Molecular characterization of *Helicobacter pylori* protein HP0986 and its involvement with mammalian innate immune system

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DECLARATION

I, **Suhail Akhtar Ansari**, hereby declare that the work presented in this thesis entitled "**Molecular characterization of *Helicobacter pylori* protein HP0986 and its involvement with mammalian innate immune system**" submitted by me under the guidance and supervision of **Dr. Niyaz Ahmed** is an original research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that the research work embodied in this thesis entitled "**Molecular characterization of *Helicobacter pylori* protein HP0986 and its involvement with mammalian innate immune system**" has been carried out by **Suhail Akhtar Ansari**, under the guidance and supervision of Dr. Niyaz Ahmed at the Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, for a full period prescribed under the PhD ordinance of this university.

We recommend this thesis for submission for the degree of Doctor of Philosophy of this university.

Dr. Niyaz Ahmed
(Supervisor)

(Head of the Department) (Dean of the School)

Dedicated to

my parents

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LIST OF ABBREVIATIONS

ATCC	American Type Culture collection
BCA	Bicinchoninic Acid
^{o}C	Degree centigrade
CD	Cluster of differentiation
cDNA	complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
FADD	Fas-associated protein with Death Domain
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Hrs	Hours
ICD	Isocitrate dehydrogenase
IgG	Immunoglobulin G
KDa	KiloDalton
LPS	Lipopolysaccharide
mg	milligram
Min	minutes
mM	millimolar
ml	millilitre

µg	microgram
µl	microlitre
MCP-1	Monocyte chemoattractant protein-1
nM	nanomolar
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
SDS	Sodium dodecyl sulphate
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TLR	Toll-like receptor
TRAF2	TNF receptor-associated factor 2
TRADD	TNF receptor type 1-associated Death Domain
k_d	Dissociation rate constant
k_a	Association rate constant

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CHAPTER 1

Introduction

1.1 Background

Helicobacter pylori is a Gram-negative, microaerophilic and spiral shaped bacterium found on the human gastric mucosa. It has been the subject of immense interest for diverse fraternity of specialists in the field of scientific inquiry including bacteriologists, gastroenterologists, epidemiologists, infection disease experts, cancer biologists and pharmaceutical scientists since after its first culture was isolated by Robin Warren and Barry Marshall from gastric biopsies of infected patients in 1982. The hostile environment of the gastric mucosal surface provides an exclusive opportunity for *H. pylori* in competition with other known microorganisms. In order to counter the harsh acidic environment of stomach, almost 300 genes from *H. pylori* are predicted to be involved in the acid regulation (Wen Y *et al.,* 2003; McGowan CC *et al.*, 2003). Among these genes, the one encoding urease protein is the most critical determinant of the breakdown of urea into ammonia and carbon dioxide. The increase in cytoplasmic pH through the generation of ammonia also buffers the bacterial periplasm. This enhances survivability of *H. pylori* in a highly acidic gastric environment of the host. However, urease activity of these bacteria provides short term resistance against the acidic gastric lumen. Further, some protection is provided by flagella dependent motility of the bacterium along the gastric mucus layer, where distribution of the bacilli is mainly governed by pH gradients with most of the bacteria located into deep mucosa and close to the epithelium. Given this, both the urease activity and motility are important parameters required for successful colonization of *H. pylori* to the gastric tissues (Eaton KA *et al.*, 1996; Schreiber S *et al.*, 1999). The bacterial

adherence to the gastric mucosa is significant in triggering the expression of many virulence associated genes (Kim N *et al.*, 2004).

Over the past 30 years since its discovery, several studies also provided more insight into the pathogenesis of the disease caused by *H. pylori* infection. Further research has established a strong correlation of *H. pylori* colonization with increased risk of gastrointestinal disorders such as chronic gastritis, peptic ulcer, gastric mucosa associated lymphoid tissue (MALT) lymphoma and gastric cancer. Such findings unleased over the years by several research groups had an immense clinical impact on gastroduodenal disease management. The discovery of the role of *H. pylori* in peptic ulcer led to the award of Nobel Prize of 2005 in physiology and medicine to Robin Warren and Barry Marshall.

1.2 Epidemiology

H. pylori is now accepted to have co-evolved with its human host and consequently resulted in tight host specificity of different strains and geographic genotypes of the bacteria. *H. pylori* infection in a human host usually occurs during childhood and persists for life. Almost 50% of world's human population is infected with *H. pylori* culminating in some populations in to a moderate to high incidence of gastric and duodenal inflammation. The inflammation thus caused progresses towards gastric or peptic ulcer disease in 10-15% of infected individuals and gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma in only about 1% of them (Suerbaum S and Michetti P, 2002).

The geographical prevalence of *H. pylori* infection throughout the world shows a large variation. Infection is more predominant in developing world where almost 80% population is *H. pylori* positive (Perez-Perez GI *et al.*, 2004). The incidence rate of infection is far less in industrialized countries which remain below 40% (Pounder RE and Ng D, 1995). However, in developed countries also, infection rate is more prevalent in migrants than the rest of population. In addition, there is an inverse correlation of infection prevalence with socioeconomic status entailing childhood (Malaty HM and Graham DY, 1994). The increased hygienic condition and intake of antimicrobial treatment in industrial world has caused a tremendous negative effect on the colonization of *H. pylori*. The prevalence of *H. pylori* in industrial settings remains very low during the early 5 years of childhood which may increase from the adult age onwards (Kosunen TU *et al.*, 1997; Rehnberg-Laiho L *et al.*, 2001; Roosendaal R *et al.*, 1997). This trend also reflects the birth cohort effect on age distribution of *H. pylori* infection. In contrast, almost all *H. pylori* infections in developing world occur before the age of 10 years and persist thereafter (Bardhan PK, 1997; Fiedorek SC *et al.*, 1991). Furthermore, many studies have shown almost the same prevalence of *H. pylori* infection in men and women in both developed and developing world (Megraud F *et al.,* 1989).

All studies on *H. pylori* prevalence suggest that infection acquisition usually occurs during early childhood and declines thereafter with increase in age (Go MF, 2002; Brown LM, 2000; Megraud F, 1992; Bardhan PK, 1997; Malaty HM, 2007; Rowland M *et al.*, 2006). Several diagnostic methods for *H. pylori* such as serological test, biopsy based test, rapid urease test (RUT), urea breath test

(UBT), or stool antigen test (SAT) have provided more insights into the infection epidemiology in different geographical regions. Reports from several groups have indicated high prevalence of infection in Asia-pacific region except for the white population of Australia and New Zealand. The children in this region showed the low prevalence of infection except in India and the children from African refugees in Western Australia. A cohort study on Indian population has reported about 57% infection prevalence among children (Mishra S *et al.*, 2008). Studies from the African population have reported the infection prevalence rate of 41.3% to 91.3% (Tanih NP *et al.*, 2010; Joutei HA *et al.*, 1998; Aje AO *et al.*, 2010; Hestvik E *et al.*, 2010). An interesting observation from USA showed up to 79% prevalence in a subpopulation of poor Americans with African ancestry whereas database from collected biopsies indicated an overall prevalence of 7.5% (Epplein M *et al.,* 2011). *H. pylori* infection prevalence in different geographical regions including developing and developed countries is summarized in table 1.

Table 1:

Author	Population Studied	Diagnostic test performed	Number of subjects or patients		*H. pylori* prevalence (%)
Asia-Pacific					
Li *et al.*,2010	Random sample of residents in Shanghai, China (aged 18–80 year olds	Serology	1022		71.7
Nam *et al.*,2010	Healthy screening population in Korea	Rapid urease test (RUT)	10102		50.8
Nguyen *et al.*,2010	Patients undergoing gastroscopy in Hanoi and Ho Chi Minh, Vietnam (aged 14–86)	Serology, RUT, culture, histology, immuno-histochemistry	270		65.6
Ozdil *et al.*,2010	Consecutive dyspeptic patients undergoing gastroscopy in Turkey	Histology	3301		71.3
Tsukanov *et al.*,2011	Consecutive patients referred for dyspepsia in Eastern Siberia	Serology, RUT, histology	689 1440	"Europoid" "Mongoloid"	93.6 94.3

Ullah *et al.*,2010	Fish handlers and non-fish handlers in Bangladesh	Serology	163 72	fish handlers non-fish handlers	77.3 37.5
Rahim *et al.*,2010	Aborigines living in north eastern Penin- sular Malaysia	Serology	480		19
Pandeya *et al.*,2011	Australian adults	Serology	1355		15.5
Fraser *et al.*,2010	Teenage females in high schools in Auckland, New Zealand	Serology	386 120 162 124	Pacific Island Maori Asian European	49.0 26.7 24.7 13.7
Al Faleh *et al.*,2010	Random sample of students aged 16–18 from 3 regions in Saudi Arabia	Serology	1157		47
Ozen *et al.*,2011	School children in Istanbul, Turkey	Serology	473		34
Thankachan *et al.*,2010	Children aged 6–10 in Bangalore, India	Urea breath test (UBT)	543		79
Cherian *et al.*,2010	African refugee children from resettlement in Australia	Stool antigen test (SAT)	163		84.0
Abdollahi *et al.*,2011	Children aged 3–18 with reflux symptoms in Iran	RUT, Histology	263		22.4
Africa					
Tanih *et al.*,2010	Consecutive dyspeptic patients at a hospital in Port Elizabeth, South Africa	Culture, PCR	254		66.1
Joutei *et al.*,2010	Dyspeptic patients in Morocco	Histology	755		69
Aje *et al.*,2010	Patients with dyspepsia and controls in Nigeria	Serology, SAT	46 dyspeptics 46 controls		67.4 78.3–91.3
Hestvik *et al.*,2010	Healthy children aged 0–12 in Kampala, Uganda	SAT	427		44.3
S. America					
Dattoli *et al.*,2010	Children aged 4–11 in north eastern Brazil	Serology	1104		28.7
Miranda *et al.*,2010	Children attending a public hospital in Sao Paulo, Brazil	Serology	326		35.6
Janjetic *et al.*,2010	Children aged 4–16 with upper gastrointestinal symptoms	UBT	395		24.3
Egorov *et al.*,2010	Children in poor suburbs of Quito, Ecuador.	SAT	124		61
Araf *et al.*,2010	Adolescents in a public school in Sao Paolo, Brazil (aged 10–16)	UBT	194		40.7
Ortega *et al.*,2010	Dyspeptic patients in Chile	RUT	5664		78
Fialho *et al.*,2010	Poor urban community in north eastern Brazil	UBT in children, serology in adults	570 members of 128 households		66.0

Adapted from Goh KL *et al.* (2011)

1.3 Transmission routes

The exact transmission mechanism of *H. pylori* is still not clear due to the difficulty in sampling of bacteria from the gastric content. The commonly used multilocus sequencing tool to assign the isolates from different individuals has also failed to address the issue of transmission mode due to high genetic diversity of *H. pylori* strains generated through rapid mutation in combination with high frequency of homologous recombination.

H. pylori has a very narrow host range and is believed to be strictly a human pathogen. However, occasionally, it could also be isolated from nonhuman primates, pigs, cats and other human pets (Brown LM *et al.*, 2001; Brown LM *et al.*, 2002; Dore MP *et al.*, 2001; Herbarth O *et al.*, 2001; Fox JG, 1995). The presence of *H. pylori* in pets could serve as reservoir for the human infection. However, there is no conclusive evidence to suggest that human contact with such animals could be implicated in widespread nature of such infection.

Human to human contact is thought to be predominant consequence of *H. pylori* infection particularly within families and local communities. The transmission mode is primarily through the faecal-oral or oral-oral routes or both. In addition, some indirect evidences also support the transmission route for *H. pylori* as waterborne besides other environmental sources (Magalhaes Queiroz DM and Luzza F, 2006; Delport W and van der Merwe SW, 2007; Khalifa MM, *et al.*, 2010). The transmission mode may also differ in different geographical regions. In developed countries, due to high sanitary measurement, the oral-oral mode is the principal mode of transmission (*via* saliva and dental plaque) (Brown LM, 2000). In developing countries, poor

quality of the sanitary management and low socio-economic status of population support the idea that the environmental sources serve as a major reservoirs for *H. pylori* transmission than the oral-oral route (Go MF, 2002).

1.4 *H. pylori* associated diseases

In contrast to other microbes, colonization with *H. pylori* in human stomach is life-long and all infected subjects develop histological gastritis. However, only minority of those infected patients develop gastric ulcer and gastric cancer. Development of a particular disease also depends on the type and pattern of gastric inflammation distribution. The risk of development of these diseases is determined by various host factors, bacterial strain specific factors and environmental factors. The interplay of these factors is related to the outcome of severity of the diseases.

1.4.1 Acute gastritis

Available data on acute gastritis are limited (Gledhill T *et al.*, 1985; Ramsey EJ *et al.,*1979, Marshall BJ *et al.*, 1985; Graham DY *et al.*, 1988; Morris A and Nicholson G, 1987; Sobala GM *et al.*, 1991; Genta RM *et al.*, 1993). Such acute infection in people is self-limiting and usually very short lived. Usually this phase of *H. pylori* colonization is characterized by some transient and nonspecific dyspeptic symptoms in patients such as nausea, fullness, vomiting etc. A marked inflammation in the antrum and body of the stomach is a characteristic feature of this phase accompanied by hypochlorhydria due to the presence of the bacteria. Impairment in secretion of ascorbic acid into the gastric juices is also accompanied by *H. pylori* colonization but its level

remains below normal during inflammation (Rokkas T *et al.*, 1995; Rood JC *et al.*, 1994). Evidence also indicates that presence of high concentration of ascorbic acid is not a favourable condition for *H. pylori* survival (Zhang H-M *et al.*, 1997). In some infected subjects, particularly during childhood, *H. pylori* is spontaneously cleared after first infection and therefore ensures the resolution of the gastritis in these individuals. However, in majority (about 60%) of *H. pylori* colonized individuals the infection may become chronic which is accompanied by the infiltration of inflammatory cells leading to chronic active gastritis (Gledhill T *et al.*, 1985; Sobala GM *et al.*, 1991).

1.4.2 Chronic gastritis

Persistent colonization with *H. pylori* in the gastric mucosa has resulted in profound effects on patterns of gastritis distribution and the levels of acid secretion in the colonized stomach. Bacterial colonization during chronic gastritis phase leads to the associated inflammations in both antrum and corpus (body) mucosa (Stolte M *et al.*, 1990; Fiocca R *et al.*, 1992; Morris A *et al.,*1988). Furthermore, gastric acid secretion is much dependent on extent of gastritis and whether it confines to antrum or body mucosa. The antrum predominant and non-atrophic gastritis usually results in hypersecretion of gastric acid which is further subjected to gastric ulceration in infected patients. The bacteria colonize superficially in gastric corpus mucosa in this case and are usually low in number as observed during histological analysis.

In some cases, impairment of acid secretion enhances the interaction of bacteria with corpus mucosa which leads to predominant gastritis (Kuipers EJ *et al,,* 1995). The acid secretion is reduced due to inflammatory process of

atrophic gastritis which in turn impairs the acid secretion capacity of parietal cells. In addition, various observations suggest that increased up-regulation of cytokines including interleukin 1-β (IL-1β) during corpus mucosa inflammation also imparts inhibition of parietal cell functions (Beales I and Calam J,1998).

1.4.3 Peptic ulcer

Establishment of gastritis in *H. pylori* infected subjects leads to the risk of other complications such as peptic ulcer disease. Approximately 15% of total *H. pylori* infected individuals possess the lifetime risk of developing peptic ulcers (Kuipers EJ *et al.*, 1995; Valle J *et al.*, 1996). The amount of inflammation caused due to colonizing *H. pylori* is usually a risk factor for peptic ulcer development. The disease mainly occurs at locations with most severe mucosal inflammation. Lesser curvature of stomach is most potential site for the development of gastric ulcer whereas duodenal ulcer is usually localised to the duodenal bulb, the area also most exposed to the gastric acid. Initial reports based on the global prevalence study carried out during 1980s has shown the association of 90% duodenal ulcer and 70% gastric ulcer cases with *H. pylori* infection (Marshall BJ *et al.*, 1985; Graham DY *et al.*, 1988). This is further supported through the fact that recent trend of decline in prevalence of *H. pylori* has also resulted in decrease in peptic ulcer incidence particularly in Western countries. However, similar link between *H. pylori* and ulcer disease is not clear in the developing world. Eradication therapy for *H. pylori* reduces the risk of ulcer recurrence in most of the cases within 6 weeks (Goh KL *et al.*, 1996). However, within 1 year of eradication, almost 50% of patients showed recurrence of ulcer disease possibly due to *H. pylori* reinfection or use of

nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin, oral bisphosphonates, potassium chloride and several immunosuppressive medications (Yuan Y and Hunt RH, 2006; Hentschell E *et al.,* 1993; Rauws EAJ and Tytgat GNJ, 1990).

1.4.4 Gastric cancer

Gastric cancer is one of the leading causes of cancer related mortality worldwide with approximately 70000 deaths per year (Hohenberger P and Gretschel S, 2003; Guggenheim, DE and Shah MA, 2013; Parkin DM *et al.*, 2002). Every year, almost 1 million people throughout the world are diagnosed with gastric cancer and thus making it fourth most common and second leading cause of deaths due to cancer worldwide (Parkin DM *et al.*, 2002). Regions with highest incidence of gastric cancer throughout the world are East Asia, Central and Southern Europe and South Africa.

H. pylori induced gastritis invariably increases the risk for development of gastric cancer in infected patients. *H. pylori* related factors are functionally involved with gastric epithelial cell signalling pathways and thus play role in promoting gastric cancer development (Segal ED *et al.*, 1999; Asahi M *et al.*, 2000; Poppe M *et al.*, 2007; Higashi H *et al.*, 2004; Tsutsumi R *et al.*, 2006). Moreover, risk for gastric cancer development is more in infected subjects with severely high gastritis, particularly in patients with chronic atrophic gastritis.

Histologically, two common types of gastric cancers have been identified according to the Lauren classification: first described in 1975 (Correa P, 1992), the intestinal type cancer is directly derived from *H. pylori* induced chronic

gastritis and its progression involves a series of histological steps such as chronic gastritis, chronic atrophic gastritis, and intestinal metaplasia. Diffused type gastric cancer is also linked with *H. pylori* infection and usually associated with poor prognosis (Kong X *et al.*, 2012; Warneke VS *et al.*, 2011; Chiaravalli AM *et al.*, 2012; Stiekema J *et al.*, 2013). However, unlike intestinal subtypes, this cancer type is not associated with chronic atrophic gastritis or intestinal metaplasia. Moreover, diffused type cancer is characterized by infiltration of single or masses of neoplastic cells into the stroma of the stomach and sometimes into the deep stomach wall. This cancer type is poorly differentiated and usually does not involve formation of any glandular structures (Werner M *et al.*, 2001).

1.4.5 MALT

Mucosa associated lymphoid tissue (MALT) lymphoma develops in lymphoid tissues of gastric mucosa and appears after *H. pylori* infection. It arises due to the proliferation of the monoclonal B-lymphocytes in MALT after their stimulation due to infection. The incidence of MALT lymphoma is a rare case in infected patients and occurs in less than 1% of *H. pylori* positive subjects. *H. pylori* induced gastritis is now regarded as a crucial factor in the process of development of MALT lymphoma. Several studies have also indicated that chronic gastric inflammation renders constant antigenic stimulation of B-lymphocytes in MALT leading to their clonal expansion and thus formation of the lymphoma (Banks PM, 2007; O'Rourke JL, 2008).

1.5 *H. pylori* virulence factors in gastric disease

As discussed before, *H. pylori* associated disease outcomes involve the interplay of multiple factors including host genetic predisposition, genetic heterogeneity of infecting *H. pylori* strains and environmental factors. *H. pylori* is now widely believed to have co-evolved with humans since they migrated out of east Africa about 60,000 years ago (Linz B *et al.*, 2007). Therefore, genetic diversity in human populations also parallels the heterogeneity of *H. pylori* strains, geographically. *H. pylori* heterogeneity with reference to the virulence factors from different strains of bacteria and their contributions to geographical differences in gastric disease outcomes have been widely investigated. Among these most notable virulence factors are the cytotoxin associated gene A product (CagA), vacuolating cytotoxin (Vac A), outer inflammatory protein (OipA) and duodenal ulcer promoting gene A (DupA).

1.5.1 Cytotoxin associated gene A (CagA)

Among all the virulence factors of *H. pylori*, CagA is the most extensively studied. Based on the status of the *cagA* gene, all clinical strains of *H. pylori* have been classified into either CagA producing or non-CagA producing strains. Detailed structural analysis of the CagA protein from different strains of *H. pylori* has confirmed the presence of multiple numbers of repeats at C-terminal variable region (Higashi H *et al.,* 2002; Higashi H *et al.*, 2002), each containing a Glu-Pro-Ile-Tyr-Ala (EPIYA) motif with a tyrosine phosphorylation site. First repeat region of all *cagA*-positive *H. pylori* strains contains EPIYA-A and EPIYA-B segments. Second repeat region contains EPIYA-C or EPIYA-D segments in Western and East-Asian strains respectively (Hatakeyama M,

2004). The variation in number of EPIYA-C segments in western strains is a direct consequence for the disease outcomes in infected patients where multiple of EPIYA-C segments in CagA protein are linked to a higher predicted risk for developing gastric cancer than strains containing a single EPIYA-C segment (Yamaoka Y *et al.,* 1998; Yamaoka Y *et al.,* 1999; Argent RH *et al.,* 2004; Azuma T *et al.,* 2002). Such association is difficult to establish in East-Asian populations where all the infecting strains of *H. pylori* are *cagA*-positive with second repeat region containing a single EPIYA-D segment (Xia Y *et al.*, 2009).

The 120-145 kDa protein, CagA was the first to be described in association with peptic ulcer disease (Cover TL *et al.*, 1990; Crabtree JE *et al.,* 1991; Covacci A *et al.*, 1993; Tummuru M K *et al.,* 1993). The genome of *H. pylori* harbours *cagA* gene at one end of the cag pathogenicity island (cag PAI) which also encodes the components for bacterial Type IV secretion System. Type IV secretion system is a molecular syringe which is involved in the delivery of the CagA protein into the host cell. The injected CagA into the gastric epithelial cells impairs the intracellular signalling system in a phosphorylation dependent or phosphorylation independent manner.

Phosphorylation dependent pathway works through phosphorylation at EPIYA motifs by members of Src and Abl family kinases (Poppe M *et al.*, 2007; Selbach M *et al.*, 2002; Stein M *et al.*, 2002; Tammer I *et al.*, 2007; Tsutsumi R *et al.*, 2003). The level of CagA phosphorylation depends on the number of EPIYA motifs present in different strains of *H. pylori* (Argent RH *et al.*, 2004; Hirata Y *et al.*, 2004). At least 10 cellular binding partners from host cell have

been identified to interact with injected CagA in a phosphorylation dependent manner (Backert S *et al.*, 2010). The phosphorylated CagA protein directly plays a role in the pathogenicity of *H. pylori*.

CagA also affects the cellular signalling system in phosphorylation independent manner through interaction with adapter molecules from host cell and/or through the self-dimerization of CagA molecules. The phosphorylation independent CagA activity leads to the loss of cell polarity, proliferation and inflammatory response in the host tissues (Argent RH *et al.,* 2004).

1.5.2 Vacuolating cytotoxin A (VacA)

VacA is the second most widely studied virulence factor from *H. pylori*. Majority of the *H. pylori* strains have *vacA* gene in their genome. As the name indicates, the secreted 95 KDa cytotoxin, VacA induces intracellular vacuolization in the cultured epithelial cells (Cover T L and Blaser MJ, 1992). VacA has been shown to induce multiple cellular effects including membrane channel formation, cytochrome C release from mitochondria leading to apoptosis, induction of proinflammatory response after binding with cell receptors (Atherton JC, 2006; Cover TL and Blanke SR 2005; Kusters JG *et al.,* 2006). VacA specifically inhibits activation and proliferation of T cells, and thus may also play a role in avoiding the clearance of highly pathogenic (CagA containing) *H. pylori* strains (Boncristiano M *et al.*, 2003; Gebert B *et al.*, 2003; Sundrud MS *et al.*,2004). Studies have also indicated the antagonistic effects of both CagA and VacA on some aspects of each other's signalling pathways. CagA has been shown to inhibit the apoptosis of epithelial cells induced by VacA (Mimuro H *et al.*, 2007; Oldani A *et al.*, 2009).

Despite having a functional VacA in all the *H. pylori* strains, they differ in their vacuolating activity. This is primarily attributed to the variation in the allelic structure of VacA at signal (s) regions (s1, s2) and middle (m) regions (m1, m2) (Rhead JL *et al.,* 2007). Experimental data suggest that s1m1 shows greatest cytotoxic or vacuolating activity than s1m2, no cytotoxic effect has been shown by s2m2, and s2m1 alleles are rarely found in *H. pylori* strains (Atherton JC *et al.*, 1995; Letley DP *et al.*, 1999). In Western population, infection with *H. pylori* strains having s1m1 alleles possesses a strong risk for developing peptic ulcer and gastric cancer (Atherton JC *et al.*, 1995; Atherton JC *et al.*, 1997; Miehlke S *et al.*, 2000).

In East-Asia, most of the *H. pylori* strains are found with s1m1 allele; therefore it is difficult to differentiate any clinical outcome in these populations based on s and m-regions (Yamaoka Y *et al.*, 1999; Yamaoka Y *et al.*, 2002).

Successful colonization of *H. pylori* requires adhesion to the gastric epithelial cells. Sequence analysis of some of the *H. pylori* strains suggests that approximately 4% of bacterial genome encodes for outer membrane proteins (OMP), some of which may be putatively involved in adhesions. BabA, SabA and OipA are some of the well characterized adhesins from *H. pylori.*

BabA is most well characterized outer membrane protein from *H. pylori.* Adhesion through BabA involves binding with fucosylated blood group antigen Lewis-b (Le^b) expressed on the surface of gastric epithelial cells (Boren T *et al.*, 1993; Gerhard M *et al.*, 1999; Ilver D *et al.*, 1998). Binding with Lewis-b is usually associated with increased *H. pylori* density in the stomach and thus enhancing the disease causing potential of bacteria (Guruge JL *et al.*, 1998).

Like BabA, Sialic acid-binding adhesin (SabA) is another adhesion factor from *H. pylori* that binds with sialyl Lewis-x antigen expressed on the surface of gastric epithelial cells.

Presence of Lewis-x is closely associated with the incidence of gastric cancer (Yamaoka Y *et al.*, 2006). The expression of Lewis-x on epithelial cells occurs during chronic inflammation due to the change in glycosylation pattern on the host cell surface structure (Mahdavi J *et al.*, 2002).

Outer inflammatory protein (OipA) is a proinflammatory outer membrane protein linked with induction of proinflammatory chemokine IL-8 in gastric epithelial cells (Yamaoka Y *et al.*, 2006). Its expression is regulated through the slip-strand mispairing due to the presence of variable number of CT repeat at 5' region of the gene. Functional OipA is associated with duodenal ulcers and gastric cancer (Yamaoka Y *et al.*, 2006).

Helicobacter pylori binding with gastric epithelial cells through BabA, SabA and other adhesins molecules and subsequent trigger of immunological events by CagA and VacA are also summarized in Figure 1.

A plasticity zone gene, *dupA,* was identified in 2005 as first disease specific virulence factor from *H. pylori*. Initial observations have also shown a strong association of DupA with IL-8 production in antral gastric mucosa *in vivo* (Lu H *et al.*, 2005). In addition to this, DupA also stimulated IL-12p40 and IL-12p70 production (proinflammatory cytokines) through infiltrated mononuclear cells (CD14+ cells) in gastric mucosa (Hussein NR *et al.*, 2010). The association of

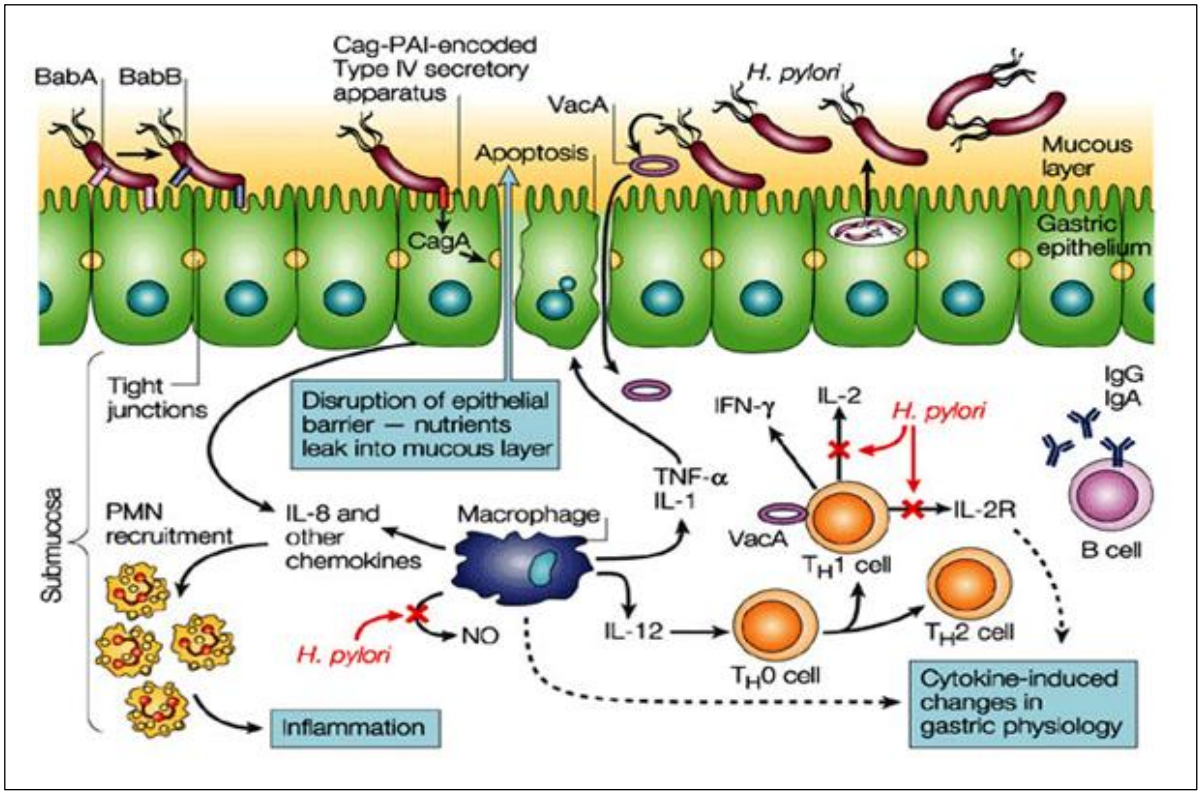

Figure 1: Binding of *H. pylori* to the gastric epithelial cells through BabA and other adhesins leads to a series of events. The injected CagA in to the epithelial cells induces the production of IL-8 and other chemokines which in turn leads to the infiltration of polymorphonuclear cells (PMNs) and thus causing inflammation. CagA also disrupts the epithelial barrier leading to the leakage of nutrients to mucus layer and also allows the entry of VacA and other factors into the submucosa. A link between adaptive and innate immunity has also been established during chronic phase of *H. pylori* gastritis. The secreted cytokine, IL-12 from macrophages recruits helper T cells (T_H0, T_H1 and T_H2), particularly a biased T_H1 response, and B cells. *H. pylori* avoid host immune response through inhibiting the production of NO by macrophages and also by down-regulating T cell activation through interfering with IL-2 signaling by VacA **(Adapted from Monack DM *et al.*, 2004**).

DupA with gastric diseases varies in different geographical populations due to the polymorphic nature of the *dupA* gene. Analysis from different populations has also indicated a population wise difference with respect to association of DupA with duodenal ulcer and/or gastric cancer (Lu H *et al.*, 2005; Arachchi HS *et al.*, 2007; Hussein NR *et al.*, 2008; Argent RH *et al.*, 2007; Gomes LI *et al.*, 2008).

1.6 Working hypothesis and objectives

Several virulence genes from *H. pylori* are linked to varied clinical outcomes in infected patients. Approximately 5-10% of a total of 1600 identified genes are *H. pylori* specific and thought to be involved in the pathogenesis of *H. pylori* in the host (Alm RA *et al.*, 1999; Tomb JF *et al.*, 1997). Many such strain specific genes such as *cagA*, *vacA*, *oipA* and *dupA* have been studied and their association with different pathological conditions in the host have been documented. However, variation also exists in the association of these virulence genes with outcomes of severe gastric diseases. This can be attributed to the genetic diversities of these virulence genes due to a high rate of recombinational and mutational events attributed to *H. pylori*. Such diversification also leads to the classification of these virulence genes into three categories. First category involves strain specific genes which are present in some but absent in other strains of *H. pylori*. Most notably among these is the Cag pathogenicity island (Cag PAI) which contains 30 genes. These genes encode for Type IV secretion system and effector protein CagA (Censini S *et al.*, 1996; Backert S *et al.*, 2000; Asahi M *et al.*, 2000). In addition to cag PAI, nearly 50 % of strain specific genes are located in the so called "Plasticity Zone" (PZ) of *H. pylori* genome. Second category is phase- variable genes. Functional status of such genes varies in different strains of *H. pylori*. Genes encoding the *oipA, sabA, babB, babC and hopZ* (all outer membrane proteins) are put in this category (Alm RA *et al.*, 1999; Oh JD *et al.*, 2006; Tomb JF *et al.*, 1997). Third group includes the genes with variable structures/genotypes. The encoded factors show structural variation in different strains of *H. pylori* and

consequently in their functions. Examples of genes in this group are *vacA* gene, 3' repeat regions of *cagA* and *alpAB* genes (East Asian and Western strains) (Atherton JC *et al.*, 1995; Lu H *et al.*, 2007; Yamaoka Y *et al.*, 1998a; Yamaoka Y *et al.*, 1998b).

Epidemiological studies showed that *H. pylori* prevalence and outcome of severe gastric diseases are always contradictory. Several efforts have been made during the last decade in understanding the role of *H. pylori* virulence factors in pathogenesis. However, such studies lead to the outcomes with controversial data and therefore difficult to establish any concrete correlation between these virulence genes (CagA, VacA and others) to the gastric disease outcomes. In this context, strain specific genes from "Plasticity Zone" of *H. pylori* are of particular interest. Several genes from this zone have been epidemiologically associated with severe clinical outcomes in some geographical populations. Genes such as *jhp 0947* and *jhp 0949* are known to be clinically linked with the outcome of gastric diseases (de Jonge R *et al.*, 2004; Occhialini A *et al.*, 2000; Santos A *et al.*, 2003). A novel virulence factor, ctkA or JHP940 has been characterized from the plasticity zone of *H. pylori* strain J99 (Rizwan M *et al.*, 2007). This important factor is described to be proinflammatory (Rizwan M *et al.*, 2007, Tenguria S *et al.*, 2014) and has been shown to function as a cell translocating Sr/Thr kinase (Kim do J *et al.*, 2010) as well as a proapoptotic protein that serves as a multi-function kinase in mouse macrophages (Tenguria S *et al.*, 2014).

Our group has been working on yet another plasticity zone protein, HP0986 (Alvi *et al.*, 2011; Devi S *et al.*, 2014; Ansari SA *et al.*, 2014) which has been

predicted to be a proinflammatory and proapoptotic protein. Much of its discovery and characterization has been the subject of this thesis.

The functional homologues for most of the PZ genes are largely unknown. Therefore, detailed functional characterization of such genes would definitely substantiate our understanding of pathogenic mechanisms operated by different strains of *H. pylori* in different human populations. Our present study has been directed at molecular and functional characterization of HP0986 with the following specific objectives:

- **Molecular characterization of *Helicobacter pylori* protein, HP0986 and its concurrent proinflammatory as well as proapoptotic activity in human macrophages**
- **Interaction of HP0986 with mouse TNFR1 and subsequent trigger of proinflammatory and proapoptotic signaling pathways in cultured mouse macrophage (RAW 264.7) cells**

CHAPTER 2: OBJECTIVE 1

Molecular characterization of *Helicobacter pylori* protein, HP0986 and its concurrent proinflammatory as well as proapoptotic activity in human macrophages

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2.1 Introduction

Infection of human gastric mucosa with *H. pylori* is associated with different forms of gastro-duodenal diseases such as gastritis, peptic ulcers and gastric carcinoma (Atherton JC, 2006). However, despite the fact that it colonizes more than 50% of the population worldwide, only a small subset of those infected develop more severe forms of gastric diseases; this may be due to various environmental and pathogen specific factors apart from different host immune responses (El-Omar EM *et al.*, 2000; Rad R *et al.*, 2003).

Establishment of successful colonization is a complex process that involves activities of several genome encoded virulence factors, aimed perhaps at survival through inflammation and defense *via* suppressing innate immune responses. Once established in the host, *H. pylori* trigger activation of transcription factors and accumulation of mucosal proinflammatory cytokines followed by cytoskeletal rearrangement, enhanced cell proliferation and apoptosis (Keates S *et al.*, 1999). The induction of proinflammatory cytokines (IL-8 and IL-6) by *H. pylori* is mediated through NF-κB *via* recognition of toll-like receptors (TLRs) (Maeda S *et al.*, 2000; Torok AM *et al.*, 2005). Translocation of NF-κB by *H. pylori* promotes either the inflammatory process through induction of proinflammatory cytokines or regulates host defense by promoting or inhibiting apoptosis (Karin M and Lin N, 2002). There are experimental evidences supporting the pro- and anti-apoptotic roles of NF-κB; its role in TNF-alpha/FasL mediated apoptosis has been described (Zheng Y *et al.*, 2001).

Given the proinflammatory responses aimed basically at gaining niche, the bacterium also appears to have evolved mechanisms to avenge primary defense maintained by the activated macrophages and lymphocytes (Blaser MJ and Atherton JC, 2004). This may involve selective inhibition of T-cell proliferation through up-regulation of Fas antigen (Jones NL *et al.*, 1999), which is possibly mediated by cytokines (TNF-α and IL-1β), reactive oxygen metabolites and iNOS (Houghton J *et al.*, 2000; Grossmann J *et al.*, 1998; Boyle JJ *et al.*, 2003). This may reveal that although the persistent infection substantially increases mucosal inflammation, loss of activated macrophages proportionately limits clearance from the host (Wang J *et al.*, 2001; Gobert AP *et al.*, 2002) leading to chronicity of inflammation.

H. pylori encodes several virulence associated molecules, including proapoptotic (such as VacA) (Kuck D *et al.*, 2001) and antiapoptotic (such as CagA) (Mimuro H *et al.*, 2007) effectors and toxins, besides important virulence factors such as OipA, Ure, flagellins and adhesins. Although the functional coordinates of these factors have been extensively determined in different studies (Backert S *et al.*, 2004; Harris PR *et al.*, 1998), discrete associations of these with different gastric disease outcomes have contradicting evidences (Yamaoka Y *et al.*, 1999). In particular, microevolution and allelic diversity of the CagPAI, and VacA do not allow robust genotype-phenotype correlations thereby posing an obvious difficulty in linking the evolving virulence factors with the pathology (Alvi A *et al.*, 2007). In view of this, it is possible that the bacterium harnesses alternative strain specific factors (Israel DA *et al.*, 2001) to achieve persistent infection. Also,

there are several hypothetical and unknown proteins coded by *H. pylori* genome whose functional role in pathogenesis is unexplored. Therefore, it is pertinent to look into the biology of novel genes/proteins to get new insights into pathogenesis and phenotypic diversification of the bacterium in a changing host. The cache of many strain specific genes (the putative virulence factors) (Janssen PJ *et al.*, 2001) comprises the 'plasticity zone' of *H. pylori* chromosome. Functional characterization of such genes and their involvement in pathogenesis of *H. pylori* could facilitate clear understanding of the development of peptic ulcer disease and gastric carcinoma. In this study, we describe efforts to systematically decipher the proinflammatory and apoptotic roles of one such putative virulence factor, HP0986, and how this observation reinforces our understanding of the biology of *H. pylori* colonization and persistence.

2.2 Materials and Methods

2.2.1 Geographic distribution of the locus *hp0986* and its genetic stability:

Distribution of HP0986 gene was analyzed in clinical isolates from different diseased subjects (gastritis n= 152, duodenal ulcers n=68, gastric cancer n=27) belonging to various geographical regions (India, Spain, South Africa, Japan, France, Peru, Ireland, England, Costa-Rica, Indo-Tibet and Bangladesh). PCR was performed using gene specific primers as described earlier (Occhialini A *et al.*, 2000).

2.2.2 Computational modelling of HP0986 protein-protein interactions: The 3D structure of query protein was predicted by automated homology modelling program, Modeller9v8 (Eswar N *et al.*, 2006). The protein template 3D structures used in the study were downloaded from RCSB Protein Data Bank (PDB). Amino acid sequences of HP0986 were aligned with PDB ID 1XMX to derive the predicted secondary structure using the online tool, ESPript (Gouet P *et al.*, 1999). The geometry of model was checked with PROCHECK tool available with PDBsum program (Laskowski RA *et al.*, 2005). Molecular visualization and general analysis were done using the program PyMOL (Delano WL, 2002). *In silico* docking experiments were performed using PatchDock (Schneidman-Duhovny D *et al.*, 2005) and then further refined and ranked with FireDock (Mashiach E *et al.*, 2008). In crystal structure, the unliganded TNFR1 (PDB ID: 1NCF) exists as a dimer, and therefore only one molecule of TNFR1 (receptor) was taken for unbound protein-protein docking with HP0986 model (ligand) under default complex-type settings.

2.2.3 Cloning, expression and purification of HP0986: The construct for recombinant protein expression was generated by cloning the PCR product spanning 711bp at site *XhoI/HindIII* of pRSETA. The chimeric construct of HP0986 was then propagated into *E.coli* BL-21 (DE3) expression host and the over expressed his-tagged protein was purified by affinity chromatography (Ni^{2+}-NTA, Qiagen or Co^{2+}-NTA, Clontech) (Banerjee S, *et al.*, 2004). Further size exclusion chromatography was performed using Superose -1210/300 GL column (GE Healthcare Ltd.) in buffer containing 20 mM Tris-Cl and 300 mM NaCl (pH

8.0). The recombinant protein was quantified using Bradford's reagent (Bradford MM, 1976). Also, purified HP0986 was treated with Polymyxin-agarose (Sigma-Aldrich, USA) to remove possible endotoxin contamination. This was also confirmed by measuring the endotoxin content with purified recombinant protein by Limulus Amebocyte Lysate (LAL) assay (Pierce LAL Chromogenic Endotoxin Quantitation Kit, Thermo Fisher Scientific, USA).

2.2.4 Analysis for humoral responses: A total of 70 human sera were collected after obtaining informed consents from different subjects having endoscopically proven gastritis, peptic ulcer and gastric carcinoma. Sera from subjects reported to be *H. pylori* negative (as ascertained by C^{14}urea breath test) were used as negative control (n=17). Humoral response against the HP0986 was determined by Enzyme linked Immunosorbent assay (ELISA) as described previously (Banerjee S, *et al.*, 2004). Concentration of recombinant protein and sera was predetermined using serial dilutions to obtain optimum antibody titers. Each ELISA experiment was repeated at least thrice with and without replicates.

2.2.5 Cell culture experiments: Approximately $1x10^6$ human monocyte cells per well (THP1) (ATCC, USA) were differentiation into adherent macrophage like phenotype using Phorbol-12 myristate 13 acetate (PMA) (Sigma-Aldrich, USA) at a concentration of 5ng/ml. These cells were induced using increasing concentration of HP0986 (0.5µg, 1.0µg, 2.5µg, 5.0µg, and 10µg/ml) and incubated for varied time interval. Cells without protein stimulus (Unstimulated cells) and the cells stimulated with proteinase K-treated HP0986 served as negative control. LPS from *Escherichia coli* (Sigma-Aldrich, USA) was used as

positive control. An irrelevant 6XHistidine-tagged protein, Isocitrate dehydrogenase (ICD), from *H. pylori* purified under similar conditions was used as an internal control. Culture medium collected at different time intervals (6hrs, 12hrs, 24hrs and 48hrs) was stored at -80^{o}C until assayed.

2.2.6 Polymorphonuclear blood monocyte cell (PBMC) culture: PBMCs were isolated from heparinized venous blood taken from a voluntary donor using Ficoll-histopaque density gradient as described previously (Savil JS *et al.*, 1989). The cell viability was checked by Trypan blue dye exclusion method and was found to be 90%. Approximately 0.5 million cells/well were seeded in 24 well plate in RPMI 1640 media supplemented with 10%FBS and 2mM glutamine. Cells were treated with HP0986 protein as described above.

2.2.7 Cytokine assay: Amount of IL-8 and TNF-α secreted in the culture medium was determined using commercially available optEIA ELISA Kit (BD Biosciences, USA) as per manufacturer's instruction. The cytokine levels were calculated using the recombinant standard provided within the kit.

2.2.8 Cell extract preparation and Electrophoretic mobility shift assay: Cytoplasmic and nuclear extracts were prepared and translocation of NF-κB complex was determined by electrophoretic mobility shift assay (EMSA) as described earlier (Visvanathan K and Goodbourn S, 1989). Unlabeled NF-κB consensus probe was used as specific competitor. For supershift assay, 5-10µg of rabbit polyclonal anti p65, p50 and C-rel antibodies (Santacruz Biotechnology, USA) were used. After electrophoresis the gel was dried and analyzed by autoradiography.

2.2.9 Immunoblotting: Immunoblotting was performed as described previously (Weih F *et al.*, 1994). Different antibodies such as those against p65, p50, c-Rel, TNFR1, TLR4 and TLR2 (Santacruz Biotechnology, USA) were used. Immunoreactive proteins were detected using enhanced chemiluminescence kit according to manufacturer's instructions (Amersham Inc., USA). β-Actin was used to confirm equal loading of the samples.

2.2.10 HP0986 binding assay by flow cytometry: Binding of HP0986 with cell surface receptors such as TLR4, TLR2 (Imgenex, USA) and TNFR1 (Santacruz Biotechnology, USA) was analyzed using specific antibodies by flow cytometry. FITC conjugated mouse IgG1 antibody (Santacruz Biotechnology, USA) was used as isotype-matched control Antibody. At least 10,000 cells were scanned per sample.

2.2.11 Immunoprecipitation: 0.1mg/ml of cell extract was incubated with HP0986 overnight at 4^{o}C and the immune complexes were trapped using Talon resin (Clontech, USA) or protein A/G agarose beads (Santacruz Biotechnology, USA). Immune complexes were separated on 10% SDS-PAGE and the immunoblot was developed using enhanced chemiluminescence kit (Amersham Inc, USA). Recombinant ICD from *H. pylori* and LPS were used as controls.

2.2.12 Kinetic analysis of interaction using Surface Plasmon Resonance (SPR): Binding kinetics of HP0986 with soluble humanTNFR1 (human TNFR1) were analyzed using BIAcore® 3000 SPR system (GE Healthcare Ltd.). Human TNFR1/Fc (Sigma-Aldrich, USA) was immobilized by an amine-coupling method over a research grade CM5 sensor chip (Biacore, Uppsala, Sweden) up to a

resonance unit of 150. A reference surface was used as a blank to correct instrumental and buffer effects prior to protein injection. During the association phase, the purified HP0986 had been serially diluted in running buffer (PBS) Biacore, Uppsala, Sweden) at 100 nM, 400 nM, 800 nM, and 1200 nM and were allowed to pass individually over the immobilized TNFR1 at a flow rate of 30µl/min for 3 minutes. During the dissociation phase, PBS buffer was applied to the sensor chip at a flow rate of 30µl/min for 4 minutes. The sensor surface was regenerated between each binding reactions by two washes of 30s each with 5M NaOH as evaluated by baseline response. The data was analyzed with BIAEVALUATION 4.1 software (GE Healthcare Ltd.) using simple 1:1 Langmuir interaction model.

2.2.13 Assay of CD95 for HP0986 mediated apoptosis: Expression of CD95 was analyzed by flow cytometry using FITC conjugated anti-Fas monoclonal antibody (CD95, BD) (Decker T *et al.*, 1987). FITC conjugated mouse IgG1 antibody (Santacruz Biotechnology, USA) was used as isotype-matched control antibody. Cells stimulated in the presence of neutralizing antibody to TNFR1 (50µg/ml) were used to check the involvement of TNFR1 in the regulation of Fas expression.

2.2.14 Analysis of CD95 expression by Immunocytochemistry: Cells were sedimented on glass slide and fixed in 1% paraformaldehyde at 4^{o}C for 30 minutes. For antigen staining, cells were incubated with rabbit polyclonal anti-Fas antibody (1:300) (Santacruz Biotechnology, USA) for 1-2 hours at 37^{o}C. Unbound antibody was washed off with PBS and the cells were further incubated

with Alexafluor (Segal ED *et al.*, 1996) conjugated anti-rabbit IgG (1:200) (Molecular probes, USA) for 30 minutes at room temperature (Jones NL *et al.*, 1999). Enhanced Fas expression was analyzed by fluorescence microscopy (Ziess epifluorescence microscope).

2.2.15 Analysis of apoptosis induction by HP0986: Apoptosis assays were performed using acridine orange/ethidium bromide staining (Houghton J *et al.*, 2000) and AnnexinV kit method (BD Biosciences, USA). For acridine orange and ethidium bromide assay, approximately 100 cells were counted in three randomly selected fields and the rate of apoptosis was expressed as mean percentage of total 300 cells counted. For AnnexinV, percentages of apoptotic cells were expressed as total % of annexinV$^+$ and PI$^+$ cells after subtracting background fluorescence (Lee SY *et al.*, 2001). Cells treated with HP0986 in presence and absence of neutralizing antibodies to TNF-α and TNFR1 were used to determine the role of HP0986 in the induction of apoptosis. Oleandrin, a known potent inducer of apoptosis (Manna SK *et al.*, 2000) was used as a positive control. Lysate of the cells not exposed to HP0986 served as negative control.

Statistical Analysis: Results were expressed as means ± the standard error (SE). Induction of cytokine levels and the rate of apoptosis were compared using a two-tailed Student's *t* test and considered significant if the *P* values were < 0.05. *P* values were calculated using the online Graph Pad scientific calculator (http://www.graphpad.com_quickcalcs_ttest1.cfm).

2.3 Results

2.3.1 Association of *hp0986* with invasive disease outcomes and its distribution in clinical isolates: *hp0986* was found to be present in more than 61% of the total isolates we screened from many different geographical regions (Figure 1A and 1C). Overall, the presence of this gene was significantly associated with invasive disease (peptic ulcer and gastric carcinoma, 72%) outcomes (Figure 1B). This apparently contrasts previous observations (Occhialini A *et al.*, 2000) that describe *hp0986* to be gastritis specific. Also, the gene was found consistently conserved in all the three strains isolated from different niches of the stomach, nine years apart (Prouzet-Mauleon V *et al.*, 2005), from a single patient (Figure 1A). This suggests that despite a genome wide trend of extensive rearrangements in *H. pylori, hp0986* is broadly conserved and maintained.

2.3.2 Protein sequence analysis and structure function prediction: HP0986 was predicted to be *H. pylori* specific with no obvious sequence similarity in the available microbial sequence databases. Functional prediction showed high antigenic indices equivalent to ~3.4 (Lasergene software, DNA Star Inc, USA). Due to the unavailability of crystallographic/solution structure of HP0986, a search for possible homologs was carried out using several programs. Sequence-based search methods (BLASTp) did not provide any significant hit but sequence searches in PDB identified a template with 22% identity. This template (PDB ID: 1XMX) was a hypothetical protein, VC1899 from *V. cholerae* and a structural model of HP0986 was built using it (Figure 2A). Total 50 solutions were obtained

using Modeller9v8 (Eswer N *et al.*, 2006) and solution No. 33 was considered the best among them on account of less energy. The quality of the structure was assessed using Ramachandran plot obtained *via* Procheck, which displayed 88.6% residues in most favored regions and 0.9% residues in disallowed regions. Consequently, ModLoop (Fiser A and Sali A, 2003) was used to re-build the two residues in the modelled region followed by energy minimization. Results of the model validation using Procheck program were as follows: 89.0% residues in the most favored regions; 10.0% residues in the additional allowed regions; 0.9% residues in the generously allowed regions and 0.0% residues in the disallowed regions. Secondary structure analysis showed ten alpha helices and seven beta sheets in the modelled structure (Figure 2B). In order to identify possible interacting domains of HP0986 with important receptors such as TNFR1, HEX (Macindoe G *et al.*, 2010), GRAMM-X (Tovchigrechko A and Vakser IA, 2006) and PatchDock (Schneidman-Duhovny D *et al.*, 2005) programs were employed for unbound protein-protein docking with TNFR1 as receptor and HP0986 as a ligand. Approximately 1000 predictions were generated using PatchDock and were submitted to FireDock (Mashiach E *et al.*, 2008) to refine 10 best solutions on the basis of global energy. Possible binding interface residues were identified using 3D2GO binding site prediction server (Kelley LA, 2009). Several of the lowest energy docking models emerging from this exercise placed the HP0986 on the side of the TNFR1. Among ten docked complexes, complexes 1 and 4 were identified as the plausible ones on the basis of minimum energy score and binding interface residues. A docking model of TNFR1-HP0986 is shown in

Figure 2C, in which loop 1 and 2 regions of TNFR1 are docked onto α helices of HP0986.

2.3.3 Expression and purification of recombinant HP0986: The over-expressed HP0986 was purified to homogeneity under native conditions as a 6XHistidine-tagged protein in *E. coli* BL21 (DE3). Homogeneity of the protein was further confirmed by fast performance liquid chromatography (FPLC) (Figure 3C). The purified protein upon fractionation on a 10% polyacrylamide gel showed a single band corresponding to ~ 29kDa on staining with Coomasie brilliant blue dye (Figure 3A).

2.3.4 Humoral responses to HP0986: A strong and significant humoral response (p<0.0001) was observed in *H. pylori* infected diseased subjects as compared to *H. pylori* negative individuals (Figure 4). Mean value of serum HP0986 levels (Mean ± SD) in *H. pylori* infected patients was 0.397± 0.081 (Mean ± SD) as compared to *H. pylori* negative subjects 0.133±0051). However, a stage wise (gastritis, peptic ulcer and gastric carcinoma) serum reactivity against HP0986 was not observed.

2.3.5 HP0986 induces proinflammatory cytokines (TNF-α and IL-8) in a dose and time dependent manner: HP0986 elicited strong cytokine response both in cultured/PMA differentiated Thp1 cells (Figure 5A and 5C) and in human polymorphonuclear blood monocytes (PBMC) in a dose dependent manner (Figure 5E, 5G). A significant increase in induction of TNF-α ($p<0.0016$) and IL-8 ($p<0.0003$) as compared to untreated cells was observed. Time kinetics revealed active production of TNF-α ($P< 0.0003$) within 6 hrs of stimulation (Figure 5D

and 5H) which decreased slowly after 12 hours. In contrast, IL-8 secretion increased during this period with peak response noted at 12 hours post stimulation; levels were more or less maintained up to 48 hours (Figure 5B and 5F). An unrelated His-tagged recombinant protein, isocitrate dehydrogenase (ICD) from *H. pylori* failed to demonstrate cytokine response even at the highest concentration of 10µg/ml (data not shown). Further, inductions of these proinflammatory cytokines by HP0986 were not affected when the protein was treated with Polymixin B. Moreover, Proteinase-K treatment confirmed loss of HP0986 induced cytokine responses suggesting that the effect was due to HP0986 (Figure 5A and 5C).

2.3.6 HP0986 induces IL-8 through NF-κB: The role of transcription factor NF-κB in regulating the expression of IL-8 is already well established (Sharma SA *et al*, 1998). We observed a significant and proportionate increase in the activation of NF-κB complex in HP0986 treated cells in a time dependent manner (Figure 6A) as compared to untreated cells and cells treated with LPS (Figure 6A). Purified recombinant (6XHis-tagged) isocitrate dehydrogenase (ICD) from *H. pylori* was used as an unrelated control (Figure 6A) and the levels of NF-κB complex corresponding to ICD were similar to those observed with untreated cells. Competition with unlabeled NF-κB DNA probe confirmed the specificity of the complex. Further, exclusive involvement of HP0986 in the activation of NF-κB complex was confirmed by using antibodies specific to NF-κB subunits, p65, p50 and c- Rel. Addition of antibodies led to the supershift of p65 and p50 subunits in the extract of cells treated with HP0986 or LPS (Figure 6B). No binding to anti-

c-Rel antibody was observed (Figure 6B). β-actin was used as an equal loading control (Figure 3K). It was thus confirmed that HP0986 up regulates NF-κB, which in turn induces IL-8 expression.

2.3.7 HP0986 functions through interaction with TNFR1: HP0986 triggered the expression of TNFR1 on the THP-1 differentiated macrophages (Figure 7A). We also tested the possible interaction of HP0986 with TLR4 and TLR2; however, HP0986 treatment did not have any effect on Toll-like receptor's expression (Figure 7B and 7C). This finding was further confirmed by analyzing the antagonist effect of TNFR1 receptor on NF-κB translocation. Pretreatment of cells with neutralizing antibodies against TLR4 and TLR2 did not abrogate NF-κB translocation (Figure 7D), however, this did happen when cells were pretreated with a neutralizing antibody against TNFR1, suggesting the possible role of HP0986 in increased TNFR1 expression (Figure 7D).

Interaction of HP0986 with TNFR1 was further validated by immunoprecipitation using anti-TNFR1 antibodies. The receptor was detected in the eluate treated with HP0986 but could not detect any corresponding signals in the cell lysate treated with either LPS or recombinant ICD (Figure 7E). Exclusive interaction of HP0986 with TNFR1 was further confirmed in competition with neutralizing antibodies against TNF α; the amount of immune complex detected was less as compared to that seen in the absence of TNF-α (Figure 7F). This suggested a direct interaction between TNFR1 and HP0986 and ruled out any possible role of endogenous TNF-α. Further, BIAcore® (Surface Plasmon Resonance) (GE Healthcare Ltd.) analysis provided insights into the interaction of HP0986 and

TNFR1 interaction. Using 1:1 Langmuir binding model to fit our binding curve, we found that HP0986 indeed binds with TNFR1 (k_a=1.26x10^4+ 5.62x10^2 Ms^{-1}) on the biosensor surface. The resultant complex was found to be highly stable as illustrated by slow dissociation rate (k_d =9.08x10^{-4}+ 3.80x10^{-5} s^{-1}). A good binding fit (x2=8.94) was obtained confirming the above values of k_a and k_d (Figure 7G).

2.3.8 HP0986 induces Fas mediated apoptosis: Considering the interaction of HP0986 with TNFR1, its possible involvement in inducing Fas expression was tested as the latter is known to function in synergy with TNFR1 and constitutively regulates downstream signaling cascade leading to apoptosis (Houghton J *et al.*, 2000). Pretreatment of PMA differentiated THP-1 cells with HP0986 effectively regulated Fas expression in a time and dose dependent manner. Comparative expression analysis with increasing protein concentration at different time points revealed significant and proportionate increase in Fas expression up to 24 hours (Figure 8A). Expression levels declined after 12 hrs in cells subjected to higher protein dose (5.0µg/ml). This could be due to possible increased cell death. Immunocytochemical staining also showed an increased expression of Fas on the surface of HP0986 stimulated cells as compared to unstimulated ones (Figure 8B).

Corroborating our findings with an earlier study (Boyle JJ *et al.*, 2003) on the synergistic function of TNFR1 and Fas in inducing apoptosis, we evaluated the potential of HP0986 as an apoptosis-inducing agent. HP0986 triggered apoptosis in cultured macrophage cells in a dose and time dependent fashion (Figure 9A). Furthermore, apoptosis as a function of Fas expression was also mechanistically

shown through binding with annexin-V and acridine orange (Figure 9C). A substantial and proportionate increase in cell death was observed when the cells were treated with increasing concentration of HP0986 (0.5µg/ml-10µg/ml) for varied time intervals; up to 48 hours (36.65% ±3.25% to 41.0%± 4.2%) as compared to untreated cells (10.65% ± 1.85%). This pro-apoptotic property of HP0986 declined significantly upon blocking its interaction with TNFR1 (Figure 9 B) indicating the involvement of HP0986 in TNFR1mediated cell death. Similar results were obtained when the cells were stimulated in the presence of neutralizing antibody against TNF-α, suggesting that the effect was not secondary to endogenous TNF-α. As expected, 6XHis-tagged ICD protein from *H. pylori* failed to induce apoptosis. A comparative analysis between oleandrin (a known inducer of apoptosis) (Manna SK *et al.*, 2000) and HP0986 also confirmed the latter being an equally potent inducer of apoptosis (Figure 9B). Collectively, all these findings confirm HP0986 to be a potent apoptosis-inducing agent.

2.4 Discussion

Novel genes constantly emerge from newly sequenced replicate genomes. This paradigm was supported by the analyses wherein the pan-genome of a true bacterial species remained 'open' and each new genome sequence would identify dozens of new genes in the existing pan-genome of *Streptococcus agalactiae*, for example (Tettelin H *et al.*, 2005). It is also clear from previous studies that a pool of strain specific genes in pathogens such as *H. pylori* termed the 'plasticity region cluster' could be useful in adaptation to a particular host population (Ge Z and Taylor DE, 1999). This pathogen shows a very strong geographic adaptation

and is known for harboring up to 45% strain specific genes with most of them gained through horizontal gene transfers (Ge Z and Taylor DE, 1999). Recently, the members of the plasticity region cluster were shown to be likely involved in promoting proinflammatory potentials of some of the strains, possibly providing a survival advantage (Rizwan M *et al.*, 2008; Peek RM Jr *et al.*, 1995).

However, a majority of the plasticity region genes /proteins are yet to be fully characterized. Earlier study from our lab has reported the functional characterization of JHP940, a novel antigen from this region that has shown potential proinflammatory activity (Rizwan M *et al.*, 2008). However, it is not clear if plasticity region proteins provide any survival advantage to the pathogen and the mechanisms thereof. The present study attempted to explore functional aspects of HP0986 as a putative virulence factor and to examine its prevalence in clinical isolates from different geographical regions. Also, we performed a series of activity experiments to elucidate its role in pathology; in particular, its proinflammatory and apoptotic activity in human macrophages. We used differentiated human macrophages since *H. pylori* considerably recruits and excites macrophages in the gastric submucosa to initiate a chronic and persistent trail of inflammatory activities leading to certain patho-physiological changes (Peek RM Jr *et al.*, 1995; Suerbaum S and Michettie P, 2002).

We observed that presence of HP0986 gene was found to be significantly associated with invasive disease outcomes (ulcer and gastric cancer) as compared to gastritis (Figure 1B); this contrasts a previous report (Occhialini A *et al.*, 2000) about its prevalence in strains linked to gastritis cases alone. In our

study, low positivity was recorded in gastritis causing strains from Peru and South Africa (Figure 1C). Moreover, the presence of HP0986 was found to be independent of *cag*A and *vac*A status of the strains tested by us.

Despite traditionally high allelic diversity in *H. pylori*, HP0986 was found to be evolutionarily conserved as observed for a period of ten years in strains isolated from different niches of the stomach of a single patient (Figure 1A) (Prouzet-Mauleon V *et al.*, 2005). This suggests conserved maintenance of the locus in the genome thereby pointing to its essential role in pathobiology of *H. pylori*.

Given our theoretical observations on high antigenicity of HP0986, *in silico* analysis showed several putative B-cell epitopes; we experimentally tested its ability to elicit humoral and cellular immune responses. Significant humoral immune responses induced by HP0986 may be important in diagnostic development given the fact that many candidate antigens have suffered due to high genetic variability across different regions and cross reactivity with other related organisms (Crabtree JE *et al.*, 1994). Our analysis showed significantly high antibody titers in *H. pylori* infected invasive disease patients when compared with healthy controls or non-infected individuals (Figure 4). These point to the extracellular abundance of HP0986 protein and its role in stimulating immune response. Further, since the NF-κB activation in *H. pylori* is often type IV dependent, we cannot rule out secretion of HP0986 protein through a type IV secretary system. Alternatively, it is possible that the protein might be directly released into the extracellular space in the aftermath of autolysis (Cao P *et at.*, 1998; Hussain MA *et al.*, 2008).

While considering the fact that *H. pylori* proteins released into extracellular space may find their way into the submucosa and augment proinflammatory signaling (Suerbaum S and Michetti P, 2002), we looked at HP0986 to potentially augment proinflammatory cytokine secretion from macrophages (IL-8 and TNF-α) consequent to NF-κB activation. These presumptions are consistent with an earlier study describing effect of *H. pylori* or its products on NF-κB (p65/p50) mediated transactivation of IL-8 (Torok AM *et al.*, 2005). Interestingly, our observed stimulation of IL-8 was found to be secondary to TNF-α secretion; maximal concentration was detected as early as 6 hrs (Figure 5D and 5H) as compared to 12 hrs for IL-8 (Figure 5B and 5F). The apparent decrease in TNF-α concentration may be due to the binding of TNF-α with the soluble TNF receptors. As a consequence, less TNF-α concentration was detected in the culture supernatant after 12 hours. Based on these findings, we propose the role of HP0986 in cytokine mediated gastric injury in a similar way as shown previously for the airway epithelial inflammation triggered by *Staphylococcus* protein-A (Gómez MI *et al.*, 2004). The effect was consistent when tested in both THP-1 differentiated macrophages (Figure 5A- D) and human PBMCs (Figure 5E-H). It is known that *H. pylori* infection disrupts tight intracellular junctions and transports its products into the gastric sub mucosal space to augment infiltration of mononuclear cells (Segal ED *et al.*, 1996). This strategy perhaps helps the bacterium to establish persistent infection as it feeds on inflammatory exudates for carbon source derived from mucosal sugars and thereby gains a niche. Also, it inhibits in parallel the expansion of antigen specific T-cells as a

mechanism of immune evasion (Gebert B *et al.*, 2003). Studies support the notion that IL-8 activity attracts mononuclear cells, and TNF-α triggers Fas mediated apoptosis of activated macrophages (Hagimoto N *et al.*, 1999; Lee SY *et al.*, 2001). TNF-α works in an autocrine fashion by up regulating Fas expression on the surface of activated macrophages by binding with TNFR1 (Boyle JJ *et al.*, 2003) and shares a hierarchy of downstream events (FADD) leading to apoptosis (Wang J *et al.*, 2001; Wang L *et al.*, 2008). Nevertheless, there are contradictory reports supporting pro and anti-apoptotic roles of Fas in different cell lineages but antigen behavior also determines cell fate (Fan X *et al.*, 2000). Our observed synergistic function of TNFR1 and Fas is consistent with the previous findings (Lee SY *et al.*, 2001; Varfolomeev EE *et al.*, 1996). Having identified the dependency of Fas on TNFR1, we anticipated that HP0986 actually binds to TNFR1 and thus mimics signaling through TNF-α. We confirmed this interaction through a series of immunological and biophysical measurement and our binding results were in accordance with computational modeling of HP0986 and TNFR1 interaction; Also, we could not observe any binding with TLR4 or TLR2.

Taken together, these observations allow us to propose a model of putative bacterial strategy (Figure 10) harnessed for survival and possibly for maintaining a balance between recruitment and activation of macrophages and their suppression by TNFR1 mediated apoptosis. This strategy potentially projects HP0986 as the central player involved in both proinflammatory and apoptotic cascades. It is therefore highly probable that HP0986 is a novel virulence factor and possibly an important effecter in gastritis and peptic ulcer disease and

various other outcomes of chronic *H. pylori* infection such as gastric adenocarcinoma. While we do not know how many such virulence factors operate behinds the pathology triggered by this manipulative pathogen, understanding of each of them in depth is necessary to devise strategies to control progression of the infection towards more serious outcomes. Future efforts are indeed necessary to understand molecular structure of this protein to gain insights into intricacies of its function and how its role is regulated *in vivo*.

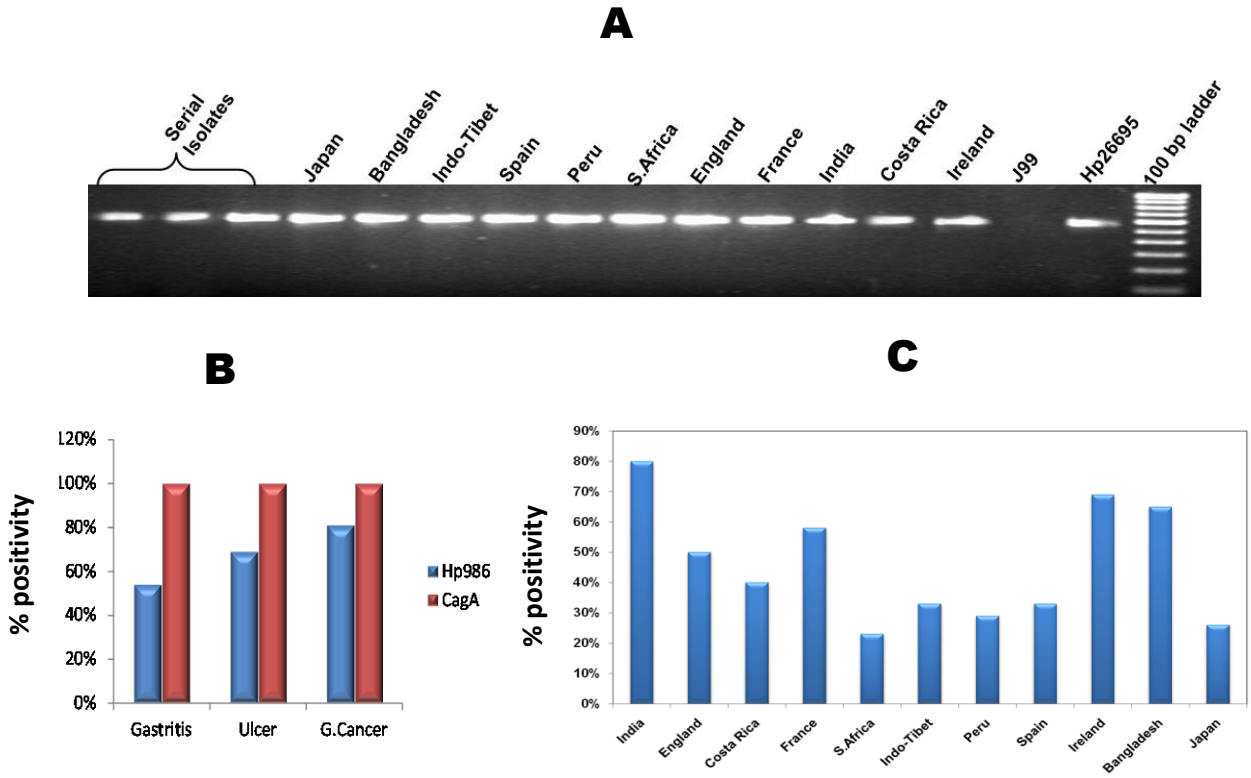


Figure 1: The locus hp0986 was found to be associated with chronic gastric disease conditions. (A) HP0986 is widely distributed in different geographical regions. (B and C) Bar diagrams to represent % prevalence of HP0986 in different disease categories and in different geographic regions, respectively.

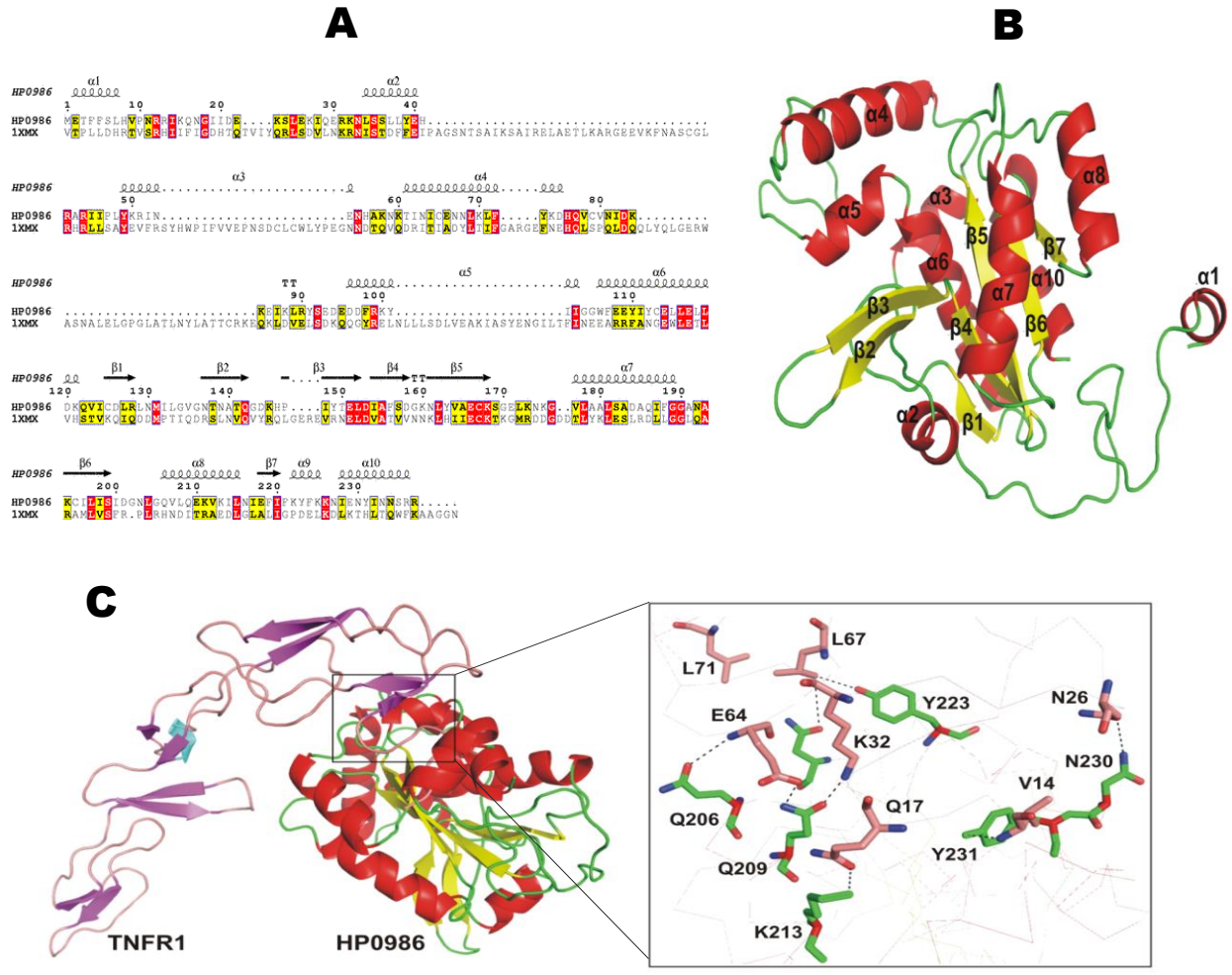


Figure 2: Sequence alignment and predicted3D-structure of HP0986. (A) Sequence alignment of HP0986 with the hypothetical protein (VC1899) from Vibrio cholerae (PDB code 1XMX). Strictly conserved residues are highlighted in red and partially conserved residues are yellow. The sequence numbering refers to HP0986. (B) Predicted 3D-structure of HP0986. The protein secondary structures elements are labelled and colored. (C) Interaction of HP0986 with TNFR1 using PatchDock and FireDock. The residues of HP0986 and TNFR1 are colored in cyan and green respectively. The residues showing interaction between both proteins are labelled and displayed as stick model in element colors (carbon colored green/pink, nitrogen colored blue, and oxygen colored red). Hydrogen bonds are represented by black dashed lines.

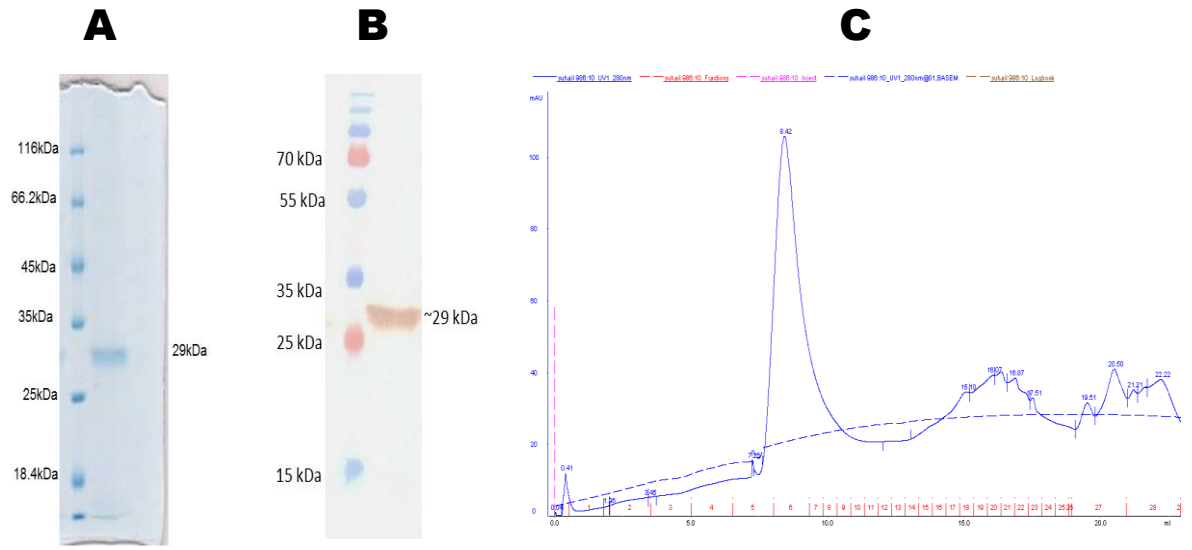


Figure 3: Purification of 6x Histidine tagged-HP0986 using column with cobalt based talon resin. Purified protein was analyzed by SDS-PAGE (A) and Western blotting using anti-His monoclonal antibody (B). Homogeneity of purified protein was confirmed by Fast Performance Liquid Chromatography (C).

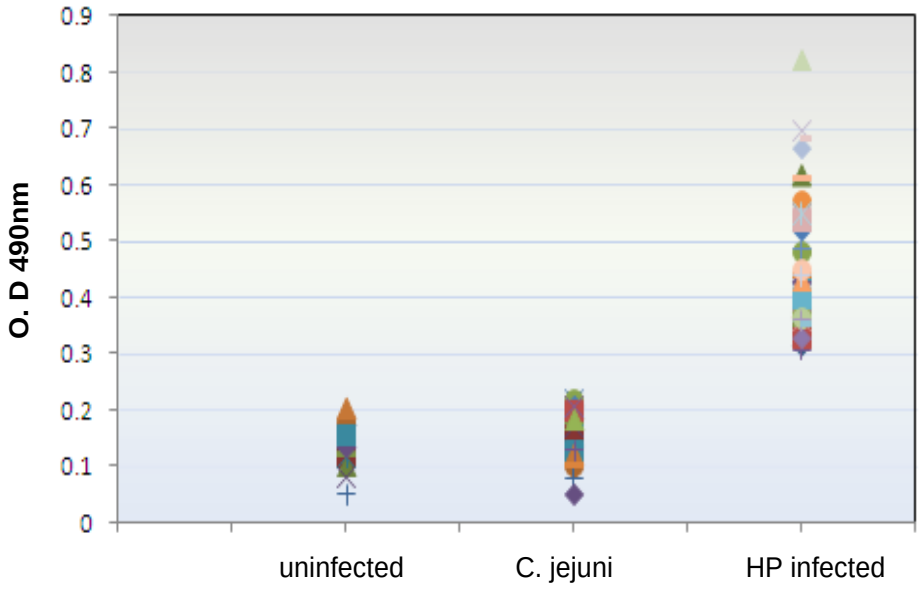


Figure 4: Humoral responses directed against HP0986 were analyzed in sera collected from *H. pylori* infected patients belonging to different diseased categories and from control individuals (healthy controls and *H. pylori* non infected subjects). Antibody titers against HP0986 were compared between infected subjects and control individuals ($P < 0.001$).

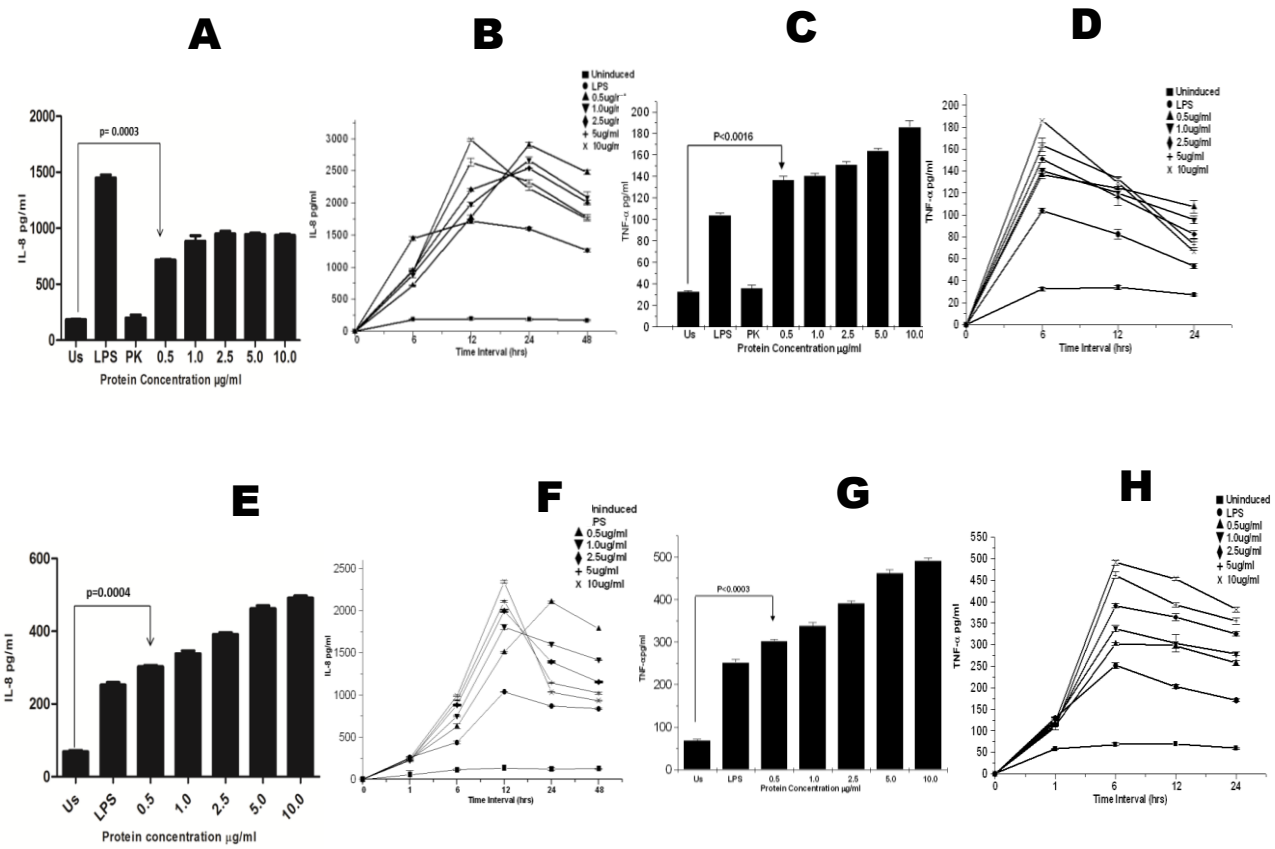


Figure 5: HP0986 stimulates the production of proinflammatory cytokines (IL-8 and TNF-α). (A and B) Bar diagram and graph representing the amount of IL-8 secreted in THP1-cells followed by HP0986 exposure. (C and D) reveal the dose and time kinetics of TNF-α induction by HP0986 treated cells. (E and F) Dose and time dependent secretion of IL-8 by human PBMCs following HP0986 stimulation. (G and H) Dose and time dependent effect on the levels of TNF-α in human PBMC treated with HP0986. Results are shown as mean ± SE and represent findings from one of the three independent experiments.

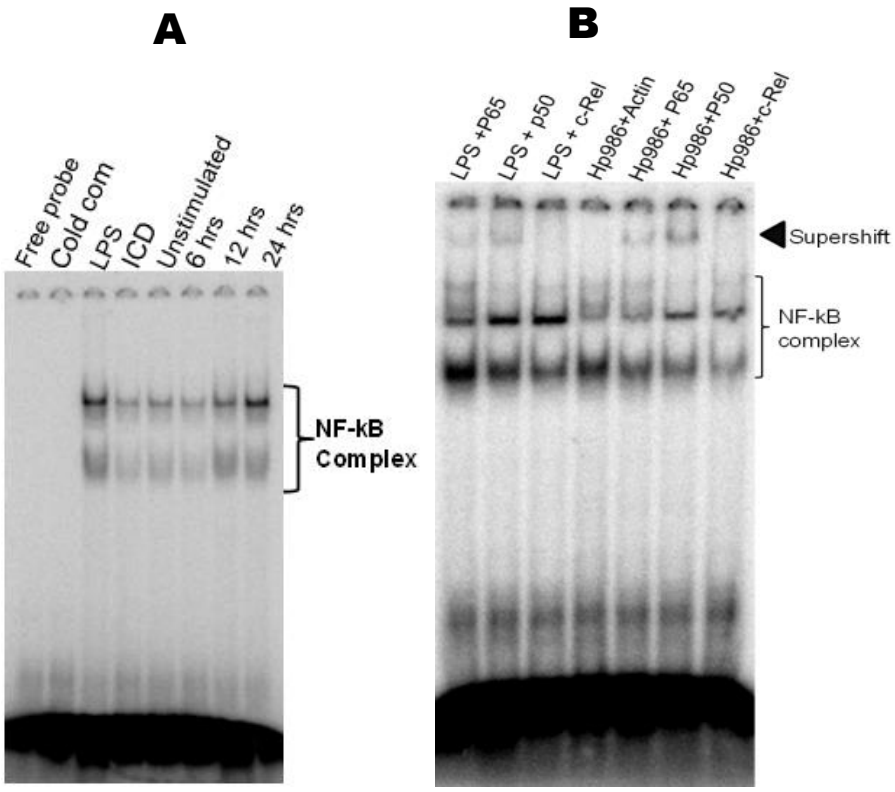
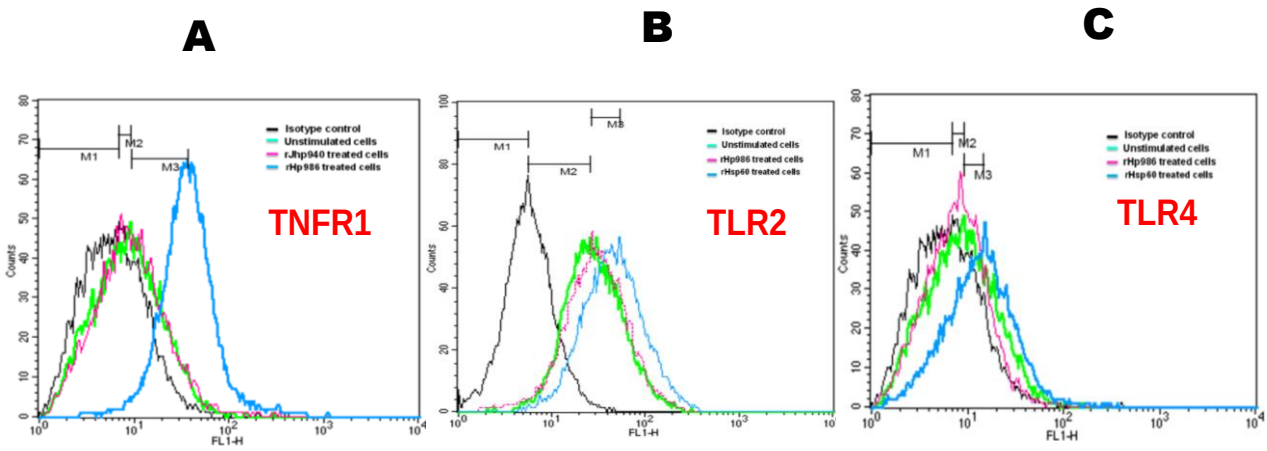
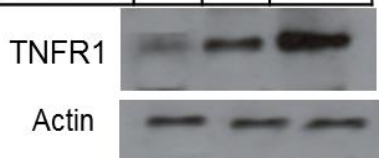


Figure 6: HP0986 induced production of proinflammatory cytokines is NF-κB dependent. (A) HP0986 mediated translocation of NF-κB complex was analyzed by electrophoretic mobility shift assay (EMSA); lane 1 – free probe, lane 2 – cold competition, lanes 3 and 4 – controls (LPS and ICD respectively), lane 5 – negative control (cells without HP0986 treatment). Cells were treated with HP0986 (0.5μg/ml) for varied time periods, lane6 - 6hrs, lane7-12 hrs and lane8 - 24 hrs. (B) Supershift assay; lane1 - probe only, lane2 - specific competitor. Specificity of HP0986 mediated activation of NF-κB complex was detected using specific antibodies against p65, p50 and c-rel. Nuclear extracts prepared from differentiated cells treated with either HP0986 (lane5-p65, lane6-p50 and lane7-c-rel) or LPS (lane1-p65, lane2-p50 and lane3-c-rel) were incubated with antibodies as described in materials and methods.



D

Beads	-	+	+
Lysate	+	+	+
Hp986 Treatm	-	+	+
TNF-α Nu-Ab	-	+	-



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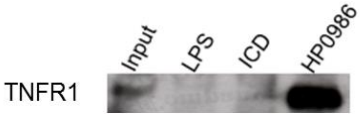

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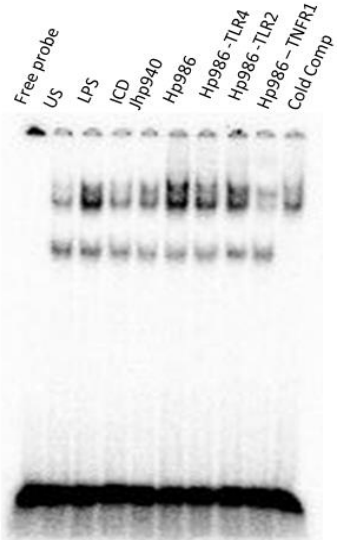

G

k_a[a] (1/Ms)	SE[b] (k_a)	k_d[c] (1/s)	SE(k_d)	Chi squire ($\chi 2$)
1.26×10^{04}	5.62×10^{02}	9.08×10^{-04}	3.80×10^{-05}	8.94
KA[d] ($=k_a / k_d$) (1/M)		KD[e] ($=k_d / k_a$) (M)		
1.39×10^{07}		7.21×10^{-08}		

Figure 7: Interaction of HP0986 with TNFR1 analyzed by flow-cytometry and immunoprecipitation. (A) Enhanced expression of TNFR1 following treatment with HP0986. (B) HP0986 did not stimulate expression of TLR2 - levels were equal to the cells that had not received any protein treatment. (C) Treatment of HP0986 had no visible effect on TLR4 expression [in the inset, lane 1 - untreated cells, lane 2 - cells treated with HP0986 and lane 3 - cells treated with rHsp60 (from *M. tuberculosis*) used as control. (D) Role of endogenous TNF-α in binding to TNFR1 was ruled out using neutralizing antibodies against TNF-α; lane 1 - whole cell lysate of cells, lane 2 - cells incubated with HP0986 in the presence of neutralizing antibody to TNF-α, lane 3 - cells incubated with HP0986 in the absence of neutralizing antibody to TNF-α. Immunoprecipitate was pulled down using Talon beads as discussed

in materials and methods section. Blot was developed using anti-TNFR1 antibody. Equal protein loading was confirmed by reprobing the blot with β-actin. (E) Immunoprecipitation assay showing interaction of HP0986 with TNFR1, lane 1 - input, lane 2 – cells treated and incubated with LPS, lane3 - cells treated and incubated with ICD, lane 4 - cells treated and incubated with HP0986. Immunoblot was developed using antibodies against TNFR1. (F) EMSA showing effects of neutralizing antibodies against TNFR1, TLR2 and TLR4 on the translocation of NF-κB complex in the cells stimulated with HP0986 [lane1- free probe, lane2 – unstimulated/untreated cells, lane 3- cells treated with LPS, lane4 – cells treated with ICD, lane5- cells treated with JHP940, lane6 - cells treated with HP0986, lane7- cells treated with HP0986 and neutralizing antibody against TLR4, lane8 - cells treated with HP0986 and neutralizing antibody against TLR2, lane9 - cells treated with HP0986 and neutralizing antibody against TNFR1 and lane 10 - specific competitor (unlabelled NF- κB consensus probe)]. **(**G) Summary of kinetic parameters for HP0986 affinity interaction with TNFR1: *a* = Association rate constant, *b* = Standard Error. *c* = Dissociation rate constant *d* = Equilibrium association constant *e* = Equilibrium dissociation constant.

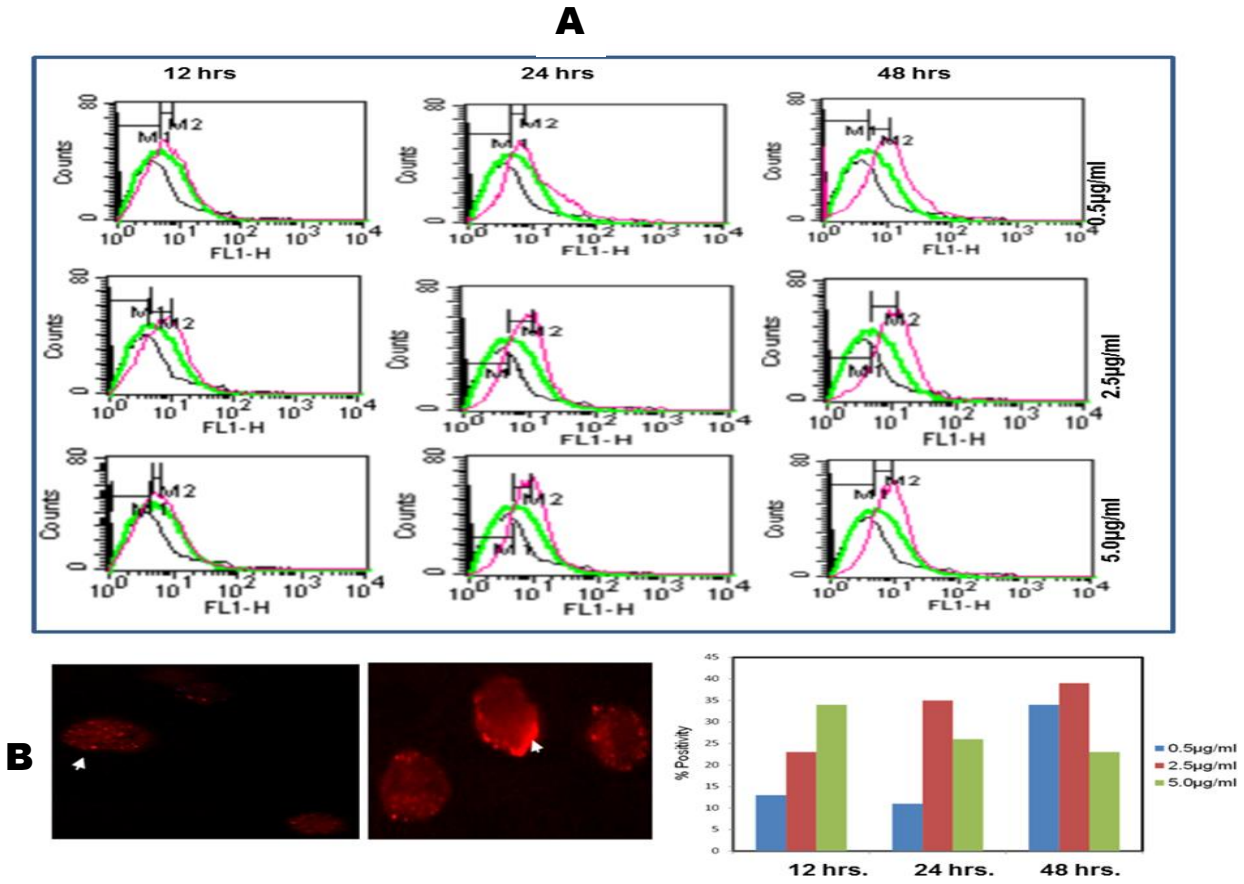

Figure 8: HP0986 induced Fas expression on the surface of differentiated macrophages as quantified by flow cytometry with anti-Fas antibody. Dose dependent increase in Fas expression in response to treatment with increasing concentration of HP0986 (0.5µg/ml, 2.5µg/ml and 5.0µg/ml) observed across varied time intervals (12 hrs, 24 hrs and 48 hrs) is indicated by graph

with pink line while the graphs with green line indicate response of cells without protein stimulation. (B) Enhanced Fas expression on cell surface after stimulation with HP0986 as compared to cells without recombinant protein stimulus.

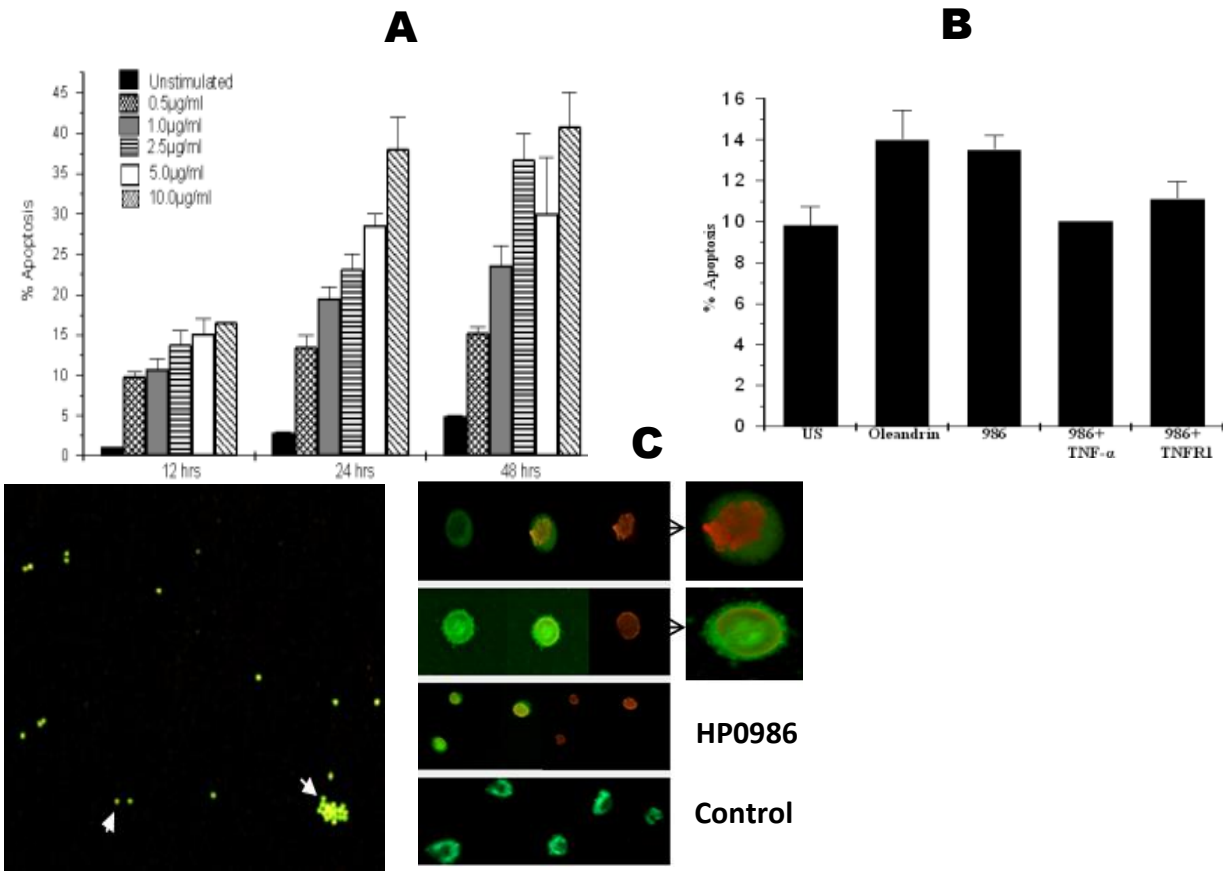

Figure 9: (A) Quantification of apoptosis in the cells treated with increasing concentration of HP0986 for varied time intervals. Results are represented as (mean±SE) percent apoptotic cells per 300 cells. (B) Bar diagram showing inhibition of apoptosis when the cells were stimulated with HP0986 in the presence of neutralizing antibody against TNFR1 (HP0986+TNFR1) as compared to cells stimulated with only HP0986. Oleandrin was used as positive control; US - unstimulated cells. Results are shown as mean ± SE. (C) Morphological identification of apoptotic cells by acridine orange and ethidium bromide staining method. Arrow head indicates live cells with normal morphology (green fluorescence) and arrow shows cells that have undergone apoptosis after stimulation with HP0986. Black arrows indicate condensed marginal nucleus, apoptotic body formation and membrane blebbing.

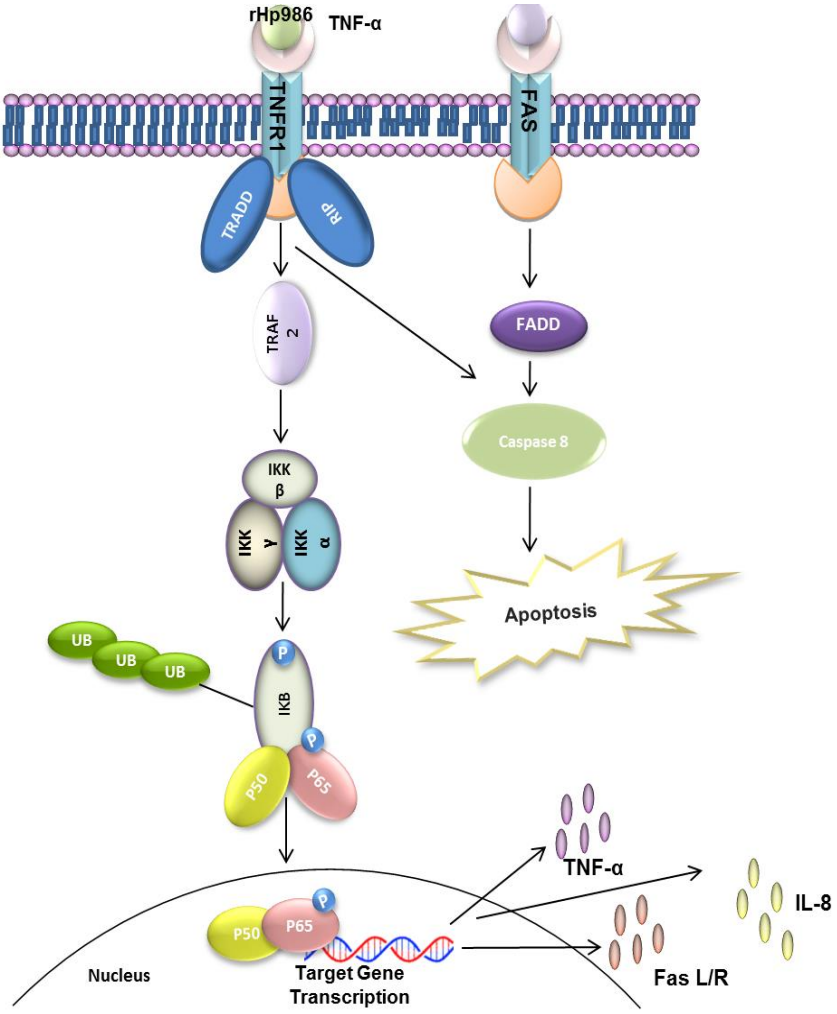

Figure 10: Schematic representation of major signaling pathways initiated following the binding of HP0986 with TNFR1. These interactions further stimulate downstream signaling cascades leading to macrophage apoptosis and induction of proinflammatory cytokines. TRADD: TNFR1 associated death domain, FADD: Fas- associated death domain, TRAF2: TNFR- associated factor 2, RIP: Receptor interacting protein.

CHAPTER 3: OBJECTIVE 2

Interaction of HP0986 with mouse TNFR1 and subsequent trigger of proinflammatory and proapoptotic signaling pathways in cultured mouse macrophage (RAW 264.7) cells

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3.1 Introduction:

Helicobacter pylori infection is the known cause of infectious gastritis, peptic ulcer disease and gastric cancer. Although more than 50% of world's population is infected with *H. pylori*, only 10-20% of total infected cases progress to form clinically significant morbidity (Suerbaum S and Michetti P, 2002). The infection initiates with the adherence of *H. pylori* to the gastric epithelium with concomitant immune responses leading to infiltration of neutrophils and lymphocytes. The inflammation developed during this process is often not sufficient enough to clear off the infection. This leads to persistence of *H. pylori* infection for decades (Day AS *et al.*, 2001). However, variations have been observed in the course and severity of infection in different individuals which can be attributed to bacterial strain heterogeneity, host genetic susceptibility and environmental factors (Sugimoto M *et al.*,2012; Ding SZ and Zheng PY, 2012; Borlace GN *et al.*, 2011).

Genetic heterogeneity of *H. pylori* is reflected in its varied functional effects occurring due to variations in the allelic structures and functional repertoires of certain virulence factors. Among the several known virulence factors, cytotoxin-associated gene (cag) Pathogenicity Island (*cag*PAI) and vacuolating cytotoxin (VacA) are most extensively studied (Montecucco C and Rappuoli R, 2001; Chattopadhyay S *et al.*, 2012; Peek Jr RM and Blaser MJ, 2002; Covacci A *et al.*, 1999). In addition, factors contributing towards the adherence of *H. pylori* to gastric epithelial cells (SabA, BabA, AlpA/B, HopZ and OipA) also play crucial roles during pathogenesis (Alm RA *et al.*, 1999; Oh JD *et al.*, 2006; Yamaoka Y *et al.*, 2006; Fujimoto S *et al.*, 2007). However, the diversity in

expression of these virulence factors in clinical strains is reflective of the existence of selection pressure on the colonizing strains infecting the same or different individuals. Such variability in strain specific virulence factors is also responsible for differences in virulence potentials of *H. pylori* isolates (Alm RA *et al.*, 1999; Delgado- Rosado G *et al.*, 2011; Alm RA and Trust TJ, 1999). Many *H. pylori* strain-specific genes that encode putative virulence factors of unknown functional mechanisms are encoded within highly variable chromosomal plasticity zones (PZs) (Sugimoto M *et al.*, 2012). Most PZ genes are predicted to be of unknown function but the encoded products of some are considered to influence the virulence potential of infecting strains. The HP0986 protein encoded within the PZ of strain 26695 is one such example. Our previous studies have shown it to be naturally expressed and presented to the immune system and addition of purified recombinant HP0986 to cultured macrophages and gastric epithelial cells were shown to activate NF-κB leading to up regulation of proinflammatory cytokines such as TNFα and IL-8 (Chapter 2, Alvi A *et al.*, 2011; Devi S *et al.* 2014). Also, HP0986 actively induced apoptosis *via* TNFR1 and Fas mediated pathways in THP-1 cells (Chapter 2, Alvi A *et al.*, 2011), although specific steps and details of these pathways have remained elusive. These pathways urgently required to be dissected. Further, there was a need to confirm HP0986 induced signaling in a murine setup in order to widen the study towards reproduction of the effects *in vivo*.

3.2 Materials and Methods*:*

3.2.1 Murine macrophage (RAW 264.7) cell culture: RAW 264.7 cells were procured from NCCS, Pune, India. The cells were grown in RPMI 1640 medium

supplemented with 10% fetal bovine serum, 2mM glutamine and 1% anti-bacterial and anti-mycotic agent (Gibco laboratories). Approximately $0.5x10^6$ cells were grown until 60-70% became confluent in 12 well culture plates. Cells were induced with increasing concentration of recombinant (His-tagged) HP0986 (0.5 µg, 1.0 µg, 2.5 µg and 5.0µg/ml). Cells without any stimulus and cells with proteinase-K treated HP0986 were used as negative controls. Cells treated with LPS (5.0 µg/ml) from *Escherichia coli* (Sigma Aldrich, USA) served as positive control. Culture supernatants from each treated set were collected after 10 hours of incubation and stored at -80°C until used for assays.

3.2.2 Cytokines assay: Cytokines/chemokines in the culture supernatant were analyzed by multiplexed bead based immunoassays using commercially available Cytometry Bead Array (CBA) Kit (BD Biosciences, USA). The assay was performed according to manufacturer's instructions. The samples' flow data were collected using a flow cytometer (BD FACS CantoII, BD Biosciences, USA) and analyzed using FCAP Array multiplex analysis software (BD Biosciences, USA). Cytokine/chemokine concentrations were determined with a standard curve plotted using the intuitive FCAP array software. The values were represented as mean±SE.

3.2.3 Real-time PCR: Approximately $2x10^6$ RAW 264.7 cells per well and per 2 ml of RPMI media were grown until 70-80% confluent in a 6 well culture plate. Cell were treated with recombinant (His-tagged) isocitrate dehydrogenase (ICD) (2.5 µg/ml), LPS (5.0 µg/ml) and HP0986 (2.5 µg/ml). An aliquot of cells was also treated with neutralizing anti-mouse TNFR1 antibody (5.0 µg/ml) before HP0986 (2.5 µg/ml) induction. The cells were incubated for 6 hours. After

incubation, cells from each well were harvested in 1 ml TRI reagent (Sigma Aldrich, USA). Total RNA was extracted as per the manufacturer's instruction. One microgram of total RNA was taken for generation of cDNA using Verso cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the protocol described in their manual. The mRNA levels of MCP-1 and TNF-α were measured with the help of SYBR green (Applied Biosystems) in a Mastercycler® ep *realplex* thermal cycler (Eppendorf, Germany). Following primers were used for the amplifications: Mouse TNF-α Forward primer: TCCCAGGTTCTCTTCAAGGGA, Reverse primer: GGTGAGGAGCACGTAGTCGG; Mouse MCP-1 Forward primer: GAAGGAATGGGTCCAGACAT, Reverse primer: ACGGGTCAACTTCACATTCA and Mouse GAPDH Forward primer: TGTGTCCGTCGTGGATCTGA and reverse primer: CCTGCTTCACCACCTTCTTGA. The relative expression levels of MCP-1 and TNF-α were normalized to expression values of GAPDH taken as a housekeeping gene reference.

3.2.4 Western blot analysis: RAW 264.7 cells were harvested and centrifuged at 500 x g for 5 minutes. The whole cell extract was prepared using non-denaturing lysis buffer and according to the protocol described earlier (Bonifacino JS *et al.*, 2001). Cytoplasmic and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, USA) according to the manufacturer's instruction. The extracts were stored at -80°C until use. Western blotting was performed as per the previous description (Weih F *et al.*, 1994). Primary antibodies used were against p65 (NF-κB subunit), procaspase-8, procaspase-3, β-actin (Santa Cruz

Biotechnology Inc., USA), lamin B and IκB-alpha (Sigma Aldrich, USA). Blots were developed using Super Signal West Pico Kit (Thermo Fisher Scientific, USA). β-actin was used as whole cell/cytoplasmic extract marker and for nuclear extract, lamin B was used as loading control.

3.2.5 Immunoprecipitation assays: For interaction analysis, cell extracts were prepared with RIPA buffer as described before (Bonifacino JS *et al.*, 2001). An aliquot of 0.5 mg of the cell lysate was incubated overnight with HP0986 (10.0 µg) and polyclonal anti- HP0986 antibody (10.0 µg) at 4°C. The immune complex was pulled down with protein-G agarose beads (Santa Cruz Biotechnology Inc., USA). The complexes were run on 12% SDS-PAGE and transferred over to PVDF membrane for western blot analysis using the primary antibodies directed against TNFR1 and TNFR2 (Santacruz Biotechnology Inc, USA), TLR2 and TLR4 (Imgenex, India). The immunoblots were developed with Super Signal West Pico Kit (Thermo Fisher Scientific, USA). Primary antibodies against TRAF2, TRADD and FADD (Santa Cruz Biotechnology Inc., USA) were used to analyze the respective co-precipitated molecules along with the HP0986-receptor complex by Immunoblotting.

3.2.6 Flow cytometry analysis: Cells were grown in 12 well plates till they became approximately 50-60% confluence. Cells were then treated with increasing doses of HP0986 (1.0, 2.5, 5.0 and 10.0 µg/ml) and were incubated in a CO_2 incubator at 37 °C for 24 hours for TNFR1 expression study, and at different time periods (12 hours, 24 hours and 48 hours) for the Fas expression analysis. Unstimulated cells were taken as negative control for both Fas and TNFR1 expressions whereas TNF-α (0.2 µg/ml) treated cells acted as positive

control. After incubation, cells were harvested in 0.2% EDTA solution (in PBS) and counted. Cells were centrifuged at 300 x g for 5 minutes and supernatant was completely removed. The cell pellet was then resuspended in appropriate volume of blocking buffer (5% horse serum in PBS) to get $1x10^6$ cells per ml of buffer. The resuspended cells were kept on ice for 30-45 minutes as this ensured blocking of the Fc receptor sites normally present on macrophage cells and thus enhance the specific binding of the primary antibody to its respective antigen on cells surface. Approximately 0.1 x 10^6 cells (100 µl) were taken in a polystyrene tube and the subsequent staining steps were performed in the same buffer. For TNFR1 expression analysis, cells were incubated with rabbit anti-TNFR1 antibody (Santacruz Biotechnology Inc. USA) for 45 minutes on ice. Cells were then kept on ice with secondary FITC-conjugated anti-rabbit antibody (Sigma Aldrich, USA) for another 45 minutes. For the Fas expression assay, approximately 0.1 x 10^6 (100 µl) cells were incubated directly with FITC-conjugated anti- CD95 antibody (BD Biosciences, USA) for 30 minutes on ice. After the incubation with labeled antibodies, the cells were washed twice and resuspended in 500 µl of the same buffer for immediate data acquisition on flow cytometer. Unstained cells were taken as internal negative control in both the assays to rule out any interference due to background fluorescence. All acquired data were analyzed with Flow Jo software (Tree Star Inc., USA).

3.2.7 MTT-assay for cell viability measurement: The RAW 264.7 cells' viability after HP0986 treatment was analyzed by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Amresco, USA) assay. The HP0986 induced cells were incubated for 48 hours in a 96 well plate. An aliquot of 20

µl of MTT (5mg/ml) was added to each well and kept for 3 hours at 37 °C. The resultant insoluble Formazan complex was dissolved in dimethyl sulfoxide (Sigma Aldrich, USA). The colored solution thus formed was measured at 570 nm (Mosmann T, 1983).

3.2.8 HP0986 mediated apoptosis Analysis: HP0986 induced apoptosis in RAW 264.7 cells was analyzed by AnnexinV-FITC/PI assay. Cells were processed and staining was performed according to manufacturer's instructions (BD Biosciences, USA) and the sample data were acquired on flow cytometer (BD FACS Canto II, BD Biosciences, USA). The percentage of cells undergoing apoptosis were expressed as total of % $AnnexinV^+$ and PI^+ cells after subtracting the background fluorescence. Furthermore, apoptosis in HP0986 treated RAW 264.7 cells could be visualized through DNA fragmentation assay. The analysis was performed as per the method described previously (Herrmann M *et al.*, 1994). In brief, 2 x 10^6 cells were washed with PBS and pelleted by centrifugation at 400 x g for 5 minutes. Cell pellets were then treated with a lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, pH, 7.5) by repeated pipetting up and down. Homogenates were then centrifuged at 13000 x g for 5 minutes. Supernatants were then treated with RNase A at 37 ^oC for 2 hours followed by proteinase-K at 37 ^oC for 2 hours. Then a ½ volume of 10 M ammonium acetate was added to DNA solutions .To further facilitate the DNA precipitation, 2.5 volume of ethanol was added and centrifuged. DNA pellets were dissolved in gel loading buffer, run and analyzed on 1% agarose gel.

Statistical analysis: Results were expressed as means ± standard error (SE). Comparisons of cytokine inductions, TNFR1 and Fas expressions were

performed by two–tailed t-test using GraphPad Prism version 5.01 for windows (Graph Pad software, San Diego, California USA: www.graphpad.com). The differences between two values were considered significant if $p < 0.05$.

3.3 Results

3.3.1 HP0986 induces expression and secretion of MCP-1 and TNF-α from murine macrophages: The N-terminal His-tagged protein was over expressed in BL-21(DE3) cells and purified under native conditions using Co^{+2}-NTA affinity column. RAW 264.7 cells treated with HP0986 and incubated for 10 hours released MCP-1 and TNF-α into the culture supernatant in a dose dependent manner. A significant secretion of MCP-1 and TNF-α was observed when 1 ml aliquot of media with about 1 million cells was treated with increasing doses of HP0986 (0.5 µg, 1.0 µg, 2.5 µg and 5.0µg/ml). Highest secretion of both MCP-1 ($p=0.0002$) and TNF-α ($p<0.0001$) were observed at the protein concentration of 2.5 µg/ml (Figure 1A). Cells treated with even higher dose (5.0 µg/ml) of HP0986 did not show any further increase in MCP-1and TNF-α levels. Proteinase-K-treated HP0986 failed to induce the production of both MCP-1 and TNF-α suggesting that the response was indeed triggered by HP0986. Cells treated with LPS (5.0 µg/ml) were taken as a positive control for the induction of both MCP-1 and TNF-α (Figure 1A). In order to further confirm the effect specific to HP0986, His-tagged isocitrate dehydrogenase (ICD) of *H. pylori* purified in a similar manner was used as an internal negative control. A relative mRNA quantification analysis using real-time PCR showed that cells treated with His-tagged ICD protein (2.5µg/ml) failed to trigger MCP-1 and TNF-α mRNA expression above the control level and therefore ruling out the

possible involvement of 6x His tag of recombinant, purified HP0986 in the above responses (Figure 1B). Thus, our data reveal HP0986 specific proinflammatory cytokine responses in RAW 264.7 cells.

3.3.2 MCP-1 and TNF-α secretion is mediated by activation of NF-κB: In order to determine whether the MCP-1 and TNF-α secretion is regulated by NF-κB activation, we analyzed the NF-κB levels in nuclear and cytoplasmic fractions of HP0986 stimulated RAW 264.7 cells. We observed an increase in nuclear translocation of p65 in HP0986 treated RAW 264.7 cells in a time dependent manner. A clear and enhanced translocation of p65 in the nuclear extract of HP0986 treated (2.5μg/ml) RAW 264.7 cells was observed with increase in time of incubation (0.5, 1.0, 2.0 and 3.0 hours). The time course followed for this study indicated that maximum level of nuclear localization of p65 was observed at 3 hr post treatment when compared to the control (Figure 2B). The corresponding observed decrease in levels of p65 from cytoplasmic extracts during the same time period was also consistent with increased translocation to the nucleus. TNF-α (50 ng/ml) was taken as positive control for NF-κB translocation analysis. In a further study, western blot analysis showed noticeable degradation of IκB-alpha in the cytosol after stimulation with HP0986 (2.5 μg/ml). A visibly clear degradation of IκB-alpha was observed at 30 min, 1 hour and 2 hours post treatment with HP0986 (Figure 2A). Taken together, we found that time course for IκB-alpha degradation preceded NF-κB activation.

3.3.3 Interaction of HP0986 with murine TNFR1: Host receptor involved in eliciting the cytokine response was analyzed by pull down assay which showed

mouse TNFR1 captured together with HP0986 when probed with anti-TNFR1 antibody. However, HP0986 did not involve in any complex formation with TNFR2/TLR2 or TLR4 as revealed by pull-down assay using anti-HP0986 antibody followed by western blot analysis with anti-TNFR2, anti-TLR2 and anti-TLR4 antibodies (to rule out any possible HP0986 binding to receptors other than TNFR1) (Figure 3A). Furthermore, an increase in cell surface TNFR1 expression was observed by flow cytometry in HP0986 treated RAW 264.7 cells in a dose (1.0 , 2.5, 5.0 and 10.0 μg/ml) dependent manner (Figure 3B). These observations confirmed the interaction between HP0986 and murine-TNFR1, an event upstream of inflammatory response, and also ruled out any interaction between HP0986 and TNFR2/TLR2 or TLR4. Moreover, RAW 264.7 cells pretreated with neutralizing anti-mouse TNFR1 antibody (5μg/ml) prior to induction by HP0986 (2.5 μg/ml) revealed reduced translocation of p65 to the nucleus (Figure 3C) and also decreased induction of MCP-1 and TNF- α (Figure 1C and 1D). These observations clearly suggest a link between TNFR1 stimulation through HP0986 binding and NF-κB activation leading to the MCP-1 and TNF-α secretion in murine macrophages.

3.3.4 HP0986 induces Fas mediated apoptosis in murine macrophages (RAW 264.7): Fas receptor on the cell surface induces apoptosis upon interaction with the ligand (Tanaka M *et al.*, 1995). According to our previous observation with human macrophages (THP-1) that suggested a link between TNFR1 activation by HP0986 and Fas expression (Chapter 2, Alvi *et al.*, 2011), we performed a similar analysis with murine macrophages. HP0986 were shown to induce increased expression of Fas on murine macrophages (RAW

264.7) in a dose (1.0, 2.5 and 10 µg/ml) and time (12, 24 and 48 hours) dependent manner. However, at highest time point of 48 hours incubation, the increment in protein doses did not reflect increase in Fas expression possibly due to apoptosis of the cells at higher concentrations of HP0986 (Figure 4). However, expression of Fas on murine macrophages was significantly higher than unstimulated cells. AnnexinV-FITC/PI assay also indicated an increase in apoptosis of HP0986 (5.0µg/ml) treated RAW 264.7 cells. In the same analysis, upon HP0986 (5.0µg/ml) stimulation, we observed decreased apoptosis of cells pretreated with neutralizing anti-mouse TNFR1 antibody (5.0µg/ml). His-tagged ICD (5.0µg/ml) failed to induce apoptosis and therefore ruling out the possible interference by His-tag attached to recombinant HP0986. Staurosporin (50 nM) treated cells in all cases were taken as positive control for apoptosis (Figure 5A). Further, we observed an increase in procaspase-8 and procaspase-3 degradation in RAW 264.7 cells induced with HP0986 for 48 hours in a dose (2.5, 5.0 and 10.0 µg/ml) dependent manner when compared with uninduced cells (Figure 5B). As a positive control, Staurosporin (50 nM) also showed an enhanced degradation of pro-caspase-8 and procaspase-3. β-actin was taken as loading control for the above analysis. This observation is also in agreement with earlier reports suggesting that Fas engagement with its ligand results in accumulation, cleavage and therefore activation of caspase-8 which further activates the effector caspases (Medema JP *et al.*, 1997; Stennicke HR *et al.*, 1998). Furthermore, apoptosis process in the HP0986 (5.0 µg/ml) treated RAW 264.7 cells was validated through DNA fragmentation in HP0986 treated cells (DNA ladder assay) as compared to unstimulated cells (Figure 5C).

3.3.5 TNFR1 activation by HP0986 and recruitment of TRADD, TRAF2 and FADD: Both TRADD and TRAF2 were co-precipitated together with TNFR1-HP0986 complex as shown in immunoblots using lysate from the RAW 264.7 cells treated with HP0986 and incubated for 30 minutes (Figure 6). This suggests that TRADD forms a complex with TNFR1 after interacting with HP0986 which in turn provides a platform for the recruitment of TRAF2 (Hsu H *et al.*, 1996) leading to the NF-κB activation. Further, we also observed in a similar study, the co-precipitation of FADD along with TNFR1-HP0986 complex in the lysate from cells treated for 24 hours (Figure 6). However since FADD interacts with both TRADD and Fas, this indicates two possible pathways for HP0986 induced apoptosis: one mediated through TNFR1 and the other involving Fas activation (Hsu H *et al.*, 1996; Micheau O *et al.*, 1999).

3.4 Discussion

HP0986 or TieA is an importantly proinflammatory protein from the plasticity region of *H. pylori* (Chapter 2, Alvi A *et al.*, 2011; Devi S *et al.* 2014). The protein has been profiled either (genotypically) using patient biopsies and clinical isolates corresponding to different populations or through (serological) testing for humoral immune responses and in (cell signaling) studies involving cultured human cells, and was also shown to induce proinflammatory cytokines through TNFR1 and NF-κB mediated signaling pathways (Chapter 2, Alvi A *et al.*, 2011; Devi S *et al.* 2014). However, its function has not been verified in an animal model such as mouse. The present study therefore assumes an important extension of the our previous works (Chapter 2, Alvi A *et al.*, 2011) and Devi *et al.* (Devi S *et al.*, 2011) to reproduce the interaction of

this protein with mouse immune apparatus (including TNFR1 and other receptors) and characterize the downstream signaling events to unravel finer details of the apoptotic pathways in order to understand the complete scenario of the proposed 'survival advantage' to the bacterium (Chapter 2, Alvi A *et al.*, 2011; Devi S *et al.* 2014) in a mammalian system.

Moreover, in order to validate the envisaged effects of HP0986, the choice of a mouse model is imminent. In that direction, the strongest observation made in this study entails consistent proinflammatory and apoptotic behavior of HP0986 in a murine macrophage cell (RAW 264.7), similar to what has been observed earlier for the human cell lines and PBMCs. Induction of proinflammatory cytokines by the mouse cells confirms HP0986 to be a 'generic' antigen that excites mammalian cells. As a potent chemoattractant for monocytes/macrophages and T- lymphocytes, MCP-1 is reported to be actively involved in the inflammation process (Villiger PM *et al.*, 1992; Sierra B *et al.*, 2010; Deng YY *et al.*, 2009; Koch AE et *al.*,1992)**.** TNF-α secreted by macrophages could have several implications during bacterial infection. TNF-α binding to TNFR1 triggers Fas mediated apoptosis of macrophages (Boyle JJ *et al.*, 2003; Hagimoto N et *al.*, 1999; Lee SY *et al.*, 2001). Reports also indicate that TNF-α down-regulates phagocytosis of apoptotic cells by macrophages (McPhillips K et *al.*, 2007). Recently, it has been shown in case of *H. pylori* infection that TNF-α accumulation during gastric injury has negative effect on the clearance of apoptotic gastric epithelial cells (Bimczok D *et al.*, 2013). The apoptotic 'cell loads' further enhance inflammatory state of gastric mucosa

through release of proinflammatory mediators from the dying cells following their secondary necrosis (Bimczok D *et al.*, 2013).

The present results reconfirmed involvement of NF-κB in induction of MCP-1 and TNF-α which are consistent with the previous reports (Deng YY *et al.*, 2009; Comstock KL *et al.* 1998). The activation of NF-κB as observed through translocation of p65 to the nucleus after HP0986 induction was also supported by the degradation of the IκB-alpha in RAW 264.7 cells (Figure 2). NF-κB activation is a key determinant of the immune cell response to the bacterial infection which in turn regulates expression of many inflammation promoting genes encoding several chemokines, cytokines and members of the IgG superfamily (Beg AA and Baldwin Jr AS, 1993; Lenardo MJ and Baltimore D, 1989; Thanos D and Maniatis T, 1995; Rizwan M *et al.*, 2008; Kumar A *et al.*, 2013).

Fas expression by RAW 264.7 cells after HP0986 induction (Figure 4) was in accordance with our previous observations in THP-1 cells (Chapter 2, Alvi *et al.*, 2011). This also suggests that the virulence potential and activity of HP0986 is similarly placed in the mouse system. In corroboration with previous reports, we also showed by immunoprecipitation method the interaction between HP0986 and murine TNFR1 (Figure 3A). Furthermore, RAW 264.7 cells pretreated with neutralizing antibody against TNFR1 revealed reduced translocation of NF-κB to the nucleus upon induction with HP0986; this confirmed the dependency of the expression of inflammatory response genes on TNFR1 (Figure 3C). This has thus indicated that HP0986 interaction with

TNFR1 leads to a downstream inflammatory response as well as Fas mediated apoptosis.

To gain further insights into the signaling events, we have shown through immunoprecipitation analysis the involvement of TNF receptor associated death domain (TRADD), Fas associated death domain (FADD) and TNF receptor associated protein2 (TRAF2) in HP0986 induced responses (Figure 6). Given these observations, we were able to propose a signaling process exerted by HP0986 in murine macrophages on the lines of the observations made in THP-1 cells and also similar to TNF-α mediated pathway (Chen G and Goeddel DV, 2002; Postal M and Appenzeller S, 2011; Wajant H *et al.*, 2003; Ea CK *et al.*, 2006; Micheau O *et al.*, 1999). In short, TNFR1 stimulation after HP0986 binding triggers the adaptor molecule (TRADD) to join in the intracellular death domain of the receptor (TNFR1). TRADD further provides a platform for the assembly of TNFR2 and other signaling proteins leading to the activation of NF-κB. TNFR1 activation also contributes to another pathway through the recruitment of FADD *via* TRADD and subsequent activation of caspase-8 leading to apoptosis. However, FADD also connects Fas to caspase-8 activation (Micheau O *et al.*, 1999) suggesting the involvement of an additional cell death pathway in HP0986 induced mouse macrophages.

In view of our observations, it is now possible to clearly portray the role of HP0986 in inflammation and apoptosis at the interface of various possible cellular pathways that conjoin TNFR1, Fas and NF-κB. These new data unleashing in a mouse macrophage background would definitely underpin future efforts directed at understanding the complexity and nature of *H. pylori*

induced cancer signaling. The immunological co-ordinates so established would also facilitate *in vivo* validation of HP0986 function in a mouse system in order to study the secretion, localization and regulation of this important virulence factor.

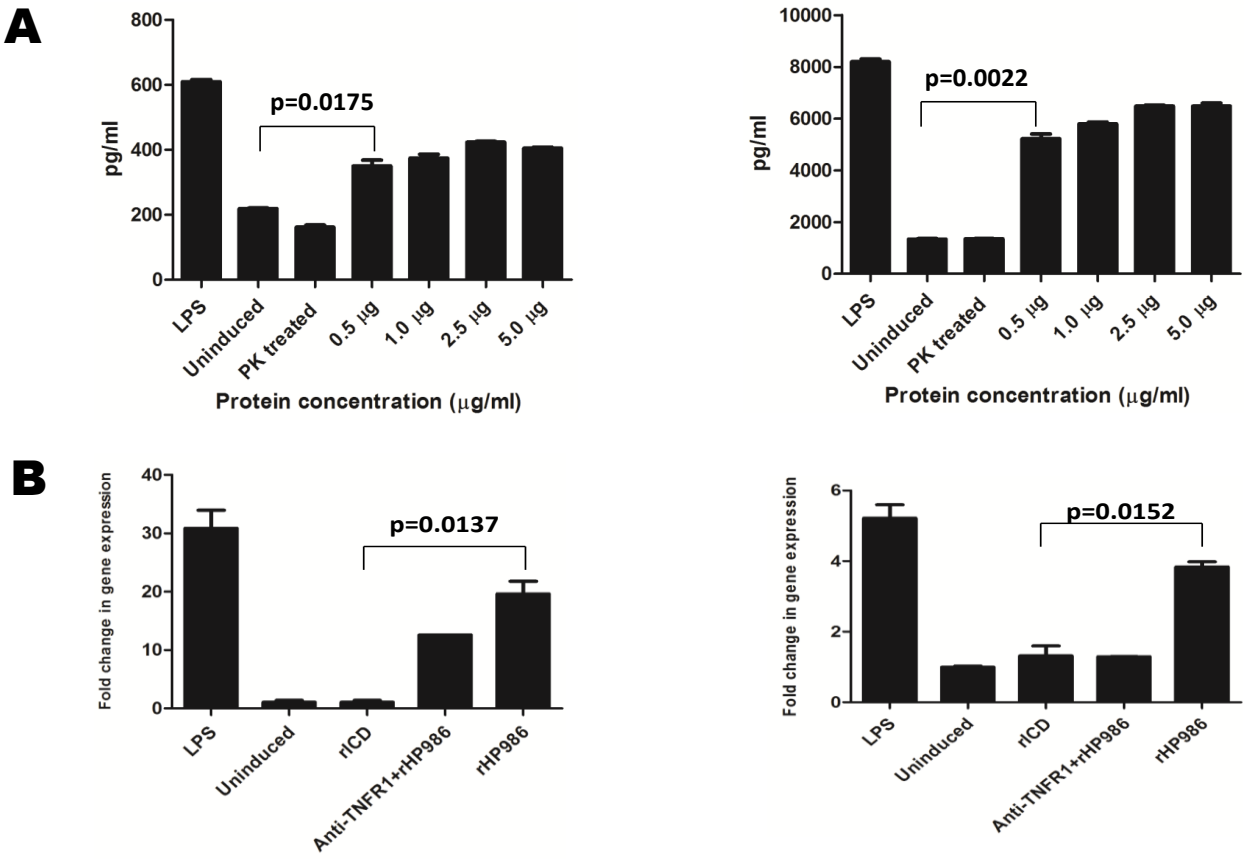

Figure 1: Secretion of the MCP-1 and cytokine TNF-α were triggered by induction of RAW 264.7 cells with recombinant HP0986 (His tagged- HP0986). Culture supernatant were collected after induction with different doses of HP0986 (0.5, 1.0, 2.5 and 5.0µg/ml) and after 10 hours incubation period. Cytokines in the supernatant were quantified by multiplex bead based immunoassay. (A) Uninduced cells, and cells stimulated with proteinase-K treated HP0986 were taken as negative control. LPS (5µg/ml) treated cells were taken as positive control. Results are shown as mean ± SE. Relative mRNA levels for MCP-1 and TNF-α in HP0986 treated cells as compared to untreated control were quantified by real time PCR. (B) Recombinant isocitrate dehydrogenase (His-tagged ICD) (2.5 µg/ml) treated cells and the cells pretreated with neutralizing anti-mouse TNFR1 (5µg/ml) before HP0986 induction also served as controls for this study.

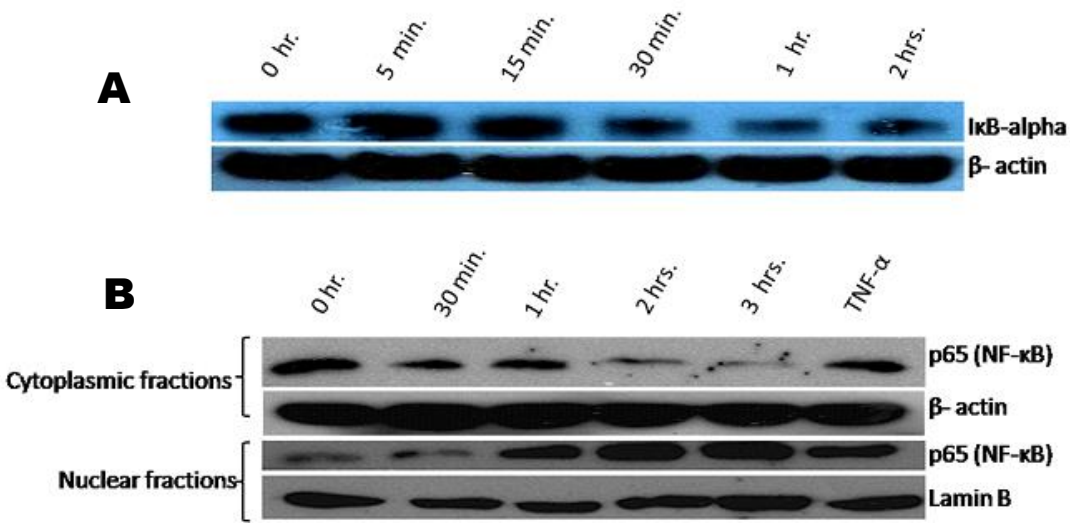

Figure 2: IκB-alpha degradation in HP0986 treated (2.5 µg/ml) murine macrophages (RAW 264.7) cells was analyzed at different time intervals (A). Total cell proteins were prepared according to the protocol described in materials and methods section. IκB-alpha level at each time interval was detected by Immunoblotting using anti-IκB-alpha antibody. Reprobing of the blot with β-actin antibody was done to ensure equal loading of the protein. Time dependent p65(NF-κB) translocation was observed in cytoplasmic and nuclear fractions from RAW 264.7 cells treated with 2.5 µg/ml protein (HP0986) for different time periods (0 minutes, 30 minutes, 1 hour, 2 hour and 3 hour). TNF- α treated cells were taken as positive control. β-actin was used as marker for cytoplasmic fractions. For the nuclear marker, Lamin B was used (B).

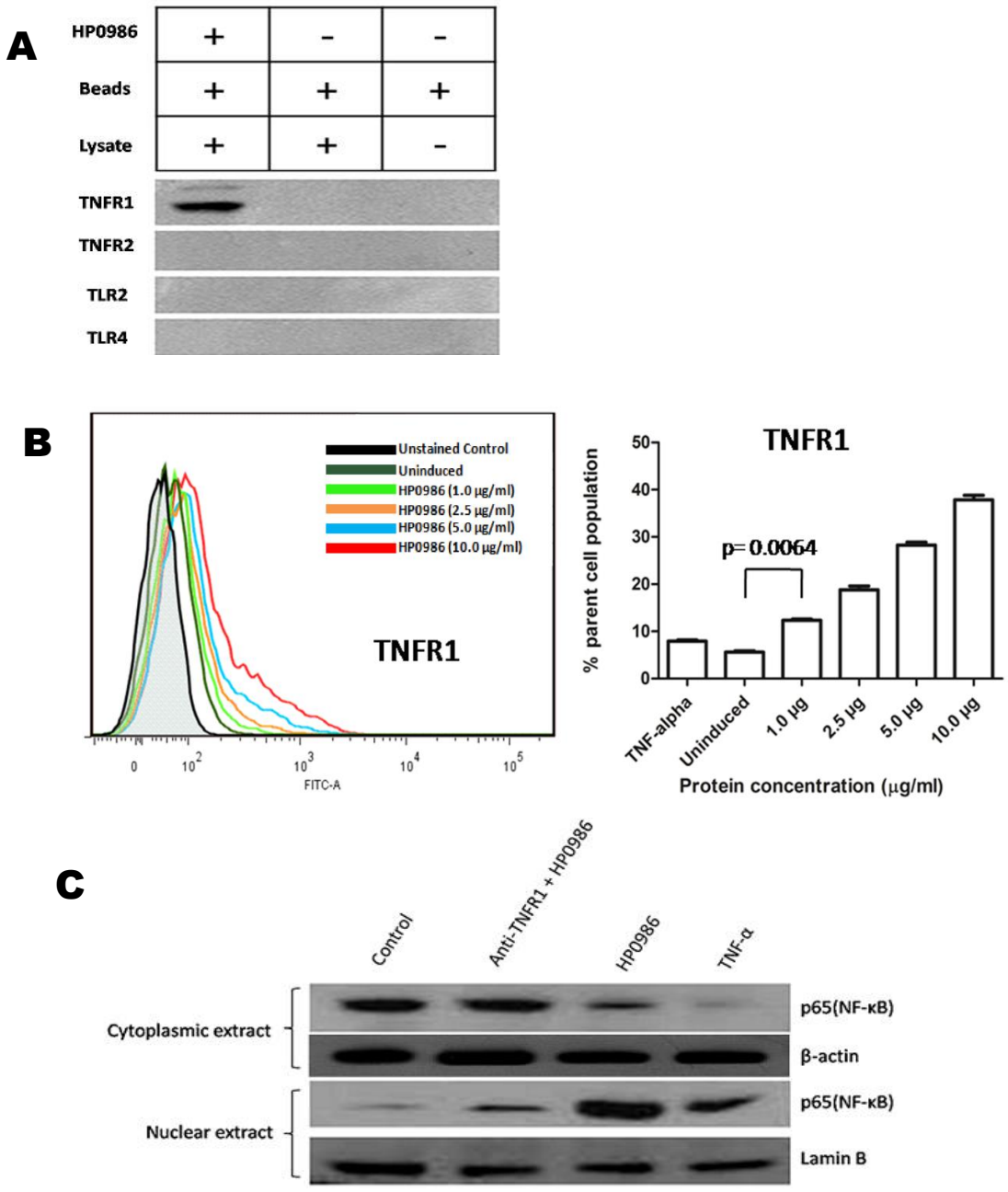

Figure 3: (A) Immunoprecipitation assay was performed using protein G-agarose beads. HP0986 bound receptor from the mouse macrophage cell lysate was pulled down with protein G-agarose beads and immunoblots were probed with anti-TNFR1, anti-TLR2, anti-TLR4 and anti-TNFR2 antibodies. Cell lysate incubated with protein-G beads, and protein-G beads alone were used as controls for this assay. (B) Flow cytometry analysis also showed the increased effect of HP0986 on TNFR1 expressions on murine macrophages (RAW 264.7). (C) Cells pretreated with neutralizing anti-TNFR1 antibody (5µg/ml) for 3 hours before the induction with HP0986 (2.5µg/ml) showed a reduced translocation of p65 (NF-κB) compared to the HP0986 (2.5µg/ml) treated cells.

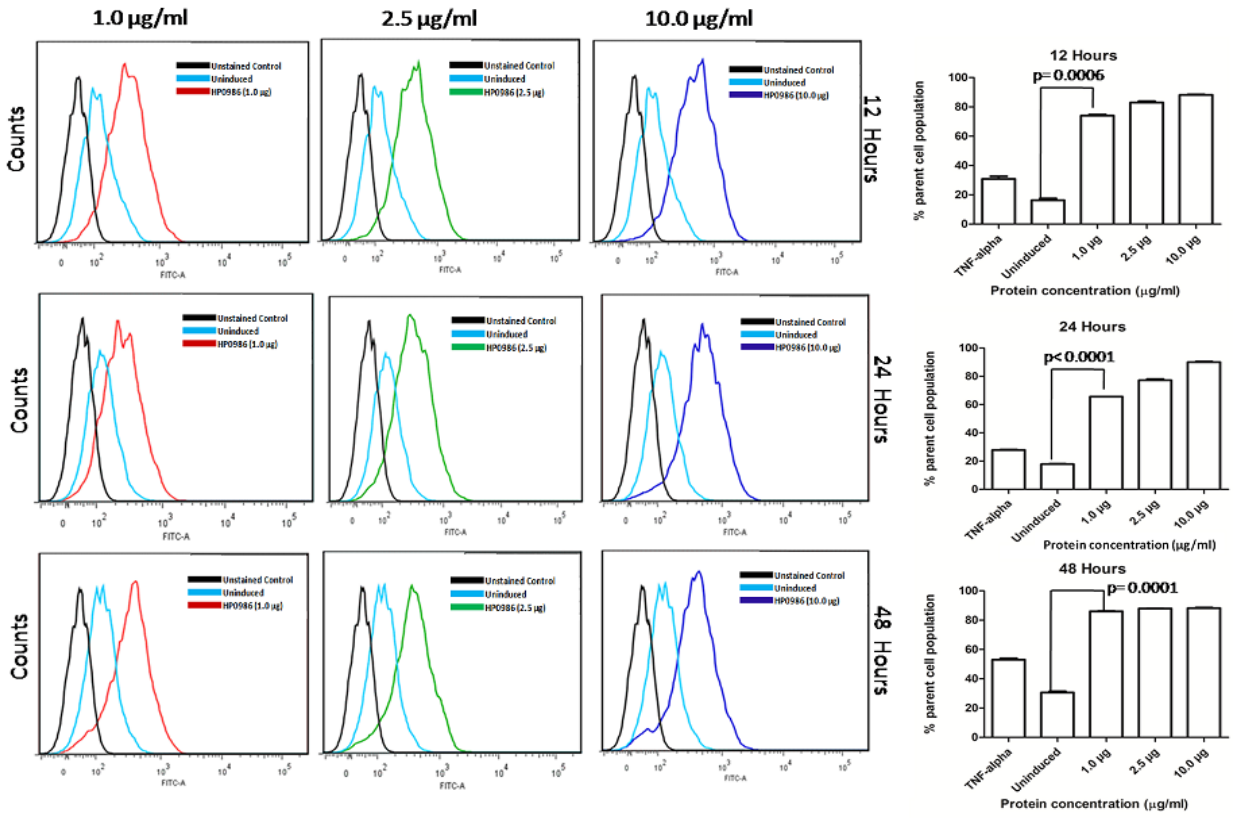

Figure 4: Fas expression on the surface of murine macrophage cells (RAW 264.7) were induced by the HP0986 and analyzed using FITC-conjugated anti-Fas antibody by flow cytometry. Dose dependent (1.0 µg/ml, 2.5 µg/ml, 10.0 µg/ml of HP0986) and time (12 hour, 24 hour and 48 hour) dependent effects of HP0986 on increased expression of Fas were observed on RAW 264.7 cells. Cells without protein stimulus and/or unstained uninduced cells were served as negative controls and TNF-α treated cells as positive control. Results are shown as mean±S.E.

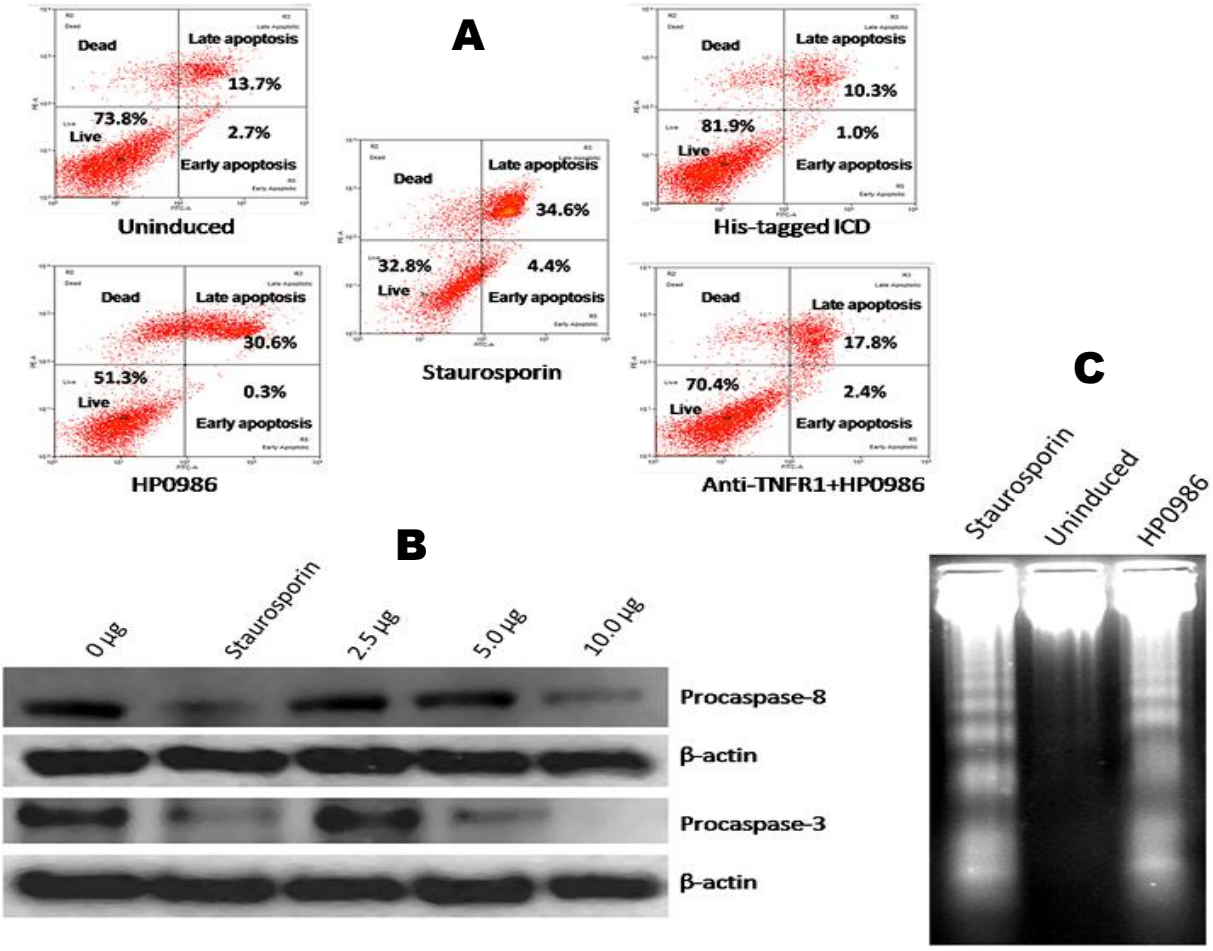

Figure 5: HP0986 induced apoptosis in murine macrophage cells (RAW 264.7) was analyzed by AnnexinV-FITC/PI staining method. In this assay, percent apoptotic cells in HP0986 (5.0 µg/ml) treated cells were compared with uninduced cells and the cells treated with His-tagged recombinant ICD were taken as negative controls. Staurosporin (50 nM) was taken as the positive control for apoptosis. A control study was also performed where the induction with HP0986 (5.0 µg/ml) after pretreatment of the cells with neutralizing anti- TNFR1 antibody (5.0µg/ml) (A). Immunoblotting was performed for procaspase-8 and procaspase-3 (B) cleavage analysis as a marker for apoptosis in murine macrophage cells in a dose dependent manner. For each analysis, cells were treated with different doses of HP0986 (2.5µg/ml, 5.0µg/ml and10.0µg/ml) and incubated for 48 hours. Cells without any stimulus were taken as negative control. Staurosporin (50 nM) treated cells used as positive control. DNA degradation assay was performed to assess the apoptotic behavior of HP0986. Total DNA was isolated from cells treated with HP0986 (5.0 µg/ml) for 48 hrs time period. Cells without protein stimulus were taken as negative control. DNA isolated from Staurosporin (50 nM) induced cells was taken as positive control for apoptosis (C). Flow cytometry analysis for apoptosis using i) HP0986 induced cells, and ii) cells treated with HP0986 and antiTNFR1 antibody; controls comprised of iii) cells without protein stimulus (negative control), iv) cells treated with recombinant His-tagged ICD protein (unrelated protein control) and v) those treated with staurosporin (positive control).

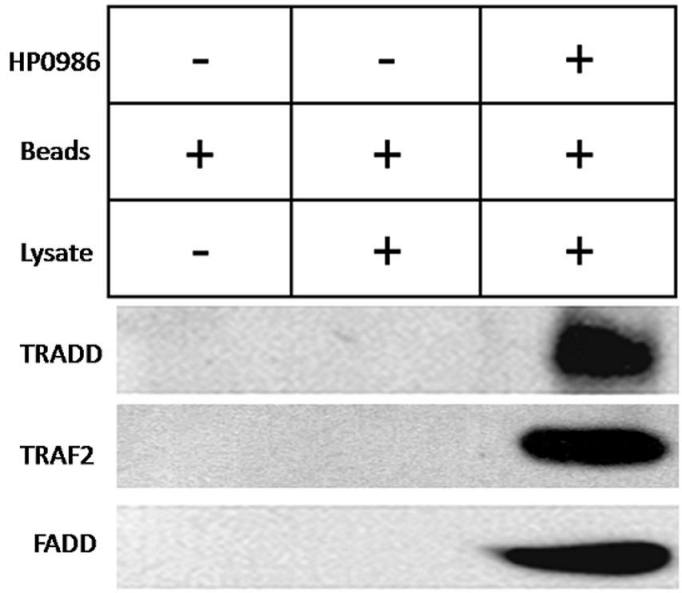

Figure 6: TRADD, TRAF2 and FADD were recruited to the activated TNFR1 interacting with HP0986. These signaling molecules were co-precipitated together with HP0986-TNFR1 complex, captured with anti-HP0986 antibody and pulled down with protein-G agarose beads. These molecules were detected by immunoblotting with anti-TRADD, anti-TRAF2 and anti-FADD antibodies respectively. Protein-G beads with cell lysate and protein-G beads only were used as controls for this analysis.

CHAPTER 4

Conclusions

The sequencing of complete genomes of *H. pylori* in 1997 (Tomb JF *et al.*, 1997; Alm RA *et al.*, 1999) had greatly accelerated research on biology of bacterial adaptation. Several *H. pylori* virulence factors have been identified and characterized in an effort to establish a concrete correlation between bacterial phenotype and disease outcomes. However, this association of hitherto known bacterial virulence factors with disease specificity always lacked a biological plausibility; therefore it becomes necessary to look into the novel virulence factors as well as to understand the mechanism of immune evasion by *H. pylori* and its subsequent impact over the human immune machinery. In the present study, we were able to identify and characterize a novel gene, *hp0986*, from the plasticity zone of *H. pylori* genome. The consistent presence of *hp0986* in the clinical isolates from global sources and in different gastric disease stages was indicative of its potential role in *H.pylori* induced gastric pathology (Chapter 2, Alvi A *et al.*, 2011). Furthermore, an enhanced humoral response against HP0986 in infected patients suggested that HP0986 was recognized by the host immune system (Chapter 2, Alvi A *et al.*, 2011). These findings also projected HP0986 to be seasoned virulence factor considering its pathological and epidemiological consistency. Further *in vitro* functional characterization of HP0986 also confirmed its role in inflammation (Devi S *et al.*, 2014; Chapter 2, Alvi A *et al.*, 2011 and Chapter 3, Ansari SA *et al.*, 2014). The inflammation induced by HP0986 appeared relevant for the bacteria to obtain the nutrients while engaging the host factors (IL-8, TNF-α and MCP-1) (Chapter 2, Alvi A *et al.*, 2011 and Chapter 3, Ansari SA *et al.,* 2014) in inflammatory processes. Such interaction of bacterial and host factors is important for successful colonization in the gastric mucosa. However, long term persistence in the

gastric niches also requires the bacteria to evolve strategies to avoid clearance by the host immune system. Therefore, downregulation of host immunity through recruitment of regulatory T cells (T_{reg}) and B cells and also the apoptosis of infiltrating immune cells could serve as hallmark of *H. pylori* induced immune response (Peek Jr RM *et al.*, 2010; Lewis ND *et al.*, 2011). In the present study, we also found HP0986 to be a potent proapoptotic molecule in addition to its proinflammatory behaviour. Also, the induction of apoptosis by HP0986 in cultured human and murine macrophages was observed to be both TNFR1 and Fas dependent (Chapter 2, Alvi A *et al.*, 2011 and Chapter 3, Ansari SA *et al.*, 2014). This mechanism of immune subversion is also relevant with regard to its clinical importance because antibiotic based treatment regimen is not effective in eradicating the disease causing *H. pylori* strains in some regions of the world where less than 50% success rate has been reported (Mégraud F H, 2004). In a nutshell, the simultaneous proinflammatory and proapoptotic roles of HP0986 might convey a survival strategy for *H. pylori*, whereby the inflammation in the gastric mucosa could be a mean to gain niche and nutrients and limiting the immune cells through apoptosis provides a survival advantage to bacteria by avoiding the host clearance. This behaviour of HP0986 could be a further addition to the similar contributions made by antagonistic roles of CagA and VacA towards the survival of the bacteria. Vac A is an effective molecule in inhibiting T cell activation/proliferation and thus inducing the immune suppression (Gebert B *et al.*, 2003). The CagA counteracts the VacA triggered apoptotic signaling and induces host cell survival and thus promotes chronic infection (Mimuro H *et al.*, 2007). Thus in

the event of missing CagA and Vac A functions, HP0986 could serve an ideal replacement.

This study has also clearly portrayed the HP0986 induced inflammation and apoptosis at the level of signaling pathways that involve TNFR1, NF-κB, Fas and caspase-8. Furthermore, similar immunological- coordinates obtained using both human and murine macrophage cells necessitated further study using a mouse model which can provide further insights into the secretion, localization and immunoregulatory mechanisms of HP0986. Therefore, *in vivo* studies are urgently needed in this direction.

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Helicobacter pylori protein HP0986 (TieA) interacts with mouse TNFR1 and triggers proinflammatory and proapoptotic signaling pathways in cultured macrophage cells (RAW 264.7)

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ABSTRACT

HP0986 protein of *Helicobacter pylori* has been shown to trigger induction of proinflammatory cytokines (IL-8 and TNF-α) through the activation of NF-κB and also to induce Fas mediated apoptosis of human macrophage cells (THP-1). In this study, we unravel mechanistic details of the biological effects of this protein in a murine macrophage environment. Up regulation of MCP-1 and TNF-α in HP0986-induced RAW 264.7 cells occurred subsequent to the activation and translocation of NF-κB to the cell nucleus. Further, HP0986 induced apoptosis of RAW 264.7 cells through Fas activation and this was in agreement with previous observations made with THP-1 cells. Our studies indicated activation of TNFR1 through interaction with HP0986 and this elicited the aforementioned responses independent of TLR2, TLR4 or TNFR2. We found that mouse TNFR1 activation by HP0986 facilitates formation of a complex comprising of TNFR1, TRADD and TRAF2, and this occurs upstream of NF-κB activation. Furthermore, FADD also forms a second complex, at a later stage, together with TNFR1 and TRADD, resulting in caspase-8 activation and thereby the apoptosis of RAW 264.7 cells. In summary, our observations reveal finer details of the functional activity of HP0986 protein in relation to its behavior in a murine macrophage cell environment. These findings reconfirm the proinflammatory and apoptotic role of HP0986 signifying it to be an important trigger of innate responses. These observations form much needed baseline data entailing future *in vivo* studies of the functions of HP0986 in a murine model.

1. Introduction

Helicobacter pylori infection is the known cause of infectious gastritis, peptic ulcer disease and gastric cancer. Although more than 50% of world's population is infected with *H. pylori*, only 10–20% of total infected cases progress to form clinically significant morbidity [1]. The infection initiates with the adherence of *H. pylori* to the gastric epithelium with concomitant immune responses leading to infiltration of neutrophils and lymphocytes. The inflammation developed during this process is often not sufficient enough to clear off the infection. This leads to persistence of *H. pylori* infection for decades [2]. However, variations have been observed in the course and severity of infection in different individuals which can be attributed to bacterial strain heterogeneity, host genetic susceptibility and environmental factors [3–5].

Genetic heterogeneity of *H. pylori* is reflected in its varied functional effects occurring due to variations in the allelic structures and functional repertoires of certain virulence factors. Among the several known virulence factors, cytotoxin-associated gene (cag) Pathogenicity Island (*cag*PAI) and vacuolating cytotoxin (VacA) are most extensively studied [6–9]. In addition, factors contributing towards the adherence of *H. pylori* to gastric epithelial cells (SabA, BabA, AlpA/B, HopZ and OipA) also play crucial roles during pathogenesis [10–13]. However, the diversity in expression of these virulence factors in clinical strains is reflective of the existence of selection pressure on the colonizing strains infecting the same or different individuals. Such variability in strain specific virulence factors is also responsible for differences in virulence potentials of *H. pylori* isolates [10,14,15]. Many *H. pylori* strain-specific genes that encode putative virulence factors of unknown functional mechanisms are encoded within highly variable chromosomal plasticity zones (PZs) [3]. Most PZ genes are predicted to be of unknown function but the encoded products of some are considered to influence the virulence potential of infecting strains.

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The HP0986 protein encoded within the PZ of strain 26695 is one such example. Our previous studies have shown it to be naturally expressed and presented to the immune system and addition of purified recombinant HP0986 to cultured macrophages and gastric epithelial cells were shown to activate NF-κB leading to up regulation of proinflammatory cytokines such as TNFα and IL-8 [16,17]. Also, HP0986 actively induced apoptosis *via* TNFR1 and Fas mediated pathways in THP-1 cells [16], although specific steps and details of these pathways have remained elusive. These pathways urgently required to be dissected. Further, there was a need to confirm HP0986 induced signaling in a murine setup in order to widen the study towards reproduction of the effects *in vivo*.

2. Materials and methods

2.1. Murine macrophage (RAW 264.7) cell culture

RAW 264.7 cells were procured from NCCS, Pune, India. The cells were grown in RPMI 1640 medium (HyClone™, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine and 1% anti-bacterial and anti-mycotic agent (Gibco, Life Technologies™). Approximately 0.5×10^6 cells were grown until 60–70% became confluent in 12 well culture plates. Cells were induced with increasing concentration of recombinant (His-tagged) HP0986 (0.5 μg, 1.0 μg, 2.5 μg and 5.0 μg/ml). Cells without any stimulus and cells with proteinase-K treated HP0986 were used as negative controls. Cells treated with LPS (5.0 μg/ml) from *Escherichia coli* 055:B5 (Sigma Aldrich, USA) served as positive control. Culture supernatants from each treated set were collected after 10 h of incubation and stored at −80 °C until used for assays.

2.2. Cytokines assay

Cytokines/chemokines in the culture supernatant were analyzed by multiplexed bead based immunoassays using commercially available Cytometry Bead Array (CBA) Kit (BD Biosciences, USA). The assay was performed according to manufacturer's instructions. The samples' flow data were collected using a flow cytometer (BD FACS CantoII, BD Biosciences, USA) and analyzed using FCAP Array multiplex analysis software (BD Biosciences, USA). Cytokine/chemokine concentrations were determined with a standard curve plotted using the intuitive FCAP array software. The values were represented as mean ± SE.

2.3. Real-time PCR

Approximately 2×10^6 RAW 264.7 cells per well and per 2 ml of RPMI media were grown until 70–80% confluent in a 6 well culture plate. Cell were treated with recombinant (His-tagged) isocitrate dehydrogenase (ICD) (2.5 μg/ml), LPS (5.0 μg/ml) and HP0986 (2.5 μg/ml). An aliquot of cells was also treated with neutralizing anti-mouse TNFR1 antibody (5.0 μg/ml) before HP0986 (2.5 μg/ml) induction. The cells were incubated for 6 h. After incubation, cells from each well were harvested in 1 ml TRI reagent (Sigma Aldrich, USA). Total RNA was extracted as per the manufacturer's instruction. One microgram of total RNA was taken for generation of cDNA using Verso cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the protocol described in their manual. The mRNA levels of MCP-1 and TNF-α were measured with the help of SYBR green (Applied Biosystems, Life Technologies™) in a Mastercycler® ep *realplex* thermal cycler (Eppendorf, Germany). Following primers were used for the amplifications: Mouse TNF-α Forward primer: TCCCAGGTTCTCTTCAAGGGA, Reverse primer: GGTGAGGAGCACGTAGTCGG; Mouse MCP-1 Forward primer: GAAGGAATGGGTCCAGACAT, Reverse primer: ACGGGTCAACTTCACATTCA and Mouse GAPDH Forward primer: TGTGTCCGTCGTGGATCTGA and Reverse primer: CCTGCTTCACCACCTTCTTGA. The relative expression levels of MCP-1 and TNF-α were normalized to expression values of GAPDH taken as a housekeeping gene reference.

2.4. Western blot analysis

RAW 264.7 cells were harvested and centrifuged at 500*g* for 5 min. The whole cell extract was prepared using non-denaturing lysis buffer and according to the protocol described earlier [18]. Cytoplasmic and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, USA) according to the manufacturer's instruction. The extracts were stored at −80 °C until use. Western blotting was preformed as per the previous description [19]. Primary antibodies used were against p65 (NF-κB), procaspase-8, procaspase-3, β-actin (Santa Cruz Biotechnology Inc., USA), lamin B and IκB-alpha (Sigma Aldrich, USA). Blots were developed using Super Signal West Pico Kit (Thermo Fisher Scientific, USA). β-actin was used as whole cell/cytoplasmic extract marker and for nuclear extract, lamin B was used as loading control.

2.5. Immunoprecipitation assays

For interaction analysis, cell extracts were prepared with RIPA buffer as described before [18]. An aliquot of 0.5 mg of the cell lysate was incubated overnight with HP0986 (10.0 μg) and polyclonal anti-HP0986 antibody (10.0 μg) at 4 °C. The immune complex was pulled down with protein-G agarose beads (Santa Cruz Biotechnology Inc., USA). The complexes were run on 12% SDS–PAGE and transferred over to PVDF membrane for western blot analysis using the primary antibodies directed against TNFR1 and TNFR2 (Santacruz Biotechnology Inc, USA), TLR2 and TLR4 (Imgenex, India). The immunoblots were developed with Super Signal West Pico Kit (Thermo Fisher Scientific, USA). Primary antibodies against TRAF2, TRADD and FADD (Santa Cruz Biotechnology Inc., USA) were used to analyze the respective co-precipitated molecules along with the HP0986-receptor complex by Immunoblotting.

2.6. Flow cytometric analysis

Cells were grown in 12 well plates till they became approximately 50–60% confluent. Cells were then treated with increasing doses of HP0986 (1.0, 2.5, 5.0 and 10.0 μg/ml) and incubated in a CO_2 incubator at 37 °C for 24 h for TNFR1 expression study. Similarly, for the Fas expression analysis, cells were treated with increasing doses of HP0986 (1.0, 2.5 and 10.0 μg/ml) and for different time periods (12 h, 24 h and 48 h). Unstimulated cells were taken as negative control for both Fas and TNFR1 expressions whereas TNF-α (0.2 μg/ml) treated cells acted as positive control. After incubation, cells were harvested in 0.2% EDTA solution (in PBS) and counted. Cells were centrifuged at 300*g* for 5 min and supernatant was completely removed. Cells were then resuspended in appropriate volume of blocking buffer (5% horse serum in PBS) to get 1×10^6 cells per ml of buffer. The resuspended cells were kept on ice for 30–45 min to ensure the blocking of the Fc receptor sites present on macrophage cells and therefore to enhance the specific binding of the primary antibodies to their respective antigenic sites on cells surface. Approximately 0.1×10^6 cells (100 μl) were taken in a polystyrene tube and the subsequent staining steps were performed in the same buffer. For TNFR1 expression analysis, cells were incubated with rabbit anti-TNFR1 antibody (Santacruz Biotechnology Inc. USA) for 45 min on ice, followed by incubation with secondary FITC-conjugated anti-rabbit antibody (Sigma Aldrich, USA) for another 45 min. For the Fas expression assay, approximately

0.1×10^6 (100 μl) cells were incubated directly with FITC-conjugated anti-CD95 antibody (BD Biosciences, USA) for 30 min on ice. After the incubation with labeled antibodies, the cells were washed twice and resuspended in 500 μl of the same buffer for immediate data acquisition on flow cytometer. Unstained cells were taken as internal negative control in both the assays to rule out any interference due to background fluorescence. All acquired data were analyzed with Flow Jo software (Tree Star Inc., USA).

2.7. MTT-assay for cell viability measurement

The RAW 264.7 cells' viability after HP0986 treatment was analyzed by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Amresco, USA) assay. The HP0986 induced cells were incubated for 48 h in a 96 well plate. An aliquot of 20 μl of MTT (5 mg/ml) was added to each well and kept for 3 h at 37 °C. The resultant insoluble Formazan complex was dissolved in dimethyl sulfoxide (Sigma Aldrich, USA). The colored solution thus formed was measured at 570 nm [20].

2.8. HP0986 mediated apoptosis analysis

HP0986 induced apoptosis in RAW 264.7 cells was analyzed by AnnexinV-FITC/PI assay. Cells were processed and staining was performed according to manufacturer's instructions (BD Biosciences, USA) and the sample data were acquired on flow cytometer (BD FACS Canto II, BD Biosciences, USA). The percentage of cells undergoing apoptosis were expressed as total of % $AnnexinV^+$ and PI^+ cells after subtracting the background fluorescence. Furthermore, apoptosis in HP0986 treated RAW 264.7 cells could be visualized through DNA fragmentation assay. The analysis was performed as per the method described previously [21]. In brief, 2×10^6 cells were washed with PBS and pelleted by centrifugation at 400*g* for 5 min. Cell pellets were then treated with a lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris–HCl, pH, 7.5) by repeated pipetting up and down. Homogenates were then centrifuged at 13,000*g* for 5 min. Supernatants were then treated with RNase A at 37 °C for 2 h followed by proteinase-K at 37 °C for 2 h. Then a ½ volume of 10 M ammonium acetate was added to DNA solutions. To further facilitate the DNA precipitation, 2.5 volume of ethanol was added and centrifuged. DNA pellets were dissolved in gel loading buffer, run and analyzed on 1% agarose gel.

2.9. Statistical analysis

Results were expressed as means ± standard error (SE). Comparisons of cytokine inductions, TNFR1 and Fas expressions were performed by two-tailed *t*-test using GraphPad Prism version 5.01 for windows (Graph Pad software, San Diego, California USA: www.graphpad.com). The differences between two values were considered significant if $p < 0.05$.

3. Results

3.1. HP0986 induces expression and secretion of MCP-1 and TNF-α from murine macrophages

The N-terminal His-tagged protein was over expressed in BL-21(DE3) cells and purified under native conditions using Co^{+2}-NTA affinity column. RAW 264.7 cells treated with HP0986 and incubated for 10 h released MCP-1 and TNF-α into the culture supernatant in a dose dependent manner. A significant secretion of MCP-1 and TNF-α was observed when 1 ml aliquot of media with about 1 million cells was treated with increasing doses of HP0986 (0.5 μg, 1.0 μg, 2.5 μg and 5.0 μg/ml). Highest secretion of both MCP-1 ($p = 0.0002$) and TNF-α ($p < 0.0001$) were observed at the protein concentration of 2.5 μg/ml (Fig. 1A, B). Cells treated with even higher dose (5.0 μg/ml) of HP0986 did not show any further increase in MCP-1and TNF-α levels. Proteinase-K-treated HP0986 failed to induce the production of both MCP-1 and TNF-α suggesting that the response was indeed triggered by HP0986. Cells treated with LPS (5.0 μg/ml) were taken as a positive control for the induction of both MCP-1 and TNF-α (Fig. 1A, B). In order to further confirm the effect specific to HP0986, His-tagged isocitrate dehydrogenase (ICD) of *H. pylori* purified in a similar manner was used as an internal negative control. A relative mRNA quantification analysis using real-time PCR showed that cells treated with His-tagged ICD protein (2.5 μg/ml) failed to trigger MCP-1 and TNF-α mRNA expression above the control level and therefore ruling out the possible involvement of 6x His tag of recombinant, purified HP0986 in the above responses (Fig. 1C, D). Thus, our data reveal HP0986 specific proinflammatory cytokine responses in RAW 264.7 cells.

3.2. MCP-1 and TNF-α secretion is mediated by activation of NF-κB

In order to determine whether the MCP-1 and TNF-α secretion is regulated by NF-κB activation, we analyzed the NF-κB levels in nuclear and cytoplasmic fractions of HP0986 stimulated RAW 264.7 cells. We observed an increase in nuclear translocation of p65 in HP0986 treated RAW 264.7 cells in a time dependent manner. A clear and enhanced translocation of p65 in the nuclear extract of HP0986 treated (2.5 μg/ml) RAW 264.7 cells was observed with increase in time of incubation (0.5, 1.0, 2.0 and 3.0 h). The time course followed for this study indicated that maximum level of nuclear localization of p65 was observed at 3 h post treatment when compared to the control (Fig. 2B). The corresponding observed decrease in levels of p65 from cytoplasmic extracts during the same time period was also consistent with increased translocation to the nucleus. TNF-α (50 ng/ml) was taken as positive control for NF-κB translocation analysis. In a further study, western blot analysis showed noticeable degradation of IκB-alpha in the cytosol after stimulation with HP0986 (2.5 μg/ml). A visibly clear degradation of IκB-alpha was observed at 30 min, 1 h and 2 h post treatment with HP0986 (Fig. 2A). Taken together, we found that time course for IκB-alpha degradation preceded NF-κB activation.

3.3. Interaction of HP0986 with murine TNFR1

Host receptor involved in eliciting the cytokine response was analyzed by pull down assay which showed mouse TNFR1 captured together with HP0986 when probed with anti-TNFR1 antibody. However, HP0986 did not involve in any complex formation with TNFR2/TLR2 or TLR4 as revealed by pull-down assay using anti-HP0986 antibody followed by western blot analysis with anti-TNFR2, anti-TLR2 and anti-TLR4 antibodies (to rule out any possible HP0986 binding to receptors other than TNFR1) (Fig. 3A). Furthermore, an increase in cell surface TNFR1 expression was observed by flow cytometry in HP0986 treated RAW 264.7 cells in a dose (1.0, 2.5, 5.0 and 10.0 μg/ml) dependent manner (Fig. 3B). These observations confirmed the interaction between HP0986 and murine-TNFR1, an event upstream of inflammatory response, and also ruled out any interaction between HP0986 and TNFR2/TLR2 or TLR4. Moreover, RAW 264.7 cells pretreated with neutralizing anti-mouse TNFR1 antibody (5 μg/ml) prior to induction by HP0986 (2.5 μg/ml) revealed reduced translocation of p65 to the nucleus (Fig. 3C) and also decreased induction of MCP-1 and TNF-α (Fig. 1C and D). These observations clearly suggest a link between TNFR1 stimulation through HP0986 binding and NF-κB activation leading to the MCP-1 and TNF-α secretion in murine macrophages.

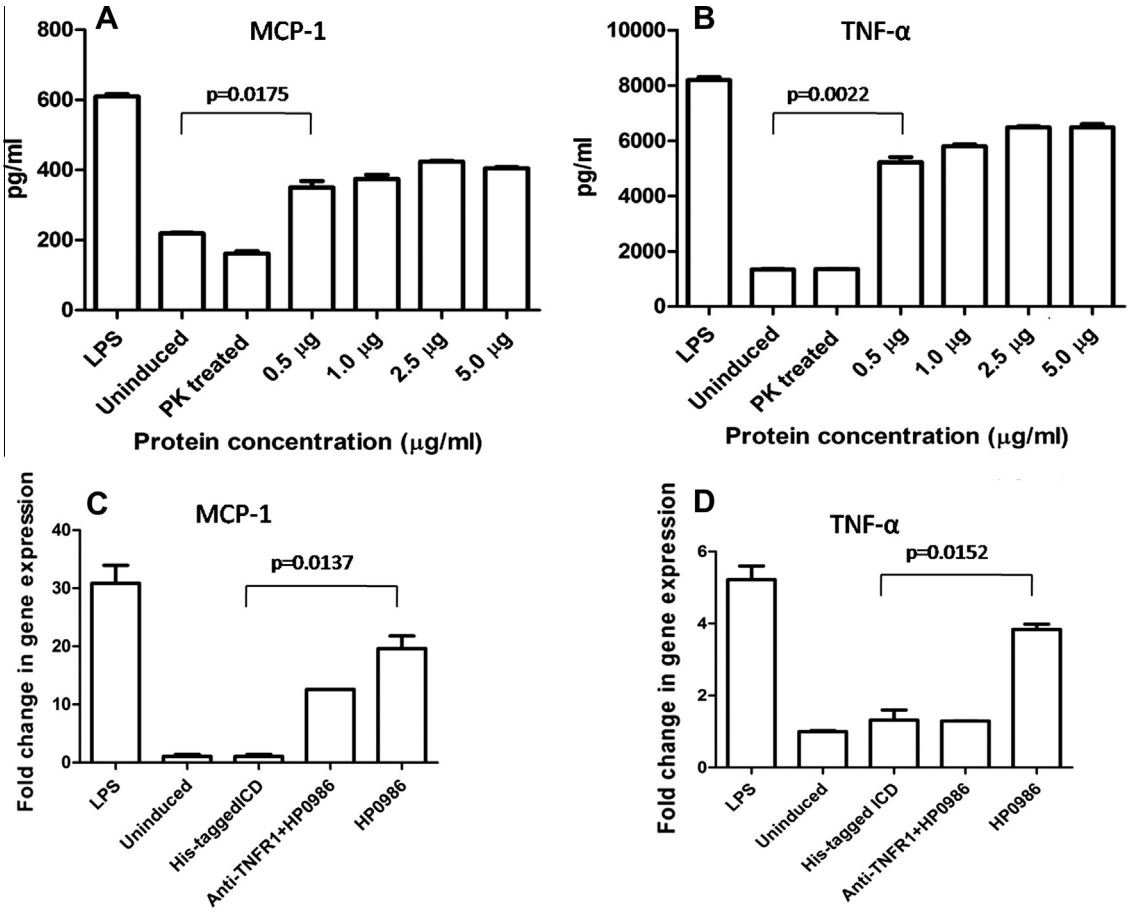

Fig. 1. (A) Secretion of MCP-1 and (B) TNF-α by murine macrophage (RAW 264.7) cells after treatment with HP0986. Uninduced cells, and cells stimulated with proteinase-K treated HP0986 were taken as negative control. LPS (5 μg/ml) treated cells were taken as positive control. Results are shown as mean ± SE. (C) Estimation of relative mRNA levels for MCP-1 and (D) TNF-α by real time PCR in HP0986 treated RAW 264.7 cells. Unstimulated cells, His-tagged ICD treated cells and the cells pretreated with neutralizing anti-mouse TNFR1 before HP0986 induction also served as negative controls for this study. Results are shown as mean ± SE.

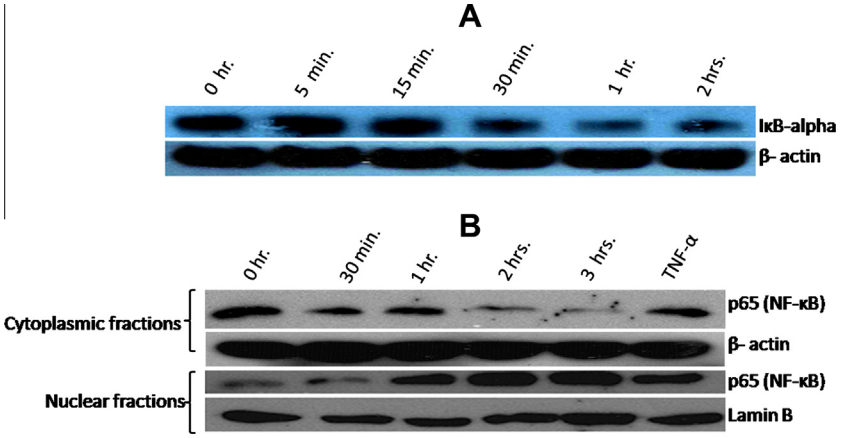

Fig. 2. (A) IκB-alpha degradation analysis in HP0986 treated murine macrophage (RAW 264.7) cells at different time intervals by western blotting. Reprobing of the blot with β-actin antibody was done to ensure equal loading of the protein. (B) Time dependent translocation analysis of p65 (NF-κB) in cytoplasmic and nuclear fractions from RAW 264.7 cells treated with HP0986. TNF-α treated cells were taken as positive control for this study. β-actin was used as a marker for equal loading of cytoplasmic fractions. Lamin B was used as a marker entailing nuclear fraction.

3.4. HP0986 induces Fas mediated apoptosis in murine macrophages (RAW 264.7)

Fas receptor on the cell surface induces apoptosis upon interaction with the ligand [22]. According to our previous observation with human macrophages (THP-1) that suggested a link between TNFR1 activation by HP0986 and Fas expression [16], we performed a similar analysis with murine macrophages. HP0986 were shown to induce increased expression of Fas on murine macrophages (RAW 264.7) in a dose (1.0, 2.5 and 10 μg/ml) and time (12, 24 and 48 h) dependent manner. However, at highest time point of 48 h incubation, the increment in protein doses did not reflect increase in Fas expression possibly due to apoptosis of the cells (Fig. 4). However, expression of Fas on murine macrophages was significantly higher than unstimulated cells. AnnexinV-FITC/PI assay also indicated an increase in apoptosis of

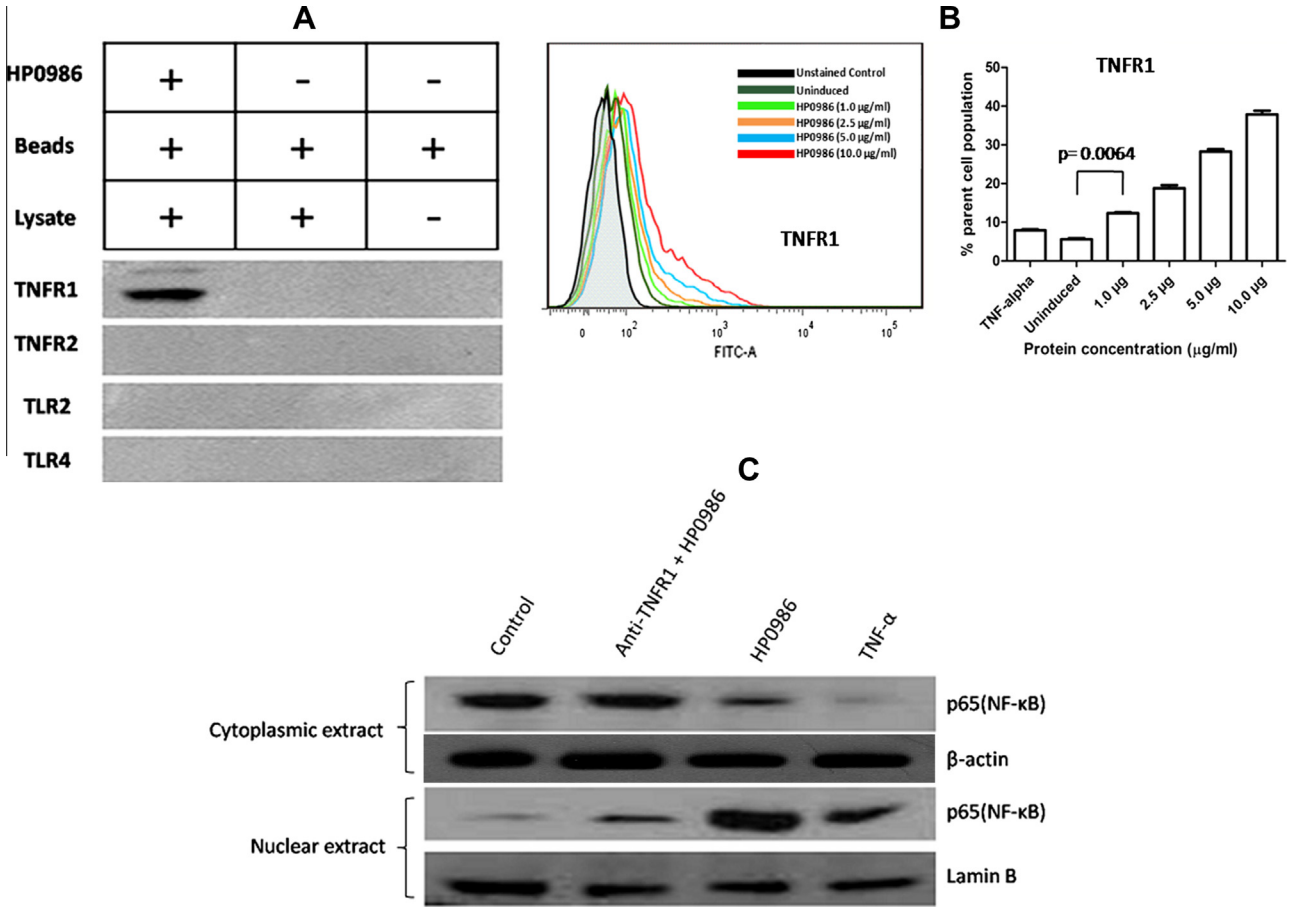

Fig. 3. (A) Pull down assays for binding analysis of HP0986 with receptors (TNFR1, TNFR2, TLR2 or TLR4). HP0986 bound receptor from the mouse macrophage cell lysate was pulled down with protein G-agarose beads and immunoblots were probed with anti-TNFR1, anti-TLR2, anti-TLR4 and anti-TNFR2 antibodies. Cell lysates incubated with protein-G beads and protein-G beads alone were used as negative controls for this assay. (B) Flow cytometry analysis showing increased effect of HP0986 on TNFR1 expression on murine macrophage (RAW 264.7) cells. Uninduced cells were taken as negative control while TNF-α treated cells were analyzed as positive control for TNFR1 expression. (C) Western blot analysis showing reduced translocation of p65 (NF-κB) in RAW 264.7 cells pretreated with neutralizing anti-TNFR1 antibody before induction with HP0986 when compared to cells treated with HP0986 alone. TNF-α treated cells served as positive control and unstimulated cells as negative control for this analysis.

HP0986 (5.0 μg/ml) treated RAW 264.7 cells. In the same analysis, upon HP0986 (5.0 μg/ml) stimulation, we observed decreased apoptosis of cells pretreated with neutralizing anti-mouse TNFR1 antibody (5.0 μg/ml). His-tagged ICD (5.0 μg/ml) failed to induce apoptosis and therefore ruling out the possible interference by 6 × His-tag attached to recombinant HP0986. Staurosporin (50 nM) treated cells in all cases were taken as positive control for apoptosis (Fig. 5A). Further, we observed an increase in procaspase-8 and procaspase-3 degradation in RAW 264.7 cells induced with HP0986 for 48 h in a dose (2.5, 5.0 and 10.0 μg/ml) dependent manner when compared with uninduced cells (Fig. 5B). As a positive control, Staurosporin (50 nM) also showed an enhanced degradation of pro-caspase-8 and procaspase-3. β-actin was taken as loading control for the above analysis. This observation is also in agreement with earlier reports suggesting that Fas engagement with its ligand results in accumulation, cleavage and therefore activation of caspase-8 which further activates the effector caspases [23,24]. Furthermore, apoptosis process in RAW 264.7 cells was validated through DNA fragmentation in HP0986 (5.0 μg/ml) treated cells (DNA ladder assay) as compared to unstimulated cells (Fig. 5C).

3.5. TNFR1 activation by HP0986 and recruitment of TRADD, TRAF2 and FADD

Both TRADD and TRAF2 were co-precipitated together with TNFR1-HP0986 complex as shown in immunoblots using lysate from the RAW 264.7 cells treated with HP0986 and incubated for 30 min (Fig. 6). This suggests that TRADD forms a complex with TNFR1 after interacting with HP0986 which in turn provides a platform for the recruitment of TRAF2 [25] leading to the NF-κB activation. Further, we also observed in a similar study, the co-precipitation of FADD along with TNFR1-HP0986 complex in the lysate from cells treated with HP0986 for 24 h (Fig. 6). However since FADD interacts with both TRADD and Fas, this indicates two possible pathways for HP0986 induced apoptosis: one mediated through TNFR1 and the other involving Fas activation [25,26].

4. Discussion

HP0986 or TieA is an importantly proinflammatory protein and putative type II endonuclease encoded by the plasticity region of *H. pylori* [16,17]. The protein has been profiled either (genotypically) using patient biopsies and clinical isolates corresponding to different populations or through (serological) testing for humoral immune responses and in (cell signaling) studies involving cultured human cells, and was also shown to induce proinflammatory cytokines through TNFR1 and NF-κB mediated signaling pathways [16,17]. However, its function has not been verified in an animal model such as mouse. The present study therefore assumes an important extension of the works of Alvi et al. [16] and Devi et al. [17] to reproduce the interaction of this protein with mouse immune apparatus (including TNFR1 and other receptors) and

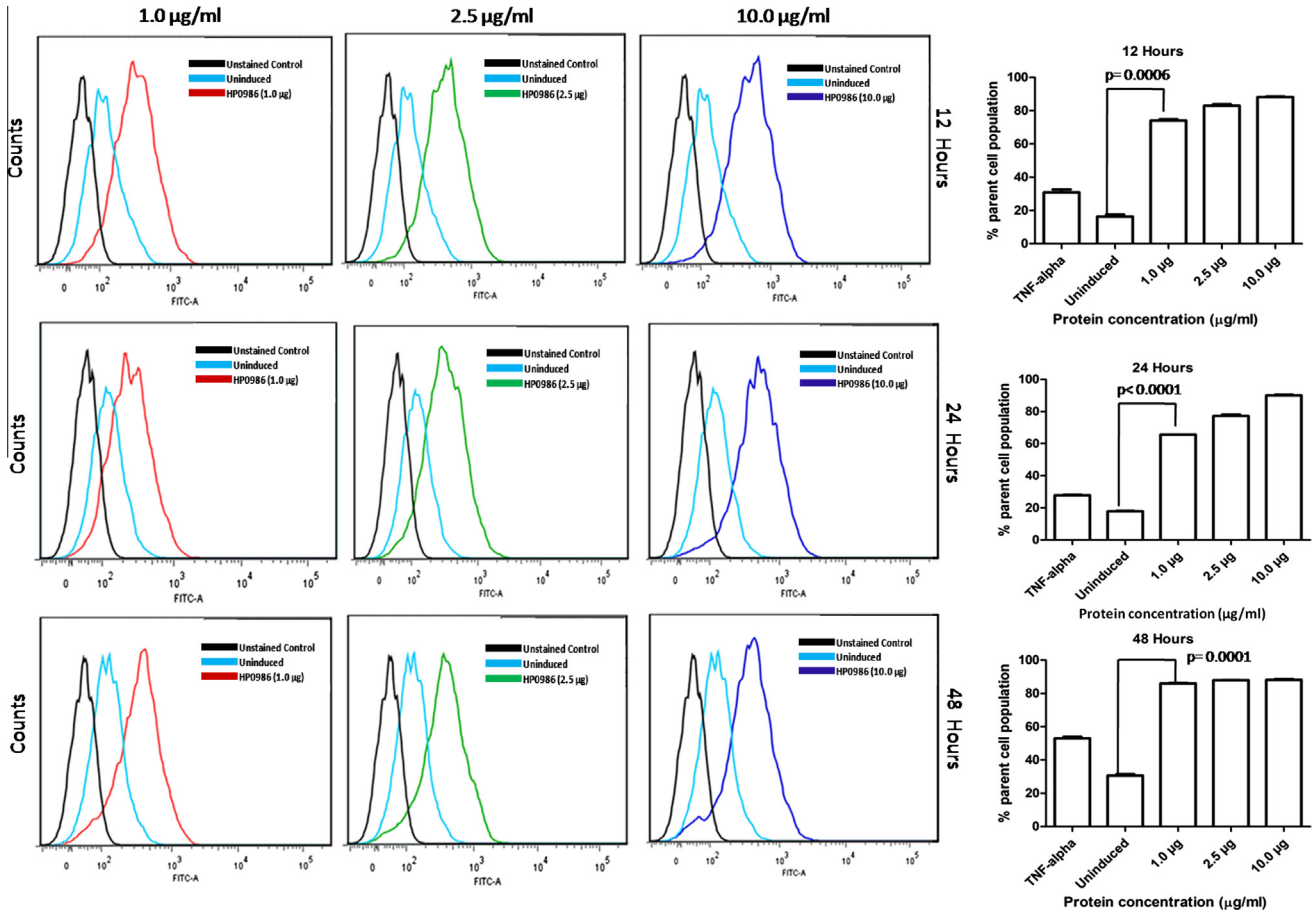

Fig. 4. Fas expression on the surface of murine macrophage cells (RAW 264.7) induced by HP0986 in a dose and time dependent fashion and analyzed using FITC-conjugated anti-Fas antibody by flow cytometry. Cells without protein stimulus and/or unstained uninduced cells served as negative controls and TNF-α treated cells as positive control. Results are also shown as mean ± S.E in bar diagrams.

characterize the downstream signaling events to unravel finer details of the apoptotic pathways in order to understand the complete scenario of the proposed 'survival advantage' to the bacterium [16,17] in a mammalian system.

In that pursuit, the strongest observation made in this study entails consistent proinflammatory and apoptotic behavior of HP0986 in a murine macrophage cell (RAW 264.7), similar to what has been observed earlier for the human cell lines and PBMCs. Induction of proinflammatory cytokines by the mouse cells confirms HP0986 to be an important antigen that excites mammalian cells. As a potent chemoattractant for monocytes/macrophages and T-lymphocytes, MCP-1 is reported to be actively involved in the inflammation process [27–30]. TNF-α secreted by macrophages could have several implications during bacterial infection. TNF-α binding to TNFR1 triggers Fas mediated apoptosis of macrophages [31–33]. Reports also indicate that TNF-α down-regulates phagocytosis of apoptotic cells by macrophages [34]. Recently, it has been shown in case of *H. pylori* infection that TNF-α accumulation during gastric injury has negative effect on the clearance of apoptotic gastric epithelial cells [35]. The apoptotic 'cell loads' further enhance inflammatory state of gastric mucosa through release of proinflammatory mediators from the dying cells following their secondary necrosis [35].

The present results reconfirmed involvement of NF-κB in induction of MCP-1 and TNF-α which are consistent with the previous reports [36,37]. The activation of NF-κB as observed through translocation of p65 to the nucleus after HP0986 induction was also supported by the degradation of the IκB-alpha in RAW 264.7 cells (Fig. 2). NF-κB activation is a key determinant of the immune cell response to the bacterial infection which in turn regulates expression of many inflammation promoting genes encoding several chemokines, cytokines and members of the IgG superfamily [38–42].

Fas expression by RAW 264.7 cells after HP0986 induction (Fig. 4) was in accordance with our previous observations in THP-1 cells [16]. This also suggests that the virulence potential and activity of HP0986 is similarly placed in the mouse system. In corroboration with previous reports, we also showed by immunoprecipitation method the interaction between HP0986 and murine TNFR1 (Fig. 3A). Furthermore, RAW 264.7 cells pretreated with neutralizing antibody against TNFR1 revealed reduced translocation of NF-κB to the nucleus upon induction with HP0986; this confirmed the dependency of the expression of inflammatory response genes on TNFR1 (Fig. 3C). This has thus indicated that HP0986 interaction with TNFR1 leads to a downstream inflammatory response as well as Fas mediated apoptosis.

To gain further insight into the signaling events, we have shown through immunoprecipitation analysis the interaction of HP0986 to TNFR1 receptor and activation of downstream signaling molecules including TRADD, TRAF2 and FADD (Fig. 6). Given these results, and on the lines of previous observations [43–46,26,16], we propose a signaling cascade similar to that mediated by TNF-α. In brief, stimulation of TNFR1 by HP0986 triggers the recruitment of the adaptor molecule TRADD to the cytoplasmic domain of TNFR1. Further, TRAF2 binds to the N-terminal domain of TRADD leading to the activation of NF-κB. TNFR1 activation also contributes to another pathway through the recruitment of FADD to the

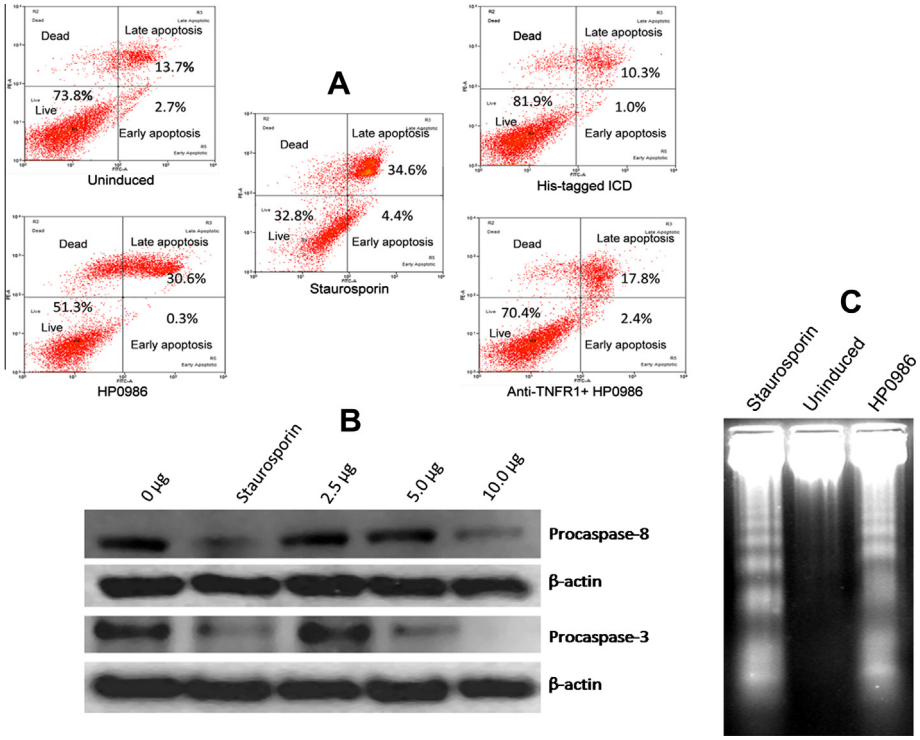

Fig. 5. (A) AnnexinV-FITC/PI staining to analyze apoptosis in murine macrophages cells by flow cytometry using (i) HP0986 induced cells, and (ii) cells treated with neutralizing antiTNFR1 antibody and HP0986; controls comprised of (iii) cells without protein stimulus (negative control), (iv) cells treated with recombinant His-tagged ICD protein (unrelated protein control) and (v) cells treated with Staurosporin (positive control). (B) Immunoblotting showing procaspase-8 and procaspase-3 cleavage analysis as a marker for apoptosis in murine macrophage cells in a dose dependent manner. Cells without any stimulus were taken as negative control. Staurosporin treated cells were used as positive control. (C) DNA ladder assay to assess the proapoptotic nature of HP0986 in RAW 264.7 cells. Cells without protein stimulation were taken as negative control. DNA isolated from Staurosporin induced cells was taken as positive control for apoptosis.

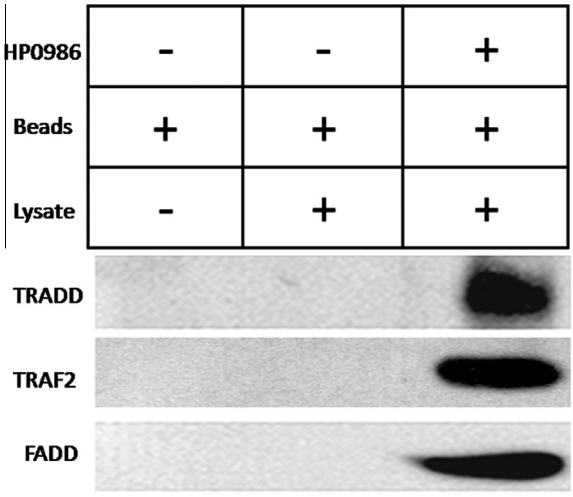

Fig. 6. Co-precipitation of TRADD, TRAF2 and FADD recruited to activated TNFR1 bound with HP0986. These adaptor molecules were pulled down together with HP0986-TNFR1 complex and detected by western blotting. Protein-G beads incubated with cell lysate, and protein-G beads alone were used as negative controls for this analysis.

C-terminal domain of TRADD and subsequent activation of caspase-8 leading to apoptosis. However, since FADD also connects Fas to caspase-8 activation [26], there could be a possibility of involvement of an additional death pathway (other than that mediated through TNFR1) in HP0986 treated mouse macrophages.

In view of our observations, it is now possible to clearly portray the role of HP0986 in inflammation and apoptosis at the interface of various possible cellular pathways that conjoin TNFR1, Fas and NF-κB. These new data unleashing in a mouse macrophage background would definitely underpin future efforts directed at understanding the complexity and nature of *H. pylori* induced cancer signaling. The immunological co-ordinates so established would also facilitate *in vivo* validation of HP0986 function in a mouse system in order to study the secretion, localization and regulation of this important virulence factor.

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Helicobacter pylori cell translocating kinase (CtkA/JHP0940) is pro-apoptotic in mouse macrophages and acts as auto-phosphorylating tyrosine kinase

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ABSTRACT

The *Helicobacter pylori* gene JHP0940 has been shown to encode a serine/threonine kinase which can induce cytokines in gastric epithelial cells relevant to chronic gastric inflammation. Here we demonstrate that JHP0940 can be secreted by the bacteria, triggers apoptosis in cultured mouse macrophages and acts as an auto-phosphorylating tyrosine kinase.

Recombinant JHP0940 protein was found to decrease the viability of RAW264.7 cells (a mouse macrophage cell line) up to 55% within 24 h of co-incubation. The decreased cellular viability was due to apoptosis, which was confirmed by TUNEL assay and Fas expression analysis by flow-cytometry. Further, we found that caspase-1 and IL-1beta were activated upon treatment with JHP0940. These results point towards possible action through the host inflammasome. Our *in vitro* studies using tyrosine kinase assays further demonstrated that JHP0940 acts as auto-phosphorylating tyrosine kinase and induces pro-inflammatory cytokines in RAW264.7 cells. Upon exposure with JHP0940, these cells secreted IL-1beta, TNF-alpha and IL-6, in a dose- and time-dependent manner, as detected by ELISA and transcript profiling by q-RT-PCR.

The pro-inflammatory, pro-apoptotic and other regulatory responses triggered by JHP0940 lead to the assumption of its possible role in inducing chronic inflammation for enhanced bacterial persistence and escape from host innate immune responses by apoptosis of macrophages.

Introduction

Even 30 years after the discovery of *Helicobacter pylori* (Marshall and Warren, 1984) and establishment of its role in gastric diseases, more than 80% of the population in developing countries (Hunt et al., 2011) as compared to 30–50% in developed countries (Covacci et al., 1999) is infected with the bacterium. However, the prevalence of *H. pylori* infection varies from geographic region to region throughout the world (Ferlay et al., 2010) which is more likely correlated with socioeconomic status of the population (Malaty and Graham, 1994) and sex; because in many populations males have 20–30% higher rates of infection than females (Replogle et al., 1995). Since *H. pylori* is the main cause of gastric cancer, WHO has classified the bacterium as a class I carcinogen in 1994 and severity of the disease in the present scenario is so high that gastric cancer is the second leading cause of cancer related deaths worldwide and constitutes the fourth most common cancer type (Ferlay et al., 2010).

H. pylori causes infection of the gastric mucosa (Marshall and Warren, 1984) and is found to be the most successful colonizer of the human stomach causing chronic gastritis, duodenal ulcer, intestinal metaplasia and gastric cancer as a consequence of long term colonization (Graham, 1997; Uemura et al., 2001). Although the severity of chronic inflammation depends on host genetic factors, environmental parameters and bacterial factors play an important role in the onset of gastric diseases. Among the bacterial factors encoded by various clinical *H. pylori* strains, one of the two most studied proteins is CagA which is encoded within the cytotoxin-associated genes (*cag*) pathogenicity island (PAI) and is about 40 kb in size (Covacci et al., 1993). It has been established that most of the genes from the *cag*PAI act as components of a type IV

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secretion system (T4SS) forming a syringe like structure that facilitates translocation of CagA and probably other macromolecular bacterial factors into the host cells (Backert et al., 2010). Moreover, the strains that harbour the *cag*PAI are associated with more severe gastric inflammation and overt outcomes as compared to *cag*PAI-negative strains. The other most studied *H. pylori* virulence factor is VacA (vacuolating cytotoxin A) which induces cytoplasmic vacuolation by forming membrane channels in epithelial membranes *in vitro* (Cover and Blaser, 1992). Besides this activity, it has a multifunctional role in colonization, virulence and immunomodulation (Cover and Blanke, 2005; Pachathundikandi et al., 2013). Apart from these two cardinal effectors, virulence genes such as BabA, SabA, SabB, OipA etc. (Basso et al., 2010; Odenbreit et al., 2009) code for the other important factors which facilitate adhesion of the bacterium to the gastric epithelium. In addition, IceA that carries a restriction endonuclease activity was also found to be associated with gastritis and duodenal ulcer (Xu et al., 2002). There is an on-going interest in another putative virulence factor, DupA, on account of its association and envisaged role in gastric diseases (Hussein et al., 2010; Shiota et al., 2010; Schmidt et al., 2009). Furthermore, findings on some other *H. pylori* gene products such as protease HtrA (Hoy et al., 2012), AnsB (asparginase), GGT (γ-glutamyl transpeptidase) (McGovern et al., 2001), HP0986 (Alvi et al., 2011), HP-NAP (Satin et al., 2000) and HorB (Snelling et al., 2007) revealed their association with inflammation and pointed to their role in gastric diseases. Given this, the data available about these new generation bacterial factors do not seem to be adequate to fully understand pathogenesis of gastric inflammation and cancer. Therefore, it seems prudent to focus on the discovery of novel virulence factors and their biological roles relevant to the development and progression of gastric diseases. JHP0940 or CtkA is one such potential virulence factor that has been discovered and partly characterized (Rizwan et al., 2008) and its crystal structure has been solved (Kim do et al., 2010).

In the present study, we attempted to investigate the functional roles of JHP0940 which is located in a plasticity region gene cluster of the chromosome. The plasticity region is a highly variable zone that was identified by comparison of complete genome sequences of the two *H. pylori* strains (26695 and J99) that contain approximately 50% strain specific genes (Alm et al., 1999). Some of the genes in this region are thought to be acquired by *H. pylori* through horizontal gene transfer from some environmental bacteria (Datta et al., 2003). Thus, the plasticity zone genes provide an assumption that the proteins encoded by them may contribute to virulence or modulation of host immune responses. The association of jhp940 gene with gastric cancer (Occhialini et al., 2000; Yakoob et al., 2010), expression of JHP940 protein in response to interaction of *H. pylori* with the gastric mucosa in Mongolian gerbils *in vivo* (Graham et al., 2002) and its antigenicity profile prompted us to look at its virulence properties in more detail. Previous observations indicated that JHP940 can induce pro-inflammatory cytokines IL-8 and TNFα and increases translocation of transcription factor NF-κB in the human macrophage cell line Thp1 (Rizwan et al., 2008). Consequently, it was reported that JHP0940 also acts as a cell-translocating Ser/Thr Kinase and could indirectly upregulate the phosphorylation of NF-κB at serine residue 276 in human gastric epithelial cells (Kim do et al., 2010).

In the present study, we further extend the functional acumen of the JHP0940 protein and report its interaction with the host immune apparatus as evident from serum antibody titres present in patients infected with JHP0940-positive *H. pylori*. We also found that JHP940 acts as an auto-phosphorylating tyrosine kinase. This is perhaps the first described tyrosine kinase in *H. pylori* and we could demonstrate that it induces strong pro-inflammatory responses as well as Fas-mediated apoptosis in murine macrophage cells.

Materials and methods

Bacterial strains

The *H. pylori* wild-type strains J99 (Alm et al., 1999), SJM180 (Kersulyte et al., 2003) and P1 (Mueller et al., 2012) were used in the study. *H. pylori* was grown in thin layers on horse serum GC agar plates supplemented with vancomycin (10 μg/ml), nystatin (1 μg/ml), and trimethoprim (5 μg/ml) as described previously (Tegtmeyer et al., 2013). All antibiotics were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bacteria were grown at 37 °C for 2 days in an anaerobic jar containing a Campygen gas mix of 5% O_2, 10% CO_2, and 85% N_2 (Oxoid, Wesel, Germany) (Tegtmeyer et al., 2011).

JHP0940 (Ctk) secretion assays

Bacterial wild-type strains were grown in BHI broth medium supplemented with 10% FCS for 12 h starting with an $OD_{600nm} \sim 0.2$. The supernatant and the cell pellets were separated by centrifugation at 4000 rpm, and the supernatant was further purified from remaining bacterial cells by passage through a 0.21 μm sterile filter (Sigma Aldrich). The resulting bacterial pellets and supernatants were analysed by immunoblotting (Boehm et al., 2012). Absence of live bacteria in the supernatant was also confirmed by incubation on agar plates showing no growth.

Cell culture

Mouse macrophage RAW264.7 cell line was obtained from National Centre for Cell Science (Pune, India) and maintained in RPMI 1640 medium (Hyclone, USA) supplemented with 10% heat inactivated fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were trypsinized and seeded for the experiments.

Expression and purification of recombinant JHP0940 protein

The *jhp0940* gene was cloned in pRSET-A expression vector and the protein was expressed in *Escherichia coli* BL21 (DE3) cells, induced with 0.1 mM IPTG. His-tagged JHP0940 was purified as described earlier (Rizwan et al., 2008) with minor modifications, using Cobalt based Talon® resin (Clontech, USA). The homogeneity of the protein was confirmed by SDS-PAGE (12%) and the amount of protein estimated by bicinchoninic acid assay (BCA). The purified protein JHP0940 was treated with polymyxin B beads for 4 h and separated by centrifugation at 10,000 rpm for 10 min. LPS contamination in polymyxin B treated protein was quantitated by *Limulus* amebocyte lysate assay using Chromogenic Endotoxin Quantitation Kit (Pierce Thermo Scientific).

Treatment of cells with JHP0940 and preparation of cell lysate

RAW264.7 cells were cultured in 60 mm culture dish at a density of 4×10^6 per plate and treated with JHP0940 protein. Before harvesting, the cells were washed in cold PBS, scraped by cold plastic cell scraper, transferred to cold microfuge tube and lysed with ice cold lysis buffer (20 mM Tris HCl pH 7.4, 137 mM NaCl, 10% Glycerol, 1% NP-40, 2 mM EDTA, PI cocktail 1 μl/1×10^6 cells, 1 mM PMSF, 1 mM DTT, 1 mM Na_3VO_4) by intermittent vortexing for 40–60 s and repeated 5 times after an interval of 10 min and followed by centrifugation at 14,000 rpm for 10 min at 4 °C. Supernatant was collected and quantified by BCA method for western blotting.

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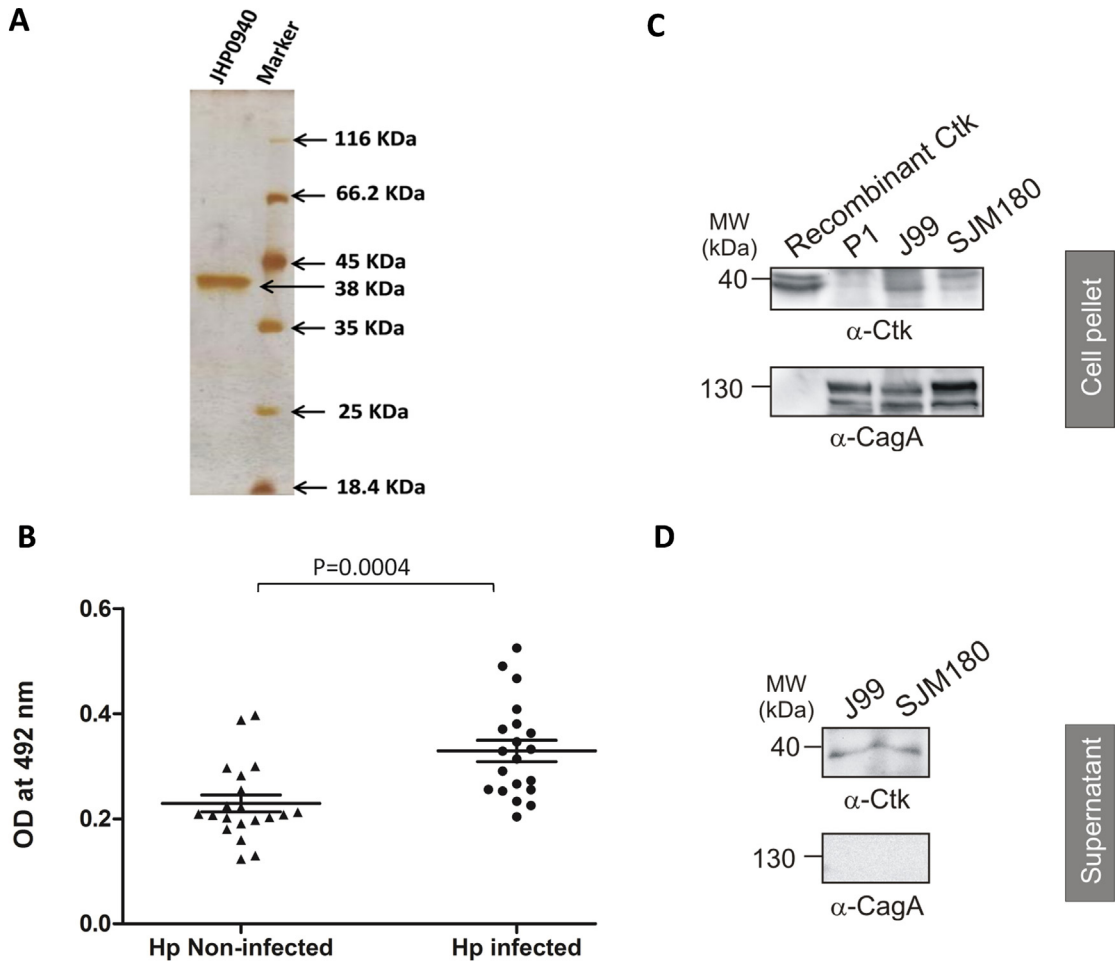

Fig. 1. (A) Purified JHP0940 in silver stained SDS gel. (B) ELISA of JHP0940 in two groups of human sera samples of 20 *H. pylori* infected and 20 healthy individuals. Statistically significant difference in *P* value (0.0004) was determined by two tailed Mann Whitney *U* test between healthy and *H. pylori* infected individuals. The data shown here represent mean ± SEM of three independent experiments. (C) Total cell lysates of *H. pylori* wild-type strains J99, SJM180 and P1 were investigated for the expression of Ctk. Equal amounts of protein per sample was confirmed by immunoblotting using an α-CagA antibody as control. (D) Ctk secretion assay in BHI medium with 10% FCS. The above *H. pylori* wild-type strains were grown for 12 h at 37 °C. Bacterial supernatants were filtered and prepared as described in the Materials and Methods section. The presence of secreted Ctk proteins in the supernatant was investigated by immunoblotting using the α-Ctk antibody and as control with α-CagA antibody. (OD: optical density; KDa: molecular weight in kilodalton).

Production of anti-JHP0940 polyclonal antibody in rabbit

A New Zealand white rabbit was given prime dose of 500 μg of JHP0940 intramuscularly after acclimatization. First intra-dermal booster of JHP0940 (300 μg) was given 21 days after the prime dose. Second intramuscular booster dose of the protein (300 μg) was followed by third intramuscular dose of JHP0940 (300 μg) 14 days after the second dose. Titre was checked 14 days after each booster dose. Serum was obtained by removing 20 ml blood from marginal ear vein of the rabbit. Serum was used in Western blot experiments to detect JHP0940.

Humoural response

Twenty human sera samples from each group of *H. pylori* infected patients and healthy subjects were obtained from the sera collection of Prof. Francis Megraud, INSERM-U853, Bordeaux (France) and used for performing ELISA. JHP0940 protein was coated in bicarbonate coating buffer@ 0.5 μg/well in 96 well plates followed by overnight incubation at 4 °C; unbound surface was blocked with 1% BSA for 2 h at 37 °C. 1:50 dilution of sera (primary antibody) were used and incubated for 2 h at 37 °C. Anti-human HRP secondary antibody was used in 1:1500 dilution followed by 1 h incubation at 37 °C. At each step, washing was done 3 times with PBST. This was followed by development of water soluble coloured reaction product with o-phenylenediamine dihydrocloride (OPD) substrate. The reaction was stopped by 3N H_2SO_4 added @ 100 μl/well and the plate was read by ELISA plate reader at 492 nm. *P* value was calculated by two tailed Mann Whitney *U* test between *H. pylori* infected and non-infected groups considering replicates of three independent experiments in each case.

ELISA for cytokine quantification

Murine macrophage RAW264.7 cells were cultured in the absence or presence of the endotoxin free JHP0940 protein for 24 h in a humidified atmosphere with 5% CO_2 at 37 °C. Culture supernatants were harvested for the estimation of various cytokines (TNF-α, IL-1β and IL-6) by two-site sandwich ELISA as described earlier (Khan et al., 2006; Kumar et al., 2013). In brief, the 96-well polyvinyl chloride microtitre plates were coated with purified anti-TNF- or anti-IL-6 or anti- IL-1β antibody at 1:250 dilutions in coating buffer (0.1 M carbonate buffer, pH 9.5) and were incubated for overnight at 4 °C. The plates were washed with wash buffer (PBS with 0.05% Tween-20) and blocked with 1% BSA in PBS followed by incubation with the test samples for overnight at 4 °C. After

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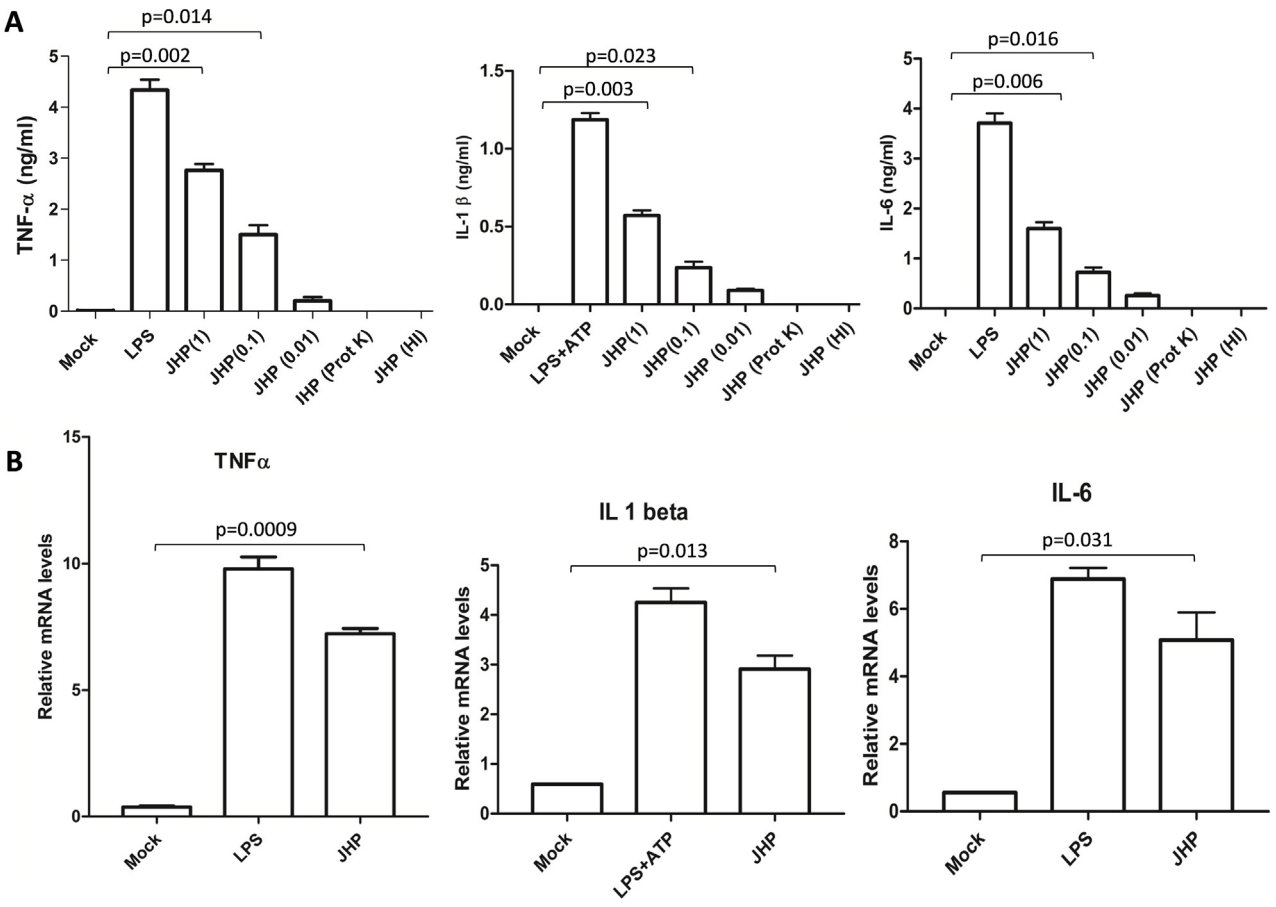

Fig. 2. (A) Treatment with JHP0940 protein in varying concentrations (0.01, 0.1 and 1 μg/ml) induced secretion of pro-inflammatory cytokines in mouse macrophage cell line (RAW264.7): TNF-α and IL-6 were measured at 24 h post treatment. For IL-1β analysis, the cells were treated (primed) with either JHP0940 (CtkA) or LPS (as positive control) for 3 h followed by 20 min of incubation with 5 mM ATP to activate the release of mature IL-1β. The cells treated with LPS (0.1 μg/ml), heat inactivated JHP0940 (100 °C for 10 min) and Proteinase K were taken as controls. The data represent means ± S.E. ($n = 3$) and P value for TNF-α, IL-6 and IL-1β was calculated separately by comparing with corresponding mock control. (B) Real time analysis of relative expression of TNF- α, IL-6 and IL-1β after 6 h of JHP0940 protein treatment (1 μg/ml) of RAW264.7 cells indicated increased relative expression of above cytokines when compared to mock. β-2 microglobulin was used as endogenous control for normalization. The data represent mean ± S.E. of duplicates ($n = 3$). P value for TNF-α, IL-6 and IL-1β was calculated separately by comparing with corresponding mock control. $P < 0.05$ was considered as significant.

washing, plates were incubated with biotin conjugated anti-TNF- or anti-IL-6 or anti-IL-1β antibody followed by incubation with streptavidin coupled to HRP. The HRP activity was detected using a chromogenic substance *o*-phenylenediamine tetrahydrochloride (Sigma) in citrate-phosphate buffer (pH 5.4) and H_2O_2. The reaction was terminated using 1 N H_2SO_4, and the absorbance values was measured at 492 nm. Standard curve for the cytokine was obtained using the recombinant standard protein provided by the manufacturer.

Real time PCR

RAW264.7 cells were cultured in the absence or presence of the JHP0940 protein in a humidified atmosphere with 5% CO_2 at 37 °C for 6 h. Following incubation, cells were harvested. For RT-PCR of cytokines, mRNA was extracted from the stimulated cells by RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed with SuperScript III reverse transcriptase (Invitrogen) according to manufacturer's recommendation. Real-time PCR was performed on a MyiQ Icycler, BioRad), using SYBR Green SuperMix. Quantification of various cytokine levels were performed by the Ct method and normalized by β-2 microglobulin level.

Cell viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out for checking cell viability of mouse macrophage RAW cells in 96 well culture plate using different concentration of JHP0940 protein. The cells were seeded at a density of 5×10^3 cells per well in 200 μl of complete medium followed by protein treatment for 24 h. 20 μl of 5 mg/ml MTT per well was added followed by 3 h incubation in dark or until the colour of the formazen product developed. Culture medium was removed and 50 μl/well DMSO was added followed by 5 min incubation in dark. The plate was measured at 570 nm.

Ladder assay

Ladder assay was performed according to method described by Gong et al. (1994) with minor modifications. Approximately one million RAW 264.7 cells were treated with JHP0940 protein (10 μg/ml) for 48 h. The cells were trypsinized and suspended in HBSS (Hank's buffer salt saline) solution and prefixed in ice cold 70% ethanol, stored in deep freezer for 24 h before the assay. After fixing, the cells were centrifuged at $800 \times g$ for 5 min to remove ethanol and DNA was extracted with 0.2 M phosphate-citrate buffer

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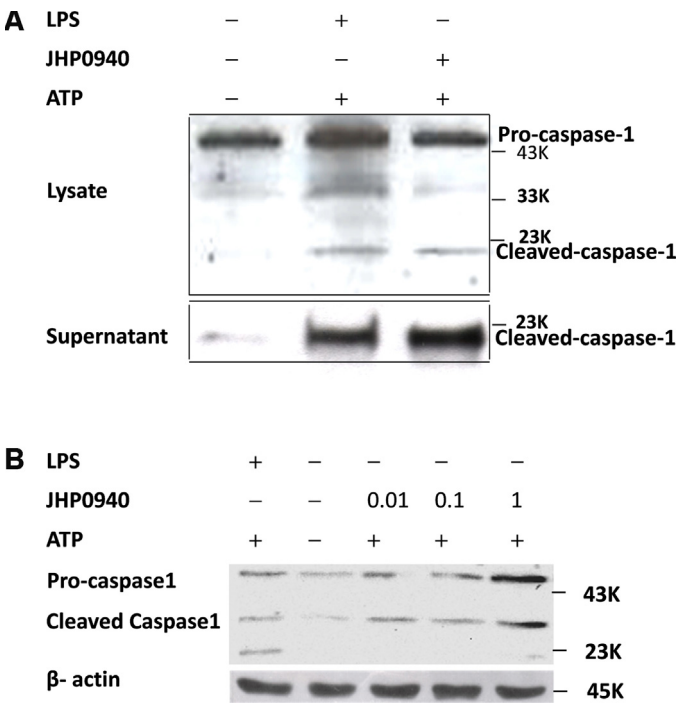

Fig. 3. Induction of caspase-1 in mouse macrophage RAW264.7 cell line on treatment with JHP0940: (A) Activation of caspase 1 was detected in whole cell lysate and in culture supernatants after priming either with JHP0940 (1 μg/ml) or LPS (as positive control) for 3 h followed by activation with 5 mM ATP for 20 min. (B) Dose dependent activation of caspase-1 in the cells pre-treated with JHP0940 and LPS for 3 h followed by 5 μM ATP treatment for 20 min. JHP0940 was used in varied doses of 0.01, 0.1 and 1 μg/ml for 3 h. LPS (positive control) was used at 100 ng/ml concentration. (K: molecular weight in kilodaltons).

(PCB) at pH 7.8 by suspending the pellet in 40 μl of PCB and incubating at room temperature for 30 min. Under these conditions, the partially degraded, oligonucleosomal DNA could be extracted selectively from the cells whereas the higher molecular weight DNA stayed associated with the nuclei. The extract (supernatant) was centrifuged at 1000 × *g* for 5 min and were treated with 1% Nonide NP40 (in water), 3 μl RNase A (5 mg/ml in water) and incubated at 37 °C for 30 min. 6 μl Proteinase K was added to it and incubated at 37 °C for 30 min. DNA loading dye was added to these samples and loaded to the 1.2% agarose gel with 0.5% EtBr. The gel was run at 4 V/cm and the bands were visualized.

Confocal microscopy

Confocal microscopy was used for visualization of DAPI stained nucleus of apoptotic cells. RAW264.7 cells were seeded at a density of 0.1×10^6/well on autoclaved coverslips in 24 well culture plate followed by protein treatment for 48 h. The cells were fixed in 4% paraformaldehyde and incubated for 15 min. After 3 washes with PBS the cells were permeabilized with pre-chilled 0.2% Triton-X followed by 15 min incubation at room temperature. DAPI with anti-fade reagent was added and the cells were mounted on slide followed by confocal microscopy for observing degraded nucleus.

For TUNEL assay, 0.05×10^6 cells/well were seeded followed by protein treatment for 48 h. The cells were fixed and permeabilized as mentioned above. After washing, the cells were equilibrated for 10 min at room temperature with equilibration buffer provided with ApoAlert DNA Fragmentation Assay Kit. (Clontech) This was followed by tailing reaction in TdT incubation buffer according to the manufacturer's protocol. Tailing reaction was terminated followed by washing with PBS. Cells were stained using DAPI with anti-fade reagent followed by observation under confocal microscope.

Flow cytometry (expression of fas receptors on RAW264.7 cells)

To determine expression of Fas receptors on RAW264.7 cells, cells were seeded followed by treatment with JHP0940 for different time intervals and in different doses. Cells were harvested by trypsinization very carefully to avoid loss of receptors, washed in PBS and suspended in wash buffer (PBS + 0.5% BSA) followed by incubation with FITC-conjugated anti-mouse CD-95 monoclonal antibody for 30 min at 4 °C. For isotype control, cells incubated with FITC-conjugated mouseIgG1 were used. The incubation was followed by washing of the cells with the wash buffer. The cells expressing Fas receptor were detected by flow cytometry analysis on BD FACS Canto II (BD Biosciences) and analysed by FlowJo software (FlowJo).

Western blotting

Equivalent amount of proteins were separated on SDS-PAGE and transferred electrophoretically to PVDF membranes. The blots were blocked with 4% BSA followed by overnight incubation with primary antibodies such as anti-CagA (Conradi et al., 2012) and various commercial antibodies (NEB and Santa Cruz) at 4 °C in TBST (0.1% v/v) and 2% (w/v) BSA. The blots were washed and incubated with secondary HRP antibody in 2% (w/v) BSA for 1 h at room temperature. After washing with TBST the blots were developed with enhanced chemiluminescence regent and exposed to X-ray film.

In vitro tyrosine kinase assay

Kinase reactions were carried out in 25 μl of volume consisting of 5 μl 2× Assay buffer (2 mM DTT, 2 mM $MnCl_2$, Na_3VO_4), 5 μl of 250 μM ATP solution, 5 μl of Src substrate (1 mg/ml), a synthetic peptide with sequence-KVEKIGEGTYGVVYK and 1 μl Src or c-Abl enzymes (NEB) at 30 °C for 15 min as described (Lind et al., 2014). Both Src substrate and Src enzyme were replaced by JHP0940 in the test reactions for showing auto phosphorylation activity of the JHP0940. Then 25 μl ADP-Glo reagent was added to deplete unused ATP, plate was shaken and allolwed 40 min incubation at room temperature 50 μl kinase detection reagent was added and incubated for 30 min at room temperature followed by reading in luminometer (Promega).

Statistical analysis

Two tailed Mann–Whitney *U* test was performed for the humoural response in *H. pylori* infected patient v/s healthy control cohorts and *P* values were calculated at 95 per cent confidence level. Student's *t*-test was performed for the analysis of results wherever required. The data were expressed as the mean of triplicates ± SEM unless or otherwise mentioned. $P < 0.05$ was considered as significant.

Results

JHP0940 is likely presented to the immune system in H. pylori infected patients

Earlier observations of our group showed that JHP0940 is associated with gastric disease in patients from different regions of the world and that enhanced activation of NF-κB occurs in cultured macrophages (Rizwan et al., 2008). This prompted us to investigate if the protein is expressed by *H. pylori* in gastric

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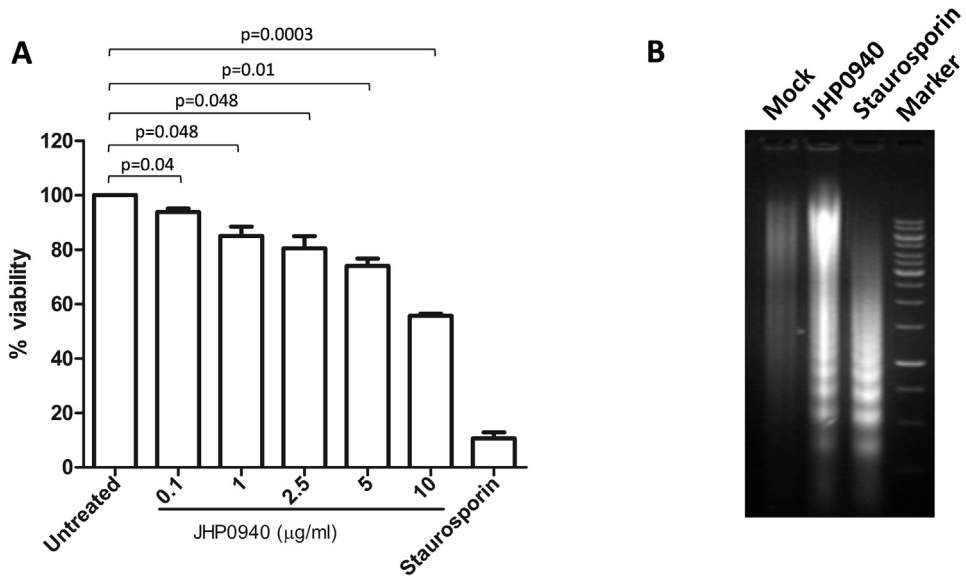

Fig. 4. (A) MTT assay of RAW264.7 cells after 24 h of treatment with different concentration of JHP0940 indicated dose dependent decrease in viability. The data are represented as means ± S.E. ($n = 3$). P value for different treatment experiments was calculated by comparing with untreated control. $P < 0.05$ was considered as significant and $P < 0.01$ as highly significant. (B) Ladder assay: The fragmentation of DNA of RAW264.7 cells on treatment with JHP0940 (10 μg/ml) was found similar to RAW264.7 cells treated with staurosporin as positive control. In mock (untreated RAW264.7 cells), DNA fragmentation was not observed. (Marker: 1Kb DNA ladder).

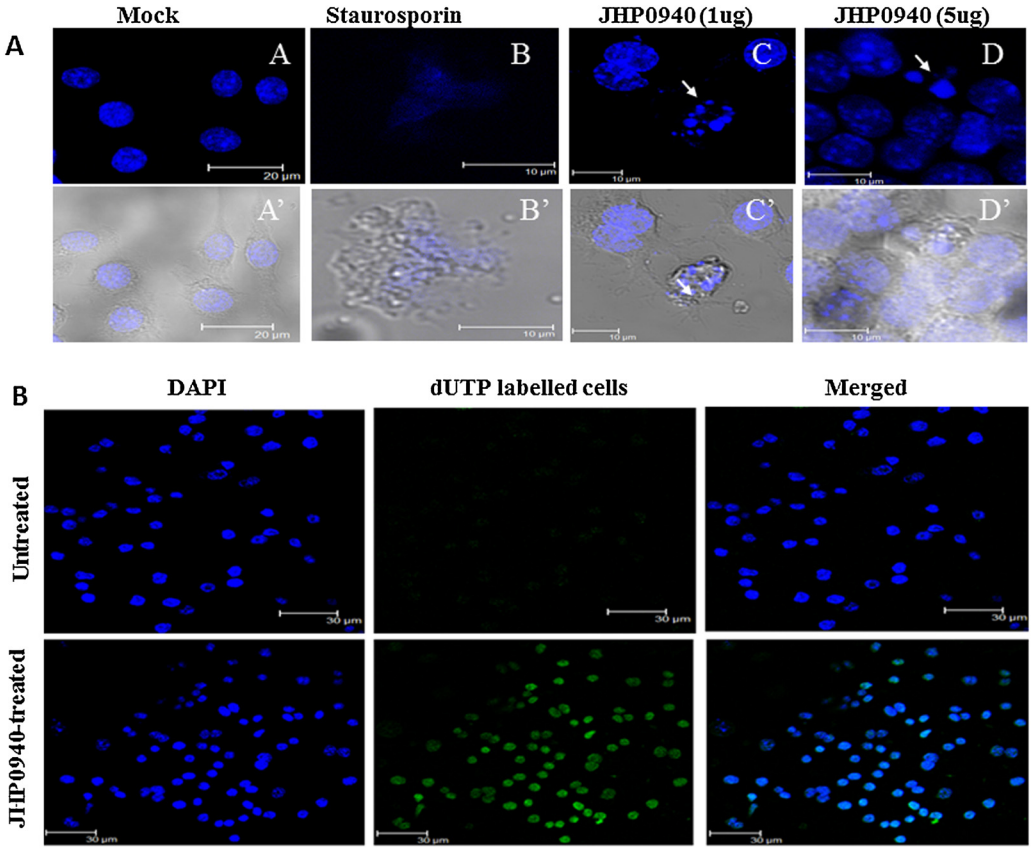

Fig. 5. (A) Confocal microscopy images indicating apoptosis of RAW264.7 cells on treatment with JHP0940 protein, after 48 h. Arrows in the images show apoptotic bodies. The RAW264.7 cells upon treatment with 1 μg/ml and 5 μg/ml of JHP0940 protein resulted in apoptosis as compared to the cells treated with staurosporin (0.1 μg/ml) used as positive control. No apoptosis was observed in the mock experiment (untreated cells). (B) TUNEL assay showing apoptosis in RAW264.7 cells upon treatment with JHP0940 protein after 48 h. Fluorescence-labelled dUTP in the fragmented DNA of cells undergoing apoptosis was observed in cells treated with JHP0940 but such fluorescence was not observed in mock (untreated cells). The figures shown are representative of three independent experiments.

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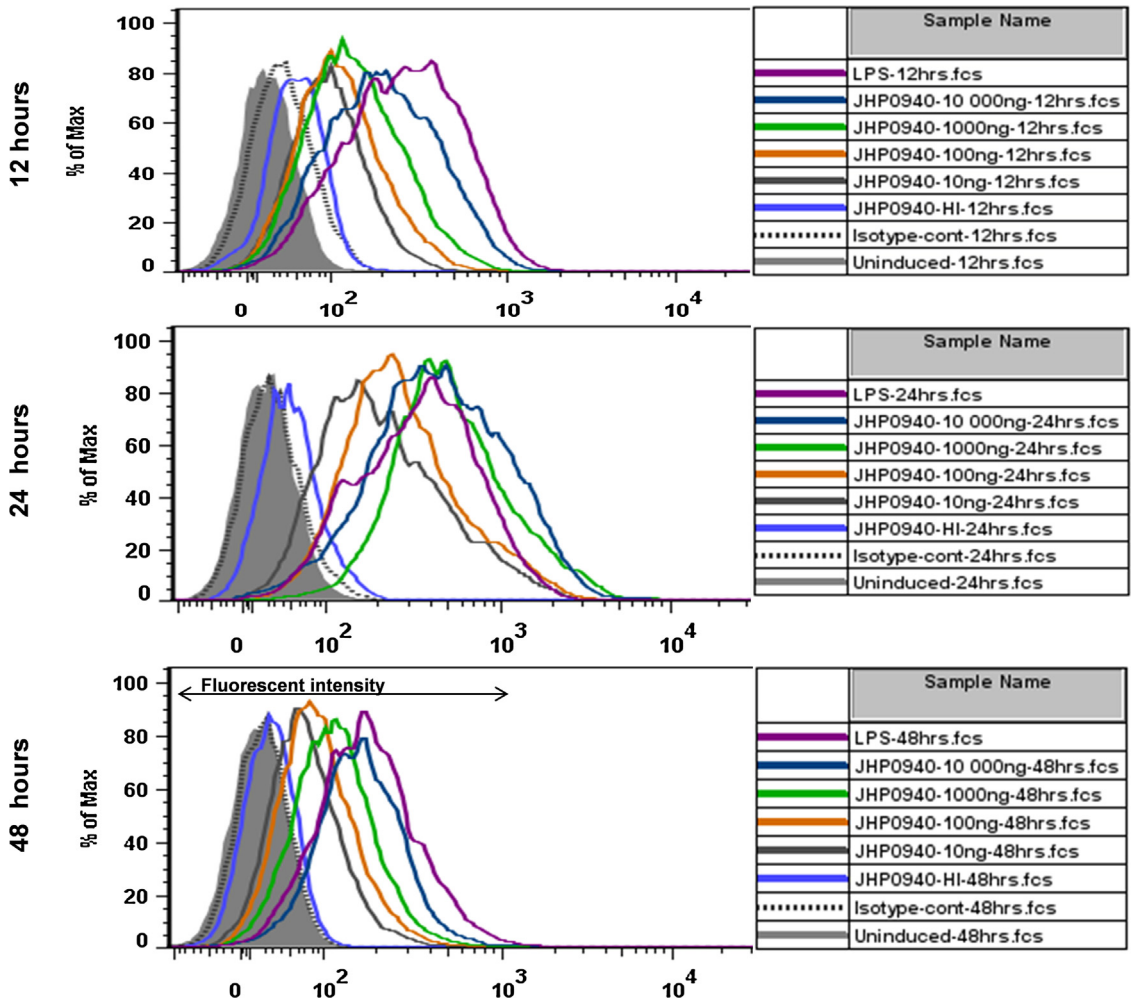

Fig. 6. Flow cytometry analysis showing expression of Fas receptors on RAW264.7 cells treated with JHP0940 in a dose dependent manner at 12, 24 and 48 h. The shift in the histogram peaks was observed in accordance with increase in concentration of JHP0940. RAW264.7 cells treated with LPS also indicated shift in histogram peak as compared to untreated. The shift in histogram peaks indicates increased expression of Fas receptors with respect to untreated.

environment and/or presented to the immune system. Since *H. pylori* colonizes the gastric epithelium, the bacterium should express some of its proteins/factors for gaining persistence while modulating the immune responses of the host. In this context, expression and exposure of factors to the immune system becomes important. To test this idea, we expressed and purified JHP0940 to homogeneity (Fig. 1A) and performed ELISA experiments with immobilized JHP0940 protein and human serum samples of *H. pylori* infected patients as compared to sera from non-infected healthy individuals. A significant difference in *P* value (0.0004) between *H. pylori* infected patients *versus* healthy group was found (Fig. 1B). This suggests that the protein expresses and presented to the immune system leading to the presence of anti-JHP0940 antibodies in the host.

JHP0940 is expressed in a subset of H. pylori strains and is secreted into the culture supernatant

Before its being presented to the immune system, we aimed to investigate if the Ctk protein is secreted into the extracellular environment. We tested this idea by fractionation studies of various wild-type *H. pylori* isolates including *ctk* gene-positive (J99 and SJM180) and *ctk*-negative (P1) strains, which were incubated for 12 h in liquid broth. Western blotting experiments showed that Ctk protein is expressed in strains J99 and SJM180, except P1 (Fig. 1C or D). In addition, bands for Ctk were also found in the culture supernatant fraction. As a control, the type IV secretion effector protein CagA, used as loading control for the *H. pylori* cell pellets (Fig. 1C) was not found in the supernatant as expected (Fig. 1D), thereby excluding artificial lysis of bacteria during experiments. Given this, our results hint at the secretory nature of the protein, allowing us to study how JHP0940 might affect signal transduction events during interaction with macrophages.

JHP0940 induces pro-inflammatory cytokines in RAW264.7 macrophage cells

RAW264.7 cells were grown and treated with recombinant JHP0940 protein at a concentration of 10 ng to 1000 ng/ml for 24 h followed by ELISA-based quantitation of cytokines in the culture supernatant. We found that the cytokines TNF-α, IL-1β and IL-6 were upregulated in a dose dependent manner (Fig. 2A). Furthermore, to confirm the pro-inflammatory properties of JHP0940, qRT-PCR was performed for mRNA profiling of the above cytokines (Fig. 2B). Our results from qRT-PCR corroborated with the ELISA results confirming the upregulation of the above cytokines (Fig. 2 B). *H. pylori* infection is recognized by the presence of chronic gastric inflammation and there is increasing evidence that caspase-1 is involved in the activation of the two important pro-inflammatory cytokines (IL-1β and IL 18), which normally mediates bacterial

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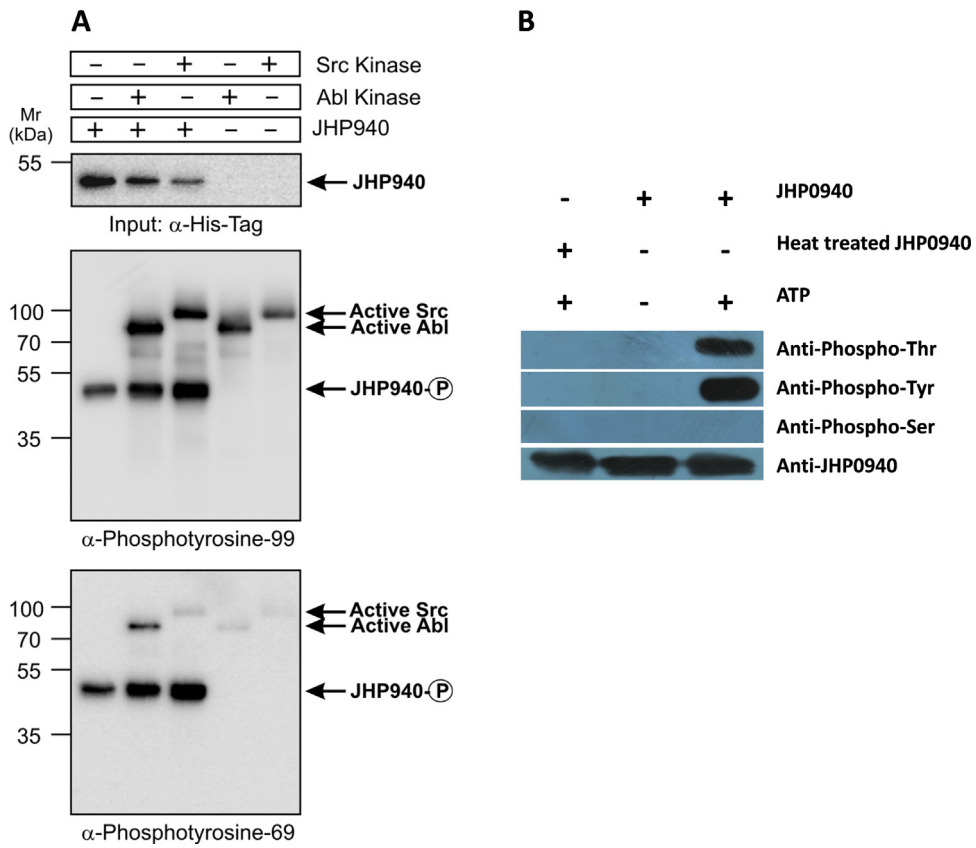

Fig. 7. (A) Western blot showing auto-tyrosine phosphorylation of JHP0940. Lane 1, from left represents kinase reaction with only JHP0940, lane 2 and 3 represent kinase reactions using JHP0940 along with Abl and Src kinase respectively. Lane 4 and 5 represent *in vitro* kinase reactions only with Abl and Src kinase respectively. Phosphorylation at tyrosine residues was probed using α-phosphotyrosine-99 and α-phosphotyrosine-69 antibodies. JHP0940 was probed with anti-His-tag antibody. (B). JHP0940 acts as auto phosphorylating kinase. *In vitro* kinase reactions were performed and phosphorylation at tyrosine and threonine residues was determined by respective anti-tyrosine and anti-threonine antibodies.

clearance after infection (Hitzler et al., 2012; van de Veerdonk et al., 2011). In this regard, we investigated the involvement of JHP0940 in caspase-1 induction. We found that caspase-1 expression was induced upon treatment of RAW264.7 cells with recombinant JHP0940. We observed that JHP0940 induces caspase-1 processing and activation in a dose dependent manner (Fig. 3B). We also observed that active caspase-1 is secreted into the cell culture supernatant (Fig. 3A). This observation partly corroborates with the above observation related to IL-1β secretion by murine macrophage cells that were treated with JHP040. Given these findings, we espouse the hypothesis that JHP0940 could most likely contribute to *H. pylori* pathogenesis by producing chronic gastric inflammatory responses and consequently ensuring *H. pylori* colonization. In our experiments, every time before treating the cells, JHP0940 protein was incubated with polymyxin B for 4 h to remove putative endotoxin contamination and to rule out the effect of bacterial endotoxin. Furthermore, we used proteinase K treated JHP0940 as control to ensure that the effect was not due to bacterial endotoxins.

JHP0940 decreases viability of RAW264.7 cells by inducing apoptosis through Fas receptors

Persistence of *H. pylori* in the gastric environment is the hallmark of this pathogen, not allowing the immune system to clear infection. The role of putative bacterial proteins/factors in immune evasion and related processes, however, is not yet fully clear. We therefore hypothesized that JHP0940 could induce caspase-1 activation to trigger macrophage apoptosis, thus facilitating enhanced bacterial survival. We therefore investigated the viability of RAW264.7 cells in response to exposure with JHP0940 protein. We observed that the protein induced cell death in these cells in a dose dependent manner leaving 56% of viable cells at 24 h upon treatment with 10 μg/ml of JHP0940. The cell viability was accessed by MTT assay for 24 h in a dose dependent manner (Fig. 4A).

Cell viability assays performed with RAW264.7 cells intrigued us to identify the cause of cell death and therefore, we performed the ladder assay. We observed that JHP0940 induced DNA fragmentation similar to the staurosporin control, explaining its role in apoptosis (Fig. 4B). To confirm these observations, we investigated the nuclear morphology of RAW264.7 cells by staining with DAPI using confocal microscopy. Our results revealed that JHP0940 induced degradation of chromosomal DNA in RAW264.7 cells (Fig. 5A). Furthermore, these observations were corroborated by TUNEL assay results (Fig. 5B). TUNEL assay revealed that most of the cells undergo apoptosis. These results were further confirmed by expression of Fas receptor on the surface of JHP0940-treated RAW264.7 cells in a time- and dose-dependent manner (Fig. 6). The expression of Fas receptors on murine macrophages intrigued us to test if JHP0940 acts as non-receptor tyrosine kinase. There are evidences where c-Abl non-receptor tyrosine kinases are involved in expression of Fas receptors (Gu et al., 2012).

JHP0940 acts as an auto-phosphorylating tyrosine kinase

In a previous study, it was shown that JHP0940 acts as cell-translocating ser/thr kinase (CtkA) and leads to phosphorylation

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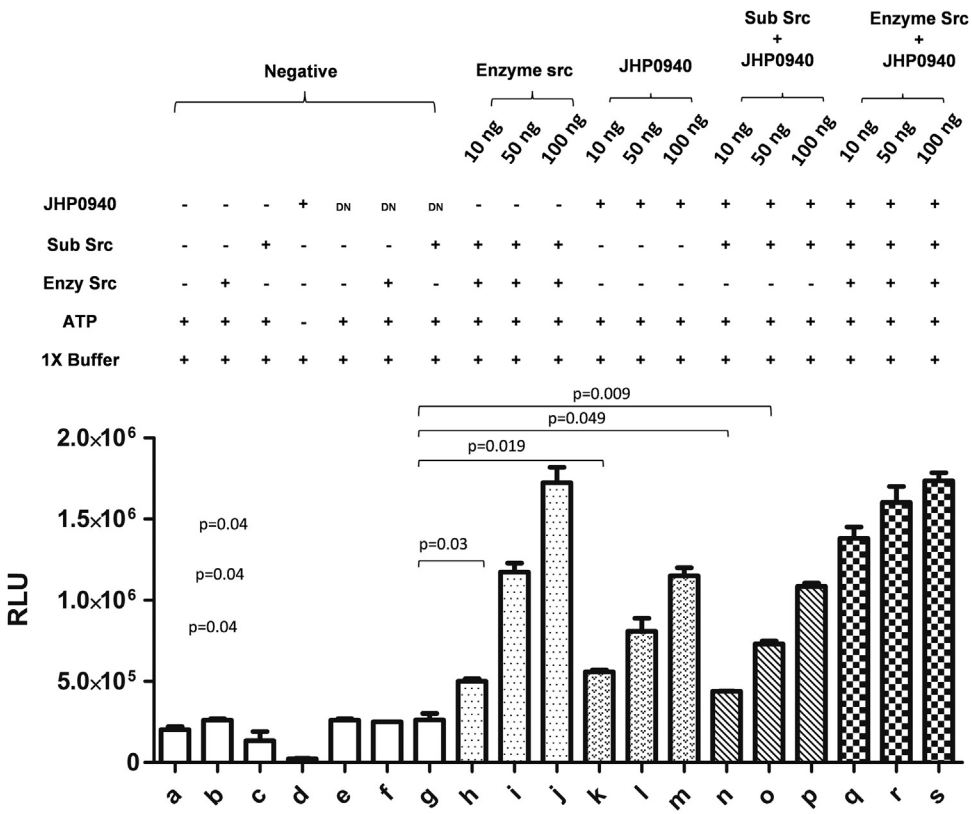

Fig. 8. *In vitro* tyrosine kinase assay. On X-axis, a to g are negative controls while *h*, *i* and *j* are reactions with increasing concentrations of Src enzyme (10, 50 and 100 ng) serving as positive controls. *k*, *l* and *m* are reactions with increasing concentrations of JHP0940 (10, 50 and 100 ng) only. *n*, *o* and *p* are reactions with increasing concentrations of JHP0940 (10, 50 and 100 ng) along with Src substrate (5 μg/reaction, the same concentration was maintained throughout the experiment). *q*, *r* and *s* are reactions with increasing concentrations of JHP0940 (10, 50 and 100 ng) along with Src enzyme (50 ng/reaction). *Y*-axis represents relative light units (RLU). The data are represented as mean ± S.E. of three independent experiments. DN represents denatured protein JHP0940. *P* values have been indicated in comparison with test and control samples.

at serine residue 276 residue of p65 subunit of NF-κB in cultured AGS gastric epithelial cells (Kim do et al., 2010). But, the association of the gene jhp0940 with gastric cancer (Santos et al., 2003) intrigued us to check if it can also act as a tyrosine kinase and hence we performed *in vitro* tyrosine kinase assay. In our results, JHP0940 was found to be an auto phosphorylating tyrosine kinase apart from its described serine/threonine kinase activity on western blot analysis (Fig. 7). In an *in vitro* tyrosine kinase assay, JHP0940 was used in enzyme range concentrations in the kinase reaction and when it was used along with the enzyme provided with the kit, it revealed cumulative enzymatic activity (Fig. 8).

Discussion

Putative virulence-associated genes of *H. pylori* have been categorized into three groups: strain specific genes, phase variable genes and genes with different structures and phenotypes (Yamaoka, 2008). The strain specific genes are further sub-classified into those encoded by two pathogenicity islands, the *cag*PAI, which encodes various virulence factors along with its T4SS and the effector protein CagA and plasticity region clusters (called plasticity zones) encoding nearly half of the strain-specific genes including another T4SS, called *tfs3* (Kersulyte et al., 2003; Fernandez-Gonzalez et al., 2014). These genes within the plasticity region are seemingly acquired extraneously and are of utmost interest because of their association with the gastric disease (Yamaoka, 2008). In previous studies, various genes of the plasticity region *i.e.*, *dupA*, *jhp*0940, *jhp0945*, *jhp0947* and *jhp0949* have been shown to be associated with various stages of gastric disease (Yamaoka, 2008). The first characterized member from this region, JHP0940 or CtkA, intrigued us to explore it in detail due to pre-existing observations such as its association with gastritis and gastric cancer in different geographical regions, high antigenicity index and *in vivo* expression in gastric mucosa of Mongolian gerbils (Graham et al., 2002). Furthermore, the mechanism of secretion of JHP0940 from *H. pylori* and its transfer to the gastric epithelial cells is not fully clear. It has also not been confirmed if JHP0940 uses T4SS-mediated injection mechanism like CagA or it harnesses some other secretory mechanism similar to other Gram-negative bacteria. However, our ELISA results portray it as a secretory protein (Fig. 1). The cell-translocating activity of the protein has been shown using GFP-fused full length recombinant protein in HeLa cells (Kim do et al., 2010). Regarding the pro-inflammatory nature of the protein, we do not rule out the involvement of kinase activity of JHP940 in IL-1β secretion since there are reports where death associated protein kinase, a type of serine/threonine kinases is required for IL-1β production (Chuang et al., 2011). But, at the same time, non-receptor tyrosine kinase members of the c-Abl family are also shown to involve in T-cell mediated inflammation (Gu et al., 2012).

It is well established that IL-1β plays a crucial role in inflammation and maintenance of infection (Dinarello, 2009). Subsequent to NF-κB activation, pro-IL-1β is synthesized in its pro-form, which is then cleaved by the action of caspase-1 to produce mature IL-1β. It is reported that the activation of caspase-1 is dependent on formation of multi-protein complexes, called inflammasome (Martinon et al., 2009; Dinarello, 2009; von Moltke et al., 2013). Recent studies identified the bacterial *cag* pathogenicity island and the cooperative interaction among host innate receptors TLR2, NOD2, and NLRP3

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as important regulators of IL-1β production in *H. pylori* infected murine dendritic cells (Kim et al., 2013). Here, our study by Western blot analysis of caspase-1 revealed its involvement in secretion of IL-1β (Fig. 3) and therefore, we propose a strong pro-inflammatory role for Ctk, with the involvement of inflammasome components, at least in murine macrophage cells.

In order to examine the effects of recombinant JHP940 on mouse macrophage cells in more detail, we observed decreased viability of host cells by MTT assay. This was followed by the visualization of apoptotic bodies in the DAPI-stained nucleus. Furthermore, the TUNEL assay confirmed occurrence of apoptosis in response to treatment of the cells with JHP940 protein. In a previous *in vitro* study, translocation of GFP-tagged JHP940 into HeLa cells was documented, indicating its cell-translocating behaviour (Kim do et al., 2010). The authors also found that transiently expressed protein translocates into the nucleus in a time dependent manner in AGS cells, although the protein doesn't contain a nuclear localization signal (NLS). These observations together with our findings based on an *in vitro* non receptor tyrosine kinase assay and its apoptotic effect on mouse macrophage cell line through Fas expression (death receptor) revealed that the activity of JHP0940 mimics the function of a non-receptor tyrosine kinase such as c-Abl. Upon caspase activation, c-Abl retained tyrosine kinase activity and triggered Fas induced apoptosis as previously shown (Barila et al., 2003). The Fas receptor activated by specific ligands, triggers assembly of a complex called as Death Inducing Signalling Complex (DISC) leading to the cleavage and activation of more downstream executive caspases and thus apoptosis (Raina et al., 2005). Moreover, epithelial and endothelial non-receptor tyrosine kinases (Etk) mediate apoptosis in small cell lung cancer cells (Cheng et al., 2010). We also found enhanced expression of Fas receptors in the mouse macrophage cell line in a time and dose dependent manner (Fig. 6).

Somehow, JHP940 does not share any sequence homology with c-Abl tyrosine kinases in any of the databases but it is predicted as cytoplasmic protein (score of 8.96 out of 10) by PSORTb v3.0.2 prediction tool (Gardy et al., 2003). The protein was found to have one SH2 domain when searched for domains in Scansite Motif Scanner database (http://scansite.mit.edu/motifscan_seq.phtml). Also, it revealed homology with one of the SH2 domains of CrkII. This SH2 domain of CrkII serves as binding site for SH3 domain of c-Abl mediated by Crk phosphopeptide and thus regulates biological functions (Donaldson et al., 2002). Despite homology with SH2 domain of CrkII, there is no experimental evidence to show that JHP0940 acts as an adaptor molecule like CrkII.

Other encoded protein from the plasticity region of *H. pylori* strain 26695, HP0986 (TieA), showed the same pro-inflammatory and pro-apoptotic behaviour but tested negative for kinase activity. However, HP0986 interacted with TNFR1, also having an endonuclease activity hinting at the potential pathobiological role of proteins/factors encoded by plasticity region genes (Alvi et al., 2011; Devi et al., 2014).

It will not be surprising that JHP0940, acting as an auto-phosphorylating tyrosine kinase and by translocating into the nucleus activates transcription factor(s) apart from NF-κB to modulate signalling in the host cells. It hints that JHP0940 may be a secretory protein, though the secretory mechanism of the protein is not established, which bacteria could use in modifying the signalling responses of the host cells. Thus, on the basis of our above observations and results, we assume that on one hand JHP0940 helps *H. pylori* to orchestrate inflammation as a survival mechanism and on the other hand it averts immune recognition by limiting macrophages through Fas mediated apoptosis. Further, it will be pertinent to investigate if JHP0940 acts as dual specific kinase or it acts as serine/threonine or tyrosine kinase in certain specific conditions and or cellular environment to help *H. pylori* in progression of gastric disease.

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Helicobacter pylori Antigen HP0986 (TieA) Interacts with Cultured Gastric Epithelial Cells and Induces IL8 Secretion *via* NF-κB Mediated Pathway

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Abstract

Background: The envisaged roles and partly understood functional properties of *Helicobacter pylori* protein HP0986 are significant in the context of pro-inflammatory and or proapoptotic activities, the two important facilitators of pathogen survival and persistence. In addition, sequence analysis of this gene predicts a restriction endonuclease function which remained unknown thus far. To evaluate the role of HP0986 in gastric inflammation, we studied its expression profile using a large number of clinical isolates but a limited number of biopsies and patient sera. Also, we studied antigenic role of HP0986 in altering cytokine responses of human gastric epithelial (AGS) cells including its interaction with and localization within the AGS cells.

Materials and Methods: For *in vitro* expression study of HP0986, 110 *H. pylori* clinical isolates were cultured from patients with functional dyspepsia. For expression analysis by qRT PCR of HP0986, 10 gastric biopsy specimens were studied. HP0986 was also used to detect antibodies in patient sera. AGS cells were incubated with recombinant HP0986 to determine cytokine response and NF-κB activation. Transient transfection with HP0986 cloned in pEGFPN1 was used to study its subcellular localization or homing in AGS cells.

Results: Out of 110 cultured *H. pylori* strains, 34 (31%) were positive for HP0986 and this observation was correlated with *in vitro* expression profiles. HP0986 mRNA was detected in 7 of the 10 biopsy specimens. Further, HP0986 induced IL-8 secretion in gastric epithelial cells in a dose and time-dependent manner *via* NF-κB pathway. Serum antibodies against HP0986 were positively associated with *H. pylori* positive patients. Transient transfection of AGS cells revealed both cytoplasmic and nuclear localization of HP0986.

Conclusion: HP0986 was moderately prevalent in clinical isolates and its expression profile in cultures and gastric biopsies points to its being naturally expressed. Collective observations including the induction of IL-8 *via* TNFR1 and NF-κB, subcellular localization, and seropositivity data point to a significant role of HP0986 in gastroduodenal inflammation. We propose to name the HP0986 gene/protein as 'TNFR1 interacting endonuclease A (TieA or *tie*A)'.

Helicobacter pylori infection is characterized by the infiltration of mononuclear and polymorphonuclear cells into the gastric mucosa in addition to the accumulation of various cytokines, including IL1β, IL6, IL-8, and TNFα secreted by gastric epithelial and immune cells [1, 2]. Among these cytokines, IL-8 is a potent pro-inflammatory mediator secreted mainly by the gastric epithelial cells and plays an important role in the recruitment and activation of neutrophils [3]. The clinical phenotype of *H. pylori* infection depends on several determinants that

include virulence factors, the host susceptibility to infection and other environmental co-factors [4–7]. It was previously believed that CagPAI was the chief virulence factor that strongly associates with severe gastric inflammation while encoding proteins that induce IL-8 secretion by epithelial cells [8]. However, subsequent studies could not identify a strong association between IL-8 induction and the presence or the functionality of the cagPAI; they found that several other genes unrelated to CagA status, such as *oip*A, flagellar proteins, heat shock proteins, and several other *H. pylori* products could also induce IL-8 secretion [9,10]. Chronic active inflammatory response is the hallmark of *H. pylori* infection and the main underlying molecule seems to be IL-8 [11]. Many putative virulence genes have been described to determine the clinical outcome; among these are the genes present in the plasticity region cluster (that is located outside the CagPAI) that correspond to nearly half of the strain-specific genes [12]. Plasticity region is recently being considered to be a novel transposable element and the genes present in this cluster possibly affect bacterial fitness and phenotypes [13]. Similar to CagPAI, plasticity region displays some characteristics of a genomic island such as its large size and a different percentage of G+C content than in the rest of the bacterial genome [14].

Most of the genes in the plasticity region of *H. pylori* are functionally unknown although they may epidemiologically associate with the strains from different disease conditions in certain human populations [15,16]. Previous studies have shown that there are regional and ethnic differences in the distribution of *H. pylori* subtypes with respect to strain variable genes; this in turn suggests that, in a given geographic area, the *H. pylori* genotypes may play a significant role in infection or progression of infection [17,18]. Moreover, plasticity region encoded proteins such as JHP0940 and HP0986 have already been reported to stimulate pro-inflammatory cytokines and activate NFκB mediated pathway in cultured mammalian cells [19–21]. In this regard, it is prudent to functionally characterize these genes/proteins with respect to their putative roles in persistence and virulence of *H. pylori*.

Our group previously reported that HP0986 was a pro-inflammatory protein that upregulates tumor necrosis factor alpha (TNF-α) and triggered IL-8 secretion and at the same time induced apoptosis through Fas-mediated pathway [21]. Although this pioneering study focused on the function of HP0986 outside the bacterial environment (as it was based on the effects of recombinant HP0986 on cultured human macrophages and peripheral blood mononuclear cells), the interaction of HP0986 with human gastric epithelial cells was not analyzed. Further, the study [21] did not explore intrinsic role of this protein (such as the predicted restriction endonuclease activity). Given this, there is a clear need to dissect the functional capacity of HP0986 in different cellular environments. We therefore, sought to extend this study to another cell type to ascertain the role of HP0986 in altering the cytokine responses by human epithelial cells (AGS cell line) and to understand the underlying mechanism. We also explored if HP0986 is presented to humoral immune system. This study also analyzed the prevalence as well as expression of HP0986 in clinical isolates and gastric biopsies obtained from an ethnically complex setting such as Malaysia. We also describe the localization of HP0986 in human gastric epithelial cells and discuss its potential to undergo a possible cytoplasmic-nuclear shuttling.

Materials and Methods

Ethics Statement

The present study was approved by the Ethics committee of the University of Malaya Hospital, Kuala Lumpur, Malaysia. Written informed consents were obtained from the patients as per the University protocol.

Patient Selection: (For *in vitro* Expression Study)

We screened more than 500 patients in the present study who underwent gastric endoscopy at the University of Malaya Hospital, Kaula Lumpur, Malaysia, during 2012–2013. In total, 110 adult patients were selected in this study, and these were the patients of functional dyspepsia ($n = 102$) (93%) and peptic ulcer disease ($n = 8$) (7%), determined on the basis of 2 inclusion criteria: those who had no history of *H. pylori* eradication therapy and those positive for rapid urease test**.** Functional dyspepsia was endoscopically and pathologically defined as *H. pylori* associated functional dyspepsia. Sixty out of 110 patients were from Indian ethnic group (mean age 48.5), 38 were of Chinese ancestry (mean age 59.7), and 12 were Malay (mean age 51.6). In all, 51% ($n = 56$) were males and 49% ($n = 54$) were females**.**

Patients and Gastric Biopsies (for *in vivo* Expression Study)

In total, 10 patients were selected in this study module; these patients underwent gastric endoscopy at the University of Malaya Hospital, Kaula Lumpur, Malaysia during 2013. All the 10 patients had functional

dyspepsia. Among these, 6 were from Chinese ethnic group (mean age 51.7) and 4 were of Indian ethnic group (mean age 59.7). Individual gastric biopsy specimens were placed in sterile vials after a positive diagnosis of *H. pylori* infection and were stored at −80°C. Out of these patients, four gastric biopsy specimens each were collected from antrum/body. One biopsy was immediately processed for bacterial culture, one for histologic examination, and two for total RNA extraction. Biopsy material was stored in formalin for histopathology and frozen in liquid nitrogen and stored at −80°C for total RNA extraction.

Helicobacter pylori Culture from Gastric Biopsies

Gastric biopsies (n = 110) were processed for *H. pylori* culture by homogenization of the tissue. Homogenates were inoculated on blood agar (Oxoid, Thermo Scientific) containing 7% horse blood and incubated at 37°C under 10% CO_2 for 5–7 days [22]. *H. pylori* growth was confirmed by microscopy and rapid urease test. Single translucent colonies that were positive for urease test were further subcultured for DNA and RNA isolation. PCR-based detection of HP0986 was performed on all *H. pylori* strains using gene-specific primers as described elsewhere [14].

Quantitative Real Time PCR assay

Helicobacter pylori cultures (n = 110) were pelleted and washed twice with 1X phosphate buffer saline and further pelleted by centrifugation at 4000 rpm for 5 minutes RNA was extracted from each pellet using Qiagen RNeasy Mini Kit as per the manufacturer's instructions and treated with DNase I (Qiagen, Hilden, Germany) on columns and further purified by RNA clean up. In a similar way, total RNA from each of the 10 frozen biopsies were also extracted. RNA samples were quantitated by Nanodrop spectrophotometer and stored at −80°C until further use. Expression analysis was carried out by IQ5 real-time PCR (BioRad Laboratories, Hercules, CA, USA). Briefly, the reaction was performed in 25 μL volume containing 12.5 μL of SYBR green (iScript^{T-M}One-Step RT-PCR kit, Qiagen), 1.25 μL of forward primer GAAAAGAGTTTA GAAAAGATACA, 1.25 μL of reverse primer CTTGAT GGTCTTTGTAAAACA, 0.25 μL of reverse transcriptase (Qiagen), 1 μL of RNA template, and 8.75 μL of Dnase/RNase free water. PCR conditions for both HP0986 and 16S RNA (control) were denaturation at 94°C for 15 minutes; 40 cycles of 94°C for 15 seconds; annealing at 45°C for 30 seconds, extension at 72°C for 30 seconds followed by 61 cycles of melting curve analysis at 65°C for 10 seconds. Reaction without RNA template was included as negative control for each primer tested and a control without reverse transcriptase was also included for each test. The analysis was performed in triplicates and IQ5 real-time PCR software (Biorad) was used to generate the quality control of the replicates, data extraction and initial analysis. Data were analyzed by ΔΔCT method as described earlier [23]. Relative expression level of HP0986 normalized to the 16SrRNA was checked in clinical isolates and in gastric biopsies when compared with the levels of HP0986 mRNA in strain P12. Also, HP0986 specific amplification was confirmed by a single amplicon on 1% agarose gel.

Cloning, Expression and Purification of HP0986 Using Prokaryotic and Eukaryotic Expression Vectors

The ORF/gene hp0986 was cloned in prokaryotic expression vector pRSETA and was purified to homogeneity as described earlier [21]. To clone hp0986 into eukaryotic expression vector, the gene was amplified from the genomic DNA of *H. pylori* strain 26,695 using the following primers: 5′CCCCTCGAGATGGTGGAACT TTTTTCTCTTTGCATGTC 3′ (xho I), 5′AATAAGCTTACG CCTAGAGTTATTAATATAATTCTCAATATTTT 3′ (Hind III). The amplified 714 bp product and TA cloning vector (Fermantas, Lafayette, CO, USA) were incubated together for an overnight ligation at 16°C. Insert was confirmed by double restriction digestion (Hind III, xho I) and was further subcloned into pEGFPN-1 (Clonetech) vector. The clone was again confirmed for the insert by double restriction digestion and sequencing.

Stimulation of AGS Cells with HP0986 and Estimation of IL-8

AGS cells were obtained from the National Center for Cell Sciences, (Pune, India). For all the experiments described below, the cells were grown in Ham's-F12 media (Sigma-Aldrich, St. Louis, USA) with 10% heat inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1X Antibiotic –Antimycotic (Invitrogen) and maintained at 37°C with 5% CO_2 in a humidified incubator.

AGS cells were seeded into a 6 -well plate at a density of 2×10^5 cells per well. They were maintained for 2 days until 70% confluent. To rule out the possibility of LPS contamination, HP0986 was incubated with Polymixin B-Agarose (Sigma-Aldrich, St. Louis, MO, USA) for 4 hours at 4°C. The cells were then treated with the following concentrations of HP0986 protein: 5 μg/mL, 10 μg/mL at different time intervals; LPS-treated AGS cells (5 μg/mL) were included as positive control. The

culture supernatants were then collected at 6, 12, 24, and 36 hours post-treatment so as to measure the levels of various cytokines such as IL6, IL-8, and TNF-α by BD CBA flex kit; acquisition of the data was carried out in the BD FACS Canto II flow cytometer (BD Biosciences, USA) using BD FACS diva software (BD Biosciences) and the analysis was performed by plotting the standard graphs for each cytokine using FCAP (BD Biosciences) array software.

Western Blot Analysis

The AGS cells were seeded and treated with HP0986 as described above. The cells were washed twice with 1X PBS for the preparation of nuclear and cytoplasmic extracts three hours after treatment with HP0986 and an equal aliquot of all the samples was loaded on 12% SDS-PAGE from respective extracts that were quantified by the MicroBCA protein assay kit (Thermo Scientific, Lafayette, CO, USA). The separated proteins were then transferred onto PVDF membrane, blocked in 5% BSA, and probed with rabbit anti phospho–p65 antibody (Santa Cruz, Dallas, TX, USA) overnight at 4°C followed by 1 hour incubation with peroxidase-conjugated goat anti-rabbit IgG (1 : 80000) (Sigma-Aldrich) to detect NF-κB. Further to detect IκBα, the blots were probed using rabbit polyclonal IκBα (Sigma-Aldrich) overnight at 4°C at a dilution of 1 : 1000 and probed with secondary antibody, anti-rabbit IgG (1 : 80,000, Sigma-Aldrich). Finally, membranes were washed thrice with 1X TBST and then developed using ECL plus chemiluminescence kit (Thermo Scientific). The membrane was blocked with 5% BSA in 1X PBST for 2 hours and then incubated in 1X TBST. It was then washed thrice with 1X TBST at room temperature and subjected to ECL plus chemiluminescence detection (Thermo Scientific).

Detection of Antibody Responses by ELISA

Antibody response against HP0986 was examined in a total of 40 human serum samples comprising of 20 *H. pylori* positive and 20 *H. pylori* negative sera which were obtained from different patients by indirect ELISA as described previously [21].

Transient Transfection of AGS Cells

AGS cells in Ham's F12 medium supplemented with fetal bovine serum were grown on 13 mm cover slips in 24-well plates until they reached 50–60% confluency. Spent medium was aspirated next day and cells were supplemented with Ham's F12 medium without fetal bovine serum and transfected with pEGFPN-1HP986 construct using Lipofectamine 2000 (Invitrogen) transfection reagent as per manufacturer's instructions. Briefly, AGS cells were treated with a mixture of lipofectamine and plasmid DNA in a ratio of 1 : 3 for 4 hours. Thereafter, the serum-free medium was aspirated and cells were grown in Ham's F12 medium with 10% fetal bovine serum for 24 hours. The coverslips were then washed thrice with 1X phosphate buffer saline and fixed in 4% paraformaldehyde and probed with anti-rabbit HP0986 antibody. This was followed by 1 hour incubation with peroxidase-conjugated goat anti-rabbit IgG. Slides were washed and mounted with Vectashield mounting medium containing DAPI (Invitrogen). Expression vector pEGFPN-1 without any insert was used as negative control.

Statistical Analysis

Two tailed student *t*-test was used to demonstrate the level of secretion of IL-8 in treated cells when compared with untreated cells. Further, Mann–Whitney's *U* test was carried out to compare antibody responses. All the data were expressed as mean ± SEM. *p* values of less than .05 were read as statistically significant.

Results

Genotyping and *in vitro* Expression of HP0986 in *H. pylori* Clinical Isolates

To investigate the *in vitro* expression of HP0986 in *H. pylori* clinical isolates and to confirm it as a virulence marker linked to disease outcome, we checked the epidemiologic consistency of HP0986 across the Malaysian patient population selected by us (see in methods). Firstly, we performed PCR-based detection of HP0986 using the genomic DNA isolated from all ($n = 110$) *H. pylori* isolates and with the help of gene specific primers as described earlier [14]. Among the 110 clinical isolates, HP0986 gene was found in 31% ($n = 34$) of the samples. To investigate if PCR-based detection of HP0986 also entails expression of HP0986, a quantitative real time PCR was performed on the above 34 isolates so as to analyze the *in vitro* expression of the gene. A single primer set, complementary to a highly conserved region, which specifically amplifies HP0986 was used to perform the *in vitro* expression analysis. This precluded the possibility of any primer mismatches. The mRNA expression of HP0986 was recorded as cycle threshold relative to the strain P12 of *H. pylori*. (Fig. 1). Our results revealed that the presence of HP0986 genotypes corroborated with the *in vitro* expression of

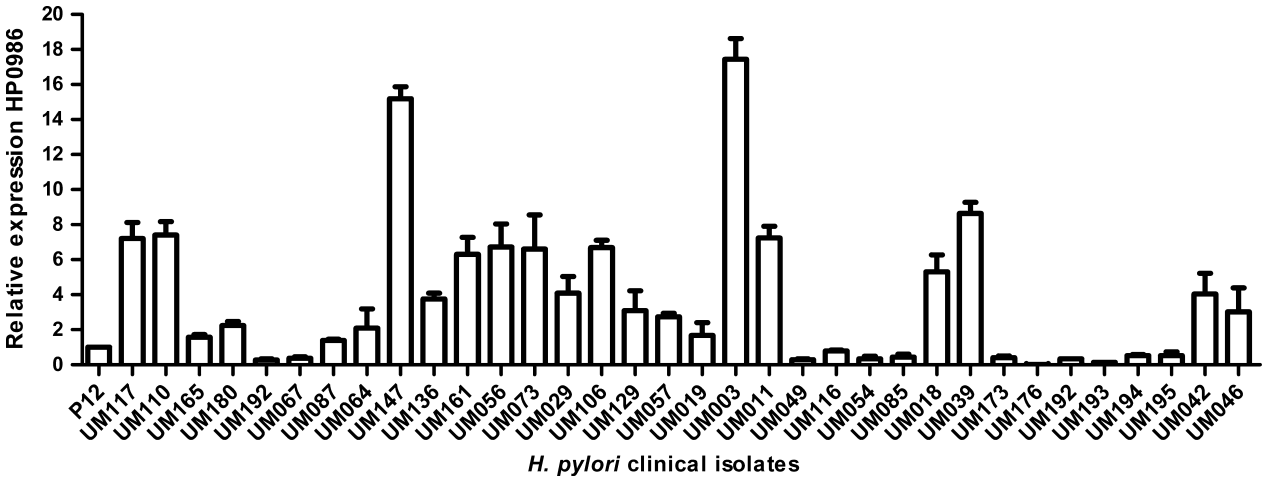

Figure 1 Relative quantitative real time PCR analysis of HP0986 gene expression in *Helicobacter pylori* clinical isolates cultured from gastric biopsies. Values represent Mean ± SEM of three replicates.

HP0986. The prevalence of HP0986 in the clinical samples varied among three ethnic groups and it was highest among the Indian origin patients (88%) followed by Chinese (10%) and Malay subjects (2%). This demonstrated that there was a differential prevalence of HP0986 in *H. pylori* clinical isolates corresponding to different ethnic groups in Malaysia.

Expression of HP0986 in Biopsy Samples

We analyzed the expression of HP0986 mRNA in gastric biopsy specimens and the analysis of relative HP0986 transcript levels was performed by quantitative RT PCR. On a pilot scale, relative mRNA was measured only in 10 gastric biopsy specimens. These 10 biopsy specimens were different from the 110 clinical isolates used for *in vitro* expression analysis. Our results demonstrated that 7 out of the 10 (70%) gastric biopsies had detectable HP0986 mRNA. To further confirm that the positive *in vivo* expression of HP0986 was reflective of presence of the gene, a PCR-based confirmation of HP0986 from the same sample was obtained. We found all the seven biopsy specimens positive for HP0986 mRNA expression and these were also positive by PCR. This indicates the specificity of our qRT-PCR in detection of *in vivo* expression. We also checked the profile of constitutively expressed 16S rRNA as an internal control. The presence of HP0986 in the biopsies correlated with the expression of HP0986 *in vivo,* wherein, all the 7 biopsies showed significant expression as determined by their cycle threshold (Fig. 2). Similar to the *in vitro* expression, among the 7 biopsies positive for HP0986, 4 were from ethnic Indians and 3 from Chinese. This observation corroborated with our *in vitro* expression results that

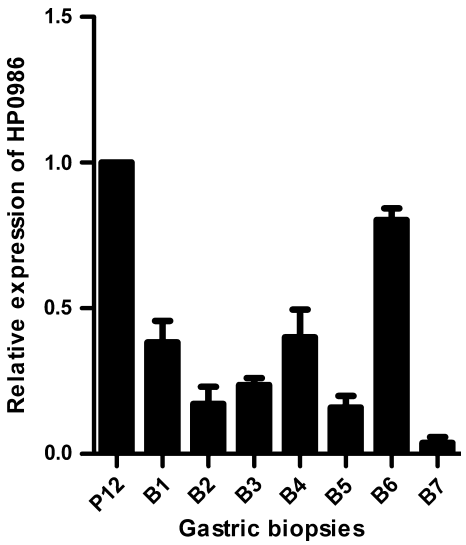

Figure 2 HP0986 expression profiles in gastric biopsies: Bar diagram represents relative expression of HP0986 mRNA transcripts in gastric biopsies. Values represent Mean ± SEM of three replicates.

HP0986 was perhaps more prevalent among Indian ethnic group followed by Chinese. In sum, expression of HP0986 under *in vivo* conditions conveys its possible role during infection and that the protein was naturally produced and presented to the immune system (see later).

HP0986 Elicits Antibody Response

Twenty *H. pylori* positive and an equal number of *H. pylori* negative patients' sera were used for an indirect ELISA to evaluate antibody response of *H. pylori* infected patients to HP0986 when compared with

healthy controls (Fig. 3). All the 20 *H. pylori* positive patients' sera showed seropositivity of HP0986. The high titers of serum IgG observed in *H. pylori* positive patients when compared with *H. pylori* negative patients ($p = .0025$) confirmed that HP0986 was expressed *in vivo* and recognized during natural infection.

HP0986 Induces IL-8 Secretion by AGS Cells

To examine the ability of HP0986 to stimulate IL-8 secretion from AGS cells, a bead-based immunoassay was performed wherein we monitored the cytokine secretion profiles in a dose and time course manner in culture supernatants of AGS cells. We observed that the IL-8 induction by HP0986 had increased in a dose- and time-dependent manner (Fig. 4), which was strongly enhanced at 36 hours post treatment (1200 pg/mL), whereas, no detectable levels of other proinflammatory cytokines such as IL6 and TNF α were observed.

To ensure that the cytokine response by the cells is specific to HP0986, we simultaneously tested proteinase K digested HP0986 preparations; as expected, they did not show any significant IL-8 secretion. Further, our previous report ensured that inclusion of another histidine tagged (6X His) protein purified in the same manner (namely, HP0023 encoding *H. pylori* isocitrate dehydrogenase) [24] did not induce any pro-inflammatory response. Secretion of IL-8 by AGS cells following stimulation with HP0986 and the previous data related to IL-8 secretion by macrophages and PBMCs [21] hints

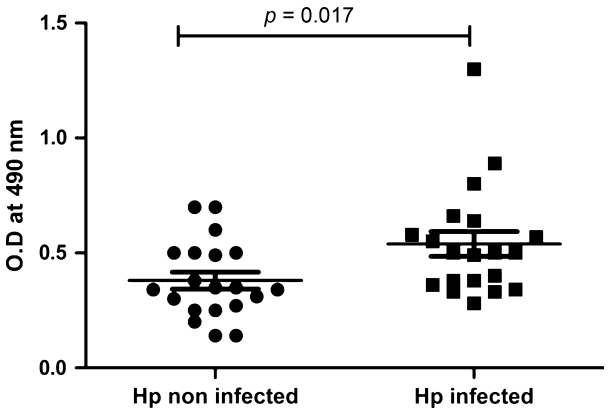

Figure 3 Detection of anti HP986 imunoglobulin G (IgG) in *Helicobacter pylori* positive patients: IgG levels in sera of 20 *H. pylori* infected patients versus 20 controls were quantitated using ELISA. We carried out a Mann–Whitney's *U* test to evaluate differences in antibody responses. Statistically significant difference was found among the levels of IgG antibody reactivity with respect to the two groups (p value: .017).

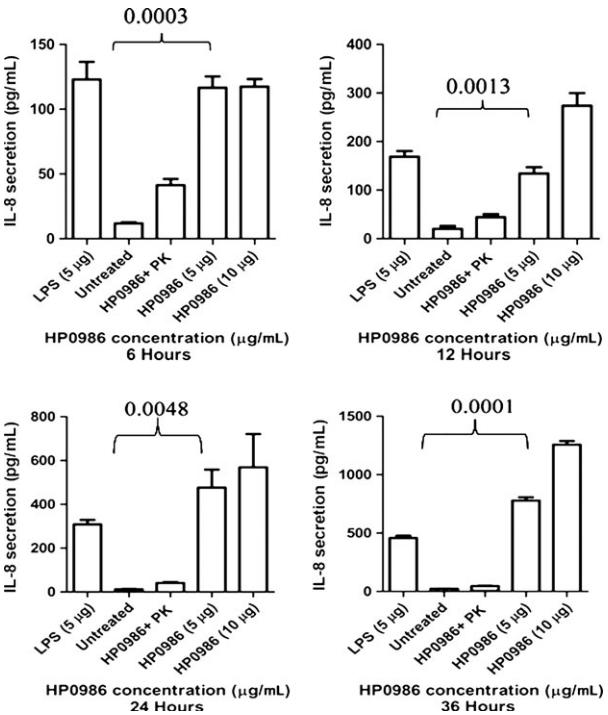

Figure 4 Time- and dose-dependent increase in IL-8 secretion by AGS cells upon treatment with HP0986: Supernatant was collected at 6, 12, 24, and 36 hours and IL-8 secretions were measured by flow cytometry. Maximum secretion was observed at 36 hours (p values are mentioned on the top of brackets in each bar chart and they indicate significant increase in induction of IL-8 when compared with untreated cells at four different time points). Each bar in the graphs shows the mean ± SEM of three replicates. PK: proteinase K.

that HP0986 is more likely to be associated with gastroduodenal inflammation.

HP0986 Triggers IL-8 Secretion by AGS Cells Through Activating NF-κB *via* Degradation of IκBα

To unravel the specific signaling pathway involved in HP0986 mediated IL-8 secretion, we treated the AGS cells with HP0986 and Western blotting was performed using antibodies specific to IκBα and phospho-p65. We were interested to determine the role of NF-κB pathway in HP0986 induced IL-8 production in gastric epithelial cells. In addition to this, we also determined the levels of IκBα in AGS cells upon treatment with HP0986. Treatment with HP0986 resulted in a decrease in the cytoplasmic levels of IκBα. The IκBα started degrading at 60 minutes post-treatment and proceeded up to 90 minutes after the treatment. We also observed the increase in the levels of NF-κB in the nucleus at 90 minutes post-treatment, followed by translocation of p65 into the nucleus of AGS cells (similar to what was observed upon LPS treatment) (Fig. 5).

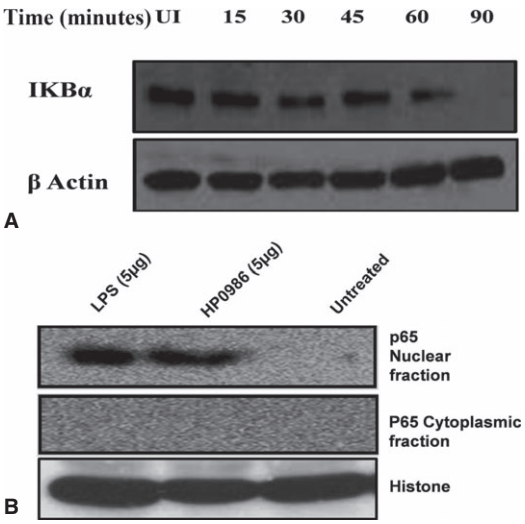

Figure 5 Western blot analysis of the effect of HP0986 (5 μg) on NF-κB activation and IκB degradation in AGS cells: (*A*) Western blot analysis for degrading levels of IκB from 0 to 90 minutes. UI indicates uninduced cell fraction used as negative control. (*B*) immunoblot representing activation of NF-κB in AGS cells upon treatment with HP0986 for 3 hours. β-actin and histone were used as equal loading controls respectively in (*A* and *B*). LPS was used as a positive control.

Cellular Localization of HP0986

Gastric epithelial surface is the main site of host pathogen interaction in *H. pylori* infection [25–28]. Several of the *H. pylori* virulence factors can enter epithelial cells either by direct injection *via* T4SS [29] or by endocytosis etc. Recent reports suggest that *H. pylori* virulence factors accumulate in the cytoplasm of several immune cells *in vitro* [30,31]. But, the cellular localization of *H. pylori* virulence factors in the gastric epithelium is sparsely studied. Therefore, to know the exact cellular localization of HP0986, we transiently transfected the AGS cells with pEGFPN1-HP0986 and the cellular location of HP0986 was visualized (Fig. 6). The fusion protein (pEGFPN-1-HP0986) was detected in the cytoplasm as well as in the nucleus. These results demonstrated that HP0986 localized both in cytoplasm and nucleus. Transfection of expression vector pEGFPN-1 alone did not produce similar localization pattern with respect to AGS cells.

Discussion

The role of strain-specific genes of the plasticity region in *H. pylori* has been of recent interest particularly concerning gastric mucosal inflammation and adaptation [32–34]. The plasticity region of *H. pylori* harbors different combination of genes and consequently, the gene content of different strains and isolates is significantly variable; this may be important in the context of different disease outcomes [35,36]. Several studies have reported the role of plasticity region genes in *H. pylori* induced gastroduodenal diseases. Some of these genes are proposed to be good candidate markers for clinical outcome, such as jhp0947and dup A etc. [37–39]. Moreover, several genes of the plasticity region have still not been characterized.

HP0986 is an important candidate antigen and a proinflammatory protein encoded by the plasticity region ORF hp0986 of *H. pylori* strain 26695. The protein has been characterized *in vitro* and was shown to be inducing proinflammatory cytokines through TNFR1- and NF-κB- mediated signaling [21]. However, the secretion, localization, and regulation of this seemingly important protein have not been worked out in an *in vivo* system. This study therefore, forms a logical extension of the work of Alvi *et al*. [21] with an emphasis on the interaction of this protein with AGS cells and its localization therein including its genotypic, serologic and expression profiling based on clinical isolates, patient sera, and biopsies. Our results broadly confirm the results of Alvi *et al*. obtained with cultured macrophages and PBMCs, by showing an upregulation of IL-8 secretion by cultured AGS cells upon stimulation with recombinant HP0986 protein. Our results also support the conclusion of Alvi *et al*. that the HP0986 protein is presented to the human immune system as demonstrated by serologic profiling of patient samples in both the studies using sera from patients with varied geographic descent.

Our results revealed presence of hp0986 locus in a significant number (31%) of clinical isolates (Fig. 1) and its prevalence was highest in strains obtained from the Indian ethnic group (88%) in Malaysia. Further, our study appears to be in agreement with the previous observation on the prevalence of *dupA*, in different ethnic groups in Malaysian and Singaporean populations [40]. Similarly, the prevalence rates of other plasticity region genes were also reported by others to be in a similar range, for example, jhp0940, jhp0945, jhp0947, and jhp0949 had a prevalence rate of 23.9, 39.1, 37.7, and 45.7%, respectively, in Colombia, United States, South Korea, and Japan [41]. As all these studies have been carried out on the basis of PCR-based genotyping, it will be necessary to stress that the absence of a PCR amplicon in *H. pylori* could be due to different allelic structures or sequences in different clinical isolates given that it is a highly recombining and geographically compartmentalized pathogen. However, our approach included consensus sequence-based primers nested within the hp0986 gene

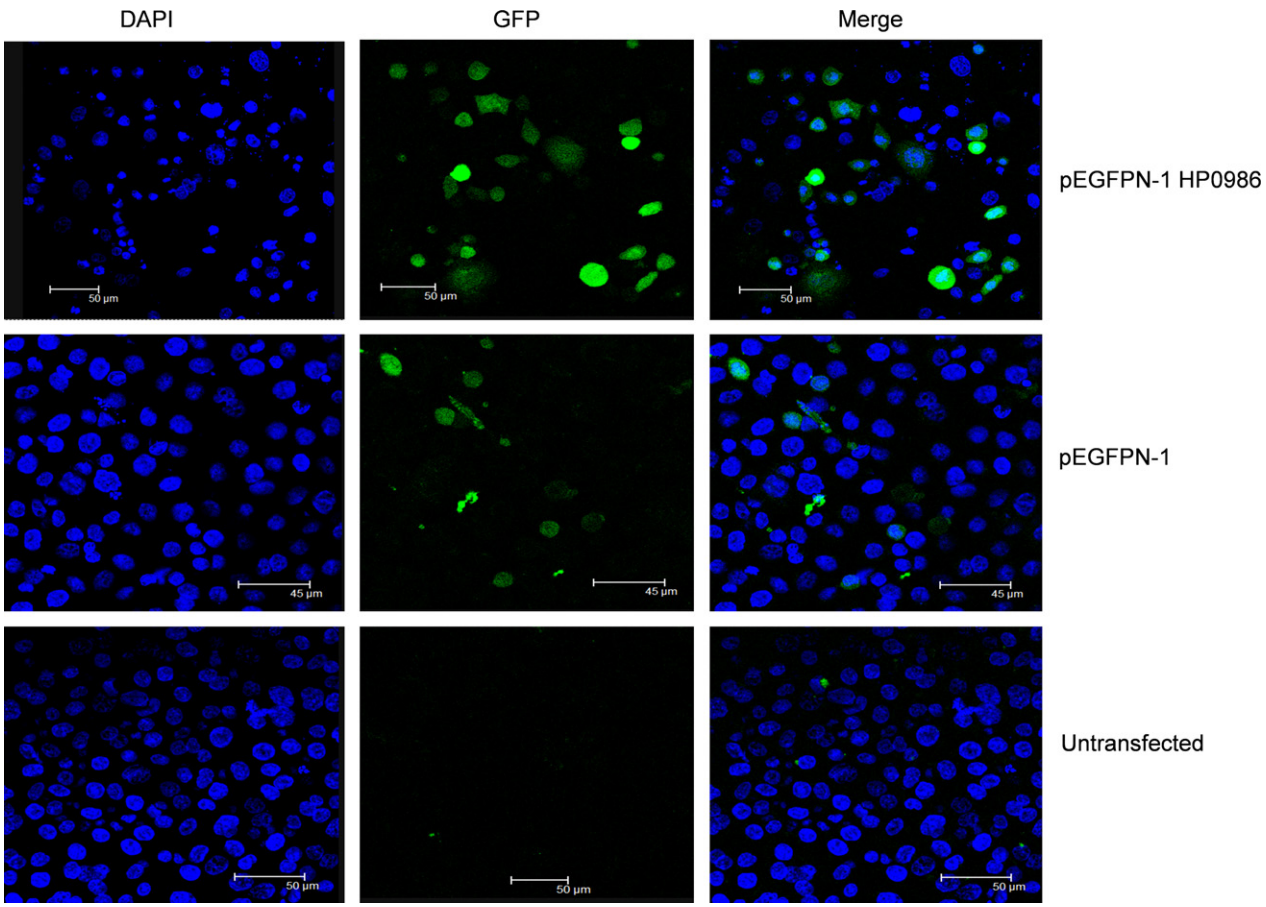

Figure 6 Expression and cellular localization of HP0986 in AGS cells: Subcellular localization of GFP+HP0986 was examined by transient transfection. Green fluorescent signal represented homing of HP0986 inside and on the surface of the AGS cells. Upper panel (pEGFPN1-HP0986) represents expression of hybrid protein GFP+HP0986 by the AGS cells. The middle panel indicates transfection of the AGS cells by the GFP vector alone whereas the lowest panel represents untreated AGS cells. DAPI indicates the position of the nuclei 24 hours after transfection. The cellular localization of HP0986 was visualized by confocal microscopy.

which appears to be highly conserved when tested in a single strain colonizing a single patient for about 10 years [42]. Although it is possible that the gene length in some strains could be larger than the gene annotated in strain 26695, but it may not affect the outcome of a PCR-based survey.

We observed variation in transcript levels of HP0986 in some *H. pylori* clinical isolates which may be due to multiple factors such as difference in environmental conditions of the stomach (pH, stress, level of mucin secretion etc.) or it may be an intrinsic property of an individual clinical isolate [25]. However, *in vivo* expression of HP0986 was limited when compared with *in vitro* expression (Fig. 2); decreased *in vivo* expression may likely be due to several reasons such as less bacterial load as the bacterium maintains low profile during infection, or it may also be due to loss of RNA during sample processing etc. [43,44]. It was also shown that *in vivo* expression of CagA in gastric biopsies was lower than its *in vitro* expression [45] which was further consistent with a DNA microarray study wherein the *in vivo* CagA expression was downregulated by low pH levels [46].

The antibody response in the sera of *H. pylori* infected patients to HP0986 was evaluated by comparing them with the healthy controls. Our results (Fig. 3) clearly demonstrate the possibility that HP0986 is expressed and presented to the immune system as *H. pylori* possibly harnesses different means of releasing its antigens into the extracellular space (T4SS, autolysis, and formation of membrane vesicles etc.). HP0986 could therefore be secreted by one of these mechanisms [47]. We have earlier shown seropositivity of HP0986 [21] in a geographically distinct and mixed patient sera collection [24]. In this study, an ethnically diverse but geographically related patient population was used to

demonstrate that HP0986 induced antibody response was not population specific.

Our observation that HP0986 induces IL-8 in a cagA-independent manner supports the notion that CagA alone may not be the sole pro-inflammatory trigger during *H. pylori* infection and that many other players could be involved in the proinflammatory activity independent of CagA. These observations are in agreement with previous reports by Selbach *et al*. and Gorrell *et al.* where they concluded that IL-8 secretion in gastric epithelial cells was independent of CagA [48,49]. This then opens up the possibility that the strains lacking cagA gene could also produce clinical symptoms linked to inflammation. It is now certain that several other genes also encode proinflammatory proteins of the sorts of flagellar antigens, outer membrane proteins and Hsp60 etc. [50]. Other investigators also reported similar findings while working on strain-specific proteins that are found outside the cagPAI; particularly, the plasticity region proteins/genes such as *dupA* and JHP0940 were shown to be able to induce IL-8 secretion [19,51]. Another plasticity region locus, jhp947-jhp949 was found to be associated with duodenal ulcer disease and IL-12 production by monocyte cells [37].

Induction of pro-inflammatory cytokine responses involving NF-κB activation is mostly described to be associated with the type IV secretion system (T4SS) in *H. pylori* [52]. Apparently, it may be possible that HP0986 is also secreted through T4SS although there is no direct evidence at this stage to show the same. We have also shown the localization of HP0986 in gastric epithelial cells using a mammalian expression vector. Our results revealed that HP0986 localizes in cytoplasm as well as in the nucleus. However, further studies are required to understand detailed mechanisms involved in HP0986 entry and regulation of host cell machinery. Nonetheless, our results appear consistent with previous observations in which CagA was also shown to localize in the inner leaflet of host cells [53]. However, as HP0986 does not have a secretion signal and that it did not offer any structural or sequence homology to some of the known effector proteins or toxins that are secreted through T4SS, such as CagA or members of any other T4SS in *H. pylori*, it will be interesting to find out its secretory mechanism and regulation.

A somewhat intriguing property of HP0986 gene/protein is its size variability. The hp0986 locus consists of an ORF encoding from 231 amino acids to 558 amino acids in various sequenced genomes of *H. pylori*. There are over 200 different sequences, available in the public domain, that show that the average size of the ORF for this protein is longer than that of the reference strain 26695. However, multiple sequence alignment revealed that an ORF of 237 amino acids corresponding to HP0986 of the strain 26695 was highly conserved in most of the sequenced genomes. Nevertheless, it will be worthwhile to study the functions of larger variants in different strains as they may be relevant to our understanding of some of the critical aspects of HP0986 (such as its mode of secretion and regulation), although they may not encode functions dramatically different than the hitherto described roles of this protein. In case of latter possibility, it will indeed be appropriate to reconsider the choice of strain 26695 as a reference strain to study HP0986 and other putative/novel gene functions.

Detailed analysis of HP0986 sequence using Pfam webserver [54] revealed that HP0986 also possesses a domain similar to type II restriction endonucleases, but whether it corresponds to functional restriction endonuclease or methylase activity needs to be proved. Given this, HP0986 could perhaps be a 'moon lighting' antigen similar to isocitrate dehydrogenase of *H. pylori* and *Mycobacterium tuberculosis* and aconitase of *M. tuberculosis*; these proteins participate in core metabolic activities such as energy cycles and also have immunological and regulatory roles respectively [24,55–58]. Perhaps, HP0986 could be very similar to another important virulence factor, IceA, which is proinflammatory and has also been shown to be a restriction endonuclease [59]. Alvi *et al*. [21] could not shed light on possible new functions of HP0986 and remained focused on TNFR1-mediated proinflammatory and proapoptotic roles of HP0986. Given their demonstration of a TNFR1-mediated signaling and our present findings, it is tempting to name this important gene/protein as 'TNFR1 interacting endonuclease A (TieA or *tie*A)'. Further, it will be possible to direct future efforts at understanding the functional promiscuity of this protein and the regulation of proinflammatory as well as methylase activities.

In conclusion, our study demonstrated that HP0986 induced IL-8 secretion in gastric epithelial cells *via* NF-κB activation and localized both in the cytoplasm as well as in nucleus of the cells. mRNA expression profiling of bacterial cultures and gastric biopsy specimens clearly conveyed that HP0986 was expressed naturally. The antibody profiles of patient sera further confirm this and point to the role of HP0986 in *H. pylori* infection-induced pathogenesis. Future studies involving mechanistic confirmation of the cellular and extracellular roles of the protein are pertinent. Our next experiments will be directed at identification of upstream and downstream signaling events with respect to NF-κB activation in epithelial cells as well as establishing the cause and effect relationships by means of isogenic knockout strains.

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Competing interests: The authors have no conflicts of interest to declare.

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Concurrent Proinflammatory and Apoptotic Activity of a *Helicobacter pylori* Protein (HP986) Points to Its Role in Chronic Persistence

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Abstract

Helicobacter pylori induces cytokine mediated changes in gastroduodenal pathophysiology, wherein, the activated macrophages at the sub-mucosal space play a central role in mounting innate immune response against the antigens. The bacterium gains niche through persistent inflammation and local immune-suppression causing peptic ulcer disease or chronic gastritis; the latter being a significant risk factor for the development of gastric adenocarcinoma. What favors persistence of *H. pylori* in the gastric niches is not clearly understood. We report detailed characterization of a functionally unknown gene (HP986), which was detected in patient isolates associated with peptic ulcer and gastric carcinoma. Expression and purification of recombinant HP986 (rHP986) revealed a novel, ~29 kDa protein in biologically active form which associates with significant levels of humoral immune responses in diseased individuals ($p<0.001$). Also, it induced significant levels of TNF-α and Interleukin-8 in cultured human macrophages concurrent to the translocation of nuclear transcription factor-κB (NF-κB). Further, the rHP986 induced apoptosis of cultured macrophages through a Fas mediated pathway. Dissection of the underlying signaling mechanism revealed that rHP986 induces both TNFR1 and Fas expression to lead to apoptosis. We further demonstrated interaction of HP986 with TNFR1 through computational and experimental approaches. Independent proinflammatory and apoptotic responses triggered by rHP986 as shown in this study point to its role, possibly as a survival strategy to gain niche through inflammation and to counter the activated macrophages to avoid clearance.

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Introduction

Infection of human gastric mucosa with *H. pylori* is associated with different forms of gastro-duodenal diseases such as gastritis, peptic ulcers and gastric adenocarcinoma [1]. However, despite the fact that it colonizes more than 50% of the population worldwide, only a small subset of those infected develop more severe forms of gastric diseases; this may be due to various environmental and pathogen specific factors apart from different host immune responses [2,3].

Establishment of successful colonization is a complex process that involves activities of several genome encoded virulence factors, aimed perhaps at survival through inflammation and defense *via* suppressing innate immune responses.

Once established in the host, *H. pylori* triggers activation of transcription factors and secretion of mucosal proinflammatory cytokines followed by cytoskeletal rearrangement, enhanced cell proliferation and apoptosis [4]. The induction of proinflammatory cytokines (IL-8 and IL-6) by *H. pylori* is mediated through NF-κB *via* recognition of toll-like receptors (TLRs) [5,6]. Translocation of NF-κB by *H. pylori* promotes either the inflammatory process through induction of proinflammatory cytokines or regulates host defense by promoting or inhibiting apoptosis [7]. There are experimental evidences supporting the pro- and anti- apoptotic roles of NF-κB; its role in TNF-alpha /FasL mediated apoptosis has been described [8].

Given the proinflammatory responses aimed basically at gaining niche, the bacterium also appears to have evolved mechanisms to avenge primary defense maintained by the activated macrophages and lymphocytes [9]. This may involve selective inhibition of T-cell proliferation through up-regulation of Fas antigen [10], which is possibly mediated by cytokines (TNF-α and IL-1β), reactive

oxygen metabolites and iNOS [11, 12, and 13]. This may reveal that although the persistent infection substantially increases mucosal inflammation, loss of activated macrophages proportionately limits clearance from the host [14,15] leading to chronicity of inflammation.

H. pylori encodes several virulence associated molecules, including proapoptotic (such as VacA) [16] and anti apoptotic (such as CagA) [17] effectors and toxins, besides important virulence factors such as OipA, Ure, flagellins and adhesins. Although the functional coordinates of these factors have been extensively determined in different studies [18,19], discrete associations of these with different disease outcomes have contradicting evidences [20]. In particular, microevolution and allelic diversity of the cagPAI, and vacA do not allow robust genotype-phenotype correlations thereby posing an obvious difficulty in linking the evolving virulence factors with the pathology [21]. In view of this, it is possible that the bacterium harnesses alternative strain specific factors [22] to achieve persistent infection. Also, there are several hypothetical and unknown proteins coded by *H. pylori* genome whose functional role in pathogenesis is unexplored. Therefore, it is pertinent to look into the biology of novel genes/proteins to get new insights into pathogenesis and phenotypic diversification of the bacterium in a changing host. The cache of many strain specific genes (the putative virulence factors) [23] comprises the 'plasticity zone' of *H. pylori* chromosome. Functional characterization of such genes and their involvement in pathogenesis of *H. pylori* could facilitate clear understanding of the development of peptic ulcer disease and gastric carcinoma. In this study, we describe efforts to systematically decipher the proinflammatory and apoptotic roles of one such putative virulence factor, HP986, and how this observation reinforces our understanding of the biology of *H. pylori* colonization and persistence.

Results

Association of HP986 with invasive disease outcomes and its distribution in clinical isolates

HP986 was found to be present in more than 61% of the total isolates we screened from many different geographical regions (Figure1 A, C). Overall, the presence of this gene was significantly associated with invasive disease (peptic ulcer and gastric carcinoma, 72%) outcomes (Figure 1B). This apparently contrasts previous observations [24] that describe HP986 to be gastritis specific. Also, the gene was found consistently conserved in all the three strains isolated from different niches of the stomach, almost a decade-apart [25], from a single patient (Figure 1 A). This suggests that despite a genome wide trend of extensive rearrangements in *H. pylori*, HP986 remains conserved.

Protein sequence analysis and structure function prediction

HP986 was predicted to be *H. pylori* specific with no obvious sequence similarity in the available microbial sequence databases. Functional prediction showed high antigenic indices equivalent to ~3.4 (DNAstar software, DNAStar Inc, USA). Due to the unavailability of crystallographic/solution structure of HP986, a search for possible homologs was carried out using several programs. Sequence-based search methods (BLASTp) did not provide any significant hit but sequence searches in PDB identified a template with 22% identity. This template (PDB ID: 1XMX) was a hypothetical protein, VC1899 from *V. cholerae* and a structural model of HP986 was built using it (Figure 2A). Total 50 solutions were obtained using Modeller9v8 [26] and solution No. 33 was considered the best among them on account of less energy. The quality of the structure was assessed using Ramachandran plot obtained *via* Procheck , which displayed 88.6% residues in most favored regions and 0.9% residues in disallowed regions. Consequently, ModLoop [27] was used to rebuild the two residues in the modelled region followed by energy minimization. Results of the model validation using Procheck program were as follows: 89.0% residues in the most favored regions; 10.0% residues in the additional allowed regions; 0.9% residues in the generously allowed regions and 0.0% residues in the disallowed regions. Secondary structure analysis showed ten alpha helices and seven beta sheets in the modelled structure (Figure 2B). HEX [28], GRAMM-X [29] and PatchDock [30] programs were employed for unbound protein-protein docking with TNFR1 as receptor and HP986 as a ligand. Approximately 1000 predictions were generated using PatchDock and were submitted to FireDock [31] to refine 10 best solutions on the basis of global energy. Possible binding interface residues were identified using 3D2GO binding site prediction server [32]. Several of the lowest energy docking models emerging from this exercise placed the HP0986 on the side of the TNFR1. Among ten docked complexes, complexes 1 and 4 were identified as the plausible ones on the basis of minimum energy score and binding interface residues. A docking model of TNFR1-HP0986 is shown in Figure 2C, in which loop 1 and 2 regions of TNFR1 are docked onto α helices of HP0986.

Expression and purification of rHP986

The over-expressed rHP986 was purified to homogeneity under native conditions as a His-tagged protein in *E. coli* BL21 (DE3). Homogeneity of the protein was further confirmed by fast performance liquid chromatography (FPLC). The purified protein upon fractionation on a 10% polyacrylamide gel showed a single band corresponding to ~29 kDa on staining with Coomasie brilliant blue dye.

Humoral responses to rHP986

A strong and significant humoral response ($p<0.0001$) was observed in *H. pylori* infected diseased subjects as compared to *H. pylori* negative individuals (Figure 1 D). Mean value of serum antibody levels (Mean ± SD) in *H. pylori* infected patients was 0.397 ± 0.081 (Mean ± SD) as compared to *H. pylori* negative subjects 0.133 ± 0051). However, rHP986 did not show disease stage wise (gastritis, peptic ulcer and gastric carcinoma) serum reactivity.

rHP986 induces proinflammatory cytokines (TNF-α and IL-8) in a dose and time dependent manner

rHP986 elicited strong cytokine response both in cultured / PMA differentiated Thp1 cells (Figure 3 A, C) and in human polymorphonuclear blood monocytes (PBMC) in a dose dependent manner (Figure 3 E, G). A significant increase in induction of TNF-α ($p<0.0016$) and IL-8 ($p<0.0003$) as compared to untreated cells was observed. Time kinetics revealed active production of TNF-α ($P<0.0003$) within 6 hrs of stimulation (Figure 3 D, H) which decreased slowly after 12 hours. In contrast, IL-8 secretion increased during this period with peak response noted at 12 hours post stimulation; levels were more or less maintained up to 48 hours (Figure 3 B, F). An unrelated His-tagged recombinant protein, isocitrate dehydrogenase (ICD) from *H. pylori* failed to demonstrate cytokine response even at the highest concentration of 10 µg/ml (data not shown). Further, inductions of these proinflammatory cytokines by rHP986 were

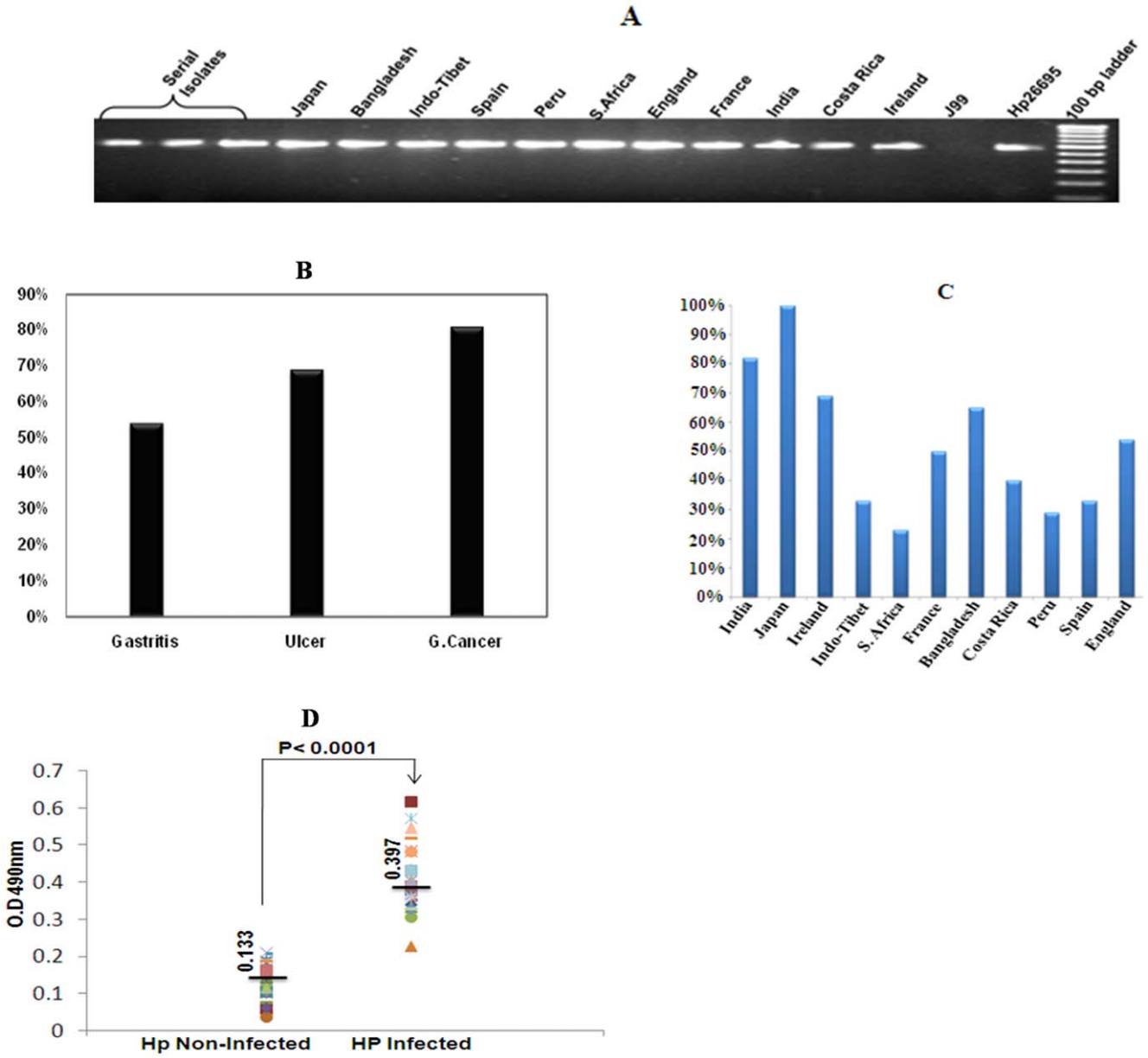

Figure 1. The locus hp986 was found to be associated with chronic gastric disease conditions. Panel A - Hp986 is widely distributed in different geographical regions; Panels B and C - Bar diagrams to represent % prevalence of hp986 in different disease categories and in different geographic regions, respectively; Panel D - Humoral responses directed against rHP986 were analyzed in sera collected from *H. pylori* infected patients belonging to different diseased categories and from control individuals (healthy controls and *H. pylori* non infected subjects). Antibody titers against rHP986 were compared between infected subjects and control individuals ($P<0.001$).
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not affected when the protein was treated with polymixin B. Additionally, proteinase-K treatment confirmed loss of rHP986 induced cytokine responses suggesting that the effect was due to rHP986 (Figure 3 A, C).

rHP986 induces IL-8 through NF-κB

The role of transcription factor NF-κB in regulating the expression of IL-8 is already well established [33]. We observed a significant and proportionate increase in the activation of NF-κB complex in rHP986 treated cells in a dose (Figure 3 I) and time dependent manner (Figure 3 J) as compared to untreated cells and cells treated with LPS (Figure 3 I,J). Recombinant ICD from *H. pylori* was used as an unrelated control (Figure 3 J) and the levels of NF-κB complex corresponding to ICD were similar to those observed with untreated cells. Competition with unlabeled NF-κB DNA probe confirmed the specificity of the complex. Further, exclusive involvement of rHP986 in the activation of NF-κB complex was confirmed by using antibodies specific to p65, p50 and c-Rel. Addition of antibodies led to the supershift of p65 and p50 subunits in the extract of cells treated with rHP986 or LPS (Figure 3 K). No binding to anti- c-Rel antibody was observed (Figure 3 K). Actin was used as an equal loading control (Figure 3

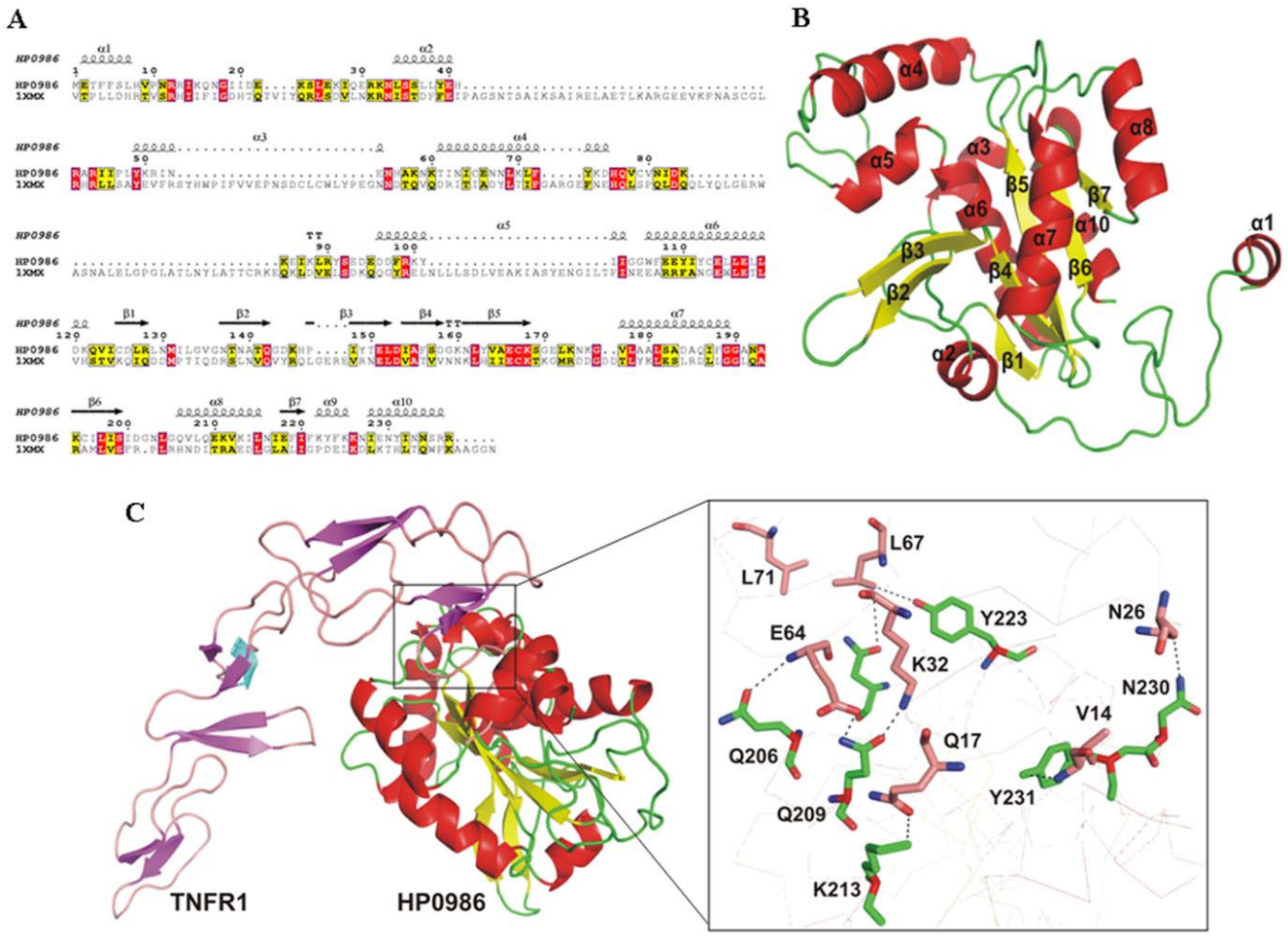

Figure 2. Sequence alignment and predicted 3D-structure of HP986. Panel A - Sequence alignment of HP986 with the hypothetical protein (VC1899) from *Vibrio cholerae* (PDB code 1XMX). Strictly conserved residues are highlighted in red and partially conserved residues are yellow. The sequence numbering refers to HP986. Final B - Predicted 3D-structure of HP986. The protein secondary structures elements are labelled and colored. Panel C - Interaction of HP0986 with TNFR1 using PatchDock and FireDock. The residues of HP986 and TNFR1 are colored in cyan and green respectively. The residues showing interaction between both proteins are labelled and displayed as stick model in element colors (carbon colored green/pink, nitrogen colored blue, and oxygen colored red). Hydrogen bonds are represented by black dashed lines.
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K). It was thus confirmed that rHP986 up regulates NF-κB, which in turn induces IL-8 expression.

HP986 functions through interaction with TNFR1

rHP986 triggered the expression of TNFR1 by the Thp-1 differentiated macrophages (Figure 4 A). We also tested the possible interaction of rHP986 with TLR4 and TLR2; however, rHP986 treatment did not have any effect on toll-like receptor expression (Figure 4 B, C). This finding was further confirmed by analyzing the antagonist effect of TNFR1 receptor on NF-κB translocation. Pretreatment of cells with neutralizing antibodies against TLR4 and TLR2 did not abrogate NF-κB translocation (Figure 4 D), however, this did happen when cells were pretreated with a neutralizing antibody against TNFR1, suggesting the possible role of rHP986 in increased TNFR1 expression (Figure 4 D).

Interaction of rHP986 with TNFR1 was further validated by immunoprecipitation using anti-TNFR1 antibodies. The receptor was detected in the eluate treated with rHP986 but could not detect any corresponding signals in the cell lysate treated with either LPS or recombinant ICD (Figure 4 E). Exclusive interaction of rHP986 with TNFR1 was further confirmed in competition with neutralizing antibodies against TNF α; the amount of immune complex detected was less as compared to that seen in the absence of TNF-α (Figure 4 F). This suggested a direct interaction between TNFR1 and rHP986 and ruled out any possible role of endogenous TNF-α. Further, BIAcore® (Surface Plasmon Resonance) (GE Healthcare Ltd.) analysis provided insights into the interaction of rHP986 and TNFR1 interaction. Using 1:1 Langmuir binding model to fit our binding curve, we found that rHP986 indeed binds with TNFR1 ($k_a = 1.26\times10^4 \pm 5.62\times10^2$ Ms^{-1}) on the biosensor surface. The resultant complex was found to be highly stable as illustrated by slow dissociation rate ($k_d = 9.08\times10^{-4} \pm 3.80\times10^{-5}$ s^{-1}). A good binding fit ($\chi 2 = 8.94$) was obtained confirming the above values of k_a and k_d (Figure 4 G).

rHP986 induces Fas mediated apoptosis

Considering the interaction of rHP986 with TNFR1, its possible involvement in inducing Fas expression was tested as the latter is known to function in synergy with TNFR1 and constitutively regulates downstream signaling cascade leading to apoptosis [11]. Pretreatment of PMA differentiated cells with rHP986 effectively

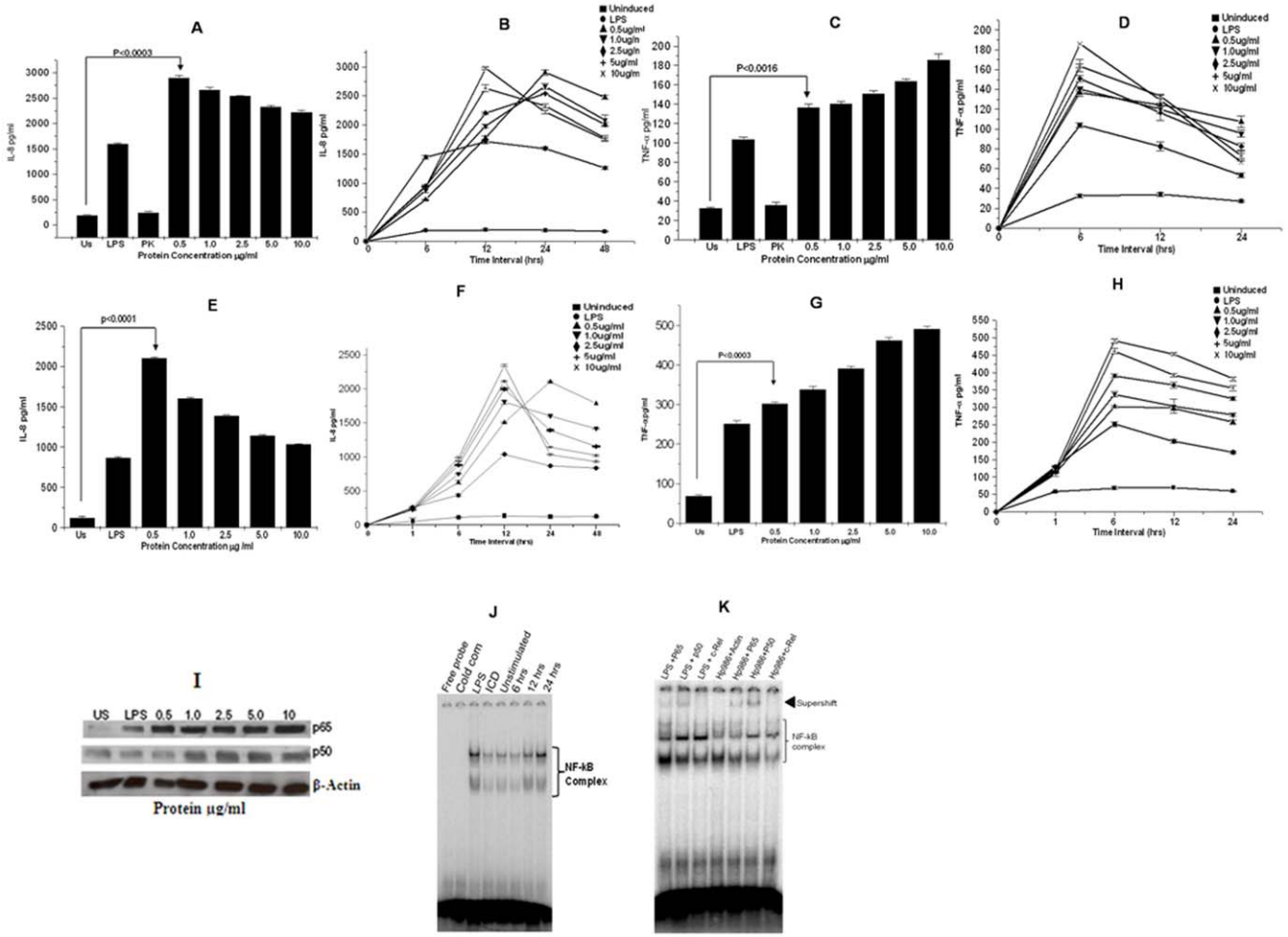

Figure 3. rHP986 stimulates the production of proinflammatory cytokines (IL-8 and TNF-α) through NF-κB. Panels A and B - Bar diagram and graph representing the amount of IL-8 secreted in Thp1-cells followed by exposure to rHP986; Panels C and D - The dose and time kinetics of TNF-α induction by rHP986 treated cells. Panels E and F - Dose and time dependent secretion of IL-8 by human PBMCs following rHP986 stimulation; Panels G and H – Dose and time dependent effect on the levels of TNF-α in human PBMC treated with rHP986. Results are shown as mean ± SE and represent findings from one of the three independent experiments. Panel I - Dose dependent effect of rHP986 on the translocation of NF-κB complex; lane1 - unstimulated cells, lane2 - cells treated with LPS, lanes 3 to 7 - cells treated with 0.5 µg/ml, 1.0 µg/ml, 2.5 µg/ml, 5.0 µg/ml and 10 µg/ml concentrations of rHP986 protein, respectively. Panel J - rHP986 mediated translocation of NF-κB complex was analyzed by electrophoretic mobility shift assay (EMSA); lane 1 – free probe, lane 2 – cold competition, lanes 3 and 4 – controls (LPS and ICD, respectively), lane 5 – negative control (cells without rHP986 treatment). Cells were treated with rHP986 (0.5 µg/ml) for varied time periods, lane6 - 6 hrs, lane7–12 hrs and lane8–24 hrs. Panel K - Supershift assay (K); specificity of rHP986 mediated activation of NF-κB complex was detected using specific antibodies against p65, p50 and c-Rel. Nuclear extracts prepared from differentiated cells treated with either rHP986 (lane5-p65, lane6-p50 and lane7-c-Rel) or LPS (lane1-p65, lane2-p50 and lane3-c-Rel) were incubated with antibodies as described in materials and methods. Nuclear extract from rHP986 treated cells incubated with β-actin was used as control - lane4.
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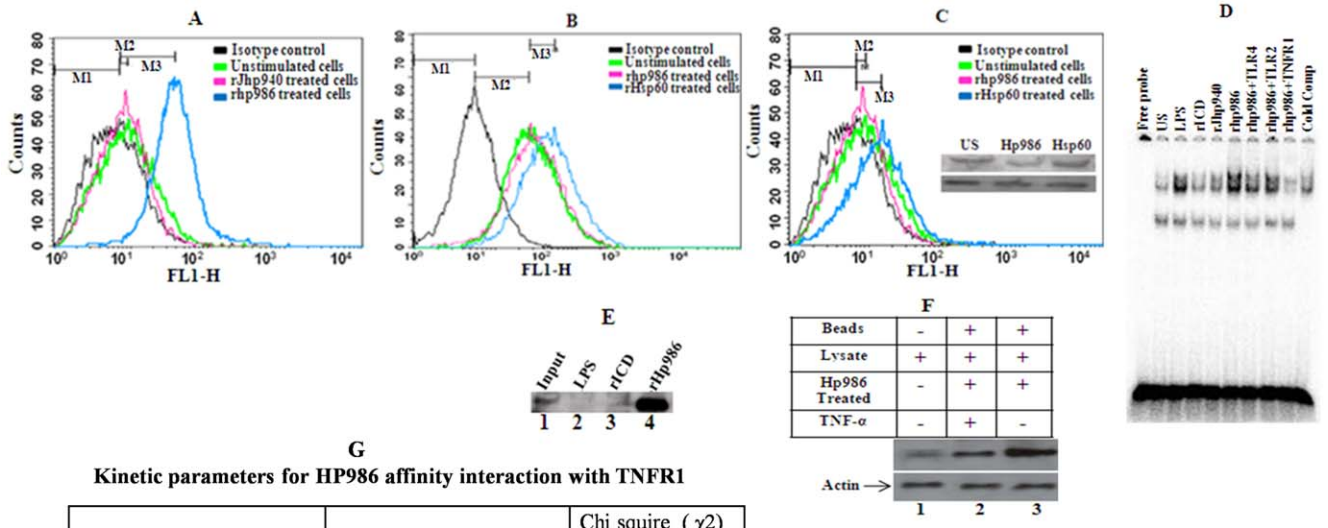
regulated Fas expression in a time and dose dependent manner. Comparative expression analysis with increasing protein concentration at different time points revealed significant and proportionate increase in Fas expression up to 24 hours (Figure 5 A). Expression levels declined after 12 hrs in cells subjected to higher protein dose (5.0 µg/ml). This could be due to possible increased cell death. Immunocytochemical staining also showed an increased expression of Fas on the surface of rHP986 stimulated cells as compared to unstimulated ones (Figure 5 B).

Corroborating our findings with an earlier study [13] on the synergistic function of TNFR1 and Fas in inducing apoptosis, we evaluated the potential of rHP986 as an apoptosis-inducing agent. rHP986 triggered apoptosis in cultured macrophage cells in a dose and time dependent fashion (Figure 5 C). Furthermore, apoptosis as a function of Fas expression was also mechanistically shown through binding with annexin-V and acridine orange (Figure 5 D). A substantial and proportionate increase in cell death was observed when the cells were treated with increasing concentration of rHP986 (0.5 µg/ml-10 µg/ml) for varied time intervals; up to 48 hours (36.65%±3.25% to 41.0%±4.2%) as compared to untreated cells (10.65%±1.85%). This pro-apoptotic property of rHP986 declined significantly upon blocking its interaction with TNFR1 (Figure 5 E) indicating the involvement of rHP986 in TNFR1mediated cell death. Similar results were obtained when the cells were stimulated in the presence of neutralizing antibody against TNF-α, suggesting that the effect was not secondary to endogenous TNF-α. As expected, His-tagged ICD protein from *H. pylori* failed to induce apoptosis. A comparative analysis between oleandrin (a known inducer of apoptosis) [34] and rHP986 also confirmed the latter being an equally potent inducer of apoptosis



G

Kinetic parameters for HP986 affinity interaction with TNFR1

k_a[a] (1/Ms)	SE[b] (k_a)	k_d[c] (1/s)	SE(k_d)	Chi squire ($\chi 2$)
1.26×10^{04}	5.62×10^{02}	9.08×10^{-04}	3.80×10^{-05}	8.94
KA[d] ($=k_a/k_d$) (1/M)		KD[e] ($=k_d/k_a$) (M)		
1.39×10^{07}		7.21×10^{-08}		

[a]Association rate constant. [b]Standard Error. [c]Dissociation rate constant. [d]Equilibrium association constant. [e]Equilibrium dissociation constant.

Figure 4. Interaction of HP986 with TNFR1 analyzed by flow-cytometry and immunoprecipitation. Panel A - Enhanced expression of TNFR1 following treatment with rHP986; Panel B - HP986 did not stimulate expression of TLR2 - levels were equal to the cells that had not received any protein treatment; Panel C - Treatment of HP986 had no visible effect on TLR4 expression [in the inset, lane 1 - untreated cells, lane 2 - cells treated with HP986 and lane 3 - cells treated with rHsp60 (from *M. tuberculosis*) used as control; Panel D - EMSA showing effects of neutralizing antibodies against TNFR1, TLR2 and TLR4 on the translocation of NF-κB complex in the cells stimulated with HP986 [lane1- free probe, lane2 – unstimulated/untreated cells, lane 3- cells treated with LPS, lane4 – cells treated with ICD, lane5- cells treated with JHP940, lane6 - cells treated with HP986, lane7- cells treated with HP986 and neutralizing antibody against TLR4, lane8 - cells treated with HP986 and neutralizing antibody against TLR2, lane9 - cells treated with rHP986 and neutralizing antibody against TNFR1 and lane 10 - specific competitor (unlabelled NF- κB consensus probe)]; Panel E -Immunoprecipitation assay showing interaction of rHP986 with TNFR1, lane 1 - input, lane 2 – cells treated and incubated with LPS, lane3 - cells treated and incubated with ICD, lane 4 - cells treated and incubated with rHP986. Immunoblot was developed using antibodies against TNFR1. Panel F - Role of endogenous TNF-α in binding to TNFR1 was ruled out using neutralizing antibodies against TNF-α; lane 1 - whole cell lysate of cells, lane 2 - cells incubated with rHP986 in the presence of neutralizing antibody to TNF-α, lane 3 - cells incubated with rHP986 in the absence of neutralizing antibody to TNF-α. Immunoprecipitate was pulled down using Talon beads as discussed in materials and methods section. Blot was developed using anti-TNFR1 antibody. Equal protein loading was confirmed by reprobing the blot with β-actin. Panel G – Summary of kinetic parameters for rHP986 affinity interaction with TNFR1: *a* = Association rate constant, *b* = Standard Error. *c* = Dissociation rate constant *d* = Equilibrium association constant *e* = Equilibrium dissociation constant.
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(Figure 5 E). Collectively, all these findings confirm rHP986 to be a potent apoptosis-inducing agent.

Discussion

Novel genes constantly emerge from newly sequenced replicate genomes. This paradigm was supported by the analyses wherein the pan-genome of a true bacterial species remained 'open' and each new genome sequence would identify dozens of new genes in the existing pan-genome of *Streptococcus agalactiae*, for example [35]. It is clear also from previous studies that a pool of strain specific genes in pathogens such as *H. pylori* termed the 'plasticity region cluster', could be useful in adaptation to a particular host population [36]. This pathogen shows a very strong geographic adaptation and is known for harboring up to 45% strain specific genes with most of them gained through horizontal gene transfers [36]. Recently, the members of the plasticity region cluster were shown to be likely involved in promoting proinflammatory potentials of some of the strains, possibly providing a survival advantage [37,38].

However, a majority of the plasticity region genes /proteins are yet to be fully characterized. We recently reported functional characterization of JHP940, a novel antigen from this region that has shown potential proinflammatory activity [37]. However, it is not clear if plasticity region proteins provide any survival advantage to the pathogen and the mechanisms thereof. The present study attempted to explore functional aspects of HP986 as a putative virulence factor and to examine its prevalence in clinical isolates from different geographical regions. Also, we performed a series of activity experiments to elucidate its role in pathology; in particular, its proinflammatory and apoptotic activity in human macrophages. We used differentiated human macrophages since

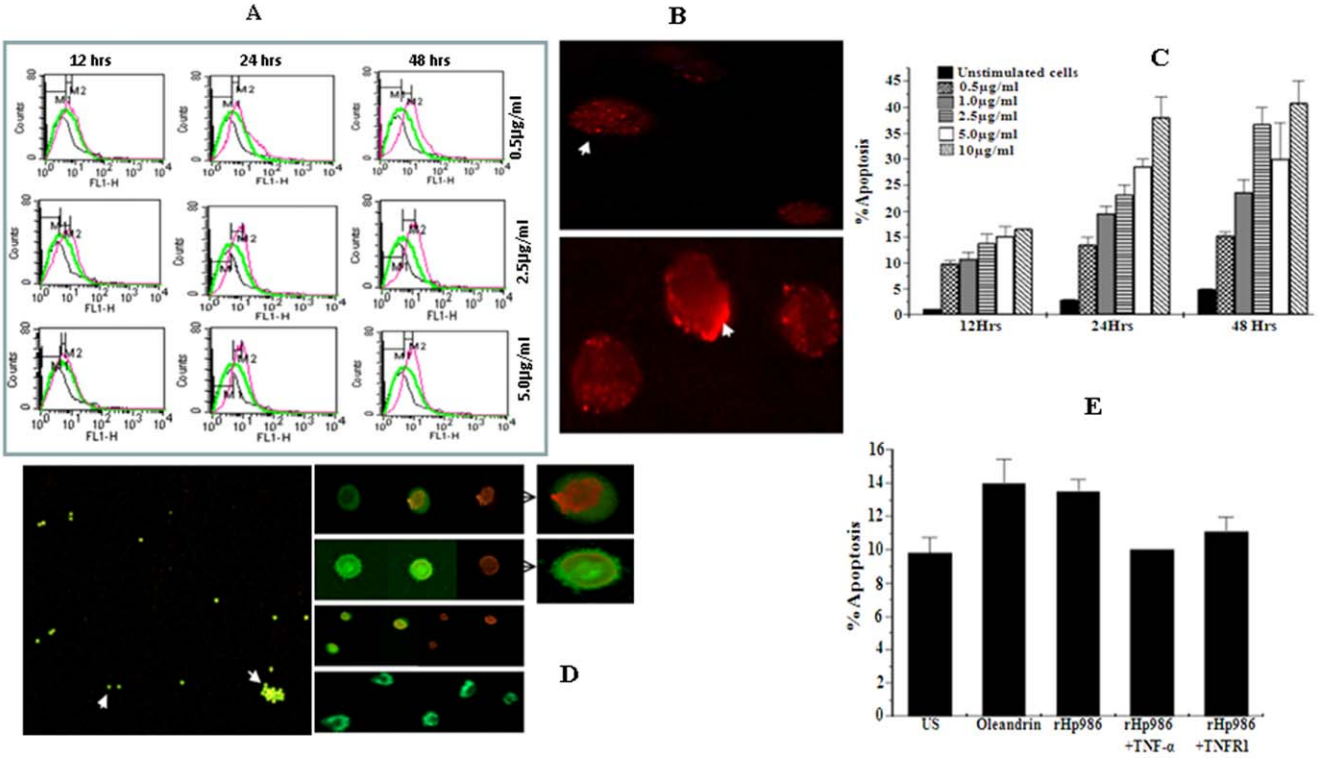

Figure 5. rHP986 induces Fas expression and apoptosis of cultured macrophages *via* a TNFR1 dependant pathway. Panel A - rHP986 induced Fas expression on the surface of differentiated macrophages as quantified by flow cytometry with anti-Fas antibody. Dose dependent increase in Fas expression in response to treatment with increasing concentration of rHP986 (0.5 µg/ml, 2.5 µg/ml and 5.0 µg/ml) observed across varied time intervals (12 hrs, 24 hrs and 48 hrs) is indicated by graph with pink line while the graphs with green line indicate response of cells without protein stimulation. Panel B - Enhanced Fas expression on cell surface after stimulation with rHP986 as compared to cells without recombinant protein stimulus. Panel C - Quantification of apoptosis in the cells treated with increasing concentration of rHP986 for varied time intervals. Results are represented as (mean±SE) percent apoptotic cells per 300 cells. Panel D - Morphological identification of apoptotic cells by acridine orange and ethidium bromide staining method. Arrow head indicates live cells with normal morphology (green fluorescence) and arrow shows cells that have undergone apoptosis after stimulation with rHP986. Black arrows indicate condensed marginal nucleus, apoptotic body formation and membrane blebbing. Panel E - Bar diagram showing inhibition of apoptosis when the cells were stimulated with rHP986 in the presence of neutralizing antibody against TNFR1 (rHP986+TNFR1) as compared to cells stimulated with only rHP986. Oleandrin was used as positive control; US - unstimulated cells. Results are shown as mean ± SE.
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H. pylori considerably recruits and excites macrophages in the gastric submucosa to initiate a chronic and persistent trail of inflammatory activities leading to certain patho-physiological changes [38,39].

In our observation, presence of HP986 gene was found to be significantly associated with invasive disease outcomes (ulcer and gastric cancer) as compared to gastritis (Figure 1 B); this contrasts a previous report [24] about its prevalence in strains linked to gastritis cases alone. In our study, low positivity was recorded in gastritis causing strains from Peru and South Africa (Figure 1 C). Moreover, the presence of HP986 was found to be independent of *cag*A and *vac*A status of the strains tested by us.

Despite traditionally high allelic diversity in *H. pylori*, HP986 was found to be evolutionarily conserved as observed for a period of ten years in strains isolated from different niches of the stomach of a single patient (Figure 1A) [25]. This suggests conserved maintenance of the locus in the genome thereby pointing to its essential role in pathobiology of *H. pylori*.

Given our theoretical observations on high antigenicity of HP986, *in silico* analysis showed several putative B-cell epitopes; we experimentally tested its ability to elicit humoral and cellular immune responses. Significant humoral immune responses induced by HP986 may be important in diagnostic development given the fact that many candidate antigens have suffered due to high genetic variability across different regions and cross reactivity with other related organisms [40]. Our analysis showed significantly high antibody titers in *H. pylori* infected invasive disease patients when compared with healthy controls or non-infected individuals (Figure 1D). This points to the extracellular abundance of HP986 protein and its role in stimulating immune response. Further, since the NF-κB activation in *H. pylori* is often type IV dependent, we can not rule out secretion of HP986 protein through a type IV secretary system. Alternatively, it is possible that the protein might be directly released into the extracellular space in the aftermath of autolysis [41,42].

While considering the fact that *H. pylori* proteins released into extracellular space may find their way into the submucosa and augment proinflammatory signaling [39], we looked at rHP986 to potentially augment proinflammatory cytokine secretion from macrophages (IL-8 and TNF-α) consequent to NF-κB activation. These presumptions are consistent with an earlier study describing effect of *H. pylori* or its products on NF-κB (p65/p50) mediated transactivation of IL-8 [6]. Interestingly, our observed stimulation of IL-8 was found to be secondary to TNF-α secretion; maximal concentration was detected as early as 6 hrs (Figure 3 D, H) as compared to 12 hrs for IL-8 (Figure 3 B, F). The apparent

decrease in TNF-α concentration may be due to the binding of TNF-α with the soluble TNF receptors. As a consequence, less TNF-α concentration was detected in the culture supernatant after 12 hours. Based on these findings, we propose the role of HP986 in cytokine mediated gastric injury in a similar way as shown previously for the airway epithelial inflammation triggered by *Staphylococus* protein-A [43]. The effect was consistent when tested in both Thp1 differentiated macrophages (Figure 3 A–D) and human PBMCs (Figure 3 E–H). It is known that *H. pylori* infection disrupts tight intracellular junctions and transports its products into the gastric submucosal space to augment infiltration of mononuclear cells [44]. This strategy perhaps helps the bacterium to establish persistent infection as it feeds on inflammatory exudates for carbon source derived from mucosal sugars and thereby gains a niche. Also, it inhibits expansion of antigen specific T-cells as a mechanism of immune evasion [15]. Studies support the notion that IL-8 activity attracts mononuclear cells, and TNF-α triggers Fas mediated apoptosis of activated macrophages [45,46]. TNF-α works in an autocrine fashion by up regulating Fas expression on the surface of activated macrophages by binding with TNFR1 [13] and shares a hierarchy of downstream events (FADD) leading to apoptosis [14,47]. Nevertheless, there are contradictory reports supporting pro and anti apoptotic roles of Fas in different cell lineages but antigen behavior also determines cell fate [48]. Our observed synergistic function of TNFR1 and Fas is consistent with the previous findings [46,49]. Having identified the dependency of Fas on TNFR1, we anticipated that rHP986 actually binds to TNFR1 and thus mimics signalling through TNF-α. We confirmed this interaction through a series of immunological and biophysical measurement and our binding results were in accordance with computational modeling of HP986 and TNFR1 interaction; Also, we could not observe any binding with TLR4 or TLR2 (see results).

Taken together, these observations allow us to propose a model of putative bacterial strategy (Figure 6) harnessed for survival and possibly for maintaining a balance between recruitment and activation of macrophages and their suppression by TNFR1 mediated apoptosis. This strategy potentially projects HP986 as an important player involved in both proinflammatory and apoptotic cascades. It is therefore highly probable that HP986 is a novel virulence factor and possibly an important effecter in gastritis and peptic ulcer disease and various other outcomes of chronic *H. pylori* infection such as gastric adenocarcinoma. While we do not know how many such virulence factors operate behinds the pathology triggered by this manipulative pathogen, understanding of each of them in depth is necessary to devise strategies to control progression of the infection towards more serious outcomes. Future efforts are indeed necessary to understand molecular structure of this protein to gain insights into intricacies of its function and how its role is regulated *in vivo*.

Materials and Methods

Ethics statement

The study was approved by the Institutional Biosafety Committee of the University of Hyderabad, India. Informed consents were obtained from all patients whose sera samples were used (all patients sampled were adults).

Geographic distribution of the locus Hp986 and its genetic stability

Distribution of hp986 gene was analyzed in clinical isolates from different diseased subjects (gastritis n = 152, duodenal ulcers n = 68, gastric cancer n = 27) belonging to various geographical regions (India, Spain, South Africa, Japan, France, Peru, Ireland, England, Costa-Rica, Indo-Tibet and Bangladesh). PCR was performed using gene specific primers as described earlier [24].

Computational modelling of HP986 protein-protein interactions

The 3D structure of query protein was predicted by automated homology modelling program, Modeller9v8 [26]. The protein template 3D structures used in the study were downloaded from RCSB Protein Data Bank (PDB). Amino acid sequences of HP986 were aligned with PDB ID 1XMX to derive the predicted secondary structure using the online tool, ESPript [50]. The geometry of model was checked with PROCHECK tool available with PDBsum program [51]. Molecular visualization and general analysis were done using the program PyMOL [52]. *In silico* docking experiments were performed using PatchDock [30] and then further refined and ranked with FireDock [31]. In crystal structure, the unliganded TNFR1 (PDB ID: 1NCF) exists as a dimer, and therefore only one molecule of TNFR1 (receptor) was taken for unbound protein-protein docking with HP986 model (ligand) under default complex-type settings.

Cloning, expression and purification of HP986

The construct for recombinant protein expression was generated by cloning the PCR product spanning 711 bp at site *XhoI/HindIII* of pRSETA. The construct was then propagated into *E.coli* BL-21 (DE3) expression host and the over expressed his-tagged protein was purified by affinity chromatography (Ni^{2+}-NTA, Qiagen) [53]. Further size exclusion chromatography was performed using Superose -1210/300 GL column (GE Healthcare Ltd.) in buffer containing 20 mM Tris-Cl and 300 mM NaCl pH 8.0. The recombinant protein was quantified using Bradford's reagent [54]. Also, purified rHP986 was treated with polymixin-B to remove possible endotoxin contamination.

Analysis for humoral responses

A total of 70 human sera were collected after obtaining informed consents from different subjects having endoscopically proven gastritis, peptic ulcer and gastric carcinoma. Sera from subjects reported to be *H. pylori* negative (as ascertained by C^{14}urea breath test) were used as negative control (n = 17). Humoral response against the rHP986 was determined by Enzyme linked immunosorbent assay (ELISA) as described previously [53]. Concentration of recombinant protein and sera was predetermined using serial dilutions to obtain optimum antibody titers (data not shown). Each ELISA experiment was repeated at least thrice with and without replicates.

Cell culture experiments

Approximately 1×10^6 human monocyte cells per well (Thp1) (ATCC, USA) were differentiation into adherent macrophage like phenotype using phorbol-12 myristate 13 acetate at a concentration of 5 ng/ml (Sigma, USA). These cells were induced using increasing concentration of rHP986 (0.5 μg, 1.0 μg, 2.5 μg, 5.0 μg, and 10 μg/ml) and incubated for varied time interval. Cells without protein stimulus (unstimulated cells) and the cells stimulated with proteinase K-treated rHP986 served as negative control. LPS (*E. coli*, Sigma) treated cells were used as positive control. A non relevant control was the His-tagged ICD from *H. pylori* purified under similar conditions. Culture medium collected at different time intervals (6 hrs, 12 hrs, 24 hrs and 48 hrs) was stored at -80°C until assayed.

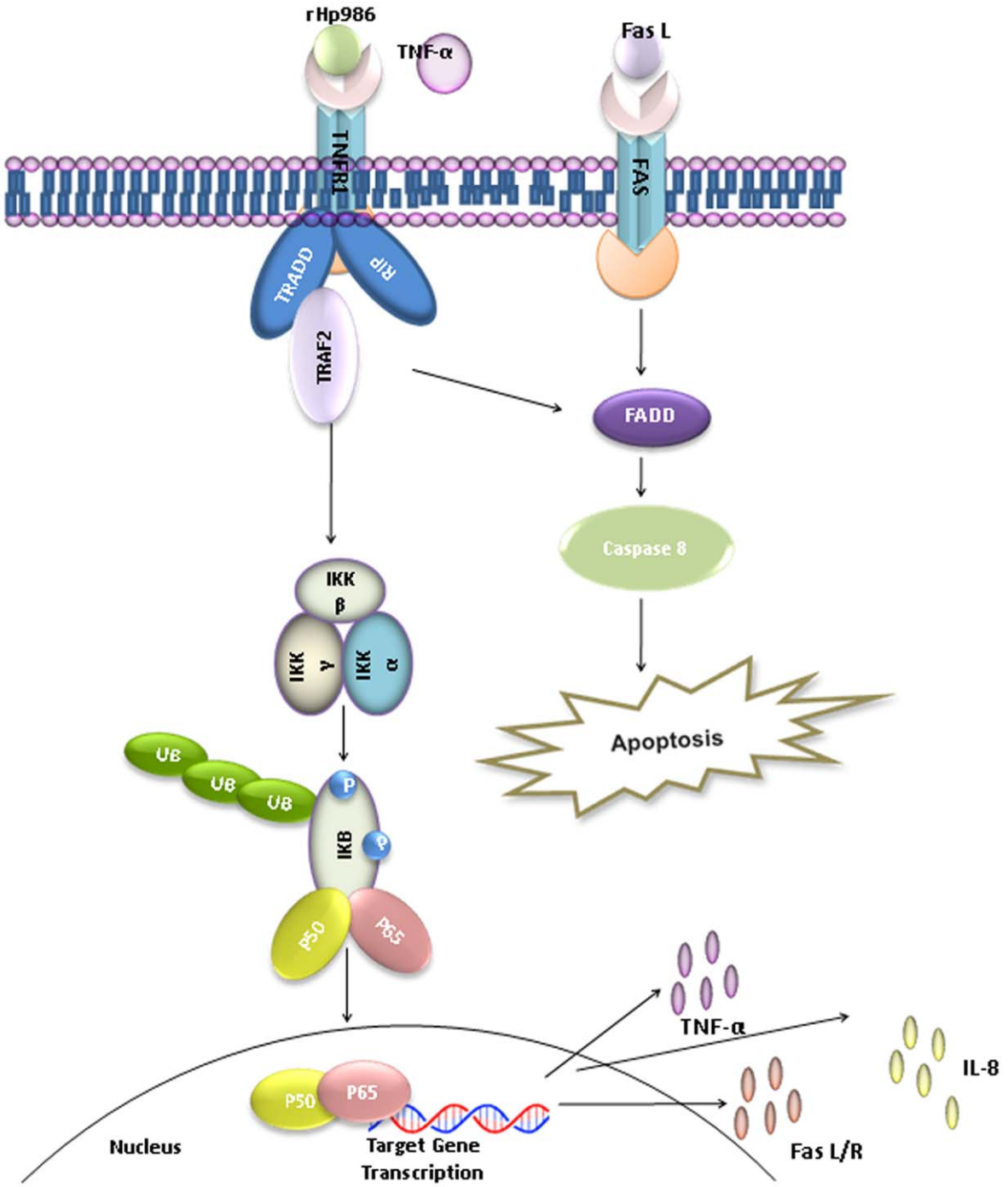

Figure 6. Schematic representation of major signaling pathways initiated following the binding of HP986 with TNFR1. The proposed interactions possibly activate downstream signaling cascades leading to macrophage apoptosis and induction of proinflammatory cytokines. TRADD: TNFR1 associated death domain, FADD: Fas- associated death domain, TRAF2: TNFR- associated factor 2, RIP: Receptor interacting protein.
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Polymorphonuclear blood monocyte cell (PBMC) culture

PBMCs were isolated from heparinized venous blood taken from a voluntary donor using ficoll-histopaque density gradient as described previously [55]. The cell viability was checked by trypan blue dye exclusion method and was found to be 90%. Approximately 0.5 million cells/well were seeded in 24 well plate in RPMI 1640 media supplemented with 10%FBS and 2 mM glutamine. Cells were treated with rHP986 protein as described above.

Cytokine assay

Amount of IL-8 and TNF-α secreted in the culture medium was determined using commercially available optEIA ELISA Kit (BD Biosciences, USA) as per manufacturer's instruction. The cytokine

levels were calculated using the recombinant standard provided within the kit.

Cell extract preparation and Electrophoretic mobility shift assay

Cytoplasmic and nuclear extracts were prepared and translocation of NF-κB complex was determined by electrophoretic mobility shift assay (EMSA) as described earlier [56]. Unlabeled NF-κB consensus probe was used as specific competitor. For supershift assay 5-10 µg of rabbit polyclonal anti p65, p50, c-Rel antibodies (Santacruz Biotechnology, USA) were used. After electrophoresis the gel was dried and analyzed by autoradiography.

Immunobloting

Immunobloting was performed as described previously [57]. Different antibodies such as those against p65, p50, c-Rel, TNFR1, TLR4 and TLR2 (Santacruz Biotechnology, USA) were used. Immunoreactive proteins were detected using enhanced chemiluminescence kit according to manufacturer's instructions (Amersham Inc., USA). β-Actin was used to confirm equal loading of the samples.

rHP986 binding assay by flow cytometry

Binding of rHP986 with cell surface receptors such as TLR4, TLR2 (Imgenex, USA) and TNFR1(Santacruz Biotechnology, USA) was analyzed using specific antibodies by flow cytometry. FITC conjugated mouse IgG1 antibody (Santacruz Biotechnology, USA) was used as isotype-matched control Antibody. At least 10,000 cells were scanned per sample.

Immunoprecipitation

0.1 mg/ml of cell extract was incubated with rHP986 overnight at 4°C and the immune complexes were trapped using Talon resin (Clontech, USA) or protein A/G agarose beads (Santa Cruz, USA). Immune complexes were separated on 10% SDS-PAGE and the immunoblot was developed using enhanced chemiluminescence kit (Amersham Inc, USA). Recombinant ICD from *H. pylori* and LPS were used as controls.

Kinetic analysis of interaction using Surface Plasmon Resonance (SPR)

Binding kinetics of rHP986 with soluble humanTNFR1 (hu TNFR1) were analyzed using BIAcore® 3000 SPR system (GE Healthcare Ltd.). Human TNFR1/Fc (Sigma, USA) was immobilized by an amine-coupling method over a research grade CM5 sensor chip (BiAcore, Uppsala, Sweden) up to a resonance unit of 150. A reference surface was used as a blank to correct instrumental and buffer effects prior to protein injection. During the association phase, the purified rHP986 had been serially diluted in running buffer (PBS, BiAcore, Uppsala, Sweden) at 100 nM, 400 nM, 800 nM, and 1200 nM and were allowed to pass individually over the immobilized TNFR1 at a flow rate of 30 µl/min for 3 minutes. During the dissociation phase, PBS solution was applied to the sensor chip at a flow rate of 30 µl/min for 4 minutes. The sensor surface was regenerated between each binding reactions by two washes of 30 s each with 5 M NaOH as evaluated by baseline response. The data was analyzed with BIAEVALUATION 4.1 software (GE Healthcare Ltd.) using simple 1:1 Langmuir interaction model.

Assay of CD95 for rHP986 mediated apoptosis

Expression of CD95 was analyzed by flow cytometry using FITC conjugated anti-Fas monoclonal antibody (CD95, BD) [58]. FITC conjugated mouse IgG1 antibody (Santa Cruz) was used as isotype-matched control antibody. Cells stimulated in the presence of neutralizing antibody to TNFR1 (50 µg/ml) were used to check the involvement of TNFR1 in the regulation of Fas expression.

Analysis of CD95 expression by Immunocytochemistry

Cells were sedimented on glass slide and fixed in 1% paraformaldehyde at 4°C for 30 minutes. For antigen staining cells were incubated with rabbit polyclonal anti-Fas antibody (1:300, Santa Cruz, USA) for 1–2 hours at 37°C. Unbound antibody was washed off with PBS and the cells were further incubated with Alexafluor [44] conjugated anti-rabbit IgG (1:200, Molecular probes, USA) for 30 minutes at room temperature [10]. Enhanced Fas expression was analyzed by fluorescence microscopy (Ziess epifluorescence microscope).

Analysis of apoptosis induction by rHP986

Apoptosis assays were performed using acridine orange/ethidium bromide staining [11] and annexinV kit method (BD pharmingen, USA). For acridine orange and ethidium bromide assay, approximately 100 cells were counted in three randomly selected fields and the rate of apoptosis was expressed as mean percentage of total 300 cells counted. For annexinV, percentages of apoptotic cells were expressed as total % of annexinV^+ and PI^+ cells after subtracting background fluorescence [46]. Cells treated with rHP986 in presence and absence of neutralizing antibodies to TNF-α and TNFR1 were used to determine the role of rHP986 in the induction of apoptosis. Oleandrin, a known potent inducer of apoptosis [34] was used as a positive control. Lysate of the cells not exposed to rHP986 served as negative control.

Statistical Analysis

Results were expressed as means ± the standard error (SE). Induction of cytokine levels and the rate of apoptosis were compared using a two-tailed Student's *t* test and considered significant if the *P* values were <0.05. *P* values were calculated using the online Graph Pad scientific calculator (http://www.graphpad.com_quickcalcs_ttest1.cfm).

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Author Contributions

Conceived and designed the experiments: NA. Performed the experiments: AA SAA MR SD. Analyzed the data: NA SD IAQ. Contributed reagents/materials/analysis tools: IAQ NZE LAS SEH. Wrote the paper: NA.

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