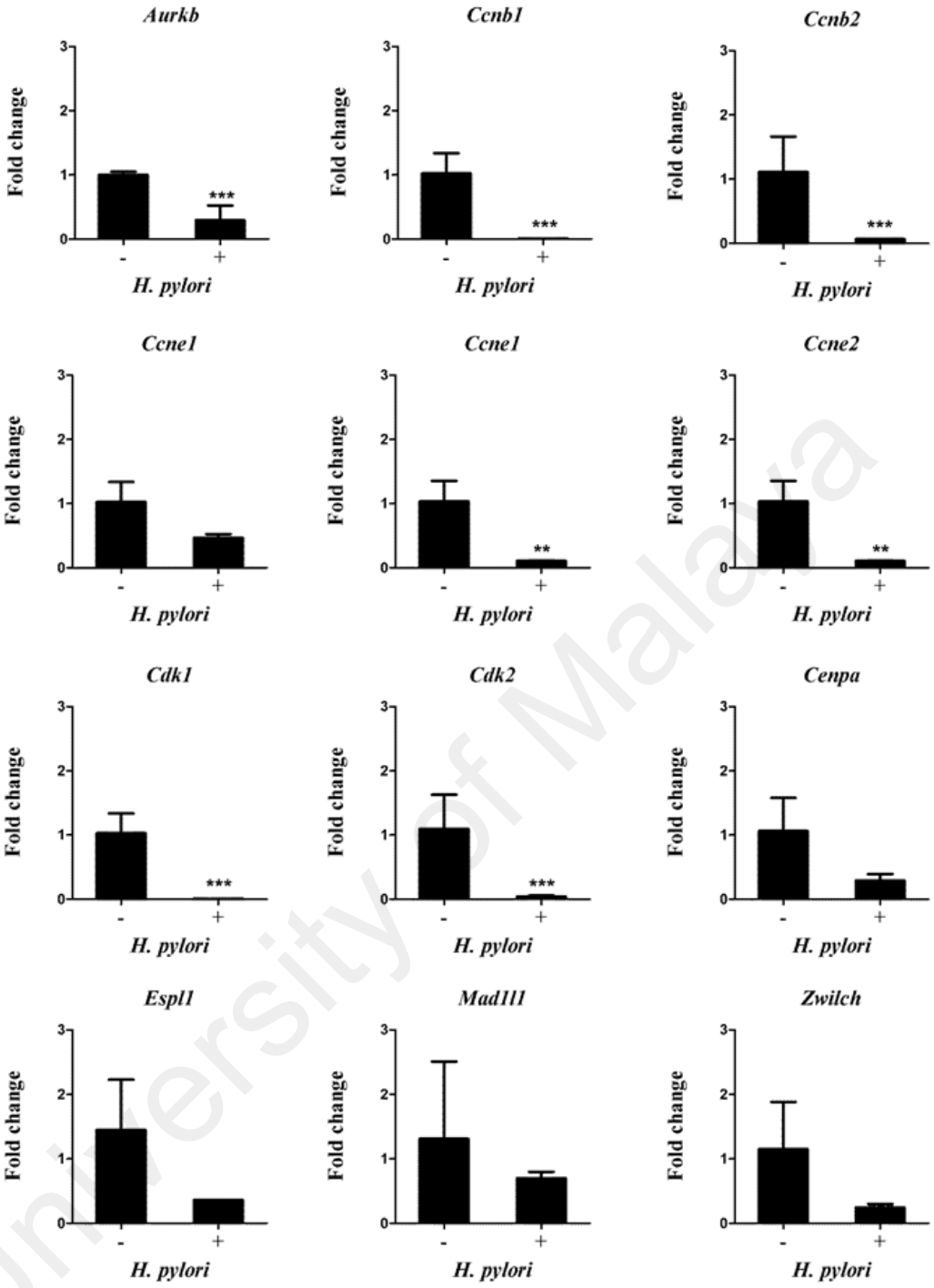


Figure 4.13: qRT-PCR analysis of cell cycle associated genes. Relative fold change shows expression of each gene relative to relative control, β actin. "-" represents non-infected control; "+" MOI 10, 24 hpi-infected cells. Data were shown as mean ± SD, from one experiment run in triplicate. Statistical significance was analyzed with unpaired Student's t-test (*P<0.05, **P<0.01, ***P<0.001)

Given that *H. pylori* infection impairs DNA replication and cell cycle-associated genes, cell cycle analysis was further examined using propidium iodide staining (Figure 4.14). As predicted, the number of cells at G0/G1 phase increased with the increase of infectivity rate (MOI). At non-infected state, only 70.4 ± 2.0 % cells were at G0/G1 phase. When the MOI increases, the cell population gradually increases to 73.2 ± 0.1 at MOI 1, 81.6 ± 0.8 at MOI 5 and 84.9 ±0.9 % at MOI 10 respectively. Similarly, percentage of cells at the S phase were reduced by approximately 3-fold upon *H. pylori* infection (6.5 ±0.1%) during MOI 10, versus 15.6 ±0.9 % in control cells. Also, this was accompanied by a reduction of G2/M population from 13.9 ±0.9 % in non-infected cells to 8.7 ±1.1% in MOI 10 infected cells. This indicates *H. pylori* infection blocks macrophage cycle progress at G1/S and G2/M phase.

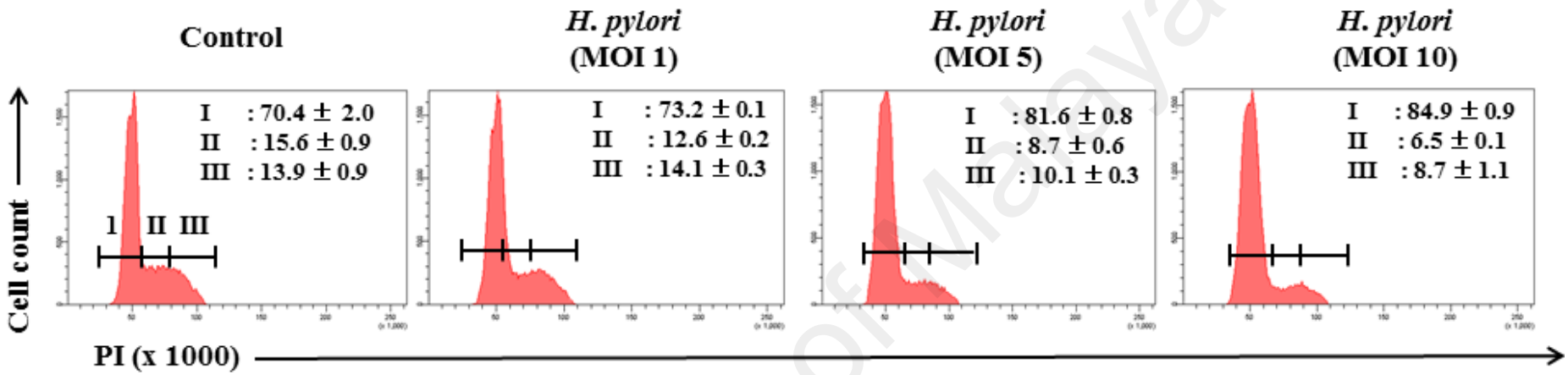


Figure 4.14: Cell cycle analysis of non-infected control RAW264.7 and *H. pylori*-infected RAW264.7 cells at indicated MOIs. Numbers represent the percentage of cells at gated phases: I represent G0/G1, II represent S and III represents G2/M respectively. Data were shown as mean ± SD from one experiment run in duplicate and were representative data of two independent experiments.

Through immunoblot analysis (Figure 4.15), protein of Aurkb was found to be diminished by *H. pylori* infection after 24 hpi and 48 hpi. Concurrently, the phosphorylation of Cdk1, a downstream molecule of Aurkb was also abolished following the infection. In addition, mitotic protein centromere protein A (Cenp-a) was reduced in *H. pylori*-infected RAW264.7 cells. Genes encoded for other mitosis-related proteins such as *Espl1* and *Zwilch* were also diminished at transcriptional level (Figure 4.13). In addition, cyclin D1, which is an important protein required for progression of G1 phase was reduced at protein level. These data suggest that *H. pylori* block macrophage progression through G1/S and G2/M transition by inhibiting formation of Aurkb and cyclin/Cdk complexes.

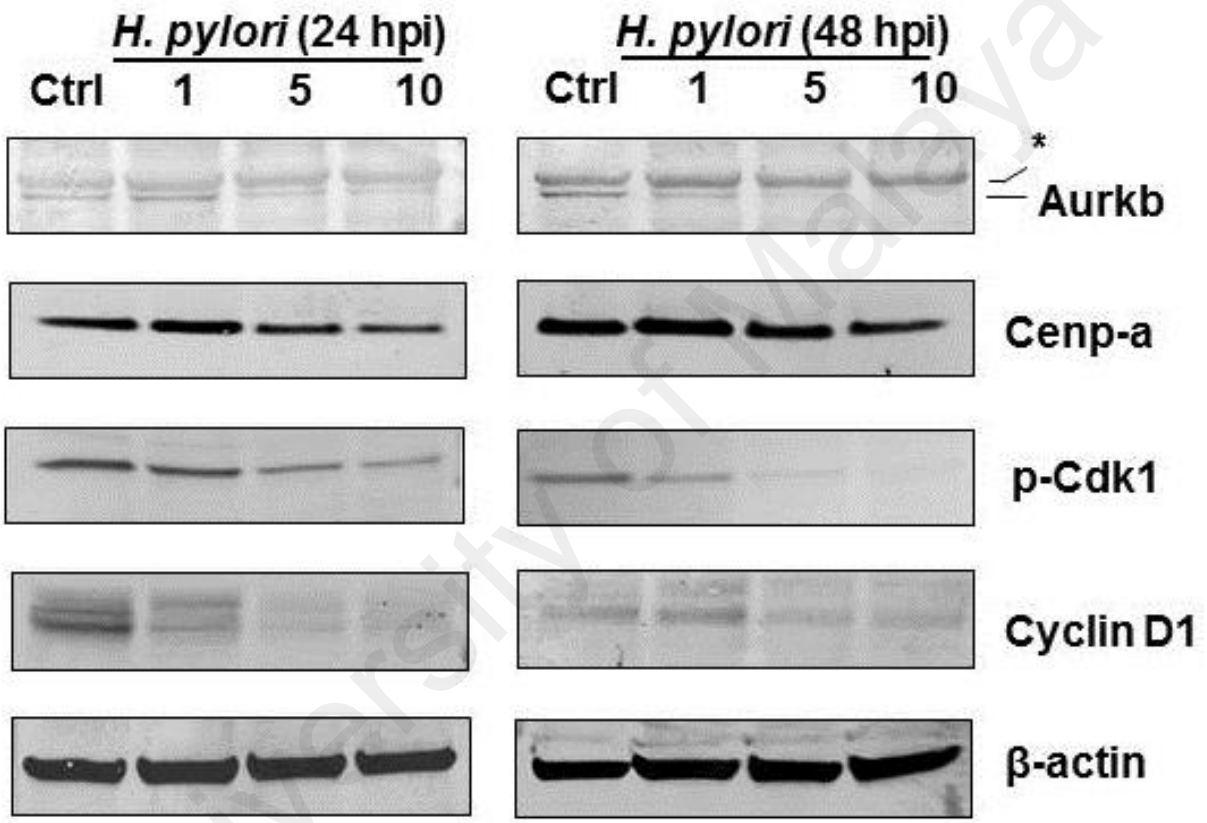


Figure 4.15: Immunoblot analysis of cell lysates prepared from non-infected control and *H. pylori*-infected RAW264.7 cells for 24 hpi and 48 hpi. Antibodies used against Aurkb, Cenp-a, phosphor-Cdk1, or Cyclin D1. β-actin was used as a loading control. Data represents of two independent experiments. Asterisks* represents non-specific bands.

4.8 Infection with differential genotypes VacA (s1m1 and s2m2) of *H. pylori* results in similar proliferation and cell cycle defect in RAW264.7 cells

The variations in the regions of *H. pylori VacA* gene are known to cause differences in vacuolating activities. Generally it is divided into two genotypes, s1m1 and s2m2. These two genotypes are associated with the production of cytotoxin, in which s2m2 is the least cytotoxic among the others (Atherton *et al.*, 1995). To address this issue, the proliferation and cell cycle analysis was compared between three strains, *H. pylori* SS1, J99 and 298. SS1 has the genotype of s2m2 while J99 and 298 both have the genotype of s1m1. All strains (SS1, J99 and 298) demonstrated comparable cell proliferation (Figure 4.16A). As seen in the figure, $Ki67^{low}$ population in non-infected control increased from 35% to 89% in SS1, 298 and 87% in J99; while $Ki67^{high}$ population decreased from 65% to 11% in SS1, 298 and 13% in J99.

All RAW264.7 cells infected with *H. pylori* strains showed inhibition in G1-to-S and G2-to-M phases (Figure 4.16B).In non-infected cells, it comprised 63.8 % of cells at G0/G1, 18.1 % of cells at S and 17.2 % of cells at G2/M phases. When RAW264.7 cells were infected with *H. pylori* strains, they showed an increase in the average (84.4 %) of cells at G0/G1 phase; a decrease of S and G2/M cells to an average of 5 % and 9.96 % respectively (Figure 4.16B)

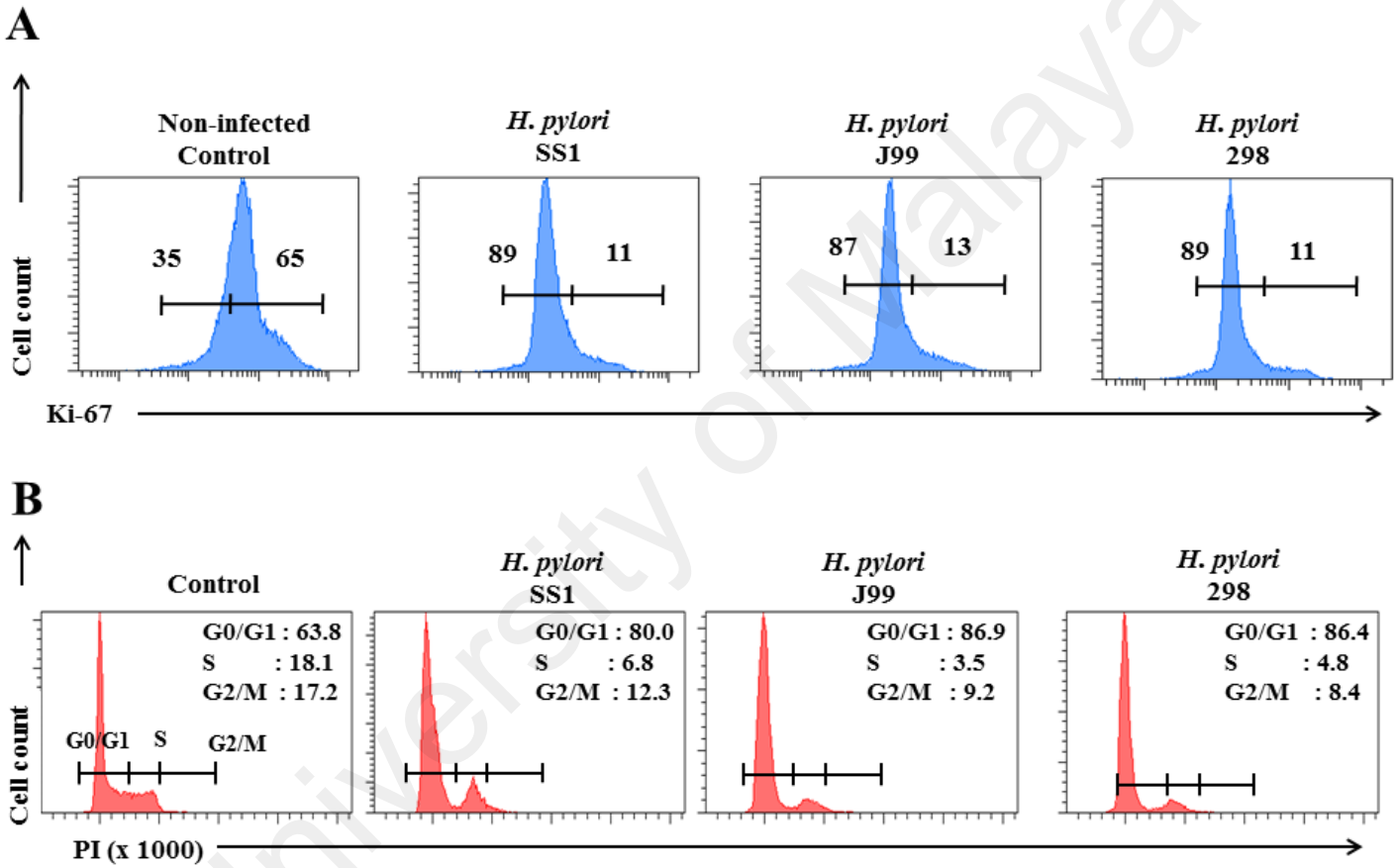


Figure 4.16: Infection with differential genotypes VacA (s1m1 and s2m2) of *H. pylori* results in similar proliferation and cell cycle defect in RAW264.7 cells. (A) Ki67 cell proliferation analysis of non-infected control versus *H. pylori*-infected RAW264.7 cells. RAW264.7 cells were infected with different strains of *H. pylori*, including SS1, J99 and 298 at MOI 100 for 24 hpi. Numbers represent the percentages of cells in the gated area. Shown were representative data of two independent experiments. (B) Cell cycle analysis of non-infected control versus $CagA^+$ and $CagA^-$ *H. pylori* infected cells. RAW264.7 cells were infected with different strains of *H. pylori* including SS1, J99 and 298 at MOI 100 for 24 h. Numbers represent the percentages of cells in the gated area. Shown were representative data of two independent experiments.

4.9 *H. pylori* infection attenuates proliferation of primary macrophage cells.

As all assays above were conducted using RAW264.7 cell line, *H. pylori* infection was further examined in primary macrophage cells. Due to the limited concentration of BMDM cells extracted from both mice, only two assays were performed. Using primary cells, the infectivity and anti-proliferative effect of *H. pylori* in the macrophages were confirmed and validated. After 24 hpi, microscopy images of BMDM indicated enlargement in size comparatively to the non-infected BMDM, (Figure 4.17). FSC and SSC of flow cytometrical analysis also supported that the population had increased in size and complexity (Figure 4.18). Similar to the results in the RAW 264.7 monocytic macrophage cell line, *H. pylori*-infected BMDM showed decreased in proliferative cells. $Ki67^{low}$ population increased from 32.7 % to 74.2 % whereas $Ki67^{high}$ population decreased from 67.2 % to 25.8 %.

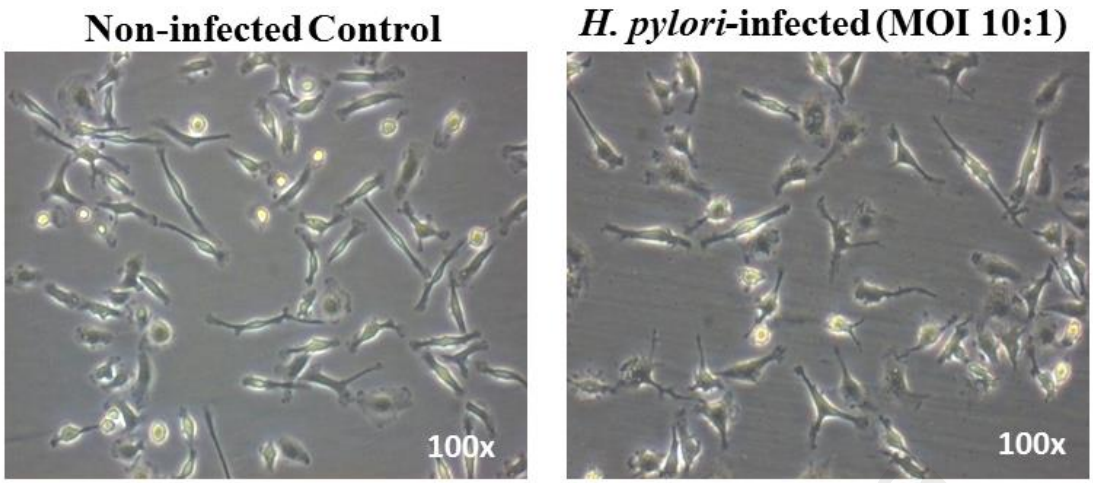


Figure 4.17: Microscopic images of non-infected BMDM and *H. pylori* (MOI 10)-infected BMDM.

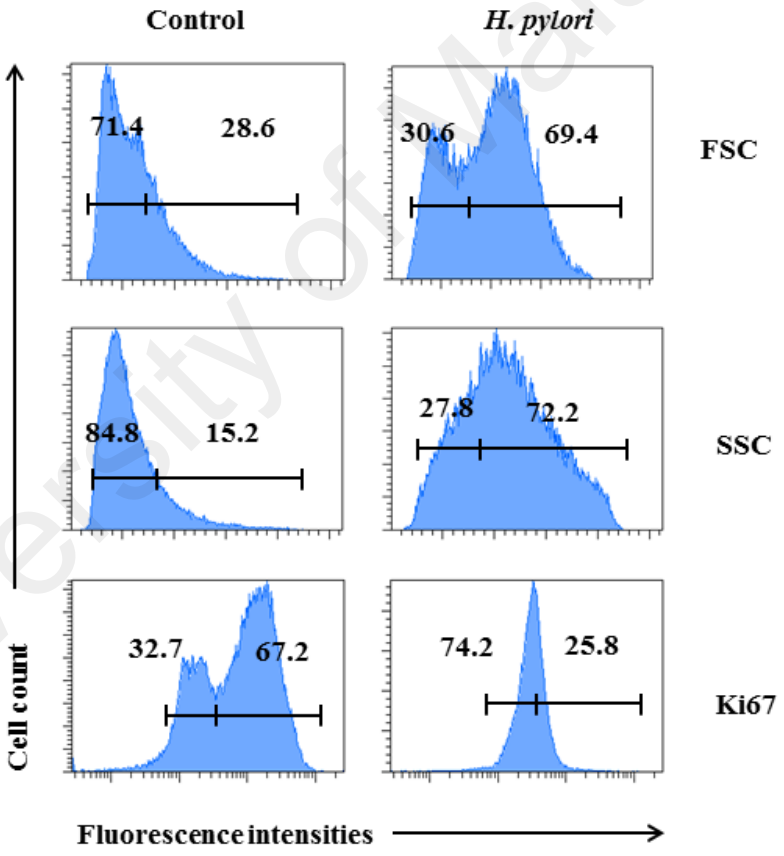


Figure 4.18: Flow cytometrical analysis of non-infected BMDM and *H. pylori*-infected BMDM at MOI 10. Forward scatter (FSC), side scatters (SSC) and intranuclear expression of Ki-67 cell proliferation marker. Numbers represent the percentages of cells in the gated area. Shown were representative data of two independent experiments.

CHAPTER 5: DISCUSSION

H. pylori is a Gram-negative bacterium that colonizes half of the gastric mucosa of human population. The chronic infection generates a state of inflammation, which occurs mainly due to the infiltration of monocytes and activation of macrophages. Macrophages are the key player of *H. pylori* pathogenesis. There are very few macrophages populated in normal gastric mucosa of healthy patients (Krauss-Etschmann *et al.*, 2005); conversely, the increase in number of neutrophils and macrophages signifies the severity and duration of *H. pylori* infection in the host (Suzuki, T. *et al.*, 2002). Although a large body of clinical and experimental observations has been provided since the discovery of *H. pylori*, a further knowledge of the mechanisms of host-pathogen interaction and of those that lead to achieve protective immunity is still needed. Understanding the interaction between *H. pylori* and the immune cells is important in delineating the pathogenesis of infection.

In this study, RAW264.7 monocytic macrophage cells were proven to be a good *in vitro* model for *H. pylori* infection studies as this study showed similar activation and induction that was done in human monocytes (Fehlings *et al.*, 2012). *H. pylori* promoted monocytes to macrophage differentiation of RAW264.7 cells. *H. pylori* infection RAW 264.7 cells displayed the increase of surface expression (F4/80, CD11b and CD40) and colony stimulating factors (*Csf1, Csf2, and Csf3*). Along with this, pro-inflammatory cytokines (IL-1α, IL-1β, IL-6 and TNF-α) and chemokines such as Ccl-2 (monocyte chemoattractant protein-1), Cxcl2 (macrophage inflammatory protein-2) (MIP), Ccl3/ (MIP-1α) and Ccl4 (MIP-1β) were also highly induced. TNF-α is a common cytokine produced by activated monocytes and macrophages during bacterial infection (Johnston & Conly, 2006). It is a key component of inflammation that induces vasodilation and triggers the expression of neutrophil-attracting chemokines such as Cxcl1, Cxcl2 and Cxcl5 which augment the expression of cell adhesion molecules (Griffin *et al.*, 2012;

Vieira, S. M. *et al.*, 2009). Similarly, IL-1β is known to activate autophagy in macrophages and transport cytoplasmic material of the cell to acidic compartment to be degraded by hydrolytic enzyme (Ravikumar *et al.*, 2009). The inflammation response is further enhanced with the activation of MHC class II co-stimulatory molecules CD80 and CD86 in RAW264.7 cells; indicating the role of macrophages as antigen presenting cells (Barker *et al.*, 2002). These series of events confirm that *H. pylori* infection activates T_H1 of the host by: activating phagocytosis process, releasing cytokines to act as chemoattractant for granulocytes (Ben-Sasson *et al.*, 2009). This is similar with the research (Fehlings *et al.*, 2012), where surface expression of *H. pylori*-infected macrophages display lower levels of CD11b and HLA-DR. Many studies have shown *H. pylori* induce pro-inflammatory cytokines such as IL-1, TNF-α and IL-6 in macrophages (Fehlings *et al.*, 2012; Gobert, A. P. *et al.*, 2004; Harris *et al.*, 1998; Zheng & Jones, 2003). CD80 and CD86, the two important co-stimulatory ligands for T cell response have also been also reported to be upregulated along with the induction of pro-inflammatory cytokines in *H. pylori*-infected macrophages (Yao *et al.*, 2015; Zhuang *et al.*, 2011).

Besides activating T_H1 subset, this research suggests *H. pylori* infected macrophages induce the differentiation of T_H17 through the secretion of cytokines IL-6 and IL-23α. T_H17 represents a novel subset of $CD4^+$T cells that is associated with chronic inflammation and protection against extracellular bacteria (Shi, Yun *et al.*, 2010). Naïve T cells differentiate into T_H17 when APC-derived cytokines such as IL-6, IL-1β and IL-23 are released (Harrington *et al.*, 2005). T_H17 produces IL-17A and IL-17F, which further triggers IL-6, M-CSF, GM-CSF and chemokines such as Cxcl1, Cxcl2, Cxcl5 and Cxcl8 to enhance the inflammation response (Kolls & Linden, 2004). In Zhuang *et al.*, (2011), co-cultured $CD4^+$ T cells with *H. pylori*-infected macrophages elevated IL-17 with the increased of IL-6, TGF-β, IL-23 and Ccl-20 secretion (Zhuang

et al., 2011). Besides, *H. pylori* infected macrophages secrete B cell activating factor (BAFF). BAFF is responsible for promoting T_H17 expansion by increasing differentiation of naïve T cell to create a pro-T_H17 milieau (Munari *et al.*, 2014). Although many previous studies have shown that T_H17 activation plays an important role in mediating mucosal host defense against *H. pylori*, other data suggest differently. Some hypothesize that T_H17 response exerts a regulatory effect as IL-17A exerts anti-inflammatory effect on *H. pylori*-induced gastritis through suppression of T_H1 differentiation (Otani *et al.*, 2009). However, this remains controversial as others show T_H17 increased the burden and inflammation of *H. pylori* infection (Shi, Y. *et al.*, 2010).

In this study, *H. pylori* disrupted gene transcriptional program in RAW264.7 monocytic macrophage cells as shown in microarray analysis. It also shifted RAW264.7 cells into $Ki67^{low}$ non-proliferative stage, arrested cells at G0/G1 phases, and impeded entry of cells into S or G2/M phases. Up to date, there is no reported study that suggests *H. pylori* cause reduction in macrophage proliferation without apoptosis. However, there are research reporting *H. pylori* VacA specifically inhibited gastric cell proliferation without affecting cell migration (Ricci *et al.*, 1996), while arginase decreases the proliferation of human T lymphocytes, paralleled by a reduced expression of CD3ζ-chain of T cell receptor, the principal signal transduction protein (Zabaleta *et al.*, 2004).

Microarray analysis results also revealed *H. pylori* infection suppressed DNA replication and repair pathway of RAW 264.7 cells. *H. pylori* infection is believed to be the driving force that leads to DNA damage in the gastric mucosa. At the early phase of infection, *H. pylori* causes neutrophil and monocyte infiltration in the gastric mucosa. This leads to the secretion of reactive oxygen species (ROS) by these inflammatory phagocytes that results DNA damage of adjacent cells (Weitzman & Gordon, 1990). DNA mutation could lead to cell death, atrophic gastritis and eventually transforms the

chronic gastritis into gastric carcinoma. This is proven when increased oxidative DNA damage was found in *H. pylori*-positive patients (Baik *et al.*, 1996). This might be the missing link between *H. pylori* infection and gastric carcinogenesis. On the contrary, there is also research which suggests that *H. pylori* activates DNA synthesis and repair in gastric epithelial cells through the secretion of ROS. The oxidative stress accumulated is predicted to be involved in tumor initiation and stimulate cell proliferation (Obst *et al.*, 2000).

To date, there is still no evidence which shows that *H. pylori* infection causes cell cycle arrest in immune cells, but it has been reported to retain gastric epithelial cell lines at G1/S and G2/M phases. In the reported study, 75% of both *H. pylori* $CagA^+$ and $CagA^-$ infected AGS cell line were attenuated at G1/S phase after 24 h; while 7.5 % to 17% of the cells showed a second checkpoint arrest at G2/M (Ahmed *et al.*, 2000). In further investigation, induction of p53, the transcription factor for cell cycle inhibition at G0-G1 checkpoint is found to be responsible for the inhibition. Furthermore, p21 which plays a major role in G1 phase arrest was significantly upregulated compared to non-infected cells. Expression of cyclin E/Cdk2 was also diminished during *H. pylori* infection. Expression of cyclin E is a critical factor which promotes cell entry into S phase and cell proliferation, as only those cells expressing cycle E over the threshold enter into S phase of the cell cycle (Qin *et al.*, 2004). Similar evidence was found in another study, where *H. pylori* also attenuates cell cycle progress of human gastric epithelial cells (MKN28, MKN7, MKN74 and AGS) through elevation of $p27^{KIP1}$, reduction in Retinoblastoma (Rb) phosphorylation and a significant decrease in cyclin E/Cdk2 (Sommi *et al.*, 2002).

The eukaryotic cell cycle is a fundamental process that regulates cell division. It is divided into four discrete phases, G1, S, G2 and M phases. DNA material is replicated during the S phase while mitosis happens in the M phase. These important

phases are separated by the gap phase (G1 and G2) that govern the readiness of cells before entering S or M phase. Strict control of S phase ensures that cells undergo one single round of chromosomal DNA replication while M phase ensures duplicated genetic material is correctly segregated into two identical cells (Norbury & Nurse, 1992).

Therefore progression of cell cycle is tightly regulated by cyclin-Cdks dimeric kinase complexes (Figure 5.1). These kinases mediate phosphorylation of specific downstream molecules, and are tightly regulated at several levels. For mammalian cells, G1 phase is regulated by Cdk4/Cdk6-D type cyclins (Matsushime *et al.*, 1992; Meyerson & Harlow, 1994) and Cdk2-cyclin E complexes (Endicott *et al.*, 1999). These two complexes are responsible for the phosphorylation of Retinoblastoma (Rb), to alleviate its inhibition on E2F, activating genes (such as: *cyclin A*, *cyclin E*, *oRC* and *Cdk1*) that are necessary for promoting S phase entry (Lim, S. & Kaldis, 2013). During the G1-to-S phase transition, Aurkb plays a role in controlling cell division by phosphorylation of Rb, Cdk1 and Cdk2. Failure of Aurkb will lead to the suppression of cyclin A; impaired the activation of Cdk1 and Cdk2 which results in cell cycle arrest (Song *et al.*, 2007). This study showed decreased Aurkb kinase in *H. pylori*-infected macrophages. This was accompanied by the suppression of cell cycle associated molecules such as cyclins (Ccna2, Ccnb1, Ccnb2, Ccnd1, Ccne1, Ccne2) coupled with the Cdks (Cdk1, Cdk2) (Figure 5.1). It is likely that *H. pylori* attenuates Aurkb kinase and its downstream molecules, of the host, leading to G1-to-S cell cycle arrest of the infected macrophages.

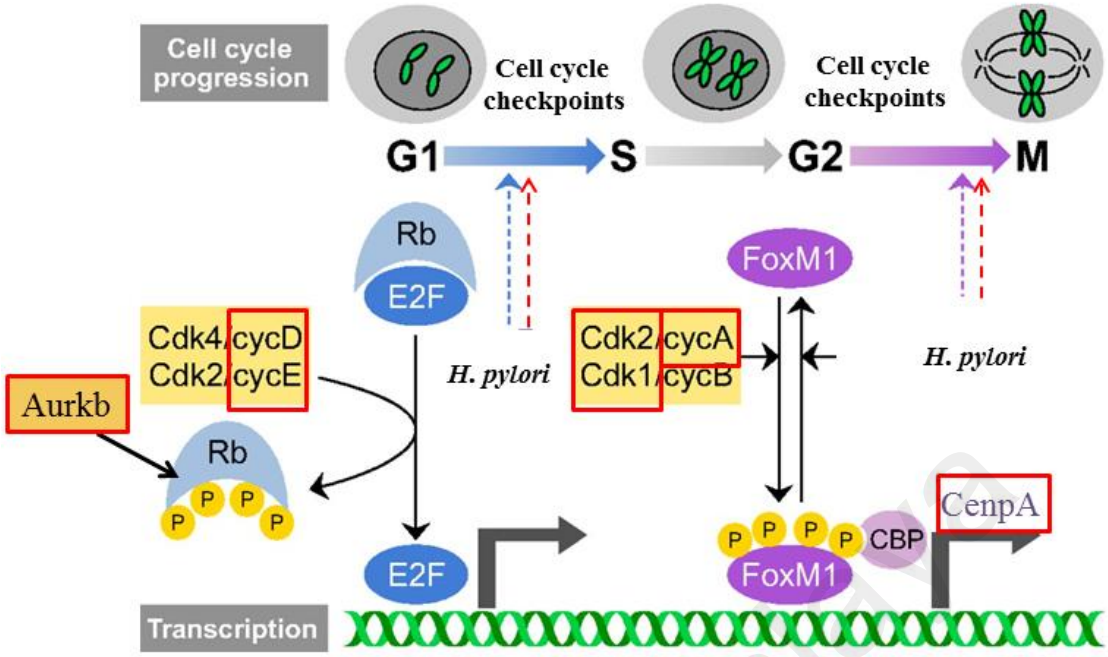


Figure 5.1: Cdk/ cyclin complexes regulate cell cycle phases. The red boxes and arrows indicate the targets of suppression by *H. pylori* in RAW264.7 cells. Figure adapted from (Lim, S. & Kaldis, 2013)

During S phase, all cells must carry out error-free DNA replication and efficiently repair any DNA damage or misincorporated nucleotides. This requires tight regulation by the replication machinery, which includes numerous enzymes and regulatory factors (Mailand *et al.*, 2013) (Figure 5.2). During initiation, minichromosome (Mcm) proteins bind to DNA at specific sites, unwind them and activate primase to form primosome (Barry & Bell, 2006). Meanwhile, proliferating cell nuclear antigen (PCNA) is opened, assembled onto the DNA template by multiple clamp loaders. These loaders are from the Replication factor C (Rfc) family; they work in ATP-dependent manner and ensure processive DNA synthesis (Kitabayashi *et al.*, 2003). PCNA then encircles DNA and topologically links Polymerase to DNA to start the elongation process (Takeda & Dutta, 2005). Our results showed the expression of genes from the Mcm families (*Mcm2, Mcm3, Mcm4, Mcm5* and *Mcm6*), *Pol* families (*Pola1, Pola2, Pold4, Pole*), *Rfc* families (*Rfc1, Rfc2, Rfc3, Rfc4*), Primase *(Prime4)* as

well as *Pcna* were inhibited during *H. pylori* infection. This suggests that *H. pylori* suppresses the replication machinery.

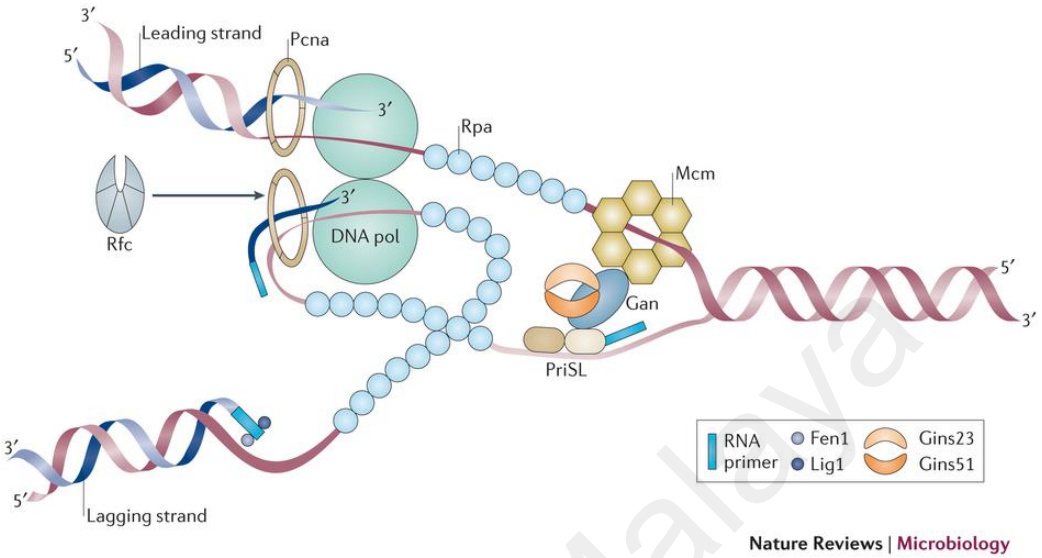


Figure 5.2: Replication machinery during synthesis process. *Pcna* is loaded onto the DNA by Rfc complex in an ATP-dependent manner. *Pcna* encircles the DNA and firmly attaches DNA polymerase to the template. Figure taken from (Lindas & Bernander, 2013)

H. pylori-mediated cell cycle inhibition is not limited to G1 to S phase, but also occurs simultaneously in G2/M phase. This is supported by our cell cycle assay and microarray data which showed significantly suppression of multiple genes associated with G2/M progress. Analogous to G1/S checkpoint, G2/M checkpoint ensures no defective DNA before cyclin-Cdk complex is activated. As shown diagram in Figure 5.1, Cdk-1 forms complex with cyclin B for the activation of G2/M phase. Upstream molecules such as Aurkb, Poll-like kinase 1 (Plk1) and Cdc25 control the activation of cyclin B-Cdk complex. In early mitosis, Plk1 is phosphorylated by Aurkb at centromeres and kinetochores (Carmena *et al.*, 2012), and Plk1 is responsible in phosphorylating Cdc25 (Lobjois *et al.*, 2009; Toyoshima-Morimoto *et al.*, 2002), the phosphatase which dephosphorylates Cdk-1 for activation. In addition, Plk1 also controls the localization of cyclin B1 by phosphorylating its nuclear export sequence (Toyoshima-Morimoto *et al.*, 2002). Our result showed that genes *Ccnb1*, *Ccnb2*, *Cdk1*,

Cdc25a and *Plk1* were all diminished at transcription level during *H. pylori* infection. Furthermore, DNA damage was unable to be repaired as *Ercc6, Ercc61, Xrcc3* and *Xrcc6* that are important for DNA repair were also downregulated.

Mitotic spindle is the last checkpoint before chromosome segregation. This checkpoint delays anaphase until all kinetochore are captured by microtubule from both spindle poles; chromosomes congress to the metaphase plate and all attached microtubules are properly sensed. All events occur at kinetochore; a structure that forms the interface between chromosomes and microtubules of the mitotic spindle (Figure 5.3). It acts as the control central of mitosis, where it is responsible in attaching chromosomes to the spindle microtubules, monitoring and activating signaling to delay cell cycle progress. In the kinetochore lies centromere, a specialized locus on chromosomes that mediates attachment of microtubules during mitosis (Maiato *et al.*, 2004). Centromere protein A (Cenp-a) is an essential histone H3-like kinetochore protein that is in direct contact with DNA (Warburton *et al.*, 1997). The function of Cenp-a phosphorylation is still unknown, but phosphorylation of other centromere proteins has been shown to control microtubule binding to chromosomes (Allshire, 1997; Liao *et al.*, 1994; Waters *et al.*, 1999). Recently, Aurkb has been found to regulate histone binding by phosphorylating Cenp-a during G2 phase (Slattery *et al.*, 2008). Failure of Cenp-a results in low viability, and mitotic arrest (Stoler *et al.*, 1995). These researches can be used to explain our results whereby *H. pylori* infection caused the disruption of Aurkb protein expression and Cenp-a. I suggest that *H. pylori* targets Aurkb, affecting the activation of downstream protein such as Cenp-a, leading to cell cycle arrest at G2/M phase. In addition, genes encoding for Zwilch kinetochore proteins and for Separase (encoded by *Espl1*) indispensable for anaphase spindle elongation were significantly suppressed by *H. pylori (*Munari *et al., 2014).* Furthermore, Mad family proteins (*Mad1l1, Mad2l1)* are also downregulated in the microarray results.

Mad family proteins are spindle checkpoint proteins that monitor the interaction between microtubules and outer kinetochore during mitosis (Maiato *et al.*, 2004). Thus, the defective of Mad proteins arrests the cell cycle in response of spindle depolymerization (Wells & Murray, 1996).

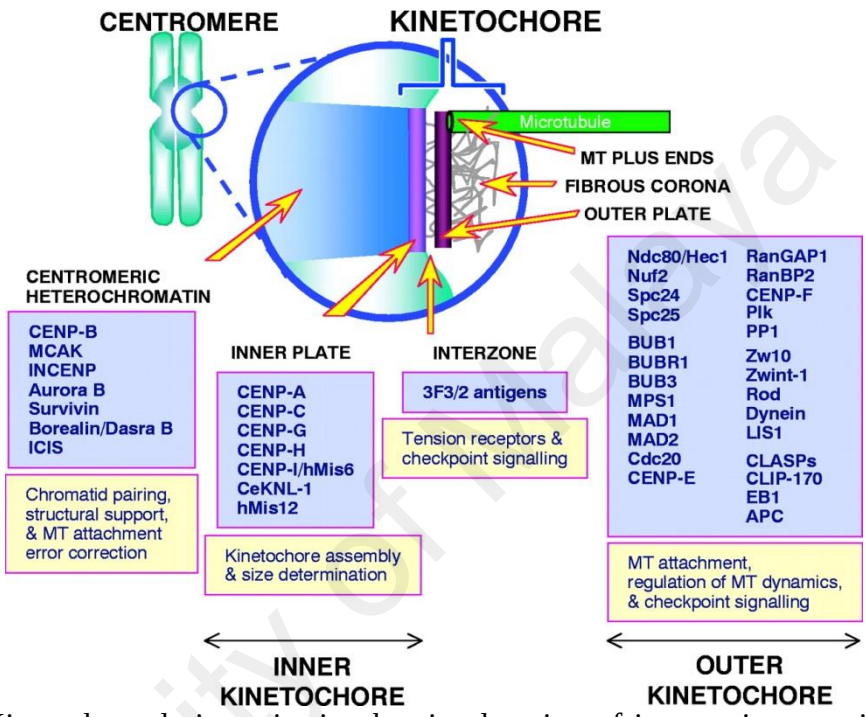


Figure 5.3: Kinetochore during mitosis, showing location of its proteins constituents. Figure taken from (Rieder & Salmon, 1998)

Previous studies showed that *H. pylori* infection in macrophages was caused by Arg2 (Lewis *et al.*, 2010) or by mitochondrial pathway (Menaker *et al.*, 2004). This study suggests a different immune evasion strategy by which the *H. pylori* is able to block various cell proliferation-associated genes thus inhibits the macrophage cell growth. Although there are many reports which suggest that the bacterial virulence might be affected by two allelic variations s1m1 and s2m2 (genotypes classified according to VacA sequence), however this study revealed that *H. pylori* SS1 (s1m1) exerted a comparable degree of inhibition of macrophage proliferation when compared to strain J99 and 298 (s2m2) strains. Similarly, *H. pylori* J99 and 298 did not exhibit

higher apoptosis in macrophages comparatively to *H. pylori* SS1 (Menaker *et al.*, 2004). This suggests that VacA may not be the main factor for attenuating host macrophage.

In summary, *H. pylori* infection impaired mitotic proliferation of RAW264.7 monocytic macrophage cells. G1-to-S cell cycle transition was inhibited in the infected cells subsequent to deplete expression of Aurkb- and cyclins/cdks-encoding genes.

CHAPTER 6: CONCLUSION

This study demonstrates the activation of RAW 264.7 monocytic macrophages cell by *H. pylori* infection. Evidences of cell activation includes cell enlargement, increased surface expression of CD80, CD86, F4/80 and CD11b and secretion of pro-inflammatory cytokines such as (TNF, IL-1β and IL-23). Subsequently, microarray analysis showed that *H. pylori* infection caused dysregulation of gene transcription machinery in RAW 264.7 cells, whereby 1341 upregulated and 1591 downregulated genes were identified. Cell proliferation and cell cycle assay showed that *H. pylori* infection impaired the mitotic proliferation of RAW 264.7 cells and arrested cell at G1-to-S phase and G2-to-M phases. This is attributed to impairment of the transcription of genes encoded for Aurkb and cyclin-Cdks. *H. pylori*-mediated interference of macrophage proliferation is possibly one of the strategies employed by *H. pylori* to limit the quantity of macrophages at the infection site, and thus evade efficient clearance by the host immune system. To summarize, this study provides a better understanding of *H. pylori* invasion and manipulation of host macrophages. This study suggests that *H. pylori* inhibits macrophages proliferation through the reduction of host Aurkb yet it does not induce cellular apoptosis. This new knowledge provides potentially useful information for future anti-bacterial peptides or vaccines design, through revitalizing the attenuated macrophages thus minimizing the damages cause by the *H. pylori* infection. In future, the investigation of *H. pylori* infection should be done in *in vivo* mice model, as the combination of *in vivo* and *in vitro* work can provide further understanding the bacterial pathogenesis mechanisms as well as the host immune response towards intestinal macrophages.

REFERENCES

Achtman, M., & Suerbaum, S. (2000). Sequence variation in *Helicobacter pylori*. *Trends Microbiology, 8*(2), 57-58.

Ahmed, Amel, Smoot, Duane, Littleton, George, Tackey, Robert, Walters, Curla S., Kashanchi, Fatah, . . . Ashktorab, Hassan. (2000). *Helicobacter pylori* inhibits gastric cell cycle progression. *Microbes and Infection, 2*(10), 1159-1169.

Akashi, S., Saitoh, S., Wakabayashi, Y., Kikuchi, T., Takamura, N., Nagai, Y., . . . Miyake, K. (2003). Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. *The Journal of Experimental Medicine, 198*(7), 1035-1042. doi: 10.1084/jem.20031076

Alberts, B.;, Johnson, A.; , & Lewis, J. (2002). *Molecular Biology of the Cell.* (4th Edition ed.). New York: Garland Science.

Allen, L. A., Schlesinger, L. S., & Kang, B. (2000). Virulent strains of *Helicobacter pylori* demonstrate delayed phagocytosis and stimulate homotypic phagosome fusion in macrophages. *The Journal of Experimental Medicine, 191*(1), 115-128.

Allshire, R. C. (1997). Centromeres, checkpoints and chromatid cohesion. *Current Opinion Genetics & Development, 7*(2), 264-273.

Alm, R. A., Bina, J., Andrews, B. M., Doig, P., Hancock, R. E., & Trust, T. J. (2000). Comparative genomics of *Helicobacter pylori*: analysis of the outer membrane protein families. *Infection and Immunity, 68*(7), 4155-4168.

Alstergren, Per, Zhu, Baoqian, Glougauer, Michael, Mak, Tak W., Ellen, Richard P., & Sodek, Jaro. (2004). Polarization and directed migration of murine neutrophils is dependent on cell surface expression of CD44. *Cellular Immunology, 231*(1–2), 146-157.

Ashkenazi, A., & Dixit, V. M. (1998). Death receptors: signaling and modulation. *Science, 281*(5381), 1305-1308.

Atherton, J. C., Cao, P., Peek, R. M., Jr., Tummuru, M. K., Blaser, M. J., & Cover, T. L. (1995). Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific vacA types with cytotoxin production and peptic ulceration. *The Journal of Biological Chemistry, 270*(30), 17771-17777.

Atherton, J. C., Peek, R. M., Jr., Tham, K. T., Cover, T. L., & Blaser, M. J. (1997). Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of *Helicobacter pylori*. *Gastroenterology, 112*(1), 92-99.

Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., . . . Geissmann, F. (2007). Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science, 317*(5838), 666-670. doi: 10.1126/science.1142883

Auffray, C., Sieweke, M. H., & Geissmann, F. (2009). Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annual Review of Immunology, 27*, 669-692. doi: 10.1146/annurev.immunol.021908.132557

Austyn, Jonathan M., & Gordon, Siamon. (1981). F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *European Journal of Immunology, 11*(10), 805-815.

Aziz, K. A. (2003). CD44 mediates polymorphonuclear leukocyte motility on hyaluronan. *Saudi Medical Journal, 24*(8), 827-831.

Bach, E. A., Aguet, M., & Schreiber, R. D. (1997). The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annual Review of Immunology, 15*, 563-591. doi: 10.1146/annurev.immunol.15.1.563

Backert, S., Moese, S., Selbach, M., Brinkmann, V., & Meyer, T. F. (2001). Phosphorylation of tyrosine 972 of the *Helicobacter pylori* CagA protein is essential for induction of a scattering phenotype in gastric epithelial cells. *Molecular Microbiology, 42*(3), 631-644.

Bafica, A., Scanga, C. A., Serhan, C., Machado, F., White, S., Sher, A., & Aliberti, J. (2005). Host control of Mycobacterium tuberculosis is regulated by 5-lipoxygenase-dependent lipoxin production. *Journal of Clinical Investigation, 115*(6), 1601-1606. doi: 10.1172/jci23949

Baik, Seung-Chul, Youn, Hee-Shang, Chung, Myung-Hee, Lee, Woo-Kon, Cho, Myung-Je, Ko, Gyung-Hyuck, . . . Rhee, Kwang-Ho. (1996). Increased Oxidative DNA Damage in *Helicobacter pylori*-infected Human Gastric Mucosa. *Cancer Research, 56*(6), 1279-1282.

Barker, R. N.;, Erwig, L. P.;, Hill, K. S. K.;, Devine, A.;, Pearce, W. P.;, & Rees, A. J. (2002). Antigen presentation by macrophages is enhanced by the uptake of necrotic, but not apoptotic, cells. *Clinical and Experimental Immunology, 127*(2), 220-225. doi: 10.1046/j.1365-2249.2002.01774.x

Barnes, M. J., & Powrie, F. (2009). Regulatory T cells reinforce intestinal homeostasis. *Immunity, 31*(3), 401-411. doi: 10.1016/j.immuni.2009.08.011

Barry, E. R., & Bell, S. D. (2006). DNA replication in the archaea. *Microbiology Molecule Biology Reviews, 70*(4), 876-887. doi: 10.1128/mmbr.00029-06

Ben-Sasson, S. Z., Hu-Li, J., Quiel, J., Cauchetaux, S., Ratner, M., Shapira, I., . . . Paul, W. E. (2009). IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Procedding National Academy Science of United States of America, 106*(17), 7119-7124. doi: 10.1073/pnas.0902745106

Bereswill, S., Waidner, U., Odenbreit, S., Lichte, F., Fassbinder, F., Bode, G., & Kist, M. (1998). Structural, functional and mutational analysis of the pfr gene encoding a ferritin from *Helicobacter pylori*. *Microbiology, 144 (Pt 9)*, 2505-2516.

Biswas, S. K., Gangi, L., Paul, S., Schioppa, T., Saccani, A., Sironi, M., . . . Sica, A. (2006). A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation). *Blood, 107*(5), 2112-2122. doi: 10.1182/blood-2005-01-0428

Blattner, F. R., Plunkett, G., 3rd, Bloch, C. A., Perna, N. T., Burland, V., Riley, M., . . . Shao, Y. (1997). The complete genome sequence of Escherichia coli K-12. *Science, 277*(5331), 1453-1462.

Boatright, K. M., Renatus, M., Scott, F. L., Sperandio, S., Shin, H., Pedersen, I. M., . . . Salvesen, G. S. (2003). A unified model for apical caspase activation. *Molecular Cell, 11*(2), 529-541. doi: 10.1016/s1097-2765(03)00051-0

Bode, G., Mauch, F., Ditschuneit, H., & Malfertheiner, P. (1993). Identification of structures containing polyphosphate in *Helicobacter pylori*. *Journal of General Microbiology, 139*(12), 3029-3033.

Boehm, U., Klamp, T., Groot, M., & Howard, J. C. (1997). Cellular responses to interferon-gamma. *Annual Review of Immunology, 15*, 749-795. doi: 10.1146/annurev.immunol.15.1.749

Bohr, V. A., Chu, E. H., van Duin, M., Hanawalt, P. C., & Okumoto, D. S. (1988). Human repair gene restores normal pattern of preferential DNA repair in repair defective CHO cells. *Nucleic Acids Research, 16*(15), 7397-7403.

Boncristiano, M., Paccani, S. R., Barone, S., Ulivieri, C., Patrussi, L., Ilver, D., . . . Baldari, C. T. (2003). The *Helicobacter pylori* vacuolating toxin inhibits T cell activation by two independent mechanisms. *The Journal of Experimental Medicine, 198*(12), 1887-1897. doi: 10.1084/jem.20030621

Borlace, Glenn N., Jones, Hilary F., Keep, Stacey J., Butler, Ross N., & Brooks, Doug A. (2011). *Helicobacter pylori* phagosome maturation in primary human macrophages. *Gut Pathogens, 3*, 3-3. doi: 10.1186/1757-4749-3-3

Brachner, Andreas, Braun, Juliane, Ghodgaonkar, Medini, Castor, Dennis, Zlopasa, Livija, Ehrlich, Veronika, . . . Foisner, Roland. (2012). The endonuclease Ankle1 requires its LEM and GIY-YIG motifs for DNA cleavage in vivo. *Journal of Cell Science, 125*(4), 1048-1057. doi: 10.1242/jcs.098392

Caldwell, J., & Emerson, S. G. (1994). IL-1 alpha and TNF alpha act synergistically to stimulate production of myeloid colony-stimulating factors by cultured human bone marrow stromal cells and cloned stromal cell strains. *Journal of Cellular Physiology, 159*(2), 221-228. doi: 10.1002/jcp.1041590205

Campuzano-Maya, Germán. (2014). Hematologic manifestations of *Helicobacter pylori* infection. *World Journal of Gastroenterology : WJG, 20*(36), 12818-12838. doi: 10.3748/wjg.v20.i36.12818

Carmena, M., Pinson, X., Platani, M., Salloum, Z., Xu, Z., Clark, A., . . . Earnshaw, W. C. (2012). The chromosomal passenger complex activates Polo kinase at centromeres. *PLoS Biology, 10*(1), e1001250. doi: 10.1371/journal.pbio.1001250

Caruso, Roberta, Fina, Daniele, Peluso, Ilaria, Fantini, Massimo Claudio, Tosti, Claudio, Blanco, Giovanna Del Vecchio, . . . Monteleone, Giovanni. (2007). IL-21 Is Highly Produced in *Helicobacter pylori*-Infected Gastric Mucosa and Promotes Gelatinases Synthesis. *The Journal of Immunology, 178*(9), 5957-5965. doi: 10.4049/jimmunol.178.9.5957

Castaño-Rodríguez, Natalia, Kaakoush, Nadeem O., & Mitchell, Hazel M. (2014). Pattern-Recognition Receptors and Gastric Cancer. *Frontiers in Immunology, 5*, 336. doi: 10.3389/fimmu.2014.00336

Chang, David W., Xing, Zheng, Capacio, Vanessa L., Peter, Marcus E., & Yang, Xiaolu. (2003). Interdimer processing mechanism of procaspase‐8 activation. *The EMBO Journal, 22*(16), 4132-4142. doi: 10.1093/emboj/cdg414

Chaturvedi, R., Asim, M., Lewis, N. D., Algood, H. M., Cover, T. L., Kim, P. Y., & Wilson, K. T. (2007). L-arginine availability regulates inducible nitric oxide synthase-dependent host defense against *Helicobacter pylori*. *Infection and Immunity, 75*(9), 4305-4315. doi: 10.1128/iai.00578-07

Cheers, C., Haigh, A. M., Kelso, A., Metcalf, D., Stanley, E. R., & Young, A. M. (1988). Production of colony-stimulating factors (CSFs) during infection: separate determinations of macrophage-, granulocyte-, granulocyte-macrophage-, and multi-CSFs. *Infection and Immunity, 56*(1), 247-251.

Chen, J., Zhao, M., Rao, R., Inoue, H., & Hao, C. M. (2005). C/EBP-beta and its binding element are required for NF-kappa B-induced COX2 expression

following hypertonic stress. *The Journal of Biological Chemistry, 280*(16), 16354-16359. doi: 10.1074/jbc.M411134200

Chen, Minjian, Divangahi, Maziar, Gan, Huixian, Shin, Daniel S.J., Hong, Song, Lee, David M., . . . Remold, Heinz G. (2008). Lipid mediators in innate immunity against tuberculosis: opposing roles of PGE2 and LXA4 in the induction of macrophage death. *The Journal of Experimental Medicine, 205*(12), 2791-2801. doi: 10.1084/jem.20080767

Clyne, M., Labigne, A., & Drumm, B. (1995). *Helicobacter pylori* requires an acidic environment to survive in the presence of urea. *Infection and Immunity, 63*(5), 1669-1673.

Correa, P. (2004). Is gastric cancer preventable? *Gut, 53*(9), 1217-1219. doi: 10.1136/gut.2004.039834

Costa, K., Bacher, G., Allmaier, G., Dominguez-Bello, M. G., Engstrand, L., Falk, P., . . . Garcia-del Portillo, F. (1999). The morphological transition of *Helicobacter pylori* cells from spiral to coccoid is preceded by a substantial modification of the cell wall. *Journal of Bacteriology, 181*(12), 3710-3715.

Cover, T. L., & Blaser, M. J. (2009). *Helicobacter pylori* in health and disease. *Gastroenterology, 136*(6), 1863-1873. doi: 10.1053/j.gastro.2009.01.073

Cover, T. L., Cao, P., Lind, C. D., Tham, K. T., & Blaser, M. J. (1993). Correlation between vacuolating cytotoxin production by *Helicobacter pylori* isolates in vitro and in vivo. *Infection and Immunity, 61*(12), 5008-5012.

Crabtree, J. E., Shallcross, T. M., Heatley, R. V., & Wyatt, J. I. (1991). Mucosal tumour necrosis factor alpha and interleukin-6 in patients with *Helicobacter pylori* associated gastritis. *Gut, 32*(12), 1473-1477.

Crameri, A., Biondi, E., Kuehnle, K., Lutjohann, D., Thelen, K. M., Perga, S., . . . Mohajeri, M. H. (2006). The role of seladin-1/DHCR24 in cholesterol biosynthesis, APP processing and A beta generation in vivo. *EMBO journal, 25*(2), 432-443. doi: 10.1038/sj.emboj.7600938

Cuervo, A. M., & Dice, J. F. (1996). A receptor for the selective uptake and degradation of proteins by lysosomes. *Science, 273*(5274), 501-503.

Curado, M.P., Edwards, B., Shin, H.R., Storm, H., Ferlay, J., Heanue, M., & Boyle, P. (2007). Cancer Incidence in Five Continents. In M. P. Curado, B. Edwards, H. R. Shin, H. Storm, J. Ferlay, M. Heanue & P. Boyle (Eds.), (Vol. 9). Lyon, IARC.

D'Elios, M. M., Amedei, A., Cappon, A., Del Prete, G., & de Bernard, M. (2007). The neutrophil-activating protein of *Helicobacter pylori* (HP-NAP) as an immune modulating agent. *FEMS Immunology Medical Microbiology, 50*(2), 157-164. doi: 10.1111/j.1574-695X.2007.00258.x

da Silva Correia, J., Soldau, K., Christen, U., Tobias, P. S., & Ulevitch, R. J. (2001). Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. *The Journal of Biological Chemistry, 276*(24), 21129-21135. doi: 10.1074/jbc.M009164200

de Bernard, M. , Moschioni, M., Habermann, A., Griffiths, G. , & Montecucco, C. (2002). Cell vacuolization induced by *Helicobacter pylori* VacA cytotoxin does not depend on late endosomal SNAREs. *Cell Microbiology, 4*(11). doi: 10.1046/j.1462-5822.2002.00163.x

de Bernard, M., & D'Elios, M. M. (2010). The immune modulating activity of the *Helicobacter pylori* HP-NAP: Friend or foe? *Toxicon, 56*(7), 1186-1192. doi: 10.1016/j.toxicon.2009.09.020

de Bernard, M., Papini, E., de Filippis, V., Gottardi, E., Telford, J., Manetti, R., . . . Montecucco, C. (1995). Low pH Activates the Vacuolating Toxin of *Helicobacter pylori*, Which Becomes Acid and Pepsin Resistant. *The Journal of Biological Chemistry, 270*(41), 23937-23940. doi: 10.1074/jbc.270.41.23937

DePamphilis, M. L., & Adler, J. (1971). Attachment of flagellar basal bodies to the cell envelope: specific attachment to the outer, lipopolysaccharide membrane and the cyoplasmic membrane. *Journal of Bacteriology, 105*(1), 396-407.

Divangahi, Maziar, Desjardins, Danielle, Nunes-Alves, Claudio, Remold, Heinz G., & Behar, Samuel M. (2010). Eicosanoid pathways regulate adaptive immunity to Mycobacterium tuberculosis. *Nature Immunology, 11*(8), 751-758.

Dixon, M. F., Genta, R. M., Yardley, J. H., & Correa, P. (1996). Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *American Journal of Surgical Pathology, 20*(10), 1161-1181.

Dlugosz, Paulina J, Billen, Lieven P, Annis, Matthew G, Zhu, Weijia, Zhang, Zhi, Lin, Jialing, . . . Andrews, David W. (2006). Bcl ‐ 2 changes conformation to inhibit Bax oligomerization. *The EMBO Journal, 25*(11), 2287-2296. doi: 10.1038/sj.emboj.7601126

Dundon, W. G., Nishioka, H., Polenghi, A., Papinutto, E., Zanotti, G., Montemurro, P., . . . Montecucco, C. (2002). The neutrophil-activating protein of *Helicobacter pylori*. *International Journal of Medical Microbiology, 291*(6-7), 545-550.

Eaton, K. A., Brooks, C. L., Morgan, D. R., & Krakowka, S. (1991). Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. *Infection and Immunity, 59*(7), 2470-2475.

Eisenreich, Wolfgang, Heesemann, Jürgen, Rudel, Thomas, & Goebel, Werner. (2013). Metabolic host responses to infection by intracellular bacterial pathogens. *Frontiers in Cellular and Infection Microbiology, 3*, 24. doi: 10.3389/fcimb.2013.00024

Endicott, Jane A., Noble, Martin E. M., & Tucker, Julie A. (1999). Cyclin-dependent kinases: inhibition and substrate recognition. *Current Opinion in Structural Biology, 9*(6), 738-744.

Eskelinen, Eeva-Liisa, Tanaka, Yoshitaka, & Saftig, Paul. (2003). At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends in Cell Biology, 13*(3), 137-145. doi: 10.1016/S0962-8924(03)00005-9

Eusebi, L. H., Zagari, R. M., & Bazzoli, F. (2014). Epidemiology of *Helicobacter pylori* infection. *Helicobacter, 19 Suppl 1*, 1-5. doi: 10.1111/hel.12165

Evans, D. J., Jr., Evans, D. G., Takemura, T., Nakano, H., Lampert, H. C., Graham, D. Y., . . . Kvietys, P. R. (1995). Characterization of a *Helicobacter pylori* neutrophil-activating protein. *Infection and Immunity, 63*(6), 2213-2220.

Falush, D., Wirth, T., Linz, B., Pritchard, J. K., Stephens, M., Kidd, M., . . . Suerbaum, S. (2003). Traces of human migrations in *Helicobacter pylori* populations. *Science, 299*(5612), 1582-1585. doi: 10.1126/science.1080857

Fehlings, Michael, Drobbe, Lea, Moos, Verena, Renner Viveros, Pablo, Hagen, Jana, Beigier-Bompadre, Macarena, . . . Ignatius, Ralf. (2012). Comparative Analysis of the Interaction of *Helicobacter pylori* with Human Dendritic Cells, Macrophages, and Monocytes. *Infection and Immunity, 80*(8), 2724-2734. doi: 10.1128/IAI.00381-12

Flo, T. H., Smith, K. D., Sato, S., Rodriguez, D. J., Holmes, M. A., Strong, R. K., . . . Aderem, A. (2004). Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature, 432*(7019), 917-921. doi: 10.1038/nature03104

Forsyth, M. H., Atherton, J. C., Blaser, M. J., & Cover, T. L. (1998). Heterogeneity in levels of vacuolating cytotoxin gene (vacA) transcription among *Helicobacter pylori* strains. *Infection and Immunity, 66*(7), 3088-3094.

Frumkin, L. R. (1997). Role of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor in the treatment of patients with HIV infection. *Current Opinion in Hematology, 4*(3), 200-206.

Fu, J., Bian, M., Jiang, Q., & Zhang, C. (2007). Roles of Aurora kinases in mitosis and tumorigenesis. *Molecular Cancer Research, 5*(1), 1-10. doi: 10.1158/1541-7786.mcr-06-0208

Fuchs, C. S., & Mayer, R. J. (1995). Gastric carcinoma. *The New England Journal of Medicine, 333*(1), 32-41. doi: 10.1056/nejm199507063330107

Garay, Jone, Piazuelo, M. Blanca, Majumdar, Sumana, Li, Li, Trillo-Tinoco, Jimena, Del Valle, Luis, . . . Zabaleta, Jovanny. (2016). The homing receptor CD44 is involved in the progression of precancerous gastric lesions in patients infected with *Helicobacter pylori* and in development of mucous metaplasia in mice. *Cancer Letters, 371*(1), 90-98.

Geissmann, F., Jung, S., & Littman, D. R. (2003). Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity, 19*(1), 71-82.

Gioannini, T. L., Teghanemt, A., Zhang, D., Coussens, N. P., Dockstader, W., Ramaswamy, S., & Weiss, J. P. (2004). Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. *Proceedings of the National Academy of Sciences of the United States of America, 101*(12), 4186-4191. doi: 10.1073/pnas.0306906101

Go, M. F., Kapur, V., Graham, D. Y., & Musser, J. M. (1996). Population genetic analysis of *Helicobacter pylori* by multilocus enzyme electrophoresis: extensive allelic diversity and recombinational population structure. *Journal of Bacteriology, 178*(13), 3934-3938.

Gobert, A. P., Bambou, J. C., Werts, C., Balloy, V., Chignard, M., Moran, A. P., & Ferrero, R. L. (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. *Journal Biology of Chemistry, 279*(1), 245-250. doi: 10.1074/jbc.M307858200

Gobert, Alain P., Cheng, Yulan, Wang, Jian-Ying, Boucher, Jean-Luc, Iyer, Ramaswamy K., Cederbaum, Stephen D., . . . Wilson, Keith T. (2002). *Helicobacter pylori* Induces Macrophage Apoptosis by Activation of Arginase II. *The Journal of Immunology, 168*(9), 4692-4700. doi: 10.4049/jimmunol.168.9.4692

Goh, K. L. (2009). Epidemiology of *Helicobacter pylori* infection in Malaysia--observations in a multiracial Asian population. *Medical Journal of Malaysia, 64*(3), 187-192.

Goh, K. L., & Parasakthi, N. (2001). The racial cohort phenomenon: seroepidemiology of *Helicobacter pylori* infection in a multiracial South-East Asian country. *European Journal of Gastroenterology & Hepatology, 13*(2), 177-183.

Goodwin, C. S., Armstrong, J.A., Chilvers, T., M. Peters, Colins, M.D., Sly, L., . . . Harper, W.E.S. (1989). Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to Helicobacter gen. nov. as *Helicobacter pylori* comb. nov. and *Helicobacter mustelae* comb. nov., respectively. *International Journal of Systemic Bacteriology, 39*, 9.

Gordon, S. (2003). Alternative activation of macrophages. *Nature Reviews Immunology, 3*(1), 23-35. doi: 10.1038/nri978

Gordon, S. (2007). The macrophage: past, present and future. *European Journal of Immunology, 37 Suppl 1*, S9-17. doi: 10.1002/eji.200737638

Gordon, S., Lawson, L., Rabinowitz, S., Crocker, P. R., Morris, L., & Perry, V. H. (1992). Antigen markers of macrophage differentiation in murine tissues. *Current Topics in Microbiology and Immunology, 181*, 1-37.

Gordon, S., & Martinez, F. O. (2010). Alternative activation of macrophages: mechanism and functions. *Immunity, 32*(5), 593-604. doi: 10.1016/j.immuni.2010.05.007

Gordon, S., & Taylor, P. R. (2005). Monocyte and macrophage heterogeneity. *Nature Reviews Immunology, 5*(12), 953-964. doi: 10.1038/nri1733

Grage-Griebenow, E., Flad, H. D., & Ernst, M. (2001). Heterogeneity of human peripheral blood monocyte subsets. *Journal of Leukocyte Biology, 69*(1), 11-20.

Green, D. R., & Reed, J. C. (1998). Mitochondria and apoptosis. *Science, 281*(5381), 1309-1312.

Gunaletchumy, Selva Perumal, Seevasant, Indran, Tan, Mun Hua, Croft, Laurence J., Mitchell, Hazel M., Goh, Khean Lee, . . . Vadivelu, Jamuna. (2014). *Helicobacter pylori* Genetic Diversity and Gastro-duodenal Diseases in Malaysia. *Scientific Reports, 4*, 7431. doi: 10.1038/srep07431

Hagemann, T., Lawrence, T., McNeish, I., Charles, K. A., Kulbe, H., Thompson, R. G., . . . Balkwill, F. R. (2008). "Re-educating" tumor-associated macrophages by

targeting NF-kappaB. *The Journal of Experimental Medicine, 205*(6), 1261-1268. doi: 10.1084/jem.20080108

Hagymási, Krisztina, & Tulassay, Zsolt. (2014). *Helicobacter pylori* infection: New pathogenetic and clinical aspects. *World Journal of Gastroenterology : WJG, 20*(21), 6386-6399. doi: 10.3748/wjg.v20.i21.6386

Hamilton, John A. (2008). Colony-stimulating factors in inflammation and autoimmunity. *Nature Review of Immunology, 8*(7), 533-544.

Hanna, Richard N., Carlin, Leo M., Hubbeling, Harper G., Nackiewicz, Dominika, Green, Angela M., Punt, Jennifer A., . . . Hedrick, Catherine C. (2011). The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes. *Nature Immunology, 12*(8), 778-785.

Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M., & Weaver, C. T. (2005). Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature of Immunology, 6*(11), 1123-1132. doi: 10.1038/ni1254

Harris, P. R., Ernst, P. B., Kawabata, S., Kiyono, H., Graham, M. F., & Smith, P. D. (1998). Recombinant *Helicobacter pylori* urease activates primary mucosal macrophages. *Journal of Infectious Disease, 178*(5), 1516-1520.

Harris, P. R., Mobley, H. L., Perez-Perez, G. I., Blaser, M. J., & Smith, P. D. (1996). *Helicobacter pylori* urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. *Gastroenterology, 111*(2), 419-425.

Hartley, J. W., Evans, L. H., Green, K. Y., Naghashfar, Z., Macias, A. R., Zerfas, P. M., & Ward, J. M. (2008). Expression of infectious murine leukemia viruses by RAW264.7 cells, a potential complication for studies with a widely used mouse macrophage cell line. *Retrovirology, 5*, 1. doi: 10.1186/1742-4690-5-1

Hashimoto, Daigo, Miller, Jennifer, & Merad, Miriam. (2011). Dendritic Cell and Macrophage Heterogeneity In Vivo. *Immunity, 35*(3), 323-335.

Higashi, H., Tsutsumi, R., Muto, S., Sugiyama, T., Azuma, T., Asaka, M., & Hatakeyama, M. (2002). SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter pylori* CagA protein. *Science, 295*(5555), 683-686. doi: 10.1126/science.1067147

Ho, M. K., & Springer, T. A. (1982). Mac-1 antigen: quantitative expression in macrophage populations and tissues, and immunofluorescent localization in spleen. *Journal of Immunology, 128*(5), 2281-2286.

Hollingsworth, J. W., Li, Z., Brass, D. M., Carantziotis, S., Timberlake, S. H., Kim, A., . . . Schwartz, D. A. (2007). CD44 regulates macrophage recruitment to the lung in lipopolysaccharide- induced airway disease. *American Journal of Respiratory Cell and Molecular Biology, 37*(2), 248-253. doi: 10.1165/rcmb.2006-0363OC

Hu, L. T., & Mobley, H. L. (1990). Purification and N-terminal analysis of urease from *Helicobacter pylori*. *Infection and Immunity, 58*(4), 992-998.

Huber, Christoph, Mårtensson, Annica, Bokoch, Gary M., Nemazee, David, & Gavin, Amanda L. (2008). FGD2, a CDC42-specific Exchange Factor Expressed by Antigen-presenting Cells, Localizes to Early Endosomes and Active Membrane Ruffles. *The Journal of Biological Chemistry, 283*(49), 34002-34012. doi: 10.1074/jbc.M803957200

Hume, D. A., Ross, I. L., Himes, S. R., Sasmono, R. T., Wells, C. A., & Ravasi, T. (2002). The mononuclear phagocyte system revisited. *Journal of Leukocyte Biology, 72*(4), 621-627.

Idris-Khodja, N., Mian, M. O., Paradis, P., & Schiffrin, E. L. (2014). Dual opposing roles of adaptive immunity in hypertension. *European Heart Journal, 35*(19), 1238-1244. doi: 10.1093/eurheartj/ehu119

Ito, T., Kobayashi, D., Uchida, K., Takemura, T., Nagaoka, S., Kobayashi, I., . . . Eishi, Y. (2008). *Helicobacter pylori* invades the gastric mucosa and translocates to the gastric lymph nodes. *Laboratory Investigation, 88*(6), 664-681. doi: 10.1038/labinvest.2008.33

Ji, X., Fernandez, T., Burroni, D., Pagliaccia, C., Atherton, J. C., Reyrat, J. M., . . . Telford, J. L. (2000). Cell specificity of *Helicobacter pylori* cytotoxin is determined by a short region in the polymorphic midregion. *Infection and Immunity, 68*(6), 3754-3757.

Johnston, B. L., & Conly, J. M. (2006). Tumour necrosis factor inhibitors and infection: What is there to know for infectious diseases physicians? *The Canadian Journal of Infectious Diseases & Medical Microbiology, 17*(4), 209-212.

Jones, D. M., Curry, A., & Fox, A. J. (1985). An ultrastructural study of the gastric campylobacter-like organism 'Campylobacter pyloridis'. *Journal of General Microbiology, 131*(9), 2335-2341.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., & Tanabe, M. (2016). KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Research, 44*(D1), D457-462. doi: 10.1093/nar/gkv1070

Kansau, I., Raymond, J., Bingen, E., Courcoux, P., Kalach, N., Bergeret, M., . . . Labigne, A. (1996). Genotyping of *Helicobacter pylori* isolates by sequencing of PCR products and comparison with the RAPD technique. *Research in Microbiology, 147*(8), 661-669.

Kaparakis, M., Walduck, A. K., Price, J. D., Pedersen, J. S., van Rooijen, N., Pearse, M. J., . . . Strugnell, R. A. (2008). Macrophages are mediators of gastritis in acute *Helicobacter pylori* infection in C57BL/6 mice. *Infection and Immunity, 76*(5), 2235-2239. doi: 10.1128/iai.01481-07

Keir, M. E., Butte, M. J., Freeman, G. J., & Sharpe, A. H. (2008). PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol, 26*, 677-704. doi: 10.1146/annurev.immunol.26.021607.090331

Khosravi, Y., Rehvathy, V., Wee, W. Y., Wang, S., Baybayan, P., Singh, S., . . . Vadivelu, J. (2013). Comparing the genomes of *Helicobacter pylori* clinical strain UM032 and Mice-adapted derivatives. *Gut Pathogens, 5*, 25. doi: 10.1186/1757-4749-5-25

Kim, Ik-Jung, & Blanke, Steven R. (2012). Remodeling the host environment: modulation of the gastric epithelium by the *Helicobacter pylori* vacuolating toxin (VacA). *Frontiers in Cellular and Infection Microbiology, 2*, 37. doi: 10.3389/fcimb.2012.00037

Kitabayashi, M., Nishiya, Y., Esaka, M., Itakura, M., & Imanaka, T. (2003). Gene cloning and function analysis of replication factor C from Thermococcus kodakaraensis KOD1. *Bioscience Biotechnology and Biochemistry, 67*(11), 2373-2380. doi: 10.1271/bbb.67.2373

Kolls, J. K., & Linden, A. (2004). Interleukin-17 family members and inflammation. *Immunity, 21*(4), 467-476. doi: 10.1016/j.immuni.2004.08.018

Konno, Mutsuko, Fujii, Nobuhiro, Yokota, Shin-ichi, Sato, Kiyoshi, Takahashi, Michiko, Sato, Kohei, . . . Sugiyama, Toshiro. (2005). Five-Year Follow-Up Study of Mother-to-Child Transmission of *Helicobacter pylori* Infection Detected by a Random Amplified Polymorphic DNA Fingerprinting Method. *Journal of Clinical Microbiology, 43*(5), 2246-2250. doi: 10.1128/JCM.43.5.2246-2250.2005

Konturek, P. C., Konturek, S. J., & Brzozowski, T. (2009). *Helicobacter pylori* infection in gastric cancerogenesis. *Journal of Physiology and Pharmacology, 60*(3), 3-21.

Krauss-Etschmann, S., Gruber, R., Plikat, K., Antoni, I., Demmelmair, H., Reinhardt, D., & Koletzko, S. (2005). Increase of antigen-presenting cells in the gastric mucosa of *Helicobacter pylori*-infected children. *Helicobacter, 10*(3), 214-222. doi: 10.1111/j.1523-5378.2005.00313.x

Kuehnle, K., Crameri, A., Kalin, R. E., Luciani, P., Benvenuti, S., Peri, A., . . . Mohajeri, M. H. (2008). Prosurvival effect of DHCR24/Seladin-1 in acute and chronic responses to oxidative stress. *Molecular and Cellular Biology, 28*(2), 539-550. doi: 10.1128/mcb.00584-07

Kuipers, Ernst J., Israel, Dawn A., Kusters, Johannes G., Gerrits, Monique M., Weel, Jan, van der Ende, Arie, . . . Blaser, Martin J. (2000). Quasispecies Development of *Helicobacter pylori* Observed in Paired Isolates Obtained Years Apart from the Same Host. *The Journal of Infectious Diseases, 181*(1), 273-282. doi: 10.1086/315173

Kusters, J. G., van Vliet, A. H., & Kuipers, E. J. (2006). Pathogenesis of *Helicobacter pylori* infection. *Clinical Microbiology Reviews, 19*(3), 449-490. doi: 10.1128/cmr.00054-05

Labigne, A., Cussac, V., & Courcoux, P. (1991). Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. *Journal of Bacteriology, 173*(6), 1920-1931.

Lee, A., O'Rourke, J., De Ungria, M. C., Robertson, B., Daskalopoulos, G., & Dixon, M. F. (1997). A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology, 112*(4), 1386-1397.

Lee, A., & O'Rourke, J. L. (1993). *Ultrastructure of Helicobacter organisms and possible revelance for pathogenesis* (I. C. S. G. a. B. W. W. (ed.) Ed.). Boca Raton, Fla: CRC Press.

Letley, D. P., Rhead, J. L., Twells, R. J., Dove, B., & Atherton, J. C. (2003). Determinants of non-toxicity in the gastric pathogen *Helicobacter pylori*. *The Journal of Biological Chemistry, 278*(29), 26734-26741. doi: 10.1074/jbc.M304071200

Leunk, R. D., Johnson, P. T., David, B. C., Kraft, W. G., & Morgan, D. R. (1988). Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. *Journal of Medical Microbiology, 26*(2), 93-99. doi: 10.1099/00222615-26-2-93

Levy, B. D., Clish, C. B., Schmidt, B., Gronert, K., & Serhan, C. N. (2001). Lipid mediator class switching during acute inflammation: signals in resolution. *Nature Immunology, 2*(7), 612-619. doi: 10.1038/89759

Lewis, N. D., Asim, M., Barry, D. P., Singh, K., de Sablet, T., Boucher, J. L., . . . Wilson, K. T. (2010). Arginase II restricts host defense to *Helicobacter pylori* by attenuating inducible nitric oxide synthase translation in macrophages. *Journal of Immunology, 184*(5), 2572-2582. doi: 10.4049/jimmunol.0902436

Liao, H, Li, G, & Yen, TJ. (1994). Mitotic regulation of microtubule cross-linking activity of CENP-E kinetochore protein. *Science, 265*(5170), 394-398. doi: 10.1126/science.8023161

Liebermann, Dan A., & Hoffman, Barbara. (2007). Gadd45 in the response of hematopoietic cells to genotoxic stress. *Blood cells, molecules & diseases, 39*(3), 329-335. doi: 10.1016/j.bcmd.2007.06.006

Lim, G.C.C., Halimah, Y., & Lim, T.O. (2002). The first report of the National Cancer Registry cancer incidence in Malaysia. In G. C. C. Lim & Y. Halimah (Eds.), (pp. 192).

Lim, S., & Kaldis, P. (2013). Cdks, cyclins and CKIs: roles beyond cell cycle regulation. *Development, 140*(15), 3079-3093. doi: 10.1242/dev.091744

Lin, D., Fiscella, M., O'Connor, P. M., Jackman, J., Chen, M., Luo, L. L., . . . Mercer, W. E. (1994). Constitutive expression of B-myb can bypass p53-induced Waf1/Cip1-mediated G1 arrest. *Proceedings of the National Academy of Sciences of the United States of America, 91*(21), 10079-10083.

Lina, Taslima T., Alzahrani, Shatha, Gonzalez, Jazmin, Pinchuk, Irina V., Beswick, Ellen J., & Reyes, Victor E. (2014). Immune evasion strategies used by *Helicobacter pylori*. *World Journal of Gastroenterology : WJG, 20*(36), 12753-12766. doi: 10.3748/wjg.v20.i36.12753

Lindas, Ann-Christin, & Bernander, Rolf. (2013). The cell cycle of archaea. *Nature Reviews of Microbiology, 11*(9), 627-638. doi: 10.1038/nrmicro3077

Lindholm, C., Quiding-Järbrink, M., Lönroth, H., Hamlet, A., & Svennerholm, A. M. (1998). Local Cytokine Response in *Helicobacter pylori*-Infected Subjects. *Infection and Immunity, 66*(12), 5964-5971.

Linz, B., Balloux, F., Moodley, Y., Manica, A., Liu, H., Roumagnac, P., . . . Achtman, M. (2007). An African origin for the intimate association between humans and *Helicobacter pylori*. *Nature, 445*(7130), 915-918. doi: 10.1038/nature05562

Lobjois, V., Jullien, D., Bouche, J. P., & Ducommun, B. (2009). The polo-like kinase 1 regulates CDC25B-dependent mitosis entry. *Biochimica Biophysica Acta, 1793*(3), 462-468. doi: 10.1016/j.bbamcr.2008.12.015

Lochhead, P., & El-Omar, E. M. (2007). *Helicobacter pylori* infection and gastric cancer. *Best Practice & Research. Clinical Gastroenterology, 21*(2), 281-297. doi: 10.1016/j.bpg.2007.02.002

Loke, P., Nair, M. G., Parkinson, J., Guiliano, D., Blaxter, M., & Allen, J. E. (2002). IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype. *BMC Immunology, 3*, 7.

Lowenstein, C. J., Alley, E. W., Raval, P., Snowman, A. M., Snyder, S. H., Russell, S. W., & Murphy, W. J. (1993). Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. *Proceedings of the National Academy of Sciences of the United States of America, 90*(20), 9730-9734.

Mach, B., Steimle, V., Martinez-Soria, E., & Reith, W. (1996). Regulation of MHC class II genes: lessons from a disease. *Annual Review of Immunology, 14*, 301-331. doi: 10.1146/annurev.immunol.14.1.301

Maiato, Helder, DeLuca, Jennifer, Salmon, E. D., & Earnshaw, William C. (2004). The dynamic kinetochore-microtubule interface. *Journal of Cell Science, 117*(23), 5461-5477. doi: 10.1242/jcs.01536

Mailand, Niels, Gibbs-Seymour, Ian, & Bekker-Jensen, Simon. (2013). Regulation of PCNA-protein interactions for genome stability. *Nature Review of Molecular Cell Biology, 14*(5), 269-282. doi: 10.1038/nrm3562

Mantovani, A., Sica, A., & Locati, M. (2005). Macrophage Polarization Comes of Age. *Immunity, 23*(4), 344-346.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., & Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology, 25*(12), 677-686. doi: 10.1016/j.it.2004.09.015

Mantovani, B., Rabinovitch, M., & Nussenzweig, V. (1972). Phagocytosis of immune complexes by macrophages : different roles of the macrophage receptor sites for complement (C3) and immunoglobin (IgG). *The Journal of Experimental Medicine, 135*(4), 780-792.

Marks, P. W., Arai, M., Bandura, J. L., & Kwiatkowski, D. J. (1998). Advillin (p92): a new member of the gelsolin/villin family of actin regulatory proteins. *Journal of Cell Science, 111 (Pt 15)*, 2129-2136.

Marshall, B. J., Armstrong, J. A., McGechie, D. B., & Glancy, R. J. (1985). Attempt to fulfil Koch's postulates for pyloric Campylobacter. *The Medical Journal of Australia, 142*(8), 436-439.

Marshall, B. J., & Langton, S. R. (1986). Urea hydrolysis in patients with Campylobacter pyloridis infection. *Lancet, 1*(8487), 965-966.

Marshall, B. J., & Warren, J. R. (1984). Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet, 1*(8390), 1311-1315.

Martinez, F. O., & Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000 Prime Reports, 6*, 13. doi: 10.12703/P6-13

Matsushime, H., Ewen, M. E., Strom, D. K., Kato, J. Y., Hanks, S. K., Roussel, M. F., & Sherr, C. J. (1992). Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins. *Cell, 71*(2), 323-334.

McClain, M. S., Cao, P., Iwamoto, H., Vinion-Dubiel, A. D., Szabo, G., Shao, Z., & Cover, T. L. (2001). A 12-amino-acid segment, present in type s2 but not type s1 *Helicobacter pylori* VacA proteins, abolishes cytotoxin activity and alters membrane channel formation. *Journal of Bacteriology, 183*(22), 6499-6508. doi: 10.1128/jb.183.22.6499-6508.2001

McColl, K. E., el-Omar, E., & Gillen, D. (1998). Interactions between H. pylori infection, gastric acid secretion and anti-secretory therapy. *British Medical Bulletin, 54*(1), 121-138.

McGaha, T. L., Chen, Y., Ravishankar, B., van Rooijen, N., & Karlsson, M. C. (2011). Marginal zone macrophages suppress innate and adaptive immunity to apoptotic cells in the spleen. *Blood, 117*(20), 5403-5412. doi: 10.1182/blood-2010-11-320028

McIlwain, D. R., Berger, T., & Mak, T. W. (2013). Caspase functions in cell death and disease. *Cold Spring Harbour Perspectives in Biology, 5*(4), a008656. doi: 10.1101/cshperspect.a008656

McKnight, A. J., & Gordon, S. (1998). The EGF-TM7 family: unusual structures at the leukocyte surface. *Journal of Leukocyte Biology, 63*(3), 271-280.

Medina-Contreras, Oscar, Geem, Duke, Laur, Oskar, Williams, Ifor R., Lira, Sergio A., Nusrat, Asma, . . . Denning, Timothy L. (2011). CX3CR1 regulates intestinal macrophage homeostasis, bacterial translocation, and colitogenic Th17 responses in mice. *The Journal of Clinical Investigation, 121*(12), 4787-4795. doi: 10.1172/JCI59150

Megraud, F., Neman-Simha, V., & Brugmann, D. (1992). Further evidence of the toxic effect of ammonia produced by *Helicobacter pylori* urease on human epithelial cells. *Infection and Immunity, 60*(5), 1858-1863.

Menaker, Rena J., Ceponis, Peter J. M., & Jones, Nicola L. (2004). *Helicobacter pylori* Induces Apoptosis of Macrophages in Association with Alterations in the Mitochondrial Pathway. *Infection and Immunity, 72*(5), 2889-2898. doi: 10.1128/IAI.72.5.2889-2898.2004

Meyerson, M, & Harlow, E. (1994). Identification of G1 kinase activity for cdk6, a novel cyclin D partner. *Molecular and Cellular Biology, 14*(3), 2077-2086. doi: 10.1128/mcb.14.3.2077

Misra, Vatsala, Pandey, Renu, Misra, Sri Prakash, & Dwivedi, Manisha. (2014). *Helicobacter pylori* and gastric cancer: Indian enigma. *World Journal of Gastroenterology : WJG, 20*(6), 1503-1509. doi: 10.3748/wjg.v20.i6.1503

Moore, K. J., Andersson, L. P., Ingalls, R. R., Monks, B. G., Li, R., Arnaout, M. A., . . . Freeman, M. W. (2000). Divergent response to LPS and bacteria in CD14-deficient murine macrophages. *Journal of Immunology, 165*(8), 4272-4280.

Morris, L., Graham, C.F., & Gordon, S. (1991). Macrophages in haemopoietic and other tissues of the developing mouse detected by the monoclonal antibody F4/80. *Development, 112*(2), 517-526.

Morrison, T. E., Simmons, J. D., & Heise, M. T. (2008). Complement receptor 3 promotes severe ross river virus-induced disease. *Journal of Virology, 82*(22), 11263-11272. doi: 10.1128/jvi.01352-08

Muller, F. J., Laurent, L. C., Kostka, D., Ulitsky, I., Williams, R., Lu, C., . . . Loring, J. F. (2008). Regulatory networks define phenotypic classes of human stem cell lines. *Nature, 455*(7211), 401-405. doi: 10.1038/nature07213

Munari, F., Fassan, M., Capitani, N., Codolo, G., Vila-Caballer, M., Pizzi, M., . . . de Bernard, M. (2014). Cytokine BAFF released by *Helicobacter pylori*-infected macrophages triggers the Th17 response in human chronic gastritis. *Journal of Immunology, 193*(11), 5584-5594. doi: 10.4049/jimmunol.1302865

Murray, P. J., & Wynn, T. A. (2011). Protective and pathogenic functions of macrophage subsets. *Nature Review of Immunology, 11*(11), 723-737. doi: 10.1038/nri3073

Musa, A.F., Yunous, M.N.M., Rahman, S.A., & Nordin, R.B. (2014). The Seroprevalence and Eradication Success of Helicobacter pylori in Indigenous People of Seletar in Southern Malaysia. *British Journal of Medicine & Medical Research, 4*(9), 10.

Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., & Dixit, V. M. (1998). An induced proximity model for caspase-8 activation. *The Journal of Biological Chemistry, 273*(5), 2926-2930.

Nasri, S., More, H., Graziano, F., Ruzzo, A., Wilson, E., Dunbier, A., . . . Humar, B. (2008). A novel diffuse gastric cancer susceptibility variant in E-cadherin (CDH1) intron 2: a case control study in an Italian population. *BMC Cancer, 8*, 138. doi: 10.1186/1471-2407-8-138

Neel, Benjamin G., Gu, Haihua, & Pao, Lily. (2003). The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. *Trends in Biochemical Sciences, 28*(6), 284-293.

Neiger, R., & Simpson, K. W. (2000). Helicobacter infection in dogs and cats: facts and fiction. *Journal of Veterinary Internal Medicine, 14*(2), 125-133.

Norbury, C., & Nurse, P. (1992). Animal cell cycles and their control. *Annual Review of Biochemistry, 61*, 441-470. doi: 10.1146/annurev.bi.61.070192.002301

O'Shea, J. J., & Paul, W. E. (2010). Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science, 327*(5969), 1098-1102. doi: 10.1126/science.1178334

Obst, B., Wagner, S., Sewing, K.F., & Beil, W. (2000). *Helicobacter pylori* causes DNA damage in gastric epithelial cells. *Carcinogenesis, 21*(6), 1111-1115. doi: 10.1093/carcin/21.6.1111

Odenbreit, S., Puls, J., Sedlmaier, B., Gerland, E., Fischer, W., & Haas, R. (2000). Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science, 287*(5457), 1497-1500.

Otani, Koji, Watanabe, Toshio, Tanigawa, Tetsuya, Okazaki, Hirotoshi, Yamagami, Hirokazu, Watanabe, Kenji, . . . Arakawa, Tetsuo. (2009). Anti-inflammatory effects of IL-17A on *Helicobacter pylori*-induced gastritis. *Biochemical and Biophysical Research Communications, 382*(2), 252-258.

Owen, D. A. (2003). Gastritis and carditis. *Modern Pathology, 16*(4), 325-341. doi: 10.1097/01.MP.0000062995.72390.14

Pagliaccia, C., de Bernard, M., Lupetti, P., Ji, X., Burroni, D., Cover, T. L., . . . Reyrat, J. M. (1998). The m2 form of the Helicobacter pylori cytotoxin has cell type-specific vacuolating activity. *Proceedings of the National Academy of Sciences of the United States of America, 95*(17), 10212-10217.

Pagliaccia, Cristina, de Bernard, Marina, Lupetti, Pietro, Ji, Xuhuai, Burroni, Daniela, Cover, Timothy L., . . . Reyrat, Jean-Marc. (1998). The m2 form of the *Helicobacter pylori* cytotoxin has cell type-specific vacuolating activity. *Proceedings of the National Academy of Sciences of the United States of America, 95*(17), 10212-10217.

Palframan, R. T., Jung, S., Cheng, G., Weninger, W., Luo, Y., Dorf, M., . . . von Andrian, U. H. (2001). Inflammatory chemokine transport and presentation in HEV: a remote control mechanism for monocyte recruitment to lymph nodes in inflamed tissues. *The Journal of Experimental Medicine, 194*(9), 1361-1373.

Palframan, Samuel L., Kwok, Terry, & Gabriel, Kipros. (2012). Vacuolating cytotoxin A (VacA), a key toxin for *Helicobacter pylori* pathogenesis. *Frontiers in Cellular and Infection Microbiology, 2*, 92. doi: 10.3389/fcimb.2012.00092

Papetti, M., & Augenlicht, L. H. (2011). MYBL2, a link between proliferation and differentiation in maturing colon epithelial cells. *Journal of Cellular Physiology, 226*(3), 785-791. doi: 10.1002/jcp.22399

Pathak, S. K., Tavares, R., de Klerk, N., Spetz, A. L., & Jonsson, A. B. (2013). *Helicobacter pylori* protein JHP0290 binds to multiple cell types and induces macrophage apoptosis via tumor necrosis factor (TNF)-dependent and independent pathways. *PLoS One, 8*(11), e77872. doi: 10.1371/journal.pone.0077872

Pegg, A. E. (2006). Regulation of ornithine decarboxylase. *The Journal of Biological Chemistry, 281*(21), 14529-14532. doi: 10.1074/jbc.R500031200

Platt, N., da Silva, R. P., & Gordon, S. (1999). Class A scavenger receptors and the phagocytosis of apoptotic cells. *Immunology Letters, 65*(1-2), 15-19.

Qin, J., Tao, D., Chen, X., Feng, Y., Hu, J., Reed, E., . . . Gong, J. (2004). Down-regulation of cyclin E expression by caffeine promotes cancer cell entry into the S-phase of the cell cycle. *Anticancer Research, 24*(5a), 2991-2995.

Quiding-Järbrink, Marianne, Raghavan, Sukanya, & Sundquist, Malin. (2010). Enhanced M1 Macrophage Polarization in Human *Helicobacter pylori*-Associated Atrophic Gastritis and in Vaccinated Mice. *Plos One, 5*(11), e15018. doi: 10.1371/journal.pone.0015018

Raes, G., Brys, L., Dahal, B. K., Brandt, J., Grooten, J., Brombacher, F., . . . Ghassabeh, G. H. (2005). Macrophage galactose-type C-type lectins as novel markers for alternatively activated macrophages elicited by parasitic infections and allergic airway inflammation. *Journal of Leukocyte Biology, 77*(3), 321-327. doi: 10.1189/jlb.0304212

Ramarao, N., Gray-Owen, S. D., Backert, S., & Meyer, T. F. (2000). *Helicobacter pylori* inhibits phagocytosis by professional phagocytes involving type IV secretion components. *Molecular Microbiology, 37*(6), 1389-1404.

Ravikumar, B., Futter, M., Jahreiss, L., Korolchuk, V. I., Lichtenberg, M., Luo, S., . . . Rubinsztein, D. C. (2009). Mammalian macroautophagy at a glance. *Journal of Cell Science, 122*(Pt 11), 1707-1711. doi: 10.1242/jcs.031773

Registry, National Cancer. (2011). Malaysia National Cancer Registry Report 2007. *Malaysian Cancer Statistics- Data and Figure Peninsular Malaysia 2006*. Kuala Lumpur: Ministry of Health (Malaysia). .

Ricci, V, Ciacci, C, Zarrilli, R, Sommi, P, Tummuru, M K, Del Vecchio Blanco, C, . . . Romano, M. (1996). Effect of *Helicobacter pylori* on gastric epithelial cell migration and proliferation in vitro: role of VacA and CagA. *Infection and Immunity, 64*(7), 2829-2833.

Rieder, C. L., & Salmon, E. D. (1998). The vertebrate cell kinetochore and its roles during mitosis. *Trends in Cell Biology, 8*(8), 310-318.

Riedl, S. J., & Shi, Y. G. (2004). Molecular mechanisms of caspase regulation during apoptosis. *Nature Reviews Molecular Cell Biology, 5*(11), 897-907. doi: 10.1038/nrm1496

Rowland, M., Daly, L., Vaughan, M., Higgins, A., Bourke, B., & Drumm, B. (2006). Age-specific incidence of *Helicobacter pylori*. *Gastroenterology, 130*(1), 65-72; quiz 211. doi: 10.1053/j.gastro.2005.11.004

Said, Elias A., Dupuy, Franck P., Trautmann, Lydie, Zhang, Yuwei, Shi, Yu, El-Far, Mohamed, . . . Sekaly, Rafick-Pierre. (2010). Programmed death-1-induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection. *Nature Medicine, 16*(4), 452-459.

Salama, Nina, Guillemin, Karen, McDaniel, Timothy K., Sherlock, Gavin, Tompkins, Lucy, & Falkow, Stanley. (2000). A whole-genome microarray reveals genetic diversity among *Helicobacter pylori* strains. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(26), 14668-14673.

Salanga, C. L., Dyer, D. P., Kiselar, J. G., Gupta, S., Chance, M. R., & Handel, T. M. (2014). Multiple glycosaminoglycan-binding epitopes of monocyte chemoattractant protein-3/CCL7 enable it to function as a non-oligomerizing chemokine. *The Journal of Biological Chemistry, 289*(21), 14896-14912. doi: 10.1074/jbc.M114.547737

Salih, B.A;. (2009). Helicobacter pylori infection in developing countries: The burden for how long? *The Saudi Journal of Gastroenterology, 15*(3), 7.

Satin, B., Del Giudice, G., Della Bianca, V., Dusi, S., Laudanna, C., Tonello, F., . . . Rossi, F. (2000). The neutrophil-activating protein (HP-NAP) of *Helicobacter pylori* is a protective antigen and a major virulence factor. *The Journal of Experimental Medicine, 191*(9), 1467-1476.

Schluger, Neil W., & Rom, William N. (1997). Early responses to infection: chemokines as mediators of inflammation. *Current Opinion in Immunology, 9*(4), 504-508.

Schwartz, Justin T., & Allen, Lee-Ann H. (2006). Role of urease in megasome formation and *Helicobacter pylori* survival in macrophages. *Journal of Leukocyte Biology, 79*(6), 1214-1225. doi: 10.1189/jlb.0106030

Serbina, N. V., Jia, T., Hohl, T. M., & Pamer, E. G. (2008). Monocyte-mediated defense against microbial pathogens. *Annual Review of Immunology, 26*, 421-452. doi: 10.1146/annurev.immunol.26.021607.090326

Sharpe, Arlene H., Wherry, E. John, Ahmed, Rafi, & Freeman, Gordon J. (2007). The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nature Immunology, 8*(3), 239-245.

Shi, Chao, & Pamer, Eric G. (2011). Monocyte recruitment during infection and inflammation. *Nature Review of Immunology, 11*(11), 762-774.

Shi, Y., Liu, X. F., Zhuang, Y., Zhang, J. Y., Liu, T., Yin, Z., . . . Zou, Q. M. (2010). *Helicobacter pylori*-induced Th17 responses modulate Th1 cell responses, benefit bacterial growth, and contribute to pathology in mice. *Journal of Immunology, 184*(9), 5121-5129. doi: 10.4049/jimmunol.0901115

Shi, Yun, Liu, Xiao-Fei, Zhuang, Yuan, Zhang, Jin-Yu, Liu, Tao, Yin, Zhinan, . . . Zou, Quan-Ming. (2010). *Helicobacter pylori*-Induced Th17 Responses Modulate Th1 Cell Responses, Benefit Bacterial Growth, and Contribute to Pathology in Mice. *The Journal of Immunology, 184*(9), 5121-5129. doi: 10.4049/jimmunol.0901115

Si, Yue, Tsou, Chia-Lin, Croft, Kelsey, & Charo, Israel F. (2010). CCR2 mediates hematopoietic stem and progenitor cell trafficking to sites of inflammation in mice. *The Journal of Clinical Investigation, 120*(4), 1192-1203. doi: 10.1172/JCI40310

Silverstein, S. C., S. Greenberg, F. Di Virgilo, and T. H. Steinberg. (1989). *Phagocytosis* (W. E. Paul Ed.). New York: Raven Press Ltd.

Simpson, K. W., Strauss-Ayali, D., Straubinger, R. K., Scanziani, E., McDonough, P. L., Straubinger, A. F., . . . Calam, J. (2001). *Helicobacter pylori* infection in the cat: evaluation of gastric colonization, inflammation and function. *Helicobacter, 6*(1), 1-14.

Sintara, K., Thong-Ngam, D., Patumraj, S., Klaikeaw, N., & Chatsuwan, T. (2010). Curcumin suppresses gastric NF-kappaB activation and macromolecular leakage in *Helicobacter pylori*-infected rats. *World J Gastroenterol, 16*(32), 4039-4046.

Skibinski, D. A., Genisset, C., Barone, S., & Telford, J. L. (2006). The cell-specific phenotype of the polymorphic vacA midregion is independent of the appearance of the cell surface receptor protein tyrosine phosphatase beta. *Infection and Immunity, 74*(1), 49-55. doi: 10.1128/iai.74.1.49-55.2006

Slattery, S. D., Moore, R. V., Brinkley, B. R., & Hall, R. M. (2008). Aurora-C and Aurora-B share phosphorylation and regulation of CENP-A and Borealin during mitosis. *Cell Cycle, 7*(6), 787-795.

Smoot, D. T., Resau, J. H., Earlington, M. H., Simpson, M., & Cover, T. L. (1996). Effects of *Helicobacter pylori* vacuolating cytotoxin on primary cultures of human gastric epithelial cells. *Gut, 39*(6), 795-799.

Solovjov, D. A., Pluskota, E., & Plow, E. F. (2005). Distinct roles for the alpha and beta subunits in the functions of integrin alphaM beta2. *The Journal of Biological Chemistry, 280*(2), 1336-1345. doi: 10.1074/jbc.M406968200

Sommi, Patrizia, Savio, Monica, Stivala, Lucia A., Scotti, Claudia, Mignosi, Paola, Prosperi, Ennio, . . . Solcia, Enrico. (2002). *Helicobacter pylori* Releases a Factor(s) Inhibiting Cell Cycle Progression of Human Gastric Cell Lines by Affecting Cyclin E/cdk2 Kinase Activity and Rb Protein Phosphorylation through Enhanced p27KIP1 Protein Expression. *Experimental Cell Research, 281*(1), 128-139.

Song, J., Salek-Ardakani, S., So, T., & Croft, M. (2007). The kinases aurora B and mTOR regulate the G1-S cell cycle progression of T lymphocytes. *Nature Immunology, 8*(1), 64-73. doi: 10.1038/ni1413

Spann, Nathanael J., Garmire, Lana X., McDonald, Jeffrey G., Myers, David S., Milne, Stephen B., Shibata, Norihito, . . . Glass, Christopher K. (2012). Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. *Cell, 151*(1), 138-152. doi: 10.1016/j.cell.2012.06.054

Stahl, P. D. (1992). The mannose receptor and other macrophage lectins. *Current Opinion in Immunology, 4*(1), 49-52.

Stein, M., Bagnoli, F., Halenbeck, R., Rappuoli, R., Fantl, W. J., & Covacci, A. (2002). c-Src/Lyn kinases activate *Helicobacter pylori* CagA through tyrosine phosphorylation of the EPIYA motifs. *Molecular Microbiology, 43*(4), 971-980.

Stoler, S., Keith, K. C., Curnick, K. E., & Fitzgerald-Hayes, M. (1995). A mutation in CSE4, an essential gene encoding a novel chromatin-associated protein in yeast, causes chromosome nondisjunction and cell cycle arrest at mitosis. *Genes & Development, 9*(5), 573-586.

Strobel, S., Bereswill, S., Balig, P., Allgaier, P., Sonntag, H. G., & Kist, M. (1998). Identification and analysis of a new vacA genotype variant of *Helicobacter pylori* in different patient groups in Germany. *Journal of Clinical Microbiology, 36*(5), 1285-1289.

Suerbaum, S., & Achtman, M. (2004). *Helicobacter pylori:* recombination, population structure and human migrations. *International Journal of Medical Microbiology, 294*(2-3), 133-139. doi: 10.1016/j.ijmm.2004.06.014

Suzuki, M., Miura, S., Suematsu, M., Fukumura, D., Kurose, I., Suzuki, H., . . . Tsuchiya, M. (1992). *Helicobacter pylori*-associated ammonia production enhances neutrophil-dependent gastric mucosal cell injury. *American Journal of Physiology, 263*(5 Pt 1), G719-725.

Suzuki, T., Kato, K., Ohara, S., Noguchi, K., Sekine, H., Nagura, H., & Shimosegawa, T. (2002). Localization of antigen-presenting cells in *Helicobacter pylori*-infected gastric mucosa. *Pathology International, 52*(4), 265-271.

Takeda, David Y., & Dutta, Anindya. (2005). DNA replication and progression through S phase. *Oncogene, 24*(17), 2827-2843.

Tay, Chin Yen, Mitchell, Hazel, Dong, Quanjiang, Goh, Khean-Lee, Dawes, Ian W., & Lan, Ruiting. (2009). Population structure of Helicobacter pylori among ethnic groups in Malaysia: recent acquisition of the bacterium by the Malay population. *BMC Microbiology, 9*, 126-126. doi: 10.1186/1471-2180-9-126

Teneberg, S., Miller-Podraza, H., Lampert, H. C., Evans, D. J., Jr., Evans, D. G., Danielsson, D., & Karlsson, K. A. (1997). Carbohydrate binding specificity of the neutrophil-activating protein of Helicobacter pylori. *The Journal of Biological Chemistry, 272*(30), 19067-19071.

Testerman, Traci L., & Morris, James. (2014). Beyond the stomach: An updated view of *Helicobacter pylori* pathogenesis, diagnosis, and treatment. *World Journal of Gastroenterology : WJG, 20*(36), 12781-12808. doi: 10.3748/wjg.v20.i36.12781

Thacker, J., & Zdzienicka, M. Z. (2003). The mammalian XRCC genes: their roles in DNA repair and genetic stability. *DNA Repair (Amst), 2*(6), 655-672.

Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., . . . Venter, J. C. (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature, 388*(6642), 539-547. doi: 10.1038/41483

Torres, J., Perez-Perez, G., Goodman, K. J., Atherton, J. C., Gold, B. D., Harris, P. R., . . . Munoz, O. (2000). A comprehensive review of the natural history of Helicobacter pylori infection in children. *Archives of Medical Research, 31*(5), 431-469.

Torroella-Kouri, M., Silvera, R., Rodriguez, D., Caso, R., Shatry, A., Opiela, S., . . . Lopez, D. M. (2009). Identification of a subpopulation of macrophages in mammary tumor-bearing mice that are neither M1 nor M2 and are less differentiated. *Cancer Research, 69*(11), 4800-4809. doi: 10.1158/0008-5472.can-08-3427

Toyoshima-Morimoto, F., Taniguchi, E., & Nishida, E. (2002). Plk1 promotes nuclear translocation of human Cdc25C during prophase. *EMBO Rep, 3*(4), 341-348. doi: 10.1093/embo-reports/kvf069

Tsou, C. L., Peters, W., Si, Y., Slaymaker, S., Aslanian, A. M., Weisberg, S. P., . . . Charo, I. F. (2007). Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *Journal of Clinical Investigation, 117*(4), 902-909. doi: 10.1172/jci29919

Uemura, N., Okamoto, S., Yamamoto, S., Matsumura, N., Yamaguchi, S., Yamakido, M., . . . Schlemper, R. J. (2001). *Helicobacter pylori* infection and the development of gastric cancer. *The New England Journal of Medicine, 345*(11), 784-789. doi: 10.1056/NEJMoa001999

Vairapandi, M., Balliet, A. G., Fornace, A. J., Jr., Hoffman, B., & Liebermann, D. A. (1996). The differentiation primary response gene MyD118, related to GADD45, encodes for a nuclear protein which interacts with PCNA and p21WAF1/CIP1. *Oncogene, 12*(12), 2579-2594.

Van Doorn, L. J., Figueiredo, C., Megraud, F., Pena, S., Midolo, P., Queiroz, D. M., . . . Quint, W. G. (1999). Geographic distribution of vacA allelic types of *Helicobacter pylori*. *Gastroenterology, 116*(4), 823-830.

Vieira, Otilia V., Botelho, Roberto J., & Grinstein, Sergio. (2002). Phagosome maturation: aging gracefully. *Biochemical Journal, 366*(Pt 3), 689-704. doi: 10.1042/BJ20020691

Wang, N., Liang, H., & Zen, K. (2014). Molecular Mechanisms That Influence the Macrophage M1–M2 Polarization Balance. *Frontiers in Immunology, 5*, 614. doi: 10.3389/fimmu.2014.00614

Wang, W.C., Wang, H.J., & Kuo, C.H. (2001). Two distinctive cell binding patterns by vacuolating toxin fused with glutathione S-transferase: one high-affinity m1-specific binding and the other lower-affinity binding for variant m forms. *Biochemistry, 40*(39), 11887-11896.

Warburton, P. E., Cooke, C. A., Bourassa, S., Vafa, O., Sullivan, B. A., Stetten, G., . . . Earnshaw, W. C. (1997). Immunolocalization of CENP-A suggests a distinct nucleosome structure at the inner kinetochore plate of active centromeres. *Current Biology, 7*(11), 901-904.

Warszawska, Joanna M., Gawish, Riem, Sharif, Omar, Sigel, Stefanie, Doninger, Bianca, Lakovits, Karin, . . . Knapp, Sylvia. (2013). Lipocalin 2 deactivates macrophages and worsens pneumococcal pneumonia outcomes. *The Journal of Clinical Investigation, 123*(8), 3363-3372. doi: 10.1172/JCI67911

Waters, J. C., Chen, R. H., Murray, A. W., Gorbsky, G. J., Salmon, E. D., & Nicklas, R. B. (1999). Mad2 binding by phosphorylated kinetochores links error detection and checkpoint action in mitosis. *Current Biology, 9*(12), 649-652.

Weeks, David L., Eskandari, Sepehr, Scott, David R., & Sachs, George. (2000). A H+-Gated Urea Channel: The Link Between Helicobacter pylori Urease and Gastric Colonization. *Science, 287*(5452), 482-485. doi: 10.1126/science.287.5452.482

Weibel, Ewald R., & Palade, George E. (1964). New cytoplasmic components in arterial endothelial. *The Journal of Cell Biology, 23*(1), 101-112.

Weischenfeldt, J., & Porse, B. (2008). Bone Marrow-Derived Macrophages (BMM): Isolation and Applications. *Cold Spring Harbour Protocol, 2008*, pdb prot5080. doi: 10.1101/pdb.prot5080

Weiss, G., Forster, S., Irving, A., Tate, M., Ferrero, R. L., Hertzog, P., . . . Kaparakis-Liaskos, M. (2013). *Helicobacter pylori* VacA suppresses Lactobacillus acidophilus-induced interferon beta signaling in macrophages via alterations in the endocytic pathway. *MBio, 4*(3), e00609-00612. doi: 10.1128/mBio.00609-12

Weitzman, S. A., & Gordon, L. I. (1990). Inflammation and cancer: role of phagocyte-generated oxidants in carcinogenesis. *Blood, 76*(4), 655-663.

Wells, W. A., & Murray, A. W. (1996). Aberrantly segregating centromeres activate the spindle assembly checkpoint in budding yeast. *Journal Cell Biology, 133*(1), 75-84.

Wilson, J. M., de Hoop, M., Zorzi, N., Toh, B. H., Dotti, C. G., & Parton, R. G. (2000). EEA1, a tethering protein of the early sorting endosome, shows a polarized distribution in hippocampal neurons, epithelial cells, and fibroblasts. *Molecular Biology of the Cell, 11*(8), 2657-2671.

Wroblewski, L. E., Peek, R. M., Jr., & Wilson, K. T. (2010). *Helicobacter pylori* and gastric cancer: factors that modulate disease risk. *Clinical Microbiology Reviews, 23*(4), 713-739. doi: 10.1128/cmr.00011-10

Wu, Ming-Hsun, Lee, Wei-Jiunn, Hua, Kuo-Tai, Kuo, Min-Liang, & Lin, Ming-Tsan. (2015). Macrophage Infiltration Induces Gastric Cancer Invasiveness by Activating the β-Catenin Pathway. *PLoS ONE, 10*(7), e0134122. doi: 10.1371/journal.pone.0134122

Xaus, Jordi, Comalada, Mònica, Valledor, Annabel F., Cardó, Marina, Herrero, Carmen, Soler, Concepció, . . . Celada, Antonio. (2001). Molecular Mechanisms Involved in Macrophage Survival, Proliferation, Activation or Apoptosis. *Immunobiology, 204*(5), 543-550. doi: 10.1078/0171-2985-00091

Xu, S., Wan, X., Zheng, X., Zhou, Y., Song, Z., Cheng, M., . . . Hou, X. (2013). Symptom improvement after helicobacter pylori eradication in patients with functional dyspepsia-A multicenter, randomized, prospective cohort study. *International Journal of Clinical Experiment Medicine, 6*(9), 747-756.

Xu, Y., & Fisher, G. J. (2012). Receptor type protein tyrosine phosphatases (RPTPs) – roles in signal transduction and human disease. *Journal of Cell Communication and Signaling, 6*(3), 125-138. doi: 10.1007/s12079-012-0171-5

Yadav, Santosh Kumar, Sah, Ajit Kumar, Jha, Rajesh Kumar, Sah, Phoolgen, & Shah, Dev Kumar. (2013). Turmeric (curcumin) remedies gastroprotective action. *Pharmacognosy Reviews, 7*(13), 42-46. doi: 10.4103/0973-7847.112843

Yamamoto, E., Suzuki, H., Takamaru, H., Yamamoto, H., Toyota, M., & Shinomura, Y. (2011). Role of DNA methylation in the development of diffuse-type gastric cancer. *Digestion, 83*(4), 241-249. doi: 10.1159/000320453

Yang, Jiyeon, Zhang, Lixiao, Yu, Caijia, Yang, Xiao-Feng, & Wang, Hong. (2014). Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomarker Research, 2*, 1-1. doi: 10.1186/2050-7771-2-1

Yao, Yongliang, Li, Guangxin, Wu, Jianhong, Zhang, Xian, & Wang, Jianjun. (2015). Inflammatory response of macrophages cultured with *Helicobacter pylori* strains was regulated by miR-155. *International Journal of Clinical and Experimental Pathology, 8*(5), 4545-4554.

Young, H. A. (1996). Regulation of interferon-gamma gene expression. *Journal of Interferon & Cytokine Research, 16*(8), 563-568.

Zabaleta, Jovanny, McGee, David J., Zea, Arnold H., Hernández, Claudia P., Rodriguez, Paulo C., Sierra, Rosa A., . . . Ochoa, Augusto C. (2004). *Helicobacter pylori* Arginase Inhibits T Cell Proliferation and Reduces the Expression of the TCR ζ-Chain (CD3ζ). *The Journal of Immunology, 173*(1), 586-593. doi: 10.4049/jimmunol.173.1.586

Zanotti, G., Papinutto, E., Dundon, W., Battistutta, R., Seveso, M., Giudice, G., . . . Montecucco, C. (2002). Structure of the neutrophil-activating protein from *Helicobacter pylori*. *Journal of Molecular Biology, 323*(1), 125-130.

Zhan, Q., Antinore, M. J., Wang, X. W., Carrier, F., Smith, M. L., Harris, C. C., & Fornace, A. J., Jr. (1999). Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. *Oncogene, 18*(18), 2892-2900. doi: 10.1038/sj.onc.1202667

Zhan, Q., Lord, K. A., Alamo, I., Jr., Hollander, M. C., Carrier, F., Ron, D., . . . Fornace, A. J., Jr. (1994). The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. *Molecular Cell Biology, 14*(4), 2361-2371.

Zhang, W., Bae, I., Krishnaraju, K., Azam, N., Fan, W., Smith, K., . . . Liebermann, D. A. (1999). CR6: A third member in the MyD118 and Gadd45 gene family which functions in negative growth control. *Oncogene, 18*(35), 4899-4907. doi: 10.1038/sj.onc.1202885

Zheng, P. Y., & Jones, N. L. (2003). *Helicobacter pylori* strains expressing the vacuolating cytotoxin interrupt phagosome maturation in macrophages by recruiting and retaining TACO (coronin 1) protein. *Cell Microbiology, 5*(1), 25-40.

Zhou, Delu, Li, Ping, Lin, Yinling, Lott, Jeremy M., Hislop, Andrew D., Canaday, David H., . . . Blum, Janice S. (2005). Lamp-2a Facilitates MHC Class II Presentation of Cytoplasmic Antigens. *Immunity, 22*(5), 571-581. doi: 10.1016/j.immuni.2005.03.009

Zhou, H., Liao, J., Aloor, J., Nie, H., Wilson, B. C., Fessler, M. B., . . . Hong, J. S. (2013). CD11b/CD18 (Mac-1) is a novel surface receptor for extracellular double-stranded RNA to mediate cellular inflammatory responses. *Journal of Immunology, 190*(1), 115-125. doi: 10.4049/jimmunol.1202136

Zhuang, Y., Shi, Y., Liu, X. F., Zhang, J. Y., Liu, T., Fan, X., . . . Zou, Q. M. (2011). *Helicobacter pylori*-infected macrophages induce Th17 cell differentiation. *Immunobiology, 216*(1-2), 200-207. doi: 10.1016/j.imbio.2010.05.005

LIST OF PUBLICATIONS AND PRESENTATIONS

Publication

1. Tan, Grace Min Yi, Looi, Chung Yeng, Fernandez, Keith Conrad, Vadivelu, Jamuna, Loke, Mun Fai, & Wong, Won Fen. (2015). Suppression of cell division-associated genes by Helicobacter pylori attenuates proliferation of RAW264.7 monocytic macrophage cells. *Scientific Reports, 5*, 11046. doi: 10.1038/srep11046

Poster presentation

1. Grace Tan Min Yi, Chung Yeng Looi, Alicia Phan Yiling, Jamunarani Vadivelu, Loke Mun Fai and Wong Won Fen. Inhibition of Aurora B kinase, cyclins and Cdks transcription by *Helicobacter pylori* attenuates cell cycle progress in monocytic macrophage cells. Presented at Immunology Symposium 2014, 16-17 October 2014, University Putra Malaysia, Malaysia.

2. Grace Tan Min Yi, Chung Yeng Looi, Keith Conrad, Loke Mun Fai and Wong Won Fen. Suppression of cell-division associated genes by *Helicobacter pylori* attenuates proliferation of RAW264.7 monocytic macrophage cells. Presented at International Congress of Malaysian Society of Microbiology (ICMSM) 2015, 7-10 December 2015, Batu Feringhi, Penang, Malaysia.

Workshops/ Courses

1. Basic Immunology Course. 23rd to 27th July 2013. University Malaya

2. Animal Experimental Unit (AEU) induction course. 2nd April 2014. University Malaya

3. BD FACS Canto II Flow Cytometer Basic Operator Training. 10th to 12th December 2014. University Malaya

Awards

1. Malaysian Toray Science Foundation Science and Technology Research Grant recipient. Malaysia Toray Science Foundation (MTSF). 2015.

2. UM Fellowship Scheme. University Malaya. 2014-2016.

3. Best poster award recipient. International Congress of Malaysian Society of Microbiology (ICMSM) 2015.

SCIENTIFIC REPORTS

OPEN

Suppression of cell division-associated genes by *Helicobacter pylori* attenuates proliferation of RAW264.7 monocytic macrophage cells

Received: 13 December 2014
Accepted: 07 May 2015
Published: 16 June 2015

Grace Min Yi Tan[1], Chung Yeng Looi[2], Keith Conrad Fernandez[1], Jamuna Vadivelu[1], Mun Fai Loke[1] & Won Fen Wong[1,3]

***Helicobacter pylori* at multiplicity of infection (MOI ≥ 50) have been shown to cause apoptosis in RAW264.7 monocytic macrophage cells. Because chronic gastric infection by *H. pylori* results in the persistence of macrophages in the host's gut, it is likely that *H. pylori* is present at low to moderate, rather than high numbers in the infected host. At present, the effect of low-MOI *H. pylori* infection on macrophage has not been fully elucidated. In this study, we investigated the genome-wide transcriptional regulation of *H. pylori*-infected RAW264.7 cells at MOI 1, 5 and 10 in the absence of cellular apoptosis. Microarray data revealed up- and down-regulation of 1341 and 1591 genes, respectively. The expression of genes encoding for DNA replication and cell cycle-associated molecules, including *Aurora-B kinase (AurkB)* were down-regulated. Immunoblot analysis verified the decreased expression of AurkB and downstream phosphorylation of Cdk1 caused by *H. pylori* infection. Consistently, we observed that *H. pylori* infection inhibited cell proliferation and progression through the G1/S and G2/M checkpoints. In summary, we suggest that *H. pylori* disrupts expression of cell cycle-associated genes, thereby impeding proliferation of RAW264.7 cells, and such disruption may be an immunoevasive strategy utilized by *H. pylori*.**

Helicobacter pylori (*H. pylori*) is a Gram-negative, microaerophilic bacterium that colonizes the human gastric and duodenal mucosal epithelium. It is a well-established causative agent of gastritis and duodenal ulcer, and is a significant risk factor of gastric adenocarcinoma[1]. *H. pylori* is often incompletely eradicated and is able to persist in host for life[2].

Gastritis in the acute *H. pylori* infection is predominantly mediated by macrophages[3]. A transient depletion of macrophages during *H. pylori* infection reduces the gastric pathology in animal model[3]. Normal gastric mucosa in an adult is populated by small population of macrophage[4]. During *H. pylori* infection, surface and secreted proteins from *H. pylori* act as chemoattractant and induce circulating monocytes to infiltrate the gastric epithelium[5,6], which subsequently differentiate to enlarge the macrophage population at the infection site. Besides, the *H. pylori*-induced gastric epithelial damage enables the bacteria to enter lamina propria and encounter macrophages[7]. Macrophages infiltration into gastric mucosa is detected in the *H. pylori*-infected patients[8], which can function to capture the bacteria[7]. Both M1 and M2 macrophages are detected in gastric biopsy specimens isolated from *H. pylori*-infected

[1]Department of Medical Microbiology, [2]Department of Pharmacology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia. [3]Tropical Infectious Disease Research and Education Center, University of Malaya, 50603 Kuala Lumpur, Malaysia. Correspondence and requests for materials should be addressed to M.F.L. (email: lokemunfai@um.edu.my) or W.F.W. (email: wonfen@um.edu.my)

Inhibition of Aurora B kinase, Cyclins and Cdks transcriptions by *Helicobacter pylori* attenuates cell cycle progress in monocytic macrophage cells

Grace Tan Min Yi, Chung Yeng Looi, Alicia Yiling Phan, Jamunarani Vadivelu, Mun Fai Loke, Won Fen Wong
Department of Medical Microbiology, Faculty of Medicine, University Malaya, Kuala Lumpur Malaysia.

Introduction

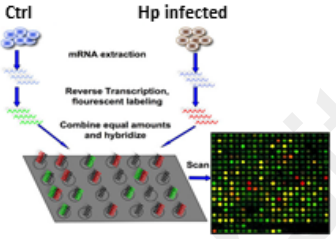
Helicobacter pylori (Hp) is a gram-negative bacterium that colonizes stomach and causes gastritis, peptic ulcer and gastric cancer. Host immune defense against *H. pylori* is predominated by macrophages and infiltrated monocytes. In this study, we investigated the response of RAW264.7 murine monocytic macrophage cells after infection with of *H. pylori*.

We demonstrated that *H. pylori* elicited cell activation, cell enlargement, surface expression of CD83 and CD86, and expression of pro-inflammatory cytokines such as TNF, IL-1βand IL-23. In genome-wide microarrray, two of the genes of top 10 downregulated genes were cell cycle regulators, namely, *Aurora B kinase* (*Aurkb*) and *Myb related protein B* (*Mybl2*). Furthermore, transcriptions of multiple Cyclins- and Cdks-encoding genes were substantially suppressed.

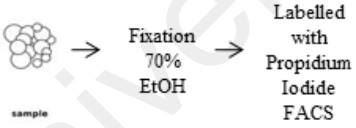
Methodology

Innoculation of Hp into RAW264.7 cells

Microarray analysis



Cell cycle analysis



Immunoblot analysis

Results & Discussion

Figure 1

Immune response expression profiles of Hp infected RAW cells

Heat map analysis of the expression of genes in cell cycle, surface markers, cytokines

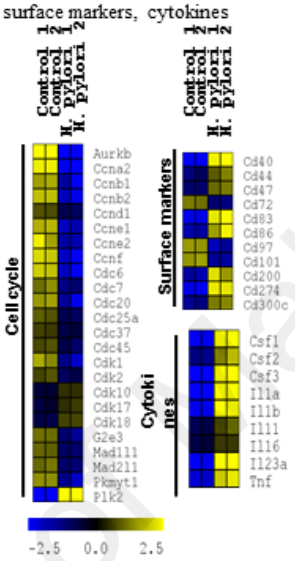


Figure 2

QPCR analysis of cell cycle progress-associated genes

Relative fold change shows expression of each gene relative to internal control β-actin. "-" is non infected control while "+" is Hp-infected cells.

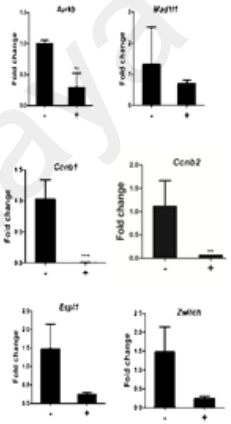
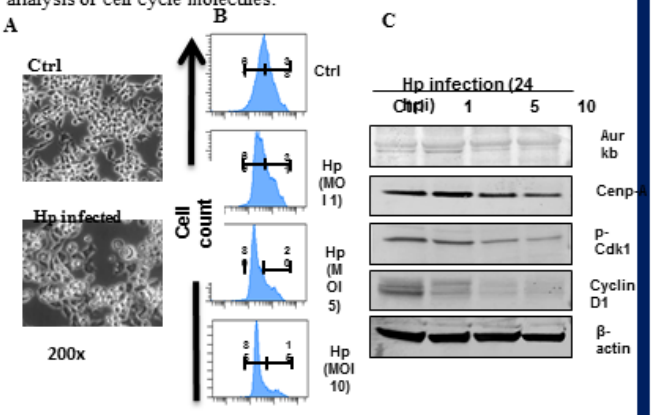


Figure 3. Hp infection impaired cell proliferation and cell cycle of RAW cells.

(A) Pictures of Control and infected RAW cells – Infected cells shown enlargement (B) Flow cytometry analysis of marker Ki67 – Hp infection dampens proliferation of macrophage cells. (C) Immunoblot analysis of cell cycle molecules.



Conclusion

Hp disrupts transactivation of cell cycle-associated genes therefore impedes the proliferation of host monocytic macrophage cells.

Acknowledgements

This research is supported by grant High Impact Research H50001-00-A000029 and RG525-13HTM

Suppression of cell division-associated genes by *Helicobacter pylori* attenuates proliferation of RAW264.7 monocytic macrophages

Grace Tan Min Yi, Chung Yeng Looi, Keith Conrad, Mun Fai Loke* and Won Fen Wong*
Department of Medical Microbiology, Faculty of Medicine, University Malaya, Kuala Lumpur Malaysia.

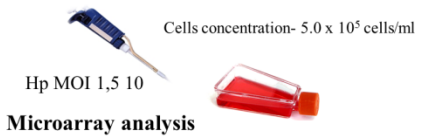
Introduction

Helicobacter pylori (Hp) is a gram-negative bacterium that colonize the stomach and causes gastritis and duodenal ulcer. Acute gastritis caused by Hp infection is predominantly mediated by macrophages. In this study, we investigated the response of Hp-infected RAW264.7 murine monocytic macrophage cells at low multiple of infection (MOI).

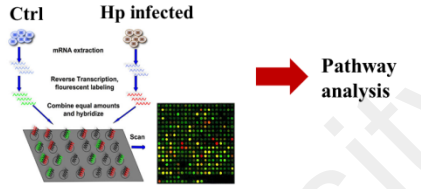
We demonstrated at low MOI, Hp is insufficient in causing apoptosis, but it suppressed the expression of genes that encode for DNA synthesis and cell-cycle associated molecules, which disrupts the cell proliferation and cell cycle progression in these Hp-infected RAW264.7 cells.

Methodology

Innoculation of Hp into RAW264.7 cells

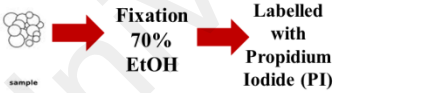


Microarray analysis

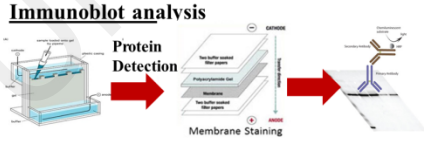


Cell proliferation assay

Cell cycle analysis



Immunoblot analysis



Results & Discussion

Figure 1

Immune response expression profiles of Hp infected RAW cells

Heat map analysis of the expression of genes in cell cycle, surface markers and cytokines

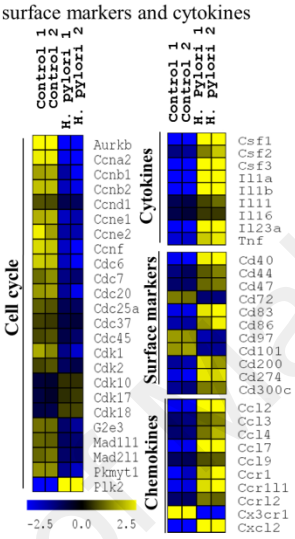


Figure 2

q-RTPCR analysis of cell cycle progress-associated genes

Relative fold change shows expression of each gene relative to internal control β-actin. "-" is non infected control while "+" is Hp-infected cells.

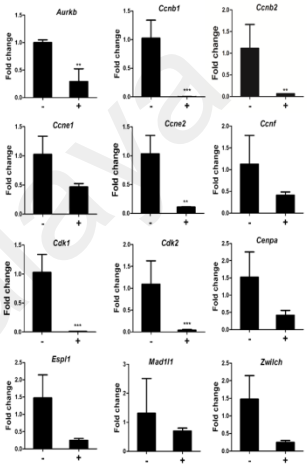
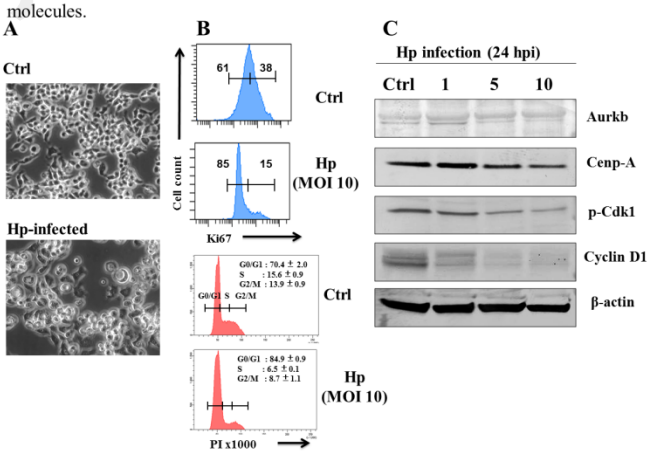


Figure 3. Hp infection impaired cell proliferation and cell cycle of RAW cells.

(A) Pictures of Control and infected RAW cells– infected cells shown enlargement (B) Flow cytometry analysis of marker Ki67 and cell cycle analysis – Hp infection dampens proliferation of macrophage cells and attenuates cell cycle at G2/M phase (C) Immunoblot analysis of cell cycle molecules.



Acknowledgements

This research is supported by grant High Impact Research H50001-00-A000029 and RG525-13 HTM

Conclusion

Hp disrupts transactivation of cell cycle-associated genes therefore impedes the proliferation of host monocytic macrophage cells.

APPENDICES

APPENDIX A: PREPARATION OF CULTURE MEDIA, REAGENTS AND CHEMICALS

A1 Bacterial culture medium

A1.1 Brain Heart Infusion (BHI) broth

Brain Heart Infusion (Difco, USA)	18.5 g
MiliQ water	500 mL

The brain heart infusion powder was dissolved in miliQ water until it completely dissolved. The solution was then autoclaved at 121^{o}C for 20 minutes. The solution was cooled down and store at 4^{o}C prior to use.

A1.2 10% glycerol (v/v) Brain Heart Infusion broth supplemented with yeast extract and fetal calf serum

Brain Heart Infusion powder (Difco, USA)	18.5 g
Yeast extract powder (Oxoid, UK)	2 g
Fetal Calf Serum (Gibco, USA)	50 mL
Glycerol (Sigma Aldrich, USA)	50 mL
miliQ water	400 mL

The brain heart infusion and yeast extract powder was dissolved in miliQ water and 50 mL of glycerol was then added into the solution. The solution was then autoclaved before adding glycerol prior use. The solution is store at 4^{o}C prior use.

A2 Cell culture media and reagents

A2.1 Dulbecco Modified Essential Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS)

Dulbecco Modified Essential Medium (Gibco, USA)	500 mL
Fetal Bovine Serum (Gibco, USA)	50 mL

Fetal Bovine serum was thawed and heat inactivated at 56^{o}C for 30 minutes with constant swirling, before adding it to Dulbecco Modified Essential Medium.

A2.2 Phosphate Buffered Saline (PBS) solution

PBS tablet (Oxoid, UK)	5 tablets
miliQ water	500 mL

The solution was autoclaved and stored at 4^{o}C.

A2.3 2x Freezing Medium

Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA)	25 mL
Fetal Bovine Serium (Gibco, USA)	20 mL
Dimethyl Sulfoxide (DMSO) (Pierce, USA)	5 mL

2x Freezing medium is stored at 4^{o}C and covered in aluminium foil. For freezing, cells were mixed with 1:1 mixture of DMEM supplemented 10% FBS.

A3 Immunoblot analysis

A3.1 Preparation of Radioimmunoprecipitation (RIPA) lysis buffer

RIPA lysis buffer (Santa Cruz Biotech, Santa Cruz, CA)	5 mL
100 x Sodium orthovanadate ($Na_3VO_{3)}$	50 µL
100 x phenlymethylsulfonyl fluoride (PMSF)	50 µL
100 x Protease Inhibitor cocktail (PI)	50 µL

RIPA lysis buffer was prepared fresh prior use. Lysis buffer solution was thawed on ice, before adding Na_3VO_3, PMSF and PI. Freshly prepared solution was kept on ice to prevent degradation.

A3.2 Preparation of 1M Tris buffer (pH 7.4)

Tris-Hydrochloric Acid (Tris-HCl) (Promega, USA)	60.7 g
miliQ water	500 mL

Tris-HCl was weigh and dissolved in 400 mL miliQ water. pH was adjusted to pH 7.4 with the appropriate volume of concentrated HCl. Solution was top up to 500 mL .

A3.3 Preparation of 1M Tris buffer (pH 9.5)

Tris (Promega, USA)	60.7 g
miliQ water	500 mL

Tris-HCl was weigh and dissolved in 400 mL miliQ water. pH was adjusted to pH 9.5 with the appropriate volume of concentrated NaOH. Solution was top up to 500 mL .

A3.4 Preparation of 5M Sodium Chloride (NaCl)

Sodium chloride (NaCl) (Nacalai Tesque, Japan)	146 g
miliQ water	500 mL

NaCl was weighed and dissolved in 500 mL miliQ water.

A3.5 Preparation of 1M Magnesium Chloride ($MgCl_2$)

Magnesium chloride ($MgCl_2$) (Nacalai Tesque, Japan)	101.65 g
miliQ water	500 mL

$MgCl_2$was weighed and dissolved in 500 mL miliQ water.

A3.6 Preparation of Tris-Buffered Saline-Tween 20 (TBS-T) solution

1M Tris pH 7.4	20 mL
5M Sodium chloride (NaCl) (Nacalai Tesque, Japan)	30 mL
miliQ water	950 mL
Tween-20 (Sigma Aldrich, USA)	1 mL

Tween-20 solution was added while stirring the solution with magnetic stirrer on a hot plate. The solution was stored at 4^oC.

A3.7 Preparation of 5% Blocking buffer

Bovine Serum Albumin (BSA) (Merck, USA)	5 g
TBS-T solution	100 mL

BSA was weighed and dissolved in 100 mL while stirring with magnetic stirrer. The solution was prepared fresh prior use.

A3.8 Staining buffer

1M Tris pH 9.5	1 mL
5M Sodium chloride (NaCl) (Nacalai Tesque, Japan)	200 µL
1M Magnesium chloride ($MgCl_2$) (Nacalai Tesque, Japan)	500µL
miliQ water	8.75 mL
Nitro-blue tetrazolium (NBT) substrate (Promega, USA)	66 µL
5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrate (Promega, USA)	33 µL

The solution was prepared fresh and covered in aluminium foil in order to protect from light.

A4 Bone marrow derived-macrophage (BMDM) isolation

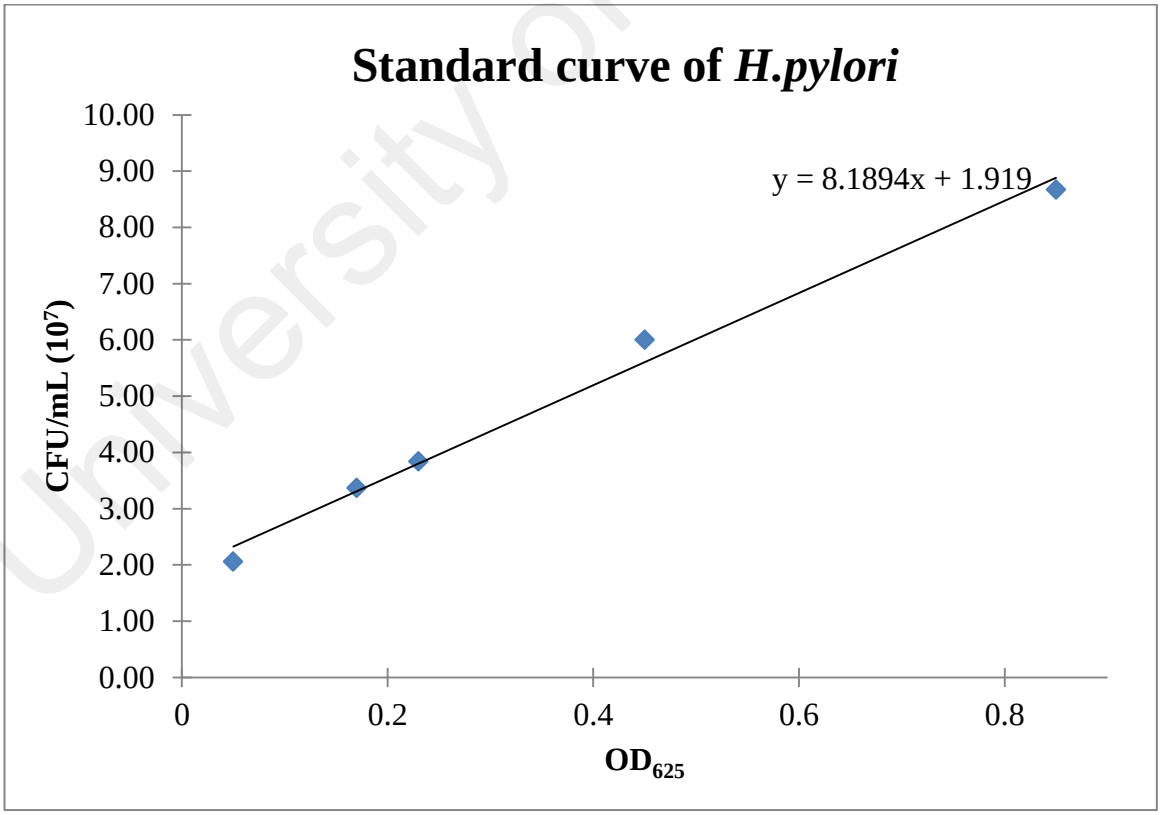
A4.1 Complete medium

RPMI 1640 supplemented with L-glutamine (Gibco, USA)	45 mL
Fetal Bovine Serum (Gibco, USA)	5 mL
10 mg /mL streptomycin (Gibco, USA)	500 µL
10000 U/mL penicillin (Gibco, USA)	500 µL
1 M HEPES (Gibco, USA)	500 µL
20 µg/mL Macrophage-Colony stimulating factor (M-CSF) (Biolegend, CA)	5 µL

The medium was prepared aseptically and kept at 4^{o}C prior use.

APPENDIX B: STANDARD CURVE OF *HELICOBACTER PYLORI*

1. A few colonies of *H. pylori* were picked up and resuspended in 1 mL of Brain Heart Infusion (BHI) broth. The mixture was vortexed to ensure no clumps of bacteria.
2. The Optical Density (OD) reading of the stock was taken and recorded at 625nm.
3. The stock was further diluted at 2x, 4x, 8x, 10x and 12x with BHI broth, and 200 µL of each of the dilution was taken for OD_{625} reading.
4. Each dilution was further diluted to 10^{-8} by adding 1 suspension: 9 BHI broth.
5. 20 µL of each dilution was dropped on the surface of the agar according to Miles and Misra method.
6. The plates were left upright on the bench to dry before inversion and incubate at $37^{o}C$, 10% $CO_{2.}$
7. Colonies that were in the range of 2-20 colonies were counted in the section after three incubation days.



APPENDIX C: FOLD CHANGE OF THE TOP 10 UPREGULATED AND DOWNREGULATED GENES IN qRT-PCR ANALYSIS

Gene	Mean fold change	P value
Car6	+11.1	0.0025
Csf3	+5.3	0.1103
Lcn2	+572.3	0.0004
Ccl7	+2738	0.006
Ptgs2	+106.6	0.229
Cxcl2	+3.4	0.042
Il1b	+4982.1	0.0057
Pappa2	+7.7	0.0005
Csf1	+23.3	0.210
Avil	+11.9	0.006
Cx3cr1	-5.4	0.2881
Fgd2	-1.5	0.611
Kbtd11	-1.5	0.189
Dhcr24	-2.43	0.022
Alox5	-19.1	0.008
Aurkb	-3.5	0.051
Ankle1	-1.6	0.440
Lrp8	-1.1	0.712

APPENDIX D: SIGNIFICANT PATHWAYS FOR DAVID ANALYSIS

Term	Genes
mmu04142:Lysosome	NAGLU, MFSD8, ATP6AP1, AP3S1, CTSA, ACP2, ATP6V0B, ATP6V0C, SLC11A2, AP1S3, SLC11A1, AP3M2, TPP1, NAGA, ATP6V0D1, ATP6V0D2, IDUA, TCIRG1, CLN3, CTSZ, PSAP, ATP6V1H, CD63, GNS, CTSL, NPC1, SLC17A5, LAMP2, GAA, ATP6V0A1, CTSD, GGA1, CLN5
mmu05340:Primary immunodeficiency	IKBKG, LCK, AICDA, IL2RG, CD40, IL7R, BLNK
mmu00970:Aminoacyl-tRNA biosynthesis	WARS, YARS, NARS, RARS, SARS, AARS, LARS, EPRS
mmu00520:Amino sugar and nucleotide sugar metabolism	AMDHD2, CYB5R1, RENBP, GNPDA1, HK3, GFPT1, GNPNAT1, FPGT
mmu04512:ECM-receptor interaction	ITGB4, ITGB5, ITGB3, COL5A3, VWF, CD47, CD36, CD44, ITGA6, ITGB7, ITGAV, ITGA7, SPP1
mmu05410:Hypertrophic cardiomyopathy (HCM)	TNF, ITGA6, TNNC1, ITGAV, ITGB7, ITGA7, MYBPC3, ITGB4, ITGB5, ITGB3, TPM1, SGCB
mmu05414:Dilated cardiomyopathy	TNF, TNNC1, MYBPC3, ITGB4, ITGB5, ITGB3, TPM1, PRKX, ITGA6, ITGB7, ITGAV, ITGA7, SGCB
mmu04060:Cytokine-cytokine receptor interaction	CSF3, CSF2, CCL3, CCL2, TNF, CSF2RB2, CCR1, CSF1, CXCL2, CCL9, TNFSF12, IL7R, CCL4, CCL7, IL11, TNFRSF1B, IL23A, IL10RB, CSF2RB, IL1B, IL2RG, FAS, IL1A, LTA, IL11RA1, CD40, OSM, VEGFC, ACVR2A, CCR1L1, ACVR1

APPENDIX D, continued

Term	Genes
mmu00190:Oxidative phosphorylation	TCIRG1, NDUFA3, ATP6AP1, ATP6V1H, ATP6V1G1, ATP6V1D, ATP6V0B, ATP6V1F, ATP6V0C, ATP6V1C1, ATP6V1A, ATP6V1E1, COX6A2, ATP6V0A1, ATP6V0D1, ATP6V0D2
mmu03030:DNA replication	POLA1, POLA2, RPA1, PRIM1, RPA2, MCM7, POLE2, PRIM2, FEN1, LIG1, POLE, MCM2, RNASEH2A, MCM3, MCM4, RNASEH2C, MCM5, MCM6, RFC5, DNA2, RFC3, RFC4, RFC1, RFC2, POLD1, POLD2, PCNA
mmu03430:Mismatch repair	EXO1, MSH6, MSH2, LIG1, RFC5, RPA1, RPA2, RFC3, RFC4, RFC1, RFC2, POLD1, POLD2, PCNA, PMS2
mmu00100:Steroid biosynthesis	TM7SF2, CYP51, EBP, SQLE, DHCR7, LSS, HSD17B7, SC4MOL, NSDHL, DHCR24, FDFT1
mmu00900:Terpenoid backbone biosynthesis	MVD, HMGCR, FDPS, HMGCS1, MVK, PMVK, ACAT2, IDI1, ACAT1, PDSS1
mmu03420:Nucleotide excision repair	LIG1, POLE, RFC5, RPA1, RPA2, RFC3, RFC4, POLE2, RFC1, RFC2, POLD1, POLD2, DDB2, PCNA
mmu00030:Pentose phosphate pathway	PGM2, PGLS, PFKL, ALDOC, PGD, TKT, PFKM, RPIA, GPI1, PRPS2
mmu01040:Biosynthesis of unsaturated fatty acids	SCD1, SCD3, SCD2, ELOVL5, FADS1, FADS2, ACOT1, ELOVL6, ACAA1A, ACAA1B
mmu01040:Biosynthesis of unsaturated fatty acids	SCD1, SCD3, SCD2, ELOVL5, FADS1, FADS2, ACOT1, ELOVL6, ACAA1A, ACAA1B

APPENDIX D, continued

Term	Genes
mmu04540:Gap junction	PRKCA, CDK1, TUBB2A-PS2, TUBB2A, GNA11, TUBB2C, ITPR3, ITPR1, HRAS1, PLCB4, TUBB5, GUCY1B2, TUBB6, TUBA4A, PLCB2, TUBA1B, TUBB4
mmu05322:Systemic lupus erythematosus	HIST2H2AA2, HIST1H2AB, HIST4H4, HIST1H4K, HIST1H2AF, HIST1H2AG, GM5196, SNRPD1, CBX3, HIST2H3C1, C1RA, H2AFV, HIST2H2AC, H2AFZ, H2AFY, H2AFX, HIST1H4C, HIST1H4D, HIST1H4I, HIST1H4J, HIST1H2BB, H2-DMB1, H2AFJ, HIST2H4, HIST2H2BB, HIST1H2AH, HIST1H2AK, HIST1H3I, HIST1H2AN
mmu05215:Prostate cancer	E2F1, HSP90AB1, E2F2, PIK3CD, IGF1, CDK2, HRAS1, CCNE2, CCNE1, CCND1, HSP90B1, CDKN1A, MTOR, GSTP2, GSTP1, PIK3R1, PIK3R2
mmu00650:Butanoate metabolism	L2HGDH, ALDH5A1, OXCT1, HMGCS1, AACS, ACAT2, HADH, ACAT1, ALDH9A1
mmu00071:Fatty acid metabolism	ACAA2, POLR2L, ACAT2, HADH, ACAA1A, ACAA1B, ACAT1, CPT1A, ALDH9A1, ACSL5
mmu00010:Glycolysis / Gluconeogenesis	LDHC, LDHA, PFKL, ALDOC, HK1, PFKM, ACSS2, GPI1, PGM2, TPI1, PKM2, ENO3, ALDH9A1
mmu00280:Valine, leucine and isoleucine degradation	ACAA2, IVD, OXCT1, HMGCS1, ACAT2, HADH, ACAA1A, ACAA1B, ACAT1, ALDH9A1
mmu05222:Small cell lung cancer	E2F1, CKS1B, E2F2, PIK3CD, SKP2, BCL2L1, COL4A6, CDK2, CCNE2, CCNE1, CCND1, RARB, TRAF4, GM6340, PIK3R1, PIK3R2
mmu05214:Glioma	E2F1, PRKCA, E2F2, CDKN1A, CCND1, CAMK2G, PIK3CD, IGF1, MTOR, PIK3R1, PIK3R2, HRAS1

APPENDIX D, continued

Term	Genes
mmu03030:DNA replication	TM7SF2, CYP51, EBP, SQLE, DHCR7, LSS, HSD17B7, SC4MOL, NSDHL, DHCR24, FDFT1
mmu03430:Mismatch repair	MVD, HMGCR, FDPS, HMGCS1, MVK, PMVK, ACAT2, IDI1, ACAT1, PDSS1
mmu00100:Steroid biosynthesis	LIG1, POLE, RFC5, RPA1, RPA2, RFC3, RFC4, POLE2, RFC1, RFC2, POLD1, POLD2, DDB2, PCNA
mmu00900:Terpenoid backbone biosynthesis	PGM2, PGLS, PFKL, ALDOC, PGD, TKT, PFKM, RPIA, GPI1, PRPS2
mmu03420:Nucleotide excision repair	SCD1, SCD3, SCD2, ELOVL5, FADS1, FADS2, ACOT1, ELOVL6, ACAA1A, ACAA1B
mmu00030:Pentose phosphate pathway	SCD1, SCD3, SCD2, ELOVL5, FADS1, FADS2, ACOT1, ELOVL6, ACAA1A, ACAA1B
mmu01040:Biosynthesis of unsaturated fatty acids	PRKCA, CDK1, TUBB2A-PS2, TUBB2A, GNA11, TUBB2C, ITPR3, ITPR1, HRAS1, PLCB4, TUBB5, GUCY1B2, TUBB6, TUBA4A, PLCB2, TUBA1B, TUBB4
mmu01040:Biosynthesis of unsaturated fatty acids	HIST2H2AA2, HIST1H2AB, HIST4H4, HIST1H4K, HIST1H2AF, HIST1H2AG, GM5196, SNRPD1, CBX3, HIST2H3C1, C1RA, H2AFV, HIST2H2AC, H2AFZ, H2AFY, H2AFX, HIST1H4C, HIST1H4D, HIST1H4I, HIST1H4J, HIST1H2BB, H2-DMB1, H2AFJ, HIST2H4, HIST2H2BB, HIST1H2AH, HIST1H2AK, HIST1H3I, HIST1H2AN