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CagA-dependent downregulation of B7-H2 expression on gastric mucosa and inhibition of Th17 responses during *Helicobacter pylori* infection

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Abstract

Gastric epithelial cells (GECs) are the primary target for *Helicobacter pylori* (*H. pylori*) infection and may act as antigen presenting cells (APC) regulating local T cell responses. We previously reported that *H. pylori* infection of GECs induces the expression of the T cell co-inhibitory molecule B7-H1 on GECs. This process contributes to the hyporesponsiveness of $CD4^+$ effector T cells and accumulation of T regulatory cells. In the studies presented herein we investigated the impact of *H. pylori* cytotoxin CagA on the modulation of the expression of the T cell co-stimulator B7-H2 by GEC. B7-H2 is involved in promoting Th17 type responses. *H. pylori* infection downregulates B7-H2 expression by GECs in a CagA dependent manner. IFNγ, which is increased in the *H. pylori* infected gastric mucosa, synergizes with *H. pylori* in downregulating B7-H2 expression by GECs. CagA-mediated modulation of B7-H2 on GEC involves p70 S6 kinase phosphorylation. The CagA-dependent B7-H2 downregulation 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*. Our 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 the *H. pylori* chronic infection.

Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative, spiral shaped bacterium that infects the gastric mucosa of more than 50% of the world's population. *H. pylori* infection initially occurs in childhood and becomes persistent. This chronic infection leads to gastric inflammation (1) and is the major cause of gastritis, gastric and duodenal ulcers as well as gastric adenocarcinoma (2–8).

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The CagA (cytotoxin associated gene A) protein is a major virulence factor of *H. pylori*. Patients infected with *cagA*$^+$ strains have higher levels of inflammatory responses and are at a higher risk of developing peptic ulcer or gastric cancer (4, 9). CagA is encoded by the *cagA* gene within the *cag* pathogenicity island (*cag* PAI), a 40-kilobase chromosomal region that encodes for a type four secretion system (T4SS).

Gastric epithelial cells (GECs) are the primary target for *H. pylori* infection. After *H. pylori* adhere to GECs, the CagA protein is translocated into their cytosol *via* a T4SS (10). Once inside GECs, CagA becomes phosphorylated and 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 (11, 12). 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 (13, 14). Studies by us and others suggested that the imbalance in CD4$^+$ T cells responses to *H. pylori* is responsible for the host's inability to clear the infection (15–18). Th17 cells, whose hallmark cytokine is IL-17A, are crucial in the clearance of extracellular bacteria (19). IL-17A is primarily associated with gastric inflammation during *H. pylori* infection and, if chronically present, may contribute to the inflammation associated carcinogenesis (19–21). On the other hand, IL-17A-initiated recruitment of neutrophils is critical for the clearance of the bacteria (22). 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 (23). 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.

During *H. pylori* infection GEC express MHC class II and may act as local APCs (24, 25). In addition to the recognition of MHC-bound peptides on APCs by T cell receptor (TCR), the outcome of APC-T cell interactions depends on a second signal provided by engagement of the B7-co-stimulatory receptor (26). Our lab has previously shown that GECs express the classical B7 co-stimulators B7–1 and B7–2, whose expression is increased during *H. pylori* infection (25). We also demonstrated that GECs express B7-H1, whose expression also increases during *H. pylori* infection and contributes to the suppression of CD4$^+$ effector T cell activity and upregulation of Treg cells (18, 27).

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 (28). Recent studies have implicated B7-H2/ICOS interaction in Th17 cell development, maintenance and function (29–31). However, the role of B7-H2 in immune responses to *H. pylori* is unknown. Thus, in this study we 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. Our *in vitro* and *in vivo* studies showed that *H. pylori* causes down-regulation of B7-H2 on GECs and this effect depends on the presence of *H. pylori* CagA *via* a process involving p70 S6 kinase activation. CagA-dependent B7-H2 downregulation 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.

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 (32). Briefly, biopsy specimens of the gastric antrum were obtained from consenting patients undergoing gastro-esophageal-duodenoscopy for various clinical indications in accordance with 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 to be infected by culture of *H. pylori* from biopsies. 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 (33, 34). *H. pylori* strain Sydney strain 1 (SS1) and PMSS1 (pre-mouse SS1) (35) used to infect mice were kind gifts from Drs. J. Pappo (Astra) and Dr. 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 maintain *cagA*$^-$ strains at 37°C under microaerophilic conditions. Bacteria were transferred after 48 hour 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 OD530 using 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 using trypan blue staining and the required number of bacteria was added after calculation. GEC were treated with IFNγ (100 U/ml) for 48 hour, 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 IgG1, κ Ab was used in the same concentration. B7-H2 expression was measured after coculture 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 (36). Correct integration of the chloramphenicol resistance gene cassette into the *H. pylori* chromosome by double crossover 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 (17). 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 where exposed to *H. pylori* strains for 8 hours, 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 till 24 hours. $CD4^+$ T cells were preactivated for 1 hour with anti-CD3/CD28 beads (Invitrogen) according to the manufacturer's instructions and added to each well at 3:1 T cell:GEC ratio, then incubated at 37°C with 5% CO_2 for 2 days.

Western blot analysis

Western blot analysis was performed as previously described (37).

Infection of mice, processing of stomach and detection of *H. pylori* in murine stomach

Experimental animal groups were inoculated by orogastric gavage with 10^8 CFU (in 100 μL of PBS) of *H. pylori* SS1 or PMSS1 strains, three times over a week. Placebo group was inoculated with PBS. Four weeks later animal serum was collected, mice were euthanized and stomach was removed. Stomachs were dissected longitudinally in 2–4 pieces and used for analysis of the *H. pylori* load, histopathology, RT-PCR and flow cytometry analysis. For histopathology analysis one longitudinal strip of stomach was placed in 10% normal buffered formalin for 24 hour at 4°C, transferred into 70% ethanol solution and stored at 4°C until needed. Tissue was then embedded in paraffin and processed by hematoxylin and eosin (H&E) staining. Stomach tissue dissociation and enzymatic digestion was performed using

gentleMACS™ Dissociator (Milteneyi Biotec, Auburn, CA) according to the manufacturer's instructions. Briefly, DTT/EDTA treatment was performed similarly as described above for human tissue. Cells were then washed twice with HBSS followed by treating with Collagenase I, II, IV (100 U/mL). After first round of dissociation cells were incubated with enzymatic mixture at 37°C for 30 min. Tissue was then subjected to the second round of dissociation and treated with DNAse (10 ug/mL, Worthington) at 37°C for 15 min. Finally, tissue was processed 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 gastric tissue was homogenized and DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) followed by a purification of the DNA according to the manufacturer's protocol. Extracted DNA was used for the detection of *H. pylori* by real time PCR using a protocol originally described by Rouessel et. al. A primer/probe set 16SHP229BP for 16S gene was used for the quantification of the *H. pylori* bacterial load. To determine bacterial load standard curves were generated by PCR of serial dilutions of extracted *H. pylori* DNA. Quantification of *H. pylori* in murine gastric mucosa and determination of absolute genome copy number was calculated according to the method described by Rouessel et. al. (38). Murine GADPH gene amplification was used to control the equal loading of total DNA used in PCR reaction.

Flow cytometry

Flow cytometry was performed as we described previously (17).

Real-Time RT-PCR

Real-time RT-PCR was performed according to the Applied Biosystems's two-step Real-time RT-PCR protocol (Applied Biosystems, Foster City, CA). The appropriate assays-on-demand™ gene expression assay primers/probe mix (Applied Biosystem) for human 18S and gene of interest (a 20X mix of unlabeled PCR primers and TaqMan® MGB probe, FAM™ dye-labeled) and 2 μL of cDNA was added to the PCR reaction step. The reactions were carried out in 20 μL final volume using BioRad Q5 real-time PCR machine according to the following protocol: 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). Triplicate cycle threshold values were analyzed in Microsoft Excel using the comparative CT (ΔΔCT) method as described by the manufacturer (Applied Biosystems).

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 using Luminex array (Millipore, Billerica, MA, USA) according the manufactures instruction. Samples were analyzed using Bio-Plex Manager software (Bio-Rad).

Statistical analysis

The results were expressed as the mean ± SD of data obtained from at least three independent experiments done with triplicate sets in each experiment, unless otherwise indicated, Differences between means were evaluated by ANOVA using Student's t-test for multiple comparisons. Values of $P<.05$ were considered statistically significant.

Results

H. pylori downregulate 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 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. 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 hours post-infection with all *H. pylori* strains (Fig. 1B, C). Fig 1B shows differential regulation of B7-H1 and B7-H2 by N87 cells infected with *H. pylori* 51B. This effect was dose-dependent (Fig. 1D). Similar results were observed when other human GEC lines (e.g. AGS, HGC-27 and HS-738 cells) were infected (not shown). Thus, our results indicate that *H. pylori* infection down-regulates B7-H2 expression on human GEC in a dose-dependent manner.

Downregulation 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, (39) we sought to determine whether downregulation of B7-H2 is influenced by *H. pylori* CagA. Infection of GEC with *H. pylori* 51B wild type (WT) strain (Fig. 2A) led to as much as 50% decrease of B7-H2 mRNA as compared to uninfected controls at the time points examined (2 and 4 hours). 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 *cagA*$^-$ mutant had a minor effect on the reduction of B7-H2 surface expression by GECs (Fig. 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 downregulation on GEC.

IFNγ and *H. pylori* have synergistic effects on B7-H2 downregulation

IFNγ is produced within the *H. pylori* infected gastric mucosa (40), and previously we showed a synergistic effect of IFNγ and *H. pylori* on B7-H1 upregulation on GEC (18). Thus, we examined whether IFNγ could modulate *H. pylori*-mediated B7-H2 downregulation 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) Blocking the IFNγ with neutralizing anti-IFNγ mAb prevented the decrease in the levels of B7-H2 expression. Our 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). This result supports the key role of CagA in B7-H2 downregulation during *H. pylori* infection.

B7-H2 downregulation involve activation of mTOR/p70 S6 kinase

Previous studies showed that *H. pylori* CagA protein can activate NFκB, MAPK, STAT 3 and PI3K pathways (41–44). In order to understand the underlying mechanisms regulating B7-H2 decreased expression during *H. pylori* infection we first globally analyzed pathways activated in *H. pylori* infected GEC using a Luminex cell signaling array. Our 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. 4A, B). In contrast, no significant phosphorylation of p70 S6 kinase was observed in GEC infected with *cagA*$^-$ strain (Fig. 4C, D). Thus, we examined the role of these pathways in *H. pylori*-mediated downregulation of the B7-H2 by using specific inhibitors. We observed that downregulation of B7-H2 by the *cagA*$^+$ *H. pylori* strain was blocked in the presence of rapamycin, a p70 S6 kinase/mTOR specific inhibitor (Fig. 4E). In contrast, inhibition of PI3K, STAT3 and NFκB pathways with pharmacological inhibitors did not affect *H. pylori* mediated downregulation of B7-H2 expression (Fig. 4F). These results suggest that p70 S6 kinase is a key signaling pathway in *H. pylori*-mediated downregulation of B7-H2 on GECs.

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

As B7-H2 has been implicated in Th17 cell differentiation, we examined whether the CagA-dependent B7-H2 downregulation impaired the capacity of GEC to induce Th17 cells differentiation from naïve CD4$^+$ T cells. Our 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. 5A). The presence of Th17 was further confirmed by measuring IL-17A in co-culture supernatants by Luminex array (Fig. 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. 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.

Downregulation 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 our observations *in vitro* to the *H. pylori* associated immunopathophysiology, we used a mouse model of *H. pylori* infection. Mice infected with *H. pylori* PMSS1, which contains a functional T4SS, had downregulated 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 (45) resulted in upregulation of B7-H2 (Fig. 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 PMSS1 strain had >100-fold higher bacterial burden when compared to those infected with SS1 strain (Fig. 6C). Further, analysis of serum cytokine profile demonstrated that in contrast to the mice infected with SS1 strain, which had significant serum levels of IL-17 and IL-21 ($P<.05$), mice infected with PMSS1 strain failed to upregulate pro-inflammatory Th17 cytokines (Fig. 7). Serum levels of IL-6, IL-22 and IL-23 were also increased significantly in *H. pylori* SS1 infected mice (Fig. 7). Taken together, our *in vivo* data correlate with our *in vitro* findings and suggest that CagA-mediated downregulation of the B7-H2 might be involved in the prevention of the Th17-mediated clearance of the *H. pylori* during onset of the infection.

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 (46–48). Recent data suggested that Th17 type responses are required for the clearance of the bacteria (49). 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 we 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 (30, 50). Stimulation of ICOS was demonstrated to be critical for the development of human IL-17-producing $CD4^+$ T cells (31). Further, Bauquet et. al., (29) 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 WT mice (51). The blockade of B7-H2/ICOS signaling inhibited Th1 and Th17 cells responses in chronic inflammatory conditions such as rheumatoid arthritis (30). 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 on intestinal epithelial cells (52) and due to its importance in activation of Th17 responses, we 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 uninfected biopsy samples. B7-H2 expression on colonic and airway epithelium was previously noted (52). However, little is known about the role of B7-H2 costimulation in the responses associated with bacterial immunopathogenesis and clearance. In the current study, we demonstrated for the first time that *H. pylori* significantly downregulated 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, (53) we sought to investigate its role in the observed B7-H2 downregulation. In this study using a CagA isogenic mutant and their corresponding parental strains, we showed that CagA plays a crucial role in downregulating B7-H2 expression on GEC. Though compared to the untreated cells *H. pylori cagA*$^-$ strains always showed some downregulation of B7-H2, suggesting that other components of *H. pylori* might also have an influence in downregulating B7-H2 expression, but it is less effective than the wild type $CagA^+$ strains. Our *in vivo* data using C57BL/6 mice also showed that *H. pylori* mediated transfer of CagA via a T4SS significantly downregulates 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 one of its important virulence factors, 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, (54) we also investigated whether IFNγ has any role in B7-H2 expression. Our results showed that IFNγ synergizes with the effect of *H. pylori cagA*$^+$ strains in downregulating B7-H2 expression by GEC. Several studies showed induction of B7–2, B7-H1, and B7-DC in different classical APCs by IFNγ (55, 56). Stanciu et. al., (57) showed a synergistic effect of Respiratory Syncytial Virus (RSV) and IFNγ in the upregulation of B7-H1 and B7-DC in respiratory epithelial cells. In addition, their study showed that treatment of RSV infected cells with IFNγ causes downregulation of B7-H2 and B7-H3 expression. Previous findings from our group showed IFNγ and *H. pylori* synergize in B7-H1 upregulation (18). Thus, the synergistic effect of IFNγ and *H. pylori* in B7-H2 downregulation could result from IFNγ mediated increase in expression by GEC receptors that are used by *H. pylori* (32).

CagA interacts with several intracellular components of signal transduction and activates NFκB, MAPK, STAT3 and PI3K/Akt pathways (41–44). Previous reports have highlighted the fact that *H. pylori* activates STAT3 to modulate host immune responses (43). Though our results showed activation of these pathways by CagA expressing *H. pylori* strains, our data implies that CagA-mediated activation of NFκB, STAT3 and PI3K pathway is not required for the *H. pylori*-mediated downregulation of B7-H2 on GEC. Furthermore, our 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 (58). Using specific cell signaling inhibitors we showed that *H. pylori* use CagA to manipulate B7-H2 expression by activating p70 S6 kinase pathway to prevent GEC from providing positive costimulation 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 (17). Herein using *in vitro* GEC-T cell co-cultures we showed that *H. pylori* uses CagA cytotoxin to downregulate Th17 cell type responses. A significant downregulation 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 support this *in vitro* observation. We have further shown that *H. pylori* CagA-mediated B7-H2 downregulation correlates with a decrease in Th17 type responses detected in murine serum and an increase in *H. pylori* colonization of the gastric mucosa. Though our data showed increased *H. pylori* bacterial load and decreased Th17 type of response in PMSS1 infected mice compared with those infected with the SS1 strain, we did not observe severe inflammatory changes in any of the *H. pylori* infected mice. This might be because *H. pylori* infection in 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 (59). Th17 cells have been suggested to have dual roles in both infection control on one hand and preneoplastic changes on the other hand. Several studies showed that protective immunity against *H. pylori* infection requires strong Th17 response (60). Our study showed reduced Th17 cell cytokines and increased bacterial load in the PMSS1 infected mice correlates with results from another recent study (49). 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 cell (29). Our study also supports the importance of B7-H2/ICOS signaling in Th17 cell development since the downregulation of B7-H2 expression in the gastric mucosa of PMSS1 *H. pylori*-infected mice correlates with decreased Th17 type responses.

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

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Abbreviations used in this paper

APC	antigen presenting cells
CagA	cytotoxin associated gene A
CFU	colony forming unit
GEC	Gastric epithelial cells
H. pylori	Helicobacter pylori
IL	interleukin
mRNA	messenger RNA
Th17	T helper 17 cells
Treg	T regulatory cells

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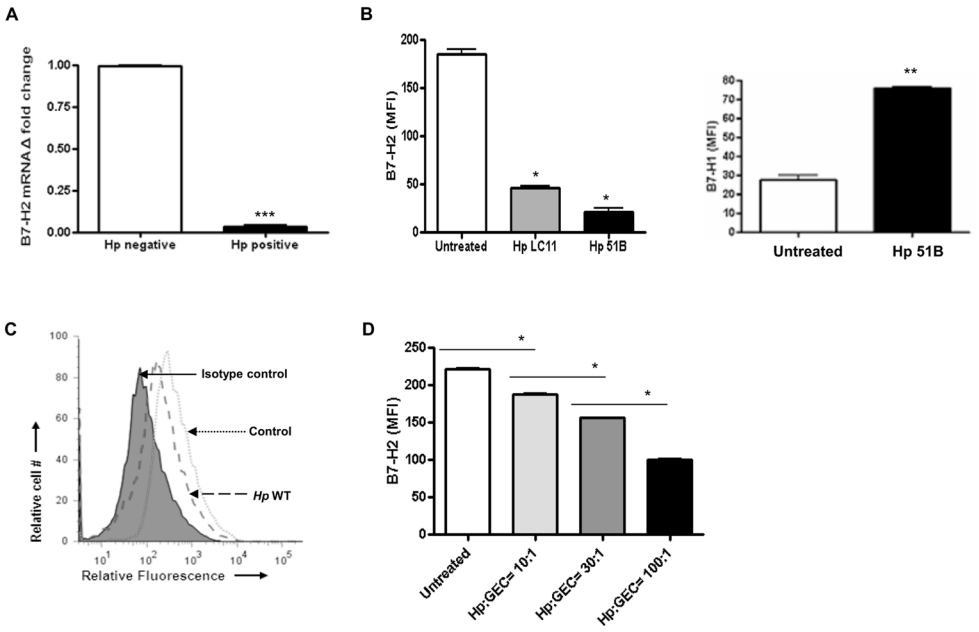

Figure 1.

Decreased expression of B7-H2 in *H. pylori* infected GEC. (A) Gastric biopsy samples were collected from five *H. pylori* negative and five positive patients, GEC was isolated and were analyzed for the B7-H2 mRNA expression by real-time RT-PCR. (B) Modulation of the B7-H2 surface expression on GEC N87 by the infection with *H. pylori* 51B and LC11 (24 hour post-infection, *H. pylori*:GEC ratio 10:1) as determined by flow cytometry analysis. Upregulation of B7-H1 on N87 cells after treating 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 hour and analyzed by flow cytometry. The data expressed as a mean of fluorescence intensity (MFI). Data represents the mean ± SD (n=8); $P < .05$.

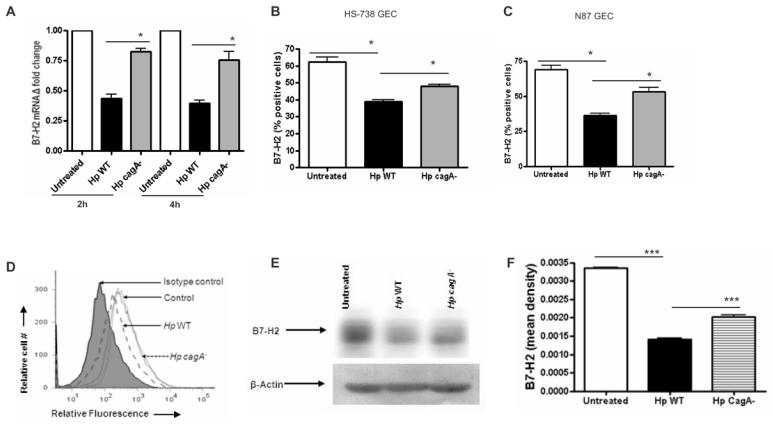

Figure 2.

H. pylori mediated downregulation of B7-H2 on GEC involves CagA. (A) B7-H2 mRNA expression in N87 cells was analyzed using real-time RT-PCR. RNA was isolated from uninfected and 2 hour, 4 hour *H. pylori cagA*$^{+}$ (WT) and *cagA*$^{-}$ strain infected GEC. mRNA level for B7-H2 was normalized to 18S and compared to the level of B7-H2 mRNA of untreated N87 cells. N=9, $P < .05$. (B) Surface B7-H2 expression was analyzed by flow cytometry on HS-738 and (C) N87 GEC cell lines after 24 hour of infection with *H. pylori cagA*$^{+}$ (WT) or *cagA*$^{-}$ mutant. The data expressed as percent 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, $P<.05$. (E) B7-H2 expression was analyzed by western blot in N87 cells treated with *H. pylori* WT and *cagA*$^{-}$ strain after 24 hour infection. (F) Quantitative analysis of B7-H2/beta-actin ratio in GEC infected sample is included (n=3).

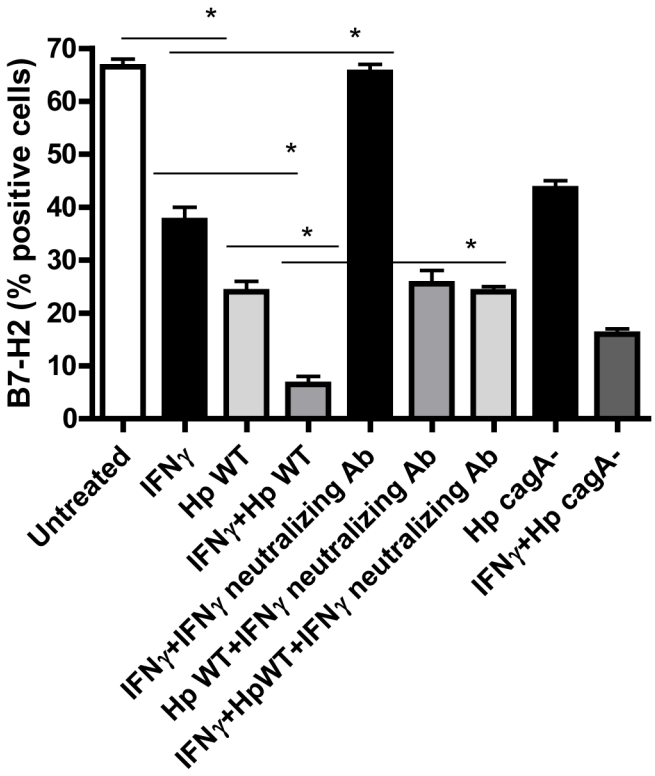

Figure 3.

IFNγ synergize *H. pylori*-mediated B7-H2 downregulation on gastric epithelium. N87 cells were treated with IFNγ (100 U/mL) for 48 hour. Cells treated with IFNγ were washed and cultured with medium for 24 hour 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 hour. Graph represents the mean ± SD (n=8); $P < .05$.

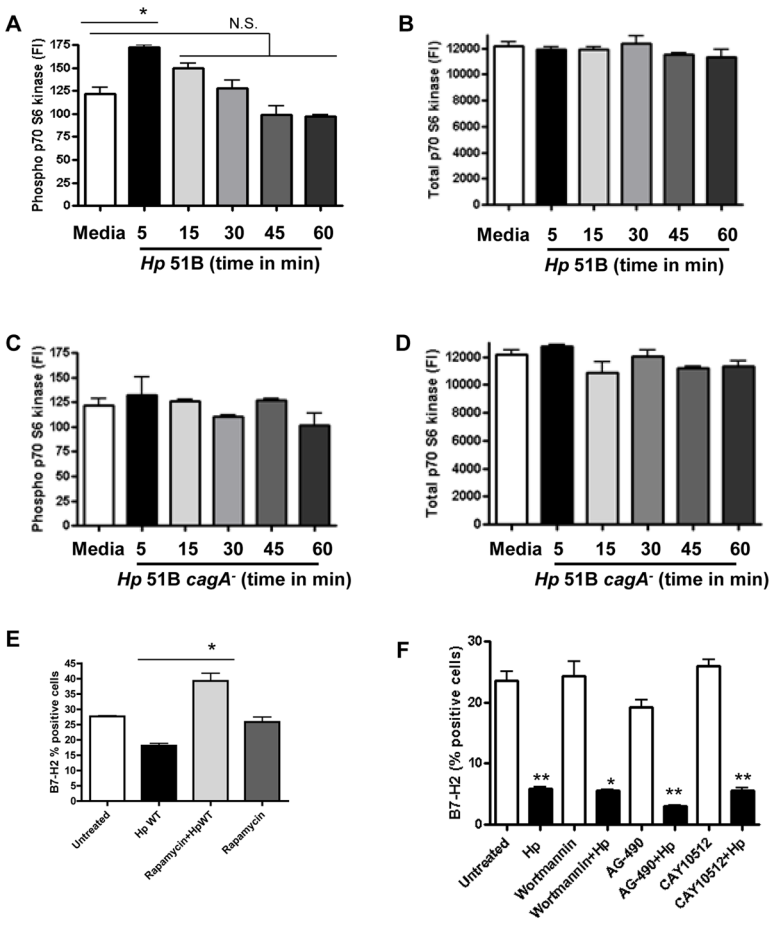

Figure 4.

Activation of mTOR/p70 S6 kinase involved in the *H. pylori* mediated downregulation of B7-H2 expression. N87 cells were incubated with *H. pylori* 51B WT and *cagA*⁻ strain. Cell were lysed after 5 min, 15 min, 30 min, 45 min and 60 min. Cell lysates from GEC exposed to *H. pylori* 51B WT and *cagA*⁻ strain was 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 mTOR/p70 S6 kinase pathway, (F) or with wortmannin (100 nM), AG-490 (100 ng/mL) and CAY10512 (10 μM) inhibitor for PI3K, STAT3 and NFκB pathway respectively, for 1 hour and then were infected with *H. pylori*. B7-H2 expression was analyzed using flow cytometry 24 hour later. Data expressed as a percentage of positive cells. Results represents as the mean ± SD (n=8); *P*=N.S. (Not significant); **P* < *.05.*

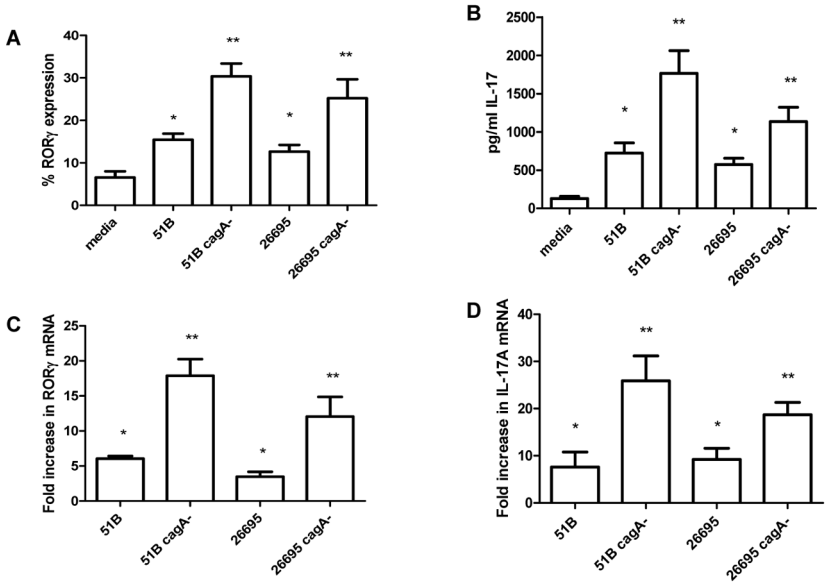

Figure 5.

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 hour. 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, $*P < .05$.

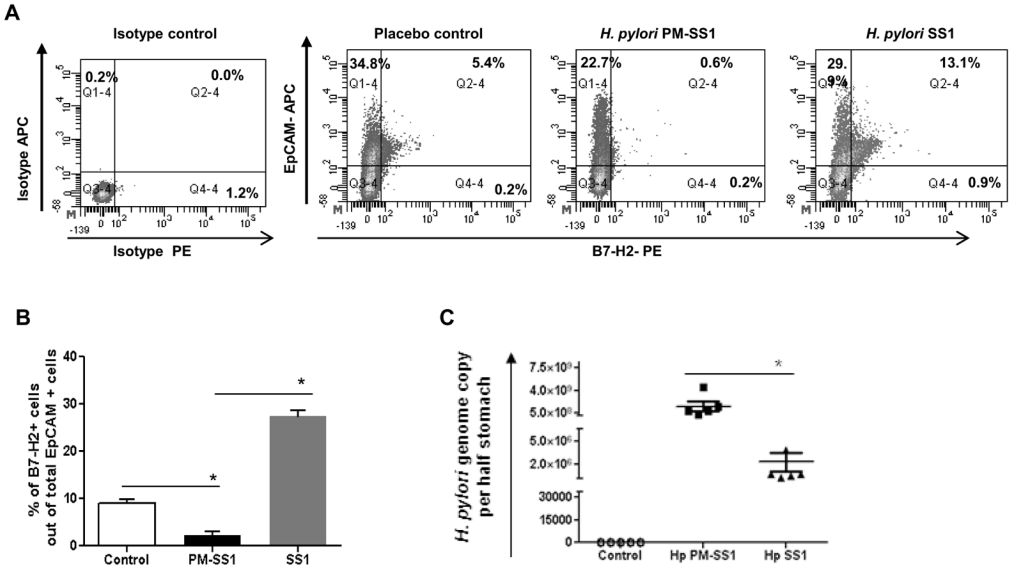

Figure 6.

H. pylori mediated downregulation of B7-H2 on GEC *in vivo* involves CagA function and inversely correlate with the bacterial clearance. (A) C57BL/6 mice were challenged with *H. pylori* strain PMSS1, which express 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 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. Average bar of infection rate were calculated from five mice per group and demonstrated as a mean ± SD.

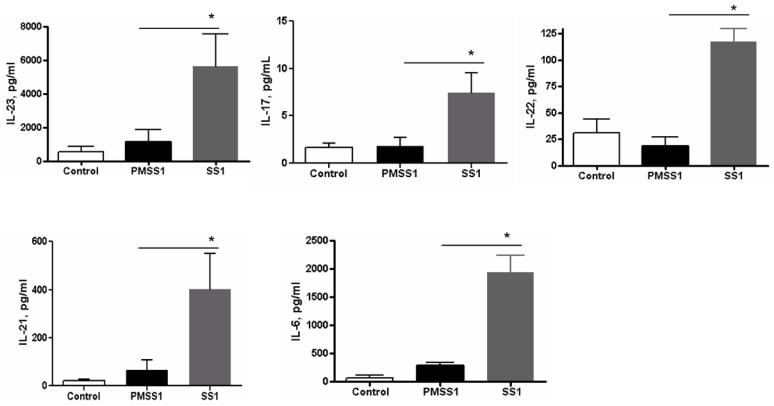

Figure 7.

In vivo infection with *H. pylori* expressing functional T4SS and can deliver CagA fails to upregulate Th17 type responses. C57BL/6 mice were challenged with *H. pylori* strain PMSS1, or with *H. pylori* SS1. Blood were collected four weeks after *H. pylori* challenge and cytokine profile was analyzed using luminex bead array. Data represents as mean ± SD (n=12); $*P < .05$.