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EXPRESSION OF INTERLEUKIN-16 IN GASTRIC MUCOSA: A POSSIBLE ROLE IN THE PERSISTANCE OF *HELICOBACTER PYLORI* INFECTION

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EXPRESSION OF INTERLEUKIN-16 IN GASTRIC MUCOSA:

A POSSIBLE ROLE IN THE PERSISTANCE OF *HELICOBACTER PYLORI* INFECTION

by

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Dedication

This thesis work is dedicated to all my family members especially my loving parents Abdurrahman Alzahrani and Latifa Aljuead for their continuous support, sacrifices and encouragement.

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Helicobacter pylori (*H. pylori*) infects the human gastric mucosa of >50% of humankind and causes chronic gastritis, peptic ulcers, and gastric adenocarcinoma. *H. pylori* infected gastric epithelial cells (GECs) produce an array of chemokines that recruit other cells. IL-16 is potentially a key chemokine not yet examined during *H. pylori* infection. IL-16 selectively recruits CD^{4+} T cells since it uses CD4 as a receptor; but makes them unresponsive to antigen, which could explain failed vaccine clinical trials. As T cells from the *H. pylori*-infected gastric mucosa are hyporesponsive, I wanted to investigate the production of IL-16 by GECs, the mechanisms underlying IL-16 production and the role of gastric IL-16 in the persistent of *H. pylori*-infection. IL-16 expression by GECs was analyzed using FACS and RT-PCR following infection with *H. pylori* as well as mediators present during infection. I observed that *H. pylori* increases expression of IL-16 in different gastric epithelial cell lines. Also, histamine, IL-8 and IFN-γ increased IL-16 expression. These findings uncover relevant information pertaining to the accumulation of $CD4^{+}$ T cells that is observed in patients' gastric mucosa with *H. pylori* and could explain why clinical trials have failed in efforts to develop a vaccine to this important pathogen**.**

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ADAM	A disintegrin and metalloproteinase)
AKT	Protein Kinase B
APCs	Antigen presenting cells
aPKC	Atypical protein kinase C
ASPP1	Apoptosis-stimulating protein of p35
BabA	Blood group antigen-binding adhesion
CagA	Cytotoxin-associated gene A
CLR's	C-type lectin receptors
Cag PAI	*cag* pathogenicity Island
DC	Dendritic cell
ECL	Enterochromaffine-like cells
EGFR	epidermal growth factor
EGFR	epidermal growth factor receptors
EPIYA motifs	Glu-Pro-Ile-Tyr-Ala
ERK	Extracellular signal regulated kinase
ETP	Ectoposide
FoxP3+	Forkhead box protein p3
GC	Gastric cancer
Gab1	Grb2-assosiated binder 1
Gbr2	Growth factor receptor bound 2
GECs	gastric epithelial cells
Git1	G protein-coupled receptor kinase-interactor 1
GM-CSF	Granulocyte-monocyte colony stimulating factor
H. pylori	*Helicobacter pylori*
Hor	Hop-related

HtrA	High Temperature Requirement A
IFN	Interferon
IL-	Interleukin-
JAM	Junctional adhesion protein
Leb	Lewis^{b} antigens
LEF	lymphoid enhancer factor
Le	Lewis
MAPs	Mitogen-activated proteins
MAPKS	Mitogen-activated protein kinases
MALToma	Mucosal associated tissue lymphoma
MCL-1	Myeloid Cell Luekemia-1
MCP-1	Monocyte chemoattractant protein-1
MHC	Class II major histocompatibility complex
MIF	Macrophages migration inhibitory factor
NFAT	Nuclear factor of activated T-cells
OMPs	Outer membrane proteins
OipA	Outer inflammatory protein A
PAMPs	Pathogen-Associated Molecular Patterns
PAR1	Partitioning-defective 1 kinases
p-ERK	phosphorylated ERK
PGE2	Prostaglandin E2
PI3K	phosphatidylinositol 3-kinase
PLC	Phospholipase C
PUD	Peptic ulcer disease
ROS	Reactive oxygen species
RT-PCR	Real time PCR
SabA	Sialic acid binding adhesion protein

SH. PYLORI-2	Src Homology **2**domains
sLex	Sialyl-Lewisx antigen
SRF	serum response factor
T4SS	Type 4-secretion system
TCF	T cell factor
TGF	Transforming growth factor
Th-	T helper cell
TLRs	Toll-like receptors
UTMB	university of Texas medical branch
VacA	Vacuolating toxin A
ZO-1	Zonula occludens

INTRODUCTION

Helicobacter pylori (*H. pylori*) is perhaps the second most common human pathogen in the world. *H. pylori* infection occurs more frequently during childhood, and untreated infections usually persist for life[2]. Low hygienic standard, poor socioeconomic level, sharing a bed and having an infected family member are common risk factors to develop *H. pylori* infection[3-6].

This Gram-negative gastroduodenal pathogen infects more than half of the world's population and responsible for 90 % of gastric and duodenal ulcer in addition to its linkage with gastric malignancy[1]. Due to the epidemiological association of *H. pylori* infection with increased risk of development of gastric cancer (GC), the World Health Organization classified *H. pylori* as a class I carcinogen[2].

Usually this pathogen is treated by antibiotic, but antimicrobial resistance is considered as the primary reason for failure of eradication therapies[3]. Because of its bacterial etiology, GC could be preventable with an effective vaccine, but the few vaccine trials done with human volunteers led to disappointing results[4] possibly due to our limited knowledge of the interplay between *H. pylori* and immune system defenses, and how *H. pylori* abates effector T cells, and consequently adaptive immunity leading to chronic infection.

Helicobacter pylori (*H. pylori*) colonization is mainly limited to the antrum of the stomach, devoid of parietal cells (**Figure 1.1**). Colonization of the gastric mucosa lead to infiltration of macrophages, neutrophils, B cells and T cells[5, 6], However, this massive immune response are not sufficient to eradicate the infection, which suggest that *H. pylori* avoid or manipulate the host response to sustain the infection.

$CD4^+$ T cells are important components of the *H. pylori*-induced immune response. Previous report showed that *H. pylori*-specific $CD4^+$ T cells preferentially home to and accumulate in the infected stomach[7], but little is currently known about how these cells are recruited to the infected mucosa. Despite increasing the numbers of $CD4^+$ T cells in the gastric lamina propria during *H. pylori* infection, these T cells are hyporesponsive, and polarized toward Th1 response[8]. These Th1 cells produce gamma

interferon, which is essential in the proinflammatory responses induced during the *H. pylori* infection. Previous reports showed that T cells exposed to *H. pylori* exhibit decreased proliferation rates[9], and *H. pylori*-exposed GEC incubated with isolated $CD4^+$ T cells resulted in impaired proliferation and IL-2 production[10]. While some studies suggest *H. pylori* may suppress human T cells, clearly *H. pylori* is not in direct contact with lamina propria (LP) T cells. *H. pylori* resides in the gastric lumen and is separated by the epithelial barrier. Thus, *H. pylori* uses the gastric epithelium as a fulcrum to convey information to T cells in the LP that affect their function and differentiation.

During infection with *H. pylori*, gastric epithelial cells (GECs) produce an array of chemokines that recruit other cells. Interleukin IL-16 (IL-16) is potentially a key chemokine not yet examined during *H. pylori* infection. This cytokine has multiple effects on $CD4^+$ T cells including induction of chemotaxis, Interleukin-2 receptor and HLA-DR expression, suppression HIV replication and subsequent $CD4^+$ T cell activation by antigens. Because of its function, IL-16 was originally described as lymphocytes chemoattractant factor. However, IL-16 requires the expression of CD4 for its functions. Unlike other chemokines, which do not differentiate among distinct cell phenotypes as long as they express the corresponding receptors, IL-16 selectively recruits $CD4^+$ T cells[11]. IL-16 is a multifunctional cytokine that uses CD4 as a receptor and is chemotactic for $CD4^+$ cells; but makes them unresponsive to antigen (reviewed in[11]). Interestingly, a recent study reported that human bronchial epithelial cells have the capacity to express IL-16 after stimulation with histamine, IL-1β and TNF-α, which suggest the involvement of IL-16 in airway mucosal inflammation, and especially in accumulation of eosinophils and $CD4^+$ T cells that are observed in patients with bronchial asthma[12].

Thus, in this study I sought to investigate the possibility that GECs produce IL-16 during *H. pylori* infection, which in turn leads to the accumulation of $CD4^+$ T cells and impairment of T cells responses that are observed in patients with *H. pylori.* Thus, our lab compared IL-16 expression by GECs isolated from biopsies from *H. pylori*-infected versus uninfected subjects and found that IL-16 expression is higher in GECs from *H. pylori* infected subjects. Subsequently, I investigated the direct impact of *H. pylori* on IL-

16 expression by human GECs. I analyzed messenger RNA (mRNA) from human GECs and IL-16 protein their culture supernatants, using real-time polymerase chain reaction RT-PCR and enzyme linked immunosorbent assay (ELISA), respectively. My study showed that human GECs express IL-16 and its expression increased upon *H. pylori* infection. The mechanisms which stimulate IL-16 production are not understood; but some studies have reported that IL-16 is released from cells upon stimulation with antigens, mitogens, histamine, and serotonin [13, 14]. Some of these factors are involved in *H. pylori* associated diseases. In an attempt to investigate the mechanisms responsible for the increased IL-16 expression in *H. pylori* infected patients, I treated GECs with different stimuli present during *H. pylori*-associated diseases and the cells and their culture supernatants were respectively analyzed by RT-PCR and ELISA for IL-16 expression. I found that inflammatory mediators, such as IFN-γ, histamine and interleukin-8 (IL-8) induced IL-16 production by GECs. Also, by using R6K, CXCR2 blocking agent antagonist of IL-8, I further confirmed that the expression of IL-16 is mediated by IL-8, which is produced by GECs during *H. pylori* infection. In conclusion, the results from these studies demonstrate that GECs have the capacity to express IL-16 during *H. pylori* infection and after stimulation with inflammatory mediators present during *H. pylori* associated diseases. These findings and the observation that IL-16 is present in the gastric mucosa of *H. pylori* infected patients, suggest that epithelial derived IL-16 protein may be involved in gastric mucosal inflammation and, especially, in the accumulation of $CD4^+$ T cells that are observed in individuals with *H. pylori* infection. These observations also suggest that therapeutics aimed at blocking IL-16 synthesis or function might be valuable to consider as adjuvants in vaccines that target *H. pylori*.

Chapter 1: Review of the Literature

1.1 MECHANISMS OF *H. PYLORI* ADHESION TO THE GASTRIC EPITHELIUM

An essential step in the colonization by *H. pylori* and its ability to mediate effects on the gastric epithelium is its selective tissue tropism leading to the establishment of intimate interactions with the epithelial surface. These interactions are largely mediated via outer membrane proteins (OMPs) that serve as adhesins. The *H. pylori* genome has more than 30 genes which encode OMPs that are divided into Hop (Helicobacter outer membrane proteins) and Hor (hop-related) subgroups. The Hop group of proteins contains *H. pylori* adhesion molecules such as BabA, SabA, AlpA/B, HopZ and OipA[15]

BabA

The blood group antigen-binding adhesion BabA was the first *H. pylori* adhesin discovered[16]. It facilitates the adherence of *H. pylori* to Lewis^b antigens (Le^b), an ABO blood group antigen that is expressed on the gastric mucosa. Binding of *H. pylori* to Le^b on the epithelial surfaces *via* BabA enhances the type 4 secretion system (T4SS)'s ability to exert the pathogencity of *H. pylori* that includes triggering production of proinflammatory cytokines[17], a well-established response of the epithelium to the infection. Therefore, the expression of BabA adhesion is closely associated with the onset of T4SS-related host cell responses, since it increases the delivery of *H. pylori* virulence factors and promotes inflammation[15].

SabA

H. pylori infection leads to an increase in gastric epithelial expression of sialyl-dimeric-Lewis^x glycosphingolipid. This molecule serves as a receptor for the bacteria, which uses sialic acid binding adhesion protein (SabA), an outer membrane protein, to bind to sialyl-Lewis^X[18]. In the early stages of infection, binding of BabA to Le^b/ABO is essential, however, with increased inflammation, the expression sialyl-Lewis^x antigen (sLe^x) also increases; thus, *H pylori* SabA enhances the adherence to the inflamed gastric

mucosa. SabA expression switches “on” or “off”, which may suggest that SabA up-regulation, may be determined by the shifting conditions of the stomach environment. Interestingly, SabA expression is closely associated with development of intestinal metaplasia, gastric atrophy and gastric cancer in U.S.[19]

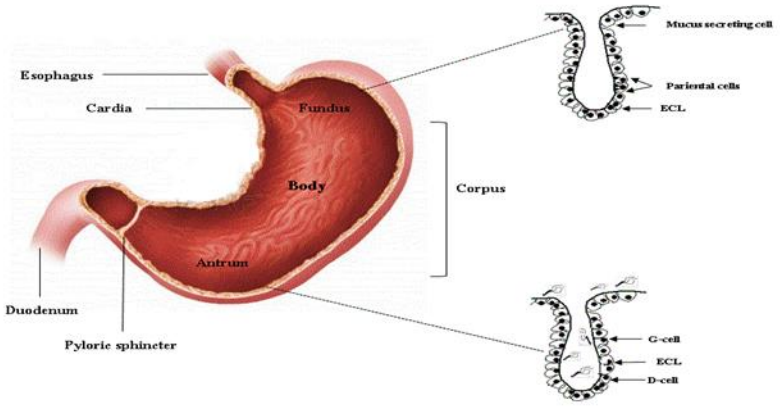

Figure1.1 *Helicobacter pylori* colonization the antrum of the stomach.

AlpA/B

AlpA and AlpB are two homologous genes that encode OMPs involved in *H. pylori* adhesion[20]. Both AlpA and AlpB proteins have been found to adhere to mouse laminin in an *in vitro* study[21], but no other adhesion receptor for AlpA/B has been recognized. Our studies with human gastric epithelial cells confirmed by flow cytometry that AlpA/B were involved in cellular adhesion and modulate proinflammatory intracellular signaling cascades[22]. This was confirmed when *alpAB* deletion mutants poorly colonized the gastric mucosa of C57BL/6 mice and induced lower mucosal levels of KC and IL-6.

HopZ

The *hopZ* gene product is another *H. pylori* adhesin protein that is located at the bacterial surface[15]. HopZ was described as a bacterial adhesin protein since the *hopZ* mutant strain reduced adherence to AGS cells. Similar to AlpA/B, the host binding

receptor for HopZ is still unknown. The *hopZ* gene is phase variable as a result of a CT dinucleotide repeat in the region that codes for the signal sequence[23]. The *hopZ* gene is also transcriptionally regulated by changing pH[24]and by contact with gastric epithelial cells[25].

OipA

The outer inflammatory protein A (OipA) is a 34kDa cell surface protein encoded by the *hopH* gene and was initially described as a promoter for IL-8 production in T4SS-independent manner and increased inflammation[26]. OipA expression was shown to be controlled by a slipped-strand repair mechanism related to the number of CT dinucleotide repeats at the 5′ end of the gene and is correlated with severe clinical outcomes, including duodenal ulcer and gastric cancer[26, 27] . Subsequent studies showed that *oipA* mutant strains have reduced adherence to gastric cancer cell lines, AGS and Kato-III although the host receptor for the OipA adhesion has not been identified[26].

2.2 *H. PYLORI* VIRULENCE FACTORS THAT AFFECT THE GASTRIC EPITHELIUM

Among the armamentarium of virulence factors expressed by *H. pylori*, in addition to the adhesins described above, there are bacterial products that are translocated into host epithelial cells *via* a type 4 secretion system (T4SS) encoded within the cag pathogenicity island (*cag* PAI) as well as factors that are secreted by *H. pylori* and exert their effects independently of the intact bacteria interacting with the epithelium. In fact, it has been estimated that at any given time only 20% of the colonizing bacteria are in direct interaction with the epithelium[28].

Cag PAI

The *H. pylori cag* pathogenicity island is perhaps the most studied virulence factor. *Cag* PAI consists of a cluster of 31 genes, most of which code for a T4SS. The T4SS is in essence a needle-like structure that penetrates the epithelial cell membrane and translocates *H. pylori* products into epithelial cells. One of the products injected is the effector protein CagA[29], which is encoded at one end of *cag*PAI and does not seem to

have homologues in other bacterial species. CagA is perhaps the most virulent factor associated with *H. pylori* and its presence in an infecting strain is regarded as a risk factor for peptic ulcer disease and gastric cancer. Once CagA is delivered into host epithelial cells, it undergoes tyrosine phosphorylation at Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs in the C-terminus by Src and Abl kinases[30, 31] and initiate signaling events described below in detail. There are four major types of EPIYA motifs (A, B, C, and D) based on the specific amino acid sequence bordering the EPIYA motif on both sides [32]. EPIYA-A, EPIYA-B and EPIYA-C motifs in tandem are found in "western strains" while CagA with EPIYA-A, EPIYA-B and EPIYA-D motifs are found in "East-Asian strains"[33]. It should be pointed out that these motifs contribute to the polymorphism in the C-terminus of the protein and are found as tandem repeats ranging in number from one to seven[34]. The quantity of EPIYA motifs is proportional to the levels of phosphorylation and the effects seen in epithelial cells[35], which will be described in detail below. These properties of CagA and its role in pathogenesis are well known and accepted by experts in the field.

In addition to the CagA protein, bacterial cell wall components such as peptidoglycan or muropeptides are also translocated via the T4SS into host epithelial cells. *H. pylori* peptidoglycan is, in turn, recognized by NOD1, an intracellular pathogen-associated molecular pattern (PAMP) recognition receptor that senses peptidoglycan which induces NF-κB activation and upregulation of proinflammatory immune responses[36].

Recently, various reports have brought to light the function of CagL, another *cag*PAI encoded protein[37-40]. Early researches defined *cagL* as a gene that is necessary for CagA delivery, therefore it was considered as a component of T4SS. More recently, it was identified as one component of pilus structures that develop at the interface between gastric epithelial cells and *H. pylori*[38] . Importantly, CagL enhances the binding of the T4SS to a5β1 integrin receptor on gastric epithelial cells,[37-40]. Other *cag*-encoded proteins, such as CagY, CagI and CagA can also bind to integrin[41]. This binding results in cellular alterations, some of which are described below, such as cell spreading, formation of focal adhesion, and activation of tyrosine kinases[38]. In light of these

important properties by these more recently described products of *cag* PAI, they represent interesting targets for translational studies in the development of a vaccine.

VacA

The vacuole-inducing toxin (VacA) is major *H. pylori* secreted protein without a known homologue in other bacterial species. VacA is initially made as a 140 kDa pro-toxin containing an N-terminal signal peptide, a central region that forms the toxin and a C-terminal domain responsible for transport function. After processing, the central region (~88 kDa), which is the mature virulent form of the toxin, is secreted and further processed into two subunits of 33 (A subunit) and 55 kDa (B subunit), respectively. It has been suggested that the p33 form is the pore forming subunit, while the p55 form was initially regarded as the cell binding component [42, 43]. However, now both subunits are known to contribute to both binding and vacuole formation[44, 45].The exact mechanism of entry is still in question as multiple receptors have been proposed, but binding to sphingomyelin appears to aid in the process[46].

Although all strains of *H. pylori* have the *vacA* gene, there is a great deal of diversity in the gene, which includes three regions: signal (s), mid-(m) and intermediate (i)-regions. There are two allelic types for each region and most virulent strains have the s1, i1, and m1 alleles, which are associated with the highest risk of gastric adenocarcinoma[47, 48].

Urease

H. pylori urease is possibly the most abundant protein expressed by the bacteria as it represents 10% of the total protein by weight[49]. Urease is a high molecular weight multisubunit enzyme made of two subunits A and B that have molecular weights of 29.5 and 66 kDa, respectively[50, 51]. The enzyme is actually a dodecameric aggregate containing six of each of the subunits resulting in a molecular weight of close to 600 kDa. Urease is best known for its enzymatic activity leading to the hydrolysis of urea into ammonia and bicarbonate, which help buffer the pH in the local microenvironment.

Urease also acts as an adhesion as it binds directly to both class II major histocompatibility complex (MHC) molecules and CD74[52, 53]. Additional support for the role of urease as a bacterial adhesin was obtained by independent studies showing that urease mutants could not colonize even in hypochloridic conditions where gastric pH was maintained at neutrality[54]. Yet another study showed that as much as 50% of the urease activity is recovered from the surface of the bacteria where it could serve as an adhesin[55]. Because of these properties, urease has been a target for vaccine design that has been included in clinical trials, which unfortunately had disappointing results.

High Temperature Requirement A

HtrA is a recently described secreted product of *H. pylori* that has enzymatic activity[56]. HtrA has been shown to possess serine protease activity and one of the substrates on which it acts is on the adhesion molecule E-cadherin[56, 57]. It cleaves the ectodomain of E-cadherin and proteolysis of E-cadherin contributes to disruption of adherence junctions. Thus, HtrA disrupts epithelial barrier integrity and permits *H. pylori* to invade the intercellular space between epithelial cells. Interestingly, the E-cadherin ectodomain that is released is a key prognostic indicator in gastric cancer[58], but more work is warranted to better understand this virulence factor and its role in disease and as target for intervention. Although a substantial body of work on the adhesins described above and the virulence factors listed has led to a greater understanding on the pathogenesis of *H. pylori*, much work is still needed on these and other yet uncharacterized virulence factors in order to develop a, thus far elusive, anti-*H. pylori* vaccine.

EPITHELIAL CELL SIGNALING INDUCED BY *H. PYLORI*

One of the outcomes of infection with *H. pylori* subsequent to its interaction either directly or indirectly via its soluble products is the activation of an array of epithelial cell signaling pathways. The various signaling pathways that are deregulated or activated by *H. pylori* will be described in this section with a brief introduction on their consequences for the host cell, and the corresponding cellular responses will be described in detail in the last section of this review.

Perhaps the best studied signaling pathways affected by *H. pylori* correspond to those affected by *cag* PAI positive strains of *H. pylori*, since those strains are more virulent inducing significant inflammatory responses. CagA as well as muropeptides translocated into the host epithelium *via* the T4SS together trigger several intracellular signaling pathways that result in epithelial cell gene expression as well as the production of proinflammatory cytokines and chemokines. A central mediator in the expression of these cytokines/chemokines is NF-kB, a transcription factor that is a convergence point for multiple pathways activated by *H. pylori*. In addition to its role in inducing proinflammatory cytokines/chemokines, one of its target genes Bcl-XL can suppress the mitochondrial apoptosis pathway which may lead to unregulated proliferation. Crabtree and Naumenn summarized the overall effects of *cag*PAI translocated products in the sequential activation of the IKK complex, JNK, p38 kinase, NF-kB, and AP-1 in gastric epithelial cells[59].

After CagA is transported into the GEC cytoplasm and undergoes phosphorylation at the EPIYA motifs by Src-family kinases and deregulates multiple signaling pathways that affect host cell shape and adhesion, as well as cell transformation. The type of EPIYA motif phosphorylated appears to determine the molecule that is then bound. CagA phosphorylated at EPIYA-C or -D binds to and activates SHP-2 phosphatase[60]. SHP-2, in turn, activates the mitogen-activated protein kinase (MAPK) and extracellular signal regulated kinase (ERK) pathway as well as dephosphorylates and inactivates focal adhesion kinase, FAK, a tyrosine kinase that regulates the turnover of focal adhesion spots[61] and whose inhibition leads to the elongated cell shape typical of the hummingbird cell phenotype. Interestingly, phosphorylation of EPIYA-A and EPIYA-B motifs leads to CagA binding to and activation of Csk kinase[62]. Activation of Csk, in turn, inhibits Src in a negative feedback loop. The EPIYA-B motif when phosphorylated can bind phosphatidylinositol 3-kinase (PI3K) whose activation results in stimulation of phosphatidylinositol-dependent kinase 1 that phosphorylates and activates AKT[63, 64].

The unphosphorylated form of CagA is able to interact with a number of intracellular proteins and those associations exert an array of responses ranging from pro-

inflammatory, mitogenic responses, disruption of cell-to-cell junctions and loss of gastric epithelial cell polarity. Perhaps the first interaction partner of unphosphorylated CagA is the adapter protein growth factor receptor bound 2 (Gbr2)[65], which in turn complexes with SOS to promote Ras-GTP formation and activation of MAPK/ERK signaling pathway. Unphosphorylated CagA can damage the barrier function of the epithelium by interacting with the scaffolding protein ZO-1 and the junctional adhesion protein (JAM), both of which are essential components of tight junctions, by mechanisms that will be detailed below. By disrupting the tight junctions *H. pylori* gains access to epidermal growth factor receptors (EGFR) as well as Her2/Neu on the basolateral membranes. CagA may also activate the calcium-dependent serine/threonine phosphatase calcineurin, which promotes mobilization of the nuclear factor of activated T cells (NFAT) from the cytoplasm into the nucleus of gastric epithelial cells. NFAT in turn activates a number of genes, such as p21Cip1, a cyclin-dependent kinase inhibitor[66]. CagA also induces cell scattering, also referred to as the "motogenic response", by binding to the hepatocyte growth factor/scatter factor receptor, c-Met[67]. This mechanism is mediated *via cag*PAI-dependent activation of Rho-GTPases Rac1 and Cdc42. In addition, this response involves ERK1/2 and MEK1/2 activation *via* extracellular *H. pylori* signaling[68, 69]. Downstream effects of binding to the c-Met receptor include tyrosine phosphorylation of Grb2-associated binder 1 (Gab1), which leads to activation of several intracellular signals like Grb2, PI3-K, PLCγ, and SHP-2. An additional signaling response that appears to depend on CagA is β-catenin translocation[70]. β-catenin is a proto-oncogene that has been shown to have numerous binding partners and plays multiple roles in cellular functions.

Since cells must undergo a highly regulated process to grow, an important question lies in the interaction of *H. pylori* and its mediators with those proteins that regulate the cell cycle. *In vitro* studies showed that cyclin D1, which controls the transition between G1 and S in the cell cycle in combination with CDK4 or CDK6[71], is induced by *H. pylori* in a partially *cag* PAI-dependent manner[72]. This then leads to increased proliferation of gastric epithelial cells. As will be described in detail below, another important effect of *H. pylori* infection is the ability of *cag* PAI^+ strains to affect the balance between proliferation and apoptosis, particularly because of the role of

*cag*PAI in triggering Fas-dependent apoptosis, reduction of Bax (pro-apoptotic factor), and overexpression of Bcl-2[73, 74].

VacA referred above as a vacuolating toxin uses the 55 kDa B subunit to bind to PTP-β (aka Ptprz), a tyrosine phosphatase receptor, leading to phosphorylation of the G protein-coupled receptor kinase-interactor 1 (Git1). This has been shown to promote ulcer formation in mice. Other studies have shown that VacA binds both PTPα and PTPβ and this results in enhanced vacuole formation[75]. Lastly, VacA can also lead to apoptosis via the caspase-3 pathway because VacA form channels that lead to release of cytochrome c release.

There are signaling processes initiated in gastric epithelial cells during infection with *H. pylori* that are not the direct result of a virulence factor, but that are triggered by the binding of *H. pylori* to receptors on the surface of gastric epithelial cells. Two notable receptors used by *H. pylori* that are known to deliver intracellular signals include CD74 and class II MHC molecules[52, 53, 76, 77]. Class II MHC molecules, when engaged, have been shown to involve several intracellular signaling processes, ranging from activation of phospholipase C (PLC), the kinases PKC, Src and Syk, to the mitogen activated kinases p38 and Erk[78],and one of the outcomes is stimulation of proinflammatory cytokine secretion and delivery of proapoptotic signals. Recent studies suggest that class II MHC molecules crosstalk with TLR receptors by amplifying their signals following association of MHC class II with the costimulatory molecule CD40 and Btk, a tyrosine kinase, in the endosomes of TLR stimulated cells[79]. However, this has not been examined in the context of *H. pylori* infection. CD74-initiated signaling has been better studied and it has been shown to activate transcription mediated by the NF-κB p65/RelA homodimer and its coactivator, TAFII105[80]. In contrast to class II MHC, CD74 activates proliferation and prosurvival pathways[81], as it induces BCL-XL transcription. Our group has shown that *H. pylori* CagA induces gastric epithelial cell production of MIF [73]and MIF, which uses CD74 as a receptor, induces the NF-κB, Erk1/2, and AP-1 signaling pathways[82, 83]. Our studies showed that the *H. pylori* urease B subunit alone, after binding to CD74, induced NF-κB activation and IL-8 production[52]. Although

preliminary, these are important insights that could be exploited in intervention against *H. pylori* infection. Thus, additional research in this area is warranted.

One last important outcome of epithelial cell signaling induced by *H. pylori* is the transactivation of EGFR in gastric epithelial cells. There are several proposed mechanisms by which this may occur. The first is via "extracellular transmembrane cleavage or proHB-EGF followed by signaling of HB-EGF." Cleavage is achieved via ADAM family metalloproteinases that are zinc dependent. Another possible mechanism is via NADPH oxidase forming ROS, leading to EGFR transactivation. This transactivation occurs in both *cag* PAI positive and negative strains. In addition, *H. pylori* expresses a γ-glutamyltranspeptidase that may be "an upregulator of HB-EGF in gastric epithelial cells." ADAM family proteins are also involved in EGFR transactivation pathways involving IL-8, PGE2, gastrin, and oxidative damage from tobacco smoke.

Understanding of the different signaling pathways activated during the infection with *H. pylori* is important not only in understanding disease mechanisms, but also in defining potential therapeutic targets as suggested above.

EFFECT OF H. *PYLORI ON* GASTRIC EPITHELIAL CELL

The induction of host epithelial signaling pathways by *H. pylori* attachment or via soluble mediators has multiple consequences on the gastric epithelium that range from epithelial barrier function to proinflammatory and procarcinogenic processes, some of which are described here in detail. Another major consequence of the deregulation/activation of epithelial signaling pathways is the subversion of mechanisms important in host immune responses and impaired immunity contributes to the chronicity, which is a hallmark feature of the infection and is reviewed elsewhere in this issue.

Disruption of epithelial cells barrier by *H. pylori*

Epithelial cells form a barrier between the lumen and interstitial space. Apical junctions are critical in maintaining essential epithelial cells functions, such as maintaining apical-basolateral cell polarity, cell-cell adhesion, cell proliferation, and cell movement[84]. The overall integrity of the epithelial barrier depends on cell-cell sealing,

which is maintained by four types of junctions including tight junctions, desmosome junctions, gap junctions and adherens junctions. Tight junctions are multiprotein complexes and represent the most common type of junction in the lumen. They are important in regulating diffusion across the epithelium. Tight junctions consist of different scaffolding proteins such as zonula occludens (ZO)-1, junctional adhesion molecules (JAM)-1, claudin, and occludin **(Figure 2)**. Tight junctions are the targets for several bacterial pathogens. Some bacteria open the cell-cell junctions, while other bacteria disorganize the epithelial cell polarity, expose the basolateral surface and cause leaks at the epithelial lining. *H. pylori* disrupts epithelial cell-cell junctions, cell polarity as well as cell proliferation via binding to the epithelial cell receptors and stimulation of different signaling pathways. *H. pylori* strains possessing cag PAI, and thus expressing T4SS, use the T4SS to translocate CagA which after phosphorylation by Src family kinases binds to and activates SHP-2 phosphatase leading to multiple cellular responses. Among the multiple effects that activated SHP-2 phosphatase has on gastric epithelial cells is the alteration of the tight junctions and adherens junctions of epithelial cells[85]. CagA contributes to these alterations through interactions with different junction proteins that include E-cadherin, ZO-1 and JAM, thus affecting the normal epithelial architecture and the normal function of both tight and adherent junctions[84]. β-catenin is localized at cell-cell junctions via interaction with E-cadherin, which is a transmembrane protein. The E-cadherin/β-catenin complex has an essential role in epithelial cell-cell interaction and maintaining the normal architecture of epithelial cell tissue. However, during *H. pylori* infection translocated CagA destabilizes the E-cadherin/β-catenin complex, in a phosphorylation independent manner[86]. The mechanism whereby CagA destabilizes the E-cadherin/β-catenin complex is still unclear, but it has been suggested that CagA may compete with β-catenin for binding with E-cadherin. Also, CagA may be connected with E-cadherin indirectly through another component of the apical-junction complex, and this connection disturbs the E-cadherin/β-catenin complex. Disturbance of the E-cadherin/β-catenin complex formation by the interaction of CagA with E-cadherin leads to the release of E-cadherin from the adherent complex, buildup of β-catenin at the cytoplasm and the nucleus, and activation of β-catenin-mediated signaling that induces transformation of gastric epithelial cells[86] **(Figure 1.2).** In addition, a study to localize

the tight junction scaffolding protein ZO-1 in CagA-expressing cells indicated that translocated CagA was associated with mislocalization of ZO-1 to the basolateral membrane[87-89]. In addition to the inducing epithelial junctional defects, *H. pylori* CagA can also cause polarity defects via interaction with PAR1/MARK kinase, which preserves the polarity of epithelial cells. Partitioning-defective 1 kinases (PAR1) play an important function in sustaining the polarity of epithelial cells through phosphorylation of microtubule-associated proteins (MAPs). PAR1 has to be released from the tight junction to maintain the epithelial polarity. The dissociation of PAR1 from tight junctions occurs via atypical protein kinase C (aPKC), which binds and phosphorylates PAR1 at tight junctions. Thus, releasing of PAR1 from the membrane inhibits the penetration of PAR1 to the apical membrane. However, during *H. pylori* infection translocated CagA binds to PAR1b[90], which is one of four members of the PAR1 family of kinases, and inhibits its activity and its phosphorylation by aPKC, eventually causing junctional and polarity defects[89] **(Figure 1.2)**. The resulting disruption in epithelial cell polarity appears to be a mechanism employed by *H. pylori* to enhance its survival and growth as it permits *H. pylori* to usurp the cell surface as a place for its growth[91].

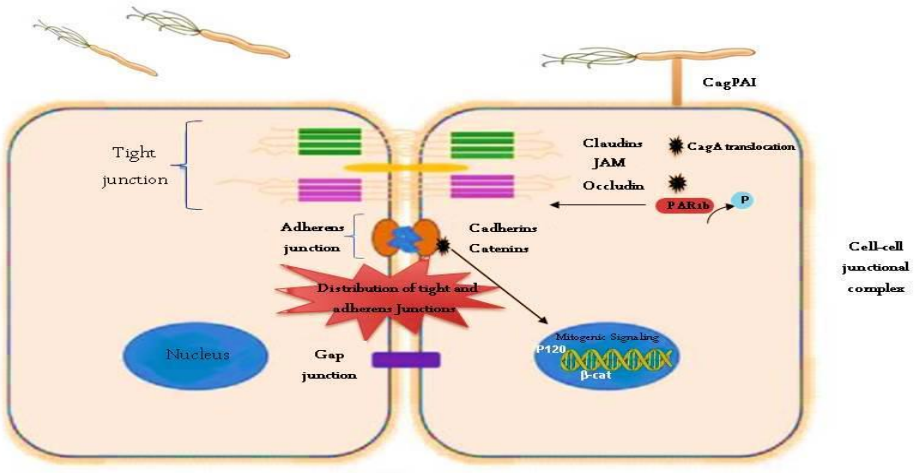

Figure 1.2 Dysregulation of epithelial junction by *Helicobacter pylori*.

Induction of gastric epithelial cell apoptosis by *H. pylori*

Continued and rapid turnover of epithelial cells is considered an innate host defense mechanisms provided by the epithelium as it contributes to reduced bacterial colonization. Since *H. pylori* inhabits the apical surfaces of gastric epithelial cells, to maintain a persistent infection *H. pylori* employs different mechanisms that favor its growth and survival. For instance, the imbalance between the epithelial turnover and proliferation could facilitate the *H. pylori* colonization and survival[92]. Several studies have shown that the development of *H. pylori*-associated gastric diseases during chronic infection involve redistribution of the equilibrium between the proliferation and the turnover of gastric epithelial cells[93]. Apoptosis is a highly conserved and regulated process in both healthy and inflamed tissue. It helps maintain tissue homeostasis by removing aged, damaged and infected cells from the tissues [94]. *H. pylori* elicits apoptosis of gastric epithelial cells in order to stimulate the cells proliferation as a compensatory mechanisms[95, 96] and *H. pylori* was found to be able to regulate these processes in order to utilize the epithelial cells as a niche for their replication[93-97]. For instance, one study detailed below reported that *H. pylori* infection triggers pro-survival mechanisms (p-ERK) as well as an antiapoptotic protein (MCL1) in the gastric epithelium of infected gerbils in order to block apoptosis and thus enhance colonization by *H. pylori* [93].

As indicated above, enzymes (i.e., urease, phospholipases) and VacA that are released by *H. pylori* cause damage the gastric mucosa, and stimulate the inflammatory and immune responses that also contribute to epithelial cell apoptosis. The inflammatory response results in the production of a significant level of free radicals by neutrophils, which damage the DNA of epithelial cells while the immune response via production of TH1 cytokines (such as TNF-a and IFN-y), which further damage the DNA of gastric epithelial cells, and induce apoptosis[72]. *H. pylori* express different adhesion molecules that aid their attachment to the epithelial surface and the overlying mucosa. As introduced earlier, one of those adhesions involves urease on the surface of *H. pylori*, which upon binding to class II major histocompatibility complex (MHC) molecules also stimulates apoptosis of gastric epithelial cells[53, 76]. Interestingly, IFN- by Th1 cells in the infected

gastric mucosa enhances apoptosis induction by increasing the density of class II MHC molecules on the surface of gastric epithelial cells to which *H. pylori* can bind and trigger apoptosis[76]. Paradoxically, *H. pylori* also has been shown to promote survival or anti-apoptotic mechanisms in gastric epithelial cells. After *H. pylori* translocates CagA via T4SS, CagA, through the signaling cascades activated inside gastric epithelial cells, induces activation of various transcription factors such as, NF-κB, nuclear factor of activated T-cells (NFAT), serum response factor (SRF), and T cell factor/lymphoid enhancer factor (TCF/LEF)[84, 85, 98]. Stimulation of these transcription factors leads to an increase in Cyclin D1 production in epithelial cells leading to their proliferation[99]. As mentioned earlier, a study that used a Mongolian gerbil model of infection to elucidate the effect of *H. pylori* on gastric epithelial cells renewal showed that *H. pylori* could actually suppress apoptotic processes of gastric epithelial cells, which when uninfected are usually shed every 2-3days, and in delaying this shedding colonization by *H. pylori* is favored. In that study, they orally administered ectoposide (ETP), an inducer of epithelial cell apoptosis. The delivery of CagA was noted to up-regulate MCL1, an anti-apoptotic protein, and the pro-survival MAPK ERK1/2 in the gastric pits. These results gave insights on the role of CagA in suppression of epithelial cells turnover while also stimulating their proliferation, which is a key strategy used by *H. pylori* to persist[93]. Our group showed that another mechanism used by *H. pylori* to induce gastric epithelial cells proliferation involved the induction of macrophage migration inhibitory factor (MIF) production by gastric epithelial cells[52]. Gastric epithelial cell proliferation was induced as a result of MIF binding to CD74, whose expression increases during infection and is polarized toward the apical surface where *H. pylori* may engage CD74 molecules[77, 100]. Interestingly, through the use of *cagA*- knockout *H. pylori* in conjuction with wild type strains, MIF production by infected gastric epithelial cells was found to depend on CagA. MIF, in turn led to decreased p53 phosphorylation as well as increased BCL-2 expression, both of which are anti-apoptotic processes[73]. Furthermore, a recent study showed that the N-terminus of CagA interacts with the tumor suppressor apoptosis-stimulating protein of p53 (ASPP2) and in doing so inhibits apoptosis[15]. In that study, it was observed that during *H. pylori* infection the N-terminus of translocated CagA interacts with ASPP2. After CagA hijacks ASPP2, it is altered and recruits and binds to

p53, and eventually leads to proteasomal degradation of p53. Thus, this represents another mechanism whereby CagA causes the infected gastric epithelial cells to become more resistant to the natural cell turnover.

Cytokines Secretion

A hallmark of the infection of the gastric mucosa with *H. pylori* is the secretion of multiple proinflammatory cytokines, several of which are secreted by gastric epithelial cells. These cytokines, in turn, play significant roles in the development of gastroduodenal diseases associated with *H. pylori* infection. One of the earliest cytokines reported to be produced by the infected gastric epithelium is interleukin (IL)-8[101-104]. It is well accepted that *H. pylori* increases the IL-8 expression both *in vivo* and *in vitro*[103]. This production of IL-8 was linked to the expression of CagA by the infecting strains[101]and is associated with the recruitment of neutrophils. Other cytokines reported to be produced by the infected gastric epithelium include IL-6, tumor necrosis factor alpha (TNF-α)[105], IL-1β, IL-1α, granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1)[106], MIF[73]and TGF-β[52] **(Figure 1.3)**. While this may not be an exhaustive list, it shows the ample repertoire of cytokines that originates from infected gastric epithelial cells, which is a well-known response to the infection.

As the understanding of underlying mechanisms responsible for proinflammatory cytokine production during infection with *H. pylori* is key in gaining important insights into pathogenesis and potential targets for therapy, various efforts have been directed at characterizing the virulence factors that are linked to cytokine induction. Various studies have examined the response of human gastric epithelial cells to purified *H. pylori* urease, since it is the most abundant protein produced by the bacteria. One of those studies reported that *H. pylori* urease induces the production of IL-6 and TNF-α by gastric epithelial cells[105]. Interestingly, mucosal IL-6 and TNF-α had been found years earlier to be increased in patients suffering from *H. pylori* chronic gastritis[107]. In more detailed studies to map the domain on urease inducing IL-8, the urease B subunit alone was found to induce the activation of NF-κB and IL-8 production subsequent to binding to CD74 on

gastric epithelial cells[77]. As CagA has multiple effects on signaling processes once it is translocated into the host epithelium, it is not surprising that it also has been shown to stimulate cytokine production. Early investigations correlated IL-8 production with CagA protein expression by *H. pylori* [101, 108]. In a detailed comparison of *H. pylori* strains that lack CagA protein with those that express the protein, CagA positive strains were reported to induce higher levels of IL-8 mRNA and IL-8 protein expression by gastric epithelial cells[101]. In a more recent study the *cag* PAI and OipA were examined for their role in IL-6 secretion by gastric epithelial cells and both virulence factors were found to increase the levels of IL-6 production by MKN-28 gastric epithelial cells through different pathways[109]. Remarkably, in one recent careful study to examine cytokine secretion by gastric epithelial cells that, unlike monocytes or lymphocytes, are polarized, cytokine secretion was reported to be polarized to the basolateral compartment, which is where the cells that would be affected by the cytokines are located[110].

While some of the cytokines produced by the gastric epithelium are induced directly by *H. pylori* virulence factors, it is important to keep in mind that in vivo there is a complex network of interactions, some of which include factors from other cells that infiltrate the infected gastric mucosa. Initially, the adaptive response was considered as a polarized Th1 response; however, more recently other $CD4^+$ T cell subsets have been reported in the infected mucosa. One of those subsets are Th17 cells, which are key in immune defense against extracellular bacteria, but are also commonly found in multiple inflammatory processes[111, 112]. Interleukin-17 (IL-17) RNA transcription and IL-17 secretion have been found to increase in the human gastric mucosa during *H. pylori* infection and its expression is up-regulated in animal stomach after 3 weeks of infection and its level elevated up to 12 months post infection[113]. An animal study that used Mongolian gerbils as a model confirmed the in vivo increased expression of IL-17 mRNA, especially during the chronic phase of *H. pylori* infection[114]. Since gastric epithelial cells express IL-17 receptors, IL-17 interaction with its receptor stimulates the epithelial cells to produce IL-8; thus confirming that gastric epithelial cells serve as important contributors for IL-17 and IL-8 synthesis during *H. pylori* infection[113]. Furthermore, IL-21 and IL-23, which are cytokines that induce and sustain IL-17 production, are found to be upregulated in the gastric mucosa of patients infected with H.

pylori. IL-23 expression does not appear to be associated with CagA protein, but it certainly plays a role in sustaining production of IL-17[113, 114].

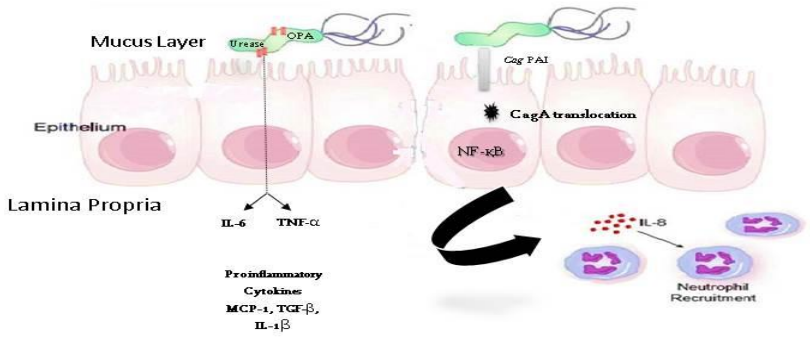

Figure1.3 Cytokines Production by Gastric Epithelial Cell during *Helicobacter pylori* Infection.

Pro-carcinogenic responses

One of the well accepted and significant clinical outcomes of *H. pylori* infection is gastric adenocarcinoma. Gastric cancer (GC) remains the second leading cause of cancer-related deaths and close to one million people succumb to GC on an annual basis. When diagnosed, the 5 year survival rate of GC patients is 15%[115]. Because of the clear evidence collected linking *H. pylori* to GC[116]., *H. pylori* is the first bacterium to have been classified as a class I carcinogen. As the infection typically results in chronic gastritis and inflammation has been linked to cancer development[117], this has been one of most accepted general mechanisms linking the infection to GC. However, not everyone who is infected develops GC. In fact, it has been reported that approximately 1 to 3% of those who are infected develop GC while about 10% develop peptic ulcer disease[116]. There are two histologically distinct types of GC. The first is a diffuse type of GC comprised of individual infiltrating neoplastic cells that fail to form glandular structures. The second type is an intestinal type GC that results from the transition of normal mucosa to chronic superficial gastritis, followed by atrophic gastritis, intestinal metaplasia, and eventual dysplasia and adenocarcinoma[118]. Another neoplasia that is

linked to infection with *H. pylori* is a non-Hodgkins type or mucosal associated tissue lymphoma (MALToma). Although *H. pylori* strains carrying cagA represent an important risk factor for GC development, GC development is thought to be the result of a multifactorial process that is orchestrated to include an array of etiological factors as well as several genetic and epigenetic modifications. As far as *H. pylori* virulence factors is concerned, CagA was reported to interact with E-cadherin and causing deregulation of β-catenin signaling and this stimulated intestinal transdifferentiation of gastric epithelial cells, a premalignant event from which gastric adenocarcinoma develops[86]. Also, a study in which CagA alone was transfected into human gastric epithelial cells showed that *H. pylori* CagA targets PAR1 and CagA–PAR1 interaction causes junctional and polarity deficits that free cells from growth-inhibitory cues and thus foster carcinogenesis[89]. A separate study by an independent group, using the transfection approach, also showed that CagA interacted with E-cadherin and induced cytoplasmic/nuclear accumulation of β-catenin by preventing its membrane association, in turn activating β-catenin signaling[86]. That same study showed that CagA-transfected cells expressed intestinal-specific molecules as a sign of intestinal transdifferentiation of gastric epithelial cells. Perhaps the most revealing study demonstrating the oncogenic potential of CagA was an in vivo study showing that genetically modified mice made to express CagA developed gastrointestinal and hematological malignancies[119]. Mice expressing wild type CagA developed gastric epithelial hyperplasia and some mice had gastric polyps as well as gastric and intestinal adenocarcinomas. Some of those CagA transgenic mice developed myeloid leukemias and B cell lymphomas. Interestingly, transgenic mice expressing phosphorylation-resistant CagA did not develop any of those pathologies, which highlight the importance of CagA phosphorylation in the deregulation of SHP-2 and subsequent cellular responses described above.

IMPACT OF *H. PYLORI* AND GASTRIC EPITHELIUM INTERACTIONS ON PATHOGENESIS

H. pylori and GECs interactions represent a complex set of multiple events, many of which induce pathogenesis and sustain the infection. Although only about 20% of *H. pylori* are attached to the epithelial cells at any point in time via different adhesions[120],

adhesion is essential for the stimulation of the inflammatory cascade. Specifically, adhesion is a prerequisite for interleukin 8 (IL8) productions by gastric epithelial cells[121]. Also, adherence induces the development of severe diseases. For example, BabA adhesion, which binds the Lewis b blood group antigen on the gastric epithelium, is associated with development of duodenal ulcer, distal gastric cancer and more severe gastritis[122]. Attachment to epithelial cells via virulence factors can also induce tissue injury. Binding of vacA to the epithelial cells leads to eventual pore formation in the lysosomal membrane, generation of vacuoles, and increases in anion permeability[123]. Patients who are infected with *H. pylori* that have a functional cag-PAI have increased IL8 levels in the gastric mucosa, resulting in significant neutrophil infiltration, and an increased risk of peptic ulcer and gastric cancer[124].

CONCLUSION

Multiple studies have been done to understand the effect *H. pylori* on the host epithelium starting from the bacterial adhesins that contribute to the interactions with the gastric epithelial cell surface, followed by the virulence factors reported to affect host cells as well as the signaling pathways that are activated, and concluding with some of the cellular responses to the infection. But, much has been learned regarding the pathogenesis of this common human pathogen since it was initially reported to be associated with human gastric disease. However, despite many years of intense investigation of the *H. pylori* interactions with the host epithelium and the consequences of those interactions, it is still unclear how it elicits PUD and GC, although recent studies have provided important clues. While this study focused on the direct effects of *H. pylori* on the epithelium, it is important to keep in mind that there are multiple other effects that *H. pylori* exerts on the host by affecting cells other than the epithelium. In turn, those cells may contribute to altering the local milieu through their products, and thus contribute to disease. Particularly, cells with immune functions are significantly increased within the infected gastric mucosa. Yet, *H. pylori* is able to evade immune-mediated clearance and persists for decades. The epithelium is surreptitiously manipulated by *H. pylori* to escape host defense mechanisms. The study of underlying mechanisms used by

H. pylori to alter the host epithelium and turn it into an immunosuppressive tool is crucial in efforts to develop a protective vaccine that is greatly needed.

Chapter 2: *Helicobacter pylori* and Its Mediators Induce IL-16 Production by Human Gastric Epithelial Cells

2.1 Introduction

Helicobacter pylori is a gram negative bacterium that colonizes approximately half of the world's population gastric mucosa. *H. pylori* infection starts in childhood and persists throughout life. This spiral shaped bacterium is a causative agent for development of gastroduodenal diseases; such as, gastric and duodenal ulcer as well as gastric cancer[125]. Due to its carcinogenic effect, The World Health Organization has classified *H. pylori* as a class I carcinogen[2].

H. pylori infection induces the infiltration of several immune cells to the infected mucosa; such as, neutrophils, macrophages[126], T cells and B cells[5, 6, 127]; however, this immune response is not adequate to eliminate the infection. As a result, the infection continues throughout life as chronic gastric inflammation[77, 128]. Several studies have demonstrated the ability of *H. pylori* to inhibit T cells proliferation *in vivo* and *in vitro* [9, 129], and described the effect of *H. pylori* on gastric epithelia cells (GECs)[130-133], but there are limited studies regarding the mechanisms involved in T cell impairment.

It has been reported that, there is an increase in the number of $CD4^+$ T cells in the antrum of *H. pylori* infected gastric mucosa. The gastric mucosa infected with *H. pylori* is infiltrated with different subsets of $CD4^+$ T cells; such as pathogenic (Th17), or inhibitory (T_{reg}), or effector Th1 cells [15, 16] .However, the homing mechanisms for these various $CD4^+$ T cells populations that infiltrate the *H. pylori*-infected mucosa are unclear.

GECs are the primary target for *H. pylori* infection, and they play an important role in the host response during the infection. They have been classified as antigen presenting cells (APCs) due to their constitutive expression of class II MHC molecules and this expression is increased during *H. pylori* infection[134]. In addition, GECs contribute to the host response *via* the secretion of multiple

cytokines/chemokines that assist in recruitment of other immune cells. For example, IL-8 is one of the earliest cytokines reported to be produced by the infected gastric epithelium. This production of IL-8 was linked to the expression of CagA by the infecting strains and is associated with the recruitment of neutrophils[101]. Other cytokines reported to be produced by the infected gastric epithelium include IL-6[109], tumor necrosis factor alpha (TNF-α)[105] IL-1β, IL-1α, granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1)[106], MIF[73]and TGF-β[52]

Interleukin-16 (IL-16) was initially described as a T cell chemoattractant. Unlike other chemokines, which do not differentiate among different cell phenotypes as long as they express the corresponding receptors, IL-16 selectively recruits $CD4^+$T cells[11] by using CD4 as a receptor, but makes them unresponsive to antigen (reviewed in [11]). Several *in vivo* studies have described IL-16 as an immunomodulatory cytokine that involves in $CD4^+$ T cell recruitment and activation at sites of inflammation in association with asthma and autoimmune diseases[135]. Interleukin-16 (IL-16) has been shown to increase during *H. pylori* infection as well as gastric cancer, but the influence of *H. pylori* on IL-16 production by GECs and its role in recruitment of different $CD4^+$ T cells subsets is unclear.

In this study I investigated IL-16 production by GECs and the mechanism underlaying this production during *H. pylori* infection.

2.2 MATERIALS AND METHODS

Cell lines and Bacterial cultures. Gastric epithelial cell lines N87, Kato III, and MKN45 were obtained from the American Type Culture Collection (ATCC). N87 and MKN-45 cell lines were maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and 2 mM L-glutamine while Kato III cell line was maintained in in IMDM with 20% fetal bovine serum (FBS) and 2 mM L-glutamine in incubator at 37^{o}C with 5% CO_2. *H. pylori* 51B strain was grown on blood-agar plates at 37^{o}C under microaerophilic conditions for 2 days.

Infection of GECs with *H. pylori.* One day before the infection with *H. pylori*, GECs media were replaced with antibiotic-free media and at the time of infection, GECs were washed with antibiotic-free media. The bacteria were re-suspended in RPMI 1640 medium and used at a cell:bacteria ratio of 1:10, unless otherwise indicated.

RNA Isolation and Real-time PCR (PT-PCR). Total RNA was extracted from cells by using RNeasy Mini Kit from QIAGEN Sample & Assay Technologies according to manufacturer's instructions. The RNS concentration was measured by a spectrophotometer at 260 nm. The RNA preparations were reverse-transcribed with 0.1 mM of random hexamer primers, 1 mM of each dNTP, and 50 U MuLV reverse transcriptase (Perkin Elmer, Branchburg, NJ) and were incubated at 42^{o}C for 1 h. The resulting cDNA was then amplified by polymerase chain reaction (PCR) in a reaction mixture consisting of 1 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl, 0.2 mM of each dNTP, 0.1 mM of each primer and 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer). All reactions were overlaid with mineral oil to prevent evaporation. Real-time RT-PCR was performed based on Applied Biosystems's two-step RT real time PCR protocol (Applied Biosystems, Foster City, CA). The appropriate assays-on-demand gene expression assay mix for human 18S and IL-16 (a 20X mix of unlabeled PCR primers and TaqMan® MGB probe, FAMTM dye-labeled) and 2 µL of cDNA were added to the PCR reaction step. The reactions were carried out in a final volume of 20 µL by using the BioRad Q5 real-time PCR machine. PCR was carried out in a programmed thermal cycler : 2 min at 50°C, 10 min at 95°C (1 cycle) and 15 sec 95°C and one min at 60°C (40 cycles).

Antibodies and Proteins. APC-conjugated Anti-human IL-16 Antibody (Clone 70720) was purchased from R&D (Minneapolis, MN). IL-8 and R6K were a generous gift from Dr. Krishna Rajarathnam (UTMB). The viability dye eFluor 780 (eBioscience, San Diego, CA, USA) was included in the experiments to control cell viability.

Flow cytometric Analysis of Intracellular IL-16. Intracellular expression of IL-16 was detected by cytoplasmic immunofluorescence staining and flow cytometry analysis. The cells were pretreated with brefeldin A and monensin for 4 h to allow accumulation of synthesized protein within the Golgi apparatus. Cells were harvested,

fixed with Fixation & Permeabilization Buffer (1 concentrate: 3 diluent) (eBioscience, Cat. No: 00-8333), and then permeabilized with Permeabilization Buffer (10X) (eBioscience, Cat. No: 00-8333-56). PBS supplemented with 2% of ethylenediaminetetra acetic acid tetrasodium salt dehydrate (EDTA) was used as washing buffer. Surface Fc receptors were blocked by incubating the cells with of 5% mouse/rabbit serum in permeabilization buffer. After washing, the cells were incubated with anti-IL-16 antibody or isotype-matched control antibody for 30 min at room temperature. After immunostaining, the cells were washed twice with permeabilization buffer and fixed with 300 µl of washing buffer (2% of EDTA in PBS). The results were analyzed by flow cytometry on a FACScan (Becton-Dickinson).

2.3 RESULTS

2.3.1 Up-regulation of IL-16 expression by gastric epithelial cells during *H. pylori* infection.

To determine whether *H. pylori* directly induced the expression of IL-16 in human gastric epithelial cells during *H. pylori* infection, the human GEC lines (N87) and (Kato III) were infected with *H. pylori* 51B or LC11 strains. RT-PCR and flow cytometry analysis were done respectively on two gastric epithelial cell lines (N87 and Kato III). IL-16 mRNA was significantly increased in both gastric epithelial cell lines at 4 h after infection with *H. pylori* 51B strain **(Fig. 2.1A).** Using intracellular cytokine staining and flow cytometry, both GEC lines were significantly up-regulated in their expression of intracellular IL-16 at 24 h after infection with *H. pylori* 51B strain when compared with uninfected GEC controls **(Fig. 2.1B).**

2.3.2 Regulation of IL-16 production by mediators present in *H. pylori*-infection associated diseases.

IFN-γ is an important cytokine produced within the *H. pylori*–infected gastric mucosa [136], and is thought to play a role as a major contributor in *H. pylori*-induced gastritis[137]. Previously, our lab reported a synergistic effect of IFN-γ and *H. pylori* on B7-H1, an immunoinhibitory molecule, upregulation[10], and B7-H2, a positive costimulatory ligand, downregulation on GECs[138]. Histamine is known to be involved in

the pathogenesis of *H. pylori* associated chronic gastritis and peptic ulcer. Gastrin-dependent enterochromaffin-like cells and gastrin-independent mast cells, the sources of histamine in the stomach have been reported to be elevated in the *H. pylori* related gastritis or peptic ulcer. Thus, I examined whether IFN-γ or histamine could induce IL-16 production by GECs. N87, Kato III and MKN-45 cells were treated with either IFN-γ or Histamine. The immune reactive IL-16 protein was detected in the culture supernatants of N87, Kato III, and MK-45 cells. The level of IL-16 secretion was significantly increased in all cell lines after treatment with histamine (10^{-4} M for 4 h) **(Fig. 2.2A)** or IFN-γ for N87 cell line **(Fig. 2.2B)** (100 U/ml for 24 h) (*$P < 0.05$, compared with corresponding medium controls).

2.3.3 Regulation of IL-16 production by IL-8.

Our lab found that *H. pylori* Binds to CD74 on GECs and induced IL-8 production[17], and this IL-8 acts in an autocrine manner and up-regulates CD74 expression[29]. Thus, an attempt to investigate the mechanisms responsible for the increased IL-16 expression in *H. pylori* infected patients I thought to determine the effect of IL-8 on IL-16 production by GECs. Two gastric epithelial cell lines, N87 and Kato III, were examined for IL-16 expression before and after treatment with different doses of IL-8 **(Fig. 2.3A)**. IL-16 mRNA levels in response to IL-8 treatment were measured by real-time PCR. As seen in **Fig 2.3A**, IL-8 treatment for 24 h resulted in 2-fold increases in IL-16 mRNA level for N87 cells compared with untreated cells and 4-fold for Kato III cells at 500 pg/ml dose of IL-8. As *H. pylori* infection induces up-regulation of the expression of IL-8 receptors, CXCR1 and CXCR2, on gastric epithelial cells, and to confirm the role of IL-8 in the noted increase of IL-16 expression by GECs, we blocked CXCR2 with an inactive CXCL8 mutant for CXCR2 receptor activation, R6K for one hour before IL-8 treatment for 23 hr. Flow cytometric analysis revealed that IL-8 treatment led to an increase in intracellular expression of IL-16 over 60 % of over basal expression by the N87 cell line at 24 h treatment time. However, adding R6K to block CXCR2 before IL-8 treatment prevented the induction of the intracellular IL-16 expression **(Fig. 2.3B).** Similar results was observed at RNA level by real-time PCR where 2-fold of decreases were seen with R6K treatment plus IL-8 **(Fig. 2.3C).**

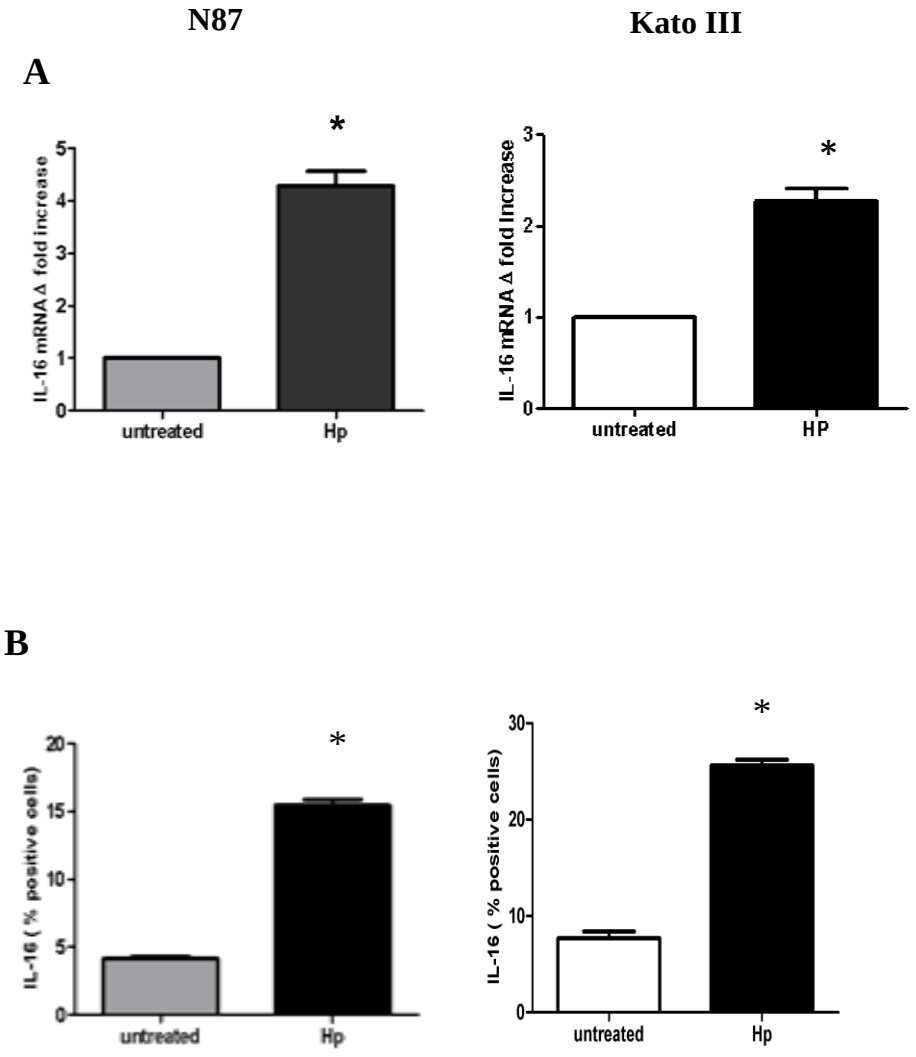

Figure 2.1 *H. pylori* upegulates IL-16 expression in GECs. (A). Kato III and N87 cells were infected with *H. pylori* 51B at 10:1 *H. pylori*:GEC ratio for 4h and IL-16 expression was measured by real-time quantitative RT-PCR. RNA was isolated from untreated and 4h *H. pylori* 51B infected GECs. The mRNA level for IL-16 was normalized to 18S and compared to the level of IL-16 mRNA of untreated GECs (N=4,* P < .05). **(B)** Flow cytometry measured IL-16 expression on N87 and Kato III cells after 24h infection with *H. pylori* LC11. The data were expressed as a percent of positive cells. Isotype control value was subtracted from the data presented. N= 4,*P < 0.05.

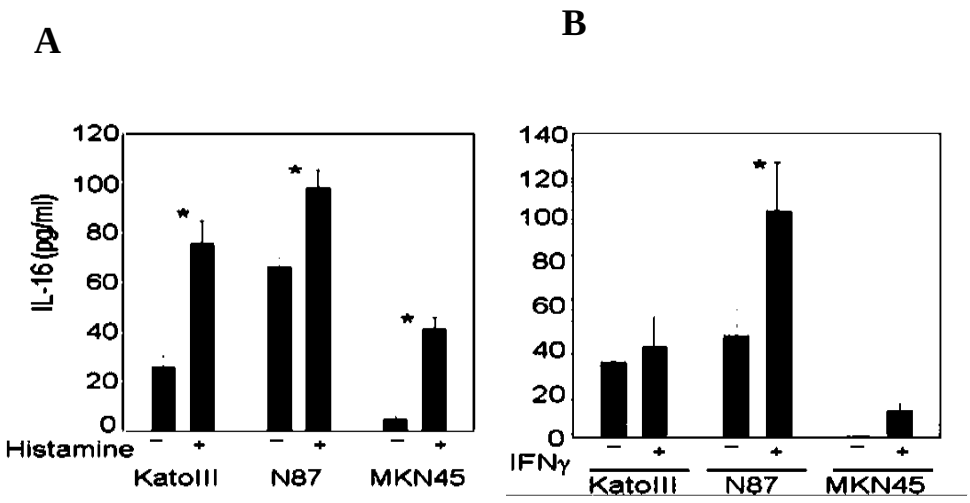

Figure 2.2 Regulation of IL-16 production by mediators present in *H. pylori*-infection associated diseases. Gastric epithelial cells Kato III, N87 and MKN45 were treated with histamine **(panel A)** or IFN-γ **(panel B)** for 24 hrs. The cell culture supernatants were collected and assayed for IL-16 production by a "sandwich" ELISA. As shown, the treatment of histamine significantly enhanced IL-16 secretion in all the three cell lines tested. IFN-γ treatment also increased IL-16 production in N87 cells. (* $P < 0.05$).

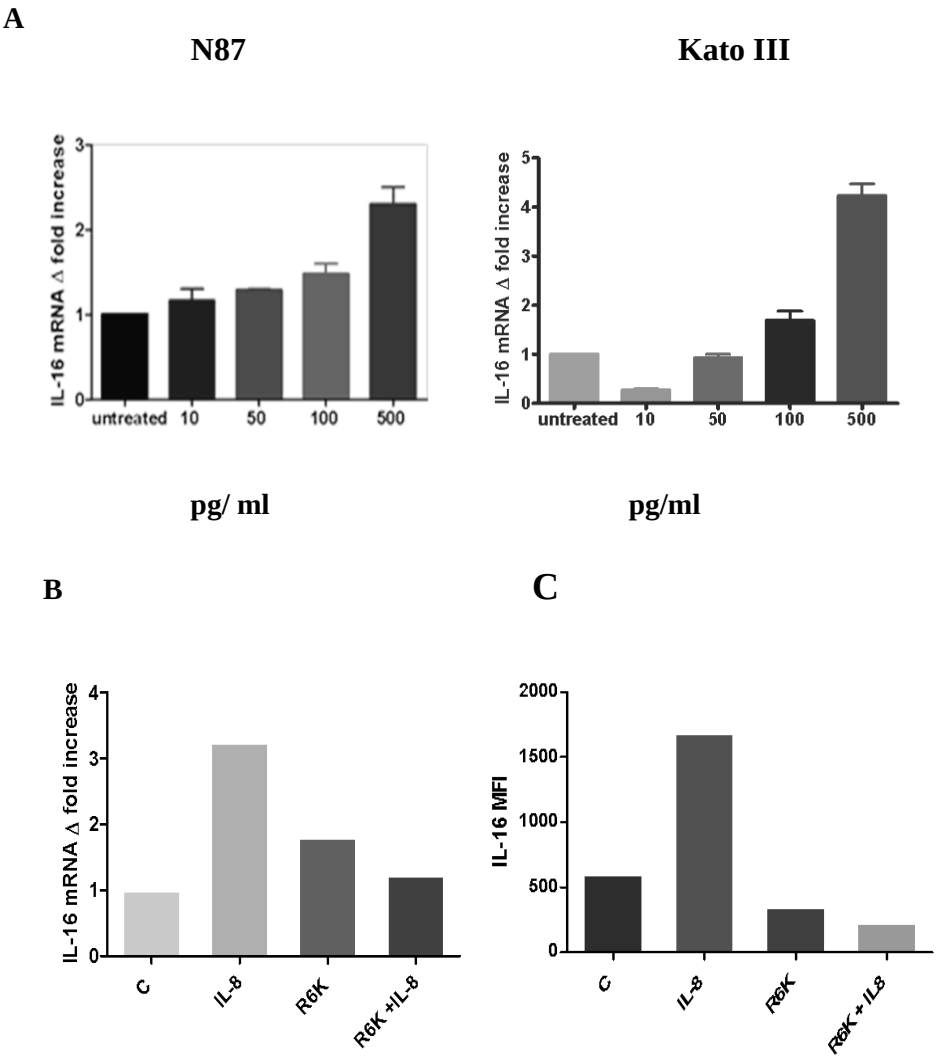

Figure 2.3 IL-8 upregulates IL-16 expression in GECs. (A) Representative data shows increases IL-16 mRNA expression in N87 and Kato III GECs after treated with different doses of IL-8. N87 cells and Kato III were treated with different doses of IL-8 based on physiological rang (10-500 pg/ml) for 24h and IL-16 expression was measured by real-time quantitative RT-PCR. RNA was isolated from untreated and 24h h IL-8 treated GECs. The mRNA level for IL-16 was normalized to 18S and compared to the level of IL-16 mRNA of untreated GECs. **(B & C)** RT-PCR and flow cytometry were done to measure IL-16 expression on N87 cells after treating the cells with 500 pg/ml of IL-8, R6k, for 24hrs and R6k for 1hrs before adding IL-8 for 23hr. flow cytometry data are expressed as mean fluorescence intensity (MFI). Isotype control value was subtracted from the presented data.

Discussion

During *H. pylori* infection, the host mounts an immune response, but this response is not sufficient to eliminate the infection leading to chronic infection. Surprisingly, T cells from infected patients are hyporesponsive and polarized toward a Th1 response. It has been reported that *H. pylori* can directly affect T cells responses *via* several mechanisms. For instance, *H. pylori* induces T-cells apoptosis by up-regulation of Fas ligand on Fas expressing T-cells[139, 140]. In addition, VacA, a virulence factor of *H. pylori*, can down-regulate IL-2 receptors on T-cells, prevent IL-2 production, and decrease cell viability[140]. Our lab has reported that *H. pylori* could inhibit T cell responses by indirect way via increased expression T cell coinhibitory molecule B7-H1 on GECs, which in turn suppress T cells proliferation in coculture experiments[10]. Recently, our lab also reported that CagA-dependent downregulation of B7-H2 expression on GECs and inhibit of Th17 responses[138]. No another mechanisms of T-cells inhibition during the infection have been reported. My study sought to investigate how *H. pylori* manipulates the host gastric epithelium to orchestrate, *via* production of IL-16, a pleotropic cytokine, which could be responsible for the recruitment of $CD4^+$T cells the inhibition of effector T cells, which are central to adaptive immunity and consequently promoting the establishment of the chronic infection.

Increased the numbers of $CD4^+$ T cell, but not of $CD8^+$ T cells, in the antrum of *H. pylori* infected gastric mucosa has been reported by a previous study[7]. This infiltration of $CD4^+$ T cells in the gastric mucosa during the infection may contribute to the damage of the mucosal epithelium and ongoing inflammation**.** Selective recruitment and inactivation of $CD4^+$ T cells in the *H. pylori* infected gastric mucosa can be a result of gastric cell-derived cytokines. Gastric cells generate a variety of cytokines during *H. pylori* infection[52, 73, 77, 101, 104, 105], but IL-16 is the only cytokine that uses CD4 as a receptor and selectively recruits $CD4^+$ cells[141, 142]. However, the influence of *H. pylori* on IL-16 expression by gastric epithelial cells was not previously considered.

IL-16 is produced by a different leukocyte subsets, including T cells[143, 144], eosinophils[145], mast cells[146, 147], monocytes[148], as well as dendritic cells[149]. Also,

bronchial epithelial cells have the capacity to express IL-16 after stimulation with histamine, TNF-α and IL-1β[12], and this expression correlates with number of infiltrating CD4+ T cells. Moreover, In Inflammatory bowel disease (IBD), IL-16 expression is significantly elevated in inflamed colonic mucosa but not in control individuals[150]. This evidence suggest that IL-16 could contribute to the inflammatory process in IBD by enhancing recruitment and activation of CD4+ cells[151, 152]. Although Nakajima and coworkers has reported that IL-16 secretion in *H. pylori* infected gastric mucosa is one of the factors for gastric cancer[153], the exact origin of the elevated IL-16 and the biological role of IL-16 in *H. pylori* infection have remained unestimated.

Since *H. pylori* has been shown to be involved directly in immune evasion mechanisms, I sought to investigate whether *H. pylori* could directly induce IL-16 production by GECs, which may be responsible for the accumulation and hyporesponsiveness of $CD4^+$ T cells during *H. pylori* infection. I found that two different human gastric epithelial cell lines, N87 and Kato III, produced increased mRNA for IL-16 and much higher levels of intracellular IL-16 after they were infected with *H. pylori* and compared to uninfected cells.

As mentioned earlier, T cells are hyporesponsive during *H. pyloi* infection and polarized toward a Th1 response[8]. These cells produce IFN-γ, which play a significant role in in the proinflammatory responses induced during the infection[154]. Because cytokines play an important role in regulating immune function, and our lab reported a synergistic effect of IFN-γ and *H. pylori* on B7-H1 upregulation[9] and B7-H2, a positive costimulatory ligand, downregulation on GECs[27], I checked if IFN-γ has an effect on IL-16 production by GEC. My results showed that immune reactive IL-16 protein was detected in the culture supernatants of N87, Kato III and MKN-45 and the level of IL-16 secretion was significantly increased in N87 cell lines after treatment with of 100 U/ml IFN-γ for 24 hr **(Figure 2.2B)**

Histamine is also involves in the pathogenesis of *H. pylori* associated chronic gastritis and peptic ulcer. The increased mast cell numbers in the gastric mucosa and epithelium in *H. pylori*-infected gastritis or peptic ulcer patients significantly decrease after eradication of *H. pylori*[155]. In my study, I detected the immune reactive IL-16

protein in the culture supernatants of N87, Kato III and MKN-45 and the level of IL-16 secretion was significantly increased in all cell lines after treatment with 10^{-4} molar of histamine for 4 hr. (**Figure 2.2A**).

A previous study showed that *H. pylori* directly increases gastric epithelial IL-8 mRNA expression and IL-8 protein production in a strain specific manner[101]. Another study confirmed the presence of IL-8Rs, CXCR1 and CXCR2, on gastric epithelia cells, and IL-8Rs expression is up-regulated during *H. pylori* infection[156]. Our lab reported that *H. pylori* binds to CD74 on gastric epithelial cells and stimulates IL-8 production[77], and IL-18 induces CD74 expression, an essential receptor for *H. pylori* on gastric epithelial cells[157]. Together, these observations led me to consider IL-8 as an important candidate responsible for up-regulation of IL-16 during *H. pylori* infection. I found that IL-16 mRNA expression on human GEC is up-regulated in dose dependent manner (**Figure 2.3A**) after GEC stimulation with IL-8. Also, I observed that addition of R6K, an inactive CXCL8 mutant, on cultures of gastric cells for one hour before adding IL-8, blocked CXCR2 and prevented the up-regulation of IL-16 mRNA (**Figure 2.3B**) as well as IL-16 protein (**Figure 2.3C**), which further approved the role of IL-8 in IL-16 production by GECs.

In conclusion, the present study showed that gastric epithelial cells have the capacity to express IL-16 after infection with *H. pylori* and after stimulation with different mediators, IFN- γ and histamine, present in *H. pylori*-mediated diseases. This study also suggests that IL-8, which induced by *H. pylori* or by general inflammatory conditions when IL-8 induced, is a mechanism for up-regulation of IL-16 production. These finding uncovers relevant information pertaining to accumulation of CD4+ T cells that is observed in patients' gastric mucosa with *H. pylori* (**Figure 2.4**).

This study has a few limitations. First, we have not yet deciphered the whether both IL-8 receptors are involved in IL-16 production by gastric epithelial cells, and we need to further delineate the biological effect of IL-16 on $CD4^+$ T cells. Second, we have so far only analyzed the IL-16 production in *in vitro* experiments, and we are in the process of confirming these findings and effects *in vivo* as well using the mouse model of H. pylori infection and IL-$16^{-/-}$ KO mice.

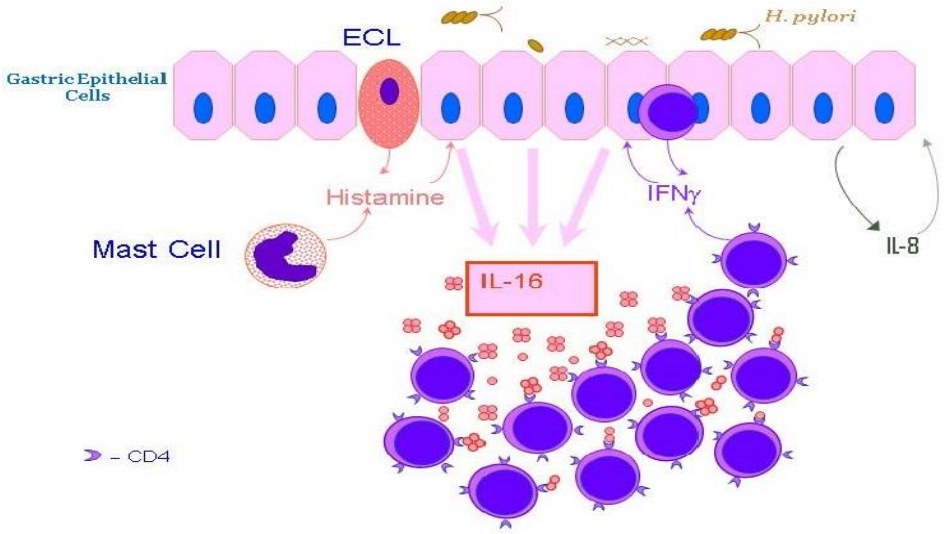

Figure 2.4 Upregulation of Il-16 productions by *H. pylori* and its mediators.

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1. Alzahrani S, Lina TT, Gonzalez J, Pinchuk IV, Beswick EJ, Reyes VE. Effect of *Helicobacter pylori* on gastric epithelial cells. *World J Gastroenterol*. 2014 Sep 28;20(36):12767-12780.

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