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The Degree of *Helicobacter pylori*-Triggered Inflammation Is Manipulated by Preinfection Host Microbiota

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Helicobacter pylori **infects over 3 billion people worldwide and is the primary risk factor for gastric cancer. Most individuals infected with *H. pylori* develop only asymptomatic gastritis; however, some develop ulcers or gastric adenocarcinoma. We demonstrate that one previously unappreciated parameter influencing *H. pylori* disease outcome is variation in the preinfection host microbiota. Utilizing a mouse model, we altered the microbiota by antibiotic treatment and found that these alterations resulted in significantly lowered *H. pylori*-triggered inflammation. Specifically, antibiotic pretreatment reduced $CD4^+$ T-helper cells and *Ifnγ* transcript levels in gastric tissue after *H. pylori* infection. The bacterial communities in mice with a reduced response to *H. pylori* displayed many differences from those in untreated mice, including significantly more cluster IV and XIVa *Clostridium* spp., bacteria known to influence inflammation via regulatory T cell populations. Our findings suggest that microbiota composition, perhaps *Clostridium* spp., contributes to the variable disease outcome of *H. pylori* infection by altering the recruitment of $CD4^+$ T cells to the gastric compartment. Our results suggest that gastric microbiota could be used as a diagnostic tool to determine which patients are at risk for developing severe disease.**

The bacterial gastric pathogen *Helicobacter pylori* colonizes more than half of the world's population (1, 2). Most infected people remain asymptomatic; however, ~10% develop either peptic ulcers, gastric adenocarcinoma, or mucosa-associated lymphoid tumors (1–3). It is not yet possible to predict who will develop disease and what form it will take (3). Additionally, *H. pylori* infections protect against diseases such as esophageal cancer and asthma (1, 4, 5). As a result, most *H. pylori* infections are not treated unless the infected individual displays symptoms. However, it would be desirable to cure *H. pylori* infections that will progress to gastric cancer as this disease has few treatment options and high mortality (6). Several variables that determine *H. pylori*-associated disease outcome have been identified, but they do not fully explain disease risk. These past studies focused on variation in *H. pylori* genetic composition (2, 3, 7), host genetics (1–3), and environmental factors (2), but there has been no examination of the role played by the host microbiota.

Microbiotas have been implicated in aspects of immune system regulation and development (8–10), and altered microbiota communities have been implicated in both ameliorating (8, 11) and enhancing (12–14) disease symptoms. Specifically, dysbiosis of microbiota has been shown to influence inflammatory bowel disease (IBD) (12), obesity (13), and immune responses to *Citrobacter rodentium* (8) and *Helicobacter hepaticus* (15). Gastric microbial communities from people infected with *H. pylori* are somewhat different from those of uninfected people (16, 17), suggesting an interaction between *H. pylori* and the gastric microbial community. Whether specific communities of the microbiota make an individual more susceptible to *H. pylori* infection or disease is unknown.

In this study, we investigate how the microbiota affects disease that develops from *H. pylori* infection using the well-established *H. pylori* mouse model. Our studies were motivated by initial observations that identical mouse strains from different vendors responded differently to *H. pylori* infection. We report that these mice have variations in their normal gastric microbiota, akin to what has been seen in mouse intestinal microbiota (8). More in-depth studies found that antibiotic-induced alterations in the normal mouse microbiota shaped the immune response to *H. pylori* in a manner that suggested that specific microbiota members can reduce *H. pylori*-initiated inflammation. Mice that displayed low *H. pylori*-initiated inflammation had high gastric abundances of *Clostridia* species. These data thus suggest that variations in specific microbiota members can have a dramatic effect on *H. pylori*-associated disease and suggest new avenues for curbing *H. pylori* inflammation-related diseases such as ulcers and gastric cancer.

MATERIALS AND METHODS

***H. pylori* strains and growth conditions.** *Helicobacter pylori* strain SS1 (18), a gift of Jani O'Rourke (University of New South Wales), was cultured on Columbia blood agar (Difco) with 5% defibrinated horse blood (Hemostat Labs, Davis, CA), 50 μg/ml cycloheximide, 10 μg/ml vancomycin, 5 μg/ml cefsulodin, 2.5 units/ml polymyxin B, and 0.2% β-cyclodextrin. Mouse stomach samples were plated on the same medium plus 5 μg/ml trimethoprim, 8 μg/ml amphotericin B, 10 μg/ml nalidixic acid, and 200 μg/ml bacitracin. For mouse infection, *H. pylori* was grown with shaking in brucella broth (Difco) with 10% fetal bovine serum (FBS; Gibco) and incubated at 37°C with 7 to 10% O_2, 10% CO_2, and 80 to 83% N_2 overnight. We inoculated mice orally intragastrically via a 20-gauge by 1.5-in. feeding needle with 500 μl containing ~1×10^7 CFU/ml *H. pylori* bacteria.

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Animal infections. The University of California, Santa Cruz (UCSC), Institutional Animal Care and Use Committee approved all animal protocols and experiments. Female C57BL/6N mice (*Helicobacter*-free; Taconic Farms [TF], Germantown, NY, or Charles River Laboratories [CRL], Hollister, CA) were housed at the animal facility of UCSC under standard conditions in sterile cages, with sterile bedding and water and with a microisolator cage top. Cages were changed under laminar-flow sterile conditions. For experiments, 0.6 g/liter of penicillin/streptomycin (Omega Scientific) was administered *ad libitum* in the water bottle for 8 days; the antibiotic was replenished every 2 days. Two days after completing antibiotic treatment, mice slated for reconstitution were orally intragastrically fed 200 μl of stomach homogenates from non-antibiotic-treated C57BL/6N mice. Ten days after receiving the stomach homogenates, mice were inoculated orally intragastrically with *H. pylori*. Mice were 7 weeks old at the time of *H. pylori* inoculation; age-matched uninfected mice were included in all experiments. Four weeks postinoculation the animals were sacrificed via CO_2 narcosis; the stomachs were dissected, opened along the lesser curvature, and divided into longitudinal strips. The tissue pieces were treated as follows: (i) homogenized using a Bullet Blender (Next Advance) with 1.0-mm zirconium silicate beads and plated to determine the number of *H. pylori* CFU/gram of stomach or used for DNA isolation for determining microbial profiles; (ii) frozen in liquid nitrogen and stored at −80°C for quantitative reverse transcription-PCR (RT-PCR); or (iii) stored in cold Hanks balanced salt solution (HBSS; Lonza) to be used in flow cytometry experiments.

Flow cytometric characterization of cells. To prepare single-cell suspensions, mouse stomachs were dissected, opened longitudinally along the lesser curvature, and placed in cold HBSS (Lonza). The stomach was cut with a razor blade into 2-cm slices and incubated for 45 min at 37°C in HBSS supplemented with 10% fetal bovine serum (FBS), 15 mM HEPES, 5 mM EDTA, and 0.014% dithiothreitol (DTT). Subsequently, the tissue was rinsed with cold HBSS and incubated for 1 h at 37°C in 10 ml of RPMI medium with 10% FBS plus 1 mg/ml Dispase (Roche) and 0.25 mg/ml collagenase (Roche). The tissue was then vortexed and filtered through a 40-μm-pore-size cell strainer (BD Biosciences). The strainer was rinsed with 10 ml of cold HBSS. After samples were filtered, the cells were collected by centrifugation and washed two times in cold phosphate-buffered saline (PBS). The cells were then incubated on ice with IgG (US Biological) for 10 min, followed by a 20-min incubation with the test antibody. Optimal concentrations for the antibodies were determined in prior experiments to be 2 μg/ml for Alexa Fluor 488-CD3 (eBioscience), phycoerythrin (PE)-Cy7-CD4 (eBioscience), allophycocyanin (APC)/Cy7-CD8 (Biolegend), and Alexa Fluor 647-CD45 (Biolegend). An isotype control was included. Subsequently, the cells were centrifuged and washed twice in PBS plus 2.0% FBS and then fixed in 2% paraformaldehyde for 10 min on ice. A total of 50,000 cells were counted on a BD LSR II instrument (BD Biosciences) and analyzed using FlowJo (BD Biosciences) software; results are presented as a percentage of the 50,000 cells counted. Two-way analysis of variance (ANOVA) with Tukey's range test was used for statistical analysis.

qPCR. Quantitative analysis of *Ifnγ*, *Il17*, *Il4*, *Foxp3*, and *Il10* in the mouse stomach was performed by real-time quantitative PCR (qPCR). RNA was isolated from mouse stomach samples using TRIzol (Gibco). Stomachs were homogenized with a Polytron (Kinematica, Switzerland) in 1 ml of TRIzol per 100 mg of stomach tissue. Following homogenization, a standard phenol-chloroform extraction was used. A total of 1 to 3 μg of RNA was converted to cDNA using an RT^2 First Strand Kit (Superarray Bioscience Corporation). The cDNA was measured in qPCRs with SYBR green master mix (Bioline). The data were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and are presented as fold increase over mock treatment using the $\Delta\Delta C_T$ (where C_T is threshold cycle) method (19). Samples were analyzed in triplicate along with controls lacking reverse transcriptase. qPCR for bacterial species in the mouse stomach was performed on DNA samples isolated from homogenized mouse stomach using a DNEasy Kit (Qiagen), and bacteria were quantified using a standard curve of plasmids containing the bacterial 16S rRNA gene. Two-way ANOVA in conjunction with Tukey's range test was used for statistical analysis.

Pathology. Gastric tissue preserved in 22-oxacalcitriol (OCT) was sectioned, stained with hematoxylin and eosin, and evaluated in a blind fashion by a pathologist (J. E. Carter). Each slide was evaluated by two grading methods as outlined by Eaton et al. (Fig. 1A and B) (20, 21). Each slide was assessed twice per method to ensure reproducibility. For the experiment shown in Fig. 1A, lymphocytic infiltration was scored as outlined by Eaton et al. (20), as follows: 0, no infiltrate; 1, mild multifocal infiltration; 2 mild widespread infiltration; 3, mild widespread and moderate multifocal infiltration; 4, moderate widespread infiltration; and 5, moderate widespread and severe multifocal infiltration. For the experiment shown in Fig. 1B, the method of Eaton et al. (21) was used to determine the percentage of fields that showed evidence of the following: (i) polymorphonuclear leukocytes (PMN), defined as a field with several clusters of three or more neutrophils; (ii) gastritis, defined as inflammatory cell infiltrate of any cell type that was sufficient to displace the gastric glands; and (iii) metaplasia, defined as loss of parietal cells with concomitant replacement by mucus-type cells.

Phylochip analysis. Bacterial DNA was isolated using a DNeasy Blood and Tissue kit (Qiagen), including pretreatment for Gram-positive bacteria. The isolated DNA was sent to Second Genome (San Francisco, CA) for bacterial 16S DNA amplification. Microbial profiles for each sample were generated by hybridizing the bacterial 16S rRNA gene amplicons to the Phylochip (Second Genome). The Phylochip has been validated to detect 90% of cloned subfamilies at a 2.5-fold higher diversity than cloning (22). The Phylochip array contains 1,016,064 probes. For analysis, the probe gene sequences are clustered into groups known as operational taxonomic units (OTUs). Each OTU summarizes an average of 0.5% sequence divergence between probes and includes an average of 37 probe pairs, each of which includes a perfectly matching probe and a control, mismatching probe. In total, the Phylochip array contains 59,959 OTUs, which represent 147 phyla, 1,123 classes, and 1,219 orders within the *Archaea* and *Bacteria* (22, 23). An Adonis test was utilized for finding significant differences among discrete categorical or continuous variables. A two-sided *t* test was used to determine the subfamilies with highly significant populations between treatment groups.

RESULTS

Mice from Taconic and Charles River Laboratories have different inflammatory responses to *H. pylori*. While studying the mouse inflammatory response to *H. pylori* infection, we noted significant differences in the degrees of inflammation that *H. pylori* strain SS1 triggered in C57BL/6N mice from Charles River Laboratories (CRL) or Taconic Farms, Inc. (TF) (Fig. 1A and B). Mice from both vendors reacted with increased inflammation compared to the uninfected controls. However, two methods of inflammation grading (20, 21) demonstrated that CRL mice had significantly higher inflammation grades than TF mice (Fig. 1A and B) even though *H. pylori* colonized mice from each group equally (Fig. 1C). This outcome suggested a difference between CRL and TF mice that could not be attributed to the *H. pylori* infecting strain, length of infection, or bacterial load or to mouse genetics, husbandry, gender, or age at infection. Studies have found differences in intestinal microbiota between C57BL/6 mice from different vendors (8). Thus, we examined differences in the gastric microbial communities in CRL versus TF C57BL/6N mice by comparing the amounts of *Lactobacillus* species ASF360 and ASF361, two resident bacteria of the mouse gastric microbiota (24). We noticed that there was significantly more ASF360 bacteria in the TF mice than in the CRL mice, while ASF361 bacteria were found in significantly higher numbers in the CRL mice than

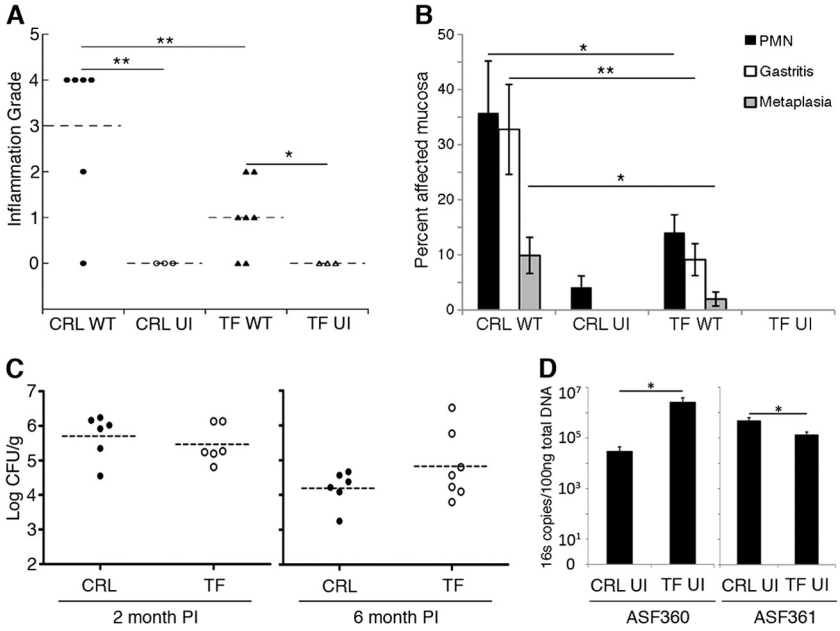


FIG 1 Female C57BL/6N mice from different vendors mount different inflammatory responses to *H. pylori* and possess various amounts of key *Lactobacillus* spp. (A) Inflammation grade scored on a scale of 0 (no lymphocytic infiltration) to 5 (moderate, widespread and severe multifocal lymphocytic infiltration) as described by Eaton et al. (20). (B) Inflammation grade of the same tissue as in panel A, scored by determining the percentage of fields that contain PMN, gastritis, or metaplasia, as outlined by Eaton et al. (21). Gastric tissue was from mice from either Charles River Laboratories (CRL) or Taconic Farms (TF) infected with wild-type *H. pylori* strain SS1 (WT) for 6 months or from uninfected (UI) controls. PMN, polymorphonuclear leukocytes (neutrophils). (C) *H. pylori* colonization levels in CFU counts per gram of stomach tissue at 2 and 6 months postinoculation. (D) Quantitative PCR on the 16S gene for *Lactobacillus* species ASF360 and ASF361 in 2-month-old uninfected mice from CRL or TF. For all panels, $n \geq 6$ mice per group infected with *H. pylori*, and $n = 3$ for uninfected groups. For panels B and D, data are presented as averages ± standard errors of the means. *, $P < 0.05$; **, $P < 0.01$, Student's *t* test.

in the TF mice (Fig. 1D). These data are consistent with the idea that mice from different vendors have different resident stomach microbial populations.

The murine stomach microbiota is refractory to *H. pylori*-triggered perturbations and similar to the human stomach microbiota. The differing levels of *Lactobacillus* ASF360 and ASF361 bacteria in CRL and TF mice suggest that different gastric microbial constituents may influence the degree of *H. pylori*-induced inflammation in the mouse model. The normal mouse stomach microbiota had not been characterized, so as a first step we identified the constituents of the normal mouse stomach microbiota. We focused our work on TF mice to eliminate complications from possible unknown variables and isolated total DNA from the stomachs of five specific-pathogen-free C57BL/6N mice (*Helicobacter*-free TF). We amplified the 16S rRNA genes and used this DNA in hybridization studies with the Phylochip microbial profiling system, a microarray-based method that identifies and measures the relative abundances of more than 59,000 individual microbial taxa (22). This analysis found that there were 10,207 species groups, termed operational taxonomic units (OTU), in any mouse stomach, with 2,056 of those OTUs found in all five mice (see Table S1 in the supplemental material). The majority of these isolates came from members of the *Firmicutes* phylum, with members from the class *Clostridia* being the most common (Fig. 2A). Members of the *Bacteroidetes* and *Verrucomicrobia* phyla were the second and third most highly represented phyla. The 2,056 OTUs represented 110 different families (see Fig. S1 in the supplemental material). The stomach phylotypes with the most members were the *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria*, a composition which is similar to what has been observed in humans except that in human samples *Fusobacteria* are also dominant (16, 17, 25).

We next used the Phylochip analysis described above to determine whether 4 weeks of infection with *H. pylori* strain SS1 changed the core gastric microbiota. We compared the microbiota differences using both abundance of bacterial species and their presence/absence. The microbiota communities from *H. pylori*-positive or -negative mice were not overall significantly different from each other (Fig. 2B; see also Fig. S2 in the supplemental material) but did display a few substantial differences in specific taxa (see Fig. S3 and Table S2). The OTUs affected by *H. pylori* colonization included decreased *Firmicutes* (class *Bacilli*), *Bacteroidetes*, and *Proteobacteria* and increased *Firmicutes* (class *Clostridia*), *Proteobacteria* (genus *Helicobacter*), and *Verrucomicrobia* (see Fig. S3).

Fewer CD4$^+$ T cells infiltrate into the stomach in response to *H. pylori* when mice have been pretreated with antibiotics. We hypothesized that different preinfection stomach microbial populations might affect the inflammatory response to *H. pylori* infection based on the inflammatory differences we observed between the TF and CRL C57BL/6N mice (Fig. 1). Thus, we performed an experiment in which we altered the starting microbial populations by antibiotic treatment of mice from a common source, TF. We included three treatment groups in this analysis: the first group was normal (not antibiotic treated), the second group received antibiotics for 8 days, and the third group received antibiotics for 8 days followed by gavage with stomach homogenates from normal mice to reconstitute the normal mouse stom-

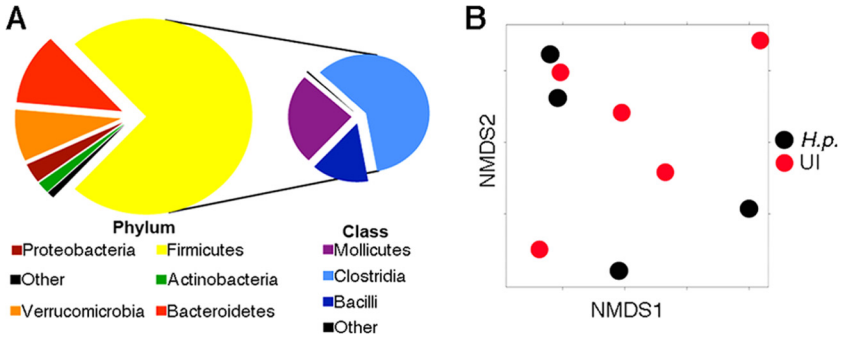


FIG 2 The murine stomach microbiota is refractory to *H. pylori*-triggered perturbations and similar to the human stomach microbiota. (A) Pie charts depicting phylum and class level distribution of 2,056 taxa that were present in all noninfected TF mice; 74% of the 2,056 taxa are *Firmicutes*, and, specifically, 44% of the total are members of the class *Clostridia*. A total of 12,032 taxa were present in at least one of five mouse stomachs. (B) Significant differences in microbiotas are not apparent between *H. pylori*-infected and noninfected samples, as indicated by nonmetric multidimensional scaling (NMDS) based on the Bray-Curtis distance between samples given the presence/absence of 12,032 taxa present in at least one of nine mouse samples; each dot represents one mouse in the study. *H.p.*, 4-week *H. pylori* infection; UI, uninfected.

ach microbiota (Fig. 3A). For each treatment group, five mice were infected with *H. pylori*, and five were left uninfected. To determine whether the pretreatment altered the total number of bacteria in the stomach, we performed qPCR for bacterial 16S rRNA genes and demonstrated no significant difference in levels between any of the groups (see Fig. S4A in the supplemental material). Furthermore, *H. pylori* colonized each experimental group similarly (see Fig. S4B), suggesting that any inflammatory differences are not due to differences in *H. pylori* colonization levels.

To determine whether there were inflammatory differences between the groups, we analyzed the mouse stomachs by flow cytometry for $CD45^+$ $CD3^+$ $CD4^+$ T-helper cells and $CD45^+$ $CD3^+$ $CD8^+$ T-cytotoxic cells (Fig. 3B and C). We chose these cells as they are known to arrive relatively early in response to *H. pylori* infection and also are reflective of the host response at later time points (26, 35). As expected, normal mice developed a $CD4^+$ response to *H. pylori* infection that was significantly above that of uninfected mice (Fig. 3B and C). Interestingly, mice treated with antibiotics did not respond immunologically to an *H. pylori* infection, displaying levels of $CD4^+$ T-helper cells that were comparable to those of uninfected controls (Fig. 3B and C). In contrast, mice with reconstituted gastric microbiota had a robust influx of $CD4^+$ T-helper cells that was comparable to the normal mouse response to *H. pylori* (Fig. 3B and C). Thus, preinfection antibiotic treatment affected the outcome of *H. pylori*-host interactions and specifically dampened the *H. pylori* immune response.

To determine what type of immune response was inhibited in the antibiotic-treated mice, we examined transcripts encoding inflammatory cytokines for T-helper type 1 (Th1) (*Ifn*γ), T-helper type 17 (Th17) (*Il17*), and T-helper type 2 (Th2) (*Il4*) responses in the mouse stomach (Fig. 3D to F). We found that the *Ifn*γ level was much lower in *H. pylori*-infected antibiotic-treated mice than in normal mice or reconstituted mice (Fig. 3D), similar to the pattern we observed with the infiltrating $CD4^+$ T cells (Fig. 3C). In contrast, the other cytokines did not display marked differences (Fig. 3E and F).

Antibiotic treatment causes significant changes to the stomach microbiota. Our finding that antibiotic treatment dampens the host Th1 response to *H. pylori* is consistent with the notion that preinfection microbial populations affect the extent of *H. pylori*-triggered immune cell infiltration. We hypothesized that there were significant differences in the microbiotas between antibiotic-treated and normal mice and thus compared the stomach microbiotas of these two groups using the Phylochip microbial profiling system. We found that the microbial populations were significantly different from each other, both in the presence/absence of specific species and in their abundances (Fig. 4A; see also Fig. S5 in the supplemental material). Specifically, over 4,400 OTUs were different between the two groups, with 55% significantly decreased in the antibiotic-treated mice and 45% significantly increased, compared to levels in normal mice (Fig. 4B and C, respectively; see also Tables S3 and S4).

Members of the *Firmicutes* phylum of Gram-positive bacteria stood out as having substantial changes between the antibiotic-treated and normal mice. Some members of the *Firmicutes* were decreased in the antibiotic-treated group, mainly members of the *Mollicutes* class (41% of all the decreased OTUs) (Fig. 4B). Other members of the *Firmicutes* were increased in the antibiotic-treated group, including members of the class *Clostridia* (46% of all the increased OTUs) (Fig. 4C); most of these fell within the order *Clostridiales*. *Faecalibacterium* species and *Clostridium* species genera with *Clostridiales* have been found to affect immune responses (8, 10, 27). We focused additional analysis on the *Clostridium* genus because a large number of these species were increased in antibiotic-treated mice (Fig. 4D). The genus *Clostridium* is subdivided into 19 clusters; members of clusters IV and XIVa are known to induce regulatory T cells (Tregs) in the colon (10). Analysis of the *Clostridium* spp. that increased in our antibiotic-treated mice determined that they fell into clusters IV and XIVa (see Fig. S6 in the supplemental material). Tregs are known to influence *H. pylori* pathogenesis. Specifically, Treg responses that arose either as a result of a *Helicobacter bilis* infection or in neonatal mice resulted in a reduced *H. pylori*-triggered pathology (28, 29). We did not, however, detect any markers of Treg-associated transcripts (*Foxp3* or *Il10*) in the antibiotic-treated mice, suggesting that elevated Treg cell levels do not underlie the inflammatory differences in our model (Fig. 4E and F).

DISCUSSION

In this study, we employed antibiotic treatment and gastric reconstitution to demonstrate that antibiotic-induced alterations in the microbiota before *H. pylori* infection reduce the number of $CD4^+$

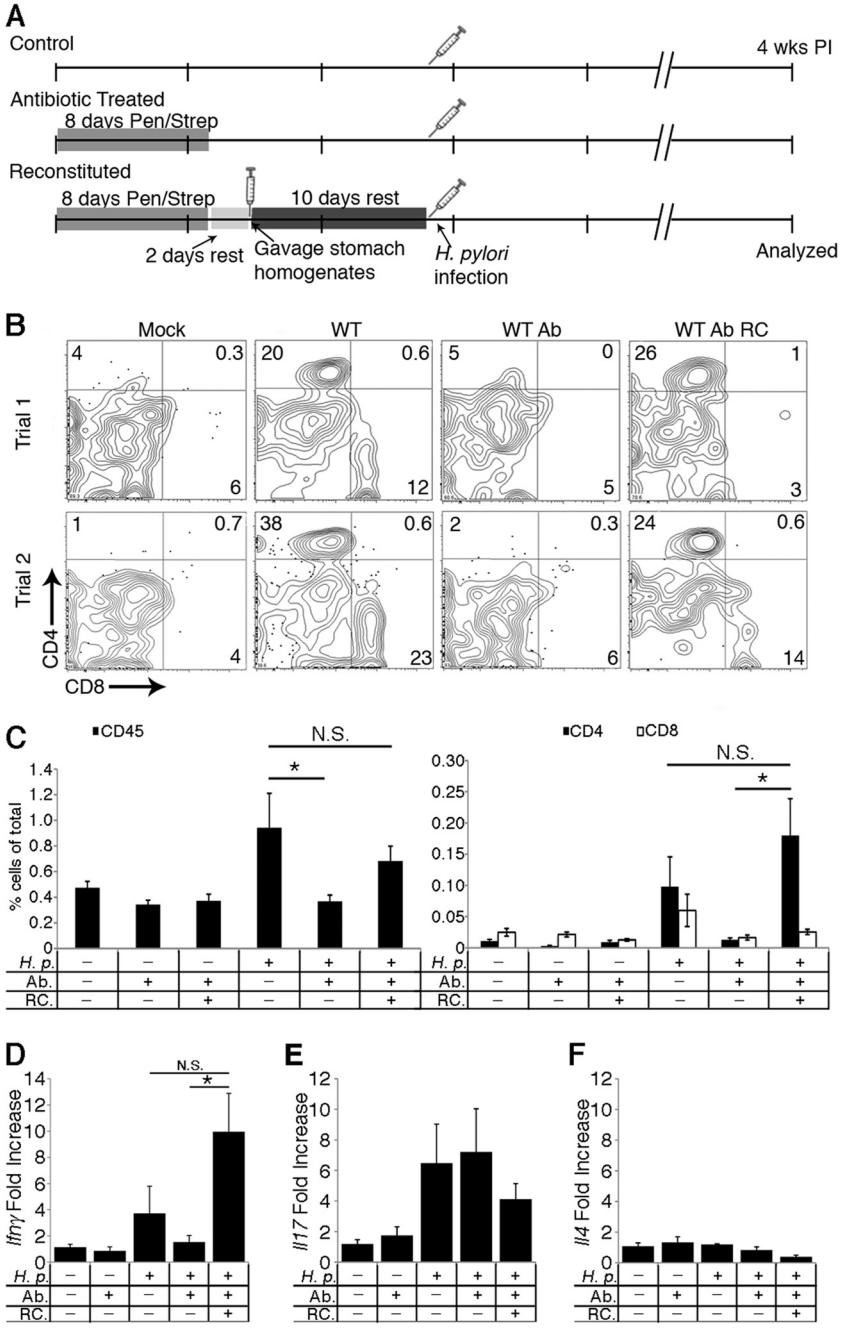
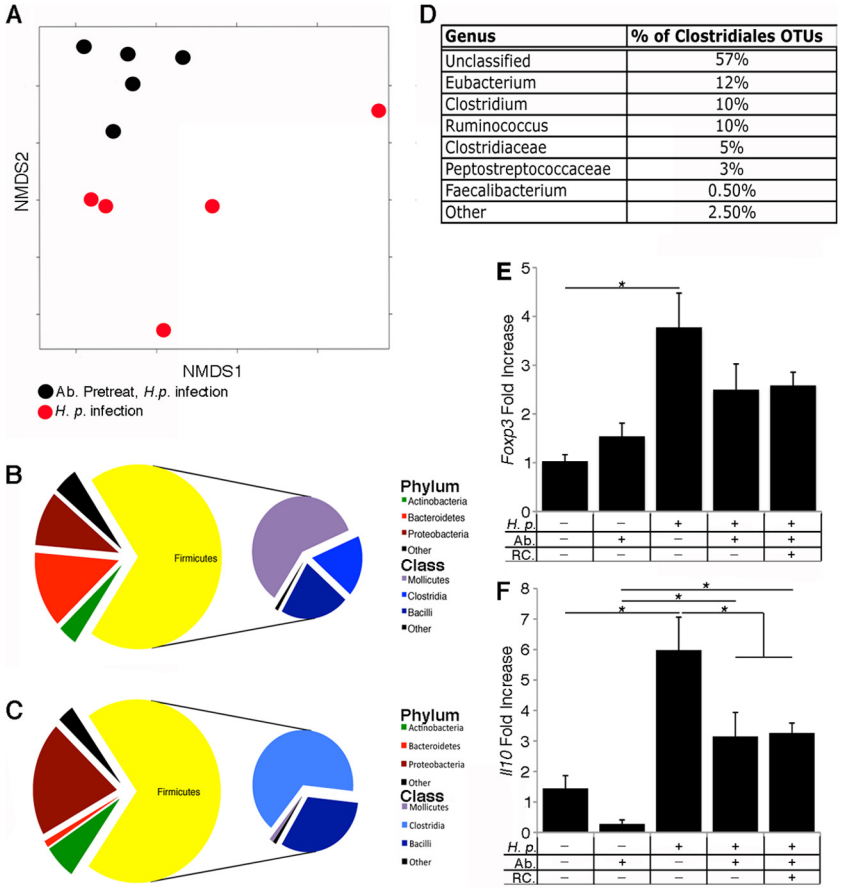


FIG 3 Fewer CD4$^+$ T cells infiltrate the stomach in response to *H. pylori* when mice have been pretreated with antibiotics. (A) Experimental setup for altering the microbiota and infecting with *H. pylori*. (B) Representative flow cytometry plots showing the percentages of CD4$^+$ and CD8$^+$ cells of the CD45$^+$ CD3$^+$ gastric lymphocytes from tissue 4 weeks postinoculation. Numbers in quadrants indicate the percentages of positively stained cells. Quadrants were determined based on isotype controls (data not shown). (C) CD45$^+$ cell percentage is presented as the percentage of CD45$^+$-stained cells out of 50,000 cells counted (left panel). CD4$^+$ and CD8$^+$ cell percentages are presented as the percentages of positively stained cells out of 50,000 cells counted (right panel). Data were obtained from two independent experiments with similar results ($n \geq 7$ mice for each group). (D to F) Total gastric mRNA expression of *Ifnγ*, *Il17*, and *Il4*. mRNA expression levels were normalized to the level of the housekeeping gene, *Gapdh*, and are expressed as fold change from mock treatment, using the $\Delta\Delta C_T$ method (19). *H.p.*, *H. pylori*-infected; Ab, antibiotic treatment; RC, reconstituted with gastric microbiota; WT, wild type; PI, postinfection; wks, weeks. Data in panels C to F are presented as averages ± standard errors of the means. N.S., not significant; *, $P < 0.05$, by two-way ANOVA and Tukey's range test.

T cells that develop in response to *H. pylori* infection. Our studies were built from the observation that C57BL/6N mice from different vendors had different levels of inflammatory responses to *H. pylori* infection and possessed different amounts of two *Lactobacillus* species known to inhabit the stomach. Microbiota differences are common between mice from different vendors. For example, other studies have reported that there are intestinal microbiota differences in C57BL/6 mice from different vendors (8).

Our results demonstrate that *H. pylori* infection does not sub-



Genus	% of Clostridiales OTUs
Unclassified	57%
Eubacterium	12%
Clostridium	10%
Ruminococcus	10%
Clostridiaceae	5%
Peptostreptococcaceae	3%
Faecalibacterium	0.50%
Other	2.50%

FIG 4 The microbiota of antibiotic-treated mice is significantly different from that of the untreated mice and is dominated by members of the *Clostridiales*. (A) Significant differences in microbiotas are apparent between antibiotic-treated and untreated *H. pylori*-infected samples, as indicated by nonmetric multidimensional scaling (NMDS) based on the Bray-Curtis distance between samples given the presence/absence of 12,765 taxa present in at least one of 10 mouse samples; each dot represents one mouse in the study. A total of 4,400 OTUs were different between the antibiotic-treated *H. pylori*-infected mice and the *H. pylori*-infected untreated mice. Of those OTUs, 55% were significantly decreased in the antibiotic-treated mice, and 45% were significantly increased. (B and C) Pie charts illustrate phylum and class level distributions between bacterial taxa that either decreased (B) or increased (C) in the antibiotic-treated mice compared to levels in the nontreated mice. The majority of the taxa that were increased in the antibiotic treated mice were members of the *Clostridia*. (D) Ninety-four percent of the *Clostridia* OTUs that were increased in the antibiotic-treated mice were in the order *Clostridiales*; the chart details those members of the *Clostridiales* at the family and genus level. (E and F) Total gastric mRNA expression of *Foxp3* and *Il10*. mRNA expression levels were normalized to the level of the housekeeping gene, *Gapdh*, and are expressed as fold change from mock treatment, using the $\Delta\Delta C_T$ method. *H.p.*, *H. pylori*-infected; Ab, antibiotic treatment; RC, reconstituted with gastric microbiota ($n = 5$ for each group). For panels E and F, data are displayed as averages ± standard errors of the means. *, $P < 0.05$, by two-way ANOVA and Tukey's range test.

stantially change the overall stomach microbiota composition but that it does affect some stomach microbiota members. Comparable findings were reported from a small-scale terminal restriction fragment length polymorphism analysis in *H. pylori*-infected C57BL/6 mice (30). Other studies similarly found that the *H. pylori*-induced changes were restricted two phylotypes—a reduction in *Bacteroidetes* and an increase in *Firmicutes* (31)—in transgenic insulin-gastrin (INS-GAS) mice. Humans are reported to show slightly more significant changes in gastric microbiota in response to *H. pylori*, with some similarities to the phylotypes that change in mice (16, 17, 25). These human studies, as well as the INS-GAS mouse studies, were all done after longer periods of *H. pylori* infection. Long-term infections may lead to more substantial changes in the microbiota over time due to accompanying immune or physiological changes, such as loss of parietal cells and increased gastric glandular atrophy. These studies are consistent with the idea, however, that the introduction of *H. pylori* into a stomach does not initially impact the microbiota.

Subsequently, we were interested in determining how the initial microbiota composition influences the immune response to *H. pylori*. Our studies found that antibiotic-treated mice were recalcitrant to *H. pylori*-triggered inflammation and lacked Th1 cytokines after 4 weeks of infection. Microbiota members can drive many different immune responses, including Th1, as recently described for *Bilophila wadsworthia* in *Il10*$^{-/-}$ mice (32). We did not detect *B. wadsworthia* in the gastric samples from our mice, but it is plausible that the microbiota of the antibiotic-treated mice had less of a Th1-promoting microbe or more of a Th1-inhibiting species. Such species remain to be discovered, however. It is tempting to speculate that the reduced inflammatory response we observe early postinoculation would drive conditions that would result in reduced inflammation long term; other work has demonstrated that the CD4 response at 4 weeks accurately reflects the long-term *H. pylori* inflammatory response (26). However, future experiments will need to examine the enduring effects on the inflammatory state.

One caveat to our studies is that the antibiotic treatment disturbed the microbiota outside the gastric compartment, which in turn might have affected the gastric immune response. Several studies have demonstrated that the microbiota in one organ can affect the immune response in another, including studies that showed that normal intestinal *Helicobacter bilis* or *Helicobacter muridarum* coinfection decreased *H. pylori* pathology (28, 33). We attempted to diminish these effects by employing mice that were *Helicobacter* free and thus lacked intestinal *Helicobacter* bacteria, including *H. bilis* and *H. muridarum*. Additionally, we reconstituted our mice with stomach microbiota, aiming to isolate the effect of the gastric microbiota on the immune response to *H. pylori*.

It is also difficult to be certain that all of our gastric microbial species are truly autochthonous members of the gastric microbiota. Mice are coprophagic, and so at least some fecal bacteria will retransit through the stomach. As suggested by Savage, we attempted to rule out nonautochthonous species by focusing on species found only in all five mouse stomachs (34). Further studies will be needed to verify the stability of our species over a longer time course.

Our results suggest that differing preinfection gastric microbial populations affect the subsequent immune response to *H. pylori*, thus contributing to the diverse outcomes of *H. pylori* infection. Our work suggests that microbial composition could be used as a marker to predict the disease outcome of *H. pylori* infection and that manipulating the stomach microbiota may be an avenue to pursue to prevent disease in response to *H. pylori* infection.

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