IN VITRO ANALYSES OF TOLL-LIKE AND NOD-LIKE RECEPTORS AND *IN VIVO* INTERLEUKIN-1 RECEPTOR ANTAGONIST POLYMORPHISM DURING *HELICOBACTER PYLORI* INFECTION

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By

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CERTIFICATE

This is to certify that the thesis entitled **'*In vitro* analyses of Toll Like and Nod Like Receptors and *In vivo* Interleukin-1 Receptor Antagonist Polymorphism during *Helicobacter pylori* Infection'** submitted by **Mr. Suneesh Kumar Pachathundikandi**, in partial fulfilment of the requirement for the award of **Doctor of Philosophy in Immunology**, to the University of Calicut is an authentic research work carried out by him under my supervision and guidance in the Department of Biotechnology and the outcome of his research under DAAD (German Academic Exchange Service) Sandwich Fellowship has become a part of the thesis. This thesis has not previously formed the basis for the award of any degree, diploma or other similar title or recognition.

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DECLARATION

I hereby declare that the work presented in this thesis entitled **'*In vitro* analyses of Toll Like and Nod Like Receptors and *In vivo* Interleukin-1 Receptor Antagonist Polymorphism during *Helicobacter pylori* Infection'** submitted to the University of Calicut for the award of the Degree of **Doctor of Philosophy in Immunology**, is original and carried out by me under the supervision of Dr. M V Joseph, Professor, Department of Biotechnology, University of Calicut. This has not been submitted earlier either in part or in full for any degree or diploma of any university.

Mr. Suneesh Kumar Pachathundikandi

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Mr. Suneesh Kumar Pachathundikandi

ABBREVIATIONS

A	Ampere
ABCA-1	ATP-binding cassette, sub-family A member-1
AKT	Serine/threonine-specific protein kinase
AlpA	Adherence-associated lipoprotein A
AlpB	Adherence-associated lipoprotein B
AP-1	Activator protein-1
APCs	Antigen presenting cells
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
ASK-1	Apoptosis-regulating Signal Kinase
ATCC	American type culture collection
ATP	Adenosine triphosphate
BabA	Blood group antigen binding Adhesin
BC	Before Christ
B cells	B lymphocytes
BCL-10	B-cell CLL/lymphoma-10
BCR	B-Cell Receptor
BD-2	Beta-defensin-2
BD-3	Beta-defensin-3
BEAS-2B	Bronchial epithelial cell line
BHI	Brain heart Infusion
BIM/BOD	Bcl-2-related ovarian death gene
BIR	Baculoviral IAP repeats
BMAP-27	Bovine myeloid antimicrobial peptide-27
BMMs	Bone Marrow-derived macrophages
BSA	Bovine serum albumin
Caco-2	Epithelial colorectal adenocarcinoma cells
CagA	Cytoxin associated gene A
*cag*PAI	*Cag* pathogenecity island
cAMP	Cyclic Adenosine monophosphate
CARD	Caspase Recruiting Domain
CARDIAK	CARD containing ICE-associated Kinase
CATERPILLER	CARD, transcription enhancer, R (purine)-binding, pyrin, lots of LRRs
CBMC	Cord Blood-derived Mast Cells
CC	CC chemokine
CCL-5	CC chemokine ligand -5
CCL-20	CC chemokine ligand -20
CCR-6	CC chemokine receptor-6
CG	Cholesterol glucosides
c-Fos	Cytoplasmic Finkel–Biskis–Jinkins murine osteogenic sarcoma virus protein
CHO	Chinese Hamster Ovary cells
CINCA	Chronic Infentile Neurological Cutaneous and Articular syndrome
CIS	Cis-acting elements
CITA	Class II, major histocompatibility complex, transactivator
c-MET	Mesenchymal-epithelial transition factor
COS-1	CV-1 (simian) in Origin, and carrying the SV40 genetic material
CpG DNA	CpG containing oligonucleotide
c-Rel	c-Rel proto-oncogene
CRK	Sarcoma virus CT10 oncogene homolog
CSK	c-SRC tyrosine kinase
CTL	Cytotoxic T-cells
CXCR-4	CXC chemokine receptor-4
CXL-9	CX chemokine ligand-9
CXL-11	CX chemokine ligand-11
CyaA	Adenylate cyclase deficient
CDT	Cytolethal distending toxin
DAP	Diaminopimetic Acid
DCs	Dendritic Cells
DC-SIGN	DC specific C-type lectin
DD	Death domain

DDCs	Dermal Dendritic Cells
DMBT-1	Deleted in Maligant Brain Tumors-1
DMP	Dsmuramylpeptides
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dotA	defect in organelle trafficking
dsRNA	Double stranded RNA
DSS	Dextran Sodium Sulfate
DTT	Dithiothreitol
DU	Duodenal Ulcer
DupA	Duodenal ulcer associated protein
ECL	Enhanced chemical luminescence
ECP	Eosinophil Cationic Protein
EGFR	Epidermal Growth Factor Receptor
EPIYA	Abi Glu-Pro-Ile-Tyr-Ala motif
ER	Endoplasmic reticulum
ERK	Extra cellular signal regulated kinase
et al	*et aliter*
ExoS	Exoenzyme S
ExoU	Exoenzyme U
FAK	Focal Adhesion Kinase
FAM	6-carboxyfluorescein
FCAS	Familial Cold Autoinflammatory Syndrome
FcRs	Fc Receptors
FITC	Fluorescein isothiocyanate
FK565	Heptanoyl-gamma-D-glutamyl-L-meso-diaminopimelyl-D-alanine
FliC	Flagellar filament structural protein
FSL-1	Pam2CGDPKHPKSF (synthetic lipoprotein)
g	gravity
GAS	Group A Streptococcus
GBS	Group B Streptococcus
G-CSF	Granulocyte colony stimulating factor
GG2EE	Mouse macrophage cell line
GlcNAc-MurNAc	N-acetylglucosamine-N-acetymuramic acid
GM-CSF	Granulocyte-Monocyte colony stimulating factor
Gp96	Glycoprotein-96 kDa
GSK-3	Glycogen Synthase Kinase-3
GSI	Gamma-secretase Inhibitor
GRB2	Growth factor receptor-bound protein-*2*
h	hour
H-60	H-60 protein
HA	Heamagglutinin
HBV	Hepatitis B Virus
HCECs	Human corneal cpithelial cells
HEK293	Human embryonic kidney cell line
HeLa cells	Cervical epithelial cell line
HeNC-2	Mouse macrophage cell line
Hes-1	Hairy and Enhancer of Split homolog-1
HIV	Human immunodeficiency virus
hopZ	Helicobacter outer membrane protein Z
HP	*Helicobacter pylori*
HRP	Horse radish peroxide
HSC	Hematopoetic Stem Cell
HSP60	Heat shock protein-60 kDa
HSP70	Heat shock protein-70 kDa
HSV-2	Herpes simplex virus type-2
HT-29	Colon epithelial cell line
HOK-16B	Human gingival epithelial cell line
IARC	International agency for cancer research
IAV	Influenza A Virus
IceA	Induced upon contact with epithelium gene
IFNγ	Interferon-gamma
IFNα	Interferon-alpha
IFNβ	Interferon-beta
IKK	Inhibitor of κB kinase
IL	Interleukin
IL-1Rs	Interleukin-1 Receptors
IP-10	Inducible protein-10

IL-RN	Interleukin-1 receptor antagonist
IP	Immunoprecipitation
IPAF	Ice Protease(caspase-1) Activatin FActor
IRAKs	IL-1 Receptor Associated Kinases
IRF	IFN-regulatory Factor
IRF-3	IFN-regulatory Factor-3
ITAM	Immunoglobullin Tyrosine Activation Motif
JNK	Jun amino-terminal Kinase
kb	Kilo base
KC	Mouse chemokine CXCL-1
kDa	Kilo Dalton
KRV	Kilham Rat Virus
LAM	Lipoarabinomannan
LC	Langerhan Cell
LC-DCs	Langerhan cells like DCs
Le^b	Lewis b antigen
LM	Lipomannans
LMW-HA	Low molecular weight Hyaluronic acid
LPS	Lipopolysaccharide
LTA	Lipotheichoic Acid
LRRs	Leucine Rich Repeats
LXR	Liver X Receptors
LYN	SRC family kinase
M	Molar
MAL	MyD-88 Adaptor Like protein
MALT	Mucosa Associated Lymphoid Tissue
MAPK	Mitogen Activated Protein Kinases
MBP-68-86	Microbacterial Peptide
MCP-1	Monocyte chemotactic protein-1
MCs	Mast Cells
MD-2	Myeloid differentiation-2
MDA	melanoma differentiation-associated gene
mDC	Myeloid DC
MDP	Muramyl dipeptide
MEKK-4	MAP kinase kinase kinase-4
MH	Mueller-Hinton
MHC	Major Histo-Compatibility
min	Minute
MIP-1	Macrophage inflammatory protein-1
MKP-1	MAPK phosphatase-1
MMP-9	Matrix Metalloproteinase
MMTV	Mouse mammary tumour virus
MOI	Multiplicity of Infection
Mo-DCs	Monocytes derived DCs
Mo-LCs	Monocytes derived LCs
mRNA	Messenger Ribonucleic acid
Muc	Mucin
MurNAc-L-Ala-D-isoGln	Muramyl dipeptide
MWS	Muckle-Wells Syndrome
MyD88	Myeloid differentiation primary response88
Nap	Neutrophil attracting protein
NACHT	A domain in NLRs
NAIP	Neuronal Apoptos Inhibitor Protein
NAIP-5	Neuronal Apoptos Inhibitor Protein-5
NBD	Necleotide-binding Domain
NEMO	NF-κB essential modulator)
NF-κB	Nuclear factor- κB
NIK	NF-kBincluding kinase
NK	Natural Killer
NKG2D	Kill cell lectin-like receptor, subfamily K, member -1
NKT	Natural Killer T-cells
NLRs	Nod-like receptors
NO	Nitric Oxide
NOD	Nucleotide Oligomerization Domain
Notch-1	Notch, Drosophila, homolog of, 1, translocation-associated
NUD	Non-ulcer or functional Dyspeptic
oipA	Outer inflammatory protein-A
OD	Optical Density

OspA	Outer surface protein -A
OVA	ovalbumin
p38	Protein 38 kDa
P65	Protein 65 kDa
PAF-R	Platelet Activating Factor Receptor
Pam3CSK4	Synthetic triacylated lipopeptide
PAMPs	Pathogen Associated Molecular Patterns
Pam3CSSNA	Bacterial lipopeptide PAM3CysSerLys4
PBMCs	Peripheral Blood Mononuclear Cells
pDC	Plasmacytoid DC
pgdA	PGN deacetylase gene
PGN	Peptidoglycan
PGRPs	Peptidoglycan Recognition Proteins
PI-3-K	Phospho inositol-3-kinase
PIR-B	Paired lg-like Receptor-B
PKA	Protein kinase-A
PKC-ζ	Protein kinase-C-ζ
PMNs	Polymorphonulcear cells
PPD	Purified Protein Derivative
PR-3	Anti proteinase-3
PRAT4A	Protein associated with Toll-like receptor 4
PRMs	Pattern Recognition Molecules
PRRs	Pattern Recognition Receptors
PSA	Polysaccharide-A
PYD	Pyrin domain
RAC-1	*Ras-related-C3-botulinum-toxin-substrate 1*
RANTES	Regulated upon activation, normal T cell expressed and secreted
RARy	Retinoic acid Receptor-hamma
RAS	*rat-sarcoma*
RAW-264	Mouse macrophage cell line
RICK	RIP-like Interacting CLARP Kinase
RIG	Retinoic acid inducible gene-1
RIP-1	Receptor Interacting Protein-1
RIP-2	Receptor Interacting Protein-2
RLR	(RIG-1)-like receptors
RNA	Ribonucleic acid
RNAi	RNA interface
RNI	Reactive Nitrogen Intermediates
ROI	Reactive Oxygen Intermediates
rpm	Revolution per minute
RP-105	Radioprotective-105
RSV	Respiratory syncitial virus
RT	Room temperature
sabA	Sialic acid binding adhesin
sabB	Sialic acid binding adhesin
SARM	Sterile Alpha and HEAT/armadillo Motif protein
SDS	Sodium dodecyl sulfate
SHP-2	Tyrosine phosphatase
sLe ^{x}	Sialyl Lewis-x
sLe ^{a}	Sialyl Lewis-a
SOCS-1	Suppressor of cytokine signalling
SPI-1	Salmonella pathogenecity island-1
SPI-2	Salmonella pathogenecity island-2
SRC	SRC family kinase
SsRNA	Single-standed RNA
STAT	Signal Transducers and Activator of Transcription
STC-1	Stanniocalcin-1
TAMRA	
T3SS	Type III Secretion System
T4SS	Type IV Secretion System
T84	Colonic crypt cell line
TAK-1	TGFβ activated kinase-1
TAB-1	TAK binding protein-1
TAB-2	TAK binding protein-2
TBST	Tris buffered saline Tween 20
T-cells	T lymphocytes
Tcps	TIR domain containing-proteins
TCR	T-Cell receptor

TFF-1	Trefoil factor Family
TGFα	Transforming growth factor-alpha
Th-0	T-helper-0
Th-1	T-helper-1
Th-2	T-helper-2
Th-17	T-helper-17
THP-1	Human monocytic leukaemia cell line
TIR	Toll/IL-1 Receptor domain
TIRAP	TIR domain containing adaptor protein
TLR	Toll Like Receptor
TMB	Tetramethylbenzidine
TNFα	Tumour necrosis factor-alpha
T reg	Regulatory T cells
TRAF-6	TNF receptor associated factor-6
TRAM	TRIF Related Adaptor Molecule
TREM-1	triggering receptor expressed on myeloid cells-1
TRIF	TIR domain containing adaptor protein inducing IFNβ
Tris	Tris (hydroxymethyl)-aminomethane
Triton-X100	Polyethyleneglycol-[4-(2´,2´,4´,4´-tetramethylbutyl)phenyl]-ether
Tween-20	Polyoxyethylen-Sorbitan-Monolaurate
U	Unit
V	Volt
vacA	Vacuolating cytotoxin
VIP	Vasoactive Intestinal Peptide
WB	Western blot
WHO	World health organization
Wt	Wild type
ZO-1	Tight junction protein

Amino Acids

Alanine	Ala	A	Methionine	Met	M
Cysteine	Cys	C	Asparagine	Asn	N
Aspartic acid	Asp	D	Proline	Pro	P
Glutaminic acid	Glu	E	Glutamine	Gln	Q
Phenylalanin	Phe	F	Arginine	Arg	R
Glycine	Gly	G	Serine	Ser	S
Histidine	His	H	Threonine	Thr	T
Isoleucine	Ile	I	Valine	Val	V
Lysine	Lys	K	Tryptophan	Trp	W
Leucine	Leu	L	Tyrosine	Tyr	Y

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To my beloved parents

1. Introduction

1.1 A Brief Account on Growth of Immunology as a Science

The word ‘immunology’ finds its root from the latin word ‘immunis’ and that was used in the context of being free of the burden of taxes or military conscription. In the early history of human civilization, pestilence and diseases are believed to be the punishment rendering as a result of “bad deeds” or “evil thoughts”. Early civilizations of humankind had the knowledge of epidemic disease from the very start of its social organization. The Babylonian Epic of Gilgamesh from about 2000 B.C. records visitations of disease and pestilence, and severe epidemics during the early dynasties of Egypt. In 430 BC, Thucydides recorded that during plague epidemics in Athens, individuals who had already contracted the disease and recovered and recognized their “immune” status had got more attention than dying persons. Thus the science of immunology grew from common knowledge that those who survived from many of the then common infectious diseases rarely contracted such diseases again. This was an observation that was made long before the establishment of the germ theory of disease. The ancient Chinese practised a form of immunization by inhaling dried powders derived from the crusts of smallpox lesions and later a practice of applying powdered smallpox "crusts" and inserting them with a pin or “poking” device into the skin became the choice. The process was referred to as variolation and became quite common in the Middle East. Tenth century Islamic physician Rhazes, Sixteenth century Italian physician Girolamo Fracastoro, and Heironymus Mercurialis were tried to explain the phenomenon of acquired immunity of small pox through some interesting ways (Silverstein AM, 1989).

In 1718 Lady Mary Wortley Montagu, the wife of the British ambassador to Constantinople, observed the positive effects of variolation on the native population and performed the technique on her own children. Edward Jenner published in 1798 his epoch-making report on a safer and even more efficacious vaccination (Latin vaccus means cow) against smallpox, derived from cowpox pustules. The Jennerian technique

vaccination became the mode of immunization for small pox in the world during that time. However, he was also not accurate on explaining the real mechanism of immunity developed by his technique. The first modern proposal of the 'germ theory of disease was introduced by Jacob Henle in 1840 and confirmed by Robert Koch in 1876 by inoculating *Bacillus antracis* which can cause the disease anthrax. Louis Pasteur played a pivotal role in the development of immunology and he was concerned about the prevention of bacterial disease and the mechanism of how human body changes to resist further infection. In 1881, the work of Emile Roux and Louis Pasteur demonstrated that chickens recovered from a mild attack of chicken cholera induced by an attenuated strain were protected from further challenge with more lethal strains. Pasteur extended these findings to other diseases, demonstrating that it was possible to attenuate a pathogen and administer the attenuated strain as a vaccine. In a classic experiment at Pouilly-le-Fort in the same year, Pasteur first vaccinated one group of sheep with heat attenuated anthrax bacillus (*Bacillus anthracis*) and then challenged the vaccinated sheep and some un-vaccinated sheep with a virulent culture of the bacillus. All the vaccinated sheep lived, and all the unvaccinated animals died. These experiments led him to test the first vaccine in human for rabies. In 1886, Theobold Smith demonstrated that *heat killed* cultures of chicken cholera bacillus were also effective in protection from cholera. Emil von Behring and Shibasaburo Kitasato demonstrated that animals immunized with diphtheria and tetanus toxins produced something in their blood that could neutralize or destroy the toxin, thus preventing disease. These substances acted against toxin were called antitoxins and later named antibodies, paved the way for serotherapy and the beginning of humoral theory of immunity. The second significant step in the expansion of the immunological research program of the nineteenth century came in 1884 with Ilya Metchnikoff's cellular theory of immunity. Metchnikoff suggested that the primitive intracellular digestive functions of lower animal forms had persisted in the capacity of the mobile phagocytes of metazoa and higher forms to ingest and digest foreign substances.

Metchnikoff proposed that the phagocytic cell is the primary element in natural immunity (the first line of defense against infection) and is critical also for acquired immunity (the heightened protection conferred by preventive immunization or prior infection). Metchnikoff suggested that the inflammatory response was in fact an evolutionary mechanism designed to protect the host organism (Silverstein AM, 1989). Both the humoral theory of immunity and cellular theory of immunity were proved to be the two pillars of mammalian immune system through extensive research carried out in the twentieth century. The advances in immunology during twentieth century have clearly identified the innate immunity as the first line of defense against diseases and paving way for specific adaptive immune responses to ward off the infection and keeping a state of immunological memory to resist further infection.

1.2 Innate Immunity

The innate immune system comprises the immune cells, epithelial cells, cytokines, chemokines and other mediators, which defend the host from microbial attack by detecting in a non-specific and primitive manner. Innate immunity represents the first line of defense against foreign body invasion and sends its message to the centres of immunological development to mount well developed and specific adaptive responses through various cellular and soluble factors. The innate immune system is evolutionarily older and phylogenetically conserved system present in almost all multicellular organisms (Hoffmann JA *et al,* 1999). Innate immune system plays a major role in detection of foreign body invasion. This process of detection and phagocytosis of foreign bodies leads to the production or release of chemical mediators such as chemokines, cytokines, histamine, bradykinin, serotonin, leukotrienes and prostaglandins. The releases of these mediators play an essential role to control the spread of infection by inflammatory reaction. At the next stage chemokines and cytokines attracts the neutrophils to the site of infection and later monocytes and lymphocytes to ward off the

infection. The professional antigen presenting cells (APCs) such as macrophages and dendritic cells (DCs) at the site of infection are involved in the phagocytosis and killing of engulfed microbes. DCs and macrophages are the most potent APCs of the immune system. DCs act very early compared to macrophages. During foreign antigen insult immature DCs in the peripheral tissues capture antigens through phagocytosis and pinocytosis and then, under micro-environmental signals (cytokines and chemokines), migrate to the draining lymph nodes while undergoing maturation. The maturing DCs with a reduced capacity for phagocytosis but with an increased ability to present antigens home to T cell rich areas and induce an antigen specific T cell response (Murphy KM *et al,* 2007).

The functioning of innate immune system must have some form of detection at the molecular level to differentiate pathogenic and non-pathogenic commensal bacteria. At the end of the 20th century, Janeway formulated a hypothesis of how such an innate immune recognition system might look like and proposed a set of factors called 'patternrecognition receptors' (PRRs) capable of detecting 'pathogen-associated molecular patterns' or PAMPs (Janeway CA, 1989). The recent identification of Toll-like receptors (TLRs) in mammals has made it clear that innate immunity plays an important role in the detection of invading pathogens. Recent evidence shows that TLRs recognize specific patterns of microbial components, especially those from pathogens, and regulates the activation of both innate and adaptive immunity. The discovery of TLRs as essential components of the innate immune system has greatly advanced our knowledge and understanding of immune responses to infection and how these are regulated. Innate immunity in general and TLRs in particular play a crucial role in the front line of host defenses against microbes and also a modulator of the immune system at large in vertebrate animals (Takeda Y, 2003).

Two additional families of PRRs have also been discovered, that can sense the molecular patterns of microorganisms intracellularly, are Nucleotide oligomerization domain (NOD)-like receptors (NLRs) and Retinoic acid inducible gene-1 (RIG-1)-like receptors (RLRs). These two families comprise the intracellular PRRs, of which NLRs recognize primarily molecules of bacterial origin and RLRs are involved in antiviral response. The general domain structure of the NLR family members includes an amino-terminal effector region that consists of a protein–protein interaction domain such as the caspase recruiting domain (CARD), Pyrin or baculovirus IAP (inhibitor of apoptosis protein) repeat (BIR) domain, a centrally located NOD domain, and carboxyl-terminal leucine rich repeats (LRRs) that are involved in microbial sensing (Inohara *et al*., 2005). NLR family of proteins composed of 23 cytosolic proteins characterized by the specific domains. NOD1 and NOD2 of NLRs are capable of detecting bacterial peptidoglycan-related molecules intrecellulary and mediate activation of NF-kB and mitogen activated protein kinases (MAPKs) lead to pro-inflammatory gene expression (McDonald *et al*., 2005). NLRs like NALP1, NALP3 and IPAF detects bacterial products and activate the caspase-1 to process the Pro-IL-1β and Pro-IL-18 to mature forms through the formation of multimeric structures called 'inflammasomes'. RIG-1 and melanoma differentiation-associated gene -5 (MDA-5), which are intracellular sensors of double-stranded (ds) RNA, are two members of the RLR family RLRs activate Interferon Responsive Factor (IRF) and NF-kB in most of the cell types after viral infection to produce type-1 Interferons (Yoneyama M *et al,* 2004; Kang DC *et al,* 2002)..

Peptidoglycan recognition proteins (PGRPs) are a novel family of pattern recognition molecules (PRMs) in innate immunity conserved from insects to mammals recognize bacterial cell wall peptidoglycan (PGN) and are suggested to act as anti-bacterial factors. In humans, four kinds of PGRPs (PGRP-L, Iα, Iβ and S) have been cloned and all four human PGRPs bind PGN. *E. coli* type tryacyl lipopeptide

(Pam3CSSNA), *E. coli* type lipid A, diaminopimelic acid containing desmuramyl peptide (gamma-D-glutamyl-meso-DAP; iE-DAP), and muramyldipeptide (MDP markedly upregulated the mRNA expression of the four PGRPs and cell surface expression of PGRP-Iα and -Iβ, but did not induce either mRNA expression or secretion of inflammatory cytokines, in oral epithelial cells. Suppression of the expression of TLR-2, TLR-4, NOD-1 and NOD-2 by RNA interference (RNAi) specifically inhibited the upregulation of PGRP mRNA expression induced by Pam3CSSNA, lipid A, iE-DAP and MDP, respectively. In addition, suppression of NF-κB activation clearly prevented the induction of PGRP mRNA expression. PAMPs induced the expression of PGRPs through TLRs and NLRs, but not proinflammatory cytokines, in oral epithelial cells, reflecting that PGRPs might be involved in host defence against bacterial invasion without accompanying inflammatory responses (Uehara A *et al,* 2005a).

PAMPs recognition by PRRs leads to different signalling events that ultimately effect the expression of many effector molecules of the immune system. The recognition in phagocytic cells leads to the production of reactive oxygen and nitrogen intermediates (ROI & RNI) and increased expression of co-stimulatory molecules which act as second stimuli for T-cells activation. PRRs also signals the production of immune mediators such as chemokines, cytokines, leukotrienes, prostaglandins and anti-microbial peptides, which ultimately determines the recruitment of leukocytes at the site of infection and the production of successful adaptive immunity to eliminate the infection.

1.3 Adaptive Immunity

Adaptive immunity detects non-self through a discrete mechanism of recognition of peptide antigens using antigen receptors expressed on the surface of B- and T-lymphocytes. In order to respond to a wide range of potential antigens, B- and T-cells rearrange their immunoglobulin and T cell receptor genes respectively to generate over 10^{11} different species of antigen receptors. With this high number of cognate receptors,

adaptive immune system acquires the capacity to eliminate or prevent many pathogenic challenges. Engagement of antigen receptors by the cognate antigen triggers clonal expansion of the lymphocyte and further production of antigen-specific antibodies. Hematopoetic stem cell (HSC) from bone marrow is the precursor of B- and T-cells. However, T-cells after differentiation leaves bone marrow for thymus and maturation happens in this lymphoid organ and thereby it got the name T-cells. In humans, approximately 1-2% of the lymphocyte pool recirculates each hour to optimize the opportunities for antigen-specific lymphocytes to find their specific antigen within the secondary lymphoid tissues. The clonal selection happens when cognate receptors such as T-cell receptor (TCR) or B-cell receptor (BCR) engage an antigen. The recognition of antigenic peptides by TCR requires specialized antigen presenting molecules called Major histo-compatibility (MHC) molecules in antigen presenting cells. Almost all of the nucleated cells are capable of presenting antigen to T-cell, however, professional antigen presenting cells such as DCs, macrophages and B-cells are only capable of activating naïve T-cells through co-stimulatory receptor engagement. B-cell receptors are membrane bound immunoglobulin molecules, which recognise the antigen in native form and doesn't need any special molecule like MHC in case of T-cells. The engagement of antigen with BCR activates the differentiation of B cells to an effector cell called plasma cells producing large quantities of immunoglobulins (Murphy KM *et al,* 2007).

T-cells are the major players of cell mediated immunity. The thymus maturation of T-cells produce CD4+ helper cell and CD8+ killer cell subsets of cell population, which varies in their effector function. CD4+ cell are called T-helper cells, which manage to maintain an immune status to clear off infection. CD4+ engagement with MHC-II loaded with processed exogenous peptides from professional antigen presenting cells induce them differentiate in to two effecter forms called T-hepler1 (Th-1) or T-helper 2 (Th-2) phenotype of CD4+ cells to eliminate different forms of disease (Aderem A and Underhill

D, 1999). Recently, it has been reported that a state of Th0 is pivotal to determine the differentiation of effecter forms, although the clear cut mechanism of this is not understood. This difference is mainly dependent on the cytokine release from antigen from T-cells during engagement with MHC-II molecules from professional antigen presenting cells. The Th-1 response is characterized by the production of Interferon-gamma (IFNγ), which activates the bactericidal activities of macrophages, and induces B-cells to make opsonizing antibodies, and leads to "cell-mediated immunity". The Th-2 response is characterized by the release of Interleukin-4 (IL-4), which results in the activation of B-cells to make neutralizing (killing) antibodies, leading to humoral immunity. Generally, Th-1 responses are more effective against intracellular pathogens (viruses and bacteria that are inside host cells), while Th-2 responses are more effective against extracellular bacteria, parasites and toxins. Th17 is the most recently identified effector T-cells forms under the influence of cytokine IL-17 (Harrington LE *et al,* 2005). Cytotoxic T-cells (CTL) or killer T-cells detect the infected cells especially with virus through the MHC-I presented endogenous peptides (Aderem A and Underhill D, 1999). CTL kills the infected cells by the release of pore forming molecules such as perforin and granulysin, which eventually leads to bursting or lysing of cells. The granzymes released from CTL has been reported to induce apoptosis in infected cells. There is another subset of T cell population known as γδ T cells which possess an alternate TCR and share the characteristics of helper T-cells, CTL and Natural killer (NK) cells. Natural killer T cells (NKT) co-express a αβ T-cell receptor (TCR), but also express a variety of molecular markers that are typically associated with NK cells such as CD161. NKT-cells TCR vary from convential αβ T-cells, that they possess a TCR with less diversity can recognize CD1 presented lipid or glycolipid molecules (Murphy KM *et al,* 2007).

B-cells derived from HSCs with BCR or membrane fixed antibodies can recognize its antigen in native form and up on engagement known as clonal selection. During this

clonal expansion process, B-cells undergo affinity maturation at germinal centres of secondary lymphoid tissues to make high affinity antibody to a particular antigen through somatic hypermutation (Forsdyke DR, 1995). Cytokines produced by the T-helper CD4+ cells enable these B- cells to rapidly proliferate to a pool of antibody-secreting plasma cells for massive synthesis and secretion of an antibody. Plasma cells are short lived cells (2-3 days) which secrete antibodies. These antibodies bind to antigens, making them easier targets for phagocytes, and trigger the complement cascade. About 10% of plasma cells will survive to become long-lived antigen specific memory B-cells. The primary response initially produces more of IgM molecules (as secreted BCR) and later replaced by IgG molecules against a particular antigen. Antibodies are the effecter molecules of the humoral immunity and that can reach the site of inflammation through blood and lymph and found most effective against bacteria, viruses and toxins before entering to cells (Murphy KM *et al,* 2007).

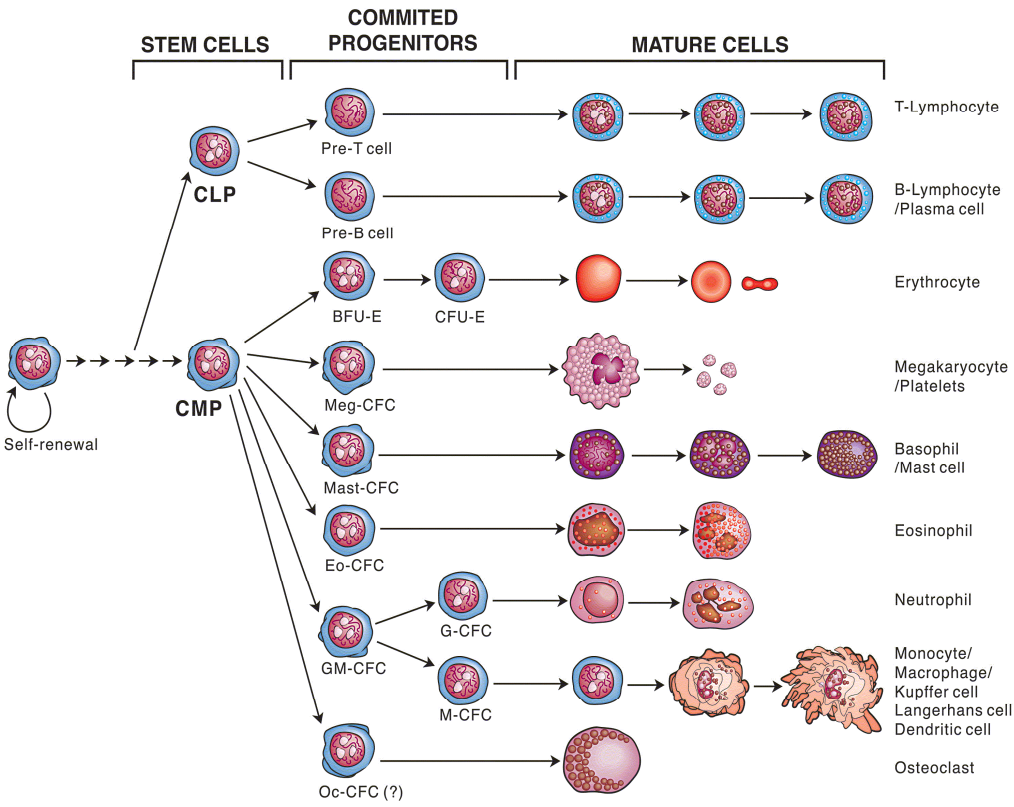


Fig.1 Diagramatic representation of haematopoetic cell lineage

1.4 Toll-Like Receptors: History, Phylogeny, Structure and Signalling

The involvement of the Toll receptors in innate immunity was first described in Drosophila and originally identified as a transmembrane receptor required for the establishment of dorso-ventral polarity in the developing embryo. Stimulation of Toll by the secreted Spatzle factor, a ligand of Toll, activates the cytoplasmic serine/threonine kinase Pelle via the adaptor protein Tube. Activation of Pelle promotes degradation of the ankyrin-repeat protein Cactus, which associates with the Rel-type transcription factor Dorsal in the cytoplasm. Once Cactus is degraded in response to the Toll-mediated signal, Dorsal is free to translocate to the nucleus, where it regulates transcription of specific target genes (Belvin MP and Anderson KV, 1996). The signalling pathway of Drosophila Toll shows remarkable similarity to the mammalian IL-1 pathway, which leads to activation of NF-κB, a transcription factor responsible for many aspects of inflammatory and immune responses.

A year after the discovery of the role of the drosophila Toll in the host defense against fungal infection, a mammalian homologue of the Drosophila Toll was identified (Medzhitov R *et al,* 1997). Subsequently, a family of proteins structurally related to Drosophila Toll was identified, collectively referred to as the Toll-like receptors (TLRs). The TLR family is known to consist of 10 members in human (TLR-1 to TLR-10), 11 members in mouse and the chromosomal location was identified for each human TLR. *TLR1, TLR6* and *TLR10* were clustered on chromosome 4p14, while *TLR2* and *TLR3* located to 4q32 and 4q35, respectively (Rock FL *et al,* 1998; Takeuchi O *et al,* 1999; Hasan U *et al,* 2005). *TLR4* and *TLR5* were located to 9q32-33 and 1q33.3, respectively (Rock FL *et al,* 1998), while *TLR7* and *TLR8* are located in tandem in Xp22, and *TLR9* occupy the position at 3p21.3 (Chuang TH and Ulevitch RJ, 2000). The TLR multigene family encodes important recognition receptors of the innate immune system that have been conserved in both the invertebrate and vertebrate lineages (Hoffman JA *et al,*

1999). TLRs recognize a variety of endogenous and exogenous ligands, many of the ligands are conserved molecules essential for pathogen survival. There are six major families of vertebrate TLRs. TLRs within a family recognize a general class of PAMPs. The family including *TLR1* has more species specific adaptations than other families.

The TLR family members are capable of recognizing several classes of pathogens and orchestrating appropriate innate and adaptive immune responses. Lipoproteins in which the N-terminal cysteine is triacylated are recognized by TLR-2 in combination with TLR-1. Diacylated lipoproteins are recognized by TLR-2 in combination with TLR-6 (Takeda Y *et al,* 2003; Ozinsky A *et al,* 2000). Double-stranded RNA, LPS and Flagellin are recognized by TLR-3, TLR-4 andTLR-5 respectively. Nucleic acids and haeme are recognized by the family consisting of TLR-7, -8, and -9 (Coban C *et al,* 2005, Diebold S *et al,* 2004; Heil F *et al,* 2004). TLRs within a family recognize a general class of PAMP associated with that family. Selective pressure for maintenance of specific PAMP recognition has clearly dominated the evolution of most of these families and often within subfamilies as well. Because *TLR1* and *TLR6* have similar genomic structures, consisting of one exon, and are located in tandem in the same chromosome, they may be the products of an evolutionary duplication. Division of TLRs into five subfamilies is also based on genomic structure. The *TLR2* gene has two exons, but all of the coding sequences are contained within, exon 2. In contrast, the TLR-9 subfamily members including TLR-7, TLR-8, and TLR-9 are encoded by two exons (Chuang TH and Ulevitch RJ, 2000). The genes for TLR-7 and TLR-8 show 42.3% identity and 72.7% similarity in their amino acid sequences, have similar genomic structures, and are located close to each other on the X chromosome (Chuang TH and Ulevitch RJ, 2000; Du X *et al,* 2000). The *TLR4* and *TLR5* genes have four and five exons, respectively. TLR-3 has a unique structure among the TLRs in that it has five exons and the protein is encoded by exons 2 through 5. An overview of the tree indicates that all of the families,

and all of the genes within each family, are about equally distant from the center of the tree, where the progenitor vertebrate TLR gene or set of genes is inferred.

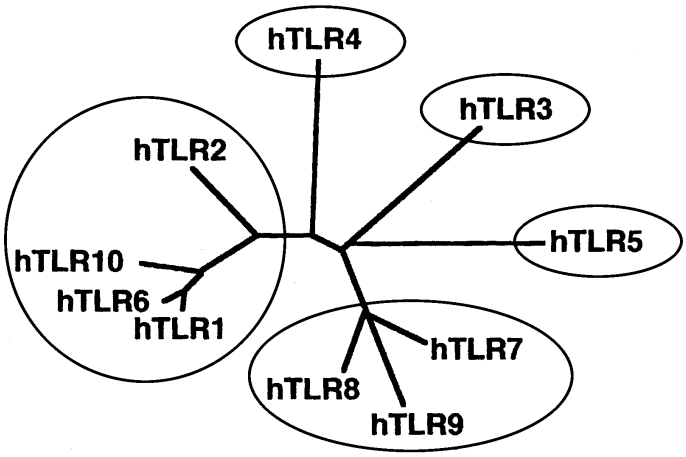


Fig.2 'Star phylogeny' chart for TLR family genes (Takeda Y *et al,* 2003)

This ''star phylogeny'' implies that all TLRs are evolving at about the same rate. This observation is somewhat unusual for multigene families, where often some members take on new functions; vertebrate TLRs are not fast-evolving genes. In addition, the discrepancies in molecular distances between species with shorter and longer generation times are relatively muted. This muting implies that selection is dominant over mutation in governing the rate of evolution of the TLRs, and thus that TLRs are under strong selection for maintenance of function.

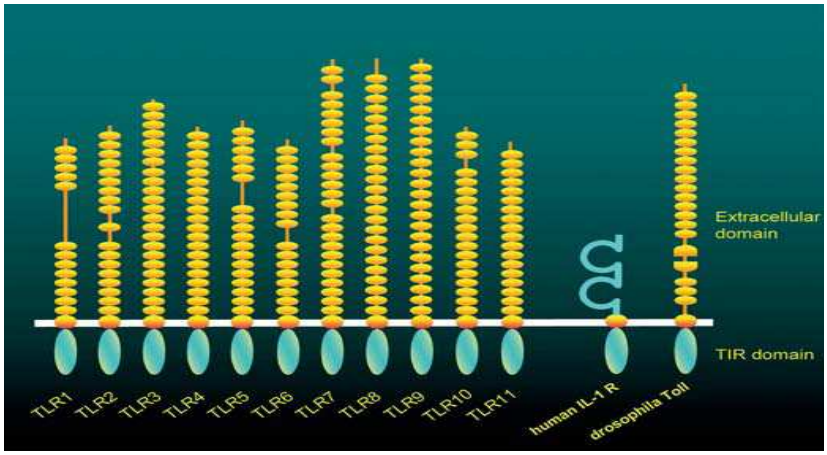


Fig.3 Basic structure of mammalian TLRs, as compared with the human IL-1 receptor and the *Drosophila* Toll protein. The extracellular portion of TLRs is defined primarily by LRRs that constitute the ligand-binding site of TLRs. LRRs are depicted as yellow ovals. The highly conserved intracellular TIR signalling domain is depicted as a blue oval. From the eleven TLRs at present identified in man, TLR 11 is truncated and probably non-functional; in mice TLR 11 is abundantly present in the uro-genital tract and appears to confer resistance to urinary tract infection (Fleer A and Krediet TG, 2007).

The innate immune response in vertebrates is the first line of defence against invading microorganisms. The main players in innate immunity are phagocytes such as epithelial cells, neutrophils, macrophages, and DCs. These cells can discriminate between pathogens and self by utilizing signals from the TLRs. The TLRs are type I integral membrane glycoproteins, and on the basis of considerable homology in the cytoplasmic region, they are members of a larger superfamily that includes the interleukin-1 receptors (IL-1Rs). However, the extracellular region of the TLRs and IL-1Rs differs markedly: the extracellular region of TLRs contains leucine-rich repeat (LRR) motifs, whereas the extracellular region of IL-1Rs contains three immunoglobulin-like domains. TLRs and IL-1Rs have a conserved region of ~200 amino acids in their cytoplasmic tails, which is known as the Toll/IL-1R (TIR) domain (Slack JL *et al,* 2000). Three conserved homologous regions in the TIR domain are crucial for signalling. The crystal structures of the TIR domains of human TLR-1 and TLR-2 have shown to contain a central five stranded parallel β-sheet, which is surrounded by five α-helices on each side (Xu Y *et al,* 2000). The extracellular domain of TLRs contains 19–25 tandem repeats of the LRR motif and each repeat consists of 24–29 amino acids (Bell JK *et al,* 2003). The repeats comprise a β-strand and a α-helix connected by loops. The LRR domains of TLRs form a horseshoe structure, and it is thought that the concave surface of the LRR domains is involved directly in the recognition of various pathogens. The sub-cellular localization of different TLRs correlates to some extent with the molecular patterns of their ligands. TLR-1, TLR-2 and TLR-4 are located on the cell surface and are recruited to phagosomes after activation by their respective ligands. On the other hand, TLR-3, TLR-7 and TLR-9, all of which are involved in the recognition of nucleic-acid-like structures, are not expressed on the cell surface (Ahmad-Nejad P *et al,* 2002; Heil F *et al,* 2003; Matsumoto M *et al,* 2003).

TLR after ligand binding recruiting signalling molecules include adaptor molecule myeloid differentiation primary response protein-88 (MyD-88), IL-1 receptor associated

kinases (IRAKs), transforming growth factor-β activated kinase –1 (TAK-1), TAK binding protein-1 (TAB-1), TAB 2 and tumour necrosis factor receptor associated factor-6 (TRAF-6) (Dunne A and O'Neill LA, 2003). The adaptor protein MyD-88 has both TIR domain and DD (death) domain and binds to TLR through TIR domain on activation. IRAKs bind to the TLR complex through DD domain and phosphorylated either by auto or cross prosphorylation. This causes its dissociation from the complex and permitting to bind with TRAF-6, TAK-1, TAB-1 and 2, causing activation of NF-κB and leads to the production of inflammatory cytokines (Medzhitov R *et al,* 1998; Akira S and Takeda K, 2004). IRAK-1, IRAK-2 and IRAK-4 are expressed ubiquitously but IRAK-M is restricted to monocytes/macrophages. IRAK-2 and IRAK-M are inactive kinases (Janssen S and Beyaert R, 2003). It has also been report that IRAK-M regulated TLR signalling negatively by preventing the dissociation of IRAK-1, -4 and MyD-88 and formation of IRAK-1 and TRAF-6 complex (Kobayashi K *et al,* 2002). The association between MyD-88 and IRAK is mediated through a DD–DD interaction. MyD-88 forms homodimers through DD–DD and TIR-domain–TIR-domain interactions and exists as a dimer when recruited to the receptor complex (Dunne A *et al,* 2003). Therefore, MyD88 functions as an adaptor linking TLRs/IL-1Rs with downstream signalling molecules that have DDs. IRAKs contain an N-terminal DD and a central serine/threonine-kinase domain. The kinase activity of IRAK-1 increases strongly following TLR/IL-1R stimulation, and its kinase domain is essential for signalling through NF-κB. However, kinase activity itself is not essential for signalling, because in IRAK-1 deficient cells, the over-expression of a kinase defective mutant of IRAK1 can strongly induce NF-κB activation (Li X *et al,* 1999). In IRAK1-deficient mice, cytokine production in response to IL-1 and LPS was diminished but not abolished (Kanakraj P *et al,* 1999; Thomas JA *et al,* 1999; Swantek JL *et al,* 2000), whereas IRAK-4deficient mice showed virtually no response to IL-1, LPS or other bacterial components, demonstrating that IRAK-4 has an important role in IL-1R/TLR signalling (Suzuki N *et al,* 2002). Patients with an inherited IRAK-4 deficiency have been

identified and they failed to respond to IL-1, to IL-18 or to stimulation of each of five TLRs (TLR-2, TLR-3, TLR-4, TLR-5 and TLR-9) (Picard C *et al,* 2003). Together, these results show that IRAK-4 and its kinase activity are required for TLR signalling and that IRAK4 functions upstream of IRAK1.

The discovery of the MyD-88-independent pathway led researchers to characterize the signalling pathways of the various TLRs, the activation of which leads to different patterns of gene expression. TLR ligand binding induces receptor dimerisation and a conformational change which recruits TIR domain containing adaptor proteins to the receptor TIR domain. Four signaling adaptor proteins have been identified to date, including MyD-88, TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor protein inducing IFNβ (TRIF), TRIF-related adaptor molecule (TRAM), while a fifth, SARM, has recently been shown to negatively regulate TLR signal transduction. TIRAP acts as a bridging adaptor between TLR4, TLR1/2, TLR2/6 and MyD-88. TRIF is associated only with TLR4 and TLR3, binding directly to TLR3 and using the bridging adaptor TRAM to bind to TLR4. TRIF is the only adaptor to activate IRF3 to mediate type I interferon induction, through the IKK related kinases IKKε and TBK1 (Kawai T and Akira S, 2006). Adaptor usage by individual TLRs has allowed the definition of two distinct signal transduction pathways downstream of TLR activation based on the requirement for MyD-88.

MyD-88 was isolated originally as a gene that is induced rapidly during the IL-6-stimulated differentiation of M1 myeloleukaemic cells into macrophages (Lord KA *et al,* 1990). MyD-88 was subsequently cloned as an adaptor molecule that functions to recruit IRAK to the IL-1R complex following stimulation with IL-1 (Muzio M *et al,* 1997; Wesche H *et al,* 1997; Burns KA *et al,* 1998). TIRAP does not have a DD and the physiological role of TIRAP was revealed by generating knockout mice: the production of inflammatory cytokines in response to LPS was found to be defective in TIRAP-deficient mice, but the expression of interferon (IFN) inducible genes and the delayed activation of NF-κB was

still observed (Fitzgerald KA *et al,* 2001; Yamamoto M *et al,* 2002). TIRAP-deficient mice also show impaired cytokine production in response to TLR2 ligands, despite having normal responses to TLR-3, TLR-7 and TLR-9 ligands (Yamamoto M *et al,* 2002; Horng T *et al,* 2002). Therefore, TIRAP is essential for MyD88-dependent signalling through TLR-2 and TLR-4.

The enforced expression of TRIF, another TIR-domain-containing adaptor, but not of MyD-88 or TIRAP, led to activation of the IFN-β promoter in human embryonic kidney-293 (HEK-293) cells, whereas a dominant negative form of TRIF inhibited TLR-3 dependent activation of the IFN-β promoter (Yamamoto M *et al,* 2002b). These *in vitro* studies indicated that TRIF functions in the MyD-88 independent pathway to induce IFN-β. The physiological role of TRIF was subsequently revealed through the targeted deletion of *Trif* in mice. In response to TLR-3 and TLR-4 ligands, these TRIF (-/-) deficient mice showed both impaired activation of IFN-regulatory factor-3 (IRF-3) and decreased expression of IFN-inducible genes (Yamamoto M *et al,* 2003). TRIF (-/-) deficient mice showed defective production of inflammatory cytokines in response to TLR-4 ligands but not to other TLR ligands. However, TLR-4 mediated activation of the MyD-88 dependent pathway was not impaired, as determined by phosphorylation of IRAK-1 and early-phase activation of NF-κB (Yamamoto M *et al,* 2003; Hoebe K *et al,* 2003).

TRAM, was recently identified through sequence homology in database searches (Bin LH *et al,* 2003; Oshiumi H *et al,* 2003). *In vitro* studies indicated that TRAM associates with TRIF and TLR-4 but not with TLR-3, and the inhibition of TRAM expression by siRNA demonstrated its important role in the TLR-4 but not TLR-3 mediated induction of IFN-β and IFN-inducible genes (Fitzgerald KA *et al,* 2003). Analysis of TRAM (-/-) deficient mice further established that TRAM has an essential role in the MyD-88 independent cascade of TLR-4 induced signals. In response to TLR-4 ligands, TRAM (-/-) deficient mice, similar to TRIF (-/-) deficient mice, showed impaired

activation of IRF-3 and reduced expression of IFN-inducible genes. However, unlike TRIF (-/-) deficient mice, TRAM (-/-) deficient mice showed a normal response to TLR-3 stimulation (Yamamoto *et al,* 2003). Thus, TRAM is involved specifically in the activation of the MyD88-independent/TRIF-dependent signalling pathway through TLR-4. In addition, similar to TRIF-deficient mice, TRAM deficient mice are defective in their production of inflammatory cytokines in response to LPS, despite the fact that the activation of IRAK-1 and early phase NF-κB activation is normal (Yamamoto *et al,* 2003).

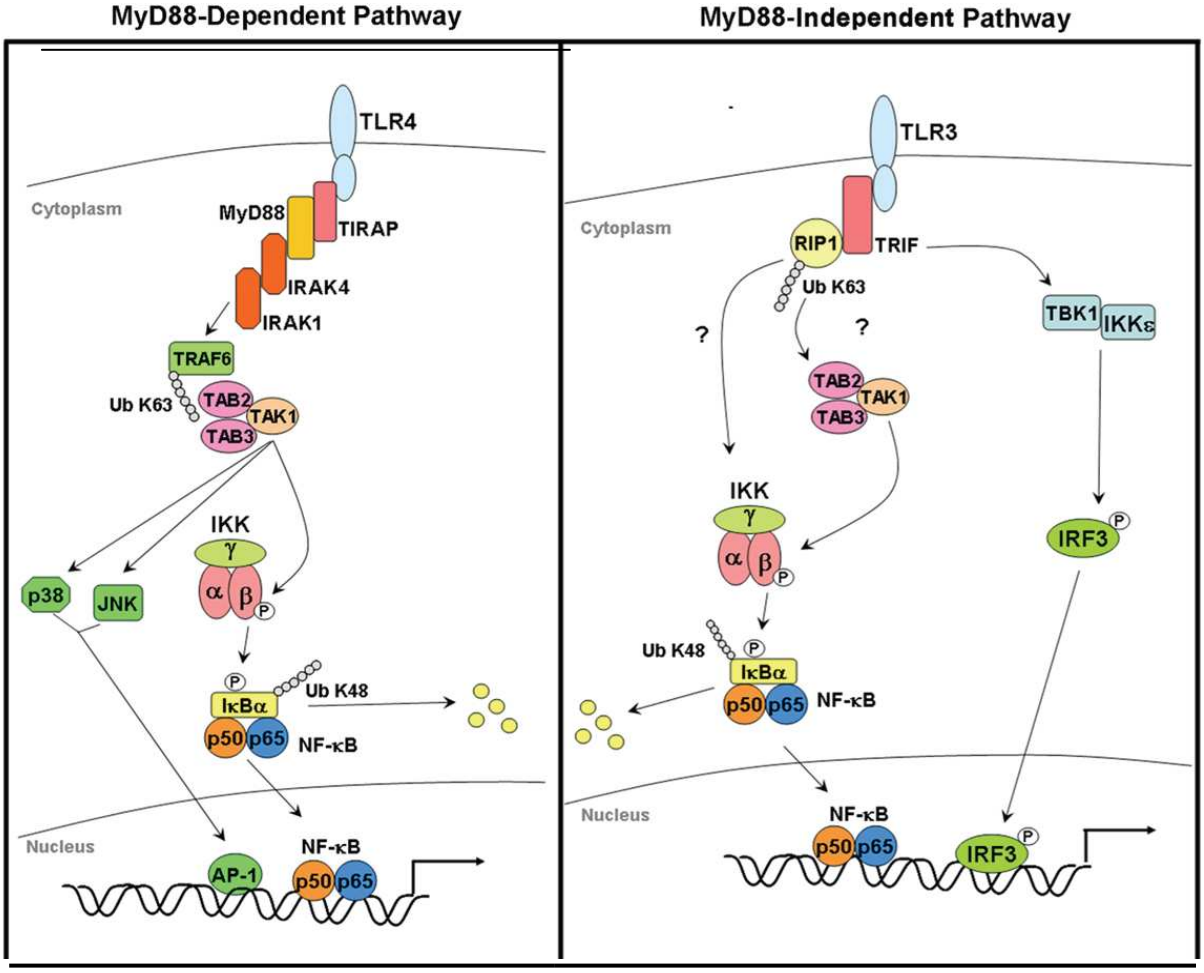


Fig.4. TLR activation of NF-κB occurs through My-D88-dependent and independent pathways. MyD-88-dependent activation of NF-κB occurs following receptor induced association of MyD-88 directly to the TIR domain of the TIR, or as in the case of TLR-4, through the bridging adaptor TIRAP. This allows the formation of a complex which includes IRAK-1, IRAK-4 and TRAF-6. K63-polyubiquitination of TRAF6 is critical for the binding of TAK-1, TAB-2 and TAB-3. Activated TAK-1 phosphorylates and activates IKKβ which in turn phosphorylates IκBα to signal for its K48-polyubiquitination and subsequent proteasomal degradation. TAK-1 activity is also required for the activation of JNK and p38, leading to the activation of the AP-1 transcription factor. MyD88-independent activation of NF-κB requires the adaptor TRIF, which binds directly to TLR-3 and uses the bridging adaptor TRAM to bind to the TLR-4 receptor. TRIF recruits RIP-1 which becomes K63-polyubiquitinated and requires TAK-1, TAB-2 and TAB-3 to activate NF-κB, although the exact mechanism is unclear. In addition, TRIF activates the IKK related kinases IKKε and TBK-1 which phosphorylate and activate the IRF-3 transcription factor (Carmody RJ and Chen YH, 2007).

1.5 The Known TLR Lignads

Table.1

TLR family	Ligands (origin)	Reference
TLR-1	Tri-acyl lipopeptides (bacteria, mycobacteria)	Takeuchi O *et al,* 2002
	Soluble factors (*Neisseria meningitides*)	Wyllie DH *et al,* 2002
TLR-2	Lipoprotein/lipopeptides (a variety of pathogens)	Aliprantis AO *et al,* 1999
	Peptidoglycan (Gram-positive bacteria)	Takeuchi O *et al,* 1999
	Lipoteichoic acid (Gram-positive bacteria)	Schwadner, R. *et al,* 1999
	Lipoarabinomannan (mycobacteria)	Means TK *et al,* 1999
	A phenol-soluble modulin (*Staphylococcus epidermidis*)	Hajjar AM *et al,* 2001
	Glycoinositolphospholipids (*Trypanosoma Cruzi*)	Coelho PS *et al,* 2002
	Glycolipids (*Treponema maltophilum*)	Opitz B *et al,* 2001
	Porins (Neisseria)	Massari P *et al,* 2002
	Zymosan (fungi)	Underhill DM *et al,* 1999
	Atypical LPS (*Leptospira interrogans*)	Werts C *et al,* 2001
	Atypical LPS (*Porphyromonas gingivalis*)	Hirschfeld M *et al,* 2001
	HSP70 (host)	Asea A *et al,* 2002
TLR-3	Double-stranded RNA (virus)	Alexopoulou L *et al,* 2001
TLR-4	LPS (Gram-negative bacteria)	Poltorak A *et al,* 1998
	Taxol (plant)	Kawasaki K *et al,* 2000
	Fusion protein (RSV)	Kurt-Jones EA *et al,* 2000
	Envelope proteins (MMTV)	Rassa JC *et al,* 2002
	HSP60 (*Chlamydia pneumoniae*)	Bulut Y *et al,* 2002
	HSP60 (host)	Vabulas RM *et al,* 2002
	HSP70 (host)	Vabulas RM *et al,* 2002
	Type III repeat extra domain A of fibronectin (host)	Okamura Y *et al,* 2001
	Oligosaccharides of hyaluronic acid (host)	Termeer C *et al,* 2002
	Polysaccharide fragments of heparan sulfate (host)	Johnson GB *et al,* 2002
	Fibrinogen (host)	Smiley ST *et al,* 2001
TLR-5	Flagellin (bacteria)	Hayashi F *et al,* 2001
TLR-6	Di-acyl lipopeptides (mycoplasma)	Takeuchi O *et al,* 2001
	Lipoteichoic acid (Gram positive bacteria)	Schwadner, R. *et al,* 1999
	Zymosan (Fungi)	Ozinsky A *et al,* 2000
TLR-7	single-stranded RNA (viruses)	Heil F *et al,* 2004
	Imidazoquinoline (synthetic compounds)	Hemmi H *et al,* 2002
	Loxoribine (synthetic compounds)	Heil F *et al,* 2003
	Bropirimine (synthetic compounds)	Heil F *et al,* 2003
TLR-8	single-stranded RNA (viruses)	Heil F *et al,* 2004
	Imidazoquinoline (synthetic compounds)	Jurk M *et al,* 2002
TLR-9	CpG DNA (bacteria and viruses)	Hemmi H *et al,* 2000
TLR-10	Unknown	
TLR-11	Unknown (Uropathogenic bacteria)	Zhang D *et al,* 2004
	Profilin like molecule (*Toxoplasma gondii)*	Yarovinsky F *et al,* 2005

1.6 Toll-Like Receptors and Its Ligands in Infection and Inflammatory Processes

Toll was identified as a Drosophila gene essential for ontogenesis and antimicrobial resistance and later several orthologues of Toll have been identified and cloned in vertebrates. These TLRs have been extensively studied thereafter and implicated in many infectious diseases and inflammatory processes. peptidoglycans (PGN) of Gram positive and LPS of Gram negative bacteria were first two ligands identified for TLR-2 and TLR-4, respectively (Zhang FX *et al*, 1999; Schwandner R *et al,* 1999; and Takeuchi O *et al,* 1999). In addition, lipoprotein from several microbes and lipoarabinomannan (LAM) from *Mycobacterium tuberculosis* found to be activated through TLR2 (Hirschfeld M *et al,* 1999; Means TK *et al,* 1999). Takeuchi *et al* (2000a) reported that MyD-88 deficient macrophages from knock out mice found to be hyporesponsive to LPS and that revealed the MyD-88 dependency of TLR mediated cellular activation. In the same year the above group have reported that mycoplasmal macrophage-activating lipopeptide-2 activated immune cells through a TLR-2 and MyD-88 dependent signalling pathway (Takeuchi O *et al,* 2000c). TLR-2 and MyD-88 (-/-) deficient mice were more susceptible to *Staphylococcus aureus* infection (Takeuchi O *et al*, 2000b). TLR-9, reported to be recognizing unmethylated bacterial CpG-DNA and that activated TLR signalling events in TLR2 (-/-) and TLR4 (-/-) deficient cells but not in MyD-88 (-/-) deficient cells (Haecker H *et al,* 2000, Bauer S *et al*, 2001). *E. coli, Klebsiella pneumoniae or S. typhimurium* LPSs have exhibited differences in their biological effects *in vivo*. In the TNFα (-/-) and TNFβ (-/-) deficient mice, *S. typhimurium* LPS induced similar responses as in wild type mice but were resistant to *E. coli* and *K. pneumoniae* LPS endotoxemia. This difference have reflected by decreased IL-1 and IFNγ and increased IL-10 levels in *E. coli* and *K. pneumoniae* LPS treated knock out mice (Netea MG *et al,* 2001).

The co-ordination among TLRs to detect a large number of PAMPs has been first reported by Ozinsky A *et al.* TLR-2 and TLR-6 heterodimer detects the PGNs while TLR-

2 homodimer detects bacterial lipopeptide. They also showed that the cytoplasmic domain of TLR-2 can form functional pairs with TLR-6 or TLR-1, and this interaction led to cytokine induction (Ozinsky A *et al,* 2000). Hajjar AM *et al* (2001) reported that phenol-soluble modulin from *Staphylococcus epidermidis* activated TLR-2 and that is enhanced by TLR-6 but inhibited by TLR-1. TLR-1 interacts with TLR-2 to recognize the lipid configuration of the native mycobacterial lipoprotein as well as several triacylated lipopeptides and enhanced the NF-κB activation (Takeuchi O *et al,* 2002). *Borrelia burgdorferi* outer-surface lipoprotein (OspA) immunization of mice genetically deficient in either TLR-2 (-/-) or TLR-1 (-/-) produced low titres of antibodies against OspA and macrophages from TLR-2 (-/-) mice were unresponsive to OspA and PGN, whereas those from TLR-1 (-/-) mice responded normally to PGN but not to OspA (Alexopoulou L *et al,* 2002). *Treponema maltophilum* glycolipids reported to be induced TNFα secretion from murine macrophage cell line RAW-264.7 through TLR-2 activation (Schroeder NW *et al,* 2000).

Epithelial cells, which considered as the first line of defence against microbes, were expressing TLRs and activated distinct Mitogen Activated Protein Kinase (MAPK) family when stimulated with LPS (Cairo E *et al*, 2000). LPS derived from *Escherichia coli* and *Salmonella minnesota* activated human cell lines through TLR-4 but not TLR-2 (Tapping RI *et al,* 2000). TLR-4 anatgonist, lipid A like molecule E5531, specifically inhibited TLR-4 dependent *M. tuberculosis* induced TNFα secretion in RAW 264.7 cells and human alveolar macrophages but Nitric oxide (NO) production was not inhibited (Means TK *et al,* 2001). The importance of accessory molecules involved in ligand binding to TLRs have recognized later and CD14 was identified as one of those molecules. Low concentrations of LPS or the LPS-mimetic, Taxol, required the participation of CD14, for the induction of TLR-4 signal induced genes, whereas high concentrations of LPS or Taxol elicited the expression of a subset of LPS-inducible genes in the absence of CD14 (Perera PY *et al,* 2001). Ploetz SG *et al* (2001) reported

that LPS induced TNFα and eosinophil cationic protein (ECP) release from Eosinophil is CD14 dependent. In a study using TLR-4 (-/-) and CD14 (-/-) deficient mice showed that they clear Gram-negative bacteria (*Escherichia coli* 0111) at least ten times more efficiently than normal mice due to early LPS-induced PMN infiltration and enhanced clearance (Haziot A *et al,* 2001). TLR-2 and TLR-4 are present on the surface of human neutrophils, which treated with GM-CSF and G-CSF dramatically up-regulated TLR-2 and CD14 surface expression. GM-CSF treatment enhanced the IL-8 secretion and superoxide priming responses of neutrophils to stimulation with TLR-2 ligands, including zymosan, PGN, and LAM (Kurt-Jones EA *et al,* 2002). Using MyD-88-GFP fusion protein expressing macrophages, Ahmad-Nejad P *et al (*2002) demonstrated that LPS and CpG-DNA trigger signaling from two different cellular locations, LPS at the cell membrane and the CpG-DNA at the lysosomal compartment. LPS did not require endocytosis to functionally associate with the membrane expressed TLR-4/MD-2 complex, while internalization and endosomal maturation of CpG-DNA was needed to activate TLR-9. TLR-4 and radioprotective-105 (RP-105) are associated with small molecules called myeloid differentiation-2 (MD-2) and MD-1, respectively, which forms hetero-dimers (TLR-4/MD-2 and RP-105/MD-1) and function as recognition and signalling molecules of LPS. RP105 or MD-1 (-/-) deficient mice showed a reduced LPS responsiveness and there may be functional associations between TLR4/MD-2 and RP105/MD-1 (Kimoto M *et al,* 2003). TLR-4 expression in live cells demonstrated pronounced expression on the plasma membrane as well in the Golgi apparatus. LPS is recognized in mammals by a receptor complex composed of CD14, TLR-4, and MD-2. Aggregation of TLR-4 by immobilized TLR4 antibodies was sufficient to induce signalling, however, pharmacological disruption of the Golgi did not inhibit LPS induced NF-κB activation (Espevik T *et al,* 2003). Frendeus B *et al* reported that *E. coli* P fimbriae activated epithelial cells by means of a TLR-4 dependent signalling pathway, and suggest that glycosphingolipid receptors for P fimbriae can recruit TLR-4 as co-receptors in CD14 (-/-)

deficient host cells (Frendeus B *et al,* 2001). LPSs of *Leptospira interrogans, Legionella pneumophila, Rhizobium species Sin-1, Bacteroides fragilis, Chlamydia trachomatis* and *Pseudomonas aeruginosa* signals through TLR-2 and all TLR-2 signalling LPS discovered to date demonstrated relatively weak endotoxicity in some models and have structural features distinct from those LPS shown to signal through TLR-4 (Erridge C *et al,* 2004). Talreja J *et al* (2004) reported that histamine up-regulated the expression of TLR-2 and TLR-4 and amplified endothelial cell inflammatory responses to Gram-negative and Gram-positive bacterial components. MD-2 is associated with the extracellular portion of TLR-4, which directly binds to LPS and regulates subsequent LPS induced TLR-4 clustering. An endoplasmic reticulum (ER) chaperone, glycoprotein 96, is required for the stability of TLR-4 and the formation of a TLR-4/MD-2 complex in ER. Recently, another molecule, a protein associated with Toll-like receptor 4 (PRAT4A), was shown to play a critical role in cell surface expression of TLR-4. These molecules control LPS responsiveness by regulating the subcellular distribution of TLR-4 (Saitoh S and Miyake K, 2006). PRAT4A knockdown inhibited trafficking of TLR-1 and TLR-4 to the cell surface and ligand-induced trafficking of TLR-9 to lysosomes (Takahashi K *et al,* 2007).

Endotoxin tolerance in HEK-293/TLR-4/MD-2 transfectants and in human monocytes markedly suppressed LPS-mediated TLR-4 tyrosine phosphorylation and recruitment of LYN kinase to TLR-4, without affecting TLR-4/MD-2 interactions. Mutations of tyrosine residues Y674A and Y680A within the TIR domains of CD4/TLR-4 impaired its ability to elicit phosphorylation of p38 and JNK MAPK, IκBα degradation, activation of NF-κB and RANTES reporters and altered MyD-88/TLR-4 interactions, increased associations with a short IRAK-1 isoform, and decreased amounts of activated IRAK-1 in complex with TLR-4 (Medvedev AE *et al,* 2007). Spiller S *et al* (2008) reported that murine splenocytes and human PBMCs released IFNγ in a TLR-4 dependent manner, leading to enhanced TLR-2 surface expression and sensitivity for TLR-2 ligands. They identified that a TLR-4/MD2 antibody (1A6) in combination with mAb to

TLR-2 after 4h of infection with clinical isolates of *S. enterica* and *E. coli* and subsequent antibiotic therapy provided robust protection in infected mice.

LPS has induced cytokine production from DCs in a MyD-88 dependent manner and also induced functional maturation of MyD-88 (-/-) deficient DCs, including up-regulation of costimulatory molecules and enhancement of APC activity. However, MyD-88 (-/-) deficient DCs could not mature in response to bacterial DNA, the ligand for TLR-9, indicating that MyD-88 is differentially required for TLR family members signalling (Kaisho T *et al,* 2001). DC maturation is induced by stimuli from Gram-positive microorganisms, such as PGN and lipotheichoic acid (LTA), with similar efficiency as by LPS. In addition, TLR-2 and TLR-4 interaction with the appropriate ligand is essential for bacteria-induced maturation of DCs (Michelsen KS *et al,* 2001). In mouse immature DC, TLR-2, TLR-4 and TLR-9 mRNA were up-regulated following LPS stimulation and inhibition of ERK and NF-κB activation suppressed the up-regulation of the expression of TLR-2, TLR-4 and TLR-9 mRNA, inhibition of p38 kinase prevented the up-regulation of TLR-2 and TLR-4 mRNA expression but enhanced the up-regulation of TLR-9 expression (An H *et al* 2002). In contrast to other TLR agonists, CpG DNA is superior in activation of dendritic dells and induction of cytokines such as IL-12 and IL-18. This qualifies CpG DNA as a Th-1 promoting adjuvant (Dalpke A *et al,* 2002). ManLAM binding to DC specific C-type lectin (DC-SIGN) prevented mycobacteria or LPS-induced DC maturation possibly through interfering with TLR-mediated signals (Geijtenbeek TB *et al,* 2003). DCs responded to TLR ligands produced IL-12 and related cytokines that are instrumental in Th-1 cell bias, and MHC class II antigen presentation to activate naive Th-cells. It has also been reported that DCs activated by TLR engagement can also provide a potent negative signal that prevents the development of Th-2 cells (Sun J *et al,* 2005). The production of IL-10, in addition to inflammatory cytokines and chemokines, was significantly reduced in DCs from TLR-4 defective C3H/HeJ mice in response to *Bordetella pertussis.* TLR-4 was required for *B. pertussis* LPS induced maturation of

DCs, but other *B. pertussis* components stimulated DC maturation independently of TLR-4. The course of *B. pertussis* infection was more severe in C3H/HeJ (TLR-4 defective) than in C3H/HeN mice. In addition, antibody and antigen specific IFNγ responses were enhanced at the peak of infection, whereas antigen specific IL-10-producing T-cells were significantly reduced in C3H/HeJ mice. This was associated with enhanced inflammatory cytokine production, cellular infiltration, and severe pathological changes in the lungs of TLR-4 defective C3H/HeJ mice (Higgins SC *et al,* 2003).). Distinct TLR ligands instruct human DCs to induce distinct Th cell responses by differentially modulating MAPK. *E. coli* LPS and flagellin, which trigger TLR-4 and TLR-5, respectively, instructed DCs to stimulate Th1 responses via IL-12p70 production, which depends on the phosphorylation of p38 and JNK-1/2. In contrast, the TLR-2 agonist, Pam3cys, and schistosome egg antigens (the Th-2 stimulus), barely induced IL-12p70, however, stimulated sustained duration and magnitude of ERK-1/2 phosphorylation, which induced stabilization of the transcription factor c-Fos, (a suppressor of IL-12 and 3) resulted in Th-2 bias (Agrawal S *et al,* 2003). Stimulation of monocyte-derived DCs with TLR ligands differentially affects the uptake and cross-presentation of cellular antigens. Activation of DCs with TLR-3 or TLR-4 inhibited phagocytosis of apoptotic tumour cells and resulted in a reduced cross-presentation of p65-derived T-cell epitopes on MHC class I molecules upon engulfment of CMV infected fibroblasts (Weck MM *et al,* 2007). Geisel J *et al* (2007) reported that TLR-4 and TLR-2 stimulation in naive DC results in homo and cross tolerance and the mechanisms involved in tolerance depend on the concentration of the TLR agonist used for DC priming and are governed by IL-6 and DC maturation. *Yersinia pestis*, the causative agent of plague, at the temperature of its flea vector (approximately 20-30°C) expressesed a profile of genes distinct from those expressed in a mammalian host (37°C). DCs exposed to *Y. pestis* grown at 26°C, secreted copious amounts of IL-12p40 homodimer. However, DCs exposed to *Y. pestis* grown at 37°C, transcribed very little IL-12p40 and secreted as IL-12p40 monomer. The absence of TLR-4 abrogated the

production of IL-12p40 homodimer in DCs exposed to *Y. pestis* grown at 26°C (Robinson RT *et al,* 2008).

Plasmacytoid DC (pDC) expressed a higher level of TLR-9 compared with myeloid DC (mDC) cultured with GM-CSF, however, stimulation with IFNγ was capable of upregulating TLR-9 expression in mDC to a level comparable with expression in pDC. IL-12 p40 and IL-6 mRNA expression and IL-12p70 secretion also enhanced in response to CpG DNA in IFNγ pretreated mDC (Uchijima M *et al,* 2005). Live or heat-killed *E. coli* in mice induces splenic DC migration, maturation, and apoptosis, whereas, DC maturation is impaired in TLR-2 (-/-), TLR-4(-/-) and TRIF (-/-) deficient mice and DC apoptosis is reduced only in TLR-4 (-/-) and TRIF (-/-) deficient mice (De Trez C *et al,* 2005). Langerhan cells like DCs (LC-DCs) and monocyte derived DCs (Mo-DCs) expressed TLR-1 to 10 mRNAs at comparable levels. Although many of the TLR-induced cytokine patterns were similar between the two cell types, stimulation with the TLR-3 agonist poly-I:C triggered significantly higher amounts of the IFN inducible chemokines CXCL-9 and CXCL-11 in Langerhans cells (LC) like DCs as compared with Mo-DCs. Supernatants from TLR-3 activated LC-DCs reduced intracellular replication of vesicular stomatitis virus in a type I IFN-dependent manner (Renn CN *et al,* 2006). Differentiations of immature to mature DCs are induced by activation of TLRs. Bartz H *et al* reported that TLR triggering deviated phenotypic and functional differentiation from CD14+ monocytes to CD1a+ DCs. TLR stimulation in DC precursor cells induced proteins of the suppressor of cytokine signalling (SOCS), which correlated with loss of sensitivity to GM-CSF. Overexpression of SOCS-1 abolished GM-CSF signal transduction. Moreover, forced SOCS-1 expression in DC precursors mimicked the inhibitory effects on DC generation observed for TLR stimulation (Bartz *et al,* 2006).

LCs, and dermal dendritic cells (DDCs) have unique functions, human DDCs have a broad TLR expression profile, whereas human LCs have a selective impaired expression of cell surface TLR-2, TLR-4, and TLR-5. LCs weakly respond to bacterial

TLR-2, TLR-4, and TLR-5 ligands in terms of cytokine production and maturation, as well as to whole Gram-positive and Gram-negative bacteria, whereas their responsiveness to viral TLR ligands and viruses is fully active and comparable to DDCs (van der Aar Am *et al,* 2007). Monocyte derived Langerin^{+}/CD1c^{+}LCs (MoLCs) secreted higher amounts of IL-6 and TNFα by stimulation through TLR-2 than by stimulation through TLR-3, -4, -5, -8, and -9, however, DCs generated from the same donor monocytes, were activated by agonists of TLRs other than TLR-2 as well. Lipopeptides triggering TLR-2 induced IRAK-1 phosphorylation and migration toward the chemokines CCL-19 and CCL-21 in epidermal LCs and MoLCs and also up-regulated CD86, CD83, and CCR-7, TNFα and IL-6, and NF-κB activation (Peiser M *et al,* 2008).

Normal and neoplastic human B-cells express a distinct TLR repertoire including TLR-9 and TLR-10 and that expression is increased upon engagement of the antigen receptor complex or ligand with TLR-9 (Bourke E *et al,* 2003). Hayashi EA *et al* (2005) demonstrated that TLR-4 signaling favors B-cell maturation, whereas TLR-2 arrests or retards maturation. RP105 (-/-) deficient B-cells proliferated poorly in response to TLR-4 ligand LPS and TLR-2 ligand lipoproteins treatment. CD138 (syndecan-1)-positive plasma cells were detected after lipid A injection in wild-type spleen but much less in RP105 (-/-) deficient spleen. The immunopotentiating activity of neisserial porins, the major outer membrane protein of the pathogenic *Neisseria*, is mediated by its ability to stimulate B-cells and up-regulate the surface expression of CD86 and this ability is dependent on TLR-2 and MyD-88 (Massari P *et al,* 2002). It has also been reported that RP105 ligation *in vivo* induced plasma cell differentiation (Nagai Y *et al,* 2005).

Regulatory CD4^{+} T-cells (T-reg) were controlled inflammatory reactions to commensal bacteria and opportunist pathogens by selectively expressing TLR-4, -5, -7, and -8. TLR-4 ligand LPS induced up-regulation of several activation markers and enhanced T-reg survival and proliferation (Caramalho I *et al,* 2003). The liver is a site for trapping and apoptosis of activated CD8^{+} T-cells during systemic immune responses and

in TLR-4 deficiency, the liver was compromised in its ability to sequester activated $CD8^+$ T-cells, and there was an inverse correlation between the frequency of activated $CD8^+$ T-cells trapped in the liver and their frequency in the circulating pool (John B and Crispe IN, 2005). *C. albicans* induced immunosuppression through TLR-2 derived signals that mediate increased IL-10 production and survival of T-reg cells (Netea MG *et al,* 2004). Vδ1 subset of γδ T cells accumulated in inflamed joints in Lyme arthritis caused by the spirochete, *B. burgdorferi*. Collins C *et al* (2008) very recently reported that *B. burgdorferi* activated murine γδ T-cells indirectly through TLR stimulation on DCs or monocytes and that also been dependent on caspases.

Mast cells (MCs) were expressed mRNAs for TLR-1, TLR-2, and TLR-6 but not TLR-4. Bacterial PGN and yeast zymosan were potent inducers of GM-CSF and IL-1β and also induced substantial short-term cysteinyl leukotriene generation in mast cells (McCurdy JD *et al,* 2003). Varadaradjalou S *et al* (2003) reported that human normal cord blood-derived mast cells (CBMC) could interact with bacterial products, especially LPS from *E. coli* and PGN from *S. aureus*, known as TLR-4 and TLR-2 agonists, respectively. They found that both LPS and PGN induced significant release of not only TNFα, but also IL-5, IL-10 and IL-13, in addition, PGN alone stimulated histamine release by human MCs. However, the release of TNFα induced by LPS required the priming of CBMC by IL-4 and the presence of serum components, in particular soluble CD14. Murine and human MCs produced type-I IFNs after exposure to double-stranded RNA through specific interactions with TLR-3 (Kulka M *et al,* 2004). Rocha-de-Souza CM *et al* (2008) reported that *S. aureus* displayed a high adherence to mast cells as well as invasive and survival abilities and production of TNFα and IL-8 when infected with CBMC. However, pretreatment of cytochalasin D or preincubation with anti TLR-2 or anti-CD48 antibodies have abrogated the invasiveness and the inflammatory response, respectively.

Mycobacterium tuberculosis, through 19-kDa lipoprotein activation of TLR-2, inhibits IFNγR signalling in human macrophages, resulting in decreased MHC-II antigen processing and recognition by MHC-II-restricted $CD4^+$ T-cells (Gehring AJ *et al,* 2003). This inhibition may promote *M. tuberculosis* evasion of T-cell responses and persistence of infection in tuberculosis. *M. tuberculosis* infections of TLR-2 deficient mice were displayed reduced bacterial clearance, a defective granulomatous response, and develop chronic pneumonia (Drennan MB *et al,* 2004). It has also been reported that glycopeptidolipids (GPLs), which are highly expressed surface molecules on *M. avium*, can stimulate the NF-κB pathway as well as MAPK p38 and JNK activation and production of proinflammatory cytokines when added to murine BMDCs. This stimulation was dependent on TLR-2 and MyD-88 but not TLR-4 (Sweet L and Schorev JS, 2006). *M. tuberculosis* 19-kDa lipoprotein and cell wall peptidoglycans (contained in the mycolylarabinogalactan peptidoglycans) inhibited macrophage responses to IFNγ at a transcriptional level. The 19-kDa lipoprotein inhibited IFNγ signalling in a TLR-2 dependent and MyD-88 dependent fashion, whereas PGNs inhibited the same signalling independently of TLR-2, TLR-4, and MyD-88 (Fortune SM *et al,* 2004). Production of IL-6 and IL-10 from DCs in response to *M. tuberculosis* is principally dependent on TLR-2. *M. tuberculosis* infection also induced IL-12 production in the absence of either TLR-2 or TLR-4 possibly through the involvement of other receptors in IL-12 production. In addition, it is also revealed that lack of TLR-2 or TLR-4 does not impact on DC maturation or on their ability to influence the polarity of differentiating naive T-cells during *M. tuberculosis* infection (Jang S *et al,* 2004). Neutrophils infected with *M. bovis* bacille Calmette Guerin (BCG) induced mRNA expression and secretion of the chemokine IL-8, by signalling through TLR-2 and TLR-4, in conjunction with MyD-88. Anti-TLR-2 antibody blocked the early phase of IL-8 and MyD-88 induction, while anti-TLR-4 antibody blocked the late phase of induction occurring 2 h after infection. The existence of a TLR/MyD-88 pathway for recognition and response to mycobacterial ligands provides neutrophils with

the ability to drive the recruitment and activation of inflammatory cells during the early phase of mycobacterial infection and immunization (Godlay G and Young DB, 2005). *M. tuberculosis* mannose-capped lipoarabinomannans (Man-LAMs) inhibited LPS-induced IL-12 p40 expression in an IL-10-independent manner through blocking IRAK-TRAF-6 interaction as well as IκBα phosphorylation leading to reduced NF-κB activation. Knockdown of IRAK-M expression by RNAi reinstated LPS-induced IL-12 production in Man-LAM-pretreated cells (Pathak SK *et al,* 2005). *M. tuberculosis* infected TLR9 (-/-) but not TLR2 (-/-) deficient mice displayed defective mycobacteria-induced IL-12p40 and IFNγ responses *in vivo*, whereas, TLR2/9 (-/-) mice displayed markedly enhanced susceptibility to infection in association with combined defects in proinflammatory cytokine production in vitro (Bafica A *et al,* 2005). Tuberculin purified protein derivative stimulation resulted in rapid activation of MAPKs and an early burst of ROIs in monocytes/macrophages in a TLR-2 dependent manner. PPD-induced ROI production led to robust activation of apoptosis-regulating signal kinase (ASK1) upstream of p38 MAPK through TLR-2. In addition, phosphorylation of the cytosolic NADPH oxidase subunit p47phox and ASK1 activation are mutually dependent on PPD/TLR-2 mediated signalling (Yang CS *et al,* 2007).

IL-32 is a newly described proinflammatory cytokine that seems likely to play a role in inflammation and host defense. TLR-4 agonist, LPS, induced moderate (4-fold) production of IL-32, whereas agonists of TLR-2, TLR-3, TLR-5, or TLR-9, each of which strongly induced TNFα and IL-6, did not stimulate IL-32 production. However, *M. tuberculosis* and *M. bovis* BCG induced high level of IL-32 in monocytes. IL-32 is cell-associated pro-inflammatory cytokine and its stimulation by mycobacteria is through a caspase-1- and IL-18 dependent production of IFNγ (Netea MG *et al,* 2006).

S. enterica serotype Typhi, the causative agent of typhoid fever, expression of the Vi capsular antigen reduced expression of the neutrophil chemoattractant IL-8 in host cells. Capsulated bacteria elicited IL-8 expression in polarized human epithelial cells

(T84) and human macrophage-like cells (THP-1) in vitro at significantly reduced levels compared to noncapsulated bacteria. HEK293 cells transfected with human TLRs demonstrated that in the presence of TLR-5 or TLR-4/MD-2/CD14, a noncapsulated serotype Typhi mutant was able to induce the expression of IL-8, while this response was significantly reduced during infection with capsulated serotype Typhi wild type (Raffatellu M *et al,* 2005). *S. typhimurium* SPI-1 and the SPI-2 type III secretion systems (T3SS) contributed to inflammation and mutants that retained only a functional SPI-1 or a SPI-2 T3SS caused attenuated colitis. SPI-1 functional mutant caused diffused cecal inflammation that did not require MyD-88 signalling, however, SPI-2 functional mutant induced focal mucosal inflammation requiring MyD-88 (Hapfelmeier S *et al,* 2005). Mouse and human TLR-5 discriminate between different flagellins and mutagenesis studies demonstrate that naturally occurring amino acid variation in TLR-5 residue-268- is responsible for human and mouse discrimination between flagellin molecules, whereas, mutations within one conserved surface identify residues D295 and D367 as important for flagellin recognition (Andersen-Nissen E *et al,* 2007). Sun J *et al* (2007) found that activation of PI-3-K and AKT by flagellin has a small damping effect in the early stages of flagellin signalling but is not responsible for tolerance in human intestinal epithelial cells and also indicated that inhibition of TLR-5 associated IRAK-4 activity occurs during the development of flagellin tolerance and is likely to be the cause of tolerance.

Mice deficient in MyD-88 (-/-) were more susceptible to *Listeria monocytogenes* and showed an inability to produce IL-12 and IFNγ during infection. TLRs/MyD-88 dependent IL-12/TNFα production and IL-12 and IL-18-mediated IFNγ production are found to be critical in early phase clearance of *L*. *monocytogenes* (Seki E *et al,* 2002). Torres D *et al* reported that the production of TNFα, IL-12, and NO and the expression of the costimulatory molecules CD40 and CD86, which are necessary for the control of infection, were reduced in TLR-2 (-/-) deficient macrophages and DCs stimulated by *L*.

monocytogenes and were almost abolished in the absence of MyD-88. In addition, there is a high susceptibility of MyD88 (-/-) deficient mice to in vivo infection. Thus, TLR-2 plays an important role in the control of *L. monocytogenes* infection, but other MyD-88 dependent signals may contribute to host resistance (Torres D *et al,* 2004). Machata S *et al* (2008) reported that lipidation of prelipoproteins in *L. monocytogenes* is required to promote NF-κB activation through TLR-2. Experiments using transiently expressing TLR-2 in HeLa cells and *L. monocytogenes* and *L. innocua* mutants lacking the prolipoprotein diacylglyceryl transferase (Δ*lgt*) gene, it has been shown that TLR-2 dependent activation of NF-κB depends on lipidation of prolipoproteins. Studies of bone marrow-derived macrophages of C57BL/6 wild-type and TLR-2 deficient mice infected with Δ*lgt* strains exhibited delayed cellular immune responses directed toward the bacterium when compared to the wild type infected mice.

Cytokine production by bone marrow-derived macrophages (BMMs) in response to *L. pneumophila* infection required the TLR adapter protein MyD-88 and is reduced in the absence of TLR-2 but not in the absence of TLR-4. *In vivo* growth of *L. pneumophila* was enhanced in the lungs of TLR-2 deficient and MyD-88 (-/-) deficient mice, which resulted in a delay in bacterial clearance, whereas *L. pneumophila* dotA mutants, which are unable to replicate intracellularly, were cleared by MyD-88 (-/-) deficient mice. However, significant differences were not observed in the growth and clearance of *L. pneumophila* in the lungs of TLR-4 (-/-) deficient mice and heterozygous littermate control mice (Archer KA and Roy CR, 2006). Bone marrow-derived macrophages and DCs from TLR-2 (-/-), TLR-4 (-/-), and wild type littermate mice infection with *L. pneumophila* have revealed that the growth of the bacteria were enhanced within TLR-2 (-/-) macrophages but no difference among DCs. The production of interleukin IL-12p40 and IL-10 after infection with *L. pneumophila* was attenuated in TLR-2 (-/-) deficient macrophages compared to wild type and TLR-4 (-/-) deficient macrophages. (Akamine M *et al,* 2005). MyD-88 dependent signalling is important for secretion of early inflammatory

cytokines and host protection in response to *L. pneumophila* infection. In addition, IFNγ deficient mice did not have a susceptibility phenotype as severe as the MyD-88 deficient mice and were able to control a pulmonary infection by *L. pneumophila* (Archer KA *et al,* 2009). Infection with the pathogen *L. pneumophila*, which utilizes a type IV secretion system (T4SS), induced an increased proinflammatory cytokine response compared to avirulent bacteria in which the T4SS was inactivated. This enhanced response involved NF-κB activation by TLR signalling as well as NOD1 and NOD2 detection of type IV secretion. In addition, a TLR and RIP-2 independent pathway leading to p38 and SAPK/JNK MAPK activation was found to play an equally important role in the host response to virulent *L. pneumophila*. Activation of this MAPK pathway was T4SS-dependent and coordinated with TLR signalling to mount a robust proinflammatory cytokine response to virulent *L. pneumophila* (Shin S *et al,* 2008).

P. aeruginosa flagella initially bound to the apical surface of polarized epithelial cells, where they prominently co-localized with asialoGM1 and induced TLR-5 expression and mobilized to the apical surface of the cells, and co-localized with superficial flagella. IL-8 expression in airway cells was activated by flagella through induction of Ca^{2+} fluxes, SRC, RAS, and ERK-1/2 MAPK and NFκB activation. TLR-2 (-/-) or TLR-5 (-/-) mutations have inhibited IL-8 induction by 78% and 35%, respectively. The participation of TLR-2 as well as TLR-5 was confirmed in CHO cells transfected with either human TLR-2 or TLR-5 in which flagella activated NFκB reporter to the same extent (Adamo R *et al,* 2004). *P. aeruginosa* virulence factor, exoenzyme S (*ExoS*) induces potent monocyte activation leading to the production of numerous proinflammatory cytokines and chemokines. *ExoS* activated monocytic cells via a MyD-88 pathway, using both TLR-2 and the TLR-4/MD-2/CD14 complex for cellular activation, whereas, the TLR-2 activity was localized to the C-terminal domain of ExoS while the TLR-4 activity was localized to the N-terminal domain (Epelman S *et al,* 2004). *P. aeruginosa* and Δ*fliC* mutant were used to study the role of TLR-2, TLR-4, TLR-5 and

MyD-88 in recognition of the pathogen. Δ*fliC* mutant infected MyD-88 (-/-) deficient mice failed to mount early cytokine and inflammatory responses or control bacterial replication, resulting in necrotizing lung injury and lethal disseminated infection. TLR-4 (-/-) and TLR-2/4(-/-) deficient mice responded to Δ*fliC* mutant infection with severely limited inflammatory and cytokine responses but intact bacterial clearance. However, TLR-2 (-/-) deficient mice had partially reduced cytokine responses but augmented inflammation and preserved bacterial killing. This indicates that TLR-4 and flagellin induced signals mediated most of the acute inflammatory response to *P. aeruginosa* and that TLR-2 has a counter-regulatory role (Skerrett SJ *et al,* 2007). MyD-88 dependent pathway is essential for the development of early (4-8 h) host response to *P. aeruginosa* lung infection, however, the development of a delayed (24-48 h) host response to *P. aeruginosa* is independent of MyD-88 (Power MR *et al,* 2006). The same group have reported recently that TRIF-deficient mouse alveolar and peritoneal macrophages showed a complete inhibition of RANTES production, severely impaired TNFα and KC (mouse equivalent of IL-8) production, and reduced NF-κB activation in response to *P. aeruginosa* stimulation (Power MR *et al,* 2007). Very recently, Ramphal R *et al* reported that interactions of either TLR-4 with LPS or TLR-5 with flagellin can effectively defend the lung from *P. aeruginosa* infection and the absence of a response by both results in hyper-susceptibility to this infection in mice (Ramphal R *et al,* 2008).

Chlamydia pneumoniae is an obligate intracellular human pathogen causing diseases such as pneumonia, bronchitis, and pharyngitis. Prebeck S *et al* (2001) reported that recognition of the Gram-negative bacterium *C. pneumoniae* depends largely on TLR-2 and only to a minor extent on TLR-4. *C. pneumoniae* stimulated maturation and cytokine secretion by bone marrow-derived dendritic cells (BMDDC) and the purified recombinant HSP60 from *C. pneumoniae* also stimulated BMDDC in a TLR-2and TLR-4 dependent way similar to the whole microorganism (Costa CP *et al,* 2002). Glucocorticoids synergistically enhanced nontypeable *Haemophilus influenzae* induced

expression of TLR-2 through specific up-regulation of the MAPK phosphatase-1 (MKP-1) that leads to dephosphorylation and inactivation of p38 MAPK. Increased expression of TLR-2 in epithelial cells greatly enhanced the nontypeable *H. influenzae* induced expression of several key cytokines, including TNFα and IL-1β and IL-8 and thereby contributing significantly to host immune and defence response (Imasato A *et al,* 2002). Rothfuchs AG *et al* (2004) reported that *C. pneumoniae* induced IFNγ expression in bone marrow derived macrophages is controlled by a TLR-4/MyD88/IFNαβ/STAT1-dependent pathway, as well as by a TLR-4 independent pathway leading to NF-κB activation.

Porin of *Shigella dysenteriae* type-1 treatment of peritoneal macrophages showed an increase of expression of mRNA for both TLR-2 and MyD-88 and IL-12 release (Ray A *et al,* 2003). Porin of *S. dysenteriae* type 1 increased the mRNA levels for TLR-2 and TLR-6 and CD80 and also induced cell-surface expression of immunoglobulin IgM, IgG2a, and IgA in peritoneal cavity B-2 cells (Ray A and Biswas T, 2005). In addition, mRNA expression of TRAF-6, MHC-II, MIP-1α, MIP-1β and RANTES and intracellular expression and release of TNFα and IL-12 in presence of porin were found to be TLR-2 and NF-κB dependent (Biswas A *et al,* 2007). *H. influenzae* type b porin induced TNFα and IL-6 production and that was eliminated in macrophages from TLR-2 or MyD-88 deficient mice. In addition, anti-TLR-2 antibodies but not anti-TLR-4 antibodies significantly reduced *H. influenzae* type b porin stimulated TNFα and IL-6 release from the human monocytic cell line THP-1 (Galdiero M *et al,* 2004).

Infection with the live vaccine strain *F. tularensis* LVS rendered cells of the murine macrophage-like cell line J774A.1 incapable of secreting TNFα or IL-1β and mobilizing an antimicrobial activity in response to bacterial lipopeptide or *E. coli* derived LPS. A mutant of *F. tularensis* LVS defective in expression of a 23kDa protein, that is upregulated during intracellular infection, restored TNFα secretion during infection with J774A.1 cells. LPS or lipopeptide induced phosphorylation of p38 MAPK and the transcription factor c-Jun were also inhibited by *F. tularensis* LVS but not by the 23kDa

protein mutant (Telepnev M *et* al, 2003). Duenas AI *et al* (2006) reported that LPS from *F. tularensis* LVS signals through TLR-4 at higher concentrations than those required for *E. coli* LPS.

LTA from Group B Streptococcus (GBS) and *S. aureus* have interacted with TLR-2 and TLR-6, but not TLR-1 and NF-κB activation by LTA required the integrity of two putative Phospho Inositol-3-Kinase (PI-3-K) binding domains within TLR-2 and was inhibited by wortmannin, indicating an essential role for PI-3-K in cellular activation by LTA. However, LTA from GBS proved to be a relatively weak stimulus of phagocytes and found to have only 20% activity when compared with LTA from *S. aureus*. LTA from GBS is a TLR-2/TLR-6 ligand but does not contribute significantly to GBS cell wall mediated macrophage activation (Henneke P *et al,* 2005). GBS, the most important pathogen in neonatal bacterial meningitis, not only activates microglia, but also induces apoptosis in these cells through TLR-2 and the TLR- daptor molecule MyD-88 and subsequent activation of caspase-8, a process that involves the transcription factor family ETS (Lehnardt S *et al,* 2007). Exposure of macrophages to heat-killed GBS activated TLR dependent pathway, whereas live GBS activated a TLR/NOD/RLR independent pathway. Phagolysosomal membrane destruction by GBS pore-forming toxins, leading to the release of bacterial DNA into the cytosol and GBS DNA in the cytosol induced IFNβ production through a pathway dependent on the activation of the serine-threonine kinase TBK-1 and phosphorylation of the transcription factor IRF-3 (Charrel-Dennis M *et al,* 2008). Gratz N *et al* (2008) reported that in mouse macrophages, the activation of MyD-88 dependent signalling by the extracellular Gram-positive human pathogen group A streptococcus (GAS; *S. pyogenes*) does not require TLR-2, TLR-4 and TLR-9. Instead of that, infection of macrophages by GAS causes IRF-3 dependent, MyD-88 independent production of type-I IFN and is induced also by GAS lacking *slo* and *sagA*, the genes encoding cytolysins that were shown to be required for IFN production in response to other Gram-positive bacteria.

LTA of *S. aureus* induced a dramatic release of cytokines, with an early appearance of TNFα and IL-1β and a delayed liberation of IL-8. The first phase of IL-8 production was induced directly by LTA, whereas the second phase was endogenously mediated by TNFα, as it was largely abrogated by neutralizing anti-TNFα antibodies in isolated human neutrophils. IL-1β was not involved in LTA induced IL-8 generation and in addition, the late phase of IL-8 generation could also be attenuated by exogenous IL-10, probably as a consequence of its down-regulatory effects on TNFα generation. Using antibodies that neutralized the activity of TLR-2 or TLR-4, it is also showed that CD14 dependent, LTA induced neutrophil activation did not proceed through TLR-2 or TLR-4 mediated pathways (Hattar K *et al,* 2006). Nakayama M *et al* (2007) identified murine-paired Ig-like receptor-B (PIR-B), and its human orthologs Ig-like transcript -2 and Ig-like transcript 5 as novel receptors for *S. aureus*. In mouse bone marrow-derived macrophages, masking of PIR-B by anti-PIR mAb or genetic deletion of PIR-B shows significantly impaired recognition of *S. aureus* and enhanced TLR mediated inflammatory responses to the bacteria. Both type-I IFNs and IRFs are well characterized in viral infections but not in bacterial. *S. aureus* LTA activated IRF-2 resulting in the up-regulation of IRF-1 and activation of STAT-1 and STAT-3 resulting in rapid secretion of IFNα (Liljeroos M *et al,* 2008).

Inhalation of *Bacillus anthracis*, a bioterrorism agent, results in a high mortality rate despite appropriate antibiotic therapy. Macrophages appear to be a key factor in *B. anthracis* pathogenesis. The burst of pro-inflammatory cytokines from macrophages could be a major cause of death in anthrax. Germination from spores to bacilli produced a substantial stimulus for the secretion of the cytokines IL-6, TNFα, IL-10, and IL-12p40 by macrophages *in vitro.* Pre-treatment of mouse macrophages with the TLR-9 ligand ISS-1018, or the TLR-7 ligands R-848 and IT-37, results in a substantial decrease in the subsequent secretion of IL-6 and TNFα in response to *B. anthracis* infection of

macrophages. In addition, the TLR-7 and TLR-9 ligands significantly decreased anthrax induced cytotoxicity in the macrophages (Sabet M *et al,* 2006).

S. pneumoniae, H. influenzae type b*, and N. meningitidis*, are the three principal causes of bacterial meningitis. Mogensen TH *et al* using HEK-293 cell lines overexpressing one type of TLR demonstrated that, *S. pneumoniae* triggered activation of the transcription factor NF-κB and expression of IL-8, only in cells expressing TLR-2 or -9. The same response was evoked by *H. influenzae* in cells expressing TLR-2 or -4 and by *N. meningitidis* in cells expressing TLR-2, -4, or -9, however, the ability of *S. pneumoniae* and *N. meningitidis* to activate TLR-9 was severely attenuated when bacteria had been heat-inactivated prior to stimulation of the cells (Mogensen TH *et al,* 2006). Expression of capsular polysaccharide by bacterial pathogens is associated with increased resistance to host clearance mechanisms, in particular by evading opsonization and uptake by professional phagocytes. Clearance of encapsulated *H. influenzae* required both TLR and NOD signaling pathways, whereas individual deficiencies in each of these signaling cascades did not affect clearance of nonencapsulated strains (Zola TA *et al,* 2008).

Zheng J *et al* (2008) reported that *Campylobacter jejuni* induced IL-8 secretion requires functional flagella and cytolethal distending toxin (CDT) and depends on the activation of NF-κB through TLR signalling and CDT in T-84 human intestinal epithelial cells. *Burkholderia cenocepacia* is known to induce a harmful inflammatory response in the airways of cystic fibrosis patients. Expression of both TLR-2 and TLR-4 was significantly up-regulated by *B. cenocepacia* infection, whereas TLR-5 expression remained unchanged in human bronchial epithelial cells. However, using epithelial cells from TLR-2 (-/-), TLR-4 (-/-) or TLR-2/4 (-/-) mice or cells over-expressing a functional form of TLR-5 revealed that TLR-5, but neither TLR-2 nor TLR-4, critically regulated *B. cenocepacia* induced lung epithelial inflammatory response (de C Ventura CM *et al,* 2008).

Commensal *E. coli* induced NF-κB DNA binding, NF-κB transcriptional activity, CCL-20 expression, and IL-8 secretion in the human IEC lines. *E. coli* MG1655 flagellin was necessary and sufficient to trigger this pro-inflammatory pathway via its interaction with TLR5 and the subsequent recruitment of the adaptor protein MyD-88 (Bambou JC *et al,* 2004). Polysaccharide-A (PSA), which is produced by the intestinal commensal *B. fragilis*, activates CD4+T-cells, resulting in a Th-1 response correcting the Th-2 cell skew of germ-free mice and optimization of the Th-1 cytokine IFNγ in PSA stimulated DC-CD4+ T-cell co-cultures depends on both TLR-2 and antigen presentation (Wang Q *et al,* 2006). Pro-biotic *E. coli* strain Nissle 1917 has been proven to be efficacious for the treatment of inflammatory bowel diseases. Sturm A *et al* reported that *E. coli* Nissle 1917 conditioned medium inhibits cell cycling and expansion of peripheral blood but not mucosal T-cells. Heat-inactivated *E. coli* Nissle 1917, LPS, or CpG DNA did not alter peripheral blood T-cell cycling. *E. coli* Nissle 1917 conditioned medium decreased cyclin D-2, B-1, and retinoblastoma protein expression, contributing to the reduction of T-cell proliferation. Using TLR-2 knockout mice, they further demonstrated that the inhibition of peripheral blood T-cell proliferation by *E. coli* Nissle 1917 conditioned medium is TLR-2 dependent (Sturm A *et al,* 2005).

The group B coxsackieviruses are ssRNA viruses that have been implicated in viral myocarditis and their viral RNA shown to persist in cardiac muscle contributing to a chronic inflammatory cardiomyopathy. Triantafilou K *et al* reported that CBV-induced inflammatory response is mediated through TLR-8 and to a lesser extent through TLR-7 (Triantafilou K *et al,* 2005). TLR-8 and TLR-7 also reported as the host sensors for human parechovirus-1, another ssRNA virus (Triantafilou K *et al,* 2005b). Craft N *et al* (2005) reported that topical imiquimod, a synthetic TLR-7 agonist, significantly enhanced the protective antitumor effects of a live, recombinant listeria vaccine against murine melanoma. Yang K *et al* (2005) showed that IFNα, β, and λ induction through TLR-7, TLR-8, and TLR-9 were abolished in IRAK-4 (-/-) deficient blood cells, whereas IFNα, β,

and λ were induced normally by TLR-3 and TLR-4 agonists. Cells lacking TRAF-3, a member of the TNF receptor-associated factor family, are defective in type-I IFN responses activated by different TLRs. In addition, TRAF-3 found to be associated with TRIF and IRAK-1, as well as downstream IRF-3/7 kinases TBK-1 and IKK-ε and therefore function as an important factor for IRF activation. TRAF-3 (-/-) deficient fibroblasts, even after TLR stimulation, are defective in their type-I IFN response to infection with vesicular stomatitis virus, indicating that TRAF-3 is also an important component of TLR independent viral recognition pathways (Oganesyan G *et al,* 2006). Hepatitis B virus (HBV) transgenic mice received a single intravenous injection of ligands specific for TLR-2, TLR-3, TLR-4, TLR-5, TLR-7, and TLR-9 have inhibited HBV replication, except for TLR-2, in the liver non-cytopathically within 24 h in a IFNα/β dependent manner (Isogawa M *et al,* 2005). IFN induction in pDC was triggered signal transduction pathways through TLR-7 and TLR-9 as well as by recognition of cytosolic virus-specific patterns. TLR-7 and TLR-9 ligands such as ssRNA and CpG DNA as well as synthetic derivatives are being evaluated as therapeutic immune modulators promoting Th-1 immune responses. Respiratory syncytial virus (RSV) and measles virus infection inhibited both TLR-dependent and independent IFN responses in human pDCs (Schlender J *et al,* 2005). HIV type 1 protease inhibitors (PI) blocked TLR-2- and TLR-4 as well as the TNFα-mediated NF-κB activation, in a dose-dependent manner. PI pretreatment also blocked the LPS induced IL-6 promoter transactivation and TNFα secretion (Equils O *et al,* 2004). TLR deficient mice and genetic complementation, it was showed that murine TLR-7 and human TLR-8 mediated species-specific recognition of GU-rich single-stranded RNA (ssRNA) (Heil F *et al,* 2004).

Lund JM *et al* (2004) showed that TLR-7 recognized ssRNA of vesicular stomatitis virus and influenza virus. The recognition of these viruses by pDCs and B-cells through TLR-7 resulted in their activation of co-stimulatory molecules and production of cytokines. Influenza A virus (IAV) is the etiological agent of a highly contagious acute

respiratory disease that causes epidemics and considerable mortality annually. Recently it is reported that TLR-3 plays a key role in the immune response of lung epithelial cells to IAV. Le Goffic R *et al* (2006) demonstrated that the pulmonary expression of TLR-3 was constitutively and markedly up-regulated following influenza infection in mice. When compared to wild-type mice, infected TLR-3 (-/-) deficient animals displayed significantly reduced inflammatory mediators, including RANTES, IL-6, and IL-12p40/p70 as well as a lower number of $CD8^+$ T-cells in the bronchoalveolar airspace. More importantly, despite a higher viral production in the lungs, mice deficient in TLR-3 had an unexpected survival advantage.

IFNα pretreatment of macrophages was required for efficient TLR-3 and TLR-4 agonist induced activation of IFNα, IFNβ, IL-28, and IL-29 genes. TLR-7/8 agonists were weakly activated IFNα, IFNβ, IL-28, and IL-29 genes, whereas TLR-2 agonist was not able to activate these genes. IFNα enhanced TLR responsiveness in macrophages by up-regulating the expression of TLR-3, TLR-4, and TLR-7. IFNα also enhanced the expression of TLR signalling molecules MyD-88, TRIF, IKK-ε, RIP-1, and IRF-7 and IRF-3 (Siren J *et al,* 2005). Fischer SF *et al* (2005) reported that stimulation with CpG-DNA resulted in a strongly increased sensitivity of TLR-9 expressing fibroblasts to apoptosis induced by staurosporine and UV-irradiation. Morris GE *et al* (2005) reported that co-culture of PBMCs with airway smooth muscle cells resulted in a marked co-operative response to TLR stimuli, and synergistic production of cytokines and chemokines. IL-1 secreted from PBMCs required for this functional activation and blocked by IL-1 receptor antagonist (IL-1RN).

TLRs as well as the TLR associated adaptor molecule MyD-88 have been implicated in the recognition of the fungal pathogens *C. albicans, Cryptococcus neoformans* and *Pneumocystis carinii.* Phospholipomannan, present in the cell surface of *C. albicans* has been shown to be recognized by TLR-2, while TLR-4 mainly interacts with glucuronoxylomannan, the major capsular polysaccharide of *C. neoformans*. MyD-

88 has been implicated in TLR signalling of linear (1, 3)-β-D-glucan, and of β-glucan from *P. carinii* (Roeder A *et al,* 2004). Peritoneal macrophages from MyD-88 (-/-) or TLR-2 (-/-) deficient mice released significantly less TNFα, compared with wild type controls during *C. neoformans* infection. However, no difference in TNFα release was noted between macrophages from C3H/HeJ mice, which have a loss of function mutation in TLR-4, relative to wild type C3H/HeN controls during *in vitro* stimulation with *C. neoformans*. In addition, when MyD-88 (-/-) or TLR-2 (-/-) deficient mice were infected with low doses of the H-99 serotype A strain, all of the control animals, but none of MyD-88 (-/-) deficient and only 38% of the TLR2 (-/-) deficient animals survived (Biondo C *et al,* 2005). Van der Graaf CA *et al* (2005) reported that TLR-4 mediated pro-inflammatory cytokine induction after *C. albicans* stimulation, whereas recognition by TLR-2 leads mainly to anti-inflammatory cytokine release and TLR-4 mediated pro-inflammatory signals are lost during germination of *C. blastoconidia* into hyphae. It has also been reported that the TLR-4 Asp299Gly/Thr399Ile polymorphisms are associated with an increased susceptibility to Candida bloodstream infections, and it was also showed that an increased production of IL-10 by PBMCs isolated from these patients during *C. albicans* infection *in vitro* (Van der Graaf CA *et al,* 2006).

As a paradigm shift in TLR research, it has been reported that host Heat shock protein 70 (HSP70) induced proinflammatory cytokine production in human monocytes is mediated via the MyD-88/IRAK/NF-κB signal transduction pathway and utilizes both TLR-2 and TLR-4 to transduce its proinflammatory signal in a CD14 dependent fashion (Asea A *et al,* 2002; Vabulas RM *et al,* 2002). Human HSP60 and Gp96 are the other examples of non-pathogen derived ligands of TLRs. Gp96 provides the first example of how the innate and adaptive immune system can be stimulated simultaneously by the same molecule which is released under physiological conditions from necrotic cells (Vabulas RM *et al,* 2002). Literature evidence suggest that TLR-mediated activation of DCs and T cells by Gp96. Gp96 or its N-terminal domain activated DCs at high

concentrations (≥ 50µg/ml) but not at lower concentrations. However, preincubation of low amounts of Gp96 with TLR-2 and TLR-4 ligands at concentrations unable to activate DCs by themselves results in the production of high levels of proinflammatory cytokines, up-regulation of activation markers, and amplification of T-cell activation (Warger T *et al,* 2006). Hyaluronic acid (HA), ubiquitous in the extracellular matrix, found to be broken down into low molecular weight HA (LMW-HA) fragments at the sites of inflammation and that have been reported to activate immunocompetent cells. LMW-HA was reported to be induced activation of keratinocytes mediated through TLR-2, TLR-4, c-Fos, PKC involved signalling pathway leading to the production of BD-2. This activation of keratinocytes found to be non-inflammatory response, because there was no production of IL-8, TNFα, IL-1β, or IL-6 (Gariboldi S *et al,* 2008).

LPS and PGN induced secretory IL-1 receptor antagonist (sIL-1Ra) gene expression in macrophages and both stimuli synergistically increased expression through distinct promoter activity (Carl VS *et al,* 2002). LPS, the mycobacterial glycolipids, and the OspC lipoprotein (TLR-2 agonist) all induced macrophages to secrete TNFα, whereas only LPS could induce NO secretion. LPS induced TNFα, MIP-1β, and RANTES production in these cells, whereas the TLR-2 agonists induced only MIP-1β production (Jones BW, 2001).

The IRAK family consists of two active kinases, IRAK-1 and IRAK-4, and two inactive kinases, IRAK-2 and IRAK-M. IRAK-M expression is restricted to monocytes/macrophages, whereas other IRAKs are ubiquitous. IRAK-M is induced upon TLR stimulation and negatively regulated TLR signalling. IRAK-M prevented dissociation of IRAK-1 and IRAK-4 from MyD-88 and formation of IRAK-TRAF6 complexes. IRAK-M (-/-) deficient cells exhibited increased cytokine production upon TLR/IL-1 stimulation and bacterial challenge, and IRAK-M (-/-) mice showed increased inflammatory responses to bacterial infection. Exposure of cells to LPS induces a hypo-responsive state to a second challenge with LPS that is termed LPS tolerance or endotoxin tolerance, a protection

mechanism against endotoxin shock. LPS tolerance was significantly reduced in IRAK-M (-/-) cells (Kobayashi *et al*, 2002). IRAK-4 (-/-) deficient animals are completely resistant to a lethal dose of LPS and severely impaired in their responses to viral and bacterial challenges (Suzuki N *et al,* 2002). Zhang G *et al* (2002) reported that Tollip suppress TLR mediated cell activation by inhibiting the activity of IRAK-1 after TLR activation. TLR cross-tolerance is inducible in the absence of IRAK-1 in peritoneal macrophages. IRAK-1-deficient macrophages respond with decreased TNFα production to a secondary TLR stimulation, but both wild type and IRAK-1 (-/-) macrophages produced increased IL-10 production. IRAK-1 (-/-) peritoneal macrophages have a defective TNFα and IL-10 production in response to LTA stimulation as well as a defective IL-10 but a normal TNFα production in response to high concentration of LPS (Berglund M *et al,* 2008). Flagellin from a number of Gram-negative bacteria induces cytokine and nitric oxide production by inflammatory cell types. Mizel SB and Snipes JA 2002) reported that prior exposure to flagellin results in a subsequent state of flagellin tolerance in human monocytes, THP1 cells, Jurkat cells, and COS-1 cells. LPS treatment of monocytes and THP1 cells resulted in a state of flagellin cross-tolerance but Flagellin did not induce tolerance to LPS. Flagellin-induced self-tolerance and LPS-induced cross-tolerance to flagellin is associated with block in release of IRAK from the TLR-5 complex in flagellin-tolerant cells. LPS signalling involves at least two pathways: a MyD-88 dependent cascade that is essential for production of inflammatory cytokines and a MyD-88 independent cascade that mediates the expression of IFN inducible genes. LPS tolerance by several microbial components in mouse peritoneal macrophages led to impaired activation of both the pathways. Mycoplasmal lipopeptides did not affect the MyD-88 independent pathway, but impaired the MyD-88 dependent signalling by inhibiting LPS mediated activation of IRAK-1. Pretreatment with double-stranded RNA (dsRNA), which triggers the activation of TLR-3, led to defective activation of the MyD-88 independent, but not the MyD-88 dependent, pathway. Imidazoquinoline compounds, which are recognized by TLR-7, had

no effect on the MyD-88 independent pathway, but inhibited LPS induced activation of MyD-88 dependent signalling through down-regulation of IRAK-1 expression (Sato S *et al,* 2002). Von Bernuth H *et al* (2008) reported nine children with autosomal recessive MyD-88 deficiency suffered from life-threatening, often recurrent pyogenic bacterial infections, including invasive pneumococcal disease. However, these patients were otherwise healthy, with normal resistance to other microbes. This is showing that MyD-88 dependent TLRs and IL-1Rs are therefore essential for protective immunity to a small number of pyogenic bacteria, but redundant for host defense to most natural infections in humans.

MyD-88 adaptor like (Mal) or TIRAP with TIR domain have been reported to be involved in TLR-4 signalling and activates NF-kB, Jun amino-terminal kinase (JNK) and extracellular signal-regulated kinase (Erk)-1 and -2 (Fitzgerald KA *et al,* 2001). In mammals, TICAM-1 dependent TLR pathways induce NF-κB and IFNβ responses. TICAM-1 activates NF-κB through two different pathways involving its interactions with TRAF-6 and RIP-1. It also activates IRF-3/7 through its interaction with TBK-1, leading to the robust up-regulation of IFNβ. Sullivan C *et al* (2007) recently reported the role of zebrafish (*Danio rerio*) TICAM-1 in activating NF-κB and zebrafish type I IFN. Zebrafish TICAM-1 activates zebrafish IFN; however, it does so in an apparently IRF-3/7 independent manner. In addition, zebrafish TICAM-1 does not interact with zebrafish TRAF-6 and NF-κB activation is dependent upon its interaction with RIP-1. Comparative genome analysis suggests that TICAM-1 and TICAM-2 evolved from a common vertebrate TICAM ancestor following a gene duplication event and that TICAM-2 was lost in teleosts following the divergence of the rayfin and lobefin fishes 450 million years ago. Macrophages from mice deficient in adaptor molecules TRIF and TRAM produced equivalent cytokines as wild-type cells, whereas responses were absent in macrophages from MyD-88 (-/-) and TIRAP/Mal (-/-) mice (Hise AG *et al,* 2007). TLR-2 and TLR-4 signalling involves two adaptor proteins MAL and TRAM. Recently, new insights into the

functioning of these two adapters have emerged. Mal is required by both TLRs to act as a bridge to recruit the adaptor MyD-88, leading ultimately to NF-κB activation and TRAM acts as a bridge to recruit TRIF to the TLR-4 complex and leading to activation of the transcription factor IRF-3. Mal and TRAM found to be regulated through phosphorylation and a variant of MAL in humans has been found to protect against multiple infectious diseases. Sterile alpha and HEAT/armadillo motif protein (SARM), another TIR domain-containing adaptor, has been shown to act as an inhibitor of TRIF dependent signalling (Sheedy FJ and O'Neill LA, 2007). Although *C. elegans* SARM up-regulates immune signalling, and its disparate role as a suppressor of TLR signalling through TRIF and not MyD-88, is well-conserved from horseshoe crab (*Carcinoscorpius rotundicauda*) to human (Belinda W *et al,* 2008).

Subversion of TLR signalling by pathogenic virulent bacteria has recently been reported and found to be directly interfering with TLR function by secreting inhibitory homologs of the TIR domain and the genes encoding TIR domain containing-proteins (Tcps) were identified in *E. coli* CFT073 (TcpC) and *Brucella melitensis* (TcpB). Tcps impede TLR signalling through direct binding of MyD-88 adaptor protein (Cirl C *et al,* 2008). Adenylate cyclase toxin (*CyaA*) of *Bordetella pertussis* binds to CD11b/CD18 on macrophages and DCs and confers virulence to the bacteria by subverting innate immune responses of the host. *CyaA* modulates TLR agonist induced IL-10 and IL-12p70 production in DCs by enhancing MAPK phosphorylation and inhibiting IRF-1 and IRF-8 expression, respectively. That is mediated by elevation of intercellular cAMP concentrations (Hickey FB *et al,* 2008). CD45 is a leukocyte-specific protein tyrosine phosphatase and an important regulator of antigen receptor signalling in lymphocytes. Cross JL *et al* reported that CD45 (-/-) DCs exhibited a negative effect of CD45 on TLR-2 and -9, MyD-88 dependent cytokine production, and a positive effect on TLR-3 and -4, MyD-88 independent IFNβ secretion (Cross JL *et al,* 2008).

Doyle SE *et al* (2003) reported that IFN regulatory factor 3 (IRF-3) mediated antiviral gene programme is induced by both TLR-3 and TLR-4 ligands and they also found that TLR-3 expression is inducible by both TLR-3 and TLR-4 ligands, while TLR-4 expression is not inducible by these same stimuli. In addition, activation of viral response gene expression in primary macrophage cells is stronger and prolonged with TLR-3 stimulation compared with that of TLR-4. Using cells derived from mice deficient in the IFN-αβR (-/-), showed that both TLR-3 and TLR-4 require IFN-β autocrine or paracrine feedback to induce TLR-3 expression and activate genes required for antiviral activity. TLR-3 and TLR-4 signalling induced IFN-β to activate antiviral gene expression, while TLR-3 used multiple mechanisms to enhance and sustain the antiviral response more strongly than TLR-4.

Expression of triggering receptor expressed on myeloid cells-1 (TREM-1) was up-regulated in response to TLR activation, an effect further enhanced by GM-CSF and TNFα but inhibited by IL-10. Functionally, primary monocytes differentiated into immature DCs following activation through TREM-1 had an improved ability to elicit T-cell proliferation and production of IFNγ (Bleharski JR *et al,* 2003). DAP-12 is an immunoglobulin tyrosine activation motif (ITAM) containing adapter that associates with receptors in myeloid and NK-cells. DAP-12 associated receptors can give activation signals leading to cytokine production; however, in some situations, DAP-12 inhibits cytokine production stimulated through TLRs and Fc receptors (FcRs). TREM-2 is responsible for the DAP-12 mediated inhibition in mouse macrophages and a chimeric receptor composed of the extra-cellular domain of TREM-2 and the cytoplasmic domain of DAP-12 inhibited the TLR and FcR induced TNFα production of DAP-12 (-/-) deficient macrophages. In wild-type macrophages, TREM-2 knockdown increased TLR induced TNFα production (Hamerman JA *et al,* 2006). DAP-12 deficient airway APCs display enhanced NF-κB activation and cytokine responses upon TLR stimulation or mycobacterial infection in vitro. Adoptive transfer of antigen loaded DAP-12 deficient

APCs alone could lead to over-activation of transferred transgenic or endogenous wild-type T-cells and uncontrolled Th-1 cell activation and severe immunopathology and tissue injury during intracellular viral infection *in vivo* (Divangahi M *et al,* 2007).

Murine macrophages exposed to the combination of apoptotic cells and archetypal ligands for TLR-2, -4, and -9 mount cytokine responses that differ importantly from those elicited by either class of stimulus alone. TLR ligands induced early and sustained secretion of TNFα, MIP-1α and MIP-2 with later secretion of IL-10, IL-12, and TGFβ (Lucas M *et al,* 2003).). Mouse Kupffer cells deficient in TLR-4 did not secrete IL-18, IL-1β, and IL-12 upon LPS stimulation. However, Kupffer cells lacking MyD-88, secreted IL-18 alone in a caspase-1-dependent and de novo synthesis-independent manner (Seki E *et al,* 2001). TLR-1 and TLR-2 together mediated strong activation of NF-κB driven luciferase activity in response to mycobacterial LAM. Posphatidylinositol mannosides, simple biosynthetic precursors of LAM, also activated cells through the combined actions of TLR-1 and TLR-2 (Tapping RI and Tobias PS, 2003). *M. tuberculosis* HSP60 preferentially induced TLR-2 without affecting TLR-4 expression on macrophages, however, interaction of HSP60 with TLR-2 resulted in significant suppression of NF-κB activation and consequently IL-12p40 levels in purified protein derivative (PPD of mycobacteria) activated macrophages. This might be playing an important role to skew the anti-PPD T-cell response towards the Th-2 type when macrophages were used as antigen presenting cells (Khan N *et al,* 2008). Castrillo A *et al* (2003) demonstrated that pathogens interfere with macrophage cholesterol metabolism through inhibition of the liver X receptors (LXR) signalling pathway. Activation of TLR-3 and TLR-4 by microbial ligands blocks the induction of LXR target genes including ABCA-1 in cultured macrophages as well as in aortic tissue *in vivo*. As a consequence of these transcriptional effects, TLR-3 and -4 ligands strongly inhibit cholesterol efflux from macrophages. In macrophages and dendritic cells, stimulation by the prototypical stimuli CpG-DNA (TLR-9), LPS (TLR-4) and LTA (TLR-2) resulted in

striking differences in expression of IL-12. However, these stimuli induced similar amounts of the common proinflammatory cytokine TNFα. An IL-12p40 promoter reporter construct was activated equally by CpG-DNA, LPS and LTA and examinations of the chromatin structure of the endogenous IL-12p40 promoter revealed that nucleosome remodelling contributed to differential IL-12 induction (Albrecht I *et al,* 2004).

It has been reported that TLR induced phagocytosis of bacteria depended on MyD-88 dependent signalling through IRAK-4 and p38 leading to the up-regulation of scavenger receptors and TLRs promote phagocytosis to varying degrees with TLR-9 being the strongest and TLR-3 being the weakest inducer of this process. TLR ligands not only amplify the percentage of phagocytes uptaking of *E. coli*, but also increase the number of bacteria phagocytosed by individual macrophages (Doyle SE *et al,* 2004). Peritoneal macrophages from MyD-88 (-/-) deficient mice exhibited a marked inability to kill *E. coli* (F18) or an attenuated strain of *S. typhimurium* (sseB). This defect in killing was due to diminished NADPH oxidase-mediated production of O^{2-} in response to bacteria by MyD-88 (-/-) deficient phagocytes as a consequence of defective NADPH oxidase assembly and that was resulted from impaired p38 MAPK activation and subsequent phosphorylation of p47phox (Laroux *et al,* 2005).

Hertz CJ *et al* (2003) reported that airway epithelial cells grown at air-liquid interface responded to bacterial lipopeptide induced the production of the antimicrobial peptide human beta defensin-2 (BD-2) in TLR-2 dependent way. LPS and PGN stimulated BD-2 promoter activation in a TLR-4 and TLR-2 dependent manner in IEC. A mutation in the NF-κB or AP-1 site within the BD-2 promoter abrogated this response and inhibition of JNK prevented up-regulation of BD-2 protein expression in response to LPS (Vora P *et al,* 2004). Funderburg N *et al* very recently reported that human BD-3, an innate antimicrobial peptide, can induce expression of the co-stimulatory molecules CD80, CD86, and CD40, on monocytes and myeloid DCs in a TLR dependent manner. Activation of monocytes by BD-3 is mediated by interaction with TLR-1 and TLR-2,

resulting in signalling that involved MyD-88 and results in IRAK-1 phosphorylation (Funderburg N *et al,* 2007).

Keratinocytes were found to constitutively express TLR-1, TLR-2, TLR-3, TLR-5, and TLR-9 but not TLR-4, TLR-6, TLR-7, TLR-8, or TLR-10. Inhibition experiments using monoclonal antibodies and the specific platelet activating factor receptor inhibitor CV3988 showed that NF-κB activation by *S. aureus* in keratinocytes were TLR-2 but not TLR-4 or platelet activating factor receptor dependent. The purified staphylococcal cell wall components LTA and PGN, known to signal through TLR-2, also showed NF-κB translocation in human keratinocytes, indicating a crucial role of the staphylococcal cell wall in the innate immune stimulation of human keratinocytes (Mempel M *et al,* 2003).

Polyriboinosinic polyribocytidylic acid (poly-I:C), which might reflect a natural genetic product from a variety of viruses during replication, has been identified as one of the critical stimuli for TLR-3. BALB/c mice were immunized with purified recombinant HIV-1 envelope gp-120 or influenza hemagglutinin (HA) protein together with poly-I:C, induced antigen specific CTL from naive $CD8^+$ T-cells *in vivo*. However, when the same proteins were immunized with LPS, agonist of TLR-4, not induced antigen specific CTL formation (Fujimoto C *et al,* 2004). PGN signalling through TLR-2 and bacterial CpG DNA signalling through TLR-9 are functionally equivalent at synergizing with IFNγ in regulating transporter associated with antigen processing (TAP-1) expression in macrophages and required activation of the p38 MAPK mediated phosphorylation of STAT-1 on serine ^{727} (Cecil AA and Klemsz MJ, 2004).

Matrix metalloproteinase-9 (MMP-9) is selectively induced through TLR-2 in human and murine monocytic cells stimulated with *B. burgdorferi*. MMP-1 was shown to be stimulated through a pathway other than TLR-2. Nuclear extracts indicated that AP-1 was reduced in TLR-2 neutralized monocytic cells, suggesting that AP-1 plays a role in the transcriptional activation of MMP-9 through TLR-2 (Gebbia JA *et al,* 2004). Mycoplasmal membrane diacylated lipoproteins initiated proinflammatory responses

through TLR-2 and TLR-6 through the activation of the transcriptional factor NF-κB and also induced apoptotic responses (Into T *et al,* 2004). Stimulation of cultured synovial fibroblasts by the TLR-2 ligand, PGN, had upregulated fourteen CC and CXC chemokine genes, which is identified by using high density oligonucleotide microarray analysis (Pierer M *et al,* 2004).

Intragastric (i.g.) and subcutaneous (s.c.) administration of probiotic and *E. coli* DNA ameliorated the severity of dextran sodium sulfate (DSS) induced colitis, whereas methylated probiotic DNA, calf thymus DNA, and DNase-treated probiotics had no effect. The colitis severity was attenuated to the same extent by i.g. delivery of nonviable gamma-irradiated or viable probiotics, while, mice deficient in MyD-88 did not respond to gamma-irradiated probiotics. The severity of DSS-induced colitis in TLR-2 (-/-) and TLR-4 (-/-) deficient mice was significantly decreased by i.g. administration of gamma-irradiated probiotics, whereas, in TLR-9 (-/-) deficient mice, gamma-irradiated probiotics had no effect (Rachmilewitz D *et al,* 2004).

TLR signalling, through the MyD-88 adaptor, found to be up-regulated transcription of the retinoic acid early inducible-1 (RAE-1) family of NKG2D ligands, but not H-60 or murine UL16-binding protein-like transcript-1. RAE-1 proteins are found on the surface of activated, but not resting, macrophages and can be detected by NKG2D on NK-cells resulting in down-regulation of this receptor both *in vitro* and *in vivo*. RAE-1-NKG2D interactions provide a mechanism by which NK-cells and infected macrophages communicate directly during an innate immune response to infection (Hamerman JA *et al,* 2004). Zanoni I *et al* reported that TLR dependent microbial stimuli typically associated with Th-1 responses confer to DCs the ability to activate NK-cells, whereas stimuli associated with Th2 responses did not have this property (Zanoni I *et al,* 2005).

Lipomannans (LM), independent of their mycobacterial origins, were potent inducers of IL-12 and apoptosis. The precursor of LM, phosphatidyl-myo-inositol dimannoside, had no activity, suggesting that the mannan core of LM was required for

the activity of LM. The specific interaction of LM with TLR-2 but not with TLR-4 suggested that these responses were mediated through the TLR-2 signalling pathway (Dao DN *et al,* 2004).

Yarovinsky F *et al* reported that a profilin-like molecule from the protozoan parasite *Toxoplasma gondii* induced potent IL-12 response in murine DCs through TLR-11 and is the first chemically defined ligand for this TLR-11 (Yarovinsky F *et al,* 2005).

Miller LS *et al* (2005) reported that TGFα, a growth and differentiation factor that is present during wound healing, increased the expression of both TLR-5 and TLR-9 on keratinocytes. In addition, TGFα regulated the function of TLR-5 and TLR-9, because activation with their respective ligands enhanced the production of IL-8 and human BDs. Expression of murine BD was upregulated by bacterial lipopeptides in wild type keratinocytes, while it was attenuated in TLR-2 (-/-) deficient keratinocytes. In addition, *S. aureus* inoculated into the tail skin from TLR-2 (-/-) deficient mice that had been grafted on the dorsal skin of syngeneic mice resulted in erosion (Sumikawa Y *et al,* 2006).

Premature newborns are highly susceptible to severe bacterial infections. This is partially due to their immature innate immune system, characterized by decreased neutrophil and monocyte activity as well as by reduced concentrations of complement factors. Foerster-Waldl E *et al* reported that very low birth weight infants have reduced TLR-4 expression was paralleled by significantly diminished *ex vivo* LPS stimulated IL-1β, IL-6, and TNFα secretion into whole blood (Foerster-Waldl E *et al,* 2005).

Martin M *et al* reported that glycogen synthase kinase 3 (GSK-3) differentially regulates TLR mediated production of pro- and anti-inflammatory cytokines. Stimulation of monocytes or PBMCs with TLR-2, TLR-4, TLR-5 or TLR-9 agonists induced substantial increases in IL-10 production, while suppressing the release of proinflammatory cytokines through GSK-3 inhibition (Martin M *et al,* 2005).

Notch signaling is a well-conserved pathway involved in cell fate decisions, proliferation and apoptosis. TLR agonists such as bacterial lipopeptide, polyI:C, LPS and

CpG DNA all induced up-regulation of Notch-1 in primary and macrophage-like cell lines. Notch-1 up-regulation was dependent on the MyD-88 pathway when stimulated through TLR-2, but not TLR-4. Activated Notch-1 and expression of the Notch target genes, Hes-1 and Deltex, were detected in activated macrophages, suggesting that Notch signalling was activated upon stimulation. Inhibiting processing of Notch receptor by gamma-secretase using a gamma-secretase inhibitor (GSI), the expression of Notch-1 was down-regulated to basal levels. This treatment significantly modulated expression of TNFα, IL-6, and IL-10. In addition, the amount of NO produced was significantly lower and the expression of MHC class-II was up-regulated in GSI treated cells. Treatment with GSI or silencing Notch-1 resulted in decreased translocation of NF-κB p50 into nucleus upon stimulation (Palaga T *et al,* 2008). SOCS-3 is a critical negative regulator of cytokine signalling and is induced by *M. bovis* BCG in mouse macrophages. *M. bovis* BCG activated the Notch-1 signalling pathway, leading to the expression of SOCS-3 and perturbation of Notch signalling in infected macrophages results in the marked reduction in the expression of SOCS-3. Enforced expression of the Notch-1 intracellular domain in RAW 264.7 macrophages was sufficient to induce the expression of SOCS-3, which can be further potentiated by *M. bovis* BCG. TLR-2 signalling was shown to play an important role in the above process and perturbation of signalling resulted in marked reduction in SOCS-3 levels and expression of the Notch-1 target gene, Hes-1. The down-regulation of MyD-88 also resulted in a significant decrease in SOCS-3 expression, implicating the role of the TLR2-MyD88 axis in *M. bovis* BCG-triggered signalling (Narayana Y and Balaji KN, 2008).

Focal adhesion kinase (FAK) is a nonreceptor protein tyrosine kinase involved in signalling downstream of integrins, linking bacterial detection, cell entry, and initiation of proinflammatory response through MAPKs and NF-κB activation. Protein-I/II from *Streptococcus mutans*, a model activator of FAK, stimulation was independent of TLR-4, TLR-2, and TLR-6. However, FAK and MyD-88 signalling pathways are interlinked and

MyD-88 dependent. In addition, LPS-induced IL-6 secretion by human and murine fibroblasts required the presence of FAK (Zeisel MB *et al,* 2005).

In human monocytic cells, both muramyldipeptide (MDP; N-acetylmuramyl-l-alanyl-d-isoglutamine) MDP and diaminopimelic acid (DAP) containing desmuramylpeptides (DMPs) induced marked synergistic IL-8 secretion in combination with synthetic TLR agonists, and suppression of the mRNA expressions of NOD-1 and NOD-2, respectively, by RNAi specifically inhibited synergistic IL-8 secretion. In human DCs, synergistic Th-1 responses are induced by combined stimulations of synthetic NOD and TLR agonists (Takada H and Uehara A, 2006).

1.7 Nod-Like Receptors

The NLR family is a group of intracellular microbial sensors, which were previously identified as CATERPILLER (CARD, transcription enhancer, R (purine)-binding, pyrin, lots of LRRs), shares structural similarity with a subclass of plant disease resistance (R) genes. The human NLR family contains more than 30 proteins, and specific ligands have been identified to date for only a minority of the NLRs. In general, members of this family share a tripartite domain structure that consists of the following: carboxy-terminal LRR domain, which is involved in ligand recognition; a central NOD (also known as a NACHT domain), which facilitates self-oligomerization and has ATPase activity; and an amino-terminal domain that is composed of protein–protein interaction cassettes, such as CARDs or pyrin domains. Although NLR family members that contain CARDs interact with different downstream adaptor molecules than those that contain pyrin domains, they are functionally related in that both types of molecule activate NF-κB and caspases; in addition, mutations in both CARD-containing and pyrin-domain-containing NLRs have been linked to inflammatory diseases (Ting JP and Davis BK, 2005; Hoffman HM *et al,* 2001). The NLR / CATERPILLER family can be separated in to subfamilies such as NOD, CIITA, NALPs and IPAF.

The CIITA is a transcription factor which activates the MHC Class II gene transcription. Three functional forms of CIITA are transcribed from three different promoters and the form which contain CARD domain specifically expressing in DCs. The NACHT and LRR domains are required for self association and function (Tschopp J *et al,* 2003).

The first NLRs reported to be intracellular microbial sensors were NOD-1/CARD-4 and NOD-2/CARD-15, and their activating ligands have been verified as subcomponents of peptidoglycans (PGN), namely D-g-glutamyl-meso-DAP (mDAP) and muramyl dipeptide (MDP), respectively (Girardin SE *et al,* 2003; Girardin SE *et al,* 2003b). NOD-1 and NOD-2 activate NF-kB through the recruitment and oligomerization of receptor-interacting protein-2 (RIP-2), also known as RIP-like interacting CLARP kinase (RICK) and CARD containing ICE-associated kinase (CARDIAK), which results in the activation of the IkB kinase complex (Bertin J *et al,* 1999; Ogura Y *et al,* 2001).

ICE protease (caspase-1) activating factor (IPAF), also known as CARD-12 or CLAN, having an amino terminal CARD, a central NACHT and carboxy terminal LRRs, however, it lacks the NOD domain. IPAF has been reported to associated with CARD containing proteins such as adaptor protein apoptosis associated speck like (ASC/PYCARD), NOD-2, BCL-10, NALP-1 and activates caspase-1 through inflammasome formation (Poyet JL *et al,* 2001; Geddes BJ *et al,* 2001). Neuronal Apoptosis Inhibitor Protein (NAIP) is also included in this subfamily because of high sequence similarities of the NACHT and LRR domains. The removal of LRRs makes these proteins constitutively active, of which IPAF is caspase activator and NAIP inhibits caspase activity (Poyet JL *et al,* 2001; Liston P *et al,* 1996).

The NALP subfamily, also known as PANs or PYPAFs, constitute the largest group among NLRs. This is composed of NALP-1 to NALP-14 and characterized by the presence of an amino terminal PYD, central NOD/NACHT and carboxy terminal LRRs.

However NALP-1 contains two additional domains at the carobxy terminal including a CARD and function to find domain (Tschopp J *et al,* 2003).

1.8 NLRs in inflammasomes

ASC or PYCARD is a protein that is essential for LPS-induced activation of caspase-1, because ASC knockout mice fail to process procaspase-1 or produce IL-1β and IL-18 following LPS and ATP stimulation, which is consistent with results in Caspase-1 null mice (Mariathasan S *et al,* 2004; Li P *et al,* 1995; Kuida K *et al,* 1995). Structurally, ASC consists of a CARD domain at its C-terminus and a PYD at its N-terminus, making it a bi-partite adaptor molecule involved in recruiting caspase-1 into activation complexes. To date, the NLRs NALP-1, NALP-3 and IPAF have been implicated in ASC-containing, caspase-1 activating complexes, termed inflammasomes (Martinon F and Tschopp J, 2004; Martinon F *et al,* 2002). Following stimulation, NALP-1 is thought to assemble an inflammasome complex consisting of NALP-1, ASC, caspase-1 and caspase-5 (Martinon F *et al,* 2002). NALP-1 contains an N-terminal PYD, an NBD followed by LRRs and a CARD domain at its C-terminus. It is thought that the LRRs of NALP1 recognize a ‘danger signal’, resulting in the self-association of NALP1 and the activation of caspases-1 and -5 and subsequent processing of IL-1b. Interestingly, a murine NALP-1 variant, NALP-1b, which lacks the N-terminal PYD, seems to become activated in response to anthrax lethal toxin (Boyden ED and Dietrich WF, 2006).

The NALP-3 protein (also known as Cryopyrin) is structurally similar to NALP-1 except it lacks the C terminal CARD sequence. NALP-3 forms an inflammasome complex through association with NALP-2, ASC, Cardinal and caspase-1 (Agostini L *et al,* 2004). The importance of NALP-3 in the inflammatory process has become apparent with the discovery that mutations within the gene encoding NALP3 are responsible for three autoinflammatory disorders: Muckle-Wells syndrome (MWS), familial cold autoinflammatory syndrome (FCAS) and chronic infantile neurological cutaneous and articular syndrome (CINCA), all of which respond well to treatment with the IL-1 receptor

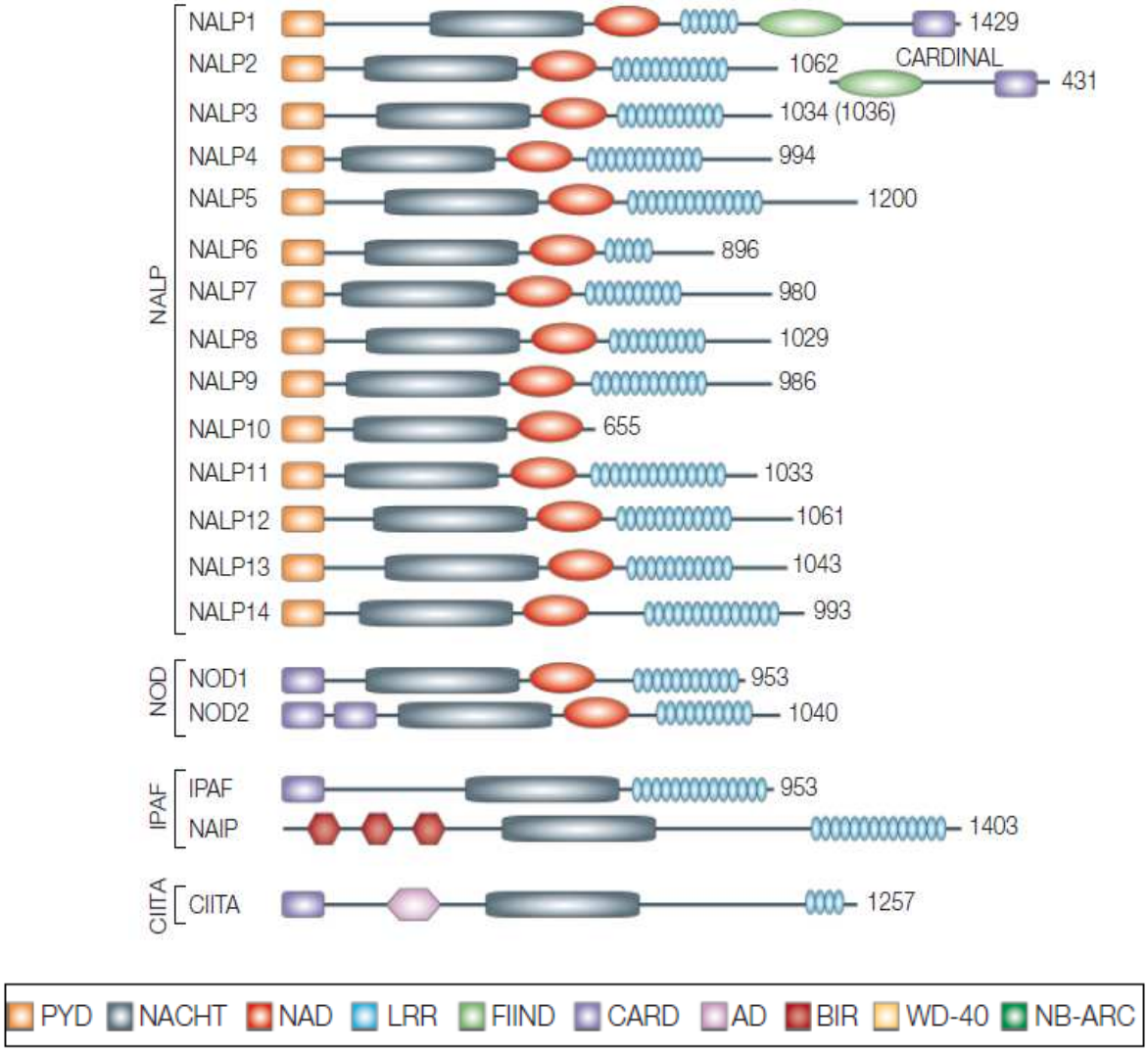


Fig.5 Domain structures of NLR/CATERPILLER proteins: AD-activation domain, BIR-baculovirus IAP repeat, CARD-caspase recruitment domain, CIITA-class II transactivator, FIIND-domain with function to find, LRRs-leucine rich repeats, NAD-NACHT associated domain, NAIP-neuronal apoptosis inhibitor protein, NB-ARC-nucleotide binding APAF-1, plant *R* gene products and CED-4 domain, NOD-nucleotide binding oligomerization domain, PYD-pyrin domain, WD-40- WD-40 repeat region (Tschopp J *et al,* 2003).

antagonist, validating the role of NALP-3 in the regulation of caspase-1 (Agostini L *et al,* 2004). Macrophages deficient in NALP-3, ASC or caspase-1 were unable to secrete IL-1b in response to all of these stimuli, indicating that each of these molecules is required for inflammasome activation in these conditions. It is unlikely that these various stimuli can all activate NALP-3 directly; more probably, a common intracellular ‘danger signal’, such as endogenously generated uric acid is activated by these stimuli to trigger NALP-3 activation (Shi Y *et al,* 2003).

IPAF, another NLR family member with a similar structure to NOD1, is the adaptor protein involved in inflammasome assembly following infection with Salmonella, because IPAF-deficient macrophages are resistant to infection by S. typhimurium (Mariathasan S *et al,* 2004). IPAF has recently been identified to be required for flagellin to activate caspase-1 and induce IL-1β production. Salmonella strains deficient in flagellin were unable to activate caspase-1 and induce IL-1β (Miao EA *et al,* 2006; Franchi L *et al,* 2006). The NLR responsible for detecting and activating caspase-1 in response to *F. tularensis* has yet to be identified, although NALP-3 and IPAF have not been involved (Kanneganti TD *et al,* 2006). Other NLRs implicated in caspase-1 activation are neuronal apoptosis inhibitory protein-5 (NAIP-5), also known as baculoviral IAP repeat-containing protein-1 (BIRC-1), and NALP-6 (Zamboni DS *et al,* 2006). NALP-6 (also known as Pyrincontaining Apaf-1-like protein, PYPAF5) synergistically activates caspase-1 following overexpression with ASC; however, this needs to be confirmed at endogenous levels (Grenier JM *et al,* 2002).

1.9 NLRs and its ligands in Bacterial Infections and Inflammations

Researches have brought forward the essential role of cytoplasmic PRRs on recognition of internalized PAMPs and transducing signals for a pro-inflammatory reaction intended to ward off infection. NOD-1/CARD4 and NOD-2/CARD15 proteins are the two early identified members of this growing family. In the beginning of this decade, Inohara N *et al* reported that HEK-293 cells expressing traces of NOD-1 and NOD-2 have activated NF-κB in a TLR-4/MyD88 independent way when treated with LPS (Inohara N *et al,* 2001). Followed by, Girardin SE *et al* (2001) reported that *S. flexneri* infection enhanced NOD-1oligomerization and expression of dominant-negative versions of NOD-1 blocked the activation of NF-κB and JNK by *S. flexneri* as well as microinjected LPS in epithelial cells. They also showed that invasive *S. flexneri* triggers the formation of a transient complex involving NOD-1, RIP-2/RICK and the IKK complex. RIP-2 (-/-)

deficient mice exhibited a profoundly decreased ability to defend against infection by the intracellular pathogen *L. monocytogenes* and also resistant to the lethal effects of LPS induced endotoxic shock. RIP-2 (-/-) deficient macrophages infected with *L. monocytogenes* or treated with LPS have decreased activation of NF-κB, whereas dominant negative RIP-2 inhibited NF-κB activation mediated by TLR-4 and NOD-1. In addition, RIP-2 deficiency resulted in impaired IFNγ production in both Th-1 and NK-cells (Chin AI *et al,* 2002). RIP-2 was reported to be recruited for downstream signalling of TLR-2/-3/-4 but not TLR-9. RIP-2 (-/-) deficient cells were also hypo-responsive to signalling through IL-1 and IL-18 receptors, and deficient for signalling through NOD proteins. It has also been noted that, RIP-2 (-/-) deficient T-cells showed severely reduced NF-κB activation, IL-2 production and proliferation on TCR engagement, and impaired differentiation to Th-1 cells. Thus RIP-2 seems to function as a crucial a signal transducer and integrator of signals for both the innate and adaptive immune systems (Kobayashi K *et al,* 2002). Girardin SE *et al* reported that human NOD-1 specifically detects a unique diaminopimelate-containing N-acetylglucosamine-N-acetylmuramic acid (GlcNAc-MurNAc) tripeptide motif found in Gram-negative bacterial PGN, resulting in activation of the transcription factor NF-κB pathway (Girardin SE *et al,* 2003). At the same time, Chamaillard M *et al* reported that NOD-1 mediates the recognition of PGN derived primarily from Gram-negative bacteria and the core structure recognized by NOD-1 is a dipeptide, gamma-D-glutamyl-meso-diaminopimelic acid (iE-DAP) (Chamaillard M *et al,* 2003). Hisamatsu T *et al* reported that IFNγ induced NOD-1 mRNA expression and thereby augmented NOD-1 protein level in an IRF-1 dependent manner in SW480 intestinal epithelial cells (Hisamatsu T *et al,* 2003). Stable expression of dominant negative NOD-1 in colon epithelial cells prevented I-κB kinase and NF-κB activation in response to infection with enteroinvasive *E. coli*. However, dominant negative NOD-1 did not eliminate IL-1 or flagellin stimulated NF-κB activation (Kim JG *et al,* 2004).

Inohara N *et al* reported that muramyl dipeptide (MurNAc-L-Ala-D-isoGln) derived from PGN is the essential structure in bacteria recognized by NOD-2 and replacement of L-Ala for D-Ala or D-isoGln for L-isoGln eliminated the ability of muramyl dipeptide to stimulate NOD-2. PBMCs from individuals homozygous for the major disease associated L1007fsinsC NOD-2 mutation, which associated with susceptibility to Crohn's disease, responded to LPS but not to synthetic muramyl dipeptide (Inohara N *et al,* 2003). NOD-2 is a general sensor of PGN through the recognition of MDP, activates the NF-κB pathway following intracellular stimulation. The 3020insC frameshift mutation, the most frequent NOD-2 variant associated with Crohn's disease patients, fully abrogates NOD-2 dependent detection of PGN and MDP (Girardin SE *et al,* 2003). Amino terminal and carboxy terminal LRRs play distinct roles in the modulation of NOD-2 activation and bacterial recognition and within the carboxy terminal LRRs, variable residues predicted to form the β-strand/β-turn structure were found to be essential for the response to MDP (Tanabe T *et al,* 2004). Netea MG *et al* demonstrated that PBMCs from patients with 3020insC frameshift-mutation in the NOD-2 gene associated with Crohn's disease results in defective release of IL-10 after stimulation with the TLR-2 ligands, PGN and Pam3Cys-KKKK, but not with TLR-4 ligand, bacterial LPS (Netea MG *et al,* 2004). NOD-2 mRNA was expressed in intestinal epithelial cell lines and primary intestinal epithelial cells and the survival of *S. typhimurium* in CACO-2 cells stably transfected with NOD-2 expression plasmid was lower than un-transfected CACO-2 cells or mock transfectant (Hisamatsu T *et al,* 2003). Mutations in NOD-1 and NOD-2 are associated with a number of human inflammatory disorders, including Crohn's disease, Blau syndrome, early-onset sarcoidosis, and atopic diseases (Tattoli I *et al,* 2007). Chron’s Disease is associated with the three main NOD-2 mutations namely R702W, G908R and 1007fs (Hugot JP *et al,* 2003). A NOD-2 variant from patients introduced in the mouse NOD-2 locus and that exhibited elevated NF-κB activation in response to MDP and more efficient processing and secretion of the cytokine IL-1β (Maeda S *et al,* 2005). NOD-2 is required for

recognition of PGN and this leads to strong synergistic effects on TLR-2 mediated production of both pro- and anti-inflammatory cytokines and also modulates signals transmitted through TLR-4 and TLR-3, but not through TLR-5, TLR-9, or TLR-7 (Netea MG *et al,* 2005).

Streptococcus pneumoniae, the major cause of community-acquired pneumonia and bacterial meningitis, has been shown to transiently invade epithelial and endothelial cells. Opitz B *et al* reported that NF-κB activation induced by *S. pneumoniae* depends on NOD-2 in HEK293 cells and over-expression of dominant negative constructs of IRAK-1, IRAK-2, TRAF-6, NF-κB inducing kinase, TAB-2 and TAK-1 also inhibited NOD-2 dependent NF-κB activation. In addition, in C57BL/6 mouse lung tissue *in vivo* as well as in the bronchial epithelial cell line BEAS-2B, NOD-1 and NOD-2 mRNA expressions were up-regulated after pneumococcal infection (Opitz B *et al,* 2004). Studies using highly purified PGs from eight bacteria such as *E. coli, P. aeruginosa, Y. pseudotuberculosis, H. pylori, B. subtilis, L. monocytogenes, S. pneumoniae and S. aureus*, have revealed that these PGs are not sensed through TLR-2, TLR-2/-1 or TLR-2/-6 and suggested PG detection occur through NOD-1/NOD-2 intracellularly (Travassos LH *et al,* 2004). Sero-epidemiological and animal studies, as well as demonstration of viable bacteria in atherosclerotic plaques, have linked *Chlamydophila pneumoniae* infection to development of chronic vascular lesions and coronary heart disease. In endothelial cells infected with viable C. *pneumoniae*, but not with heat inactivated bacteria, activated the cell and RNAi of NOD-1 gene reduced *C. pneumoniae* induced IL-8 release markedly. In HEK-293 cells, over-expressed NOD-1 or NOD-2 amplified the capacity of *C pneumoniae* to induce NF-κB activation and heat-inactivated bacteria were able to induce a NF-κB reporter gene activity through NOD proteins when internalized, but not from the extracellular side (Opitz B *et al,* 2005).

In vitro analysis showed that NOD-2 expression levels did not change significantly in mononuclear cells stimulated with *M. tuberculosis* antigens. There was a variable

result for NOD-2 expression among patients, no significant differences in NOD-2 transcriptional responses were detected in patients and controls but expression was markedly increased in some patients. However, NOD-2 mRNA levels increased significantly after completion of anti-tuberculosis treatment (Lala S *et al,* 2007). Macrophages and DCs from NOD-2 (-/-) deficient mice were impaired in the production of proinflammatory cytokines and NO following infection with live, virulent *M. tuberculosis*. Mycolylarabinogalactan peptidoglycan (PGN), the cell wall core of *M. tuberculosis*, stimulated macrophages to release TNFα and IL-12p40 in a partially NOD-2 dependent manner, and *M. tuberculosis* PGN required NOD-2 for the optimal induction of TNFα. In addition, mice deficient for NOD-2 (-/-) and doubly deficient for TLR-2 (-/-) and NOD-2 (-/-) were similarly able to control an *M. tuberculosis* infection (Gandotra S *et al,* 2007). Divangahi M *et al* (2008) reported that analysis of the lungs after four weeks of *M. tuberculosis* BCG infection demonstrated that NOD-2 (-/-) mice had decreased production of type 1 cytokines and reduced recruitment of $CD8^+$ and $CD4^+$-T cells. Antigen specific T-cell responses in both the spleens and thoracic lymph nodes were diminished in NOD-2 (-/-) deficient mice, indicating impaired adaptive antimycobacterial immunity. In addition, bacterial burden after six months of aerosol infection with *M. tuberculosis* in NOD-2 (-/-) deficient mice were high and succumbed to death sooner than did wild-type controls.

Mice deficient in NOD-2 are more susceptible to *S. aureus* and increased susceptibility is due in part to defective neutrophil phagocytosis, elevated serum levels of Th-1 cytokines, and higher bacterial tissue burden (Deshmukh HS *et al,* 2009). Clearance of encapsulated *H. influenzae* required both TLR and NOD signalling pathways, whereas individual deficiencies in each of these signaling cascades did not affect clearance of non-encapsulated strains (Zola TA *et al,* 2008). Depletion of the gut microbiota suppressed tumour development in NOD-1 (-/-) deficient mice, thus highlighting a link between the commensal bacteria within the intestine and the host

innate immune NOD-1 signalling pathway in the regulation inflammation mediated colon cancer development (Chen GY *et al,* 2008).

PBMCs preincubated with NOD-2 ligands were specifically down-regulated the production of TNFα induced by the TLR-4 ligand LPS, as well as by intestinal microorganisms, whereas the production of anti-inflammatory cytokines was not modulated. PBMCs from patients with Crohn's disease having the wild type NOD-2 allele, the NOD-2 engagement led to a similar cross-tolerance to TLR-4 dependent stimulation of TNFα, however, the cross-tolerance between NOD-2 and TLR-4 was absent in the cells of five patients homozygous for the 3020insC NOD-2 mutation, leading to uninhibited release of TNFα by TLR-4 ligands and intestinal bacteria (Kullberg BJ *et al,* 2008). Hedl M *et al* (2007) very recently reported that pretreatment with MDP, a ligand for NOD-2, significantly decreased production of the proinflammatory cytokines TNFα, IL-8, and IL-1β upon NOD-2, TLR-4, and TLR-2 re-stimulation in primary human monocyte-derived macrophages from a large cohort of individuals. In addition, acute NOD-2 stimulation induced IRAK-1 activation, and that chronic MDP treatment down-regulated IRAK-1 activation upon NOD-2 or TLR-4 re-stimulation and in a subset of individuals, chronic NOD-2 stimulation induced expression of the IRAK-1 inhibitory protein IRAK-M.

Synthetic peptidoglycan fragments, diaminopimelic acid (DAP)-containing desmuramylpeptides (DMP) and muramyldipeptide (MDP), induced secretion of IL-8 in a dose-dependent manner in human monocytic THP-1 cells, although high concentrations of compounds are required as compared with chemically synthesized TLR agonists mimicking like TLR-2 agonistic lipopeptide (Pam3CSSNA), TLR-4 agonistic lipid A (LA-15-PP) and TLR-9 agonistic bacterial CpG DNA. MDP or DAP-containing DMP in combination with synthetic TLR agonists synergistically induced the secretion of IL-8. NOD-1 and NOD-2 silencing through RNAi specifically inhibited the synergistic IL-8 secretion, through suppression of NF-κB activation, induced by DMP and MDP with

these TLR agonists respectively (Uehara A *et al,* 2005). The stimulation of DCs with NOD-2 agonist (MDP) and NOD-1 agonist (FK565) in combination with TLR-4 agonist (lipid A), TLR-3 agonist (polyI:C), and TLR-9 agonist (CpG DNA), but not with TLR-2 agonist (Pam3CSSNA), synergistically induced IL-12p70 and IFNγ, but not IL-18, in culture supernatants and induced IL-15 on the cell surface (Tada H *et al,* 2005). Synthesized meso-DAP itself activated human epithelial cells from various tissues, through NOD-1 to generate antibacterial factors, PGN recognition proteins and BD-2, and cytokines in specified cases, although the activities of meso-DAP were generally weaker than those of known NOD agonists. In human monocytic cells, in the presence of cytochalasin-D, meso-DAP induced slightly but significantly increased production of cytokines, although the cells did not respond to meso-DAP in the absence of cytochalasin-D (Uehara A *et al,* 2006). IFNγ primed murine macrophages induced NO production when stimulated with synthetic MDP and depends on IFNγ induced expression and dissociation from the actin cytoskeleton of NOD-2 within the cell (Toetemeyer S *et al,* 2006).

Pan Q *et al* reported that NOD-2 bound NF-κB inducing kinase (NIK) mediated induction of specific changes by the NOD-2 activator, MDP, and also revealed a role for NIK in the synergistic effect of both NOD-2 and TLR-4 pathways when activated by their respective agonists. NIK induction through NOD-2 agonist has specifically induced B-cell chemoattractant known as BLC (Pan Q *et al,* 2006). Bacteria, but not virus, primed human DCs induced IL-17 production in Th cells through NOD-2 ligand, MDP and this also enhanced obligate bacterial TLR agonist induction of IL-23 and IL-1, which promoted IL-17 expression in T-cells and thereby the development of antibacterial Th-17 cells (van Beelen AJ *et al,* 2007). Kim YG *et al* (2008) reported that macrophages or mice made insensitive to TLRs by previous exposure to microbial ligands remained responsive to NOD-1 and NOD-2 stimulation and also found that NOD-1 and NOD-2 mediated signalling and gene expression are enhanced in TLR-tolerant macrophages.

This study revealed that innate immune responses induced by bacterial infection relied on NOD-1 and NOD-2 and their adaptor RIP2/RICK in macrophages pretreated with TLR ligands but not in naive macrophages.

Kim JY *et al* (2008) reported that TAK-1 is an essential intermediate of NOD-2 signalling and found that TAK-1 deletion completely abolished MDP-NOD-2 signalling, activation of NF-κB and MAPKs, and subsequent induction of cytokines/chemokines and also MDP induced NOD-2 expression in keratinocytes. Mice with epidermis specific deletion of TAK-1 developed severe inflammatory conditions and this showing that TAK-1 and NOD-2 signalling are important for maintaining normal homeostasis of the skin.

Complexing of NOD-2 with Erbin reported to function as a negative regulator of NOD-2 and show that bacterial infection has an impact on NOD-2/Erbin complex formation within cells. Ectopic expression of increasing amounts of Erbin or short hairpin RNA-mediated knockdown of Erbin showed a negative influence of Erbin on NOD-2/MDP mediated NF-κB activation (Kufer TA *et al,* 2006). Using *S. flexneri* as a model for physiological activation of NOD-1, it has been reported that NOD-1 is recruited to the site of bacterial entry and there it colocalizes with NEMO. Signalling-inactive mutants of NOD-1 or disruption of the actin cytoskeleton interferes with this localization pattern and impacts on downstream NF-κB activation (Kufer TA *et al,* 2008).

RIP2/RICK is a kinase that has early been implicated in NOD-1 and NOD-2 signalling. Park JH *et al* showed that macrophages and mice lacking RIP2/RICK are defective in their responses to NOD-1 and NOD-2 agonists but exhibited unimpaired responses to synthetic and highly purified TLR agonists. Stimulation of macrophages with muramyl dipeptide, the NOD-2 activator, enhanced immune responses induced by LPS, IFNγ, and heat-killed *Listeria* in wild-type but not in RIP2/RICK or NOD-2-deficient macrophages (Park JH *et al,* 2007). Werts C *et al* reported that activation of both NOD-1 and NOD-2 led to substantial secretion of CCL-5 by murine macrophages and the

intraperitoneal injection of murine NOD-1 or NOD-2 agonists resulted in a rapid secretion of CCL-5 into the bloodstream (Werts C *et al,* 2007).

Other cytosolic NLRs assemble caspase-1 activating multiprotein complexes termed inflammasomes and caspase-12 reported to modulate the caspase-1 inflammasome. Caspase-12 dampens mucosal immunity to bacterial infection independent of its effects on caspase-1. Caspase-12 deficiency enhanced production of antimicrobial peptides, cytokines, and chemokines to enteric pathogens, an effect dependent on bacterial T3SS and the NOD pathway. It was also showed that caspase-12 binds to RIP-2, displacing TRAF-6 from the signalling complex, inhibiting its ubiquitin ligase activity, and thereby blunting NF-κB activation (LeBlanc PM *et al,* 2008).

Human peripheral blood NK-cells expressed NOD-2 and responded to MDP through internalization of MDP, leading to direct cell activation, including signalling through NF-κB. MDP synergizes with IFNα and IL-12 to activate NK-cells and stimulated IFNγ secretion. Although IL-12 co-stimulation leads to a greater IFNγ response by NK-cells, higher levels of CD69 in response to MDP are induced in the presence of IFNα. However, MDP alone or in combination with either IFNα or IL-12 poorly increased NK-cell cytotoxicity (Athie-Morales V *et al,* 2008).

Increased cell surface CD-14, TLR-2, TLR-4 and intracellular TLR-3, TLR-7, TLR-8, TLR-9, NOD-1 and NOD-2 expressions were found in human monocytic THP-1 cells treated anti proteinase-3 (PR-3) and markedly promoted the release of IL-8 induced by chemically synthesized TLR and NOD ligands. The anti-PR-3 antibody mediated cell activation was significantly abolished by RNAi targeted at PR-3 mRNA and by inhibition of phospholipase-C and NF-κB (Uehara A *et al,* 2007).

Bacillus anthracis infection and anthrax lethal toxin induced IL-1β secretion in a manner that depended on caspase-1 and NOD-2 (Hsu LC *et al,* 2008). Macrophages rendered refractory to TLR-4 and NOD-2 signalling by exposure to LPS and MDP exhibit impaired TNFα and IL-6 production in response to pathogenic *L. monocytogenes* and *Y.*

pseudotuberculosis as well as commensal bacteria including *E. coli* and *Bacteroides fragilis*. Whereas, NOD-2 null macrophages tolerant to LPS and MDP showed enhanced production of TNFα and IL-6 as well as increased NF-κB and MAPK activation in response to the dipeptide KF1B, the NOD-1 agonist. In addition, reduced tolerization of NOD-2 (-/-) deficient macrophages in response to bacteria was abolished when mutant macrophages were also rendered tolerant to the NOD-1 ligand (Kim YG *et al,* 2008). Clark NM *et al* (2008) reported that a MAP-3-K, MEKK-4, bound to RIP-2 to sequester RIP-2 from the NOD-2 signalling pathway. This MEKK-4/RIP-2 complex dissociated upon exposure to the NOD-2 agonist, MDP, allowing NOD-2 to bind to RIP-2 and activate NF-κB. Interestingly, Crohn's-disease-associated NOD-2 polymorphisms cannot compete with MEKK-4 for RIP-2 binding. MEKK-4 deficient macrophages exposed to MDP exhibited increased NF-κB activity, absent p38 activity, and hypo-responsiveness to TLR-2 and TLR-4 agonists.

A crucial part of the innate immune response is the assembly of the inflammasome, a cytosolic complex of proteins that activated caspase-1 to process the proinflammatory cytokines IL-1β and IL-18. Caspase-1 cleaves the inactive pro IL-1β and IL-18 precursors into active inflammatory cytokines in *Salmonella* infected macrophages and also mediates a pathway of proinflammatory programmed cell death termed "pyroptosis." Caspase-1 diffusely distributed in the cytoplasm and localized in discrete foci within macrophages responding to either *Salmonella* infection or intoxication by *B. anthracis* lethal toxin. *B. anthracis* lethal toxin activation of caspase-1 is known to require the inflammasome adapter NALP-1, while *Salmonella* infection activated caspase-1 through an independent pathway requiring the inflammasome adapter IPAF. Pyroptosis, caspase-1-dependent cell death, featuring DNA cleavage, cytokine activation, and, ultimately and cell lysis resulting from the formation of membrane pores between 1.1 and 2.4 nm in diameter and pathological ion fluxes (Fink SL *et al,* 2008). In the inflammasome complex, NALP-3 or NALP-1 binds to ASC and activates caspase-1

which induces IL-1β. Infiltrated leukocytes producing IL-1β in the anterior chamber were found after 12-hour of LPS treatment and up-regulated expression of NALP-3, ASC, caspase-1, IL-1β, and IL-18 was also observed (Gonzalez-Benitez JF *et al,* 2008).

Live *Fusobacterium nucleatum* induced both BD-2 and -3 efficiently, whereas heat-killed bacteria induced only BD -3 at a reduced level in immortalized human gingival epithelial HOK-16B cells. Silencing of NALP-2, the most abundant intracellular PRR in HOK-16B cells, by RNAi significantly reduced the induction of BD -3 but not BD-2 and IL-8 (Ji S *et al,* 2008).

Caspase-1 activation and cytokine processing and release were late events inhibited by elevated levels of KCl and sucrose, by potassium channel blockers, and by proteasome inhibitors, suggesting that inflammasome formation requires a protein-degradation event and occurs downstream of anthrax lethal toxin mediated potassium efflux. NALP-3 inflammasome formation in anthrax lethal toxin resistant macrophages did not sensitize cells to the same, and this shows that general caspase-1 activation cannot account for sensitivity to anthrax lethal toxin and that a NALP-1b mediated event is specifically required for death (Wickliffe KE *et al,* 2008). *Anaplasma phagocytophilum* is an obligate intracellular pathogen that resides within neutrophils and can cause fever, pancytopenia, or death. ASC and Caspase-1 regulates the IFNγ during *A. phagocytophilum* infection, which plays a critical role in the control of infection. In addition, caspas-1 (-/-) and ASC (-/-) deficient mice were more susceptible than control animals to *A. phagocytophilum* infection due to the absence of IL-18 secretion and reduced IFNγ levels in the peripheral blood and also exhibited reduced $CD4^+$T-cell mediated IFNγ after in vitro restimulation with *A. phagocytophilum* (Pedra JH *et al,* 2007).

Muruve DA *et al* (2008) very recently reported that internalized adenoviral DNA induces maturation of pro-IL-1β in macrophages, which is dependent on NALP-3 and ASC, components of the inflammasome. Inflammasome activation also occurs as a result of transfected cytosolic bacterial, viral and mammalian (host) DNA, but in this case

sensing is dependent on ASC but not NALP-3 and this DNA sensing proinflammatory pathway functions independently of TLRs and IRFs.

L. monocytogenes escapes from the phagosome of macrophages and replicates within the cytosolic compartment. Cytosolic *L. monocytogenes* activates caspase 1, resulting in post-translational processing of the cytokines IL-1β and IL-18 as well as caspase 1-dependent cell death (pyroptosis) through multiple NOD-like receptors, including IPAF and NALP-3. Flagellin expression by cytosolic *L. monocytogenes* was detected through IPAF and this promoted bacterial clearance in a murine infection model (Warren SE *et al,* 2008).

Exocytosis of lysosomes from macrophages has been described as a response to microbial cytotoxins and haemolysins, as well as for releasing proinflammatory cytokines IL-1β and IL-18 during inflammasome activation. The mycobacterial ESX-1 secretion system, encoded in part by the Region of Difference-1, is a virulence factor necessary for phagosome escape and host cell lysis by a contact-dependent haemolysin in *M. marinum*. Recently it has been reported that ESX-1 from *M. marinum* and *M. tuberculosis* was required for Ca^{2+}dependent induction of lysosome secretion from macrophages. Mycobacteria induced lysosome secretion was concurrent to release of IL-1β and IL-18, dependent on phagocytosis of bacteria containing ESX-1. Synthesis but not release of IL-1β and IL-18 occurred in response to dead bacilli and bacteria lacking ESX-1, indicating that only cytokine release was regulated by ESX-1 and inflammasome components caspase-1, ASC and NALP-3, but not IPAF, were also required for release of IL-1β and IL-18 (Kool IC *et al,* 2008).

IPAF was initially identified as autocatalytic activator of procaspase-1 and caspase-1-dependent apoptosis in transfected cells in response to pro-inflammatory and apoptotic stimuli (Poyet JL *et al,* 2001). IPAF (-/-) deficient macrophages activated caspase-1 in response to TLR plus ATP stimulation but not with *S. typhimurium* (Mariathasan S *et al,* 2004). Two years later, Franchi L *et al* reported that Salmonella

strains either lacking flagellin or expressing mutant flagellin were deficient in activation of caspase-1 and in IL-1β secretion, although transcription factor NF-κB dependent production of IL-6 or the chemokine MCP-1 was unimpaired. It was found that detection of flagellin through IPAF induced caspase-1 activation independently of TLR-5 in salmonella infected and LPS tolerized macrophages (Franchi L *et al,* 2006). Stimulation of the IPAF pathway in macrophages after infection required a functional Salmonella pathogenicity island-1 T3SS but not the flagellar T3SS and also IPAF activation could be restored by the introduction of purified flagellin directly into the cytoplasm (Miao EA *et al,* 2006). Mouse macrophages lacking the NLR protein IPAF or its downstream effector caspase-1 were permissive to intracellular Legionella replication. The presence of flagellin and a competent T4SS are critical for Legionella to activate caspase-1 and a Legionella mutant lacking flagellin did not activate caspase-1. In the absence of IPAF or caspase-1 activation, the Legionella containing phagosome acquired endoplasmic reticulum-derived vesicles, avoided fusion with the lysosome, and allowed Legionella replication (Amer A *et al,* 2006). Similar to IPAF and caspase-1, the NLR protein NAIP-5 restricted intracellular proliferation of *L. pneumophila*, the causative agent of a severe form of pneumonia known as Legionnaires' disease. However, cytosolic delivery of recombinant flagellin activated caspase-1 in A/J macrophages carrying a mutant NAIP-5 allele, and in C57BL/6 (B6) macrophages congenic for the mutant NAIP-5 allele (B6-NAIP-5(A/J), but not in IPAF (-/-) deficient cells (Lamkanfi M *et al,* 2007). Flagellin deficient *L. pneumophila* replicate more efficiently in human THP-1 macrophages, primary monocyte-derived macrophages, and alveolar macrophages, and in A-549 lung epithelial cells compared with wild-type bacteria. Gene silencing of NAIP or IPAF in macrophages or of NAIP in lung epithelial cells leads to an enhanced bacterial growth, and overexpression of both molecules strongly reduces Legionella replication (Vinzing M *et al,* 2008).

1.10 *Helicobacter pylori*: History, Taxonomy, Genome and Culture

The real history of gastric pathogen *H. pylori* is very short and starts in 1983 when Barry Marshall and Robin Warren described the successful isolation and culture of a spiral bacterial species from the human stomach (Warren JR and Marshall BJ, 1983). However the presence of bacteria in stomach of animlas and humans were reported in the late 19th and early 20th centuries by several investigators (Bizzozero G, 1893; Pel PK, 1899; Krienitz W, 1906; Luger A, 1917). The etiological role of these bacteria in the development of peptic ulcer disease and gastric cancer was considered at the time, and patients were sometimes even treated with high doses of the antimicrobial compound bismuth. This possibility was later discarded as irrelevant, probably because of the high prevalence of these spiral bacteria in the stomachs of persons without any clinical signs. The bacteria observed in human stomachs were thus considered to be bacterial overgrowth or food contaminants until the early 1980s. At this time, Warren and Marshall performed their groundbreaking experiments, leading to the identification of a bacterium in 58 of 100 consecutive patients, with successful culture and later demonstration of eradication of the infection with bismuth and either amoxicillin or tinidazole (Marshall BJ *et al,* 1985a; Marshall BJ *et al,* 1985b; Marshall BJ *et al,* 1987; Marshall BJ and Warren JR, 1984). The organism was initially named "*Campylobacter*- like organism," "gastric *Campylobacter*-like organism," "*Campylobacter pyloridis*," and "*Campylobacter pylori*" and finally named *Helicobacter pylori* in recognition of the fact that this organism is distinct from members of the genus *Campylobacter* (Goodwin CS *et al,* 1989). Self-ingestion experiments by Marshall and Morris and later experiments with volunteers demonstrated that these bacteria can colonize the human stomach, thereby inducing inflammation of the gastric mucosa. Marshall developed a transient gastritis after ingestion of *H. pylori*; the case described by Morris developed into a more persistent gastritis, which resolved after sequential therapy with first doxycycline and then bismuth

subsalicylate (Marshall BJ *et al,* 1985b; Morris A and Nicholson G, 1987; Morris AJ *et al,* 1991). Extensive research on this bacterium after the initial identification showed that gastric colonization with *H. pylori* can lead to variety of upper gastrointestinal disorders, such as chronic gastritis, peptic ulcer disease, gastric mucosa associated lymphoid tissue (MALT) lymphoma, and gastric cancer. All these data generated in few decades have changed the way how the world looks for gastric pathologies and greatly advanced the treatment and curing of these pathologies. In honour of this great achievement, 2005 Nobel Prize in Physiology or Medicine was awarded to Robin Warren and Barry Marshall for their "discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease."

H. pylori belongs to ε subdivision of the *Proteobacteria*, order *Campylobacterales*, family *Helicobacteraceae*. *H. pylori* is gram-negative, measuring 2 to 4 µm in length and 0.5 to 1 µm in width, usually spiral-shaped, the bacterium can also appear as a rod, while coccoid shapes appear after prolonged in vitro culture or antibiotic treatment (Kusters JG *et al,* 1997). The organism has 2 to 6 unipolar, sheathed flagella of approximately 3 µm in length, which often carries a distinctive bulb at the end. The flagella confer motility and allow rapid movement in viscous solutions such as the mucus layer overlying the gastric epithelial cells (O' Toole PW *et al,* 2000). *H. pylori* is microaerophilic and prefers O_2 concentration of 2 to 5% and a CO_2 concentration of 5 to 10% for optimal growth at 37°C and neutral pH of the medium, however it can also grow at the relatively narrow pH range of 5.5 to 8.0 and temperature range of 34 to 40°C. *H. pylori* is a fastidious organism and requires complex media for growth. Solid media for *H. pylori* growth usually based on Colombia or Brucella agar supplemented with sheep blood or horse serum or, alternatively, newborn or fetal calf serum. Supplements such as β-cyclodextrins or IsoVitaleX, or activated charcoal were also using to protect against the toxic effects of long-chain fatty acids (Taneera J *et al,* 2002). Primary isolation and routine culture often used antibiotic supplements such as Dent supplement, which

consists of vancomycin, trimethoprim, cefsoludin, and amphotericin B (Dent JC and McNulty CA, 1988), or Skirrow supplement, which consists of vancomycin, trimethoprim, polymyxin B, and amphotericin B (Skirrow MB, 1977). Liquid media usually consist of brucella, Mueller-Hinton, or brain heart infusion broth supplemented with 2 to 10% calf serum or horse serum 0.2 to 1.0% β-cyclodextrins, often together with either Dent or Skirrow supplement. Most of the commercially available tissue culture media do not support the growth of *H. pylori* without the addition of serum, with the exception of Ham's F-12 nutrient mixture (Testerman TL *et al,* 2001).

The size of the two sequenced *H. pylori* genomes is approximately 1.7 Mbp, with a G+C content of 35 to 40%. The *H. pylori* strain 26695 genome includes 1,587 genes, whereas the genome of strain J99 includes only 1,491 genes (Tomb JF *et al,* 1997; Alm RA *et al,* 1999; Boneca IG *et al,* 2003). Both genomes contain two copies of the 16S, 23S, and 5S rRNA genes. Many strains carry one or more cryptic plasmids, which do not seem to carry antibiotic resistance genes or virulence genes (Heuermann D and Haas R, 1995). *H. pylori* is genetically heterogeneous and this is possibly an adaptation of *H. pylori* to the gastric conditions of its host, as well as to the distinct patterns of the host-mediated immune response to *H. pylori* infection (Kuipers EJ *et al,* 2000). This high level of genetic heterogeneity thought to occur due to DNA exchanges and thereby addition or deletion of nucleotide sequences. These portions have aberrant G+C content and often carry virulence genes and the *cag* Pathogenecity Island (*cag*PAI) of *H. pylori* encodes 31 proteins is thought to be acquired through that way and other plasticity regions also reported in the pathogenesis of *H. pylori* (Santos A *et al,* 2003; Lehours P *et al,* 2004; de Jonge R *et al,* 2004; Suerbaum S and Achtman M, 2004). It has been reported that virulence genes, such as adhesion factor and outer membrane protein encoding genes and LPS biosynthetic enzymes display reversible phenotypic diversity through transcriptional and translational phase variation and mutation (Appelmelk BJ *et al,* 1999; Aspholm-Hurtig M *et al,* 2004; de Jonge R *et al,* 2004b; Mahdavi J *et al,* 2002).

1.11 Epidemiology and Clinical Conditions Associated with *H. pylori*

Over half of the world's population is infected with *H. pylori*, with the highest rates in developing countries (Rothenbacher D and Brenner H, 2003). Infections occur in early childhood and persist for decades in the absence of targeted antimicrobial therapy. In developing world childhood prevalence is more than developed country children. However, the colonization of *H. pylori* with increasing age increased in developed countries. Within geographical areas, the prevalence of *H. pylori* inversely correlates with socioeconomic status, in particular in relation to living conditions during childhood (Perez-Perez GI *et al,* 2004; Perez-Perez GI *et al,* 2005; Malaty HM and Graham DY, 1994). This low prevalence in developed countries might be attributed to the improved hygiene and sanitation and the active elimination of carrier ship through antimicrobial treatment. However, the prevalence of this bacterium is often considerably higher among first and second generation immigrants in western countries from the developing world (Tsai CJ *et al,* 2005).

The exact mode of transmission of *H. pylori* is not clearly known till today. New infections thought to occur through human to human contact either in oral-oral or faecal-oral route or both. *H. pylori* is reported to be colonized in few hosts such as humans and some non-human primates and there has been few reports of isolation of the organism from pet animals (Brown LM *et al,* 2001; Brown LM *et al,* 2002; Dore MP *et al,* 2001; Herbarth O *et al,* 2001). Presence of *H. pylori* DNA in water sources has been reported in several studies but only one report was published on successful culture of the organism from water (Hegarty JP *et al,* 1999; Enroth H and Engstrand L, 1995; Queralt N *et al,* 2005; Lu Y *et al,* 2002). Occurrence of *H. pylori* infection among institutionalized individuals during outbreaks of gastroenteritis supports the faecal route of transmission (Laporte R *et al,* 2004). Another possible route of transmission of *H. pylori* is through the use of contaminated food (Poms RE and Tatini SR, 2001).

Although *H. pylori* colonized individuals are high in population, only a few percentage shows clinical symptoms of *H. pylori* associated diseases. In the infamous self ingestion experiments by Marshall developed a transient gastritis after ingestion of *H. pylori*, whereas the case described by Morris developed into a more persistent gastritis. Among the *H. pylori* positive individuals 10 to 20% develop ulcer diseases and 1 to 2% having risk of leading to gastric cancer (Ernst PB and Gold BD, 2000). The initial colonization by *H. pylori* to gastric mucosa invites the infiltration of neutrophils and mononuclear cells to the site of infection. The acute phase of colonization with *H. pylori* may be associated with transient nonspecific dyspeptic symptoms, such as fullness, nausea, and vomiting, and with considerable inflammation of both proximal and distal stomach mucosa, otherwise pangastritis. This phase is often associated with hypochlorhydria, which can last for months (Graham DY *et al,* 2004). If initial colonization is not cleared, it leads to chronic gastritis. In chronic gastritis, the distribution of *H. pylori* colonization is correlated with the acid secretion and regulation. In persons with active acid secretion, *H. pylori* colonize gastric antrum, where acid secreting parietal cells are scarce and this condition is associated with antrum-predominant gastritis. Individuals with impaired acid secretion have a more even distribution of bacteria in antrum and corpus, and bacteria in the corpus are in closer contact with the mucosa, leading to a corpus-predominant pangastritis (Kuipers EJ *et al,* 1995). *H. pylori* corpus gastritis is often associated with hypochlorhydria, and eradication therapy leads to increased acid secretion in these individuals (El-Omar EM *et al,* 1997). In addition, individuals with proinflammatory genotypes have a higher risk of corpus-predominant pangastritis, predisposing them to atrophic gastritis, intestinal metaplasia, and gastric cancer (El-Omar EM *et al,* 2000).

Gastric and duodenal ulcers, collectively known as peptic ulcer, are also associated with long term colonization of *H. pylori.* Gastric or duodenal ulcers are defined as mucosal defects with a diameter of at least 0.5 cm penetrating through the muscularis

mucosa. Gastric ulcers mostly occur along the lesser curvature of the stomach, in particular, at the transition from corpus to antrum mucosa. Duodenal ulcers usually occur in the duodenal bulb, which is the area most exposed to gastric acid (Veldhuyzen van Zanten, SOJ *et al,* 1999). Epidemiological data from different parts of the world have shown that *H. pylori* is associated with approximately 95% of duodenal ulcers and 85% of gastric ulcers (Kuipers EJ *et al,* 1995). Introduction of *H. pylori* eradication regimens completed the evidence for a causal relation between *H. pylori* and ulcer disease by showing that eradication of this bacterium strongly reduced the risk of recurrent ulcer disease (Rauws EAJ and Tytgat GNJ, 1990). Ulcer development in the presence of *H. pylori* is influenced by a variety of host and bacterial factors. Ulcers mostly occur at sites where mucosal inflammation is most severe. In subjects with decreased acid output, this usually is the gastric transitional zone between corpus and antrum, giving rise to gastric ulcer disease. If acid production is normal to high, the most severe inflammation usually is found in the distal stomach and proximal duodenum, giving rise to juxtapyloric and duodenal ulcer disease (Veldhuyzen van Zanten, SOJ *et al,* 1999). Complications of ulcer disease include bleeding, perforation, and stricture formation. Bleeding is the most common complication of ulcer disease and is estimated to occur in 15 to 20% of ulcers.

Non-ulcer or functional dyspepsia (NUD) is a condition with the presence of symptoms of upper gastrointestinal distress without any identifiable structural abnormality during upper gastrointestinal endoscopy. Dyspeptic symptoms may have a reflux-like character, with heartburn and regurgitation as predominant signs with early satiety and nausea or may be ulcer-like, with pain and vomiting. About 30 to 60% of patients with functional dyspepsia carry *H. pylori* (Talley NJ and Hunt RH, 1997).

Chronic *H. pylori*-induced inflammation can eventually lead to loss of the normal gastric mucosal architecture, with destruction of gastric glands and replacement by fibrosis and intestinal-type epithelium. This process of atrophic gastritis and intestinal metaplasia occurs in approximately half of the *H. pylori*-colonized population (Kuipers EJ

et al, 1995b). The risk for atrophic gastritis depends on the distribution and pattern of chronic active inflammation and areas of gland loss and intestinal metaplasia extend with time multifocally, and although they do not give rise to any specific symptoms, they increase the risk for gastric cancer by 5 to 90 fold depending on the extent and severity of atrophy (Sipponen P *et al,* 1985). *H. pylori* increases the risk of gastric cancer development through the sequence of atrophy and metaplasia and it was shown that *H. pylori* positive subjects develop these conditions more often than do uninfected controls (Kuipers EJ, 1998). *H. pylori* colonization increases the risk of gastric cancer approximately 10 fold and thus it was designated as a class I carcinogen by the WHO (IARC, 1994). As reported in clinical studies, the Mongolian gerbil model of *H. pylori* infection also induced atrophic gastritis and gastric cancer (Honda S *et al,* 1998; Rieder G *et al,* 2005). The risk of development of atrophy and cancer in the presence of *H. pylori* is again related to host and bacterial factors, which influence the severity of the chronic inflammatory response. The risk is increased in subjects colonized with cytotoxin associated gene-A (cagA) positive strains, but also in those with a genetic predisposition to higher IL-1 production in response to colonization (Parsonnet J *et al,* 1997; El-Omar EM *et al,* 2000). Gastric ulcer patients are characterized by reduced gastric acid secretion, corpus predominant pangastritis, and accelerated progression toward atrophic gastritis and intestinal metaplasia and these individuals are reported to be at higher risk for gastric cancer than duodenal ulcer patients (Hansson LE *et al,* 1996).

The gastric mucosa does not normally contain lymphoid tissue, but MALT nearly always appears in response to colonization with *H. pylori*. In rare cases, a monoclonal population of B cells may arise from this tissue and slowly proliferate to form a MALT lymphoma. Almost all MALT lymphoma patients are *H. pylori* positive, and *H. pylori*-positive individuals have a significantly increased risk for the development of gastric MALT lymphoma, however, MALT lymphomas occur in less than 1% of *H. pylori* positive individuals (Eidt S *et al,* 1994; Parsonnet J *et al,* 1994; Parsonnet J and Issacson PG,

2004). It has been reported that eradication can lead to complete remission in patients with stage IE MALT lymphoma confined to the stomach (de Mascarel A *et al,* 2005; Fischbach W *et al,* 2004; Wundisch T *et al,* 2005).

H. pylori has also been linked to a variety of extragastric disorders. These include coronary heart disease, dermatological disorders such as rosacea and idiopathic urticaria, autoimmune thyroid disease and thrombocytopenic purpura, iron deficiency anemia, Raynaud's phenomenon, scleroderma, migraine, and Guillain-Barre´ syndrome. It has been hypothesized that chronic low grade activation of the coagulation cascade, accelerating atherosclerosis, and antigenic mimicry between *H. pylori* and host epitopes leading to autoimmune disorders (Gasbarrini A *et al,* 2004).

1.12 Pathogenesis and Virulence Factors of *H. pylori*

The long term colonization of *H. pylori* cause chronic inflammatory state and the intragastric distribution and severity of this process depend on a variety of factors, such as characteristics of the colonizing strain, host genetics and immune response, diet, and the level of acid production. *H. pylori* induced ulcer disease, gastric cancer, and lymphoma are all complications of this chronic inflammation; ulcer disease and gastric cancer in particular occur in those individuals and at those sites with the most severe inflammation. Infection is associated with production of proinflammatory cytokines which lead to chronic or chronic active gastritis. The activity of the gastritis is commonly considered an indicator of severity, and chronic active gastritis is associated with more severe manifestations of disease, such as peptic ulceration and neoplasia (Blaser MJ *et al,* 1995; Crabtree JE, 1998; Peek RM *et al,* 1995). *H. pylori* pathogenesis and virulence factors such as cagPAI and its effector protein cagA (Covacci A *et al,* 1993), vacuolating cytotoxin (vacA) (Cover TL, 1996), Urease (van Vliet AHM *et al,* 2001), adhesins such as blood group antigen binding adhesin (BabA), sialic acid binding adhesin (sabA and SabB) (Prinz C *et al,* 2001), adherence-associated lipoprotein (AlpA and AlpB),

Helicobacter outer membrane protein Z (HopZ), outer membrane inflammatory protein A (OipA) (Yamaoka Y *et al,* 2000) and neutrophil activating protein (Nap) (Tonello F *et al,* 1999; Namavar F *et al,* 1998), other factors associated with ulcer such as induced upon contact with epithelium gene (IceA) (Figueiredo C *et al,* 2000) and duodenal ulcer associated protein (DupA) (Lu H *et al,* 2005), LPS and flagella contributes to the outcome of the gastric pathologies independently or collectively. Many of these defined *H. pylori* virulence factors, including flagella, the stomach acid neutralizing enzyme urease and the multifunctional VacA, are found in all strains (Monack DM *et al*, 2004). However, the *cag* pathogenicity island (PAI), a 40 kb stretch of DNA-encoding homologues of components of a T4SS, positive strains are associated with severe disease (Censini S *et al*,1996).

T4SS have been found in a number of bacterial pathogens and symbionts and are evolutionarily related to bacterial conjugation systems (Cascales E and Christie PJ, 2003). The best-studied model is the T4SS of *Agrobacterium tumefaciens*, a secretion system used by this promiscuous plant pathogen to deliver DNA and proteins into plant cells in a process that introduces bacterial DNA into the plant genome and causes tumour formation. *L. pneumophila* and *Bartonella henselae* are the other two pathogens which can secrete the effector proteins in to the host cells through T4SS. Eighteen of the *cag* PAI-encoded proteins serve as building blocks of a T4SS apparatus, which forms a pilus, capable of penetrating the gastric epithelial cells and facilitating the translocation of CagA, peptidoglycan, and possibly other bacterial factors into host cells (Fischer W *et al,* 2001; Odenbreit S *et al*, 2001). This attachment is accomplished by a specialized adhesin of the pilus surface, the CagL protein, which binds to and activates host cell integrin β1 for subsequent delivery of CagA across the host cell membrane (Kwok T *et al*, 2007; Backert S and Selbach M, 2008) Once delivered, CagA interacts with a large number of host proteins and has multiple effects on host signal transduction pathways,

the cytoskeleton and cell junctions (Segal ED *et al*, 1999; Asahi M *et al*, 2000; Backert S *et al*, 2000; Odenbreit S *et al*, 2000; Stein M *et al*, 2000).

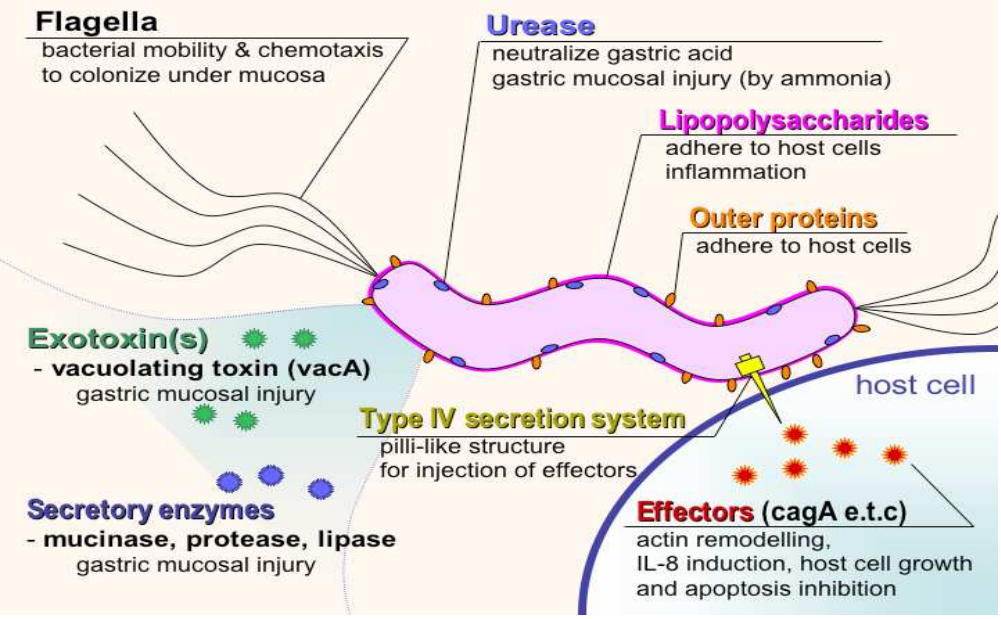


Fig.6 Diagramatic representation of virulence and pathogenecity factors of *H. pylori* (source: www.wikimedia.org)

CagA localizes to the cell membrane at the point of bacterial attachment (Segal ED *et al*, 1999; Tanaka J *et al*, 2003) and colocalizes with the cell junctional proteins ZO-1 and JAM (Amieva MR *et al*., 2003). CagA is recognized as a substrate by SRC and ABL family kinases that phosphorylate it on tyrosine residues in unique C-terminal region Glu–Pro–Ile–Tyr–Ala (EPIYA) motifs (Selbach M *et al*, 2002; Stein M *et al*, 2002; Poppe *et al*, 2007; Tammer *et al*, 2007). CagA, the effector protein of *cag*PAI, can influence the host cell machinery in phosphorylated and non-phosphorylated forms. These host cell manipulations have been identified *in vivo* and *in vitro* which include the induction of membrane dynamics, actin-cytoskeletal rearrangements and the disruption of cell-to-cell junctions as well as proliferative, pro-inflammatory and antiapoptotic nuclear responses (Blaser and Atherton, 2004; Backert and Selbach, 2008; Hatakeyama, 2008). The non-phosphorylated CagA can directly interact with multiple host cell proteins such as cell adhesion proteins E-cadherin and ZO-1, the hepatocyte growth factor receptor c-MET, the adaptor protein GRB2 and the kinase PAR1 (Mimuro *et al*.,2002; Amieva *et al*., 2003;

Churin *et al*., 2003; Murata-Kamiya *et al*., 2007; Saadat *et al*., 2007; Zeaiter *et al*.,2007). The phosphorylated CagA were reported to be binding three SH2 domain containing host cell proteins such as tyrosine phosphatase SHP-2, the carboxy-terminal SRC kinase (CSK) and the adaptor protein CRK (Higashi *et al*., 2004; Tsutsumi *et al*., 2003; Suzuki M *et al*., 2005; Brandt *et al*., 2007). CagA phosphorylation inactivates proteins controlling cell-to-matrix adhesion, which includes SHP-2-dependent dephosphorylation of focal adhesion kinase FAK (Tsutsumi *et al*., 2005) and CSK-dependent inactivation of SRC which leads to tyrosine dephosphorylation of the actin binding protein cortactin, vinculin and ezrin (Selbach *et al*., 2003; 2004; Tsutsumi *et al*., 2003; Moese *et al*., 2007) and also induce cellular elongation *in vitro* by causing a cell retraction defect in focal adhesions (Bourzac *et al*.,2007). *H. pylori* T4SS induces several signalling events such as the activation of epidermal growth factor receptor EGFR (Keates *et al*, 2007; Wallasch *et al*, 2002; Du Y *et al*, 2007) and the small Rho GTPase RAC-1 (Churin *et al*, 2001; Suzuki M *et al*, 2005; Brandt *et al*, 2007) and the induction of proinflammatory cytokines in epithelial cells independent of CagA phosphorylation (Odenbreit *et al,* 2000; Segal ED *et al,* 1999).

H. pylori VacA protein plays an important role in the pathogenesis of both peptic ulceration and gastric cancer (Atherton JC *et al,* 1995; Marchetti M *et al,* 1995; Ogura K *et al,* 2000; Wada A *et al,* 2004). The gene encoding *vacA* is present in virtually all of the *H. pylori* strains, which suggests that the production of VacA plays an important role in the colonization or persistence of *H. pylori* in the human stomach (Blaser and Atherton, 2004). The VacA protein is produced as a 140-kDa protoxin that is cleaved into the 95-kDa mature form when secreted. Although all strains carry a functional *vacA* gene, there is considerable variation in vacuolating activities among strains (Cover TL, 1996; Cover TL and Blaser MJ, 1992; de Bernard M *et al,* 1997). This is due to the sequence heterogeneity within the *vacA* gene at the signal region (s) and the middle region (m). The s region of the gene, which encodes the signal peptide, occurs as either an s1 or s2

type, whereas the m region, which contains the p58 cell binding domain, exists as an m1 or m2 type. Vacuolating activity is high in s1/m1 genotypes, intermediate in s1/m2 genotypes, and absent in s2/m2 genotypes. In line with this, *vacA* s1/m1 genotypes are more frequently associated with peptic ulceration and gastric carcinoma (Atherton JC *et al,* 1995; van Doorn LJ *et al,* 1998). Secreted VacA can be further processed into a 33-kDa Nterminal fragment and a 55-kDa C-terminal fragment through proteolytic cleavage. The N-terminal protein performs an essential function in the formation of anion channels, while the C-terminal protein mediates cell binding (Ji X *et al,* 2000; Kim S *et al,* 2004; Torres VJ *et al,* 2005). VacA acts as a pore-forming toxin and induces both structural and functional alterations in the cells, the most prominent being mitochondrial damage, apoptosis and the formation of cytoplasmic vacuolation (Galmiche A *et al*, 2000; Cover TL and Blanke SR, 2005). Protein tyrosine phosphatase receptors RPTP-α and RPTP-β as well as sphingomyelin act as receptors for VacA in gastric epithelial cells (Fujikawa A *et al*., 2003; Yahiro K *et al*., 2003; Nakayama M *et al*., 2006; Gupta VR *et al*., 2008). It has also been reported that VacA can bind to other surface molecules such as heparan sulphate, RACK1, fibronectin and EGFR (Utt M *et al*, 2001; Seto K *et al*, 1998; Hennig EE *et al*, 2001; Hennig EE *et al*, 2005). The vacuoles induced by vacA contain both late endosomal and lysosomal markers, and the internalized vacA in cells forms anion-selective membrane channels, and the vacuoles then arise due to swelling of the endosomal compartments (Molinari M *et al,* 1997; Blaser and Atherton, 2004; Cover and Blanke, 2005).

Bacterial adhesins play an important role in colonization to the host organism. *H. pylori* is also ultilize certain adhesins and outer membrane proteins to establish an infection and subsequent inflammatory processes. *H. pylori* adhesins identified so far includes BabA, SabA, SabB, AlpA, AlpB, HopZ and OipA. Lewis b antigen (Le^b) and related fucosylated ABO blood group antigens are recognized by BabA, whereas sialyl-Lewis x and sialyl-Lewis a antigens (sLe^x and sLe^a) are recognized by SabA (Ilver D *et*

al, 1998; Mahdavi J *et al,* 2002). The corresponding receptors for AlpA, AlpB, OipA, and HopZ are not known till today. BabA and SabA are the two well characterized adhesins of *H. pylori* having an important role in the pathogenesis. The 78 kDa BabA protein is encoded by the *H. pylori* gene *BabA.* BabA mediates binding to fucosylated Lewis b (Le^b) blood group antigens on the human host cells (Boren T *et al,* 1993; Ilver D *et al,* 1998). There are two distinct *babA* alleles, *babA1* and *babA2*, but due to a 10-bp insertion in the 3' end of the gene, only *babA2* can encode a full-sized (active) bacterial adhesion protein. The adherence of *H. pylori* to the gastric mucosa is widely assumed to play an important role in the initial colonization and long-term persistence in the human gastric mucosa. The mucins secreted by gastric mucous cells form a mucous gel layer covering the gastric mucosa. This gel layer is considered the first line of gastric mucosal defense against luminal noxious agents (Allen A *et al,* 1993; Ho SB *et al,* 2004). The gastric surface mucous cells and gland mucous cells express the secretory mucins MUC5AC and MUC6/MUC5B, respectively (Ho SB *et al,* 1995; Van de Bovenkamp *et al,* 2005). The majority of *H pylori* reside in the gastric mucus overlying the epithelium. It is reported that *H pylori* could be colocalized with MUC5AC gastric mucin, but not with MUC6-producing cells in the glandular areas and the adhesion is predominantly toward MUC5AC specific ligands in gastric mucosa (Van den Brink GR *et al,* 2000). However, additional epitopes and/or adhesins must be involved, as BabA binding to Le^b-negative MUC5AC of nonsecretors also reported recently (Van de Bovenkamp *et al,* 2003).

It has been reported that *H. pylori babA* deletion mutant cannot bind ABO/Le^b blood group antigens but that was able to bind gastric epithelium. This has been achieved through another *H. pylori* adhesin, sabA, which binds the sialyl-Lewis x and sialyl-Lewis a antigens (sLe^x and sLe^a) (Mahdavi J *et al,* 2002). The sLe^x and sLe^a glycans are better known as the glycan binding sites for the selectin family of cell adhesion molecules and they have important roles in recruitment of white blood cells from circulation to the during inflammation (Alper J, 2001). Infection of the gastric

mucosa by *H. pylori* results in inflammatory responses, with concomitant expression of sialylated glycans and thereby utilizes these molecules for epithelial binding and colonization.

The 34-kDa OipA protein expression in *H. pylori* is strongly associated with increased in vitro and in vivo IL-8 expression in host cells and may also serve as adhesin (Yamaoka Y *et al,* 2002). Neutrophil-activating protein (Nap), have important roles in the recruitment and activation of inflammatory cells in the lamina propria and activates neutrophils and monocytes to produce ROIs by activating the plasma membrane NADPH oxidase through a signaling pathway involving the activation of heterotrimeric G proteins, phosphatidylinositol 3-kinase, SRC family tyrosine kinases and calcium mobilization (Satin B *et al,* 2000).

The first barrier *H. pylori* must overcome for the colonization is the low pH in the lumen of the stomach. *H. pylori* express urease enzyme, which breaks down urea to form NH_3 and CO_2 and that buffer the microenvironment and the cytosol of the bacteria (Weeks DL *et al,* 2000; Stingl K *et al,* 2002). Powerful flagella then help the bacterium to swim through the viscous mucous layer covering the gastric epithelium, where bacterial adhesins mediate a close interaction with the host cells (Montecucco C and Rappuoli R, 2001).

The cholesteryl-α-glucosides of *H. pylori* support the pathogenicity of this organism, because inhibition of the cholesterol glucosyltransferase (*Cgt*) by *O*-glycans of the human gastric mucosa suppresses growth of the bacterium. This enzyme catalyzes the first step in the biosynthesis of four unusual glycolipids such as cholesteryl-α-glucoside,cholesteryl-6-*O*-acyl-α-glucoside, cholesteryl-6-*O*-phosphatidyl-α-glucosideand cholesteryl-6-*O*-lysophosphatidyl-α-glucoside (Kawakubo M *et al,* 2004). Deletion of the *HP0421* gene in *H. pylori* resulted in the loss of cholesteryl-α-glucoside and all of its three derivatives (Lebrun A *et al,* 2006). Morphological changes of the bacterium or changes in colony variants are accompanied by alterations in the total amount of steryl

glucosides and the relative proportions of cholesteryl-α-glucoside and cholesteryl-6-*O*-acyl-α-glucoside and cholesteryl-6-*O*-phosphatidyl-α-glucoside (Shimomura H *et al,* 2004). *H. pylori* follows a cholesterol gradient and extracts the lipid from plasma membranes of epithelial cells for subsequent glucosylation. Excessive cholesterol promotes phagocytosis of *H. pylori* by antigen-presenting cells, such as macrophages and DCs, and enhances antigen specific T -cell responses. A cholesterol-rich diet during bacterial challenge leads to T-cell dependent reduction of the H. pylori burden in the stomach. However, intrinsic α-glucosylation of cholesterol abrogates phagocytosis of *H. pylori* and subsequent T-cell activation (Wunder C *et al,* 2006).

The majority of *H. pylori* strains express LPS that contains fucosylated oligosaccharide antigens that are structurally and immunologically closely related to human blood group antigens. Compared to other gram-negative bacteria, the LPS of *H. pylori* is a poor activator of the innate immune response (Muotiala A *et al,* 1992; Bliss CM *et al,* 1998). However, It has recently been reported that *H. pylori* LPS stimulates NFκB and IL-8 production in both epithelial cells and immune cells (Bhattacharyya A *et al,* 2006; Maeda S *et al,* 2001). It has been reported that *H pylori* bind to the dimeric form of human recombinant trefoil factor family 1 (TFF-1) (Clyne M *et al,* 2004). Trefoil factors are small cysteine-rich proteins localized within mucin granules in mucus-secreting epithelial cells. In the gastrointestinal tract TFF1 is coexpressed with MUC5AC in foveolar cells of the stomach, TFF2 is coexpressed with MUC6 in the fundus and deep antral glands of the stomach and in Brunner's glands of the duodenum and TFF3 is coexpressed with MUC2 in goblet cells throughout the large and small bowel mucosa (Longman RJ *et al,* 2000). Very recently, it has been shown that *H. pylori* core oligosaccharide portion of LPS bound to TFF1 and to a lesser extent TFF3 and this interaction was inhibited by incubation of LPS portion with mannosidase, glucosidase, or mixed monosaccharides (Reeves EP *et al,* 2008). This finding attributes an important

role for *H. pylori* LPS as an adhesin that helps colonization along with the established BabA and SabA.

1.13 Immune Responses to *Helicobacter pylori* infection

H. pylori infection was sufficient to stimulate the immune system at the site of colonization and subsequent inflammatory process and epithelial damage, which has been seen in the active chronic gastritis. However, in most of the individulals *H. pylori* keeps a balanced situation of low immune reaction that was not able to eliminate organism but definitely causing tissue damage and that may help the bacterium to get sufficient nutrients to survive in a hostile situation like stomach. *H. pylori* infection attracts more neutrophils and monocytes to the site of infection through induction of IL-8 release and *H. pylori* Nap protein has also been implicated for the neutrophil activating process by inducing NADPH oxidase (Tonello F *et al,* 1999; Namavar F *et al,* 1998; Satin B *et al,* 2000; Naumann M and Crabtree JE, 2004). The phagocytic cells at the site of infection try to eliminate the microbes by engulfment subsequent killing by the help of of reactive oxygen and nitrogen intermediates (ROIs and RNIs). However, *H. pylori* possess catalase and superoxide dismutase enzymes involved in the ROI scavenging, this might help the organism to cotrol its elimination by phagocytotic killing (McGee DJ and Mobley HL, 1999). It also produces the arginase enzyme which converts arginine to ornithine and thereby competitively inhibiting the inducible nitric oxide synthase enzyme of the host and control the production of NO. *H. pylori* also induce eukaryotic arginase II expression in macrophages, which might further counteract NO production by these cells (Gobert AP *et al,* 2002). These mechanisms might limit the availability of L-arginine at the site of infection and therby control phagocytic killing. These all are showing that *H. pylori* have evolved mechanisms to control the elimination by immune responses even at the level of detection.

H. pylori infection result in an induction of a Th-1 polarized response, however, that was not sufficient to eliminate the infection (Smythies LE *et al,* 2000). *H. pylori* have been reported to induce rapid maturation and activation of monocyte derived DCs, which also secreted high levels of Th-1 cytokine IL-12. *cag*PAI and plasticity regions of *H. pylori* have been implicated for this activity in addition to other unidentified factors (Guiney DG *et al,* 2003; Hafsi N *et al,* 2004; de Jonge R *et al,* 2004a). Transfer of unfractionated splenocytes from *H. pylori* infected mice induces gastritis, delayed-type hypersensitivity, and even metaplasia in mice and this supporting the notion that *H. pylori*-induced pathology being predominantly a T-cell mediated disease (Eaton KA *et al,* 2001). It has also been reported that protection against infection through immunization with *H. pylori* proteins has been achieved in antibody-deficient mice and requires an intact MHCII-restricted $CD4^+$ T-cell compartment (Ermak TH *et al,* 1998). *H. pylori* has also evolved strategies to control T-cell function, Zabaleta J *et al* reported that *H. pylori* causes a reduction in expression of the TCR–CD3ζ subunit in activated T cells (Zabaleta J *et al,* 2004). Induction of arginase in macrophages by *H. pylori* could be one mechanism reported to be crucial for immunosuppressive effects on T-cells including reduction in expression of the TCR–CD3ζ subunit (Bronte V *et al,* 2003). *H. pylori* VacA protein found to be inhibiting IL-2 production and proliferation of peripheral blood lymphocytes and that is attributed to the inhibition of translocation of the transcription factor NF-AT to the nucleus (Gebert B *et al,* 2003; Hogan PG *et al,* 2003).

Humoral immunity against *H. pylori* is evident by the presence of antibodies in serum and gastric juice of individuals infected with the bacterium but that was not enough to eliminate the infection (Mattsson A *et al,* 1998; Perez-Perez GI *et al,* 1999). However, a report correlated well with *H. pylori* antibodies in the milk of Gambian mothers and the absence of *H. pylori* infection in their breast-fed children (Thomas JE *et al,* 2004). *H. pylori* has a clear role in the development of gastric lymphoma of MALT. Gastric MALT lymphoma results from the uncontrolled polyclonal expansion of a subset

of IgM memory B cells that eventually leads to the emergence of a monoclonal tumour. This has been attributed to bacterial factors such as CpG DNA that can induce mitogenic effect on IgM memory B-cells (Bernasconi NL *et al,* 2002). *H. pylori* specific T-cells from MALT lymphoma appear to be deficient in the cytolytic mechanisms that normally ensure the deletion of activated B-cells and this inability to delete these proliferating B-cells might contribute to the uncontrolled growth in the MALT (D'Elios MM *et al,* 1999).

1.14 Host Gene Polymorphisms and Susceptibility to *H. pylori* associated Pathologies

Several studies in the past were clearly shown the influence of host genetics along with the bacterial factors in *H. pylori* related pathologies. Genetic polymorphisms can directly affect the expression levels of gene products by generation or deletion of transcription factor sites or by affecting RNA splicing and subsequent translation. Many of the pathogenic effects of *H. pylori* infection are related to chronic active inflammation, which is controlled and maintained by the complex interplay of proinflammatory and anti-inflammatory mediators (Macarthur M *et al,* 2004). The level of gastric acid secretion and the presence of a proinflammatory response contribute significantly to the development of either duodenal ulcer disease or atrophic gastritis. The IL-1 cytokine is encoded by a gene cluster that contains the polymorphic *IL-1B* (encoding the IL-1βcytokine) and *IL-RN* (encoding the IL-1 receptor antagonist) genes. IL-1β is a potent proinflammatory cytokine and the most potent known inhibitor of acid secretion (El-Omar EM *et al,* 2000). The *IL-1* gene cluster contains several polymorphisms, such as *IL-1B*-31C, IL-1B*-511T*, and *IL-1RN*2/*2*, which lead to high-level expression of IL-1β. This subsequently leads to reduced acid output, which is associated with corpus-predominant colonization by *H. pylori*, resulting in pangastritis, formation of atrophic gastritis, and increased risk of gastric cancer (El-Omar EM, 2001; El-Omar EM *et al,* 2000; El-Omar EM *et al,* 2003; Furuta T *et al,* 2002, Hwang IR *et al,* 2002; Rad R *et al,* 2003; Zambon CF *et al,* 2002). TNFα is also a proinflammatory cytokine, and several polymorphisms in the *TNF-A* gene

are known. The *TNFA* 308A* genotype is associated with increased TNFα production, which, together with IL-1, influences gastrin production and thus acid production by gastric parietal cells (Suzuki T *et al,* 2001). The *TNF-A*308A* genotype is therefore associated with *H. pylori* infection and increased risk of gastric cancer (El-Omar EM *et al,* 2003; Ohyama I *et al,* 2004; Rad R *et al,* 2004; Yea SS *et al,* 2001; Zambon CF *et al,* 2005). Similarly, expression of the anti-inflammatory cytokine IL-10 is affected by the haplotypes described for the IL-10 gene. The GCC haplotype is associated with a higher expression level of IL-10 and hence favors an anti-inflammatory response, whereas the ATA haplotype results in lowered IL-10 levels and a shift toward a proinflammatory response (El-Omar EM *et al,* 2003; Hamajima N *et al,* 2003, Lu W *et al,* 2005; Hellmig S *et al,* 2005; Wu MS *et al,* 2003; Zambon CF *et al,* 2005). The GCC haplotype is associated with colonization with more-virulent *H. pylori* strains, whereas the ATA haplotype is associated with increased risk of gastric cancer (Rad R *et al,* 2004; El-Omar EM *et al,* 2003). *CD14*-159T* genotype has bee reported to be associated with higher *H. pylori* infection rates (Karhukorpi J *et al,* 2002) Polymorphisms in the fucosyltransferase genes which affect the synthesis of Lewis antigens and *FUT2 (Se)* and *FUT3 le/le* genotypes has been reported to increase risk of *H. pylori* infection (Ikehara Y *et al,* 2001). Presence of the *IL-8 -251A* allele results in increased expression of IL-8 and this genotype increased the risk of gastric cancer and gastric ulcer in Japanese population and increased risk of duodenal ulcers in a Hungarian population (Gyulai Z *et al,* 2004; Lu W *et al,* 2005). Studies have shown that while single polymorphisms may increase the risk of development of gastric cancer only two- to threefold, the presence of multiple proinflammatory genotypes increases this risk substantially.

1.15 Pattern Recognition Receptors in *Helicobacter pylori* Infection

The role of innate immune response to *H. pylori* through PRRs is not completely understood till today. Few prevoius studies have reported the involvement of some TLRs

and NOD proteins in the detection of *H. pylori.* Gastric epithelial cells are reported to be not enough to provide all the TLR molecules expressed on its surface for detection of *H. pylori.* Smith MF *et al* reported that gastric epithelial cells recognize and respond to H. pylori infection at least in part through TLR-2 and TLR-5 (Smith MF *et al,* 2003). TLR-4 mediated recognition of bacterial LPS is a key activator of the innate immune response in epithelial cells, while *H. pylori* LPS is a relatively weak inducer. However, *H. pylori* LPS does activate NF-κB, but this is via TLR-2 rather than TLR-4 (Smith MF *et al,* 2003; Maeda S *et al,* 2001; Backhead F *et al,* 2003). In contrast to this, Kawahara T *et al* (2001) reported that *H. pylori* LPS activated NF-κB in association with the expression of Mitogen oxidase-1 (MOX-1), cyclooxygenase-II (COX II) and TNFα transcripts in gastric pit cells, which express more TLR-4 but no TLR-2. Immunocytochemical studies using gastric mucosal biopsies have revealed that TLR-5 and TLR-9 expression on the gastric epithelium changed to an exclusive basolateral localization without detectable expression at the apical pole in *H. pylori* gastritis, however, TLR-4 expression was highly polarized in an apical and a basolateral compartment identical to the non-inflamed mucosa (Schmausser B *et al,* 2004). Interestingly, Mandell L *et al* reported that *H. pylori* LPS induced cytokine production was mediated through TLR-4, but the response to the whole Helicobacter bacteria such as *H. pylori, H. hepaticus, and H. felis* were mediated through TLR-2 (Mandell L *et al,* 2004). *H. pylori* infection with HEK-293 cells have differentially regulated 46 genes more than 2-fold and three genes less than 2-fold, while infection with HEK-293 cells transfected with TLR-2 differentially regulated 54 genes more than 2-fold and only 1 gene was repressed less than 2-fold. It was identified that 28 genes expression were dependent on the presence of TLR-2 during infection with HEK-293 cells and 8 of the selected genes showed distinct expression patterns in epithelial cell lines (Ding SZ *et al,* 2005). Ogawa T *et al* reported chemically synthesized lipid A, mimicking the natural lipid A portion of lipopolysaccharide from *Helicobacter pylori* strain 206-1, has a low endotoxic potency and immunobiological activities, and is recognized

by TLR-4 (Ogawa T *et al,* 2003). A study using recombinant *flaA* and *flaA* mutant of *H. pylori* revealed the less potent activity of TLR-5 mediated IL-8 secretion in epithelial cells (Gewirtz AT *et al,* 2004). A recent report attributed the *flaA* evasion of TLR-5 due to amino acids 89-96 of the N-terminal D1 domain and that may be responsible for low TLR5 mediated activity on IL-8 secretion (Andersen-Nissen E *et al,* 2005).

It has been reported that Syndecan-4 expression was induced in gastric epithelial cells and macrophages in response to infection with live *H. pylori* and purified TLR agonists. Syndecan-4, heparan sulphate proteoglycan, has been implicated in a wide variety of biological functions including the regulation of growth factor signaling, adhesion, tumorigenesis, and inflammation (Smith MF *et al,* 2006). HSP60 of *H. pylori* have been implicated for IL-8 secretion through ERK and p38 MAPK signalling linked to the TLR-2 recognition receptor in human monocytes (Zhao Y *et al,* 2007).

It has been shown that DCs from Myd88 (-/-) deficient mice have impaired adaptive immune responses during *H. pylori* infection and that was found to be through genes involving in maturation, antigen presentation and effector cell recruitment. In addition, *H. pylori* infection of Myd88 (-/-) deficient mice displayed reduced gastric inflammation, increased bacterial colonization and reduced Helicobacter-specific IgG2c/IgG1 ratios (Rad R *et al,* 2007). *H. pylori* LPS has earlier reported to be signalling through TLR-4 and Uno K *et al* affirmed this finding and showed that TLR-4 signaling initiated by *H. pylori* LPS and propagated through ERK and NF-κB activation induced TLR-2 expression in gastric epithelial and induced TLR-2 cooperated with TLR-4 to amplify iNOS induction (Uno K *et al,* 2007). Very recently, it has been reported that probiotic bacteria, *Lactobacillus bulgaricus,* and its culture supernatant prevented *H. pylori* LPS induced IL-8 production through inhibition of TLR-4 signalling (Zhou C *et al,* 2008).

NOD-1 and NOD-2 expression levels were induced in the gastric epithelium in *H. pylori* positive patients and expression of NOD-1 and NOD-2 in HEK-293 cells activated

NF-κB during *H. pylori* infection in a *cag*PAI dependent manner. However, Crohn's disease associated NOD-2 variant R702W inhibited NF-κB activation significantly and this genotype was significantly associated with gastric MALT lymphoma (Rosenstiel P *et al,* 2006). It has been reported that E266K-NOD1, but not the TLR-4 gene polymorphism (ASP/299/Gly and Thr/399/Ile) increased the risk of peptic ulceration in *H. pylori* infected patients. In addition, 251-IL-8 polymorphism was significantly associated with either gastritis or duodenal ulcer in *H. pylori* infection (Hofner P *et al,* 2007). Viala J *et al* (2004) reported that *H. pylori* PGN has been sensed by NOD-1 in epithelial cells and that required the delivery of PGN through T4SS encoded by *cag*PAI. NOD-1 appears to be implicated in the production of β-defensins (BD) from *H. pylori* infected epithelial cells and dominant-negative NOD-1 constructs were able to block *H. pylori* induced expression of a human β-defensin-reporter transgene (Boughan PK *et al,* 2006).

As the literature review has exposed the deficiency of information regarding the role of PRRs during *H. pylori* infection and also the lack of host gene polymorphism data among the *H. pylori* infected patients of this area, the present study envisaged to conduct research on the following **objectives**.

1. Analysis of TLRs mRNA and protein expression in THP-1 monocytes during infection with *H. pylori* and isogenic mutants of Δ*cagA,* Δ*vacA,* Δ*flaA,* Δ*cgt,* and Δ*cag*PAI to ascertain the role of these virulence and pathogenecity factors on recognition through TLRs, if any.
2. TLR mRNA expression in THP-1 derived DCs during *H. pylori* infection to find out any difference between these two important cell types of the immune system.
3. Analysis of IRAK-1 phosphorylation in THP-1 cells during infection with *H. pylori* and isogenic mutants to find out any role for CagA, VacA, FlaA, Cholesterol glucoside and *cag*PAI in TLR signalling.

4. Analysis of the role of few individual TLR molecules during *H. pylori* infection using stably transfected HEK293 cells, which is known to have less or no TLR expression.
5. Analysis of pro-inflammatory cytokines such IL-1β, IL-6, IL-8, IL-18 and TNFα expression and secretion during *H. pylori* infection with THP-1 cells and HEK293 cells stably transfected with TLR molecules in comparison with HEK293 cells.
6. Analysis of NLRs and ASC mRNA expression and functional activation of inflammasome in THP-1 cells during infection with *H. pylori* and isogenic mutants.
7. Analysis of IL-1 receptor antagonist polymorphism and genotyping of *H. pylori* in colonized patients of the Calicut area.

2. Materials & Methods

2.1 Materials

2.1.1 Chemicals

Most of the chemicals used in this study were of analysis grade reagents purchased from M/S Genei (Bangaluru, India), Merck (Mumbai, India), SRL (Mumbai, India), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Sigma (Taufkirschen, Germany), if not stated otherwise.

2.1.1.1Antibiotics

Antibiotic and Antimycotic solutions for cell culture were purchased from Sigma (Taufkirschen, Germany) and Blasticidin S purchased from Invivogen (France)

2.1.1.2 Enzyme Inhibitors

Pan-caspase inhibitor Z-VAD-FMK, c-Src Kinase inhibitor PP2 and EGFR Kinase inhibitor AG1478 were purchased from Calbiochem, Merck (Darmstadt, Germany).

2.1.2 Clinical Specimen

42 gastric mucosal biopsy specimens collected from patients positive for Rapid Urease Test (RUT) during diagnostic upper intestinal endoscopy in the Department of Gastroenterology, Malabar Institute of Medical Sciences were used for the present investigation (Approved by the Institutional Ethics committee).

2.1.3 Custom Made PCR Primers

The following custom made oligonucleotide primers are synthesized by Genei, Bangalore, India

Human Gene		**Nucleotide Sequence**	**Reference**
IL-1RN	*Forward*	5' CTCAGCAACACTCCTAT 3'	Joos *et al.,* 2001
	Reverse	5' TCCTGGTCTGCAGGTAA 3'	
***H. pylori* Gene**		**Nucleotide Sequence**	**Reference**
GlmM	*Forward*	5' AAGCTTTTAGGGGTGTTAGGGGTTT 3'	Bickley J *et al,* 1993
	Reverse	5' AAGCTTACTTTCTAACACTAACGC 3'	
CagA	*Forward*	5' GATAACAGGCAAGCTTTTGAGG 3'	NCBI database
	Reverse	5' CCATGAACTTTTGATCCGTTCGG 3'	
VacA	*Forward*	5' ACAAACACACCGCAAAATCA 3'	NCBI database
	Reverse	5' CCTCTGCCTGCTTGAA 3'	
IceA1	*Forward*	5' GGATTGCAGCTAGGTGTTCC 3'	NCBI database
	Reverse	5' ACCCATCACCATAGCCTTTT 3'	
IceA2	*Forward*	5' TGCTGCTGTTACCACAAAGG 3'	NCBI database
	Reverse	5' CAAGTCTTAACCCCCAACGA 3'	

2.1.4 Real Time Taqman PCR Primers

The following custom made oligonucleotide primers are synthesized by MWG Biotech, Ebersberg, Germany.

Human Gene		Nucleotide Sequence	Reference
TLR1	*Forward*	5'CAGTGTCTGGTACACGCATGGT 3'	Zarember *et al,* 2002
	Reverse	5'TTTCAAAAACCGTGTCTGTTAAGAGA 3'	
TLR2	*Forward*	5'GGCCAGCAAATTACCTGTGTG 3'	Zarember *et al,* 2002
	Reverse	5'AGGCGGACATCCTGAACCT 3'	
TLR3	*Forward*	5'CCTGGTTTGTTAATTGGATTAACGA 3'	Zarember *et al,* 2002
	Reverse	5'TGAGGTGGAGTGTTGCAAAGG 3'	
TLR4	*Forward*	5'GCAGTGAGGATGATGCCAGGAT 3'	Zarember *et al,* 2002
	Reverse	5'GCCATGGCTGGGATCAGAGT 3'	
TLR5	*Forward*	5'TGCCTTGAAGCCTTCAGTTATG 3'	Zarember *et al,* 2002
	Reverse	5'CCAACCACCACCATGATGAG 3'	
TLR6	*Forward*	5'GAAGAAGAACAACCCTTTAGGATAGC 3'	Zarember *et al,* 2002
	Reverse	5'AGGCAAACAAAATGGAAGCTT 3'	
TLR7	*Forward*	5'TTTACCTGGATGGAAACCAGCTA 3'	Zarember *et al,* 2002
	Reverse	5'TCAAGGCTGAGAAGCTGTAAGCTA 3'	
TLR8	*Forward*	5'CTTCGATACCTAAACCTCTCTAGCA 3'	Nishimura *et al,* 2005
	Reverse	5'AAGATCCAGCACCTTCAGATGA 3'	
TLR9	*Forward*	5'GGACCTCTGGTACTGCTTCCA 3'	Zarember *et al,* 2002
	Reverse	5'AAGCTCGTTGTACACCCAGTCT 3'	
TLR10	*Forward*	5' TGTTATGACAGCAGAGGGTGATG 3'	Zarember *et al,* 2002
	Reverse	5'GAGTTGAAAAAGGAGGTTATAGGATAAATC 3'	
GAPDH	*Forward*	5'GAAGGTGAAGGTCGGAGTC 3'	Zarember *et al,* 2002
	Reverse	5'GAAGATGGTGATGGGATTTC 3'	

2.1.4.1 Real Time PCR Dual Labelled Probes

Human Gene		Nucleotide Sequence	Reference
TLR1	*Probe*	FAM- 5'TGCCCATCCAAAATTAGCCCGTTCC 3'-TAMRA	Zarember *et al,* 2002
TLR2	*Probe*	FAM-5'TCCATCCCATGTGCGTGGCC 3'TAMRA	Zarember *et al,* 2002
TLR3	*Probe*	FAM-5'ACCCATACCAACATCCCTGAGCTGTCAA 3'TAMRA	Zarember *et al,* 2002
TLR4	*Probe*	FAM-5'TGTCTGCCTCGCGCCTGGC 3'TAMRA	Zarember *et al,* 2002
TLR5	*Probe*	FAM-5'CCAGGGCAGGTGCTTATCTGACCTTAACA 3'TAMRA	Zarember *et al,* 2002
TLR6	*Probe*	FAM5'TGCAACATCATGACCAAAGACAAAGAACCT3'TAMRA	Zarember *et al,* 2002
TLR7	*Probe*	FAM-5'AGAGATACCGCAGGGCCTCCCG 3'TAMRA	Zarember *et al,* 2002
TLR8	*Probe*	FAM-5'CCCTCAGGAAGATTAATGCTGCCTGGTT 3'TAMRA	Nishimura*et al,* 2005
TLR9	*Probe*	FAM-5'ACGATGCCTTCGTGGTCTTCGACAAA 3'TAMRA	Zarember *et al,* 2002
TLR10	*Probe*	FAM-5' TGACCCCAGCCACAACGACACT 3'TAMRA	Zarember *et al,* 2002
GAPDH	*Probe*	JOE-5'CAAGCTTCCCGTTCTCAGCC 3'TAMRA	Zarember *et al,* 2002

2.1.5 Applied Biosystems (AB) Taqman Gene Expression Assays

The following pre-made Gene Expression Assay reagents were purchased from Applied Biosystems, Darmstadt, Germany

Human Gene	Assay ID	Reporter Dye	NCBI Reference
NOD1	Hs00196075_m1	FAM	NM_006092.1
NOD2	Hs00223394_m1	FAM	NM_022162.1
NALP1	Hs00248187_m1	FAM	NM_033004.3
NALP2	Hs00215284_m1	FAM	NM_017852.2
NALP3	Hs00366461_m1	FAM	NM_004895.3
IPAF	Hs00368367_m1	FAM	NM_021209.3
ASC	Hs00203118_m1	FAM	NM_145183.1
IL-1β	Hs00174097_m1	FAM	NM_000576.2
IL-6	Hs00985639_m1	FAM	NM_000600.2
IL-18	Hs00155517_m1	FAM	NM_001562.2
TNFα	Hs00174128_m1	FAM	NM_000594.2

2.1.6 Antibodies

The antibodies used in this study, purchased from different vendors are listed below.

2.1.6.1 Primary Antibodies

Antibodies	Type	Origin	Provider
α-TLR5	Monoclonal	Mouse	Imgenex, Germany
α-TLR10	Monoclonal	Mouse	Imgenex, Germany
α-phospho IRAK-1ser376	Polyclonal	Rabbit	Cell Signalling, USA
α-IRAK-M	Polyclonal	Rabbit	Cell Signalling, USA
α-Caspase-1	Polyclonal	Rabbit	Cell Signalling, USA
α-IL-1β	Polyclonal	Rabbit	Cell Signalling, USA
α-GAPDH	Polyclonal	Goat	Santa Cruz, Germany
α-HA	Monoclonal	Mouse	Cell Signalling, USA

2.1.6.2 Secondary Antibodies

Antibodies	Type	Source	Provider
Anti Mouse Immunoglobulin-HRP	Polyclonal	Rabbit	Dako, Germany
Anti Goat Immunoglobulin-HRP	Polyclonal	Rabbit	Dako, Germany
Anti Rabbit Immunoglobulin-HRP	Polyclonal	Pig	Dako, Germany

2.1.7 Molecular Biology Reagents and Kits

The following are the pre-made molecular biology reagents and kits purchased from different vendors

Technique	Product	Provider
RNA isolation	RNeasy Kit	Qiagen, Hilden, Germany
Real Time -PCR	Taqman Universal Master Mix (20X)	Applied Biosystems, Darmstadt, Germany
RT-PCR	Reverse Transcriptase	Invitrogen, Karlsruhe, Germany
	5X First strand buffer RNase inhibitor DTT	Genei, Bangalore, India
	dNTPs	Applied Biosystems, Darmstadt, Germany
Immunoprecipitation	Protein G-Sepharose	Amersham Pharmacia Freiburg, Germany
	Lysis Buffer	Roche Molecular Biochemicals Mannheim, Germany
ELISA	OptEIA™ Human IL-1β OptEIA™ Human IL-8 OptEIA™ Human TNFα	BD Bioscience, Heidelberg, Germany

2.1.8 Bacteria

2.1.8.1 *Helicobacter pylori* strains

Strain	Knock Down Gene	Resistance	Source/Type	Reference
26695			Clinical isolate	ATCC 700392
P12			Clinical isolate	Schmitt and Haas, 1994
P12 Δ*cagA*	HP0547	Cam^R	*cagA* knock down mutant	Prof. Dr. Steffen Backert, Magdeburg, Germany
P12 Δ*vacA*	HP0887	Kam^R	*vacA* knock down mutant	Prof. Dr. Steffen Backert, Magdeburg, Germany
P12 Δ*flaA*	HP0601	Cam^R	*flaA* knock down mutant	Prof. Dr. Steffen Backert, Magdeburg, Germany
P12 Δ*cgt*	HP0421	Kam^R	Cholesterol glucosyl transferase (*cgt*) knock down mutant	Prof. Dr. Steffen Backert, Magdeburg, Germany
P12 Δ*cag*PAI	HP0520-48	Kam^R	*cag*PAI knock down mutant	Prof. Dr. Steffen Backert, Magdeburg, Germany

2.1.8.2 Bacterial Culture Media and Plates

***H. pylori* culture media and plates**

BHI Culture Medium – BHI medium supplemented with 10%FCS, 1% Vitaminmix and 0.1% Vancomycin (10mg/l)

GC-Agar Base Plates – Prepared by dissolving 36g/l GC-agar base and 15g/l Peptose Peptone (Glaxo India Ltd, Mumbai; Difco/ Becton Dickenson, Maryland, USA) in double distilled water and sterilized by autoclaving. After cooling, the media were supplemented with 10ml/l Vitaminmix, 100ml/l horse serum, 10mg/l vancomycin and 4mg/l chloramphenicol or 8mg/l kanamycin before pouring to sterile petri dishes.

Vitaminmix Preparation – Prepared by mixing Solution-I and Solution-II and making up to 1000ml using double distilled water.

Solution-I (500 ml in ddH_2O)

100 g/l Dextrose; 10 g/l L-Glutamin; 26 g/l L-Cystein; 0,1 g/l Cocarboxylase; 0,02 g/l $Fe(NO_3)_3$; 0,003 g/l Thiamin-HCl; 0,013 g/l p-Aminobenzoic acid; 0,250 g/l NAD^+; 0,010 g/l Vitamin B_{12}

Solution-II (300 ml in dd. H2O + 15 ml HCl)

1,1 g/l L-Cystein; 1 g/l Adenine; 0,03 g/l Guanine-HCl; 0,150 g/l L-Arginine; 0,5 g/l Uracil

2.1.9 Eukaryotic cell lines

Cell line	Origin/Type	Reference
THP1	Human Monocytic leukaemia cells	ATCC TIB 202
HEK293	Human Embryonic Kidney cells	ATCC CRL-1573
HEK293-TLR2	HEK293 cells stably transfected with pUNO-hTLR2, Blasticidin resistant	Invivogen, France
HEK293-TLR5	HEK293 cells stably transfected with pUNO-hTLR5, Blasticidin resistant	Invivogen, France
HEK293-TLR10-HA	HEK293 cells stably transfected with pUNO-hTLR10-HA, Blasticidin resistant	Invivogen, France

2.1.9.1 Eukaryotic Cell Culture Media

Cell line	Medium
THP1	Roswell Park Memorial Institute Media (RPMI) 1640 containing 2 mM L-glutamine, 25 mM HEPES, 10% FBS (Invitrogen, Germany) supplemented with 1% Antibiotic and Antimycotic solution (Sigma, Germany)
HEK293	Dulbecco's Modified Eagle Medium (D-MEM) containing 4.5g/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10% FBS (Invitrogen, Germany) supplemented with 1% Antibiotic and Antimycotic solution (Sigma, Germany)
HEK293-TLR2	Dulbecco's Modified Eagle Medium (D-MEM) containing 4.5g/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10% FBS (Invitrogen, Germany) supplemented with 1% Antibiotic and Antimycotic solution (Sigma, Germany) and 10 µg/ml blasticidin (Invivogen, France)
HEK293-TLR2	Dulbecco's Modified Eagle Medium (D-MEM) containing 4.5g/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10% FBS (Invitrogen, Germany) supplemented with 1% Antibiotic and Antimycotic solution (Sigma, Germany) and 10 µg/ml blasticidin (Invivogen, France)
HEK293-TLR10-HA	Dulbecco's Modified Eagle Medium (D-MEM) containing 4.5g/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10% FBS (Invitrogen, Germany) supplemented with 1% Antibiotic and Antimycotic solution (Sigma, Germany) and 10 µg/ml blasticidin (Invivogen, France)

2.2 Methods

2.2.1 Collection and transport of Clinical Specimen

The gastric mucosal biopsy samples were collected from patients who have under gone upper gastrointestinal endoscopy in the Department of Gastroenterology, Malabar Institute of Medical Sciences, Kozhikode, Kerala, India (Approved by the institutional Ethics Committee). These samples were trasferred to sterile eppendorf tubes and transported to the Department of Biotechnology, University of Calicut in a refrigerated flask within 2h of collection.

2.2.2 Biochemical and Molecular Biological Methods

2.2.2.1 Rapid Urease Test (RUT)

Rapid urease solution was prepared using K_2HPO_4 (11 mM), NaCl (85 mM), urea (0.33 M) and phenol red (100 µM) (Merck, India) in water and pH was adjusted to 6.8. It was then filter (0.2 µm) sterilized and stored at 4ºC. Small piece of gastric mucosal biopsy specimen from patients were incubated in 0.5 ml of the medium in a test tube and positive reaction reported by change in colour from golden yellow to pink within 6 hr and such specimens only used for the present study.

2.2.2.2 DNA Isolation from Clinical Biopsy Samples

DNA was isolated from gastric mucosal biopsies by boiling in 100 µl of sterile double distilled water for 10 minutes and cooling it on ice for 5 min. It was then centrifuged at 10,000 rpm for 10 minutes. The supernatant containing DNA was used for PCR study.

2.2.2.3 PCR for *H. pylori* genotyping

PCR was performed using 5 µl of DNA solution at a final volume of 25 µl containing 60mM Tris-HCl, 1.5 mM $MgCl_2$, 25mM KCl, 250µM dNTPs, 20pM each of forward and reverse primers and 1U of *Taq* DNA polymerase (Biogene, USA) in a MJ Ressearch PTC 150 thermocycler. All negative reactions were ruled out by checking again in a larger scale (50µl) reaction. The DNA isolated from *H pylori* ATCC 700392 was used as positive control. Custom made oligonucleotide primers for *glmM* gene (Bickley *et al.,* 1993), *cagA, vacA-S* region, and *iceA1 and A2* of *H pylori* (Genei, Bangalore, India) were used for this study. Amplified products were separated by agarose gel (1.5%) electrophoresis and visualized using ethidium bromide staining in a gel documentation system (Alpha Imager 2200, USA)

2.2.2.4 PCR for IL1-Receptor Antagonist polymorphism

PCR reaction was performed as mentioned in the above case. The following primers flanking polymorphic variable nucleotide tandem repeats (VNTR) region were used for PCR amplification: 5' CTCAGCAACACTCCTAT 3' (forward) and 5' TCCTGGTCTGCAGGTAA 3'(reverse). Polymerase chain reaction conditions were as follows: 40 cycles at 94℃ for 60 seconds, 60℃ for 60 seconds, and 72℃ for 45 seconds (Joos *et al.,* 2001).

2.2.2.5 RNA Isolation and DNase Digestion

The eukaryotic cell lines such as THP1 cells and HEK293 cells infected with *H. pylori* were used for the isolation of mRNA. The THP1 cells infected with *H. pylori* for the required time periods were separated from bacteria by centrifuging at 150g for 5 minutes at 4°C. The supernatant having most of the bacteria were removed carefully and the pellet washed with PBS, pH 7.4 before adding lysis buffer of RNeasy Mini Kit (Qiagen).

In case of HEK293 cells infected with *H. pylori*, the supernatant was carefully removed and the monolayer washed with PBS, pH 7.4 before adding 300 µl of lysis buffer directly to the wells. The cells were homogenized by pipetting for several times and then added equal amount of 70% ethanol and mixed together by repeated pipetting. Then the mixture was loaded on Qiagen RNeasy Mini Kit Spin columns having a specialized silica membrane for RNA binding and subsequent isolation. The RNAs having more than 200 nucleotides only were bound to the membrane, all other small RNAs, genomic DNA and proteins were removed by centrifuging at 8000 rpm for 1 min at RT. The filtrates collected in the attached collection tube of the spin column were removed and the spin columns with bound RNAs were placed on a new collection tube. Then added 350 µl of RW1 solution of the kit to the spin columns with RNA and centrifuged at 8000 rpm for 1min at RT. The columns washed with RW1 solution were placed on a new collection tube and 80 µl of DNase solution (see below) carefully loaded to the membrane without touching the brim of the column to spread evenly on the membrane. Then the columns were incubated for 20 minutes at RT to digest the genomic DNA, if any. After DNase digestion, the spin columns with bound RNA were washed again with 350 µl of RW1 solution. In the next step, the spin columns with bound RNAs were washed with 500 µl of RP1 solution of the kit for two times by centrifuging at 8000 rpm for 1 min at RT and followed by a free run of the column at same rpm for 1 min to remove traces of residual washing solutions, if any. After that, 50 µl of RNase free water was loaded on to the membrane of each RNA bound columns and centrifuged at 8000 rpm for 1 min at RT to collect the RNA in an RNase free collection tube supplied with the kit. The RNA solutions were stored at $^{-}20^{\circ}C$ till further use.

2.2.2.6 Quantitative and qualitative Assessment of RNA using Nanodrop™ Method

All the RNA preparations using RNeasy kit were analyzed spectrophotometrically using Nanodrop™ 1000 spectrophotometer. The absorbance values of nucleotides at

230 nm, 260 nm, and 280 nm were measured by this system by loading 1 µl of the RNA solution.

A260/280 Ratio

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as“pure” for DNA; a ratio of ~2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

A260/230 Ratio

This ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for “pure” nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm. EDTA, carbohydrates and phenol all have absorbance near 230 nm.

RNA preparations which have A260/280 and A260/230 values above 2.0 were used for cDNA preparation and subsequent Real Time PCR.

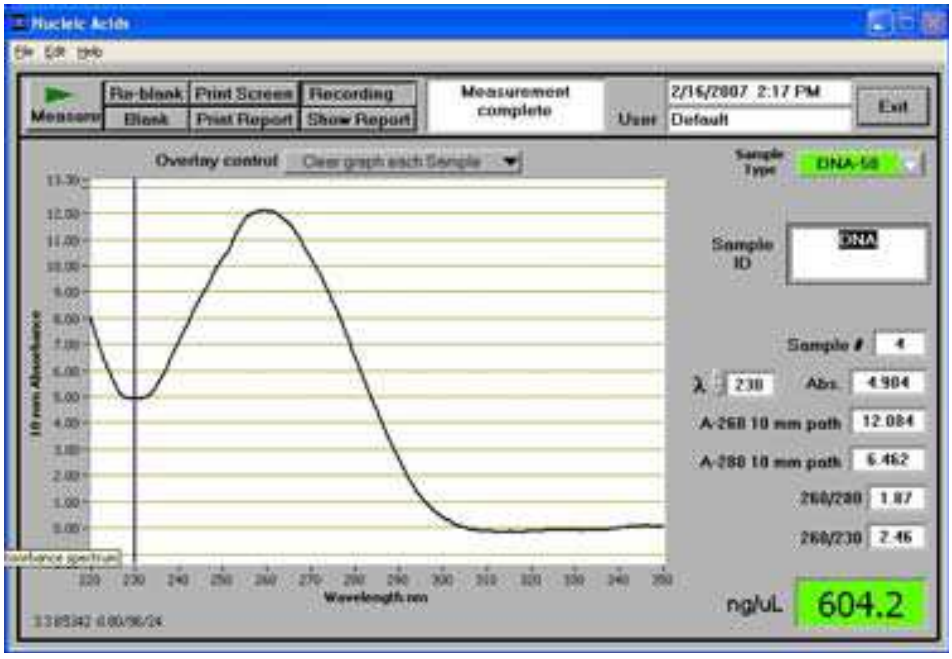


Fig.1 Typical spectral pattern for Nucleic Acids in Nanodrop^{TM}1000

2.2.2.7 Agarose Gel Electrophoresis for Checking Integrity of RNA

Native agarose gel electrophoresis found to be sufficient to analyze the integrity and overall quality of a total RNA preparation by inspection of the 28S and 18S rRNA bands. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band. This 2:1 ratio (28S:18S) is a good indication that the RNA is intact. 1% agarose was prepared in 0.5M TBE buffer containing 0.1 µg/ml ethidium bromide by heating in a microwave oven. The gel solution was then poured on to the gel making tray (BioRad, Munich, Germany) and kept for 20 min at RT to form the gel. Then the samples were mixed with 6X loading dye (Fermentas, Germany) and loaded on to the gel with known molecular weight markers. Then the gel submerged in 0.5M TBE buffer were run at 5-6 V/cm until the bromophenol blue dye has migrated as far as 2/3 the length of the gel. After the run, RNA integrity on gel with ethidium bromide was visualized in a digital gel documentation system DNA Molecular Weight Marker XIV™ (Roche Diagnostics GmbH, Mannheim).

2.2.2.8 Reverse Transcriptase PCR

The purified mRNAs from *H. pylori* infected cell lines were used to prepare cDNA by RT-PCR. The RT-PCR in principle synthesizing the first strand complimentary DNA from mRNA with help of Oligo dT primers, which binds to Poly A tail of all eukaryotic mRNAs, and Moloney Murine Leukaemia Virus (M-MLV) Reverse Transcriptase. The reaction mixture should contain deoxynucleotide triphosphate (dNTPs), dithiothreitol (DTT), RNase Inhibitor in 1X First strand buffer. The following table gives the details of the reaction mixture. The reaction mixture was incubated at 37°C for 1h to prepare cDNA

Materials	Volume (µl)	Final Concentration
RNAse free water	28.4	
5X First strand buffer	10.0	1x
dNTPs (25mM)	4.0	2.0mM
Oligo dT Primer (0,5µg)	1.0	25.0µM
DTT (0.1M)	5.0	25.0mM
Rnase Inhibitor (170 U/µl)	0,6	2.6 U
Reverse Transcriptase (200 U/µl)	1.0	4.0 U
RNA solution	10.0	1-5 µg
Total	50.0	

The cDNA prepared from infected cell transcriptome were stored at ˉ20°C till further use.

2.2.2.9 Taqman Real Time PCR

Reverse Transcriptase PCR is the most sensitive method widely used for the detection and quantitation of mRNA expression. RT-PCR has the advantage that it can be used to quantify mRNA from much smaller samples or even from a single cell when compared to other quantifying methods such as Northern blot analysis and RNase protection assay. The development of reagents and instrumentation to detect the real time detection of PCR product formation has made Real Time PCR as the best method of choice for gene expression studies. The Taqman Real Time PCR uses the fluorescent oligonucleotide probes labeled with one reporter dye at 5' end of the probe and quencher dye at the 3' end along with the usual sense and anti-sense primers used in PCR. These probes are designed to hybridize to an internal region of a PCR product. The probe when attached to the template or unattached, the quencher dye reduces the fluorescence from reporter dye by the mechanism of Fluorescence Resonance Energy Transfer (FRET), which is the inhibition of one dye caused by another without emission of a proton. When the Taqman PCR proceed, the probe annealed to the template will be removed by the exonuclease activity of Taq polymerase and this separates the reporter dye from the quencher and that increases the emission of fluorescence. DNase treated mRNA samples isolated from *H. pylori* infected cells were used for first strand cDNA preparation using reverse transcriptase. Taqman probes and primers prepared using Primer express software (Applied Biosystems, CA) were used for the analysis (12).

All primers were used at concentration of 100 pM for forward and reverse primers and 10 pM for probes in a 25 µl reaction volume containing Taqman Universal Master Mix (Applied Biosystems, Germany). In case of Applied Biosystems Gene Expression Assays, 1µl of primer mix (20X) was mixed with cDNA solution and Taqman Master Mix in a final volume of 20 µl. The reactions were performed in Applied Biosystems sequence detection system 5700 and data analyzed using SDS software. PCR reaction parameters

were as following: 52°C for 10min for activation, 95°C for 2min as one cycle and 95°C for 15s and 58 or 60°C for 1min in 45 cycles. The fold changes in gene expression were analyzed using $2^{-\Delta\Delta Ct}$ method using house keeping gene GAPDH as internal control.

$$\Delta\Delta Ct = \Delta Ct \text{ sample} - \Delta Ct \text{ control}$$

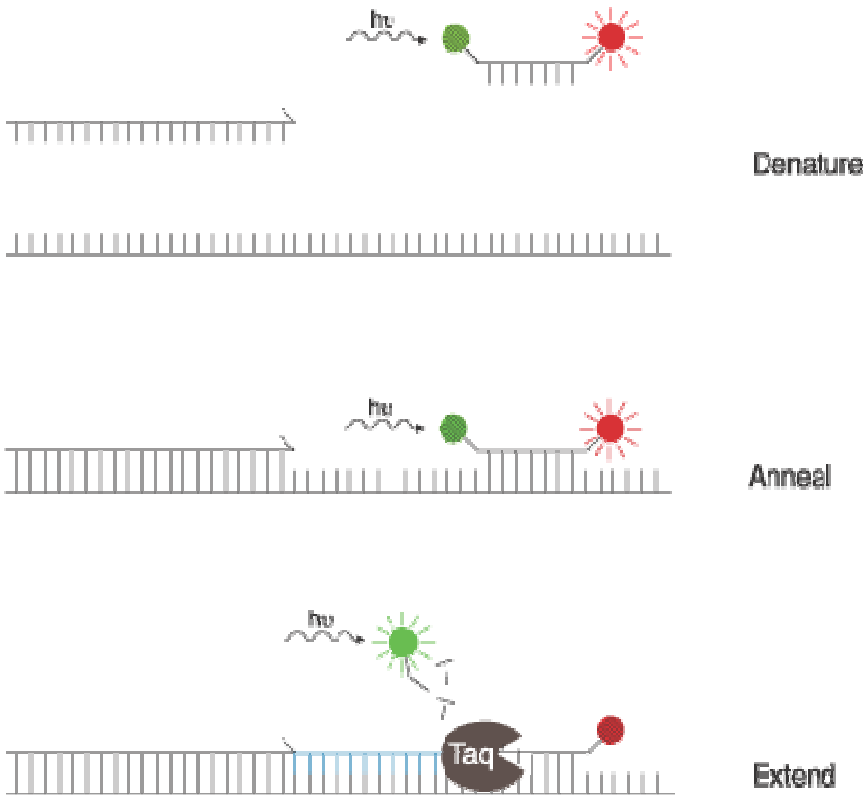


Fig.2 Diagramatic representation of Taqman Real Time PCR

Here, ΔCt sample is the C_t value for any infected cell samples normalized to the endogenous GAPDH and ΔCt control is the C_t value for the uninfected control cells also normalized to the endogenous GAPDH.

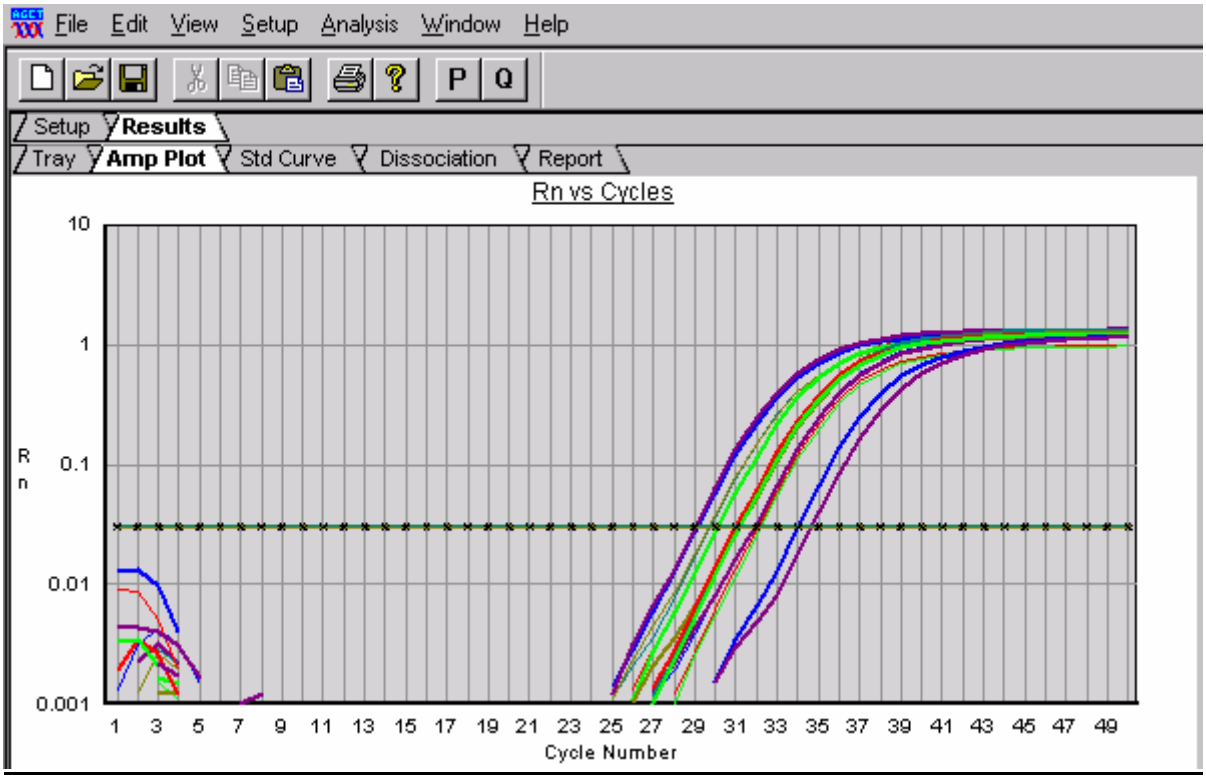


Fig.3 Real time analysis of Taqman PCR using SDS software and *Ct* value calculation

2.2.2.10 Immunoprecipitation

Immunoprecipitation is the technique used to isolate protein antigens using specific antibodies and a solid support. In this technique, the antibodies against a particular protein of interest are treated with crude cell lysate to precipitate as immune complexes. The immune complexes can be recovered to a solid support by using antibody bound (direct) or protein G bound (indirect) agarose beads. This method is also used to precipitate protein complexes, in which one protein antigen binds to its antibody, can be able to pull down the entire molecular complex from crude cell lysates known as co-immunoprecipitation. Chromatin Immunoprecipitation (ChIP) uses to precipitate the DNA binding proteins such as transcription factors to understand the protein-DNA interaction occur inside the nucleus of a cell. RNA Immunoprecipitation (RIP) is a technique using to study the RNA binding proteins,

The whole cell extract was prepared using lysis buffer containing pan protease inhibitors. Then the whole cell extracts were pre-cleared with Protein-G sepharose beads

(Amersham Pharmacia, Germany) for 2 h at 4°C. After pre-clearing the beads were removed by centrifugation at 5000 rpm for 5min at 4°C. The monoclonal antibodies against human TLR-5 or TLR-10 (Imgenex, USA) were added to pre-cleared lysate in each case and incubated overnight at 4°C in a rotatory shaker at low speed. The immune complexes were precipitated using Protein-G sepharose beads by incubating for 4h at 4°C and subsequent centrifugation at 5000 rpm for 5min. The Protein-G sepharose beads bound immune complexes were washed three times with PBS, pH 7.4 and then added SDS lysis buffer (2%SDS, 10% Glycerol, 0.01% Bromophenol blue, 62.6 mM Tris-HCl, 0.1M DTT) and boiled for 5 min before using for SDS-PAGE and Western blot analysis.

Lysis Buffer

Tris	20mM pH 7.2
NaCl	150mM
EDTA	5mM
Triton X-100	1%
Glycerol	10%
Na_3VO_4	1mM
Protease inhibitor cocktail (Roche)	

2.2.2.11 SDS- Polyacrylamide Gel Electrophoresis and Western Blot Analysis

SDS-PAGE is using for the separation of proteins based on their size (Laemmli UK, 1970). The solution of proteins to be analyzed is first mixed with SDS, an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. When loaded onto a gel matrix and placed in an electric field, the negatively charged protein molecules migrate towards the positively charged electrode and are separated by a molecular sieving effect. SDS binds in a ratio of approximately 1.4 g SDS per 1.0 g protein (although binding ratios can vary from 1.1-2.2 g SDS/g protein), giving an approximately uniform mass:charge ratio for most proteins, so that the distance of migration through the gel can be assumed to be directly related to only the size of the protein. Besides the

addition of SDS, proteins may optionally be briefly heated to near boiling in the presence of a reducing agent, such as DTT or 2-mercaptoethanol, which further denatures the proteins by reducing disulfide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (oligomeric subunits). The denatured proteins are subsequently applied to one end of a layer of polyacrylamide gel submerged in a suitable buffer. An electric current is applied across the gel, causing the negatively-charged proteins to migrate across the gel towards the anode. Following electrophoresis, proteins in a polyacrylamide gel can be visualized by staining (*e.g.* Coomassie staining) or blotted onto a positively charged membrane and probed with protein-specific antibodies. With the semi-dry electro-blotting method, the gel and membrane are sandwiched between two stacks of filter paper that have been pre-wet with transfer buffer. The membrane is placed near the anode, and the gel is placed near the cathode. Proteins are transferred to the membrane when an electric current is applied. The specificity of the antibody-antigen interaction enables a single protein to be identified in the midst of a complex protein mixture that has been immobilized on a membrane. The membrane is blocked to prevent any non-specific binding of antibodies to its surface and then primary antibody is added. In order to locate it, a secondary antibody is applied which binds to all IgG antibodies from animal species in which primary antibody was generated. The secondary antibody is chemically coupled to a reporter, *e.g.* to an enzyme that after addition of appropriate substrate produces luminescent reaction products which allows its detection.

The protein samples prepared from THP1 infected cells were lysed in SDS buffer (2%SDS, 10% Glycerol, 0.01% Bromophenol blue, 62.6 mM Tris-HCl, 0.1M DTT) (Fermentas, St. Leon Rot, Germany) and boiled at 95°C for 5 min. Then the protein samples were separated on 6-15% SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) depending on the size of the protein of interest. Prestained Page Ruler Plus (Fermentas, St. Leon Rot, Germany) was used as standard protein markers during the

run. After the run, the gel separated proteins were blotted to poly vinylidene difluoride (PVDF) membrane and blocked with 5% milk powder in PBS with Tween (PBST). Then, the membrane was probed with primary antibody against protein of interest diluted in 5% BSA or Milk Powder in TBST. After washing the membrane with PBST for three times, probed secondary antibody conjugated with horse radish peroxidase at a dilution of 1:1000. Then the membrane washed three times with PBST and the protein bands were visualized using ECL Plus detection reagents (Amersham Biosciences, Germany) and Lumi-Image F1 (Roche, Germany) imaging system. Preparation, running, blotting and staining of the gels was performed according to Sambrook *et al.,* 1989 with use of Mini-Protean 3 gel system, (120 V, 1.5-2 hrs, RT) (Bio-Rad Laboratories, Hercules, USA), semi-dry blotting apparatus (0.8 mA/cm^2, 2 hrs, RT) (Roth, Karlsruhe, Germany) and buffers listed below.

Gel running buffer pH 8.3

Tris	3.20 g/l
Glycine	18.80 g/l
10% (v/v) SDS	10.00ml/l

Transfer buffer pH 8.4

Tris	3.00 g/l
Glycine	14.50 g/l
10% (v/v) SDS	10 .00ml/l
Methanol	200.00 ml/l

10xTBS buffer pH 7.4

Tris	24.20 g/l
NaCl	80.00 g/l

TBS-T buffer pH 7.4

10xTBS pH 7.4	100.00ml/l
Tween 20	1.00ml/l

To re-probe the membrane with another primary antibody, membrane was incubated in stripping buffer for 45 min at 60℃, washed exte nsively in TBS-T, blocked and probed again as described above.

Stripping buffer pH 6.7

1M Tris	62.50 ml/l
10% (v/v) SDS	200.00ml/l
ß-merkaptoethanol	8.33 ml/l

2.2.2.12 ELISA (Enzyme Linked Immunosorbent Assay)

ELISA is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. The sample with an unknown amount of antigen is immobilized on a solid support (usually 96 well plate) either non-specifically or specifically. After the antigen is immobilized the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody which is linked to an enzyme through bioconjugation. Between each step the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample. In sandwich ELISA, proteins are immobilized on a solid support and used as capture molecules to bind the protein that is being assayed. After a wash step to remove non-specifically bound material, a secondary antibody, specific for the target protein, is added. This secondary antibody is usually conjugated to an enzyme that allows its detection by chromogenic or chemiluminescent methods. In parallel, series of dilutions of known concentration of a standard are assayed alongside and the concentration of protein is determined based on standard curve obtained from this measurement.

IL-1β, IL-8 and TNFα secreted into the cell culture supernatants during *H. pylori* infection with cells were determined by ELISA using the OptEIA™ human IL-8 and TNFα kits (BD Biosciences, Heidelberg, Germany) and according to the manufacturers' instructions. For this purpose, 96-well plates were coated with 100 µl/well of a human IL-8 monoclonal antibody diluted (1:250) in Coating Buffer (0.1 $NaCO_3$ pH 9.5) and incubated overnight at 4℃. Wells were aspirated and washed three times with 300 µl/well of Wash Buffer (PBS-0.05% Tween 20). Plates were blocked with 200 µl/well of Assay Diluent (PBS-10% FBS) and incubated for 1 h at RT. Wells were aspirated and

washed three times with 300 µl/well of Wash Buffer. Standard and sample dilutions (1:20) in Assay Diluent were prepared. 100 µl of standard or sample was pipetted into appropriate wells and incubated for 2 hrs at RT. Wells were aspirated and washed three times with 300 µl/well of Wash Buffer. 100 µl of Working Detector (biotinylated anti-human IL-1β, IL-8 or TNFα monoclonal antibody, streptavidin-horseradish peroxidase conjugate reagent) was added to each well and incubated for 1 h at RT. Wells were aspirated and washed three times with 300 µl/well of Wash Buffer. 100 µl of Substrate Solution (tetramethylbenzidine (TMB) and hydrogen peroxide) was added to each well and incubate for 30 min at RT in the dark. The reaction was stopped by adding of 50 µl of Stop Solution (2 N H_2SO_4) to each well. The absorbance was measured at λ=450 nm with λ correction at 570 nm in a microplate reader (Spectrafluor Plus, TECAN, Crailsheim, Germany). Values were calculated from a standard curve. IL-1β, IL-8 and TNFα secreted into the cell culture supernatants during infection with *H. pylori* were quantified according to procedure described above.

2.2.3 Bacterial Culture Methods

2.2.3.1 *Helicobacter pylori*

H. pylori were cultivated on GC-Agar plates supplemented with vitamix and antibiotics under microaerophilic (5% O_2, 10% CO_2 and 85% N_2) conditions prepared using Campygen (Oxoid, Hampshire, England) in an anaerobic jar at 37°C for 48h. The colonies grown on plates were resuspended in 1 ml of BHI (Oxoid, Hampshire, England) medium using a sterile wet cotton swab and spreaded on a new GC-agar-plate. Then, the used swab immersed in another 1 ml of BHI medium and spreaded on another plate and this step was repeated once more using a third GC-agar plate. All the plates were grown under the conditions mentioned earlier. The colonies grown on third plate was used for the infection assay. The knocks out mutants of H. pylori were grown on selective GC-agar plates supplemented with specific antibiotics such as Chloramphenicol or Kanamycin.

2.2.4 Eukaryotic Cell Culture Methods

2.2.4.1 THP1 Monocytic Leukaemia Cell line Culture

THP1 is a human monocytic leukaemia cell line. THP1 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) supplemented with 1% antibiotic and antimycotic solution (Sigma, Taufkirschen, Germany) in an atmosphere of 5% CO2 at 37°C in a humidified incubator. The cells were sub-cultured using 1/4 of the 3 days grown culture with 3/4 of the fresh medium. This method is used for maintaining continuous passage of cells, normally. Otherwise, cells cultured for the log phase of the growth were used for cryopreservation for long time storage. The cells were isolated by centrifugation at 150g for 10 min at RT and the pellet resuspended in cryopreservation medium (RPMI-1640 medium-60%, FBS-20% and DMSO-10%) and made up to 1.8 ml at a cell concentration of $2x10^6$ cells in a cryovial (Greiner-Bio-One, Frickenhausen, Germany). Then the cryovials were stored at $^{-}$70°C for 24h before transferring to liquid nitrogen for long term storage.

The cells kept in liquid nitrogen were revived, when needed, by thawing the vial immediately at 37°C water bath and the vial contents were transferred to 12 ml of fresh RPMI medium taken in a 15 ml tube to wash the cells by centrifugation at 150g for 5 min. The pellet was resuspended in 2 ml cRPMI medium and then transferred to 8 ml warm media taken in a 25 cm^2 culture flask (Greiner-Bio-One, Frickenhausen, Germany) and incubated in an atmosphere of 5% CO2 at 37°C in a humidified chamber for 3 days before subculturing.

2.2.4.2 THP1 Derived Dendritic Cell Culture

THP1 cells were reported to be used as a source for preparing monotypic dendritic cells (Berges C *et al,* 2005). In this study, we have differentiated THP1 monocytes in to DCs by culturing in complete RPMI 1640 medium containing 10% FBS and supplemented with 100 µg /ml of rhIL-4 and rhGM-CSF (Peprotech, Rocky Hill, NJ,

USA) and 1% Antibiotic and Antimycotic solution for 5 days. After every 2 days, cultures were replenished with cytokine added medium. THP1 derived DCs were analyzed microscopically for their characteristic morphology before using for the infection assay.

2.2.4.3 HEK293 Cell Line Culture

HEK293 cells purchased from invivogen were maintained in DMEM containing 4.5g/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10% FBS (Invitrogen, Germany) supplemented with 1% Antibiotic and Antimycotic solution (Sigma, Germany) in an atmosphere of 5% CO2 at 37°C in a humidified incubator. The cells were grown 75% confluent within 2 days of culture in DMEM and then detached from the adherent surface by gentle tapping. The subculturing was done by adding 1/3 of the detached cells in DMEM to 2/3 of the fresh media and the mixture incubated in an atmosphere of 5% CO2 at 37°C in a humidified chamber for normal passage. Otherwise, the detached cells were suspended properly in culture medium and then centrifuged to remove the supernatant. The cell pellet then dissolved in cryopreseravtive medium (DMEM-60%, FBS-20% and DMSO-10%) and made up to 1.8 ml at a cell concentration of $2x10^6$ cells in a cryovial (Greiner-Bio-One, Frickenhausen, Germany). Then the cryovials were stored at ¯70°C for 24h before transferring to liquid nitrogen for long term storage.

The frozen cells were thawed at 37°C immediately and the content transferred to 12 ml of fresh DMEM media in a 15 ml and washed by centrifugation at 150g for 5 min. The cell pellet was resuspended in 2 ml complete DMEM and then transferred to 3 ml warm fresh media taken in a $25cm^2$ culture flask (Greiner-Bio-One, Frickenhausen, Germany) and incubated in an atmosphere of 5% CO2 at 37°C in a humidified chamber for 2 days before subculturing.

2.2.4.4 HEK293-THLR2 Cell Line Culture

HEK293 cells stably transfected with pUNO-hTLR2, Blasticidin resistant, purchased from invivogen were maintained in DMEM containing 4.5g/L D-glucose, 4 mM

L-glutamine, 110 mg/L sodium pyruvate, 10% FBS (Invitrogen, Germany) supplemented with 1% Antibiotic and Antimycotic solution (Sigma, Germany) and 10 µg/ml Blasticidin (Invivogen) in an atmosphere of 5% CO2 at 37°C in a humidified incubator. The cells were grown 75% confluent within 2 days of culture in DMEM and then detached from the adherent surface by gentle tapping. The subculturing was done by adding 1/3 of the detached cells in DMEM to 2/3 of the fresh media and the mixture incubated in an atmosphere of 5% CO2 at 37°C in a humidified chamber for normal passage. Otherwise, the detached cells were suspended properly in culture medium and then centrifuged to remove the supernatant. The cell pellet then dissolved in cryopreseravtive medium (DMEM-60%, FBS-20% and DMSO-10%) and made up to 1.8 ml at a cell concentration of $2x10^6$ cells in a cryovial (Greiner-Bio-One, Frickenhausen, Germany). Then the cryovials were stored at $^{-}$70°C for 24h before transferring to liquid nitrogen for long term storage.

The frozen cells were thawed at 37°C immediately and the content transferred to 12 ml of fresh DMEM media in a 15 ml and washed by centrifugation at 150g for 5 min. The cell pellet was resuspended in 2 ml complete DMEM and then transferred to 3 ml warm fresh media taken in a $25cm^2$ culture flask (Greiner-Bio-One, Frickenhausen, Germany) and incubated in an atmosphere of 5% CO2 at 37°C in a humidified chamber for 2 days before subculturing.

2.2.4.5 HEK293-THLR5 Cell Line Culture

HEK293 cells stably transfected with pUNO-hTLR5, Blasticidin resistant, purchased from invivogen were maintained in DMEM containing 4.5g/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10% FBS (Invitrogen, Germany) supplemented with 1% Antibiotic and Antimycotic solution (Sigma, Germany) and 10 µg/ml Blasticidin (Invivogen) in an atmosphere of 5% CO2 at 37°C in a humidified incubator. The cells were grown 75% confluent within 2 days of culture in DMEM and then detached from the

adherent surface by gentle tapping. The subculturing was done by adding 1/3 of the detached cells in DMEM to 2/3 of the fresh media and the mixture incubated in an atmosphere of 5% CO2 at 37°C in a humidified chamber for normal passage. Otherwise, the detached cells were suspended properly in culture medium and then centrifuged to remove the supernatant. The cell pellet then dissolved in cryopreseravtive medium (DMEM-60%, FBS-20% and DMSO-10%) and made up to 1.8 ml at a cell concentration of $2x10^6$ cells in a cryovial (Greiner-Bio-One, Frickenhausen, Germany). Then the cryovials were stored at $^{-}$70°C for 24h before transferring to liquid nitrogen for long term storage.

The frozen cells were thawed at 37°C immediately and the content transferred to 12 ml of fresh DMEM media in a 15 ml and washed by centrifugation at 150g for 5 min. The cell pellet was resuspended in 2 ml complete DMEM and then transferred to 3 ml warm fresh media taken in a $25cm^2$ culture flask (Greiner-Bio-One, Frickenhausen, Germany) and incubated in an atmosphere of 5% CO2 at 37°C in a humidified chamber for 2 days before subculturing.

2.2.4.6 HEK293-THLR10-HA Cell Line Culture

HEK293 cells stably transfected with pUNO-hTLR10-HA, Blasticidin resistant, purchased from invivogen were maintained in DMEM containing 4.5g/L D-glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10% FBS (Invitrogen, Germany) supplemented with 1% Antibiotic and Antimycotic solution (Sigma, Germany) and 10 µg/ml Blasticidin (Invivogen) in an atmosphere of 5% CO2 at 37°C in a humidified incubator. The cells were grown 75% confluent within 2 days of culture in DMEM and then detached from the adherent surface by gentle tapping. The subculturing was done by adding 1/3 of the detached cells in DMEM to 2/3 of the fresh media and the mixture incubated in an atmosphere of 5% CO2 at 37°C in a humidified chamber for normal passage. Otherwise, the detached cells were suspended properly in culture medium and then centrifuged to

remove the supernatant. The cell pellet then dissolved in cryopreseravtive medium (DMEM-60%, FBS-20% and DMSO-10%) and made up to 1.8 ml at a cell concentration of $2x10^6$ cells in a cryovial (Greiner-Bio-One, Frickenhausen, Germany). Then the cryovials were stored at $^{-}$70°C for 24h before transferring to liquid nitrogen for long term storage.

The frozen cells were thawed at 37°C immediately and the content transferred to 12 ml of fresh DMEM media in a 15 ml and washed by centrifugation at 150g for 5 min. The cell pellet was resuspended in 2 ml complete DMEM and then transferred to 3 ml warm fresh media taken in a $25cm^2$ culture flask (Greiner-Bio-One, Frickenhausen, Germany) and incubated in an atmosphere of 5% CO2 at 37°C in a humidified chamber for 2 days before subculturing.

2.2.5 Infection Assay

2.2.5.1 *Helicobacter pylori* infection with THP1 Cells

THP1 cells maintained in complete RPMI supplemented with Antibiotic and Antimycotic cells were washed with fresh RPMI media to remove the residual before using for the infection assay. The washed cells were suspended in cRPMI without antibiotics and antimycotics and aliquoted to 12 well or 6 well plates (Greiner-Bio-One, Frickenhausen, Germany) at a concentration of $5x10^5$ cells/ml and $1x10^6$ cells/ml, respectively, and incubated for atleast 5h before infection.

The *H. pylori* grown on the third plate (see section 2.2.2.1) were resuspended in Phosphate Buffered Saline (PBS), pH 7.4 using a sterile wet cotton swab ((Raucotupf, Lohmann & Rascher, Rengsdorf, Germany) and the bacterial concentration was measured using Absorbance at 550nm using a spectrophotometer with reference to the known growth curve measurement. This was also cross checked with colonies grown on GC-Agar plates after serial dilution of the bacterial suspension.

The THP1 cells grown in cRPMI without antibiotics and antimycotics were infected with *H. pylori* at a multiplicity of infection (MOI) =1:50. The uninfected cells were added with equal amount of PBS as control. The infected cells and uninfected control cells after required time of incubation were harvested from tissue culture plates and centrifuged at 150g for 5 min at 4°C, this process keeps most of the bacteria in the supernatant. Supernatant was saved after centrifugation at 3000 g for 10 min to remove the bacteria and stored at -80°C for measuring secreted cytokines by ELISA. Otherwise, removed the bacteria rich supernatant completely and the cells pellet were lysed using respective lysis buffer used in the RNA isolation or SDS-PAGE methods.

2.2.5.2 *Helicobacter pylori* infection with THP1 derived DCs

THP1 derived DCs maintained in complete RPMI supplemented with 100 µg /ml of rhIL-4 and rhGM-CSF (Peprotech, Rocky Hill, NJ, USA) and Antibiotic and Antimycotic were washed with fresh RPMI media to remove the residual before using for the infection assay. The washed cells were suspended in cRPMI without antibiotics and antimycotics and aliquoted to 12 well plates at a concentration of $2x10^5$ cells/ml and incubated for atleast 5h before infection.

The infection of DCs with *H. pylori* and cell isolation by centrifugation were done as in the case of THP1 cells as described above. The cell pellet was lysed in lysis buffer of Qiagen RNeasy Kit and used for RNA isolation.

2.2.5.3 *Helicobacter pylori* infection with HEK293 cell lines (HEK293, HEK293 TLR2, HEK293-TLR5 and HEK293-TLR10-HA)

HEK293 cell lines maintained in DMEM supplemented with Antibiotic and Antimycotic or/and Blasticidin were harvested by gently tapping the tissue culture flask. The cells harvested were washed with antibiotic and antimycotic free DMEM and the pellet resuspended in fresh medium. The cells were then subcultured in the antibiotic and

antimycotic free media in 6 well culture plates and when it reaches 70-80% confluent cultures used for infection assay with *H. pylori*.

The infections with *H. pylori* were done as in the case of THP1 cells as described above. The cells after infection were harvested by removing the overlaying medium. Then the cells were washed with fresh warm DMEM. In the case of analysis of cytokine secretion analysis, supernatant was saved after centrifugation at 3000 g for 10 min to remove the bacteria and stored at -80°C for measuring secreted cytokines by ELISA. The *H. pylori* infected monolayer were lysed using respective lysis buffer used in the RNA isolation or SDS-PAGE methods by directly adding to the well.

2.2.6 Inhibitor Assay

THP1 cells maintained in complete RPMI supplemented with 1% antibiotic and antimycotic cells were washed with fresh RPMI media to remove the residual before using for the infection assay. The washed cells were suspended in cRPMI without antibiotics and antimycotics and aliquoted to 6 well plates at a concentration of $1x10^6$ cells/ml and incubated for atleast 5h before infection.

The THP1 cells were treated with Src-kinase inhibitor PP2 (20 µM), EGFR-kinase inhibitor AG1478 (20 µM) and pan-caspase inhibitor Z-VAD-FMK (20 µM) in DMSO and control cells with equivalent amount of DMSO alone for 30 min before infecting with *H. pylori* to study the influence on cytokine release from infected cells.

2.2.7 Statistical Analysis

All the data were expressed as mean ± SD and the level of significance was analysed by the method of paired T-test using the software SPSS-11.5 (SPSS Inc. USA)

3. Results

3. Results

3.1 THP1cells infection with *H. pylori* and isogenic mutants

THP1 cells cultured in complete RPMI-1640 medium were used for the infection with *H.pylori* and isogenic mutants. The cells were analyzed under phase contrast microscope after 6h of infection with *H. pylori* and isogenic mutants at MOI (multiplicity of infection) of 1:50. The uninfected cells were monotypic and rounded in shape under phase contrast microscopic examination at a magnification of 40x. It has been reported that U937 macrophage cell lines have formed homotypic cellular aggregates during infection with *H. pylori* and that correlated with the expression of ICAM-1 and receptor LFA-1 and depended on *cag*PAI status (Moese S *et al,* 2002). *H. pylori* strains are also reported to be exhibiting haemagglutination properties (Valkonen KH *et al,* 1997). In the present study, we have analyzed the morphological features of THP1 cells after 6 h of infection with *H. pylori* wild type and isogenic mutants of Δ*cagA,* Δ*vacA,* Δ*flaA,* Δ*cgt* and Δ*cag*PAI. Infection of THP-1 cells with *H. pylori* wild type and isogenic mutants resulted in the aggregate formation of THP1 cells. However, Δ*cag*PAI infected cells, which showed the least induction of aggregate formation and the aggregates found to be small in size when compared to the aggregate induction by wild type and other isogenic mutants studied (Fig.1).

3.2 TLR mRNA expression in THP-1 cells during infection with *H. pylori* and isogenic mutants

Toll like receptors are one of the major molecules of the innate immune system for detecting different molecular patterns of the invading microorganisms. The active involvements of pattern recognition receptors (PRRs) such as TLRs are paving way for a successful adaptive immunity to ward off the infection. TLR molecule engagement with ligands will induce different cellular signalling pathways to produce inflammatory mediators at the site of infection (Takeda Y *et al,* 2003). These molecules are capable of regulating the normal cellular events of the epithelial cells and attracting the immune

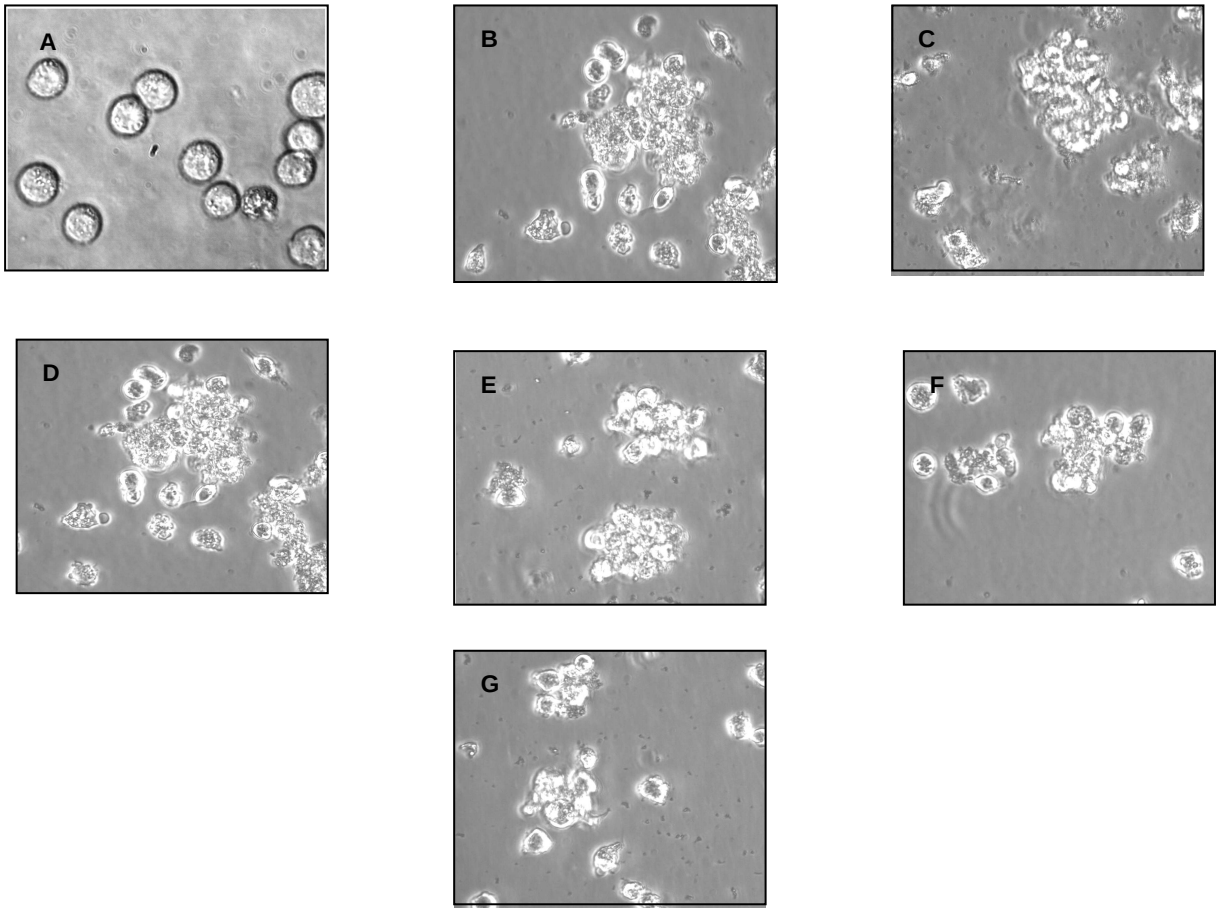


Figure.1 THP1 monocytic leukaemia cells (A) infected with *H. pylori* wild type (B) and isogenic mutants of Δ*cagA* (C), Δ*vacA* (D), Δ*flaA* (E), Δ*cgt* (F) and Δ*cag*PAI (G) at 6h of infection (MOI - 1:50). Magnification – 40x

cells to the site of infection. Although epithelial cells forms the natural barrier against infection, the combined activities of immune cells such as phagocytosis, antigen presentation and production of soluble mediators like antibodies, cytokines and chemokines making the removal of microorganisms or containing of infection at particular sites only. *H. pylori* is a gastric pathogen and a known causative factor for gastritis, peptic ulcer and gastric cancer. It has been reported earlier that *H. pylori* is able to colonize the gastric epithelium for long period even after the successful initiation of adaptive immune responses (Smythies LE *et al,* 2000; Guiney DG *et al,* 2003; Hafsi N *et al,* 2004; de Jonge R *et al,* 2004). It has also been reported that TLR-2 and TLR-5 mRNA expressions were increased during *H. pylori* infection in the epithelial cells and initiated signalling pathways that enhanced activation of NFκB (Smith MF *et al,* 2004; Maeda S *et al,* 2001; Backhead F *et al,* 2003). TLR-1 to 10 mRNA expression dynamics during *H. pylori* infection with human THP-1 monocytes and THP-1 derived DCs were analyzed in this study at the mRNA level and TLR-5 and TLR-10 at the protein level. THP-1 cell is one of the best systems to study all the 10 TLR molecules as it is reported to be expressing all TLRs constitutively (Zarember KA and Godowski PJ, 2002). The THP-1 cells maintained in complete RPMI 1640 medium were used for infection assays with *H. pylori* strain P12 and its isogenic mutants such as Δ*cagA,* Δ*vacA,* Δ*flaA,* Δ*cgt* and Δ*cag*PAI. The dynamics of mRNA expression of TLR-1 to TLR-10 were determined by Taqman Quantitative RT-PCR relative to the expression of house keeping gene GAPDH. TLR expression pattern in THP1 cells at MOI of 1:50 of *H. pylori* and mutant strains were analyzed after 6h and 24h of infection in both cell types.

TLR-1, TLR-2 and TLR-6 have been phylogenetically grouped as one of the five subfamilies among TLRs and reported to be detecting lipopeptides, lipoproteins and peptidoglycans (PGN) molecules in general (Takeda Y *et al,* 2003; Ozinsky A *et al,* 2000). The present study revealed that TLR-1 and TLR-2 mRNA expression have moderately increased after 6h and 24h of infection with *H. pylori* wild type when

compared to uninfected cells. Δ*flaA* and Δ*vacA* isogenic mutant strains were the least inducers of TLR-1 and TLR-2 mRNAs in THP-1 cells. TLR-6 mRNA expression was more than 10 fold after 6h and increased to 15 fold after 24h of infection with *H. pylori* when compared to uninfected control cells. Δ*flaA,* Δ*vacA,* Δ*cgt* and Δ*cag*PAI isogenic mutants were not potent inducers of TLR-6 mRNA expression in THP-1 cells at 6 h of infection when compared to wild type and Δ*cagA* isogenic mutant. However, Δ*cgt* and Δ*cag*PAI isogenic mutants have induced similar level of TLR-6 mRNA expression induced by wild type strain after 24h of infection with THP-1 cells (Fig.2,3 and Table.1,2)..

The second subfamily of TLRs includes TLR-7, TLR-8 and TLR-9 and all are known to be activated upon binding with nucleic acid ligands (Coban C *et al,* 2005, Diebold S *et al,* 2004; Heil F *et al,* 2004). TLR-7 mRNA expressions in THP-1 cells have been increased to 30 fold after 6h and 70 fold after 24h of infection with *H. pylori* when compared to uninfected control cells. TLR-7 is the highly induced molecule among all the TLRs in THP-1 cells analyzed during infection with *H. pylori*. Δ*cagA* isogenic mutant strain has increased TLR-7 mRNA expression higher than wild type strain (34 fold), though not significantly, after 6h of infection. No further increase in the TLR-7 mRNA expression was noted even after 24h of infection. However, wild type strain induced TLR-7 mRNA expression has elevated to 72 fold after 24h of infection when compared to uninfected control cells. Δ*cag*PAI*,* Δ*cgt,* and Δ*vacA* isogenic mutant strains were found to be less potent than wild type strain on inducing TLR-7 mRNA expression. Δ*flaA* isogenic mutant was the least inducer of TLR-7 mRNA expression in THP-1 cells among all the mutant strains tested and the expression was increased to 4 fold after 6h and this level was reduced to 2 fold after 24h of infection. TLR-8 mRNA expression was induced more than 15 and 12 fold in THP-1 cells by *H. pylori* wild type and Δ*cagA* isogenic mutant, respectively. All the other isogenic mutants strains were found to be less potent to induce

TLR-8 mRNA expression after 6h of infection with THP-1 cells. However, Δ*cgt* and

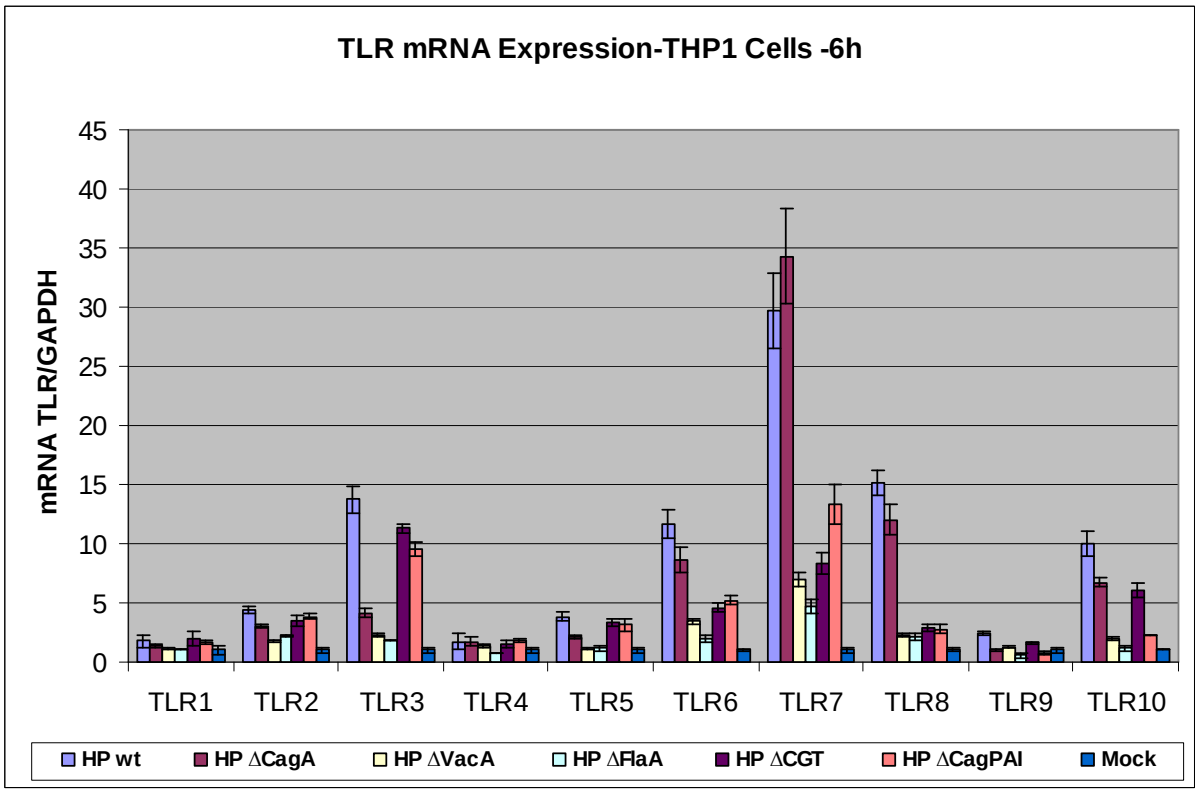


Table.1

	TLR1	±SD	TLR2	±SD	TLR3	±SD	TLR4	±SD	TLR5	±SD
HP wt	1.76	0.49	4.40*	0.30	13.73*	1.08	1.74	0.61	3.83*	0.34
HP Δ*CagA*	1.39	0.19	3.03*#	0.21	4.11*#	0.36	1.72	0.39	2.11*#	0.18
HP Δ*VacA*	1.08	0.06	1.76#	0.11	2.28*#	0.13	1.31	0.13	1.13#	0.11
HP Δ*FlaA*	1.10	0.01	2.24#	0.09	1.87#	0.02	0.78	0.04	1.14#	0.18
HP Δ*CGT*	1.96	0.60	3.48*	0.47	11.29*	0.44	1.52	0.36	3.31*	0.26
HP Δ*Cag*PAI	1.64	0.20	3.85*	0.27	9.53*#	0.63	1.82	0.18	3.11*	0.46
Mock	1.00	0.39	1.00	0.25	1.00	0.21	1.00	0.26	1.00	0.21

	TLR6	±SD	TLR7	±SD	TLR8	±SD	TLR9	±SD	TLR10	±SD
HP wt	11.63*	1.25	29.65*	3.20	15.14*	1.03	2.38*	0.16	10.05*	1.08
HP Δ*CagA*	8.63*#	1.01	34.29*	4.04	12.04*#	1.27	1.00#	0.13	6.72*	0.39
HP Δ*VacA*	3.41*#	0.30	6.96*#	0.61	2.26*#	0.20	1,27#	0.11	1.94*#	0.17
HP Δ*FlaA*	2.00#	0.27	4.72*#	0.56	2.17#	0.30	0.54#	0.27	1.14#	0.17
HP Δ*CGT*	4.60*#	0.40	8.31*#	0.89	2.91*#	0.28	1.60#	0.12	6.09*	0.60
HP Δ*Cag*PAI	5.19*#	0.40	13.34*#	1.70	2.78#	0.38	0.75#	0.12	2.30#	0.00
Mock	1.00	0.11	1.00	0.20	1.00	0.15	1.00	0.23	1.00	0.20

Figure.2 Fold changes of TLR mRNA expression in THP1 cells after 6h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions of TLRs were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*

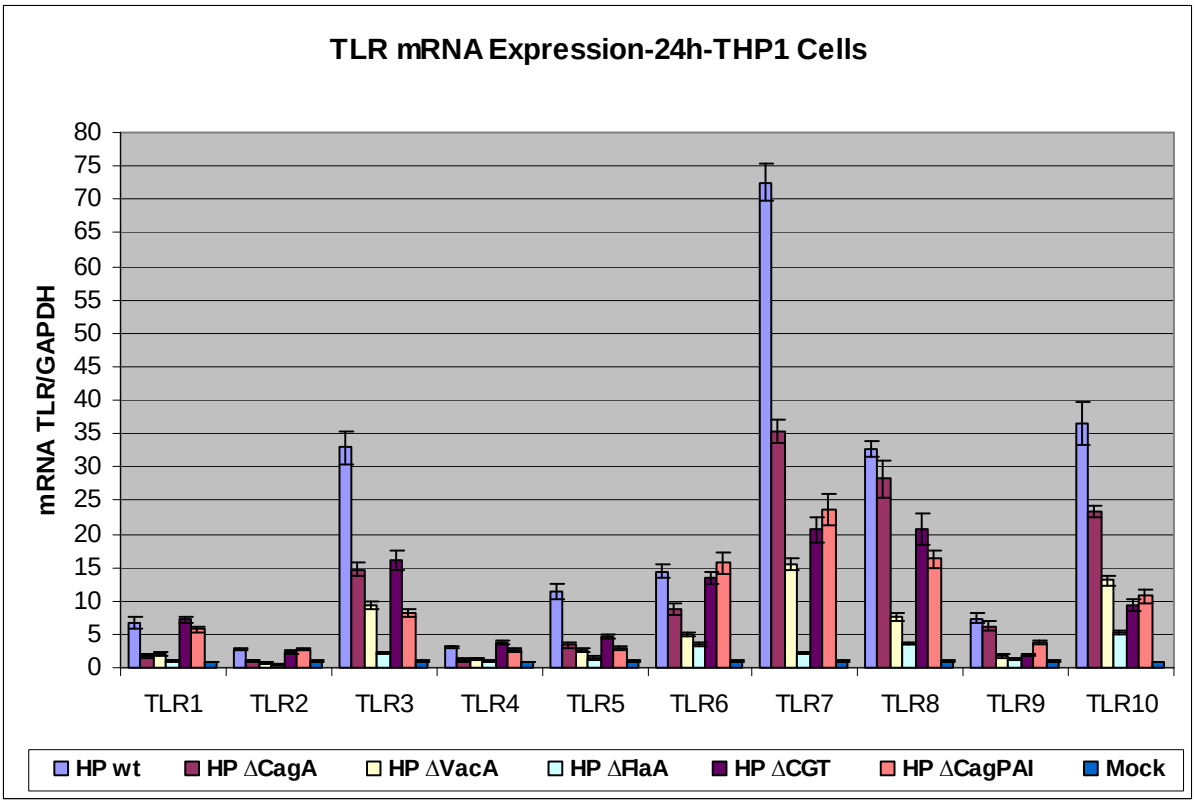


Table.2

	TLR1	±SD	TLR2	±SD	TLR3	±SD	TLR4	±SD	TLR5	±SD
HP wt	6.68*	0,78	2.73*	0.15	32.89*	2.58	3.16*	0.18	11.31*	1.22
HP Δ*CagA*	1.74#	0,42	1.10#	0.18	14.72*#	1.01	1.30#	0.30	3.41*#	0.37
HP Δ*VacA*	2.08#	0,29	0.67#	0.18	9.38*#	0.55	1.34#	0.13	2.73*#	0.30
HP Δ*FlaA*	1.05#	0,24	0.46*#	0.03	2.19*#	0.19	0.91#	0.13	1.51#	0.25
HP Δ*CGT*	7.23*	0,49	2.32*#	0.18	16.14*#	1.42	3.73*	0.30	4.57*#	0.27
HP Δ*Cag*PAI	5.71*	0,45	2.75*	0.21	8.18*#	0.64	2.62#	0.23	2.89*#	0.25
Mock	1.00	0,01	1.00	0.06	1.00	0.22	1.00	0.01	1.00	0.15

	TLR6	±SD	TLR7	±SD	TLR8	±SD	TLR9	±SD	TLR10	±SD
HP wt	14.42*	1.13	72.50*	2.84	32.67*	1.28	7.41*	0.72	36.50*	3.22
HP Δ*CagA*	8.81*#	0.95	35.26*#	1.73	28.24*#	2.77	6.27*#	0.74	23.43*	0.92
HP Δ*VacA*	4.92*#	0.29	15.56*#	0.92	7.57*#	0.59	1.69#	0.28	13.08*#	0.77
HP Δ*FlaA*	3.46*#	0.31	2.23*#	0.09	3.58*#	0.18	1.35#	0.17	5.28*#	0.21
HP Δ*CGT*	13.35*	0.92	20.60*#	2.01	20.70*#	2.23	1.86*#	0.20	9.40*#	0.93
HP Δ*Cag*PAI	15.66*	1.53	23.74*#	2.32	16.27*#	1.27	3.79*#	0.30	10.73*#	1.05
Mock	1.00	0.12	1.00	0.16	1.00	0.13	1.00	0.10	1.00	0.12

Figure.3 Fold changes of TLR mRNA expression in THP1 cells after 24h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions of TLRs were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*

Δ*cag*PAI isogenic mutants have induced TLR-8 mRNA expression to 20 and 16 fold after 24h of infection, respectively, when compared to uninfected THP1 cells. Δ*flaA* and Δ*vacA* isogenic mutants have induced 3.5 and 7.5 fold after 24h of infection and become the least inducers of TLR8 mRNA expression in THP1 cells among the strains tested (Fig.2, 3 and Table.1,2)..

TLR-3, the sole member of third subfamily, is known to be detecting double stranded RNAs (Takeda Y *et al,* 2003). *H. pylori* wild type, Δ*cgt,* Δ*cag*PAI and Δ*cagA* mutants have increased TLR-3 mRNA expression to 14, 11, 9.5 and 4 folds, respectively, after 6h of infection in THP-1 cells. The fold changes of TLR-3 mRNA expression were 33, 16 and 15 after 24h of infection with wild type, Δ*cgt* and Δ*cagA* isogenic mutants of *H. pylori*, respectively. However, Δ*cag*PAI strain did not make significant change in mRNA expression from the above level even after 24h of infection. It is observed that, Δ*flaA* and Δ*vacA* isogenic mutants were the least inducers of TLR-3 mRNA expression in THP1 cells when compared to uninfected cells (Fig.2, 3 and Table.1,2).

TLR-4 constitutes the sole member of fourth subfamily of TLRs. TLR-4 has less specificity towards *H. pylori* LPS and hence it has been reported that this receptor having less role in the detection of *H. pylori* infection (Takeda Y *et al,* 2003). In this study, TLR-4 mRNA expression was one of the least induced TLRs after 24h of infection with *H. pylori* and isogenic mutant strains. TLR-5 known to detect flagellin of bacteria and it is grouped as another subfamily in the 'star phylogeny'. *H. pylori* flagellin-A has been reported to have less affinity with TLR-5 and the site responsible for TLR5 evasion mapped to amino acids 89-96 of the N-terminal D1 domain and that may be the reason for its poor response in TLR-5 mediated IL-8 secretion from epithelial cells (Andersen-Nissen E *et al,* 2005; Gewirtz AT *et al,* 2004). However, other factors from *H. pylori* that can activate TLR-5 mediated signalling and gene expression were not known. Infection of *H. pylori* with THP-1 cells have increased TLR-5 mRNA expression moderately after 6h and reached 11 fold after 24h of when compared to uninfected control cells, indicating

that some factors from *H. pylori* are involved in the mRNA level expression of TLR-5. Δ*flaA* and Δ*vacA* isogenic mutants are the least inducers of TLR-5 mRNA expression in THP1 cells (Fig.2, 3 and Table.1,2).

TLR-10, which is known as the orphan receptor among TLRs, is grouped with the subfamily including TLR-1, TLR-2 and TLR-6 due to structural similarity (Takeda Y *et al,* 2003). The ligand for TLR-10 has not been reported. No data is available in the literature on microbes that inducing TLR-10 mRNA expression. The present study revealed that *H. pylori* infection of THP-1 cells has increased the mRNA level expression of TLR-10 in THP-1 cells after 6h and continued to increase even after 24h. The enhancement of TLR-10 mRNA expression was 10 and 36.5 fold after 6 and 24h of infection with THP-1 cells by *H. pylori* wild type. Δ*cagA*, Δ*cgt,* Δ*cag*PAI and Δ*vacA* isogenic mutants were also induced TLR-10 mRNA expression in THP-1 cells significantly after 24h, although not at the level exhibited by *H. pylori* wild type. Δ*flaA* isogenic mutant found to be the least inducer of TLR-10 mRNA expression as in the case of other TLRs analyzed in the present study (Fig.2, 3 and Table.1,2).

All the isogenic mutant strains tested were less active in inducing TLR expression than wild type strain and this is an important finding of the present study. The genes which are important for virulence and pathogenecity of *H. pylori* such as *cagA, vacA, flaA, cag*PAI and *cgt* acting even at the first level of detection of the innate immune system namely TLRs.

3.3 Immunoprecipiation and Western blot analysis of TLR-5 and TLR-10 from THP1 cells during infection with *H. pylori* and Δ*flaA* mutant

The protein level expressions of TLR-5 and TLR-10 were studied using whole cell extract immunoprecipitation and Western blot. *H. pylori* wild type and Δ*flaA* isogenic mutant, the least inducer of TLR mRNA expression, were used to infect THP-1 cells to study the variation in protein level expression. After 6h of infection, the whole cell extract of THP-1 cells was prepared for immunoprecipitation of TLR-5 and TLR-10 protein

molecules. The whole cell extract were pre-cleared with Protein-G sepharose for 2 h and the TLRs were precipitated using appropriate antibodies by incubating for overnight on a rotatory shaker at 4°C. SDS lysis buffer was added to Protein-G bound immunecomplexes and boiled for 5 min. The immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blot method. TLR-5 was found to be moderately increased in expression at protein level as similar to mRNA expression after 6h of infection with *H. pylori* when compared to uninfected control cells. TLR-10 was highly expressed at the protein level in *H. pylori* infected cells which correlated with mRNA expression as shown earlier (Fig.4). Δ*flaA* mutant of *H. pylori* was a least inducer of TLR-5 and TLR-10 proteins in THP1 monocytes after 6h of infection when compared to wild type strain. These results clearly ascertain that *H. pylori* virulence and pathogenecity factors have an important role on quantitative and qualitative properties of TLR molecules and that will be a determining factor for successful adaptive immune response.

3.4 TLR mRNA Expression in THP1 monocyte derived Dendritic Cells during Infection with *H. pylori* and Δ*flaA* isogenic mutant

THP-1 derived DCs represent monotypic DCs and reported to be function as antigen presenting cells to activate T cells when matured through stimulation (Berges C *et al,* 2005). In this study, THP-1 derived immature DCs were generated and the characteristics were analyzed under phase contrast microscope. The cells exhibited the formation of a ruffled and lobular cytoplasm with spikes and semicircular extrusions (Fig.5). These THP1 derived DCs were used to study mRNA expression of TLRs during infection with *H. pylori* and Δ*flaA* isogenic mutant. Similar to THP1 cells, the THP-1 derived immature DCs also expressed mRNAs of all the 10 TLRs. *H. pylori* wild type and Δ*flaA* isogenic mutant, the least inducer of TLR mRNA expression, were used for the infection to study the differences in mRNA level expression in infected THP-1 cells and THP1 derived immature DCs. TLR-3, TLR-7 and TLR-10 were prominently expressed in

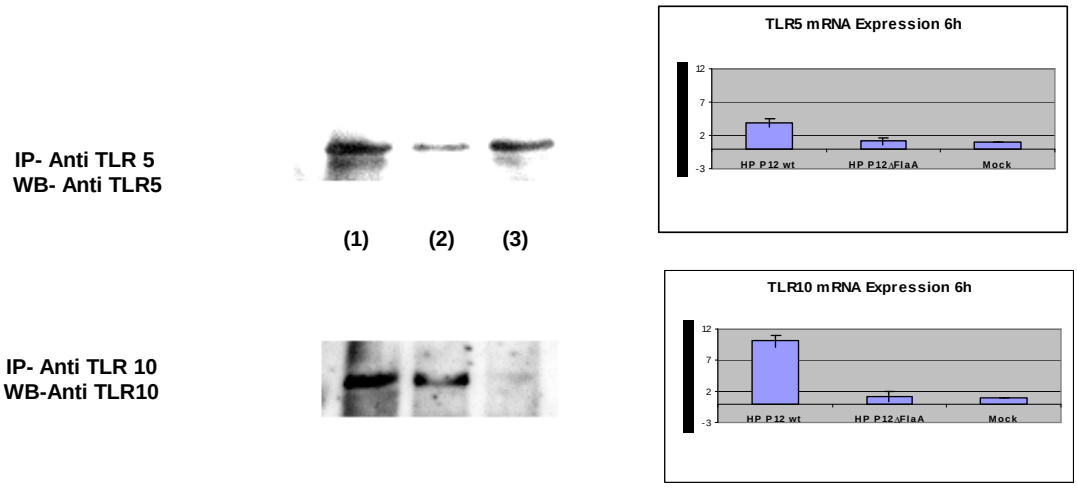


Figure.4 Western blot analysis of immunoprecipitated TLR-5 and TLR-10 proteins from THP1 cells infected with *H. pylori* and isogenic Δ*flaA* mutant for 6h (**lane.1** -*H. pylori* wild type infected, **lane.2**-isogenic *flaA* mutant infected and **lane.3**-uninfected control). The histogram shows the corresponding fold changes of mRNA level expression analyzed by Taqman Real Time PCR method.

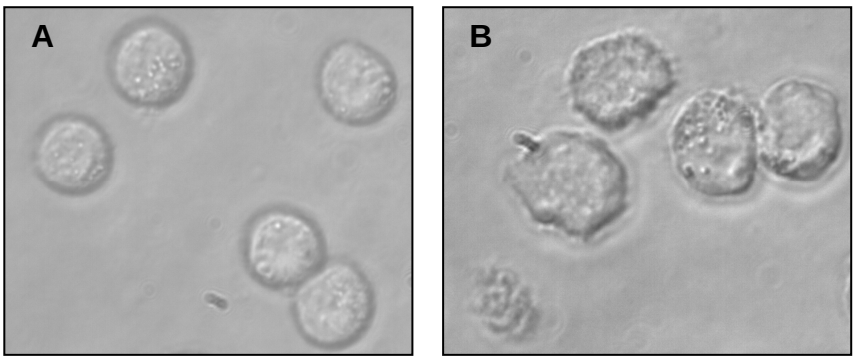


Figure.5 THP1 monocytic leukaemia cells (A) and THP1 derived Dendritic Cells (B). Magnification – 40x

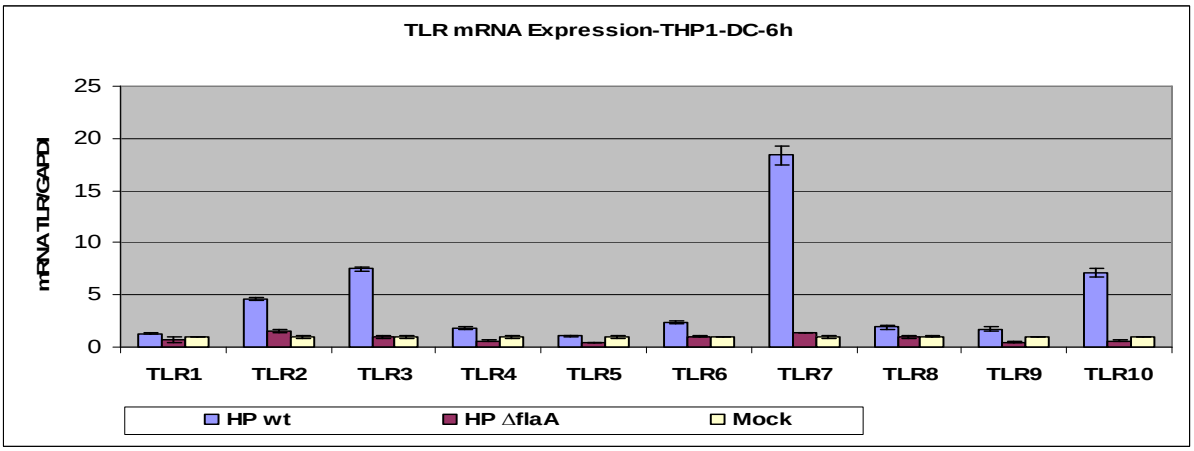


Table.3

	TLR1	±SD	TLR2	±SD	TLR3	±SD	TLR4	±SD	TLR5	±SD
HP wt	1.31	0.10	4.56*	0.13	7.51*	0.23	1.82	0.08	1.08	0.08
HP Δ*fla*A	0.74#	0.30	1.52*#	0.18	1.03#	0.14	0.62#	0.06	0.41#	0.03
Mock	1.00	0.03	1.00	0.16	1.00	0.15	1.00	0.18	1.00	0.15

	TLR6	±SD	TLR7	±SD	TLR8	±SD	TLR9	±SD	TLR10	±SD
HP wt	2.39*	0.16	18.37*	0.89	1.90*	0.18	1.74	0.18	7.16*	0.42
HP Δ*fla*A	1.02#	0.11	1.40#	0.04	1.02#	0.14	0.48#	0.03	0.61*#	0.03
Mock	1.00	0.03	1.00	0.14	1.00	0.08	1.00	0.03	1.00	0.04

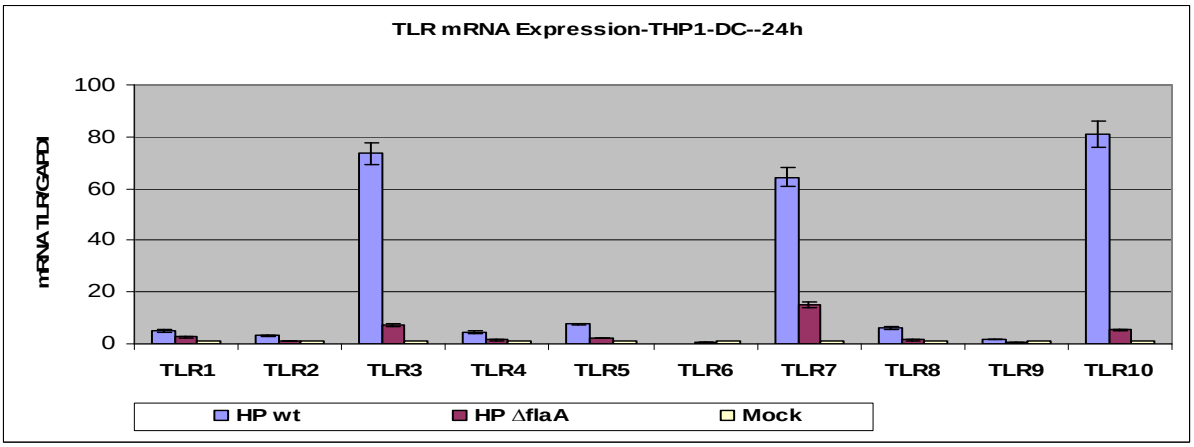


Table.4

	TLR1	±SD	TLR2	±SD	TLR3	±SD	TLR4	±SD	TLR5	±SD
HP wt	5.02*	0.29	3.20*	0.23	73.51*	4.33	4.40*	0.35	7.67*	0.30
HP Δ*fla*A	2.58*#	0.18	1.18#	0.18	7.11*#	0.56	1.43#	0.26	2.29*#	0.18
Mock	1.00	0.12	1.00	0.23	1.00	0.07	1.00	0.16	1.00	0.07

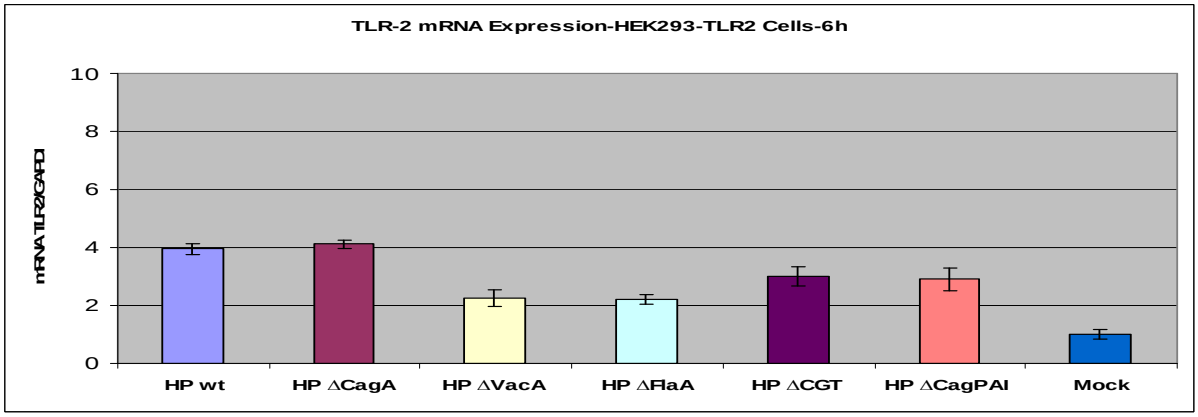
	TLR6	±SD	TLR7	±SD	TLR8	±SD	TLR9	±SD	TLR10	±SD
HP wt	1.85*	0.09	64.44*	3.79	6.19*	0.42	1.68*	0.15	81.00*	4.76
HP Δ*fla*A	0.46*#	0.08	15.13*#	1.03	1.42#	0.37	0.31#	0.06	5.42*#	0.37
Mock	1.00	0.07	1.00	0.10	1.00	0.16	1.00	0.13	1.00	0.16

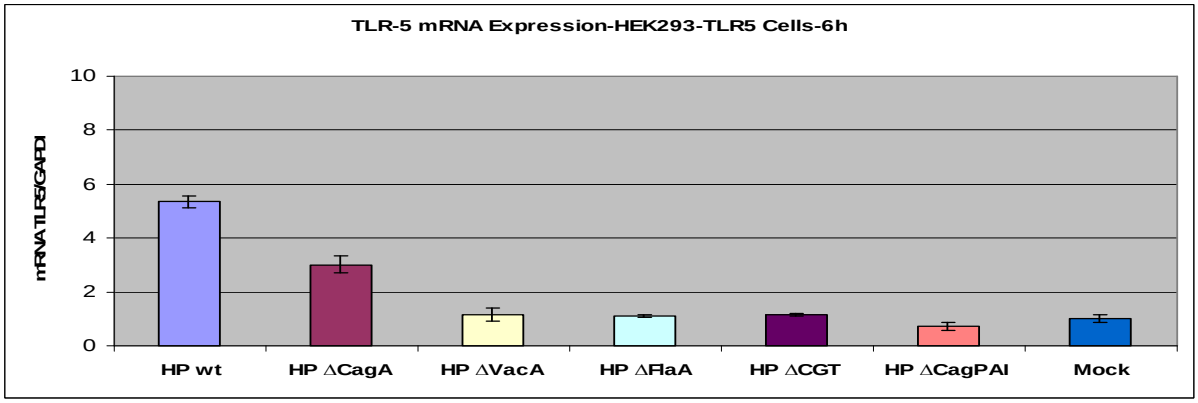
Figure.6 Fold changes of TLR mRNA expression in THP1-DCs after 6h and 24h of infection with *H. pylori* and isogenic mutant of *flaA*. The mRNA expressions of TLRs were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*

THP1-derived dendritic cells after 6h and 24 h of infection with *H. pylori* when compared to uninfected control cells. TLR-2 expression was moderately increased after 6h of infection with *H. pylori* but found to be decreased after 24h of infection. TLR-1, TLR-4, TLR-5, TLR-6, TLR-8 and TLR-9 mRNA expressions in THP-1 derived DCs were not induced after 6h of infection with *H. pylori.* However, the expression levels of above TLRs, except TLR6 and TLR-9, reached 5, 4, 8, and 6 folds, respectively, after 24h of infection. Δ*flaA* isogenic mutant strain which was found to be the least inducer of TLRs mRNA expression in THP-1 cells and also in THP-1 derived immature DCs (Fig. 6 and Table 3, 4).

3.5 TLR mRNA expression in HEK293 cells stably transfected with TLR-2, TLR-5 and TLR-10, respectively, during infection with *H. pylori* and isogenic mutants

Previous studies have shown the involvement of TLR-2 and TLR-5 in *H. pylori* infection with gastric epithelial cells (Smith MF *et al,* 2004; Maeda S *et al,* 2001; Backhead F *et al,* 2003). Among all TLR molecules mRNA expression in THP-1 cells the TLR-10 is considered as a promising molecule due to its high expression at mRNA and protein level. Further to confirm the above findings HEK-293 cells stably transfected with TLR-2 (HEK293-TLR-2 cells), TLR-5 (HEK293-TLR-5 cells) and TLR-10 (HEK293-TLR-10 cells) were used for *H. pylori* infection. In the previous experiments, it was observed that TLR-2 mRNA expression during *H. pylori* infection with human THP-1 cells was increased moderately after 6h. HEK-293-TLR2 cells infected with *H. pylori* for 6h have also exhibited moderate increase of TLR-2 mRNA expression when compared to uninfected control. As observed in THP-1 cells, Δ*flaA* and Δ*vacA* isogenic mutant strains were the least inducers of TLR-2 mRNAs in HEK-293-TLR2 cells among all the strains tested. THP-1 cells infected with *H. pylori* after 6h have also induced an increase in TLR-5 mRNA expression. After 6 h of infection of HEK293-TLR-5 cells with *H. pylori* an increase in the TLR5 mRNA expression was observed similar to that of THP1 cells. *H. pylori* wild type and Δ*cagA* isogenic mutant strain have induced 5 and 3 fold increases in





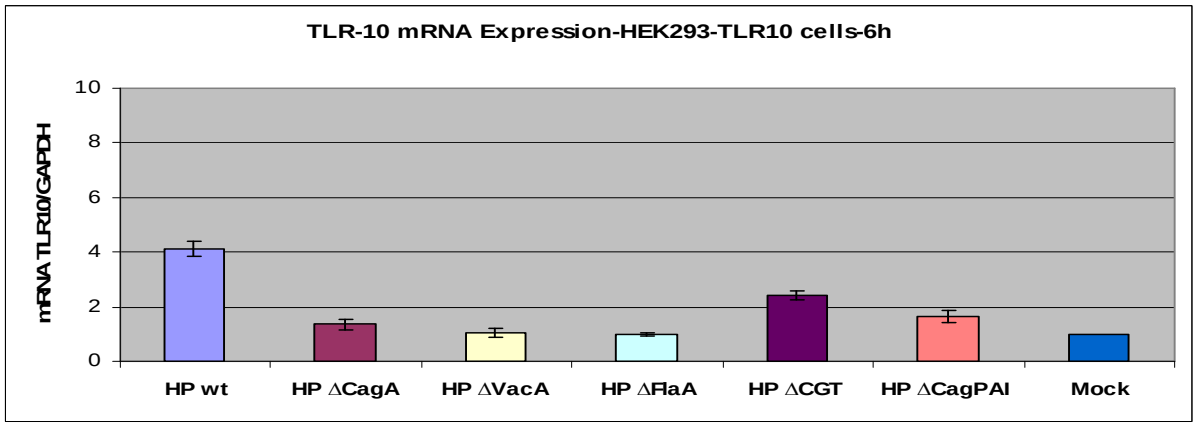


Table.5

	TLR2	±SD	TLR5	±SD	TLR10	±SD
HP wt	3.95*	0.19	5.35*	0.21	4.11*	0.28
HP Δ*CagA*	4.11*	0.16	3.01*	0.30	1.35#	0.17
HP Δ*VacA*	2.24*#	0.30	1.16#	0.23	1.06#	0.16
HP Δ*FlaA*	2.21*#	0.15	1.11#	0.04	0.98#	0.07
HP Δ*CGT*	3.01*	0.33	1.15#	0.04	2.42*#	0.18
HP Δ*Cag*PAI	2.90*	0.39	0.73*#	0.13	1.64#	0.23
Mock	1.00	0.18	1.00	0.15	1.00	0.13

Figure. 7. Fold changes of TLR-2, TLR-5 and TLR-10 mRNA expression in HEK293-TLR-2, HEK293-TLR-5 and HEK293-TLR-10 cell lines, respectively, after 6h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*

TLR-5 mRNA expression, respectively, in HEK-293-TLR-5 cells after 6h of infection. Δ*flaA,* Δ*vacA,* Δ*cgt* and Δ*cag*PAI isogenic mutants have not shown the induction of TLR-5 mRNA expression in HEK-293-TLR-5 cells after 6h of infection. An increase In the TLR-10 mRNA expression was noted in THP1 cells during *H. pylori* infection. HEK-293-TLR-10 cells infected with *H. pylori* have also exhibited an increase in the TLR-10 mRNA expression to 4 fold level after 6 h of infection. Infection of Δ*cgt* isogenic mutant infection resulted an increase of 2.5 fold of TLR-10 mRNA expression in HEK-293-TLR-10 cells. However, Δ*cagA,* Δ*flaA,* Δ*vacA* and Δ*cag*PAI isogenic mutants were not able to increase TLR-10 mRNA expression more than two folds in HEK-293-TLR-10 cells after 6h of infection (Fig.7 and Table 5).

3.6 Induction of phosphorylation of IRAK-1 (ser376) in THP-1 cells during infection with *H. pylori* and isogenic mutants

TLR receptor ligand binding activates the MyD-88 interaction to the cytoplasmic tail of these receptors through its TIR domain and that in turn recruits IRAK-4 and thereby induces association of IRAK-1. TRAF-6 also gets recruited to the above complex and this module induces phosphorylation of few Threonine molecules in the IRAK-1 that activates the massive autophosphorylation of IRAK-1 (Kollewe C *et al,* 2004). Phosphorylated IRAK-1 and TRAF-6 dissociates from the complex and bind to cell membrane protein TAB-1 (TAK1 binding protein-1) and subsequent binding of TAK-1 (transforming growth facor-β-activated kinase) and TAB-2. IRAK-1 ubiquitinylation and degradation take place quickly and the remaining complex leaves to cytoplasm. There it associates with ubiquitin ligases such as UBC-13 (ubiquitin conjugating enzyme 13) and UEV-1a (ubiquitin conjugating enzyme E2 variant-1) and this leads to ubiquitinylation and degradation of TRAF-6. This activates TAK-1 and phosphorylation of IKK (inhibitor of κB kinase) complex (IKKα, IKKβand IKKγ) and MAPK. The IKK complex then phosphorylates IκB (inhibitor of κB) and leads to ubiquitinylation and degradation. This

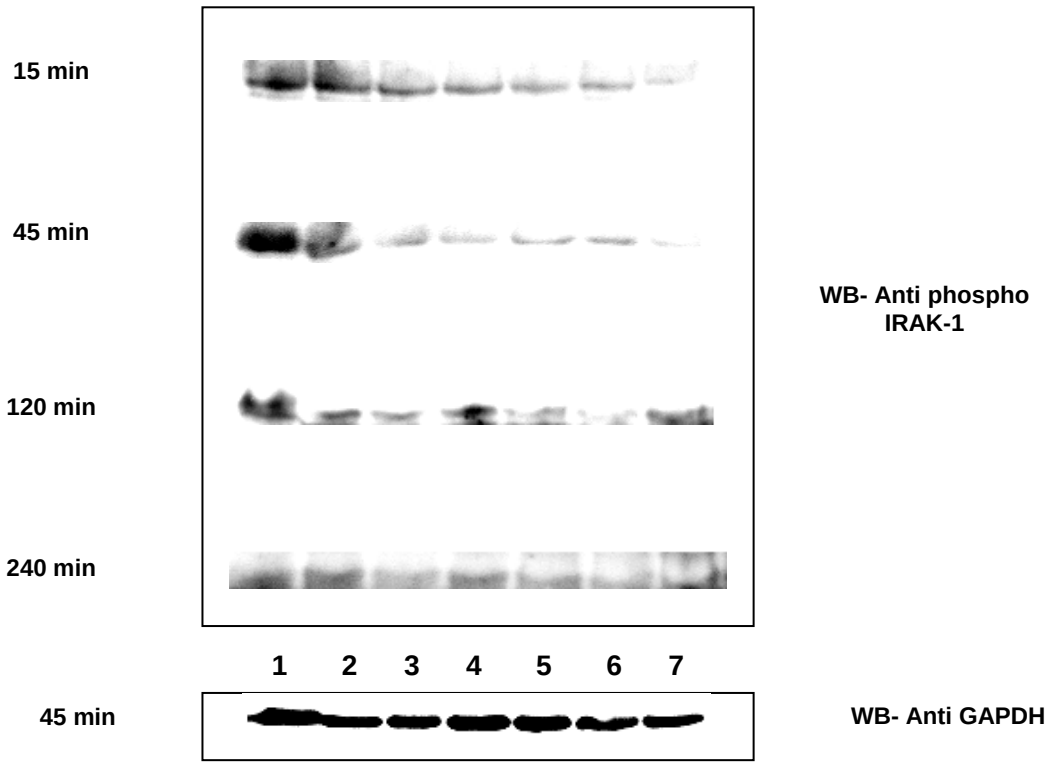


Figure.8 Western blot analysis of phospho IRAK-1 (Ser 376) from THP1 cells infected with *H. pylori* and mutants of *cagA, vacA, flaA, cgt and cag*PAI for 15 min, 45 min, 120 min and 240 min. (**lane.1** -*H. pylori* wild type, **lane.2** Δ*cagA*, **lane.3**-Δ*vacA*, **lane.4** Δ*flaA*, **lane.5** Δ*cgt*, **lane.6** Δ*cag*PAI and **lane.7** uninfected control). Western blot analysis for house keeping gene GAPDH was used as loading control, represented by GAPDH concentration in THP1 cells at 45 min of infection with *H. pylori.*

ultimately frees NFκB (Nuclear factor κB) to translocate to nucleus and induction of transcription of pro-inflammatory genes (Akira S and Takeda K, 2004).

In this study, the ser-376 phosphorylation of IRAK-1 induced by *H. pylori* infection in THP-1 monocytes at various time points was analyzed by Western blot. Analysis of IRAK-1 phosphorylation at various time points can be very useful to understand the initiation of TLR signalling and subsequent ubiquitinylation and degradation ensures the continuation of down stream signalling. It was observed that *H. pylori* wild type induced phosphorylation of IRAK-1 at 15 min and reached the peak level at 45 min. The reduction of phosphorylation was observed at 120 min and lost almost completely after 240 min of infection in THP1 cells (Fig.8). The Δ*cagA,* Δ*vacA,* Δ*flaA,* Δ*cgt* and Δ*cag*PAI isogenic mutants induced very less psophorylation of IRAK-1. This clearly shows that low level of induction of TLR mRNAs and proteins can regulate the downstream signalling of TLRs in THP-1 cells.

3.7 IRAK-M, a TLR signalling negative regulator, expression in THP-1 cells during infection with *H. pylori* and isogenic mutants

The IRAK family consists of two active kinases, IRAK-1 and IRAK-4, and two inactive kinases, IRAK-2 and IRAK-M. IRAK-M expression is restricted to monocytes/macrophages, whereas other IRAKs are ubiquitous. IRAK-M is induced upon TLR stimulation and negatively regulates TLR signalling. IRAK-M prevented dissociation of IRAK-1 and IRAK-4 from MyD88 and formation of IRAK-TRAF6 complexes (Kobayashi K *et al,* 2002). IRAK-M (-/-) deficient cells exhibited increased cytokine production upon TLR/IL-1 stimulation and bacterial challenge, and IRAK-M(-/-) mice showed increased inflammatory responses to bacterial infection. Endotoxin tolerance, a protection mechanism against endotoxin shock, was significantly reduced in IRAK-M (-/-) cells. Recent study showed that LPS tolerance was associated with decreased IRAK-1 and elevated IRAK-M expression (van't Veer C *et al,* 2007). No significant change in the expression of IRAK-M protein in THP-1 cells when infected with *H. pylori* and isogenic

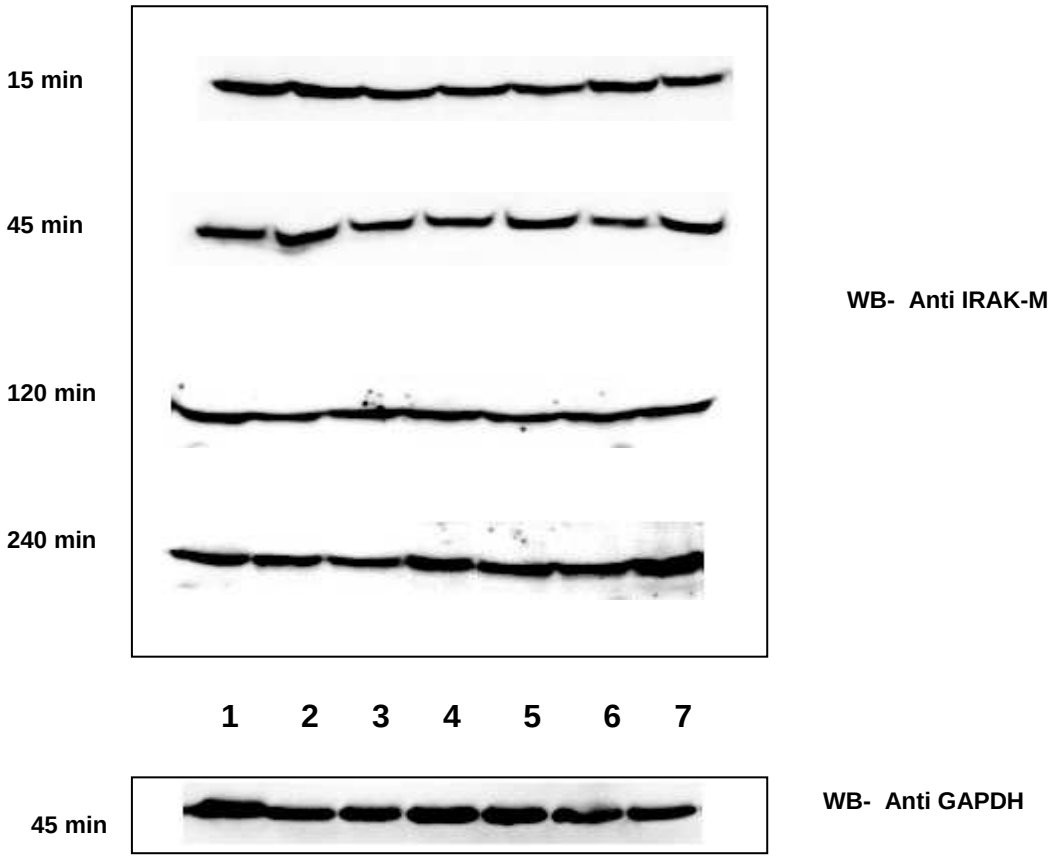


Figure.9 Western blot analysis of IRAK-M from THP1 cells infected with *H. pylori* and mutants of *cagA, vacA, flaA, cgt and cag*PAI for 15 min, 45 min, 120 min and 240 min. (**lane.1** -*H. pylori* wild type, **lane.2** Δ*cagA*, **lane.3**-Δ*vacA*, **lane.4** Δ*flaA*, **lane.5** Δ*cgt*, **lane.6** Δ*cag*PAI and **lane.7** uninfected control). Western blot analysis for house keeping gene GAPDH was used as loading control, represented by GAPDH concentration in THP1 cells at 45 min of infection with *H. pylori.*

mutant strains was noted in Western blot at various time points such as15 min, 45 min, 120 min and 240 min. This indicatess that IRAK-M protein is not elevated to counter the high IRAK-1 phosphorylation during *H. pylori* infection and hence IRAK-M was not involved in the low level phosphorylation of IRAK-1 in isogenic mutants infected cells (Fig. 9).

3.8 NLRs and ASC/PYCARD expression in THP-1 monocytic cells during infection with *H. pylori* and isogenic mutants

Nod-like Receptors are the cytoplasmic pattern recognition receptors for detecting microbial pathogen associated molecular patterns and transducing signals to mount a pro-inflammatory reaction. NLR family of proteins are recognized with a modular organization of a carboxy-terminal leucine rich repeats (LRR), central Nucleotide binding domain (NOD/NACHT) and an amino-terminal CARD domain, pyrin domain or BIR domain for protein-protein interaction (Tschopp J *et al,* 2003). These proteins are able to form the high molecular weight structures called 'inflammasome' and recruit caspase1 that leads to proteolytic activation of pro IL-1 β and pro IL-18 (Martinon F and Tschopp J, 2004; Martinon F *et al,* 2002). NOD-1 and NOD-2 proteins among NLRs are reported to detect molecular patterns from bacteria. NOD-1 senses PGN containing meso-diaminopimelic acid from Gram negative bacteria and NOD-2 found to detect muramyl dipeptide of PGN present in both Gram negative and positive bacteria (Girardin SE *et al,* 2003; Girardin SE *et al,* 2003b). The other members of NLRs include NALPs (NACHT, LRR and Pyrin domain containing proteins), IPAF (ICE protease activating factor) and NAIPs (Neuronal apoptosis inhibitor proteins). In the present study, NOD-1, NOD-2, NALP-1, NALP-2, NALP-3, IPAF and ASC mRNA expressions during infection with *H. pylori* and isogenic mutants were analyzed.

Infection of THP-1 monocytes with *H. pylori* and isogenic mutants have not significantly changed the mRNA level expression of NOD-1 and NOD-2 after 6h and it was found to be down-regulated after 24h when compared to uninfected control cells.

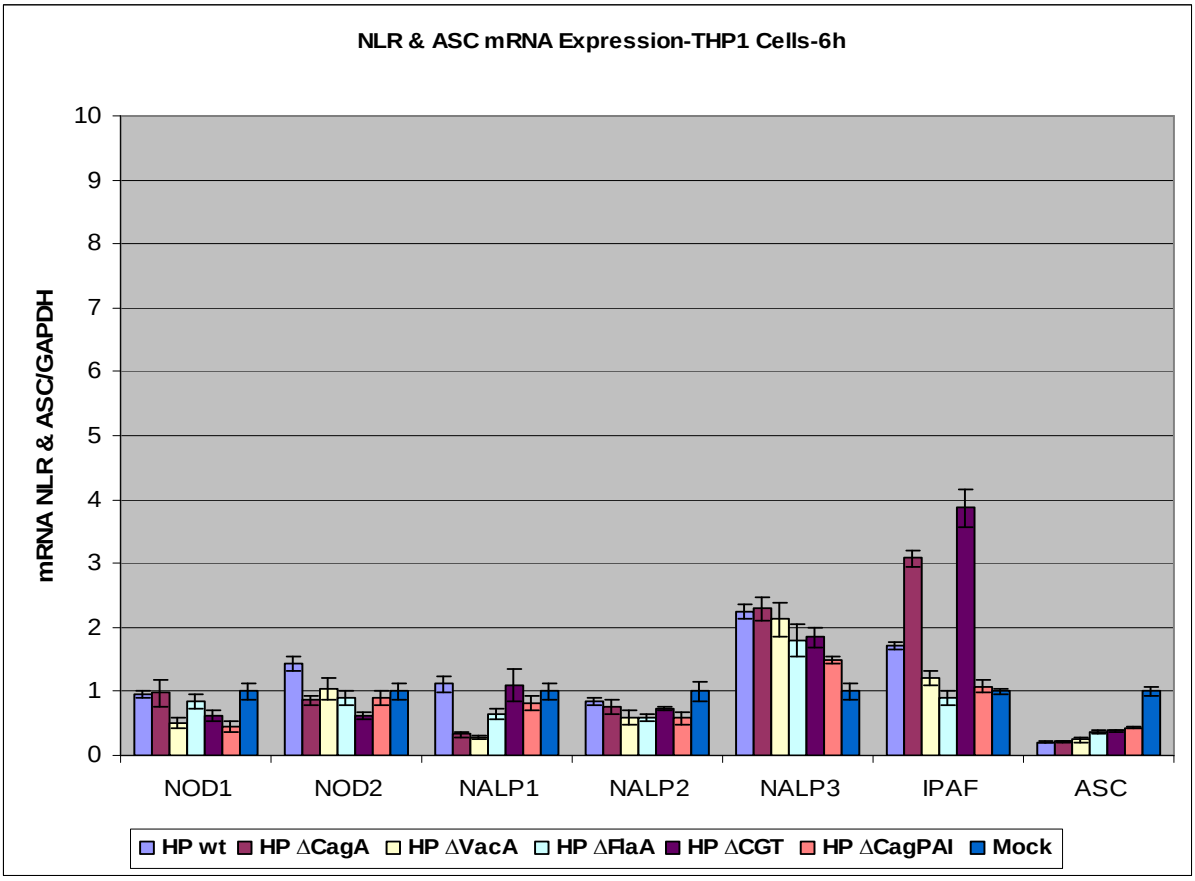


Table.6

	NOD1	±SD	NOD2	±SD	NALP1	±SD	NALP2	±SD
HP wt	0.95	0.06	1.44*	0.11	1.11*	0.12	0.85	0.06
HP Δ*CagA*	0.97	0.20	0.86#	0.08	0.33#	0.04	0.76	0.11
HP Δ*VacA*	0.51*#	0.09	1.04	0.18	0.28#	0.03	0.60*	0.11
HP Δ*FlaA*	0.85	0.11	0.90	0.12	0.65#	0.08	0.59#	0.07
HP Δ*CGT*	0.62#	0.08	0.61#	0.06	1.10	0.25	0.72	0.03
HP Δ*Cag*PAI	0.45*#	0.08	0.91	0.11	0.81*#	0.11	0.58	0.09
Mock	1.00	0.13	1.00	0.13	1.00	0.13	1.00	0.15

	NALP3	±SD	IPAF	±SD	ASC	±SD
HP wt	2.25*	0.11	1.71*	0.05	0.21*	0.02
HP Δ*CagA*	2.30*	0.18	3.08*#	0.13	0.22*	0.01
HP Δ*VacA*	2.13*	0.27	1.20#	0.11	0.24*	0.03
HP Δ*FlaA*	1.79	0.25	0.90#	0.12	0.36*	0.03
HP Δ*CGT*	1.84*	0.16	3.87*#	0.30	0.38*	0.01
HP Δ*Cag*PAI	1.48#	0.06	1.08	0.10	0.43*	0.01
Mock	1.00	0.13	1.00	0.04	1.00*	0.08

Figure. 10 Fold changes of NLRs and ASC mRNA expression in THP1 cells after 6h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions of TLRs were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*

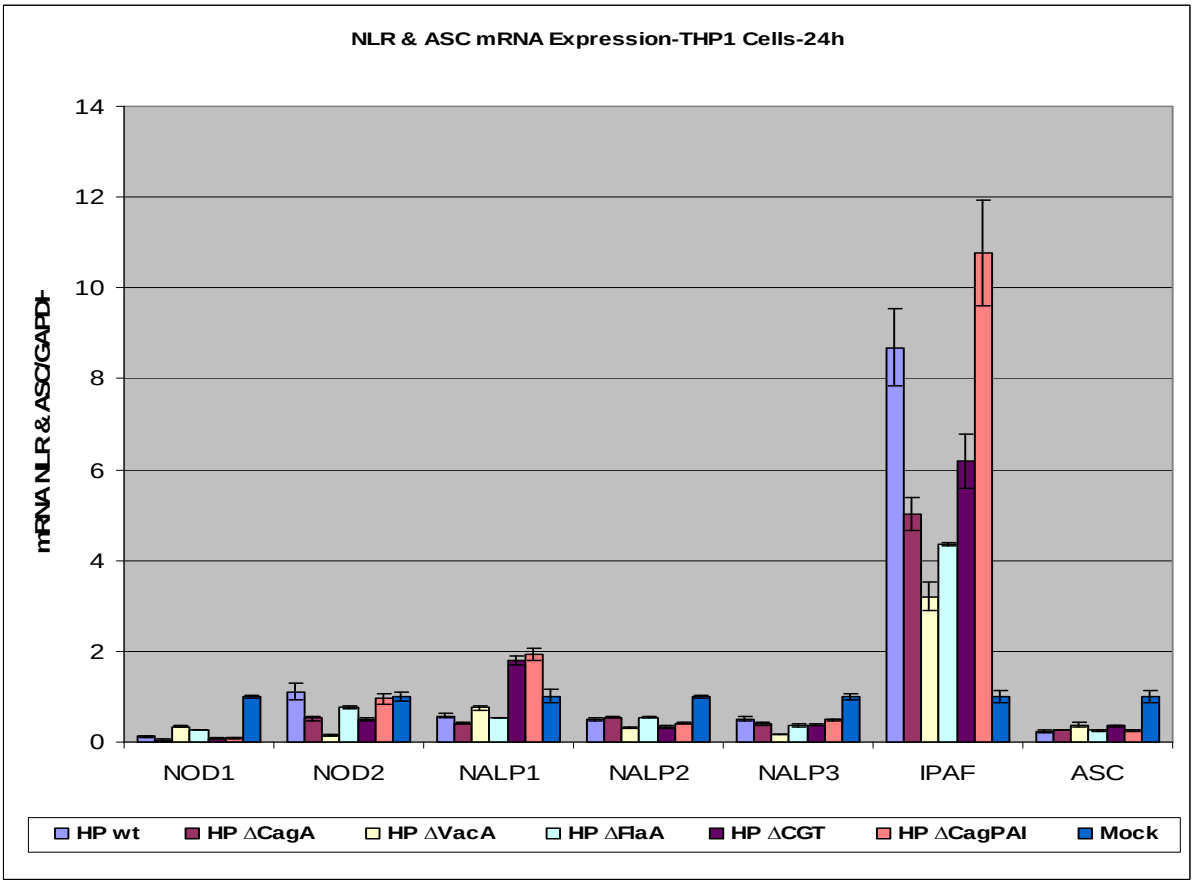


Table.7

	NOD1	±SD	NOD2	±SD	NALP1	±SD	NALP2	±SD
HP wt	0.12*	0.01	1.11	0.18	0.58	0.05	0.49*	0.04
HP Δ*CagA*	0.04*	0.01	0.52	0.04	0.41	0.01	0.54*	0.01
HP Δ*VacA*	0.34*	0.01	0.14*	0.01	0.76	0.05	0.31*#	0.01
HP Δ*FlaA*	0.26*#	0.01	0.76	0.03	0.53	0.01	0.54*	0.01
HP Δ*CGT*	0.08*	0.01	0.50	0.04	1.79*#	0.11	0.33*	0.04
HP Δ*Cag*PAI	0.09*	0.01	0.96	0.12	1.92*#	0.13	0.41*	0.01
Mock	1.00	0.03	1.00	0.10	1.00	0.15	1.00	0.03

	NALP3	±SD	IPAF	±SD	ASC	±SD
HP wt	0.51*	0.05	8.69*	0.85	0.24*	0.03
HP Δ*CagA*	0.39*	0.03	5.02*	0.37	0.26*	0.01
HP Δ*VacA*	0.16*#	0.01	3.20*#	0.31	0.37*	0.05
HP Δ*FlaA*	0.36*#	0.03	4.35*	0.04	0.25*	0.02
HP Δ*CGT*	0.38*	0.03	6.19*	0.61	0.35*	0.03
HP Δ*Cag*PAI	0.49*	0.01	10.77*	1.16	0.25*	0.03
Mock	1.00	0.07	1.00	0.13	1.00	0.12

Figure. 11 Fold changes of NLRs and ASC mRNA expression in THP1 cells after 24h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions of TLRs were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*

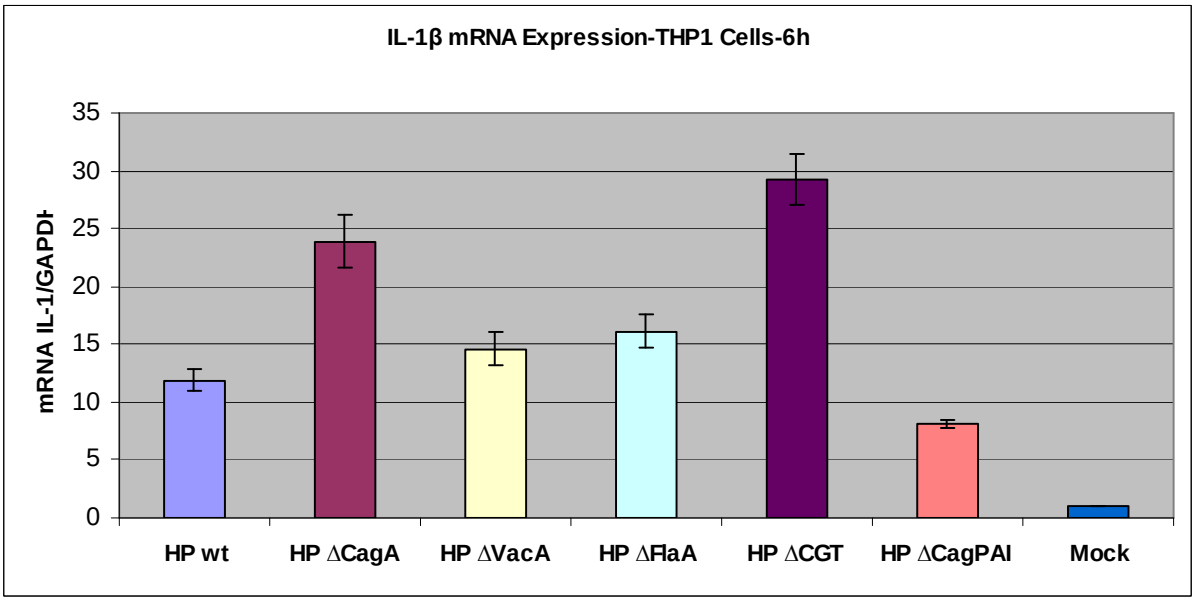
NALP-1 and NALP-2 mRNAs expression in THP-1 cells were also not significantly changed after 6h of infection with *H. pylori* and isogenic mutants. NALP-3 mRNA expression in THP1 cells have been increased above 2 folds during infection with *H. pylori* wild type, Δ*cagA* and Δ*vacA* isogenic mutant after 6h of infection. However, the fold changes of mRNA expression of NALP-3 in THP-1 cells were down regulated after 24h of infection with *H. pylori* and isogenic mutants. It has been reported that, detection of flagellin through IPAF induces activation of caspase-1 and IL-1β independently of TLR-5 in salmonella-infected and LPS-tolerized macrophages (Franchi L *et al,* 2006). IPAF mRNA expression in THP-1 cells were significantly increased above 3 folds during infection with Δ*cgt* and Δ*cagA* mutants after 6h. The fold changes of IPAF mRNA expression in THP-1 cells were reached 11, 9, 6, 5, 4 and 3 after 24h of infection with Δ*cag*PAI, wild type, Δ*cgt,* Δ*cagA,* Δ*flaA* and Δ*vacA,* respectively. ASC mRNA expression in THP-1 cells have down regulated significantly after 6h and 24h of infection with *H. pylori* and isogenic mutants (Fig. 10, 11 and Table. 6, 7).

3.9 Interleukin 1β and IL-18 gene expression, Caspase-1 activation and Interleukin 1β processing and secretion from THP1 monocytic cells during infection with *H. pylori* and isogenic mutants

IL-1β and IL-18 are proinflammatory cytokines important in the host defence against infection and in the pathogenesis of various inflammatory disorders. Both IL-1β and IL-18 are synthesized as inactive cytoplasmic precursors that are proteolytically processed to biologically active mature forms in response to various proinflammatory stimuli by caspase-1 (Yu HB and Finlay BB, 2008). Although the intracellular signalling pathways leading to caspase-1 activation remain poorly defined, studies have suggested involvement of members of the NLRs in the regulation of caspase-1 activation. The N-terminal regions of NALP-3 and NALP-1 comprise a pyrin domain, whereas NOD-1, NOD-2 and IPAF contain a caspase-recruitment domain. The pyrin and caspase-recruitment domains of NALP-3 and IPAF associate with those of ASC, an adaptor

molecule that interacts with the caspase recruitment domain of pro-caspase-1. NALP-3 NALP-1 and IPAF can form an endogenous multiprotein complex 'inflammasome' containing ASC and caspase-1, that promotes caspase-1 activation and processing of pro- IL-1β. ASC is critical for caspase-1 activation and secretion of mature IL-1β and IL-18 in response to several purified microbial components as well as intracellular bacteria (Martinon F and Tschopp J, 2004; Martinon F *et al,* 2002).

As analysis of functional status of inflammasome formation, IL-1β processing and secretion along with the IL-1β mRNA expression were analyzed. IL-1β mRNA expressions was increased to 12, 24, 15, 16, 29 and 8 folds in THP-1 cells after 6h of infection with *H. pylori* wild type and isogenic mutants Δ*cagA,* Δ*vacA,* Δ*flaA,* Δ*cgt* and Δ*cag*PAI, respectively. The level of IL-1β mRNA expressions has subsequently been increased to 55, 50, 44, 39, 32 and 30 folds after 24h of infection with *H. pylori* and isogenic mutants, respectively. IL-18 mRNA expressions were not significantly changed after 6h of infection with *H. pylori* and isogenic mutants. However, IL-18 mRNA expression was significantly down-regulated after 24h of infection with *H. pylori* and isogenic mutants (Fig.12, 13 and Table 8, 9). The intracellular IL-1 β in THP-1 cells during infection with *H. pylori* and isogenic mutants were analyzed by Western blot. The protein level of IL-1 β was not changed significantly, during the infection of *H. pylori* and isogenic mutants with THP-1 cells, after 8h (Fig.14). To answer the question, whether caspase-1 activation involved in the processing of Pro- IL-1 β in THP-1 cells, analysis were performed using antibody that detect the Pro-caspase-1 and p20 fragment of activated caspase-1. It was observed that Pro-caspase-1 cleavage was inhibited during infection with *H. pylori* and isogenic mutant strains Δ*vacA* and Δ*flaA*. On the contrary, Pro-caspase-1 cleavage was not inhibited in the case of Δ*cagA,* Δ*cgt* and Δ*cag*PAI infected cells (Fig.15). However, there was no significant change in the process of Pro-IL-1β cleavage to mature IL-1β in THP-1 cells during infection with *H. pylori* and all isogenic mutants tested. This clearly reflects the severe derangement of inflammasome



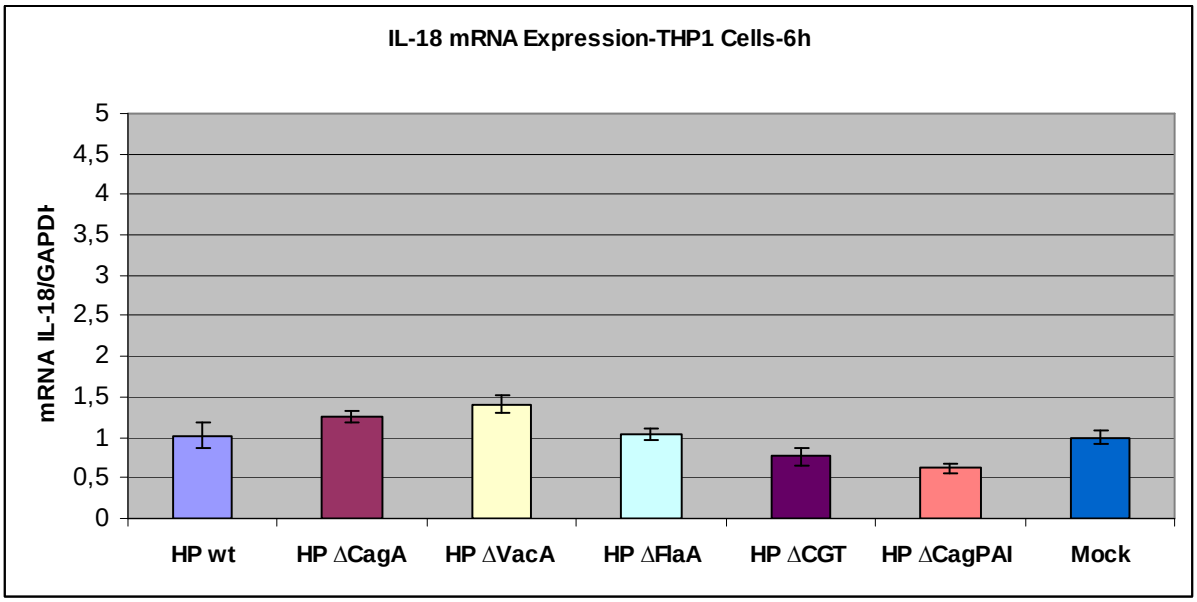
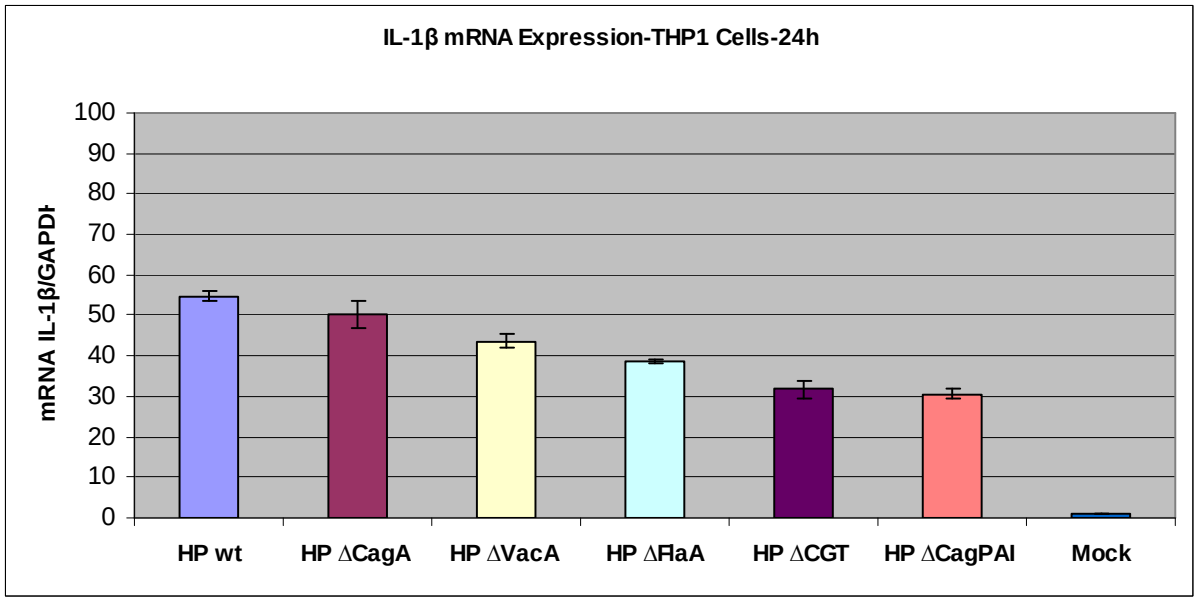


Table.8

	IL-1β	±SD	IL-18	±SD
HP wt	11.87*	0.93	1.02	0.16
HP Δ*CagA*	23.91*#	2.34	1.25*	0.07
HP Δ*VacA*	14.62*	1.43	1.41*	0.11
HP Δ*FlaA*	16.11*	1.42	1.04	0.07
HP Δ*CGT*	29.30*#	2.23	0.77*	0.11
HP Δ*Cag*PAI	8.16*	0.32	0.62*	0.06
Mock	1.00	0.04	1.00	0.08

Figure. 12 Fold changes of IL-1β and IL-18 mRNA expression in THP1 cells after 6h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*



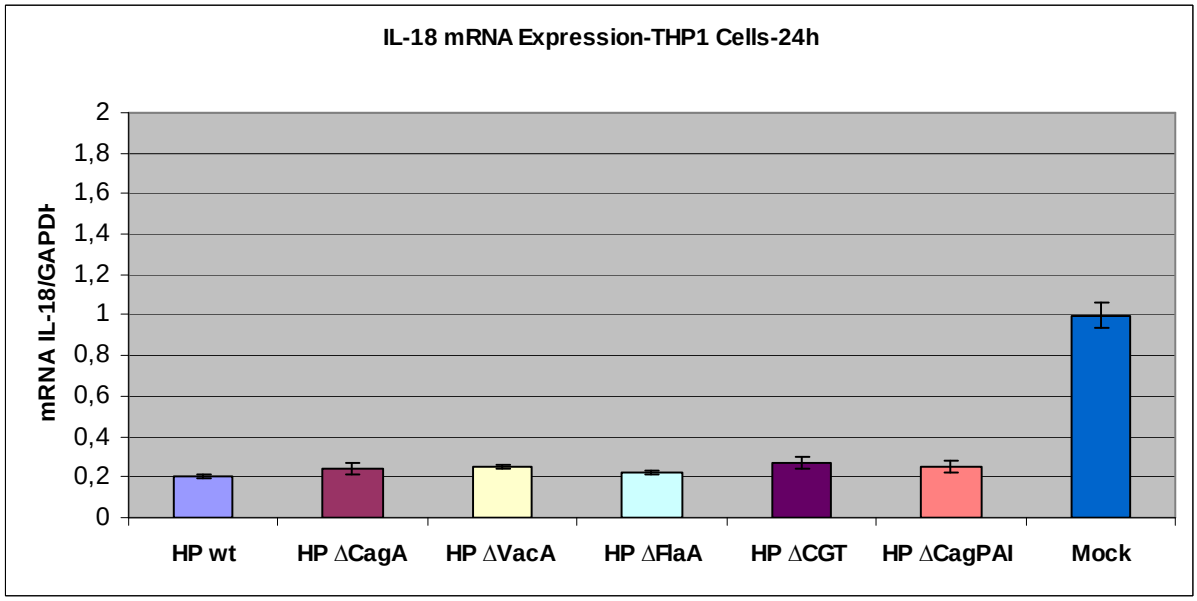


Table.9

	IL-1β	±SD	IL-18	±SD
HP wt	54.76*	1.07	0.20*	0.01
HP Δ*CagA*	50.04*	3.42	0.24*	0.03
HP Δ*VacA*	43.71*#	1.71	0.25*	0.01
HP Δ*FlaA*	38.58*#	0.37	0.22*	0.01
HP Δ*CGT*	31.86*#	2.19	0.27*	0.03
HP Δ*Cag*PAI	30.66*#	1.20	0.25*	0.03
Mock	1.00	0.07	1.00	0.06

Figure. 13 Fold changes of IL-1β and IL-18 mRNA expression in THP1 cells after 24h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylor*

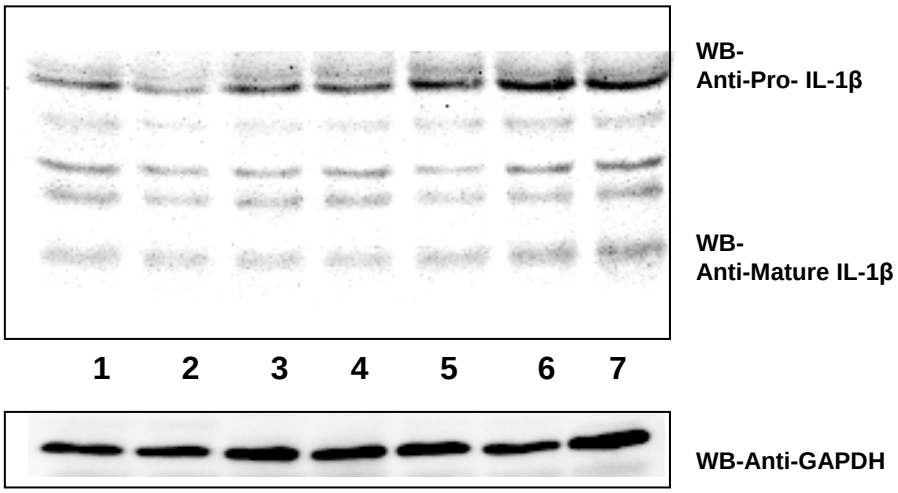


Figure.14 Western blot analysis of Pro-IL-1β and mature form of IL-1β from THP1 cells infected with *H. pylori* and mutants of *cagA, vacA, flaA, cgt and cag*PAI for 8h (**lane.1** -*H. pylori* wild type, **lane.2** Δ*cagA*, **lane.3**-Δ*vacA*, **lane.4** Δ*flaA*, **lane.5** Δ*cgt*, **lane.6** Δ*cag*PAI and **lane.7** uninfected control (Mock)). Western blot analysis for house keeping gene GAPDH was used as loading control.

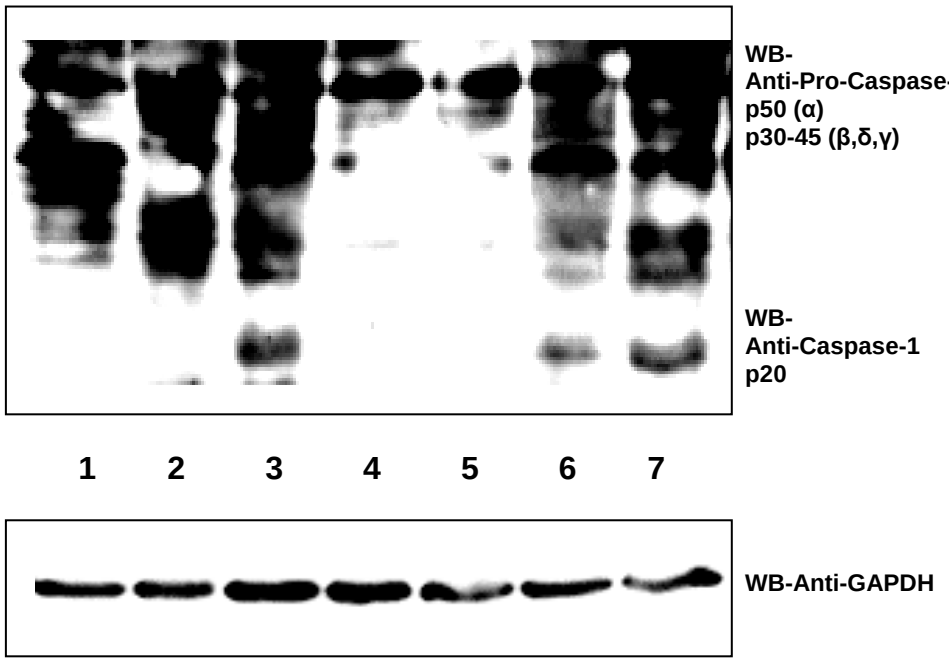


Figure.15 Western blot analysis of Pro-Caspase-1 and the Caspasw-1 p20 subunit from THP1 cells infected with *H. pylori* and mutants of *cagA, vacA, flaA, cgt and cag*PAI for 8h (**lane.1** uninfected control (Mock), **lane.2** -*H. pylori* wild type, **lane.3** Δ*cagA*, **lane.4**-Δ*vacA*, **lane.5** Δ*flaA*, **lane.6** Δ*cgt*, and **lane.7** Δ*cag*PAI). Western blot analysis for house keeping gene GAPDH was used as loading control.

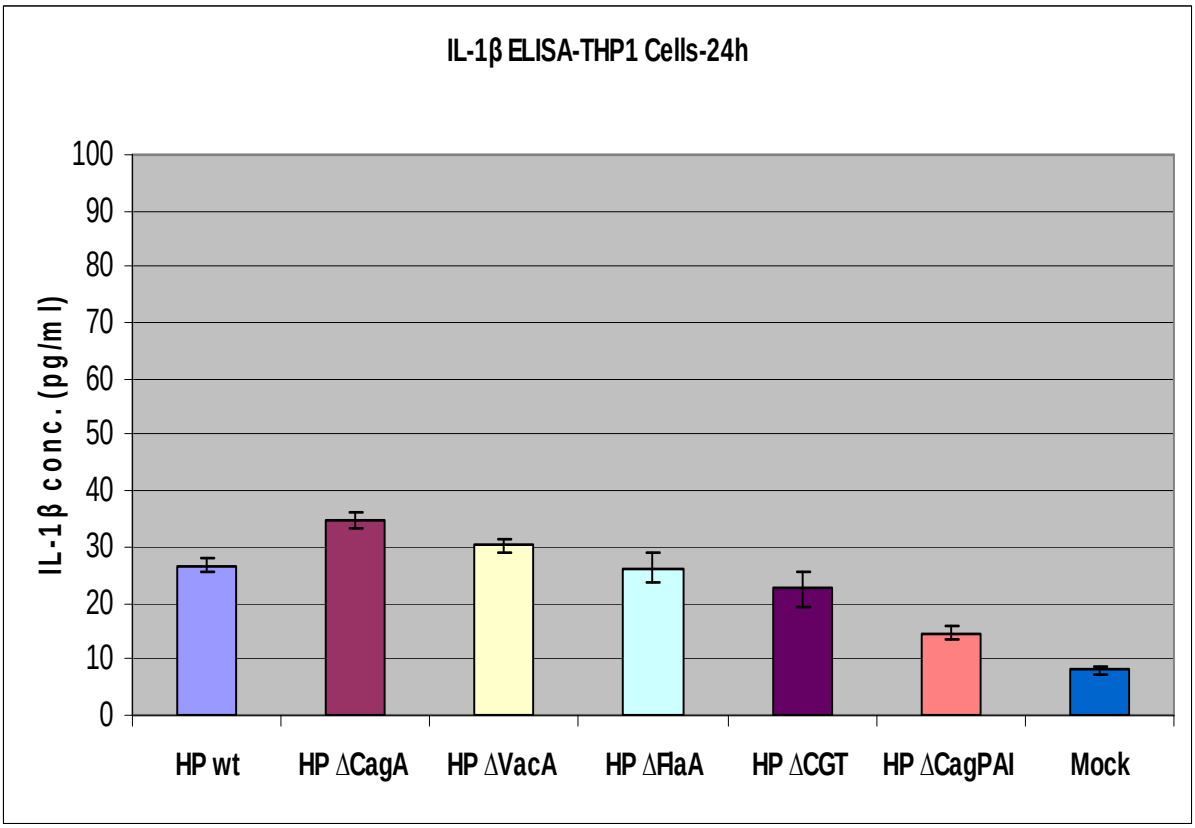


Table.10

	IL-1β (pg/ml)	±SD
HP wt	26.76*	1.32
HP Δ*CagA*	34.87*#	1.32
HP Δ*VacA*	30.23*	1.32
HP Δ*FlaA*	26.32*	2.65
HP Δ*CGT*	22.55*	3.28
HP Δ*Cag*PAI	14.72*#	1.14
Mock	8.13	0.66

Figure. 16 Concentration of IL-1β secreted from THP1 cells after 24h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. IL-1β concentration in the culture supernatants were analyzed by ELISA. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.005 when compared to the uninfected mock cells to the infected cells. # = P≤0.005 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*

mediated caspase-1 activation and mature IL-1β formation during *H. pylori* infection in THP-1 cells.

The secretion of mature IL-1β from THP-1 cells after infection with *H. pylori* and isogenic mutants was analyzed. The level of IL-1β secretions was not high, although

Δ*cagA* and Δ*vacA* isogenic mutants infected cells significantly increased the secretion of IL-1β than wild type infected cells. The other isogenic mutants such as Δ*flaA*, Δ*cgt*, Δ*cag*PAI infected THP-1 cells secreted less amounts of IL-1β than wild type infected cells and Δ*cag*PAI exerted the least induction of IL-1β secretion (Fig.16 and Table 10).

3.10 Effect of Src Kinase inhibitor PP2, Pan-caspase Inhibitor Z-VAD-FMK and EGFR Kinase inhibitor-AG1478 on Interleukin 1 beta secretion from THP1 monocytic cells during infection with *H. pylori* P12 and isogenic mutants

H. pylori use the type IV secretion system to inject *cagA* protein to the cytoplasm of host cells during infection. *cagA* being phosphorylated within the host cells and initiate the cytoskeletal rearrangements leading to hummingbird or scattered phenotype. c-SRC and ABL family kinase are responsible for *cagA* phosphorylation within the host cell and PP2, c-Src kinase inhibitor, specifically blocks cytoskeletal rearrangements and thereby hummingbird or scattered phenotype in epithelial cells (Selbach M *et al*, 2002; Stein M *et al*, 2002; Poppe *et al*, 2007; Tammer *et al*, 2007). The epidermal growth factor receptor (EGFR; ErbB1) belongs to the ErbB group of receptor tyrosine kinases, which also include ErbB2, ErbB3, and ErbB4. The EGFR is a 170-kDa transmembrane glycoprotein that normally plays an important role in cell proliferation, differentiation, and growth; however, abnormal expression of the EGFR has been described in many human tumors and has been implicated in the development and prognosis of malignancies (Zhang H *et al,* 2007). *H. pylori* infection with MKN-1, ST42, and MKN-28 gastric epithelial tumour cells results in the activation of HB-EGF gene expression and EGFR tyrosine phosphorylation, which enhanced IL-8 production (Wallasch C *et al,* 2002). *H. pylori* induce increased production of EGFR through various signal transduction pathways, including those mediated by the EGFR and AP-1 (Ashktorab H *et al,* 2007). Incubation of gastric epithelial cells with *H. pylori* causes a time and concentration dependent induction of DNA fragmentation, cleavage of BID, release of cytochrome c and a concomittant sequential activation of caspase-9, caspase-8, caspase-6, and caspase-3 but no effect on caspase-1 and -7 were observed in an earlier study (Potthoff A *et al,* 2002).

H. pylori is known to regulate some of the host cellular signalling mechanisms and thereby able to change the phenotype of the cells and that may be a reason for causing MALT lymphoma and gastric cancer. The inhibitory effect of *H. pylori* on IL-1β production and secretion in THP-1 cells was not reported earlier. In the present study, *H. pylori* induced high level of IL-1β mRNA expression in THP-1 cells; however, the secreted levels determined by ELISA were below 50pg/ml during infection with *H. pylori* and isogenic mutants. The influence of known *H. pylori* induced kinase activities in host cells and apoptosis on IL-1β processing and secretion by treating the cells with specific inhibitors, such as c-Src kinase inhibitor-PP2, EGFR kinase inhibitor-AG1478 and pan-caspase inhibitor-Z-VAD-FMK, thirty minutes prior to infection with *H. pylori* wild type was investigated. The IL-1β secretion from THP1 cells, treated with inhibitors as stated above, after 8h, 24h and 32h of infection with *H. pylori* wild type was measured. c-Src inhibitor-PP2 treated cells had secreted significantly higher amount of IL-1β early after 8h of infection, whereas, levels of IL-1β remain unchanged in cells treated with EGFR kinase inhibitor-AG1478, pan-caspase inhibitor-Z-VAD-FMK and wild type *H. pylori* treated cells. It was also observed that a significant level of increase in secretion of IL-1β after 24h of infection with wild type strain. Pan-caspase inhibitor treated cells infected with *H. pylori* also secreted higher IL-1β at 24h of infection. However EGFR kinase inhibitor treated cells infected with *H. pylori* had not enhanced IL-1β secretion after 24h of infection. c-Src kinase inhibitor treated cells infected with *H. pylori* continued to increase the IL-1β secretion after 24h of infection. Pan-caspase inhibitor treated cells infected with *H. pylori* P12 have secreted slightly higher amount than the untreated cells infected with *H. pylori* after 32h of infection. On the contrary, EGFR kinase inhibitor treated cells infected with *H. pylori* had down-regulated the secretion of IL-1β after 32 h of infection. However, c-Src kinase inhibitor treated cells continued to enhance IL-1β secretion even after 32h of infection (Fig.17).

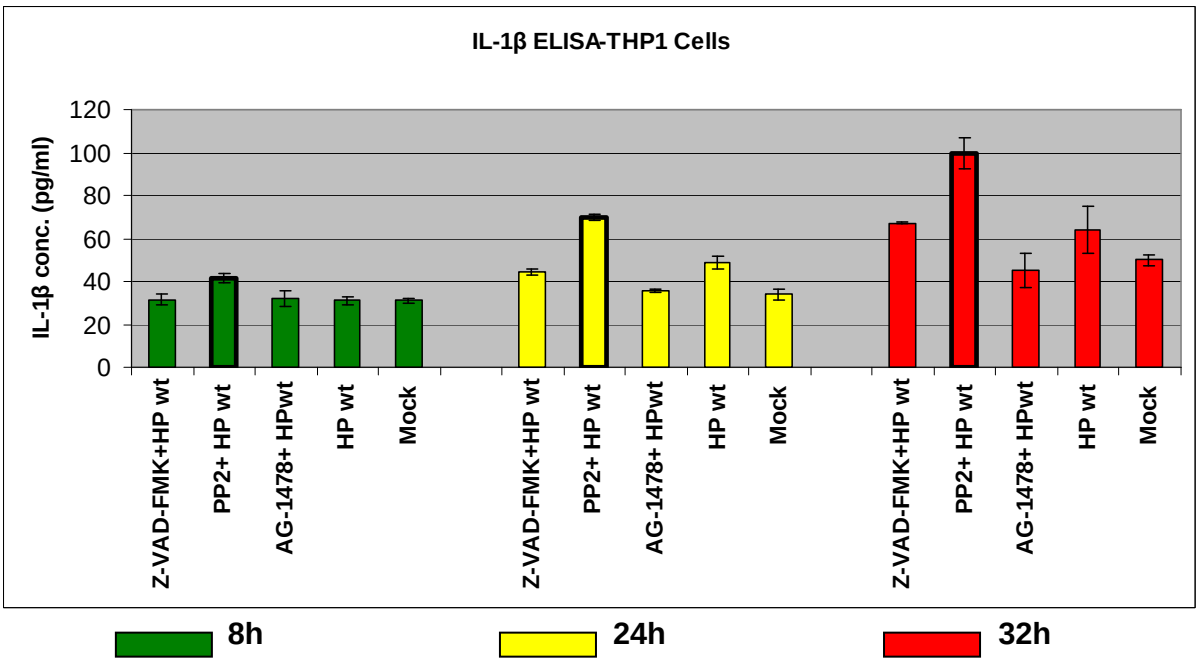


Table.11

	IL-1β (8h) (pg/ml)	±SD	IL-1β (24h) (pg/ml)	±SD	IL-1β (32h) (pg/ml)	±SD
Z-VAD-FMK+HP wt	31.50	2.37	44.29*	1.74	67.20*	0.63
PP2+ HP wt	41.23*	2.16	69.98*#	1.50	99.70*#	7.55
AG-1478+ HPwt	32.2	3.73	35.39#	0.83	45.12	8.15
HP wt	31.09	1.96	48.59*	3.12	63.87	10.83
Mock	31.09	1.04	34.01	2.51	49.97	2.60

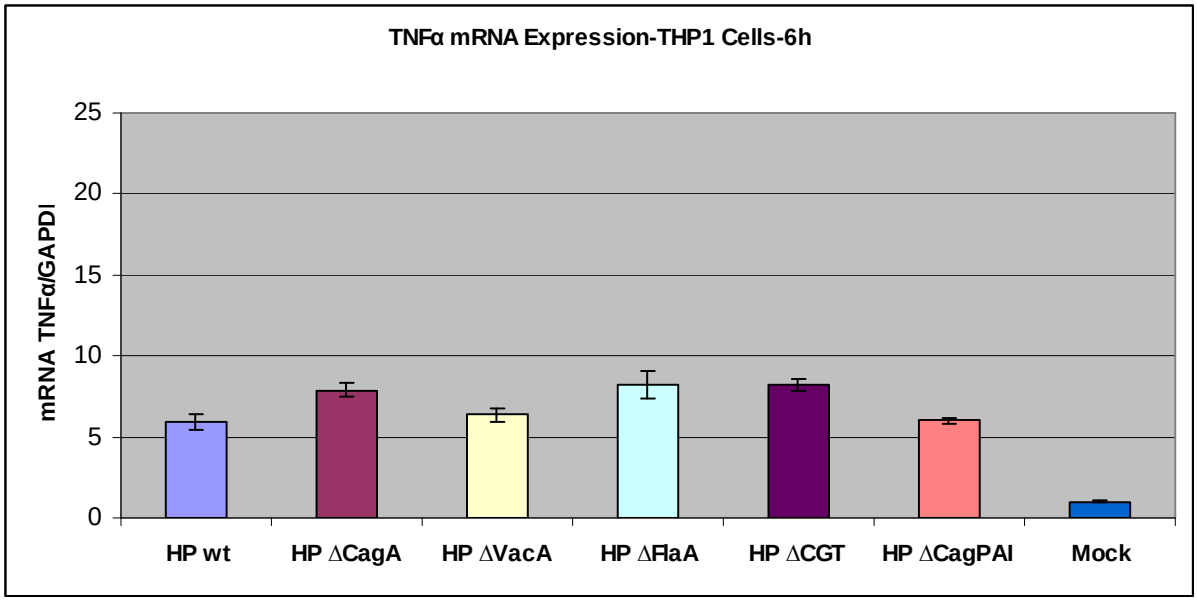
Figure. 17 Concentration of IL-1β secreted from THP1 cells infected with *H. pylori* wild type and THP1 cells pre-treated with pan caspase inhibitor (Z-VAD-FMK-20µM), SRC inhibitor (PP2-20µM) and EGFR kinase inhibitor (AG1478-20µM) infected with *H. pylori* wild type after 8h, 24h and 32h of infection. IL-1β concentration in the culture supernatants were analyzed by ELISA. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.005 when compared to the uninfected mock cells to the infected cells. # = P≤0.005 when compared to *H. pylori* wild type infected cells with inhibitor treated cells infected with *H. pylori*. HP = *H. pylori*

3.11 Pro-inflammatory cytokines such as IL-6 and TNFα mRNA expression, TNFα and chemokine IL-8 secretion from THP1 monocytic cells during infection with *H. pylori* and isogenic mutants

H. pylori infection has been reported to be inducing pro-inflammatory changes through the secretion of mediators such as cytokines and chemokines. Pro-inflammatory cytokines such as IL-6, TNFα and chemokine IL-8 were reported to be increased in the gastric tissues of *H. pylori* infected patients. There are no reports regarding the influence of *H. pylori* virulence and pathogenecity factors on cytokine and chemokine gene expression and secretion during infection. In this study, the IL-6 and TNFα gene

expression in THP1 cells during infection with *H. pylori* and isogenic mutants Δ*cagA,* Δ*vacA,* Δ*flaA,* Δ*cgt* and Δ*cag*PAI were analyzed. The IL-6 mRNA expression in THP1 cells remain unchanged during infection with *H. pylori* and isogenic mutants Δ*vacA,* Δ*flaA,* Δ*cag*PAI, whereas, Δ*cagA* and Δ*cgt* mutants have induced 3.2 and 3.8 fold changes in IL-6 mRNA expression after 6h of infection, respectively. IL-6 mRNA expression levels were increased to 22, 14, 11 and 16 folds in THP1 cells infected with *H. pylori* wild type and isogenic mutants Δ*vacA,* Δ*flaA,* Δ*cag*PAI after 24h of infection. Δ*cagA* and Δ*cgt* mutant strains continued to increase IL-6 mRNA expression and this was found to be 33 and 32 folds, respectively, after 24h of infection. TNFα mRNA expressions in THP1 cells were observed to be 6, 8, 6, 8, 8 and 6 folds, respectively, after 6h of infection with wild type, Δ*cagA,* Δ*vacA,* Δ*flaA,* Δ*cgt* and Δ*cag*PAI. A decrease in the expression TNFα mRNA was noted with all strains tested except for Δ*cag*PAI infected cells (Fig.18, 19 and Table 12, 13).

The secretion of TNFα and IL-8 from THP1 cells infected with *H. pylori* and isogenic mutants after 24h were analyzed by ELISA. *H. pylori* wild type and Δ*cgt* mutant infected THP1 cells have secreted significantly high amount of TNFα after 24h of infection. Although, Δ*cagA* mutant infected cells also secreted significant amount of TNFα after 24h of infection but it was less than that of wild type strain. However, Δ*vacA* and Δ*flaA* mutant infected cells were secreted TNFα almost 5 times less than wild type infected cells and Δ*cag*PAI mutant was the least inducer of TNFα secretion. IL-8 secretion from THP1 cells during infection with *H. pylori* and isogenic mutants were also analyzed. *H. pylori* wild type and isogenic mutants Δ*cagA,* Δ*vacA* and Δ*cgt* infected THP1 cells secreted almost similar amounts of IL-8 after 24h of infection and Δ*flaA* mutant infected cells secreted significantly less amount than wild type infected cells. In this case also Δ*cag*PAI was the least inducer of IL-8 from infected THP1 cells (Fig.20 and Table 14).



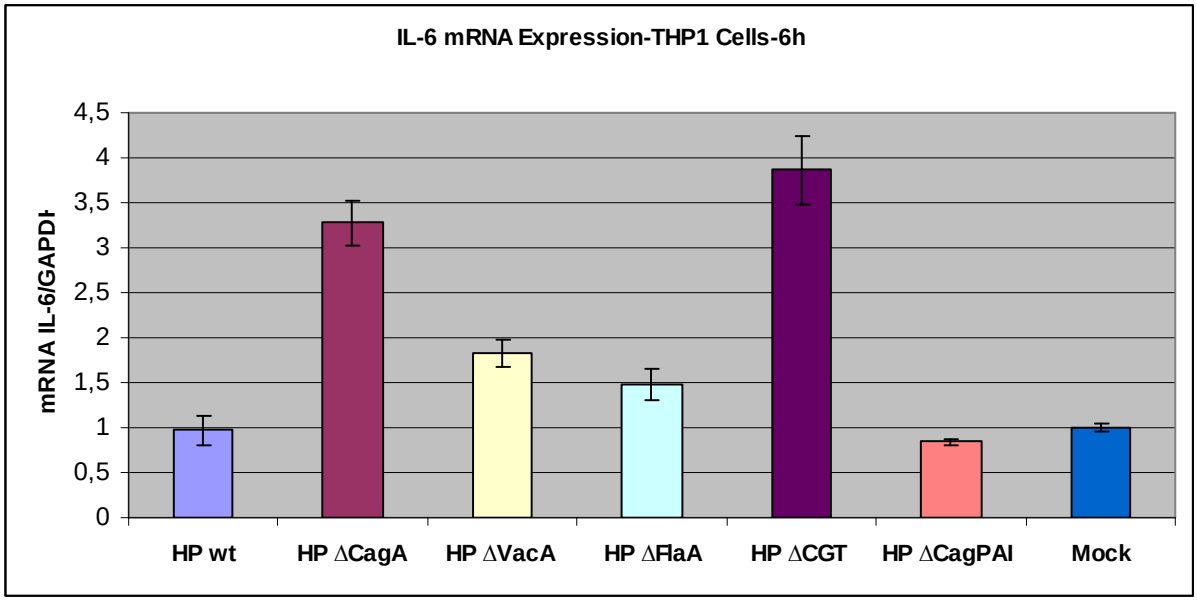
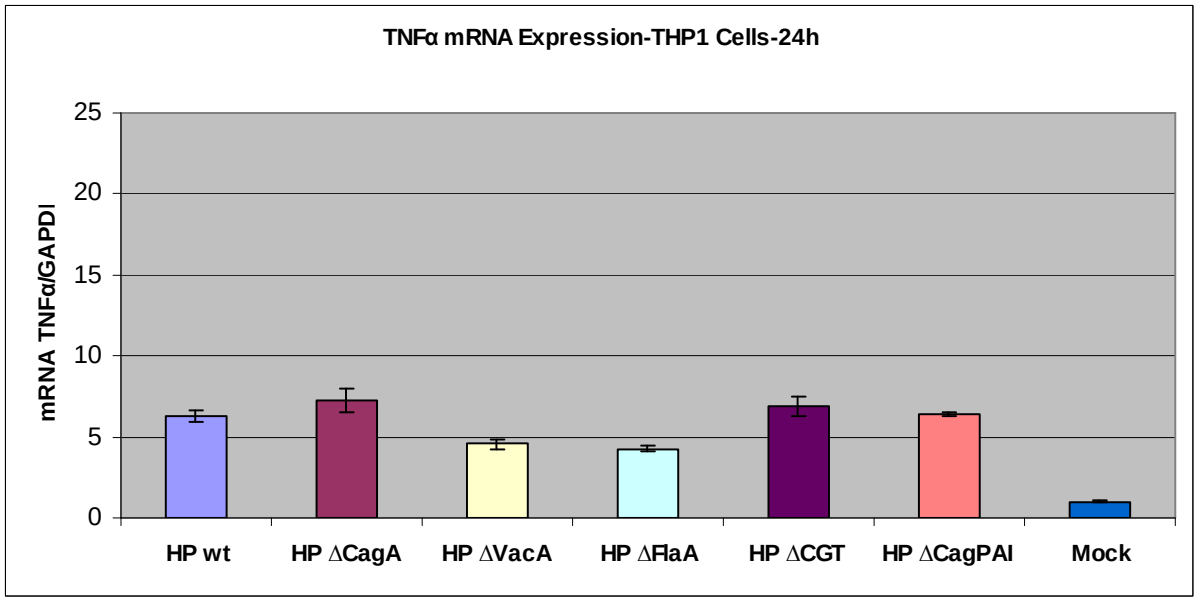


Table.12

	TNFa	±SD	IL-6	±SD
HP wt	5.93*	0.53	0.97	0.17
HP Δ*CagA*	7.91*#	0.39	3.28*#	0.25
HP Δ*VacA*	6.36*	0.44	1.82#	0.15
HP Δ*FlaA*	8.22*#	0.81	1.48#	0.18
HP Δ*CGT*	8.21*#	0.32	3.86*#	0.38
HP Δ*Cag*PAI	5.98*	0.23	0.84*	0.03
Mock	1.00	0.04	1.00	0.04

Figure. 18 Fold changes of TNFα and IL-6 mRNA expression in THP1 cells after 6h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*



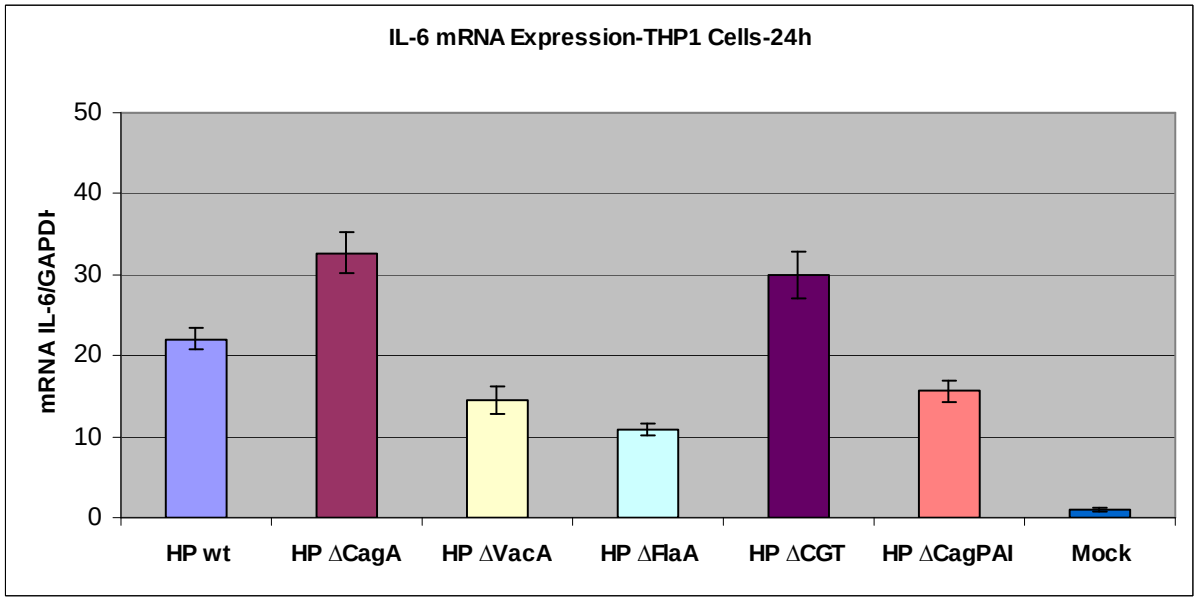
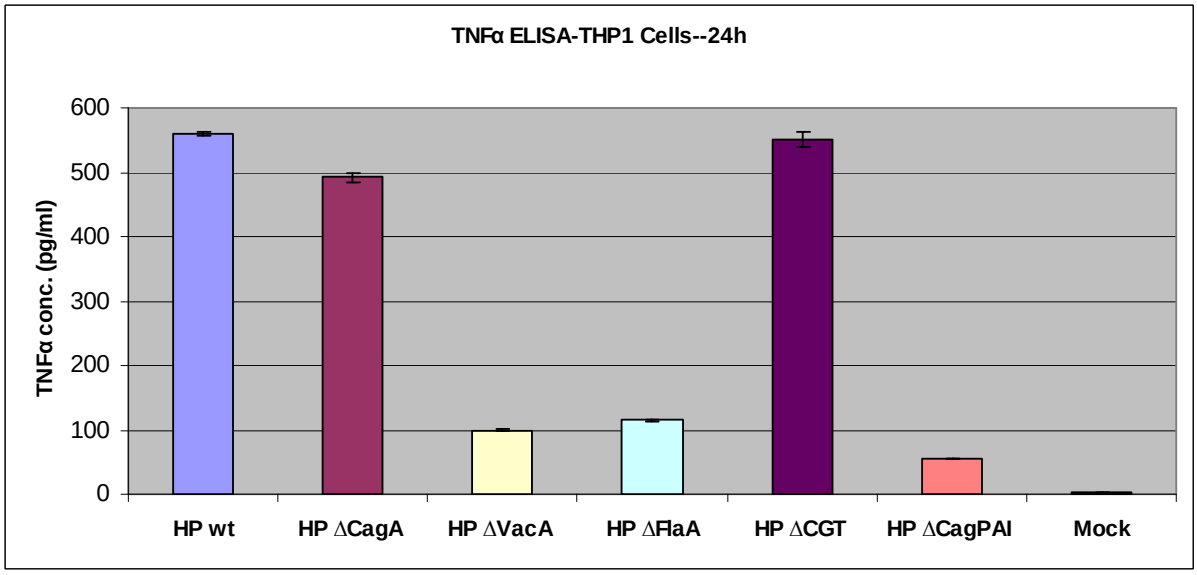


Table.13

	TNFa	±SD	IL-6	±SD
HP wt	6.25*	0.37	22.08*	1.29
HP Δ*CagA*	7.28*	0.71	32.67*#	2.56
HP Δ*VacA*	4.57*#	0.31	14.42*#	1.70
HP Δ*FlaA*	4.24*#	0.17	10.85*#	0.74
HP Δ*CGT*	6.90*	0.61	30.02*#	2.95
HP Δ*Cag*PAI	6.40*	0.13	15.60*#	1.37
Mock	1.00	0.08	1.00	0.21

Figure. 19 Fold changes of TNFα and IL-6 mRNA expression in THP1 cells after 24h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*



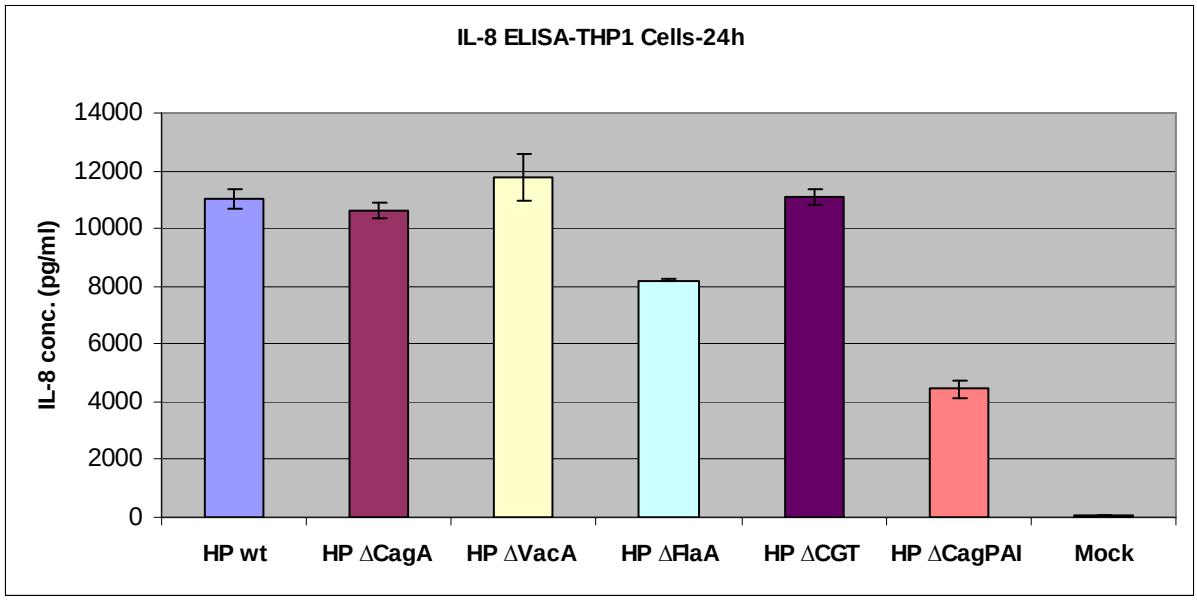


Table.14

	TNFα (pg/ml)	±SD	IL-8 (pg/ml)	±SD
HP wt	560.08*	2.34	11,020*	332
HP Δ*CagA*	491.39*#	6.83	10,628*	256
HP Δ*VacA*	98.99*#	1.62	11,773*	788
HP Δ*FlaA*	115.06*#	2.20	8,213*#	16.7
HP Δ*CGT*	550.93*	10.61	11,090*	300
HP Δ*Cag*PAI	54.57*#	0.46	4,460*#	307
Mock	1,47	0.26	68.5	14.4

Figure. 20 Concentrations of TNFα and IL-8 secreted from THP1 cells after 24h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. TNFα and IL-8 concentrations in the culture supernatants were analyzed by ELISA. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.005 when compared to the uninfected mock cells to the infected cells. # = P≤0.005 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*

3.12 IRAK1 (ser376) phosphorylation and IL-1beta, IL-6, IL-18, TNFalpha and IL-8 gene expression and secretion in HEK293, HEK293-TLR2, HEK293-TLR5 and HEK293-TLR10-HA cells during infection with *H. pylori* and isogenic mutants

HEK293 cells are reported to be having less or no expression of TLR molecules and which gives a clear background for studying the effect of individual TLR molecules through gene overexpression mechanism. In the present investigation, the analysis of TLR2, TLR5 and TLR10 expression and signalling initiation through IRAK1ser376 phosphorylation in HEK293-TLR2, HEK293-TLR5 and HEK293-TLR10 were performed. The role of some individual TLR molecules over-expressed in HEK293 cells and their involvement in IRAK1ser376 phosphorylation and subsequent pro-inflammatory gene expression during infection with *H. pylori* P12 and isogenic mutants were analyzed.

HEK293-TLR2 cells have induced IRAK1ser376 phosphorylation when infected with *H. pylori* and isogenic mutants almost with the same intensity as shown by western blot (Fig. 21). This reveals that the ligand responsible for TLR-2 induction is not significantly affected by mutations in the virulence or pathogenecity genes tested. In concordance with IRAK-1 phoshorylation, TNFα mRNA expression was also significantly increased in cells infected with *H. pylori* and isogenic mutants (Fig.23). TNFα secretions from HEK293-TLR2 cells were also significantly increased during infection with *H. pylori* and isogenic mutants (Fig.24). The IL-1β mRNA expressions were also increased in HEK293-TLR2 cells during infection with *H. pylori* and isogenic mutants (Fig.22). However, IL-6 and IL-18 mRNA expressions were significantly down-regulated during infection *H. pylori* (Fig.22, 23 and and Table 15, 16). The IL-8 secretion from HEK293-TLR2 cells were also significantly enhanced during *H. pylori* infection (Fig.24 and and Table 17).

IRAK1 phosphorylation is increased in HEK293-TLR5 cells during *H. pylori* infection and found to be of similar intensity except in the case of Δ*cag*PAI mutant (Fig.21).

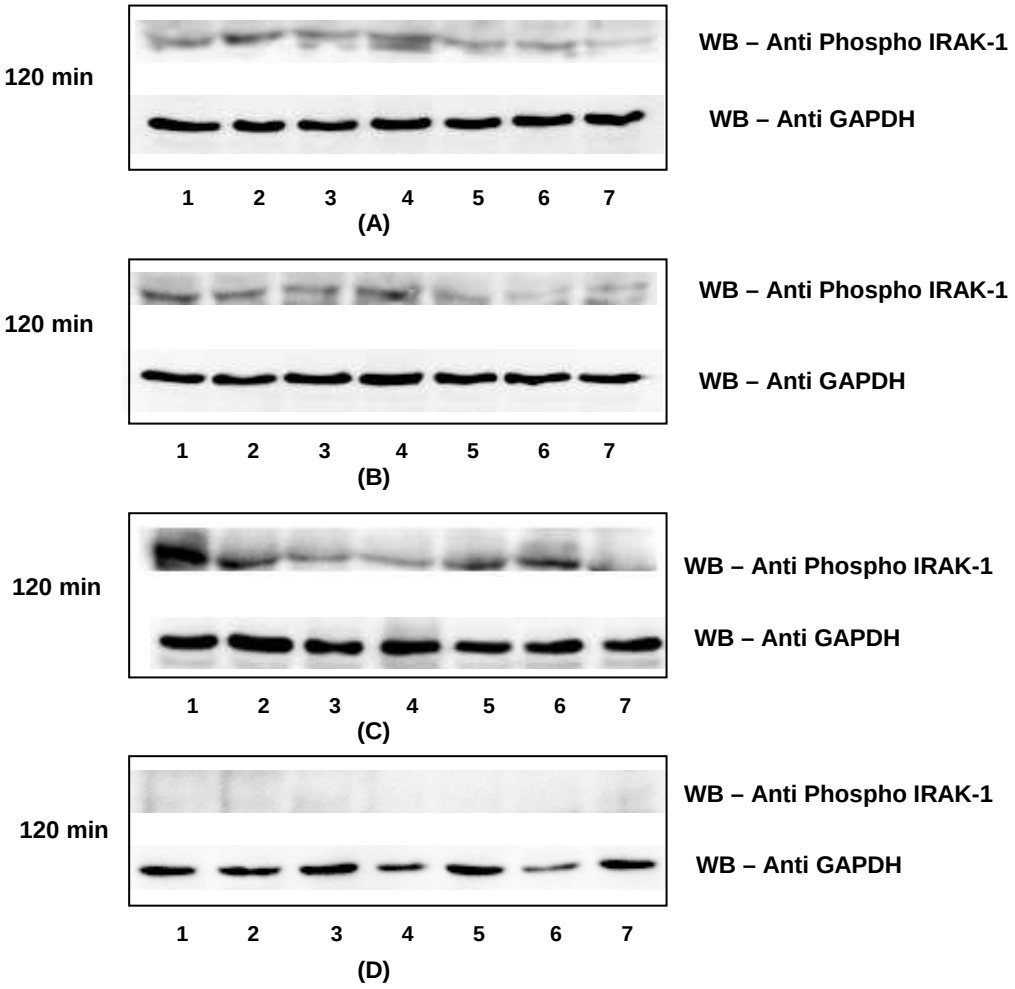
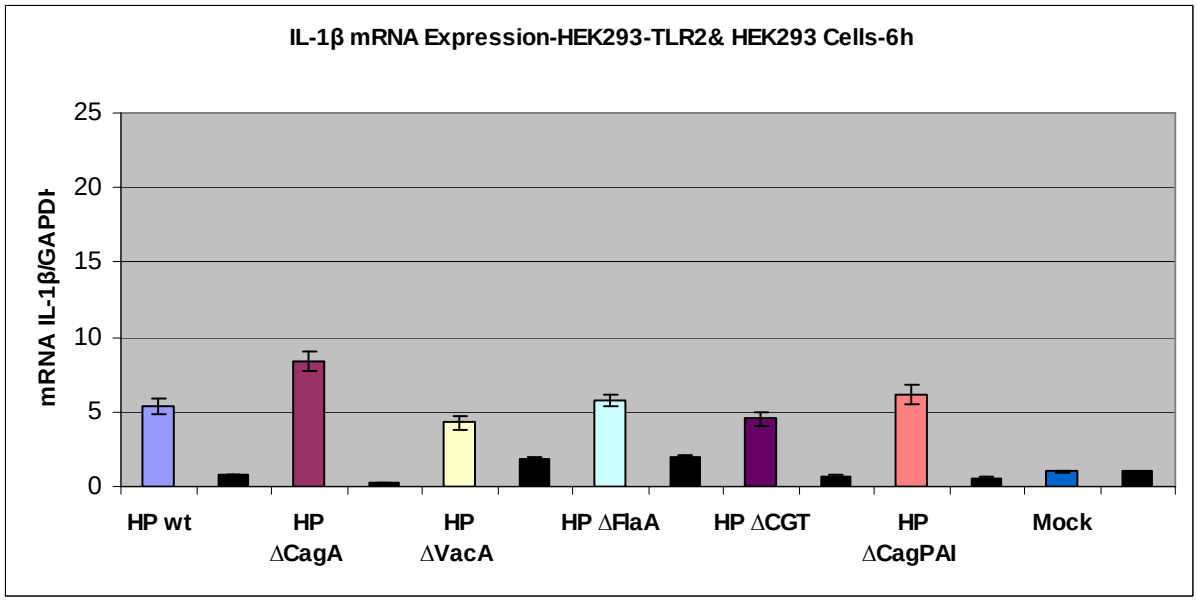


Figure.21 Western blot analysis of phospho IRAK-1 (Ser 376) from HEK293-TLR2 (A), HEK293-TLR5 (B), HEK293-TLR10-HA (C) and HEK293 (D) cells infected with *H. pylori* and mutants of *cagA, vacA, flaA, cgt and cag*PAI for 2h. (**lane.1** -*H. pylori* wild type, **lane.2** Δ*cagA*, **lane.3**-Δ*vacA*, **lane.4** Δ*flaA*, **lane.5** Δ*cgt*, **lane.6** Δ*cag*PAI and **lane.7** uninfected control). Western blot analysis for house keeping gene GAPDH was used as loading control.

TNFα mRNA expression was also significantly increased in HEK293-TLR5 cells during *H. pylori* infection and the lowest induction was found in Δ*cag*PAI mutant infected cells. IL-1β, IL-18 and IL-6 mRNA expressions were down-regulated in HEK293-TLR5 cells during *H. pylori* infection (Fig.25, 26 and and Table 18, 19). TNFα secretion was not significantly enhanced during *H. pylori* infection in HEK293-TLR5 cells. IL-8 secretions from HEK293-TLR5 cells were significantly high during infection with *H. pylori* and isogenic mutants. However Δ*flaA* infected cells was the least inducer of IL-8 among all the mutants tested (Fig.27 and and Table 20).

HEK293-TLR10 cells infected with *H. pylori* have significantly increased IRAK1 phosphorylation when compared to other isogenic mutants infected cells and uninfected cells, of which Δ*flaA* and Δ*vacA* mutants infected cells were least inducers of phosphorylation (Fig.21). In concordance with IRAK1 phosphoryaltion, TNFα mRNA expressions were significantly increased in HEK293-TLR10 cells infected with *H. pylori* when compared to other isogenic mutants infected cells. TNFα secretions were significantly increased in *H. pylori* wild type, Δ*cagA,* and Δ*cag*PAI infected cells in comparison with Δ*flaA*, Δ*vacA* and Δ*cgt* infected cells (Fig.30). However, IL-1β, IL-18 and IL-6 mRNA expressions were downregulated in HEK293-TLR10 cells during *H. pylori* infection (Fig. 28, 29 and Table 21, 22). IL-8 secretions were also significantly increased in HEK293-TLR10 cells during infection with *H. pylori* (Fig. 30 and Table 23).

HEK293 wild type cells infected with *H. pylori* and isogenic mutants have not shown any signal for phospho IRAK-1 in western blot analysis (Fig.21). This is confirms the less or no expression of TLR molecules in HEK293 cell lines. IL-1β, IL-6 and IL-18 mRNA expressions were down-regulated in HEK293 cells during infection with *H. pylori* and isogenic mutants. However, TNFα mRNA expressions in HEK293 cells were significantly increased during *H. pylori* infection and secreted double amount of TNFα in *H. pylori* infected cells. IL-8 secretions were also significantly increased in HEK293 during *H. pylori* infection (Figure. 22, 23 , 24 and Table 15, 16, 17). TNFα and IL-8 expression and its secretions increased during *H. pylori* infection in HEK293 cells and that was substantially enhanced in stably transfected HEK293-TLR2, HEK293-TLR5 and HEK293-TLR10 cells. In addition to the above HEK293-TLR2 cells exhibited increased expression of IL-1β mRNA in *H. pylori* and isogenic mutants infection. This reflects the essential role of different TLR molecules for a pro-inflammatory change during *H. pylori* infection.



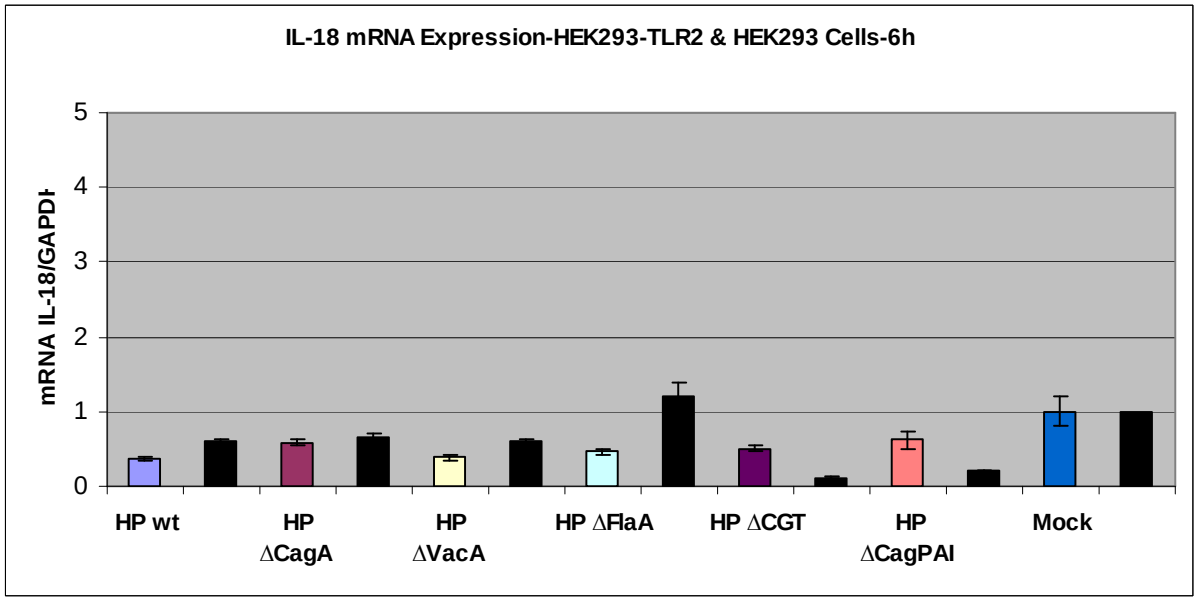
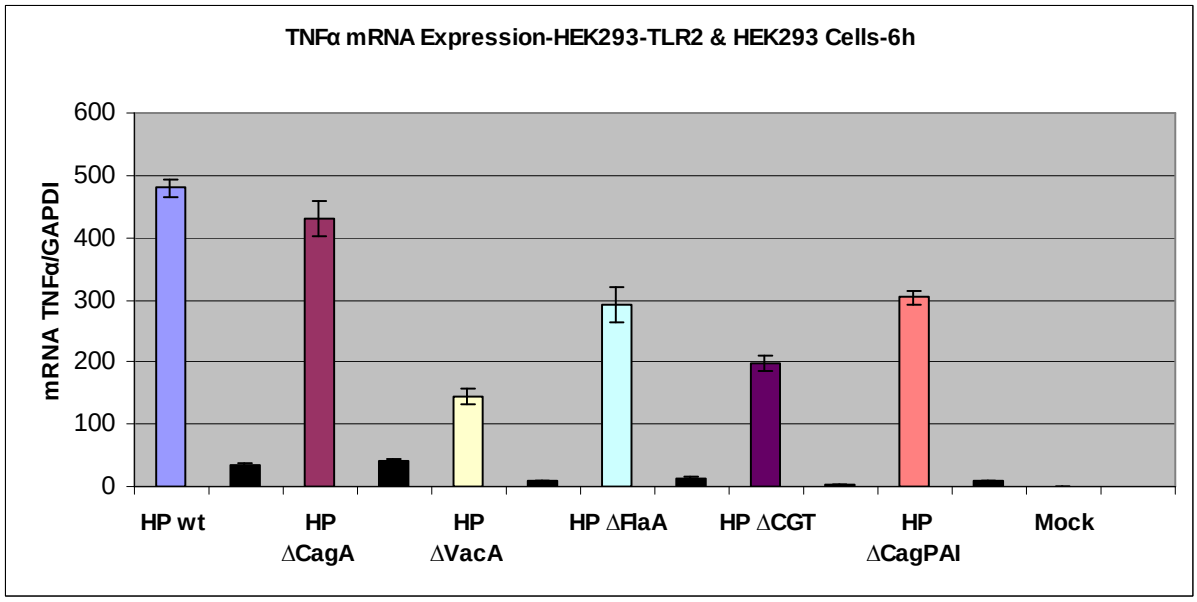


Table.15

	HEK293-TLR2		HEK293		HEK293-TLR2		HEK293	
	IL-1β	**±SD**	**IL-1β**	**±SD**	**IL-18**	**±SD**	**IL-18**	**±SD**
HP wt	5.35*	0.48	0.78	0.03	0.36	0.02	0.60	0.04
HP Δ*CagA*	8.33*#	0.65	0.21*#	0.03	0.58#	0.04	0.65	0.06
HP Δ*VacA*	4.28*#	0.42	1.77	0.19	0.39	0.04	0.61	0.03
HP Δ*FlaA*	5.73*#	0.39	2.02*#	0.10	0.46	0.04	1.21	0.18
HP Δ*CGT*	4.53*#	0.44	0.70*	0.07	0.50	0.04	0.11*#	0.01
HP Δ*Cag*PAI	6.10*	0.65	0.58*	0.07	0.62	0.12	0.21*#	0.01
Mock	1.00	0.04	1.00	0.08	1.00	0.20	1.00	0.11

Figure. 22 Fold changes of IL-1β and IL-18 mRNA expression in HEK293-TLR2 cells in comparison with HEK293 wild type cells (black bar) after 6h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*



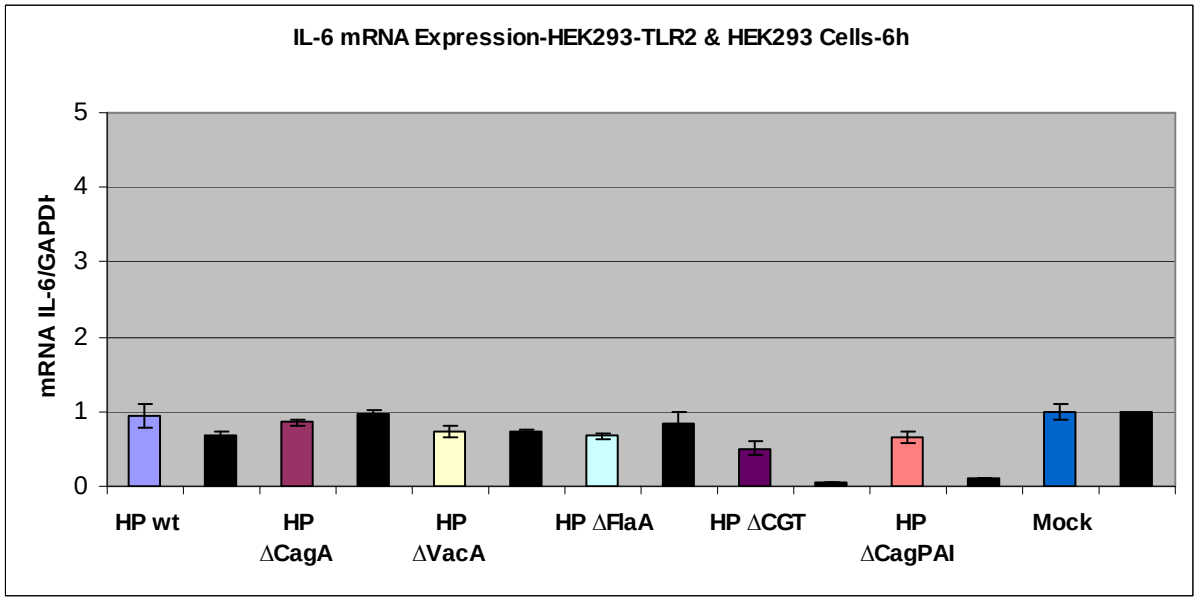
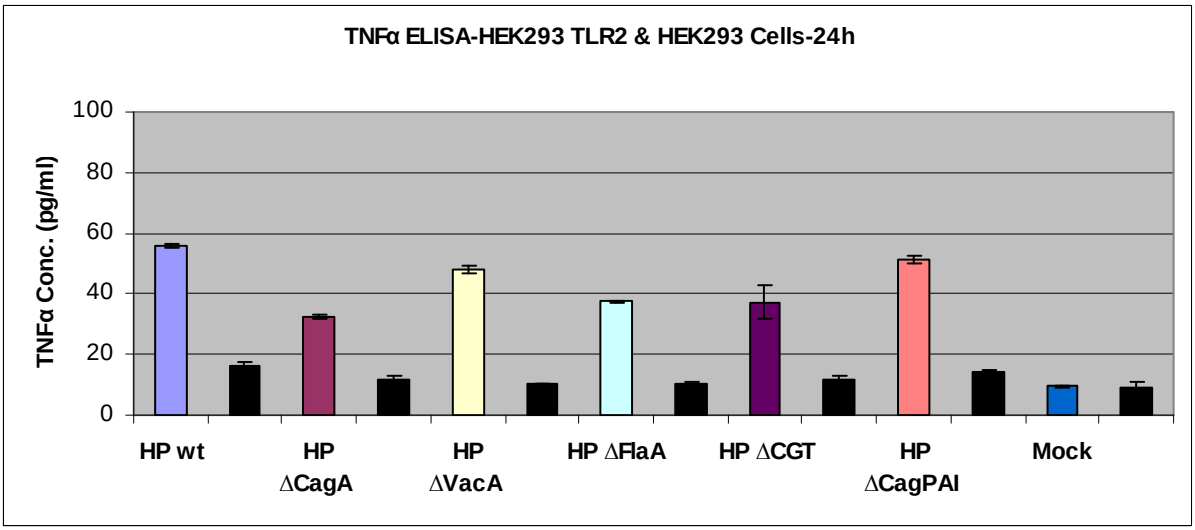


Table.16

	HEK293-TLR2		HEK293		HEK293-TLR2		HEK293	
	TNFα	**±SD**	**TNFα**	**±SD**	**IL-6**	**±SD**	**IL-6**	**±SD**
HP wt	479.30*	14.05	33.82*	3.32	0.94	0.16	0.69*	0.05
HP Δ*CagA*	430.50*	29.56	40.50*	2.38	0.86	0.04	0.96#	0.07
HP Δ*VacA*	143.01*#	12.62	9.81*#	0.38	0.73*	0.08	0.74	0.01
HP Δ*FlaA*	291.02*#	28.45	13.83*#	0.68	0.67*	0.04	0.83	0.16
HP Δ*CGT*	198.08*#	11.67	2.82*#	0.25	0.51*	0.08	0.05*#	0
HP Δ*Cag*PAI	303.38*#	11.86	9.06*#	1.07	0.66*	0.08	0.11*#	0
Mock	1.00	0.16	1.00	0.08	1.00	0.11	1.00	0.08

Figure. 23 Fold changes of TNFα and IL-6 mRNA expression in HEK293-TLR2 cells in comparison with HEK293 wild type cells (black bar) after 6h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*



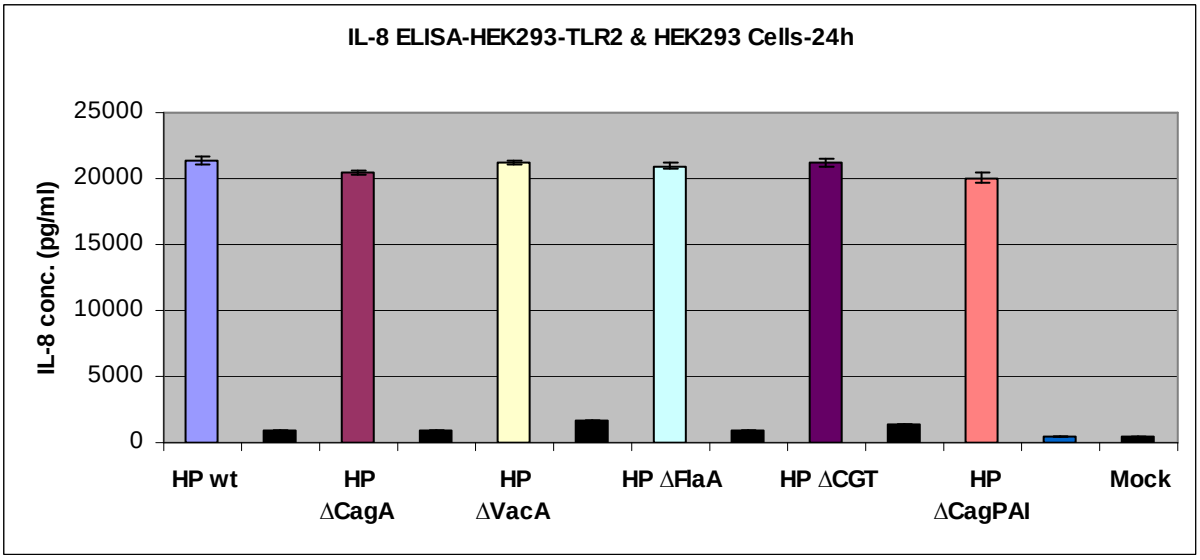
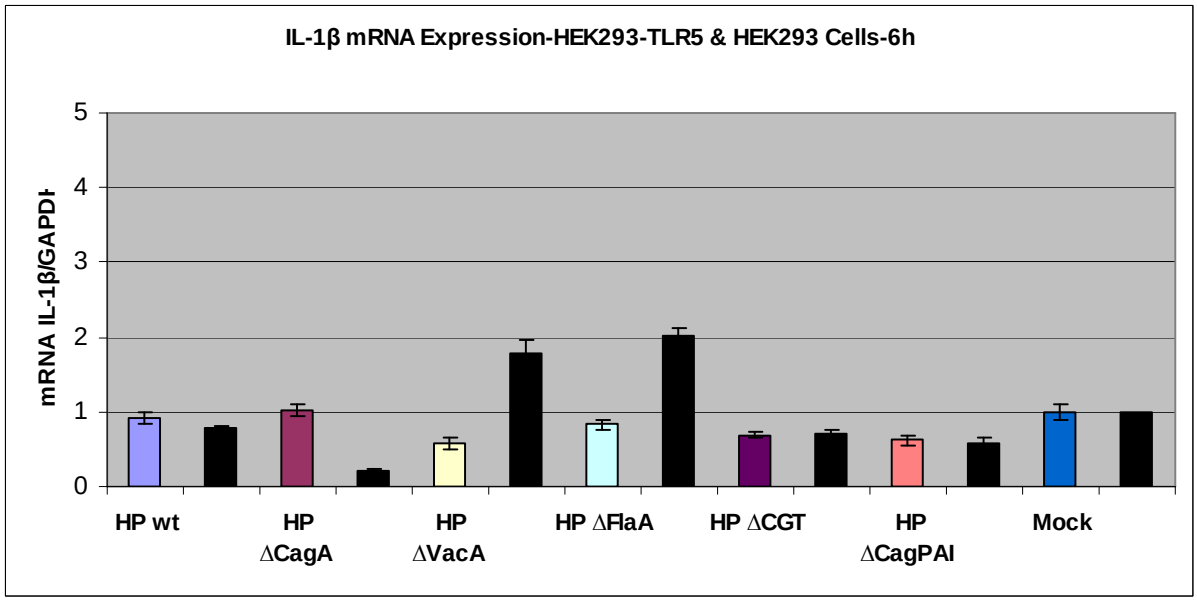


Table.17

	HEK293-TLR2		HEK293		HEK293-TLR2		HEK293	
	TNFα (pg/ml)	±SD	TNFα (pg/ml)	±SD	IL-8 (pg/ml)	±SD	IL-8 (pg/ml)	±SD
HP wt	55.56*	0.67	16.32*	1.37	21,325*	308	891*	28
HP Δ*CagA*	32.56*#	0.49	11.64#	1.13	20,474*#	158	924*	21
HP Δ*VacA*	47.82*#	1.34	10.35#	0.23	21,233*	156	1608*#	10
HP Δ*FlaA*	37.40*#	0.49	10.68#	0.22	20,935*	220	928*	17
HP Δ*CGT*	37.18*#	5.40	11.64#	1.13	21,230*	277	1391*#	11
HP Δ*Cag*PAI	51.37*	1.13	14.38	0.45	20,046*#	405	445*#	27
Mock	9.44	0.18	8.90	1.82	394	24	74	7

Figure. 24 Concentrations of TNFα and IL-8 secreted from HEK293-TLR2 cells in comparison with HEK293 wild type cells (black bar) after 24h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. TNFα and IL-8 concentrations in the culture supernatants were analyzed by ELISA. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.005 when compared to the uninfected mock cells to the infected cells. # = P≤0.005 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*



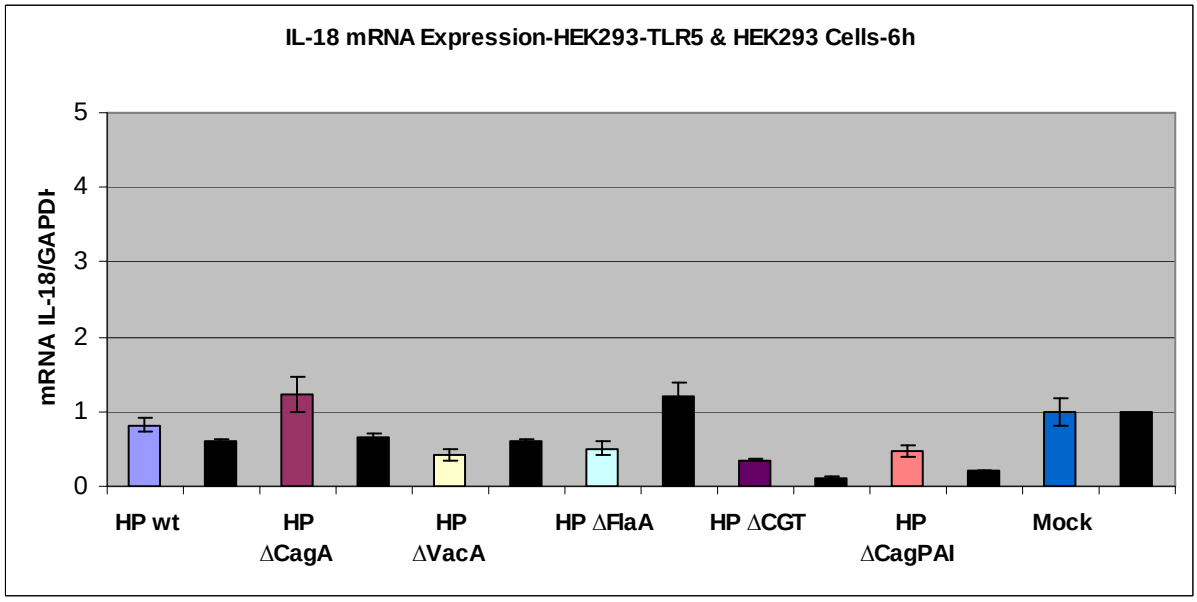
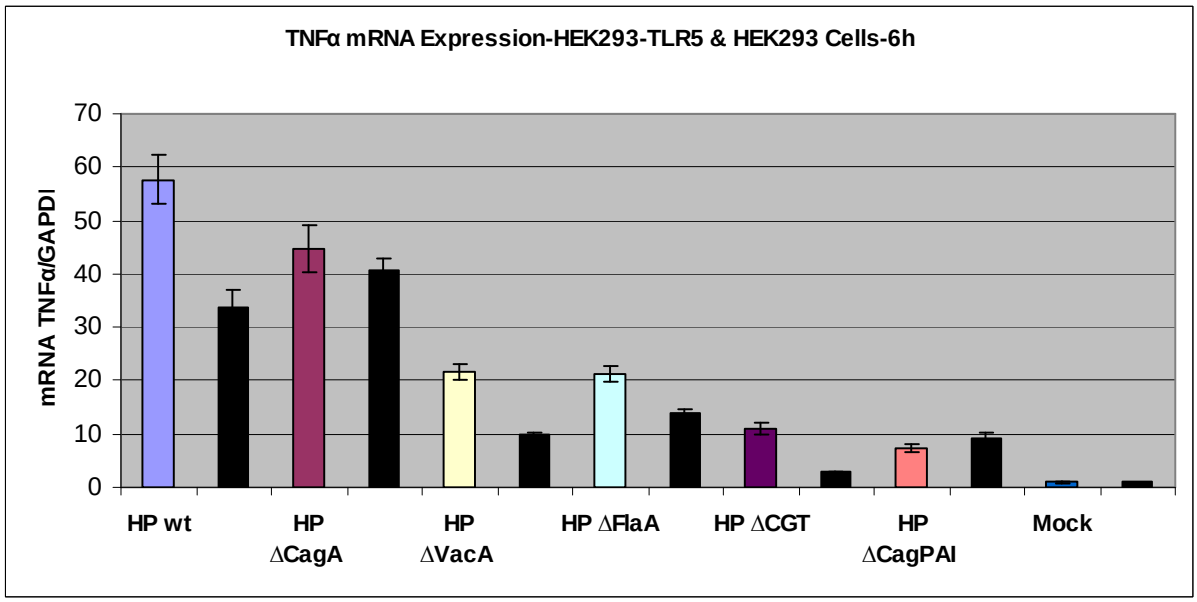


Table.18

	HEK293-TLR5		HEK293		HEK293-TLR5		HEK293	
	IL-1β	**±SD**	**IL-1β**	**±SD**	**IL-18**	**±SD**	**IL-18**	**±SD**
HP wt	0.91	0.08	0.78	0.03	0.82	0.10	0.60	0.04
HP Δ*CagA*	1.01	0.08	0.21*#	0.03	1.23	0.24	0.65	0.06
HP Δ*VacA*	0.58*	0.07	1.77	0.19	0.43#	0.08	0.61	0.03
HP Δ*FlaA*	0.83	0.06	2.02*#	0.10	0.51#	0.08	1.21	0.18
HP Δ*CGT*	0.69	0.03	0.70*	0.07	0.35	0.01	0.11*#	0.01
HP Δ*Cag*PAI	0.62*	0.07	0.58*	0.07	0.47#	0.07	0.21*#	0.01
Mock	1.00	0.10	1.00	0.08	1.00	0.18	1.00	0.11

Figure. 25 Fold changes of IL-1β and IL-18 mRNA expression in HEK293-TLR5 cells in comparison with HEK293 wild type cells (black bar) after 6h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*



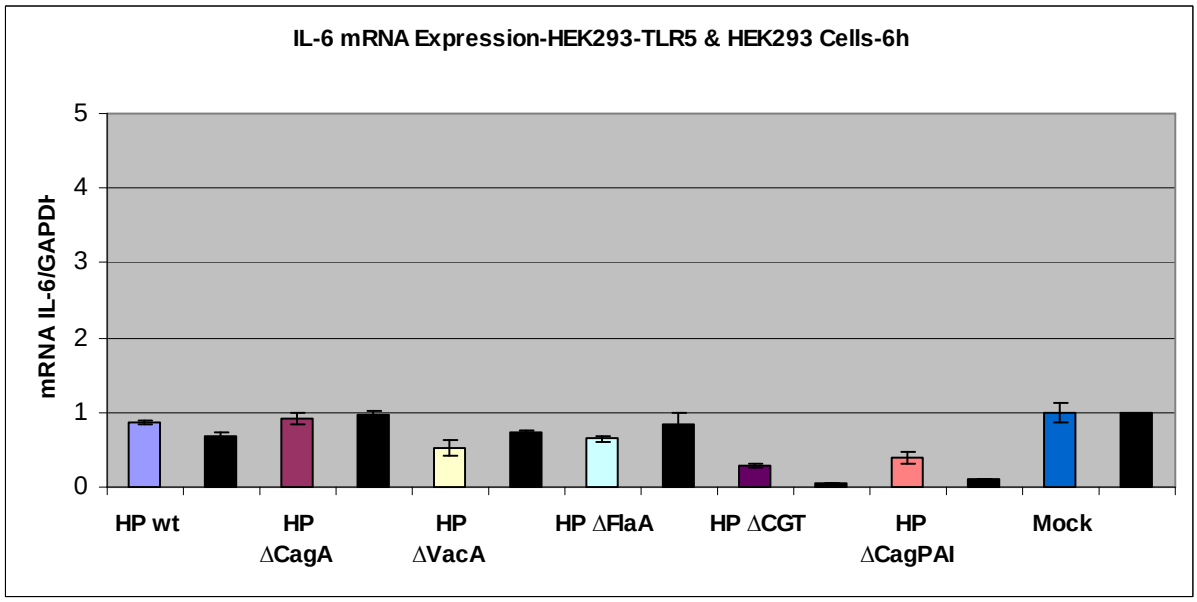
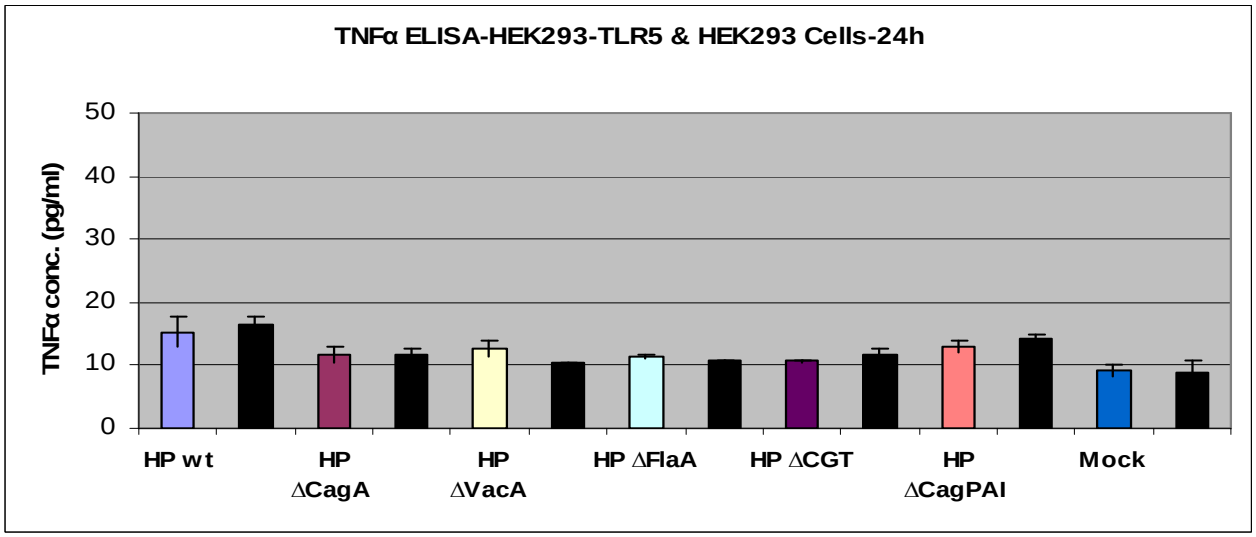


Table.19

	HEK293-TLR5		HEK293		HEK293-TLR5		HEK293	
	TNFα	**±SD**	**TNFα**	**±SD**	**IL-6**	**±SD**	**IL-6**	**±SD**
HP wt	57.68*	4.52	33.82*	3.32	0.86	0.03	0.69*	0.05
HP Δ*CagA*	44.78*#	4.38	40.50*	2.38	0.92	0.07	0.96#	0.07
HP Δ*VacA*	21.55*#	1.48	9.81*#	0.38	0.53*#	0.10	0.74	0.01
HP Δ*FlaA*	21.18*#	1.45	13.83*#	0.68	0.65#	0.04	0.83	0.16
HP Δ*CGT*	10.96*#	1.18	2.82*#	0.25	0.28*#	0.03	0.05*#	0
HP Δ*Cag*PAI	7.33*#	0.78	9.06*#	1.07	0.40*	0.08	0.11*#	0
Mock	1.00	0.10	1.00	0.08	1.00	0.13	1.00	0.08

Figure. 26 Fold changes of TNFα and IL-6 mRNA expression in HEK293-TLR5 cells in comparison with HEK293 wild type cells (black bar) after 6h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*



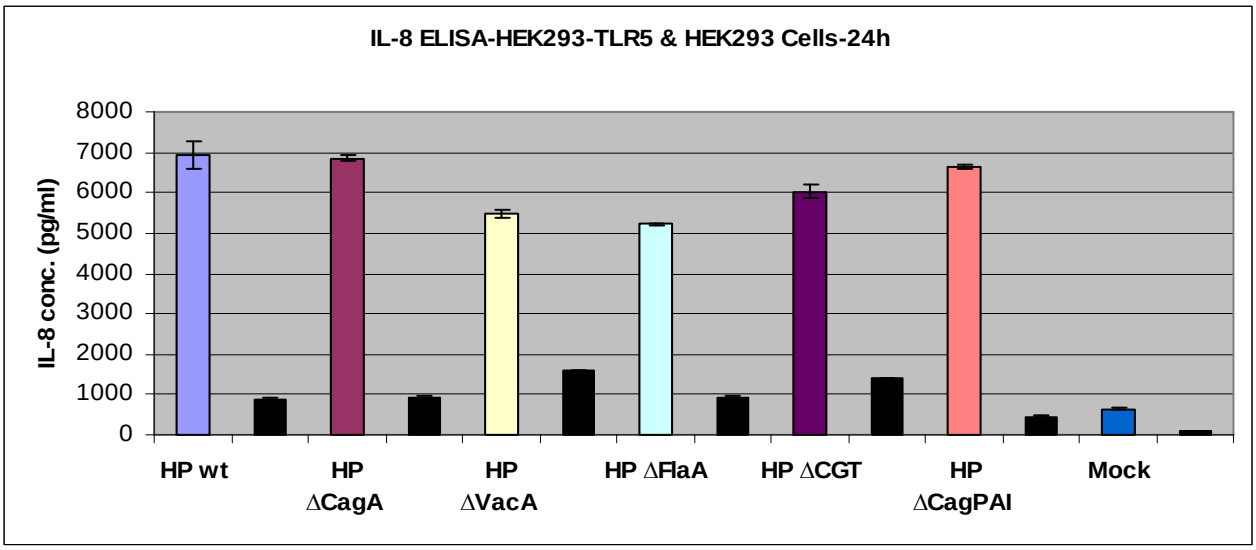
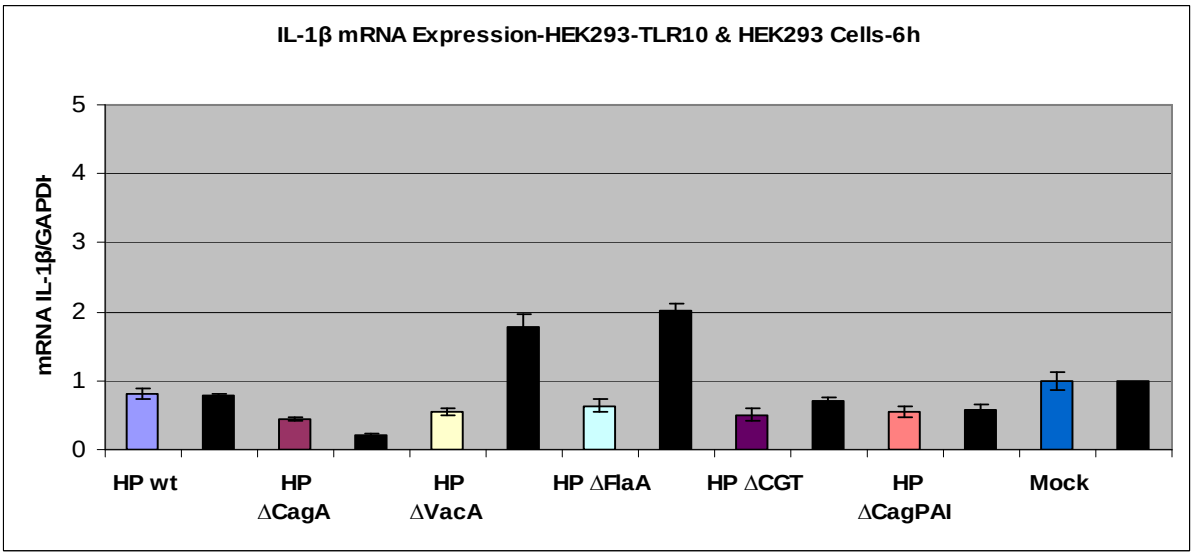


Table.20

	HEK293-TLR5		HEK293		HEK293-TLR5		HEK293	
	TNFα (pg/ml)	**±SD**	**TNFα (pg/ml)**	**±SD**	**IL-8 (pg/ml)**	**±SD**	**IL-8 (pg/ml)**	**±SD**
HP wt	15.25*	2.37	16.32*	1.37	6,924*	351	891*	28
HP Δ*CagA*	11.69	1.30	11.64#	1.13	6,846*	80	924*	21
HP Δ*VacA*	12.71	1.29	10.35#	0.23	5,488*#	87	1608*#	10
HP Δ*FlaA*	11.37	0.37	10.68#	0.22	5,221*#	24	928*	17
HP Δ*CGT*	10.73	0.18	11.64#	1.13	6,030*#	187	1391*#	11
HP Δ*Cag*PAI	12.94*	1.00	14.38	0.45	6,641*	44	445*#	27
Mock	9.22	0.85	8.90	1.82	644	25	74	7

Figure. 27 Concentrations of TNFα and IL-8 secreted from HEK293-TLR5 cells in comparison with HEK293 wild type cells (black bar) after 24h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. TNFα and IL-8 concentrations in the culture supernatants were analyzed by ELISA. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.005 when compared to the uninfected mock cells to the infected cells. # = P≤0.005 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*



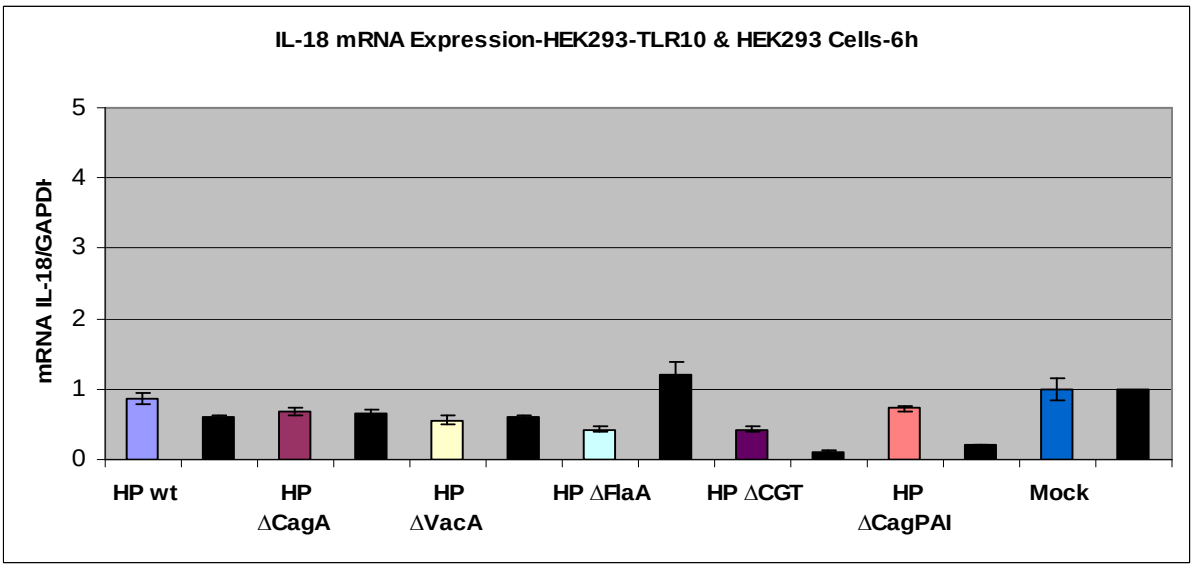
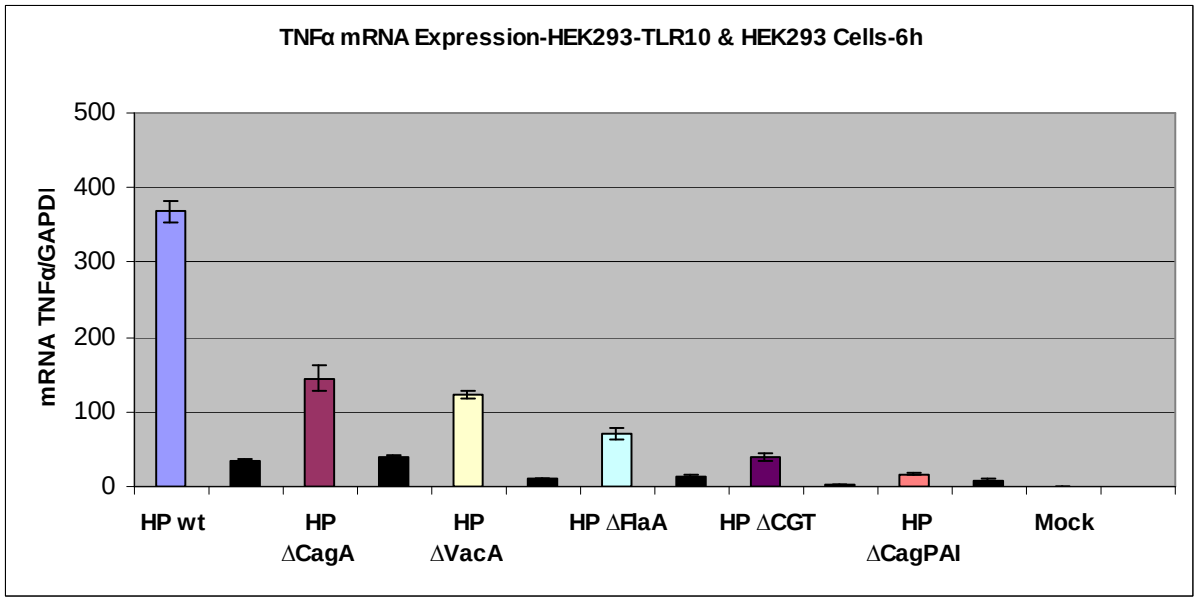


Table.21

	HEK293-TLR10		HEK293		HEK293-TLR10		HEK293	
	IL-1β	**±SD**	**IL-1β**	**±SD**	**IL-18**	**±SD**	**IL-18**	**±SD**
HP wt	0.80	0.08	0.78	0.03	0.86	0.07	0.60	0.04
HP Δ*CagA*	0.44#	0.03	0.21*#	0.03	0.68	0.06	0.65	0.06
HP Δ*VacA*	0.55#	0.04	1.77	0.19	0.56	0.06	0.61	0.03
HP Δ*FlaA*	0.64#	0.09	2.02*#	0.10	0.43*	0.03	1.21	0.18
HP Δ*CGT*	0.51*#	0.09	0.70*	0.07	0.42*	0.04	0.11*#	0.01
HP Δ*Cag*PAI	0.55#	0.08	0.58*	0.07	0.72	0.03	0.21*#	0.01
Mock	1.00	0.13	1.00	0.08	1.00	0.15	1.00	0.11

Figure. 28 Fold changes of IL-1β and IL-18 mRNA expression in HEK293-TLR10 cells in comparison with HEK293 wild type cells (black bar) after 6h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*



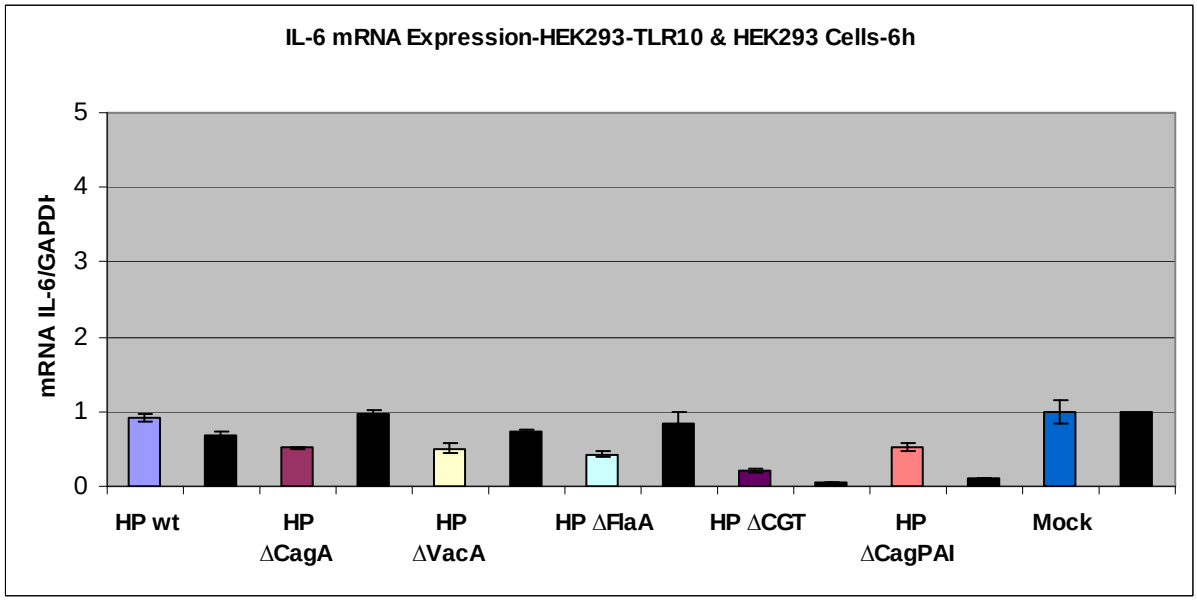
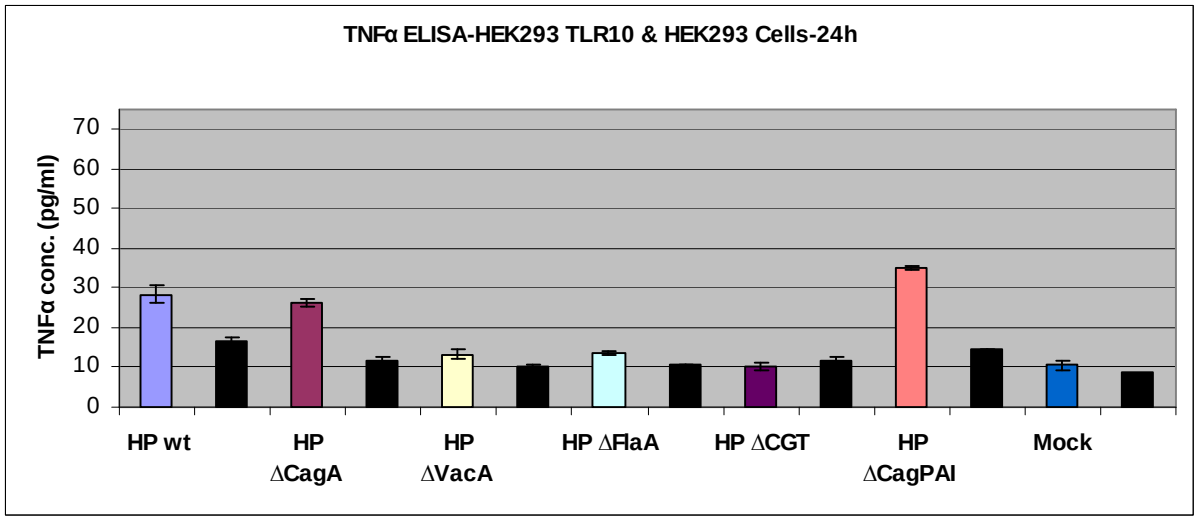


Table.22

	HEK293-TLR10		HEK293		HEK293-TLR10		HEK293	
	TNFα	**±SD**	**TNFα**	**±SD**	**IL-6**	**±SD**	**IL-6**	**±SD**
HP wt	368.30*	14.40	33.82*	3.32	0.92	0.06	0.69*	0.05
HP Δ*CagA*	145.00*#	17.08	40.50*	2.38	0.52#	0.01	0.96#	0.07
HP Δ*VacA*	122.36*#	4.78	9.81*#	0.38	0.51#	0.06	0.74	0.01
HP Δ*FlaA*	70.52*#	6.92	13.83*#	0.68	0.43#	0.03	0.83	0.16
HP Δ*CGT*	39.80*#	4.67	2.82*#	0.25	0.21#	0.02	0.05*#	0
HP Δ*Cag*PAI	16.56*#	1.30	9.06*#	1.07	0.52#	0.06	0.11*#	0
Mock	1.00	0.13	1.00	0.08	1.00	0.16	1.00	0.08

Figure. 29 Fold changes of TNFα and IL-6 mRNA expression in HEK293-TLR10 cells in comparison with HEK293 wild type cells (black bar) after 6h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. The mRNA expressions were analysed by Taqman Real Time PCR relative to the house keeping gene GAPDH using $2^{-\Delta\Delta Ct}$ method. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.05 when compared to the uninfected mock cells to the infected cells. # = P≤0.05 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*



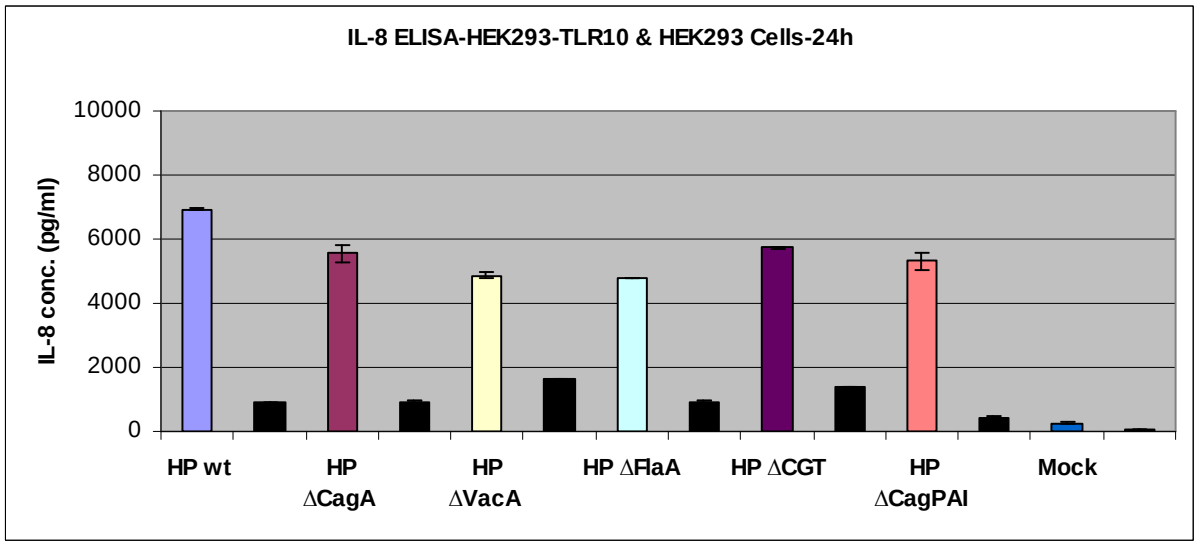


Table.23

	HEK293-TLR10		HEK293		HEK293-TLR10		HEK293	
	TNFα (pg/ml)	**±SD**	**TNFα (pg/ml)**	**±SD**	**IL-8 (pg/ml)**	**±SD**	**IL-8 (pg/ml)**	**±SD**
HP wt	28.44*	2.37	16.32*	1.37	6,924*	22	891*	28
HP Δ*CagA*	26.15*	1.00	11.64#	1.13	5,546*#	271	924*	21
HP Δ*VacA*	13.35#	1.19	10.35#	0.23	4,871*#	81	1608*#	10
HP Δ*FlaA*	13.63#	0.67	10.68#	0.22	4,808*#	8	928*	17
HP Δ*CGT*	10.40#	0.92	11.64#	1.13	5,738*#	48	1391*#	11
HP Δ*Cag*PAI	34.96*#	0.50	14.38	0.45	5,310*#	282	445*#	27
Mock	10.62	1.29	8.90	1.82	258	28	74	7

Figure. 30 Concentrations of TNFα and IL-8 secreted from HEK293-TLR10 cells in comparison with HEK293 wild type cells (black bar) after 24h of infection with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt and cag*PAI. TNFα and IL-8 concentrations in the culture supernatants were analyzed by ELISA. The values were expressed as mean ± SD and the significance of difference were analysed by paired samples T-test using SPSS software. * = P≤0.005 when compared to the uninfected mock cells to the infected cells. # = P≤0.005 when compared to *H. pylori* wild type infected cells with isogenic mutant infected cells. HP = *H. pylori*

3.13 IL-1 Receptor Antagonist (IL-RN) gene polymorphism among the *H pylori* colonized patients

Several studies in the past were clearly shown the influence of host genetics along with the bacterial factors in *H. pylori* related pathologies. Genetic polymorphisms can directly affect the expression levels of gene products by generation or deletion of transcription factor sites or by affecting RNA splicing and subsequent translation. Many of the pathogenic effects of *H. pylori* infection are related to chronic active inflammation, which is controlled and maintained by the complex interplay of proinflammatory and anti-inflammatory mediators (Macarthur M *et al,* 2004). The IL-1 cytokine is encoded by a gene cluster that contains the polymorphic *IL-1B* (encoding the IL-1βcytokine) and *IL-RN* (encoding the IL-1 receptor antagonist) genes. IL-1β is a potent proinflammatory cytokine and the most potent known inhibitor of acid secretion (El-Omar EM *et al,* 2000). The *IL-1* gene cluster contains several polymorphisms, such as *IL-1B*-31C, IL-1B*-511T*, and *IL-1RN*2/*2*, which lead to high-level expression of IL-1β. This subsequently leads to reduced acid output, which is associated with corpus-predominant colonization by *H. pylori*, resulting in pangastritis, formation of atrophic gastritis, and increased risk of gastric cancer (El-Omar EM, 2001; El-Omar EM *et al,* 2000; El-Omar EM *et al,* 2003; Furuta T *et al,* 2002, Hwang IR *et al,* 2002; Rad R *et al,* 2003; Zambon CF *et al,* 2002).

IL-1 Receptor Antagonist (IL-1RN) is structurally related to IL-1 (Carter DB *et al.,* 1990; Eisenberg SP *et al.,* 1990; Eisenberg SP *et al.,* 1991) but specifically blocks the binding of IL-1α and IL-1β to cell surface receptors without itself activating target cells (Arend WP *et al.,*1998; Dripps DP *et al.,* 1991). IL-1 stimulates the synthesis of IL-8, which is a potent chemotactic agent for neutrophils, and it also induces release of neutrophil elastase. IL-1 promotes the adhesion of neutrophils and other cells by enhancing the expression of adhesion molecules such as ICAM-1, VCAM-1, and L-selectin (Abdelaziz MM *et al.,* 1995; Tsang YT *et al.,* 1997). The IL-1RN gene showing polymorphism by

penta allelic 86 bp tandem repeat in intron 2, of which allele 2 (*IL-*

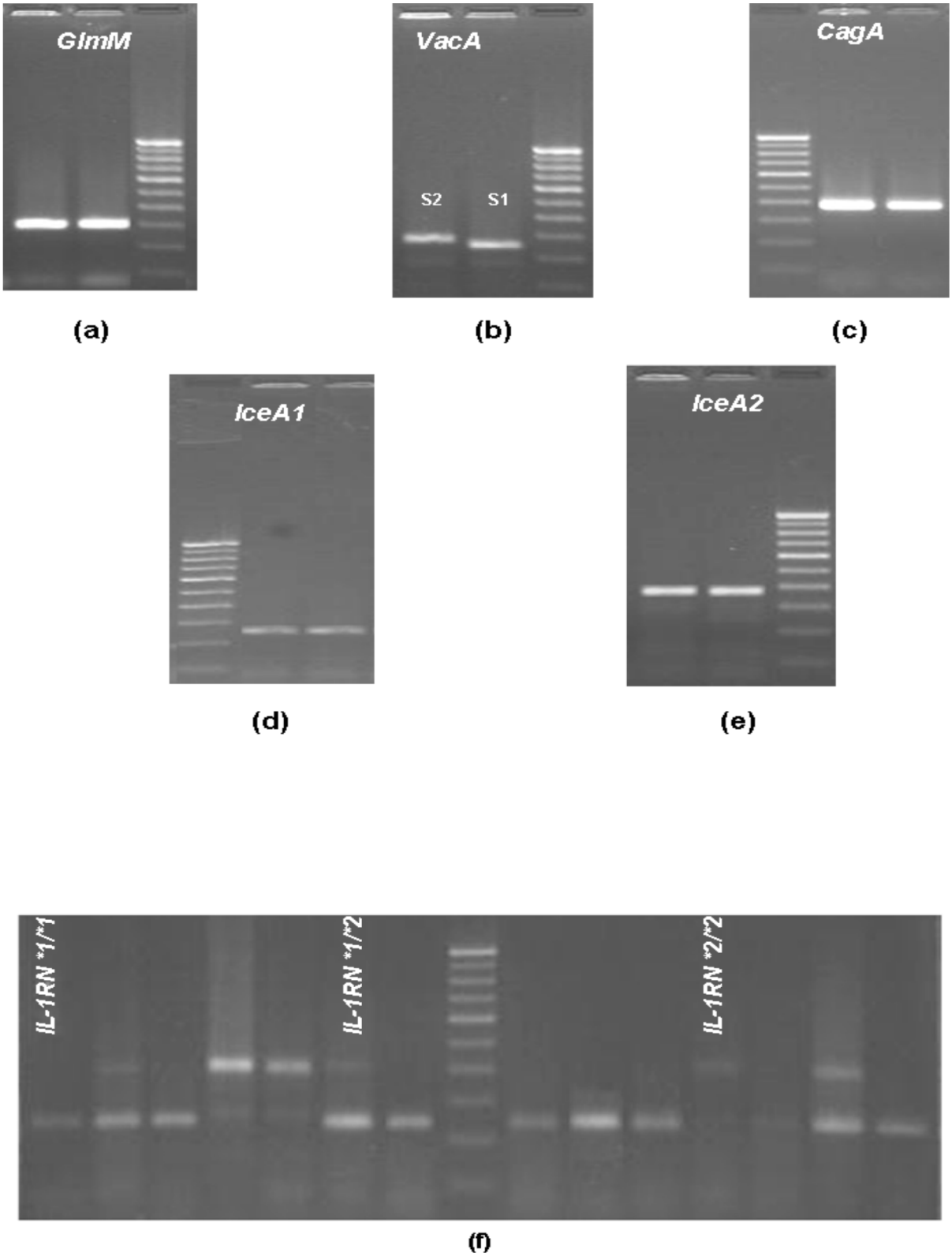


Figure. 31 PCR amplification of *H. pylori* genes *Glm* **(a)** *VacA* –S region **(b)** *CagA* **(c)** *IceA1* **(d)** *IceA2* **(e)** and host *IL-1RN* gene polymorphism **(f)** from gastric mucosal biopsies collected from dyspepsia patients. The PCR product were electrophoresed on 1.5% agarose gel along with 100bp DNA ladder and visualized by ethidium bromide staining.

*1RN*2/*2*) associated with enhanced IL-1 β production and proinflammatory responses more severe and more prolonged than those of other *IL-1RN* genotypes (Hurme M and Helminen M, 1998; Hwang IR *et al.,* 2002). Among population *IL-1RN*2/*2* homozygous individuals are a distinct minority in every population examined to date, most of them are either homozygous for allele 1 (*IL-1RN*1/*1*), which contains four tandem repeats, or heterozygous for IL-1RA*1 and allele 2 (*IL-1RN 1/2*), which contains two repeats (Tarlow JK *et al.,* 1993; Bioque G *et al.,* 1995; Jeremias J *et al.,* 2000). *IL-1RN*2/*2* genotype may be beneficial in the immune defense against infection by promoting a prolonged Th1 cell-mediated immune response. The T helper cell response towards H pylori is generally considered to be of the Th1 phenotype, leading to a cell-mediated immune response (Ernst PB, 1999; Shimoyama T and Crabtree JE, 1998). But prolonged Th1 cell mediated immunity is a causing factor for inflammatory conditions. There is increasing evidence that the H pylori induced Th1 response contributes to cancer development (Ernst PB and Gold BD, 2000).

The analysis pertaining to *IL-1RN* allele polymorphism and genotyping of virulence genes such as *CagA, VacA* and *IceA1* and *A2* were done in 42 patients colonized with *H pylori,* as confirmed by RUT and *GlmM* gene by PCR. *VacA* S1/*CagA*/*IL-1RN*2*-allele combination was found to be high risk factors for inflammatory changes in gastric mucosa of patients leading to metaplasia and later to gastric cancer. *IL-RN*2/*2* allele were found in 43% (18/42), *IL-RN*1/*1* allele in 21% (9/42) and heterozygous allele *IL-RN *1/*2* in 36% (15/42) of patients analysed by PCR amplification using specific primers for VNTR region of the intron 2 which yielded 240bp (two repeats) and 412bp (four repeats) product for *IL-RN*2/*2* and *IL-RN*1/*1* allele respectively (Fig.31). The high risk *VacA* S1/*CagA*/ *IL-RN*2/*2* -allele combination was found in 6 patients (14%), *VacA* S1/*CagA*/ *IL-RN*1/*1* allele in 3 patients (7%) and *VacA* S1/*CagA*/ *IL-RN *1/*2* allele in 7 patients (16%).

Table.24 *IceA* genotype pattern in association with *Vac s1, CagA* and *IL-1RN* allele in *H pylori* infected patients

Bacterial and Host genes	**No. of patients**	***IceA* pattern (No.)**		
Vs1C-IL-1RN*2/*2	6	A1A2 (4)	A1 (2)	A2 (0)
Vs1-IL-1RN*2/*2	8	A1A2 (1)	A1 (6)	A2 (1)
Vs1C-IL-1RN*1/*2	7	A1A2 (1)	A1 (4)	A2 (2)
Vs1-IL-1RN*1/*2	6	A1A2 (4)	A1 (1)	A2 (1)
Vs1C-IL-1RN*1/*1	3	A1A2 (0)	A1 (2)	A2 (1)
Vs1-IL-1RN*1/*1	3	A1A2 (0)	A1 (2)	A2 (1)
Vs2C-IL-1RN*2/*2	0	0	0	0
Vs2-IL-1RN*2/*2	4	A1A2 (1)	A1 (2)	A2 (1)
Vs2C-IL-1RN*1/*2	2	A1A2 (1)	A1 (1)	A2 (0)
Vs2-IL-1RN*1/*2	0	0	0	0
Vs2C-IL-1RN *1/*1	2	A1A2 (1)	A1 (1)	A2 (0)
Vs2-IL-1RN*1/*1	1	A1A2 (0)	A1 (0)	A2 (1)

IceA1 and *IceA2* are two different genes, which are present at the same genomic locus among various strains. *IceA1* expression is up regulated by contact with epithelium and in some populations this phenotype is associated with peptic ulcer. In this study, we have analyzed the *IceA1* and *IceA2* status along with *VacA* S, *CagA* and IL-1RA status among patients (Fig.31). Among these patients, 81% infected with *IceA1* genotype, whereas only 19% exhibited *IceA2* genotype. In this group of patients 31% (13/42) were infected with both *IceA1* and *IceA2* genotypes of *H pylori.* The association with *VacA* S1/*CagA*/IL-1RN allele has been given in table-27. Both *IceA1* and *IceA2* status were observed in all four cases of ulcer diagnosed endoscopically (3 duodenal ulcer and 1 antral ulcer), however, two DU and one AU are also associated with *VacA* S1genotype, while remaining DU is infected with *VacA* S2 genotype. *H pylori* infected patient with antral ulcer was also associated with *CagA* genotype. IL-RA allele polymorphisms in two DU were IL-RA1/2 type (heterozygous) and IL-RA*2 in the remaining DU and AU (Table.27)

4. Discussion

4.0 Discussion

Helicobacter pylori is the causative factor for gastritis, gastric ulcer and MALT lymphoma and gastric cancer in humans. This bacterium finds its niche as the acid coated gastric epithelium and survives there with its special mechanisms. In most of the individulals colonized *H. pylori* keeps a balanced situation of low immune reaction that was not able to eliminate organism but definitely causing tissue damage and which help the bacterium to get sufficient nutrients to survive in the unfavourable environment of the stomach. It is known that over half of the world's population is infected with *H. pylori*, with the highest rates in developing countries (Rothenbacher D and Brenner H, 2003). Infections occur in early childhood and persist for decades in the absence of targeted antimicrobial therapy. The exact mode of transmission of *H. pylori* is not clearly known till today. New infections thought to occur through human to human contact either in oral-oral or faecal-oral route or both. *H. pylori* is reported to be colonized in few hosts such as humans and some non-human primates and there exists a few reports of isolation of the organism from pet animals (Brown LM *et al,* 2001; Brown LM *et al,* 2002; Dore MP *et al,* 2001; Herbarth O *et al,* 2001). Although *H. pylori* colonized individuals are high in population, only a few percentage shows clinical symptoms of *H. pylori* associated diseases. Among the *H. pylori* positive individuals 10 to 20% develop ulcer diseases and 1 to 2% having risk of developing gastric cancer or MALT lymphoma (Ernst PB and Gold BD, 2000; Parsonnet J and Issacson PG, 2004). Gastric ulcer patients are characterized by reduced gastric acid secretion, corpus predominant pangastritis, and accelerated progression toward atrophic gastritis and intestinal metaplasia such individuals are reported to be at higher risk for gastric cancer than are duodenal ulcer patients (Hansson LE *et al,* 1996). Chronic *H. pylori*-induced inflammation can eventually lead to loss of the normal gastric mucosal architecture, with destruction of gastric glands and replacement by fibrosis and intestinal-type epithelium. This process of atrophic gastritis and intestinal

metaplasia occurs in approximately half of the *H. pylori*-colonized population (Kuipers EJ *et al,* 1995b). The risk of development of atrophy and cancer in the presence of *H. pylori* is again related to host and bacterial factors, which influence the severity of the chronic inflammatory response. The risk is not only increased in individuals colonized with cytotoxin associated gene (cagA) positive strains, but also in those with a genetic predisposition to higher IL-1 production in response to colonization (Parsonnet J *et al,* 1997; El-Omar EM *et al,* 2000).

H. pylori induced ulcer disease, gastric cancer, and lymphoma are complications of chronic inflammation; ulcer disease and gastric cancer in particular occur in those individuals and at those sites with the most severe inflammation. Infection is associated with production of proinflammatory cytokines which lead to chronic or chronic active gastritis. The activity of the gastritis is commonly considered an indicator of severity, and chronic active gastritis is associated with more severe manifestations of disease, such as peptic ulceration and neoplasia (Blaser MJ *et al,* 1995; Crabtree JE, 1998; Peek RM *et al,* 1995). The *cag* pathogenicity island (PAI), a 40 kb stretch of DNA-encoding homologues of components of a T4SS, positive strains are associated with severe disease conditions (Censini S *et al*,1996). *H. pylori* pathogenesis and virulence factors such as cagPAI and its effector protein cagA (Covacci A *et al,* 1993), vacuolating cytotoxin (vacA) (Cover TL, 1996), Urease (van Vliet AHM *et al,* 2001), adhesins such as blood group antigen binding adhesin (BabA), sialic acid binding adhesin (sabA and SabB) (Prinz C *et al,* 2001), adherence-associated lipoprotein (AlpA and AlpB), *Helicobacter* outer membrane protein Z (HopZ), outer membrane inflammatory protein A (OipA) (Yamaoka Y *et al,* 2000) and neutrophil activating protein (Nap) (Tonello F *et al,* 1999; Namavar F *et al,* 1998), other factors associated with ulcer such as induced upon contact with epithelium gene (IceA) (Figueiredo C *et al,* 2000) and duodenal ulcer associated protein (DupA) (Lu H *et al,* 2005), LPS and flagella contributes to the outcome of the gastric pathologies independently or collectively. The cholesteryl-α-

glucosides of *H. pylori* support the pathogenicity of this organism, because inhibition of the cholesterol glucosyltransferase (*Cgt*) by *O*-glycans of the human gastric mucosa suppresses growth of the bacterium (Kawakubo M *et al,* 2004). Intrinsic α-glucosylation of cholesterol abrogates phagocytosis of *H. pylori* and subsequent T-cell activation (Wunder C *et al,* 2006). Many of these defined *H. pylori* virulence factors, including flagella, the stomach acid neutralizing enzyme urease and the multifunctional VacA, are found in all strains (Monack DM *et al*, 2004).

The components of innate immunity such as PRRs play an important role on detecting PAMPs on microbes and orchestrating the adaptive immunity against infection. Toll like receptors constitute a major component of the innate immune system by detecting molecules like lipopolysaccharides (LPS), lipoprotein, lipotheichoic acid, peptidoglycan, lipoarabinomannan (LAM), flagellin, CpG containing DNA, single and double stranded RNA of viruses, HSP60, Porins, Zymosan, Glycolipids are known examples of PAMPs (Janeway CA and Medzhitov R, 2002; Takeda Y *et al,* 2003).. PAMPs recognition by PRRs leads to different signalling events that ultimately exert the expression of many effector molecules of the immune system. The recognition in phagocytic cells leads to the production of reactive oxygen and nitrogen intermediates (ROI & RNI) and increased expression of co-stimulatory molecules which act as second stimuli for T-cells activation. PRRs also signals the production of immune mediators such as chemokines, cytokines, leukotrienes, prostaglandins and anti-microbial peptides, which ultimately determines the recruitment of leukocytes at the site of infection and the production of successful adaptive immunity to eliminate the infection.

Gastric epithelial cells do not express all ten TLR molecules in humans. The invading immune cells are capable of providing the missing receptors for detecting important molecular patterns of many bacteria. *H. pylori* infection attracts more neutrophils and monocytes to the site of infection through induction of IL-8 release and *H. pylori* Nap protein has also been implicated for the neutrophil activating process by

inducing NADPH oxidase (Tonello F *et al,* 1999; Namavar F *et al,* 1998; Satin B *et al,* 2002; Naumann M and Crabtree JE, 2004). The phagocytic cells at the site of infection try to eliminate the microbes by engulfment and subsequent killing by the help of ROIs and RNIs. However, *H. pylori* possess catalase and superoxide dismutase enzymes involved in the ROI scavenging, which might help the organism to evade its elimination by phagocytotic killing (McGee DJ and Mobley HL, 1999). It also produces the arginase enzyme which converts arginine to ornithine and thereby competitively inhibiting the inducible nitric oxide synthase enzyme of the host and control the production of NO. *H. pylori* also induce eukaryotic arginase II expression in macrophages, which might further counteract NO production by these cells (Gobert AP *et al,* 2002). These mechanisms might limit the availability of L-arginine at the site of infection and therby control phagocytic killing.

The role of innate immune response to *H. pylori* through PRRs is not completely understood till today. A few studies have reported the involvement of some TLRs in the detection of *H. pylori.* Smith MF *et al* reported that gastric epithelial cells recognize and respond to H. pylori infection at least in part through TLR-2 and TLR-5 (Smith MF *et al,* 2004). TLR-4 mediated recognition of bacterial LPS is a key activator of the innate immune response in epithelial cells, while *H. pylori* LPS is a relatively weak inducer. However, *H. pylori* LPS activates NF-κB through TLR-2 rather than TLR-4 (Smith MF *et al,* 2004; Maeda S *et al,* 2001; Backhead F *et al,* 2003). In the present study, the THP1 monocytic leukaemic cell line reported to be expressing all ten TLRs (Zarember KA and Godowski PJ, 2002), THP-1 derived DCs, HEK293 cells having less or no TLR expression and HEK293 cells stably transfected with TLR2, TLR5 and TLR10 have been used to delineate the role of *H. pylori* virulence genes on TLR detection and signalling through in vitro infection assays involving wild type strain and isogenic mutants of *cagA, vacA, flaA, cgt* and *cag*PAI.

4.1 Toll like receptors Expression and IRAK-1 phosphorylation during *H pylori* infection in THP-1 cells, THP-1-DCs, HEK-293, HEK293-TLR2, HEK293-TLR5 and HEK293-TLR10 cells

The complete profile of TLR-1 to 10 mRNA expression pattern during *H. pylori* infection is not available. Hence one of the objectives of the present study was to analyze the TLR-1 to 10 mRNA expressions in human monocytic THP1 cell line during *H. pylori* infection. The mRNA expressions of genes give a rough idea of their involvement in cellular activities at a particular event. The higher expression of mRNA of a particular gene attributes to higher turn over or continuous signal transduction of particular pathway that regulate the gene expression. TLRs are involving in the recognition of PAMPs present on microbes or invading organisms and subsequent signalling to activate the pro-inflammatory changes to control infection. Several known ligands from different sources have been reported to bind TLRs and transducing signals. Smith MF *et al* (2004) reported that gastric epithelial cells recognize and respond to *H. pylori* infection at least in part through TLR-2 and TLR-5. *H. pylori* LPS activates NF-κB, through TLR-2 rather than TLR-4 (Smith MF *et al,* 2004; Maeda S *et al,* 2001; Backhead F *et al,* 2003). In contrast to this, Kawahara T *et al* reported that *H. pylori* LPS activated NF-κB in association with the expression of Mitogen oxidase-1 (MOX-1), cyclooxygenase-II (COX II) and TNFα transcripts in gastric pit cells, which express more TLR-4 but no TLR-2 (Kawahara T *et al,* 2003).

The infection of THP-1 cells with *H. pylori* and isogenic mutants of *cagA, vacA, flaA, cgt,* and *cag*PAI at a MOI of 1:50 for 6 and 24h have revealed that virulence factors of *H. pylori* significantly altering the quantitative expression of TLR mRNAs. TLR-1 mRNA expression after 6h was not significantly changed during *H. pylori* infection. However, this level was significantly increased with the infection by wild type, Δ*cgt* and Δ*cag*PAI after 24h of infection (Fig.3). Some investigations have shown that Lipoproteins in which the N-terminal cysteine is triacylated are recognized by TLR-2 in combination with TLR-1. Similarly, diacylated lipoproteins are recognized by TLR-2 in combination

with TLR-6 (Takeda Y *et al,* 2003; Ozinsky A *et al,* 2000). CagA, VacA and FlaA proteins of *H. pylori* are required for the increase in cellular mRNA expression of TLR-1 in THP-1 cells. It has been reported that LTA from Group B Streptococcus (GBS) and *S. aureus* have interacted with TLR-2 and TLR-6, but not TLR-1 (Henneke P *et al,* 2005). TLR-2 expression has been significantly increased by wild type strain but Δ*vacA* and Δ*flaA* mutants showed no significant change after 6 and 24h of infection. However, the mRNA expression level after 6h of infection was reduced by half after 24h of infection with *H. pylori* wild type. This may be due to the overall control mechanisms of expression of TLR-2 molecules in THP-1 cells or due to tolerance as reported earlier in the case of TLR-4 and TLR-5 mediated detection of LPS and flagellin, respectively (Mizel SB *et al,* 2002). In addition, *H. pylori* LPS found to be the ligand for TLR-2 (Smith MF *et al,* 2004; Maeda S *et al,* 2001; Backhead F *et al,* 2003). The above observation has been confirmed by analyzing the TLR-2 mRNA expression in HEK293-TLR2 cells by showing increased expression of TLR-2 by *H. pylori* infection. This experiment demonstrated that Δ*vacA* and Δ*flaA* mutants were the least inducer of TLR-2 mRNA expressions. Hence, it can be assumed that TLR-2 lignad (s) from *H. pylori* may need the virulence factor VacA and FlaA for maximum potency and which may be mediated through the changes exerted by VacA on host cells or changes in surface properties of *H. pylori* by FlaA molecules, since *H. pylori* FlaA is a UDP-GlcNAc-inverting 4,6-dehydratase enzyme catalyzes the first step in the biosynthetic pathway of a pseudaminic acid derivative, which is implicated in protein glycosylation (Ishiyama N *et al,* 2006).

TLR-3 binds known lignad dsRNA from virus and induces IRF-3/7 dependent upregulation of Type-I IFNs (Oganesyan G *et al,* 2006). However, there are only few reports on bacteria dependent Type-I IFNs production. Gratz N *et al* (2008) reported that in mouse macrophages, extracellular Gram-positive human pathogen group A streptococcus (GAS; *S. pyogenes*) causes IRF-3 dependent, MyD-88 independent production of type-I IFN and which is also induced by GAS lacking *slo* and *sagA*, the

genes encoding cytolysins that were shown to be required for IFN production in response to other Gram-positive bacteria (Gratz N *et al,* 2008). THP-1 monocyte infection with *H. pylori* significantly increased the TLR-3 mRNA expression after 6h and continued to increase even after 24h. This shows the important role for TLR-3 in the detection of *H. pylori.* Among the iosgenic mutant studied, Δ*flaA* demonstrated least significant change in TLR-3 mRNA expression after 6h and 24h of infection in THP-1 cells. All the other mutants significantly increased the TLR-3 mRNA expression but found to be less potent than wild type strain.

The data regarding the involvement of TLR-4 in *H. pylori* infection are contradictory. *H. pylori* LPS activates NF-κB, through TLR-2 rather than TLR-4 (Smith MF *et al,* 2004; Maeda S *et al,* 2001; Backhead F *et al,* 2003). In contrast to this, Kawahara T *et al* (2003) reported that *H. pylori* LPS activated NF-κB in association with the expression of Mitogen oxidase-1 (MOX-1), cyclooxygenase-II (COX II) and TNFα transcripts in gastric pit cells, which express TLR-4 without TLR-2 (Kawahara T *et al,* 2003). Mandell L *et al* reported that the purified form of *H. pylori* LPS induced cytokine production was mediated through TLR-4, but the response to Helicobacters such as *H. pylori, H. hepaticus, and H. felis* was mediated through TLR-2 (Mandell L *et al,* 2004). THP-1 cells have not significantly increased TLR-4 mRNA expression after 6h of infection with *H. pylori*. However, there was a significant increase in TLR-4 mRNA expression after 24h of infection with the wild type and Δ*cgt*. On the contrary, other mutants clearly reduced TLR-4 mRNA expression compared to wild type strain. This indicates that *H. pylori* up-regulate TLR-4 at the late phase of infection, where TLR-2 found to be down-regulated from the initial phase of infection (Fig.2, 3). This would suggest that TLR-4 up-regulation might be occurred due to the LPS released from the bacteria lysis at the late phase of the infection. LPS on the surface of *H. pylori* may have special features which inhibit the interaction with TLR-4, conversely it is able to bind with TLR-2 and induce signal transduction.

Bacterial flagellins are the known ligands for TLR-5. A study using recombinant *flaA* and ∆*flaA* mutant of *H. pylori* revealed the less influence of TLR-5 mediated IL-8 secretion in epithelial cells (Gewirtz AT *et al,* 2004). A recent report showed the site responsible for low TLR-5 mediated activity to amino acids 89-96 of the N-terminal D1 domain of *H. pylori flaA* (Andersen-Nissen E *et al,* 2005). THP-1 cells infected with *H. pylori* have up-regulated the TLR-5 mRNA expression significantly. Among the mutants tested, Δ*flaA* has no significant influence on TLR-5 expression compared to uninfected cells. However, other mutants were less potent than the wild type on inducing TLR-5 mRNA expression. In this study, we found that TLR-5 mRNA expression in THP-1 cells was moderately increased after *H. pylori* infection. This finding was confirmed at the protein level by western blot analysis of immunoprecipitated TLR-5 from THP-1 cells infected with *H. pylori* wild type and Δ*flaA* mutant. This data strongly suggest the involvement of TLR-5 in *H. pylori* detection and pro-inflammatory changes. In the experiment using HEK293-TLR5 cells infected with *H. pylori* revealed that wild type and *ΔcagA* mutant up-regulated the TLR-5 mRNA expression significantly, leading to suggest the involvement of TLR-5 in *H. pylori* infection.

TLR-6 binds to ligands such as diacyl lipopeptides from mycoplasma, LTA from Gram positive bacteria and zymosan from fungi (Takeuchi O *et al,* 2001; Schwander R *et al,* 1999; Ozinsky A *et al,* 2000). THP-1 cells infected with *H. pylori* and isogenic mutants significantly up-regulated mRNA expression of TLR6, except in the case of *ΔflaA* mutant. The wild type strain induced the up-regulation of TLR-6 expression significantly compared to other mutant strains after 6h of infection. However, *Δcgt* and *Δcag*PAI mutants have up-regulated TLR-6 to the level of wild type after 24h of infection. Ozinsky A *et al* (2000) reported that TLR-2 and TLR-6 heterodimer detect the PGNs while TLR-2 homodimer detects bacterial lipopeptide. They also showed that the cytoplasmic domain of TLR-2 can form functional pairs with TLR-6 or TLR-1, and this interaction led to cytokine induction. Ray A and Biswas T (2005) observed that porin of *S. dysenteriae*

type 1 increased the mRNA levels for TLR-2 and TLR-6 and CD80 and also induced cell-surface expression of immunoglobulin IgM, IgG2a, and IgA in peritoneal cavity B-2 cells. Mycoplasmal membrane diacylated lipoproteins initiated proinflammatory responses through TLR-2 and TLR-6 through the activation of NF-κB and also induced apoptotic responses (Into T *et al,* 2004).

TLR-7 and -8- are involved in the binding of synthetic compounds imidazoquinoline and ssRNA from viruses. TLR-7 also binds synthetic compounds Loxoribine and Bropirimine (Heil F *et al,* 2003; Heil F *et al,* 2004; Hemmi H *et al,* 2002; Jurk M *et al,* 2002). IFN induction in pDC was triggered by signal transduction pathways through TLR-7 and TLR-9 as well as by recognition of cytosolic virus-specific patterns (Schlender J *et al,* 2005). No reports are available in the literature showing that bacterial products can bind and induce TLR-7 and -8- mRNA expression. The present study shows that THP-1 cells infected with *H. pylori* very significantly up-regulated the TLR-7 mRNA expression even after 6h of infection and reached more than 70 fold in case of wild type infected cells compared to uninfected cells. Δ*cagA* mutant have exhibited an increase in the TLR-7 mRNA expression after 6h when compared to wild type infected cells. However, after 24h of infection, TLR-7 mRNA expression level reached almost double in wild type infected cells when compared Δ*cagA* mutant infected cells. Δ*flaA* mutant was the least inducer of TLR-7 mRNA expression. Triantafilou K *et al* (2005) reported that group B coxsackievirus induced inflammatory response is mediated through TLR-8 and to a lesser extent through TLR-7. TLR-8 and TLR-7 also function as the host sensors for human parechovirus-1, a ssRNA virus (Triantafilou K *et al,* 2005b). Both type-I IFNs and IRFs are well characterized in viral infections but not in bacterial. *S. aureus* LTA activated IRF-2 resulted in the up-regulation of IRF-1 and activation of STAT-1 and STAT-3 yielded rapid secretion of IFNα. (Liljeroos M *et al,* 2008). Interestingly, THP-1 cells infected with *H. pylori* have up-regulated TLR-8 mRNA expression significantly after 6h of infection and continued even after 24h. Δ*flaA* mutant

has also significantly up-regulated TLR-8 mRNA expression but weakly after 24h of *H. pylori* infection.

CpG DNA is the known and well studied ligand for TLR-9. LPS has induced cytokine production from DCs in a MyD-88 dependent pathway and also induced functional maturation of MyD-88 (-/-) deficient DCs, including up-regulation of costimulatory molecules and enhancement of APC activity. However, MyD-88 (-/-) deficient DCs could not mature in response to bacterial DNA, the ligand for TLR-9, indicating that MyD-88 is differentially required for signalling of TLR family (Kaisho T *et al,* 2001). Schmausser B *et al* (2004) reported that immunocytochemical studies using gastric mucosal biopsies have revealed TLR-5 and TLR-9 expression on the gastric epithelium changed to exclusive basolateral localization without detectable expression at the apical pole in *H. pylori* gastritis. Although the level of mRNA expression was very low, the wild type significantly up-regulated the TLR-9 mRNA expression in THP-1 cells after 6h of infection. Other mutants studied have not significantly changed the mRNA expression after 6h of infection. Δ*cagA* and Δ*cag*PAI significantly increased the TLR-9 mRNA expression only after 24h of infection, whereas the wild type infected cells continued to up-regulate TLR-9 expression from 6h onwards. This indicates that the *H. pylori* induced TLR-9 expression moderately increased during *H. pylori* infection and the virulence factors such as VacA, FlaA and CG were found to be essential for up-regulation of TLR-9 expression. *M. tuberculosis* infected TLR9 (-/-) but not TLR2 (-/-) deficient mice displayed defective mycobacteria-induced IL-12p40 and IFNγ responses in vivo, whereas, TLR2/9 (-/-) mice displayed markedly enhanced susceptibility to infection in association with combined defects in proinflammatory cytokine production (Bafica A *et al,* 2005).

TLR-10 is known as orphan receptor among TLRs. The ligand for TLR-10 is not known till today. No published data is available describing TLR-10 involvement in any infectious and inflammatory process. Normal and neoplastic human B-cells express a

distinct TLR repertoire including TLR-9 and TLR-10 and such expression is increased upon engagement of the antigen receptor complex or ligand with TLR-9 (Bourke E *et al,* 2003). It has also been reported that TLR-10 is a functional receptor, which can form homodimers and heterodimers with TLR-1 and TLR-2, transducing signals in MyD-88 dependent pathway (Hasan U *et al,* 2005). THP-1 cells express low levels of TLR-10 mRNA. Infections of THP-1 cells with H. pylori have very significantly increased the TLR-10 mRNA expression after 6 and 24h. All the mutants used in this study have also significantly increased the TLR-10 mRNA expression in THP-1 cells after 24h of infection. As observed in other TLR molecules expression, Δ*flaA* found to be the least inducer of TLR-10 mRNA expression. The mRNA level expression during *H. pylori* infection was checked at the protein level by western blot of immunoprecipitated TLR-10 from THP-1 cells infected with *H. pylori* wild type and Δ*flaA* mutant. Western blot analysis revealed the high level expression of TLR-10 protein during *H. pylori* infection. This observation was again confirmed at the mRNA level in HEK293-TLR10 cells infected with *H. pylori.* In this experiment, *H. pylori* wild type significantly increased the TLR-10 mRNA expression after 6h of infection. However, among the mutants Δ*cgt* only increased TLR-10 mRNA expression significantly in HEK293-TLR10 cells although not to the level induced by wild type. This attributes that TLR-10 is playing an important role in the detection of *H. pylori* infection and is the first report on TLR-10 mRNA and protein which can be induced by a bacterium.

THP-1 derived DCs were also used in this study to understand the differential expression of TLRs compared to THP-1 monocytes. LTA and CpG DNA were additive in induction of TNFα, IL-6 and NO in RAW-264 macrophages, peritoneal macrophages and DCs. In contrast, LTA suppressed IL12p40 secretion induced by CpG DNA in RAW-264 cells and peritoneal macrophages but not in DCs. These findings indicated that the consequences of interaction of innate immune cells with microbial pattern depend on the responding cell type and might be differential for certain effector mechanisms (Dalpke

AH *et al,* 2002). Infection of THP1-DCs with wild type *H. pylori* and Δ*flaA* revealed that TLR-3, TLR-7 and TLR-10 are the three main TLRs very significantly induced in the THP1-DCs during *H. pylori* infection. TLR-1, TLR-2, TLR-5 and TLR-8 mRNA expressions were significantly increased, although the induction was moderate in THP1-DCs during *H. pylori* infection. TLR-6 and TLR-9 mRNA expressions were very weak in THP1-DCs even after 24h of infection with *H. pylori*. TLR-2 expression was moderately increased after 6h of infection with *H. pylori* but found to be decreased after 24h of infection. TLR-6 and TLR-8 were drastically down-regulated in THP-1 derived DCs in comparison with THP-1 cells during *H. pylori* wild type infection. Δ*flaA* mutant was again found to be a weak inducer of TLR mRNA expression. This affirms the earlier findings that TLR expression can be varied in different cell types during the infection with same bacterium

The TLR family members are capable of recognizing several classes of pathogens and orchestrating appropriate innate and adaptive immune responses. The role of TLRs in innate immunity to *H. pylori* infection was poorly understood. The present study focused to examine the role of all ten known TLRs expression in THP-1 cells and in particular the role of few individual TLRs by stably transfected in HEK293 cells. mRNA expression has revealed that TLR-3, TLR-6, TLR-7, TLR-8 and TLR-10 transcripts were very significantly up-regulated after 6h and 24h of infection in THP-1 cells. TLR-2 and TLR-9 were weakly up-regulated by *H. pylori* in THP-1 cells after 6h of infection. However, TLR-1 and TLR-9 mRNA expressions were moderately up-regulated after 24h of infection. Among the mutants studied, Δ*flaA* and Δ*vacA* found to be the least inducers of TLR mRNA expression in THP-1 cells. Infection of *H. pylori* with HEK293-TLR2, HEK293-TLR5 and HEK293-TLR10 cell lines have also revealed the similar pattern of the aforementioned TLRs and the role of *H. pylori* virulence factors in the establishment of pathogenesis.

IRAK1 phosphorylation is an important event in the downstream signaling after activation of TLRs through ligand binding. Initial phosphorylation of two threonine residues of IRAK1 by IRAK4 induces massive autophosphorylation and dissociation from the MyD88 adapter complex to induce further downstream signaling that allows NFκB activation and pro-inflammatory gene expression (Kollewe C *et al,* 2004; Akira S and Takeda K, 2004). The IRAK-1 phosphorylation is a critical step in the signalling of TLR proteins. After analysing mRNA expressions of all ten TLRs qualitative changes in the TLR signalling during *H. pylori* infection were also studied. It has been revealed that *H. pylori* virulence and pathogenecity factors such as CagA, VacA, FlaA, Cgt and *cag*PAI status are essential for IRAK-1 massive phosphorylation in THP-1 cells. IRAK-M level, negative regulator of TLR signalling, was also examined. It was found that IRAK-M levels were not changed significantly in THP-1 cells after infection with *H. pylori*. HEK293-TLR2 cells infected with *H. pylori* and mutants have induced a low level IRAK-1 phosphorylation. In HEK293-TLR5 cells infection, Δ*cgt* and Δ*cag*PAI mutants did not induce IRAK-1 phosphorylation at all, however, wild type, Δ*cagA,* Δ*vacA* and even ΔflaA induced low level of IRAK-1 phosphorylation. Interestingly, HEK293-TLR10 cells had induced massive IRAK-1 phosphorylation during infection with *H. pylori* wild type strain, whereas very low level of phosphorylation was noted with ∆*flaA* and ∆*vacA* mutants. HEK293 cells having no or less TLR expression did not activate IRAK-1 phosphorylation during *H. pylori* infection. This implies that *H. pylori* induce IRAK-1 massive phosphorylation through TLR molecules in a host cell type dependent mechanism.

4.2 Nod like receptors Expression and Functional Activation of Inflammasome for IL-1β maturation and secretion during *H pylori* infection with THP-1 monocytes

Nod-like Receptors are the cytoplasmic pattern recognition receptors for detecting microbial molecular patterns and transducing signals to mount a pro-inflammatory reaction. NLR family of proteins are recognized with a modular organization of a C-terminal leucine rich repeats (LRR), central Nucleotide binding domain (NACHT) and an

N-terminal CARD domain, pyrin domain or Bir domain for protein-protein interaction (Tschopp J *et al,* 2003). These proteins are able to form the high molecular weight structures called 'inflammasome' and recruit caspase1 that leads to proteolytic activation of pro IL-1 β and pro IL-18 (Martinon F and Tschopp J, 2004; Martinon F *et al,* 2002). The mRNA expressions of NLRs molecules were not up-regulated more than 2 fold in THP-1 cells during *H. pylori* infection, except IPAF. The mRNA expression of IPAF was significantly increased by *Δcgt, ΔcagA* and wild type after 6h of infection. However, wild type and isogenic mutants infected cells have significantly increased the IPAF expression after 24h. The *ΔvacA* mutant was the least inducer of IPAF in *H. pylori* infected THP-1 cells. This reveals that IPAF might be playing an important role in the intracellular detection of *H. pylori* or its derived molecules in THP-1 cells. It has been reported that NLRs such as NALP-1, NALP-3 and IPAF forms ASC-containing inflammasomes, which activates Pro-caspase-1 to process Pro-IL-1β to mature form (Martinon F and Tschopp J, 2004; Martinon F *et al,* 2002). ASC or PYCARD is a protein that is essential for LPS-induced activation of caspase-1, because ASC knockout mice fail to process procaspase-1 or produce IL-1β and IL-18 following LPS and ATP stimulation, which is consistent with the results obtained in Caspase-1 null mice (Mariathasan S *et al,* 2004; Li P *et al,* 1995; Kuida K *et al,* 1995). However, ASC mRNA expression was very significantly down-regulated in THP-1 cells infected with *H. pylori.* This might have seroius implications on the inflammsome Pro-IL-1β and Pro-IL-18 processing and subsequent secretion. ASC was found to be essential for the secretion of IL-1β/IL-18, but dispensable for IL-6, TNFα and IFNγ production, in macrophages infected with *L. monocytogenes*. Activation of caspase-1 was abolished in ASC deficient macrophages, whereas activation of NF-κB and p38 was unaffected. In contrast, secretion of IL-1β, IL-6, and TNFα was reduced in TLR2-deficient macrophages infected with *L. monocytogenes* (Oezoeren N *et al,* 2006). The secretory forms of these cytokines, IL-1β and IL-18, are synthesized as biologically inactive precursor molecules

inside cells and are cleaved by the enzyme caspase-1 to the biologically active mature forms that are released from cells. IL-1β is synthesized by multiple cells including monocytes, macrophages, neutrophils, hepatocytes, and tissue macrophages throughout the body (Arend WP *et al*, 2008). Western blot analysis had revealed that mature IL-1β were not significantly changed in THP-1 cells during infection with *H. pylori*. However, western blot analysis had shown that Δ*cag*PAI, Δ*cagA* and Δ*cgt* mutants were able to atleast activate Pro-caspase-1 cleavage to active caspase-1, this step is necessary for Pro-IL-1β cleavage to form mature IL-1β. The above data would suggest that inflammasome components expression and functional process have been severely deranged in THP-1 cells during infection with *H. pylori.*

IL-1β lack signal peptides, or leader sequences, and are not released from cells by the usual mechanism of vesicular transport form the Golgi apparatus. The secretion of IL-1β from THP-1 cells infected with *H. pylori* found to be very less amount and Δ*cagA* mutant infected cells have significantly increased the secretion when compared wild type and other mutant infected cells. This is showing that CagA molecule is some way inhibiting the secretion of IL-1β. It has been reported that SRC family tyrosine kinase inhibited the neurotransmitter release from neuronal cells (Ohnishi H *et al,* 2001). CagA is recognized as a substrate by SRC and ABL family kinases that phosphorylate it on tyrosine residues in unique C-terminal region EPIYA motifs (Selbach M *et al*, 2002; Stein M *et al*, 2002; Poppe *et al*, 2007; Tammer *et al*, 2007). The role of SRC in IL-1β secretion was examined by specifically inhibiting the SRC activity using the inhibitor PP2. Additional inhibitors such as pan-caspase inhibitor Z-VAD-FMK and EGFR kinase inhibitor AG1478 were also used to check whether these enzymes are involved in the processing and secretion of IL-1β. It was observed that PP2 treated THP-1 cells had significantly increased the IL-1β secretion even after 8h of infection and continued up to 32h. This would suggest that CagA protein and SRC kinase are involved in the processing and secretion stage of IL-1β. The earlier reported role of IL-1β on inhibition

gastric acid secretion and pangastritis formation may be due to the release of Pro- IL-1β from dead cells and cleaved by some non-specific proteases to mature form.

4.3 Pro-inflammatory cytokine and chemokine expression and secretion in relation to TLR Expression and Signalling during *H. pylori* infection with THP-1 monocytes

Immune mediators such as IL-1β, IL-8 and TNFα proteins and gene polymorphisms have been implicated in the *H. pylori* associated pathologies (El-Omar EM *et al,* 2000; Rad R *et al,* 2003; El-Omar EM *et al,* 2003; Gyulai Z *et al,* 2004). The involvements of innate immune receptors such as TLRs role in cytokine and chemokine gene expression have not been studied extensively. In this study, attempts were made to analyze the pro-inflammatory cytokines such as IL-1β, IL-6, IL-8 IL-18 and TNFα gene expression and secretion in THP-1 cells in relation to TLR expression and signalling during *H. pylori* infection. The particular role of few TLRs on inducing the above mentioned cytokine and chemokine genes have also been analyzed using HEK293-TLR2, HEK-TLR5 and HEK-TLR10 cell lines infected with *H. pylori.*

Three decades ago, TNFα and TNFβ or lymphotoxins (LT) were identified as products of lymphocytes and macrophages that caused the lysis of cells, especially tumor cells (Granger GA *et al*, 1969; Carswell EA *et al*, 1975). TNF secretion can be induced by conserved structural elements common to microbial pathogens, including cell wall moieties such as PGN, LPS, and CpG DNA motifs on binding with TLRs (Aderem A and Ulevitch RJ, 2000). TLRs transcriptionally induce proinflammatory cytokines, including TNF, through the convergence of NF-kB and NF-AT activating pathways, and enhance translational efficiency by a mechanism targeting consensus 39-untranslated AU-rich elements (ARE) in mRNA (Dumitru CD et al., 2000). The ARE is common to many cytokine mRNAs and is bound by tristetraprolin (TTP), a zinc finger–containing protein that accelerates the turnover of ARE-containing mRNAs (Carballo E et al,1998). TTP is induced by TNF as a negative feedback loop that limits TNF activity. Therapeutics to inhibit TNF have been developed which control previously recalcitrant inflammatory

conditions such as rheumatoid arthritis and inflammatory bowel disease (Maini RN and Taylor PC, 2000; Papadakis KA and Targan SR, 2000). THP-1 cells infected with *H. pylori* and isogenic mutants had significantly increased the mRNA expression of TNFα after 6h of infection. However, the level of mRNA expression of TNFα has been significantly decreased in Δ*vacA* and Δ*flaA* mutants after 24h of infection when compared to wild type infected cells. TNFα secretion from THP-1 cells after 24h of infection was also analyzed. The data showed that Δ*vacA* and Δ*flaA* mutants significantly reduced the secretion of TNFα from THP-1 cells after 24h of infection when compared to wild type infected cells which correlated with mRNA expressions after 24h. In addition, Δ*cag*PAI mutant also reduced TNFα secretion from THP-1 cells when compared to wild type infected cells. However, secretion of TNFα secretion from THP-1 cells infected with Δ*vacA,* Δ*flaA,* and Δ*cag*PAI significantly increased in comparison with uninfected cells. This suggests that VacA and FlaA molecules, which have previously been shown to induce significantly less TLR expression and signalling, may be partially responsible for the induction of TNFα secretion from THP-1 cells during infection with *H. pylori.* TLR expression and signalling might be playing an important role along with other signal transduction pathways in TNFα gene expression and secretion during *H. pylori* infection. It has been reported that activation of all four MAP kinase pathways showed a dramatic synergistic effect on TNF reporter gene expression. The MAP kinases appear to target unknown *cis*-element(s) in the TNF promoter and ARE in the 3′-UTR (Zhu W *et al,* 1999).

The IL-1β and IL-18 are activated by inflammasome and have similarity in receptor structure and signal transduction. The receptors for IL-1 are IL-1 receptor-I (IL-1R-I), IL-1R-II and the IL-1R accessory protein (IL-1RAcP). IL-18 has IL-18 receptor and the IL-18R accessory protein (IL-18RAcP). IL-1β is synthesized as 31 kDa biologically inactive precursor molecules inside cells and are cleaved by the enzyme caspase-1 to the biologically active 17 kDa mature form that are released from cells. It is synthesized by multiple cells including monocytes, macrophages, neutrophils, hepatocytes, and

tissue macrophages throughout the body. IL-1β lack signal peptides, or leader sequences, and are not released from cells by the usual mechanism of vesicular transport form the Golgi apparatus (Arend WP *et al*, 2008). IL-18 is produced by monocytes/macrophages in the presence of different microbial components and plays a major role in the innate immune responses to pathogens. IL-18 is particularly important for the clearance of intracellular pathogens and viruses (Gracie JA *et al,* 2003). The IL-1β mRNA expressions in THP-1 cells were significantly increased after 6h of *H. pylori* infection. In addition, Δ*cagA* and Δ*cgt* mutants infected cells had very significantly increased the IL-1β mRNA expression when compared to wild type infected cells. This may be due to direct involvement of CagA and Cholesterol glucosides on transcriptional regulation of IL-1β during the early phase of infection. However, the wild type strain exhibited more mRNA expression of IL-1β after 24h of infection with THP-1 cells. This type of increase in IPAF mRNA expression by Δ*cagA* and Δ*cgt* mutants has been observed in the previous experiments describing the NLR expression. This suggests that CagA and cholesterol glucoside of *H. pylori* may be involved at the transcriptional regulation of host genes. Although *H. pylori* induced IL-1β mRNA expression significantly, the production, processing and secretion of IL-1β at the protein level have been significantly deranged in *H. pylori* infection. In addition, CagA mutant and inhibition of SRC through PP2 treatment and subsequent infection resulted in the increase of IL-1β secretion significantly.

IL-18 was originally identified as an IFNγ inducing factor (IGIF), which circulated during endotoxemia in mice primed with *Propionibacterium acnes* (67). Like IL-1β, IL-18 is synthesized as a 23 kDa biologically inactive precursor peptide, which is subsequently cleaved by caspase-1 (68). Pro-IL-18 is expressed in macrophages, DCs, Kupffer cells, keratinocytes, chondrocytes, synovial fibroblasts, and osteoblasts (Arend WP, 2008). THP-1 monocyte is also expressing IL-18 mRNA and infection with *H. pylori* has not significantly increased the mRNA expression after 6h of infection. However, the IL-18

mRNA expression was significantly down-regulated after 24h of infection of *H. pylori* with THP-1 cells. IL-18 secretion as analysed by ELISA (data not shown) after 24h infection was negligible since no detectable any amount was present in the supernatant collected from THP-1 cells infected with *H. pylori* and isogenic mutants. Hence it can be assumed from the above data, IL-18 expression and secretion are not playing important role in THP-1 cells during *H. pylori* infection.

IL-6 is traditionally considered an activator of acute phase responses and a lymphocyte stimulatory factor (Kishimoto T *et al,* 1995). IL-6 belongs to a family of cytokines that promote cellular responses through a receptor complex consisting of at least one subunit of the signal-transducing glycoprotein gp130 (Heinrich PC *et al,* 2003). Many of the biological activities assigned to IL-6 are mediated through naturally occurring soluble IL-6 receptors (IL-6R). This soluble receptor forms an agonistic complex with IL-6 that binds gp130 to trigger cellular responses. Regulation of this activity is termed “IL-6 *trans*-signaling” (Jones SA and Rose-John S, 2002; Jones SA *et al,* 2005). In contrast to the ubiquitous expression of gp130, the cognate IL-6R exhibits a highly defined pattern of expression and is largely confined to hepatocytes and leukocytes (Jones SA *et al*, 2001). The mRNA expression of IL-6 in THP-1 cells during *H. pylori* infection was studied and it was found that Δ*cagA* and Δ*cgt* infected cells significantly increased the expression after 6 of infection. However, after 24h of infection not only the mutants but also the wild type increased the mRNA expression of IL-6 and the induction by Δ*cagA* and Δ*cgt* were more than wild type. Δ*vacA,* Δ*flaA* and Δ*cag*PAI infected cells were the least inducers of IL-6 mRNA expression in THP-1 cells among the strains tested. As seen in the case of IL-1β and IPAF, CagA and Cholesterol glucosides are directly involving in the regulation of mRNA expression and that continued even in the late phase of infection.

Interleukin-8 (IL-8) is a potent neutrophil-activating chemokine, central to the immunopathogenesis of *H. pylori* induced tissue injury (Crabtree J and Lindley I, 1994)

and high tissue expression is dependent on the presence of a complete *cag*PAI (Nilsson *et al*, 2003). In gastric cell lines, co-culture with *H. pylori* induces IL-8 via activation of NF-κB a transcriptional regulator (Sharma *et al*, 1998; Nozawa *et al*, 2002). In the present study, the IL-8 secretion from THP-1 cells after 24h of infection with *H. pylori* was analyzed. *H. pylori* wild type strain induced very high level of IL-8 secretion from THP-1 monocytes. However, Δ*flaA* and Δ*cag*PAI mutants significantly reduced IL-8 secretion to 74% and 40%, respectively, in comparison with wild type. It can be concluded that reduced TLR expression and signalling by Δ*flaA* and Δ*cag*PAI mutants may be one of the reasons for the reduced IL-8 expression and secretion in THP-1 cells.

4.4 Pro-inflammatory cytokine and chemokine expression and secretion in relation to TLR Expression and Signalling during *H. pylori* infection with HEK293, HEK293-TLR2, HEK293-TLR5 and HEK293-TLR10 cells

HEK293 cells are having less or no TLR expression and therefore an ideal cell line to study the role of individual TLRs during a particular infection or inflammatory process. We have used HEK293 cells and HEK293 cells stably transfected with TLR-2, TLR-5 and TLR-10. In this study, we analyzed the mRNA level expression of TNFα, IL-1β, IL-18 and IL-6 after 6h infection of *H. pylori* with HEK293, HEK293-TLR2, HEK293-TLR5 and HEK293-TLR10 cells. HEK293 cells infected with *H. pylori* and isogenic mutants up-regulated IL-8 secretion and TNFα mRNA expression and secretion significantly when compared to uninfected cells. However, the mRNA expression of IL-1β, IL-18 and IL-6 were not increased during infection with *H. pylori* and isogenic mutants. The role of TLR-2, TLR-5 and TLR-10 in pro-inflammatory gene expression and secretion was also studied by infecting stably transfected TLRs in HEK293 cells.

HEK293-TLR2 cells substantially enhanced the mRNA expression and secretion of TNFα in comparison with HEK293 cells infected with *H. pylori* and isogenic mutants. IL-8 secretion from HEK293-TLR2 cells during *H. pylori* infection was reached more than 20 fold of the secretion exhibited by HEK293 cells. The TNFα mRNA expression was highly induced in HEK293-TLR2 cells when compared to HEK293 cells during infection

with *H. pylori* and isogenic mutants. HEK293-TLR2 cells secreted 3.5 to 5 times TNFα than by HEK293 cells. This enhancement of TNFα expression and secretion in HEK293-TLR2 cells may be due to the increased signal transduction through TLR-2 activation. IL-1β mRNA expression was increased in HEK293-TLR2 cells in comparison with HEK293 cells during infection with *H. pylori* and isogenic mutants. However, IL-6 and IL-18 mRNA expression increased only below two folds in both cell lines and no further enhancement was noted during *H. pylori* infection. This supports the earlier findings that TLR-2 is involved in *H. pylori* infection mediated pro-inflammatory changes. In the present study, IL-1β, IL-8 and TNFα expressions were enhanced in HEK293-TLR2 cells in comparison with HEK293 cells during *H. pylori* infection. Δ*cagA* and Δ*cag*PAI mutants infected HEK293-TLR2 cells have exhibited a significant level of reduction in IL-8 secretion when compared to wild type strain. Δ*cagA,* Δ*vacA,* Δ*flaA* and Δ*cgt* infected HEK293-TLR2 cells have shown significant reduction in TNFα mRNA expression and secretion when compared to the wild type strain. However, TNFα mRNA expression change was not significantly in Δ*cagA* infected cells (Fig.23,24). IL-1β mRNA expression has significantly reduced in HEK293-TLR2 cells infected with Δ*vacA,* Δ*flaA* and Δ*cgt* mutants, on the contrary, Δ*cagA* infected cells significantly increased the expression when compared to wild type strain (Fig 22, 23, 24).

HEK293-TLR5 cells infected with *H. pylori* and isogenic mutants enhanced IL-8 secretion almost seven folds when compared to infected HEK293 cells. TNFα mRNA expression increased in HEK293-TLR5 cells in comparison with HEK293 cells during infection with *H. pylori* and isogenic mutants. However, this enhancement was not as high as exhibited by HEK293-TLR2 and HEK293-TLR10 cell lines infected with *H. pylori* and isogenic mutants. TNFα secretion from HEK293-TLR5 was almost similar to the HEK293 cells secretion. This implies that TLR-5 signalling during *H. pylori* infection is not playing much role on inducing TNFα expression. IL-1β, IL-6 and IL-18 mRNA expressions were not increased in HEK293-TLR5 and HEK293 cells during infection with

H. pylori. Δ*vacA,* Δ*flaA* and Δ*cgt* mutants infected cells have significantly reduced the IL-8 secretion from HEK293-TLR5 cells when compared to the wild type strain (Fig.25, 26, 27).

The present study revealed the fact that TLR-10 is highly expressed during *H. pylori* infection. The involvement of TLR-10 on cytokine gene expression and secretion has not been studied earlier. HEK293-TLR10 cells infected with *H. pylori* and isogenic mutants have significantly increased the IL-8 secretion, which was observed to be more than 5-7 folds in comparison with HEK293 cells, suggesting that TLR-10 has increased the IL-8 expression and secretion through its signalling. TNFα mRNA expression has significantly increased in HEK293-TLR10 cells infected with *H. pylori* and isogenic mutants. However, TNFα secretion from HEK293-TLR10 in comparison with HEK293 cells was significantly increased in wild type, Δ*cagA* and Δ*cag*PAI infected cells only. IL-1β, IL-6 and IL-18 mRNA expressions in HEK293-TLR10 cells were increased not more than two fold in HEK293-TLR10 cells in comparison with HEK293 cells during *H. pylori* and isogenic mutants infection. This indicates that TLR-10 is a functional receptor and enhancing the IL-8 and TNFα secretion significantly, but not IL-1β, IL-6 and IL-18 mRNA expression, during infection with *H. pylori* and may be playing an important role in the pro-inflammatory changes during this peculiar bacterial infection.

4.5 IL-1 Receptor Antagonist polymorphism and genotyping of *CagA, VacA, IceA1* and *A2* among a group of patients colonized with *H. pylori*

The host genetic background in relation to colonization of *H pylori* was analyzed in the present study. Gastric mucosal biopsy samples collected from patients who have tested positive for rapid urease test under routine endoscopic examination were included as the subjects. The presence of *H pylori* infection was further confirmed by PCR amplification for *GlmM* gene. 42 *H pylori* positive samples diagnosed by above method were further analyzed for genotying of *VacA* S, *CagA* and *IceA1* and *IceA2*. *VacA* S region was very important in determining the vacoulating action of *VacA* protein of *H*

pylori. VacA S1 is found to be more toxic than *VacA* S2, which is having an additional 27 bp nucleotide sequence probably affecting the hydrophobic characteristic of mature protein and its secretion. This showed that 74% of patients are infected with *VacA* S1 genotype and associated with 3 peptic ulcer diseases and 29 NUDs, in which one sample is infected with both *VacA* S1 and S2. But there is no significant change was observed in this patient to discuss the importance of this co-existence as reported in few earlier studies (Rahman M *et al.,* 2003). *CagA* gene is a marker of cag pathogenecity island found in certain *H pylori* strains and a crucial virulence factor associated with peptic ulcer diseases and gastric cancer in various studies (Rugge M *et al.,* 1999; Miehlke S *et al.,* 2000, Van Doorn LJ *et al.,* 1998). *CagA* genotype was also analyzed among this group of patients infected with *H pylori*. *VacA* S1/*CagA* genotype is more virulent due to the synergistic effect of these toxic proteins on the host system. Among the patients 45% found to be infected with *CagA* genotype, of which 79% associated with *VacA* S1 genotype and thereby increasing the severity of toxic effects. The only patient having antral ulcer is associated with more virulent *VacA* S1/*CagA* genotype. This genotype is present in 13 other NUDs showing these patients are at more risk for developing ulcers and gastric cancer in later stages if the infection persists.

Host genetic background is also implicated as a putative factor on determining the severity of *H. pylori* associated diseases. Interleukin-1 Receptor Antagonist (*IL-1 RN*) gene is reported to be associated with inflammatory conditions due to its polymorphic region within the second intron containing 2–6 tandem repeats of an 86 bp sequence. In which, the *IL-RN*2/*2* genotype has been associated with proinflammatory responses more severe and more prolonged than those of other *IL-RN* genotypes (Hurme M and Helminen M, 1998). This analysis of *IL-RN* gene polymorphism using specific primers differentiating the alleles among patients was performed. The pro-inflammatory *IL-RN*2/*2* allele was present in 43% of patients studied, while the intermediary heterozygous allele *IL-RN *1/*2* in 36% of patients. *IL-RN*1/*1* allele was present in the

remaining 21% of patients. The high risk *VacA* S1/*CagA*/IL-1RA*2-allele were present in 14% of patients studied and *VacA* S1/*CagA*/ *IL-RN*1/*1* allele in 7% patients, while *VacA* S1/*CagA*/ *IL-RN *1/*2* allele in 16% of patients. The host pro-inflammatory factors along with bacterial virulence factors may increase the risk for pathological changes leading to gastric cancer among this group of patients. The highest prevalence of gastric abnormalities were reported in patients with both host and bacterial high-risk genotype such as *VacA* S1/*CagA*/ *IL-RN*2/*2* /*IL-1B-511T* (Rad R *et al.,* 2003).

IceA was identified following transcriptional up regulation on contact with gastric epithelial cells (Peek RM *et al*., 1998). *IceA* exists as two distinct genotypes, *iceA1* and *iceA2*, and only *iceA1* mRNA is induced following adherence in vitro. *H pylori iceA1* demonstrates strong homology to a restriction endonuclease *nla*IIIR in *Neisseria lactamica* (Xu Q *et al.,* 2002), and in vivo carriage of *H pylori iceA1* strains has been reported to be associated with peptic ulceration and enhanced acute neutrophilic infiltration. It has been reported that *IceA1* genotype is the predominant in the East Asia, while *iceA2* genotype is predominant in the USA and Columbia (Yamaoka Y *et al.,* 1999). The present study examined the *IceA1* and *IceA2* genotypes along with the high-risk host and bacterial factors. Although the protein product of *IceA1* and *IceA2* has no significant homology to known proteins and its structure reveals patterns of repeated protein cassettes. *IceA1* strains have been reported to be associated with peptic ulceration and enhanced acute neutrophilic infiltration, while *iceA2* strains are more prevalent among patients with asymptomatic gastritis and non-ulcer dyspepsia. Further analysis revealed that 81% and 19% infected with *IceA1* and *IceA2* genotype, respectively, and among these 31% patients infected with both strains. *IceA1* and *IceA2* strains were present in all four peptic ulcer patient samples included in the study. Kidd *et al*. reported that the *vacA* S1b, m1 and *iceA1* were closely linked to gastric cancer and 40% of patients in their study had infected with *iceA1 and iceA2* strains, although higher prevalence of gastric cancer patients infected with *iceA1/VacA* S1genotype observed in

South African population. Further studies are needed to establish the synergistic effect of VacS1/CagA+/IL-1RA*2/IceA1/IceA2 combination on establishing gastric pathologies in *H pylori* infected patients.

5. Conclusion

5.0 Conclusion

1. THP-1 cell is one of the best model systems to study all ten TLR molecules as it is expressing these molecules constitutively (Zarember KA and Godowski PJ, 2002). The THP-1 cells maintained in complete RPMI 1640 medium were used for infection assays with *H. pylori* strain P12 and its isogenic mutants such as Δ*cagA,* Δ*vacA,* Δ*flaA,* Δ*cgt* and Δ*cag*PAI.The dynamics of mRNA level expression of TLR-1 to TLR-10 were determined by Taqman Quantitative RT-PCR relative to expression of house keeping gene GAPDH. TLR expression pattern at a multiplicity of infection (MOI) 1:50 of *H. pylori* and isogenic mutant strains were analyzed at 6h and 24h after infection. Quantitative RT-PCR data showed that TLR-3, TLR-5, TLR-6, TLR-7, TLR-8 and TLR-10 were highly expressed in THP1 cells after infection with *H. pylori* wild type. TLR-1 and TLR-9 mRNA expressions were induced moderately, whereas TLR-2 and TLR-4 were at very low level after 24h of infection. All the isogenic mutants used in this study were less potent on inducing TLR mRNA expression in comparison with wild type strain. Of which, Δ*flaA* and Δ*vacA* mutants were shown to be the least inducer of TLR mRNA expression in THP-1 cells.
2. THP-1 derived DCs represent monotypic DCs and function as antigen presenting cells to activate T cells when matured through stimulation (Berges C *et al,* 2005). THP1 derived DCs were used to study mRNA expression of TLRs during infection with *H. pylori* and Δ*flaA* isogenic mutant. TLR-3, TLR-7 and TLR-10 were prominently expressed in THP-1 derived DCs during infection with *H. pylori.* Δ*flaA* mutant was again found to be the least inducer of TLR mRNA expression. Taken together the data obtained in THP-1 and THP-1 derived DCs showed that there exists differential induction of TLRs in different populations of immune cells during *H. pylori* infection.
3. The protein level expressions of TLR-5 and TLR-10 were studied using whole cell extract immunoprecipitation and Western blot. *H. pylori* wild type and Δ*flaA*

isogenic mutant, the least inducer of TLR mRNA expression, were used to infect THP-1 cells to study the variation in protein level expression. TLR-5 was found to be moderately increased in expression at protein level as similar to mRNA expression after 6h of infection with *H. pylori* when compared to uninfected control cells. TLR-10 was highly expressed at the protein level in *H. pylori* infected cells which correlated with mRNA expression as shown in Fig.4 of the results. Δ*flaA* mutant of *H. pylori* was a least inducer of TLR-5 and TLR-10 proteins in THP1 monocytes after 6h of infection when compared to wild type strain. These results clearly ascertain that *H. pylori* virulence and pathogenecity factors have an important role on quantitative and qualitative properties of TLR molecules and that will be the determining factors for successful adaptive immune response.

4. HEK-293 cells stably transfected with TLR-2 (HEK293-TLR-2 cells), TLR-5 (HEK293-TLR-5 cells) and TLR-10 (HEK293-TLR-10 cells) were used for *H. pylori* infection. HEK-293-TLR2 cells infected with *H. pylori* for 6h have also exhibited moderate increase of TLR-2 mRNA expression when compared to uninfected control. Δ*flaA* and Δ*vacA* isogenic mutant strains were the least inducers of TLR-2 mRNAs in HEK-293-TLR2 cells. *H. pylori* wild type and Δ*cagA* isogenic mutant strain have induced 5 and 3 fold increases in TLR-5 mRNA expression, respectively, in HEK-293-TLR-5 cells after 6h of infection. Δ*flaA,* Δ*vacA,* Δ*cgt* and Δ*cag*PAI isogenic mutants have not shown the induction of TLR-5 mRNA expression in HEK-293-TLR-5 cells after 6h of infection. HEK-293-TLR-10 cells infected with *H. pylori* have also exhibited an increase in the TLR-10 mRNA expression to 4 fold level after 6 h of infection. Infection of Δ*cgt* isogenic mutant infection resulted an increase of 2.5 fold of TLR-10 mRNA expression in HEK-293-TLR-10 cells. However, Δ*flaA,* Δ*vacA* and Δ*cag*PAI isogenic mutants were not able to increase TLR-10 mRNA expression more than two folds in HEK-293-TLR-10 cells after 6h of infection.

5. The IRAK-1 phosphorylation is a critical step in the signalling of TLR proteins. After analysing mRNA expressions of all ten TLRs, qualitative changes in the TLR signalling during *H. pylori* infection were also studied. It has been revealed that *H. pylori* virulence and pathogenecity factors such as CagA, VacA, FlaA, Cgt and *cag*PAI status are essential for IRAK-1 massive phosphorylation in THP-1 cells. IRAK-M level, negative regulator of TLR signalling, was also examined. It was found that IRAK-M levels were not changed significantly in THP-1 cells after infection with *H. pylori*. HEK293-TLR2 cells infected with *H. pylori* and mutants have induced a low level IRAK-1 phosphorylation. In HEK293-TLR5 cells infection, Δ*cgt* and Δ*cag*PAI mutants did not induce IRAK-1 phosphorylation at all, however, wild type, Δ*cagA,* Δ*vacA* and even ΔflaA induced low level of IRAK-1 phosphorylation. Interestingly, HEK293-TLR10 cells had induced massive IRAK-1 phosphorylation during infection with *H. pylori*, whereas very low level of phosphorylation was noted with ∆*flaA* and ∆*vacA* mutants. HEK293 cells having no or less TLR expression did not activated IRAK-1 phosphorylation during *H. pylori* infection. This implies that *H. pylori* induce IRAK-1 massive phosphorylation through TLR molecules in a host cell type dependent mechanism.

6. Nod-like Receptors are the cytoplasmic pattern recognition receptors for detecting microbial molecular patterns and transducing signals to mount a pro-inflammatory reaction. NLR family of proteins are recognized with a modular organization of a C-terminal leucine rich repeats (LRR), central Nucleotide binding domain (NACHT) and an N-terminal CARD domain, pyrin domain or Bir domain for protein-protein interaction (Tschopp J *et al,* 2003). These proteins are able to form the high molecular weight structures called ‘inflammasome’ and recruit caspase1 that leads to proteolytic activation of pro IL-1 β and pro IL-18 (Martinon F and Tschopp J, 2004; Martinon F *et al,* 2002). Apoptosis-associated speck-like protein containing a CARD (ASC) or PYCARD is a protein that is essential for LPS-

induced activation of caspase-1, because ASC knockout mice fail to process procaspase-1 or produce IL-1β and IL-18 following LPS and ATP stimulation, which is consistent with results in Caspase-1 null mice(Mariathasan S *et al,* 2004; Li P *et al,* 1995; Kuida K *et al,* 1995). NLRs such as NOD-1, NOD-2, NALP-1, NALP-2, NALP-3, IPAF and inflammsome adaptor ASC mRNA expression molecules were not up-regulated more than 2 fold in THP-1 cells during *H. pylori* infection, except IPAF. IPAF was significantly increased by *Δcgt, ΔcagA* and wild type in the descending order after 6h of infection. However, wild type and isogenic mutants infected cells have significantly increased the IPAF expression after 24h. The *ΔvacA* mutant was the least inducer of IPAF in *H. pylori* infected THP-1 cells. This reveals that IPAF might be playing an important role in the intracellular detection of *H. pylori* or its derived molecules in THP-1 cells.

7. IL-1β and IL-18 are proinflammatory cytokines important in the host defence against infection and in the pathogenesis of various inflammatory disorders. Both IL-1β and IL-18 are synthesized as inactive cytoplasmic precursors that are proteolytically processed to biologically active mature forms in response to various proinflammatory stimuli by caspase-1 (Yu HB and Finlay BB, 2008). Although the intracellular signalling pathways leading to caspase-1 activation remain poorly defined, studies have suggested involvement of members of the NLRs in the regulation of caspase-1 activation. NALP-3 NALP-1 and IPAF can form an endogenous multiprotein complex 'inflammasome' containing ASC and caspase-1, that promotes caspase-1 activation and processing of pro- IL-1β. ASC is critical for caspase-1 activation and secretion of mature IL-1β and IL-18 in response to several purified microbial components as well as intracellular bacteria (Martinon F and Tschopp J, 2004; Martinon F *et al,* 2002). The IL-1β mRNA expression was high during *H. pylori* and isogenic mutants infection of THP-1 cells and IL-18 mRNA expression was significantly down-regulated. The protein

level expression of mature IL-1β was not changed significantly, after 8h of infection with *H. pylori* and isogenic mutants. Pro-caspase-1 cleavage was inhibited during infection with *H. pylori* and isogenic mutant strains Δ*vacA* and Δ*flaA*. On the contrary, Pro-caspase-1 cleavage was not inhibited in the case of Δ*cagA,* Δ*cgt* and Δ*cag*PAI infected cells (Fig.15). However, there was no significant change in the process of Pro-IL-1β cleavage to mature IL-1β in THP-1 cells during infection with *H. pylori* and all isogenic mutants tested. The level of IL-1β secretions was very low, although Δ*cagA* and Δ*vacA* isogenic mutants infected cells significantly increased the secretion of IL-1β than wild type infected cells. The other isogenic mutants such as Δ*flaA*, Δ*cgt*, Δ*cag*PAI infected THP-1 cells secreted less amounts of IL-1β than wild type infected cells and Δ*cag*PAI exerted the least induction of IL-1β secretion. This implicates that inflammasome mediated Pro IL-1β processing was severly deranged in THP-1 cells during *H. pylori* infection.

8. The influence of known *H. pylori* induced kinase activities in host cells and apoptosis on IL-1β processing and secretion by treating the cells with specific inhibitors, such as c-SRC kinase inhibitor-PP2, EGFR kinase inhibitor-AG1478 and pan-caspase inhibitor-Z-VAD-FMK, thirty minutes prior to infection with *H. pylori* wild type was investigated. The IL-1β secretion from THP1 cells, treated with inhibitors as stated above, after 8h, 24h and 32h of infection with *H. pylori* wild type was measured. c-SRC inhibitor-PP2 treated cells had secreted significantly higher amount of IL-1β after 8h of infection, whereas, levels of IL-1β remain unchanged in cells treated with EGFR kinase inhibitor-AG1478, pan-caspase inhibitor-Z-VAD-FMK and wild type *H. pylori* treated cells. This suggests that c-SRC is involved in the retardation of IL-1β secretion during *H. pylori* infection.

9. Pro-inflammatory cytokines such as IL-6, TNFα and chemokine IL-8 were reported to be increased in the gastric tissues of *H. pylori* infected patients. There are no reports regarding the influence of *H. pylori* virulence and pathogenecity factors on cytokine and chemokine gene expression and secretion during infection. In this study, the IL-6 and TNFα gene expression in THP1 cells during infection with *H. pylori* and isogenic mutants Δ*cagA,* Δ*vacA,* Δ*flaA,* Δ*cgt* and Δ*cag*PAI were analyzed. TNF secretion can be induced by conserved structural elements common to microbial pathogens, including cell wall moieties such as PGN, LPS, and CpG DNA motifs on binding with TLRs (Aderem A and Ulevitch RJ, 2000). TLRs transcriptionally induce proinflammatory cytokines, including TNF, through the convergence of NF-kB and NF-AT activating pathways, and enhance translational efficiency by a mechanism targeting consensus 39-untranslated AU-rich elements (ARE) in mRNA (Dumitru CD et al., 2000). THP-1 cells infected with *H. pylori* and isogenic mutants had significantly increased the mRNA expression of TNFα after 6h of infection. However, the level of mRNA expression of TNFα has been significantly decreased in Δ*vacA* and Δ*flaA* mutants after 24h of infection when compared to wild type infected cells. TNFα secretion from THP-1 cells after 24h of infection was also analyzed. The data showed that Δ*vacA* and Δ*flaA* mutants significantly reduced the secretion of TNFα from THP-1 cells after 24h of infection when compared to wild type infected cells and that correlated with mRNA expressions after 24h. In addition, Δ*cag*PAI mutant also reduced TNFα secretion from THP-1 cells when compared to wild type infected cells. These data suggests that VacA and FlaA molecules, which have previously been shown to induce significantly less TLR expression and signalling, may be partially responsible for the induction of TNFα secretion from THP-1 cells during infection with *H. pylori.*

10. IL-6 is traditionally considered an activator of acute phase responses and a lymphocyte stimulatory factor (Kishimoto T *et al,* 1995). IL-6 belongs to a family of cytokines that promote cellular responses through a receptor complex consisting of at least one subunit of the signal-transducing glycoprotein gp130 (Heinrich PC *et al,* 2003). Many of the biological activities assigned to IL-6 are mediated through naturally occurring soluble IL-6 receptors (IL-6R). This soluble receptor forms an agonistic complex with IL-6 that binds gp130 to trigger cellular responses. Regulation of this activity is termed “IL-6 *trans*-signaling” (Jones SA and Rose-John S, 2002; Jones SA *et al,* 2005). The mRNA expression of IL-6 in THP-1 cells during *H. pylori* infection was studied and it was found that Δ*cagA* and Δ*cgt* infected cells significantly increased the expression after 6 of infection. However, after 24h of infection not only the mutants but also the wild type increased the mRNA expression of IL-6 and the induction by Δ*cagA* and Δ*cgt* were more than wild type. Δ*vacA,* Δ*flaA* and Δ*cag*PAI infected cells were the least inducers of IL-6 mRNA expression in THP-1 cells.

11. Interleukin-8 (IL-8) is a potent neutrophil-activating chemokine, central to the immunopathogenesis of *H. pylori* induced tissue injury (Crabtree J and Lindley I, 1994) and high tissue expression is dependent on the presence of a complete *cag*PAI (Nilsson *et al*, 2003). In gastric cell lines, co-culture with *H. pylori* induces IL-8 via activation of NF-κB a transcriptional regulator (Sharma *et al*, 1998; Nozawa *et al*, 2002). *H. pylori* wild type strain induced very high level of IL-8 secretion from THP-1 monocytes. However, Δ*flaA* and Δ*cag*PAI mutants infection with THP-1 cells significantly reduced IL-8 secretion to 74% and 40%, respectively, in comparison with wild type. It can be concluded that reduced TLR expression and signalling by Δ*flaA* and Δ*cag*PAI mutants may be one of the reasons for the reduced IL-8 expression and secretion in THP-1 cells.

12. HEK293-TLR2 cells substantially enhanced the mRNA expression and secretion of TNFα in comparison with HEK293 cells when infected with *H. pylori* and isogenic mutants. IL-8 secretion from HEK293-TLR2 cells during *H. pylori* infection was reached more than 20 fold of the secretion by HEK293 cells. TNFα and IL-1β mRNA expressions were significantly induced in HEK293-TLR2 cells when compared to HEK293 cells during infection with *H. pylori* and isogenic mutants. However, IL-6 and IL-18 mRNA expression were increased not more than two folds in both cell lines during *H. pylori* infection.

13. HEK293-TLR5 cells infected with *H. pylori* and isogenic mutants enhanced IL-8 secretion almost seven folds when compared to infected HEK293 cells. Δ*vacA,* Δ*flaA* and Δ*cgt* mutants infected cells have significantly reduced the IL-8 secretion from HEK293-TLR5 cells when compared to the wild type strain. TNFα mRNA expression increased in HEK293-TLR5 cells in comparison with HEK293 cells during infection with *H. pylori* and isogenic mutants, however, this enhancement was not enough to make changes in TNFα secretion when compared to HEK293 cells. IL-1β, IL-6 and IL-18 mRNA expressions were not increased in HEK293-TLR5 and HEK293 cells during infection with *H. pylori* infection. It can be concluded from the above data that TLR-5 signalling is not enough to induce enhanced IL-1β, IL-6 and IL-18 mRNA expressions and TNFα secretion in HEK293 cells.

14. The present study disclosed the fact that TLR-10 is highly expressed during *H. pylori* infection. HEK293-TLR10 cells infected with *H. pylori* and isogenic mutants have significantly increased the IL-8 secretion, which was observed to be more than 5-7 folds in comparison with HEK293 cells. TNFα mRNA expression has significantly increased in HEK293-TLR10 cells infected with *H. pylori* and isogenic mutants. However, TNFα secretion from HEK293-TLR10 in comparison with HEK293 cells was significantly increased in wild type, Δ*cagA* and Δ*cag*PAI

infected cells only. IL-1β, IL-6 and IL-18 mRNA expressions in HEK293-TLR10 cells were increased not more than two fold in HEK293-TLR10 cells in comparison with HEK293 cells during *H. pylori* and isogenic mutants infection. This indicates that TLR-10 is a functional receptor and enhancing the IL-8 and TNFα secretion significantly, but not IL-1β, IL-6 and IL-18 mRNA expression, during infection with *H. pylori.*

15. *In vivo* analysis of high risk host and bacterial genotypes using 42 biopsy samples from patients colonized with *H. pylori* revealed that *VacA* S1/*CagA*/*IL-1RN*2/*2*-allele in 14%, *VacA* S1/*CagA*/ *IL-RN*1/*1* allele in 7%, and *VacA* S1/*CagA*/ *IL-RN *1/*2* allele in 16% of patients. *IceA1* and *IceA2* status were observed in all four cases of gastric ulcer diagnosed endoscopically (3 duodenal ulcer and 1 antral ulcer), however, two DU and one AU are also associated with *VacA* S1genotype, while remaining DU is infected with *VacA* S2 genotype. Of the 42 samples studied, 81% infected with *IceA1* genotype, whereas only 19% exhibited *IceA2* genotype. In this group of patients 31% (13/42) were infected with both *IceA1* and *IceA2* genotypes of *H pylori.*

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