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Helicobacter pylori TYPE 4 SECRETION SYSTEM MODULATES HOST T CELL RESPONSES

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Dedication

This dissertation is dedicated to my parents Mohammad Abu Taher and Azmeri Begum for their continuous support and sacrifices. I also dedicate this dissertation to my husband Bijay K. Khajanchi for his constant support and encouragement and to my one-year-old daughter Shanika A. Khajanchi for being my inspiration.

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Infection with *Helicobacter pylori* (*H. pylori*) bacteria is associated with gastritis, peptic ulcer, and gastric cancer. During *H. pylori* infection $CD4^+$ T cells in the gastric lamina propria are hyporesponsive and polarized to Th1/Th17 cell responses controlled by Treg cells. Gastric epithelial cells (GECs) are the primary target for *H. pylori* infection and may act as antigen-presenting cells (APCs) regulating local T cell responses. Previously our lab showed that *H. pylori* up-regulates B7-H1 (a T cell co-inhibitory molecule) expression on GEC, which, in turn, suppress T cell proliferation and effector function, and induce Treg cells *in vitro*. My studies showed that, along with B7-H1, GEC expresses B7-H2 and B7-H3 molecules. B7-H2 is a T cell co-stimulatory molecule, and B7-H3 has both co-stimulatory and inhibitory functions. Moreover, their expression is modulated during *H. pylori* infection. In addition, I investigated the underlying mechanisms of these responses-and demonstrated that *H. pylori* requires its type 4 secretion system (T4SS) translocated components: effector protein CagA and cell-wall peptidoglycan (PG) fragments for up-regulating B7-H1 as well as B7-H3, and for down-regulating B7-H2 on GEC. These data were validated *in vivo* by using a mouse model of infection. My study demonstrated that, along with T4SS, cytokines produced by Th17 and Treg cells also induce B7-H3 expression. I evaluated the underlying cell signaling pathways and showed that *H. pylori* uses the p38 MAPK pathway to up-regulate B7-H1 and B7-H3 expression and the p70S6/mTOR pathway for B7-H2 down-regulation in GEC. By using *in vitro* and *in vivo* systems, I demonstrated that *H. pylori* T4SS-

mediated up-regulation of B7-H1 expression by GEC caused Treg cell induction and increased bacterial loads whereas *H. pylori* CagA-mediated B7-H2 down-regulation in GECs correlated with a decrease in Th17-type responses. Furthermore, the modulation of Th17 responses inversely correlated with the *H. pylori* colonization levels. Finally, the up-regulation of B7-H3 expression resulted in induction of Th2 cells *in vitro* and *in vivo*. In conclusion, these studies revealed some novel regulatory mechanisms employed by *H. pylori* to influence the type of T cell response that develops in the infected gastric mucosa and help in establishing chronic infection there.

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APCs	Antigen presenting cells
CagA	Cytotoxin-associated gene A
CLR's	C-type lectin receptors
DC	Dendritic cell
FoxP3^{+}	Forkhead box protein p3
GECs	gastric epithelial cells
GGT	γ-glutamyltranspeptidase
HP-NAP	*H. pylori* neutrophil-activating protein
ImSt	Immortomouse stomach epithelium
IFN	Interferon
IL-	Interleukin-
LPS	Lipopolysaccharide
Le	Lewis
MAPKS	Mitogen-activated protein kinases
MLN	Mesenteric lymph node
Nod1	Nucleotide-binding oligomerization domain containing 1
PAMPs	Pathogen-Associated Molecular Patterns
PRR	Pattern-recognition receptor
PD-L1	Programmed death-1 ligand 1
PD-1	Programmed death-1
PMSS1	Pre-mouse SS1

ROS	Reactive oxygen species
RORγt	Retinoic acid-related orphan receptor gamma t
RT-PCR	Real time PCR
SS1	Sydney strain 1
TSA	Tryptic soy agar
T4SS	Type 4 secretion system
T-bet	Tbx-21
Treg	regulatory T cells
TCR	T cell receptor
TGF	Transforming growth factor
Th-	T helper cell
TLRs	Toll-like receptors
VacA	Vacuolating toxin A
WT	Wild type

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative gastroduodenal pathogen. It is perhaps the main constituent of the human gastric microbiome since it infects >50% of the world's population and is linked to chronic gastritis, peptic ulcer disease and gastric cancer [1,2,3,4,5,6,7]. *H. pylori* is responsible for ~90% of cases of peptic ulcer formation [8,9]. Additionally, the World Health Organization classified *H. pylori* as a class I carcinogen because of the epidemiological link of *H. pylori* infection with a higher risk of development of gastric malignancy [10].

H. pylori infection is typically acquired during childhood and usually becomes a lifelong infection, if left untreated [11]. Effective treatment of *H. pylori* infection requires multidrug regimens. Patient compliance, re-infection and resistance against antibiotics have become an emerging problem [12,13]-[14]. Thus, a vaccine is clearly needed. However, most efforts to develop an *H. pylori* vaccine that protects humans have been unsuccessful [15]. A major knowledge gap which prevents development of an efficient anti-*H. pylori* vaccine is the absence of a full understanding of the mechanisms that allow *H. pylori* to escape from immune system defenses, leading to chronic infection. Thus, *H. pylori* successfully establishes a chronic infection by achieving a delicate balance between inducing immune responses and surviving in the inflammatory milieu by using an array of important virulence factors. Additionally, multiple lines of evidence suggest that the immune response during *H. pylori* infection plays an important role in pathogenesis.

Among the multiple virulence factors expressed by *H. pylori*, one that is noteworthy is encoded within a 40-kilobase chromosomal region known as the *cag* pathogenicity island (PAI), which is composed of more than 30 genes that encode for a type 4 secretion system (T4SS). Also, this island of genes includes the *cagA* gene that codes for the cytotoxin-associated gene A (CagA) protein, which is the only known effector protein encoded in *cag* PAI and is a key virulence factor of *H. pylori*. Epidemiological studies showed that $CagA^+$ *H. pylori* strains are associated with an increased risk of gastric cancer compared to strains of *H. pylori* lacking CagA [3,16,17].

The CagA protein is translocated into gastric epithelial cells (GECs) *via* the *H. pylori* T4SS [18,19] and once inside GECs the tyrosine residue at specific C-terminal Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs of CagA is phosphorylated [20,21]. The activated CagA interacts with several intracellular signaling mediators, mainly in the tyrosine phosphorylated mode [20,21], and activates important signaling pathways to manipulate host immune regulation and deregulate GECs homeostasis for their survival [22,23]. CagA elicits multiple cell responses, including disruption of epithelial tight junctions, cytoskeleton rearrangement, changes in cellular adhesion properties and polarity as well as secretion of proinflammatory mediators [24,25]. In addition to CagA effector protein, T4SS also delivers *H. pylori* peptidoglycan (PG) cell wall fragments into host cells, which are recognized by the intracytoplasmic pattern-recognition receptor (PRR) nucleotide-binding oligomerization domain containing 1 (NOD1). The sensing of *H. pylori* PG by NOD1 activates NFκB and mitogen-activated protein kinases (MAPKs) leading to IL-8 production and associated pathogenesis [26,27,28]. Despite the marked inflammatory response within the *H. pylori*-infected gastric mucosa, the host immune response is unable to clear *H. pylori* resulting in persistent infection and development of chronic gastric inflammation [29,30]. Although *H. pylori'*s T4SS importance in virulence is recognized because of its multiple effects on GECs, its role in modulating T cell function during *H. pylori* infection has not been well investigated.

$CD4^+$ T helper (Th) cells are major effector cells in the immune responses to *H. pylori.* Although the numbers of $CD4^+$ T cells with a memory phenotype increase in the gastric lamina propria during *H. pylori* infection, these T cells are hyporesponsive [31]. Because this hyporesponsiveness contributes to chronicity, there have been targeted efforts to understand the mechanisms employed by *H. pylori* to down-regulate T cell responses. *H. pylori* also manipulate T cell function by eliciting regulatory T cells (Treg) which are frequently found in *H. pylori*-infected patients [32,33]. Because of their suppressive effect on T effector cells, Treg cells further assist in the chronicity of infection. Treg cells are also a probable cause of gastric tumor progression [34]. Th17 cells, whose hallmark cytokine is IL-17A, represent a recently described subset of inflammatory T helper (Th) cells, which appear to be crucial in the clearance of extracellular bacteria (22). Though increased IL-17A expression is observed during

chronic gastric inflammation, the levels produced are not sufficient to clear the infection. The mechanisms responsible for the increased Treg cells and reduced Th17 responses during *H. pylori* infection remain poorly understood.

Activation of $CD4^+$ T cells is regulated by antigen-presenting cells (APCs). Their activation and function not only depends on TCR-MHC class II interaction between APC and T cells, but also on the B7 co-stimulatory molecules signaling on the surface of APC. It has been demonstrated that professional APCs such as monocyte-derived dendritic cells (DCs) and macrophages are important in regulating immune responses against *H. pylori* [35]. In contrast, the contribution of the mucosal non-professional APCs, such as GECs is unknown. GECs are a major target for *H. pylori* infection, and express cytokines and receptors that influence T cell responses during *H. pylori* infection [36,37]. Our lab previously reported that GECs express the classical B7 co-stimulators B7-1 and B7-2, whose expression is increased during *H. pylori* infection [38]. Our lab also showed that *H. pylori*-increases the expression of the T cell inhibitory molecule B7-H1 on GEC upon infection, which leads to a decrease in T cell proliferation and contributed to the induction of Treg cells [36,37]. In the current study, I investigated the underlying mechanisms behind *H. pylori*-mediated B7-H1 induction by GEC and its functional relevance to chronic infection both *in vitro* and *in vivo*. By using *H. pylori* wild-type (WT), *cag* pathogenicity island (*cag* PAI^-) and *cagA*$^-$ isogenic mutant strains, I showed that *H. pylori* required its type 4 secretion system (T4SS) as well as its effector protein CagA for B7-H1 up-regulation. My data established a significant role of *H. pylori* PG, an additional mediator translocated by T4SS and recognized by NOD1, in increased B7-H1 expression by GECs. By using pharmacological inhibitors I showed that *H. pylori* is able to use the p38 MAPK pathway to up-regulate B7-H1 expression in GEC. *In vivo* confirmation was obtained when infection of C57BL/6 mice with *H. pylori* PMSS1 strain, which has a functional T4SS delivery system, but not with *H. pylori* SS1 strain lacking a functional T4SS, led to a strong up-regulation of B7-H1 expression in the gastric mucosa, increased bacterial load, induction of Treg cell in the stomach, increased IL-10 in the serum and less gastric inflammation. Interestingly, B7-$H1^{-/-}$ mice showed less Treg cells, increased inflammation and reduced bacterial loads after infection. These studies demonstrate how *H. pylori* T4SS components activated the p38 MAPK pathway,

up-regulated B7-H1 expression by GEC, and caused Treg cell induction, and accordingly, contribute to establishing a persistent infection which is characteristic of *H. pylori*.

In my subsequent study, I investigated the impact of *H. pylori* cytotoxin CagA on the modulation of the T cell co-stimulator B7-H2, which was implicated in Th17 cell responses. My study showed that *H. pylori* infection down-regulates B7-H2 expression by GECs in a CagA-dependent manner. IFNγ, which has been shown to be increased in the *H. pylori*-infected gastric mucosa, synergizes with *H. pylori* in down-regulating B7-H2 expression by GECs. By using pharmacological inhibitors, I showed that CagA-mediated modulation of B7-H2 on GEC involves p70 S6 kinase phosphorylation. The CagA-dependent B7-H2-down-regulation in GEC correlates with a decrease in Th17-type responses *in vitro* and *in vivo*. Further, CagA-dependent modulation of Th17 responses inversely correlated with the *H. pylori* colonization levels *in vivo*. My data suggest that CagA contributes to the ability of *H. pylori* to evade Th17-mediated clearance by modulating expression of B7-H2 and, thus, to the establishment of *H. pylori* chronic infection.

Finally, since B7-H3 has recently been implicated in Th2 responses and appears to play a role in inhibition and costimulation, I examined the expression of the B7-H3 molecule on GEC and how the expression is regulated by *H. pylori* during infection. My study showed that both murine and human GEC express the B7-H3 molecule and its expression increased upon *H. pylori* infection. My study also showed that the bacterium uses its type 4 secretion system (T4SS) components CagA and cell wall PG fragments to up-regulate B7-H3. Th17 and Treg cells, which infiltrate the gastric mucosa during *H. pylori* infection, also affect B7-H3 induction, because IL-17, IL-10 and TGF-β, produced by these cells, induced expression of B7-H3 in GEC. I next investigated the underlying cell signaling pathway and found that *H. pylori*-uses the p38MAPK pathway for B7-H3 up-regulation. As B7H3 have been shown to up-regulate Th2 responses, I characterized the phenotype of T cell subpopulations in mice infected with *H. pylori* PMSS1 or SS1 strains. *H. pylori* infected mice showed a mixed Th1/Th2 response. Consistent with our previous findings, the PMSS1 infected mice showed increased Treg cells and decreased Th17 cells in the MLN compared to SS1 infected mice. By using T cell: GEC co-cultures I demonstrated that only an *H. pylori* strain associated with gastritis but not cancer or

peptic ulcer induced Th2 cells. Using anti-B7-H3 blocking Ab I further confirmed that the induction of Th2 is mediated by B7-H3. Measurement of B7-H3 and GATA3 expression in patient biopsy samples showed a strong correlation between increased B7-H3 and Th2 responses in gastritis associated *H. pylori* strains associated with gastritis. In conclusion, these studies revealed some novel regulatory mechanisms employed by *H. pylori* to influence the type of T cell response that develops in the infected gastric mucosa. Future studies will examine how these findings may be implemented in designing a vaccine against *H. pylori*.

Chapter 1: Review of the Literature

1.1 INHIBITION OF INNATE IMMUNE RECOGNITION

Evasion of Recognition by Pattern Recognition Receptors (PRRs)

H. pylori evades the innate immune system by a variety of mechanisms. One of those mechanisms is avoidance of detection by Pattern Recognition Receptors (PRR), which are proteins that recognize Pathogen-Associated Molecular Patterns (PAMPs). PAMPs include a large group of molecules that are part of microbes and can vary from microbial surface molecules to nucleic acids. When PRR's recognize PAMP's they induce several extracellular activation cascades such as the complement pathways and various intracellular signaling pathways, leading to inflammatory responses that are essential for clearance of pathogens [39].

H. pylori eludes identification by PRRs by multiple methods, including: avoidance of recognition by Toll-like receptors (TLRs) and inhibition of c type lectin (DC-SIGN)-mediated signaling. To avoid recognition by TLRs the bacterium modulates its surface molecules, including lipopolysaccharide (LPS) and flagellin. LPS is a glycolipid found on the outer membrane of Gram negative bacteria [40]. It has three distinct subunits: lipid A, which is responsible for the toxic effects; a core polysaccharide of five sugars linked through ketodeoxyoctulonate (KDO) to lipid A; and the O-antigen, an outer polysaccharide consisting of up to 25 repeating units of three-to-five sugars [41]. *H. pylori* expresses O-antigens with great variability; the bacterium also has Lewis antigens, which are made of carbohydrates, that resemble human blood group antigens [42]. By exploiting this form of molecular mimicry, the bacterium is able to evade TLR's because the normally detectable O-antigen is recognized as a "self" molecule by this type of PRR. In addition to having variable O-antigens, the bacterium also modifies the lipid A portion of the LPS molecule. Modification of this unit is achieved through several pathways, resulting in alteration of the net charge of the microbial surface. This leads to an inability of cationic antimicrobial peptides (CAMP's) to bind to typically negatively charged structures like lipid A [40]. Lipid A, within LPS, is recognized by the human toll-like receptor 4-myeloid differentiation factor 2 (hTLR4-MD2) complex. *H. pylori* expresses a modified Kdo (3-deoxy-d-mannoctulosonic acid)-lipid A structure tetra-

acylated with a phosphoethanolamine added at the 1 position of the disaccharide [43], which might promote high resistance to CAMP and decreased activation of the hTLR4-MD2 complex [43]. The 1' and 4'-phosphatases involved in lipid A synthesis in *H. pylori* act synergistically to produce a bacterial surface that is highly resistant to CAMP attack. In addition, de-phosphorylation of *H. pylori* lipid A at the 1' and/or 4' position results in LPS with attenuated hTLR4-MD2 activation [40]. Moran and colleagues [44] proposed that reduced immunogenicity of *H. pylori* LPS could be due to uncommon phosphorylation and acylation of *H. pylori* lipid A. *H. pylori* LPS binds poorly and at a slower rate to LPS-binding proteins (LBP's), which are acute phase reactants that aid in LPS binding to CD14 and TLR4 on monocytes/macrophages. This reduced binding of LPS to its receptors results in decreased activation of monocyte-macrophages, preventing their contribution to an innate immune response. Interestingly, *H. pylori* LPS has also been shown to possess anti-phagocytic properties *in vitro* [45].

Flagellin is the protein component of bacterial flagella needed for motility and colonization [46]. *H. pylori* rely on five or six polar flagella made of two separate subunits, FlaA and FlaB, to enable movement within the gastric mucus and to counteract peristalsis [47]. TLR5 is a PRR that recognizes flagellin. However, studies showed that *H. pylori* flagellin was not recognized by TLR5, and thus failed to induce NF-κB activation [48]. The study also reported that an 8 amino acid stretch in the N-terminal D1 domain of flagellin differed from that of flagellin from bacteria that activated TLR5. One study showed that flagellin, especially FlaA, is not "shed" by the bacteria and thus could not be detected by western blots in supernatants of infected gastric epithelial cells [47]. There were no evident traces of flagellin, which diminished the probability of it interacting with TLR5, allowing for evasion of this mechanism of bacterial recognition. Most flagellated bacteria are able to induce a proinflammatory state by promoting production of IL-8, but *H. pylori* flagellin seems unable to induce IL-8 production in GECs [47].

H. pylori LPS is important not only for the activation of TLR4 but also because the bacterium expresses Lewis (Le) blood group antigens in the O-antigen portion of the LPS molecule. As mentioned above, this polysaccharide area of the molecule is a clear method of evasion of the innate immune response because the Le group antigen system is

biochemically related to carbohydrates present in ABO blood groups. The bacterium employs molecular mimicry to evade recognition by the innate immune system. The group of Le antigens is divided into type 1 (Le^a and Le^b) and type 2 antigens (Le^x and Le^y). Approximately, 80-90% of *H. pylori* strains express Le^x and/or Le^y antigens, whereas GECs also express $Le^{x/y}$ antigens [44], [49]. *H. pylori* uses phase variation in the synthesis of LPS, including Le antigens. Phase variation in this context refers to a high frequency of LPS phenotype changes, like a reversible on-and-off switch, that results in loss/gain of certain LPS epitopes, as well as a heterogeneous population of LPS.

It has been suggested that although *H. pylori* LPS is not a strong activator of TLR4, it may be modulating the immune response via interactions with DC-specific, ICAM3-grabbing non-integrin (DC-SIGN). DC-SIGN belongs to a subset of PRR's termed C-type lectin receptors (CLRs), which are involved in inducing specific genes within cells in response to pathogens as well as in modulating TLR signaling [50]. When CLRs are expressed on dendritic cells (DCs) they detect carbohydrates like mannose, fucose, and glucan, which are common on bacterial surfaces. Ligand binding to these receptors initiate signaling pathways which induce phosphorylation of a subunit of the NF-κB complex and result in an increased rate of transcription of proinflammatory cytokine and chemokine mRNAs, such as IL-8 [50]. DCs possessing these receptors are found on all mucosal surfaces as well as in lymphoid organs. Miszczyk et al. showed that *H. pylori* LPS was able to bind to recombinant human DC-SIGN *in vitro* and that this binding was abolished in the presence of monoclonal antibodies against the Le antigens and when fucose was added [51]. By binding to this receptor it may be possible that the presence or absence of certain carbohydrates at the O-antigen end of the molecule could determine how DCs help T cells mature. Another independent study showed that Le^+ variants from clinical isolates were able to bind to DC-SIGN and have effects on the polarization of the T cell response (Th1 vs. Th2) [52]. It seems that *H. pylori* targets DC-SIGN to block a polarized Th1 response by phase-variable expression of Le antigens. In addition, this study provided evidence that *H. pylori* strains without Le^x and Le^y were able to evade recognition by DC-SIGN and possibly evade detection by any other mechanism [52].

Inhibition of phagocytic killing

H. pylori infection activates an inflammatory response within the human gastric mucosa, which leads to the recruitment of macrophages, neutrophils, and lymphocytes to the gastric tissue [53]. *H. pylori* can efficiently inhibit its own uptake by these professional phagocytes. This anti-phagocytic phenotype depends on type IV secretion components encoded by the *cag* PAI [54,55]. Macrophages can engulf *H. pylori,* but the bacterium has developed mechanisms to avoid killing upon phagocytosis [56,57,58]. In a study where ingestion of *H. pylori* by human and murine macrophages was monitored by using immunofluorescence and electron microscopy, *H. pylori* type I strains (*cag* PAI^+ and vacuolating toxin A^+, $VacA^+$) were shown to employ an unusual mechanism to avoid phagocytic killing. Once inside the macrophage, *H. pylori* actively delayed actin polymerization and phagosome formation. *H. pylori*-containing phagosomes then underwent extensive clustering and fusion, resulting in the formation of "megasomes" containing multiple bacteria, which caused resistance to intracellular killing [56,59]. Studies also showed enhanced survival of *H. pylori* type I strains in macrophages compared to type II strains, which lack *cag* PAI and VacA. *H. pylori* type I strains were shown to reside in compartments with early endosomal properties and did not fuse with lysosomes. The study also showed that retention of TACO, a tryptophan aspartate-containing coat protein on phagosomes, inhibited fusion of phagosomes and lysosomes in macrophages infected with *H. pylori* type I strains. It is worth noting that VacA alone plays a significant role in the interruption of the phagosome maturation [58]. By interfering with the phagosome function, VacA might prevent phagocytic killing of *H. pylori*. In fact, a study showed that, by interfering with endosomal traffic, VacA altered the presentation of antigens by B cells [60]. This mechanism would be expected to result in impaired adaptive responses, as presentation of antigens to T cells is critical for the initiation of protective immune responses, as will be described in detail below. A related recent study provided evidence that the effects of VacA on endosomal traffic may prevent the development of a strong Th1 response. The study showed that *H. pylori* VacA could redirect the endocytic pathway of the probiotic bacterium *Lactobacillus acidophilus*, which induces a polarized Th1 response, and does this by blocking the

induction of key innate cytokines such as IFN-β and IL-12 [61]. Like other pathogenic bacteria, *H. pylori* also regulate host trafficking pathways by the selective modification of GTPases in macrophages during infection. *H. pylori* has been shown to disrupt the actin cytoskeleton by suppressing *Rgs1*/*2*, *Fgd2*, and *Dock8*, which are the key regulators of the Rho, Rac, and Cdc42 GTPases, respectively [61]. These are required for the organization and dynamics of actin cytoskeleton needed for proper cell function. This is another mechanism that disrupts phagocyte function and helps *H. pylori* survival in its host [61].

Inhibition of killing by reactive oxygen species and nitric oxide

A major proinflammatory factor produced by *H. pylori* is neutrophil-activating protein (NAP) [62]. *H. pylori* -NAP (HP-NAP) is a 150-kDa oligomeric protein, which increases adhesion of PMNs to endothelial cells, stimulates phagocyte chemotaxis, and activates NADPH oxidase to produce reactive oxygen species (ROS) [63,64]. However, *H. pylori* produces catalase and superoxide dismutase to detoxify ROS [65,66]. *H. pylori* can also down-regulate CXCR1 and CXCR2 expression in human neutrophils, which act as receptors for the neutrophil-recruiting chemokine, IL-8, and thereby result in an inhibitory effect on neutrophil migration and reduced bacterial killing [67]. *H. pylori* also disrupt NADPH oxidase targeting, which was shown to result in the release of superoxide anions in the cytoplasmic membrane instead of the accumulation inside *H. pylori* phagosomes [68].

One antimicrobial host defense mechanism is the generation of NO through the enzyme inducible NO synthase (iNOS). *H. pylori* activates the inducible iNOS in macrophages [69]. A mechanism employed by *H. pylori* to activate iNOS involves urease, an important virulence factor of *H. pylori*. Despite the presence of iNOS, *H. pylori* infection persists, which suggests that the iNOS production may be at a suboptimal level. *H. pylori* arginase was shown to be an important factor that affords protection of the bacteria against NO-mediated killing since macrophages infected with *H. pylori* produce significantly less NO than do arginase isogenic mutants [70]. A recent study showed that induction of macrophage arginase II (Arg2) restricts iNOS protein expression, elicits apoptosis of macrophages as well as proinflammatory cytokine

production, and limits bacterial killing [71], suggesting another mechanism this bacteria uses to escape macrophage-mediated killing. Interestingly, the study used a chronic infection mouse model to show that $Arg2^{-/-}$ mice infected with *H. pylori* had reduced bacterial colonization and increased gastritis compared with similarly infected, wild-type mice. $Arg2^{-/-}$ mice infected with *H. pylori* had more $iNOS^{+}$ macrophages in the gastric mucosa expressing higher levels of iNOS and more robust cytokine responses, which led to the suggestion that *H. pylori* induction of Arg2 is part of the bacterial mechanism to escape host innate immunity together with other mechanisms that target adaptive immunity [71].

1.2 MODULATION OF APC FUNCTIONS IN ADAPTIVE IMMUNITY

H. pylori have evolved an array of mechanisms to actively dodge adaptive immunity by interfering with antigen presentation and modulation of T cell responses. Antigen-presenting cells (APCs), represented by macrophages, DCs and B cells, internalize antigen by phagocytosis or endocytosis, process the antigens and present them to $CD4^{+}$ T cells *via* class II MHC molecules. This leads to the initiation of an antigen-specific T cell response. In the gastric mucosa of *H. pylori*-infected individuals, there is an increase in activated macrophages and DCs. Activated macrophages produce IL-6, IL-1β, IL-12 and TNF-α, which cause inflammation and help to initiate Th1 type responses. In spite of the presence of these effector cells, *H. pylori* successfully establish a persistent infection, which may mean that these effector cells are unable to clear the pathogen. *H. pylori* has also been shown to cause the polarization of APCs. For instance, during atrophic gastritis macrophages are polarized to an M1 subtype [72]. *H. pylori* can even control the functions of these APCs differently. A study showed that *H. pylori*-mediated activation of DCs and M1 macrophages leads to induction of T cell proliferation and decreased phagocytosis. On the other hand, upon *H. pylori* infection the M2 macrophages, when compared to M1 macrophages, produced fewer pro-inflammatory cytokines and increased anti-inflammatory cytokines [73]. As alluded to earlier, several studies show that *H. pylori* uses mechanisms to avoid killing by APC and those will be discussed in detail below.

Apoptosis of macrophages

H. pylori causes apoptosis of macrophages by using several mechanisms. Inside a macrophage, *H. pylori* activates the ERK1/2 pathway, leading to formation of the activation protein (AP-1) complex. The AP-1 complex was reported to induce c-Myc gene expression and nuclear translocation, leading to increased ornithine decarboxylase (ODC) expression and apoptosis of macrophages [74,75,76]. Another recent study showed an important role for an unknown gene, HP986, which is associated with peptic ulcer and gastric carcinoma in the apoptosis of macrophages through a Fas-mediated pathway [74]. *H. pylori* VacA protein also causes apoptosis of monocytes. The underlying mechanism of this process involves the amino-terminal 476 residue fragment (p52) of VacA, which activates the NFκB pathway and induces pro-inflammatory cytokine production, e.g. TNF-α, IL-1β, and induction of NO, ROS and subsequently causes apoptosis of monocytes [77].

Inhibition of DC maturation and function

DCs are important APCs in initiating T cell responses, particularly to mucosal pathogens. *H. pylori* control maturation of DCs and, consequently, limit their ability to present antigens. Transcription factor E2F1, is an important regulator of DC maturation. When LPS was used as a stimulator, E2F1 expression was reported to be down-regulated during DC maturation. However, *H. pylori* VacA was shown to inhibit DC maturation via restoration of E2F1 since transfection of murine DCs with E2F1 siRNA showed recovery of the inhibited maturation of DCs caused by *H. pylori* VacA [78]. VacA caused reduced expression of surface co-stimulatory molecules, e.g. CD40, CD80, CD86, MHC class II molecule and decreased secretion of IL-1β, IL-12p70 and TNF-α by DCs [78]. Reduced expression of co-stimulatory molecules could, in turn, dampen effector T cell activation or promote tolerance. In addition to VacA, *H. pylori* CagA also plays a key role in regulating DCs and in inhibiting $CD4^+$ T cells' differentiation towards Th1 type cells. Once inside the APC, CagA protein was shown to be phosphorylated leading to the activation of SHP-2. Activated SHP-2 then suppresses the enzymatic activation of TBK-1, IRF-3 phosphorylation and nuclear translocation, resulting in reduced interferon production by DC [79]. Long-term infection with *H. pylori* $cagA^+$ strains causes

increased expression of the T cell co-inhibitory molecule B7-H1 (also known as PDL-1) as well as increased IL-10 and IL-23 production by DC. The simultaneous inhibition of DC maturation and IL-12 secretion led to suboptimal Th1 development and activation [80]. *H. pylori* were shown to multiply in DC and impair their function by inhibiting the production of the pro-inflammatory cytokine IL-12 and increasing IL-10 production [81]. A separate study showed that the *H. pylori*-mediated inhibition of DC maturation is independent of the presence of *cag* PAI, but direct contact with the bacteria was required for this inhibitory mechanism [82]. When expression of different co-stimulatory molecules was evaluated after treating DCs with TLR ligands and subsequent infection with *H. pylori,* it was found that *H. pylori* inhibited TLR ligand induced DC maturation by inhibiting CD80, CD86, CD40 expression, IL-12, IL-6 secretion and increasing production of the anti-inflammatory cytokine IL-10 [82]. These studies showed that inhibition of DCs is another mechanism used by *H. pylori* to deter its clearance by the host immune system.

Inhibition of antigen presentation

The proliferation of human $CD4^+$ T cells is triggered by recognition of antigenic epitopes bound to MHC class II molecules exposed on the surface of APCs. Antigen presentation by APCs plays an essential role in the initiation of adaptive immune responses. As another approach to inhibit APC function, *H. pylori* uses several mechanisms to interrupt antigen presentation, some of which were mentioned previously. *H. pylori* inhibits antigen processing by APC by interfering with late endocytic membrane trafficking. *H. pylori* VacA interferes with the proteolytic processing of antigens and the generation of T cell epitopes loaded on newly synthesized MHC class II molecules (the Ii-dependent pathway of antigen presentation), but it does not affect generation and presentation of epitopes by mature class II molecules that recycle from the cell surface (Ii–independent pathway) [60]. Also, possibly linked directly to this event, *H. pylori* can cause impaired antigen presentation by DC by inhibiting the export of MHC-class II molecules to the cell surface [81]. This observation is directly related to the inability of these DCs exposed to *H. pylori* VacA to degrade Ii (aka, CD74), which requires the action of cathepsins activated by acidic pH [83,84,85].

Apoptosis of GECs

GEC may act as non-professional APCs, as they express all the elements associated with conventional APCs, including class II MHC, CD74, cathepsins and co-stimulatory molecules [38,86]. Their importance in *H. pylori* infection is obvious as they are the first cell types that come in direct contact with the bacteria and are strategically situated to interact with *H. pylori* and its antigens as well as with lamina propria lymphocytes. In fact, GEC separate the lamina propria immune cells from direct contact with *H. pylori* in the lumen. One of the multiple ways *H. pylori* has been shown to induce apoptosis of GEC is by up-regulation of the Fas receptor leading to increased interaction with Fas ligand and increased apoptosis [87]. This interaction has also been shown to induce production of ROS [88]. Another mechanism that our group showed previously is *via* the engagement of MHC class II molecules on GEC by *H. pylori*, which use urease on its surface as an adhesin and bind to and crosslink MHC class II molecules to induce apoptosis in GEC [89]. In yet another mechanism of induction of GEC apoptosis, VacA was shown to induce apoptosis via disruption of mitochondrial membranes [90,91].

Using GEC as orchestrators of T cell responses

Activation of T cells requires two signals triggered by (1) recognition by T cell receptor (TCR) of peptides/MHC complexes and (2) a co-stimulation by special receptor molecules on APCs. Recognition of antigen by T cells in the absence of the second signal renders T cells unresponsive or anergic. The B7 family of co-stimulatory/co-inhibitory receptors provides this second signal to initiate responses and some members of this family of receptors serve to regulate or attenuate responses. In addition to their role as on/off switches for T cell activity, recent studies from multiple groups also suggest their role in influencing T cell differentiation and phenotype [92,93,94]. Our group showed that *H. pylori* can subvert GECs and use them as mediators to inhibit T cell proliferation and cause Treg cell induction from naïve T cells by inducing increased expression of the T cell co-inhibitory molecule B7-H1 on GEC [36,37]. Interaction of B7-H1 with programmed death-1 (PD-1) receptor is also known to cause down-regulation of T cell activation and promote the induction of T regulatory cells (Treg) as we have previously shown [36,37]. Because of their suppressive effect on T effector cells, Treg cells may

assist in the chronicity of infection. *H. pylori*-mediated B7-H1 up-regulation also contributes to apoptosis of effector T cells by engaging PD-1 on their surface [95]. My study uncovered another mechanism by which *H. pylori* uses its *cag* PAI encoded T4SS, translocated effector protein CagA to down-regulate B7-H2 (ICOS-L), which is the only positive T cell co-stimulatory molecule known among the newer members of this family of receptors. Down-regulation of B7-H2 caused decreased Th17 cell response, which correlated with increased bacterial load in the stomach of *H. pylori*-infected mice [96]. As Th17 cells play a very important role in immune protection against extracellular bacteria, *H. pylori* hinders Th17-mediated clearance by preventing B7-H2 expression on the surface of GECs to establish chronic infection.

1.3 INHIBITION OF EFFECTIVE T CELL RESPONSE

T helper $CD4^+$ cells (Th) are major effector cells in the immune response to *H. pylori.* The response was initially characterized as a Th1-polarized response [97,98] but more recently other $CD4^+$ T cell subsets have been found in *H. pylori*-infected patients, and those include Treg and Th17 cells [34,99,100,101]. To explain the initial observations regarding Th1 polarity of the response, the neutrophil-activating protein of *H. pylori* (HP-NAP) was shown to increase IL-12 and IL-23 production by neutrophils and monocytes, which promote Th1 responses. Addition of HP-NAP to antigen-induced T cell lines caused a shift from a predominant Th2 to a Th1 phenotype of specific T cells. HP-NAP also elicited an antigen-specific, Th1-polarized T cell response in the gastric mucosa of *H. pylori*–infected patients [102]. Increased production of gamma interferon (IFN-γ) by Th1 cells was shown to cause chronic gastric inflammation [97,103]. On the other hand increased Treg cells produced during *H. pylori* infection suppress mucosal effector T cell responses, which contribute to bacterial persistence, and are also a probable cause of gastric tumor progression [34]. Th17 cells, which produce IL-17A, appear to be crucial in the clearance of extracellular bacteria such as *H. pylori* [104]. IL-17 also acts on GEC to release IL-8, a chemokine that recruits neutrophils, and thus promotes gastric inflammation. On the other hand, this IL-17-initiated recruitment of neutrophils is critical for the clearance of the bacteria [105]. A hallmark of *H. pylori* infection is that effector T cell responses are generally impaired during *H. pylori*

infection, and T cells from *H. pylori*-infected individuals are hyporesponsive [31]. As this is an important issue in vaccine design development, there has been significant effort to address mechanisms that impair T cell responsiveness. *H. pylori* virulence factors that have been reported to play a role in interfering with T cell responses are VacA, γ-glutamyltranspeptidase (GGT), and arginase (RocF) [106,107,108,109,110,111]. My study showed *H. pylori* CagA also plays important role in modulating Th17 cell responses indirectly by modulating expression of B7-H2 on GEC [96].

Inhibition of T cell proliferation and signaling

As described earlier, the vacuolating cytotoxin, VacA, induces cellular vacuolation in epithelial cells. *H. pylori* disrupt tight junctions between GECs and VacA secreted by *H. pylori* can reach the lamina propria. Once in the lamina propria, VacA can interact directly with T cells. *H. pylori* exploits the recycling of the heterodimeric transmembrane receptor lymphocyte function–associated antigen 1 (LFA-1) by cells for VacA uptake. VacA enters activated primary human T lymphocytes by binding to the β2 (CD18) integrin subunit of LFA-1 [112]. Once VacA is inside the cytoplasm of T cells it inhibits their proliferation and activation by several mechanisms. One approach is by interrupting IL-2 signaling, which is required for lymphocyte activation and proliferation. *H. pylori* VacA induces cell cycle arrest [113]. VacA also blocks IL-2 at the transcription level by inhibiting translocation of nuclear factor of activated T cells (NFAT), an essential transcription factor required for IL-2 promoter activation [113]. Further study into the mechanism of action of VacA on T cell impairment showed that VacA requires its intact N-terminal hydrophobic domain for membrane channel formation and inhibition of T cell proliferation [109]. Furthermore, VacA may reduce the mitochondrial membrane potential of $CD4^+$ T cells to inhibit their proliferation [110]. In addition to preventing calcium influx from the extracellular milieu by formation of anion-specific channels and inhibiting NFAT translocation, VacA also uses a channel-independent mechanism to activate intracellular signaling *via* mitogen-activated protein kinases MKK3/6 and p38 as well as the Rac-specific nucleotide exchange factor, Vav, which results in actin rearrangement and defects in T cell activation [106]. VacA-mediated apoptosis in T cells is another possible mechanism of immune evasion. There are two

pathways of apoptosis initiation. One pathway depends on the death receptor and is called the extrinsic pathway, whereas a second pathway depends on mitochondrial activation and is known as the intrinsic pathway. *H. pylori* mediated apoptosis of T cells is independent of death receptor. A mitochondrial pathway was shown to play a critical role in *H. pylori* induced apoptosis since higher expression of anti-apoptotic protein Bcl-2 in T cells showed reduced apoptosis by *H. pylori.* Bcl-2 inhibits apoptosis by stabilizing the mitochondrial membrane [107].

GGT is another secreted protein of *H. pylori* which mediates the extracellular cleavage of glutathione, leading to ROS production and induction of a cell cycle arrest in lymphocytes. A study showed that *H. pylori* uses GGT to inhibit T-cell proliferation since site-directed mutagenesis of GGT in different *H. pylori* strains and inhibition of GGT by acivicin abrogated the inhibitory effect, while recombinant expression of GGT showed inhibition of T cell proliferation. GGT was found to inhibit T cell proliferation by inducing G1 cell cycle arrest through disruption of the Ras signaling pathway [108].

Though most studies have shown involvement of VacA and GGT in T cell inhibition, other reports also show involvement of *cag* PAI in T cell apoptosis. *H. pylori cag* PAI causes apoptosis in T cells in a Fas-dependent manner concurrently with induction of Fas ligand (FasL) in T cells leading to apoptosis [111]. Another virulence factor that impairs T-cell function during *H. pylori* infection is arginase, which is important for urea production. Arginase hydrolyzes L-arginine to urea and ornithine. L-arginine is also required for T cell activation and function. Co-culturing of *H. pylori* wild-type and arginase mutant bacteria with T cells revealed that arginase caused a significant decrease in T cell proliferation and reduced expression of the chief signal transduction protein CD3ζ-chain of the TCR by decreasing L-arginine availability [114]. Decreased expression of the CD3ζ-chain partially explains T cell anergy status in the host, which is a hallmark of *H. pylori* infection.

H. pylori mediated skewing of T cell response towards Treg cells

$CD4^{+}CD25^{high}$ Treg cells can inhibit infection-induced immunopathology, but may also allow for an increase in the bacterial load and facilitate chronicity of the infection by suppressing protective immune responses [33]. Treg cells are found in increased amounts

in the gastric tissue of *H. pylori*-infected patients compared to healthy controls [32,33]. Several studies have shown the immunosuppressive roles of Treg cells during *H. pylori* infection [33,37]. Induction of Treg cells appears to depend on the age of the host when they get the infection, since *H. pylori*-infected children have increased levels of FoxP3-expressing Treg cells and reduced gastric pathology compared to adults [115]. A study in mice showed that mice that were infected during the neonatal period were did not developed gastritis and were protected from gastric cancer precursor lesions, in spite of having increased bacterial loads while adult mice infected with *H. pylori* developed those lesions. Neonatally infected mice developed tolerance, were unable to induce T cell responses and were protected from T cell-mediated immunopathology [116]. In contrast to Treg cells, Th17 cells play a crucial role in *H. pylori* clearance as suggested by vaccine studies [117,118]. However, *H. pylori* may utilize several mechanisms to induce Treg responses while keeping a suboptimal level of Th17 cells in the host, which helps to establish a chronic infection. There is an increased recruitment of DCs in the gastric lamina propria of *H. pylori*-infected mice [119]. A study by Kao JY and colleagues (2010) showed that DCs stimulated with *H. pylori* inhibit the Th17 response and skew the response toward Treg cells. This mechanism depends on development of Treg cells with the required cytokines, TGF-β and IL-10, and this mechanism was independent of *H. pylori* virulence factors VacA and CagA [120]. This study further showed that Treg depletion enhanced the *H. pylori*-specific Th17 response, and correlated with decreased bacterial colonization in mice [120]. In another mechanism of T cell suppression, *H. pylori* interfered with DC maturation process and converted immature DCs to tolerogenic DCs. Increased numbers of these semi-mature DCs were found in the gastric mucosa of humans chronically infected with *H. pylori. H. pylori*-induced tolerogenic DCs were incapable of activating effector functions in naive T cells; however, these cells became very efficient in inducing Treg, and this process depended on DC derived IL-18 production [82].

Bone marrow-derived mesenchymal stem cells (BM-MSCs) also play an important role in the *H. pylori*-induced immunosuppressive response. Transplantation of BM-MSCs into the stomachs of mice with *H. pylori* infection fostered significant stimulation of

systemic and local IL-10-secreting T cells, which may inhibit other T cells. There was also an increased percentage of $CD4^+IL\text{-}10^+$ cells and $CD4^+CD25^+FoxP3^+$ cells in splenic mononuclear cells. BM-MSC-transplanted mice showed elevated Treg/Th17 ratios [121]. These studies demonstrated that *H. pylori*-uses several mechanisms to skew the T cell response towards Treg cells, which helps *H. pylori* to successfully establish a chronic infection.

1.4 EVASION OF HUMORAL RESPONSE

The majority of people infected with *H. pylori* develop a specific antibody response. This response is not sufficient to clear infection. Some studies suggest that infected children produce fewer antibodies, which may be concurrent with more Treg cells and fewer activated $CD4^+$ cells to acts as helper cells in the induction of B cell responses [122]. Although most or all infected individuals are thought to mount an antibody response to *H. pylori*, differences in this response have been noted between those who develop gastritis or duodenal ulcers compared to those who develop gastric cancer [123]. When patient serum antibody levels were examined, infected individuals who developed gastritis or duodenal ulcers were shown to have a greater IgG response than those who developed gastric cancers. In turn, gastric cancer patients mounted a more vigorous IgA response than did those with gastritis and duodenal ulcers. In another study of serum antibody responses to *H. pylori* in Japan, the authors suggested that a weak antibody response was linked to a high risk of developing gastric cancer by infected individuals [124]. Another study suggested that development of antibodies specific to virulence factors of *H. pylori* may be linked to gastric cancer [125]. In that study, gastric cancer patients were found to be more likely to develop antibodies to CagA and heat shock protein B, while no significant differences were found in the levels of VacA-specific antibodies between individuals with gastric cancer in comparison to other disease manifestations. These studies suggest that differences in humoral responses to infection may be linked to disease in infected individuals, but the mechanisms behind these differing responses remain elusive.

Although most people respond to *H. pylori* with a high serum antibody titer, this response is not efficient in reducing bacterial burden as evidenced by various vaccine

studies and reports in mice that lack B cells. In the latter study, mice were protected against *H. pylori* challenge meaning possibly that the humoral response is dispensable in protection against *H. pylori.* In further support of these findings, another B cell knockout study showed that with an *H. pylori* urease vaccine, mice deficient in B cells had equal protection to that in wild-type mice, and stomach $CD4^+$ T cells were equal in both mouse strains [126]. This study further indicated a correlation between the number of T cells in the gastric mucosa and the level of protection, again suggesting that the humoral response is less crucial in protection against *H. pylori*. In addition to the viewpoint that protection against *H. pylori* challenge is independent of the B cell response, there is also compelling evidence that antibodies elicited against *H. pylori* may be harmful to the host. One group has shown in mice that specific antibody responses to *H. pylori* may actually aid in bacterial colonization and impair other immune responses against *H. pylori* [127]. This study showed that T cells, not B cells, were responsible for gastritis induced by infection and suggested the possible role for antibodies in inhibiting host resistance, which improved elimination of bacteria in the absence of antibodies in B cell-deficient mice. B cell-deficient mice were able to clear bacteria at 12-16 weeks post infection, whereas wild-type mice still had a robust infection coupled with gastritis. Another compelling study showed that *H. pylori* evade antibody-mediated recognition because of a lack of surface binding of host-elicited antibodies [128]. This study consisted of incubating bacteria with sera from patients who had detectable antibody responses to *H. pylori.* There was very little binding of antibodies to the surface of the bacteria, thus indicating another way the host immune response may be evaded.

Another intriguing aspect of the humoral response to *H. pylori* is a series of reports of autoantibodies that are induced during infection. These antibodies were against self epitopes and potentially caused damage in the host. For instance, one group showed that *H. pylori* induced antibodies against parietal cells in the stomach, which persisted after bacterial eradication and were linked to intestinal metaplasia [129]. In support of these results, another study examined autoantibodies in infected patient sera, revealing a prevalence of autoantibodies during gastritis associated with gland destruction and stomach atrophy [130]. There has also been indication of disease-specific autoantibodies induced by *H. pylori* infection. A study in duodenal ulcers showed that

autoantibodies impair gastric secretory functions [131]. Decreased acid secretion, but increased gastrin secretion, was seen along with increased gastritis. This was shown in 20% of duodenal ulcer patients coupled with a more severe disease manifestation. Likewise, detrimental effects of autoantibodies have been seen in gastric cancer as well. In a small panel of gastric cancer patients, spleen cells were isolated, immortalized with human hybridoma technologies, which allowed for characterization of 11 *H. pylori*-induced autoantibodies that reacted with gastric cancer cell specific proteins [132]. Several of these antibodies stimulated gastric cancer cells to proliferate, interestingly enough, in contrast to normal epithelial cells. These studies represent an intriguing aspect to *H. pylori* immune evasion in humans that may still require further investigation to clarify the mechanisms involved.

1.5 GENOMIC DIVERSITY IN IMMUNE EVASION

H. pylori is one of the most genetically diverse bacterial species. Initial insights into this diversity were apparent when the first strains of *H. pylori* were first sequenced. When 26695 and J99 *H. pylori* strains were compared at the genome level, it was observed that 6% of the genome represented strain-specific genes, which are mostly located in a region now referred to as the plasticity zone [133]. Since then, multiple other strains have supported the observation that such diversity occurs at the site of the genome, gene arrangement and alleles. The extensive genetic diversity of *H. pylori* is the result of high mutation rates and high recombination frequency [134,135]. An array of *H. pylori* isolates have been noted to differ in the rate with which they have the *cag* PAI in their genome [136], which was recently supported by a study that included 877 isolated from diverse populations and which highlighted the variability in the carriage of *cag* PAI by *H. pylori* strains. When *H. pylori* adheres to GECs, the T4SS effector protein CagA is translocated via the T4SS into the host cell cytoplasm where it becomes phosphorylated by host cell kinases and interacts with various signaling proteins [20,137]. As a result of the multiple interactions of CagA with host cell signaling proteins, multiple processes are affected leading to cell transformation. This effector protein also has a significant level of diversity, particularly in the C-terminal EPIYA repeat motifs where CagA is phosphorylated once it is inside the host cell. These EPIYA motifs differ between Asian

and Western isolates. A study of *H. pylori* isolates from experimentally infected mice and non-human primates showed that they have rearrangements in CagY [138], which in turn result in gain or loss of function in the *H. pylori* T4SS. These observations may be reflective of the overall variability in *H. pylori* strains, which in turn contribute to immune escape and the establishment of chronic infection.

1.6 IMMUNE SYSTEM-BASED THERAPY

To get an effective immune response against *H. pylori*, T cells must be activated into an effector state. Co-stimulatory and inhibitory molecules regulate T-cell activation, and works as "immune checkpoints". *H. pylori* have been shown to upregulate B7-H1 expression and down-regulate B7-H2 expression on GEC and, thus, not only to decrease the effector T cell response but also to alter T cell sub-population balances by increasing Treg and decreasing Th17 cell numbers [37,95,96]. This novel information may permit control of *H. pylori* infection by targeting the inhibitory receptor/ligand axis. The anti-PD-1 monoclonal antibody nivolumab, (also known as MDX-1106 or BMS-936558) and lambrolizumab have already been used in a phase I trial and have shown promising results in patients with melanoma and other cancers [139,140,141], and conceivably could have an effect on the outcome of *H. pylori* infection and/or immunization. Since we have shown previously that *H. pylori* uses mTOR/p70 S6 kinase pathway to down-regulate B7-H2 expression [96], another approach to control *H. pylori* could involve the use of rapamycin, which inhibits this pathway. Analogues of this drug have been used already and shown promising results in renal cell carcinoma and breast cancer treatments [142]. The immune modulatory properties of this bacterium could be exploited therapeutically to control other diseases. For example, recombinant HP-NAP has been shown to be beneficial in the treatment of allergic diseases and immunotherapy of cancer due to its ability to induce Th1 responses. It was shown to inhibit the growth of bladder cancer [143] and has also been used as an immune modulating agent to suppress Th2 responses in allergic asthma and *Trichinella spiralis* infection [144,145].

1.7 CONCLUSIONS

H. pylori has co-existed with human host for at least 30,000 years [146]. During this long period of co-existence, the bacterium has undergone evolutionary adaptation and established a comfortable niche in the human host. Unlike most other pathogenic bacteria which are cleared by the host adaptive immune response, *H. pylori* successfully establishes a persistent infection in its host in spite of the presence of vigorous innate and adaptive immune responses. *H. pylori* has evolved an array of mechanisms to evade both innate and adaptive immune responses. Host-mediated immune responses not only fail to clear the bacterium but also help it colonize providing increased availability of adhesion sites such as MHC II and CD74, both of which are induced by IFN-γ and IL-8 during *H. pylori* infection [29,147]. *H. pylori* virulence factors VacA, HP-NAP, Cag T4SS have been shown to cause damage in the gastric epithelium which results in peptic ulcer or even gastric cancer, if left untreated. Bacterial virulence factors together with host factors determine the severity of disease. Though multiple studies have examined how the bacterium interacts with its host, there is still a lack of clear knowledge about how it avoids host mediated immune responses. Furthermore little is currently known about the role of T cell subsets in controlling *H. pylori* infection and associated immunopathogenesis, particularly in humans. A better understanding of the mechanisms it uses to evade or subvert host immune response is crucial to designing a therapeutic or a successful vaccine to eliminate this highly prevalent and deadly pathogen.

Chapter 2: *H. pylori cag* Pathogenicity Island's Role in B7-H1 Induction and Immune Evasion.

2.1 INTRODUCTION

GECs express cytokines and receptors that influence T cell responses during *H. pylori* infection [36,37]. B7-H1, also known as programmed death-1 ligand 1 (PD-L1), interacts with programmed death-1 (PD-1) receptor and causes down-regulation of T cell activation. *H. pylori* can also use GECs as a fulcrum to inhibit T cell proliferation and cause Treg cell induction from naïve T cells by inducing increased expression of the T cell co-inhibitory molecule B7-H1 on GEC [36,37]. However, the mechanism that is used by *H. pylori* to increase B7-H1 molecule expression on GECs is unknown. In this study, by using *in vitro* and *in vivo* systems, I investigated the role of *H. pylori* T4SS and two mediators, CagA and PG, translocated into GECs in their increased expression of B7-H1. As both CagA and PG can activate several cell signaling pathways, I also investigated the cell-signaling pathways involved in B7-H1 up-regulation by *H. pylori*. My results showed that *H. pylori* uses the p38 MAPK pathway to up-regulate B7-H1 expression in GEC. My data also highlighted the *in vivo* correlation of the presence of functional T4SS delivery system and B7-H1 up-regulation with induction of Treg cells in *H. pylori* infected mice.

2.2 MATERIALS AND METHODS

Cell lines and bacterial cultures. Human GECs N87 and AGS were obtained from the American Type Culture Collection (ATCC) and the GEC line HGC-27 was obtained from RIKEN, The Institute of Physical and Chemical Research, Japan. These cell lines were maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Immortomouse stomach epithelium (ImSt) cells were derived from C57/Bl6 and maintained in media described by Whitehead et al. [148]. *H. pylori* strains 51B and 26695 as well as their corresponding isogenic *cagA* and *cag* PAI mutants were described previously [96,149]. *H. pylori* strains were grown on tryptic soy agar (TSA) plates supplemented with 5% sheep's blood (Becton Dickinson, San Jose, CA) or on blood agar

plates with 2.5 μg/ml of chloramphenicol (Technova, Hollister, CA) to maintain *cagA*$^-$ [96] and *cag* PAI$^-$ strains at 37°C under microaerophilic conditions. *H. pylori* strain Sydney strain 1 (SS1) and PM-SS1 (pre-mouse SS1) [116] were used to infect mice. These strains were provided by Drs. J. Pappo (Astra) and Richard Peek (Vanderbilt Univ.), respectively.

Animals. Female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). B7-H1$^{-/-}$ mice [150] in the C57BL/6 background were obtained from Dr. Arlene H. Sharpe (Harvard Medical School, Boston). Animals were tested negative for the intestinal *Helicobacter* spp. prior to use in the experiments. Six-to-eight week old mice were used in the model of gastric *H. pylori* infection.

Antibodies and cell signaling inhibitors. PECγ7-conjugated anti-human B7-H1 (clone M1H1), APC-conjugated anti-murine epithelial cell marker EpCAM (clone G8.8) and their isotype controls were purchased from eBioscience. Brilliant violet-conjugated B7-H1 (clone 10F.9G2) and the corresponding isotype control was purchased from Biolegend. The viability dye eFluor 780 (eBioscience, San Diego, CA, USA) was included in the experiments to control cell viability. For cell-signaling inhibition the following inhibitors were used: CAY10512 (10 μM; Cayman chemical, MI), AG-490 (100 ng/mL; Enzo Life Sciences, Farmingdale, NY), Wortmannin (100 nM; Calbiochem, Billerica, MA), and PD169316 (10 μM/mL; Cayman chemical, MI). PG-like molecule-NOD1 ligand-iEDAP (InvivoGen, San Diego, USA) was used to investigate the role of PG in B7-H1 expression.

Infection of GEC with *H. pylori*. Before infecting with *H. pylori*, GEC were washed and their media replaced with antibiotic-free medium. The bacteria were re-suspended in RPMI 1640 medium and used at a cell:bacteria ratio of 1:10, unless otherwise indicated.

Flow cytometry. Flow cytometry was used for surface staining of B7-H1 on cultured GEC lines. Samples were collected after 24 h of incubation with the bacteria or after 12, 24 and 48 h incubation to examine the kinetics of B7-H1 expression after iEDAP treatment. Prior to performing flow cytometry, cells were harvested, counted, their concentration/tube adjusted (10^6 cells), washed and pre-incubated with normal mouse serum for 15 minutes on ice. Cells were washed again and incubated with the

corresponding conjugated antibodies or with isotype controls for 30 min on ice. After immunostaining the cells were washed twice and fixed with paraformaldehyde (1% in PBS). Cells were analyzed by flow cytometry on a LSRII instrument, where at least 10^4 live events were analyzed on cultured human GEC and 10^5 cells isolated from murine stomach in order to get 90% interval of confidence. The data were analyzed with BD FACSDiva software (BD Biosciences, San Jose, CA) and FlowJo (Tree Star, Inc, Ashland, OR).

Real-Time RT-PCR. Real-time RT-PCR analysis was performed as previously described [96]. Briefly, RT real-time PCR was done according to the 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 B7-H1 (a 20X mix of unlabeled PCR primers and TaqMan® MGB probe, FAM™ 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. The cycling parameters were as follows: 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).

NOD1 siRNA transfection. To knock down NOD1 in GEC, the cells were transfected with siRNA for NOD1 by using the basic nucleofection kit for epithelial cells (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer's instructions with a cocktail of 0.2 μM of siRNA or a negative control siRNA (Santa cruz Biotechnology, INC). For N87 cells, program T-005 was used. Knockdown of expression of NOD1 was verified by real–time RT-PCR.

Murine infection and detection of B7-H1 expression, IL-10 production, FoxP3 expression, bacterial load and histopathology. C57BL/6 mice were orogastrically inoculated with 10^8 CFU (in 100 μL of PBS/inoculation) of *H. pylori* SS1 or PMSS1 strains, three times over a week. Four weeks later, animal serum was collected, mice were euthanized and their stomachs removed, dissected longitudinally in 2-4 pieces and used for analysis of the *H. pylori* load, histopathology, RT-PCR and flow cytometry analysis as described before [96]. Stomach tissue dissociation and enzymatic digestion were performed by using gentleMACS™ Dissociator (Milteneyi Biotec, Auburn, CA) according to the manufacturer's instructionsThe tissue was then placed in HBSS

containing 0.1 mM EDTA and 0.1 mM DTT and agitated at 37°C for 15–30 min to remove the mucus. Cells were then washed twice with HBSS, which was followed by treatment with Collagenase I, II, IV (100 U/mL). After the first round of dissociation, cells were incubated with an enzymatic mixture at 37°C for 30 min. Tissue was then subjected to the second round of dissociation and treated with DNAse (10 μg/mL, Worthington) at 37°C for 15 min. Finally, tissue was processed by using the dissociator and washed twice with HBSS. The digested tissue was passed through a 40-μm cell strainer. Recovered cells were counted and used for immunostaining followed by flow cytometry analysis**.**

Murine sera collected from *H. pylori*-infected mice were examined for IL-10 by using Luminex array (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Samples were analyzed by using Bio-Plex Manager software (Bio-Rad). After homogenization of mouse stomach tissue, mRNA was isolated and expression of IL-10 and FoxP3 were determined using RT-PCR.

Murine gastric tissue was homogenized, and DNA was extracted by using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). After purification, the extracted DNA was used to detect *H. pylori* DNA by real time PCR using a protocol originally described by Rouessel *et. al.* [151]. A primer/probe set 16SHP229BP for 16S gene was used to quantify the *H. pylori* bacterial load. To determine bacterial load, standard curves were generated by PCR of serial dilutions of extracted *H. pylori* DNA. *H. pylori* in murine gastric mucosa was quantified and absolute genome copy number was calculated by previously described methods [96]. Murine GAPDH gene amplification was used to control the equal loading of total DNA used in the PCR reaction.

For histopathology analysis, one longitudinal strip of stomach was placed in 10% normal buffered formalin for 24 h at 4°C, transferred into 70% ethanol solution the next day, and stored at 4°C. Tissue was then embedded in paraffin and processed by H&E staining. The corresponding tissue sections were then evaluated and scored by a pathologist.

Statistical analysis. Unless otherwise indicated, the results were expressed as the mean ± SE of data obtained from at least three independent experiments done with triplicate sets per each experiment. Differences between means were evaluated by

analysis of variance (ANOVA) by using student's *t* test for multiple comparisons and considered significant if *p* was <0.05.

2.3 RESULTS

2.3.1 *H. pylori* uses its T4SS to up-regulate B7-H1 expression in GEC.

Our lab previously showed using gastric biopsy samples from *H. pylori*-infected and uninfected subjects as well as different human GEC lines that *H. pylori* infection causes up-regulation of B7-H1 expression by GEC. This increased expression of B7-H1 contributes to the inhibition of T cell proliferation and IL-2 production [36]. Interestingly, this increased B7-H1 expression by GECs was also noted to cause Treg cell induction *in vitro* [37]. These responses are important contributors to the chronicity of *H. pylori* infection. Herein, I sought to determine the underlying mechanisms leading to B7-H1 up-regulation by *H. pylori*. As the *H. pylori cag* PAI-encoded T4SS is important in delivering bacterial products (i.e., CagA) that alter multiple properties of the gastric epithelium, I hypothesized that this virulence factor could influence B7-H1 up-regulation. To that end, I infected the human GEC lines (AGS, N87 and HGC-27 cells) with the *H. pylori* 51B wild-type (WT) strain, which has an intact *cag* PAI and with an isogenic mutant strain lacking the *cag* PAI, *H. pylori* 51B *cag* PAI$^-$. Flow cytometry results showed that while the *H. pylori* WT strain caused a significant up-regulation of B7-H1 expression in GECs, the mutant strain lacking *cag* PAI failed to up-regulate B7-H1 expression **(Fig. 2.1A)**, which suggests a *cag* PAI-dependent up-regulation of B7-H1 expression by *H. pylori*. Similar data were obtained with a different set of WT and *cag* PAI$^-$ strains (*H. pylori* 26695). All experiments *in vitro* were performed with the three human GEC lines listed earlier to confirm that the results were consistent and not cell line-dependent.

2.3.2 *H. pylori* T4SS translocated products CagA and PG both play a significant role in B7-H1 up-regulation.

H. pylori uses its T4SS to translocate into GECs the effector protein CagA and cell wall PG fragments [27,152], and each of these bacterial products has the ability to influence cell signaling pathways, pathogenesis, and modulation of the physiology of

GEC [27,153]. As my data showed, *H. pylori*-mediated up-regulation of B7-H1 depends on the presence of T4SS. To dissect the role of T4SS components in B7-H1 up-regulation, I investigated the role of T4SS translocated CagA and PG fragments on B7-H1 increased expression. To examine whether CagA plays a role in B7-H1 up-regulation the human AGS cells were infected with *H. pylori* 51B *cagA*$^{+}$ (WT) and a *cagA*$^{-}$ isogenic mutant strain and assessed B7-H1 mRNA expression by using real time RT-PCR. **Fig. 2.1B** indicates more than a two-fold increase of B7-H1 mRNA expression from cells infected with *H. pylori* WT strain over the levels for untreated controls after 2 h of infection. In contrast, the *H. pylori cagA*$^{-}$ strain did not affect B7-H1 mRNA expression. Flow cytometry data also showed that *H. pylori* 51B *cagA*$^{+}$ strains induced a significant up-regulation of B7-H1 expression on the surface of AGS cells compared to AGS infected with a *cagA*$^{-}$ mutant strain (**Fig. 2.1C**). Similar data were obtained with the GEC lines N87 and HGC-27 and *H. pylori* 26695 WT and its isogenic *cagA*$^{-}$ mutant strains. These results further confirm that *H. pylori* uses CagA protein for the up-regulation of B7-H1 expression. As my data showed, a partial dependence of *H. pyl*ori on CagA for B7-H1 up-regulation, suggesting involvement of other components in this process, I also examined the role of PG in B7-H1 up-regulation by GEC. GEC (AGS) stimulated with the synthetic PG analogue, iEDAP, that is a NOD1 ligand, had more than two-fold up-regulation of B7-H1 mRNA expression within two hours of incubation **(Fig. 2.2A)**. These results were confirmed by using an independent method to examine surface expression of the B7-H1 protein by flow cytometry, which showed significant up-regulation of B7-H1 expression on the surface of GECs after iEDAP stimulation **(Fig. 2.2B)**. These findings were further confirmed by inhibiting NOD1 expression by using siRNA nucleofection in GECs and examined B7-H1 expression after iEDAP (PG fragment which works as NOD1 ligand) stimulation. **Fig. 2.2C** showed that GECs in which NOD1 expression was silenced with siRNA, iEDAP stimulation failed to up-regulate B7-H1 expression. Similar data were obtained with other GEC lines (N87 and HGC-27). Taken together, our data suggest that *H. pylori* uses T4SS delivered components CagA and PG to up-regulate the T cell co-inhibitory molecule B7-H1 on GEC.

2.3.3. Dose response and kinetics of PG mediated B7-H1 up-regulation.

To understand the involvement of PG in B7-H1 up-regulation the dose response and the kinetics of the response were also examined. To determine the dose response, GEC (AGS) were treated with ten-fold different concentrations of iEDAP, and B7-H1 expression was analyzed by flow cytometry. The lowest concentration of iEDAP which elicited a significant up-regulation of B7-H1 was 10 μg/ml, but it did not change much by increasing it to 100 μg/ml iEDAP, as the response was similar to that with 10 μg/ml iEDAP **(Fig 3A)**. Similar data were obtained with other GEC lines (N87 and HGC-27). Since 10 μg/ml iEDAP appeared to be optimal for the up-regulation of B7-H1 in GEC, I used this concentration of iEDAP to examine the kinetics of the response. Both AGS and N87 (data not shown for N87 cells) cell lines, showed a progressive increase of B7-H1 expression detected as early as 12 h after stimulation with iEDAP, peaked at 24 h and stayed at the same level at 48 h **(Fig. 2.3B)**. However, HGC-27 cells showed a decrease at 48-h incubation **(Fig. 2.3C)**.

2.3.4. B7-H1 up-regulation in GEC involves the p38 MAPK pathway.

CagA can activate several important cell signaling pathways, including NFκB, MAPK, STAT3, and PI3K and cause pro-inflammatory cytokine production and modulation of GEC homeostasis [19,154,155,156]. PG fragments released by *H. pylori* and other Gram-negative bacteria are recognized by the intracellular NOD1 receptors and cause activation of NFκB and MAPK pathways [157]. Since this study showed a role for both CagA and PG in B7-H1 expression, I investigated the underlying cell signaling pathways used by these components that could influence the modulation of B7-H1 expression. To that end, I used different pharmacological inhibitors directed against NFκB (CAY10512), p38 MAPK (PD169316), STAT3 (AG-490) and PI3K (wortmannin) pathways, which are known to be activated by *H. pylori* [20,154,158,159,160,161]. Inhibition of NFκB, STAT3 and PI3K pathways had no effect in B7-H1 up-regulation by *H. pylori* **(Fig. 2.4A)**; however, treating GECs with an inhibitor of the p38 MAPK pathway inhibited *H. pylori*-mediated up-regulation of B7-H1 expression **(Fig. 2.4B)**. These results suggest that *H. pylori* uses the p38 MAPK pathway to modulate B7-H1 expression in GEC.

2.3.5. Up-regulation of B7-H1 and induction of Treg cells during murine *H. pylori* infection depends on T4SS.

In order to confirm the role of *H. pylori* T4SS in B7-H1 increased expression *in vivo*, I used the mouse model of infection in this study. First,, I sought to determine whether murine GECs express B7-H1 and whether their expression of B7-H1 is modulated by *H. pylori* infection. Thus, I used the ImSt murine gastric epithelial cell line and infected the cells with the *H. pylori* PMSS1 strain, which is $CagA^+$ and has functional T4SS. Using different *H. pylori* PMSS1: ImSt cell ratios (1:1, 10:1, 30:1) I showed that infection with this strain causes significant up-regulation of B7-H1 expression in murine GECs, and the response is dose dependent **(Fig. 2.5A)**. Since my studies with human GEC showed involvement of CagA and PG in B7-H1 up-regulation, I investigated the role of these components in the up-regulation of B7-H1 by murine GECs by infecting ImSt cells with the *H. pylori* PMSS1 strain, which showed significant up-regulation of B7-H1 expression by flow cytometry, in parallel with ImSt cells infected with the *H. pylori* SS1 strain, whose T4SS is defective [162], and was found to be less effective at increasing B7-H1 **(Fig. 2.5B)**. These results were validated *in vivo* since GECs isolated from *H. pylori* PMSS1-infected mice showed significant up-regulation of B7-H1 expression after four weeks of infection compared to the control mock-infected mice while GECs isolated from the *H. pylori* SS1-infected mice showed a minimal increase in B7-H1 expression **(Fig. 2.5 C)**.

To investigate the role of B7-H1 in the induction of Treg cells *in vivo*, the Treg cell population in WT and $B7\text{-}H1^{-/-}$ mice infected with PMSS1 and SS1 strains were examined after four weeks of infection. Serum cytokine analysis demonstrated that, in contrast to SS1-infected mice, PMSS1-infected mice have increased levels of IL-10, a cytokine that is associated with Treg cell function **(Fig. 2.6A)**. The Treg cell cytokine IL-10 and transcription factor FoxP3 mRNA expression were also higher in both WT and $B7\text{-}H1^{-/-}$ mice infected with PMSS1 compared to SS1-infected mouse. However, PMSS1-infected $B7\text{-}H1^{-/-}$ mice had a lower expression of both IL-10 and FoxP3 mRNA in their stomachs compared to that found in the WT mice (Fig. 2.6B and C). Analysis of bacterial loads in the stomachs showed increased bacterial loads in PMSS1-infected mice compared to SS1-infected mice and significantly reduced bacterial loads in the $B7\text{-}H1^{-/-}$

mice compared to WT mice **(Fig 2.6D)**. To investigate the functional relevance of the B7-H1-mediated increased Treg cell response, the gastric inflammatory response was analyzed after 4 weeks of infection. An early sign of chronic inflammation is the infiltration of mononuclear cells and eosinophils. A very scarce gastric infiltration of these cells could be observed in *H. pylori* PMSS1-infected compared to SS1-infected mice. Interestingly, the B7-H1$^{-/-}$ mice also showed an increased presence of the eosinophils compared to the WT mice. However, the observed differences did not reach significant levels (data not shown). Taken together, my *in vivo* data correlated with our *in vitro* data and showed that *H. pylori* T4SS and its ability to translocate CagA and PG play an important role in up-regulating T-cell, co-inhibitory molecule B7-H1 on GEC, which in turn promotes induction of a Treg cell type of anti-inflammatory response and aids in bacterial persistence.

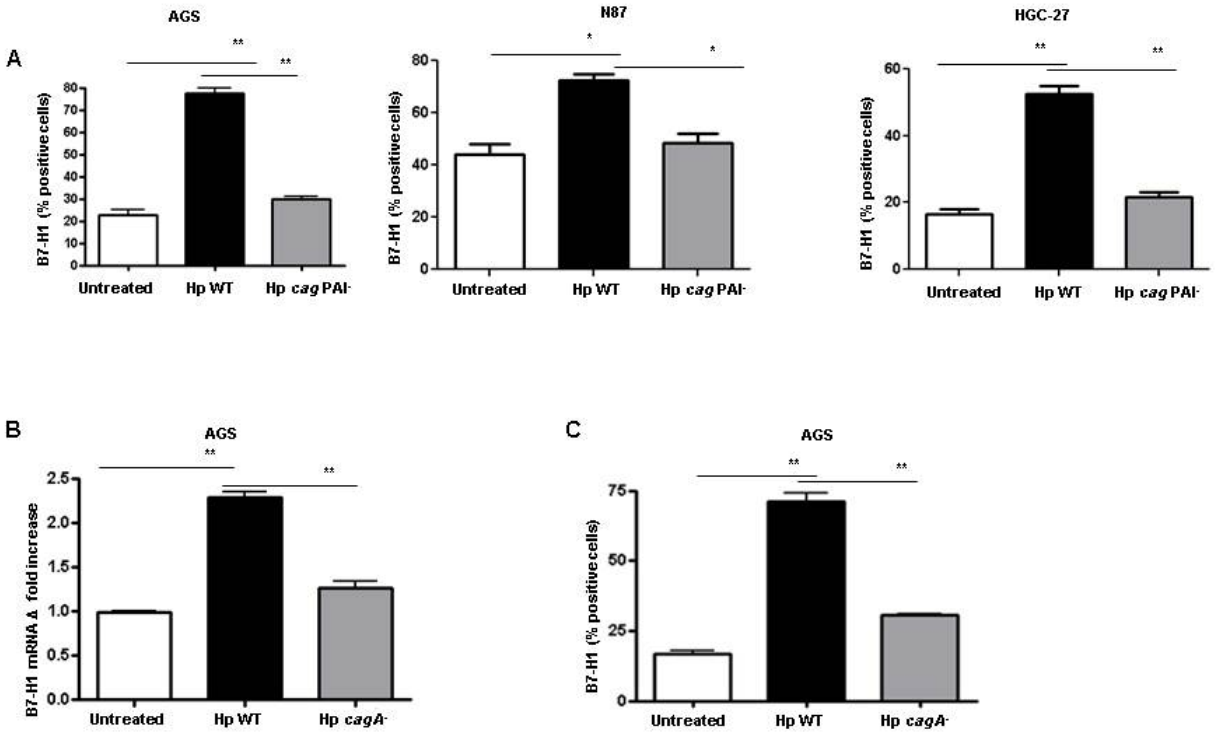

Figure 2.1: *H. pylori* uses T4SS to upregulate B7-H1 expression in GEC. (A) AGS, N87 and HGC-27 cells were infected with *H. pylori* 51B WT or 51B *cag* PAI - negative strains at 10:1 *H. pylori*:GEC ratio for 24h and B7-H1 expressed was measured by immunostaining followed by flow cytometry. (B) B7-H1 mRNA expression was analyzed using real-time quantitative RT-PCR in AGS cells. RNA was isolated from untreated and 2h *H. pylori* 51B *cagA*$^{+}$ and *cagA*$^{-}$ infected GEC. The mRNA level for B7-H1 was normalized to 18S and compared to the level of B7-H1 mRNA of untreated AGS cells (N=9,* $P < .05$). (C) Flow cytometry measured B7-H1 expression on AGS cells after 24h infection with *H. pylori* 51B *cagA*$^{+}$ wild-type (WT) and *cagA*$^{-}$ mutant strain. The data were expressed as a percents of positive cells. Isotype control value was subtracted from the data presented. N=8,*$P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

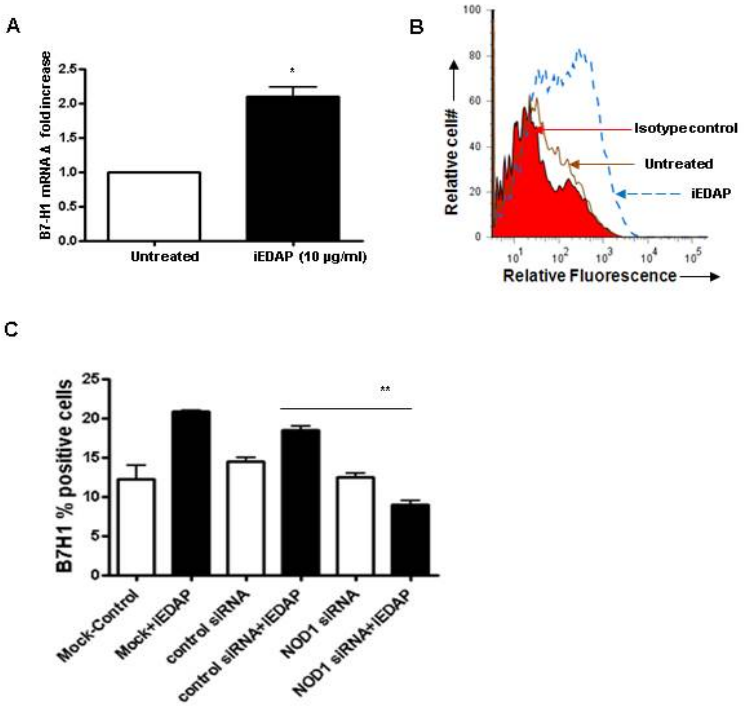

Figure 2.2: Recognition of PG by NOD1 causes induction of B7-H1 expression by GEC. (A) B7-H1 mRNA expression was analyzed by using real-time quantitative RT-PCR in AGS cells. RNA was isolated from untreated and 2 h iEDAP (dipeptide present in PG) treated (10 µg/mL) cells. The mRNA level for B7-H1 was normalized to 18S and compared to the level of B7-H1 mRNA of untreated AGS cells. N=9, $*P < 0.05$. (B) Flow cytometric analysis of AGS cells stained for B7-H1 after exposure to 10 µg/mL iEDAP for 24 h showed increased expression in a representative histogram for AGS cells where the solid peak is the isotype control and (C) GECs were treated with NOD1 siRNA to knock down NOD1 or with control siRNA, and B7-H1 expression was analyzed by flow cytometry after iEDAP (10 µg/mL) stimulation. The means are shown as the results of duplicates in four experiments, n= 8,$*P < 0.05$.

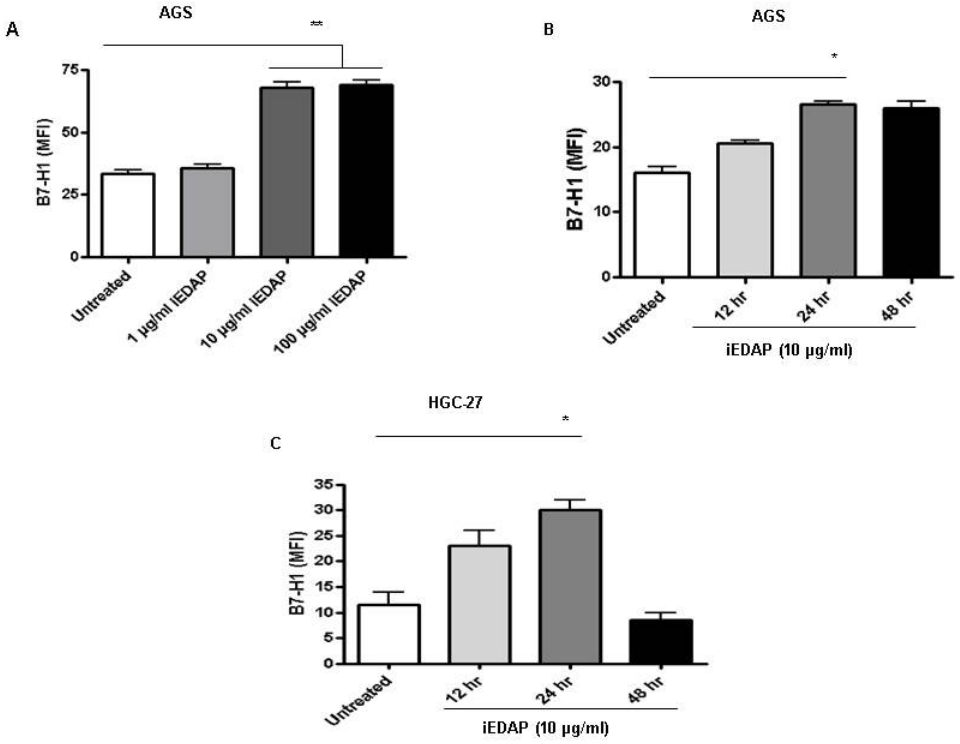

Figure 2.3: Kinetics and dose response of PG-mediated B7-H1 up-regulation . (A) Flow cytometry was done to measure B7-H1 expression on AGS cells after treating the cells with different concentrations (1, 10 and 100 µg/ml) of iEDAP for 24 h. (B) AGS and (C) HGC-27 cells expressing B7-H1 at different time points after iEDAP treatment. B7-H1 expression was assayed by flow cytometry. The data are expressed as mean fluorescence intensity (MFI). Isotype control value was subtracted from the presented data. The means ± SD are shown as the results of duplicates in four experiments, n=8, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

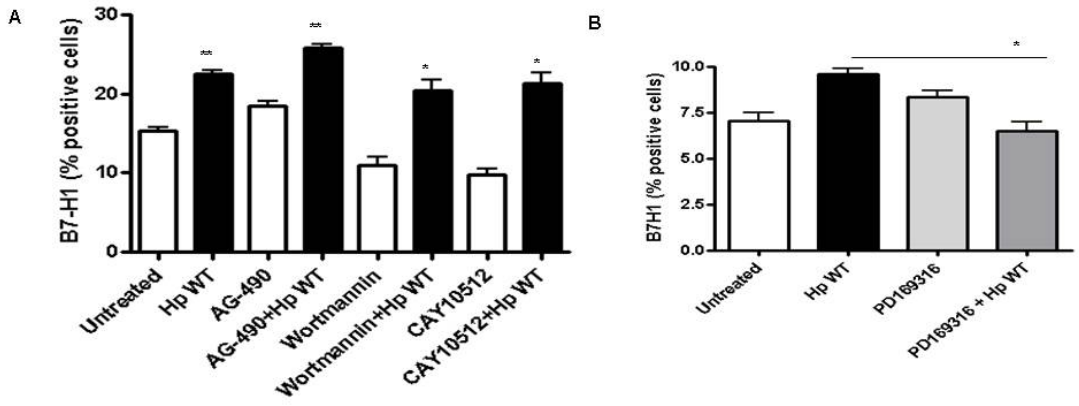

Figure 2.4: B7-H1 up-regulation by *H. pylori* depends on the p38 MAPK pathway. B7-H1 expression on AGS cells was measured by flow cytometry after treating the cells with (A) STAT3 inhibitor (AG-490), PI3K inhibitor (Wortmannin), NFκB inhibitor (CAY10512) and (B) p38 MAPK inhibitor (PD169316; 10 μM/ml) for 1 h and infecting the cells with *H. pylori* for 24 h. The means ± SD are shown as the results of duplicates in four experiments, n=8, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

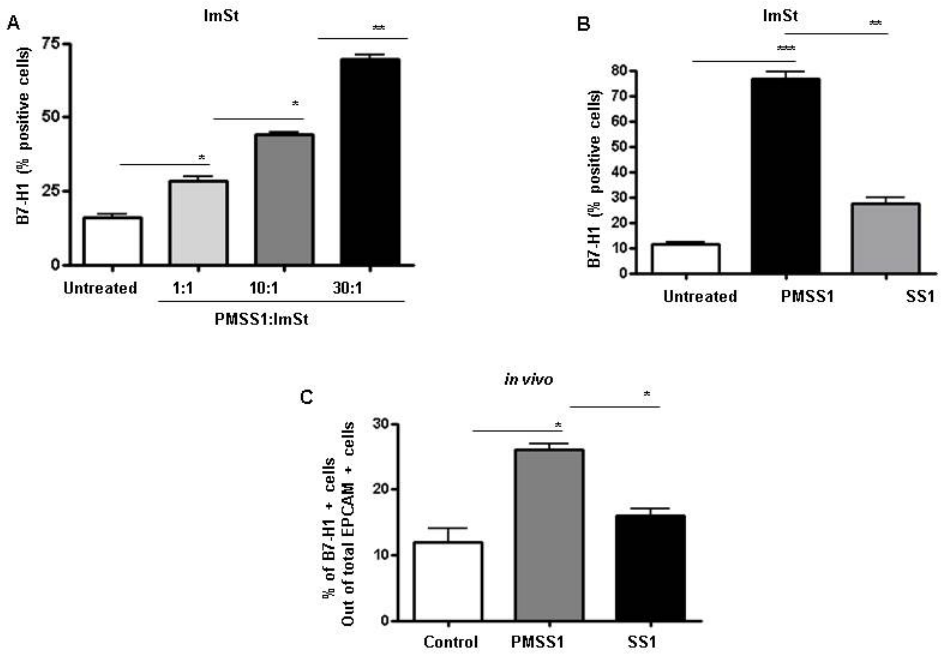

Figure 2.5: B7-H1 expression in mouse GEC and the *in vivo* model depends on *H. pylori* T4SS. (A) ImSt cells infected with different ratios of *H. pylori* PMSS1:ImSt (1:1. 10:1, 30:1) for 24 h exhibited dose-dependent up-regulation of B7-H1 when analyzed by flow cytomery. (B) ImSt cells infected with PMSS1 strain (contains functional T4SS) had higher expression of B7-H1 compared with cells infected with the SS1 strain (lacks functional T4SS) as analyzed by flow cytometry. (C) C57BL/6 mice were challenged with *H. pylori* strain PMSS1, or with *H. pylori* SS1. Gastric mononuclear cells were isolated four weeks after *H. pylori* challenge by using enzymatic digestion, and levels of the B7-H1-expressing epithelial cells ($EpCam^+$) in the gastric mucosa from the cells were measured by flow cytometry.

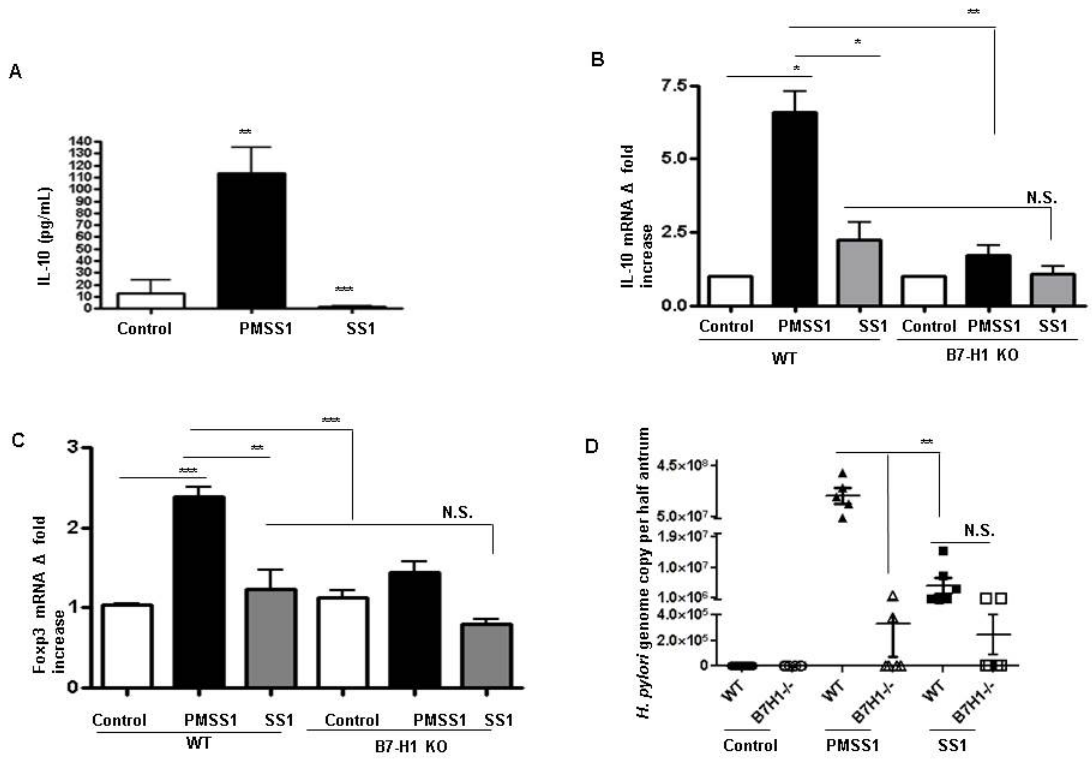

Figure 2.6: *H. pylori* uses its T4SS for Treg cell induction and bacterial persistence. WT and B7-H1$^{-/-}$ C57BL/6 mice were challenged with *H. pylori* strain PMSS1, or with *H. pylori* SS1. Mice were sacrificed after 4 weeks of infection. (A) Blood were collected and IL-10 analyzed by using a luminex bead array. Data represent mean ± SD (n=12); $*P < 0.05$. Expression of (B) IL-10 and (C) FoxP3 mRNA in the mouse stomach was done by RT-PCR. (D) Infection rate was determined by quantification of *H. pylori* genome copy per half of antrum based on the analysis of *H. pylori* 16S gene amplification by real time PCR. Average bars of infection rates were calculated from five mice per group and demonstrated as a mean ± SD.

2.4 DISCUSSION

H. pylori persistently colonizes the human stomach and elicits both humoral and cellular immune responses [97,163,164]. However, these immune responses do not clear the bacteria. $CD4^{+}CD25^{hi}$ $FoxP3^{+}$ regulatory T cells (Treg) are present in higher numbers in the gastric mucosa of *H. pylori*-infected persons and play an important role in regulating the inflammatory response and inhibiting bacterial clearance [33,99]. Previously, our lab showed that *H. pylori* up-regulates the expression of the T cell co-inhibitory molecule B7-H1 by human GEC, which in turn inhibit T cell proliferation [36] and cause induction of Treg cells [37]. However, the mechanisms whereby *H. pylori* up-regulate B7-H1 were unknown and could represent important targets in vaccine design. Using human GEC in an *in vitro* model complemented with an *in vivo* mouse model of infection which included both WT and $B7\text{-}H1^{-/-}$ mice, I showed that *H. pylori* uses T4SS to translocate the effector protein CagA and PG cell wall fragments to up-regulate B7-H1 expression by GECs. This increased expression of B7-H1 by GEC promotes the expansion of Treg cells which may foster bacterial persistence. My study also explored the underlying cell signaling pathways involved in this mechanism and showed that *H. pylori* uses the p38 MAPK pathway for the up-regulation of B7-H1 expression.

B7-H1 (PDL-1) is a member of the B7 family which interacts with its putative receptor PD-1 and plays an important role in cell-mediated immune responses [165,166]. B7-H1/PD1-mediated signaling plays a significant role in the regulation of T cell activation, tolerance, inhibition of T cell function and survival [167,168]. This pathway is targeted by various pathogens as it was noted that up-regulation of B7-H1 occurs during *Porphyromonas gingivalis* in oral squamous carcinoma cells and also hepatitis B virus-infected myeloid dendritic cells have been reported to increase B7-H1 [169,170]. Our group also showed up-regulation of B7-H1 in GEC by *H. pylori* [36]. Since *H. pylori* T4SS has been shown to play an important role in inflammation, pathogenesis and immune evasion mechanisms during *H. pylori* infection [153], I investigated its role in B7-H1 up-regulation. First using *H. pylori* WT, *cag* PAI^{-} and *cagA*$^{-}$ mutant strains, I showed that the up-regulation of B7-H1 by *H. pylori* depends on the presence of T4SS and its effector protein CagA. Though in our initial studies I did not observe a significant difference in B7-H1 expression levels in GEC infected with *H. pylori* WT and *cag* PAI

mutant strain, this time using several GECs and infecting them with *H. pylori* clinical strain and its corresponding mutant I showed that *H. pylori cag* PAI and its effector protein CagA play an important role in up-regulating B7-H1 at both the mRNA and protein levels. The previous attempt to determine the role of *H. pylori* T4SS in B7-H1 up-regulation may not have revealed differences between WT and *cagA* mutants in B7-H1 induction due to differences in the growth kinetics of the WT and mutant strains, which had not been studied. This time I studied the growth kinetics of the mutant and its corresponding parental strain and infected the GEC with the same CFU of the mutant and parental strains.

However, my data showed that *H. pylori*-mediated B7-H1 up-regulation was only partially dependent on CagA injection. This finding suggested that other components of T4SS are also involved in this mechanism. Previous studies highlighted the importance of *H. pylori* T4SS-secreted component PG in activation of MAPK and NFκB pathway and induction of inflammation [26]. Though recognition of PG by NOD1 is considered as important for host defense [27], the activation of these signaling pathways and production of IL-1β links them to the pathogenesis of several inflammatory diseases [157]. A recent study showed that *H. pylori* uses its HP310 protein for PG N-deacetylation which contributes to *H. pylori*'s survival in the host [171]. In this study I looked at the role of *H. pylori* PG in B7-H1 expression. My flow cytometry and RT-PCR data clearly showed that *H. pylori* PG plays an important role in the up-regulation of B7-H1 in GEC. By silencing NOD1 expression in GEC using siRNA nucleofection, I confirmed that recognition of PG by NOD1 contributes to this modulation of B7-H1 by *H. pylori*. This is the first report showing the ability of *H. pylori* PG in modulating the immunoregulatory properties of GEC, specifically in contributing to inhibition of host T cell function.

As *H. pylori*-mediated activation of host cell signaling pathways plays an important role in changing the homeostasis of GEC, which is very important for regulation of local T cell responses, I also determined the cell signaling pathways used by *H. pylori* to modulate B7-H1 expression in GEC. I focused on the cell-signaling pathways that are known to be activated by *H. pylori* T4SS, e.g. NFκB, STAT3, MAPK and PI3K pathways [20,154,158,159,160,161]. Previous reports have highlighted the fact that *H. pylori* activates STAT3 to modulate host immune responses [22,155]. A recent

study showed that CagA-dependent IL-8 mRNA induction also partially depends on STAT3. In that study, *H. pylori* CagA was reported to increase the bacterial lectin regenerating islet-derived (REG)3γ expression in GECs via activation of the STAT3 pathway, which allows *H. pylori* to manipulate host immunity to favor its own survival in the gastric environment [155]. MAP kinase activation is required for *H. pylori* IL-8 production [172]. MAPKs also regulate cell proliferation, differentiation, programmed death, stress, and inflammatory responses [173]. These observations suggest that through the activation of these pathways, *H. pylori* T4SS manipulates host immune regulation and deregulates gastric epithelial homeostasis for their survival. By using cell-signaling inhibitors, I showed that *H. pylori* uses the p38 MAPK pathway to up-regulate B7-H1 expression in GEC, since treating cells with the MAPK p38 inhibitor PD169316 prior to infecting the GEC with *H. pylori* showed inhibition of B7-H1 induction by the pathogen. Thus my study revealed another novel mechanism whereby *H. pylori* uses host cell signaling pathways to change the properties of GEC and thus makes a safer environment for their survival.

The role of T4SS was further confirmed in a murine GEC line and *in vivo* using a mouse model. Infection with the *H. pylori* PMSS1 strain, which contains a functional T4SS and can deliver CagA and PG into GEC, showed up-regulation of the B7-H1 molecule in both the murine GEC line (ImSt) and the mouse model. However, the *H. pylori* SS1 strain, which lacks this delivery system, failed to up-regulate B7-H1 in both ImSt murine epithelial cells and in GEC isolated from infected murine gastric mucosa. Taken together my *in vitro* and *in vivo* data confirmed that *H. pylori* uses its T4SS component CagA and PG to increase T cell–coinhibitory molecule B7-H1 on GEC, which plays an important role in controlling T cell activation.

H. pylori-infected patients have increased numbers of Treg cells ($CD4^+$ $CD25^{high}$ $FoxP3^+$) in their gastric mucosa [99]. Though Treg cells are important for suppressing overall T cell response during infection and cancer, they also inhibit protective effector T cell activity by producing anti-inflammatory cytokines IL-10 and TGF-β [174,175]. Treg cells also inhibit memory T cell response in the periphery [33]. A previous study showed that vascular endothelial cells can function as non-professional APC promoting the generation of Treg cells from naïve $CD4^+$ T cells in a B7-H1-dependent manner. $Treg_s$

cause inhibition of T cell proliferation and this process depends on B7-H1 [176]. By using B7-H1$^{-/-}$ APC, Francisco LM et al., showed that B7-H1 regulates Treg cell development from naïve CD4^{+} T cells [177]. Previously by co-culturing *H. pylori* infected GEC with naïve T cells our lab showed that *H. pylori*-mediated induction of B7-H1 co-related with induction of Treg cell development from naïve T cells [37]. In the present study, I validated these findings *in vivo* and also determined the bacterial virulence factor involved in this mechanism. My study showed that mice infected with the *H. pylori* PMSS1 strain have increased levels of circulating IL-10, an important mediator of Treg cell function and increased Treg cell transcription factor FoxP3 mRNA expression in their stomachs after four weeks of infection. In contrast, infection with the SS1 strain, which lacks a functional T4SS, failed to up-regulate IL-10 production and FoxP3 expression. IL-10 produced by Treg cells is critical for their function, as FoxP3^{+} T cells lacking IL-10 are unable to suppress the development of gastritis and colitis [178]. In addition, by using B7-H1$^{-/-}$ mice, I showed that *H. pylori* uses B7-H1 to foster Treg cell development since the B7-H1$^{-/-}$ mice infected with *H. pylori* had reduced Treg cell numbers compared to the WT mice. My study also showed less inflammatory response in the PMSS1-infected WT mice compared to the SS1-infected mice. I noticed less infiltration of mononuclear cells, lymphocytes and eosinophils in the PMSS1-infected mice. Interestingly the B7-H1$^{-/-}$ mice showed an increased inflammatory response compared to the WT mice. This finding correlates with other studies because, as in those studies, I also noticed increased Treg cell frequency is directly related to a decreased inflammatory response. One study showed that Treg cells play a protective role against gastric inflammation by reducing inflammation and ulceration in children [115]. In contrast, a study by Goll et al [179] suggested that IL-10 produced by Treg cell contribute to the chronicity of *H. pylori* infection. Work by Rad *et al.* [180] showed that Treg cells have a dual function in inhibiting gastric inflammation and facilitating bacterial colonization. In the current study, I also showed that PMSS1-infected mice have an increased Treg type of response, reduced inflammation and increased bacterial loads in their stomachs. Though inhibition of bacterial-induced inflammation is important to reduce the extent of gastritis and ulcer formation, it is also important to clear the bacteria from the system to inhibit chronic infection. My data further support our previous

findings *in vitro* and showed that *H. pylori*-mediated modulation of B7-H1 expression in GEC causes an increased Treg type response and validates the hypothesis that Treg induction aids bacterial survival in the gastric mucosa and further demonstrated that the pathogen is using its T4SS components for this mechanism. Taken together, my studies showed for the first time how *H. pylori* use GEC as mediators to manipulate local Treg cell response by using its T4SS components which helps its survival. These findings have significant importance as they provide insights into the *H. pylori* virulence factor involved in inhibiting host immune response and the underlying cell signaling pathway involved in this mechanism and could be used as potential targets for a therapeutic or a vaccine to circumvent this highly prevalent human pathogen.

CHAPTER 3: CagA-dependent down-regulation of B7-H2 expression on gastric mucosa and inhibition of Th17 responses during *Helicobacter pylori* infection

3.1 INTRODUCTION[1]

H. pylori causes a chronic infection in its host. In spite of the presence of $CD4^+$ T cells the bacteria still can cause a persistent infection. One of the reason is there is imbalance in $CD4^+$ T cells responses during the infection [36,80,115,181]. Th17 cells, whose hallmark cytokine is IL-17A, are crucial in the clearance of extracellular bacteria [104]. IL-17A is primarily associated with gastric inflammation during *H. pylori* infection and, if chronically present, may contribute to the inflammation-associated carcinogenesis [104,182,183]. On the other hand, IL-17A-initiated recruitment of neutrophils is critical for the clearance of the bacteria [105]. Although increased IL-17A expression is observed during chronic gastric inflammation, the levels produced are not sufficient to clear the infection. The mechanisms responsible for the reduced Th17 responses during the establishment of *H. pylori* persistence in the gastric mucosa remain poorly understood. DC-mediated skewing of T cell balance toward suppressive regulatory T cells (Treg) has been suggested to be important in the down regulation of Th17 and the establishment of the *H. pylori* persistence [120]. However, it is unknown whether and how GEC, as primary targets for *H. pylori* infection, contribute to suboptimal Th17 cell responses during the establishment of the chronic infection.

B7-H2 (ICOS-L) is among the newer members of the B7-family of receptors and is known to have a co-stimulatory function on T cell activity upon binding to its receptor, ICOS [184]. Recent studies have implicated B7-H2/ICOS interaction in Th17 cell development, maintenance and function [93,94,185]. However, the role of B7-H2 in immune responses to *H. pylori* is unknown. Thus, in this study I investigated the impact of *H. pylori* and its major virulence factor CagA on the modulation of B7-H2, as an important regulator of Th17 cell responses. Both *in vitro* and *in vivo* studies showed that *H. pylori* causes down-regulation of B7-H2 on GECs, and this effect depends on the

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presence of *H. pylori* CagA *via* a process involving p70 S6 kinase activation. CagA-dependent B7-H2 down-regulation on GEC correlated with the decrease in Th17 responses and was inversely correlated with the level of *H. pylori* colonization *in vivo*. Thus, this study points out a novel strategy used by *H. pylori* to impair Th17 responses, and this impairment could contribute to the establishment of persistent infection in the host.

3.2 MATERIALS AND METHODS

Animals. Female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Six-to-eight-week old mice, which were tested negative for intestinal *Helicobacter* spp., were used in the experiments. The UTMB Institutional Animal Care and Use Committee-approved protocol was followed.

Human tissue and cell lines. Gastric epithelial cells were isolated from biopsy specimens as described previously [186]. Briefly, biopsy specimens of the gastric antrum were obtained from consenting patients undergoing gastro-esophageal-duodenoscopy for various clinical indications in accordance with an Institutional Review Board-approved protocol. Patients were considered infected if *H. pylori* was detected by both rapid urease testing and histopathology, and for these studies were confirmed from biopsies to be infected by cultures of *H. pylori*. Biopsy tissue was placed in calcium- and magnesium-free HBSS, supplemented with 5% FCS and penicillin plus streptomycin, and transported immediately to the laboratory. The tissue was then placed in HBSS containing 0.1 mM EDTA and 0.1 mM DTT and agitated at 37°C for 15–30 min to remove the mucus. The biopsy tissue was placed in dispase solution (2.4 U/ml; Boehringer Mannheim, Mannheim, Germany) and agitated at 37°C for 30 min with a change of fresh dispase solution after 15 min. The supernatant was collected, and the cells were pelleted by centrifugation at 200 g for 5 min. The cells isolated were mostly (>90%) epithelial cells according to the morphology with May-Grunwald-Giemsa staining (Sigma Chemical Co., St. Louis, MO) and flow cytometry with immunofluorescence staining of anticytokeratin mAb. Human gastric carcinoma epithelial cells (GECs) N87 and AGS, as well as Hs738.st/int human gastric epithelial cells, were obtained from the American Type Culture Collection (ATCC). HGC-27 was obtained from RIKEN, The Institute of

Physical and Chemical Research, Japan and was maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and 2 mM L-glutamine.

Bacterial cultures and infection of GEC. *H. pylori* LC11 (*cagA*$^+$) and RD26 (*cagA*$^+$) strains were originally isolated from duodenal ulcer and peptic ulcer patients, respectively [187,188]. *H. pylori* strain Sydney strain 1 (SS1) and PMSS1 (pre-mouse SS1) [116] used to infect mice were kind gifts from Drs. J. Pappo (Astra) and Richard Peek (Vanderbilt Univ.), respectively. These bacterial strains were grown on tryptic soy agar (TSA) plates supplemented with 5% sheep's blood (Becton Dickinson, San Jose, CA) or on blood agar plates with 2.5 μg/ml of chloramphenicol (Technova, Hollister, CA) to grow *cagA*$^-$ strains at 37°C under microaerophilic conditions. Bacteria were transferred after 48 h into *Brucella* broth containing 10% FBS for overnight. After centrifugation at 3000 RPM for 10 min, bacteria were resuspended in normal saline. The concentration of bacteria was determined by measuring the OD_{530} on a spectrophotometer (DU-65; BD Biosciences) and comparing the value to a standard curve generated by quantifying viable organisms from aliquots of bacteria at varying concentrations that were also assessed by OD and colony formation. For specific MOI the numbers of GEC were determined by using trypan blue staining, and the required number of bacteria was added after calculation. GEC were treated with IFNγ (100 U/ml) for 48 h, and then washed and incubated an additional day in regular medium without IFNγ before infection. When IFNγ needed to be neutralized, anti-IFNγ-neutralizing Ab was added at an optimal concentration (10 μg/ml). As an isotype control, mouse IgG1κ Ab was used in the same concentration. B7-H2 expression was measured after co-culture with IFNγ or *H. pylori*-infected GEC in the presence of either anti-IFNγ -neutralizing Ab or isotype control Ab.

Construction of *cagA* isogenic mutant. For isogenic *cagA* mutants, portions of the genes were amplified by PCR, and the amplified fragment was inserted into the pBluescript SK (+) (Stratagene, La Jolla, CA). After mutagenesis by insertion of a chloramphenicol resistance gene cassette (a gift from Dr. D. E. Taylor, University of Alberta, Edmonton, Canada) in the *cagA* gene, the obtained plasmids (1 to 2 μg) were used for inactivation of chromosomal genes by natural transformation as previously described [189]. Correct integration of the chloramphenicol resistance gene cassette into

the *H. pylori* chromosome by double cross-over recombination was confirmed by PCR amplification, followed by Southern blot hybridization.

Antibodies, recombinant proteins and cell signaling inhibitors. PE-conjugated anti-human B7-H2 (clone M1H12), PE-conjugated anti-murine B7-H2 (clone HK5.3), APC-conjugated anti-murine epithelial cell marker EpCAM (clone G8.8), and PE-conjugated RORγτ (clone AFKJS-9) were purchased from eBioscience as were the isotype controls. The viability dye eFluor 780 (eBioscience, San Diego, CA, USA) was included in the experiments to control cell viability. Human rIFNγ (Roche) was used at 100 U/ml. Neutralizing Abs for IFNγ included the purified functional grade anti-human IFNγ from eBioscience. The isotype control Ab used for IFNγ studies was functional grade mouse IgG1 κ from eBioscience. For cell signaling inhibition the following inhibitors were used: CAY10512 (10 μM; Cayman chemical, Michigan, USA), AG-490 (100 ng/mL; Enzo Life Sciences, Farmingdale, NY), Wortmannin (100 nM; Calbiochem, Billerica, MA), and Rapamycin (100 ng/mL; Calbiochem, Germany).

GEC:T cell co-culture. For GEC:T cells co-culture experiments naïve $CD4^+$ T cells were isolated from peripheral blood as previously described [181]. Briefly, heparinized venous blood samples were collected from healthy volunteers negative for *H. pylori* (IRB-approved protocol 06-122 at the University of Texas Medical Branch). Peripheral blood mononuclear cells (PBMC) were prepared from collected blood by density gradient centrifugation over Ficoll-Paque Plus. Naïve $CD4^+$ T cells were isolated from the peripheral blood by negative selection using MACS Naïve $CD4^+$ T isolation kit II (Miltenyi Biotec, Germany). GEC were exposed to *H. pylori* strains for 8 h, then supernatants from *H. pylori*-treated cultures were filtered to remove bacteria, and GEC cells were washed twice with PBS to remove attached bacteria and replaced with filtered supernatants for 24 h. $CD4^+$ T cells were pre-activated for 1 h with anti-CD3/CD28 beads (Invitrogen) according to the manufacturer's instructions and added to each well at 3:1 T cell:GEC ratio and then incubated at 37°C with 5% CO_2 for 2 days.

Western blot analysis. Western blot analysis was performed as previously described [190].

Infection of mice and processing of stomach sample. Infection of mice and processing of the stomach were described in the previous chapter.

Detection of *H. pylori* in murine stomach. Detection of *H. pylori* was done by the protocol originally described by Rouessel et. al. A primer/probe set 16SHP229BP for 16S gene was used to quantify the *H. pylori* bacterial load. To determine bacterial load, standard curves were generated by PCR of serial dilutions of extracted *H. pylori* DNA. *H. pylori* in murine gastric mucosa was quantified and absolute genome copy number determined according to the method described by Rouessel et. al. [151].

Bio-Plex. The levels of total and phosphorylated cell signaling proteins in N87 cells infected with *H. pylori* strains; IL-17A from T cell-GEC co-culture and IL-17, IL-21, IL-22, IL-23 and IL-6 from murine serum collected from *H. pylori* infected mice was measured by using a Luminex array (Millipore, Billerica, MA, USA) according to the manufacturer's instruction. Samples were analyzed by using Bio-Plex Manager software (Bio-Rad).

Flow cytometry and Real-Time RT-PCR. Flow cytometry and Real-time RT-PCR was performed as we described in the previous chapter.

Statistical analysis. Statistical analysis was done as described in the previous chapter.

3.3 RESULTS

3.3.1. *H. pylori* down-regulates B7-H2 expression on Gastric Epithelial Cells.

To assess B7-H2 expression during *H. pylori* infection, mRNA expression was examined in GECs isolated from gastric mucosa biopsy samples from *H. pylori*-infected and uninfected individuals by using real-time RT-PCR. B7-H2 mRNA expression was significantly decreased in GECs from *H. pylori*-positive subjects when compared to GECs from uninfected controls **(Fig. 3.1A)**. To determine whether *H. pylori* directly induced a reduction of B7-H2 by GECs, a human GEC line (N87) was infected with *H. pylori* 51B, LC-11 or RD26 strains. A significant decrease in surface B7-H2 expression on GECs was observed at 24 h post-infection with all *H. pylori* strains **(Fig.3. 1B, C)**. Fig 1B shows the differential regulation of B7-H1 and B7-H2 by N87 cells infected with *H. pylori* 51B. This effect was dose-dependent **(Fig. 3.1D)**. Similar results were observed

when other human GEC lines (e.g. AGS, HGC-27 and HS-738 cells) were infected (not shown). Thus, these results indicate that *H. pylori* infection down-regulates B7-H2 expression on human GEC in a dose-dependent manner.

3.3.2. Down-regulation of B7-H2 expression depends on the presence of *H. pylori* CagA.

Since CagA is an important *H. pylori* virulence factor capable of eliciting multiple host cell responses, [19] I sought to determine whether down regulation of B7-H2 is influenced by *H. pylori* CagA. Infection of GEC with *H. pylori* 51B WT strain **(Fig. 3.2A)** led to as much as a 50% decrease in B7-H2 mRNA as compared to uninfected controls at the time points examined (2 and 4 h). In contrast, *H. pylori cagA*$^-$ mutant had a very limited effect on B7-H2 expression compared to the controls. These data were confirmed at the protein level. In contrast to the *cagA*$^+$ *H. pylori* strain, infection with the *cagA*$^-$ mutant had a minor effect on the reduction of B7-H2 surface expression by GECs **(Fig. 3.2B-D)**. Western blot analysis of N87 cells treated under the same conditions provided an independent approach to validate decreased B7-H2 protein levels in cells infected with *H. pylori* WT as compared to cells infected with *cagA*$^-$ strain **(Fig. 2E-F)**. These results suggested that the major *H. pylori* virulence factor CagA is involved in the B7-H2 down-regulation on GEC.

3.3.3. IFNγ and *H. pylori* have synergistic effects on B7-H2 down-regulation.

IFNγ is produced within the *H. pylori*-infected gastric mucosa [191], and previously our lab showed a synergistic effect of IFNγ and *H. pylori* on B7-H1 up-regulation on GEC [36]. Thus, I examined whether IFNγ could modulate *H. pylori*-mediated B7-H2 down-regulation on GECs. N87 cells treated with either IFNγ or *H. pylori* alone showed significant decreases in B7-H2 expression. However, treatment with both IFNγ and *H. pylori* resulted in complete abrogation of B7-H2 expression. B7-H2 expression decreased after culturing GECs with IFNγ, and decreased expression was more prominent when GEC cells were pretreated with IFNγ prior to infection with *H. pylori* **(Fig. 3.3).** Blocking the IFNγ with neutralizing anti-IFNγ mAb prevented the decrease in the levels of B7-H2 expression. These results clearly showed a synergistic

effect of IFNγ and *H. pylori* in reduced B7-H2 expression by GECs. Interestingly, the synergism of IFNγ and *H. pylori* in decreasing B7-H2 expression was less obvious when cells were infected with a *cagA*$^-$ strain **(Fig. 3.3)**. This result supports the key role of CagA in B7-H2 down regulation during *H. pylori* infection.

3.3.4. B7-H2 down regulation involves activation of mTOR/p70 S6 kinase.

Previous studies showed that *H. pylori* CagA protein can activate NFκB, MAPK, STAT 3 and PI3K pathways [154,158,192,193]. In order to understand the underlying mechanisms regulating B7-H2 decreased expression during *H. pylori* infection, I first globally analyzed pathways activated in *H. pylori* infected GEC by using a Luminex cell signaling array. My data demonstrated that, in addition to NFκB and STAT3 pathways, *H. pylori* infection of GEC also leads to the activation of mTOR/p70 S6 kinase within the first 5 minutes of infection **(Fig.3. 4A, B)**. In contrast, no significant phosphorylation of p70 S6 kinase was observed in GEC infected with the *cagA*$^-$ strain **(Fig. 3.4C, D)**. Thus, I examined the role of these pathways in *H. pylori*-mediated down regulation of the B7-H2 by using specific inhibitors. I observed that down regulation of B7-H2 by the *cagA*$^+$ *H. pylori* strain was blocked in the presence of rapamycin, a p70 S6 kinase/mTOR-specific inhibitor **(Fig. 3.4E)**. In contrast, inhibition of the PI3K, STAT3 and NFκB pathways with pharmacological inhibitors did not affect *H. pylori*-mediated down regulation of B7-H2 expression **(Fig. 3.4F)**. These results suggest that p70 S6 kinase is a key signaling pathway in *H. pylori*-mediated down regulation of B7-H2 on GECs.

3.3.5. CagA$^+$ *H. pylori* infection reduces induction of Th17 by human gastric epithelium.

As B7-H2 has been implicated in Th17 cell differentiation, I examined whether the CagA-dependent B7-H2 down regulation impaired the capacity of GEC to induce Th17 cells differentiation from naïve CD4$^+$ T cells. My results showed that there is a small induction of RORγ-expressing CD4$^+$ T cells when naïve CD4$^+$ T cells were co-cultured with N87 cells infected with *H. pylori* WT, but this induction was significantly increased when cells were infected with a *cagA*$^-$ strain **(Fig. 3.5A)**. The presence of Th17 was further confirmed by measuring IL-17A in co-culture supernatants by a Luminex

array **(Fig. 3.5B)**. Analysis of mRNA levels in parallel cultures confirmed these findings by showing increased RORγ and IL-17A mRNA levels in $CD4^+$ T cells **(Fig. 3.5C and D)**. These data demonstrate the critical role of CagA in maintaining low level Th17 responses, and this may contribute to *H. pylori* immune evasion.

3.3.6. Down-regulation of B7-H2 on GEC during murine *H. pylori* infection depends on CagA and correlates with the decrease of Th17 responses and increase in *H. pylori* colonization.

To understand the relevance of the observations *in vitro* to the *H. pylori*-associated immunopathophysiology, I used a mouse model of *H. pylori* infection. Mice infected with *H. pylori* PMSS1, which contains a functional T4SS, had down-regulated B7-H2 expression by gastric epithelial (EpCAM+) cells. Interestingly, infection of mice with *H. pylori* SS1 strain in which the T4SS is defective and cannot deliver CagA in GEC [162] resulted in up regulation of B7-H2 **(Fig. 3.6A-B)**. No significant difference was observed in gastric mucosal inflammation in mice infected with either strain at week four. However, the *H. pylori* load was drastically different between the mice infected with SS1 and PMSS1 strains. Mice infected with the PMSS1 strain had a >100-fold higher bacterial burden when compared to those infected with the SS1 strain **(Fig. 3.6C)**. Further, analysis of the serum cytokine profile demonstrated that in contrast to the mice infected with the SS1 strain, which had significant serum levels of IL-17 and IL-21 ($P<0.05$), mice infected with the PMSS1 strain failed to up regulate pro-inflammatory Th17 cytokines **(Fig. 3.7)**. Serum levels of IL-6, IL-22 and IL-23 were also increased significantly in *H. pylori* SS1-infected mice **(Fig. 3.7)**. Taken together, my *in vivo* data correlate with my *in vitro* findings and suggest that CagA-mediated down regulation of the B7-H2 might be involved in the prevention of the Th17-mediated clearance of *H. pylori* during onset of the infection.

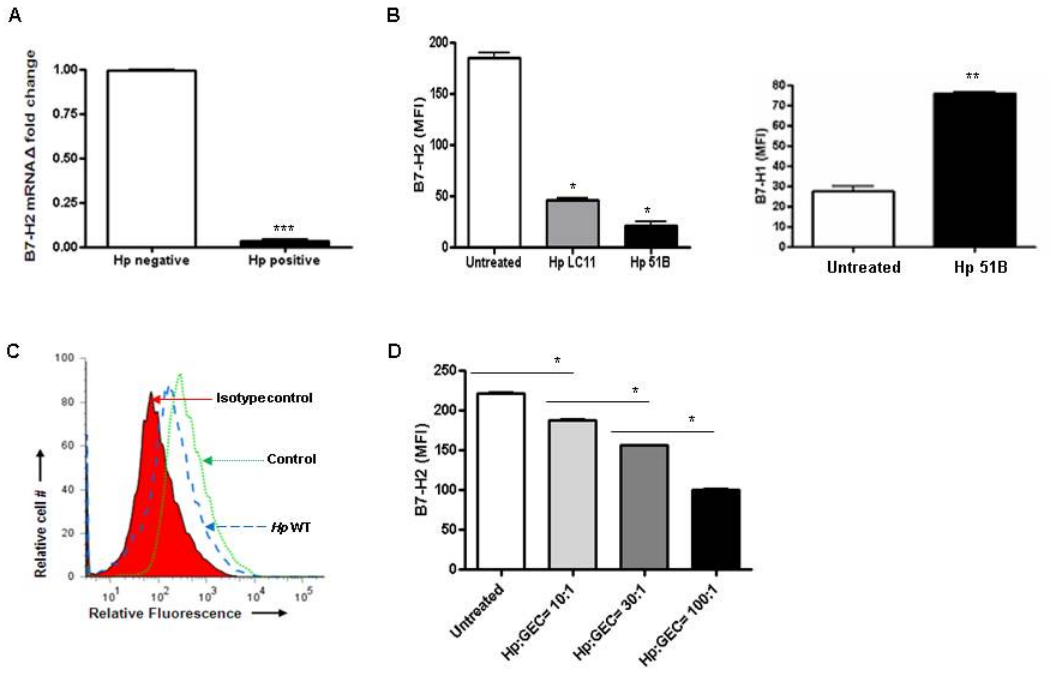

Figure 3.1: Decreased expression of B7-H2 in *H. pylori*-infected GEC. (A) Gastric biopsy samples were collected from five *H. pylori*-negative and five *H. pylori* positive patients. GEC was isolated and analyzed for the B7-H2 mRNA expression by real-time RT-PCR. (B) Modulation of the B7-H2 surface expression on GEC N87 by infection with *H. pylori* 51B and LC11 (24 h post- infection, *H. pylori*:GEC ratio 10:1) as determined by flow cytometry analysis. Up regulation of B7-H1 on N87 cells after treatment with *H. pylori* 51B was shown as a positive control. (C) One of representative flow cytometry histogram obtained for GEC N87 infected with *H. pylori* RD26. (D) Dose-response analysis. N87 cells were infected with *H. pylori* RD26 strain at different ratio of *H. pylori*:GEC (10:1, 30:1 and 100:1) for 24 h and analyzed by flow cytometry. The data expressed as a mean of fluorescence intensity (MFI). Data represents the mean ± SD (n=8); $P < 0.05$.

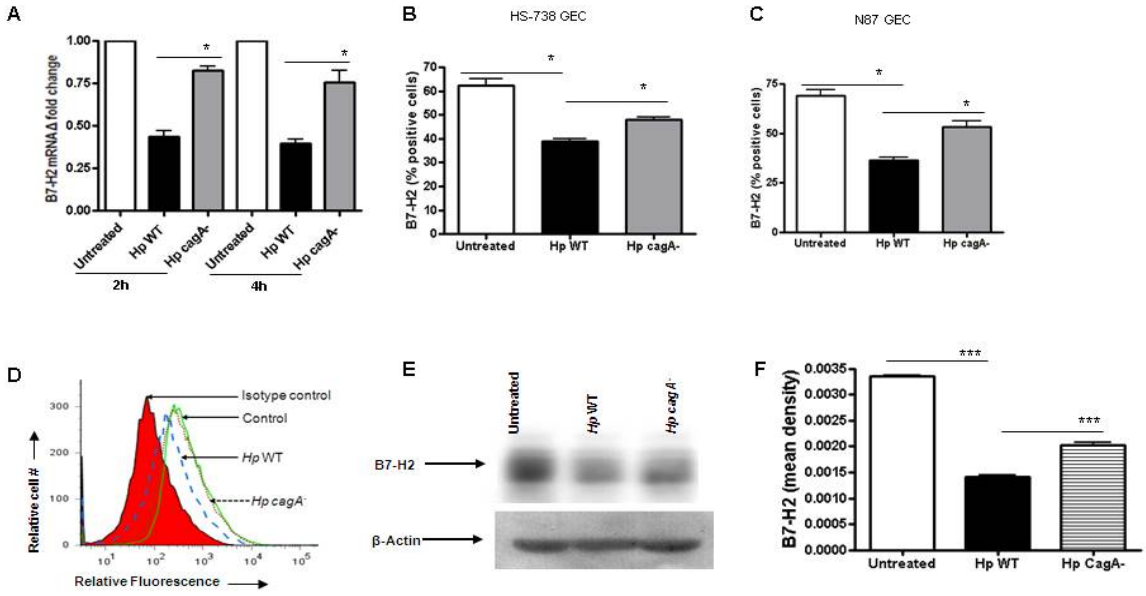

Figure 3.2: *H. pylori*-mediated down regulation of B7-H2 on GEC involves CagA. (A) B7-H2 mRNA expression in N87 cells was analyzed by using real-time RT-PCR. RNA was isolated from uninfected and at 2 and 4 h of *H. pylori cagA*$^+$ (WT) and *cagA*$^-$ strain infected GEC. The mRNA level for B7-H2 was normalized to 18S and compared to the level of B7-H2 mRNA in untreated N87 cells. N=9, $P < 0.05$. (B) Surface B7-H2 expression was analyzed by flow cytometry on HS-738 and (C) N87 GEC cell lines after 24 h of infection with *H. pylori cagA*$^+$ (WT) or *cagA*$^-$ mutant. The data are expressed as percentage of positive cells expressing B7-H2. (D) One representative histogram is shown for GEC N87 infected with *H. pylori* 51B WT or *cagA*$^-$ mutant. N=8, $P<0.05$. (E) B7-H2 expression was analyzed by western blot in N87 cells treated with *H. pylori* WT and *cagA*$^-$ strain after 24 h infection. (F) Quantitative analysis of B7-H2/beta-actin ratio in GEC infected sample is included (n=3).

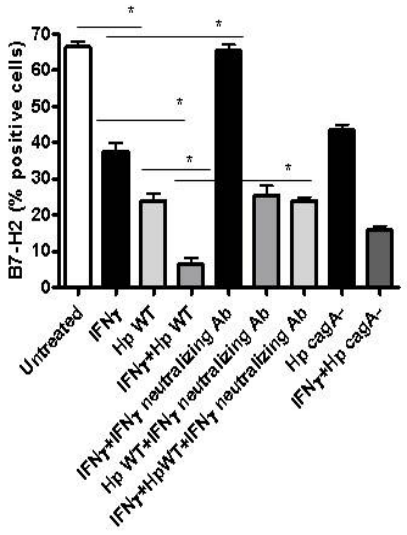

Figure 3.3: IFNγ synergize *H. pylori*-mediated B7-H2 down regulation on gastric epithelium. N87 cells were treated with IFNγ (100 U/mL) for 48 h. Cells treated with IFNγ were washed and cultured with medium for 24 h before they were either infected with *H. pylori* 51B WT and *cagA*$^{-}$ strain in the presence or absence of IFNγ-neutralizing Ab. B7-H2 expression on N87 GEC was measured by flow cytometry after 24 h. Graph represents the mean ± SD (n=8); $P < 0.05$.

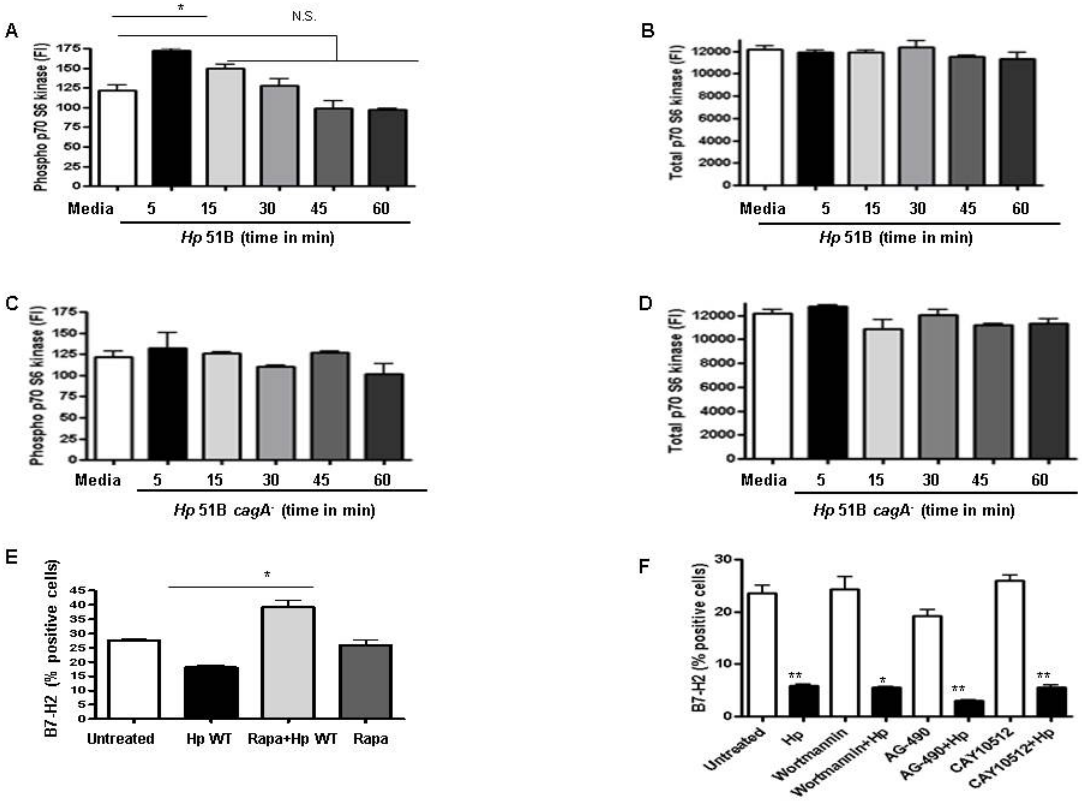

Figure 3.4: Activation of mTOR/p70 S6 kinase involved in the *H. pylori*-mediated down-regulation of B7-H2 expression. N87 cells were incubated with *H. pylori* 51B WT and *cagA*$^-$ strain. Cell were lysed after 5 15, 30, 45 and 60 min. Cell lysates from GEC exposed to both *H. pylori* 51B WT and *cagA*$^-$ strain were analyzed for (A, C) phospho and (B, D) total p70 S6 kinase using Luminex bead array, respectively. (E) GECs were treated with rapamycin (100 ng/ml), an inhibitor of the mTOR/p70 S6 kinase pathway, (F) or with wortmannin (100 nM), AG-490 (100 ng/mL) and CAY10512 (10 μM) inhibitor for the PI3K, STAT3 and NFκB pathways, respectively, for 1 h and then were infected with *H. pylori*. B7-H2 expression was analyzed by using flow cytometry 24 h later. Data expressed as a percentage of positive cells. Results are represented as the mean ± SD (n=8); *P*=N.S. (Not significant); **P < 0.05.*

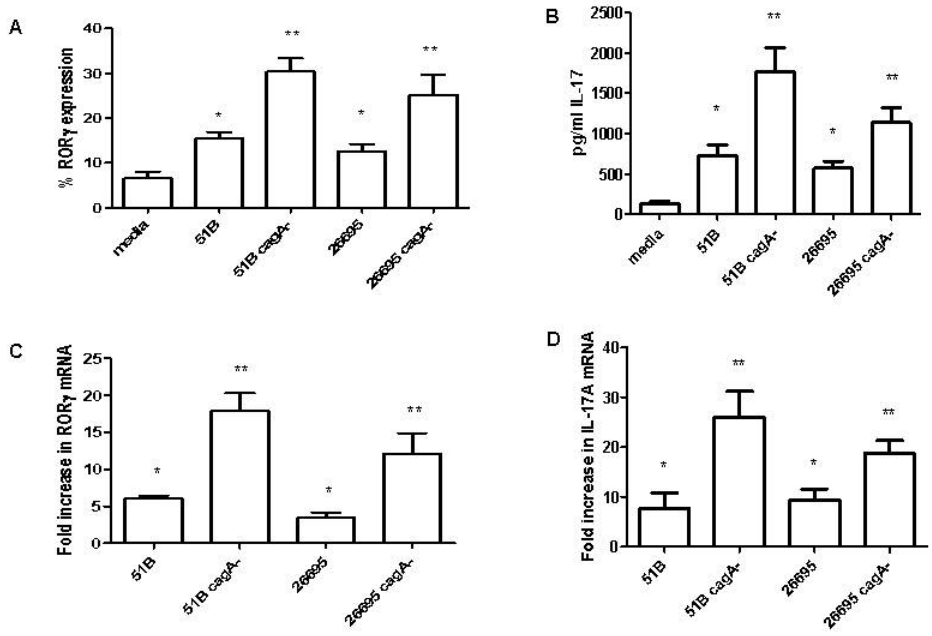

Figure 3.5: *H. pylori*-mediated Th17 development from activated naïve T cells in co-culture with N87. N87 cells were treated with either media, with *H. pylori* WT, or with *H. pylori cagA*$^-$ strain (*H. pylori*:GEC=10:1) for 8 h. After treatment, the GECs were washed and co-cultured with preactivated human CD4$^+$ T cells (3:1 T cell: GEC) for 2 days and were analyzed for (A) RORγ expression in T cells by flow cytometry, (B) IL-17A production in supernatant by Luminex array, (C) RORγ mRNA levels, and (D) IL-17A mRNA levels in T cells by Real Time PCR. mRNA was normalized to 18S and compared to untreated cells. N=6, $*P < 0.05$.

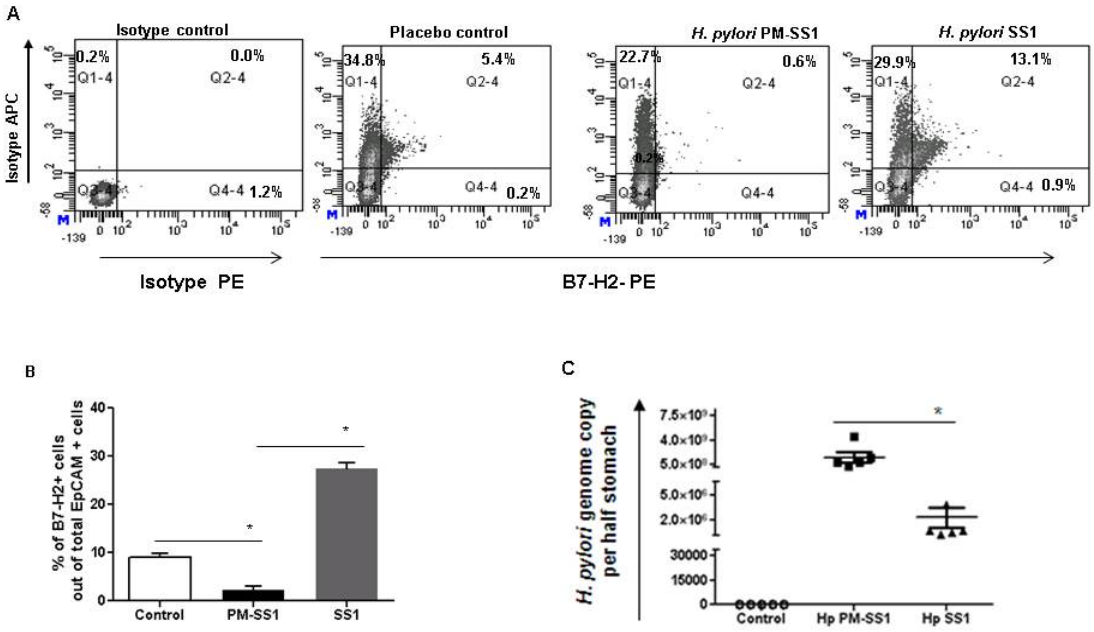

Figure 3.6: *H. pylori*-mediated down regulation of B7-H2 on GEC *in vivo* involves CagA function and inversely correlates with the bacterial clearance. (A) C57BL/6 mice were challenged with *H. pylori* strain PMSS1, which expresses functional T4SS and can deliver CagA or with *H. pylori* SS1, which does not. Gastric mononuclear cells were isolated four weeks after *H. pylori* challenge by using enzymatic digestion and expression of B7-H2 and epithelial cell marker EpCAM was analyzed by flow cytometry. (B) Level of the B7-H2-expressing epithelial cells ($EpCam^{+}$) in the gastric mucosa from the cells was measured by flow cytometry. (C) Infection rate was determined by quantification of *H. pylori* genome copy per half of stomach based on the analysis of *H. pylori* 16S gene amplification by real time PCR. Each data point represents a single mouse tested in quadruplicate. The average bar of infection rates was calculated from five mice per group and demonstrated as a mean ± SD.

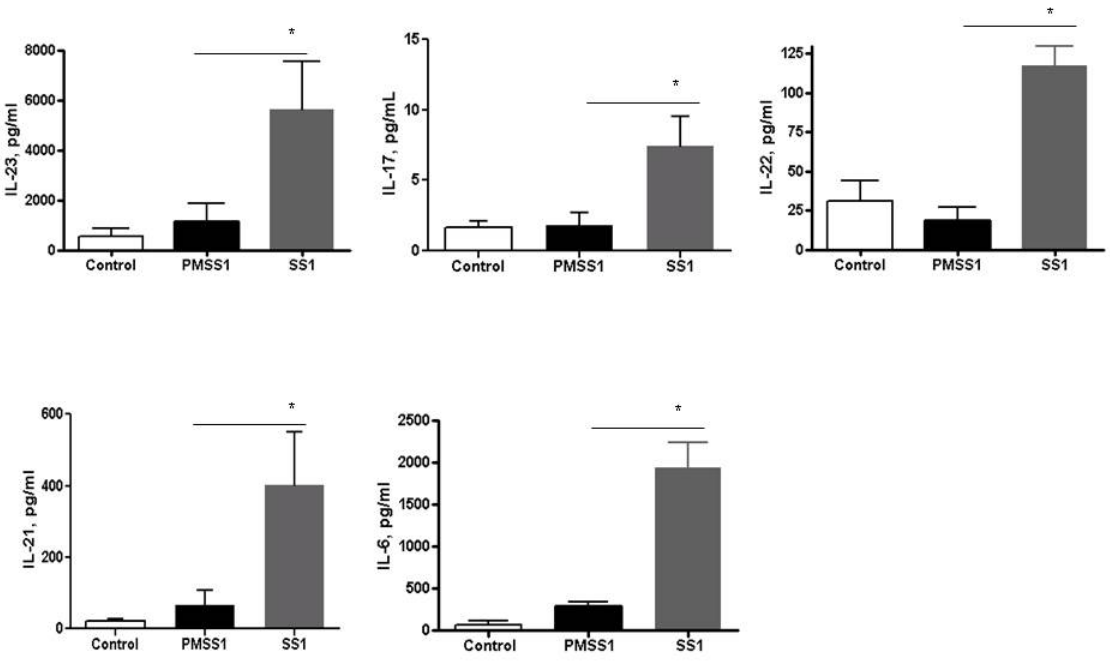

Figure 3.7: *In vivo* infection with *H. pylori* expressing functional T4SS and which can deliver CagA fails to up regulate Th17 type responses. C57BL/6 mice were challenged with *H. pylori* strain PM-SS1, or with *H. pylori* SS1. Blood was collected four weeks after *H. pylori* challenge and cytokine profile analyzed by using a luminex bead array. Data are represented as mean ± SD (n=12); **P <0 0.05*.

3.4 DISCUSSION

During *H. pylori* infection, the host mounts an immune response, but this response is insufficient to clear the infection leading to the establishment of a persistent infection and development of chronic inflammation. Infiltration of $CD4^+$ T cells into the gastric mucosa is among the major factors contributing to the ongoing inflammation. At the same time, these cells are required for the immunization-induced protective responses [194,195,196]. Recent data suggested that Th17 type responses are required for the clearance of the bacteria [197]. The exact mechanism(s) implicated in the *H. pylori*-mediated escape of host immunity to prevent clearance of *H. pylori* remains far from understood. Here I demonstrated that *H. pylori* infection of GEC leads to a decrease of B7-H2, which is a positive co-stimulatory ligand, and that this process might be important in *H. pylori*-mediated escape of Th17-mediated bacterial clearance.

Co-stimulatory interactions between B7 family ligands and their receptors play important roles in the growth, development and differentiation of T cells. Recent data demonstrated that interaction between B7-H2 on APCs with its putative receptor ICOS on T and B cell regulate adaptive immune responses [185,198]. Stimulation of ICOS was demonstrated to be critical for the development of human IL-17-producing $CD4^+$ T cells [94]. Further, Bauquet *et. al.*, [93] demonstrated that ICOS was critical for maintaining effector-memory Th17 cells. B7-$H2^{-/-}$ knock out (KO) mice also were noted to have lower Th17 responses to chlamydial infection than had WT mice [199]. The blockade of B7-H2/ICOS signaling inhibited Th1 and Th17 cells responses in chronic inflammatory conditions such as rheumatoid arthritis [185]. Together these findings implicate B7-H2/ICOS signaling as an important mediator in the activation of Th17 cells in inflammation. Since expression of ICOS ligand B7-H2 was previously reported in intestinal epithelial cells [200] and, due to its importance in activation of Th17 responses, I measured the expression of B7-H2 in human biopsy samples and found that epithelial cells in gastric biopsies isolated from *H. pylori*-infected patients had decreased levels of B7-H2 expression compared to those in uninfected biopsy samples. B7-H2 expression in colonic and airway epithelium was previously noted [200]. However, little is known about the role of B7-H2 co-stimulation in the responses associated with bacterial immunopathogenesis and clearance. In the current study, I demonstrated for the first time

that *H. pylori* significantly down regulated B7-H2 expression in gastric mucosa, particularly on GEC.

Since CagA has been shown to play an important role in *H. pylori*-mediated pathogenesis and immune evasion mechanisms [153], I sought to investigate its role in the observed B7-H2 down regulation. In this study using a CagA isogenic mutant and their corresponding parental strains, I showed that CagA plays a crucial role in down regulating B7-H2 expression on GEC. Though compared to the untreated cells, *H. pylori cagA*$^-$ strains always showed some down regulation of B7-H2, suggesting that other components of *H. pylori* might also have an influence in down regulating B7-H2 expression, but it is less effective than the WT CagA$^+$ strains. *In vivo* data also showed that *H. pylori*-mediated transfer of CagA via a T4SS significantly down regulates B7-H2 expression in the GEC in the murine gastric mucosa. These *in vitro* and *in vivo* data reveal a novel mechanism whereby *H. pylori* uses CagA, to create a favorable environment for its persistence via suppression of the positive costimulators required for an efficient effector T cell response.

As cytokines play an important role in regulating immune function and IFNγ has been detected in *H. pylori*-infected gastric tissues in both humans and mice, [103] I also investigated whether IFNγ has any role in B7-H2 expression. My results showed that IFNγ synergizes with the effect of *H. pylori cagA*$^+$ strains in down regulating B7-H2 expression by GEC. Several studies showed induction of B7-2, B7-H1, and B7-DC in different classical APCs by IFNγ [201,202]. Stanciu *et al.* [203] showed a synergistic effect of respiratory syncytial virus (RSV) and IFNγ in the up regulation of B7-H1 and B7-DC in respiratory epithelial cells. In addition, their study showed that treatment of RSV infected cells with IFNγ causes down-regulation of B7-H2 and B7-H3 expression. Previous findings from our group showed IFNγ and *H. pylori* synergize in B7-H1 up regulation [36]. Thus, the synergistic effect of IFNγ and *H. pylori* in B7-H2 down regulation could result from IFNγ-mediated increase in expression by GEC receptors that are used by *H. pylori* [186].

CagA interacts with several intracellular components of signal transduction and activates the NFκB, MAPK, STAT3 and PI3K/Akt pathways [154,158,192,193]. Previous reports have highlighted the fact that *H. pylori* activates STAT3 to modulate

host immune responses [192]. Though my results showed activation of these pathways by CagA-expressing *H. pylori* strains, my data implies that CagA-mediated activation of the NFκB, STAT3 and PI3K pathways is not required for the *H. pylori*-mediated down regulation of B7-H2 on GEC. Furthermore, these data for the first time demonstrate that CagA contributes to the *H. pylori*-mediated activation of the mTOR/p70 S6 kinase pathway. Serine/threonine protein kinase mTOR acts in a signaling pathway downstream from PI3K/Akt and regulates the activation of the p70 S6 kinase, which is required for translational regulation of ribosomal proteins [204]. By using specific cell signaling inhibitors, I showed that *H. pylori* uses CagA to manipulate B7-H2 expression by activating the p70 S6 kinase pathway to prevent GEC from providing the positive co-stimulation needed for protective Th17 cells.

Previously, our group showed that *H. pylori* uses its CagA and VacA proteins to induce TGF-β production from GEC, which causes inhibition of $CD4^+$ effector T cell proliferation and induction of Treg cells [181]. Herein using *in vitro* GEC-T cell co-cultures we showed that *H. pylori* uses CagA cytotoxin to down regulate Th17 cell type responses. A significant down regulation of Th17 cell transcription factor RORγτ and IL-17A was observed when GEC were exposed to the *H. pylori* strains expressing CagA, but not in presence of the *cagA*$^-$ mutant. Our *in vivo* data with the *H. pylori* mouse model supported this *in vitro* observation. Further, I showed that *H. pylori* CagA-mediated B7-H2 down regulation correlates with a decrease in Th17-type responses detected in murine serum and an increase in *H. pylori* colonization of the gastric mucosa. Though my data showed an increased *H. pylori* bacterial load and decreased Th17 type of response in PMSS1-infected mice, compared with those infected with the SS1 strain, I did not observe severe inflammatory changes in any of the *H. pylori*-infected mice. This might be because *H. pylori* infection in this mouse model results mostly in lymphocytic gastritis which does not progress to severe inflammation. An optimum induction of chronic gastritis can be achieved using *H. felis* as a model [205]. Th17 cells have been suggested to have dual roles in both infection control on one hand and pre-neoplastic changes on the other. Several studies showed that protective immunity against *H. pylori* infection requires a strong Th17 response [15]. This study showed reduced Th17 cell cytokines and increased bacterial load in the PMSS1-infected mice which correlates with results from

another recent study [197]. Horvath et al. showed that mice lacking IL-23 when infected with *H. pylori* showed reduced IL-17 production and increased bacterial load in their stomachs. Another study suggested that ICOS-induced signaling is essential for IL-21-mediated regulation of IL-23R expression in differentiated Th17 cells and for IL-23-driven expansion of Th17 cells [93]. This study also supports the importance of B7-H2/ICOS signaling in Th17 cell development, since the down-regulation of B7-H2 expression in the gastric mucosa of PMSS1 *H. pylori*-infected mice correlates with decreased Th17 type responses.

These data suggest a novel CagA-dependent mechanism, which involves down-regulation of B7-H2 on GEC, a primary target for *H. pylori* infection, used by the bacteria to avoid a Th17 cell-mediated clearance. Thus, my *in vitro* and *in vivo* studies suggest that *H. pylori* uses the T4SS to down regulate Th17 cell responses, which are critical for clearing the pathogen. Therefore, the *H. pylori* CagA delivery system may be an important target in vaccine development for achieving acceptable levels of immune protection and thereby useful for designing a therapeutic strategy to treat patients infected with this prevalent and deadly human pathogen.

CHAPTER 4: *H. pylori* Induces B7H3 Expression by Gastric Epithelial Cells: Implications for Local T cell Regulation and Subset Development.

4.1 INTRODUCTION

B7-H3 (CD276) is a new member of the B7 family that shares 20–27% identical amino acids with other members of this family of receptors [206]. Human B7-H3 protein is not constitutively expressed but can be induced in activated dendritic, B, T, and NK cells and in some tumor cell lines [206,207,208,209]. B7-H3 has been found to be strongly expressed in unstimulated tracheal, bronchial, and alveolar epithelial cells and the expression is induced by respiratory syncytial virus (RSV) infection [203]. B7-H3 was initially identified as a co-stimulatory molecule that was shown to promote T-cell proliferation and IFN-γ production [206]. However, recent studies have presented contradictory roles for B7-H3, since they suggest that B7-H3 has both immunological stimulatory and inhibitory functions [206,207,208,209,210,211,212,213]. For instance, in conjunction with anti-CD3, B7-H3-Ig fusion protein co-stimulates $CD4^+$ and $CD8^+$ T cells and induces IFN-γ production. Another study demonstrated that acute and chronic cardiac allograft rejection can be reduced in B7-H3 knockout mice, which supports a stimulatory role for B7-H3 on T cells [213]. In contrast, B7-H3 has been reported to impair T-helper 1 (Th1) cell responses and inhibits cytokine production [210]. An *in vivo* study also showed B7-H3 negatively regulates T cells [208,210,212]. Moreover, recent study in an asthma model demonstrated that B7-H3 also plays role in the induction of Th2 cells [214]. Not only does B7-H3 regulates T cell activity and subset development but also works as a biomarker for tumor progression and development of cancer. Higher expression of B7-H3 have been shown in different cancers [215,216,217,218,219]. An increased expression of B7-H3 was reported to lead to an increased risk of recurrence of some cancers [215,216,217], while alternatively increased B7-H3 sometimes showed links with the prospective survival in other cancers [218,219]. Recently, increased B7-H3 expression was found in circulating tumor cells in gastric cancer patients compared to those in healthy volunteers. Moreover, patients with increased B7-H3 levels showed lower survival rates [220]. However, a separate study reported that increased B7-H3

during gastric cancer was associated with increased survival rate [219]. These suggested that B7-H3 might be also involved in cancer immunity.

During *H. pylori* infection, patients have a mixed Th1/Th2 response [179], with increased Treg [32,33] and Th17 cells in their circulation [104,182,221,222]. Though there are reports showing the type of T cell responses elicited by *H. pylori* infection, there is knowledge gap regarding the mechanism that *H. pylori* uses to induce different T cell phenotypic subsets. Previously our laboratory group has shown that *H. pylori* modulates B7 molecule expression in GECs, which not only help restrain T cells responses, but also induce T regulatory cells to facilitate *H. pylori* survival [36,37,96]. More recently, my data showed that *H. pylori* uses its T4SS to down-regulate B7-H2 expression in GECs, which act to keep Th17 cells at a suboptimal level, since Th17 are important in the control of extracellular bacterial infections, this down-regulation of B7-H2 helps *H. pylori* to persist [96]. Our study also showed that *H. pylori*-mediated up-regulation of B7-H1 expression in GEC causes induction of Treg cells, which also contributes to the establishment of a chronic infection [37]. Herein, I studied another important B7 molecule, B7-H3, and showed that *H. pylori* up-regulates the expression of this molecule on GEC. The up-regulation of B7-H3 is regulated not only by the T4SS but also by the cytokines produced by Th17 and Treg cells. I further evaluated the underlying cell signaling pathway and demonstrate that *H. pylori* uses the p38 MAPK pathway for B7-H3 up-regulation. *H. pylori* is one of the most genetically diverse bacterial species. *H. pylori* strains differ in the rate with which they have *cag* PAI in their genome. The EPIYA motifs in *cagA* gene also differs between Asian and western countries. Moreover, *H. pylori* infection may result in gastritis, ulcer and gastric cancer development. I tested how *H. pylori* strains isolated from these three types of gastric disease modulates B7-H3 expression on epithelial cells. In my study I was curious to determine whether the increase of B7-H3 is consistent with all strains or not. I have shown that only *H. pylori* strains associated with gastritis causes increased B7-H3 expression and induction of the $GATA3^+$ Th2 cell response. This finding was further confirmed by co-culturing GECs infected with different *H. pylori* strain with naïve $CD4^+$ T cells. This is a novel finding which shows how *H. pylori* manipulate GECs to differentially express the B7-H3

molecule and thus regulates T cell response involved in the *H. pylori* associated immune-pathogenesis to promote their survival/persistence.

4.2 MATERIALS AND METHODS

Human tissue. For evaluation of B7-H3 expression, biopsy specimens of the gastric antrum were obtained from consenting patients undergoing gastro-esophageal-duodenoscopies in accordance with an Institutional Review Board-approved protocol. GECs were isolated from biopsy specimens as described previously [96]. Patients were considered infected if *H. pylori* was detected by rapid urease testing, histopathology, and by culture of *H. pylori* from biopsies. For examination of the relative expression of B7-H3 and Th2, biopsy samples from patients with gastritis and tumors from patient with gastric tumors were collected by surgical resections (under IRB protocol numbers 10-513 and 10-514).

Cell lines, bacterial cultures and small peptides. Human GECs N87 and AGS were obtained from the American Type Culture Collection (ATCC) and HGC-27 from RIKEN, The Institute of Physical and Chemical Research, Japan. All cell lines were maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Immortalized mouse stomach epithelium (ImSt) cells were maintained in media described by Whitehead et al. [148]. *H. pylori* strains 51B and 26695, as well as their corresponding isogenic *cagA* and *cag* PAI mutants were described previously [96,149]. *H. pylori* LC-11 and CA8 strain were originally isolated from the antral mucosa of a patient with duodenal ulcer and gastric cancer, respectively, as previously described [187,223]. Tryptic soy agar (TSA) plates supplemented with 5% sheep's blood (Becton Dickinson, San Jose, CA) were used to grow *H. pylori* strains. Blood agar plates with 2.5 μg/ml of chloramphenicol (Technova, Hollister, CA) were used to maintain *cagA*$^-$ [96] and *cag* PAI$^-$ strains at 37°C under microaerophilic conditions. For mouse infection, *H. pylori* Sydney strain 1 (SS1) and PM-SS1 (pre-mouse SS1) [116] were used, which were provided by Drs. J. Pappo (Astra) and Richard Peek (Vanderbilt Univ.), respectively. PG-like molecule that is a NOD1 ligand iEDAP (InvivoGen, San Diego, USA) was used to investigate the role of PG in B7-H3 expression.

Animals. Female, six-to-eight week old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were used in the model of gastric *H. pylori* infection. Animals were tested and found negative for the intestinal *Helicobacter* spp. prior to use in the experiments.

Flow Cytometry. APC-conjugated anti-human B7-H3 (clone 185504) and isotype controls were purchased from R&D Systems. T cells from co-culture assays were stained for CD25, FoxP3, RORγ, Tbet and GATA3 for analysis by flow cytometry as previously described earlier [37]. Mouse anti-human CD25-PE-Cy7, FoxP3-Alexafluor 488, Tbet-PerCP-Cy5.5 and Gata3-eFluor 660 were used for staining. The viability dye eFluor 780 (eBioscience, San Diego, CA, USA) was included in the experiments to gate on viable cells. Cells were analyzed by flow cytometry on an LSRII instrument. The data were analyzed with BD FACSDiva software (BD Biosciences, San Jose, CA) and FlowJo (Tree Star, Inc, Ashland, OR).

Cell signaling inhibitors. Cell signaling inhibitors were described in chapter 1.

Real-Time RT-PCR. Real-time RT-PCR analysis was performed as previously described [96].

Murine infection and detection of different T cell subsets. C57BL/6 mice were orogastrically inoculated with 10^8 CFU (in 100 μL of PBS/inoculation) of *H. pylori* SS1 or PMSS1 strains, three times over a week. Four weeks later, mice were euthanized and mesenteric lymph nodes (MLN) were removed and homogenized. Then, mRNA was isolated and expression measured of different T cell transcription factors and corresponding cytokines e.g. FoxP3, IL-10 (Treg); RORγt, IL-17 (Th17); Tbet, IFN-γ (Th1) and GATA3, IL-4 (Th2) were determined by using RT-PCR.

T cell isolation and co-culture with GEC. Naïve $CD4^+$ T cells were isolated from human peripheral blood as previously described [181]. GEC-T cell co-cultures were established as described earlier [96]. Briefly, GECs were preinfected with *H. pylori* CA8 (cancer strain), *H. pylori* 51B (gastritis strain) and *H. pylori* LC-11 (ulcer strain). After 8 h of infection cells were washed and co-cultured with $1X10^6$ T cells to obtain 3:1 T cell:GEC ratio and incubated for 5 days at 37º C with 5% CO_2. For blocking, anti-B7-H3 blocking antibody (1 μg/mL, functional grade from eBioscience) was added to GECs 1 h

before co-culture. As an isotype control rat IgG2a κ (1 μg/mL, functional grade from eBioscience) was used.

Statistical analysis. The results were expressed as the mean ± SE of data obtained from at least three independent experiments done with triplicate sets per each experiment unless otherwise indicated. Differences between means were evaluated by analysis of variance (ANOVA) by using student's *t* test for multiple comparisons and considered significant if *p* was <0.05.

4.3 RESULTS

4.3.1. Expression of B7-H3 on gastric biopsies.

To determine the expression of B7-H3 in relation to *H. pylori* infection, GECs were isolated from biopsy samples collected from *H. pylori*-infected or healthy individuals. B7-H3 expression was measured by real time RT-PCR after mRNA was extracted. RT-PCR data showed a strong up-regulation of B7-H3 expression in the *H. pylori*-infected biopsies when compared to uninfected samples **(Fig. 4.1A)**.

4.3.2. *H. pylori* T4SS regulate B7-H3 expression on GEC during infection.

To evaluate whether increase of B7-H3 expression is a direct effect of *H. pylori* infection and not an indirect result of inflammatory changes in the host, I used GEC lines and infected them with *H. pylori*. Since *H. pylori* T4SS has the capacity to modulate GEC homeostasis and because previously I have seen their effect in the modulation of B7-H1 and B7-H2 molecules, I used *H. pylori* 51B wild-type (WT) and an *H. pylori* 51B *cag* PAI mutant strain to infect GEC (N87 cells). B7-H3 expression was measured after 24 h infection by using flow cytometry. My data showed a significant up-regulation of B7-H3 expression in GEC infected with *H. pylori* WT, but not with *H. pylori cag* PAI negative strains, which suggested that *H. pylori* T4SS plays a role in B7-H3 induction **(Fig. 4.1B)**. To further dissect the role of the effector protein CagA, *H. pylori* 51B *cagA* mutant was used to infect GECs, along with the *H. pylori* 51B WT strain. Both flow cytometry and RT-PCR data showed that CagA influences B7-H3 up-regulation **(Fig. 4.1C, D)**. These results were also confirmed in two additional epithelial cell lines (AGS, HGC-27) and by

using another *H. pylori* strain 26695 WT and the corresponding isogenic mutant strains (not shown). Furthermore, a murine cell line was used to confirm this finding. To this end, murine GEC lines (ImSt) were infected with *H. pylori* PMSS1, which contains a functional T4SS, and with the *H. pylori* SS1 strain, in which the T4SS is defective and cannot deliver CagA in GECs. Then, I used flow cytometry to measure the surface expression of B7-H3. A significant up-regulation of B7-H3 expression was seen in murine GECs infected with the *H. pylori* PMSS1 strain, but not with the SS1 strain **(Fig. 4.1E)**. Overall, these data showed a strong correlation between the presence of T4SS and induction of B7-H3 expression on GEC.

4.3.3. PG induces B7-H3 expression in GEC.

Along with CagA, *H. pylori* also translocates PG fragments into GECs, which are recognized by NOD1 and cause activation of cell signaling pathways that result in inflammatory mediator release [26,27,28,158]. To determine the involvement of PG in B7-H3 up-regulation, GECs were treated with iEDAP, which is a PG analogue recognized by the NOD1 ligand. B7-H3 expression was significantly up-regulated in terms of mRNA levels **(Fig. 4.2A)** after iEDAP stimulation. These RT-PCR data were confirmed by using flow cytometry as an independent approach to measure the surface expression of B7-H3 **(Fig. 4.2B)** My kinetics data showed a progressive up-regulation of B7-H3 as early as 18 h of stimulation which peaked at 24h (**Fig. 4.2C**), and is mediated by *H. pylori* T4SS component CagA and PG (**Figure 4.2D**).

4.3.4. *H. pylori* uses p38MAPK pathway for B7-H3 up-regulation.

Further analysis was done to determine the cell signaling pathway used by *H. pylori* for B7-H3 up-regulation. To that end, the cells were treated with different pharmacological inhibitors of NFκB, MAPK, STAT3, PI3K and mTOR pathways. These pathways were chosen because *H. pylori* is known to activate them [references]. My data indicated that B7-H3 up-regulation by *H. pylori* was inhibited by PD169316, which is a p38 MAPK-specific inhibitor **(Fig. 4.3)**. In contrast, inhibition of the PI3K, mTOR, STAT3 and NFκB pathways did not affect *H. pylori*-mediated up-regulation of B7-H3

expression. These results suggested that the p38 MAPK pathway is a key signaling pathway in *H. pylori*-mediated up-regulation of B7-H3 on GECs.

4.3.5. B7-H3 expression is regulated by Th17 and Treg cells.

Cytokines regulate the expression of immunoregulatory molecules, which allows for fine tuning of the immune response. During *H. pylori* infection, there is induction of Th17 cells [182,221,222]. Since patients have increased circulating levels of IL-17, I sought to investigate the effect of this molecule on B7-H3 expression. In RT-PCR analysis **(Fig. 4.4A)**, I observed a significant induction of B7-H3 after IL-17 (10 ng/ml) stimulation. Further, the experiments showed that the expression of B7-H3 on the GECs in response to IL-17 stimulation was increased in a dose-dependent manner (1-100 ng/ml) **(Fig. 4.4B)**. The surface expression of this ligand was also analyzed at different time points (18, 24 and 48 h) after IL-17 treatment. The expression was significantly increased in GECs after 18 h of incubation with IL-17, which remains constant after 24 h but decreases after 48 h incubation **(Fig. 4.4C)**.

Treg cells, which are frequently found in *H. pylori*-infected patients, produce IL-10 and TGF-β [32,33]. Since there is bidirectional regulation of Treg cells and B7-H1 [224], I evaluated whether the hallmark cytokines produced by these cells have any effect on B7-H3 expression. To that end, GECs were stimulated with either IL-10 or TGF-β alone or with both. Both IL-10 and TGF-β induced B7-H3 expression on GECs **(Fig. 4.5A and B)**. My study also showed a cumulative effect of IL-10 and TGF-β in B7-H3 expression **(Fig. 4.5C)**.

4.3.6. Different T cell subset development during *H. pylori* infection.

Besides the known function of T cell activation and inactivation recent studies by Nagashima et al., showed B7-H3 can up-regulate Th2 responses [214]. Since my study demonstrated that *H. pylori* up-regulates B7-H3 expression, I sought to investigate whether the modulation of B7-H3 expression affects local T cell responses. To that end, I collected MLN from mice infected with PMSS1 and SS1 strains and analyzed the T cell subsets present by measuring mRNA expression of the different T cell transcription factors considered "master regulators" for each $CD4^+$ T cell subset, such as GATA3,

Tbet, RORγ and FoxP3 for Th2, Th1, Th17 and Treg cells, respectively. Mice infected with the SS1 strain showed an increased GATA3, Tbet and RORγ expression, compared to those infected with PMSS1 strain. However, the PMSS1 group showed increased FoxP3 expression compared to SS1-infected mice **(Fig 4.6)**. I further measured the mRNA expression of the corresponding cytokines produced by Th2, Th1, Th17 and Treg cells, e.g., IL-4, IFN-γ, IL-17A and IL-10 in MLN. The cytokine data correlate with the transcription factors found in mice infected with the different strains **(Fig. 4.6)**.

4.3.7. Increased B7-H3 and GATA3 expression in gastritis patients.

There are several publications reporting the presence of Th2 cells during *H. pylori* infection [179]. Moreover, in this study, the mouse model showed induction of $GATA3^+$ Th2 cell in MLN after *H. pylori* infection. Since B7-H3 has been shown to influence Th2 cell development, we sought to determine the influence of B7-H3 induction by GECs during *H. pylori* infection in Th2 response cells and whether it depends on the infecting strain. To this end, patient samples from gastritis and gastric tumors were evaluated. Biopsy samples from gastritis and tumor samples from gastric tumors were evaluated for the relative expression of B7-H3 and GATA3. Interestingly, samples collected from gastritis patients showed increased B7-H3 and GATA3 expression compared to those from healthy individuals. However, in the case of patients with gastric tumors the expression of both B7-H3 and GATA3 was decreased, which suggested B7-H3 and Th2 induction during H. pylori infection might be a characteristic of gastritis strains (Fig. 4.7).

4.3.8. B7-H3 expressed by GEC after *H. pylori* infection induces development of Th2 cells.

To further confirm whether the induction of B7-H3 and Th2 is only associated with *H. pylori* gastritis strains, N87 cell lines were treated with either medium alone or with different *H. pylori* strains: CA8 (from a gastric cancer case [reference]), 51B (from a gastritis case [reference]) and LC-11 (from an ulcer case [reference]). After 8 h of infection, the cells were washed extensively and incubated with isolated $CD4^+$ naïve T cells for 5 days. T cells were harvested and stained for CD25, Tbet, GATA3, RORγ and

FoxP3 monoclonal antibodies and analyzed by flow cytometry from which the data showed increased $GATA3^+$ cells in T cells co-cultured with GECs pre-infected with the gastritis strain (*H. pylori* 51B), but not with the other strains **(Fig 4.8A)**. A significant increase in $GATA3^+$ $Tbet^+$ double-positive cells was also observed in T cells co-cultured with GECs pre-infected with the gastritis strain, suggesting conversion of Th1 cells to Th2 cell type (data not shown). Interestingly, incubation of the T cells with GECs pretreated with blocking B7-H3 antibody resulted in reduced Th2 frequency. This data suggested that induction of Th2 is influenced by B7-H3 **(Fig. 4.8B)**.

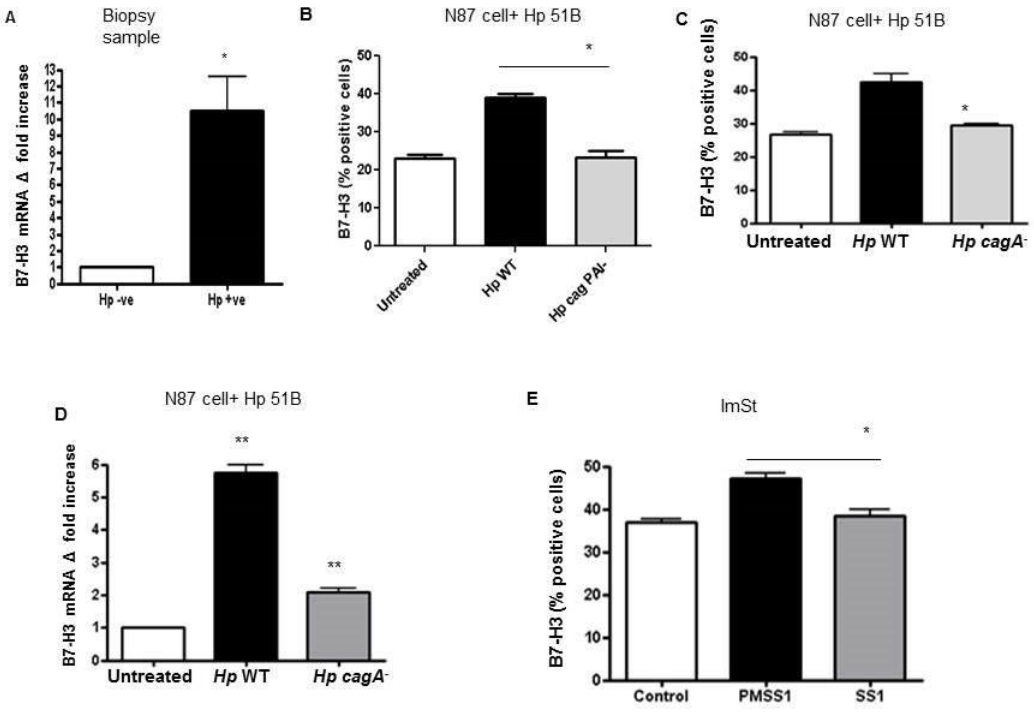

Figure 4.1: *H. pylori* T4SS up-regulates B7-H3 expression on GECs. Gastric biopsy samples were collected from *H. pylori*-positive patients and healthy individuals, GECs were collected and analyzed for B7-H3 mRNA expression by real-time RT-PCR. N87 cells were infected with (B) *H. pylori* 51B WT and *cag* PAI$^-$ or with (C) *H. pylori* 51B WT and *cagA*$^-$ for 24 h. The surface expression of B7-H3 was determined by using immunostaining followed by flow cytometry. (D) N87 cells were infected with *H. pylori* 51B WT and *cagA*$^-$ for 2 h, and B7-H3 mRNA expression was analyzed by using RT-PCR. mRNA levels for B7-H3 were normalized to 18S and compared to the level of B7-H3 mRNA of untreated N87 cells. (E) Murine GECs (ImSt) were infected for 24 h with *H. pylori* PMSS1, which has a functional CagA delivery system, or with *H. pylori* SS1, lacking a CagA delivery system. Surface expression of B7-H3 was determined by flow cytometry. The data were expressed as a percentage of positive cells. The means ± SD are shown as the results of duplication of one of four representative experiments, n=8, **P* < 0.05.

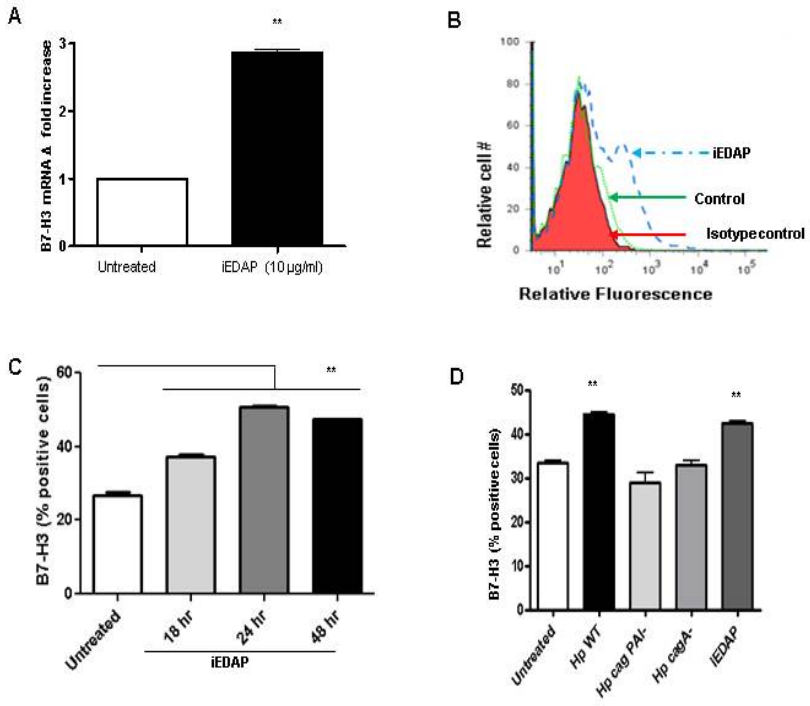

Figure 4.2: *H. pylori* T4SS translocated PG causes induction of B7-H3 expression by GECs. (A) B7-H3 mRNA expression was analyzed by using real-time quantitative RT-PCR in N87 cells. RNA was isolated from untreated and 2 h iEDAP (dipeptide present in peptidoglycan) treated (10 µg/mL) cells. mRNA levels for B7-H3 were normalized to 18S and compared to the levels of B7-H3 mRNA in untreated N87 cells. N=9, $*P < 0.05$. (B) Flow cytometric analysis of GEC (N87) cells stained for B7-H3 after exposure to 10 µg/mL iEDAP for 24 h (in a representative histogram for AGS cells where the solid peak is the isotype control) or (C) for different times (18, 24 and 48 h) showed increased expression. (D) N87 cells were infected with *H. pylori* WT, *H. pylori cag* PAI$^-$, and *H. pylori cagA*$^-$ and stimulated with iEDAP for 24 h and B7-H3 expression was measured by flow cytometry. The means are shown as the results of duplicates in four experiments, n= 8,$*P < 0.05$.

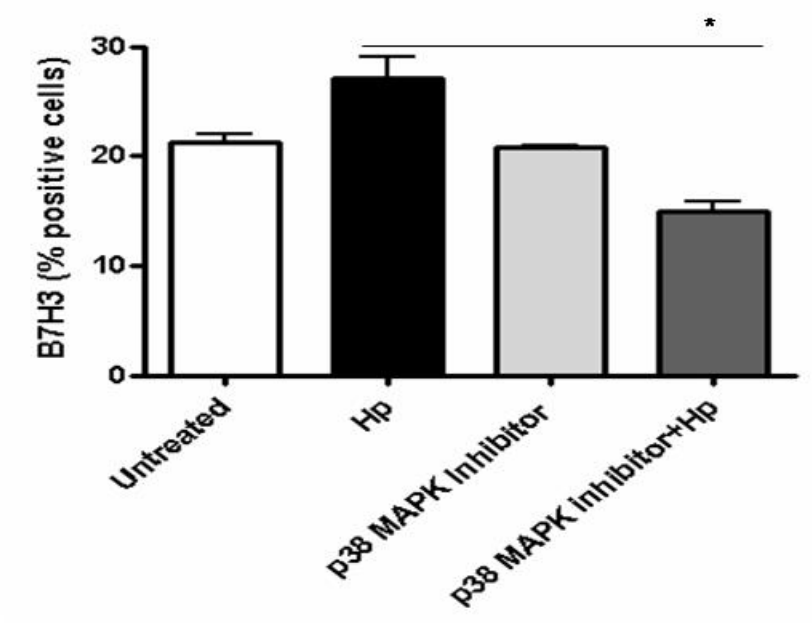

Figure 4.3: B7-H3 up-regulation by *H. pylori* depends on p38 MAPK pathway. B7-H3 expression on GEC was measured by flow cytometry after treating the cells with p38 MAPK inhibitor (PD169316, 10 μM/ml) for 1h and infected with *H. pylori* for 24 h. The means ± SD are shown as the results of duplicates in four experiments, n=8, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

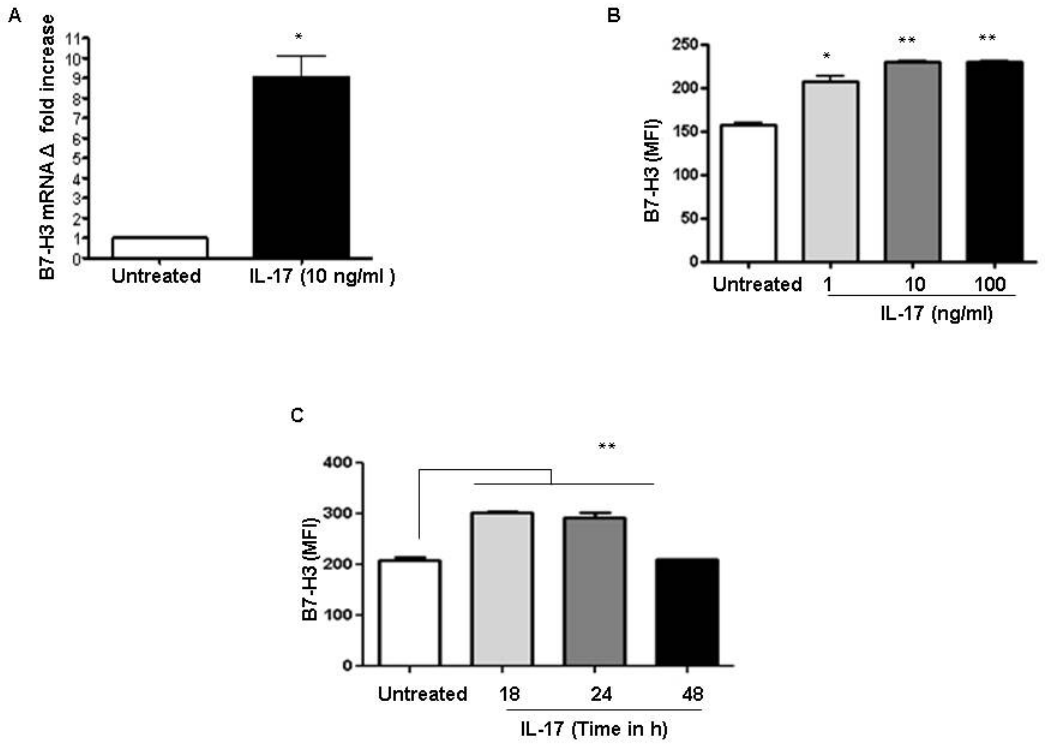

Figure 4.4: B7-H3 expression is regulated by IL-17. (A) GEC (N87) cells were treated with IL-17 (10 ng/ml) for 2 h and B7-H3 expression was measured by RT-PCR. mRNA levels for B7-H3 was normalized to 18S and compared to the levels of B7-H3 mRNA of untreated N87 cells. Kinetics and dose response of IL-17-mediated B7-H3 up-regulation was determined by treating GEC (N87) cells with (B) different concentrations (1, 10 and 100 ng/ml) of IL-17 for 24 h or (C) exposing the GEC (N87) cells to IL-17 (10 ng/ml) for different time points (18, 24 and 48 h) and measuring the B7-H3 expression by flow cytometry. The data were expressed as mean fluorescence intensity (MFI). The means ± SD are shown as the result of duplicates of one of four representative experiments: n=8, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

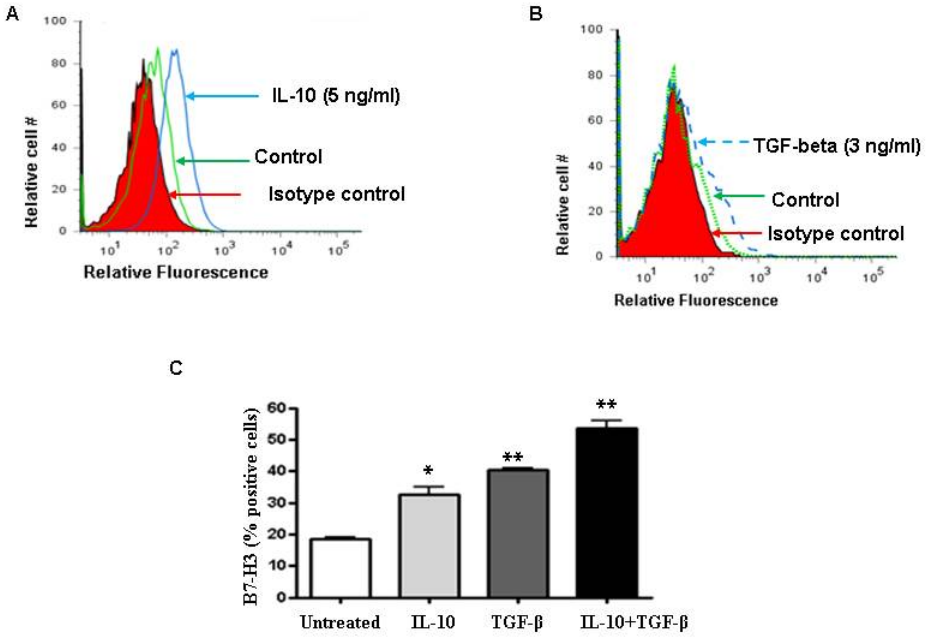

Figure 4.5: B7-H3 expression is regulated by Treg cell cytokines. (A) Flow cytometry analysis of GEC cells stained for B7-H3 after exposure to 5 ng/mL IL-10 for 24 h showed increased expression in a representative histogram where the solid peak is the isotype control (B) Flow cytometry was done to measure B7-H3 expression on GECs after treating the cells with TGF-β (3 ng/ml) for 24 h. (C) Flow cytometry was done to measure B7-H3 expression on GECs treated with either IL-10 (5 ng/mL) or TGF-β (3 ng/ml) or both IL-10 and TGF-β. The data were expressed as the percentage of positive cells. The means ± SD are shown as the results of duplicates of one of four representative experiments: n=8, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

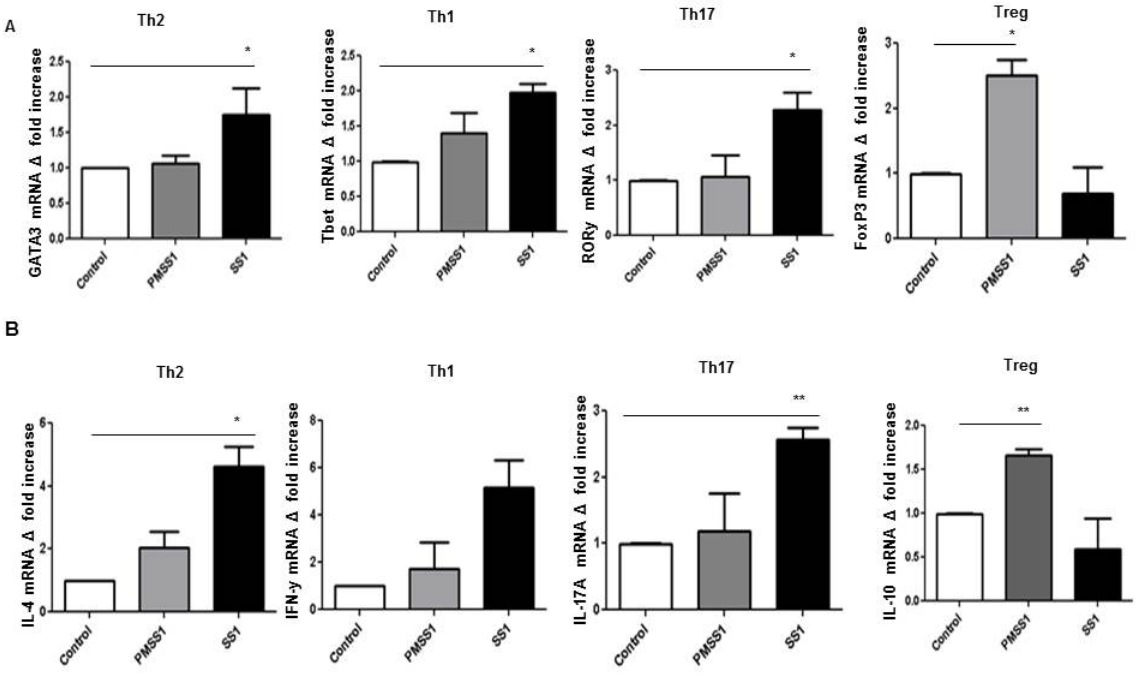

Figure 4.6: Different T cell subsets developed during *H. pylori* infection. C57BL/6 mice were challenged with *H. pylori* strain PMSS1 or with *H. pylori* SS1. Mice were sacrificed after 4 weeks of infection, MLN were collected, and expression measured of (A) GATA3, Tbet, RORγ, FoxP3 and (B) IL-4, IFN-γ, IL-17A, IL-10 mRNA by RT-PCR. Five mice per group were used in this experiment.

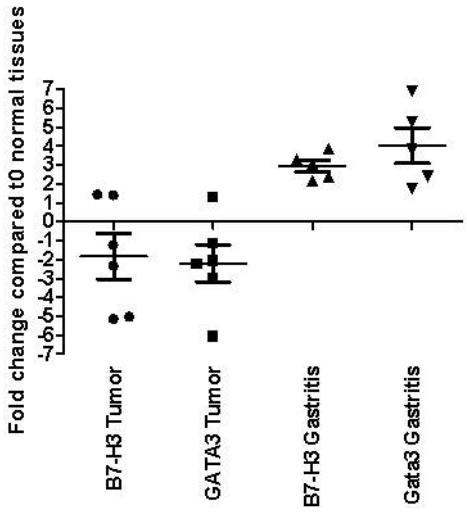

Figure 4.7: B7-H3 and Th2 induction is associated with gastritis. B7-H3 and GATA3 expression in biopsy and tumor samples isolated from gastritis or gastric tumor patients with a history of *H. pylori* infection.

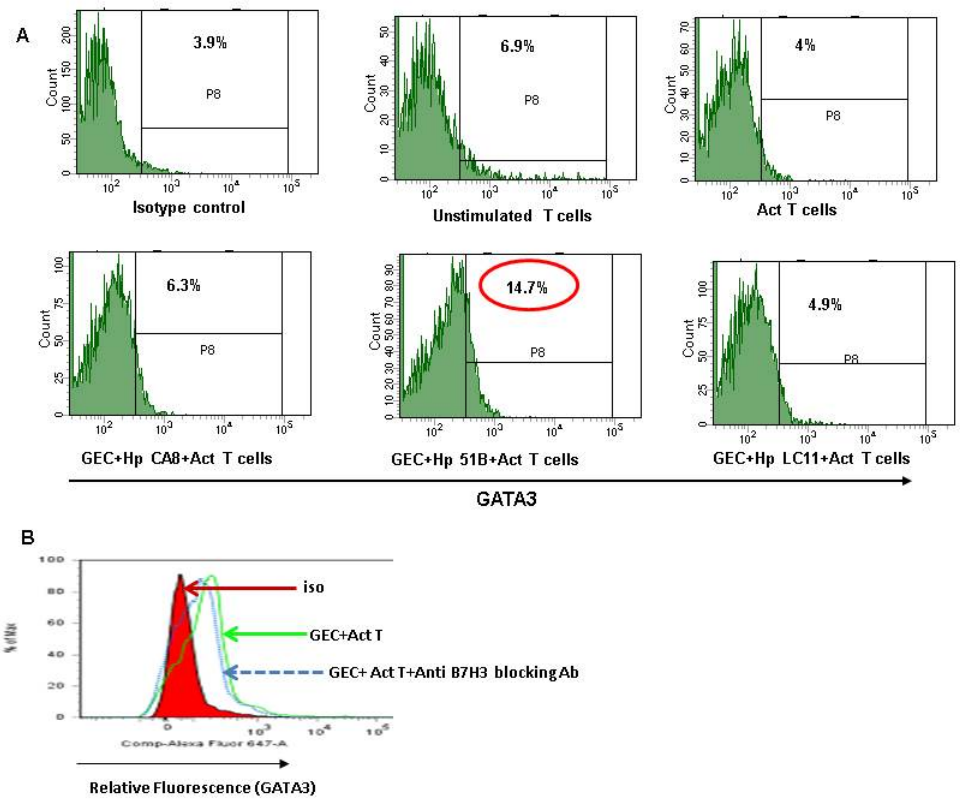

Figure 4.8: Increased B7-H3 expression and Th2 induction by H. pylori gastritis strain. (A) GECs were infected with either H. pylori CA8/51B/Lc-11 strains for 8 h, washed and co-cultured with T cells. Cells were stained and GATA3 expression analyzed by flow cytometry. (B) A representative histogram showing GATA3 expression by GEC co-cultured with activated T cells in the presence or absence of ant-B7-H3 blocking Ab.

4.4. DISCUSSION

B7-H3 has been thought previously to be a co-stimulatory molecule which promotes T cell proliferation [206]. However, later studies have shown that B7-H3 can also function as a co-inhibitory molecule [206,207,208,209,210,211,212,213]. B7-H3 is expressed by an array of cell types. Also, a previous study showed that RSV (respiratory syncytial virus) causes induction of B7-H3 in tracheal, bronchial, and alveolar epithelial cells [203]. Our laboratory previously showed that B7-H3 is expressed by GEC [36]. In this study I found increased expression of B7-H3 was demonstrated in the GECs isolated from biopsies of *H. pylori*-infected patients. This observation was further confirmed *in vitro* by findings of increased B7-H3 mRNA levels and surface expression in a panel of human GEC lines (N87, AGS and HGC-27) after infection with *H. pylori* 51B and 26695 strains. Though, previously our lab reported that GECs can be expressed in B7-H3, the expression was unchanged after infection with *H. pylori* LC-11 strain [36]. This observation suggested that this cellular response to infection might depend on the infecting *H. pylori* strain since both 51B and 26695 are were isolated from gastritis patients, and LC-11 was isolated from apeptic ulcer patient. These observations were further confirmed in this study by using gastric tissue samples from patients with different gastric diseases associated with *H. pylori* infection.

H. pylori T4SS influences GEC homeostasis [22,23]. My recent findings, which confirmed the use of T4SS by *H. pylori* in the modulation of B7 molecule expression [96], led me to consider *H. pylori* T4SS as a virulence factor responsible for the up-regulation of B7-H3 by GECs. By using *H. pylori* WT and *cag* PAI mutant, I found in the present study that B7-H3 induction depends on *H. pylori* T4SS. This expression pattern was observed both in human GECs and murine GECs (ImSt). Besides using a mutant which lacks the whole *cag* PAI, I also used another *H. pylori* mutant which lacks the *cagA* gene to determine the role of this effector protein, which is translocated by the T4SS, in B7-H3 induction. My study showed induction of B7-H3 depends on the presence of CagA. PG, the other component translocated to GEC by T4SS, can work as an inflammatory molecule and induces IL-8 production by GEC [26,27,28]. As my data demonstrated a complete dependence of *H. pylori cag* PAI, but partial involvement of CagA on B7-H3 induction, I hypothesized that PG might also influence B7-H3 induction.

The addition of the PG fragment iEDAP, which is recognized by NOD1, resulted in the induction of B7-H3, both at the mRNA and protein levels. My kinetics data showed B7-H3 can be increased within 18 h of stimulation by PG fragments. This study also highlighted the involvement in B7-H3 induction of the p38 MAPK pathway, which is known to be activated by both PG and CagA [26,172]. Previously it was reported that PG can modify itself to resist the action of lysozymes, and this mechanism helps in *H. pylori* survival [171]. However, this is the first study showing the role of PG in GEC modification and T cell regulation.

Cytokines play important roles in influencing the expression of different immune regulatory molecules. Since IL-17, IL-10 and TGF-β produced by Th17 and Treg cells have been shown to be present in increased amounts in *H. pylori*-infected patients [32,33,182,221,222], I hypothesized that these cytokines may play a role in induction of B7-H3 on GEC. My data showed that, stimulation of GECs by both Th17 (IL-17) and Treg cell cytokines (IL-10 and TGF-β) causes an increased expression of the B7-H3 molecules on GECs. Regulation of B7 molecules by IL-10 and TGF-β has been reported previously. For example, several studies showed IL-10 inhibits B7 molecule expression in macrophages [225] and B7-2 expression in DCs [226]. TGF-β has been found to inhibit B7-1 expression in APCs [227]. Another study reported that IL-10 down-regulated B7-1 and B7-2 expression on *Mycobacterium tuberculosis*-infected monocytes to a greater extent than did TGF-β [228]. However, IL-10 and TGF-β did not show any additive or synergistic inhibition in their study, whereas, in this study, I found TGF-β is a better inducer of B7-H3 than is IL-10, and they have cumulative effects in B7-H3 induction.

By using a mouse model and measuring different transcription factors considered as master regulators for different $CD4^+$ T cell subsets and cytokines in MLN, I showed that *H. pylori*-infected mice develop a mixed population of both Th1 and Th2 cells. These data correlated with previous findings [179]. Compared to SS1, PMSS1-infected mice showed less induction of the Th1 and Th2 cell population. Additionally, Th17 and Treg cell data correlated with our previous findings, since *H. pylori* PMSS1 infection causes increased Treg cells and a lesser Th17 cell response when compared with findings with SS1 infection [96]. Our lab has previously shown that *H. pylori*-mediated modulation of

Th17 and Treg cell responses depends on the change of B7-H2 and B7-H1 molecules on GECs [37,96]. Besides being a positive/stimulator for T cell activation, B7-H3 has also been shown to contribute to pathogenic Th2 cell development during asthma in a mouse model [214]. However, studies also reported a negative regulatory effect of B7-H3 in Th1 and Th2 immune responses [229]. A major question that remains for the Th2 cell response that we found in *H. pylori*-infected mice is whether or not this induction of Th2 is mediated by a B7-H3 molecule.

To answer this question and investigate whether this response depends on the *H. pylori* strain, I collected samples from *H. pylori* infected patients with gastritis and tumor and compared B7-H3 and GATA3 expression on those samples with samples collected from healthy individuals. Interestingly, the samples collected from gastritis patients, and not from the gastric tumor patients, had increased B7-H3 and $GATA3^{+}$ cells. Though our study showed a strong association between B7-H3 induction and Th2 development during *H. pylori* infection, further studies are required to determine the link between disease condition and B7-H3 expression. To further evaluate this finding, I used a GEC: T cell co-culture, in which the GECs were pre-exposed to either *H. pylori* strains associated with gastritis, gastric cancer or peptic ulcer in the presence of anti-B7-H3-blocking antibody or control antibody. The flow cytometry data indicated the induction of Th2 cells and Th1/Th2 double-positive cells in the T cells co-cultured with *H. pylori* 51B (from a gastritis case) pre-treated cells, suggesting a shift of Th1 towards Th2 cells. Moreover, by using anti-B7-H3 blocking antibody, I showed that induction of Th2 depends on B7-H3.

In conclusion, this study revealed a novel mechanism that *H. pylori* uses to cause in its host chronic inflammation, which causes gastritis. This is an important finding which helps to better understand the interaction of *H. pylori* with GECs and how it manipulates T cell response. The relationship of *H. pylori*-mediated B7-H3 induction and disease conditions must be further defined.

Chapter 5: Summary and Conclusions

In summary, in the first part of this study, I analyzed the mechanism behind *H. pylori*-mediated B7-H1 induction and how it affects increased Treg cell responses and persistent infection that are usually seen in *H. pylori*-infected patients. By using both *in vitro* and *in vivo* systems, I demonstrated that *H. pylori* uses its T4SS components: effector protein CagA and cell and PG fragments, both of which are translocated by this secretion system to GECs, to up-regulate B7-H1 expression. This study also provided information about the underlying cell-signaling pathway involved in B7-H1 induction. My study showed that *H. pylori* uses the p38 MAPK pathway for the up-regulation of B7-H1. By using a mouse model, I further showed that *H. pylori* requires a functional T4SS for up-regulation of B7-H1 expression in the gastric mucosa, induction of Treg cell in the stomach, increased IL-10 in the serum, and increased bacterial load in the stomach. To determine whether the increased production of Treg cells is associated with B7-H1 induction, I used B7-H1$^{-/-}$ mice. By infecting WT and B7-H1$^{-/-}$ mice with *H. pylori* PMSS1 and the SS1 strain, I showed that the increase in Treg cells and bacterial load depends on *H. pylori*-mediated induction of B7-H1 expression (Illustration 5.1).

During *H. pylori* infection, T cell hyporesponsiveness and down-regulation of the Th17 response hamper the immune system's attempts to clear the pathogen. By using biopsy samples from *H. pylori*-infected patients, I showed that T cell co-stimulatory molecule B7-H2 expression is down-regulated by *H. pylori*. Further, by using different GEC cell lines and *in vivo* model, I found that CagA modulates the expression of B7-H2 on GECs. IFNγ, produced during *H. pylori* infection, synergized with the bacterium to decrease GEC B7-H2 expression. This down-regulation was CagA dependent and required p70 S6 kinase phosphorylation. Since, B7-H2/ICOS interactions have been shown to be required for Th17 cell development I studied the effect of B7-H2 down-regulation in Th17 development. My *in vitro* and *in vivo* data demonstrated a direct correlation between B7-H2 down-regulation by CagA and a decrease in the magnitude of Th17 responses. In addition, the decreased Th17 response was followed by an increase in *H. pylori* colonization of the gastric mucosa of *H. pylori*-infected mice. Thus, through the

action of CagA, *H. pylori* decreases B7-H2 expression and dampens Th17 responses, thereby facilitating bacterial persistence (Illustration 5.2).

These data led me to consider the effect of *H. pylori* on the B7-H3 molecules on GECs, which have both T cell stimulatory and inhibitory function. Also recent studies showed that B7-H3 can regulate Th2 development. My study demonstrated that B7-H3 expression is increased in *H. pylori*-infected patients. To further dissect the mechanism of B7-H3 induction, I examined, in the third part of the study, the role of T4SS and cytokines produced by Th17 and Treg cells in B7-H3 expression and how the modulation of B7-H3 regulates local T cell responses. I found that T4SS encoded CagA, as well as PG, Th17, and Treg cell cytokines up-regulates B7-H3 expression in GECs. I further demonstrated *H. pylori*-infected mice have increased Th2 in the MLN, and by using GEC:T cell co-culture, I showed the induction of Th2 is mediated by B7-H3 and depends on the *H. pylori* strain. Additionally, the only strain which causes gastritis is associated with the induction of B7-H3 and Th2. By using patient samples, I further confirmed this finding and showed that gastritis is associated with increased B7-H3 and Th2 response (Illustration 5.3).

In conclusion, my study demonstrated how *H. pylori* manipulates GECs to differentially express various B7 molecules and thus regulate T cell response. Future studies will examine how these findings may be implemented in vaccine efforts against *H. pylori*.

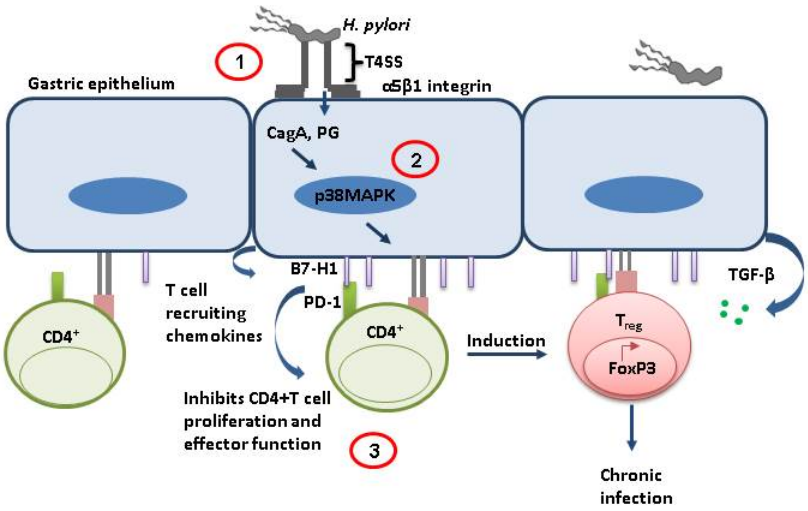

Figure 5.1: *H. pylori* mediated up-regulation of B7-H1 on GEC induces Treg cell development and facilitates bacterial persistence. (1) *H. pylori* T4SS interacts with host receptor integrin α5β1 and translocates effector protein CagA and PG. (2) This causes activation of p38 MAPK pathway and induction of B7-H1 on GEC. (3) Induction of T cell co-inhibitory molecule B7-H1 further inhibits $CD4^+$ T-cell proliferation and effector function. It also facilitates induction of Treg cells from naïve $CD4^+$ T cells. This mechanism helps to establish a chronic infection.

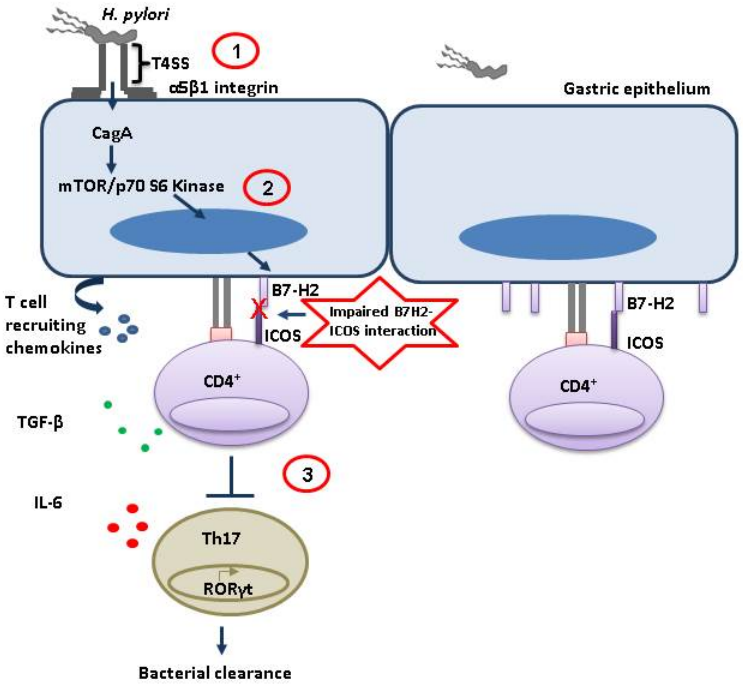

Figure 5.2: *H. pylori* mediated down-regulation of B7-H2 on GEC inhibits Th17 cell development and facilitates bacterial persistence. (1) *H. pylori* T4SS interacts with host receptor integrin α5β1 and translocates effector protein CagA. (2) The CagA protein activates the mTOR/p70 S6 kinase pathway and down-regulates T cell co-stimulatory molecule B7-H2 expression on GEC. (3) Decreased B7-H2/ICOS signaling further inhibits Th17 cell development from naïve $CD4^+$ T cells and Th17 cell-mediated bacterial clearance.

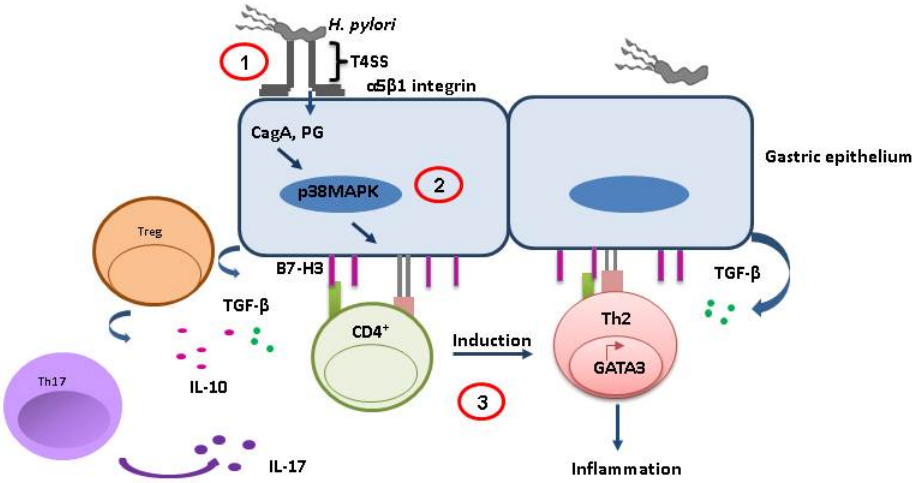

Figure 5.3: *H. pylori* mediated up-regulation of B7-H3 on GEC induces Th2 cell development. (1) *H. pylori* T4SS interacts with host receptor integrin α5β1 and translocate effector protein CagA, PG. (2) This activates p38 MAPK pathway and up-regulates B7-H3 expression on GEC. (3) Increased B7-H3 further induces Th2 cell development from naïve $CD4^+$ T cells.

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VITA

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Education

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Publications

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Abstracts and Posters

1. **Lina, T. T.,** I. V. Pinchuk, E. J. Beswick, V. E. Reyes. CagA-dependent modulation of B7-H2 expression on gastric epithelial cells: Effects on Th17 responses during

Helicobacter pylori infection. April 24th 2013. 54th National Student Research Forum, UTMB, Galveston.

2. **Lina, T. T.,** I. V. Pinchuk, E. J. Beswick, V. E. Reyes. Role of *Helicobacter pylori* Type IV secretion system in regulating B7-H1 (PDL-1) and B7-H2 (ICOSL) expression in gastric epithelial cells: effect on Th17, Treg cell response and bacterial persistence. April 12th 2013. IHII/McLaughlin Colloquium, UTMB, Galveston.

3. **Lina, T. T.,** I. V. Pinchuk, E. J. Beswick, V. E. Reyes. Role of *Helicobacter pylori* Type IV secretion system in regulating B7-H1 (PDL-1) and B7-H2 (ICOSL) expression in gastric epithelial cells: effect on Th17, Treg cell response and bacterial persistence. March 22, 2013 Molecular Basis of Infectious Diseases (MBID) Retreat, UTHealth (University of Texas Health Science Center at Houston), Houston.

4. **Lina, T. T.,** I. V. Pinchuk, E. J. Beswick, V. E. Reyes. CagA-dependent modulation of B7-H2 expression on gastric epithelial cells: Effects on Th17 responses during *Helicobacter pylori* infection. Dec 5-6, 2012 IHII Retreat, UTMB, Galveston.

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6. Gonzalez, J, **T. T. Lina,** I. V. Pinchuk, V. E. Reyes. *H. pylori* Cag Pathogenicity Island: More than a secretion apparatus. June 27, 2013, Medical Student Summer Research Program Poster Session, UTMB, Galveston, Tx.

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8. Gonzalez, J, **T. T. Lina,** I. V. Pinchuk, V. E. Reyes. *H. pylori* Cag Pathogenicity Island: More than a secretion apparatus. April 25-26, 2013, National Student Research Forum UTMB, Galveston, Tx.

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