# Prior *Helicobacter pylori* Infection Ameliorates *Salmonella Typhimurium*-Induced Colitis: Mucosal Crosstalk Between Stomach and Distal Intestine*

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**Background:** *Helicobacter pylori* infection is associated with a lower risk of chronic autoimmune diseases including inflammatory bowel disease (IBD). *H. pylori* modulates the gastric immune response, decreasing the local inflammatory response to itself. In mice, chronic *Salmonella typhimurium* infection induces colitis similar to Crohn's disease, characterized by inflammation, which progresses toward fibrosis. The aim of this study was to determine whether prior *H. pylori* infection acts at a distance to modulate the immune response of *S. typhimurium*-induced colitis.

**Methods:** Mice were infected with the mouse-adapted strain of *H. pylori* (SS1), followed by infection with *S. typhimurium*. The effect of *H. pylori* on colitis was determined by gross pathology, histopathology, cytokine response, and development of fibrosis in the cecum. Gastritis and systemic immune response was measured in response to infection.

**Results:** *H. pylori* suppresses the Th17 response to *S. typhimurium* infection in the mouse cecum, but does not alter the Th2 or T-regulatory response or the development of fibrosis. *H. pylori* infection induces IL-10 in the mesenteric lymph nodes, suggesting an extragastric mechanism for immunomodulation. *H. pylori* / *S. typhimurium* coinfection decreases inflammation in both the cecum and the stomach.

**Conclusions:** This study demonstrates a potential mechanism for the negative association between *H. pylori* and IBD in humans. *H. pylori* represses the lower gastrointestinal tract Th17 response to bacterially induced colitis via extragastric immunomodulatory effects, illustrating immunological crosstalk between the upper and lower gastrointestinal tract.

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**Key Words:** inflammatory bowel disease, Helicobacter pylori, Salmonella typhimurium, colitis, gastritis

*Helicobacter pylori* is a Gram-negative bacterium that frequently and chronically colonizes the human gastric mucosa. *H. pylori*'s capacity to cause chronic infection stems from its ability to modulate the gastric immune response. Recent data demonstrate that *H. pylori* upregulates the production of antiinflammatory T-regulatory (Treg) cells in the stomach, thereby decreasing the inflammatory response against the bacteria.[1] In fact, *H. pylori*-colonized patients express higher levels of gastric Foxp3, a Treg cell marker.[2] Whether this upregulation affects systemic immune responses in addition to the local response against *H. pylori* is unknown. However, colonization with *H. pylori* has been inversely associated with certain chronic inflammatory diseases, such as asthma and lupus.[3] This suggests that immune regulation by *H. pylori* may have systemic effects.

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the intestinal tract, resulting in part from a dysregulated immune response. Previous work in animal models has illustrated the importance of Treg cells in the pathogenesis of colitis. For example, mice deficient in IL-10, a key regulatory cytokine of Treg cells, develop spontaneous colitis.[4] Additionally, adoptive transfer of Treg cells can inhibit the development of colitis in certain animal models.[5–7] Furthermore, a meta-analysis of studies investigating the relation between *H. pylori* colonization and IBD found a significant inverse association between the two, suggesting a possible protective role for *H. pylori*[8] colonization against the development of IBD. However, these clinical data have various confounders, limiting the certainty of the findings.

In this study we aimed to examine the effect of *H. pylori* on an experimental model of colitis and further define the relationship between *H. pylori* and IBD. We used the *Salmonella typhimurium* model of colitis in order

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to illustrate the effect of gastric *H. pylori* colonization on a distant bacterial–host immune system interaction in the colon.[9] We found that *H. pylori* infection decreased cecal inflammatory cytokine expression in response to *S. typhimurium* infection. We also found that prior *H. pylori* infection influences distal mucosal immune responses, as evidenced by an increased interleukin (IL)-10 expression measured in the mesenteric lymph nodes of mice infected with *H. pylori*. Our data provide the first evidence that *H. pylori* infection in the stomach alters the immunological environment of the lower gastrointestinal (GI) tract, providing mechanistic support for the epidemiological observation of a negative association between *H. pylori* status and risk of IBD.

## MATERIALS AND METHODS

### Mice

Female 8–12-week-old C57/BL6 mice (Jackson Laboratories, Bar Harbor, ME) were infected with either *H. pylori* SS1, *S. typhimurium* ΔaroA or *H. pylori* SS1 + *S. typhimurium* ΔaroA. Twenty-four hours prior to oral infection with $3 \times 10^6$ colony-forming units (CFU) of *S. typhimurium* strain ΔaroA in 100 μL 0.1 M HEPES buffer (pH = 8.0), mice received 20 mg of streptomycin in 0.1 M Hank's balanced salt solution (HBSS) by oral gavage. All animal experiments were conducted with the approval and oversight of the University of Michigan UCUCA (University Committee on Use and Care of Animals).

### Bacterial Strains

*Salmonella typhimurium* strain ΔaroA (a gift from Guntram Grassl, University of British Columbia, Vancouver, BC, Canada) which is naturally resistant to streptomycin was grown in LB broth containing 100 μg/mL streptomycin at 37°C. The mouse-adapted *H. pylori* SS1 strain (a gift from Kathryn Eaton, University of Michigan, Ann Arbor, MI) was grown on Campylobacter-selective agar (BD Diagnostics, Bedford, MA) supplemented with 5% sterile horse blood, trimethoprim (5 μg/mL), vancomycin (10 μg/mL), and nystatin (10 μg/mL) for 2 days at 37°C in a humidified microaerophilic chamber (BBL Gas System, with CampyPak Plus packs; BD Microbiology, Sparks, MD).

### Animal Studies

Mice were infected with *H. pylori* SS1 with an oral gavage of $10^8$ CFU of live organisms thrice over 1 week. Control animals received HBSS by oral gavage. Thirty-four days after the *H. pylori* infection, half of the *H. pylori*-infected animals (*H. pylori*/*S. typhimurium*) received 20 mg streptomycin by oral gavage followed by infection with $3 \times 10^6$ CFU *S. typhimurium* ΔaroA in 100 λ 0.1 M HEPES buffer (pH = 8.0) (Supporting Fig. 1). The other half of the *H. pylori*-infected cohort (*H. pylori* group) was not infected with *S. typhimurium* but received streptomycin and HBSS on the same schedule. Of the animals uninfected with *H. pylori*, half received streptomycin and subsequent *S. typhimurium* infection (*S. typhimurium* group). Negative control (HBSS group) animals were not infected with *S. typhimurium* but received streptomycin and HBSS. Mice were euthanized 21 days post *S. typhimurium* infection.

### Gross Pathology and Tissue Collection

Mice were euthanized at 21 days post-*S. typhimurium* infection. Cecum and distal colon were collected, photographed, measured, and weighed. Cecal area was determined from digital photographs using ImageJ (NIH, Bethesda, MD) and a defined region of interest (ROI) to delineate the cecum. To control for differences in images, the cecal area was normalized against a 1-cm marker in the photographic image. Tissues were snap-frozen in liquid nitrogen and stored at −80°C prior to molecular analysis. Cecal contents were collected and serially diluted before plating onto LB-streptomycin plates to determine bacterial titers.

### Histology

Formalin-fixed and paraffin-embedded tissues were stained with hematoxylin and eosin (H&E, inflammatory histology) and Masson's trichrome (fibrosis) by the University of Michigan CCGC Research Histology and Immunoperoxidase Laboratory (Ann Arbor, MI). Digital photomicrographs of tissue sections were taken with an Olympus BX microscope (University of Michigan Microscopy and Image Analysis Laboratory). Histological scoring was performed by two blinded observers (J.K., J.L.). Tissue measurements were quantitated with ImageJ analysis software.

### Histological Scoring

Gastric tissue was scored for inflammation on a scale of 0 = none to 3 = marked by the Modified Sydney Approach.[10] Cecal inflammation was determined using a separate scoring system[11]: (0) no inflammation; (1) low level of inflammation with scattered infiltrating mononuclear cells; (2) moderate inflammation with multiple foci; (3) high level of inflammation with increased vascular density and marked wall thickening; (4) maximal severity of inflammation with transmural leukocyte infiltration and loss of goblet cells.

### Immunoblotting

Immunoblotting was utilized for the detection of α-smooth muscle actin (αSMA). Whole tissue was pulverized under liquid nitrogen and lysed in ice-cold RIPA buffer with a cocktail of proteinase inhibitors (Roche, Indianapolis, IN). Protein content was determined using a modified

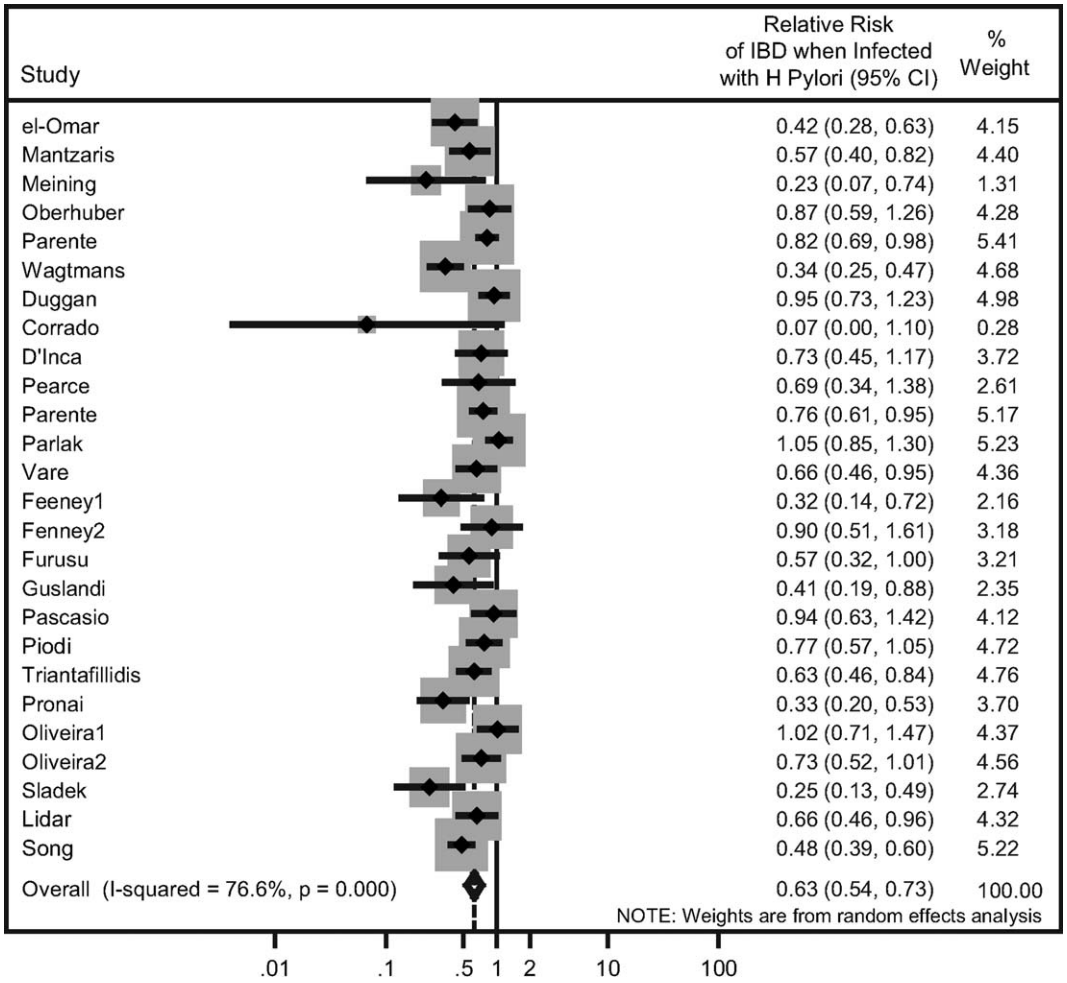



**FIGURE 1**. *H. pylori* infection is negatively associated with the relative risk for developing IBD in human populations. The summary RR was calculated from 26 studies based on a random effects model. Horizontal lines delineate the upper and lower confidence limits with diamonds indicating the point estimates. The area of the gray rectangular boxes corresponds to the weight of the study in the meta-analysis. RR is shown in the right column with upper and lower 95% CIs in parentheses. The pooled RR estimate (Overall) is shown at the bottom ($P < 0.0001$). The solid vertical line represents no effect.

Bradford assay kit (BioRad, Hercules, CA). Total protein was separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ). Membranes were blocked in 5% milk solution for 1 hour at room temperature or overnight at 4°C. α-SMA was detected by incubating the membrane overnight at 4°C with mouse antihuman monoclonal antibody (Sigma, St. Louis, MO) at 1:5000 dilution in 5% milk/TBST. As a loading control, a mouse antibody for GAPDH (Chemicon, Temecula, CA) was used after a stripping procedure. Secondary antibody antimouse IgG+ HRP (Amersham, Piscataway, NJ) was incubated for one hour at room temperature and the signal was detected by the Pierce detection system (Rockford, IL). Autoradiographs were scanned and quantitated using Image J analysis software.

## Meta-analysis

The relative risk (RR) of *H. pylori* infection in IBD versus controls was determined from 26 observational studies as previously described.[8] Briefly, RR was used to describe the ratio of the probability of the *H. pylori* infection occurring in IBD patients versus the controls. The RR was calculated with a 95% confidence interval (CI) based on a random-effects model as described by Mantel-Haenszel.[12] Meta-analysis was performed with the *metan* command in Stata 10.1 (StataCorp, College Station, TX).

## Real-time Quantitative Polymerase Chain Reaction (PCR)

RNA was extracted from the cecum using the RNeasy kit (Qiagen, Valencia, CA). Reverse transcription of 2 μg of total RNA was performed with the Superscript

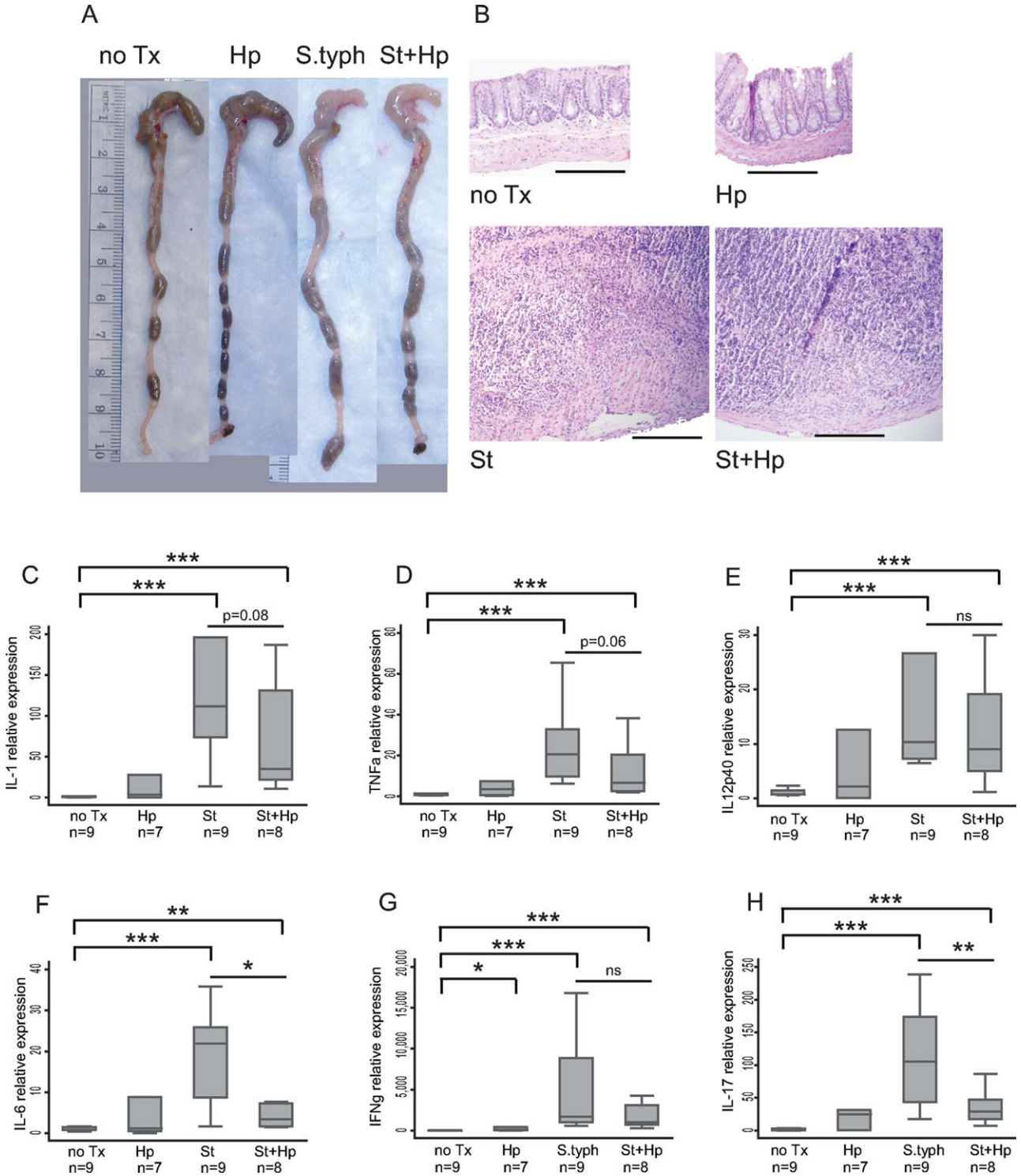



**FIGURE 2**. *H. pylori* infection suppresses the Th17 cytokine response in the cecum. (A) Representative gross appearance of the cecum and distal colon of *H. pylori*-infected mice compared to uninfected (no Tx), *S. typhimurium*-infected (St) and *H. pylori / S. typhimurium* (St+Hp) infected with a 1-cm reference ruler. (B) H&E-stained histological sections (100× magnification). Infection with *S. typhimurium* induces substantial submucosal expansion, collagen deposition, epithelial destruction, and inflammatory infiltration. Scale bar = 200 $\mu$m. (C–H) qRT-PCR gene expression of Th1 and inflammatory cytokines in the cecum of uninfected (no Tx) compared to *H. pylori* (Hp), *S. typhimurium* (St), and *H. pylori / S. typhimurium* (St+Hp) infected mice. The markers of innate immune response IL-1$\beta$ (C) and TNF-$\alpha$ (D) are compared to markers of the adaptive immune response IL-12p40 (E), IL-6 (F), IFN-$\gamma$ (G), and IL-17 (H). Gene expression was normalized to GAPDH expression. The results are from two independent animal experiments. Numbers (*n*) below the graph labels represent number of animals per experimental group (ns = not significant, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

First Strand RT kit (Invitrogen, Carlsbad, CA). Quantitative real-time PCR (qPCR) of IL-1$\beta$, tumor necrosis factor alpha (TNF-$\alpha$), interferon gamma (IFN-$\gamma$), IL-17, IL-6, IL-13, IL-12p40, Foxp3, IL-10, IL-4, TGF-$\beta$, and GAPDH was performed with the TaqMan gene expression assays (ABI, Foster City, CA). Quantitative PCR was performed using a Stratagene Mx3000P real-time PCR system (La Jolla, CA). Cycling conditions were 95°C 10 minutes, followed by 40 cycles of 95°C 15 seconds and 62°C 60 seconds. $\Delta\Delta$Ct were calculated from GAPDH expression.

### Statistical Analysis

Comparisons between several groups of mice and associated tissues or RNA expression were analyzed with analysis of variance (ANOVA), while pairwise comparisons of two groups were performed with Student's *t*-test.

## RESULTS

### H. pylori Infection Is Negatively Associated with the RR for Developing IBD in Human Populations

An update of our previously published meta-analysis of 24 observational studies was performed to include an additional two new studies.[1] The overall RR of developing IBD in patients infected with *H. pylori* was 0.63 (95% CI; 0.54–0.73, $P < 0.001$, Fig. 1). This observation suggests that *H. pylori* infection is protective against the development of IBD.

### H. pylori Infection Suppresses the Th17 Cytokine Response in the Cecum

To examine the effect of *H. pylori* gastric infection on distal gut inflammation, *H. pylori*-infected mice were coinfected with *S. typhimurium*. Chronic infection with *S. typhimurium* is characterized by a dramatic reduction in cecal size and a concurrent increase in tissue inflammation.[9] Mice infected with either *S. typhimurium* or coinfected with *H. pylori* / *S. typhimurium* developed marked changes in cecal appearance compared to either the uninfected or *H. pylori*-infected mice (Fig. 2A). H&E histological sections of cecal tissue from *S. typhimurium* or *H. pylori* / *S. typhimurium*-infected animals exhibited extensive crypt loss, inflammatory infiltration, transmural inflammation, and hypertrophy in the muscularis propria, findings that were not present in uninfected or *H. pylori*-infected controls (Fig. 2B). *S. typhimurium* infection elicits both innate (IL-1$\beta$ and TNF-$\alpha$) and adaptive (IL-12p40, IL-6, IFN-$\gamma$, and IL-17) responses in the cecum (Fig. 2C–H). *H. pylori* / *S. typhimurium* coinfection significantly repressed IL-6 and IL-17 mRNA expression (3-fold and 5.3-fold, respectively) in the cecum, with a trend toward lower IL-1$\beta$ and TNF-$\alpha$ in the cecum compared to infection by *S. typhimurium* alone. As expected, *H. pylori* infection alone did not significantly induce these proinflammatory cytokines in the cecum except for a small but significant rise in IFN-$\gamma$ expression. Overall, gastric *H. pylori* infection significantly downregulates the cecal Th17 response to *S. typhimurium* infection.

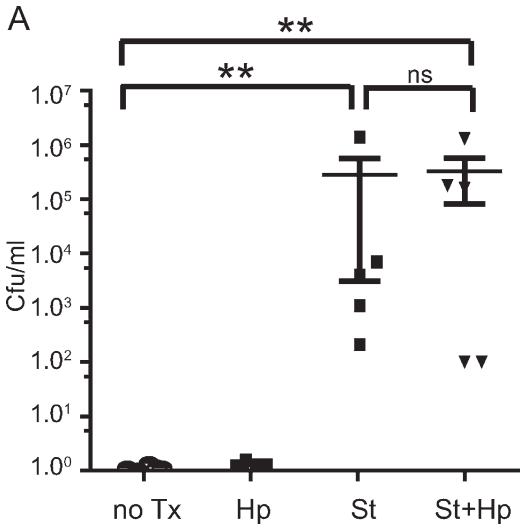



**FIGURE 3.** *H. pylori* gastric colonization and *S. typhimurium* cecal colonization are not altered by coinfection. (A) *S. typhimurium* colonizes the mouse cecum, irrespective of *H. pylori* coinfection. Colonization was determined from serial dilutions of 100 $\mu$L of cecal contents of uninfected (no Tx), *H. pylori* (Hp), *S. typhimurium* (St), and *H. pylori* / *S. typhimurium*-infected mice cultured on LB/streptomycin plates. The results are from two independent animal experiments. Statistical comparisons are made to the uninfected group (ns = not significant, $^{**}P < 0.01$).

### H. pylori Gastric Colonization and S. typhimurium Cecal Colonization Are Not Altered by Coinfection

To examine whether *H. pylori* suppression of *S. typhimurium* colitis is caused by a reduction in the level of *S. typhimurium* cecal colonization, the effect of *H. pylori* infection upon *S. typhimurium* cecal colonization was determined. *S. typhimurium* heavily colonizes the mouse cecum, persisting at day 21 postinfection. Luminal cecal contents were isolated from uninfected, *S. typhimurium, H. pylori*, and *H. pylori* / *S. typhimurium* coinfected mice. Despite prior *H. pylori* infection, *S. typhimurium* cecal colonization did not differ between *S. typhimurium*-infected and *H. pylori* / *S. typhimurium* coinfected mice (Fig. 3). Therefore, *H. pylori* suppression of the Th17 response to *S. typhimurium* was not due to a decrease in *S. typhimurium* colonization of the cecum.

To investigate further the mechanism of *H. pylori*'s effect on distal GI tract, we studied whether *H. pylori* could colonize the mouse cecum, allowing it to directly

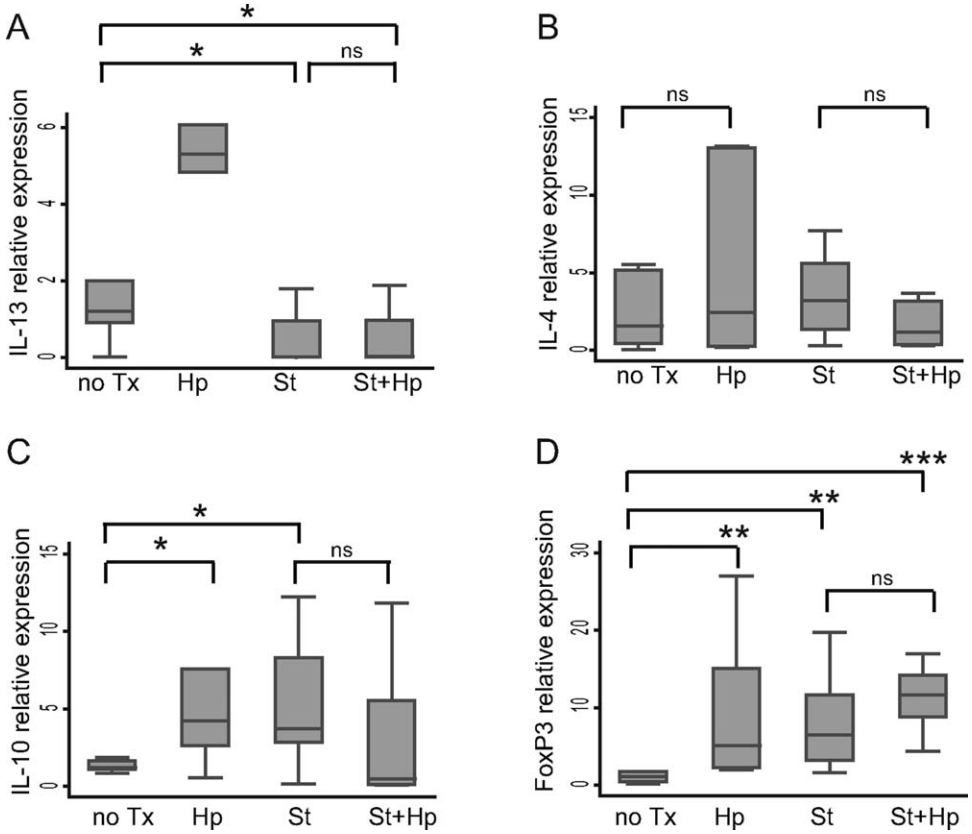



**FIGURE 4.** *H. pylori* infection does not alter *S. typhimurium*-induced Th2 or Treg cytokine responses in the mouse cecum. (A) qRT-PCR gene expression of Th2 cytokines, IL-13 (A), and IL-4 (B) in the cecum of uninfected (no Tx) mice compared to *H. pylori* (Hp), *S. typhimurium* (St), and *H. pylori / S. typhimurium* (St+Hp) infected mice. The qRT-PCR expression of Treg cytokines IL-10 (C) and Foxp3 (D) in the cecum. Gene expression was normalized to GAPDH expression. Results are from two independent animal experiments (ns = not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

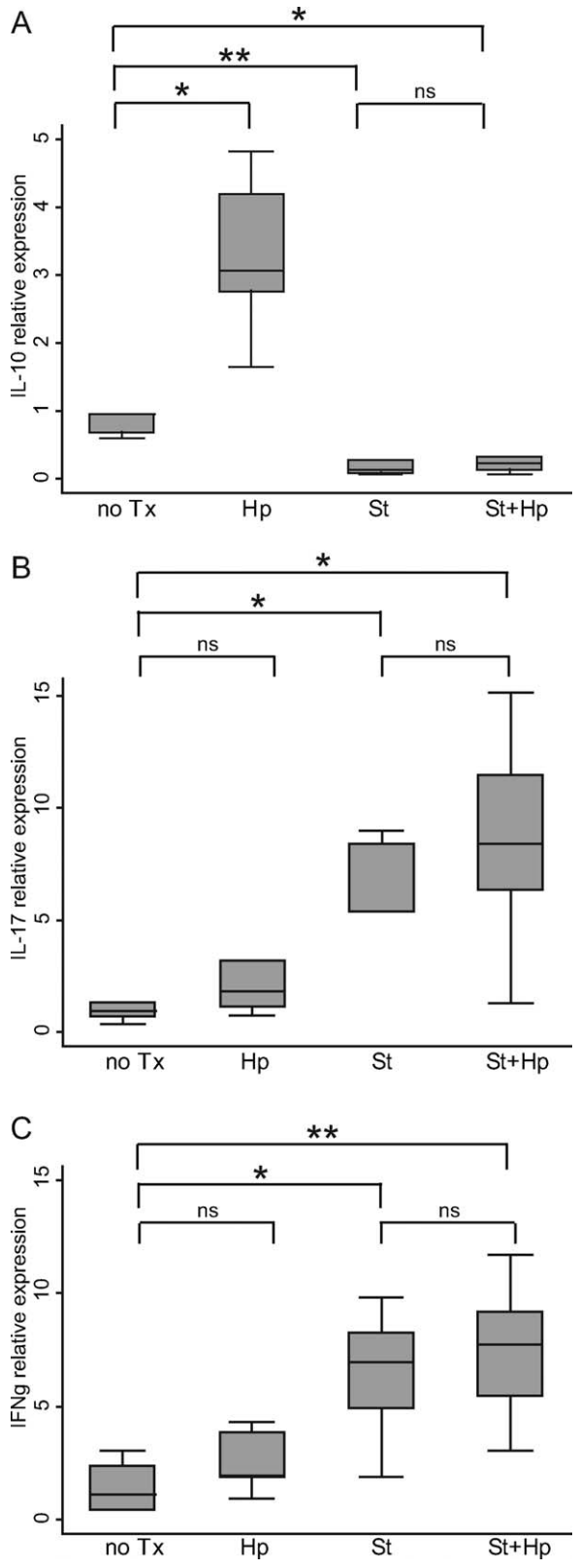
suppress local mucosal immune responses. First, levels of gastric *H. pylori* colonization were compared and no difference was found between *H. pylori* infection alone versus coinfection with *S. typhimurium* by qRT-PCR (Supporting Fig. 2A). Our data also showed that *H. pylori* did not colonize the cecum as cecal tissue samples had undetectable levels of *H. pylori* urease A measured by quantitative RT-PCR from either *H. pylori* or *H. pylori* / *S. typhimurium* coinfected mice (Supporting Fig. 2B).

## H. pylori Infection Does Not Alter S. typhimurium-induced Th2 or Treg Cytokine Responses in the Mouse Cecum

Another potential mechanism of immune suppression by *H. pylori* is by induction of Th2 or Treg responses in the cecum. *H. pylori* infection alone did not induce significant cecal expression of Th2 cytokines IL-4 and IL-13, but did induce higher levels of IL-10 and Foxp3 in the cecum (Fig. 4). During coinfection with *S. typhimurium*, however, the *H. pylori* coinfection was not associated with an increased cecal IL-10 expression but a trend towards increased Foxp3 was observed (Fig. 4C,D). *H. pylori* infection alone induced significant increases in the expression of IL-10 and Foxp3 in the cecum, and a mild increase in IL-13, but had no effect on IL-4 (Fig. 4A,B). During coinfection with *H. pylori* and *S. typhimurium*, there was no further increase in cecal IL-10 and Foxp3. Addition of *S. typhimurium* did reverse the induction of IL-13 by *H. pylori*, to either monoinfection with *H. pylori* or *S. typhimurium*. These findings suggest that gastric *H. pylori*'s immunosuppressive effect at a distance in the cecum may be related to the immunoregulatory modulation of the Th17 cecal mucosal immune response.

## H. pylori Infection Induces IL-10 Expression in the Mesenteric Lymph Nodes.

To examine whether the immunoregulatory effect of *H. pylori* infection in the cecum also occurs in the secondary draining lymph nodes, mesenteric lymph nodes (MLNs) were collected and cytokine profiles compared. As




shown in Figure 5A, *H. pylori* infection increased IL-10 mRNA expression in the MLNs compared to uninfected mice. During *S. typhimurium* infection, the levels of MLN IL-10 decreased and the levels of IL-17 and IFN-$\gamma$ were increased compared to uninfected mice (Fig. 5B,C). No significant differences in these cytokine levels were measured with *H. pylori* / *S. typhimurium* coinfection, suggesting a greater influence of *H. pylori* infection in the cecum than in the MLNs. This could be due to the presence of *H. pylori* antigen traveling through the intestinal lumen and augmenting the immunoregulatory effect of *H. pylori* at the cecal mucosa.

## H. pylori Infection Does Not Affect S. typhimurium-induced Cecal Fibrosis

Chronic *S. typhimurium* infection produces an intestinal fibrosis phenotype similar to human Crohn's disease.[9] Fibrosis is largely confined to the cecum and is characterized by excessive extracellular matrix deposition, submucosal hypertrophy, and myofibroblast activation. *S. typhimurium* infection induces a marked increase in extracellular collagen deposition as determined by trichrome staining of cecal tissue compared to uninfected or *H. pylori*-infected mice (Fig. 6A). *H. pylori* / *S. typhimurium* coinfection induces a similar response. Cecal submucosal expansion, a measure of both edema and fibrosis, was induced 4.7-fold by *S. typhimurium* infection compared to uninfected mice (Fig. 6B). *H. pylori* / *S. typhimurium* coinfection induced a similar 4.5-fold increase in submucosal thickness, indicating *H. pylori* did not alter the fibrogenic response induced by *S. typhimurium* ($P = 0.01$ versus uninfected), while *H. pylori* infection alone had no effect. Collagen deposition is effected by activated resident myofibroblasts which express $\alpha$SMA that is mediated by TGF-$\beta$ during the wound-healing response. *S. typhimurium* induces higher $\alpha$SMA protein and TGF-$\beta$ mRNA expression compared to uninfected mice as determined by Western blotting (Fig. 6C,D). *H. pylori* / *S. typhimurium* coinfection induces similar levels of $\alpha$SMA protein TGF-$\beta$ mRNA expression, comparable to *S. typhimurium* alone. Therefore, gastric *H. pylori* coinfection does not reduce *S. typhimurium*-induced intestinal fibrosis.

**FIGURE 5.** *H. pylori* infection induces IL-10 expression in the MLNs. qRT-PCR expression of IL-10 (A), IL-17 (B), and IFN-$\gamma$ (C) in *H. pylori*-infected mice (Hp) compared to uninfected (no Tx), *S. typhimurium*-infected (St) and *H. pylori / S. typhimurium* (St+Hp) infected. Results are from five animals per experimental group. Cytokine expression was normalized to GAPDH expression (ns = not significant, $*P < 0.05$, $**P < 0.01$).

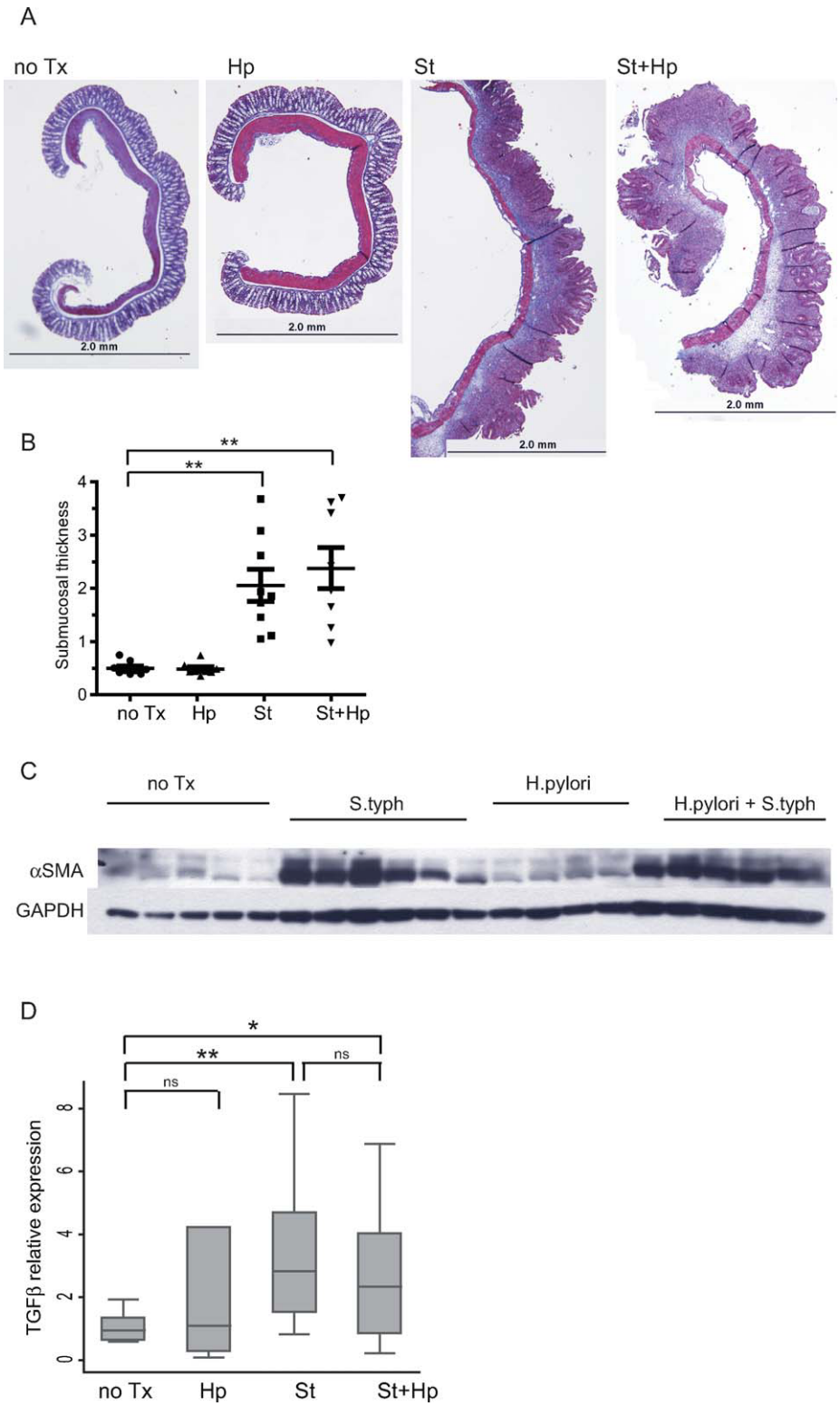
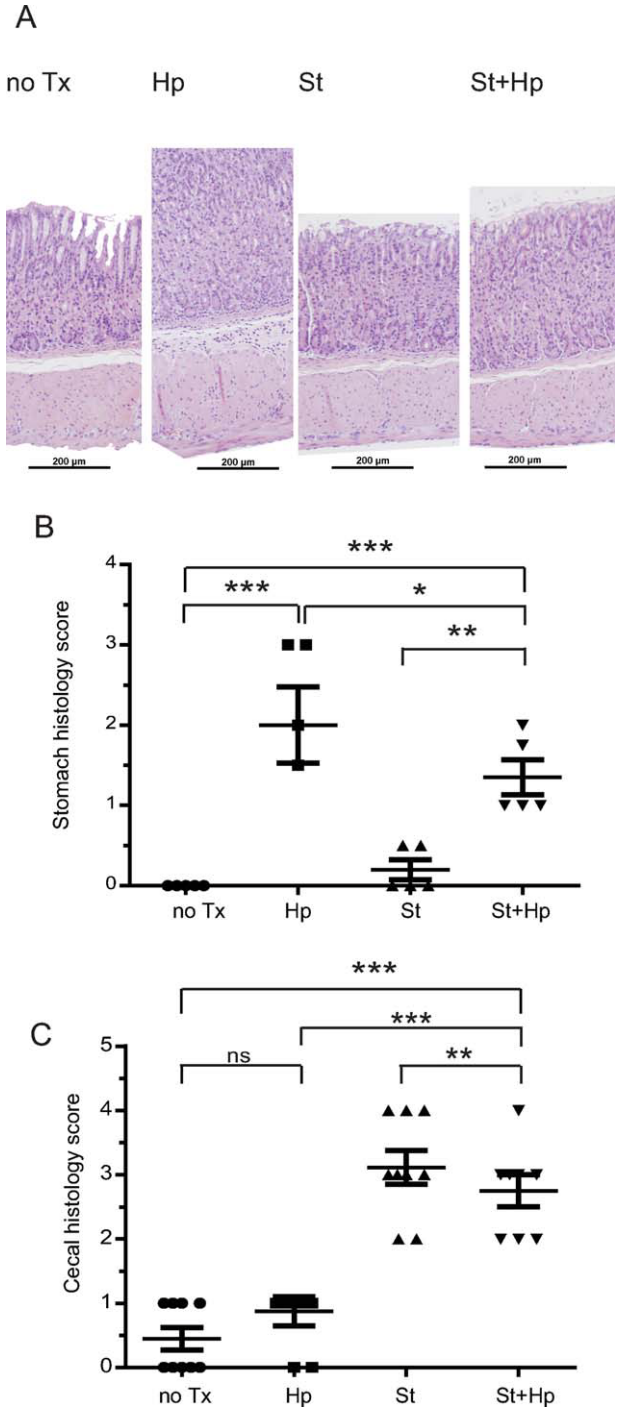



**FIGURE 6**. *H. pylori* infection does not affect *S. typhimurium*-induced cecal fibrosis. (A) Trichrome stained histological sections (5× magnification). Infection with *S. typhimurium* (St) or *H. pylori / S. typhimurium* (St+Hp) induces substantial increase in the extracellular matrix characterized by collagen deposition (blue staining) compared to uninfected (no Tx) or *H. pylori*-infected (HP). Scale bar = 2 mm. (B) Cecal submucosal thickness measurements. The submucosal thickness was determined at three reference points from photomicrographs above. Each point represents an individual animal. Horizontal bars indicate the average submucosal thickness for each experimental group. (C) Representative Western blot illustrating αSMA protein expression in cecal extracts. GAPDH protein expression was used as a loading control. (D) qRT-PCR gene expression of TGF-$\beta$ in the cecum of uninfected (no Tx) compared to *H. pylori* (Hp), *S. typhimurium* (St), and *H. pylori / S. typhimurium* (St+Hp) infected. Gene expression was normalized to GAPDH expression. Results are from two independent animal experiments (ns = not significant, $*P < 0.05$, $**P < 0.01$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

### H. pylori and S. typhimurium Coinfection Decreases Inflammation in Both the Stomach and Cecum

*H. pylori* infection is characterized by gastric hyperplasia and inflammatory infiltration in the gastric mucosa. H&E-stained sections of stomach from *H. pylori*-infected mice exhibit hyperplasia and leukocyte infiltration compared to uninfected mice (Fig. 7A). *S. typhimurium* infection does not induce gastritis. To assess the extent of *H. pylori* induced gastritis, H&E-stained sections of mouse stomach were scored by two blinded observers using the Modified Sydney Approach for inflammation.[10] Mice infected with *H. pylori* developed significant gastritis compared to uninfected mice (2.0 versus 0 points, $P < 0.01$). *H. pylori* / *S. typhimurium* coinfected mice have a lower gastritis score than *H. pylori*-infected mice (Fig. 7B).

While *H. pylori* infection induces gastritis, *H. pylori* effects on the lower GI tract are unknown. H&E-stained cecal tissue sections were scored by a blinded observer on a 0 to 4 scale[11] for mononuclear infiltration, vascular density, wall thickening, transmural leukocyte infiltration, and loss of goblet cells (Fig. 7C). Surprisingly, *H. pylori* infection alone induces mild cecal inflammation compared to uninfected mice (2.4 versus 3.6, $P = 0.0009$). While mice infected with *S. typhimurium* (3.6 versus 0.4, $P < 0.0001$) or *H. pylori /S. typhimurium* (2.4 versus 0.4, $P = 0.004$) developed marked cecal inflammation compared to uninfected controls, *H. pylori* / *S. typhimurium* coinfected mice exhibited less cecal inflammation than *S. typhimurium*-infected alone (2.4 versus 3.6, $P = 0.009$). This indicates immunological crosstalk between the upper and lower GI tract.




## DISCUSSION

The incidence of *H. pylori* infection is highest among individuals from developing countries and lower in developed countries. The incidence of IBD has an opposite trend, raising the possibility of immunological crosstalk between the stomach and the distal GI tract. In fact, an update of our previously published meta-analysis[8] indicates

**FIGURE 7.** *H. pylori* and *S. typhimurium* coinfection decreases inflammation in both the stomach and cecum. (A) H&E histological sections (100× magnification). Infection with *H. pylori* (Hp) induces gastric hyperplasia and inflammatory infiltration compared to uninfected (no Tx), *S. typhimurium* (St), and *H. pylori / S. typhimurium* (St+Hp) infected mice. Scale bar = 200 µm. (B) *H. pylori*-induced inflammation in the stomach is repressed by *S. typhimurium* infection. Gastritis was scored from H&E-stained sections of stomach using the Modified Sydney Approach on a scale of 0 (normal) to 3 (marked inflammation). (C) *H. pylori* infection represses *S. typhimurium*-induced inflammation in the cecum. Cecal inflammation was determined from H&E-stained sections of cecum were scored (0) no inflammation, (1) low level of inflammation with scattered infiltrating mononuclear cells, (2) moderate inflammation with multiple foci (3) high level of inflammation with increased vascular density and marked wall thickening, (4) maximal severity of inflammation with transmural leukocyte infiltration and loss of goblet cells. Results are from two independent animal experiments (ns = not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the overall relative risk (0.63) of developing IBD in patients infected with *H. pylori* remains significantly less than uninfected individuals. To investigate the mechanism for the inverse association observed between *H. pylori* status and the risk of developing IBD, we examined the effect of *H. pylori* infection on the immunological responses in the lower GI tract and in a mouse model of *S. typhimurium* typhlitis. We showed that mice chronically infected with *H. pylori* had a significantly elevated IL-10 mRNA expression in the cecum and in the MLNs, indicating *H. pylori* induces immunoregulatory responses in the lower GI tract. Additional supporting evidence is the finding that a lower cecal Th17 response and a repressive trend in the innate immune response was measured in mice coinfected with *H. pylori* and *S. typhimurium* compared to infection with *S. typhimurium* alone. This was not due to a difference in *Salmonella* colonization of the cecum nor to differences in IL-13, IL-4, or IL-10 cytokine levels. No significant difference in fibrosis was detected between the two groups, suggesting selective immunomodulation. Of note, we did observe decreased gastric inflammation in mice coinfected with *H. pylori* and *Salmonella,* indicating a potential crosstalk between distant GI sites colonized by different bacteria.

The ability of *H. pylori* to colonize the stomach depends on its expression of BabA, which enables it to anchor on gastric epithelium.[13] There is no evidence that *H. pylori* colonizes the intestine, except in regions with gastric heterotypia,[14] which further supports the gastric specificity of *H. pylori* colonization. We further verified by PCR the absence of *H. pylori* in the cecal specimens of *H. pylori* colonized mice. Thus, we speculate that *H. pylori* potentially influences the distal GI tract through systemic immunomodulation. Epidemiological data also supports this concept as a negative association between *H. pylori* status and asthma has been reported.[15]

A potential mechanism for the observed *H. pylori* immune modulation in the distal gut is the regulation of mucosal immune response owing to the tolerogenic properties of *H. pylori*.[1] *H. pylori*-infected patients were found to have higher gastric expression of Foxp3, a regulatory T cell marker, than uninfected individuals.[2] In fact, *H. pylori*-infected children with a lower degree of gastritis expressed higher levels of Foxp3 than infected adults with more severe gastritis,[16] indicating that the *H. pylori*-induced regulatory T-cell response might play a critical role in downmodulating mucosal inflammation. This response is not limited to the gastric mucosa, as it has been shown to play a critical role in peripheral T-cell anergy in *H. pylori*-infected patients.[17] Our study further supports the systemic nature of *H. pylori* induced Treg responses by showing an increased IL-10 expression in the MLNs of *H. pylori*-infected mice. This provides the critical missing mechanism to explain the observed negative association between *H. pylori* status and the development of IBD. We showed further that *H. pylori* infection reduces *S. typhimurium*-induced cecal inflammatory cytokines and histologic inflammation, but a histological difference in fibrosis was difficult to demonstrate using this chronic model, especially in a model that induced fibrosis.

As *H. pylori* was shown to influence distant intestinal immune responses, *S. typhimurium* numerically reduced the severity of gastritis in *H. pylori*-infected hosts. This is consistent with the report by Lemke *et al*[18] that a lower bowel bacterium can influence gastritis. Whether this represents a systemic endotoxic tolerance induced by *S. typhimurium* remains to be seen. Another potential mechanism is the alteration of the gut microbiota by *S. typhimurium,* which could alter the immune responses at a different site. This concept has been previously demonstrated, in that antibiotic-induced dysbiosis can alter pulmonary responses to mucosal pathogens.[19] This crosstalk may be historically and evolutionarily important, as monoinfection is uncommon in areas with poor public hygiene, and colonization with multiple organisms is the rule, rather than the exception. In fact, colonization with *H. pylori* might have conferred a selective advantage in the millennium when a pandemic of typhoid fever shifted the Greek balance of power from Athens to Sparta,[20] and the conquests of Alexander the Great may have come to an end due to typhoid.[21] The potential power of the evolutionary selective pressure of *Salmonella typhi* is demonstrated by the prevalence of heterozygosity for CFTR mutations, as the heterozygous state for cystic fibrosis confers a selective advantage through protection from *S. typhi*.[22] It is speculative, but attractive, to consider the possibility that lethal gut infections like *Salmonella* may have selected for the survival of humans who were colonized with and immunologically tolerant to *H. pylori*.

In summary, our study demonstrated a potential mechanism to explain the observed negative association between *H. pylori* and IBD. *H. pylori*-infected hosts demonstrated an increased level of IL-10 in mesenteric lymph nodes and a repressed lower GI Th17 response to *S. typhimurium*. These findings provide mechanistic support for the protective role of *H. pylori*, but prospective studies of the effect of *H. pylori* eradication on the risk of developing IBD will be required to prove the beneficial role of *H. pylori* for IBD prevention.

## REFERENCES

1. Kao JY, Zhang M, Miller MJ, et al. Helicobacter pylori immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. *Gastroenterology*. 2010;138:1046–1054.
2. Rad R, Brenner L, Bauer S, et al. CD25+/Foxp3+ T cells regulate gastric inflammation and Helicobacter pylori colonization in vivo. *Gastroenterology*. 2006;131:525–537.
3. Sawalha AH, Schmid WR, Binder SR, et al. Association between systemic lupus erythematosus and Helicobacter pylori seronegativity.

*J Rheumatol*. 2004;31:1546–1550.
4. Leach MW, Davidson NJ, Fort MM, et al. The role of IL-10 in inflammatory bowel disease: "of mice and men." *Toxicol Pathol*. 1999;27:123–133.
5. De Winter H, Cheroutre H, Kronenberg M. Mucosal immunity and inflammation. II. The yin and yang of T cells in intestinal inflammation: pathogenic and protective roles in a mouse colitis model. *Am J Physiol*. 1999;276:G1317–1321.
6. Izcue A, Coombes JL, Powrie F. Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol Rev*. 2006;212:256–271.
7. Gad M. Regulatory T cells in experimental colitis. *Curr Top Microbiol Immunol*. 2005;293:179–208.
8. Luther J, Dave M, Higgins PD, et al. Association between Helicobacter pylori infection and inflammatory bowel disease: a meta-analysis and systematic review of the literature. *Inflamm Bowel Dis*. 2010; 16:1077–1084.
9. Grassl GA, Valdez Y, Bergstrom KS, et al. Chronic enteric salmonella infection in mice leads to severe and persistent intestinal fibrosis. *Gastroenterology*. 2008;134:768–780.
10. Dixon MF, Genta RM, Yardley JH, et al. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol*. 1996; 20:1161–1181.
11. Wirtz S, Neufert C, Weigmann B, et al. Chemically induced mouse models of intestinal inflammation. *Nat Protoc*. 2007;2:541–546.
12. Mantel N, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. *J Natl Cancer Inst*. 1959;22: 719–748.
13. Aspholm-Hurtig M, Dailide G, Lahmann M, et al. Functional adaptation of BabA, the H. *pylori ABO blood group antigen binding adhesin*. *Science*. 2004;305:519–522.
14. Harris AW, Baron JH. Helicobacter pylori and gastric metaplasia of the duodenum. *Gut*. 1995;37:446.
15. Reibman J, Marmor M, Filner J, et al. Asthma is inversely associated with Helicobacter pylori status in an urban population. *PLoS One*. 2008;3:e4060.
16. Harris PR, Wright SW, Serrano C, et al. Helicobacter pylori gastritis in children is associated with a regulatory T-cell response. *Gastroenterology*. 2008;134:491–499.
17. Lundgren A, Suri-Payer E, Enarsson K, et al. Helicobacter pylori-specific CD4+ CD25high regulatory T cells suppress memory T-cell responses to H. *pylori in infected individuals*. *Infect Immunity*. 2003;71:1755–1762.
18. Lemke LB, Ge Z, Whary MT, et al. Concurrent Helicobacter bilis infection in C57BL/6 mice attenuates proinflammatory H. *pylori-induced gastric pathology*. *Infect Immunity*. 2009;77:2147–2158.
19. Noverr MC, Falkowski NR, McDonald RA, et al. Development of allergic airway disease in mice following antibiotic therapy and fungal microbiota increase: role of host genetics, antigen, and interleukin-13. *Infection and immunity*. 2005;73:30–38.
20. Papagrigorakis MJ, Yapijakis C, Synodinos PN, et al. DNA examination of ancient dental pulp incriminates typhoid fever as a probable cause of the Plague of Athens. *Int J Infect Dis*. 2006;10:206–214.
21. Oldach DW, Richard RE, Borza EN, et al. A mysterious death. *N Engl J Med*. 1998;338:1764–1769.
22. Pier GB, Grout M, Zaidi T, et al. Salmonella typhi uses CFTR to enter intestinal epithelial cells. *Nature*. 1998;393:79–82.