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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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Gastric epithelial cells (GECs) are the primary target for Helicobacter pylori infection and may act as APCs regulating local T cell responses. We previously reported that H. pylori infection of GECs induces the expression of the T cell coinhibitory molecule B7-H1 on GECs. This process contributes to the hyporesponsiveness of CD4+ effector T cells and accumulation of regulatory T cells. In the present study, we investigated the impact of H. pylori cytotoxin-associated gene A (CagA) on the modulation of the expression of the T cell costimulator B7-H2 by GECs. B7-H2 is involved in promoting Th17 type responses. IFN-γ, 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 GECs involves p70 S6 kinase phosphorylation. The CagA-dependent B7-H2 downregulation in GECs correlates with a decrease in Th17 type responses in vitro and in vivo. Furthermore, CagA-dependent modulation of Th17 responses was 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.

Helicobacter pylori is a Gram-negative, spiral-shaped bacterium that infects the gastric mucosa of >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).

The cytotoxin-associated gene A (CagA) 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, a 40-kb chromosomal region that encodes for a type IV secretion system (T4SS).

Gastric epithelial cells (GECs) are the primary target for H. pylori infection. After H. pylori adheres 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, when chronically present, may contribute to the inflammation-associated carcinogenesis (19–21). Alternatively, 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. Dendritic cell–mediated skewing of T cell balance toward suppressive regulatory T cells (Tregs) has been suggested to be important in the downregulation of Th17 and the establishment of the H. pylori persistence (23). However, it is not known whether and how GECs, as primary targets for H. pylori infection, contribute to regulation of Th17 cell responses during the establishment of the chronic infection.

During H. pylori infection GECs express MHC class II and may act as local APCs (24, 25). In addition to the recognition of MHC-bound peptides on APCs by TCR, the outcome of APC/T cell

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Abbreviations used in this article: CagA, cytotoxin-associated gene A; EpCAM, epithelial cell adhesion molecule; GEC, gastric epithelial cell; PMSS1, premouse Sydney strain 1; ROR, retinoic acid-related orphan receptor; SS1, Sydney strain 1; Treg, regulatory T cell; T4SS, type IV secretion system; WT, wild-type.

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interactions depends on a second signal provided by engagement of the B7 costimulatory receptor (26). Our laboratory has previously shown that GECs express the classical B7 costimulators 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 Tregs (18, 27).

B7-H2 (ICOS ligand) is among the newer members of the B7 family of receptors and is known to have a costimulatory 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 establishment of persistent infection in the host.

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 8-wk-old mice, which were tested negative for intestinal Helicobacter spp., were used in the experiments. The University of Texas Medical Branch Institutional Animal Care and Use Committee–approved protocol was followed.

Human tissue and cell lines

GECs were isolated from biopsy specimens as described previously (32). Briefly, biopsy specimens of the gastric antrum were obtained from consenting patients undergoing gastroesophageal duodenoscopy for various clinical indications in accordance with Institutional Review Board–approved protocol. Patients were considered infected when H. pylori was detected by both rapid urease testing and histopathology, and for these studies they were confirmed to be infected by culture of H. pylori from biopsies. Biopsy tissue was placed in dispase solution (2.4 U/ml; Boehringer Mannheim, Mannheim, Germany) and agitated at 37˚C for 30 min with 0.1 mM EDTA (Sigma-Aldrich, St. Louis, MO) and flow cytometry with May–Grunwald–Giemsa staining (Sigma-Aldrich). GECs were isolated from biopsy specimens as described previously (32). GECs were isolated from biopsy specimens as described previously (32). H. pylori strains were grown on tryptic soy agar plates supplemented with 5% sheep’s serum. Bacterial cultures and infection of GECs

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 premouse SS1 (PMS11) (35) used to infect mice were gifts from Dr. J. Pappo (Astra Research Center) and Dr. Richard Peek (Vanderbilt University), respectively. These bacterial strains were grown on tryptic soy agar plates supplemented with 5% sheep’s blood (Becton Dickinson, San Jose, CA) or on blood agar plates with 2.5 μg/ml chloramphenicol (Technova, Hollister, CA) to maintain 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 OD600 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 multiplicity of infection, the numbers of GECs were determined using trypan blue staining, and the required number of bacteria was added after calculation. GECs were treated with IFN-γ (100 U/ml) for 48 h, then washed and incubated an additional day in regular medium without IFN-γ before infection. When IFN-γ was neutralized, anti–IFN-γ-neutralizing Ab was added at an optimal concentration (10 μg/ml). As an isotype control, mouse IgG1 Ab was used in the same concentration. B7-H2 expression was measured after coculture with IFN-γ or H. pylori–infected GECs in the presence of either anti–IFN-γ-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, AB, Canada) in the cagA gene, the obtained plasmids (1–2 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.

Abs, recombinant proteins, and cell signaling inhibitors

PE-conjugated anti-human B7-H2 (clone M1H12), PE-conjugated anti-murine B7-H2 (clone H5K3.2), allophycocyanin-conjugated anti-murine epithelial cell adhesion molecule (EpCAM; clone G8.8), and PE-conjugated retinoic acid–related orphan receptor (ROR)γT (clone AFKJS9) were purchased from eBioscience, as were the isotype controls. The viability dye eFluor 780 (eBioscience, San Diego, CA) was included in the experiments to control cell viability. Human rIFN-γ (Roche) was used at 100 U/ml. Neutralizing Abs for IFN-γ included both purified functional grade anti-human IFN-γ from eBioscience. The isotype control Ab used for IFN-γ studies was functional grade mouse IgG1 from eBioscience. For cell signaling inhibition the following inhibitors were used: CAY10512 (10 μM; Cayman Chemical, Ann Arbor, MI), AG-490 (100 ng/ml; Enzo Life Sciences, Farmingdale, NY), wortmannin (100 nM; Calbiochem, Billerica, MA), and rapamycin (100 ng/ml; Calbiochem, Darmstadt, Germany).

GEC/T cell coculture

For GEC/T cell coculture experiments, naive 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 (Institutional Review Board–approved protocol 06-122 at the University of Texas Medical Branch). PBMCs were prepared from collected blood by density gradient centrifugation over Ficoll-Paque Plus. Naive CD4+ T cells were isolated from the peripheral blood by negative selection using a MACS naive CD4+ T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). GECs where exposed to H. pylori strains for 8 h, and then supernatants from H. pylori–treated cultures were filtered to remove bacteria, and GECs were washed twice with PBS to remove attached bacteria and replaced with filtered supernatants until 24 h. CD4+ T cells were preactivated 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% CO2 for 2 d.

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 108 colony-forming units (in 100 μl PBS) of H. pylori SS1 or PMS11 strains three times during a week. The 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 two to four 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 h at 4˚C, transferred into 70% ethanol solution, and stored at 4˚C until needed. Tissue was then embedded in paraffin and processed by H&E.
staining. Stomach tissue dissociation and enzymatic digestion was performed using a gentleMACS dissociator (Miltenyi 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, and IV (100 U/ml). After the 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 μg/ml; Worthington Biochemical) at 37˚C for 15 min. Finally, tissue was processed using the dissociator and washed twice with HBSS. The digested

FIGURE 1. Decreased expression of B7-H2 in *H. pylori*–infected GECs. (A) Gastric biopsy samples were collected from five *H. pylori* negative and five positive patients. GECs were 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 strains (24 h after infection, *H. pylori*/GEC ratio of 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 the representative flow cytometry histograms 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*/GECs (10:1, 30:1, and 100:1) for 24 h and analyzed by flow cytometry. The data are expressed as mean fluorescence intensity (MFI). Data represent the means ± SD (n = 8). *p < 0.05, **p < 0.01.

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, 2 h, and 4 h *H. pylori* cagA+ (WT) and cagA− strain–infected GECs. mRNA level for B7-H2 was normalized to 18S and compared with the level of B7-H2 mRNA of untreated N87 cells (n = 9). (B) Surface B7-H2 expression was analyzed by flow cytometry on HS-738 and (C) N87 GEC lines after 24 h of infection with *H. pylori* cagA+ (WT) or cagA− mutant. 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). (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/β-actin ratio in GEC–infected sample is included (n = 3). *p < 0.05, ***p < 0.001.
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 a 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 Roussel et al. (38). A primer/probe set 16SF229BP 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 were calculated according to the method described by Roussel et al. (38). Murine GADPH gene amplification was used to control the equal loading of total DNA used in the PCR reaction.

**Flow cytometry**

Flow cytometry was performed as described previously (17).

**Real-time RT-PCR**

Real-time RT-PCR was performed according to the two-step real-time RT-PCR protocol (Applied Biosystems, Foster City, CA). The appropriate Assays-on-Demand gene expression assay primers/probe mixes (Applied Biosystems) for human 18S and gene of interest (a 20 × mix of unlabeled PCR primers and Taqman MGB probe, FAM dye-labeled) and 2 μl cDNA was added to the PCR reaction step. The reactions were carried out in 20 μl final volume using a Bio-Rad Q5 real-time PCR machine according to the following protocol: 2 min at 50°C, 10 min at 95°C (1 cycle) and 15 s at 95°C, and 1 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—were measured using a Luminex array (Millipore, Billerica, MA) according to manufacturer’s instructions. Samples were analyzed using Bio-Plex Manager software (Bio-Rad).

**Statistical analysis**

The results were expressed as the means ± 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 a Student’s t test for multiple comparisons. The p values < 0.05 were considered statistically significant.

**Results**

**H. pylori downregulates B7-H2 expression on GECs**

To assess B7-H2 expression during *H. pylori* infection, mRNA levels were 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*-infected subjects when compared with 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 h after infection with all *H. pylori* strains (Fig. 1B, 1C). 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 downregulates B7-H2 expression on human GECs in a dose-dependent manner.

**Downregulation of B7-H2 expression depends on the presence of *H. pylori* CagA**

Because 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 GECs with *H. pylori* 51B wild-type (WT) strain (Fig. 2A) led to as much as 50% decrease of B7-H2 mRNA as compared with uninfected controls at the time points examined (2, 4 h). In contrast, the *H. pylori* cagA mutant had a very limited effect on B7-H2 expression compared with 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. 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 with cells infected with cagA* strain (Fig. 2E, 2F). These results suggested that the major *H. pylori* virulence factor CagA is involved in the B7-H2 downregulation on GECs.

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

IFN-γ is produced within the *H. pylori*-infected gastric mucosa (40), and previously we showed a synergistic effect of IFN-γ and *H. pylori* on B7-H1 upregulation on GECs (18). Thus, we examined whether IFN-γ could modulate *H. pylori*-mediated B7-H2 downregulation on GECs. N87 cells treated with either IFN-γ or *H. pylori* alone showed significant decreases in B7-H2 expression. However, treatment with both IFN-γ and *H. pylori* resulted in complete abrogation of B7-H2 expression. B7-H2 expression decreased after culturing GECs with IFN-γ, and decreased expression was more prominent when GECs were pretreated with IFN-γ prior to infection with *H. pylori* (Fig. 3). Blocking the IFN-γ with neutralizing anti–IFN-γ mAb prevented the decrease in the levels of B7-H2 expression. Our results clearly showed a synergistic effect of IFN-γ and *H. pylori* in reduced B7-H2 expression by GECs. Interestingly, the synergism of IFN-γ and *H. pylori* in decreasing B7-H2 expression was less obvious when cells were infected with a cagA* strain (Fig. 3). This result supports a 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 NF-κB, MAPK, STAT3, and PI3K pathways (41–44). To understand the underlying mechanisms regulating B7-H2 decreased expression during H. pylori infection, we first globally analyzed pathways activated in H. pylori–infected GECs using a Luminex cell signaling array. Our data demonstrated that, in addition to NF-κB and STAT3 pathways, H. pylori infection of GECs also leads to the activation of mTOR/p70 S6 kinase within the first 5 min of infection (Fig. 4A, 4B). In contrast, no significant phosphorylation of p70 S6 kinase was observed in GECs infected with cagA− strain (Fig. 4C, 4D). Thus, we examined the role of these pathways in H. pylori–mediated downregulation of 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 NF-κB 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

Because B7-H2 has been implicated in Th17 cell differentiation, we examined whether the CagA-dependent B7-H2 downregulation impaired the capacity of GECs to induce Th17 cell differentiation from naive CD4+ T cells. Our results showed that there is a small induction of RORγ expressing CD4+ T cells when naive CD4+

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. Cells were lysed after 5, 15, 30, 45, and 60 min. Cell lysates from GECs exposed to H. pylori 51B WT and cagA− strain were analyzed for (A, C) phosphorylated and (B, D) total p70 S6 kinase using Luminex bead arrays, respectively. (E) GECs were treated with rapamycin (100 ng/ml), an inhibitor of mTOR/p70 S6 kinase pathway, or (F) with wortmannin (100 nM), AG-490 (100 ng/ml), and CAY10512 (10 μM) inhibitors of PI3K, STAT3, and NF-κB pathways, respectively, for 1 h and then were infected with H. pylori. B7-H2 expression was analyzed using flow cytometry 24 h later. Data are expressed as a percentage of positive cells. Results represents as the means ± SD (n = 8). *p < 0.05, **p < 0.01.
T cells were cocultured 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 coculture supernatants by a 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, 5D). These data demonstrate the critical role of CagA in maintaining low-level Th17 responses, and this may contribute to *H. pylori* immune evasion.

### 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 *H. pylori* remain far from understood. In this study, we demonstrated that *H. pylori* infection of GECs leads to a decrease of B7-H2, which is a positive costimulatory ligand, and that this process might be important in *H. pylori*-mediated escape of Th17-mediated bacterial clearance.

Costimulatory interactions between B7 family ligands and their receptors play important roles in the growth, development, and differentiation of T cells. Recent data demonstrated that interactions between B7-H2 on APCs with its putative receptor ICOS on T and B cells 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). Furthermore, Bauquet et al. (29) demonstrated that ICOS was critical for maintaining effector memory Th17 cells. B7-H2−/− knockout mice also were noted to have lower Th17 responses to chlamydial infection than do 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). Taken together, our in vivo data correlate with our in vitro findings and suggest that CagA-mediated downregulation of B7-H2 might be involved in the prevention of the Th17-mediated clearance of the *H. pylori* during onset of the infection.
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 present study, we demonstrated for the first time, to our knowledge, that *H. pylori* significantly downregulated B7-H2 expression in gastric mucosa, particularly on GECs.

Because 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 GECs. Compared with 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 they are less effective than the WT 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 GECs in the

**FIGURE 6.** *H. pylori*–mediated downregulation of B7-H2 on GECs in vivo involves CagA function and is inversely correlated with 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 4 wk 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 datum point represents a single mouse tested in quadruplicate. Average bars of infection rate were calculated from five mice per group and are demonstrated as means ± SD. *p < 0.05.

**FIGURE 7.** In vivo infection with *H. pylori* expressing functional T4SS and that 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 was collected 4 wk after *H. pylori* challenge, and cytokine profile was analyzed using a Luminex bead array. Data are represented as means ± SD (n = 12). *p < 0.05.
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.

Because cytokines play an important role in regulating immune function, and IFN-γ has been detected in *H. pylori*–infected gastric tissues in both humans and mice (54), we also investigated whether IFN-γ has any role in B7-H2 expression. Our results showed that IFN-γ synergizes with the effect of *H. pylori* cagA+ strains in downregulating B7-H2 expression by GECs. Several studies showed induction of B7-2, B7-H1, and B7-DC in different classical APCs by IFN-γ (55, 56). Stanciu et al. (57) showed a synergistic effect of respiratory syncytial virus and IFN-γ in the upregulation of B7-H1 and B7-DC in respiratory epithelial cells. Additionally, their study showed that treatment of respiratory syncytial virus infected cells with IFN-γ causes downregulation of B7-H2 and B7-H3 expression. Previous findings from our group showed that IFN-γ and *H. pylori* synergize in B7-H1 upregulation (18). Thus, the synergistic effect of IFN-γ and *H. pylori* in B7-H2 downregulation could result from an IFN-γ-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 NF-κB, 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). Although our results showed activation of these pathways by CagA-expressing *H. pylori* strains, our data imply that CagA-mediated activation of the NF-κB, STAT3, and PI3K pathways is not required for the *H. pylori*–mediated downregulation of B7-H2 on GECs. Furthermore, to our knowledge, our data demonstrate for the first time 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* uses CagA to manipulate B7-H2 expression by activating p70 S6 kinase pathway to prevent GECs 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 GECs, which causes inhibition of CD4+ effector T cell proliferation and induction of Tregs (17). In this study, using in vitro GEC/T cell cocultures we showed that *H. pylori* uses CagA to cytotoxin to downregulate Th17 cell type responses. A significant downregulation of Th17 cell transcription factor ROR-γ and IL-17A was observed when GECs 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. Although our data showed increased *H. pylori* bacterial load and decreased Th17 type 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 the mouse model results mostly in lymphocytic gastritis, which does not progress to severe inflammation. An optimum induction of chronic gastritis can be achieved using *Helicobacter felis* as a model (39). Th17 cells have been suggested to have dual roles in both infection control on the one hand and preneoplastic changes on the other hand. Several studies showed that protective immunity against *H. pylori* infection requires a strong Th17 response (60). Our study showed reduced Th17 cell cytokines and increased bacterial load in the PMSS1-infected mice correlated with results from another recent study (49). Horvath et al. (49) 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 (29). Our study also supports the importance of B7-H2/ICOS signaling in Th17 cell development because 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 GECs, 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, the *H. pylori* CagA delivery system may be an important target in vaccine development for achieving acceptable levels of immune protection and for designing a therapeutic strategy to treat patients infected with this prevalent and deadly human pathogen.

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**Disclosures**

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**References**


