Attenuation of H-Pylori-Induced Gastric Pathology in C57bl/6 Mice by Co-Infection with Enterohepatic Helicobacters Is Helicobacter Species-Dependent

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Attenuation of Helicobacter pylori-induced gastric pathology in C57BL/6 mice by co-infection with enterohepatic helicobacters is species-dependent

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Running title: EHS-dependent attenuation of Hp-induced gastric diseases

Key words: H. pylori, H. muridarum, H. hepaticus, gastritis, heterologous immunity, mice

Non-standard abbreviations: Helicobacter pylori (Hp), Helicobacter bilis (Hb), H. bilis / H. pylori (HbHp), H. muridarum (Hm), H. hepaticus (Hh), H. muridarum / H. pylori (HmHp), H. hepaticus/H. pylori (HhHp)

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Abstract

We previously demonstrated that concurrent infection with an enterohepatic helicobacter species (EHS) Helicobacter bilis (Hb) can attenuate Helicobacter pylori (Hp)-induced gastritis in C57BL/6 mice. To investigate whether this Hb-mediated attenuation of gastric pathology is also applied to other members of EHS, C57BL/6 mice were mono-infected with H. hepaticus (Hh) or H. muridarum (Hm), and co-infected with HhHp or HmHp. Compared to Hp-infected mice, HmHp-infected mice at 6 and 11 months postinoculation (MPI) developed markedly less histopathologic activity index (HAI) (P< 0.0001), whereas HhHp-infected mice developed more severe HAI (P=0.01) at 6 MPI and had similar HAI (P=0.8) at 11 MPI. Hm-mediated HAI attenuation was associated with significant down-regulation of proinflammatory Th1 (Il-1β, Ifn-γ, and Tnf-α) and Th17 (Il-17A) cytokine mRNA levels in murine stomachs compared to the Hp-infected mice. Co-infection with Hh also suppressed Hp-induced elevation of these gastric Th1 cytokines but increased Th17 cytokine mRNA levels. Colonization levels of gastric Hp increased in HhHp- to HmHp-infected mice compared to mono-Hp-infected mice. Furthermore, the mRNA levels of Il-17A were positively correlated with the severity of helicobacter-induced gastric pathology (HhHp>Hp>HmHp). Our data collectively suggest that enhancement of gastric IL-17 response to Hp infection can compensate down-regulation of Th1 response for inducing severe gastric pathology and that EHS-mediated attenuation of the Hp-induced gastric pathology depends on the ability of the individual EHS to suppress both Th1 and IL17 proinflammatory responses.
Introduction

*Helicobacter pylori* (Hp) establishes a persistent infection of stomachs in over 50% of the human population (Amieva and El-Omar, 2008). This persistent colonization can lead to chronic active gastritis, peptic ulcer disease, and is also linked to gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma in a subset of infected individuals. It has been classified by the World Health Organization as a Class I carcinogen (Anonymous, 1994). It has been documented in animal models that eradication of Hp by use of antibiotics, particularly in the earlier stage of infection, can protect mice and gerbil from the development of Hp-induced gastric carcinoma (Lee et al., 2008; Romero-Gallo et al., 2008). However, only less than 5% of the Hp-infected population develops gastric tumors; the underlying mechanisms governing clinical outcome of Hp infection are poorly understood (Cheung and Wong, 2008; Fox and Wang, 2007). It is generally accepted that host immunity, environmental factors, and pathogenicity of *H. pylori* strains play an important role in disease development. In addition, it has been reported that the presence of endemic parasites could be linked to relatively lower than expected rates of gastric cancer in Hp-positive patients in some African countries as well as Colombia and South America with especially high prevalence rates of Hp infection (Whary et al., 2005; Maizels and Yazdanbakhsh, 2003; Bravo et al., 2002; Fox et al., 2000).

The gastrointestinal tract (GIT) of mammals is colonized by $10^{12-14}$ microbes and various parasites which can be mutualistic or pathogenic to human health (Hooper and Gordon, 2001). The interplay among certain organisms can lead to attenuation or aggravation of infectious pathology. Our previous study showed that co-infection with *Heligmosomoides polygyrus*, a natural murine nematode parasite, attenuated gastric atrophy induced by gastric *Helicobacter felis* in C57BL/6 mice, a relative of Hp; the pathological attenuation was associated with reduced
elevation of proinflammatory Th1 cytokine mRNA levels and as well as with increased Th2 cytokine mRNA levels (Fox et al., 2000). By contrast, dual infection with H. felis and an obligate intracellular protozoan parasite Toxoplasma gondii led to severe colitis associated with increased production of proinflammatory Th1 cytokines IFNγ and IL-12 in BALB/c mice that have minimal gastric inflammation when infected with H. felis infection alone (Stoicov et al., 2004). Mice were protected from Helicobacter hepaticus-induced inflammatory bowel disease by administration of polysaccharide A from the human symbiont Bacteroides fragilis; this protection resulted from increased production of IL-10 that suppressed expression of proinflammatory cytokines TNFα (Th1) and IL17A (Th17) (Mazmanian et al., 2008). Concurrent infection with H. hepaticus delayed recovery and prolonged weight loss of acutely diarrheal disease caused by a self-limiting pathogen Citrobacter rodentium, which was associated with up-regulation of IL17 mRNA levels (McBee et al., 2008). A recent report showed that prior H. pylori infection attenuated Salmonella typhimurium-induced colitis in C57BL/6 mice, which associated with down-regulation of cecal Th17 response to S. typhimurium (Higgins et al.). These lines of evidence indicate that co-infection with different organisms in the mammalian gastrointestinal tract can modulate host proinflammatory response to a given pathogenic microbe in the same niches and through remote anatomical sites, thereby leading to different pathological outcomes: pathological aggravation via enhancement of these proinflammatory responses or attenuation/protection from diseases via their suppression of these responses.

Recently, we showed that co-infection with enterohepatic Helicobacter bilis significantly decreased severity of Hp-induced gastritis and premalignant lesions in C57BL/6 mice (Lemke et al., 2009). Attenuation of Hp-induced gastric pathology was correlated with reduced elevation of
proinflammatory mediators induced by Hp in the dually infected mice. In the present study, we investigated whether the protective effect by co-infection with *H. bilis* is applicable to other enterohepatic helicobacters as well as further dissected the mechanisms operable in the development or suppression of Hp-induced gastric diseases.

**Methods**

**Bacterial strains**

*Helicobacter pylori* strain SS1, *Helicobacter hepaticus* 3B1 (ATCC 51449) and *Helicobacter muridarum* strain ST1 were cultured for 2-3 days on Blood agar plates (Remel, Lenexa, KS) at 37°C under microaerobic conditions (10% H₂, 10% CO₂, 80% N₂). Bacteria were harvested from the plates with freezing medium (Brucella broth containing 30% glycerol), centrifuged at 6000 rpm for 10 min. The pellets were resuspended in the freezing medium at 10⁹ organisms/ml estimated by OD₆₀₀nm in the DU 640 Spectrophotometer (Beckman).

**Experimental Infections**

Five-week-old, female C57BL/6 mice obtained from Taconic Farms (Germantown, NY) were housed in groups of five in polycarbonate microisolator cages on hardwood bedding (PharmaServ, Framingham, MA) under specific pathogen free (SPF) conditions (free of *Helicobacter* spp., *Citrobacter rodentium*, *Salmonella* spp., endoparasites, ectoparasites and known murine viral pathogens) in an Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility. Mouse rooms were maintained at constant temperature and humidity on a 12:12 hour light to dark cycle, and mice were provided standard rodent chow (Purina Mills, St. Louis MO) and water *ad libitum*. All protocols were approved by the MIT Committee on Animal Care.
Groups of 30 mice were either orally inoculated with mono-Hm, mono-Hh, Hm or Hh followed by oral dosing of Hp in 2 weeks. Mice were dosed with 0.2 mL (~2 X 10⁸ organisms) of the above bacterial suspensions of each helicobacter every other day for a total of three doses. At six and 11 months post inoculation (MPI), 15 mice from each group were euthanized with CO₂ and necropsied.

Necropsy and Histopathology

At necropsy, stomach samples from the lesser curvature extending from the squamous forestomach through the duodenum were collected and processed as described previously (Rogers et al., 2005; Fox et al., 2003). Tissues were graded by a comparative pathologist (S.M) blinded to sample identity for inflammation, epithelial defects, atrophy, hyperplasia, pseudopyloric metaplasia, dysplasia, hyalinosis and mucous metaplasia as defined elsewhere (Rogers et al., 2005; Fox et al., 2003). Gastric lesions were scored on an ascending scale from 0 to 4 using criteria previously described (Fox et al., 2007; Rogers et al., 2005).

Quantitative PCR for \textit{H. pylori} SS1, \textit{H. hepaticus} and \textit{H. muridarum}

To quantify colonization levels of Hp SS1, Hh and Hm within the gastric mucosa as well as Hh and Hm in cecal tissue, a real-time quantitative PCR assay (Q-PCR) was utilized (Maurer et al., 2006; Fox et al., 2003). A standard curve was generated using serial 10-fold dilutions of the respective helicobacter genomic DNA (from $1 \times 10^6$ to 10 genome copies). The copy number for Hp SS1 and Hh was calculated based on an average Hp genome size of 1.66 Mb of two sequenced isolates and the Hh 3B1 genome size of 1.8 Mb, respectively (Suerbaum et al., 2003;
Alm et al., 1999; Tomb et al., 1997); the genome size of Hm was represented by 1.73 Mb averaged from the genome sizes of two Hp isolate and Hh 3B1 (Alm et al., 1999; Tomb et al., 1997). Primers and probes for quantifying Hp and Hh were previously described (Maurer et al., 2006; Ge et al., 2001). A forward primer (5’-AAGAGTGCACCCCGGGCTAAT-3’) and a reverse primer (5’-CGTTAGCTGCATTACTGCCCTG TC-3’), which hybridize nucleotides 529 to 550 and 800 to 823 of Hm strain ST1 16S rRNA gene (M08205) respectively, were evaluated and selected for measuring quantities of Hm. All Q-PCR assays were performed in the 7500 Fast detection system (Applied Biosystems). Genome copy numbers of the Hp, Hh or Hm were express per micrograms of murine chromosomal DNA which were measured by Q-PCR using a mammalian 18S rRNA gene-based primer and probe mixture (Applied Biosystems, Foster City, CA) as described previously (Haggerty et al., 2005; Whary et al., 2001).

Gastric Cytokines

RNA from murine stomachs was prepared using Trizol Reagents following the supplier’s instructions (Invitrogen); the RNA samples were further purified for removing the contaminated DNA using the RNAeasy kit (Qiagen). cDNA from gastric mRNA (2 μg) was reverse-transcribed using the High Capacity cDNA Archive kit following the supplier’s instructions (Applied Biosystems, Foster City, CA). Q-PCR assays were performed in the 7500 Fast Real-Time PCR System (Applied Biosystems). First, mRNA expression of mouse genes involved in innate and adaptive immunity was measured with RT² Profiler PCR arrays (Super Array Bioscience Corporation). In this assay, 3 mice from each groups of this study at 11 MPI with pathological index scores close to a median score were used. In addition, 3 mice from the non-infected and Hp-infected groups at 11 MPI, which were described in our previous study (Lemke et al., 2009),
were also included in this assay as controls. Second, mRNA levels of proinflammatory Th1 cytokines interferon-gamma (Ifn-γ), tumor necrosis factor-alpha (Tnf-α), and interlukin-1beta (Il-1β) as well as Foxp3 were measured in the gastric tissues of all the mice for each group at 6 MPI (also at 11 MPI for Foxp3). Third, transcript levels of proinflammatory gastric Il-17A was measured and compared for all mice from this study at both 6 and 11 MPI. The mice in the sham control, mono-Hp-, HbHp- and mono-Hb-infected groups from our previous study were used as control, since the mice used in these two studies were conducted at the same time, and were age-, gender-, and time point-matched; mRNA levels of these genes were not determined in our previous publication (Lemke et al., 2009). The detected genes were normalized to the endogenous control glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA, and expressed as fold change in reference to sham-dosed control mice using the Comparative C_T method (Applied Biosystems User Bulletin no. 2).

Statistics
Gastric HAI scores were compared across groups by the Kruskal-Wallis one-way analysis of variance with Dunn’s post-test, and between groups by the Mann-Whitney U-test using Prism software (Graphpad, San Diego, CA). Data on the colonization levels of Helicobacter species, cytokine mRNA levels in the tissues were analyzed using the two-tailed Student’s t test. Values of p<0.05 were considered significant.

Results
Co-infection with H. muridarum but not H. hepaticus attenuated H. pylori –induced gastritis and gastric premalignant lesions
We previously demonstrated that concurrent infection of *H. pylori* with *H. bilis* attenuated Hp-induced gastric diseases (Lemke *et al.*, 2009). In this study, we tested whether this effect is attributable to other enterohelicobacters such as Hh and Hm. Because dual infection of Hp with Hh or Hm were performed concurrently with the HpHb infection, the sham dosed and Hp-infected mice in the previous report (Lemke *et al.*, 2009) also served negative and positive controls in this setting, respectively. C57BL/6 mice infected with Hp exhibited moderate gastritis at 6 MPI and severe gastritis with early dysplasia at 11 MPI, whereas there was no infection-associated histopathological changes developed in the sham controls (Lemke *et al.*, 2009). Lesions were characterized by a lymphocyte-predominant mucosal and submucosal infiltrates, multifocal surface erosions and glandular ectasia, oxyntic atrophy, hyperplasia, pseudopyloric metaplasia and dysplasia (Figure 1A). Hp-infected mice also exhibited mucous metaplasia of the oxyntic mucosa that contributed to parietal cell atrophy, although mucus metaplasia was not included in the gastric HAI because it is not a helicobacter-specific lesion (Fox *et al.*, 2007; Rogers *et al.*, 2005). As expected, mono-infection with enterohepatic Hm or Hh did not produce overt gastritis nor did persistent colonization of the lower bowel with Hm or Hh, led to lower bowel inflammation (data not shown). Mice colonized with HmHp developed a significantly lower gastric HAI at both 6 and 11 MPI than mice infected with Hp alone (Figure 1B, n=15, p<0.0001 for both time points) or HbHp mice at 11 MPI (P=0.023, data not shown). In contrast, co-infection with HhHp did not attenuate gastric pathology at 6 MPI, and rather the dual infection significantly increased severity of gastric HAI compared to mono-Hp infection at 6 MPI (Figure 1B, P=0.01); there was no significant difference in the scores of HAI between dual HhHp- and mono-Hp infected mice at 11 MPI (Figure 1B, P=0.81). Lesions in the co-infected group were of a similar character as those induced by Hp alone. Therefore, concurrent
colonization with Hm but not Hh in the lower bowel significantly abrogated the histologic severity of stomach lesions induced by Hp.

*H. pylori*-induced up-regulation of gastric proinflammatory Th1 cytokine mRNAs was attenuated by co-infected *H. muridarum* or *H. hepaticus*

We previously showed that Hb-mediated attenuation of Hp-induced gastric pathology in the dually infected mice was associated with down-regulation of gastric mRNA levels of proinflammatory Th1 cytokines Ifn-γ, Tnf-α, and IL-1β compared to the mono-Hp-infected mice (Lemke *et al.*, 2009). Thus mRNA expression of these cytokines was examined in all mice of this study at 6 MPI. The levels of gastric Ifn-γ, Tnf-α, and IL-1β mRNAs were significantly decreased in the HmHp mice compared to mono-Hp (P<0.0001) or HhH (P<0.0001) mice (Figure 2A). The HmHp mice also contained significantly lower gastric mRNA levels of these three proinflammatory cytokines than the HbHp-infected mice (P≤0.0002, data not shown), which was consistent with histological evidence (Hm >Hb, data not shown). When compared to the sham controls, the HmHp-infected mice contained similar mRNA levels for gastric Ifn-γ and IL-1β and lower mRNA levels of Tnf-α (P=0.015). In addition, mono-Hm infection significantly decreased mRNA expression of gastric Ifn-γ and Tnf-α (P<0.0001), whereas mono-Hh infection significantly reduced mRNA levels of all these three Th1 cytokines (Figure 2).

Despite that the HhHp-infected mice developed more severe gastric pathology than mono-Hp-infected mice, the mRNA levels of gastric Ifn-γ and Tnf-α were significantly lower in the HhHp mice than mono-Hp mice (P<0.0001, Figure 2); mRNA levels of IL-1β was also decreased with
trend ($P=0.076$). Interestingly, mono-Hh mice produced significantly less mRNA of these gastric Th1 cytokines compared to the sham controls ($P \leq 0.0002$).

To obtain an overview of expression of mouse genes involved in innate and adaptive immunity in response to helicobacter infection, we measured mRNA levels of 84 relevant genes using a Superarray (SABsciences, Frederick, MD) in the selected mice at 11 MPI by which gastric dysplasia was developed in the mono-Hp and HhHp mice. The summary of the results for this assay are presented as supplemental table 1. The genes, for which mRNA levels were elevated in correlation with gastric pathology, include all three chains ($\delta$, $\epsilon$, $\gamma$) of CD3, CD28, and proinflammatory cytokines $\text{Ifn-}\gamma$, $\text{Tnf-}\alpha$, and $\text{Il-1}\beta$. The CD3 and CD28 are T cell surface receptors involved in T cell activation and cell-mediated immunity. The mRNA levels of these genes were significantly up-regulated by Hp or HhHp infection ($P<0.05$) compared to the controls, whereas there was no significant changes in their levels in the gastric tissues of mice infected with HmHp, Hm or Hh ($P>0.2$). The enhanced expression of $\text{Ifn-}\gamma$, $\text{Tnf-}\alpha$, and $\text{Il-1}\beta$ mRNA in mono-Hp or HhHp mice with severe gastric inflammation was consistent with previous results in Hp-infected patients or experimentally infected mice and gerbils with gastric helicobacters (MORE REFERENCES) (Fox et al., 2000, Fox, 2007 #18, Fox, 2003 #16, Lemke, 2009 #76). However, when compared to the Hp-infected mice, the average of these three gene mRNA levels in the stomachs of the HhHp-infected mice tended to be relatively lower but the difference was not statistically significant.

mRNA levels of gastric $\text{Il-17A}$ were positively correlated with the increased severity of helicobacters-induced gastric disease
It has been reported that expression of *IL-17A* produced by proinflammatory Th17 cells was significantly increased in the Hp-colonized human gastric mucosa (Luzza *et al.*, 2000). Experimental infection of Hp in mice and gerbils up-regulated mRNA levels of gastric *IL-17A* (Sugimoto *et al.*, 2009; Shiomi *et al.*, 2008). Thus, we measured and compared mRNA levels of *IL-17A* among the infection groups in this study (HmHp, HhHp, Hm, Hh) as well as our previous study (sham control, Hp, HbHp, Hb) (Figure 3A). At both 6 and 11 MPI, all the groups infected with Hp regardless of co-infection status expressed significantly higher levels of gastric *IL-17A* mRNA than the sham controls (P<0.0001); there was no significant difference in gastric *IL-17A* mRNA levels among the sham controls, mono-Hm and mono-Hb groups (P>0.2). However, the mono-Hh mice expressed significantly higher mRNA levels of gastric *IL-17A* than the mice for the sham controls, mono-Hm infection or mono-Hb infection at both 6 and 11 MPI (Figure 3A).

For the dually infected groups, the mice infected with HhHp contained significantly higher mRNA levels of gastric *IL-17A* when compared to the mono-Hp (P<0.05), HmHp (P<0.001), or HbHp (P<0.05) mice. There was a higher level of gastric *IL-17A* mRNA in the mono-Hp mice than the HmHp mice (P<0.01). At 11 MPI, the HhHp mice produced significantly higher levels of gastric *IL-17A* mRNA than HbHp mice (P=0.028) and trended to be higher when compared to the mice infected with HmHp (P=0.059). Correlation analysis indicated that gastric *IL-17A* mRNA levels were significantly correlated with severity of gastric pathology (Fig. 3B).

Higher levels of gastric *Foxp3* mRNA were associated with more severe gastric pathology and *H. muridarum* induced stronger T_{REG} response than other EHS. *Foxp3* encodes a transcription factor essential for differential development of inflammation-suppressive natural regulatory T cells (Sayi *et al.*, 2009; Fontenot *et al.*, 2003; Hori *et al.*, 2003).
Larger numbers of CD4+CD25+ Foxp3+ TREG cells were present in the gastric tissues of Hp-positive patients and mice infected experimentally with Hp (Harris et al., 2008; Rad et al., 2006). We previously documented that the mono-Hp-infected mice contained more Foxp3+ cells and higher mRNA levels of Foxp3 in the gastric tissue compared to the HbHp mice (Lemke et al., 2009). In this study, HhHp mice contained higher mRNA levels of gastric Foxp3 than mono-Hp (P=0.0005) or HmHp mice (P=0.019) at 6 MPI and HmHp mice at 11 MPI (P<0.05) (Figure 4). The gastric Foxp3 mRNA levels in the mono-Hp mice were significantly higher than those in the HmHp mice at 11 MPI (P<0.05). There was no significant difference in gastric Foxp3 mRNA levels between the mono-Hp and the HmHp mice at 6 MPI (P=0.439) as well as between the mono-Hp and the HhHp mice at 11 MPI (P=0.628). All the mice infected with Hp contained higher mRNA levels of gastric Foxp3 than the sham controls (P<0.05) at both time points.

Despite the lack of overt gastric or intestinal pathology in the mono-EHS-infected mice, the mono-Hm mice contained significantly higher mRNA levels of gastric Foxp3 at both time points than the sham controls (P<0.05). By contrast, there was no significant difference in gastric Foxp3 mRNA levels between the mono-Hh mice and the sham controls at both time points (P>0.5). The levels of gastric Foxp3 mRNA in the mono-Hm mice were similar to at 6 MPI (P=0.2) and higher at 11 MPI (P=0.001) compared to the mono-Hh mice. When compared to the respective groups of the dually infected mice, mono-Hm mice contained lower levels at 6 MPI (P=0.0007) and similar levels (P=0.34) of gastric Foxp3 mRNA, whereas there were significantly lower levels at both time points (P<0.0001) in mono-Hh mice.
Colonization of gastric *H. pylori* was enhanced in mice concurrently infected with *H. hepaticus* or *H. muridarum*

The levels of Hp were higher by ~280-fold at 6 MPI (P < 0.05) and approximately by 40-fold at 11 MPI (P=0.14) in the HhHp mice when compared to the mono-Hp mice (Figure 5). No statistical significance at 11 MPI was attributed to exceptional higher numbers of gastric Hp in two mice of the mono-Hp group. The HmHp mice contained significantly higher levels of gastric Hp than the mono-Hp mice at both 6 and 11 MPI (P < 0.01) mice. Between the dually infected groups, co-infection with Hm significantly increased Hp levels at 6 MPI (P = 0.019) compared to co-infection with Hh; there are no significantly difference in Hp colonization levels at 11 MPI (P = 0.49) between these two groups (Figure 5).

*H. muridarum* and *H. hepaticus* established persistent infection in the lower bowel of C57BL/6 mice

All mice inoculated with Hm or Hh were colonized with the respective inoculums. The overall colonization levels of cecal Hh were approximately 2x 10^7 for all the groups except for Hh-infected group at 11 MPI (an average of 2 x 10^6). At MPI, there was no significant difference in colonization levels of Hh in the cecum between mono-Hh and HhHp mice (Figure 6A, p=0.32).

At 11 MPI, the mono-Hh mice contained significantly less cecal Hh levels than the HhHp mice (P<0.0001). There was significantly a lower level of cecal Hh at 6 MPI than at 11 MPI in the mono-Hh mice (P<0.0001). Colonization levels of Hh in the ceca of the dually infected mice were similar between 6 and 11 MPI (P=0.699).
Average levels of Hm colonization in the cecum were approximately $2-5 \times 10^6$ (Figure 6A). There was significant lower levels of cecal Hm in the mono-Hm mice at 11 MPI than 6 MPI (P<0.05). The remaining three groups had similar levels of cecal Hm (Figure 6A, P≥0.2).

Co-infection increased the presence of gastric *H. muridarum* and *H. hepaticus* over time

Gastric Hb was detected in approximately 50% of the Hb-infected mice (Lemke et al., 2009). We measured levels of gastric Hm and Hh in the respectively infected mice. Despite that the ceca serve as a primary site for colonization of Hm and Hh, both EHS established persistent infection in the stomach (Figure 6B). The percentage of the dually infected mice positive for gastric Hh or Hm increased at 11 MPI (73%, 11/15) compared to 6 MPI (42%, 6/14) as well as 73% at 11 MPI versus 57% at 6 MPI for Hm. By contrast, percentage of gastric Hm or Hh in the mono-infected groups decreased at 11 MPI compared to 6 MPI (61% versus 80% for Hh; 33% versus 60% for Hm). Average numbers of gastric Hh or Hm were comparable among the groups except for HmHp group at 11 MPI, in which 6 of 11 gastric Hm-positive mice contained relatively higher levels of gastric Hm.

Discussion

In this study, we demonstrated that *H. muridarum* significantly attenuated Hp-induced gastric pathology in the dually infected mice. In addition, this effect with Hm was more profound than the attenuation of Hp-associated gastric pathology noted in the mice co-infected with enterohepatic helicobacter Hb previously published by our group (Lemke *et al.*, 2009). In contrast, co-infection with Hh, the prototype of enterohepatic helicobacters, did not suppress (both 6 and 11 MPI) but rather aggravated the development of gastric diseases caused by Hp at 6
MPI. Despite Hm or Hh colonizing the murine stomachs in a subset of the dually or mono-infected mice, there were no correlations noted between the severity of gastric pathology (or levels of proinflammatory cytokine) and the numbers of EHS colonizing the stomach of these mice, suggesting that the effects on Hp-induced gastric disease by co-infection resulted from intestinal colonization of EHS. In addition, our data indicate that the attenuation of Hp-induced premalignant gastric lesions by co-infection of an EHS is species-dependent; the potency of this effect is attributed to the ability of the particular EHS in suppressing both Th1 and Th17 pathways. Thus, the lines of evidence from this study suggest that marked enhancement of the Th17 pathway by an EHS can compensate for suppression of the Th1 pathway, thereby aggravating Hp-induced gastric disease or providing gastric milieu where attenuation of Hp-induced gastric pathology is not noted. Thus, we developed a model system for delineating molecular mechanisms underlying the distinct interactions between genetically closely related bacterial species through the remote anatomic sites of the gastrointestinal tract.

In contrast to Hm or Hb, co-infection with Hh, did not attenuate but aggravated Hp-induced gastritis at 6 MPI. Intriguingly, HhHp mice expressed lower mRNA levels of gastric Th1 cytokines Tnf-α, Ifn-γ and Il-1β and higher mRNA levels of gastric Il-17A compared to mono-Hp mice; the mRNA levels of these gastric Th1 cytokines in HhHp mice were similar to those in HbHp mice with attenuated gastric pathology (Lemke et al., 2009). Previous studies have established a role of a proinflammatory Th17 pathway in the development of Hp-induced gastric disease in mouse and gerbil models (DeLyria et al., 2009; Sugimoto et al., 2009; Shiomi et al., 2008). Thus, our results indicated that the Hh-associated aggravation of Hp-induced gastric pathology was not mediated by up-regulation of the Th1 response but instead resulted from a
robust Th17 response to HhHp infection. Prior Hh infection likely potentiate a Th17 response to subsequent Hp infection, which is supported by the finding that there was significantly higher mRNA levels of gastric Il-17A in mono-Hh mice compared to the sham control, mono-Hm or mono-Hb mice. Also suppression of Th1 cytokines in HhHp mice compared to mono-Hp mice, particularly Ifn-γ, could also contribute in part to the up-regulation of gastric Th17 responses, because Ifn-γ has an inhibitory effect on Th17 pathway (Harrington et al., 2005; Jiang and Chess, 2004). However, a distinct factor(s) from Hh must have played a pivotal role in enhancing gastric Th17 responses, since the mRNA levels of gastric Ifn-γ in the HbHp- and HmHp-infected mice were similar (HbHp) or significantly lower (HmHp) than the HhHp mice. We postulate that intestinal Hh initiate “memory” Th17 cells that migrate to stomach where they produce a robust Th17 response to following Hp infection. This postulation is suggested by our previous study demonstrating that partial deletion of the Hh pathogenicity island did not affect colonization levels of the mutant but abolished the ability of Hh to causing colitis in Il-10−/− mice; the mutant-infected mice contained significantly lower mRNA levels of cecal Il-17A compared to the wild-type 3B1-infected mice (Ge et al., 2008). Whether proteins encoded within HhPAI involve enhancement of Th17 responses in the stomachs of the HhHp mice remains being characterized.

Our results showed that elevated Th17 responses were positively correlated with severity of Hp-induced gastric pathology and also increased colonization levels of gastric Hp when compared to mono-Hp mice. These findings are consistent with the recent data showing that overexpression of IL-17A in mouse stomachs elevated gastric Th17 response to Hp infection, which was associated with increased colonization of gastric Hp as well as severe Hp-induced gastric pathology in female BALB/c and C57BL/6 mice (Shi et al.). In addition, Hp colonization levels
and severity of Hp-induced gastric inflammation were not decreased in \textit{IL17}^{+} mice compared to infected wild-type mice (Shi \textit{et al.}). It has been documented that, \textit{Ifn-\gamma} was essential for clearing gastric pathogen \textit{Helicobacter felis} which is a relative of Hp and cause gastric cancer in mouse models (Sayi \textit{et al.}, 2009). Thus, our results indicate that lower levels of gastric \textit{Ifn-\gamma} mRNA in the HhHp mice compared to mono-Hp mice contributed to the relatively higher colonization levels of Hp and that higher levels of gastric \textit{Il-17A} mRNA may promote differentiation of navel CD4\(^{+}\) T cells towards Th17 cells, thereby leading to more severe gastric pathology at 6 MPI. Recently, two studies reported that Th17 cells played an important role in reducing colonization levels of Hp in mice. Kao \textit{et al.} (2010) showed that colonization levels of Hp were increased by suppressing Th17 response via Hp-specific dendritic cell-mediated \textsubscript{REG} skewing at 2 weeks post infection (WPI); however, his Hp reduction effect was diminished at 6 WPI, suggesting that the role of Th17 cells in clearing Hp was limited in the early phase of infection. Similarly, vaccination of female C57BL/6 mice with Hp SS1 cell lysate followed by challenge with the same Hp strain drove strong Th-17 response and gastric inflammation, which significantly reduced Hp levels compared to unimmunized mice by 13 days post infection (DeLyria \textit{et al.}, 2009). This discrepancy could partially result from difference in experimental designs between our study and these previous studies: (1) coinfection with live EHS cells in this study versus either adoptive transfer of a large number of Hp-stimulated DCs (10\(^{6}\)) (Kao \textit{et al.}, 2010) or immunization with cell lysate of Hp ((DeLyria \textit{et al.}, 2009); (2) long-term infection duration (6 to 11 MPI) in this study compared to 14 days (Kao \textit{et al.}) or 13 days (DeLyria \textit{et al.}, 2009).

Natural regulatory T cells (Foxp3\(^{+}\) \textsubscript{REG}) are actively involved in suppressing host inflammatory responses to infectious agents for preventing tissue injury as well as maintaining physiological
homeostasis of host immunity (Curotto de Lafaille and Lafaille, 2009). Foxp3 expression is considered the most specific marker for natural T_{REG} cells (Demengeot et al., 2006). An association between \textit{H pylori} infection and the induction of T_{REG} has been established. For example, larger numbers of Foxp3^{+} cells were located in the inflamed gastric tissues in Hp-positive patients and mice experimentally with Hp when compared to uninjected controls; depletion of T_{REG} by treatment with C61 antibody led to enhanced expression of gastric proinflammatory cytokines as well as the development of a severe gastritis (Harris et al., 2008; Rad et al., 2006). \textit{H pylori}–specific CD4^{+}CD25^{+} T_{REG} cells were shown to suppress memory T-cell responses to \textit{H pylori} in infected persons (Raghavan et al., 2004; Lundgren et al., 2003). In \textit{Rag}^{2/−} mice lacking T and B lymphocytes, we previously reported that Hp-induced gastritis was suppressed by adoptive transfer of T_{REG} harvested from IL10-competent C57BL/6 donor mice, demonstrating that T_{REG} play a crucial role in suppressing Hp-induced gastric disease (Lee et al., 2007). Consistent with these previous findings, our data showed that mRNA levels of gastric \textit{Foxp3} were significantly higher in the mono-Hp-infected or HhHp-infected mice compared to the sham controls or the HmHp-infected mice. Furthermore, the HbHp-infected mice with attenuated gastritis had fewer number of Foxp3-positive cells and lower levels of gastric \textit{Foxp3} mRNA compared to the mono-Hp-infected mice (Lemke et al., 2009). Thus, it is reasonable to surmise that the higher levels of gastric \textit{Foxp3} mRNA in the infected mice with a severe Hp-induced gastritis represent an attempt by the host to suppress proinflammatory responses to Hp infection. We propose that co-infection with Hb or Hm sensitizes T_{REG} cells with higher efficacy to suppress Hp-induced proinflammatory responses. This hypothesis is supported by the finding of Eaton et al. who reported that splenocytes from Hp-infected C57BL/6 mice were more efficient in attenuating Hp-induced gastritis and premalignant lesions in \textit{scid} mice lacking
functional T and B cells (Peterson et al., 2003). In addition, we demonstrated that the “sensitized” T\textsubscript{RAG} cells from Hh-infected C57BL/6 mice had more potency to inhibit intestinal inflammation in Apc\textsuperscript{min/+} mice (Rao et al., 2006), further strengthening our proposal. Our data suggest that stronger attenuation of Hp-induced gastric pathology by Hm is ascribed to its ability in potentiating T\textsubscript{REG} function, because mono-Hm mice contained higher levels of gastric Foxp3 mRNA than mono-Hb mice or sham controls. We hypothesize that T\textsubscript{REG} cells are sensitized by specific antigens shared between Hm or Hb and Hp, probably by “heterologous immunity”, a phenomenon initially described for protective immunity against subsequent infection with a different virus through “memory T cells” sensitized by shared antigens from a previous virus infection (Welsh and Fujinami, 2007). Further investigations are needed using adoptive transfer of T\textsubscript{REG} to identify which ESH antigens are required to sensitize T\textsubscript{REG} cells by co-infection with an EHS and suppress Hp-induced gastric diseases.

Taken together, we propose a conceptual model explaining our finding that attenuation of Hp-induced gastric diseases by co-infection with EHS is species-dependent (Fig. 7). In this model, Hp infection up-regulates proinflammatory Th1 and Th17 pathways which lead to gastric pathology. “Sensitizing” antigens from EHS with anti-Hp inflammatory antigenic properties such as Hb or Hm are presented presumably by antigen-presenting cells (such as dendritic cells and macrophages) to natural T\textsubscript{REG} cells. “Sensitized” T\textsubscript{REG} cells with high anti-inflammatory properties migrate to the stomach where they dampen host proinflammatory response to subsequent Hp infection by decreasing expression of proinflammatory cytokines including IFN\textgamma, TNF\alpha, and IL-1\beta as well as IL-17a. This down-regulation, despite higher colonization levels of gastric Hp, attenuates Hp-induced gastritis and premalignant lesions. By contrast, Hh with
minimal anti-Hp inflammatory properties, could sensitize both $T_{REG}$ to a less extend, and Th17 cells (strongly); these sensitized cells can migrate to the stomach. Upon Hp infection, “sensitized” $T_{REG}$ cells moderately down-regulate proinflammatory Th1 cytokines such as IFN$\gamma$ which play an important role in helicobacter clearance (Sayi et al., 2009), thereby permitting higher colonization levels of Hp in HhH-infected mice compared to mono-Hp infected mice. “Sensitized” Th17 cells as well as relatively lower levels of Ifn-$\gamma$ markedly increase signals of the Th17 pathway, which leads to a severe Hp-induced gastric pathology in the HhHp-infected mice compared to mono-Hp-infected mice at 6 MPI. Our data showed that alteration of these Hp-induced proinflammatory cytokines by co-infection with an EHS were more apparent at 6 MPI than 11 MPI, indicating that the interplay among Th1, $T_{REG}$, Th17 pathways have occurred at the early phase of infection, even prior to overt clinical manifestations. In conclusion, we here developed a model in which the interaction between bacteria colonizing the lower bowel can either attenuate or aggravate bacterially induced gastric diseases. Additional studies using this model with identification of specific bacterial antigens, mechanisms involving antigen presentation, “sensitized” T cell trafficking and interventions of key proinflammatory pathways will help delineate how the interaction among pathogenic and/ or commensal microbes in the gastrointestinal tract can affect human disease.

**Acknowledgements**

This work was supported by NIH grants R01CA67529 (JGF), R01AI51404 (JGF), T32RR07036 (JGF), P30 ES02109 (JGF).
Figure Legends

Figure 1. Gastric histology.

A. Representative histopathology of gastric tissues from mice infected with *H. pylori* (Hp), *H. muridarum* (Hm), *H. hepaticus* (Hh), or co-infected with HmHp, HhHp for 6 to 11 months (MPI). Lesions were characterized by lymphocyte-predominant mucosal and submucosal infiltrates, multifocal surface erosions and glandular ectasia, oxyntic atrophy, hyperplasia, pseudopyloric metaplasia and dysplasia.

B. Gastric histologic activity index. Tissues from infected with *H. pylori* (Hp), *H. muridarum* (Hm), *H. hepaticus* (Hh), or co-infected with HmHp, HhHp for 6 to 11 months (MPI) (n=15 for all groups) were graded for inflammation, epithelial defects, atrophy, hyperplasia, pseudopyloric metaplasia, dysplasia, hyalinosis and mucous metaplasia. A gastric histologic activity index (HAI) was generated by combining scores for all criteria except hyalinosis and mucous metaplasia which may develop irrespective of helicobacter infection.

Figure 2. Gastric Th1 cytokine mRNA expression levels. Gastric tissues (n=13 to 15 per group) from mice infected with *H. pylori* (Hp), *H. muridarum* (Hm), *H. hepaticus* (Hh), or co-infected with HmHp, HhHp for 6 to 11 months (MPI) were evaluated by Q-PCR for expression levels of mRNA for pro-inflammatory cytokines, all normalized to the expression of the housekeeping gene GAPDH. The Y axis represents the mean fold change (± standard deviation) of the mRNA levels in reference to uninfected controls. HmHp or HhHp mice expressed lower levels of pro-inflammatory mediators *I fq-γ, Il1-β, Tnf-α* at 6 MPI. (p values in figures). P values when compared to the sham controls: * <0.05, ** <0.01, *** <0.001.
Figure 3. Gastric Il-17A mRNA levels and their correlation with severity of Hp-induced gastric pathology. A, The Il-17A mRNA levels in gastric tissues from mice (n=13 to 15 per group) infected with H. pylori (Hp), H. muridarum (Hm), H. hepaticus (Hh), or co-infected with HmHp, HhHp for 6 to 11 months (MPI) were evaluated by Q-PCR. The gastric tissues from mice infected with H. bilis (Hb) alone or co-infected with HbHp, which were described previously (Lemke et al., 2009), were also included in this assay, since these mice were matched with the mice used in the present study for age, gender, and infection paradigm. The expression levels of gastric Il-17A mRNA were normalized to the gastric house-keeping gene Gapdh mRNA level in the respective samples. The Y axis represents the mean fold change (± standard deviation) of the mRNA levels in reference to uninfected controls. B, linear regression between gastric Il-17A mRNA levels (fold change in reference to the sham controls, X axis) and degrees of gastric historic activity index (HAI, Y axis) for all the groups in A. P values when compared to the sham controls: * <0.05, ** <0.01, *** <0.001.

Figure 4. Gastric Foxp3 mRNA levels in the mice (n=13 to 15 per group) infected with H. pylori (Hp), H. muridarum (Hm), H. hepaticus (Hh), or co-infected with HmHp, HhHp for 6 to 11 months (MPI) were evaluated by Q-PCR. The expression levels of gastric Foxp3 mRNA were normalized to the gastric house-keeping gene Gapdh mRNA level in the respective samples. The Y axis represents the mean fold change (± standard deviation) of the mRNA levels in reference to uninfected controls. P values when compared to the sham controls: * <0.05, ** <0.01, *** <0.001.
Figure 5. Quantitation of gastric *H. pylori*. Copy numbers of gastric *H. pylori* (Hp) SS1 genome were estimated by Q-PCR of gastric samples from mice (n=13 to 15 per group) infected with *H. pylori* (Hp) and dually infected with either *H. muridarum* and Hp (HmHp) or *H. hepaticus* and Hp (HhHp) for 6 to 11 months.

Figure 6. Colonization level of *H. muridarum* (Hm) and *H. hepaticus* (Hh) in cecum (A) and stomach (B) using Q-PCR. Cecal and gastric tissues were collected from mice (n=13 to 15 per group) infected with either Hm, Hh, Hm/ *H. pylori* (HmHp) or Hh/Hp (HhHp).

Figure 7. Proposed working model deliberating possible mechanisms underlying species-dependent effects on *H. pylori*-induced gastric pathology by concurrent enterohepatic helicobacter infection in C57BL/6 mice.


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